วิทยาสารเกษตรศาสตร์

สาขาวิทยาศาสตร์

ปีที่ ๓๕ ฉบับที่ ๒

เมษายน - มิถุนายน ๒๕๔๔

ISSN 0075-5192

THE KASETSART JOURNAL

Natural Sciences

http://www.rdi.ku.ac.th

April – June 2001

Volume 35 Number 2

THE KASETSART JOURNAL

Natural Sciences
Volume 35, Number 2, 2001

ISSN 0075-5192

The Official Journal of Kasetsart University
Published by the Kasetsart University, Bangkok 10900, Thailand

วิทยาสารเกษตรศาสตร์

Home pages : http://www.ku.ac.th และ http://www.rdi.ku.ac.th

สำนักงาน : สถาบันวิจัยและพัฒนาแห่งมหาวิทยาลัยเกษตรศาสตร์
มหาวิทยาลัยเกษตรศาสตร์ จตุจักร กรุงเทพฯ 10900

วิทยาสารเกษตรศาสตร์ เป็นวารสารของมหาวิทยาลัยเกษตรศาสตร์ ซึ่งพิมพ์เผยแพร่ผลงานทางวิชาการแขนงต่างๆ ทางด้านวิทยาศาสตร์และสังคมศาสตร์ ทั้งจากภายในและภายนอกมหาวิทยาลัย โดย**วิทยาสารเกษตรศาสตร์ สาขาวิทยาศาสตร์** มีกำหนดออกปีละ 4 ฉบับ คือ เดือนมีนาคม มิถุนายน กันยายน และธันวาคม และ**วิทยาสารเกษตรศาสตร์ สาขาสังคมศาสตร์** มีกำหนดออกปีละ 2 ฉบับ คือ เดือนมิถุนายน และธันวาคม

การแลกเปลี่ยนวารสาร ติดต่อสำนักหอสมุด มหาวิทยาลัยเกษตรศาสตร์ จตุจักร กรุงเทพฯ 10900

The Kasetsart Journal

Business Office : Kasetsart University Research and Development Institute (KURDI)
Kasetsart University, Chatuchak, Bangkok 10900.

The Kasetsart Journal is a publication of Kasetsart University intended to make available the results of technical work in the natural and the social sciences. Articles are contributed by Kasetsart University faculty members as well as by those from other institutions. **The Kasetsart Journal : Natural Sciences** edition is issued four times per year in March, June, September and December while **The Kasetsart Journal : Social Sciences** edition is issued twice a year in June and December.

Exchange publications should be addressed to
The Librarian,
Main Library,
Kasetsart University,
Bangkok 10900, Thailand.

Kasetsart J. (Nat. Sci.) 35 : 115 - 121 (2001)

Cypermethrin Insecticide Residues in Vegetable Soybean, *Glycine max* (L.) Merrill, at Different Days of Pre-harvest Interval

Md. Abdullah[1], Ouab Sarnthoy[1] and Suratwadee Jiwajinda[2]

ABSTRACT

The experiments were conducted to investigate cypermethrin insecticide residues in vegetable soybean at different days of pre-harvest interval for two growing seasons. Soybean pods were collected at 0, 1, 3, 5 and 10 days after last application with cypermethrin. Residues were extracted from pods and determined using gas liquid chromatography with an electron capture detector. Cypermethrin when applied at the rate of 100 g ai/ha using a knapsack sprayer showed a mean residue level of 0.71 and 0.52 mg/kg at 0 and 1 day, respectively, in dry season (December- February) whereas the residues were 1.62 and 1.17 mg/kg at 0 and 1 day, respectively, in early wet season (April-June). A pre-harvest interval of 3 days was found safe (0.42 mg/kg) for consumers in dry season whereas early wet st season soybean exhibited residue level of 0.48 mg/kg at 10 day which fell below maximum residue limit of 0.5 mg/kg as prescribed by Food and Agriculture Organization/World Health Organization. Therefore, a pre-harvest interval of 10 days should be considered for cypermethrin residues in vegetable soybean to avoid any health risk of consumers. The dissipation rates of cypermethrin on soybean pods were more or less similar at the same days for both seasons which was 70% (based on 0 day) at 10 days.

Key words: vegetable soybean, cypermethrin, pre-harvest interval, residue, gas liquid chromatography

INTRODUCTION

Vegetable soybean, *Glycine max* (L.) Merrill, is an important cash crop in Thailand. It is harvested after the R_6 and before R_7 growth stage while the pods are still green and the seeds have developed to fill 80-90% of the pod width (Fehr *et al*., 1971). Vegetable soybean is popular in China, Japan and Korea for its unique taste and high nutritional value as a source of vitamins A, B_1, B_2 and C, protein, fat, fiber and mineral such as phosphorous, calcium and iron. The on-farm yield of this crop in Asia is much lower than the potential yield. One of the major reasons for low yield is the infestation by diverse insect pests from germination to harvest. Control of these pest is essential because infestation by certain species could result in total crop loss (Talekar, 1997). As a consequence of pesticide use, the presence of residues in field crop is unavoidable.

Cypermethrin or (RS)-∝-cyano-3-phenoxybenzyl (1RS,3RS; 1RS,3SR)-3-(2,2-dichloro-vinyl)-2,2-dimethylcyclopropanecarboxylate is a digestive and contact insecticide effective against a wide range of insect pests, particularly leaf- and fruit-eating Lepidoptera and Coleoptera in cotton, fruit, vegetables, vines, tobacco

1 Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.

2 Central Laboratory and Greenhouse Complex, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand.

and other crops (Worthing, 1987). Cypermethrin is widely used widely by farmers to control insect pests of vegetables.

There is currently an increasing concern and awareness on the hazards of pesticides to consumers. Even with the adoption of integrated pest management, farmers still believe in the control of pests using pesticide because of its quick effect. The application of pesticides pre- or post-harvest could, however, leave residues on food products which pose a potential risk to the health of consumer (Lindsay, 1997). Insecticide use in controlling insect pest complex of vegetable soybean should, therefore, be examined for residues. Hence an attempt has been made to estimate the quantity of residue of cypermethrin left in vegetable soybean at different days of pre-harvest intervals for safe consumption.

MATERIALS AND METHODS

Spray treatments

Field experiments were conducted during dry (December, 1999 to February, 2000) and early wet (April to June, 2000) seasons at Kamphaeng Saen Campus of Kasetsart University, Nakhon Pathom, Thailand. Vegetable soybean variety AGS292 was planted in a plot consisted of 10 rows, 5 m long and 50 cm apart. Plants within the rows were 20 cm apart maintaining 25 hills/row. Three plots (each plot was considered as a replication) were sprayed with cypermethin 10% EC at the concentration of 100 g ai/ha using a knapsack sprayer. Adjuvant (IBA spreader) was added at the concentration of 0.5 ml/l of spray volume. Three untreated plots (each plot was considered as a replication) were also maintained to observe insecticidal contamination. Since vegetable soybean is attacked by insect pests from germination to harvesting, cypermethrin was sprayed at 10 days interval until harvest. Conditions of rainfall and sunshine data were recorded throughout the experimental periods.

Sampling procedure

Soybean pods were collected from treated and untreated plots at 0 (3 hr), 1, 3, 5 and 10 days after last spraying. Samples (600 g each) were randomly collected from the plots and poured into polyethylene bags. These bags were tightly closed with rubber band, labeled and kept in a deep frceeger at -65°C until extracted.

Analytical procedure

The procedures for cypermethrin residue estimation in vegetable soybean were as follows:

Extraction of residues from pods

The frozen samples were taken out from deep frceeger after 2 months of sample collection and allowed to set room temperature. A 100 g of sample was weighed and poured into a blender jar. Then 25 g sodium sulfate was added into the sample and blended at low speed for a few seconds. One hundred and fifty ml of petroleum ether was added into the blender jar and blended at high speed for 2 min. Decant petroleum ether was filtered through a perforated Buchner funnel using Whatman No. 1 filter paper. The blended sample was re-extracted twice with 100 ml petroleum ether in blender jar and blended at high speed for 1 min. The sides of blender jar were scraped down to break up caked materials between extractions. After last blending, blender jar and materials were rinsed with 50 ml petroleum ether. The combined extracts were then filtered through anhydrous sodium sulfate into 500 ml round-bottom flask. The extract was then evaporated by a rotary evaporator maintaining at 40°C to make a final volume of 2 ml.

Acetonitrile partitioning

Two ml extract was adjusted to 30 ml using petroleum ether, partitioned with acetonitrile saturated with petroleum ether (40 ml) in 125 ml separatory funnel, and shaken vigorously for 1 min. After allowing the layer to separate, acetonitrile was drained into 1 l separator containing 650 ml

distilled water, 40 ml saturated sodium chloride solution, and 100 ml petroleum ether. Petroleum ether layer was re-extracted three times with additional 30 ml saturated acetonitrile and combined acetonitrile layers in the same separatory funnel as above. The separator was then shaken for 30-45 sec, the layer was allowed to separate and the aqueous layer was drained into 1,000 ml conocal flask. The upper petroleum ether was then transferred into a conical flask. Aqueous layer was re-extracted with 100 ml petroleum ether. The petroleum ether was combined with previous one and filtered through anhydrous sodium sulfate into 500 ml round-bottom flask. The volume of extract was reduced to 2 ml using rotary evaporator.

Clean-up with Florisil

A glass column, 50 cm × 2.5 cm id, fitted with stopcock was used where activated Florisil (90 g) (over night at 130°C) was filled to column followed by sodium sulfate (10 g). Filled column was pre-wetted with 40 ml petroleum ether. Extract solution was transferred to column with controlled flow rate (5 ml/min). The column was eluted with 200 ml 6% ethyl ether/petroleum ether followed by the same volume of 15% and 50% eluants. All of these eluates were collected separately in a 500 ml round-bottom flask and then evaporated to final volume of 0.5 ml. These were rinsed with several small portions of petroleum ether and poured into small vials to a final volume of 2 ml. All vials were kept in a refrigerator at 4°C before subjecting to GC analysis.

Gas chromatographic analysis

Sample solution in vials were reduced to a volume of 1 ml before subjecting to analysis by GC. Working standard solutions of 10 and 20 ppm were prepared in petroleum ether by dilution of appropriate volumes of the stock solution. Cypermethrin was analyzed by gas liquid chromatography using an electron capture detector. A gas chromatograph (Shimadzu Model GC-9A) was used for this purpose. All samples were injected at 1µl. The analysis conditions were as follows:

Column type: Widebore DB-1701P (1.00 µm, 30 m × 0.530 mm)
Column temperature: 250°C
Injector temperature: 270°C
Detector temperature: 270°C
Carrier gas: N_2
Flow rate: 20 ml/min.

Recovery experiment

Recovery experiment was followed to observe the efficiency of extraction, clean-up and estimation procedures, by fortifying soybean pods (100 g) with 20 µg cypermethrin on two samples.

Calculation of residues

The residue R, expressed in mg/kg were calculated using the following formula:

$$R = \frac{H_A \cdot V_{end} \cdot W_{sd}}{H_{sd} \cdot V_i \cdot G}$$

where G = sample weight (in g), V_{end} = terminal volume of sample solution (in ml), Vi = volume of sample solution injected into gas chromatography (in µl), W_{sd} = amount of cypermethrin for standard solution (in µg), H_{sd} = Peak area from W_{sd} (in cm^2), H_A = Peak area from V_i (cm^2).

RESULTS AND DISCUSSION

Recovery experiment exhibited the efficiency of procedures used for cypermethrin residues in vegetable soybean, when 20 µg cypermethrin were added to 100 g soybean pods showed an average recovery of 11.97 µg (59.85%). Cypermethrin residues on vegetable soybean at different days of pre-harvest interval were investigated for two growing seasons. Cypermethrin 10% EC when sprayed at the concentration of 100 g ai/ha showed a residue level of 0.71 mg/kg at 0 day followed by 0.52 mg/kg at 1 days in dry season

(Table 1) which were higher than the recommended maximum residue limit (MRL) of 0.5 mg/kg set for beans with pods (FAO/WHO, 1985). The residue levels at 3, 5 and 10 days decreased to 0.42, 0.30 and 0.22 mg/kg, respectively which were all below the maximum residue level in the same season. Cypermethrin residues were higher in early wet season (Table 1). Residues were 1.62 mg/kg at initial level (0 day) followed by 1.17, 0.94 and 0.69 mg/kg at 1, 3 and 5 days, respectively, in early wet season which were all above the maximum residue limit. At 10 day, the residues were 0.48 mg/kg which was below the maximum residue level (Table 1) set by FAO/WHO. No significant difference of residues were observed between different days of pre-harvest intervals in dry season. Cypermethrin residues decreased gradually in all pre-harvest intervals. The degradation of cypermethrin residue in dry season were 26.76, 40.84, 57.75 and 69.01% for 1, 3, 5 and 10 days interval, respectively, on initial residue level (0 day). In case of early wet season, the initial residue (0 day) level was 1.62 mg/kg which differed significantly from all pre-harvest intervals. No significant differences were observed between 1 and 3 day interval, 3 and 5 day interval, and 5 and 10 day interval but 10 day interval differed significantly over 1 and 3 day interval. The degradation of cypermethrin in early wet season were 27.78, 41.98, 57.40 and 70.37% at 1, 3, 5 and 10 day interval, respectively, on initial residue level (0 day). The reduction rates of cypermethrin residues were very close at the same days for all pre-harvest intervals in both seasons.

A total rainfall of 12.5 and 217.6 mm were recorded during growth period of dry and early wet season, respectively. But after last spray, dry and early wet season soybean received a total rainfall of 12.5 and 12.4 mm (Table 2), respectively, which might be the cause of the same reduction rate of residue at all pre-harvest intervals. There was no rainfall within 3 hrs of sample collection at 0 day for both seasons. So residue washing out by rain did not occur at the initial level. Cypermethrin was applied to each plot with the same amount of spray in both seasons. The higher residues in early wet season might be due to the less canopy with poor yield (5.75 t/ha) which allowed more insecticide and residues on the pods. The higher canopy in dry season with higher yield (9.65 t/ha) might be responsible for receiving less spray deposit resulting in less residues on pods. On the other hand, a total sunshine period of 593.6 and 463.7 hrs were recorded from planting to harvesting in dry and early wet season, respectively, whereas after the last spray the dry season received 81.7 hrs of sunshine while the early wet season received only 54.4 hrs sunshine period (Table 2). This might have an influence on having higher residues in early wet season. Tajeda *et al.*(1983) reported that the disappearance of residues in and on plants is the effect of the interactions of environmental conditions such as wind, rain, sun, humidity and temperature and chemical and physical factors such as volatilization and growth of the plants.

Tejada *et al.* (1989) reported an initial deposit of 0.36 mg/kg on string bean when cypermethrin was applied at the rate of 1 ml/l under field conditions. This amount is close to the residue obtained in dry season soybean of the present investigation. They also observed detectable (0.11mg/kg) residues up to 4 days after last spraying. Since in the present study the recommended dose was higher (2.5 ml/l), and gave an initial deposit of 0.71 mg/kg in dry season. The average residue level of any insecticides depends primarily on the quantity of its active ingredient. Field studies were conducted by Kumar *et al*. (1998) on chick pea where cypermethrin was applied either at 60 or 90 g ai/ha (low and high dose, respectively). Initial mean residue level on green pods were 0.42 and 0.62 mg/kg at low and high doses, respectively. These dissipated by 73.81 and 70%, respectively after 15 days which was also close to the residues at initial level (dry season) and degradation rate of both seasons. Residues of cypermethrin were determined by Singh and Kalra (1992) in the fruits and leaves

of aubergines and in the soil when applied at 50 and 100 g ai/ha. After eight applications at lower dosage, initial deposits on the fruits were 0.73 mg/kg which declined to 0.08 mg/kg in 10 days. On leaves the initial deposits averaged 1.16-1.69 mg/kg which declined by 68% in the 10 days. These results are close to the present investigation where there was an initial deposit (0 day) of 1.62 mg/kg on pods which declined by 70.37% in the 10 days of early wet season. Singh and Udeaan (1989) investigated the persistence and dissipation of cypermethrin in okra fruits where 50 or 100 g ai/ha were applied three times. After 2nd and 3rd spray, the initial residues were 0.76 and 0.65 mg/kg for 50 g ai/ha

Table 1 Cypermethrin residues in vegetable soybean applied at 100 g ai/ha in dry season (February) and early wet season (June) 2000 at Kamphaeng Sean, Nakhon Pathom.

Pre-harvest interval (Days)	Cypermethin residues (mg/kg) (Mean ± S.D)	
	Dry season	Early wet season
0	0.71 ± 0.44	1.62 ± 0.21a*
1	0.52 ± 0.09	1.17 ± 0.19b
3	0.42 ± 0.36	0.94 ± 0.13bc
5	0.30 ± 0.05	0.69 ± 0.14cd
10	0.22 ± 0.14	0.48 ± 0.11d
F-test	NS	**
C. V (%)	54.2	16.6

* Means followed by the same letters are not significantly different at the 5% level by Duncun's multiple range test.

Table 2 Rainfall and sunshine data at different days of pre-harvest intervals in dry season (February) and early wet season (June) 2000 at Kamphaeng Sean, Nakhom Pathom.

Pre-harvest interval (Days)	Dry season		Early wet season	
	Rainfall (mm)	Sunshine (hrs)	Rainfall (mm)	Sunshine (hrs)
0	2.0	5.7	2.5	8.7
1	0	6.7	0	3.8
2	0	8.8	T*	0.2
3	3.9	9.3	6.0	0.2
4	0	6.1	T	4.1
5	0	9.0	0.1	5.7
6	0	9.9	T	10.4
7	0	9.7	0	7.0
8	0	9.2	0.3	4.3
9	0.1	4.2	T	2.7
10	6.5	3.1	3.5	7.3

* Trace of rainfall less than 0.1 mm

dose, respectively and 1.53 and 1.43 mg/kg for 100 g ai/ha dose, respectively. They recommended a waiting period of at least one day to let them fall below the maximum limit of 0.5 mg/kg. In the present study, the results of dry season showed a pre-harvest interval of three days for the safe use by consumers where residue fell below the maximum residue limit. Rai *et al*. (1986) observed the persistence of cypermethrin for 11 days which was below prescribed maximum residue limit within eight days on cauliflower which was close to the safe period for wet season. Kumar *et al*. (2000) reported a safe waiting period of 1.8 days when cypermethrin was applied at 300 g ai/ha on chilli which is close to the waiting period for dry season. Persistence and safe period on okra fruits were investigated by Khan *et al*. (1999). They observed an initial concentration of 1.31 mg/kg which dissipated to a mean concentration of 0.05 mg/kg after 10 days where residues existed below the maximum residue limit set in India for cypermethrin (0.2 mg/kg) at 5.91 days after final application. Awasthi (1994) reported the waiting periods for cypermethrin which was between 0 to 5 days based on the FAO/WHO prescribed tolerance limits for chilli. All of these reviews support the investigation of cypermethrin residues in vegetable soybean. In the control samples (collected from the plots where no pesticide was applied) no peaks were observed for both seasons indicating no contamination of residues on soybean pods.

From the present investigation of cypermethrin residues in vegetable soybean it was concluded that this application dose was safe for the consumption after three days of application in dry season where it fell below maximum residue limit. So a pre-harvest interval of three days might be considered for dry season. But in case of early wet season where crop canopy was smaller allowing higher deposition of cypermethrin residues on pods, it showed a pre-harvest interval of 10 days for the safe use. So vegetable soybean should be harvested after 10 days of cypermethrin application to avoid any health risk of consumers.

ACKNOWLEDGEMENTS

The authors would like to express their sincere thanks to Miss Lukkhana Benjawan, Environmental Science Laboratory, Central Laboratory and Greenhouse Complex, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom for her co-operation during analysis of the samples. We are also grateful to other staffs sharing the same laboratory.

LITERATURE CITED

Awasthi, M. 1994. Studies on the dissipation and persistence of pyrethroid residues on chilli fruits for safety constants. Pesticide Research Journal 6 (1) : 80-83.

Fehr, W. R., C. C. Caviness, D. T. Burmood, and J. S. Pennington. 1971. Stages of development descriptions for soybean, *Glycine max* (L.) Merrill. Crop Science 11 : 929-931.

FAO/WHO. 1985. Evaluation of some pesticide residue in food. FAO Plant Production and Protection Paper No. 67. Rome, Italy. 730 p.

Khan, M. A. M., D. J. Reddy, and S. V. Rao. 1999. Dissipation of cypermethrin residue in okra fruits. Pesticide Research Journal 11 (1) : 84-85.

Kumar, P. K., D. J. Reddy, K. N. Reddy, and T. R. Babu. 2000. Dissipation of cypermethrin residues in chilli. Pesticide Research Journal 12 (1) : 130-132.

Kumar, P., S. P. Singh, R. S. Tanwar, and P. Kumar. 1998. Dissipation of cypermethrin residue on chickpea. Pesticide Research Journal 10 (2) : 242-245.

Lindsay, D. G. 1997. Pesticide residue in food: the need for fairer cost-benefit analysis. Pesticide Outlook 8 : 6-10.

Rai, S., N. P. Agnihotri, and H. K. Jain. 1986. Persistence of residues of synthetic pyrethroids

on cauliflower and their residual toxicity against aphids. Indian J. Agricultural Science 59 (9) : 667-670.

Singh, B. and A. S. Udeaan. 1989. Estimation of cypermethrin residues in the fruits of okra, *Abelmoschus esculentus* (Linn.) Moench. J. Insect Science 2 (1) : 49-52.

Singh, I. P. and R. L. Kalra. 1992. Determination of residues of cypermethrin in brinjal fruits, leaves and soils. Indian J. Entomology 54 (2) : 207-216.

Talekar, N. S. 1994. Sources of resistance to insect pests of soybean in Asia, pp. 161-165. *In* B. Napompeth (ed.). Soybean feeds the world. Proceedings of World Soybean Research Conference V, February 21-27, 1994. Chiang Mai.

Tejada, A. W., E. D. Magallona, and E. B. Lakan-Ilaw. 1983. Insecticide residues in vegetables: application of the modified approach to organophosphate insecticide residues in string bean (*Vigna sesquipedalis* Fruw.). The Philippine Agriculturist 66 : 405-416.

Tejada, A. W., S. M. F. Calumpang, M. J. V. Barredo, and E. D. Magallona. 1989. Insecticide residues in string beans (*Vigna sesquipedalis* Fruw.). I. Monocrotophos, fenvalerate, cypermethrin, BPMC and chloropyrifos. The Philippine Agriculturist 72 (3) : 391-400.

Worthing, C. R. 1987. Pesticide Manual. A World Compendium. British Crop Protection Council, UK. 1081p.

Received date : 23/01/01
Accepted date : 22/06/01

Kasetsart J. (Nat. Sci.) 35 : 122 - 131 (2001)

Effects of Mating Ratio, Cock Number in the Flock and Breeder Age on Fertility in Thai Native Chicken Flock

Ratana Chotesangasa

ABSTRACT

Trials were conducted to determine general semen characteristics of the male native chicken and effects of mating ratio, cock number in the flock, and breeder age on fertility in the native chicken flock. The results revealed that the mature native male had the semen volume of about 0.4 ml/bird/ejaculation, spermatocrit value of about 13.86-15.60%, semen concentration of about 7031-8001 million cells/ml, and total sperm of about 2870-3200 million cells/ejaculation. The motility score and pH value were around 4-5 and 7.7-8.4, respectively. The effect of mating ratios on fertility of the flock was not significantly different ($P>0.05$). The mean fertility rates measured during 35-44 weeks of (hen) age of the mating ratios of 1:7, 1:10, 1:13, and 1:16 were 88.21, 91.20, 88.20 and 79.82% ; and during 34-44 weeks of age of the mating ratios of 1:8, 1:12, and 1:16 were 84.31, 90.84, and 81.76% , respectively.

The evidence which the lower trend of fertility rate occurring in the 1:16 flock disappeared after the exchange of cocks between the flocks indicated that sexual competency of the male played a major role in fertility of the breeding flock. Continuous surveillance on egg incubation results and immediate replacement of the ill with the healthy cocks wherever needed were suggested. Housing two cocks within a flock showed ambiguous influence. While it caused a decrease in fertility percentage on the 1:8 flock, it showed no ill effect at all on the 1:16 flock. Furthermore, the incidence of low fertility observed in the 1:12 flock when its sole male was ill confirmed that the high fertility of the flock depended heavily on sexual competency of the male and it could possibly have been secured if there were two cocks in the flock. The results revealed more of the benefit of housing two cocks within the same flock. The declining fertility rate of the old hens in their third year of age was greatly improved from the mean value of 58.32% for the flock with one cock to 81.20% for the flock with two cocks. For the effect of breeder age on fertility, the overall results of these experiments indicated that the native cocks aged from nine months to two years had similar fertilizing ability of about 80-90% whereas the native hens had their fertilizing ability decreased annually from about 80-90% in the first year, to about 70-80% in the second year, and 60-70% in the third year.

Key words: native chicken, mating ratio, fertility, semen characteristics, breeder age

INTRODUCTION

Being successful in poultry breeding depends not only on basic reproductive characteristics of breeders, testicular and reproductive tract growth in the male or ovary and oviduct performance in the female and their hormonal-controlling system, but also on efficient management of the breeder flock. Application of additional daylight has long been accepted as an important practice required for the

Department of Animal Science, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.

most efficient reproductive performance of the chicken flock. The photoperiod of about 15-16 hours a day was reported appropriate for the breeder (North and Bell, 1990). Mating ratio of males to females within a flock was another factor to be considered. The mating ratios of 1:14-16 and 1:10-12 were recommended for layer and broiler breeders, respectively (Appleby *et al*., 1992). Moreover, the breeder age was also important to the fertility of flock. The evidence of decreasing in fertility with increasing in age of the breeders was reported. It was due more to the male than to the female (Brillard and McDaniel, 1986). Becoming less often in mating activity of the older male was suggested to be the cause of lower fertility of the flock (Duncan *et al*., 1990).

For Thai native chickens which are popular for their not-too-soft and good-flavoured meat, their typical characteristics of being wild, aggressive and possessive of their mates and occupying areas were relatively distinct comparing with their commercial meat-type counterpart. Handling of the native breeder flock may need an extra caution and the outcome could be different from those had been reported in the modern breeds. These experiments were, therefore, designed to study general characteristics of semen in the native male and also the effects of mating ratio, cock number in the flock and breeder age on fertility of the flock.

MATERIALS AND METHODS

Experiment 1

The first experiment was carried out to determine general semen quality of the native chicken at weekly intervals during 35-52 weeks of age. Fourty-four healthy Thai native cocks were randomly allocated into 4 groups of 11 each. The chickens were reared in cages with free access to feed and water and under the photoperiod of 15 hours a day. Pooled semen of each group was obtained twice a week, by means of the conventional abdominal massage technique. Mean volume of the two ejaculations within a week of each group was regarded as the average pooled semen volume of the group from which the average semen volume/bird/ejaculation at that age level was calculated. Spermatocrit value of each pooled semen sample was determined by the method of microcapillary such as that used for the blood haematocrit determination. For each determination, duplicate semen samples were centrifuged at 15,000 g, for 5 minutes. The average spermatocrit value was then substituted for x value in the regression equation of y (semen concentration) = -0.732+0.56x acquired earlier from the previous experiment (Chotesangasa and Gongrattananun, 1999). The total number of sperm/ejaculation was obtained by multiplying the semen concentration value by the average semen volume/bird/ejaculation.

Microscopic examination of motility of the fresh semen was performed immediately after completion of each round of semen collection. Determination of pH value of the seminal fluid was also conducted.

Experiment 2

The objective of this experiment was to study the effect of different mating ratios on fertility in the native breeder flock. Twelve healthy males and 138 females of the same age of 35 weeks were randomly arranged into 4 groups of mating ratios of 3 replications each. The 4 mating ratios of male : female were 1:7, 1:10, 1:13, and 1:16, respectively. Fertility was evaluated in term of percentage of fertilized eggs, at weekly intervals, during 35-44 weeks of age. For each batch of incubation, the hatching eggs were collected daily and accumulated for 7 days before placing them in the incubators. On the seventh day of incubation, all incubated eggs were candled to identify live or dead or infertile eggs. All eggs identified as infertile or dead embryo were then break-opened to examine the germinal disc areas to verify the result of egg candlings. Eggs with live and dead embryos were regarded as fertile eggs. The fertility rate was expressed as

percentage of the fertile eggs out of the total number of incubated eggs.

Experiment 3

This experiment was aimed to compare the effect of different mating ratios on fertility in the breeder flock similar to what had been done in the experiment 2. The male used in this experiment was, however, at more mature age of 74 weeks, whereas the female was about the same age of 34 weeks as previously used in the experiment 2. Twelve males and 144 females were allocated into 3 groups of mating ratios of 1:8, 1:12, and 1:16 of 4 replications each. Fertility rates of the groups measured at 7 days of incubation were recorded at two-week intervals during 34-68 weeks of the female age.

Experiment 4

The effects of two experimental factors, the mating ratios with 3 levels of 1:8, 1:12 and 1:16 and the cock number in the flock with 2 levels of 1 cock and 2 cocks, were simultaneously studied. Eighteen cocks aged 36 weeks and 216 hens aged 56 weeks were grouped into six treatment combinations with 2 replications each. The six treatment combinations were 1:8, 1:12, 1:16, 2:16, 2:24 and 2:32. Fertility rates of the flocks measured at 7 days of incubation were recorded at two-week intervals for the whole experimental period of 30 weeks.

Experiment 5

This experiment was to test whether the age of breeder hens and the cock number in the flock affected fertility of the breeder flocks of the same mating ratio of 1:12. The hens were in their second year (age 72 weeks), and third year (age 110 weeks) whereas the cocks were 72 weeks of age. A total of 18 cocks, 108 hens in their second year of laying and 108 hens in their third year of laying were randomly allocated into 4 treatment combinations of 3 replications each. The four treatment combinations were as follows : 12 second-year hens + 1 cock, 24 second-year hens + 2 cocks, 12 third-year hens + 1 cock, and 24 third-year hens + 2 cocks. Fertility rates were measured at two-week intervals for the period of 20 weeks.

Statistical methods

For experiments 1, 2, and 3, a completely randomized design (CRD) was used. Statistical designs used for experiments 4 and 5 were 3x2 factorial experiment in CRD and 2x2 factorial experiment in CRD, respectively. All of the data obtained were analysed by means of analysis of variance and the differences between means were compared by the method of Duncan's new multiple range test (Snedecor and Cochran, 1980).

RESULTS AND DISCUSSION

Semen characteristics of the native chicken

Semen characteristics of the native chickens measured at various age levels are shown in Table 1. It was found that the native cocks during 35-52 weeks of age had similar quality of semen, though the semen volume and total sperm/ejaculation tended to be lower in the younger ones. Generally, it could be concluded that the native cocks from the age of 38 weeks upwards were able to give high quality semen similar to the more mature chickens. The results of semen measurements were as follows : the semen volume was about 0.4 ml/bird/ejaculation, spermatocrit was in the range of 13.86-15.60%, semen concentration was about 7031-8001 million cells/ml, and the number of total sperm was in the range of 2870-3200 million cells/ejaculation. The rather low values observed at the 52 weeks of age were, however, not included into the calculations since they could by erred by the long pause period (8 weeks) prior to the last collection time. There was clear evidence that the semen collected at a rather low frequency usually had lower quality than that obtained at a higher frequency (Sexton, 1983). Apart from the characteristics mentioned above, sperm motility

Table 1 Semen characteristics of native cocks aged 35 to 52 weeks, (experiment 1).

Cock age (w)	Semen volume (ml./bird/ejac.)	Spermatocrit (%)	Semen concentration (million cells/ml.)	Total sperm (million cells/ejaculation)
35	0.35	13.93	7067.4	2473.59
36	0.37	13.39	6767.80	2504.09
37	0.40	13.66	6917.60	2717.04
38	0.41	14.54	7411.80	3038.84
39	0.41	13.86	7031.00	2882.71
40	0.40	14.12	7176.6	2870.64
41	0.37	15.52	7956.4	2943.87
42	0.40	15.11	7726.8	3090.72
43	0.40	15.55	7977.4	3190.96
44	0.40	15.60	8001.2	3200.48
⋮				
52	0.29	14.91	7617.6	2209.10

Apart from the Characteristics shown in the table, pH values and motility scores of the pooled semen were also evaluated. It was found that both qualities of the pooled semen did not change in accordance with age of the cock but rather scattered in nature. The pH values of seminal plasma obtained from this experiment were ranged from 7.7 - 8.4. The motility scores varied within the range of 4 - 5 of the 0 - 5 scale. Each figure was the mean of 4 replications of 11 cocks each.

scores and pH values of the semen were also similar among the various ages studied. The motility scores were mainly at the level 4 or 5 and occasionally at the level 3 of the 0-5 scale (Wishart and Wilson, 1997). The semen pH varied within the range of 7.7-8.4. The rather high alkalinity of semen in this experiment was possibly due to high amount of CO_2 loss during the long waiting period prior to the measuring time (more than an hour). Furthermore, these measurements were taken place at rather warm room temperatures, the changes of pH values were then even more rapid. Lake (1984) revealed that the pH value of chicken semen kept at room temperature under paraffin sheet was about 7.1, and after removing the paraffin sheet it turned rapidly into 7.8 because of CO_2 loss into the air. The evidence indicated the difficulty of acquiring a true pH value of the chicken semen. What could be concluded from the results of this experiment was that the pH value of the chicken fresh semen measured in an open-air condition, at room temperature, within 1-2 hours after the collection time, was about 7.7-8.4.

Mating ratio

The effects of different mating ratios on fertility of the breeder flock were studied separately in two different conditions. In one condition, both male and female breeders were started from the same age of 35 weeks (experiment 2) and in the other one, the male and female breeders were from 74 and 34 weeks of age, respectively (experiment 3).

Results obtained from the two experiments were in agreement and revealed that the different mating ratios did not affect ($P>0.05$) the fertility of flock. In experiment 2, the average fertility rates measured during 35-44 weeks of age of the 1:7,

1:10, 1:13, and 1:16 groups were 88.21, 91.20, 88.20, and 79.82%, respectively (Table 2). The results of experiment 3 showed that the average fertility rates measured during 34-44 weeks of hen age of the 1:8, 1:12, and 1:16 groups were 84.31, 90.84, and 81.76%, respectively (Table 3). Though the two experiments did not show any significant difference between the mating ratios, both reflected the same trend of having relatively low fertility rate in the 1:16 group. The previous suggestion that the ratio of 1:16 was too large to give a satisfactory fertility rate should be reconsidered because it was found that the fertility of the very same female flock was considerably improved after the exchange of the males between groups. The average fertility rate of the 1:16 group measured after the male exchange, during 48-68 weeks of the (hen) age, was increased to 89.41% which tended to be higher than the 81.16% of the 1:8 group and the 75.80% of the 1:12 group. The difference was, however, not significant ($P>0.05$). The evidence of exchanging of the males resulted in altering in fertility of the flock indicated the importance of sexual competency of individual males on the flock fertility. Though the sexual competency was, in general, related to the age of the cock, it was probably not the case of those under 2 years of age since a significant difference in fertility rates between the cocks of different ages was not observed (Table 2, 3).

On the fertility basis, the evidence suggested that the mating ratio of 1:16 in the native breeder flock was not too large and could bring a satisfactory result as long as the male breeder was staying sexually competent. To maintain the satisfactory fertility, a close watch on egg incubation results of each breeder flock was suggested to be carried out at intervals so that the incompetent males could be replaced with the competent ones whenever considered necessary. In facts, the incidence of low fertility occurring within a rather short period of time after the onset of flock mating was not unusual and could happen even in the modern-commercial hybrid flock. Practically, the deteriorating situation could be improved by means of "**spiking the**

Table 2 Fertility percentage measured at 7 days of incubation in native chicken flocks of different mating ratios, (experiment 2).

Hen-Cock age (w)	Mating ratio			
	1 : 7	1 : 10	1 : 13	1 : 16
	%			
35 - 35	85.24	90.70	84.11	77.06
36 - 36	89.68	91.77	78.75	73.81
37 - 37	82.38	91.36	87.68	95.69
38 - 38	87.70	91.65	94.80	91.43
39 - 39	90.41	91.83	93.80	84.07
40 - 40	93.93	88.46	87.32	74.22
41 - 41	87.31	91.60	92.00	75.65
42 - 42	90.37	94.62	86.66	69.36
43 - 43	90.70	86.21	88.21	78.47
44 - 44	84.41	93.83	88.63	78.47
Average	88.21	91.20	88.20	79.82

Each figure, other than the average value, was the mean of 3 replications.

flock" which could be done either by the way of adding some supplementary males into the flock or replacing partially or entirely the males of low-fertility flock with the freshly new males. To clarify how the spiking technique worked to improve the flock fertility, Peterson *et al.* (2000) explained that the spiking technique caused a double action to occur simultaneously. That was while the supplementary or replacement males themselves were more active and had higher mating frequency than the original males, their frequent mating behavior also stimulated mating activity of the original males which still remained in the flock.

For the native cock which still had relatively high instinct of being ferocious, aggressive and possessive of their mates and occupying areas, the only spiking practice considered appropriate was the replacement of the whole set of cocks with a new one. It was also important to note that all replacement cocks to be housed in the same flock should have been together and be acquainted with each other from the beginning. The spiking practice by adding some supplementary cocks into the flock reared in a confined pen which its original cocks still existed, must be avoided. Fighting between unfamiliar native cocks would bring about not only

Table 3 Fertility percentage measured at 7 days of incubation in native chicken flocks of different mating ratios, (experiment 3).

Hen - cock age (w)	Mating ratio		
	1 : 8	1 : 12	1 : 16
	%		
34 - 74	71.40	81.45	77.06
36 - 76	84.39	93.86	76.81
38 - 78	80.30	87.74	71.54
40 - 80	83.51	94.84	86.34
42 - 82	93.45	94.22	89.75
44 - 84	92.78	92.90	89.06
Average	84.31	90.84	81.76
Cocks of the different mating ratios were exchanged			
48 - 88	90.16	88.18	92.98
50 - 90	80.57	84.87	96.63
52 - 92	83.93	65.95	91.86
54 - 94	75.11	87.69	92.41
56 - 96	91.01	76.47	92.40
58 - 98	81.98	83.44	87.80
60 - 100	77.32	80.61	80.53
62 - 102	68.18	60.70	96.43
64 - 104	77.26	62.39	92.92
66 - 106	94.87	79.30	88.75
68 - 108	72.38	64.23	70.84
Average	81.16	75.80	89.41

Each figure, other than the average value, was the mean of 4 replications.

emotional stress leading to the decreased fertility rate of the flock but also injury and/or death of the male breeders.

Mating ratio and cock number in the flock

The effects of two factors, mating ratio and cock number in the flock, on flock fertility were examined in experiment 4. Results of each factor and their interaction effects are shown in Table 4. Although there were exceptions at some age levels, the mating ratio and the cock number in the flock did not have any significant difference on the flock fertility. Furthermore, the result of the mating ratio in this experiment was also in agreement with those obtained from the experiments 2 and 3. The interaction of mating ratio by cock number (MR x CN) was found significant ($P<0.05$) at several age levels.

The significant effect of the MR x CN interaction implied that the effect of either factor depended on the condition of the other factor. For the flock of 1:8 mating ratio, it could be concluded

Table 4 Fertility percentage measured at 7 days of incubation in native chicken flocks of different mating ratios and with one or two cocks, (experiment 4).

Hen-cock age (w)	Mating ratio						Factor effect		
	1 : 8		1 : 12		1 : 16				
	One cock	Two cocks	One cock	Two cocks	One cock	Two cocks	MR	CN	MRxCN
	%								
56-36	95.00	59.02	75.00	64.04	75.42	85.65	NS	NS	NS
58-38	96.88	71.43	83.34	75.73	83.73	80.14	NS	NS	NS
60-40	94.12	65.00	93.93	77.84	98.57	93.84	NS	*	NS
62-42	93.75	62.16	81.20	71.91	97.92	97.22	*	*	*
64-44	97.06	75.42	87.21	89.48	88.10	89.98	NS	NS	NS
66-46	95.00	84.79	93.75	88.10	93.75	93.59	NS	NS	NS
68-48	81.25	77.17	82.26	78.24	100.00	89.66	NS	NS	NS
70-50	90.00	76.80	88.24	76.04	96.16	90.26	NS	NS	NS
Average	92.88	71.47	85.62	77.67	91.71	90.04	*	*	NS
Cocks of the different mating ratios were exchanged									
74-54	100.00	79.81	72.12	81.53	97.06	86.67	*	*	*
76-56	100.00	74.38	71.15	82.84	87.55	76.40	NS	NS	*
78-58	100.00	65.71	59.97	87.35	91.67	90.04	NS	NS	*
80-60	100.00	66.67	64.17	84.72	84.52	97.41	*	NS	*
82-62	99.00	68.75	60.72	78.82	75.95	86.26	NS	NS	NS
84-64	93.75	61.07	66.73	87.50	85.00	80.48	NS	NS	NS
86-66	86.50	61.58	53.69	86.21	82.94	84.93	NS	NS	NS
Average	97.04	68.28	64.08	84.14	86.38	86.03	*	*	*

Each figure, other than the average value, was the mean of 2 replications

MR = mating ratio, CN = cock number in the flock, MRxCN = interaction between the two factors

NS = nonsignificant difference ($P>0.05$)

* = significant difference ($P<0.05$)

that keeping two cocks in the flock would yield lower fertility than keeping only one cock. Fighting for hens between the two cocks was assumed to be the cause of lower fertility. A similar but with less degree of effect also occurred in the flock of 1:12 mating ratio. The experiment 4 revealed the good side of having more than one cock in a flock. It was found that when one of the two became weak or ill, the flock fertility could be sustained at a certain level by the remainder cock whereas there was no such compensation occurred in the flock with only one cock. For the flock of 1:16 mating ratio, no significant difference in fertility was observed between the flocks of one cock and two cocks. It was possible that the number of hens in the flock of 1:16 mating ratio was sufficient to maintain a peaceful mating activity without fighting between the two cocks and was also not too many for one cock to mate for the high fertility of the flock.

The results observed in this experiment indicated that the native chickens reared in a confined pen with two cocks would yield a negative effect on fertility if the mating ratio was rather low. The ill effect was decreased as the mating ratio was increased. At the mating ratio of 1:16, the negative effect of keeping two cocks in a flock was not observed. On the other hand, it helped securing the high fertility of the flock.

Hen age and cock number in the flock

The results of experiment 5 studying the effects of two factors, hen age and cock number in the flock, on the flock fertility are shown in Table 5. In general, the third-year hen showed a tendency of

Table 5 Fertility percentage measured at 7 days of incubation in native chicken flocks of old hens in their second or third year of age and with one or two cocks in the flock, (experiment 5).

