CHAPTER 3

RESULTS

3.1) SCREENING AND CHARACTERIZATION OF GLUCOSINOLATE-DEGRADING MICROORGANISMS

3.1.1) Screening and preliminary identification of glucosinolate-degrading microorganisms

Forty-five decayed mustard seed meal samples suspected to contain glucosinolate-degrading microorganisms were collected. According to physical appearance, these samples could be classified into 4 groups, solid meal, semi-solid meal, liquid meal and soil samples (Table 3.1). Each sample was suspended in sterile distilled water, and the supernatant was then inoculated in sinigrin agar plate, as described in 2.3.2.

After incubation at 30 °C for 24 h, colorless convex circular bacterial colonies were formed on some plates, whose inoculants were originated from semi-solid meals no. 1-5, 8-10, 14, 16-18 and liquid meal no. 1-9 (Table 3.1). Average colony diameters between 0.5 and 1 mm were observed. In order to obtain pure isolates, selected colonies were picked and then further streaked on nutrient agar plate. After incubation at 30 °C for 24 h, the pure isolates exhibited milky convex circular shape with colony diameters between 2 and 4 mm (Figure 3.1). Gram staining revealed that the bacterium was a gram-negative rod. In cases of solid meal and soil samples, no bacterial growth was observed in sinigrin agar plate, even after 72-h incubation.

Table 3.1 Summaries of glucosinolate-degrading microorganisms isolated from decayed mustard seed meal samples, obtained in Lamphun, Thailand.

Samples	Bacteria	Fungi
1) Solid meal no.1	-	A, B
2) Solid meal no. 2	-	A, B
3) Solid meal no. 3	-	A, B
4) Solid meal no. 4	-	A, B
5) Solid meal no. 5	-	A, B
6) Solid meal no. 6	-	A, B
7) Solid meal no. 7	-	A, B
8) Solid meal no. 8	-	A, B
9) Solid meal no. 9	-	A, B
10) Solid meal no. 10	-	A, B
11) Solid meal no. 11	-	A, B
12) Solid meal no. 12	-	A, B
13) Semi-solid meal no. 1	X	A, B, C
14) Semi-solid meal no. 2	X	A, B, C
15) Semi-solid meal no. 3	X	A, B, C
16) Semi-solid meal no. 4	X	A, B, C
17) Semi-solid meal no. 5	X	A, B, C
18) Semi-solid meal no. 6	-	C
19) Semi-solid meal no. 7	-	C
20) Semi-solid meal no. 8	X	A, B, C
21) Semi-solid meal no. 9	X	A, B, C
22) Semi-solid meal no. 10	X	A, B, C
23) Semi-solid meal no. 11	-	A, B, C
24) Semi-solid meal no. 12	-	A, B, C
25) Semi-solid meal no. 13	-	C

Table 3.1 (continued)

Samples	Bacteria	Fungi
26) Semi-solid meal no. 14	X	A, B, C
27) Semi-solid meal no. 15	-	A, B, C
28) Semi-solid meal no. 16	X	C
29) Semi-solid meal no. 17	X	C
30) Semi-solid meal no. 18	X	C
31) Liquid meal no. 1	X	C
32) Liquid meal no. 2	X	C
33) Liquid meal no. 3	X	C
34) Liquid meal no. 4	X	-
35) Liquid meal no. 5	X	C
36) Liquid meal no. 6	X	C
37) Liquid meal no. 7	X	-
38) Liquid meal no. 8	X	-
39) Liquid meal no. 9	X	-
40) Soil sample no. 1	-	-
41) Soil sample no. 2	-	-
42) Soil sample no. 3	-	-
43) Soil sample no. 4	-	-
44) Soil sample no. 5	-	-
45) Soil sample no. 6	-	-

Bacterium X : gram negative rod bacterium

Fungus A : *Rhizopus* sp.

Fungus B : *Mucor* sp.

Fungus C : *Aspergillus* sp.