Cock age (w)	Hen age				Factor effect		
	Second year (72-92 w.)		Third year (110-130 w.)				
	One cock	Two cocks	One cock	Two cocks	HA	CN	HAxCN
	%						
72	79.47	81.33	69.10	93.01	NS	NS	NS
74	81.57	82.09	66.67	92.83	*	*	*
76	86.76	79.35	65.37	92.15	NS	NS	*
78	84.16	71.51	43.27	84.17	NS	NS	*
80	84.62	77.14	54.91	82.80	NS	NS	NS
82	83.05	65.73	56.56	80.70	NS	NS	NS
84	59.21	75.16	68.18	55.56	NS	NS	NS
86	74.19	76.47	57.88	50.00	NS	NS	NS
88	94.45	84.13	63.40	86.91	NS	NS	*
90	81.49	82.84	42.86	86.97	*	*	*
92	67.86	87.10	53.33	88.09	NS	NS	NS
Average	79.71	78.44	58.32	81.20	*	*	*

Each figure, other than the average value, was the mean of 3 replications

Each flock (treatment combination) had the same mating ratio of 1 : 12

HA = hen age, CN = cock number in the flock, HAxCN = interaction between the two factors

NS = nonsignificant difference ($P>0.05$)

* = significant difference ($P<0.05$)

having lower fertility than the second-year hen with significant differences were observed only at some age levels. Regardless of hen age, housing one cock or two cocks in the flock virtually gave similar results. Only at some age levels that the difference was significant. However, when one confined only to the third-year hen, the positive effect of housing two cocks in the flock was more distinct. The average fertility rates during the 20 weeks of the experimental period, of the flocks of third-year hens with one and two cocks were 58.32% and 81.20%, respectively. Therefore, if could be concluded that housing two cocks could improve fertility of the flock of the older hens. The reason why the older hens usually had lower fertility rate was explained in such a way that the older hens had higher releasing rate of sperm from the sperm storage tubules (SST) located in the uterovaginal junction area into the fertilization site in the infundibulum of the ovidict. The higher sperm releasing rate brought about a rapid emptying of the SST and then a shorter period of rendering the fertilized eggs (Brillard, 1993). Another evidence indicated that eggs obtained from the older hens had less binding ability to spermatozoa than those obtained from the younger hens (Fairchild *et al*., 2000). The binding ability to spermatozoa of eggs was highly related to fertilizing ability of the eggs (Wishart, 1995). The evidence mentioned above suggested that the deterioration in fertility of the older hens was due mainly to the hens themselves. The higher benefit of housing two cocks over one cock could be clarified in such a way that the mating activity of either cock would psychologically stimulate the mating activity of the other cock which then help elevating mating frequency within the flock. The deterioration in fertility occurring in the old hens due to the rapid release of sperm from the SST would then be compensated. Therefore, when the cocks used in this experiment were still in their prime time which the frequent mating activity was likely, the improvement in fertility of the third-year hens with two cocks in the flock was understandable.

In conclusion, the native cocks aged from 9 months to 2 years had similar quality of semen. Mating capability of the cocks was proved important to determine the fertility of the breeder flock since the mating ratios of 1:8, 1:12, and 1:16 gave similar results in fertility as long as the cocks of the flocks were healthy and competent. Assuring high fertility by housing two cocks with hens reared in a confined pen would give a satisfactory result only when the number of hens was large enough to provide peaceful mating without fighting between the two cocks for the hens. The reverse was true if the number of hens was too small. Additionally, housing more than one cock in the breeder flock could help elevating fertility of the old hens.

LITERATURE CITED

Appleby, M.C., B.O. Hughes, and H.A. Elson. 1992. Poultry Production Systems : Behaviour, Management and Welfare. C.A.B. International, United Kingdom, 238 p.

Brillard, J.P. and G.R. McDaniel. 1986. Influence of spermatozoa numbers and insemination frequency on fertility in dwarf broiler breeder hens. Poult. Sci. 65 : 2330 - 2334.

Brillard, J.P. 1993. Sperm storage and transport following natural mating and artificial insemination. Poult. Sci. 72 : 923-928.

Chotesangasa, R. and N. Gongrattananun. 1999. Reproductive development and performance of male native chickens raised under natural day length and photoperiod of fifteen hours a day. Kasetsart J. (Nat. Sci.) 33 : 530-542.

Duncan, I.J.H., P.M. Hocking, and E. Seawright. 1990. Sexual behaviour and fertility in broiler breeder domestic fowl. Appl. Anim. Behav. Sci. 26 : 201 - 213.

Fairchild, B.D., V.L. Christensen, and L.G. Bagley. 2000. The relationship of insemination, sperm concentration and hen age on the number of holes hydrolyzed in the perivitelline membrane.

p. 51. *In* H.S. Siegel (ed.) Poultry Science Annual Meeting Abstracts. Poultry Science Association, Inc., Illinois.

Lake, P.E. 1984. The male in reproduction, pp. 381-405. *In* B.M. Freeman (ed.). Physiology and Biochemistry of the Domestic Fowl., Vol. 5 . Academic Press Inc., London.

North, M.O. and D.D. Bell. 1990. Commercial Chicken Production Mannual, 4th ed. Van Nostrand Reinhold, New York, 913 p.

Peterson, A.R., P. Lui, and W.D. Berry. 2000. Effects of male replacement on fertility in the broiler breeder flock, p. 111. *In* H.S. Siegel (ed.). Poultry Science Annual Meeting Abstracts. Poultry Science Association, Inc., Illinois.

Sexton, T.J. 1983. Maximizing the utilization of the male breeder : A review. Poult. Sci. 62 : 1700-1710.

Snecdecor, G.W. and W.G. Cochran. 1980. Statistical Methods. The Iowa State University Press, Ames, Iowa. 593p.

Wishart, G. J. 1995. New Approaches to evaluating male and female fertility, pp. 207 – 223. *In* M.R. Bakst and G.J. Wishart (eds.). Proceedings of First International Symposium on the Artificial Insemination of Poultry. The Poultry Science Association, Inc., Savoy, Illinois.

Wishart, G.J. and Y.I. Wilson. 1997. 4. Sperm motility and metabolism I. Visual scoring of motility using the hanging drop method, pp. 46-47. *In* M.R. Bakst and H.C. Cecil (eds.) Techniques for Semen Evaluation, Semen Storage, and Fertility Determination. The Poultry Science Association, Inc., Savoy, Illinois.

Received date : 14/05/01
Accepted date : 29/06/01

Kasetsart J. (Nat. Sci.) 35 : 132 - 138 (2001)

Some Biological Aspects of *Sternocera ruficornis* Saunder, 1866 in Dry Dipterocarp Forest at Sakaerat Environmental Research Station

Nantasak Pinkaew

ABSTRACT

The buprestid, *Sternocera ruficornis* in dry dipterocarp forest at Sakaerat Environmental Research Station was investigated. The life cycle from egg to adult was approximately two years in the soil. The adults were short-lived, about one month. Eggs were oval, yellowish and deposited singly in the soil one centimeter deep at the base of *Arundinaria pusilla* Cheval. & A. Camus. Each female laid 5-12 eggs with an incubation period of 57.32 ± 2.25 days. The larva was legless and with a reduced head that was sunken into laterally expanded prothorax. The abdomen was cylindrical. There were five larval instars with the first, second, third and fourth remaining in soil and feeding on the rhizome of *A. pusilla*. The fifth instar built an earthen cell or cocoon in the soil. The last quiescent larva required at least 14 to 15 months inside the earthen cell to transform to the pupal stage. Pupation took place in earthen cell. Adults emerged from the soil, after the heavy rain during the rainy season and were active in the daytime. After copulation, the female oviposit the egg in the soil.

Key words: biology, morphology, hostplants, *Sternocera ruficornis*

INTRODUCTION

The family Buprestidae, consists of a group of insects of about eight thousand species. The larvae are borers in plant tissue, some mine in leaves, other bore into twigs, branches, woody stems or beneath the bark of trees. The eggs are usually laid in crevices in the bark. The larvae tunnel under the bark, and some species eventually bore into the wood. Pupation takes place in the gallery. Development from egg to adult can require several years (Chunram, 1974)

In Thailand there are two species of buprestid belonging to genus *Sternocera.* They are red legged *Sternocera ruficornis* and green legged *Sternocera aequisignata* which can be found in all regions of Thailand (Ohmomo and Akiyama, 1972). Their colors are metallic dark green, copper green, bluish green, and golden green according to its beautiful and durable wings (Viravaidya and Annes, 1994). Since both species are edible and their wings are made into jewellery and other maketable objects, their numbers have decilned. Also the red legged metallic beetles are quite popular among insect collectors.

They live in big groups in dry dipterocarp forest around the North-East of Thailand where *Arundinaria pusilla* grows and can be found during the rainy season. The Sakaerat Environmental Research Station situated in Wangnamkeaw District, Nakornratchasima Province was the study site because *A. pusilla* was in abundance. It is necessary

Department of Entomology, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.

to investigate the biology of *Sternocera ruficornis* Saunder, 1866 in an attempt to successfully rear the insect in the future. The purpose of this study is to examine external morphological characteristics of adults, to study the life cycle, and to investigate the relationship of the host plant to the larval and adult stages.

MATERIALS AND METHODS

This study took place at both the Department of Entomology, Faculty of Agriculture, Kasetsart University and Sakaerat Environmental Research Station, Wangnamkaew, Nakornratchasima from January 1997 to December 1999.

Morphological study of adult *S. ruficornis*

Morphological features of the body and genitalia of 25 males and 25 females were investigated. A stereomicroscope and electronic digital caliber were used to make measurements.

Life cycle study

Ten cages of $25 \times 25 \times 25$ cm^3 in size were set up. Each cage contained one male and one female. Soil from dry dipterocarp forest was put on the bottom of the cage. The number of eggs laid each day, size of egg, egg period, and percentage of hatchability were recorded. After measuring the larval size, they were brought to rear on *A. pusilla*. The larval growth was measured every week under laboratory and field conditions. The larval instars were seperated by the mandibular width.

To study the pupa stage, the fifth instar larva was place in a 20 ml vial and the vials were placed in the styrafoam boxes with soil at the bottom. They were kept in the growth chamber at 25°C and 75% RH and the development time to adult stage was recorded.

Earthen cells from the forest were also studied. Pupae in earthen cells were buried in the soil with leaf and litter of *Shorea roxburgii*, and *Xylia xylocarpa* from the Dry Dipterocarp Forest. A cage (6x6x6 m^3) was placed over the buried pupae. After, the adults emerged, their feeding, mating, egg laying, natural enemies, and longevity were recorded. The results of lab study and nature study were compared.

Host plant study

There were two groups of host plants, one for larval stage and the other for adult stage. Larvae were found digging in the vicinity of *A. pusilla* and feeding on the roots of this tree. Adult host plants were determined by visual observations with the aid of binoculars. These observations were made on forest paths and forest fire prevention boarders.

RESULTS AND DISCUSSION

Morphological study of adult *S. ruficornis*

Head

Metallic green, with punctures, convex. Frons furrowed medially. Eyes oval, brown. Antenna red-brown, serrate, eleven-segmented. First segment clubbed, the second short, the fifth to the tenth serrate, last segment truncate. Labrum distinct, with setae along margin. Mandible black, stout, margin ridged. Maxillary palpus with four segments.

Thorax

Metallic green. Pronotum convex with deep punctures, anterior margin shorter than posterior margin, posterior angles pointed. Scutellum absent. Mesosternum short, broad. Metasternum long and board, anterior margin extends to mesosternum, arrow shape.

Wings

Elytra metallic green, slightly broader than pronotum, small punctures, humeral angle round, tip serrated, one yellow spot near humeral angle on each elytron. Hindwing membranous folded.

Legs

Red-brown, fore and middle coxa oval, hind coxa wide and expanded; trochanter small and short; femur swollen; tibia long, slender; tarsi five

segments last segment bearing a pair of claws.

Abdomen

Abdominal sternum metallic green, only five segments visible, sternite I and II fused, sternite VI with posterior margin emarginated in male, rounded in female.

Genitalia

Male. Aedeagus symmetrical, long, slender composed of penis and tegmen. Penis tubular, long and flat with acute apex, paramere length about three times of width, tapering toward both ends.

Female. Modified saccular type, a pair of hemisternites present posteriorly, each hemisternite bearing sensory process, stylus.

Body width, male 14.40±0.95 mm.female 15.89±1.43 mm. Body length, male 34.30±2.17 mm. female 38.02±3.37 mm.

The morphological characters of the male and the female were similar only that females were larger than males. Borer and Delong (1954) reported that female beetles were usually bigger than male beetles but sometimes in nature some females were found to be smaller than male due to their greater variation.

The genitalia (Figure 1) and abdominal sternite VI with posterior margin emarginated in male, rounded in female were used to separate male from female of *S. ruficornis* the same way as Chunram (1974) who used both characters to identify sexes in the genus *Chrysochroa* family Buprestidae.

The adult of *S. ruficornis* was quite similar to *Sternocera aequisignata* with some different morphological characters such as punctures on the elytra. The ones on elytra of *S. ruficornis* were deeper than those of *S. aequisignata.* Saunder (1866) reported that the colors of antennae and legs of *S. ruficornis* were red-brown whereas those of *S. aequisignata* were dark green hence the name, green legged metallic beetle. In nature, sometimes they were found together so it was difficult to separate the species.

Life cycle study (Figure 2)

Egg

Oval, yellow, smooth and no sculpturing, inner layer metallic brown. Two hard black spots on chorion. Diameter width 4.40±0.17 mm, length 6.40±0.29 mm.

The two hard black spots on eggshell were

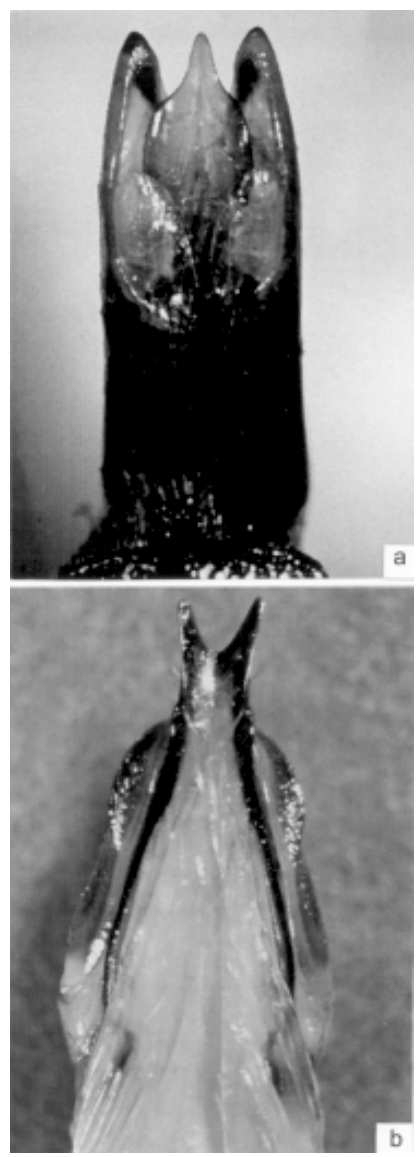


Figure 1 *Sternocera ruficornis* genitalia.
a. male
b. female

aeropyles. Beeson(1961) suggested that they were used by the embryo for respiration because they originated from the body wall of embryo through the surface of chorion and were important for development of embryo.

The eggs were usually laid singly in the soil at the base of *A. pusilla* about one centrimeter deep to prevent being preyed upon by ants. When first laid, the egg was pale yellow and later becoming dark yellow. Development from egg to larva was 57.32±2.25 days (Table 1). Females in cages could lay 5 eggs per day. On average females laid 5-12 eggs in her oviposition period and the average percentage of hatch was 92.29.

Larva

Yellow-brown with pubescence of brown hairs. Apodous larva. Small head sunk into the prothorax. No eyes, antenna 3 segments, mouthparts

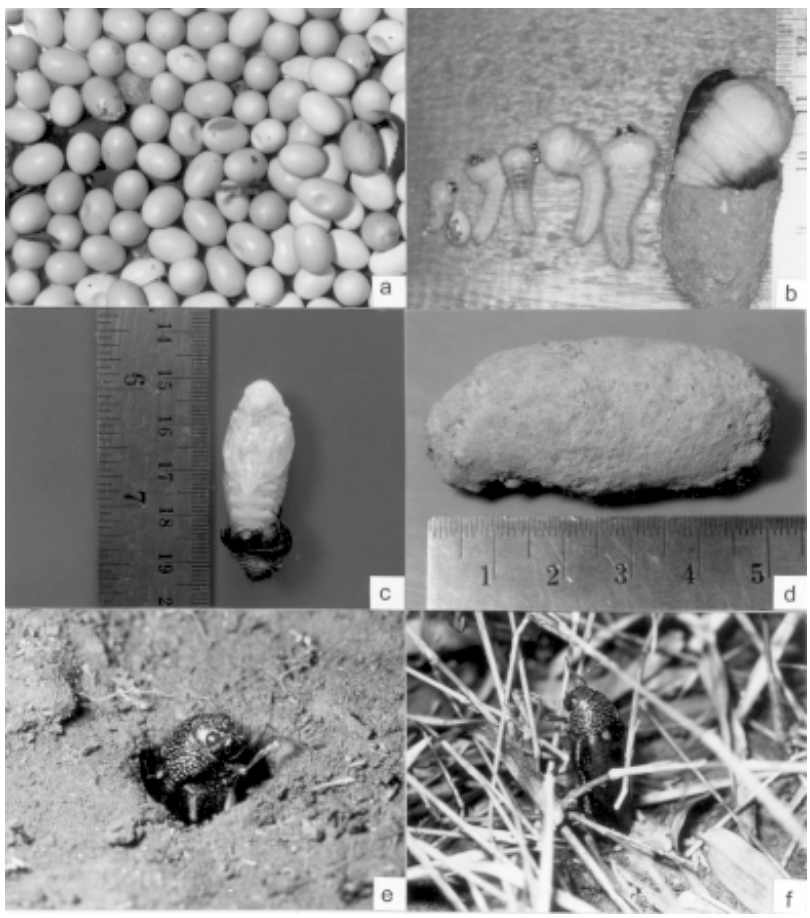


Figure 2 Life cycle of *Sternocera ruficornis.*

a. egg
b. larva in each stage
c. pupa
d. cocoon
e. adult emerge to soil surface
f. female lay egg

chewing type, large black mandibles. Prothorax expanded and flattened, mesothorax and metathorax small. Abdomen 9 segments, soft.

Since the larval characters of *S. ruficornis* were similar to those of beetles in subfamily Lamiini family Cerambycidae, it was hard to recognize the studied larva in nature as Beeson (1961) suggested that both larvae were encounted in same habitat and some morphological characters were the same, such as yellow color, legless, and cylindrical abdomen. Buprestid larvae are known as flat headed borers and cerambycids round head borers. There were five instars, each one was seperated by mandible width (Table 2), similarly as reported by Vincent (1983) for the Malaysian buprestid larva.

Upon hatching the first instar larva made its way out of the eggshell and moved directly to feed on root of *A. pusilla*. Newly hatched larvae were found from December to January. Every stage of larval development is underground. When the larva of each instar molted, they would build an earthen cell. At each molt, the width of mandible increased, especially at the last instar when the larva consumed a large amount of root tissue. Then it built cocoon from saliva mixed with soil. The cocoon was difficult to observe because its color blended with soil and was buried 10-15 cm from the base of *A. pusilla.* The larva remained in the cocoon for 12 – 13 months. The depth at which the cocoon was located in the soil provided protection from the damaging effects of forest fires.

Larval growth in nature was the same as in the laboratory. Development from larva to pupa was 565.90±8.75 days (Table 1).

Pupa

Exarate pupa, yellow, soft. Body width 16.29±1.70 mm, length 36.55±1.54 mm.

At first, the pupa was yellow and soft, then it gradually became green and hardened except for antennae and legs which turned red. The pupal stage took 75.73±4.48 days.

The adult emerged from the cocoon and

Table 1 Development time of *S. ruficornis* at each stage.

Stage	Period Avg ± S.D. (days)	Range (days)	Number of sample
Egg	57.32 ± 2.25	53 – 67	77
Larva	565.90 ± 8.75	553 – 572	10
Pupa	75.73 ± 4.48	70 – 83	15
Adult - male	22.40 ± 4.06	15 – 28	10
- female	24.20 ± 2.53	21 – 29	10

Table 2 Mandibular length and body length of *S. ruficornis* larva.

Larval instar	Mandible width Avg ± S.D. (mm.)	Body length Avg ± S.D. (mm.)	Number of sample
First instar	1.80 ± 0.10	13.17 ± 1.75	20
Second instar	2.38 ± 0.25	24.27 ± 3.61	10
Third instar	3.68 ± 0.30	37.37 ± 2.64	10
Fourth instar	4.80 ± 0.25	44.88 ± 2.79	10
Fifth instar	5.61 ± 0.28	54.21 ± 2.64	20

came out of the ground during rainy season due to the rain water seeping into the ground and softening the cocoon. The emergences holes on the soil were round and 16.42±1.00 mm in diameter. During the day, the beetles were active and this is when copulation normally occurred. The mating took place in the trees when the male approached the female. Female could mate more than once, and then would lay the eggs at the base of *A. pusilla*. Generally *S. ruficornis* laid eggs at the base of *A. pusilla* which was different from other buprestids in other genus such as genus *Chrysochroa* that laid eggs in crevices in the bark of host plants (Chunram, 1974). The larva of *Chrysochroa* hatched from egg on the bark, then burrowed into the stem and growth and development took place in the stem.

On the other hand, while in the cage females that are on a plant and allow the eggs to drop to the ground. Other behaviors were the same as found in the nature. The males life span was shorter than the females. In nature, *S. ruficornis* laid eggs at the base of *A. pusilla* but in cage it laid eggs while on the tree or cage wall. The change in behavior occurred because in the cage the adults had limited space to fly and often flew directly into the cage wall. This impact could cause stress resulting in an earlier oviposition.

Figure 3 Host plant of *Sternocera ruficornis.*
a. host plant of larva (*Arundinaria pusilla)*
b. host plant of adult (*Albizia odoratissima)*

Host plant study (Figure 3)

The larva was found only feeding on the rhizome of *A. pusilla* at a depth of 5-10 centimeters. The larva ate on the outside of the rhizome then burrowed into the rhizome. The rhizomes were one year or less. The larvae were not found feeding on the large roots of the tree. It is the ground covered plant in dry dipterocarp forest. There were enough food resource for all larvae.

Adults were found feeding on leaves of terminal branches. There were eleven host plants in the Dry Dipterocarp Forest (Table 3), representing 11 species from 6 families which is similar to the report by Viravaidya and Annes (1994). Hutacharern and Tubtim (1995) suggested that *S. ruficornis* was the forest insect pest causing damage to trees, but this study has showed that *S. ruficornis* larvae did not damage the trees like other larvae in Buprestidae therefore *A. pusilla* is not considered on economically important pest.

The natural enemies besides ants which ate their eggs on the ground included the spiders, and some species of birds.

CONCLUSION

This study provides information on the morphology, post-embryonic development and host plants of *S. ruficornis* in Dry Dipterocarp Forest. The results can be used as an aid in studying the life

Table 3 Host plants in different families of adult *S. ruficornis.*

Number	Scientific name	Family
1	*Shorea roxburgii* G. Don	Dipterocapaceae
2	*Shorea obtusa* Wall.	Dipterocapaceae
3	*Shorea siamensis* Miq.	Dipterocapaceae
4	*Sindora siamensis* Teijsm. Ex. Miq.	Caesalpiniaceae
5	*Erythrophleum succirubrum* Gagnep.	Caesalpiniaceae
6	*Dendrolobium triangulare* Schindl.	Caesalpiniaceae
7	*Albizia odoratissima* Benth.	Mimosaceae
8	*Xylia xylocarpa* Taub.	Mimosaceae
9	*Pterocarpus macrocarpus* Kurz.	Papilionaceae
10	*Irvingia malayana* Olive. ex. A. Benn	Ixonanthaceae
11	*Phyllanthus emblica* Linn.	Euphorbiaceae

cycle of *S. aequisignata* which is a closely related species that does not lived in Dry Dipterocarp Forest.

ACKNOWLEDGEMENT

The author would like to thank Prof. Gerald T. Baker from Mississippi State University for reviewing this manuscript.

LITERATURE CITED

Anderson, R.F. 1966. Forest and Shade Tree Entomology. John Wiley & Son Company, New York. 482 p.

Beeson, C.F.C. 1961. The Ecology and Control of Forest Insect of India and Niegbouring Countries. Government Printing Office, New Delhi. 767 p.

Bily, S. 1982. The Buprestid (Coleoptera) of Funnoscandia and Denmark. Scandinavian Science Press Ltd., Copenhaken. 109 p.

Borer, D.J. and D.M. Delong. 1954. An Introduction to the Study of Insects. Holt, Rinehart and Winston, Incs, New York. 819 p.

Chunram, S. 1974. Taxonomic Studies of Thai Metallic Wood Boring Beetle in Tribe *Chrysochroini* (Coleoptera: Buprestidae). Entomology and Zoology Division, Department of Agriculture, Bangkok. 85 p.

Hutacharern, C and N. Tubtim. 1995. Checklist of Forest Insects in Thailand. Office of Environmental Policy and Planning, Bangkok. 392 p.

Ohmomo, S and K. Akiyama, 1972. Jewel Beetles. Sansho Co., Ltd., Tokyo. 59 p.

Saunder, E. 1866. Catalogue of Buprestidae Collected by the Late M. Mouhot, in Siam with Description of a New Species. Transactions of the Entomological. Society of London 3(5) : 297-322.

Vincent, W.Y.T. 1983. Common Malaysian Beetles, Art Printing Work Sdn. Bhd., Kuala Lumper. 142 p.

Viravaidya, P. and P. Annes. 1994. Some Splendid Crafts. Bangkok Printing Co., Ltd., Bangkok. 187 p.

Received date : 2/04/01
Accepted date : 28/06/01

Kasetsart J. (Nat. Sci.) 35 : 139 - 143 (2001)

Histochemical Detection of Glycoconjugates in Colonic Epithelium of the Goat

Pakawadee Pongket[1], Suppalak Romrattanapun[2] , Maleewan Liumsiricharoen[1], Dollada Srisai[1] and Apinun Suprasert[1]

ABSTRACT

The distribution of glycoconjugates in the goat descending colon was studied by means of light microscopic histochemical methods. The staining procedures employed were horseradish peroxidase conjugated lectin, alcian blue (AB) pH 2.5, periodic acid-Schiff (PAS), ABpH 2.5-PAS, high iron diamine (HID), HID-ABpH 2.5. The lectins used in the present study were concanavalin A (ConA), *Ricinus communis* agglutinin I(RCA-I), *Solanum tuberosum* agglutinin (STL) and *Limulus polyphemus* agglutinin(LPA) . The result revealed that descending colonic epithelium of the goat contained acid sulfated glycoconjugates and neutral glycoconjugates with ∝-D-mannose, ∝-D-glucose, β-D-galactose, N-acetylglucosamine and sialic acid residues. The quantity of neutral glycoconjugates with ∝-D-mannose, ∝-D-glucose, β-D-galactose and N-acetylglucosamine increased from mucosal crypt up to mucosal surface of the epithelium, while sialic acid residues and sulfated glycoconjugates decreased from mucosal crypt to mucosal surface.

Key words: histochemistry, glycoconjugate, colon, goat

INTRODUCTION

One of the main function of the gastrointestinal epithelium is the production of the protective and lubricant mucus. Such mucus has been shown to be biochemically and a heterologous entity being composed of high molecular weight glycoconjugates. Previous histochemical studies have revealed that the secretory glycoconjugates of the gastrointestinal tract are varied in property from species to species, in addition to regional variations within a particular species (Sheahan and Jervis, 1976 ; Freeman *et al*.,1980). In different mammals, furthermore, a series of histology and biochemistry has so far been made on glycoconjugates involved in the colonic epithelium and a wealth of information is available (Sheahan and Jervis, 1976; Thomopolous *et al*., 1983). However, glycoconjugates histochemical studies of the goat epithelium have been reported only with conventional method. The present study was designed to clarify glycoconjugates components in the goat colon by using currently available methods of lectins and correlated procedures.

MATERIALS AND METHODS

Goats of different ages and sexes were sacrified by exsanquination under deep anesthesia. Tissue material from middle part of descending colon was dissected out and fixed immediately by immersion in 10% formaline containing 2% calcium acetate for 24 hrs. at 4°C After that the tissue

1 Department of Anatomy, Faculty of Veterinary Medicine, Kasetsart University, Bangkok 10900, Thailand
2 Department of Zoology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

samples were processed routinely for embedding in paraplast and sectioned serially at 3 μm. in thickness. The tissue sections were then subjected to stain with the following staining procedures.

Conventional staining procedures

1. Haematoxylin and eosin for the general observation of histological structures.

2. AB pH 2.5 for acidic glycoconjugates (Spicer, 1960)

3. Periodic acid-Schiff (PAS) for vicinal diol containing glycoconjugates (Pearse, 1968)

4. AB pH 2.5-PAS for demonstrating of acidic and neutral glycoconjugates (Spicer *et al*., 1967)

5. High iron diamine (HID) for sulfated glycoconjugates (Lev and Spicer, 1964)

6. HID-AB pH 2.5 for demonstrating of sulfated glycoconjugates and carboxylated glycoconjugates (Spicer *et al*., 1967)

Lectin staining procedures

Briefly, deparaffinized sections were treated with 1% bovine serum albumin(BSA) in 10 mM phosphate buffer saline (PBS) pH 7.4 and then incubated with biotinyl lectins (25 ug/ml, Vector Lab Inc. USA) in 0.1% BSA-PBS for 30 min. After rinsing with PBS, the sections were incubated in avidin-biotinyl peroxidase complex (ABC Vector lab Inc.USA) for 30 min. rinsed with distilled water, dehydrated and mounted.

The lectins use in this study,along with their reported carbohydrate binding specificities are listed in table 1.

Control experiments

For lectin staining, control procedures were performed : Tissue sections were preincubated in appropriated hapten sugars for each lectin and then incubated in lectin solutions containing hapten sugars, non specific staining was also checked by incubation in the ABC and DAB-H_2O_2 solutions.

RESULTS

The results of the stainings are summarized in Table 2

The mucosal surface and crypts of the goat descending colon are lined with simple columnar epithelium. The epithelium in the crypts consists mainly of goblet cells whereas absorptive columnar cells and some goblet cells occur in the upper half of the crypts and the mucosal surface. When the epithelium was reacted with PAS, mucous granules of goblet cells and striated border of columnar cells stained moderately at lower crypts and stained intensely at the mucosal surface. By AB pH 2.5 staining, mucous granules of goblet cells and striated border of columnar cells were moderate to deep blue. When the colonic epithelium was reacted with AB pH 2.5-PAS,both structures at lower crypts were blue or red while those in mucosal surface were deep purple. (Fig. 1) The HID procedure resulted in strong positive reaction in the two

Table 1 List of lectins employed histochemical studies and their binding specificities according to references cited.

Lectins binding	Specificities	References
Concanavalin A	α-D-Man., α-D-glu.	Kiernan, 1975
Ricinus communis RCA-I	β-D-gal	Yamada and Shimizu, 1977
Solanum tuberosum STL	β-D-GlcNAc	Goldstein and Hayes,1978
Limulus polyphemus LPA	NeuAc	Goldstein and Hayes, 1978

structures. When stained with the HID-ABpH2.5 sequence, the surface goblet cells and crypt goblet cells turned to a mixture of black and blue coloration.

The mucous granules of goblet cells and striated border of columnar cells in the colonic epithelium reacted strongly with ConA (Fig. 2), RCA-I and STL. Both the Con A and RCA-I staining of goblet cells and striated border of columnar cells increased in intensity with the upward cell migration along the crypts. Only mucous granules of goblet cells and striated border of columnar cells at the lower crypts showed affinity against LPA while those at mucosal surface showed negative stain.

DISCUSSION

The precise identification of glycoconjugates and their localization when correlated with changes in morphogenesis, tumorigenesis and physiological function may help us to understand valuable agents for the demonstration of various saccharide residues. Using biotinyl lectins we examined the distribution of glycoconjugates in the goat descending colon by light microscopic histochemical methods.

In the present study we find the different distribution of glycoconjugates between the mucosal crypt and mucosa surface of the goat colon. PAS is believed to stain sugars with vicinal diol group and neutral charges including β-D-galactose, α-D-mannose, α-D-glucose and N-acetyl derivatives of the hexosamines while AB pH 2.5 stains the carboxyl groups of sialic glyconjugates or other glycoconjugates with a strong negative charge (Spicer *et al*., 1967). Pattern of staining for acid and neutral glycoconjugates with PAS at the mucosal surface correlate well with our finding that lectins which recognize these residues(ConA,RCA-I) stain this region moderately to poorly. Alcian blue stains

Table 2 Histochemical reactions of glycoconjugates in the goat colonic epithelium.

Staining procedures	Mucosal crypt		Mucosal surface	
	Crypt goblet cells	Striated border of columnar cells	Surface goblet cells	Striated border of columnar cells
AB pH 2.5	2-3 B	2B	3 B	1-2B
PAS	2M	1-2M	3-4 M	2-3M
ABpH 2.5-PAS	2BM	2 BM	3MB	2BM
HID	3BL	2 BL	2BL	3BL
HID-ABpH2.5	3BLB	2 BLB	2BBL	2B
ConA	1-2 Br	2 Br	3 Br	1Br
RCA-I	1-2 Br	2 Br	3Br	2-3Br
STL	2 Br	2 Br	3Br	3Br
LPA	1-2 Br	1-2 Br	0	0

Key to symbols in table

B : AB positive , M : PAS positive , BL : HID positive , Br : Lectin positive
MB : A mixture of PAS and AB-positive glycoconjugates with PAS positive glycoconjugates predominanting.
BM : A mixture of AB-and PAS-positive glycoconjugates with AB positive glycoconjugates predominanting
BLB : A mixture of HID and AB-positive glycoconjugates with HID positive glycoconjugates predominanting
BBL : A mixture of AB and HID-positive glycoconjugates with AB positive glycoconjugates predominanting
0 : Negative reaction
1-3 : Numerical values correspond to increasing intensity of staining

glycoconjugates throughout the colon suggesting the presence of acid mucins in all of these segments. The absence of staining by LPA in the mucosal surface suggests that the acid mucins recognized by AB are propbably not sialomucins and may represent sulfated residues or proteoglycans.

In view of the staining specificities of the AB pH 2.5, PAS, AB pH.2.5-PAS, HID, HID-ABpH 2.5 (Spicer *et al*.,1967) the present results can be comprehended consistently in the goblet cell colonic epithelium, glycoconjugates are provided primarily with acid sulfated and vicinal diol groupings. However the glycoconjugates in the villus epithelium appear to be higher in acidity as compared with those in the crypt epithelium.

If the staining mechanisms underlying the positive staining reaction of Con A,RCA-I and STL at the mucous granules of goblet cells and striated

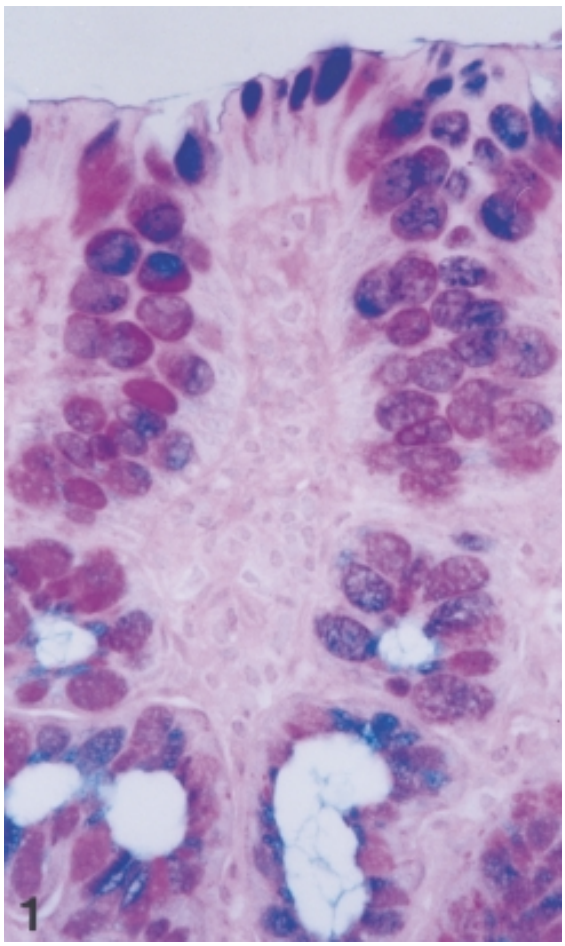


Figure 1 The dual staining with ABpH2.5-PAS result in blue or red at crypt goblet cells but stain deep purple or primarily blue at surface goblet cells. X260

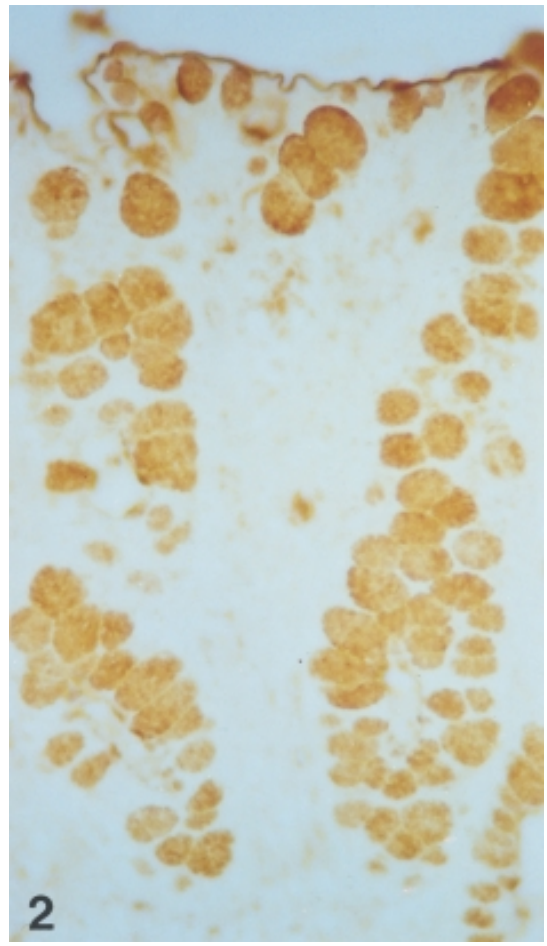


Figure 2 Mucous granules of goblet cells exhibit strong to moderate positive reaction. However the positive reactions of mucous granules of goblet cells at the surface are stronger in intensity than those at the crypt Con A. staining. X260

border of columnar cells are taken into consideration; the present conclusion can be made : carbohydrates in the epithelial cells of the goat colon are glycoconjugates with ∝-D-glucose, ∝-D-mannose, β-D-galactose and N-acetylglucosamine. In addition, glycoconjugates with terminal sialic acid residues were also presence in the goblet cells mucous granules at mucosal crypt as judged from their positive staining with LPA.

In a similar way to that described in many mammalian species, the epithelial cells of the goat colon contain both sialomucin and sulfomucins.Such acid glycoconjugates may play an important role in resisting the invasion of potential pathogens (Schauer, 1982). Sialic acid and sulfated groups are also believed to play an essential role of lubrication and protection of digestive tract (Werner *et al*., 1982). Functional activities of other sugar residues of glycoconjugates are still unknown.

LITERATURE CITED

Freeman, H.J., R. Lotan, and Y.S. Kim. 1980. Application of lectin for detection of goblet cell glycoconjugates differences in proximal and distal colon of the rat. Lab. Invest. 42 : 405-412.

Goldstein, I.J., and C.E. Hayes. 1978. The lectins : Carbohydrate-binding proteins of plants and Animal. Adv. Carbohyd. Chem. Biochem. 35 : 127-340.

Kiernan,J.A.1975. Localization of α-D-glucosyl and α-D-mannosyl groups mucosubstances with concanavalin A and horseradish peroxidase.Histochemistry 44 : 39-45.

Lev, R., and S.S. Spicer. 1964. Specific staining of sulphate groups with alcian blue at low pH. J. Histochem. Cytochem. 12 : 309.

Pearse, A.G.E. 1968. The periodic acid-Schiff technique, pp. 659-660. *In* Histochemistry. Theorectical and applied. Vol 1. 3rd ed. J and A Churchil, Edinburgh.

Schauer, R. 1982. Chemistry, metabolism and biological functions of sialic acid. Adv. Carbohyd. Chem. Biochem. 40 : 131-234.

Sheahan, D.G. and H.R. Jervis. 1976. Comparative histochemistry of gastrointestinal mucosubstances. Am. J. Anat. 146 : 103-132.

Spicer, S.S. 1960. A correlative study of the histochemical properties of rodent acid mucopoly-saccharides. J. Histochem. Cytochem. 8 : 18-36.

Spicer, S.S., R.G. Horn, and T.J. Leppi. 1967. Histochemistry of connective tisssue mucopoly saccharides, pp. 251-303. *In* B.M. Wagner and D.E. Smith (eds.). The connective tissue. Williams & Wilkins, Baltimor.

Thomopolous, G.N., B.A. Schulte, and S.S. Spicer. 1983. Light and electron microscopic cytochemistry of glycoconjugates in the rectosigmoid colonic epithelium of the mouse and rat. Am. J. Anat. 168 : 239-256.

Yamada, K. and S. Shimizu. 1977. The histochemistry of galactose residues of complex carbohydrates as studied by peroxidase-labeled Ricinus communis agglutinin. Histochem. 53 : 143-156.

Werner, R., K. Eckart, B. Christian, and G. Wolfgang. 1982. Biological significance of sialic acid, pp. 263-305. *In* R. Schauer (ed.) Sialic acid : Chemistry, Metabolism and Function, Cell Biology Monograph. Vol 10 Springer Verlag, Vien, New York.

Received date : 20/02/01
Accepted date : 29/06/01

Kasetsart J. (Nat. Sci.) 35 : 144 - 148 (2001)

Effect of Age on Serum Cholesterol and Triglyceride Levels in the Experimental Rats

Wanpen Mesomya, Duangchan Hengsawadi, Yaovadee Cuptapun, Pongsri Jittanoonta and Vipaporn Na Thalang

ABSTRACT

The feeding of stock diet to different ages, four, eight and twelve months of, rats (Sprague – Dawley) showed that serum cholesterol level of 19 female rats was statistically significant difference at 5 % level among three ages 85.42, 112.11, 122.21 mg/dl in four, eight and twelve months rats respectively. In 14 male rats, the results showed that serum cholesterol level was statistically significant difference between four and eight monthed rats whereas there was no significant difference between eight and twelve monthed rats. The result of serum triglyceride level was fluctuate in both male and female rats among four, eigth, twelve months of age. The results of this study indicated that age of the experimental rats may effect on the serum cholesterol level especially in the female rat.

Key words: cholesterol, triglyceride, serum, age, rats

INTRODUCTION

Edible fats are important food components that enhance palatability by providing texture and enhancing flavour. They also provide essential fatty acids and fat-soluble vitamins. We enjoy eating foods containing fat, but there is a negative side; excessive consumption may not be good for health. The question is what are the right amounts and types of fat we should use and eat ? The average consumer is somewhat aware of the relationship between dietary fat and health. Despite this, there is still a great deal of confusion formulate hypotheses and express themselves in terms of probabilities. Journalists look for news, which is not always balanced. Governments and industry translate complex research findings into information that can be used make policies and to educate the consumer. A balance must be achieved between complicated scientific communication and information that can be more easily communicated and understood by the public.

More than 20 years ago the research groups of Keys and Hegsted in the United States independently published the results of their many experiments on healthy men eating a wide variety of dietary fats. They found that saturated fat raised total blood cholesterol levels compared with a nutritionally equivalent carbohydrate intake. It had been thought since 1956 that polyunsaturated fats (linoleic acid rich) reduced blood cholesterol, whereas monounsaturated fats (oleic acid rich) had no effect. Although techniques to measure the levels of LDL cholesterol and HDL cholesterol were not well developed at that time, it was concluded that these changes in total cholesterol appeared to be the result of changes in LDL cholesterol. These findings formed the basis for

Institute of Food Research and Product Development, Kasetsart University, Bangkok 10900, Thailand.

dietary guidelines and recommendations in the Western industrialised countries in the 1970s and 1980s. (Truswell, 1995).

Dietary principles aimed at reducing the risk of coronary heart disease

- Total fat intake should be equal to or less than 30 % of total energy intake.
- Saturated fat intake should be less than 10 % of total energy intake.
- Polyunsaturated fat should constitute up to10 % of total energy intake (of this, 10 - 20 %, i.e., 1-2 % of total energy, should be n-3 and the rest n-6), and it should contain adequate vitamin E, at least 0.6 mg/g polyunsaturated fat. The balance of dietary fat should be monounsaturated.
- Dietary cholesterol intake should be less than 300 mg/day.
- Dietary fiber (including soluble fiber) intake should be 25-30 g/day.
- Sodium intake should be less than 100 mg (6 g salt) / day.
- Energy intake should be achieve and maintain desirable body weight.
- Eat plenty of fish (including fatty fish), vegetables, fruits and whole-grain cereal.

Plus, exercise regularly and do not smoke

Most dietary fats and oils are triacylglycerols (also called triglycerides), which consist of three fatty acids attached to a glycerol molecule. Their impact on coronary heart disease (CHD) risk depends to a large extent on the types of fatty acids they contain. Fatty acids are differred in their chain length (number of carbon atoms) and degree of saturation (number of double bonds in the carbon chain) and they can effect blood cholesterol levels and, hence, risk for CHD (Kris–Etherton, 1995).

All dietary fats and oils consist of mixtures of three categories of fatty acids:

- saturated – no double bonds.
- monounsaturated – one double bond.
- polyunsaturated – more than one double bond.

The predominant saturated fatty acids in food include lauric acid, myristic acid, palmitic acid, and stearic acid. With the exception of stearic acid, which is believed to have no effect on blood cholesterol levels. These saturated fatty acids raise blood cholesterol and low-density lipoprotein (LDL) cholesterol levels (Grundy and Denke, 1990).

Monounsaturated fat was once believed to have a neutral effect on blood cholesterol levels but recently epidemiologic and clinical intervention studies indicated that prudent diets rich in oleic acid, the predominant monounsaturated fatty acid, lower blood total cholesterol and LDL cholesterol levels when substituted for diets rich in saturated fatty acids (Loscalzo and Stunchi, 1991).

Polyunsaturated fatty acids may be classified by the location of the first double bond from the methyl (CH_3) end. Omega-6 fatty acids include the essential fatty acid linoleic acid; omega-3 fatty acids include the essential fatty acid α - linolenic acid. When substituted for saturated fatty acids in the diet, polyunsaturated fatty acids-especially linoleic acid-actively lower blood total cholesterol and LDL cholesterol levels (Kris-Etherton et al., 1990).

Public health recommendations for the treatment of high blood cholesterol levels include decreasing the consumption of saturated fatty acids total fat. Reducing total fat and/or emphasizing fats that are rich sources of monounsaturated and polyunsaturated fatty acids facilitates decreasing the amount of saturated fat in the diet. Americans consume on average 12-14 % of calories from saturated fatty acids and 34-36 % of calories from total fat (Wright et al., 1991; Tippett and Goldman, 1994; Daily dietary fat and total food-energy intakes-third National Health and Nutrition Examination Survey, 1994). Dietary recommendations advise that no more than 30 % of calories come from all fats and that less than 10 % of calories come from saturated fatty acids.

From reviewing literature, the research

papers about effect of age on blood lipid levels is very rare, so in this paper we have tried to present information about effect of age on serum lipid levels in the experimental rats.

MATERIALS AND METHODS

Animals : Nineteen female and fourteen male Sprague – Dawley rats of the following ages were used : four, eight and twelve months, the corresponding mean weights of the females in these age groups were 265, 307 and 332 g ; and 409, 485 and 510 g, respectively for the males. The animals had free access to water and experimental diet having the following composition and chemical analysis of experimental diet according to the deliver shown in Table 1 and Table 2.

The studies were carried out in the mornings. The animals were anesthetized with ether and blood was collected by heart puncture. Serum was removed after centrifuging with 2500 rpm and was analysed for cholesterol and triglyceride levels.

Statistical analysis

Datas were statistically analysed using Analysis of Variance (ANOVA) and Duncan's New Multiple Range Test. A value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Table 3 showed that the level of cholesterol in serum was statistical significantly increased from four to eight and from eight to twelve months in female rats. The result in Table 4 showed that serum cholesterol levels significantly changed from four to eight months and remained unchanged from eight to twelve months. In both of female and male young rats (four months) the cholesterol level was thus below 100 mg/dl where as serum cholesterol level of the older rats (eight and twelve months) were higher than 100 mg/dl.

The level of serum triglyceride both in female and male rats were fluctuate among four, eight, twelve months of age.

Table 1 Composition of experimental diet.

Composition	g/100 g
Corn meal	24
Fish meal	20
Soybean extract	12
Wheat bran	15
Rice flour	20
Mineral mixture	3
Vitamin mixture	2
Sugar	2
Vegetable oil	2

Table 2 Chemical analysis of experimental diet.

Composition	g/100 g	mg/g
Protein	22.86	
Fat	12.95	
Moisture	12.33	
Ash	9.07	
Dietary fiber	15.45	
Cholesterol		0.96

The serum triglycerides showed a different course with age. They increased slightly from four to eight months and strikingly from a value of 113.86 mg/dl on the eight months to 88.43 mg/dl at the age of twelve months in male rat. In female rats, serum triglycerides levels showed significantly increased from four months to eight months and significantly decreased at the age of twelve months.

In agreement with the previous findings by Carlson et al (1968), the level of cholesterol in plasma remained essentially unchanged from 1 to 4 months in order to increase significantly from 4 to 9 and from 9 to 18 months. In young rats (1 and 4 months) the cholesterol level was there around 100 my/dl which a level of around 300 was reached in the oldest (18 months).

Table 3 Means of serum cholesterol, triglyceride levels and body weight in nineteen female rats of the following ages : four, eight and twelve months.

Age (month)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	Body weight (g)
4	85.42[a]	88.58[a]	264.79
8	112.11[b]	127.89[c]	307.00
12	122.21[c]	106.74[b]	332.53

Values in a column with different superscripts are significant different, $P < 0.05$.

Table 4 Means of serum cholesterol, triglyceride levels and body weight in fourteen male rats of the following ages : four, eight and twelve months.

Age (month)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	Body weight (g)
4	79.79[a]	108.29[a]	408.64
8	156.43[b]	113.86[b]	485.14
12	157.71[b]	88.43[a]	510.07

Values in a column with different superscripts are significant different, $p < 0.05$.

The plasma triglycerides showed a different course with age. They increased slightly from 1 to 4 months and strikingly from a level of 0.8 m mole/l on the fourth month to 2.5 m mole/l at the age of 9 months and then they remained constant.

In conclusion this study showed that the level of cholesterol in serum was significantly increased from four to eight and from eight to twelve months in female rats. In male rats, serum cholesterol levels significantly changed from four to eight and remained unchanged from eight to twelve months. The results of this experiment indicate that age of experimental rats may effect on the serum cholesterol level especially in the female rat.

ACKNOWLEDGMENTS

The authors are deeply grateful to Institute of Food Research and Product Development, Kasetsart University, Bangkok, Thailand in supporting both of fund for this research project and for attending in 52nd International Congress and Expo. Finally we would like to sincerely thanks Miss Supamas Chotmethepirom and Associated Professor Vorasak Patchimasiri, Department of Physiology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand for kindly co-operation and supplying the instruments.

LITERATURE CITED

Carlson L.A., S.O. Fröberg and E.R. Nye. 1968. Effect of age on blood and tissue lipid levels in the male rat. Gerontologia 14 : 65–79.

Daily dietary fat and total food – energy intakes – third National Health and Nutrition Examination Survey. 1994. Phase 1, 1988-91. Morb Mortal Wkly Rep. 43 (7) : 116-117, 123 – 125.

Grundy S.M., M.A. Denke. 1990. Dietary influences on serum lipids and lipoprotiens. J. Lipid Res.

31 : 1149 – 1172.

Kris – Etherton P.M. and R.J. Nicolosi. 1995. Trans Fatty Acids and Coronary Heart Disease Risk. ILSI Press, Washington, D.C. 24 p.

Kris – Etherton P.M., P. Volz – Clarke, and K. Clark. 1990. Cardiovascular Disease : nutrition for prevention and treatment. Chicago : American Dietetic Association. 349 p.

Loscalzo J. and A.F. Stunchi. 1991. Effect of dietary fat saturation on low density lipoprotein metabolism. *In* Nelson G.J. (ed), Health Effects of Dietary Fatty Acids. Champaign, IL : American Oil Chemists Society, chap 7. 274 p.

Tippett K.S. and J.D. Goldman. 1994. Diets more healthful, but still fall short of dietary guidelines. Food Rev. Jan – April : 8 -14.

Truswell A.S. 1995. Dietary Fat : Some Aspects of Nutrition and Health and Product Development. LISI Europe, Avenue E. Mounier 83, Box 6, B 1200 Brussels, Belgium. 37 p.

Wright H.S., H.A. Guthrie, M.Q. Wang, and V. Bernardo. 1991. The 1987-88. Nationwide Food Consumption Survey: an update on the nutrient intake of respondents. Nutr. Today. May – June : 21 – 27.

Received date : 29/03/01
Accepted date : 29/06/01

Kasetsart J. (Nat. Sci.) 35 : 149 - 156 (2001)

Hematozoa of Snakes in Queen Saovabha Memorial Institute

Jarernsak Salakij [1], Chaleow Salakij[1], Nual-Anong Narkkong[2], Lawan Chanhome[3], Nirachra Rochanapat[1] and Piyawan Suthunmapinunta[1]

ABSTRACT

During December 1997 to March 2001, 275 blood samples of snakes from eighteen species of four families, including Pythonidae, Colubridae, Elapidae and Viperidae, at Queen Saovabha Memorial Institute were examined for blood parasites. 111 samples (40.4%) were positive for hematozoa infections. The single infection (38.2%) were following genera : *Hepatozoon* (32.4%), *Haemogregarina* (4.0%) and trypanosome (1.8%). The incidence rates of mixed infections between *Hepatozoon* and *Haemogregarina* and between *Hepatozoon* and trypanosome were 0.4% and 1.8% respectively. *Hepatozoon* infections were found in mangrove snake (*Boiga dendrophila melanota)*, mask-faced water snake *(Homalopsis buccata)*, rainbow water snake *(Enhydris enhydris)*, copper head racer snake *(Elaphe radiata)*, common rat snake (*Ptyas mucosus*), Siamese spitting cobra *(Naja siamensis)*, golden spitting cobra *(Naja sumatrana)*, king cobra *(Ophiophagus hannah)*, banded krait *(Bungarus fasciatus)*, Malayan krait (*Bungarus candidus*), mangrove pit viper *(Trimeresurus purpureomaculatus)* and Siamese russell's viper *(Daboia russellii siamensis)*. There were three types of gamont of *Hepatozoon* sp., including small gamont (3.0×10.9 μm, n=120), medium gamont (3.3×14.8 μm, n=60) and the large gamont (3.9×15.0 μm, n=140). Haemogregarina infections were found in Burmese python *(Python molurus bivittatus)*, mangrove snake, mask-faced water snake and rainbow water snake. The gamonts of *Haemogregarina* sp. were very large (7.6×15.2 μm, n=40). Trypanosomes were found only in mask-faced water snake and rainbow water snake. They were large flagelate hematozoa with 10-20 μm body width (14.1 ± 3.0 μm, n=120).

Key words: *Haemogregarina*, hematozoa, *Hepatozoon*, Queen Saovabha Memorial Institute, snake, trypanosome

INTRODUCTION

There are 163 species of snake in Thailand. The 48 venomous species of snake are in the family Elapidae and Viperidae (Jintakune and Chanhome, 1996). The number of poisonous snakes for antiserum production in Queen Saovabha Memorial Institute (QSMI) was decreasing. Establishing of snake farm in QSMI was started in 1994. Hematological examinations were performed in these snakes for general health examination.

Hematological examination has been used in the lower vertebrates during the last 20 years (Frye, 1991). There were a number of hemoparasites in reptiles (Campbell, 1996). The major hematozoas found in reptiles were trypanosomes, *Plasmodium* and haemogregarines. The *Hepatozoon*, *Haemogregarina* and *Karyolysus* were difficult to

1 Faculty of Veterinary Medicine, Kasetsart University, Kamphaengsaen, Nakorn Pathom 73140, Thailand.
2 Center of Scientific Equipment and Laboratory, Kasetsart University, Bangkok 10900, Thailand.
3 Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok 10330, Thailand.

differentiate when found in the blood so were grouped as haemogregarines. In general, *Haemogregarina* is found in aquatic reptiles, *Hepatozoon* is occured in terrestial snakes and *Karyolysus* is existed in old world lizards and possibly tree snakes (Campbell, 1996).

In this study, the percentage of hematozoa infection in snakes from QSMI and morphological features of the hematozoa are described.

MATERIALS AND METHODS

During December 1997 to March 2001, 275 blood samples of snakes from eighteen species in Queen Saovabha Memorial Institute were collected from the ventral caudal vein using a 22-gauge needle. Some snakes were captured from different locations in Thailand, some were bred in the Institute. Blood samples were anticoagulated with ethylene diaminetetraacetic acid (EDTA) except for samples from copper head racer snakes were anticoagulated with heparin sodium because EDTA causes hemolysis in this snake. Blood smears were prepared immediately and air dried. Then they were fixed in absolute methanol and stained with Wright-Giemsa for determination of differential leukocyte counts and hematozoa examination. Buffy coat examination for moving blood parasite was done on microhematocrit tubes prepared for hematocrit determination under 100X magnification (Schalm *et al*., 1975)

Grading of *Hepatozoon* sp. infection was quantitated by the number of infected erythrocytes per 200X microscope field. The degree of infection was shown as 1+ to 4+, in which 1+ is equal to 1 to 20 infected erythrocytes; whereas 2+, 3+ and 4+ are equal to 21 to 40; 41 to 60; and more than 60 infected erythrocytes per field, respectively. Hematozoa body width and length were measured with eyepiece micrometer of Olympus®, microscope Bx50 (Japan) at 1,000X field. Means and other statistic data were generated for each hematozoa.

RESULTS

There were two kinds of blood parasites moving in the plasma above the buffy coat, including trypanosome and *Hepatozoon* sp. According to blood smear examination, there were three hematozoas in the snakes; *Hepatozoon* sp., *Haemogregarina* sp. and trypanosome. There were no hematozoa in the 5 studied species including reticulated python, fishing salt-water snake, monocled cobra, Malayan pit viper and white-lipped green pit viper. The percentage of hematozoa infection in the snakes is summerized in Table 1. The highest rate of infection was seen in the banded krait. The grading of *Hepatozoon* sp. infected erythrocytes in three species of snake with high percentage of infection is shown in Table 2. The highest rate of parasitemia was seen in the mangrove snake.

Hepatozoon sp.

Among the twelve species of snake which were infected with *Hepatozoon* sp., there were three distinct forms of intraerythrocyte gamont (Table 3). The small gamonts were averaged 3.0 ± 0.3 by 10.9 ± 0.6 μm. They were oval, slightly curved with round ends; round to oval band nucleus, nearly centrally located. Some hepatozoas had blue inclusions and small granules in the cytoplasm (Figure 1). The parasitized erythrocytes were slightly enlarged and had some halo-like appearances were seen in the cytoplasm. The small gamonts were found in seven species of snakes, including mask-face water snake, copper head racer snake, common rat snake (Figure 2), Siamese spitting cobra, king cobra (Figure 3) banded krait and Malayan krait. In heavier infections, two gamonts may infect the same erythrocyte (Figure 4).

The medium gamonts were averaged 3.3 ± 0.6 by 14.8 ± 0.9 μm. Gamonts were slender, stained dense basophilia at the periphery with bipolar stretching (Figure 5). The infected erythrocyte

were intact and with no hemolysis. The medium gamonts were found in mask-faced water snakes and rainbow water snakes.

The large gamonts, averaged 3.9 ± 0.6 by 15.0 ± 1.1 μm, were found in mangrove snakes, golden spitting cobras, king cobras, mangrove pit vipers (Figure 6) and common rat snakes. Gamonts were oval, slightly curve with smooth round ends

Table 1 Percentage of hematozoa infection (number) in 257 snakes.

Snake species (common name)	Number	*Hepatozoon*	*Haemogregarina*	Trypanosome
Python reticulatus (Reticulated python)	16	0	0	0
Python molurus bivittatus (Burmese python)	14	0	14.3 (2)	0
Boiga dendrophila melanota (Mangrove snake)	17	72.3 (34)	2.1 (1)[1]	0
Homalopsis buccata (Mask-faced water snake)	47	10.6 (5)	17.0 (8)	14.9 (7)[2]
Enhydris enhydris (Rainbow water snake)	13	7.7 (1)	7.7 (1)	23.1 (3)[1]
Elaphe radiata (Copper head racer snake)	12	8.3 (1)	0	0
Cerberus rhynchops (Fishing salt-water snake)	1	0	0	0
Ptyas mucosus (Common rat snake)	5	80.0 (4)	0	0
Naja kaouthia (Monocled cobra)	17	0	0	0
Naja siamensis (Siamese spitting cobra)	12	41.5 (5)	0	0
Naja sumatrana (Golden spitting cobra)	6	50.0 (3)	0	0
Ophiophagus hannah (King cobra)	22	68.2 (15)	0	0
Bungarus fasciatus (Banded krait)	16	93.8 (15)	0	0
Bungarus candidus (Malayan krait)	6	16.7 (1)	0	0
Calloselasma rhodostoma (Malayan krait)	6	0	0	0
Trimeresurus albolabris (White-lipped green pit viper)	4	0	0	0
Trimeresurus purpureomaculatus (Mangrove pit viper)	6	66.7 (4)	0	0
Daboia russellii siamensis (Siamese russell's viper)	25	4.0 (1)	0	0
Total	275	32.4 (89)	4.4 (12)	3.6 (10)

[1] One snake was co-infected with *Hepatozoon* sp.

[2] Four snakes were co-infected with *Hepatozoon* sp.

Table 2 Grading percentage of *Hepatozoon* sp. infected erythrocytes in three species of snake with high percentage of infection.

Snakes species	Total snakes	+ (n)	2+ (n)	3+ (n)	4+ (n)
Boiga dendrophila melanota	47	59.6 (28)	6.4 (3)	2.1 (1)	4.3 (2)
Bungarus fasciatus	16	68.9 (11)	25 (4)	-	-
Ophiophagus hannah	22	59.1 (13)	9.1 (2)	-	-

and had oval nucleus, located from central to the subterminal; and its cytoplasm contained small granules. The host cell nucleus was usually intact except in some infections in mangrove snakes (Figure 7) which caused hypochromic. In heavy infections, more than one gamonts were found (Figure 8) and likely appeared as vermicules in the plasma leaving the parasitophorous vacuole membrane in the hemolytic erythrocyte (Figure 9). Vermicules were detected by moving in the plasma under light microscope examination. In some infections, there were stout microgamonts (6x14 μm, n = 20) with the smaller number than those of the macrogamonts.

Usually there was one type of gamont in each infection but small and large gamonts were detected in the same infection in the common rat snake, king cobra and banded krait (Figure 10).

***Haemogregarina* sp.**

Intraerythrocyte gamonts of *Haemogregarina* sp. were found in mask-faced water snakes and rainbow water snakes. The gamonts were larger than those of hepatozoa gamonts (Table 3) that made host cells enlarged and laterally displaced of the nuclei. They had big oval nuclei, that were as wide as the body, and cytoplasm filled with red fine granules and vacuolated blue granules (Figure 11). In some gamonts, a long tail at one end of the gamont was observed (Figure 11, arrowhead).

Trypanosome

Trypomastigote forms of trypanosome was found in mask-faced water snakes and rainbow water snakes. They were large, broad body width (Table 3), light blue cytoplasm, well defined undulating membrane, free fragellum and kinetoplast (Figure 12).

DISCUSSION

There were three types of hematozoa in thirteen of the eighteen species of snakes in this study. The total rate of hematozoa infection was very high (40.4%) with *Hepatozoon* and *Haemogregarina* infections being 35%. So the haemogregarines were the most common blood parasites of snakes in Thailand which is agreed with other reports (Smith, 1996; Wozniak *et al.*, 1998).

The movement of the vermicule in the plasma was called traveler by Sambon and Seligmann (1908) and Plimmer (1912). The vermicule was identical to type 4 haemogregarines identified by Hull and Camin (1960) or identical to E-type haemogregarines identified by Toshioka (1970). The size of the vermicule in this study was different from the vermicule of *Hepatozoon rarefaciens* in indigo snake, *Drymarchon corais* (Frye, 1991).

In this study, the hepatozoa were classified by size into three types; small, medium and large gamonts. The small gamonts was identical with type 1 haemogregarines identified by Hull and Camin (1960). The three types of gamont in this study were different from the gamont found in the southern water snake, *Nerodia fasciata pictiventris*

Table 3 Measurement (mean ± SD) of the hematozoa size in snake.

	Hepatozoon			*Haemogregarina*	Trypanosome
	Small	Medium	Large		
Number	120	60	140	40	30
Body width (μm)	3.0 ± 0.3	3.3 ± 0.6	3.9 ± 0.6	7.6 ± 1.2	14.1 ± 3.0
Body length (μm)	10.9 ± 0.6	14.8 ± 0.9	15.0 ± 1.1	15.2 ± 2.6	ND

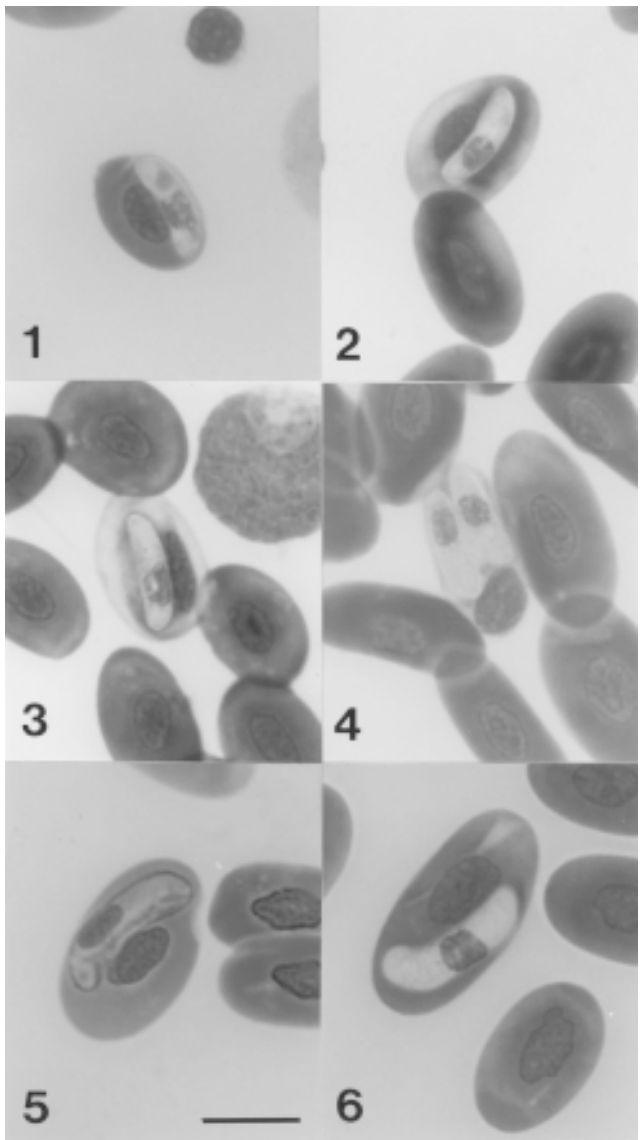


Figure 1 Small gamont (3×11 μm) of *Hepatozoon* in 9×13 μm erythrocyte of the Siamese spitting cobra. Note the prominent nucleus and blue inclusion in the cytoplasm of the hepatozoa.

Figure 2 Small gamont (3×12 μm) of *Hepatozoon* in 10×16 μm erythrocyte of the common rat snake. Note the halo appearance of the erythrocyte cytoplasm.

Figure 3 Small gamont (3×11 μm) of *Hepatozoon* sp. in the king cobra. Note the diplacement of intact nucleus of the erythrocyte.

Figure 4 Two small intraerythrocyte gamonts of *Hepatozoon* are packed in the erythrocyte of the banded krait. Note the displacement of the host nucleus.

Figure 5 Medium gamont of *Hepatozoon* sp. in the mask-faced water snake. Note the dense basophilia at the peripheral of the gamonts.

Figure 6 Large gamont (4×16 μm) of *Hepatozoon* sp. in 12×23 μm erythrocyte of the mangrove pit viper.

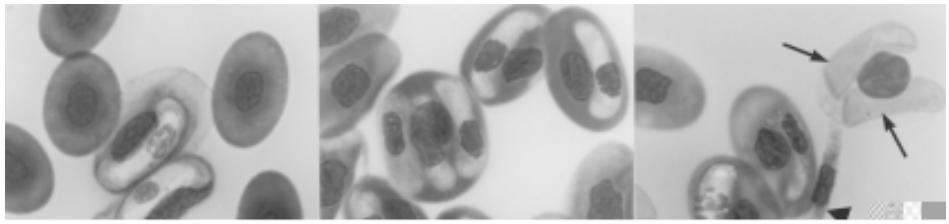


Figure 7 Two large gamonts of *Hepatozoon* sp. infected two erythrocyte of the mangrove snake. Note the halo appearance of the erythrocyte cytoplasm and bipolar stretching of the hepatozoa.

Figure 8 Three large gamonts infecting an erythrocyte of the mangrove snake with 4+ infection.

Figure 9 An exerythrocytic or vermicule form of a *Hepatozoon* (arrowhead) in the mangrove snake. Note the stout microgametocyte (5×13 μm) and the slender macrogametocyte (3×17 μm) in different erythrocytes and the parasitophorous vacuole membrane (arrows) left in the hamolytic erythrocyte.

Figure 10 Small (5×11 μm) and large (5×15 μm) hepatozoa infected different erythrocyte of the banded krait. Bar = 10 μm

Figure 11 Two gamont of *Haemogregarina* sp. in two erythrocyte of mask-faced water snake. Note the displacement of the host nucleus and well-pigmentation of the parasite. The long tail at one end of the gamont (arrowhead) was easily observed.

Figure 12 Trypomastigote of trypanosome in the blood from the mask-faced water snake. Note the well-defined kinetoplast (arrowhead), undulating membrane and free flagellum.

(Wozniak *et al.*, 1998) or the gamont of *Hepatozoon sipedon* in the northern water snake (*Nerodia sipedon sipedon*) collected from Frontenac Country in the eastern part of Ontario (Smith *et al.*, 1994).

Among the seven species infected with the small gamont, some infected erythrocytes were intact but some were hypochromic and had displaced nuclei (Figure 1-4). Among the five species infected with the large gamont, most of the infected erythrocytes were intact (Figure 6, 8) except in some infections of mangrove snakes (Figure 7). The different in host cell cytopathological effects may indicate a difference in the species of the hepatozoa.

Species differentiation of *Hepatozoon* sp. was usually classified by complete life cycle or parasite developmental patterns within invertebrate vectors, including oocyst and sporocysts dimensions, the number of sporocyst/oocyst, the number of sporozoite/sporocyst (Smith, 1996).

Despite the appearance and wide spread distribution of the intraerythrocytic gamonts of *Hepatozoon* sp. from many species of snakes, only 13 species of *Hepatozoon* have been described from North America, with only 4 species having complete life cycle. The description of the 4 species needs morphological and morphometric features at the oocyst stage (Smith, 1996) to identification of their species. The *Hepatozoon* infection in snakes is a 3-host life cycle which was proved by Landau *et al.* (1972). They fed mosquitoes containing oocysts of *H. domerguei* to the insectivorous lizards *Oplurus sebae* and *Lacerta muralis*. Snakes became infected after ingesting these lizards.

The gamonts of *Haemogregarina* sp. were larger than those of *Hepatozoon* sp. which appeared with well-pigmentation of the cytoplasm. Some gamonts revealed a long-tailed folding at one end. These characteristics were identical to type 3 haemogregarines identified by Hull and Camin (1960) even though they were larger in size. The well-pigmentation and vacuolated cytoplasm of the *Haemogregarina* sp. gamont was easily differentiated from those gamont of *Hepatozoon* sp. Wozniak and Telford (1991) differentiated genera by the developmental pattern in invertebrate host : *Haemogregarina* produces sporocyst within the vector gut while *Hepatozoon* forms oocysts containing sporocyst within the vector hemocoel. Siddall (1995) suggested that all haemogregarines infecting snakes, lizards, crocodilians, birds and mammals that remained in the genus *Haemogregarina* were transferred to genus *Hepatozoon*.

The study of trypanosome infections in reptiles was limited because the finding of these flagellates in the hematological examination was difficult due to low parasitemia (Chia and Miller, 1984) The size of trypomastigote in the mask-faced water snakes and rainbow water snakes was larger than those *Trypanosoma hydrae*, trypanosome in mammals or Atkinson and Valkalis found in broad-banded water snake (*Nerodia fasciata confluens*) from Louisiana which were 1.8-2.6 μm body width (Chia and Miller, 1984). Anyhow, trypanosome infections were only found in water snakes.

The naturally infected hematozoa in the reptiles were slightly pathogenic or non-pathogenic (Campbell, 1996; Hawkey and Dennett, 1989) but there was a report of granulomatous hepatitis associated with *Hepatozoon* sp. in the southern water snake (Wozniak *et al.*, 1998). In the case of heavy infection, the hemolysis of infected erythrocyte was observed in the blood smear (Figure 9) and hemolytic anemia may have occurred.

In conclusion, there were hematozoa in 13 species/18 species of studied snakes. Hepatozoas were the most common hematozoa of snake in Thailand. There were no hematozoa in reticulated pythons, fishing salt-water snakes, monocled cobras, Malayan pit vipers and white-lipped green pit vipers. This is the preliminary study of hematozoa of snake in Thailand.

ACKNOWLEDGEMENTS

The authors wish to thank the Kasetsart University Research and Development Institute for supporting fund in this investigation.

LITERARURE CITED

Campbell, T.W. 1996. Hemoparasites, pp. 379-381. *In* D.R. Mader (ed.). Reptile Medicine and Surgery. W.B. Saunders Company. Philadelphia.

Chia, N.M. and J.H. Miller. 1984. Morphological and developmental studies of the snake trypanosome *Trypanosoma hydrae* Ayala, Atkinson, Vakalis, 1983 in experimentally infected hosts and in culture. J. Protozool. 31 : 352-356.

Frye, F.L. 1991. Hematology as applied to clinical reptile medicine, pp. 211-263. *In* F.L. Frye (ed.).Reptile Care : An Atlas of Disease and Treatment. TFH Publication, Inc. Neptune City,

New Jersey.

Hawkey, C.M. and T.B. Dennett. 1989. A Color Atlas of Comparative Veterinary Haematology. Wolfe Publishing Limited. Ipswich. 192 p.

Hull, R. W. and J. H. Camin. 1960. Haemogregarines in snakes: The incidence of the erythrocyte stage. J. Parasitol. 46 : 515-523.

Jintakune, P. and L. Chanhome. 1996. Venomous Snake in Thailand. Queen Saovabha Memorial Institute, Bangkok. 175 p.

Landau, I., J.C. Michel, A.G. Chabaud, and E.R. Brygoo. 1972. Cycle biologique *d' Hepatozoon domerguei*; discussion surles caracteres fondamentaux d'un cycle de coccidie. Zeitschrift fur Parasitenkunde. 38 : 250-270.

Plimmer, H.G. 1912. On the blood parasites found in animals in the zoological gardens during the four years 1908-1911, pp. 406-419. *In* Proceedings of Zoological Society of London. London.

Sambon, L. W. and C. G. Seligmann. 1908. The haemogregarines of snakes. J. Tropical Med and Hygiene 11 : 355-358.

Schalm, O.W., N.C. Jain, and E.J. Carroll. 1975. Veterinary Hematology. 3rd ed. Lea and Febiger, Philadelphia. 807 p.

Siddall, M.E. 1995. Phylogeny of adeleid blood parasite with a partial systemic revision of the haemogregarinae complex. J. of Eukaryotic Microbiology 42 : 116-125.

Smith, T.G. 1996. The genus Hepatozoon (Apicomplexa :Adeleina). J. Parasitol. 82 : 565-585.

Smith, T.G., S.S. Desser, and D.S. Martin. 1994. The development of *Hepatozoon sipedon* n.sp. (Apicomplexa : Adeleina : Hepatozoidae) in its natural host, the northern water snake (*Nerodia sipedon sipedon*), the culicine vectors, *Culex pipiens* and *Culex territans,* and an intermediate host, the northern leopard frog (*Rana pipiens*). Parasitology Research 80 : 559-568.

Toshioka, S. 1970. Natural infection of protozoal blood parasite captive Japanese snake (Preliminary report) The SNAKE 2 : 53-56.

Wozniak, E.J. and S.R. Telford, Jr., 1991. The fate of *Hepatozoon* species naturally infecting Florida black racers and watersnakes in potential mosquito and soft tick vectors, and histological evidence of pathogenicity in unnatural host species. International J. Parasitol. 21 : 511-516.

Wozniak, E.J., S.R. Telford, Jr., D.F. Denardo, G.L. McLaughlin, and J.F. Butler. 1998. Granulomatous hepatitis associated with *Hepatozoon* sp. meronts in a southern water snake (*Nerodia fasciata pictiventris*). J. Zoo Wildl. Med. 29 : 68-71.

Received date : 5/06/01
Accepted date : 2/07/01

Kasetsart J. (Nat. Sci.) 35 : 157 - 163 (2001)

Effects of Root Extract from Derris (*Derris elliptica* Benth) on Mortality and Detoxification Enzyme Levels in the Diamondback Moth Larvae (*Plutella xylostella* Linn.)

Suraphon Visetson[1] and Manthana Milne[2]

ABSTRACT

Two types of ethanol extraction methods, Soxhlet and stirring soaking, were carried out. The rotenone content determined by the HPLC was 8.6 % w/w for the former method compared to 5.2% w/w for the latter one. Third instar larvae of the diamond back moth gave LD_{50} of 24.25 PPM and 89.07 PPM for the Soxhlet and stirring methods, respectively. Triphenyl phosphate (TPP) and Piperonylebutoxide (PB) reduced the LD_{50} upto ca. 4 folds and also significantly lowered the number of larvae in the field.
The optimal medium for the detoxification enzymes, esterase, GSH-S-transferses and monooxygenases, was found to contain 0.1 M phosphate buffer pH 7.5 with 10 mM glutathione (reduced forms), 1 mM EDTA and 50% w/w PVPP. Protein concentrations (BSA as a standard protein) between 50-100 mg protein/g larvae/ml extracted from 2 – 4 instar larvae were used for all enzyme assays.

Derris extracts induced ca. 10-20% of all enzyme activities. By adding TPP to the extracts, esterase activity was reduced by 20%. The coefficient of correlation, r^2 (mortality against esterase activity), was ca. 0.9. The addition of DEM showed a lower in r^2 value (0.62 – 0.77) (mortality against GSH-S-transferases activity). The highest fluctuation of r^2 (0.48 – 0.97) (mortality against monooxygenases activity) was observed by the addition of PB to both extracts.

Key words: derris extracts, detoxification enzymes, diamondback moth, *Derris elliptica* Benth, *Plutella xylostella* Linn.

INTRODUCTION

Many types of insecticides have been imported to control insect pests in Thailand. The improper use of insecticides by farmers may lead to pest resistance. Although many measures have been introduced to control insect pests, many problems, such as high cost of production and pollution, are among the controversial issues in vegetable producing areas (Waleeluck and Visetson, 1995). Of all insect pests of vegetables, Diamondback moth (*Plutella xylostella* Linn.) is one of the most serious pests found in Thailand. It is resistant to many insecticides in the central part of Thailand where insecticides are frequently used.

Thailand is one of the most diverse countries in terms of plants and animals in Southeast Asia. Some plants show high ability to adapt to and tolerate herbivores and their environment. The adapting ability derived from the production of special chemicals is called allelochemicals, which are parts of secondary plant substances (Yu and

1 Department of Zoology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
2 Institute of Natural Products, Department of Agriculture, Bangkhen, Bangkok 10900, Thailand.

Hsu, 1985). Plant active ingredients show hormonal inhibition in insects such as azadirachtin from neem seed kernels (Sombatsiri *et al*. 1995). The others show repellent properties such as citronellal from citrus leaves (Visetson, 1996). Feeding inhibition from plant substances such as eupathal in Siam weed and nimbidin from neem seed kernels were carried out (Lange and Schmutterer, 1982). As a result, Thailand will be the one of many Southeast Asian countries where plant extracts are increasingly important as pest control alternatives.

Derris (*Derris elliptica* Benth) is another pesticide alternative. Thai farmers used to grow it widely in the eastern part of Thailand. It is called "Lotin". They used it to kill some insect pests especially in the Order Homoptera. Derris has not appeared on the insecticide market for two decades because more highly effect, synthetic pesticides have been introduced. However, with the development of resistant insects, the threat of contaminated food and high production cost problems, derris came back again in 1995.

Derris belongs to the family Leguminosae. It's principal secondary plant substance is called rotenone (1,2, 12a-tetrahydro-8,9-dimethoxy-2 (1-menthylethenyl-(1) benzopyrano (2,4-b) furo (2,3-h) (1) benzophyran-6 (6H)-one with M.W. at 394.41). Its mechanism of action in higher organisms is on interference with the electron transport chain at the inner mitochondrial membrane. It has low toxicity to mammals, but is extremely toxic to fish (Matsumura, 1975). The detoxification enzyme mechanisms in insects have not been investigated.

The aims of this research are to investigate the toxicity of rotenone extracts (LD_{50}) and synergistic effects as well as the activity of detoxification enzymes, namely general esterases, glutathione-S- transferases and monooxygenases, in diamondback moth larvae after exposure to the extracts. Their results could be beneficial for farmers as well as businessmen who are concerned of the environmental deterioration.

MATERIALS AND METHODS

Insect larvae and plant samples

Diamondback moth larvae were collected from the vegetable producing area in Kanchanaburi province, 150 km west of Bangkok. Larvae were reared under the laboratory at 23 ± 2°C following the method of Leckprayun *et al*. (1999). Two-year old derris root was collected from Chonburi province, 150 km east of Bangkok. Only the diameter size of roots less than 1 cm were used. They were oven- dried at 45°C for 7 days and then ground to powder.

Plant extraction and efficacy tests

Two types of ethanolic extractions; the Soxhlet extraction at 70°C and stirring soaking at room temperature were administered for 8 hours. The crude extracts then were evaporated and analyzed for the rotenone content by Thin Layer Chromatography and then HPLC-UV detector using methods modified from Pitiyon and Sangwanit (1997). The extracts were diluted into various concentrations and were conducted toxicity test for 10 – 90% mortality of 3^{th}- instar larvae of diamondback moths. Three replicates comprised 20 larvae in each replicate. A 5% emulsifier, triton X-100, was mixed into each concentration before the trials commenced. Following Raffa and Priester (1985), the synergists, triphenyl phosphate (TPP), diethyl maleate (DEM) and piperonyl butoxide (PB) were used in separate experiments. A no-choice leaf dipping method using a leaf circle disk of Chinese kale with a diameter of 5 cm was placed to feed the larvae. Mortality was checked after 24 hours exposure. A Completely Randomized design with 3 replicates was used. All experiments were run at 23 ± 2°C. In case of control mortality, Abbott's formula (Matsumura,1975) was employed. LD_{50} were calculated by using regression equation with cypermethrin as a control.

Detoxification enzyme assays

The surviving larvae from the treatment were in vitro assays to optimize enzyme activity of esterases, GSH-S-transferases and monooxygenases activities following the methods of Visetson (1991) by using paranitrophenyl acetate (PNPA) , diethyl maleate (DEM) and aldrin for their substrates, respectively. The enzyme optimization included some stabilized chemicals in extracting buffers such as polyvinyl polypyrrolidine (PVPP), glutathione, ethylenediaminetetraacetic acid tetra sodium salt (EDTA), phenylmethylsulfonyl flouride (PMSF) and bovine serum albumin (BSA) and pH were carried out. The larval stages were also tested for their optimal activities. Protein measurement followed the method of Lowry *et al*., (1951) and BSA was used as a standard protein. The best condition was used to quantify all enzyme activity.

The coefficient of determination (r^2) was determined for both insect's larval mortality and enzyme activity. Synergist ratios (SR) and change factors (CF) were quantified to measure the effectiveness of synergists and changes in enzyme levels, respectively.

Field experiments

Field trials using Chinese kale were separately undertaken to confirm the laboratory results. The experimental design was allocated in a Randomized Complete Block design with 3 replicates. Plot size was given as 6×2 square meters. Spraying was done once a week by beginning on 10-day-old Chinese kale. There were 7 sprays for the whole experiment. Larval numbers were regularly checked at fixed points before and after spraying for 24 hours. DMRT was employed for mean comparisons with probability > 95% following Finney (1964).

RESULTS AND DISCUSSIONS

Extraction and rotenone content

The Soxhlet extraction and stirring soaking methods showed ca. 8.6 and 5.2% w/w of rotenone respectively. The former method gave a 1.6 fold higher amount of rotenone than the latter which indicated that the high temperature during Soxhlet extraction had a greater influence on rotenone solubility in ethanol than did the stirring soaking method. This result was similar to that found for the extraction of azadirachtin from neem seed kernels (Visetson, 1994) and euphathal and α-pinine extracted from Siam weed (Leckprayun *et al*. 1999). However, temperatures above 70°C may cause decomposition of plant active ingredients (Visetson and Chuchoui, 1999). Although the use of chloroform as a solvent (Pitiyon and Sangwanit, 1997) resulted in a higher amount of rotenone being extracted; the chloroform was proved to be dangerous to human health. Ethanol, therefore, is a more suitable solvent for rotenone extraction for Thai farmers.

The LD_{50} values obtained from the Soxhlet and stirring soaking methods without any synergists were 24.25 PPM and 89.07 PPM, respectively (Table 1). These results also indicated that higher temperature of Soxhlet extraction yielded a higher quality of rotenone extracted than the stirring soaking method.

The addition of the synergists, TPP and PB, showed synergist ratio (SR) of ca. 3 fold, whereas addition of DEM showed the SR of ca. 1.0 (Table 1). The results indicated that esterase and monooxygenase played major roles in detoxifying rotenone (Table 3). These indications differ to those from experiments with other plant extracts. Leckprayun *et al*. (1999) indicated no correlation with these enzymes in the detoxification of Siam weed extracts. Although the inefficient of DEM showing increased SR in these experiments that did not mean GSH –S-transferases were unimportant in rotenone detoxification. Rotenone might be broken down in the phase I reaction (energy requiring reaction) before conjugation took place in phase II (glutathione conjugating reaction), indicating the increase of SR by DEM. This interpretation was

supported by the results of field trial. Field applications revealed that the addition of TPP and PB to the extracts was found to lower the larval number to levels similar to the control plot treated with cypermethrin. It was also noticed that the addition of DEM to both extracts did not give any significant change in larval number (P>0.05) (Table 2).

The homogenizing medium, composed of 0.1 M phosphate buffer (pH 7.5) with 10 mM glutathione (reduced form), 1 mM EDTA and 50% PVPP (w/w) was suitable for detoxification enzyme extraction in this study. Although further addition of PMSF and BSA gave higher monooxygenase activity, but this reduced GSH-S-transferase activity (Figure 1a). The compounds might help stabilizing pH of monooxygenase (Powis and Schenkman, 1977), but stable pH may not be sufficient to enhance GSH-S-transferase levels. The homogenizing media without PVPP showed very little enzyme activity indicating quinone to be produced whilst extraction was in process. The inhibition of GSH-S-transferase activity by quinone was known by a number of research workers (Kotze and Rose, 1989).