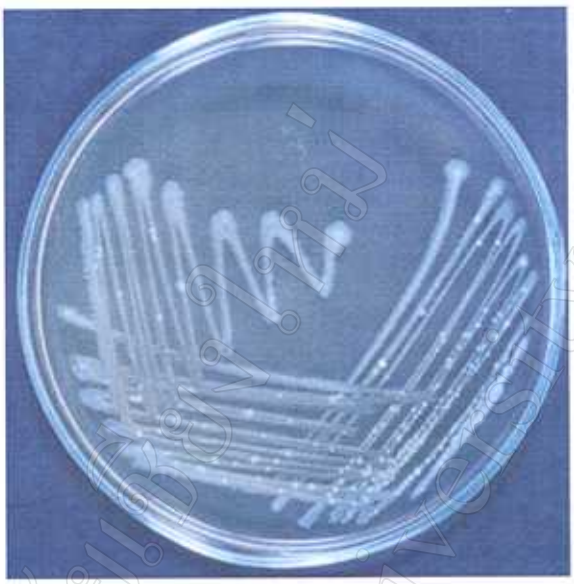

Figure 3.1 Growth of gram negative rod bacterium in nutrient agar plate for 2 days.

After incubation at 30 °C for 48 h, mycelial growth was formed embedding in the agar medium inoculated with samples from solid meals (no. 1-12) and semi-solid meals (no. 1-5, 8-12 and 14-15). Even though, the incubation was extended up to 72 h, only mycelium embedded in the medium and spread around sinigrin agar plate (Figure 3.2). Fungal colony did not form on the agar surface even in the incubation period for 7 days. These microbes were then picked and further plated in PDA that later gave two distinct fungal isolates. The first isolate exhibited spongy-cottony mycelium. Sporulation with numerous black spores was observed after 2 days (Figure 3.4). Microscopic examination with lactophenol-blue staining revealed that the strain was a non-septate filamentous fungus. Rhizoide formation was observed at the corresponding position of sporangiophores. In addition, the existing sporangiophores appeared with no branch, of which the top-end connected to sporangium.

Figure 3.2 Growth of glucosinolate-degrading microorganisms in sinigrin agar plate for 4 days. Mycelium embedding in the agar medium were later identified as *Rhizopus* sp. and *Mucor* sp.

Figure 3.3 Growth of glucosinolate-degrading microorganisms in sinigrin agar plate for 5 days. White and yellowed-green colonies were later identified as *Aspergillus* sp.

These morphologies were typical of *Rhizopus* sp. [117, 118].

The second isolate of fungi also developed spongy-cottony mycelium in PDA. However, sporulation was found to be delayed until about 5-6 days (Figure 3.5). It was shown that numbers of spore forming were relatively less than that of the first isolate. Examination with lactophenol-blue staining indicated that this fungal strain was a non-septate filamentous fungus with no rhizoide formation. Zygospores were found. Sporangium appeared at the top-end sporangiophores which existed with no branch. These were the characteristics of *Mucor* sp. [117, 118].

Another fungal strain was isolated from semi-solid meals no. 1-18 and liquid meals no. 1-3 and 5-6 (Table 3.1). The strain exhibited white colonies in sinigrin agar plates after 48 h (Figure 3.3). Growth in PDA, the fungus exhibited yellowed-green velvety colonies within 4 days (Figure 3.6). Formation of sclerotia was seen after 7 days (Figure 3.7). Result from microscopic observation indicated that the strain was a septate filamentous fungus. Conidiospores formed around conidial heads (phialides). The strain was consequently identified as *Aspergillus* sp. according to Raper and Fennell's manual [119].