Enzyme activity was quantified the highest at 3rd instar larvae and the least activity was detected in pupae (Figure 1b). Furthermore, pH 7.5 showed optimum activity occurring for all enzyme systems with little variation (Figure 1c). This pH corresponded with that determined by the previously

Table 1 Comparisons of linear regression on LD_{50} values and synergistic ratio (SR) among different synergist treatments of derris extracts on larvae of diamond back moth under the laboratory condition[1]

Synergists added[2]	Soxhlet (LD_{50})[SR][3,4]	Stirring-soaking (LD_{50})[5][SR]
None	Y = 20.89 + 1.2X (24.25)	Y = 14.37 + 0.4X (89.07)
TPP[5]	Y = 34.77 + 2.3X (6.62) [3.66]	Y = 22.97 + 1.2X (22.52)[3.95]
DEM	Y = 18.01 + 1.2X (26.65)[0.90]	Y = 7.07 + 0.4X (107.32)[0.83]
PB	Y = 37.85 + 1.2X (10.12)[2.39]	Y = 16.23 + 1.3X (25.97)[3.43]

[1] n = 3, 20 of 3th instar each batch, assay at 23 ± 2°C with leaf dipping method, 24 hrs. check.
[2] TPP , DEM and PB stand for triphenyl phosphate, diethyl maleate and piperonyl butoxide, respectively.
[3] Y and X represents % mortality and concentration, respectively.
[4] SR = LD_{50} none/ LD_{50} with synergists.
[5] Linear regression for cypermethrin showed LD50 = 17.2 ppm (Y = 13.89 + 2.1X)

Table 2 Mean ±SD of diamond back moth lavae found on the leaves of Chinese kale after application of the different derris extracts and cypermethrin.

10% Synergists added[1]	10% cypermethrin	Extract I[2]	Extract II
None	25.23 ± 6.12 b[3]	30.16 ± 1.23 b	49.16 ± 4.17 c[3]
TPP	10.33 ± 3.17a	15.12 ± 2.61 a	28.16 ± 2.17 a
DEM	23.45 ± 4.35b	30.13 ± 2.74 b	39.21 ± 1.89 c
PB	14.43 ± 5.13a	18.27 ± 3.32 a	29.23 ± 2.32 a

[1] TPP, DEM and PB stand for triphenyl phosphate, diethyl maleate and piperonyl butoxide, respectively. SD is a standard deviation.
[2] Extracts I and II derived from soxhlet extraction and stirring soaking method, respectively.
[3] Means following by the same letter in the same collumn are not significantly different, P>0.05, DMRT

work of Visetson (1991) on the red-rust flour beetles. Protein contents ranging from 50-70 mg protein/g larvae/ml resulted in a little variation of activities (CV<20%) (Figure 1d). These results are similar to those obtained for the Australian blow fly larvae (Kotze and Rose, 1989) the American ball worm (Rose, 1985) and fall armyworm (Yu, 1984).

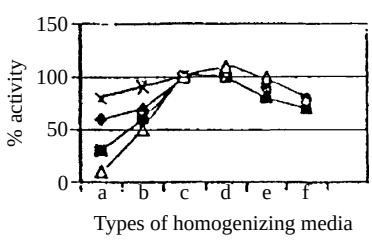
With the Soxhlet method, all CFs (controled enzyme activity versus treated enzyme activity) were less than 1 (Table 3). After addition of DEM into the Soxhlet extracts the CFs ratio found to increase to 2.61. Additions of PB resulting in CFs of ca. 2.05 indicated that the inhibition of monooxygenase occurred. On the other hand, the addition of synergists did not produce any effect on the efficacy of the stirring soaking extract.

In short, synergists actively inhibited the enzymes proportionately. The results showed that the specificity of TPP was as an esterase inhibitor (r^2 = 0.88-0.97). DEM and PB fluctuated (r^2 = 0.68 - 0.70 and r^2 = 0.36 – 0.98, respectively) in inhibiting GSH-S-transferases and monooxygenases, respectively. The correlation between the synergist mortality and enzyme activity resulted from addition of PB and monooxygenases activity may be the indication of nonspecificity of PB (Collins, 1990). The synergist was reported to be an esterase inhibitor as well. However, before certain conclusion can be made, an experiment using purified enzymes should be conducted for further verification.

CONCLUSION

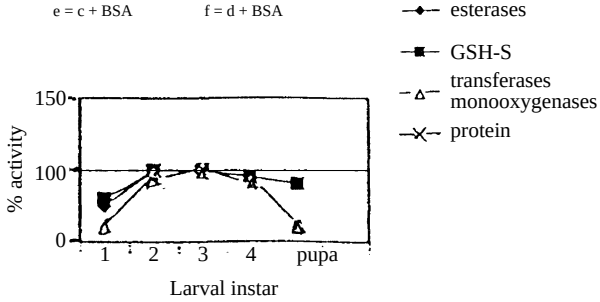
The highest rotenone content determined using HPLC showed 8.6 % w/w yield for the Soxhlet's method. Third instar larvae of the diamond back moth gave LD_{50} of 24.25 PPM and 89.07 PPM for the Soxhlet and stirring methods, respectively. The addition of Triphenyl phosphate (TPP) and Piperonylebutoxide (PB) found reduced the LD_{50}significantly.

Adding of TPP and PB to the extracts also reduced esterase as well as monooxygenase activity.

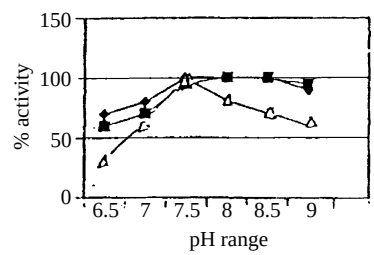


(a). Enzyme activity for different homogenizing media

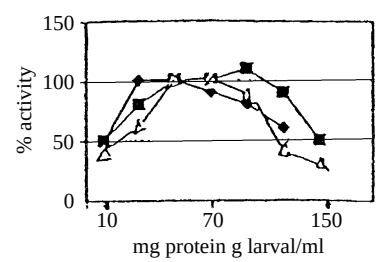
a = buffer alone b = buffer + PVPP + glutathione
c = b + EDTA d = c + PMSF
e = c + BSA f = d + BSA



(b). Enzyme activity of different larval instars and pupae



(c). Effects of assay pH on enzyme activity



(d). Effect of protein concentration on enzyme activity

Figure 1 Effects of homogenizing media (a), larval instars and pupae (b), pH (c) and protein concentration (d) on activity of detoxification enzymes.

Table 3 Mean ±SD activity of esterase, GSH-S-transferase and monooxygenase of diamondback moth larvae survived after being exposed 24 h to derris extracts and cypermethrin treated with synergists. (n = 3)

Type of pesticides	Synergist added	Enzyme activity (CF)[r^2] Gen. Esterases	GSH-S-transferases	monooxygenase
None	None	12.14 ± 2.23 a [1]	32.13 ± 2.46 b	4,320.12 ± 126.22b
Cypermethrin		15.21 ± 1.64 b	40.21 ± 6.41c	5,120.45 ± 123.31b
		(0.79)[0.98]	(0.79)[0.95]	(0.84)[0.90]
Extract I	None	16.42 ± 1.24b	40.16 ± 1.67c	4,100.43 ± 213.66b
		(0.73)[0.45]	(0.80)[0.58]	(1.05)[0.97]
	TPP	10.32 ± 1.23a	34.12 ± 1.42b	4,321.45 ± 126.77b
		(1.18)[0.97]	(0.94)[0.92]	(0.99)[0.68]
	DEM	15.12 ± 1.41b	12.33 ± 1.46a	4,273.78 ± 177.09b
		(0.80)[0.68]	(2.61)[0.62]	(1.01)[0.94]
	PB	10.23 ± 1.41a	33.14 ± 1.24b	2,100.87 ± 231.60a
		(1.19)[0.98]	(0.97)[0.95]	(2.05)[0.48]
Extract II	None	16.12 ± 1.76b	40.16 ± 1.67c	4,100.75 ± 213.58b
		(0.75)[0.77]	(0.80)[0.57]	(1.05)[0.71]
	TPP	12.14 ± 2.16a	32.16 ± 1.72b	4,000.67 ± 161.96b
		(1.00)[0.88]	(0.99)[0.89]	(1.08)[0.14]
	DEM	13.14 ± 1.23a	30.12 ± 1.47b	4,121.54 ± 123.89b
		(0.92)[0.70]	(1.07)[0.77]	(1.05)[0.93]
	PB	13.12 ± 1.42a	31.16 ± 1.26b	3,214.67 ± 162.56a
		(0.92)[0.36]	(1.03)[0.66]	(1.34)[0.83]

[1] Means followed by the same letters within the same collumn are not significantly different, P>0.05, DMRT

Further steps of complete enzyme purification data including of isoelectric emphasizing on the methods from both controlled and induced forms in the larvae must be conducted. These steps will elucidate the unbiased mechanisms and be important steps to modify the synergists as well as the structure of rotenone in perfect binding at the exact biding side of the enzymes leading of better rotenone formulations in the future.

ACKNOWLEDGMENTS

This research was supported by the Kasetsart University Research and Development Institute-KURDI, KU., Thailand. I especially would like to thank Dr. John Milne, Faculty of Science, Mahidol University for kindly reviewing this manuscript.

LITERATURE CITED

Collins, P.J. 1990. A new resistance to pyrethroids in *Tribolium castaneum* (Herbst). Pestic. Sci. 28 :101- 115.

Finney, D.J. 1964. Statistical Methods in Biological Assay. 2nd ed. Charls Griffin and Company Limitted, London. 356 p.

Kotze, A.C. and H. A. Rose. 1989. Purification and properties of glutathione S-transferases from the larvae of the Australian sheep blowfly, *Lucilia cuprina* (Wiedemann). Insect Biochem. 19 : 703-713.

Leckprayun, J, S. Visetson, and M. Pianjalearn. 1999. Effects of Siam grass extracts (*Chromolaena odorata* L) on detoxification enzymes in the diamond back moth(*Plutella xylostella* L.), pp. 870-873.*In* the 3rd Annual Conference in Biodiversity in Thailand (BRT). October, 11-14, 1999. Hatyai, Songkla, Thailand. (in Thai).

Lange, W. and H. Schmutterer. 1982. Experiments with synergists to improve the effects of the growth disturbing properties of methanolic extract of seeds of the neem tree (*Azadirachta indica*). J. Plant Diseases and Protection. 89 : 258-265.

Lowry, O.H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193 : 265-275.

Matsumura, F. (1975). Botanical insecticides, pp. 94-98. *In* F. Matsumura. (ed.). Toxicology of Insecticides. Plenum, New York.

Pitiyon, W. and A. Sangwanit. 1997. The study of derris extracts in insect control, pp. 42-48. *In* Agricultural Toxic Substances Conference. Department of Agriculture. Ministry of Agriculture and Cooperatives. July, 2 – 10, 1997. The First Riverkaew Hotel. Kanchanaburi. (in Thai).

Powis, G., I. Jansson, and J. B. Schenkman. 1977. The effects of albumin upon the spectral changes and metabolism by the hepatic microsomal fraction. Arch. Biochem. Biophys. 179 : 34-42.

Raffa, K.F. and T. M. Priester. 1985. Synergists as research tools and control agents in agriculture. J. Agric. Ent. 2 : 27-45.

Rose, H.A. 1985. The relationship between feeding specialization and host plants to aldrin epoxidase activity of three blowfly species and its induction by phenobabital and B-naphtholflavone. Pestic. Biochem. Physiol. 14 : 275-281.

Sombatsiri, K., K. Kongkatip, S. Visetson, A. Sankwarapong, and K. Anukoonwata, K. 1995. Neem: Pesticides. J. of Navakaset. 11(11) : 103-104. (in Thai).

Visetson, S. 1991. Insecticide resistance mechanisms in the red rust flour beetle (*Tribolium castaneum* Herbst). PhD. Thesis. The University of Sydney. Sydney.

Visetson, S. 1994. The effects of neem extracts on the levels of some insect detoxification enzymes. J. of Toxic Substances. 21(1) : 11-23. (in Thai).

Visetson, S. 1996. The alternatives for vegetable pest control. J. of Toxic Substances. 23(1) : 46-47. (in Thai)

Visetson, S. and L. Chuchoui. 1999. The efficiency of lemon grass (*Cymbopogon winterianus* Jewitti) and neem (*Azadirachta indica* var. siamensis, Valeton) extracts on the changes in detoxification enzymes in the dog tick (*Rhipicephalus sanguineus* Latreille), pp. 847-851. *In* the 3rd Annual Conference in Biodiversity in Thailand (BRT). October, 11-14, 1999. Hatyai, Songkla. (inThai).

Waleeluck, N. and S. Visetson. 1995. Plant extracts as insecticide alternatives. J. of Navakaset. 11(11) : 89-102. (in Thai).

Yu, S.J. 1984. Interactions of allelochemicals with detoxification enzymes of insecticide-susceptible and resistant fall armyworms. Pestic. Biochem. Physiol. 22 : 60-68.

Yu, S.J. and E. L. Hsu. 1985. Induction of hydrolases by allelochemicals and host plants in fall armyworm (Lepidoptera: Noctuidae) larvae. Environ. Ent. 14 : 512-515.

Received date : 28/12/00
Accepted date : 30/06/01

Kasetsart J. (Nat. Sci.) 35 : 164 - 170 (2001)

Production of L-Lactic Acid from Raw Cassava Starch by *Rhizopus oryzae* NRRL 395

Nuanphan Naranong and Duangduan Poocharoen

ABSTRACT

Lactic acid, which is commonly used in food, chemical and pharmaceutical industries, has recently received much attention for the production of biodegradable plastic. L-lactic acid production by *Rhizopus oryzae* NRRL 395 from raw cassava starch as a sole carbon source was studied. The optimum production medium was as follows (g/l) : raw cassava starch, 120; $(NH_4)_2SO_4$, 3.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.25; $ZnSO_4.7H_2O$, 0.04; pH 6.0. The maximum L-lactic acid production in shake flasks was 68.32 g/l on day 5 with the shaking speed of 200 rpm at 30°C. L – lactic acid yield and productivity were 0.59 g/g substrate and 0.57 g/lh, respectively. In a jar fermentor, the maximum L-lactic acid production was 54.62 g/l on day 4 of cultivation at the agitation speed of 400 rpm and the aeration rate of 1.5 vvm. The L – lactic acid yield was 0.49 g/g substrate with the productivity of 0.56 g/lh.

Key words: L-lactic acid, *Rhizopus oryzae*, cassava starch

INTRODUCTION

Lactic acid is an important organic acid widely used not only in the food industry as a preservative, a food acidulant and flavour agent but also in chemical industry as solvent. More-over, various lactic acid salts are also used in formulation of pharmaceutical products (Kascak *et al*., 1996 ; Vick Roy, 1985). Recently, lactic acid is used as a starting material of polylactic acid (PLA), a polymer used in the manufacture of new biodegradable plastics (Vert *et al*., 1992). Lactic acid has been produced by lactic acid bacteria (Bibal *et al*., 1991 ; VickRoy *et al*., 1982) and the fungus *Rhizopus oryzae* (Soccol *et al*., 1995 ; Yu and Hang, 1989). *Rhizopus oryzae* can produce large amounts of L-lactic acid and utilize both various sugars and starch as carbon sources (Yin *et al*., 1997 : Soccol *et al*., 1995 ; Yu and Hang, 1989). Lactic acid production using *Rhizopus oryzae* seems to be a viable alternative because it can grow on minimal liquid medium and on solid medium (Soccol *et al*., 1994).

Starch has been considered for use as a raw material for various fermentation because of its abundance and low price. However, when high concentration of starch is used in medium, an increase in viscosity of the medium due to gelatinization by heat will reduce the microbial growth. Recently, Yahiro *et al*. (1997) reported that itaconic acid could be produced from the medium consisting of 140 g/l corn starch hydrolyzed by nitric acid and higher than 60 g/l of itaconic acid was produced from *Aspergillus terreus*. The lactic acid production from *Rhizopus oryzae* using corn starch partially hydrolyzed with α-amylase or diluted hydrochloric acid as the sole carbon source was studied by Yin *et al*. (1997). The highest lactic acid concentration of 82 g/l was obtained from the

Department of Applied Biology, Faculty of Science, King Mongkut's Institute of Technology Ladkrabang. Bangkok 10520, Thailand.

medium containing 120 g/l corn starch (hydrolyzed with α-amylase) on day 4 of cultivation in a jar fermentor.

In this study, the optimum medium for the production of L-lactic acid in shake flasks was investigated when raw cassava starch pretreated with hydrochloric acid was used as a sole carbon source. Moreover, the influence of aeration rate and agitation speed on the L-lactic acid production in a jar fermentor was also determined.

MATERIALS AND METHODS

Microorganism and inoculum

Rhizopus oryzae NRRL 395 was obtained from the Northern Regional Research Center, Peoria, Illinois, and it was used throughout this study. The fungus was maintained on PDA slant and transferred to fresh slants every 4 months.

An inoculum was a spore suspension obtained by suspending spores from seven-day-old culture grown on PDA in 250 ml Erlenmeyer flask at 30°C with sterile distilled water containing 0.05% Tween 80 and filtering through a pad of sterile cotton wool. Spore concentration was determined using haemocytometer. Both flask culture and fermentor were inoculated with the inoculum so that the final concentration was 2-5 $\times 10^5$ spores/ml.

Medium and cultivation

Culture medium was as follows (g/l): carbon source, 120.0; $(NH_4)_2SO_4$, 2.0; K_2HPO_4, 0.6; $MgSO_4.7H_2O$, 0.25; and $ZnSO_4.7H_2O$, 0.04. The initial pH was adjusted to 6.0. When raw cassava starch was used as a carbon source, it was partially hydrolyzed with 3 N HCL. The acid was added to the starch solution until pH 2.0 was reached and then the solution was autoclaved at 121°C for 30 min. The medium was sterilized at 121°C for 15 min.

In shake flasks, the fungus was grown in 250 ml Erlenmeyer flasks containing 50 ml of the medium. Four flasks were used in each experiment. The flasks were incubated for 5 days at 30°C on a rotary shaker with the shaking speed at 200 rpm. In order to prevent a decrease in pH, 3 g of sterilized calcium carbonate was added to each flask after 24 h of cultivation. Various concentrations of raw cassava starch, nitrogen sources, and K_2HPO_4 were investigated for L-lactic acid production. The optimum parameter from each experiment was used in the next experiment.

In a controlled fermentor, a 5-l jar fermentor (BIOSTAT-B, B.Braun, Germany) with instruments for the control of agitation speed, aeration rate, pH and temperature were used in this study. The fermentor was filled with 2.5 l of optimum medium and then autoclaved at 121°C for 40 min. The fermentation temperature was controlled at 30°C. The influence of aeration rate and agitation speed on lactic acid production was evaluated at 0.5, 1.0 and 1.5 vvm under 200, 300 and 400 rpm. After 24 h of cultivation, 150 g of sterilized calcium carbonate was added.

Samples were taken daily for analyses of pH, dry mycelial weight, total sugar, reducing sugar and lactic acid concentrations.

Analytical methods

pH value of culture broth was measured by a pH meter (Model HM-7E, TOA). Dry mycelial weight was measured by filtering the culture broth through filter paper, washing the mycelium with 3N HCl, following by distilled water and then drying at 90°C to constant weight. The supernatant fluid was used for total sugar, reducing sugar and lactic acid determinations. For the total sugar, the phenol – sulfuric acid method (Dubois, *et al*., 1956) was used. The DNS method (Bernfeld, 1955) was used for the reducing sugar measurement. The concentration of lactic acid was measured by the Barker – Summerson method (Barker, 1957).

RESULTS AND DISCUSSION

Effect of raw cassava starch on L- lactic acid production

Raw cassava starch concentrations of 50, 100, 120, 150 and 180 g/l were used as carbon source in the medium for flask cultivation. The results are shown in Figure 1. An increase of initial raw cassava starch concentration from 50-120 g/l, higher levels of L-lactic acid were produced. However, a further increase of the initial concentration over 120 g/l reduced the L-lactic acid production. The highest L-lactic acid concentration of 58.57 g/l was obtained in the medium containing 120 g/l of raw cassava starch on day 5 of cultivation. This result was lower than that reported by Yin *et al.* (1997) showing that L-lactic acid concentration of 95.10 g/l was produced by *R. oryzae* in the medium containing 120 g/l of corn starch (hydrolyzed with hydrochloric acid). This might be due to the difference in the compositions of cassava starch and corn starch.

Effect of nitrogen sources on L- lactic acid production

The effect of various nitrogen sources on L-lactic acid production was investigated using 2 g/l of ammonium sulfate, ammonium chloride, ammonium nitrate, yeast extract and corn steep liquor. The results are shown in Figure 2. The highest L-lactic acid concentration of 57.00 g/l was produced when ammonium sulfate was used as a nitrogen source. In case of ammonium chloride, 51.30 g/l of L-lactic acid was produced. When corn steep liquor was used as nitrogen source, L-lactic acid production was very low.

The effect of initial ammonium sulfate concentration was also determined. As shown in Figure 3, the optimum concentration of ammonium sulfate providing L-lactic concentration of 60.80 g/l on day 5 of cultivation was 3.0 g/l. It could be concluded that ammonium sulfate was the most effective nitrogen source for L-lactic acid production in *R. oryzae*. This is in agreement with Soccol *et al*. (1994) and Kosakai *et al.* (1997). However, Yin *et al*. (1997) showed that ammonium sulfate at the concentration of 1.35 g/l resulted in the maximum L-lactic acid production by *R.oryzae* when corn starch was used as a sole carbon source.

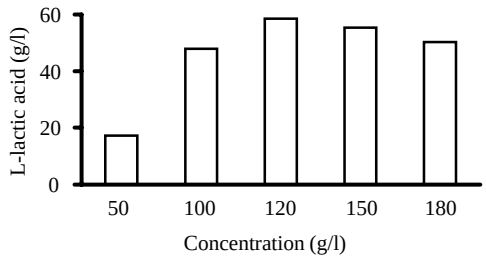


Figure 1 Effect of raw cassava starch concentration on L-lactic acid production.

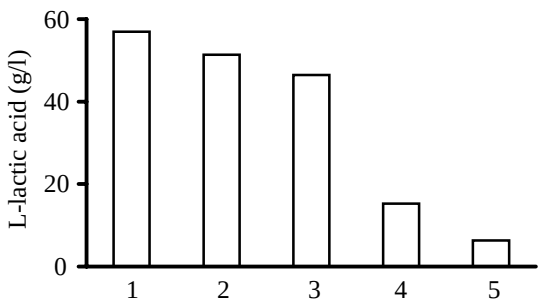


Figure 2 Effect of various nitrogen sources on L-lactic acid production.
(1, $(NH_4)_2 SO_4$; 2, NH_4Cl: 3, NH_4NO_3; 4, yeast extract; 5, corn steep liquor)

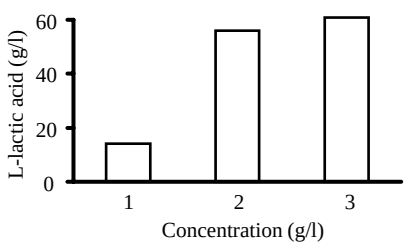


Figure 3 Effect of $(NH_4)_2 SO_4$ concentration on L-lactic acid production.

Effect of KH_2PO_4 concentration on L-lactic acid production

KH_2PO_4 concentrations of 0, 0.3, 0.6 and 1.0 g/l were investigated. The results are shown in Figure 4. The maximum L-lactic acid with the concentration of 63.33 g/l was obtained with 1.0 g/l KH_2PO_4. As the concentrations of L-lactic acid were reduced to 58.80 g/l and 59.47 g/l in the medium containing 0.3 g/l and 0.6 g/l KH_2PO_4, respectively.

In conclusion, an optimized medium for L-lactic acid production by *R.oryzae* NRRL 395 contained (g/l) : raw cassava starch, 120.0; $(NH_4)_2SO_4$, 3.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.25; $ZnSO_4.7H_2O$, 0.04 and pH 6.0. Figure 5 shows time courses of growth and L-lactic acid production of *R.oryzae* cultivated in the optimized medium using shake flasks. The production of L-lactic acid appeared related to the mycelial growth and reached the maximum concentration of 68.32 g/l on day 5 of cultivation. Then it gradually decreased. L – lactic acid yield (based on an initial cassava starch concentration) and productivity were 57% and 0.57 g/lh, respectively.

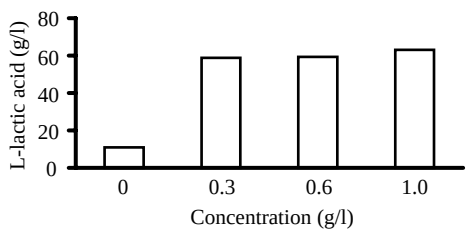


Figure 4 Effect of KH_2PO_4 concentration on L-Lactic acid production.

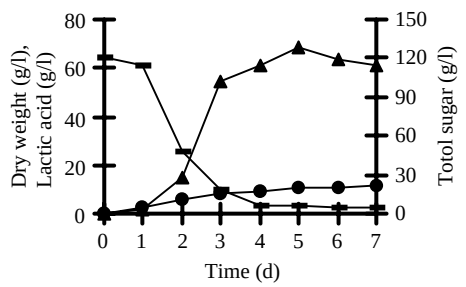


Figure 5 Time courses of L-lactic acid fermentation by *R.oryzae* in shake flasks. (▲, L-lactic acid concentration; ●, dry weight; –, total sugar)

Effect of aeration rate and agitation speed on L-lactic acid production in a jar fermentor

Three aeration rates of 0.5, 1.0 and 1.5 vvm and three agitation speeds of 200, 300 and 400 rpm were combined giving nine conditions for L-lactic production. The results are summarized in Table 1. An increase in aeration rate increased specific growth rate (μ) at all agitation conditions studied. The maximum specific growth rate of 0.06 h^{-1} was obtained at the agitation rate of 400 rpm with 1.5 vvm. At the agitation rate of 400 rpm, L- lactic acid production, L – lactic acid yield ($Y_{P/S}$) and productivity were increased when the aeration rate was raised from 0.5 to 1.5 vvm. L – lactic acid production was highest (54.62 g/l) at the aeration rate of 1.5 vvm on day 4 of cultivation. At agitation rate of 200 rpm, L – lactic acid production was increased by an increase in aeration rate of 0.5 to 1.0 vvm (36.33 and 41.68 g/l with 0.5 and 1.0 vvm, respectively) while an increase to 1.5 vvm only 42.41 g/l of L – lactic acid was obtained. It was found that L – lactic acid yield and productivity were independent of the aeration rate tested. For the aeration rate of 300 rpm, L- lactic acid production, L – lactic acid yield and productivity were not different when the aeration rate was increased from 0.5 to 1.5 vvm. Comparing to various conditions, it was elucidated that the maximum L-lactic acid concentration was 54.62 g/l and L – lactic acid yield of 0.49 g/g substrate with a corresponding the productivity of 0.56 g/lh were obtained on day 4 of cultivation when the fermentation was carried out at the agitation speed of 400 rpm and the aeration rate of 1.5 vvm (Figure 6).

From these results, the values of specific growth rate increased with increasing the aeration rate demonstrating that growth was dependent on

Table 1 Kinetic parameters of L-lactic acid fermentation by R.oryzae in a jar fermentor.

Agitation (rpm)	Aeration (vvm)	μ (h^{-1})	L-lactic acid (g/l)	$Y_{p/s}$ (g/g)	Productivity (g/lh)
200	0.5	0.026	36.33	0.37	0.38
200	1.0	0.030	41.68	0.36	0.43
200	1.5	0.035	42.41	0.38	0.44
300	0.5	0.036	40.35	0.40	0.42
300	1.0	0.042	41.94	0.41	0.44
300	1.5	0.046	42.32	0.41	0.44
400	0.5	0.048	44.88	0.44	0.46
400	1.0	0.054	49.80	0.46	0.51
400	1.5	0.060	54.62	0.49	0.56

μ = specific growth rate; Productivity = g L – lactic acid produced per liter per hour
$Y_{p/s}$ = L-lactic acid yield (g L – lactic acid produced per g total sugar consumed)

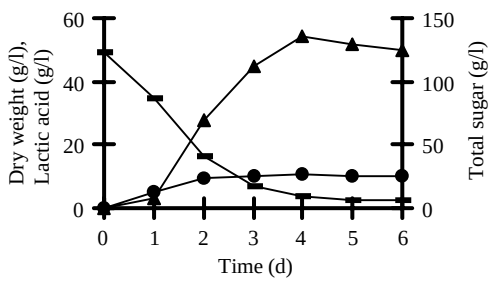


Figure 6 Time courses of L-lactic acid fermentation by *R.oryzae* in a jar fermentor at agitation speed of 400 rpm and aeration rate of 1.5 vvm.
(▲, L-lactic acid concentration; ●, dry weight; –, total sugar)

oxygen supply. In case of the agitation rate of 400 rpm, production, yield and productivity of L – lactic acid were higher than those obtained at the aeration rates of 200 and 300 rpm. This might be due to the medium homogeneity and higher transfer rates of substrate and oxygen resulting from more thorough mixing. In addition, an increase in aeration rate also increased oxygen supply in the medium which promoted not only mycelial growth but also L – lactic acid production. *Rhizopus oryzae* NRRL 395 produced high level of L-lactic acid in aerobic culture while ethanol was produced instead in oxygen limited culture (Soccol *et al*., 1995). Similar result reported by Skory *et al*. (1998) showed that ten-fold increase in alcohol dehydrogenase activity was obtained under oxygen limited condition compared to aerobic condition during L-lactic acid fermentation by *R. oryzae.*

Comparison of L-lactic acid production by *R. oryzae* NRRL 395 between shake flasks and a jar fermentor

The results are summarized in Table 2. For flask culture, the highest L-lactic acid concentration of 68.32 g/l was obtained on day 5 of cultivation with a productivity of 0.57 g/lh and L-lactic acid yield of 0.59 g/g substrate. In jar fermentor, the maximum L-lactic acid production was 54.62 g/l on day 4 of cultivation with a productivity of 0.56 g/lh and a product yield of 0.49 g/g substrate. The reason for L-lactic acid production in the jar fermentor was lower than in the shake flasks might

Table 2 Comparison of L-lactic acid production by *R. oryzae* in shake flasks and in the fermentor.

Fermentation type	Time (day)	μ (h^{-1})	L-lactic acid (g/l)	$Y_{p/s}$ (g/g)	Productivity (g/lh)
Shake flasks	5	0.038	68.32	0.59	0.57
Fermentor@	4	0.060	54.62	0.49	0.56

@ = agitation speed of 400 rpm and aeration rate of 1.5 vvm.

be due to a difficulty in morphological problems and wall growth of mycelium in the fermentor. In the early growth phase of *R. oryzae*, fluffy mycelia were formed. After 2-3 days of culture, the mycelia clung to baffles and impellers causing clumped mycelia form. This might be due to diffusional limitation of oxygen and substrate transfer to the inner aggregated mycelia resulting in low L-lactic acid production. *R. oryzae* was characterized by hyphal growth, formation of a big pellet – like cake, and twisting of hyphae in baffles and impellers of a jar fermentor (Kosakai *et al*., 1997; Yin *et al*., 1997) and Kosakai *et al*. (1997) also reported that L-lactic acid production in a jar fermentor by *R.oryzae* was enhanced by addition of mineral support and polyethylene oxide in the medium. It was found that the yield of lactic acid was 1.7-fold higher than that without the support and polyethylene oxide. This might be due to the formation of dispersed mycelia in the culture broth. Yin *et al*. (1998) reported that small mycelial pellets were effective for the production of lactic acid by *R.oryzae* in an air-lift bioreactor.

CONCLUSION

In this study, we found that *R.oryzae* NRRL 395 has the ability to produce L-lactic acid by using partially hydrolyzed raw cassava starch and ammonium sulfate as carbon and nitrogen sources, respectively. The production of L-lactic acid in the jar fermentor by *R.oryzae* is affected by both agitation and aeration. However, in the fermentor L-lactic acid production was lower than obtained in the shake flask culture. So, it is difficult to produce high yield of L-lactic acid in the stirred tank bioreactor. Further investigation will be required to use proper type of bioreactor and to optimize bioreactor operation for mass production of L-lactic acid by *R.oryzae*

ACKNOWLEDGEMENTS

This research was supported by a grant from Faculty of Science, KMITL (No. 04103722 - 0022). The authors also wish to thank Miss Patcharin Khaosuai for help in preparing manuscript.

LITERATURE CITED

Barker, S.B. 1957. Preparation and colorimetric determination of lactic acid. Method Enzymol. 3 : 241 – 246.

Bernfeld, P. 1955. Amylase α and β. Method Enzymol. 1 : 149 – 158.

Bibal, B., Y. Vayssier, G. Goma, and A. Pareilleux. 1991. High - concentration cultivation of *Lactococcus cremoris* in a cell-recycle reactor. Biotechnol. Bioeng. 37 : 746-754.

Dubois, M., K.A. Gilles, J.K Hamilton, P.A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28 : 350 – 356.

Kascak, J.S., J. Kominek, and M. Roehr. 1996. Lactic acid, pp. 293 – 306. *In* H.J. Rehm, G. Reed, A. Puhler and P. Stadler (eds.).

Biotechnology, Vol. 6. VCH Press, Weinheim.

Kosakai, Y., Y.S. Park, and M. Okabe. 1997. Enhancement of L(+)-lactic acid production using mycelial flocs of *Rhizopus oryzae*, Biotechnol. Bioeng. 55 : 461 – 470.

Skory, C.D., S.N. Freer, and R.J. Bothast. 1998. Production of L-lactic acid by *Rhizopus oryzae* under oxygen limiting conditions. Biotechnol. Lett. 20 : 191 – 194.

Soccol, C.R., B. Marin, M. Raimbault, and J.M. Lebeault. 1994. Potential of solid state fermentation for production of L(+)-lactic acid by *Rhizopus oryzae*., Appl. Microbiol. Biotechnol. 41 : 286 – 290.

Soccol, C.R., V.I. Stonoga, and Raimbault. 1995. Production of L-lactic acid by *Rhizopus* species, World J. Microbiol. Biotechnol. Vol. 10 : 433 – 435.

Vert, M., S.M. Li, G. Spenlehauer, and P. Guerin. 1992. Bioresorbability and biocompatibility of aliphatic polyesters. J. Mater. Sci. Mater. Med. 3 : 432 – 446.

VickRoy, T.B. 1985. Lactic acid. pp. 761 – 776. *In* H.W. Blanch, S. Drew and D.I.C. Wang (eds.). Comprehensive Biotechnology. Pergamon Press, Oxford.

VickRoy, T.B., H.W. Blanch, and C.R. Wilke. 1982. Lactic acid production by *Lactobacillus delbreuckii* in a hollow fiber fermentor. Biotechnol. Lett. 4 : 483 – 488.

Yahiro, K., S. Shibata, S. Jia, Y.S. Park, and M. Okabe. 1997. Efficient itaconic acid production from raw corn starch. J. Ferment. Bioeng. 84 : 375 – 377.

Yin, P., K. Yahiro, T. Ishigaki, Y.S. Park, and M. Okabi. 1998. L(+)-Lactic acid production by repeated batch culture of *Rhizopus oryzae* in air-lift bioreactor, J. Ferment. Bioeng. 85 : 96 – 100.

Yin, P., N. Nishina, Y. Kosakai,K. Yahiro, Y.S. Park, and M. Okabe. 1997. Enhanced production of L(+)-lactic acid from corn starch in a culture of *Rhizopus oryzae* using an air-lift bioreactor, J. Ferment. Bioeng. 84 : 249 – 253.

Yu, R.C. and Y.D. Hang. 1989. Kinetics of direct fermentation of agricultural commodities to L(+)-lactic acid by *Rhizopus oryzae*. Biotechnol. Lett. 11 : 597 – 600.

Received date : 22/03/01
Accepted date : 19/06/01

Kasetsart J. (Nat. Sci.) 35 : 171 - 178 (2001)

Production of Keratinase by *Bacillus* sp. FK 28 Isolated in Thailand

Dakrong Pissuwan and Worapot Suntornsuk

ABSTRACT

Screening for keratinase-producing bacteria and their keratinase production were investigated. Fifty two keratinase-producing bacterial strains were isolated from soils in Thailand on a semi-solid agar medium containing 5% feather powder as a substrate at 37°C. They produced keratinase in a range of 0.7-2.6 U/ml by shaking cultivation. The best keratinase producer was designated as FK 28 closely related to *Bacillus.* Keratinase was produced maximally in the medium containing 1% feather meal with an initial pH of 7.5 under cultivation conditions at 37°C and 150 rpm shaken.

Key words: *Bacillus* sp., feather, keratinase production

INTRODUCTION

Chicken feather mainly contains keratin, which is an insoluble protein with high stability and is indigestible by common proteases (Goddard and Michaelis, 1934; Papadopoulos, 1986). It is recognized as an abundant waste generated from poultry processing industry in Thailand (Anonymous, 1999). The feather can be hydrolysed by keratinase which is a proteolytic enzyme specific to keratins (Friedrich *et al*., 1999). The enzyme is a potential enzyme for removing hair and feather in the poultry industry (Takami *et al*., 1992), for nutritional upgrading of feather meal and conversion of feathers into a feed protein in feed industry (Williams *et al*., 1991), and for clearing obstructions in the sewage system during waste water treatment (Godfrey, 1996). This enzyme has been produced by fungi, including the species of *Aspergillus*, *Onygena*, *Absidia* and *Rhizomucor* (Friedrich *et al*., 1999), some species of dermatophytes, including *Trichophyton mentagrophytes*, *T. rubrum*, *T. gallinae*, *Microsporum canis* and *M. gypseum* (Wawrzkiewicz *et al.*,1991), a few actinomycetes such as *Streptomyces pactum, S. albus, S. fradiae* and *S. thermoviolaceus* (Bockle *et al.*, 1995; Chitte *et al*., 1999; Letourneau *et al*., 1998; Noval and Nickerson, 1959) and bacteria such as some *Bacillus* strains (Takami *et al*., 1992; Williams *et al.*, 1990) and the thermophilic *Fervidobacterium pennovorans* (Friedrich and Antranikian, 1996). In this paper we successfully isolated keratinase-producing bacteria from soils in Thailand and evaluated factors affecting bacterial keratinase production.

MATERIALS AND METHODS

Chemicals

Chicken feather and feather meal were supplied by a local poultry processing factory. The feather was ground by a ball mill to a feather powder. Standard proteins and tyrosine including important chemicals were purchased from Sigma Company.

Enrichment and isolation

Twenty soil samples were collected from

Department of Microbiology, King Mongkut's University of Technology Thonburi, Bangkok 10140, Thailand.

areas in Bangkok and nearby provinces. To enrich keratinase-producing bacteria, five grams of soil were added to 250 ml-Erlenmeyer flasks containing 45 ml enrichment medium [1% (w/v) chicken feather in 0.1% (w/v) peptone]. The flasks were cultivated at the temperature of 30°C on a rotary shaker at 150 rpm for 30 days. During this enrichment, fresh medium was aseptically added every 10 days. The suspension was then plated onto a screening medium that contained (%, w/v): NH_4Cl 0.05, NaCl 0.05, K_2HPO_4 0.03, KH_2PO_4 0.04, $MgCl_2.6H_2O$ 0.024, yeast extract 0.01, agar 2, and feather powder 5, pH 7.5. The inoculated medium was incubated at 37° C for 3-5 days. A pure keratinase-producing culture was obtained by selecting a single colony surrounded by a clear zone. The cultures were kept on nutrient agar at 4°C until used.

Test for keratinase production

A full loop of each pure culture was added to 50 ml liquid medium containing (%, w/v): NH_4Cl 0.05, NaCl 0.05, K_2HPO_4 0.03, KH_2PO_4 0.04, $MgCl_2.6H_2O$ 0.024, yeast extract 0.01 and feather meal 1, pH 7.5 in a 250 ml-Erlenmeyer flask. The culture was grown on a rotary shaker at 150 rpm and incubated at 37°C for 24 h and used as an inoculum.

The enzyme production was done by inoculating 1 ml of each bacterial inoculum into 500 ml-Erlenmeyer flasks containing the same 100 ml liquid medium. The flasks were shaken at 150 rpm and incubated at 37°C for 3 days. The submerged cultures were carried out in duplicate. After 3 days of incubation, the broth was filtered through Whatman No.4 filter paper and the filtrate was collected to examine for keratinase activity and protein content.

Bacterial identification

Several bacterial strains producing high keratinase were identified. Bacterial cells were observed under light microscopy after Gram's and endospore stainings. Physiological and biochemical characteristics were studied followed Bergey's Manuals of Systematic Bacteriology (Kreig and Holt, 1984; Sneath *et al.*, 1986).

Factor affecting keratinase production

The best keratinase-producing strain was cultured under conditions described above for 5 days to examine the bacterial growth and its keratinase production. Factors affecting the keratinase production were investigated under the same conditions previously described. They were initial pH of the medium (pH 4.0-9.0), feather meal concentration (0.5-5.0%, w/v), cultured temperature (25-65°C) and shaking speed (100-250 rpm).

Keratinase assay

The keratinase activity was assayed by the modified method of Cheng *et al.* (1995). The mixture of 10 mg of feather powder suspended in 1 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM $CaCl_2$ and 1 ml of culture filtrate was incubated at 45°C with shaking at 300 rpm for 30 min in a water bath shaker. This elevated temperature was used for the enzyme incubation to accelerate substrate hydrolysis. The enzyme reaction was terminated by adding 2 ml of trichloroacetic acid (TCA) solution (0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid) into the reaction mixture. The mixture was then centrifuged at 3000 x g, 4°C for 30 min and the absorbance of the supernatant was spectrophotometrically measured at wavelength of 275 nm. The enzyme inactivated by TCA solution was used as a control. One unit (U) of keratinase activity was expressed as 1 μmol of tyrosine released per minute under the specific conditions.

Protein and bacterial growth determination

Protein content was analysed using the Lowry method with bovine serum albumin as standard protein (Lowry *et al*, 1951). Bacterial growth was determined by total plate count on nutrient agar.

RESULTS AND DISCUSSION

Isolation of keratinase-producing bacteria

A total of fifty two keratinase-producing bacterial strains was isolated from soil samples collected in Bangkok and nearby provinces (Phetchaburi, Nakhon Pratom and Suphanburi). They showed a clear zone around their colonies on the screening agar medium containing feather powder. The amount and particle size of feather powder in the medium affected the clarity of the clear zone around a colony. Five percent of feather powder (300 meshes) gave the most visible zone (data not shown). Soluble keratin was reported to employ as a substrate in an agar medium to select keratinase-producing fungi (Wawrzkiewicz *et al.*, 1991).

From preliminary study, an isolate produced the maximum keratinase after 3 days of cultivation (data not shown). All isolates yielded keratinase in a range of 0.7-2.6 U/ml as shown in Table 1. The strain FK 28 was able to produce the highest

Table 1 Keratinase production by bacterial isolates from soils in Bangkok and nearby provinces, Thailand.

Isolate	Keratinase (U/ml)	Isolate	Keratinase (U/ml)
B	2.12 ± 0.17	FK 31	1.23 ± 0.13
FK 1	1.56 ± 0.11	FK 32	1.25 ± 0.10
FK 2	1.62 ± 0.10	FK 33	2.38 ± 0.03
FK 3	1.32 ± 0.61	FK 36	1.23 ± 0.02
FK 4	0.73 ± 0.17	FK 39	1.62 ± 0.01
FK 5	1.35 ± 0.42	FK 40	1.91 ± 0.03
FK 6	1.05 ± 0.04	FK 41	1.35 ± 0.05
FK 7	0.99 ± 0.04	FK 42	2.21 ± 0.13
FK 8	1.45 ± 0.04	FK 43	1.72 ± 0.23
FK 9	1.10 ± 0.16	FK 44	1.18 ± 0.05
FK 10	1.79 ± 0.03	FK 45	0.98 ± 0.44
FK 11	1.80 ± 0.00	FK 46	2.05 ± 0.02
FK 13	0.89 ± 0.09	FK 47	1.10 ± 0.04
FK 14	1.94 ± 1.08	FK 48	1.09 ± 0.13
FK 15	1.12 ± 0.07	FK 49	1.58 ± 0.25
FK 16	1.34 ± 0.45	FK 50	1.75 ± 0.01
FK 17	1.68 ± 0.45	FK 51	1.34 ± 0.09
FK 18	0.94 ± 0.08	FK 52	1.32 ± 0.05
FK 19	1.24 ± 0.04	FK 53	1.95 ± 0.65
FK 20	0.77 ± 0.02	FK 54	2.12 ± 0.00
FK 21	1.60 ± 0.04	FK 55	1.79 ± 0.20
FK 23	1.56 ± 0.16	FK 56	2.00 ± 0.00
FK 24	0.98 ± 0.09	FK 57	1.80 ± 0.01
FK 26	2.23 ± 0.11	FK 58	1.47 ± 0.03
FK 27	1.06 ± 0.11	FK 62	1.63 ± 0.13
FK 28	2.60 ± 0.03	FK 65	1.87 ± 0.18
FK 29	1.61 ± 0.23		

keratinase activity of 2.6 U/ml with specific activity of 1.87 U/mg. It occurred singly or in chains and were straight rods (0.6 to 1.2 μm wide by 2.5 to 5.0 μm long in size), Gram positive and endospore-forming strain. It was aerobic, motile, and oxidase and catalase positive. From the morphological studies, stainings and biochemical examinations, the strain FK 28 was identified as the genus *Bacillus* (Table 2).

Bacillus species have been known to produce a number of hydrolytic enzymes including keratinase, which is able to degrade feathers, wool and hair. Williams *et al.* (1990) found a straight rod shaped, aerobic and endospore-forming bacterium appeared in singly and in chains, catalase positive and Gram variable, classified as *Bacillus licheniformis* PWD-1. This bacterium was isolated from a high temperature poultry waste digestor and was able to degrade feather keratin when using feathers as a primary source of carbon and energy. Atalo and Gashe (1993) described a thermophilic *Bacillus* species that could produce protease to degrade various fibrous protein such as feather, hair, sheep skin or horn. In addition, Takami *et al.* (1989, 1992) reported a rod-shaped and spore-forming bacterium, identified as *Bacillus* sp. No. AH-101, that produced a thermostable alkaline protease to degrade human hair. Friedrich and Antranikian (1996) described that a keratin-degrading strain of a thermophilic anaerobic bacteria, rod-shaped with an outer sheath-like structure of 2.0-20 μm long, occurred singly or in pairs, Gram negative and no endospore-forming. Lal *et al*. (1999) reported *Bacillus* spp. were able to

Table 2 Some characteristics of the strain FK 28 and a species of *Bacillus*.

Characteristics	FK 28	*Bacillus* sp.
Rod-shaped	+	+
Diameter over 2.5 μm	-	-
Gram reaction	+ or variable	+
Endospore produced	+	+
Motility	+	+
Anaerobic growth	-	-
Catalase	+	+
Oxidase	+	D
Voges-Proskauer reaction	-	-
Indole production	-	-
Nitrate reduction	-	D
Growth in 5% NaCl	+	D
7% NaCl	+	D
10% NaCl	-	D
Starch hydrolysis	-	-
Casein hydrolysis	+	+
Temperature for growth (°C)	RT to 45	RT to 65
Citrate utilization	+	D
Urea hydrolysis	+	D

Symbols: + positive; - negative; D substantial proportion of species differ;
RT room temperature

degrade keratin substrates from human hair, human nails, cow horn and cow hooves *in vitro*.

Factors affecting keratinase production

Bacterial growth and keratinase production by *Bacillus* sp. FK 28 during cultivation were illustrated in Figure 1. The keratinase activity was maximal during late exponential growth after 48 h cultivation (2.6 U/ml) and rapidly decreased during the stationary phase. Similar result was found in protease production by other *Bacillus* strains (Atalo and Gashe, 1993; Cheng *et al*., 1995).

The amount of keratinase production depended on substrate concentrations and cultivation conditions. Feather meal concentration, initial pH, shaking speed and culture temperature were affected keratinase production (Figures 2-5). The highest keratinase production was obtained at 1% feather meal (Figure 2). Cheng *et al*. (1995) also reported that 1% feather powder gave the highest keratinase activity for *B. licheniformis* PWD-1. Keratinase produced at a level of 0.5% feather meal was ten times less than that produced at 1% feather meal. This is because the amount of substrate supplied for growth and enzyme production was insufficient, while 2.5% and 5% feather meal showed substrate repression on keratinase production. High feather meal concentration also increased medium viscosity which possibly resulting in oxygen limitation for bacterial growth. Similar finding was found with high feather concentration (2%) but lower protease production by *Streptomyces thermonitrificans* (Mohamedin, 1998).

Maximal keratinase production was obtained when an initial pH of medium was 7.5 (Figure 3). Poor growth was observed when initial pHs were at 4.0, 6.0 and 9.0. Altalo and Gashe (1993) found the optimum pH of keratinase production by *Bacillus* species P-001A at pH 9.5. Cheng *et al* (1995) also reported the highest keratinase production by *B. licheniformis* PWD-1 was at an initial pH of 8.7.

The shaking speed at 150 rpm yielded maximum keratinase production (Figure 4). Generally, increased shaking speed provided high oxygen transfer rate supporting cell growth. However, high shaking speed (200-250 rpm) gave good bacterial growth but low keratinase production possibly because of too high dissolved oxygen and too much shear stress repressed keratinase synthesis and excretion. Perlman (1969) reported that vitamin

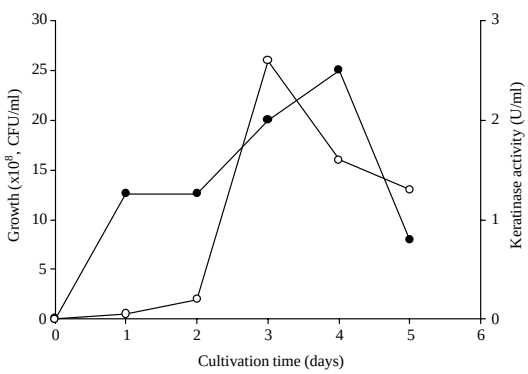


Figure 1 Growth (•) and keratinase (o) production by *Bacillus* sp. FK 28 cultivated in liquid medium containing 1% feather meal as a substrate at pH 7.5, 37°C and 150 rpm shaken.

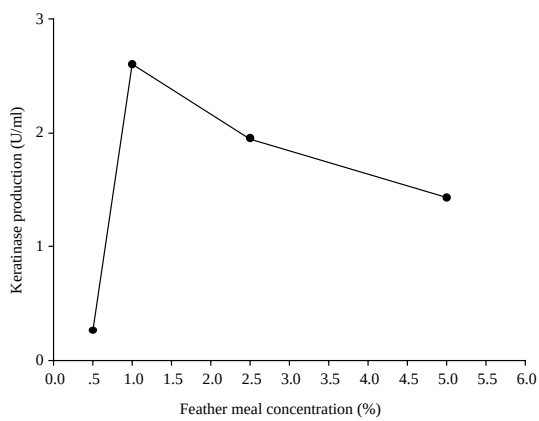


Figure 2 Effect of feather meal concentration (%) on keratinase production by *Bacillus* sp. FK 28 at pH 7.5, 37°C and 150 rpm shaken.

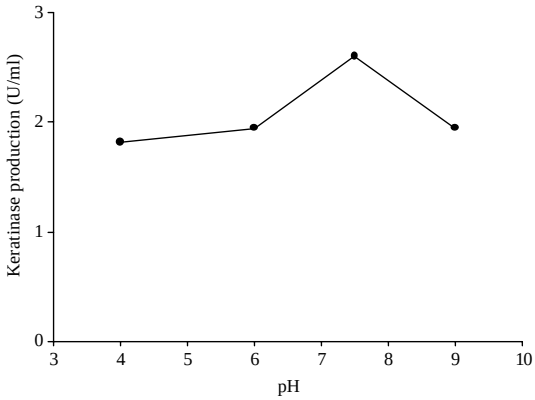


Figure 3 Effect of initial pH on keratinase production by *Bacillus* sp. FK 28 in 1% feather meal at 37°C and 150 rpm shaken.

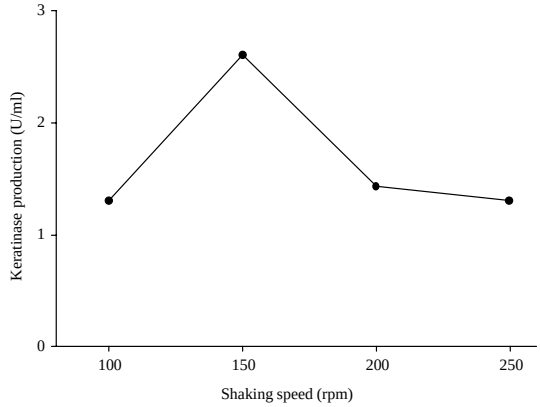


Figure 4 Effect of shaking speed on keratinase production by *Bacillus* sp. FK 28 in 1% feather meal at pH 7.5 and 37°C.

B_{12}-producing enzyme was inhibited by high aeration. At low shaking speed (100 rpm), bacterial cells and substrate were not well mixed with heterogeneous distribution and lower oxygen dissolved resulting in low keratinase production.

The highest bacterial growth and keratinase production were found at the incubation temperature of 37°C (Figure 5). No keratinase production was observed at 50°C and 65°C because of an absence of bacterial cell growth at such high temperatures. Poor growth and 50% less keratinase production were found at 25°C. Compared with *B. licheniformis* PWD-1, the maximum growth was reported at 50° C (Cheng *et al*., 1995; William *et al*., 1990), while the maximum enzyme production was obtained at 45°C (Cheng *et al*., 1995).

In conclusion, a bacterial strain designated as FK 28, isolated from soils in Thailand, is closely related to *Bacillus*. It was able to produce keratinase and could be applied for feather degradation into feather protein hydrolysate used in feed industry. Its keratinase production was maximal in a medium containing 1% feather meal as a substrate, pH 7.5, and under cultivation conditions at the temperature of 37°C and the shaking speed of 150 rpm. Identification of bacterial isolates by a rapid biochemical test kit and 16s rRNA analysis is under investigation.

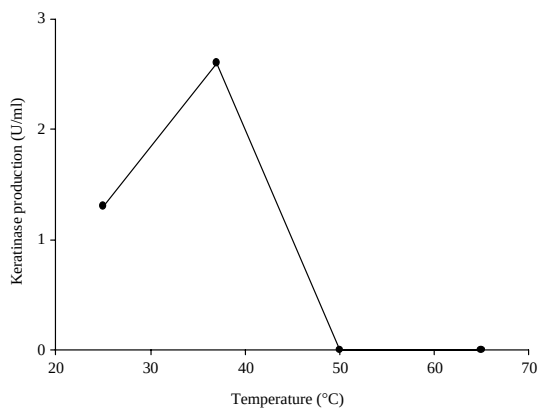


Figure 5 Effect of cultivation temperature on keratinase production by *Bacillus* sp. FK 28 in 1% feather meal at pH 7.5 and 150 rpm shaken.

ACKNOWLEDGEMENT

The authors would like to thank the Royal Thai Government and International Foundation for Science for the financial support.

LITERATURE CITED

Altalo, K. and B.A. Gashe. 1993. Protease production by a thermophilic *Bacillus* species (P-001A) which degrade various kinds of fibrous protein. Biotechnol. Lett. 15: 1151-1156.

Anonymous. 1999. Agricultural Statistics of Thailand Crop Year 1999. Office of Agricultural Economics, Bangkok. 275p.

Bockle, B., B. Galunsky, and R. Muller. 1995. Characterization of a keratinolytic serine proteinase from *Streptomyces pactum* DSM 40530. Appl. Environ. Microbiol. 61 : 3705-3710.

Cheng, S.W., H.M. Hu, S.W. Shen, H.Takagi, M. Asano, and Y.C. Tsai. 1995. Production and characterization of keratinase of a feather-degrading *Bacillus licheniformis* PWD-1. Biosci. Biotechnol. Biochem. 59 : 2239-2243.

Chitte, R. R., V.K. Nalawade, and S. Dey. 1999. Keratinolytic activity from the broth of a feather-degrading thermophilic *Streptomyces thermoviolaceus* strain SD8. Lett. Appl. Microbiol. 28 : 131-136.

Friedrich, A.B. and G. Antranikian. 1996. Keratin degradation by *Fervidobacterium pennavorans*, a novel thermophilic anaerobic species of the order *Thermotogales*. Appl. Environ. Microbiol. 62 : 2875-2882.

Friedrich, J., H. Gradisar, D. Mandin, and J.P. Chaumont. 1999. Screening fungi for synthesis of keratinolytic enzymes. Lett. Appl. Microbiol. 28 : 127-130.

Goddard, D.R. and L. Michaelis. 1934. A study of keratin. J. Biol Chem. 106 : 604-614.

Godfrey, T. 1996. Protease in waste treatment, pp. 315-316. *In* T. Godfrey and S. West (eds.) Industrial Enzymology. Macmillan Press, London.

Kreig, N. R. and J. G. Holt. 1984. Bergey's Manual of Systematic Bacteriology Vol.1. Williams and Wilkins, Baltimore. 964 p.

Lal, S., R. C. Rajak, and S. K. Hasija. 1999. *In vitro* degradation of keratin by two species of *Bacillus*. J. Gen. Appl. Microbiol. 45 : 283-287.

Letourneau F., V. Soussotte, P. Bressollier, P. Branland, and B. Verneuil. 1998. Keratinolytic activity of *Streptomyces* sp. $S.K_{1-02}$: a new isolated strain. Lett. Appl. Microbiol. 26 : 77-80.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193 : 265-275.

Mohamedin, A.H. 1998. Isolation, identification and some cultural conditions of a protease-producing thermophilic *Streptomyces* strain grown on chicken feather as a substrate. Int. Biodeterioration and Biodegradation 43 : 13-21.

Noval, J.J. and W.J. Nickerson. 1959. Decomposition of native keratin by *Streptomyces fradiae*. J. Bacteriol. 77 : 251-263.

Papadopoulos, M.C. 1986. The effect of enzymatic treatment on amino acid content and nitrogen characteristics of feather meal. Anim. Feed Sci. Technol. 16 : 151-156.

Perlman, D. 1969. Fermentation Advances. Academic Press, New York. 909 p.

Sneath, P.H.A., N.S. Mair, M.E. Sharpe, and J.G. Holt. 1986. Bergey's Manual of Systematic Bacteriology Vol.2. Williams and Wilkins, Baltimore. 1599 p.

Takami, H., N. Satoshi, R. Aono, and K. Horikoshi. 1992. Degradation of human hair by a thermostable alkaline protease from alkaliphilic *Bacillus sp.* no. AH-101. Biosci. Biotechnol. Biochem. 56 : 1667-1669.

Takami, H., T. Akiba, and K. Horikoshi. 1989. Production of extremely thermostable alkaline protease from *Bacillus sp.* no. AH-101. Appl. Microbiol. Biotechnol. 30 : 120-124.

Wawrzkiewicz, K., T. Wolski, and J. Lobarzewski.

1991. Screening the keratinolytic activity of dermatophytes *in vitro*. Mycophthologia 114 : 1-8.

Williams, C.M., C. G. Lee, J. D. Garlich, and J.C.H. Shih. 1991. Evaluation of a bacterial feather fermentation product, feather-lysate, as a feed protein. Poultry Sci. 70 : 85-94.

Williams, C.M., C.S. Richter, J. M. MacKenzie, and J.C.H. Shih. 1990. Isolation, identification and characterization of a feather-degrading bacterium. Appl. Environ. Microbiol. 56 : 1509-1515.

Received date : 6/03/01
Accepted date : 29/06/01

Kasetsart J. (Nat. Sci.) 35 : 179 - 187 (2001)

Isolation and Characterization of Extracellular Halophilic Ribonuclease from Halotolerant *Pseudomonas* species

Werasit Kanlayakrit, Takahiro Ikeda, Somporn Tojai, Mangkorn Rodprapakorn and Sarote Sirisansaneeyakul

ABSTRACT

A halotolerant bacterium isolated from Thai fish sauce, obtained in Surathani province, was identified as a *Pseudomonas* sp. No. 3241. This strain showed halophilic ribonuclease activity. When casamino acids (CA) and yeast extract (YE) were used as the nitrogen source in a mini jar fermenter, a ratio concentration of CA to YE of 15:20 g/l in Sehgal and Gibbons Complex (SGC) medium, without NaCl, gave maximum growth and ribonuclease activity (18.18 U/ml). The ribonuclease enzyme was optimal at pH 10.0 and at the temperature of 50°C. It had marked halophilic enzyme properties that required an optimal NaCl concentration of 3 M. The ribonuclease was stable between pH 6.0 and 9.0 and at temperatures between 30 and 40°C.

Key words : halophilic, nuclease, ribonuclease, halotolerant, *Pseudomonas* sp.

INTRODUCTION

As halophilic means "salt loving" halophilic enzymes or bacteria by definition require NaCl for activity or growth. In contrast, halotolerant forms do not require NaCl for growth. Nucleases comprise of deoxyribonucleases (DNase) and ribonucleases (RNase). The latter are enzymes that catalyze RNA to 5'-ribonucleotides (5'-GMP, 5'-AMP, 5'-CMP and 5'-UMP) or to 2', 3'-nucleotides. DNase, on the other hand, catalyzes DNA to 5'-deoxyribonucleotides (5'-GMP, 5'-AMP, 5'-CMP and 5'-TMP) or 2', 3'-nucleotides. According to Kuninaka *et al*. (1961), nucleoside-5'-monophosphates, especially 5'-IMP and 5'-GMP, are flavoured, and there is a specific synergistic action as regard taste between monosodium glutamate (MSG) and nucleoside–5'-monophosphate. Kamekura and Onishi (1974) first reported a halophilic nuclease produced by the moderately halophilic bacterium, *Micrococcus varians* var. *halophilus.* This enzyme has marked halophilic properties, requiring an optimal level of 2.9 M NaCl. Furthermore, Onishi *et al*. (1983) reported a halophilic nuclease from a moderately halophilic *Bacillus* sp. N23-2, which was later identified by Ventosa (1991) as *Bacillus halophilus.* Maeda and Taga (1976a, b) also studied an extracellular deoxyribonuclease from *Vibrio* sp. strain No. 2, which was isolated from seawater, but they did not report any halophilic nuclease properties. These halophilic and marine bacteria nucleases showed both ribonuclease and deoxyribonuclease activities in the presence of salt (Kamekura and Onishi, 1974; Onishi *et* al., 1983; Maeda and Tage, 1976b). Since then, no further halophilic ribonucleases from halotolerant microorganisms have been reported. Concerning

Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok 10900, Thailand.

the production of fish source, the application of halophilic ribonuclease may be expected that the synergistic action between 5'-GMP and L-glutamate will give better taste (Kurihara 1987). This paper describes the strain identification, enzyme production and characterization of a crude halophilic ribonuclease from a halotolerant *Pseudomonas* sp. No. 3241.

MATERIALS AND METHODS

Organism preparation

Strain No. 3241, isolated from fermented fish sauce in Surathani province Thailand, was stored at 4°C on an agar slant of Sehgal and Gibbons Complex (SGC) medium (Sehgal and Gibbons, 1960), supplemented with 2.0 M NaCl.

Nuclease activity test on plate agar

Firstly, Bacto DNase activity was tested using deoxyribonucleic acid as a substrate in petri dishes. DNase test medium was supplemented with 1, 2 and 3 M of NaCl. Control cultures lacked NaCl. The strain was spotted onto test agar and then incubated at 37°C for 10 days. A positive reaction for the DNase test was indicated by the disappearance of methyl green around the colony, and the width of this clear zone was considered to be directly related to the amount of extracellular deoxyribonuclease produced. The enzyme production and growth ratio was defined as A/B, where A was the diameter of clear zone (cm), and B the diameter of the colony size (cm).

Ribonuclease production in shaken flasks

An inoculum was prepared by transferring one loop of the strain into SGC seed medium (pH 7.0) with 2.0 M NaCl. This was then agitated on a rotary shaker at 30°C, 250 rpm for 18 h. Later, 5 ml of this seed culture was inoculated into 50 ml SGC production medium, containing various concentrations of NaCl, in 250-ml Erlenmeyer flasks. Cultivation conditions were the same as that for the seed culture.

Ribonuclease production in mini jar fermenter

A volume of 1,350 ml of SGC production medium, supplemented with twice the nitrogen source without NaCl was placed into a 2-L mini jar fermenter (Biostat B, B. Braun Biotech International GmbH, Germany) and autoclaved at 121°C for 30 min. After being cooled, it was inoculated with 150 ml of SGC seed culture containing 2.0 M NaCl. Fermentation conditions were maintained at 30°C, 600 rpm-agitation and air supply at 1vvm. The pH of the broth was not controlled during this process. After cultivation and cooling, the culture broth was centrifuged and the supernatant was subjected to partial purification (see below).

Ribonuclease activity

Halophilic ribonuclease activity was measured by a modificaiton of the method used by Kamekura and Onishi (1974). The assay substrate contained 1 mg/ml ribonucleic acid (from *Torula* yeast), 1.8 mM NaCl, 0.04 mM Tris-HCl buffer (pH 8.0), and 0.01 mM $MgSO_4 .7H_2O$. This was mixed with 1 ml enzyme solution and incubated at 40°C for 2 h. Enzyme activity was terminated with 6 ml 99.9% cold ethanol for 20 min at 0°C. The precipitate was removed by centrifugation at 8,000 × g at 4°C for 20 min. The supernatant (1 ml) was diluted 5 times with distilled water and absorbance was measured at 260 nm. A blank measure was carried out using 1 ml of substrate solution alone, followed by incubation at 40°C for 2 h. This was then supplemented with 1 ml of enzyme solution followed immediately with 6 ml 99.9% cold ethanol. One unit of the ribonuclease activity was defined as the amount of enzyme catalyzing an increase of 1.0 in absorbance at 260 nm under the above conditions. Ribonuclease activity was calculated from the following equation :

Ribonuclease activity (U/ml, where S and B refer to sample and blank, respectively.)

$$= \frac{Abs(S) - Abs(B)}{Abs(B)} \times dilution$$

Identification of bacterial strain

Identification of the strain No. 3241 producing nuclease was based first on Gram staining, followed by physiological properties determined using standard methods and the API system.

Partial purification of crude halophilic ribonuclease

All steps were carried out at 5°C in a refrigerated room. The supernatant from the mini jar fermenter (see above) was mixed slowly with an equal volume of 99.9% cold ethanol at 0°C on ice. After standing overnight, the mixture was centrifuged at 12,000 × g for 20 min. The precipitate was dissolved in a minimum volume of 0.01M Tris-HCl buffer pH 8.0, then 2.0 M NaCl was added, followed by dialysis against the previously mentioned Tris-Hce buffer for 24 h and subsequent lyophilization.

Protein determination

Protein concentration was determined by the Folin-Phenol method (Lowry *et al*., 1951) with bovine serum albumin as a standard.

Nitrogen determination

The defermination of nitrogen concentration was carried out by the ninhydrin method (Moore and Stein, 1954) with a mixture of casamino acids and yeast extract as a standard.

Carbohydrate determination

Total carbohydrate was determined by the phenol-sulphuric acid method (Dubois *et al*., 1956) with glucose as a standard.

Determination of dry cell weight

Three ml of the sample was filtered with using 0.2-μm filter membrane under vacuum. After drying at 105°C for 24 h, the dry cell weight was calculated as follows:

$$\text{Dry cell weight} = \frac{(W_1 - W_0) \times 1,000}{3}$$

where, W_o = weight (g) of membrane before filtration ; W_1 = weight (g) of membrane after filtration and drying.

Chemicals

Bacto DNase test agar, with methyl green and casamino acids, was purchased from Difco Laboratories, USA. Yeast extract was purchased from E. Merck, Germany. *Torula* yeast ribonucleic acid, dialysis tubing, bovine serum albumin and Tris (hydroxymethyl) aminomethane wree obtained from Sigma Chemical Co., Ltd. USA. The API system was purchased from bioMerieux, France. Filtration membranes (pore size 0.2 μm) were purchased from Sartorius Co., Ltd. Germany.

RESULTS

Effect of NaCl concentrations on DNase test plates

The first screening of nuclease was carried out using DNase test plates, with deoxyribonucleic acid as a substrate with various concentrations of NaCl. Figure 1 shows a strongly positive ratio of deoxyribonuclease activity at about 6.7 at 0 M NaCl for 4 days. However, at 1 and 2 M, NaCl had little effect, while 3 M NaCl gave a negative ratio. Strain No. 3241 could produce deoxyribonuclease over a range of 0 to 2 M of NaCl. This shows that strain No. 3241 has halotolerant DNase activity, as the activity at 0 M NaCl is higher than those supplemented with 1, 2 and 3 M.

Strain identification

Table 1 shows the physiological properties of the strain, determined by standard methods, in the absence of NaCl. At the first stage of identification the organism was characterized as Gram-negative rods, motile, aerobic (facultative),

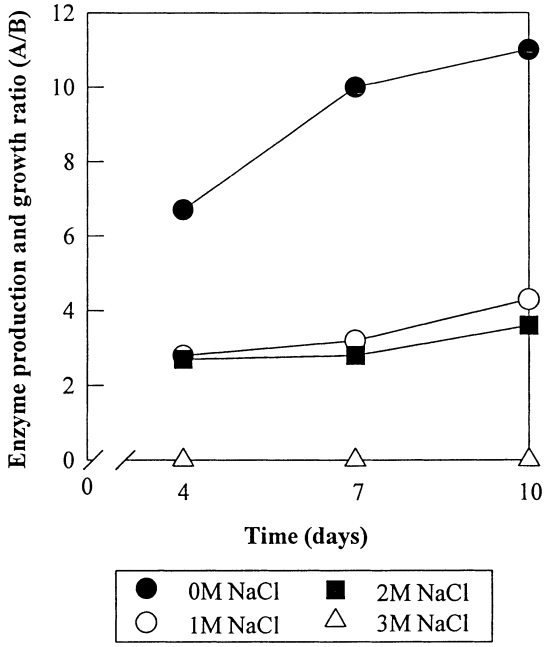


Figure 1 Effect of NaCl concentrations on DNase plate test of strain No. 3241. A : clear zone diameter (cm); B: colony size diameter (cm).

catalase positive and oxidase positive. From these above characteristics, strain No. 3241 was placed into the genus *Pseudomonas*. The second stage of identification using the API system characterized it as the genus *Pseudomonas* with a reliability of 79.5%. In this case, species identification was insufficient, as the API system does not identify all kinds of *Pseudomonas* species. The growth range in 3.0 M NaCl was less than that of at 0 M, so strain No. 3241 was classified as a halotolerant *Pseudomonas* sp. No. 3241. This identification, however, is still preliminary.

Effects of temperature and NaCl concentration on specific growth rate and enzyme productivity

Figure 2A demonstrates that there was no difference in specific growth rates at various NaCl concentrations at 30°C and 37°C. At higher temperature (45°C) without NaCl, *Pseudomonas* sp. No. 3241 could not grow. Generally, only

Table 1 Physiological characteristics of strain No. 3241.

Determination	Reaction
Gram stain	–
Morphology	Rod
Pigment	CR
Catalase	+
Cytochrome oxidase	+
Growth on MacConkey agar	+
Motility	+
Spore stain	–
Starch hydrolysis	–
Casein digestion	+
Oxidative-Fermentative (OF) medium	N
Anaerobic growth	+
Growth in 0 M NaCl	+
1 M NaCl	+
2 M NaCl	+
3 M NaCl	+
4 M NaCl	–
Growth at 42°C	–
API test	
ONPG (β-galactosidase)	WK
ADH (Arginine dihydrolase)	–
LDC (Lysine decarboxylase)	–
ODC (Ornithine decarboxylase)	–
CIT (Citrate utilization)	–
H_2S (H_2S production)	–
URE (Urease)	–
TDA (Tryptophan deaminase)	–
IND (Indole production)	–
VP (Acetoin production)	+
GEL (Gelatinase)	–
NO_2 production	
Reduction to N_2 gas	–
Sugar fermentation	
GLU (Glucose)	–
MAN (Mannitol)	–
INO (Inositol)	–
SOR (Sorbitol)	–
RHA (Rhamnose)	–
SAC (Sucrose)	–
MEL (Melibiose)	–
AMY (Amygdalin)	–
ARA (Arabinose)	–

Key : rod = rod shape, CR = cream, N = no acid production WK = weak positive.

thermophilic bacteria can grow in such high temperature. However, in this case NaCl enhanced the growth at 45°C. We hypothesize that the sodium cation may stabilize and protect the protein from denaturation in the cell membrane even at such high temperature. Figure 2B demonstrates that increasing of salt concentration and temperature caused a decrease in enzyme productivity. The maximum value was observed using 0 M NaCl at 30°C (0.16 U/ml/h). Ribonuclease from *Pseudomonas* sp. No. 3241 was denatured at 45°C. One conclusion from these results is that *Pseudomonas* sp. No. 3241 can produce a ribonuclease enzyme both in the presence or absence of salt. The best production, however, was observed in SGC medium without NaCl.

Effect of nitrogen sources on specific growth rate and enzyme productivity during shaking

Specific growth rates and enzyme productivity did not increase with increasing nitrogen source (Figure 3), and optimal conditions were observed at a CA:YE ratio of 15:20 g/l in SGC medium without NaCl.

Effect of glucose on specific growth rate and enzyme productivity

Figure 4 shows the effect of glucose as carbon source on the specific growth rate and enzyme productivity in SGC medium containing a ratio concentration of CA:YE at 15:20 g/l. Increasing the glucose concentration resulted in the reduction of specific growth rate and enzyme productivity. These results might stem from the repression of respiratory enzymes by glucose acting as a catabolite repersor (the "Crabtree" effect). (Blanch and Clark, 1997).

Growth and enzyme production in mini jar fermenter

Using nitrogen sources of CA:YE at 15:20 g/l in SGC medium without NaCl, the fermentation process was classified as follows: phase 1 (0 ~ 22 h) ; phase 2 (22 ~ 32 h) ; phase 3 (32 ~ 48 h) (Figure 5). In phase 1, both carbon and nitrogen sources were slightly utilized and decreased in amount ; maximum specific growth rate (μ_{max}) and growth-associated ribonuclease production were observed in this phase (logarithmic growth phase). In the

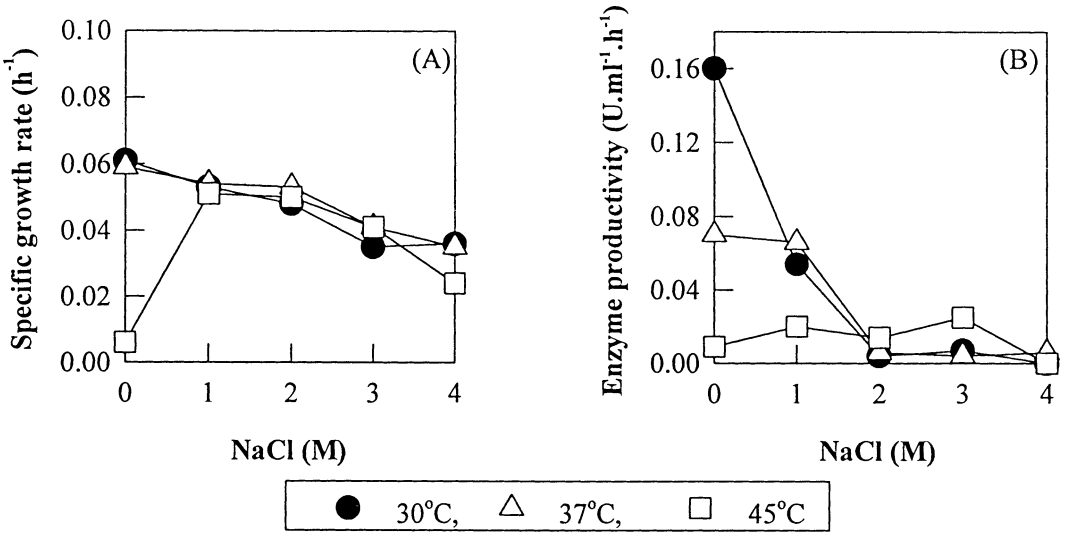


Figure 2 Effects of temperature and NaCl concentration on specific growth rate and enzyme productivity of *Pseudomonas* sp. No. 3241. Key (A) : Specific growth rate; (B): Enzyme productivity.

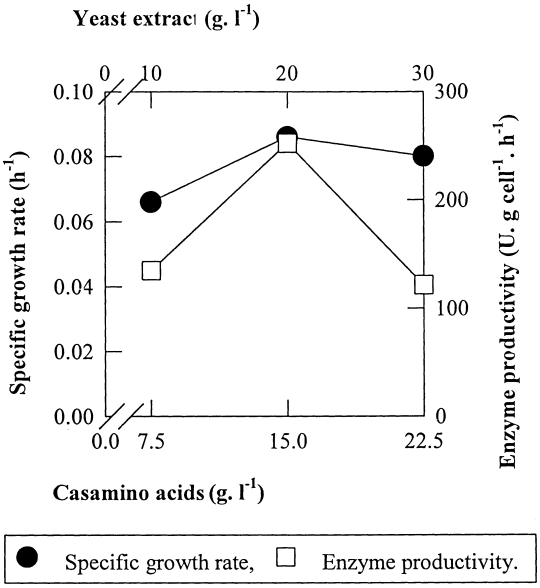


Figure 3 Effect of nitrogen sources on specific growth rate and enzyme productivity of *Pseudomonas* sp. No. 3241.

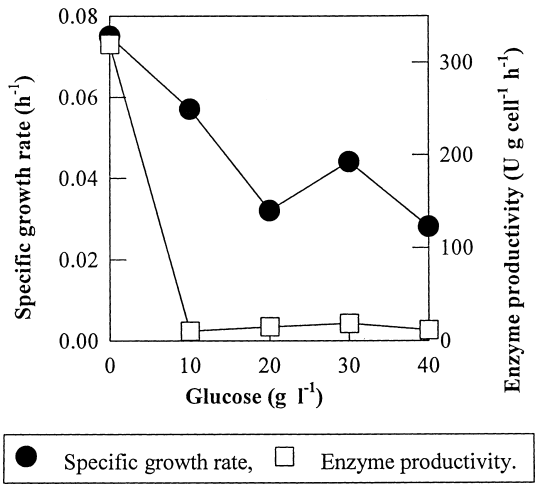


Figure 4 Effect of glucose on specific growth rate and enzyme productivity of *Pseudomonas* sp. No. 3241.

second (stationary) phase, the carbon source was not utilized completely, but on the other hand nitrogen sources were still consumed. The maximum enzyme production (18.18 U/ml) of halophilic ribonuclease was observed at 32 h. In the last phase, growth was still stationary, but enzyme activity decreased. It is possible that cell lysis occurred, or that lost of activity due to a high concentration of ribonuclease (product inhibition).

Partial purification

A summary of the partial purification of the halophilic ribonuclease enzyme from *Pseudomonas* sp. No. 3241 is shown in Table 2. The best fractionation of the enzyme was obtained at a final concentration of 50% of ethanol. The final specific activity of ribonuclease was 77.5 U/mg protein with 95% total yield.

Crude enzyme characteristics

A quantity of 270 ml of crude ribonuclease enzyme was obtained through batch fermentation and stored at –20°C. The crude enzyme activity had an optimal pH and temperature of 10.0 and 50°C, respectively (Figure 6A and B). The enzyme was stable between pH 6.0 and 9.0 at 40°C for 24 h and was stable for 30 min between 30 and 40°C at pH 8.0 (Fig. 7 A and B). The effect of NaCl on the activity of crude ribonuclease enzyme is presented in Figure 8 This enzyme had marked halophilic properties, which required an optimal NaCl level of 3.0 M. In addition, activity at 3.5 M NaCl was higher than that at 2.5 M. From these properties, it is concluded that ribonuclease from halotolerant *Pseudomonas* sp. No. 3241 enzyme is a form of halophilic ribonuclease.

DISCUSSION

There are only a few reports of halophilic nuclease production by halophiles, including *Micrococcus varians* var. *halophilus* (Kamekura and Onishi, 1974) and *Bacillus halophilus* (Onishi *et al*., 1983, Ventosa, 1991). In these experiments, halophilic nuclease from slightly or moderately

Table 2 Partial purification of ribonuclease from halotolerant *Pseudomonas* sp. No. 3241.

Purification steps	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)
Culture filtrate	1,320	4,224	10,982.4	2.6	100
50% ethanol precipitation	220	176	10,467.6	59.48	95.31
Dialysis with 0.01 M Tris-HCl buffer+2M NaCl	270	135	10,459.8	77.48	95.24

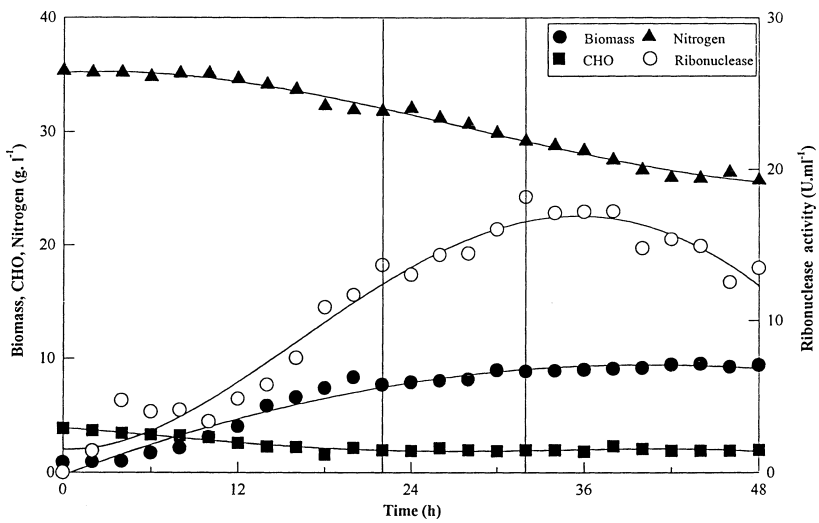


Figure 5 Batch cultivation of halotolerant *Pseudomonas* sp. No. 3241 in 2-L mini jar fermenter.

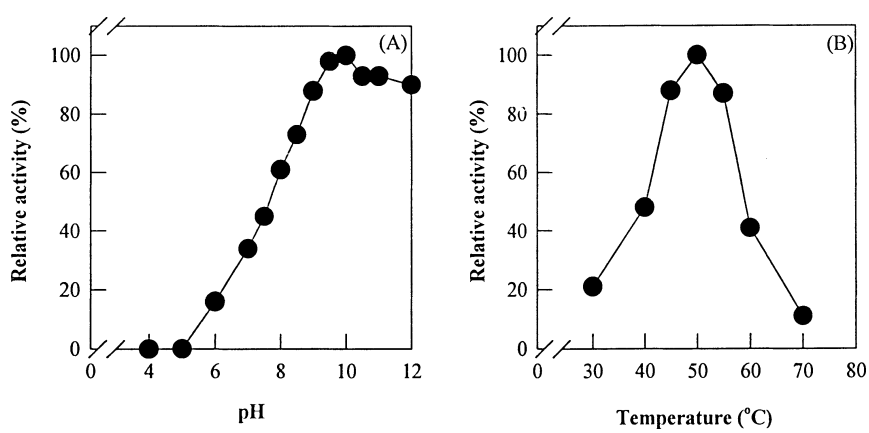


Figure 6 Effects of pH and temperature on activity of the crude ribonuclease from halotolerant *Pseudomonas* sp. No. 3241. Key (A) : Optimal pH; (B) : Optimal temperature.

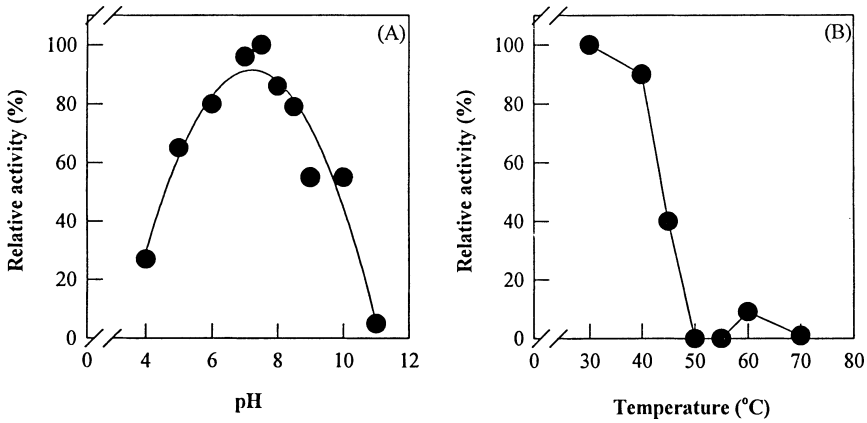


Figure 7 pH and temperature stability of crude ribonuclease from halotolerant *Pseudomonas* sp. No. 3241. Key (A) : pH stability; (B) : Temperature stability.

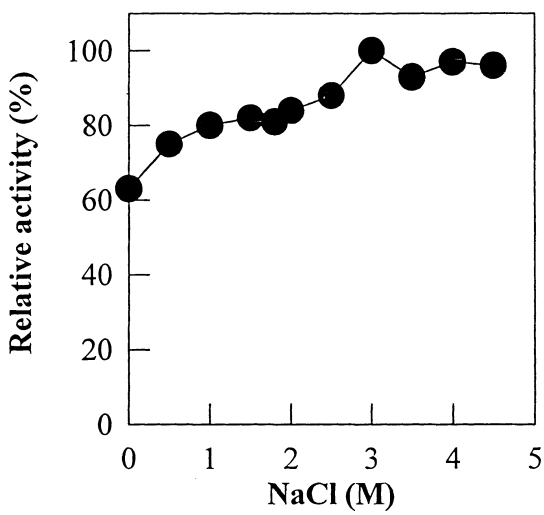


Figure 8 Effect of NaCl on activity of crude ribonuclease from halotolerant *Pseudomonas* sp. No. 3241.

halophilic bacteria, but not from halotolerant bacteria, were used. In the present study a halophilic ribonuclease from a halotolerant bacterium was isolated and investigated for the first time. Halotolerant DNase from halotolernat *Pseudomonas* No. 3241 was first found by the plate test, then halophilic RNase from halotolerant *Pseudomonas* No. 3241 was investigated following partial purification of enzyme.

One unusual outcome from these experimental results is that growth medium containing a high concentration of NaCl enabled the bacterium to grow even at high temperature (Figure 2A). Interactions between the effects of temperature and NaCl concentration on growth of halophilic bacterium have been reported by Ohno *et al.* (1979). In the case of moderately halophilic species, *Pseudomonas halosaccharolytica*, one possible explanation of the regulatory mechanism of membrane structure and function in high NaCl concentration is as follows. The total amount of negatively charged phospholipids in cells increases with higher concentrations of NaCl in the medium. High content of negatively charged phospholipids may contribute to the regulation of cation permeability in a salty environment. Negatively charged phospholipids may be increased selectively in the permeability of cations in a cell. One reason for this is the presence of a regulatory mechanism to maintain suitable proportions of individual phospholipids for cation shielding, and to adjust adequately the internal concentration of cations in the presence of NaCl at high temperature. It was observed that, while the growth of *Pseudomonas* No. 3241 was rescued or stabilized in the presence of NaCl at high temperature, enzyme production

was inhibited. Formerly, there were no other reports of halophilic nucleases from halotolerant *Pseudomonas* species, and this is the first research reported on a halophilic ribonuclease from halotolerant *Pseudomonas* sp. isolated from fish sauce. In future research it is proposed to purify this enzyme and apply the crude enzyme to Thai fish sauce production for taste improvement.

LITERATURE CITED

Blanch, H.W. and D.S. Clark. 1997. Biochemical Engineering. Marcel Dekker, Inc. New York. 702 p.

Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Roberts, and F. Smith, 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28 : 350-356.

Kamekura, M. and H. Onishi, 1974. Halophilic nuclease from a moderately halophilic *Micrococcus varians*. J. Bacteriol. 119 : 339-344.

Kuninaka, A., M. Kibi, H. Yoshino, and K. Sakaguchi. 1961. Studies on 5'-phosphodiesterases in microorganisms. Part 2: Properties and application of *Penicillium citrinum* 5'-phosphodiesterase. Agric Biol. Chem. 25 : 693-701.

Kurihara, K. 1987. Recent progress in the taste receptor mechanism, pp. 3-39. *In* Y. Kawamura and M.R. Kare (eds.). Umami : A Basic Taste. Marcel Dekker, New York.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193 : 265-275.

Maeda, M. and T.Taga. 1976a. Extracellular nuclease produced by a marine bacterium. 1. Extracellular deoxyribonuclease formation by a marine *Vibrio* sp. Can. J. Microbiol. 22 : 1437-1442.

Maeda, M. and T. Taga. 1976b. Extracellular nuclease produced by a marine bacterium. 2. Purification and properties of extracellular nuclease from a marine *Vibrio* sp. Can. J. Microbiol. 22 : 1443-1452.

Moore, S. and W.H. Stein. 1954. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J. Biol. Chem. 211 : 907-913.

Ohno, Y., I. Yano, and M. Masui. 1979. Effect of NaCl concentration and temperature on the phospholipid and fatty acid compositions of a moderately halophilic bacterium, *Pseudomonas halosaccharolytica*. J. Biochem. 85 : 413-421.

Onishi, H., T. Mori, S. Takeuchi, K.Tani, T. Kobayashi, and M. Kamekura. 1983. Halophilic *Bacillus* sp.: production, purification and characterization. Appl. Environ. Microbiol. 45 : 24-30.

Sehgal, S.N. and N.E. Gibbons. 1960. Effect of some metal ions on the growth of *Halobacterium cutirubrum*. Can. J. Microbiol. 6 : 165-169.

Ventosa, A. 1991. Taxonomy of new species of moderately halophilic eubacteria, pp. 45-51. *In* F.R. Valera, (ed.). General and Applied Aspects of Halophilic Microorganisms. Plenum Press, New York.

Received date : 1/07/99
Accepted date : 29/06/01

Kasetsart J. (Nat. Sci.) 35 : 188 - 194 (2001)

Feasibility Study on Snack Production by Using Dietary Fiber Concentrate from Soymilk Residue

Kulvadee Trongpanich[1], Pracha Boonyasirikool[1], Sumalai Srikumlaitong[2], Chowladda Taengpook[1] and Udom Kanjanapakornchai[1]

ABSTRACT

The production of high dietary fiber snack was studied by using corn grit and broken rice as the bases and composed of dietary fiber concentrates (DFC) from soy milk residue which were defatted and non-defatted. The amounts of the dietary fiber concentrates were 5 %, 10 % and 15 %, respectively. The mixtures were extruded by using a twin screw extruder. It was found that the total dietary fiber and the protein contents of the snack samples were increased with the increasing quantity of the DFC in the products. The snack samples that contained 5 % DFC were equal to or even more bulky than the control sample and more bulky than the snack samples that contained 10 % DFC and up . The results were confirmed by measuring the bulk density of the snacks. From the color measurement of the snack samples, it was found that the lightness (L*) of the snacks were decreased with the increasing amount of DFC in the samples. Snacks made from corn grit as the base had a golden yellow color, while snacks made from broken rice as the base were white.

From the sensory evaluation, it was found that there was no significant difference in the preferential scores in color, odor and taste between the snack samples that contained 5 – 15 % DFC and the control sample at $p < 0.05$. However, adding DFC in the snacks could improve the snack's texture as the texture preferential scores of all the snack samples which contained 10 %DFC were higher than of the control ones.

The high dietary fiber snacks made from the defatted DFC contained more protein and total dietary fiber than the snacks made from the non-defatted DFC.

Key words : dietary fiber concentrate, high dietary fiber snack

INTRODUCTION

Nowadays, snack is a popular food item which still get a steady increase in the market size. In fact, snack have many varieties which are different in shape, size, taste, aroma and food compositions. Normally their bases are dehulled cereals, either in the forms of flour or grit, and tubers from which the main composition is starch and low in protein and fiber content. For example, potato chips contain 45.9 % carbohydrate, 3.6 % protein and 0.9 % fiber (FAO and US. Dept. of Health, Education and Welfare, 1972), while the composition of extruded snacks varies greatly with the kinds of the raw materials being used. However, the main composition of the extruded snacks is still carbohydrate with only 3.3 – 8.3 % protein (Boonyasirikool *et.al.*, 1986). Lee (1994) studied on the effects of extrusion conditions on the solubility and cholesterol – lowering potential of dietary

1 Institute of Food Research and Product Development, Kasetsart University, Bangkok. 10900, Thailand.

2 Thailand Institute of Scientific and Technological Research, Bangkok. 10900, Thailand.

fiber. She found that soluble dietary fiber (SDF) was maximized at low dough moisture content, high barrel temperature and high screw speed. These high shear conditions produced a greater than 3 fold SDF in soy (to 11.0 %) and wheat (to 8.3 %) snacks as compared with SDF in the raw mixes (3.4 and 2.3 % respectively)

The amount of soymilk residue in Thailand is increasing the same time as the drinking of soymilk becomes more and more popular. This could be due to the knowledge of people to avoid the diseases that are caused from animal products . Trongpanich *et al*. (2000) studied the production of dietary fiber concentrate (DFC) from soymilk residue by water extraction process. They found that the DFC they produced contained more than 40 % total dietary fiber (TDF) and more than 45 % protein content. The shelflife of the DFC was about 3 months and a longer shelflife could be obtained if the DFC is defatted. In order to increase the protein content of the extruded snack., many researches have been done and have had some success by adding of a limited amount of defatted soy flour in the base of raw mixes. However, none have been done yet on the DFC from soymilk residue from which properties might change after passing the soymilk extraction process.

The objective of this study is to find the feasibility of using DFC from soymilk residue as a raw material in the extruded snack production, in order to increase the TDF and protein content of the snack, and to utilize the soymilk residue, as well.

MATERIALS AND METHODS

1. Materials

1.1 DFC and defatted DFC powder from water extraction process .

1.2 Corn grit (yellow color with particle size about 30 – 40 mesh)

1.3 Broken rice (Kaw – tha – hang) with the size particle about 30 – 60 mesh.

1.4 Barbecue flavor and soybean oil for coating.

1.5 Other flavoring agents such as sugar, salt and minerals.

2. Methods

2.1 Formulation

Corn grit and broken rice were used as the bases. Each base was mixed with either DFC or defatted DFC powder at 5 %, 10 % and 15 % DFC or defatted DFC concentration, respectively. Each raw mix was mixed with 1 % flavoring agents (item 1.5). For the controls, 2 % vegetable oil was added in each formula, and for the defatted formulas 0.725 % vegetable oil was added. Fourteen formulas were used for this study.

2.2 Extrusion

The raw mixes were extruded by using Hermann Berstorff Laboratory Twin Screw Extruder (Co – rotating ZE 25 x 33 D). The samples were fed at the speed of 375 gm./min., and with a screw speed of 300 rpm. Water was fed to the control and the composite raw mixes at the speed of 19.2 gm./min. The highest barrel temperature was 165°C at the barrel 6. The melting temperature was at the range 155 – 160°C.

After extrusion, the products were dried with an electric carbinet dryer at 80°C for 15 – 20 min., and coated with the barbecue flavor at the concentration of 10 % by wt. Then they were packed in plastic bags.

2.3 Sensory evaluation

Twenty food scientists were involved for the sensory evaluations. The tasters evaluated the products by using the hedonic scale method, from which the scores were 1 – 9. The low scores showed, undesirable or dislikable products' characters, while the high scores showed more desirable or preferable products' characters. The scores were analysed for the statistical significant difference by using the Analysis of Variance and Duncan's Multiple Range Test.

2.4 Chemical and other characters analysis

The samples were analysed for moisture, protein, fat, fiber and ash (AOAC, 1990), total dietary fiber (AOAC, 1995), and color was measured from the ground samples by a Data Color International Spectroflash. Bulk density (10 replications) was calculated from the volume of replacement and the weight of the samples, and texture (10 replications) by the Instron 1140 with 5 kg. weighing, head speed of 50 mm/min. and chart speed of 200 mm/min.

RESULT AND DISCUSSION

Table 1 shows the results from the proximate analysis, TDF and the color of the main raw materials, i.e. corn grit, broken rice and defatted and non-defatted soymilk residue (DFSR and SR respectively). The DFSR and SR showed a higher protein, fiber, ash and TDF content than corn grit and broken rice. The color of DFSR and SR had the same shade of the broken rice, i.e., white and pale yellow,respectively. The color of the corn grit was golden yellow.

Table 2 shows the results from the proximate analysis, the TDF and the color of the snack samples made from the corn base and SR at 5, 10 and 15 % concentration. It was found that the protein, fiber and TDF content of the samples increased with the steady increase of SR in the raw mixes, while the lightness (L*) of the sample decreased with the increase of SR in the samples. However, since the amount of SR in the sample was not much, the color of all samples were still yellow.

Table 3 shows the sensory evaluation of the snacks made from corn grit and SR. The scores of the samples in color, odor, taste and acceptability

Table 1 The proximate analysis (based on dry basis) and the color of the corn grit, broken rice, soymilk residue and defatted soy milk residue.

Samples	Moisture	Protein	Fat	Fiber	Ash	TDF	Color		
	%	%	%	%	%	%	L	a	B
Corn grit	12.46	7.50	2.22	0.63	0.62	3.24	84.04	9.70	38.49
Broken rice	10.57	8.75	0.91	0.56	0.45	1.53	92.73	-0.13	7.61
Soymilk residue (SR)	9.86	39.34	23.31	6.89	2.59	43.10	91.22	0.64	16.13
Defatted soymilk residue (DFSR)	11.73	48.50	3.11	8.17	2.70	46.12	94.99	0.23	7.02

Table 2 The proximate analysis (based on dry basis) TDF and the color of the snacks made from corn grit and the soymilk residue (SR).

Samples	Moisture	Protein	Fat	Fiber	Ash	TDF	Color		
	%	%	%	%	%	%	L	a	B
100 % corn	5.33	7.19	4.31	0.63	1.58	3.12	85.60	5.33	39.15
5 % SR	3.84	8.41	3.16	0.84	1.71	4.82	84.03	5.78	37.54
10 % SR	5.16	9.75	4.10	0.98	1.68	6.23	82.78	6.29	38.93
15 % SR	3.39	11.06	5.32	1.01	1.82	8.64	82.07	6.10	37.57

were not significantly different. However, the adding of SR seemed to have an advantage in improving the texture of the product. The taster preferred the samples with SR more than the control with significant difference ($p < 0.05$). The sample with 10 % SR had the highest score, although there was no significant difference with the other samples with SR.

Table 4 shows the results from the proximate analysis, TDF and color of the snacks made from corn grit and defatted soymilk residue (DFSR). It was found that the protein, fiber, and TDF of the samples increased with the increasing amount of DFSR in the products, while the lightness (L*) of the samples decreased with the increasing amount of DFSR. All samples were yellow. The preference test of the samples were evaluated and the result are shown in Table 5. Scores of all characters of the control sample were not significantly different than the samples of DFSR. However, the scores of the controls were lower than the scores of the samples with DFSR at 5 and 10 % concentration in all

Table 3 The sensory evaluation of the snacks made from corn grit and soy milk residue (SR).

Samples	Texture	Color	Odor	Taste	Acceptability
100% corn	6.55^{b}	7.47^{a}	6.72^{a}	7.19^{a}	6.94^{a}
5% SR	6.71^{a}	7.45^{a}	6.92^{a}	7.08^{a}	6.94^{a}
10% SR	6.74^{a}	7.42^{a}	6.68^{a}	7.00^{a}	6.97^{a}
15% SR	6.68^{a}	7.10^{a}	6.47^{a}	6.47^{a}	6.16^{b}

In a column, means followed with the same letter are not significantly different at $p < 0.05$

Table 4 The proximate analysis (based on dry basis), TDF and the color of the snacks made from corn grit and the defatted soy milk residue (DFSR).

Samples	Moisture	Protein	Fat	Fiber	Ash	TDF	Color		
	%	%	%	%	%	%	L	a	b
100% Corn	5.33	7.19	4.31	0.63	1.58	3.12	85.60	5.33	39.15
5% DFSR	4.23	8.60	3.09	1.07	1.87	5.09	84.55	5.54	37.92
10% DFSR	3.75	10.69	4.01	1.11	1.98	7.19	84.03	5.15	36.69
15% DFSR	3.37	11.50	4.81	2.13	1.78	9.11	83.23	5.10	35.89

Table 5 The sensory evaluation of the snacks made from corn grit and defatted soymilk residue (DFSR).

Samples	Texture	Color	Odor	Taste	Acceptability
100 % corn	6.68^{a}	7.08^{a}	6.60^{a}	6.85^{a}	6.63^{ab}
5 % DF-SR	6.78^{a}	7.08^{a}	6.93^{a}	6.90^{a}	6.85^{a}
10 % DF-SR	6.90^{a}	7.00^{a}	6.90^{a}	6.90^{a}	6.75^{ab}
15 % DF-SR	6.45^{a}	7.08^{a}	6.80^{a}	6.55^{a}	6.25^{b}

In a column, means followed with the same letter are not significantly different at $p < 0.05$

characters, except color.

Table 6 and 8 shows the proximate analysis and TDF content, and color of the snacks made from broken rice and SR, and broken rice and DFSR, respectively. The protein, fiber, TDF of the samples increased with the increasing amount of SR and DFSR, while the addition of SR and DFSR had a reverse effect to the lightness (L*) of the samples.

Table 7 shows the scores from the sensory evaluation of the snacks made from broken rice and soymilk residue. There was no significant difference between scores of the samples in color, odor, taste and acceptability, but there was a significant difference ($p < 0.05$) between the texture scores of the control and 15 % SR. SR improved the texture of the products, from which it let us know that the snacks can have an addition of SR up to 15 % and

Table 6 The proximate analysis (based on dry basis), TDF and the color of the snacks made from broken rice and the soymilk residue (SR).

Samples	Moisture	Protein	Fat	Fiber	Ash	TDF	Color		
	%	%	%	%	%	%	L	a	b
100% rice	4.08	8.44	2.24	0.56	2.29	1.01	90.16	-0.96	11.87
5% SR	4.07	10.31	2.73	0.57	1.52	2.40	88.86	-0.69	13.66
10% SR	3.35	11.56	3.23	0.74	1.22	3.24	87.13	-0.23	14.96
15% SR	4.01	13.31	4.12	0.89	1.48	4.78	84.96	0.44	16.27

Table 7 The sensory evaluation of the snacks made from broken rice and soymilk residue (SR).

Samples	Texture	Color	Odor	Taste	Acceptability
100% rice	5.80^{b}	6.50^{a}	6.75^{a}	6.60^{a}	6.00^{a}
5% SR	6.25^{ab}	6.50^{a}	6.65^{a}	6.60^{a}	6.35^{a}
10% SR	6.00^{ab}	6.55^{a}	6.65^{a}	6.60^{a}	6.10^{a}
15% SR	6.43^{a}	6.55^{a}	6.60^{a}	6.85^{a}	6.55^{a}

In a column, means followed with the same letter are not significantly different at $p < 0.05$

Table 8 The proximate analysis (based on dry basis), TDF and the color of the snacks made from broken rice and the defatted soymilk residue (DFSR).

Samples	Moisture	Protein	Fat	Fiber	Ash	TDF	Color		
	%	%	%	%	%	%	L	a	b
100% rice	4.08	8.44	2.24	0.56	2.29	1.01	90.16	-0.96	11.87
5% DFSR	4.88	10.63	1.14	0.58	1.48	3.84	89.51	-0.04	14.08
10% DFSR	4.12	12.50	1.25	0.78	1.21	5.12	87.77	0.18	14.68
15% DFSR	3.75	15.56	1.29	0.85	1.46	7.49	85.94	0.40	15.41

receive the higher preference test than the control one.

Table 9 shows the result from the sensory evaluation of the samples made from broken rice and DFSR. Again, there was no significant difference between the snacks in color, odor and taste characters. DFSR improved the texture and acceptability of the product from which the scores of these characters of the DFSR added samples were higher than of the control.

There was a significant differences between the control and the DFSR-added samples ($p < 0.05$) in texture and in acceptability scores and the results showed that a 10 % addition of DFSR was the best.

Table 10 shows the bulk density of the snack samples. The bulk density had a relationship with the size of the snack. The less density sample received a bigger size,and a 5 % addition of SR and DFSR increased the size and decreased the bulk density of the products with a significant difference ($p < 0.05$), except the samples of corn with DFSR. Upon increasing the added amount of SR and DFSR, the size decreased with the increasing bulk density.

Table 11 shows the result from the texture testing of the products. There was no significant difference among the corn base samples. For the rice base samples, there was a significant differences ($p < 0.05$) between the control and the 10 % and 15 % SR samples, and between the control and the DFSR- added samples. Snacks from 100 % rice were more crispy than 100 % corn, thus were easily broken. The SR and DFSR addition improved the structure and texture of the rice base products.

Table 9 The sensory evaluation of the snacks made from broken rice and the defatted soymilk residue (DFSR).

Samples	Texture	Color	Odor	Taste	Acceptability
100% rice	5.15^{c}	6.50^{a}	6.53^{a}	6.45^{a}	5.55^{c}
5% DFSR	5.98^{b}	6.55^{a}	6.35^{a}	6.60^{a}	6.05^{bc}
10% DFSR	6.75^{a}	6.65^{a}	6.70^{a}	6.70^{a}	6.65^{a}
15% DFSR	6.48^{ab}	5.95^{b}	6.30^{a}	6.55^{a}	6.33^{ab}

In a column, means followed with the same letter are not significantly different at $p < 0.05$

Table 10 The bulk density (gm/ml) of the snacks samples.

Samples	Corn base with SR	Corn base with DFSR	Rice base with SR	Rice base with DFSR
Control	0.054^{b}	0.054^{b}	0.074^{a}	0.074^{a}
5 % adding	0.042^{c}	0.054^{b}	0.068^{b}	0.064^{b}
10 % adding	0.056^{b}	0.056^{b}	0.073^{a}	0.073^{a}
15 % adding	0.077^{a}	0.067^{a}	0.074^{a}	0.075^{a}

In a column, means followed with the same letter are not significantly different at $p < 0.05$

Table 11 The texture (kg.) of the snack samples using the Instron measurement.

Samples	Corn base with SR	Corn base with DFSR	Rice base with SR	Rice base with DFSR
Control	1.50[a]	1.50[a]	1.39[c]	1.39[b]
5 % adding	1.46[a]	1.41[a]	1.61[c]	1.82[a]
10 % adding	1.46[a]	1.54[a]	1.93[b]	1.85[a]
15 % adding	1.71[a]	1.70[a]	2.58[a]	1.93[a]

In a column, means followed with the same letter are not significantly different at $p < 0.05$

CONCLUSION

Dietary fiber concentrate from soymilk residue either defatted or non-defatted, can be used to increase the dietary fiber and protein content of snacks from cereals. However, in order to improve the texture of the products and receive the consumer acceptability, the amount of its use should not be more than 10 % by wt.

ACKNOWLEDGEMENT

The researchers wish to thank Research and Development Institute of Kasetsart University for supporting the research fund for this project.

LITERATURE CITED

AOAC 1990. Official Methods of Analysis. 15th ed. The Association of Official Analytical Chemists. Arlington, Virginia. 1298 p.

AOAC 1995. Official Methods of Analysis. 16th ed. The Association of Official Analytical Chemists. Arlington, Virginia. 45.4.07 Chapter 45, p. 70-73.

Boonyasirikool, P., S. Reungmaneepaitoon, S. Thippayang, and S. Prabhawat. 1986. Research on the Production of High Protein Snack Foods. ASEAN – Thailand Food Technology Research and Development 1982 – 1985. IFRPD, Kasetsart University, Bangkok. 67 p.

FAO and US. Dept. of Health, Education, and Welfare. 1972. Food Composition Table For Use in East Asia. USA. 334 p.

Lee, J.T. 1994 Production and nutritional evaluation of an extruded snack containing soy fiber and plant protein. Ph.D. Dissertation, Kansas State Univ., USA.

Trongpanich, K., Chidchom Hiraga, Duangchan Hengsawadi and Urai Phawsungthong. 2000. Feasibility study on production of dietary fiber concentrate from soymilk residue, pp. 95-96. *In* Proceedings of The Third International Soybean Processing and Utilization Conference. Oct. 15 – 20, Tsukuba, Ibaraki.

Received date : 02/05/01
Accepted date : 29/06/01

Kasetsart J. (Nat. Sci.) 35 : 195 - 204 (2001)

Study of Parameters Affecting Drying Kinetics and Quality of Corns

Somboon Wetchacama[1], Somchart Soponronnarit[1], Somkiat Prachayawarakorn[1], Adisak Pongpullponsak[1], Wuttitat Tuntiwetsa [2] and Suprarat Kositcharoeankul[3]

ABSTRACT

The objectives of this research are to investigate factors affecting on drying rate of high moisture corn at high temperature with fluidisation technique and to develop a mathematical model for predicting drying rate. There were three following steps of drying process: 1) rapid drying using fluidised bed dryer at inlet drying air temperature of 130-170°C, 2) corn tempered for period of 40-180 minutes under the same temperature as drying from the step 1 and 3) drying with ambient air. Drying kinetic shows the inlet air temperature and the specific airflow rate significantly affecting the drying rate. Amongst three semi-empirical drying equations (Wang and Singh, Page and Lewis), Page's equation provides the best prediction.

This study also aims to study the quality of corn dried in each step. Corn qualities in terms of *aflatoxin* content, percentages of breakage and stress crack, and colour change have been considered. Experimental results show that *aflatoxin* content in dried corn does not change. Breakage and cracking depend strongly on final moisture content and are relatively dependent to temperature. Tempering provides the improvement of colour while inlet air temperature has no effect.

Key words: drying kinetics, grain, quality

INTRODUCTION

One of the most important agricultural products in Thailand is corn. The need of corn in the feed mill and the other food industries tend to be increased considerably. Corn can be produced in two seasons. The first one is grown in the period from April-May to July-August-September, which falls in the rainy season. The second crop is started from July-August-September to October-November-December and harvested at the end of the rainy season. Since a very large amount of corn produces in the first crop, a serious problem of poor corn quality has been faced if corn could not be immediately dried. This is by virtue of the fact that fresh corn is usually harvested at moisture content more than 23% wet basis. The micro-organism already infecting the corn can grow up easily under conditions of such moisture levels coupled with a suitable water activity especially higher than 0.85 (Wongurai *et al.*, 1992). Most species found in corn are likely to *A.flavus and A.parasiticus.* Both moulds can yield the poison substances known as *aflatoxin B-1* within 2-8 days (Laecy *et al*., 1986) if the environmental conditions are suitable. This substance causes seriously the cancer at different organs of human. Drying can contribute to corn quality since moisture content is the most significant

1 King Mongkut's University of Technology Thonburi, Bangkok 10140, Thailand.

2 Division of Plant Pathology Section and Microbiology, Department of Agriculture, Ministry of Agriculture and Co-operatives, Bangkok. 10900, Thailand.

factor affecting changes in quality of corn. Numerous approaches can be applied for reducing moisture content. Each method has the advantage and disadvantage. The use of ambient air is the simplest one. When corn with moisture content higher than 23% wet basis was dried by blowing the ambient air through the bulk of corn. It showed that after its moisture content reduced to the desired value of 14 % wet basis, the level of *aflatoxin B-1* significantly increased to 250 ppb while the amount of *aflatoxin B-1* at the beginning was nil (Prachayawarakorn *et al*., 1995). This technique seems to spend so long drying time that some amounts of corn were infected and hence the *aflatoxin* could be occurred. The artificial drying method would therefore be an appropriate choice because of rapid removal of moisture contents in the process.

There are many types of artificial dryer being used in grain industries, for example LSU dryer (LSU = Louisiana State University), cross-flow dryer, spouted bed dryer and fluidised bed dryer. The latter one is of the main interest in this work. By fluidised technique, grains are transformed into a like fluid after thoroughly mixed with a sufficiently high air velocity. Under the fluidised state, the drags force on the grain particles balance the gravitational force pulling on them. Therefore, the grains remain in a semi-suspended condition. This inherent phenomenon provides the main advantages over the other types of dryer. The intensively mixed solids throughout the bed provide almost uniform temperature and moisture content. High heat and mass transfer rates are possible because of very good contact between air and solid. Thus, at high moisture level, a large proportion of water concentrated near the surface of solid particles can be removed quickly whilst the small one existing deeply inside the kernel still remains and is extremely very difficult to vaporise it although the capable of drying air allows to be powerful. There are some workers studying the corn drying using the fluidised bed technique (*e.g*. Soponronnarit *et al*., 1997a). They investigated the factors affecting the drying rate and the quality. They reported that moisture movement inside the kernel was controlled by diffusion and constant drying rate period was absent. The inlet air temperature is strongly influence to moisture reduction whilst the air velocity and the bed depth become relatively significant factor. In addition, when the drying was proceeded successively, the physical change was virtually found. The amount of corn tracing the stress crack and the breakage significantly increased following with the increased temperature and the reduced moisture content whilst it did not change with air humidity (Soponronnarit *et al.*, 1997b). So far, the colour was relatively changed of which the value of "a" representing red increased with the increase of temperature and of drying time whilst the value of "b" representing yellow decreased.

Such changes lead to a serious problem in that the micro-organism, then inducing the occurrence of toxin substances, can easily attack the broken or cracked corn. In addition to easy infection, such physical damage is also an important measure of quality in processing operations such as cereal and snack food measuring. This cause took us to explore the way of improving the dried corn quality. One of the common approaches that can be improved its quality is a tempering process. This process allows the reduced moisture gradient inside the grain and eventually the moisture concentration at local positions inside the corn kernel becomes relatively identical. Foster (1973) showed that tempering process could reduce the degree of stress cracking during artificial drying of corn. For the paddy drying, Steffe and Singh (1980) also concluded that the additional tempering stage in the drying process was able to sustain head rice yield as compared the conventional one where the paddy was dried in a single pass. Despite its importance to grain quality, the research works have been less interest to provide an important information of tempering time. This fact may be useful not only for grain quality improvement but also for energy

consumption.

The objectives of this work are to explore the effect of tempering period on the corn quality in terms of colour, stress crack and breakagibility and the operating parameters such as temperature, bed depth and airflow rate affecting the removal of moisture content of corn. Its moisture content was eventually reduced to 14% wet basis in order to inhibit the growths of *A.flavus* and *A.parasiticus*.

MATERIALS AND METHODS

Corn was dried by a batch fluidised bed dryer mainly composed of a cylindrical shaped stainless chamber with a 20 cm diameter and a 140 cm height, as shown in Figure 1. In order to save economically energy consumption, some proportions of the exhausted air are recycled and then mixed with the fresh air. The mixed air after reheated with 4 element heaters, each of elements having 3 kW power (total 12 kW), is flowed through the dryer again. A PID controller with an accuracy of ± 1°C was used to control the inlet temperature. In the fluidised bed system, it requires a high air flow rate and a high-pressure drop, so that a backward-curved blade centrifugal fan driven by a motor power of 1.5 kW was selected. In order to obtain a desired inlet air velocity and maintain performance of the system, a mechanical variable speed unit was chosen to control the revolution of fan.

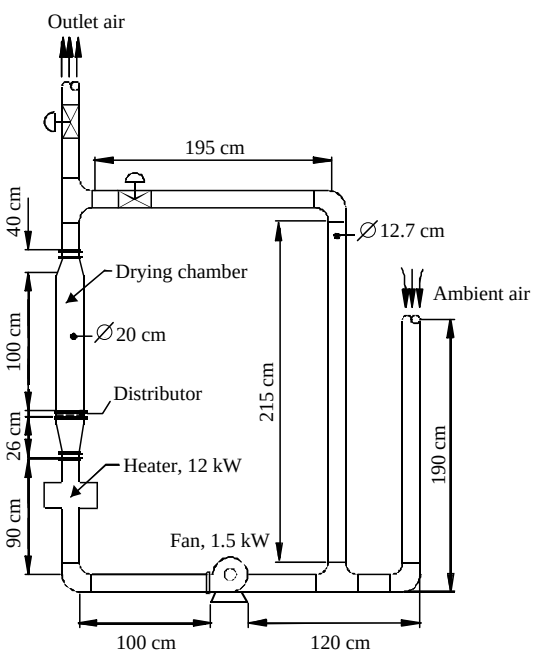


Figure 1 Schematic of experimental fluidized bed corn dryer.

Drying kinetics

Freshly harvested corn, type Suwan 1, was rewetted to obtain desired moisture content of 43% dry basis. The rewetted corn was then kept in a temperature-controlled room at 5-10°C for 5 or 7 days in order to get the uniform moisture content inside the grain kernel. The following rewetted corn was dried to moisture content of 23% wet basis. The experiments were carried out at the following conditions: inlet temperatures of 130, 150 and 170°C and bed depths of 4, 6 and 8 cm. The air velocity was kept at a constant value of 3 m/s throughout these experiments. This value is approximately 1.6 times higher than the minimum fluidisation velocity for corn of which the value was approximately 1.8 m/s (Soponronnarit *et al.*, 1997a). Such a value beyond the minimum velocity was certainly sure that every moist corn could rigorously mix with the drying air. The relatively similar moisture content throughout the bed is a consequent result. If the air velocity is set too high, then the corn is increasingly agitated due to the formation of large bubble size, detrimental effect on the interchange of heat and mass transfers between gas and solid phases. The samples drawn from the dryer in every minute were then kept in a hot air oven at a constant temperature of 103°C for 72 hours to determine the moisture content

Thin layer drying equations

Development of mathematical models to describe the drying of porous solids is of important

topic in literature. Models are needed to enable process design and to minimise energy consumption and total costs subject to quality constraints. In the fluidisation technique, the sufficiently high air velocity provides the body of corns behaving like a liquid, so that at this stage the grains feel "weightless" and move randomly and severely within the bed. Every kernel is therefore suspended completely in the air. Such phenomenon results in the insignificant change of the air properties along the bed depth and the grain temperature increased rapidly and then approached to the inlet air temperature for a short period of time. So far, the moisture content at surface is reduced speedily and consequently equilibrated with the existing air. Under this condition, the theoretical diffusion model can explain the moisture transfer inside single kernel. Unfortunately, the theoretical model predicts inaccurately since the grain often has an irregular shape and the mechanism of moisture movement inside the grain has been being questioned. In practice, the empirical thin layer drying equation has therefore been applied. Three following forms of thin layer drying equation were proposed in this work. First, the Lewis' equation (1921) is given by

$$MR = \exp(-bt) \quad (1)$$

where b = drying constant depending on the operating parameter

t = drying time, min

$$MR = \text{moisture ratio} = \frac{M(t) - M_{eq}}{M_{in} - M_{eq}}$$

M(t) = moisture content at time t

M_{in} = initial moisture content

M_{eq} = equilibrium moisture content

The equation (1), known as Newton's law of cooling, is assumed the negligible moisture gradient inside the grain kernel, indicating that the resistance of diffusion is insignificant. Wang and Singh (1978) was then simply modified equation (1) by adding one more drying constant. Wang and Singh's equation can then expressed as

$$MR = a \cdot \exp(-bt) \quad (2)$$

where a is drying constant similar to the constant b in equation (1). Finally, the purely empirical equation is demonstrated. Page's equation (1952) also expresses moisture ratio as a form of exponential function of drying as shown in equation (3),

$$MR = \exp(-bt^a) \quad (3)$$

Equation (3) is often described favourably the experimental data (Soponronnarit and Prachayawarakorn, 1994).

Drying constants determined from all these equations were analysed using the non-linear regression approach in the commercial package SPSS (Statistical Package for the Social Science). The equation to describe them is not based on the theoretical formulation. Simple or complex one may be chosen arbitrarily by fitting it with parameters influencing to drying rate such as specific airflow rate and temperature. Details will be discussed in the following section. However, the appropriate expressions proposed for *a* and *b* are given by

$$a = A_1+A_2T+A_3S_P+A_4T.S_P+A_5\text{In}(S_P) \quad (4)$$

$$b = B_1+B_2T+B_3S_P+B_4T.S_P+B_5\text{In}(S_P) \quad (5)$$

where A_1-A_5 and B_1-B_5 = constant

T = inlet air temperature

S_p = specific air flow rate

Quality test

The successful or failure in drying process can be justified from the grain quality obtained. The criterion for considering the grain quality in each species is very different. However, for corn, one of the most important qualities that market is often used is the amount of *aflatoxin* B-1 in corn, besides breakage, stress crack and colour. Four quality aspects for examples *aflatoxin*, breakage and stress crack were therefore subject to consider in this work. The amount of *aflatoxin* was detected by using HPLC with the corn sample of 50 g. In each condition, three samples were tested. One-way analysis of variance (ANOVA) was performed to examine whether the amount of *aflatoxin* before and after drying in each stage is changed or not by

using 95% confident level.

For the stress crack and the breakage, the visual inspection under light was employed with 200 g of corn sample. Each kernel was classified into three classes: broken, cracked (multiple and single) and undamaged. The percentage for each class was normalised by dividing the weight of corn kernel in each category by the total weight. Multiple cracking was often found in this work. A colour meter, Juki JP 7100p, determines grain surface colour. The values of a, b and L in Hunter system corresponding to red, yellow and lightness, respectively were read. The corn samples after final drying stage were checked carefully the above mentioned qualities.

In testing quality, after the corn was dried to 23% dry basis, it was then tempered in an airtight container for periods of 40, 120 and 180 minutes respectively under the same inlet air temperature. Finally, the corn was dried again using ambient air until the moisture content reached 16% dry basis. To obtain the accurate results, the experiment in each condition was three replicates.

RESULTS AND DISCUSSION

Effects of parameter on drying rate

The inlet air temperatures used in this experiment as shown in Figure 2 are very high compared to other techniques that are normally operated at temperatures between 50°C and 70°C for drying grains. This may have crossed the minds of many scientists and innovators, but Soponronnarit and Prachayawarakorn (1994) showed that it was possible to dry grain at high temperature range without significant loss of grain quality if the drying process was controlled effectively. Figure 2 represents the influence of inlet air temperatures on the reduction of moisture ratio at 6 cm bed depth and 3 m/s air velocity indicating that faster moisture removal relates closely to higher inlet temperatures. This effect is increasingly important during the final period of drying at which the moisture content

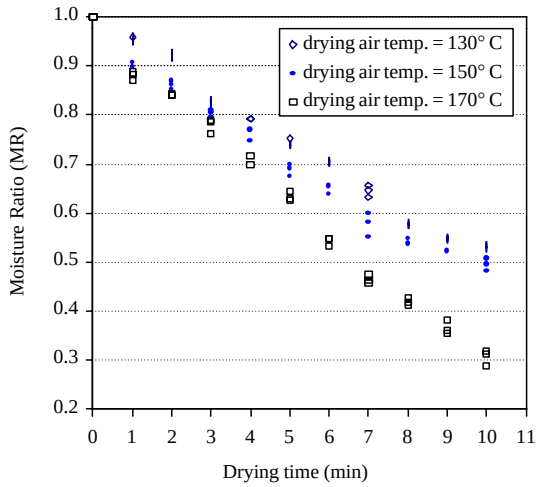


Figure 2 Effect of drying air temperature on moisture ratio (initial moisture content 43% d.b., drying air velocity 3 m/s and bed height 6 cm).

is rather low; more release of moisture content requires higher temperature difference between the solid and drying medium. As can be seen in Figure 2, the degree of moisture content at different temperatures starts remarkably different after 6 minutes whilst at the early period, the temperature is less important effect.

The drying rate is also relatively affected by the specific airflow rate, defined as the ratio of mass of drying air to dry mass of corn loaded into the chamber. As shown in Figure 3, moisture extraction relatively increases with the increase of specific airflow rate under the identical operating condition. In the configuration investigated, the quantity of corn in each case was different whilst the airflow rate was kept a constant value and the change in the amount of corn did not effect behaviour of fluidised corn. When corn was subjected to the drying air, some proportions of energy were transferred to the grains, resulting in some evaporation. By such a situation, the surrounding conditions inside the chamber were therefore changed with changing the amount of corn; the quantity of water vapour around the corn kernel directly related to the capacity of

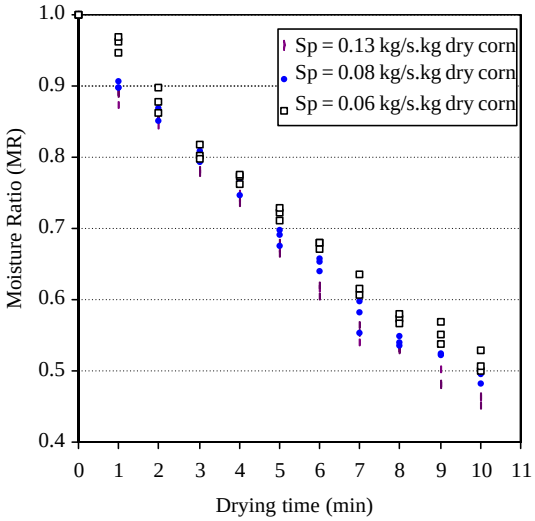


Figure 3 Effect of specific air flow rate (Sp) on moisture ratio (initial moisture content 43% d.b., drying air velocity 3 m/s and drying air temperature 150°C).

corn in the dryer. Hence, this effect results in drying rate.

Comparison of thin layer equations

Three thin layer-drying equations were used to validate the experimental data. The fitted data were obtained from the single pass of corn drying, starting from 43% dry basis until to 16% dry basis. All these equations can predict the moisture content insignificantly different at the beginning period of drying particularly higher moisture range of 23% dry basis as represented in Figure 4 whereas their predictions have a small difference near the end of drying period at which the moisture content is relatively low. Only Page's equation is found to yield the best fit to the experimental data. This may be because when the moisture content start reducing from such a high level to lower level, the large moisture gradient exists inside the corn, so that the equation (1) and (2) predict inaccurately.

Drying constants in those equations fitted statistically with the above-mentioned factors are thus given by the following equations:

Lewis's equation

$$b = 737 + 0.0006000T - 0.8420S_p + 0.003700T.S_p + 0.06590\ln(S_p) \quad (R^2 = 0.9778) \quad (6)$$

Wang and Singh's equation

$$a = 0.3019 - 0.0002000T + 2.001S_p + 0.002000T.S_p - 0.2112\ln(S_p)$$

$$b = 0.09332 - 0.0005000T - 0.60888S_p + 0.003900T.S_p + 0.04171\ln(S_p) \quad (R^2 = 0.9782) \quad (7)$$

Page's equation

$$a = -3.089 - 0.001000T + 2.703S_p + 0.1474T.S_p - 1.586\ln(S_p)$$

$$b = 0.6017 + 0.002100T - 0.07010S_p - 0.01790T.S_p + 0.2510\ln(S_p) \quad (R^2 = 0.9832) \quad (8)$$

Based on the R^2 values, Page's equation is the most suitable equation that can describe the corn dying in the fluidised bed dryer. For the other conditions, the results also show similar trend to Figure 4.

Corn quality

In testing the corn quality, the experiments were performed at the conditions of 43% dry basis initial moisture content, 3.0 m/s air velocity, 8 cm bed depth and 170-180°C inlet air temperatures.

Aflatoxin

In testing the change of *aflatoxin* quantity by heat treatment, the corn samples were dried to 23 and 16% dry basis under the previously mentioned temperature range. One way-ANOVA shows that although the inlet temperature is increased, the *aflatoxin* is insignificantly changed in amount after completed the process of drying.

In fact, the *aflatoxin* could be removed by heat treatment (temperature of 250°C), at which it is the melting point (Feull, 1966). In contrast, Cucullu *et al*. (1966) reported that even though the

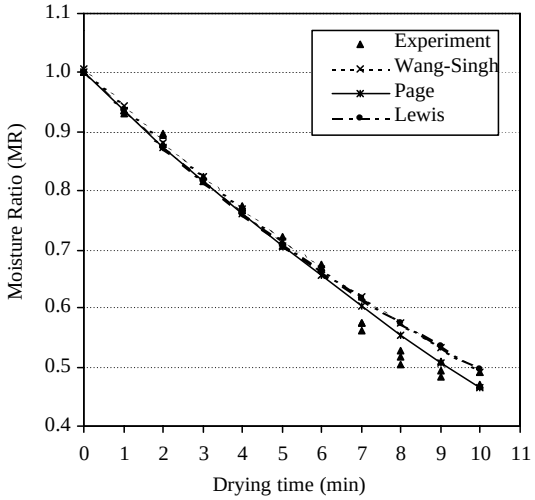


Figure 4 Comparison between thin layer drying equations and experimental results (initial moisture content 43% d.b., drying air velocity in drying chamber 3 m/s, bed height 4 cm and inlet air temperature 130°C).

temperature is not high enough to melt the *aflatoxin*, the quantity of the *aflatoxin* substance in peanut could possibly be reduced by roasting at a temperature of 150°C for 90 minutes. From that point of view, we tried exploring the heat treatment approach using the fluidised bed technique. But, corn can be not contacted with high drying air temperature for a long drying period due to the serious physical damage, as we will discuss in the following section. Thus, the corn was initially dried at temperature of 160°C and then followed with tempering for periods of 120 and 180 minutes under the corresponding temperature. The results still show the same trend as the previous ones, indicating invariable amount of *aflatoxin*. This is likely to treat the *aflatoxin* by such an approach ineffectively.

Breakageability and stress crack

Figure 5 shows the relationship between breakageability or stress crack and inlet temperatures under various final moisture levels. Major contribution of drying induced the stresses is the non-uniform moisture content. The stresses are tensional force near the boundary of dried material, so that they give the rise to the crack of dried body (Musielak, 2000). Refer to Figure 4, at the early stage of corn drying during which moisture content is higher than 19% wet basis, the moisture gradient inside the corn kernel expected to be very small, as indicated by Lewis's equation, so that such a small difference can not encourage the stresses. As a result of this, the percentage of stress crack is almost constant in spite of temperature increased as shown in Figure 5. In addition, the percentage of breakageability is not changed. When the corn was dried further and then approached to 14% wet basis, the percentage of stress crack increases to approximate 11.5% with respect to the previous one with showing value of 9.5% (see the cross symbol). The increase of stress cracking also contributes importantly to the breakagibility. The percentage of breakagibility in this case increases more than twice as compared to the one that corn dried to 19% wet basis. When using drying air at

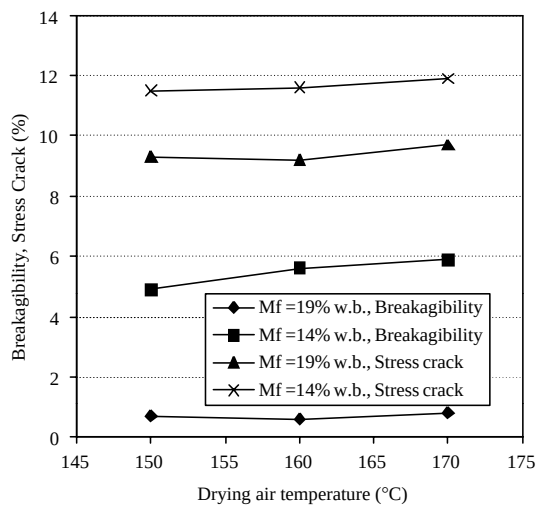


Figure 5 Influence of drying air temperature on the stress crack and breakage at difference final moisture contents, Mf (initial moisture content 30% w.b.).

170°C, the broken corn has a shape similar to the popcorn. From this figure, the temperatures seem to insignificant effect on the stress crack and the breakagibility for all cases whereas the moisture content plays an important role. Similarly, Peplinski *et al*. (1994) reported that the total number of kernel stress cracks did not change significantly over a wide range of drying temperatures.

However, the tempering period was then included between the drying stage by starting tempering since the corn was reduced to 23% dry basis. After that, it was dried to 14% wet basis with the ambient air. The corn quality is now improved. As shown in Figure 6, the number of cracked corn is relatively reduced following the increase of tempering time. By contrast, the percentage of breakagibility insignificantly changes despite the tempering time is increased with showing the value of approximate 5.5%. This value is almost exactly the same amount as the previous case that corn was dried to 14% wet basis shown in Figure 5. The explanation of this cause has not been cleared yet and it is subjected to more work being investigated.

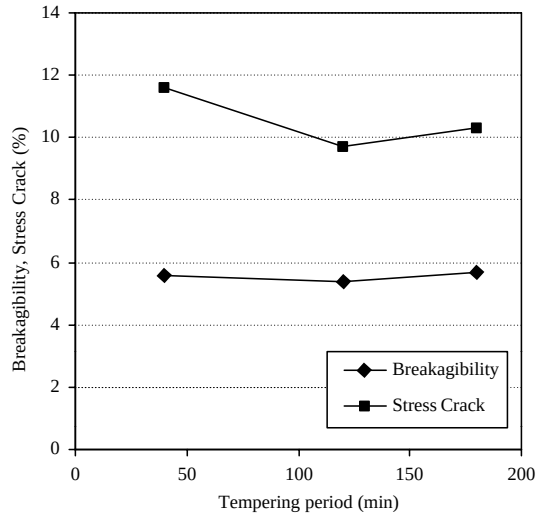


Figure 6 Effect of tempering periods on the breakage and stress crack (initial moisture content 30% w.b. and inlet air temperature 160°C).

Colour

Following previous work by Soponronnarit *et al*. (1997b) showed that a and b values changed with drying times and temperatures. In their work, the corn was dried continuously until its moisture content reached 16% dry basis. In order to improve the colours, the concept similar to the above-mentioned case was used, that is, the additional tempering period between drying stage. Figure 7 shows the evolution of a, b and L values with tempering periods at 160°C. The a values linearly increase with tempering time whilst b and L values monotonically decrease. At the tempering time more than 120 minutes, the tempered corn becomes much more intense colour than the normal level, related to the resulting change of a, b and L values. This result is similar to the work reported by Chotijukdikul (1997). On the contrary, the colour is acceptable at tempering period of 40 minutes. It

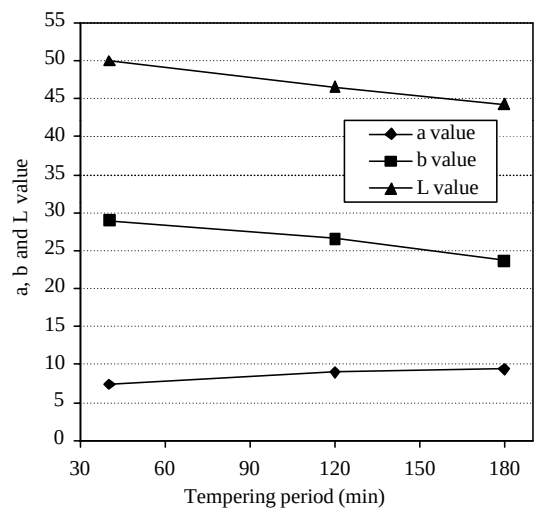


Figure 7 Effect of tempering periods on the value of a, b and L (initial moisture content 30% w.b. and inlet air temperature 160°C).

was visually observed that the colours were insignificantly changed after the corn was tempered for period of 40 minutes in spite of the fact that before tempering, the corn dried at higher

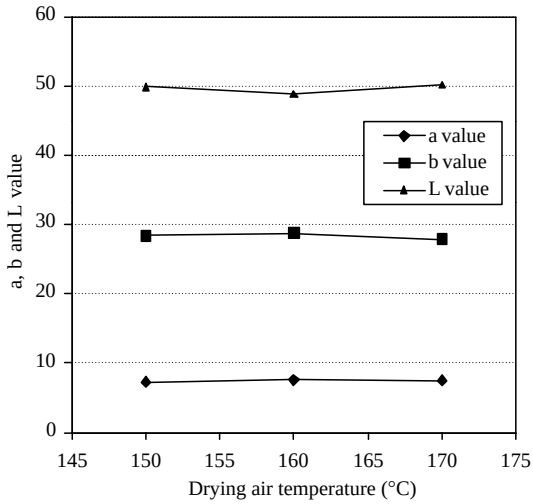


Figure 8 Effect of drying air temperature on the value of a, b and L (initial moisture content 30% w.b., final moisture content 19% w.b. and tempering period 40 minutes).

temperatures, resulting in lower moisture contents, had more intense colour. This can be seen in Figure 8 showing the values for a, b and L are all insignificant deviations with temperatures for the tempering period of 40 minutes. The explanation of such a cause may be because when the dried corn was subjected to temper in the airtight container only for a short period, some evaporation can occur, resulting in grain temperature reduced, and at the same time, the change of the colours cannot be transformed completely. The colours can, therefore, be recovered again. However, if it was corrected for a longer time, then the grain temperature increased and approached to a fixed temperature for tempering. Hence, the grain colours became poorer.

CONCLUSIONS

The results of this study can be concluded as the followings:

1) Specific airflow rate and inlet drying air temperature are important factors influencing drying rate of corn in fluidised bed dryer.

2) Amongst three popular thin layer equations, Page's equation is the most suitable equation predicting in agreement with the experiments.

3) The fluidised bed drying technique can inhibit the increase of *aflatoxin* level. With this technique, the *aflatoxin* is not enabled to destroy by heat treatment although the temperature range used is very high.

4) Final moisture content of corn is a main effect on breakagibility and stress crack whilst inlet air temperature is a less significant factor. Over range of high temperature, the corn should not be dried to 14% wet basis in a single pass. Tempering is recommended for corn drying.

5) Tempering period in drying process play a key role in improving the corn colours in terms of a, b and L values.

ACKNOWLEDGEMENTS

The authors would like to thank the Thailand Research Fund for financial support and the Division of Plant Pathology Section and Microbiology in Department of Agriculture for their kind assistance in testing the *Aflatoxin*.

LITERATURE CITED

Chotijukdikul, A. 1997. Managing moist corn by fluidized bed drying, tempering and ambient air ventilation. Master Thesis of Energy Management Technology. King Mongkut's University of Technology Thonburi, Bangkok.

Cucullu. A.F., L.S. Lee, R.Y. Mayue, and L.A. Goldblatt. 1966. Determination of *aflatoxins* in individual peanuts and peanut sections. J.Am.Oil Chemist's Soc. 43 : 89.

Feull, A.J. 1966. Aflatoxin in groundnuts. IX problem of detoxification. Trop. Sci. 8 : 61.

Foster, G.H. 1973. Dryeration: Heated air drying and corn quality, Ann. Technol. Agric. 22 :

238-241.

Laecy, J., N. Magan, R.G. Cuero and J.E. Smith. 1986. The influence of water and temperature on moulding and *Mycotoxin* production, pp. 63-76. *In* 4th International Working Conference on Stored-Product Protection, Tel. Aviv, 21-26 September.

Lewis, W.K. 1921. The rate of drying of solid materials, J. Ind. and Eng. Chem. 13(5) : 427-432.

Musielak, G. 2000. Influence of the drying medium parameters on drying induced stresses, Drying-Technology-An International Journal 18(3) : 561-581.

Page, G.W. 1952. Factor influencing the maximum rate of drying shell corn in layer. Master Thesis of Science. Purdue University. Purdue.

Peplinski, A.J., J.W. Paulis, A. Biets, and R.C. Pratt. 1994. Drying of high moisture corn: changes in properties and physical quality, Cereal Chemistry 71(2) : 129-133.

Prachayawarakorn, S., S. Soponronnarit, A. Nathakaranakule, and S. Inchan. 1995. Preventing *Aflatoxin* production in corn by in-store drying. Kasetsart J. (Nat. Sci.) 29 : 404-415. (in Thai)

Siriacha, P., P. Tonboon-ek, and D. Buangsuwon. 1991. Aflatoxin of maize in Thailand. In Proceeding of an International Conference, 23-26 April. Bangkok.

Soponronnarit, S. and S. Prachayawarakorn. 1994. Optimum strategy for fluidised bed paddy drying. Drying Technology –An International Journal 12(7) : 1667-1686.

Soponronnarit, S., A. Pongtornkulpanich, and S. Prachayawarakorn. 1997a. Drying characteristics of corn in fluidised bed dryer. Drying Technology – An International Journal 15(5) : 1603-1615.

Soponronnarit, S., A. Pongtornkulpanich, and S. Prachayawarakorn. 1997b. Corn quality after drying by fluidization technique at high temperature. Drying Technology – An International Journal 15(10) : 2577-2586.

Steffe, J.F. and R.P. Singh. 1980. Theoretical and practical aspects of rough rice tempering. Trans. ASAE 23 : 775-781.

Wang, C.Y. and R.P. Singh. 1978. A single layer drying equation for rough rice. ASAE. No. 78-3001. St. Joseph, MI.

Wongurai, A., O. Tsuruta, and K. Arai. 1992. Water activity of Thai maize and growth of *Aspergillus flavus*, pp. 7-9. *In* Research report on maize quality improvement research centre project (JICA).

Received date : 28/02/01
Accepted date : 4/06/01

Kasetsart J. (Nat. Sci.) 35 : 205 - 209 (2001)

Irrationality of some Series with Rational Terms

Pairit Kuhapatanakul and Vichian Laohakosol

ABSTRACT

An irrationality criterion due to Diananda and Oppenheim states that a Cantor series of rational terms is rational except possibly when the rational terms are of certain special shapes. In the first part, this excepted case is analyzed and conclusions are drawn for two specific classes of series. Badea in 1993 established very strong irrationality tests for series of positive rational terms and applied them to settle some previous open problems. Later Brown, Pei and Shiue extended Badea's applications to those series whose terms satisfy linear recurrence relations. Extensions of these results are derived in the second part.

AMS Mathematics Classification: 11J72,11B37

Key words: irrationality, Cantor series, linear recurrences

INTRODUCTION

It is known, see e.g. Theorem 1.6 p. 7 of Niven (1967) that any real number x is expressible as a Cantor series, i.e. in the form

$$x = a_0 + \sum_{i=1}^{\infty} \frac{a_i}{b_1 b_2 \ldots b_i} = a_0 + \frac{a_1}{b_1} + \frac{a_2}{b_1 b_2} + \frac{a_3}{b_1 b_2 b_3} + \ldots,$$

where $b_i > 1$ are integers, a_i are integers satisfying $0 \le a_i \le b_i - 1$ for all $i \ge 1$, and $a_i < b_i - 1$ for infinitely many i. Diananda and Oppenheim (1955) proved the following result giving a criterion for irrationality of certain numbers written as Cantor series: If every limit of $c_i = a_i/b_i$ is a rational number h/k where $0 < h < k$, $(h,k) = 1$, then x is irrational except possibly when $a_i = [hb_i/k]$ for all large i in the subsequence for which $c_i \to h/k$. In the excepted case x may be rational or irrational. Diananda and Oppenheim gave two examples illustrating that in the excepted case both possibilities do exist. Our first objective is to determine explicitly some excepted cases embracing these examples.

Badea (1993) proved some very strong theorems yielding irrationality criteria for series of the form $\Sigma\, b_n/a_n$, where a_n and b_n are positive integers and applied them to settle certain irrationality assertions of Erdos and Graham regarding series of the forms $\sum 1/_{F_{2^n+1}}$ and $\sum 1/_{L_{2^n}}$ where F_n and L_n are Fibonacci and Lucas numbers, respectively, as well as those of Sierpinski regarding series of the form $\Sigma(-1)^{n+1}/a_n$. Later Brown *et al* (1995) employed one of Badea's criteria to establish sufficient irrationality conditions for series of the form $\Sigma 1/H(f(n))$, where (H(k)) is a sequence of integers, positive from some point on, satisfying a homogeneous linear recurrence relation with integer coefficients and f is a strictly increasing function from the set of positive integers to the set of nonnegative integers. Our second objective is to extend a number of these results in the direction of irrationality criteria and their applications.

Department of Mathematics, Faculty of Science Kasetsart University, Bangkok 10900, Thailand.

MATERIALS AND METHODS

I. Cantor series

Let the notation pertaining Cantor series, save that the elements a_n are also allowed to be negative, be as expounded in the introduction. The following two lemmas were proved in Oppenheim (1954).

Lemma 1. Given $b_i \geq 2$, $0 \leq a_i \leq b_i - 1$. Then $x = a_0 + \sum_{i=1}^{\infty} \frac{a_i}{b_1 \cdots b_i}$ is irrational if $a_i > 0$ infinitely often and if there is a subsequence (i_n) such that $c_{i_n} = a_{i_n} / b_{i_n} \to 0$ and $b_{i_n} \to \infty$ $(n \to \infty)$.

Lemma 2. Given $b_i \geq 2$, $|a_i| \leq b_i - 1$ and $a_m a_n < 0$ for some $m > i$, $n > i$ when i is any assigned integer. Then $x = a_0 + \sum_{i=1}^{\infty} \frac{a_i}{b_1 \cdots b_i}$ is irrational if there exists a subsequence (i_n) such that one of the following two conditions holds: 1) $c_{i_n} = a_{i_n} / b_{i_n} \to 0$ and $b_{i_n} \to \infty$, or 2) $c_{i_n} = a_{i_n} / b_{i_n} \to 1$ or -1 $(n \to \infty)$.

II. Badea's criteria

Let (a_n) and (b_n) $(n \geq 1)$ be two sequences of positive integers and let $N = (n(k); k \geq 1)$ be an increasing sequence of positive integers. Define

$d(k) := n(k+1) - n(k)$, $S_k(N) := a_{n(k)+1} \ldots a_{n(k+1)}$,

$$R_k(N) := \sum_{j=1}^{d(k)} \frac{b_{n(k)+j}}{a_{n(k)+j}} S_k(N).$$

The next two lemmas were proved in Badea (1993).

Lemma 3. Assume that $\sum_{n=1}^{\infty} \frac{b_n}{a_n}$ is rational and that

$$S_{k+1}(N) \geq \frac{R_{k+1}(N)}{R_k(N)} S_k(N)\{S_k(N) - 1\} + 1.$$

Then $S_{k+1}(N) = \frac{R_{k+1}(N)}{R_k(N)} S_k(N)\{S_k(N) - 1\} + 1$ when k is sufficiently large.

Lemma 4. Assume that $\sum_{n=1}^{\infty} \frac{b_n}{a_n}$ is rational and that

$$a_{n+1} \geq \frac{b_{n+1}}{b_n} a_n^2 - \frac{b_{n+1}}{b_n} a_n + 1.$$

Then $a_{n+1} = \frac{b_{n+1}}{b_n} a_n^2 - \frac{b_{n+1}}{b_n} a_n + 1$ when n is sufficiently large.

Finally, we list here some more notation to be used in the last part.

$$F(k) = \frac{b_{n(k)+1}}{a_{n(k)+1}} + \frac{b_{n(k)+2}}{a_{n(k)+2}} + \ldots + \frac{b_{n(k+1)}}{a_{n(k+1)}},$$

$$F_o(2,k) = \frac{b_{2n(k)+1}}{a_{2n(k)+1}} + \frac{b_{2n(k)+3}}{a_{2n(k)+3}} + \ldots + \frac{b_{2n(k+1)-1}}{a_{2n(k+1)-1}},$$

$$F_e(2,k) = \frac{b_{2n(k)+2}}{a_{2n(k)+2}} + \frac{b_{2n(k)+4}}{a_{2n(k)+4}} + \ldots + \frac{b_{2n(k+1)}}{a_{2n(k+1)}},$$

$\Pi(k) = a_{n(k)+1} \ldots a_{n(k+1)}$, $\Pi(2,k) = a_{2n(k)+1} \ldots a_{2n(k+1)}$.

RESULTS

Theorem 1. Let x be a real number whose Cantor series is $\sum_{i=1}^{\infty} \frac{a_i}{b_1 b_2 \ldots b_i}$, $b_i \geq 2$, $0 \leq a_i \leq b_i - 1$. Assume that every limit of (a_i/b_i) is a rational number. Let h/k where $0 < h < k$, $(h,k) = 1$ be one such limit corresponding to a subsequence (j), called (h/k)-subsequence. Let $ka_i/h - b_i + 1 = \xi_i$ $(i \geq 1)$. Assume further that $b_j \to \infty$ $(j \to \infty)$ in the (h/k)-subsequence.

A) If i) $|\xi_i| \leq b_i - 1$ $(i \geq 1)$,

ii) $\xi_m \xi_n < 0$ for some $m > i$, $n > i$, when i is any assigned positive integer

and iii) $\xi_j / b_j \to 0$ as $j \to \infty$ in the (h/k)-subsequence, then x is irrational.

B) Suppose that ξ_i is constant, equal to a rational ξ for all sufficiently large i. Then x is rational if and only if $\xi = 0$.

Proof. Write

$$x = \sum_{i=1}^{\infty} \frac{a_i}{b_1 b_2 \ldots b_i} = \frac{h}{k} \sum_{i=1}^{\infty} \frac{b_i - 1 + \xi_i}{b_1 b_2 \ldots b_i}$$

$$= \frac{h}{k}\left(\left(\frac{b_1 - 1}{b_1} + \frac{b_2 - 1}{b_1 b_2} + \frac{b_3 - 1}{b_1 b_2 b_3} + \ldots\right) + \sum_{i=1}^{\infty} \frac{\xi_i}{b_1 b_2 \ldots b_i}\right) = \frac{h}{k}\left(1 + \sum_{i=1}^{\infty} \frac{\xi_i}{b_1 b_2 \ldots b_i}\right).$$

To prove A), note that by Lemma 2, $\sum_{i=1}^{\infty} \frac{\xi_i}{b_1 b_2 \ldots b_i}$ is irrational and so is x.

To prove B), since there is an index i_0 such that $\xi_i = \xi$ $(i \geq i_0)$, then

$$x = \frac{h}{k}\left(\alpha + \xi \sum_{i=i_0}^{\infty} \frac{1}{b_1 b_2 \ldots b_i}\right),$$

for some rational α. Now $\sum_{i=i}^{\infty} \frac{1}{b_1 b_2 \ldots b_i}$ is irrational by Lemma 1 and this immediately yields the conclusion.

Theorem 2. Let the notation be as set out in Part II of the last section. If $1 > \frac{F(k+1)}{F(k)}\{\prod(k) - 1\} + \frac{1}{\prod(k+1)}$ for sufficiently large k, then $\sum_{n=1}^{\infty} \frac{b_n}{a_n}$ is irrational.

Proof. Note first that

$$R_k(N) = \sum_{j=1}^{d(k)} S_k(N) \frac{b_{n(k)+j}}{a_{n(k)+j}} = \prod(k)F(k),$$

and $S_k(N) = a_{n(k)+1} \ldots a_{n(k+1)} = \prod(k)$.

From $1 > \frac{F(k+1)}{F(k)}\{\prod(k) - 1\} + \frac{1}{\prod(k+1)}$, we get

$$\prod(k+1) > \frac{F(k+1)}{F(k)} \prod(k+1)\{\prod(k) - 1\} + 1,$$

i.e. $S_{k+1}(N) > \frac{R_{k+1}(N)}{R_k(N)} S_k(N)\{S_k(N) - 1\} + 1$ for sufficiently large k. By Lemma 3, $\sum_{n=1}^{\infty} \frac{b_n}{a_n}$ is irrational.

Theorem 3. If $\lim_{k\to\infty} \frac{F(k+1)}{F(k)} \prod(k) < 1$ and $\lim_{k\to\infty} \prod(k) = \infty$, then $\sum_{n=1}^{\infty} \frac{b_n}{a_n}$ is irrational.

Proof. Let $\lim_{k\to\infty} \frac{F(k+1)}{F(k)} \prod(k) = \alpha < 1, \varepsilon = \frac{1-\alpha}{2} > 0$. Then for sufficiently large k, we have

$$0 < \frac{F(k+1)}{F(k)} \prod(k) - \frac{F(k+1)}{F(k)} < \varepsilon + \alpha - \frac{\alpha - \varepsilon}{\prod(k)} \leq \alpha + 2\varepsilon = 1.$$

Since $\lim_{k\to\infty} \prod(k) = \infty$, then $0 < \frac{F(k+1)}{F(k)} \prod(k) - \frac{F(k+1)}{F(k)} + \frac{1}{\prod(k+1)} < 1$ for sufficiently large k, and Theorem 2 yields the desired result.

Theorem 4. Let $(n(k); k \geq 1)$ and (x_n) be increasing sequences of positive integers.

Assume that 1) $n(k+1) \geq 2n(k) - 1$ for all k sufficiently large, and 2) $x_n \sim A\beta^n$ where $\beta > 1$, $A > 0$ and $A\beta \leq 1$.

Then $\sum_{k=1}^{\infty} \frac{1}{x_{n(k)}}$ is irrational.

Proof. From $\frac{x_{2n-1}}{x_n^2} \sim \frac{1}{A\beta} \geq 1$, we get $x_{2n-1} \geq x_n^2$, and so $x_{n(k+1)} \geq x_{2n(k)-1} \geq x_{n(k)}^2$ when k is sufficiently large. Since $x_{n(k)} > 1$, then $x_{n(k+1)} > x_{n(k)}^2 - x_{n(k)} + 1$ when k is sufficiently large. Considering $x_{n(k)}$ as a_k when k is sufficiently large, the result now follows from Lemma 4.

Theorem 5. Let $(n(k); k \geq 1)$ and (x_n) be increasing sequences of positive integers. Let $y_n = x_{n+r} + \ldots + x_{n+3} + x_{n+1} + x_{n-1} + x_{n-3} + \ldots + x_{n-r}$, $r = 2m + 1$ for some nonnegative integer m and $n \geq r$. Assume that

1) $n(k+1) \geq 2n(k)$ for all k sufficiently large

2) $x_n \sim A\beta^n$ where $\beta > 1$, $A > 0$, and

3) $A\beta^r \left\{1 + \frac{1}{\beta^2} + \ldots + \frac{1}{\beta^{2r}}\right\} \leq 1.$

Then $\sum_{k=1}^{\infty} \frac{1}{y_{n(k)}}$ is irrational.

Proof. We have $y_{2n} = x_{2n+r} + \ldots + x_{2n+3} + x_{2n+1} + x_{2n-1} + x_{2n-3} + \ldots + x_{2n-r}$

$$\sim A\beta^{2n+r} + \ldots + A\beta^{2n+1} + A\beta^{2n-1} + \ldots + A\beta^{2n-r} = A\beta^{2n+r}\left(1 + \frac{1}{\beta^2} + \ldots + \frac{1}{\beta^{2r}}\right),$$

and similarly, $y_n \sim A\beta^{n+r}\left(1 + \frac{1}{\beta^2} + \ldots + \frac{1}{\beta^{2r}}\right)$. Thus

$$\frac{y_{2n}}{y_n^2} \sim \frac{1}{A\beta^r\left(1+\frac{1}{\beta^2}+\ldots+\frac{1}{\beta^{2r}}\right)} \geq 1,$$

and so $y_{2n} \geq y_n^2$ for n sufficiently large. Since $n(k+1) \geq 2n(k)$, then

$y_{n(k+1)} \geq y_{2n(k)} \geq y_{n(k)}^2$ for k sufficiently large.

As $y_{n(k)} > 1$, we get $y_{n(k+1)} > y_{n(k)}^2 - y_{n(k)} + 1$. The result again follows from Lemma 4.

Theorem 6. Let $(b_n/a_n ; n \geq 1)$ be a decreasing sequence of positive rationals satisfying

$$1 > \frac{F_o(2,k+1)-F_e(2,k+1)}{F_o(2,k)-F_e(2,k)}\{\Pi(2,k)-1\} + \frac{1}{\Pi(2,k+1)}. \qquad (*)$$

If $\sum_{n=1}^{\infty}(-1)^{n+1}\frac{b_n}{a_n}$ is rational, then (*) becomes equality when k is sufficiently large.

Proof. Put $\frac{B_n}{A_n} = \frac{b_{2n-1}}{a_{2n-1}} - \frac{b_{2n}}{a_{2n}}$, with $A_n := a_{2n}a_{2n-1}$. Now

$$F_o(2,k) - F_e(2,k) = \left(\frac{b_{2n(k)+1}}{a_{2n(k)+1}} - \frac{b_{2n(k)+2}}{a_{2n(k)+2}}\right) + \ldots + \left(\frac{b_{2n(k+1)-1}}{a_{2n(k+1)-1}} - \frac{b_{2n(k+1)}}{a_{2n(k+1)}}\right) = \frac{B_{n(k)+1}}{A_{n(k)+1}} + \ldots + \frac{B_{n(k+1)}}{A_{n(k+1)}},$$

and $\Pi(2,k) = a_{2n(k)+1}\ldots a_{2n(k+1)} = A_{n(k)+1}\ldots A_{n(k+1)}$. From the hypotheses, we deduce that

$$1 > \frac{\frac{B_{n(k+1)+1}}{A_{n(k+1)+1}} + \ldots + \frac{B_{n(k+2)}}{A_{n(k+2)}}}{\frac{B_{n(k)+1}}{A_{n(k)+1}} + \ldots + \frac{B_{n(k+1)}}{A_{n(k+1)}}}\{A_{n(k)+1}\ldots A_{n(k+1)} - 1\} + \frac{1}{A_{n(k+1)+1}\ldots A_{n(k+2)}}.$$

Since (b_n/a_n) is a decreasing sequence of positive rationals, then (B_n/A_n) is a sequence of positive rationals and by Theorem 2, $\sum_{n=1}^{\infty}\frac{B_n}{A_n} = \sum_{n=1}^{\infty}(-1)^{n+1}\frac{b_n}{a_n}$ is irrational.

Theorem 7. Let f be a function from the positive integers to the nonnegative integers, G and H be functions from the nonnegative integers to the positive integers. Assume that

1) f is strictly increasing, and

2) there are real numbers $A > 0$, $c \geq 0$ and $\beta > 1$ such that $\lim_{k\to\infty}\frac{H(k)}{G(k)k^c\beta^k} = A$, and $\lim_{k\to\infty}\frac{G(f(k)f(k)^{2c}}{f(k+1)^c\beta^{f(k+1)-2f(k)}} = 0.$

Then $\sum_{n=1}^{\infty}\frac{G(f(n))}{H(f(n))}$ is irrational.

Proof. By Lemma 4, we must show that for sufficiently large n,

$$H(f(n+1)) > \frac{G(f(n+1))}{G(f(n))}\{H(f(n))^2 - H(f(n))\} + 1.$$

From $\lim_{n\to\infty}\frac{H(f(n+1))}{G(f(n+1))f(n+1)^c\beta^{f(n+1)}} = A > 0$, $c \geq 0$ and $\beta > 1$, we deduce that

$$\frac{H(f(n+1))}{G(f(n+1))f(n+1)^c\beta^{f(n+1)}} > \varepsilon A,$$

for fixed $0 < \varepsilon < 1$ and sufficiently large n. Now

$$\frac{\frac{H(f(n))^2 - H(f(n))}{G(f(n))} + \frac{1}{G(f(n+1))}}{f(n+1)^c\beta^{f(n+1)}} = \frac{G(f(n))f(n)^{2c}}{f(n+1)^c\beta^{f(n+1)-2f(n)}}\left(\frac{H(f(n))^2}{G(f(n))^2 f(n)^{2c}\beta^{2f(n)}} - \frac{H(f(n))}{G(f(n))^2 f(n)^{2c}\beta^{2f(n)}}\right) + \frac{1}{G(f(n+1))f(n+1)^c\beta^{f(n+1)}}.$$

Using

$$\lim_{n\to\infty}\frac{G(f(n))f(n)^{2c}}{f(n+1)^c\beta^{f(n+1)-2f(n)}} = 0,$$

$$\lim_{n\to\infty}\frac{H(f(n))^2}{G(f(n))^2 f(n)^{2c}\beta^{2f(n)}} = A^2,$$

we get for sufficiently large n,

$$\frac{H(f(n))^2 - H(f(n))}{G(f(n))} + \frac{1}{G(f(n+1))} < \varepsilon A/2, \text{ i.e.}$$

$$\frac{H(f(n+1))/G(f(n+1))}{\frac{H(f(n))^2 - H(f(n))}{G(f(n))} + \frac{1}{G(f(n+1))}} > \frac{\varepsilon A}{\varepsilon A/2} = 2 > 1.$$

Hence, for sufficiently large n, $H(f(n+1)) > \frac{G(f(n+1))}{G(f(n))}\{H(f(n))^2 - H(f(n))\} + 1$, yielding the desired result.

The following examples illustrate a wide applicability of Theorem 7.

Examples. Take $G(0) = 1, G(1) = 1, G(k+2) = G(k+1) + G(k), H(0) = H(1) = 1, H(k+2) = 3H(k+1) + 4H(k)$. Then $G(k) \sim \frac{\sqrt{5}+1}{2\sqrt{5}}\left(\frac{1+\sqrt{5}}{2}\right)^k$, $H(k) \sim \frac{2}{5}4^k$, and so $\frac{H(k)}{G(k)} \sim \frac{4}{5+\sqrt{5}}\left(\frac{8}{1+\sqrt{5}}\right)^k$. Taking $\beta = \frac{8}{1+\sqrt{5}}$, $c = 0$, we get $\lim_{k\to\infty} H(k)/G(k)k^c\beta^k = \frac{4}{5+\sqrt{5}}$, and $I(k) = \frac{G(f(k))f(k)^{2c}}{f(k+1)^c\beta^{f(k+1)-2f(k)}} \sim \frac{\left((1+\sqrt{5}/2\right)^{f(k)+1}/\sqrt{5}}{\left(8/(1+\sqrt{5})\right)^{f(k+1)-2f(k)}}$.

1) For $f(k) = 3^k$, we see that $I(k) \to 0$ $(k \to \infty)$, and so $\sum_{k=1}^{\infty} \frac{G(3^k)}{H(3^k)}$ is irrational.

2) For $f(k) = k!$, we conclude similarly that $\sum_{k=1}^{\infty} \frac{G(k!)}{H(k!)}$ is irrational.

DISCUSSION AND CONCLUSION

The two examples of Diananda and Oppenheim mentioned in the introduction are special cases of our Theorem 1 above. The first example corresponds to $a_i = i$, $b_i = 2i + 1$, which gives $a_i/b_i \to h/k = {}^1/_2$, $\xi_i = 2i - 2i - 1 + 1 = 0$ and so x is rational. The second example corresponds to $a_i = 1$, $b_i = 3i + 2$, which gives $a_i/b_i \to h/k = 1/3$, $\xi_i = 3i - 3i - 2 + 1 \neq 0$ and so x is irrational.

Our Theorem 2 is a modification of Theorem 2.1' in Badea (1993), while our Theorem 3 gives a simplification of Theorem 2. Theorems 4, 5, 6 are extensions of Corollaries 3.2, 3.4, 3.5 and 3.6 of Badea (1993).

Theorem 1 of Brown *et al*. (1995) is a special case with $G(k) = 1$ of our Theorem7,because $\lim_{k\to\infty}\{f(k+1) - 2f(k)\} = \infty$ implies $\lim_{k\to\infty} \frac{1}{\beta^{f(k+1)-2f(k)}} = 0$ and $f(k+1) \geq f(k)^2$ implies $\lim_{k\to\infty} \frac{f(k)^{2c}}{f(k+1)^c\beta^{f(k+1)-2f(k)}} = 0$.

LITERATURE CITED

Badea, C. 1993. A theorem on irrationality of infinite series and applications. Acta Arith. 58 : 313-323.

Brown, T.C., D.Y. Pei, and P.J.S. Shiue. 1995. Irrational sums. Rocky Mountain J. Math. 25 : 1219-1223.

Diananda, P.H. and A. Oppenheim. 1955. Criteria for irrationality of certain classes of numbers II. Amer. Math. Monthly 62 : 222-225.

Niven, I. 1967. Irrational Numbers. The Mathematical Association of America. Washington DC. 161 p.

Oppenheim, A. 1954. Criteria for irrationality of certain classes of numbers. Amer. Math. Monthly 61 : 235-241.

Received date : 29/05/01
Accepted date : 29/06/01

Kasetsart J. (Nat. Sci.) 35 : 210 - 223 (2001)

Some Estimates Involving Density of Algebraic Numbers and Integer Polynomials

Chanon Chuntra and Vichian Laohakosol

ABSTRACT

Nymann in 1970 derived an asymptotic formula for the probability that k positive integers, chosen at random from the first n natural numbers, are relatively prime. In 1996, Arno *et al.* introduced a new concept called the denominator of an integer polynomial. Using this concept, Arno *et al.* proved theorems establishing formulae for determining the denominator of any algebraic number and the density of algebraic numbers whose denominators are equal to the leading coefficients in their minimal polynomials. The proofs of Arno *et al.* made use of the result of Nymann . The first part of this paper is an extension of the work of Nymann done by relaxing the condition that the chosen numbers are relatively prime. In the second part, the formulae derived in the first part are employed to find asymptotic estimates and the density of the set of integer polynomials refining the work of Arno *et al.*

Key words: algebraic numbers, integer polynomials, denominators, asymptotic value

INTRODUCTION

Nymann (1970) derived an asymptotic formula for the probability of k integers chosen at random from the set $\{1, 2, \dots, n\}$ to be relatively prime. Nymann's result says that this probability is approximately equal to $1/\varsigma(k)$, where ς is the Riemann zeta function. An integer polynomial is a polynomial with integral coefficients. A complex number is an algebraic number if it is a root of a nonzero polynomial with rational coefficients. Among the polynomials with rational coefficients which have an algebraic number α as a root, the one which is monic, and has the least degree is called the minimal polynomial of α over $\mathbb{Q}$ and its degree is called the degree of α. Multiplying this minimal polynomial of α by the least common multiple of the denominators of its coefficients, we get what we call the **minimal polynomial of α over $\mathbb{Z}$.** If α is an algebraic number with p(x) as its minimal polynomial, then all the roots of p(x) are called conjugates of α. An algebraic integer is an algebraic number whose minimal polynomial over $\mathbb{Q}$ has all its coefficients integral. By the **denominator** of an algebraic number α, written **den(α),** we mean the least positive integer n such that $n\alpha$ is an algebraic integer. Denominators are useful in various approximation problems because they satisfy certain multiplicative and additive properties, yet their exact calculation is difficult. Arno *et al.*(1996), see also Laohakosol *et al*.(2000), introduced a new concept of the **denominator of an integer polynomial** A of degree d and with roots α_k ($1 \le k \le d$), as the least positive integer n, written **den(A)**, for which $n\alpha_k$ is an algebraic integer for all such k. Working through this concept, Arno *et al.*(1996) established formulae for computing the denominator of an algebraic number and the density of those

Department of Mathematics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.

algebraic numbers whose denominators equal to the leading coefficients of their minimal polynomials. Their results say for example that this density is about 83%. The proofs of Arno *et al.*(1996) make essential use of the asymptotic estimates of Nymann (1970) mentioned above.

There are two main objectives in this paper. First, the work of Nymann (1970) is extended by relaxing the condition that the chosen numbers are relatively prime. Second, the formulae derived in the first part are used to find asymptotic estimates and the density of the set of integer polynomials refining the work of Arno *et al.* (1996). In the first part, it is found that the probability for k integers chosen at random from the first n natural numbers to have their greatest common divisor equal to g is approximately $1/g^k\zeta(k)$. In the second part, it is found in particular that the probability that an integer polynomial, with its last two coefficients having their greatest common divisor equal to a square-free integer g, has its denominator equal to its leading coefficient is approximately equal to $|\mu(g)g|/\Pi(p+1)$, where μ is the Möbius function and the product extends over all primes p dividing g.

MATERIALS AND METHODS

Definition. Let α be an algebraic number whose minimal polynomial over $\mathbb{Z}$ is $A(x) = a_d x^d + \ldots + a_0 \in \mathbb{Z}[x]$. The **height** of a is defined as

$$H(\alpha) = \max \{|a_i| : 0 \le i \le d\}.$$

The following terminology will be kept standard throughout the entire paper.

$Z_k^{(g)}(t)$ the number of k-tuples $<m_1,\ldots,m_k>$ of integers such that all $|m_i| \le t$, $(m_1,\ldots,m_k) = g$ and $m_k \ne 0$

$Z_k^{(1,g)}(t)$ the number of k-tuples $<m_1,\ldots,m_k>$ of integers such that all $|m_i| \le t$, $(m_1,\ldots,m_k) = 1$, $(m_k,m_{k-1}) = g$ and $m_k \ne 0$

$Prob_k^{(g)}(n)$ the probability that k integers chosen randomly from the set $\{0,\pm1,\pm2,\ldots,\pm n\}$ have $(m_1,\ldots,m_k) = g$ and $m_k \ne 0$

$Prob_k^{(1,g)}(n)$ the probability that k integers chosen randomly from the set $\{0,\pm1,\pm2,\ldots,\pm n\}$ have $(m_1,\ldots,m_k) = 1$, $(m_k,m_{k-1}) = g$ and $m_k \ne 0$

$$P_d(H) = \{A(x) = \sum_{i=0}^{d} a_i x^i \in \mathbb{Z}[x] : a_d \ne 0, |a_i| \le H, (a_0,\ldots,a_d) = 1\}$$

$$P_d^{(g)}(H) = \{A(x) = \sum_{i=0}^{d} a_i x^i \in P_d(H) : (a_d,a_{d-1}) = g\}$$

$$\hat{P}_d(H) = \{A(x) = \sum_{i=0}^{d} a_i x^i \in P_d(H) : den(A) = |a_d|\}$$

$$\hat{P}_d^{(g)}(H) = \{A(x) = \sum_{i=0}^{d} a_i x^i \in P_d^{(g)}(H) : den(A) = |a_d|\}$$

$$S_r = \{A(x) \in P_d(H) : r^2 \mid a_d, r \mid a_{d-1}\}$$

$$S_r^{(g)} = \{ A(x) = \sum_{i=0}^{d} a_i x^i \in P_d^{(g)}(H) : r^2 \mid a_d, r \mid a_{d-1}\}$$

$$A_d(H) = \{\alpha : \alpha \text{ is an algebraic number, } \deg(\alpha) = d,\ H(\alpha) \le H\}$$

$$\hat{A}_d(H) = \{\alpha \in A_d(H) : \text{den } \alpha = \text{leading coefficient of } \alpha \text{ over } \mathbb{Z}\}$$

$$U_d(H) = \{A(x) = \sum_{i=0}^{d} a_i x^i \in \mathbb{Z}[x] : a_d \ne 0,\ |a_i| \le H\ (0 \le i \le d)\}$$

$\hat{U}_d(H) = \{A(x) = \sum_{i=0}^{d} a_i x^i \in U_d(H) : \text{den}(A) = |a_d|\}$

Lemma 1. Let $g \geq 1$ be fixed. For $t \geq 1$, we have

$$Z_k^{(g)}(t) = \frac{(2t)^k}{g^k \varsigma(k)} + O(\frac{t^{k-1}}{g^{k-1}}) \quad (k \geq 3), \text{ and } \quad Z_2^{(g)}(t) = \frac{(2t)^2}{g^2 \varsigma(2)} + O(\frac{t}{g}\log\frac{t}{g}).$$

Proof. We first treat the case $g = 1$. Observe that

$$Z_k^{(1)}(t) = \sum_{\substack{(m_1,\ldots,m_k)=1 \\ -t\leq m_i \leq t, m_k \neq 0}} 1 \tag{1}$$

and

$$2[t](2[t]+1)^{k-1} = \sum_{\substack{-t\leq m_i \leq t, m_k \neq 0 \\ i=1,2,K,k}} 1 = \sum_{1\leq d\leq t} \sum_{\substack{(m_1,\ldots,m_k)=d \\ -t\leq m_i \leq t, m_k \neq 0}} 1 \tag{2}$$

Since $(m_1,\ldots,m_k) = d$ if and only if $(m_1/d,\ldots,m_k/d) = 1$, then there is a one-to-one correspondence between the k-tuples $<m_1,\ldots,m_k>$ with $(m_1,\ldots,m_k) = d$, $-t \leq m_i \leq t$, $m_k \neq 0$ and the k-tuples $<m_1',\ldots,m_k'>$ with $(m_1',\ldots,m_k') = 1$, $-t/d \leq m_i' \leq t/d$, $m_k' \neq 0$. By definition, the number of such k-tuples $<m_1',\ldots,m_k'>$ is $Z_k^{(1)}(\frac{t}{d})$. From (1) and (2) we get

$$2[t](2[t]+1)^{k-1} = \sum_{1\leq d\leq t} Z_k^{(1)}\left(\frac{t}{d}\right). \tag{3}$$

Applying the Möbius inversion formula to (3) yields

$$Z_k^{(1)}(t) = \sum_{1\leq d\leq t} \mu(d)\{2[t/d]+1\}^{k-1} 2[t/d] = \sum_{1\leq d\leq t} \mu(d)\{2t/d + O(1)\}^k$$

$$= (2t)^k \sum_{1\leq d\leq t} \frac{\mu(d)}{d^k} + (2t)^{k-1} O(\sum_{1\leq d\leq t} \frac{\mu(d)}{d^{k-1}}) + \ldots + (2t) O(\sum_{1\leq d\leq t} \frac{\mu(d)}{d}) + O(\sum_{1\leq d\leq t} 1) \tag{4}$$

From Apostol (1976), we know that

$$\sum_{1\leq d\leq t} \frac{\mu(d)}{d^k} = \frac{1}{\varsigma(k)} + O(\frac{1}{t^{k-1}}), \tag{5}$$

and then the first term on the right-hand side of equation (4) is equal to $\frac{(2t)^k}{\varsigma(k)} + O(2^k t)$. From equation (5), we have

$$\sum_{1\leq d\leq t} \frac{\mu(d)}{d^i} = O(1) \qquad (2 \leq i \leq k-1), \tag{6}$$

while, see Apostol (1976),

$$|\sum_{1\leq d\leq t} \frac{\mu(d)}{d}| \leq \sum_{1\leq d\leq t} \frac{1}{d} = \log t + \gamma + O(1/t), \tag{7}$$

where γ is the Euler's constant, and

$$\sum_{1\leq d\leq t} 1 = [t] = O(t). \tag{8}$$

Using equations (4)-(8), we arrive at

$$Z_k^{(1)}(t) = \frac{(2t)^k}{\varsigma(k)} + O(t) + O(t^{k-1}) + O(t^{k-2}) + \ldots + O(t^2) + O(t \log t) + O(t).$$

$$= \begin{cases} \frac{(2t)^k}{\varsigma(k)} + O(t^{k-1}), & k \geq 3 \\ \frac{(2t)^2}{\varsigma(2)} + O(t \log t), & k = 2 \end{cases}.$$

Next, we observe that $Z_k^{(g)}(t) = \sum_{\substack{(m_1,\ldots,m_k)=g \\ -t \leq m_i \leq t, m_k \neq 0}} 1 = \sum_{\substack{(\frac{m_1}{g},\ldots,\frac{m_k}{g})=1 \\ -\frac{t}{g} \leq \frac{m_i}{g} \leq \frac{t}{g}, \frac{m_k}{g} \neq 0}} 1 = \sum_{\substack{(m_1',\ldots,m_k')=1 \\ -\frac{t}{g} \leq m_i' \leq \frac{t}{g}, m_k' \neq 0}} 1..$

Replacing t by t/g in the preceding discussion, we get

$$Z_k^{(g)}(t) = Z_k^{(1)}(\frac{1}{g}) = \begin{cases} \frac{(2t)^k}{(g)^k \varsigma(k)} + O(\frac{t^{k-1}}{g^{k-1}}), k \geq 3 \\ \frac{(2t)^k}{(g)^2 \varsigma(2)} + O(\frac{t}{g} \log \frac{t}{g}), k = 2 \end{cases}$$

as to be proved. Q.E.D.

Lemma 2. Let $g \geq 1$ be fixed. For $t \geq 1$, we have

$$Z_k^{(1,g)}(t) = \frac{(2t)^k}{(g)^2 \varsigma(2)} \prod_{p \mid g} (1 - \frac{1}{p^{k-2}}) + O(\frac{t^{k-1}}{g} \log \frac{t}{g}) \quad (k \geq 3), \text{ and}$$

$$Z_2^{(1,g)}(t) = \frac{(2t)^2}{\varsigma(2)} + O(t \log t).$$

Proof. Observe that

$$Z_k^{(1,g)}(t) \sum_{\substack{(m_1,\ldots,m_k)=1 \\ (m_k,m_{k-1})=g \\ -t \leq m_i \leq t, m_k \neq 0}} 1, \tag{9}$$

and, applying the case k = 2 of Lemma 1 to the last two coordinates, we have

$$\{\frac{(2t)^2}{g^2 \varsigma(2)} + O(\frac{t}{g} \log \frac{t}{g})\}(2[t]+1)^{k-2} = \sum_{\substack{(m_k,m_{k-1})=g \\ -t \leq m_i \leq t, m_k \neq 0 \\ i=1,2,\ldots,k}} 1 = \sum_{1 \leq d \leq t} \sum_{\substack{(m_1,\ldots m_k)=d \\ (m_k,m_{k-1})=g \\ -t \leq m_i \leq t, m_k \neq 0}} 1. \tag{10}$$

Since $(m_1,\ldots,m_k) = d$ if and only if $(m_1/d,\ldots,m_k/d) = 1$, then there is a one-to-one correspondence between the k-tuples $\langle m_1,\ldots,m_k \rangle$ with $(m_1,\ldots,m_k) = d$, $-t \leq m_i \leq t$, $m_k \neq 0$, $(m_k, m_{k-1}) = g$ and the k-tuples $\langle m_1',\ldots,m_k' \rangle$ with $(m_1',\ldots,m_k') = 1$, $-t/d \leq m_i' \leq t/d$, $m_k' \neq 0$, $(m_k', m_{k-1}') = g/d$, $d \mid g$. By definition, the number of such k-tuples $\langle m_1',\ldots,m_k' \rangle$ is equal to $Z_k^{(1,\frac{g}{d})}(\frac{t}{d})$. By equations (9) and (10), we obtain

$$\{\frac{(2t)^2}{g^2\varsigma(2)}+O(\frac{t}{g}\log\frac{t}{g})\}(2[t]+1)^{k-2}=\sum_{\substack{1\le d\le t\\ d\,|\,g}} Z_k^{(1,\frac{g}{d})}(\frac{t}{d}). \tag{11}$$

Applying the Möbius inversion formula to equation (11), we deduce that

$$Z_k^{(1,g)}(t)=\sum_{\substack{1\le d\le t\\ d\,|\,g}}\mu(d)\{2[t/d]+1\}^{k-2}\{\frac{1}{\varsigma(2)}(\frac{2(t/d)}{g/d})^2+O(\frac{t/d}{g/d}\log\frac{t/d}{g/d})\}$$

$$= \{(2t)^{k-2}\sum_{\substack{1\le d\le t\\ d\,|\,g}}\frac{\mu(d)}{d^{k-2}}+(2t)^{k-3}O(\sum_{\substack{1\le d\le t\\ d\,|\,g}}\frac{\mu(d)}{d^{k-3}})+\ldots+2tO(\sum_{\substack{1\le d\le t\\ d\,|\,g}}\frac{\mu(d)}{d})+O(\sum_{\substack{1\le d\le t\\ d\,|\,g}}1)\}\times$$

$$\times\{\frac{1}{\varsigma(2)}(\frac{2t}{g})^2+O(\frac{t}{g}\log\frac{t}{g})\}\cdot \tag{12}$$

Since, see Apostol (1976),

$$\sum_{\substack{1\le d\le t\\ d\,|\,g}}\frac{\mu(d)}{d^{k-2}}=\prod_{p\,|\,g}(1-\frac{1}{p^{k-2}}), \tag{13}$$

$$\sum_{\substack{1\le d\le t\\ d\,|\,g}}\frac{\mu(d)}{d^{i}}=O(1)\qquad (2\le i\le k\text{-}3), \tag{14}$$

$$\sum_{\substack{1\le d\le t\\ d\,|\,g}}\frac{\mu(d)}{d}=O(\log t), \tag{15}$$

and

$$\sum_{\substack{1\le d\le t\\ d\,|\,g}}1=[t/g]\quad=\quad O(t/g), \tag{16}$$

then from equations (12)-(16), we have for $k\ge 3$

$$Z_k^{(1,g)}(t)=\frac{(2t)^k}{g^2\varsigma(2)}\prod_{p\,|\,g}(1-\frac{1}{p^{k-2}})+O(\frac{t^{k-1}}{g^2})+K+O(\frac{t^3}{g^2}\log t)+O(\frac{t^3}{g^3})+O(\frac{t^{k-1}}{g}\log\frac{t}{g})$$

$$+O(\frac{t^{k-2}}{g}\log\frac{t}{g})+\ldots+O(\frac{t^2}{g}\log\frac{t}{g}\log t)+O(\frac{t^2}{g^2}\log\frac{t}{g})$$

$$=\frac{(2t)^k}{(g)^2\varsigma(2)}\prod_{p\,|\,g}(1-\frac{1}{p^{k-2}})+O(\frac{t^{k-1}}{g}\log\frac{t}{g}),$$

while for k = 2, from equation (12), we have

$$Z_2^{(1,g)}(t)=\sum_{\substack{1\le d\le t\\ d|g}}\mu(d)\,(\frac{1}{\varsigma(2)}\,(\frac{2t}{g})^2+O(\frac{t}{g}\log\frac{t}{g}))=\frac{(2t)^2}{g^2\varsigma(2)}\sum_{\substack{1\le d\le t\\ d|g}}\mu(d)+O(\frac{t}{g}\log\frac{t}{g}),$$

which can be rewritten by using $\sum_{\substack{1\le d\le t\\ d|g}} \mu(d) = [1/g]$ as

$$Z_2^{(1,g)}(t) = \begin{cases} 0, & g>1 \\ \dfrac{(2t)^2}{\varsigma(2)} + O(t\log t), & g=1 \end{cases}. \qquad \text{Q.E.D.}$$

Lemma 3. Let $d \ge 2$ and g be fixed integers. Then

$$\left| P_d^{(g)}(H) \right| = \frac{(2H)^{d+1}}{\varsigma(2)g^2} \prod_{p|g} \left(1 - \frac{1}{p^{d-1}}\right) + O_d\left(\frac{H^d}{g}\log\frac{H}{g}\right).$$

Proof. From its definition, see also Pólya and Szegö (1976), $P_d^{(g)}(H) = Z_k^{(1,g)}(t)$, with $t = H$, $d = k-1$. Using Lemma 2, we get

$$\left| P_d^{(g)}(H) \right| = \frac{(2H)^{d+1}}{\varsigma(2)g^2} \prod_{p|g} \left(1 - \frac{1}{p^{d-1}}\right) + O_d\left(\frac{H^d}{g}\log\frac{H}{g}\right). \qquad \text{Q.E.D.}$$

Lemma 4. Let $d \ge 2$, $g \ge 1$ be fixed integers, and $1 \le r \le H$. Then for $u = (r, g/mr)$, we have

$$\left| S_r^{(g)} \right| = \frac{(2H)^{d+1} u}{\varsigma(2)g^2 r} \prod_{p|\frac{r}{u}} \left(\frac{1}{1+\frac{1}{p}}\right) \prod_{p|g} \left(1 - \frac{1}{p^{d-1}}\right) + O_d\left(\frac{H^d u}{gr}\log\frac{H}{g}\right).$$

Proof. First consider the case $r = 1$. Since $S_1^{(g)} = P_d^{(g)}(H)$, then the theorem is true in this case. Assume $r \ge 2$. For brevity, we omit writing the hypothesis $0 \le i \le d$ underneath the summation sign. Thus

$$\left| S_r^{(g)} \right| = \sum_{\substack{|a_i|\le H, a_d\ne 0\\ r^2|a_d, r|a_{d-1}\\ (a_d,\ldots,a_0)=1\\ (a_d,a_{d-1})=g}} 1 = \sum_{\substack{|a_i|\le H, a_d\ne 0\\ r^2|a_d, r|a_{d-1}\\ (a_d,a_{d-1})=g}} \sum_{k|(a_d,\ldots,a_0)} \mu(k) = \sum_{\substack{|a_i|\le H, a_d\ne 0\\ r^2|a_d, r|a_{d-1}\\ (a_d,a_{d-1})=g}} \sum_{k|a_i(\forall i)} \mu(k)$$

$$= \sum_{\substack{k\le H\\ k|g}} \mu(k) \sum_{\substack{|b_i|\le \frac{H}{k}, b_d\ne 0\\ r^2|kb_d, r|kb_{d-1}\\ (b_d,b_{d-1})=\frac{g}{k}}} 1, \quad \text{(writing } a_i = kb_i\text{)}$$

$$= \sum_{s|r} \sum_{\substack{k\le H\\ (k,r)=s\\ k|g}} \mu(k) \sum_{\substack{|b_i|\le \frac{H}{k}, b_d\ne 0\\ r^2|kb_d, r|kb_{d-1}\\ (b_d,b_{d-1})=\frac{g}{k}}} 1 = \sum_{s|r} \sum_{\substack{m\le \frac{H}{s}\\ (m,\frac{r}{s})=1\\ m|g}} \mu(ms) \sum_{\substack{|b_i|\le \frac{H}{ms}, b_d\ne 0\\ \frac{r^2}{s}|mb_d, \frac{r}{s}|mb_{d-1}\\ (b_d,b_{d-1})=\frac{g}{ms}}} 1,$$

with $k = ms$. Note that the term $\mu(ms)$ ensures that $(m,s) = 1$. This together with $(m, r/s) = 1$ is equivalent to $(m,r) = 1$, and so $(r^2/s)|b_d$ and $(r/s)|b_{d-1}$. Put $(r^2/s)c_d = b_d$, $(r/s)c_{d-1} = b_{d-1}$ and $c_i = b_i$ $(0 \le i \le d-2)$. Thus,

$$\left| S_r^{(g)} \right| = \sum_{s|r} \mu(s) \sum_{\substack{m \le \frac{H}{s} \\ (m,r)=1 \\ m|g}} \mu(m) \sum_{\substack{|c_i| \le \frac{H}{ms} \ (0 \le i \le d-2) \\ 0 < |c_d| \le \frac{H}{mr^2} \ |c_{d-1}| \le \frac{H}{mr} \\ (rc_d, c_{d-1}) = \frac{g}{mr}}} 1. \qquad (17)$$

Claim 1: For $x,y \in \mathbb{Z}$, $r,g \in \mathbb{Z}$, $r \ge 2$, with $0 < |x| \le C_1$ and $|y| \le C_2$, the number of ordered pairs $<x,y>$ satisfying

$(rx,y) = g$ is $\frac{4C_1C_2u}{\varsigma(2)g^2} \prod_{p|\frac{r}{u}} \left(\frac{1}{1+1/p}\right) + O\left(\frac{C_2}{g}, \frac{C_1u}{g} \log(C_2/g)\right)$,

where $u = (r,g)$ and $O(\lambda_1, \lambda_2) = \max(O(\lambda_1), O(\lambda_2))$.
To see this, consider x,y for which $(rx, y) = g$. There are two possible cases.
Case 1: $(r, g) = 1$.

If $y = \pm kg$ and $(k,r) = 1$, then, see Pólya and Szegö (1976), the total number of possible x's is $2\left[\frac{\varphi(k)}{k}\frac{C_1}{g}\right]$

$= 2\left(\frac{\varphi(k)}{k}\frac{C_1}{g}\right) + O(1)$, and possible values of k are $\pm 1, \ldots, \pm[C_2/g]$. Thus, the number of possible ordered

pairs $<x, y>$ is $2 \sum_{\substack{k=1 \\ (k,r)=1}}^{[\frac{C_2}{g}]} \left(2\frac{\varphi(k)}{k}\frac{C_1}{g} + O(1)\right)$

$$= 4\left(\frac{C_1}{g}\right) \sum_{\substack{k=1 \\ (k,r)=1}}^{[\frac{C_2}{g}]} \frac{\varphi(k)}{k} + O\left(\frac{C_2}{g}\right) = 4\left(\frac{C_1C_2}{\varsigma(2)g^2}\right) \prod_{p|r} \left(\frac{1}{1+1/p}\right) + O\left(\frac{C_2}{g}, \frac{C_1}{g} \log\frac{C_2}{g}\right).$$

Case 2: $u = (r,g) > 1$.
Let $r = uR$ and $g = uG$ where $R,G \in \mathbb{Z}$, $(R,G) = 1$. Similar to the arguments used in Case 1, the number of

possible ordered pairs $<x, y>$ is $2 \sum_{k=1}^{[C_2/g]} \left\{2\frac{\varphi(k)}{k}\frac{C_1}{g} + O(1)\right\}$

$$= (4C_2/G) \sum_{\substack{k=1 \\ (k,R)=1}}^{[\frac{C_2}{g}]} \frac{\varphi(k)}{k} + O(C_2/g) = 4\left(\frac{C_1C_2u}{\varsigma(2)g^2}\right) \prod_{p|\frac{r}{u}} \left(\frac{1}{1+1/p}\right) + O\left(\frac{C_2}{g}, \frac{C_1u}{g} \log\frac{C_2}{g}\right).$$

From Claim 1, let $x = c_d$ and $y = c_{d-1}$. Then the number of ordered pairs $<c_d, c_{d-1}>$ with $(rc_d, c_{d-1}) = g/mr$, $0 < |c_d| \le H/mr^2$ and $|c_{d-1}| \le H/mr$ is equal to

$$\frac{4}{\varsigma(2)}\frac{H^2u}{rg^2} \prod_{p|\frac{r}{u}} \left(\frac{1}{1+1/p}\right) + O\left(\frac{H}{g}, \frac{Hu}{rg} \log\frac{H}{g}\right) = \frac{4}{\varsigma(2)}\frac{H^2u}{rg^2} \prod_{p|\frac{r}{u}} \left(\frac{1}{1+1/p}\right) + O\left(\frac{Hu}{rg} \log\frac{H}{g}\right),$$

with $u = (r, g/mr)$, and since $s \le r$ and $g \ge s$, we have

$$\sum_{\substack{|c_i|\le \frac{H}{ms}(0\le i\le d-2)\\ 0<|c_d|\le \frac{H}{mr^2},|c_{d-1}|\le \frac{H}{mr}\\ (rc_d,c_{d-1})=\frac{g}{mr}}} 1 = \left(\frac{4}{\varsigma(2)}\frac{Hu^2}{rg^2}\prod_{p|\frac{r}{u}}\left(\frac{1}{1+1/p}\right)+O\left(\frac{Hu}{rg}\log\frac{H}{g}\right)\right)\left(\frac{2H}{ms}+O(1)\right)^{d-1}.$$

$$= \frac{(2H)^{d+1}u}{\varsigma(2)rg^2(ms)^{d-1}}\prod_{p|\frac{r}{u}}\left(\frac{1}{1+1/p}\right)+O_d\left(\frac{H^d u}{rg(ms)^{d-1}}\log\frac{H}{g}\right).$$

Substituting into equation (17) and separate into two terms $M_r^{(g)}$ and $R_r^{(g)}$, we have

$$\left|S_r^{(g)}\right| = \sum_{s|r}\mu(s)\sum_{\substack{m\le\frac{H}{s}\\(m,r)=1\\m|g}}\mu(m)\left\{\frac{(2H)^{d+1}u}{\varsigma(2)rg^2(ms)^{d-1}}\prod_{p|\frac{r}{u}}\left(\frac{1}{1+1/p}\right)+O_d\left(\frac{H^d u}{rg(ms)^{d-1}}\log\frac{H}{g}\right)\right\}$$

$$= \frac{(2H)^{d+1}u}{\varsigma(2)rg^2}\prod_{p|\frac{r}{u}}\left(\frac{1}{1+1/p}\right)\sum_{s|r}\frac{\mu(s)}{s^{d-1}}\sum_{\substack{m\le H/s\\(m,r)=1\\m|g}}\frac{\mu(m)}{m^{d-1}}+\sum_{s|r}\mu(s)\sum_{\substack{m\le H/s\\(m,r)=1\\m|g}}\mu(m)\,O_d\left(\frac{H^d u}{rg(ms)^{d-1}}\log\frac{H}{g}\right).$$

$= M_r^{(g)} + R_r^{(g)}$,
where

$$M_r^{(g)} = \frac{(2H)^{d+1}u}{g^2\varsigma(2)r}\prod_{p|\frac{r}{u}}\left(\frac{1}{1+1/p}\right)\sum_{s|r}\frac{\mu(s)}{s^{d-1}}\sum_{\substack{m\le H/s\\(m,r)=1\\m|g}}\frac{\mu(m)}{m^{d-1}}$$

$$= \frac{(2H)^{d+1}u}{g^2\varsigma(2)r}\prod_{p|\frac{r}{u}}\left(\frac{1}{1+1/p}\right)\sum_{s|r}\frac{\mu(s)}{s^{d-1}}\sum_{\substack{m=1\\(m,r)=1\\m|g}}^{\infty}\frac{\mu(m)}{m^{d-1}}-\frac{(2H)^{d+1}u}{g^2\varsigma(2)r}\prod_{p|\frac{r}{u}}\left(\frac{1}{1+1/p}\right)\sum_{s|r}\frac{\mu(s)}{s^{d-1}}\sum_{\substack{m>H/s\\(m,r)=1\\m|g}}\frac{\mu(m)}{m^{d-1}}.$$

Claim 2: $\sum_{\substack{m>H/s\\(m,r)=1\\m|g}}\frac{\mu(m)}{m^{d-1}} = O\left(g\left(\frac{s}{H}\right)^{d-1}\right)$.

To see this, put $g = p_1^{\alpha_1}\dots p_t^{\alpha_t}C(r)$. As $r|g$, $(p_i, r) = 1$, $C(r)$ is the factor of g relatively prime to r. Let $r = q^{r_1}\dots q_s^{r_s}$. Since $r|g$, then $g = q_1^{t_1}\dots q_s^{t_s}\ p_1^{\alpha_1}\dots p_t^{\alpha_t}$, $r_i \le t_i$, and so $C(r) = q_1^{t_1}\dots q_s^{t_s}$. If $(m, r) = 1$ and $m|g$, then $m = p_1^{\beta_1}\dots p_t^{\beta_t}$ where $0 \le \beta_i \le \alpha_i$. Now

$$\left|\sum_{\substack{m>H/s\\(m,r)=1,m|g}}\frac{\mu(m)}{m^{d-1}}\right| = \left|\sum_{\substack{0\le\beta_i\le\alpha_i\\p_1^{\beta_1}\dots p_t^{\beta_t}>H/s}}\frac{\mu(p_1^{\beta_1}\dots p_t^{\beta_t})}{(p_1^{\beta_1}\dots p_t^{\beta_t})^{d-1}}\right| \le \left(\frac{s}{H}\right)^{d-1}\sum_{\beta_1=0}^{1}\dots\sum_{\beta_t=0}^{1}1 \le g(s/H)^{d-1},$$

as claimed. Using $\sum_{s|r} \frac{\mu(s)}{s^{d-1}} = \prod_{p|r} (1-1/p^{d-1})$ and $\sum_{\substack{m=1 \\ (m,r)=1 \\ m|g}}^{\infty} \frac{\mu(m)}{m^{d-1}} = \frac{\prod_{p|g} (1-1/p^{d-1})}{\prod_{p|r} (1-1/p^{d-1})}$, we have

$$\sum_{s|r} \frac{\mu(s)}{s^{d-1}} \sum_{\substack{m>\frac{H}{s} \\ (m,r)=1 \\ m|g}} \frac{\mu(m)}{m^{d-1}} = \sum_{s|r} \frac{\mu(s)}{s^{d-1}} \; O(g\,(\frac{s}{H})^{d-1}) = O(\frac{g}{H^{d-1}}), \text{ and so}$$

$$M_r^{(g)} = \frac{(2H)^{d+1}u}{\varsigma(2)g^2r} \prod_{p|\frac{r}{u}} (\frac{1}{1+1/p}) \prod_{p|g} (1-1/p^{d-1}) + O_d(\frac{H^2u}{gr}).$$

Similarly for $d \geq 2$, we get

$$R_r^{(g)} = \sum_{s|r} \mu(s) \sum_{\substack{m\leq H/s \\ (m,r)=1 \\ m|g}} \mu(m)\, O(\frac{H^d u}{gr(ms)^{d-1}} \log(H/g)) = O_d(\frac{H^d u}{gr} \log(H/g) \sum_{s|r} \frac{\mu(s)}{s^{d-1}} \sum_{\substack{m\leq H/s \\ (m,r)=1 \\ m|g}} \frac{\mu(m)}{m^{d-1}})$$

$$= O_d(\frac{H^d u}{gr} \log(H/g) \prod_{p|g} (1-1/p^{d-1}) + \frac{H^d u}{gr} \cdot \log \frac{H}{g} \cdot \frac{g}{H^{d-1}}) = O_d(\frac{H^d u}{rg} \cdot \log \frac{H}{g}),$$

and the desired result follows. Q.E.D.

Lemma 5. If $A(x) = a_d x^d + \ldots + a_0 \in \mathbb{Z}[x]$ has content $c > 1$, then $den(A) \neq |a_d|$ and $\hat{U}_d(H) = \hat{P}_d(H)$.

Proof. Since $A(x), (1/c)A(x) \in \mathbb{Z}[x]$ both have the same set of roots and $(1/c)A(x)$ is primitive, then $den(A) = den((1/c)A)$. By Theorem 1 of Arno *et al.* (1996), $den((1/c)A)$ divides a_d/c and $den(A) = den((1/c)A) \leq |a_d|/c < |a_d|$ implying that $den(A) \neq |a_d|$. Next, let $A(x) \in \mathbb{Z}[x]$ have c as its content. Assume that $A(x) \in \hat{U}_d(H)$. If $c > 1$, then $den(A) \neq |a_d|$, and so $A(x) \notin \hat{U}_d(H)$, which is a contradiction. Thus, $c = 1$ and so $A(x) \in \hat{P}_d(H)$ yielding $\hat{U}_d(H) \subseteq \hat{P}_d(H)$. Finally, we assume that $A(x) \in \hat{P}_d(H)$. Thus, $A(x) \in \hat{U}_d(H)$ which gives $\hat{P}_d(H) \subseteq \hat{U}_d(H)$. Hence, $\hat{U}_d(H) = \hat{P}_d(H)$. Q.E.D.

RESULTS

Theorem 1. Let $g \geq 1$ be fixed. Then for $n \in \mathbb{N}$, we have

(i) $$Prob_k^{(g)}(n) = \frac{1}{g^k \varsigma(k)} (\frac{2n}{2n+1})^k + O(\frac{n^{k-1}}{g^{k-1}(2n+1)^k}), \text{ when } k \geq 3,$$

(ii) $$Prob_2^{(g)}(n) = \frac{1}{g^2 \varsigma(2)} (\frac{2n}{2n+1})^2 + O(\frac{n}{g(2n+1)^2} \log \frac{n}{g}), \text{ and}$$

(iii) $$\lim_{n\to\infty} Prob_k^{(g)}(n) = \frac{1}{g^k \varsigma(k)}.$$

Proof. Since $\text{Prob}_k^{(g)}(n) = \dfrac{Z_k^{(g)}(n)}{(2n+1)^k}$, then from Lemma 1, for $k \geq 3$, we have

$$\text{Prob}_k^{(g)}(n) = \frac{\frac{(2n)^k}{g^2\varsigma(k)} + O(\frac{n^{k-1}}{g^{k-1}})}{(2n+1)^k} = \frac{1}{g^k\varsigma(k)}(\frac{2n}{2n+1})^k + O(\frac{n^{k-1}}{g^{k-1}(2n+1)^k}),$$

while for $k = 2$, we have

$$\text{Prob}_2^{(g)}(n) = \frac{\frac{(2n)^2}{g^2\varsigma(2)} + O(\frac{n}{g}\log\frac{n}{g})}{(2n+1)^2} = \frac{1}{g^2\varsigma(2)}(\frac{2n}{2n+1})^2 + O(\frac{n}{g(2n+1)^2}\log\frac{n}{g}),$$

and (iii) follows immediately from (i) and (ii). Q.E.D.

Theorem 2. Let $g \geq 1$ be fixed and p be a prime. Then for $n \in \mathbb{N}$, we have

(i) $\text{Prob}_k^{(1,g)}(n) = \dfrac{1}{g^2\varsigma(2)}(\dfrac{2n}{2n+1})^k \prod_{p|g}(1-\dfrac{1}{p^{k-2}}) + O(\dfrac{n^{k-1}}{g(2n+1)^k}\log\dfrac{n}{g})$, when $k \geq 3$,

(ii) $\text{Prob}_2^{(1,g)}(n) = \dfrac{1}{\varsigma(2)}(\dfrac{2n}{2n+1})^2 + O(\dfrac{n}{(2n+1)^2}\log n)$,

(iii) $\lim\limits_{n\to\infty} \text{Prob}_k^{(1,g)}(n) = \dfrac{1}{g^2\varsigma(2)} \prod_{p|g}(1-\dfrac{1}{p^{k-2}})$, when $k \geq 3$, and

(iv) $\lim\limits_{n\to\infty} \text{Prob}_2^{(1,g)}(n) = \dfrac{1}{\varsigma(2)}$.

Proof. Since $\text{Prob}_k^{(1,g)}(n) = \dfrac{Z_k^{(1,g)}(n)}{(2n+1)^k}$, then from Lemma 2 for the case $k \geq 3$, we get

$$\text{Prob}_k^{(1,g)}(n) = \frac{\frac{(2n)^k}{g^2\varsigma(2)} \prod_{p|g}(1-\frac{1}{p^{k-2}}) + O(\frac{n^{k-1}}{g}\log\frac{n}{g})}{(2n+1)^k}$$

$$= \frac{1}{g^2\varsigma(2)}(\frac{2n}{2n+1})^k \prod_{p|g}(1-\frac{1}{p^{k-2}}) + O(\frac{n^{k-1}}{g(2n+1)^k}\log\frac{n}{g}),$$

while for the case $k = 2$, we get

$$\text{Prob}_2^{(1,g)}(n) = \frac{\frac{(2n)^2}{\varsigma(2)} + O(n\ \log n)}{(2n+1)^2} = \frac{1}{\varsigma(2)}(\frac{2n}{2n+1})^2 + O(\frac{n}{(2n+1)^2}\log n).$$

Lastly , (iii) and (iv) follow directly from (i) and (ii). Q.E.D.

Theorem 3. Let $d \geq 1$ and $g \geq 1$ be fixed. For $H \geq 2$, we have

$$\left| \hat{P}_d^{(g)}(H) \right| = \frac{|\mu(g)|(2H)^{d+1}}{\varsigma(2)g^2} \prod_{p|g}(1-\frac{1}{p^{d-1}})(1-\frac{1}{p+1}) + O_d(\frac{H^d}{g}\log\frac{H}{g}).$$

Proof. For d = 1, we have $\hat{P}_1^{(g)}(H) = P_1^{(g)}(H)$. Therefore, the theorem is true in this case. Assume $d \geq 2$. Using Theorem 1 (i) and the proof of Theorem 2 of Arno et al. (1996), we get den (A) $\neq |a_d| \Leftrightarrow$ there is a prime p such that $p \mid a_d$ and $n = a_d/p$ satisfies

$$(n^d/a_d)A(x/n) \in \mathbb{Z}[x]$$

$\Leftrightarrow$ there is a prime $p \mid a_d$ and $(1, a_{d-1}/p, a_{d-2}a_d/p^2, a_{d-3}a_d^2/p^3, \ldots, a_0 a_d^{d-1}/p^d) \in \mathbb{Z}^{d+1}$.
Since A(x) is primitive, then den (A) $\neq |a_d|$ is equivalent to $p^2|a_d$ and $p|a_{d-1}$. Thus ,

$$\{A(x) = \sum_{i=0}^{d} a_i x^i \in P_d^{(g)}(H) : \text{den}(A) \neq |a_d|\} = \bigcup_{p \leq H} S_p^{(g)},$$

where $S_p^{(g)} = \{A(x) = \sum_{i=0}^{d} a_i x^i \in P_d^{(g)}(H)\colon p^2|a_d, p|a_{d-1}\}$. Observe from its definition that

$$S_r^{(g)} = \{A(x) = \sum_{i=0}^{d} a_i x^i \in P_d^{(g)}(H)\colon r^2|a_d, r|a_{d-1}\}.$$

Thus, $S_1^{(g)} = P_d^{(g)}(H)$, $S_r^{(g)} = \bigcap_{p|r} S_p^{(g)}$, with $r \geq 2$, r square-free, and $S_r^{(g)} = \varnothing$ when

$r > \sqrt{H}$. Since $\hat{P}_d^{(g)}(H) = S_1^{(g)} - \bigcup_{p \leq H} S_p^{(g)}$, then by the inclusion-exclusion principle,

$$|\hat{P}_d^{(g)}(H)| = |S_1^{(g)} - \bigcup_{p \leq H} S_p^{(g)}| = |S_1^{(g)}| + \sum_{2 \leq r \leq H} \mu(r) |\bigcap_{p|r} S_r^{(g)}| = \sum_{1 \leq r \leq H} \mu(r) |S_r^{(g)}|.$$

Apply Lemma 4 and separate the sum into two parts M_g and R_g to get

$$|\hat{P}_d^{(g)}(H)| = \sum_{\substack{1 \leq r \leq H \\ r|g}} \mu(r)\left(\frac{(2H)^{d+1}u}{\varsigma(2)g^2 r} \prod_{p|g}(1-\frac{1}{p^{d-1}}) \prod_{p|g}(1-\frac{1}{p^{d-1}}) + O_d(\frac{H^d u}{gr}\log\frac{H}{g})\right)$$

$$= M_g + R_g, \quad \text{where } M_g = \sum_{\substack{1 \leq r \leq H \\ r|g}} \mu(r)\left\{\frac{(2H)^{d+1}u}{\varsigma(2)g^2 r} \prod_{p|\frac{r}{u}}(\frac{1}{1+1/p}) \prod_{p|g}(\frac{1}{p^{d-1}})\right\}$$

$$= \frac{(2H)^{d+1}}{\varsigma(2)g^2} \prod_{p|g}(1-\frac{1}{p^{d-1}}) \sum_{\substack{1 \leq r \leq H \\ r|g}} \frac{\mu(r)u}{r} \prod_{p|\frac{r}{u}}(\frac{1}{1+1/p}).$$

Set $F_g^{(r)} = \sum_{\substack{1 \leq r \leq H \\ r|g}} \frac{\mu(r)u}{r}\pi(\frac{r}{u})$ and $\pi(\frac{r}{u}) = \prod_{p|\frac{r}{u}}(\frac{1}{1+1/p})$.

Let $g = p_1^{a_1} \cdots p_k^{a_k}$. As r|g, then $r = p_1^{b_1} \cdots p_k^{b_k}$, $0 \leq b_i \leq a_i$. Thus,

$$F_g^{(r)} = \sum_{b_1=0}^{1} \frac{\mu(p_1^{b_1})}{p_1^{b_1}}(p_1^{a_1-b_1}, P_1^{b_1}) \cdots \sum_{b_k=0}^{1} \frac{\mu(p_k^{b_k})}{p_k^{bk}}(p_k^{a_k-b_k}, p_k^{b_k})\pi(\frac{p_1^{b_1} \cdots p_k^{b_k}}{(p_1^{a_1-b_1} \cdots p_k^{a_k-b_k}, p_1^{b_1} \cdots p_k^{b_k})}).$$

$$= \begin{cases} 0, & \exists a_i > 1 \Leftrightarrow g \text{ not square - free} \Leftrightarrow \mu(g) = 0 \\ \prod_{p|g}(1-\frac{1}{p+1}), & \forall a_i = 1 \Leftrightarrow g \text{ square - free} \Leftrightarrow |\mu(g)| = 1 \end{cases}$$

$$= |\mu(g)| \prod_{p|g}(1\frac{1}{p+1}).$$

Thus, $M_g = \frac{|\mu(g)|(2H)^{d+1}}{\varsigma(2)g^2}\prod_{p|g}(1-\frac{1}{p^{d-1}})(1-\frac{1}{p+1})$, and

$$R_g = \sum_{\substack{1\le r\le H \\ r|g}} \mu(r) O_d(\frac{H^d u}{gr}\log\frac{H}{g}) = O_d(\frac{H^d}{g}\log\frac{H}{g}),$$

using $\frac{H^d u}{g}\log\frac{H}{g}\sum_{\substack{1\le r\le H \\ r|g}}\frac{\mu(r)}{r} = O_d(\frac{H^d}{g}\log\frac{H}{g})$, and the result follows. Q.E.D.

Theorem 4. Let $d \ge 1$ and $g \ge 1$ be fixed. For $H \ge 2$, we have $\frac{|\hat{P}_1^{(g)}(H)|}{|P_1^{(g)}(H)|} = 1$, and when $d \ge 2$, $\frac{|\hat{P}_d^{(g)}(H)|}{|P_d^{(g)}(H)|}$

$= |\mu(g)| g \prod_{p|g}(\frac{1}{p+1}) + O_d(\frac{g}{H}\log\frac{H}{g})$, where g is square-free.

Proof. For $d = 1$, we have $\hat{P}_1^{(g)}(H) = P_1^{(g)}(H)$, i.e. the result is true in this case.

Assume $d \ge 2$. From Theorem 3, and Lemma 3 we see that

$$\frac{|\hat{P}_d^{(g)}(H)|}{|P_d^{(g)}(H)|} = \frac{\frac{\varsigma(2)g^2}{(2H)^{d+1}}}{\prod_{p|g}(1-\frac{1}{p^{d-1}})}\{\frac{|\mu(g)|(2H)^{d+1}}{\varsigma(2)g^2}\prod_{p|g}(1-\frac{1}{p^{d-1}})(1-\frac{1}{p+1}) + O_d(\frac{H^d}{g}\log\frac{H}{g})\}\times$$

$$\times\{\frac{1}{1+O_d(\frac{1}{H}\log\frac{H}{g})}\}$$

$$= |\mu(g)| \prod_{p|g}(\frac{p}{p+1}) + O_d(\frac{g}{H}\log\frac{H}{g}) = |\mu(g)| g \prod_{p|g}(\frac{1}{p+1}) + O_d(\frac{g}{H}\log\frac{H}{g}),$$

as g is square-free. Q.E.D.

Theorem 5. Let $d \ge 1$, $H \ge 2$ and p be prime. Then $\lim_{d\to\infty}\lim_{H\to\infty}\frac{|\hat{U}_d(H)|}{|U_d(H)|} = \frac{1}{\varsigma(3)}$.

Proof. From Lemma 5, $\hat{U}_d(H) = \hat{P}_d(H)$, and from Theorem 3,

$$|\hat{P}_d(H)| = \frac{(2H)^{d+1}}{\varsigma(d+1)}\prod_p(1-\frac{1}{p^3}\frac{(1-1/p^{d-1})}{(1-1/p^{d+1})}) + O_d(H^d \log^2 H).$$

Using $|U_d(H)| = (2H)^{d+1} + O_d(H^d)$, we deduce that

$$\frac{|\hat{U}_d(H)|}{|U_d(H)|} = \frac{1}{(2H)^{d+1}}\{\frac{(2H)^{d+1}}{\varsigma(d+1)}\prod_p(1-\frac{1}{p^3}\frac{(1-1/p^{d-1})}{(1-1/p^{d+1})}) + O_d(H^d \log^2 H)\}\frac{1}{1+O_d(1/H)}$$

$$= \frac{1}{\varsigma(d+1)}\prod_p(1-\frac{1}{p^3}\frac{(1-1/p^{d-1})}{(1-1/p^{d+1})}) + O_d(\frac{\log^2 H}{H}),$$

and so $$\lim_{H\to\infty}\frac{|\hat{U}_d(H)|}{|U_d(H)|} = \frac{1}{\varsigma(d+1)}\prod_p(1-\frac{1}{p^3}\frac{(1-1/p^{d-1})}{(1-1/p^{d+1})})$$

$$= \prod_p(1-\frac{1}{p^{d+1}})\prod_p(1-\frac{1}{p^3}\frac{(1-1/p^{d-1})}{(1-1/p^{d+1})}) = \prod_p(1-\frac{1}{p^3}-\frac{1}{p^{d+1}}+\frac{1}{p^{d+2}}).$$

Consequently, $$\lim_{d\to\infty}\lim_{H\to\infty}\frac{|\hat{U}_d(H)|}{|U_d(H)|} = \prod_p(1-\frac{1}{p^3}) = \frac{1}{\varsigma(3)}.$$ Q.E.D.

DISCUSSION

The first group of results obtained in this paper is

(I) $$\text{Prob}_k^{(g)}(n) = \frac{1}{g^k\varsigma(k)}(\frac{2n}{2n+1})^k + O(\frac{n^{k-1}}{g^{k-1}(2n+1)^k}),\ k \geq 3.$$

(II) $$\text{Prob}_2^{(g)}(n) = \frac{1}{g^2\varsigma(2)}(\frac{2n}{2n+1})^2 + O(\frac{n}{g(2n+1)^2}\log\frac{n}{g}).$$

(III) $$\lim_{n\to\infty}\text{Prob}_k^{(g)}(n) = \frac{1}{g^k\varsigma(k)}.$$

This extends the results of Nymann (1970) which correspond to the case g = 1.
The second group of results obtained in this work is

(1) $$\frac{|\hat{P}_d^{(g)}(H)|}{|P_d^{(g)}(H)|} = |\mu(g)|\,g\prod_{p|g}(\frac{1}{p+1}) + O_d(\frac{g}{H}\log\frac{H}{g}) \text{ for } d \geq 2.$$

(2) $$\lim_{d\to\infty}\lim_{H\to\infty}\frac{|\hat{U}_d(H)|}{|U_d(H)|} = \prod_p(1-\frac{1}{p^3}) = \frac{1}{\varsigma(3)};$$

both provide refinements to the following results of Arno *et al.* (1996)

(i) $$\frac{|\hat{P}_d(H)|}{|P_d(H)|} = \prod_p\{1-\frac{1}{p^3}\frac{(1-1/p^{d-1})}{(1-1/p^{d+1})}\} + O_d(\frac{\log^2 H)}{H}),\ d \geq 2.$$

(ii) $$\lim_{d\to\infty}\lim_{H\to\infty}\frac{\left|\bigcup_{k\leq d}\hat{A}_k(H)\right|}{\left|\bigcup_{k\leq d}A_k(H)\right|} = \frac{1}{\varsigma(3)}.$$

LITERATURE CITED

Apostol, T.M. 1976. Introduction to Analytic Number Theory. Springer-Verlag, New York. 338 p.

Arno, S., M.L. Robinson and F.S. Wheeler. 1996. On denominators of algebraic numbers and integer polynomials. J. Number Theory 57 : 292-302.

Laohakosol , V , N. Sukvaree, and M. Maiya . 2000. Denominators of algebraic numbers. Kasetsart J. (Nat. Sci.) 34 : 431-437.

Nymann, J.E. 1970. On the probability that k positive integers are relatively prime. J. Number Theory 4 : 469-473.

Pólya, G. and G. Szegö. 1976. Problems and Theorems in Analysis II. Springer-Verlag, New York. 392 p.

Received : 9/03/01
Accepted : 29/06/01

ใบสมัครสมาชิกวิทยาสารเกษตรศาสตร์

ชื่อผู้สมัคร ข้าพเจ้า นาย/นาง/นางสาว.....

☐ ขอสมัคร ☐ ขอต่ออายุสมาชิกวิทยาสารเกษตรศาสตร์ เลขที่.....

สาขา ☐ วิทยาศาสตร์ ☐ สังคมศาสตร์ ประจำปี พ.ศ.....

การชำระค่าบำรุงการจัดส่ง ข้าพเจ้าชำระค่าบำรุงการจัดส่งจำนวน.....บาท

(.....) โดยจ่ายเป็น ☐ เงินสด ☐ ธนาณัติ

สั่งจ่ายในนาม สถาบันวิจัยและพัฒนาแห่งมหาวิทยาลัยเกษตรศาสตร์ ป.ท. เกษตรศาสตร์ (นักเรียน นิสิต นักศึกษา โปรดแนบสำเนาบัตรประจำตัวหรือจดหมายรับรองของอาจารย์ที่ปรึกษาดังรายละเอียดด้านหลัง)

ใบเสร็จรับเงิน โปรดกรอกใบเสร็จรับเงินในนาม ☐ ข้าพเจ้า ☐ นิติบุคคลชื่อ.....

.....

การจัดส่งสิ่งพิมพ์ โปรดจัดส่งสิ่งพิมพ์ไปที่ ☐ ที่อยู่ปัจจุบัน ☐ สถานที่ศึกษา ☐ สถานที่ทำงาน

ตามรายละเอียดดังนี้.....

.....จังหวัด.....รหัสไปรษณีย์.....

โทรศัพท์.....

ลงชื่อ.....ผู้สมัคร

(.....)

สถานที่ติดต่อ ผู้จัดการวิทยาสารเกษตรศาสตร์
สถาบันวิจัยและพัฒนาแห่ง มก. มหาวิทยาลัยเกษตรศาสตร์ บางเขน กรุงเทพฯ 10900

ค่าบำรุงการจัดส่งต่อปี	สาขาวิทยาศาสตร์ ๔ ฉบับ/ปี	สาขาสังคมศาสตร์ ๒ ฉบับ/ปี
นักเรียน นิสิต นักศึกษา	๖๐	๔๐
บุคคลและนิติบุคคลที่ไม่ใช่ส่วนราชการ	๑๒๕	๘๐

สำหรับเจ้าหน้าที่

สมาชิกเลขที่.....

ใบเสร็จรับเงินเลขที่.....ลงวันที่.....

ลงชื่อ.....เหรัญญิก

(.....)

วันที่.....

ใบรับรอง

ข้าพเจ้า นาย/นาง/นางสาว.....

ตำแหน่ง.....สถานที่ทำงาน.....

.....

จังหวัด.....รหัสไปรษณีย์.....ขอรับรองว่า นาย/นาง/นางสาว

.....เป็นนักเรียน/นิสิต/นักศึกษา ปีที่.....ปีการศึกษา พ.ศ.....ในสถาน

ศึกษา.....จริง

ลงชื่ออาจารย์ที่ปรึกษา.....

(.....)

วันที่.....เดือน.....พ.ศ.....

หมายเหตุ : การแลกเปลี่ยนวารสาร โปรดติดต่อ ผู้อำนวยการสำนักหอสมุด มหาวิทยาลัยเกษตรศาสตร์ จตุจักร กรุงเทพฯ 10900

Text and Journal Publication Co., Ltd.

54 Chulalongkon 42 Wangmai Phatumwon Bangkok 10330 Tel. 611-7312, 215-4470, 215-0155

Close