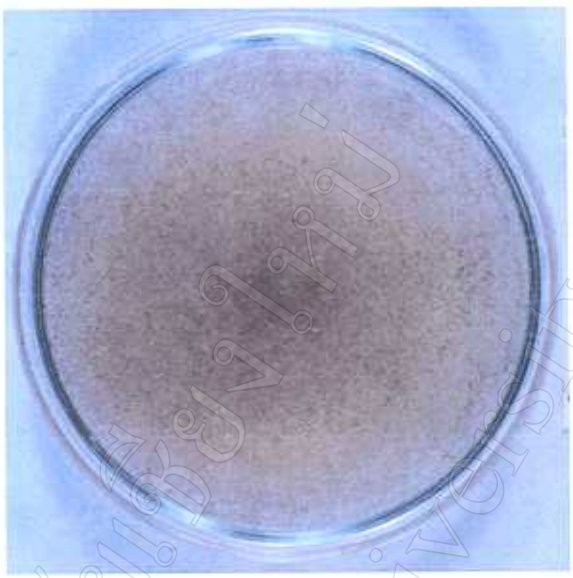

Figure 3.4 Growth of *Rhizopus* sp. in potato dextose agar plate for 4 days.

Figure 3.5 Growth of *Mucor* sp. in potato dextose agar plate for 4 days.

Figure 3.6 Growth of *Aspergillus* sp. in potato dextrose agar plate for 4 days.

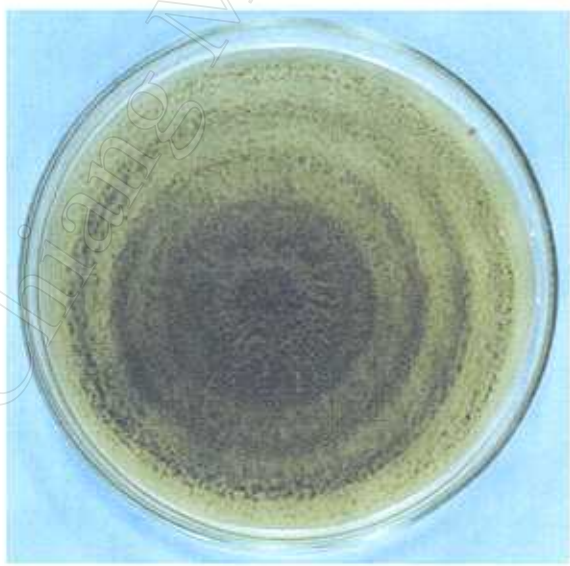

Figure 3.7 Growth of *Aspergillus* sp. in potato dextrose agar plate for 7 days.

3.1.2) Preliminary examination of sinigrin degradation and myrosinase-producing ability of glucosinolate-degrading microorganisms in liquid culture

The gram negative rod bacterium and three fungal strains, *Rhizopus* sp., *Mucor* sp. and *Aspergillus* sp. were assessed for their abilities on glucosinolate degradation and myrosinase production. These were carried out by inoculating the pre-cultured cells (bacteria) or spore suspension (fungi) in sinigrin-glucose medium and incubated at 30 °C (as described in 2.3.3). The bacterium showed considerable growth in sinigrin-glucose medium as monitored by measuring an absorbance at 660 nm (Figure 3.8). Glucose was consumed completely within 24 h. However, no degradation of sinigrin was observed in the incubation period of 72 h.

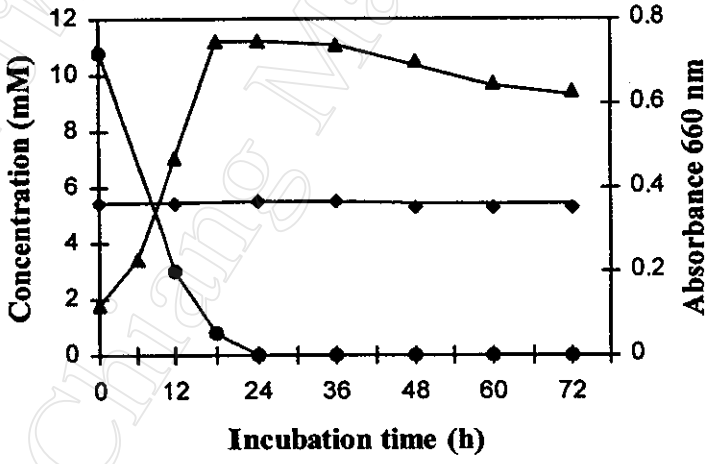

Figure 3.8 Growth profiles of gram negative rod bacterium in sinigrin-glucose medium. Growth (▲), consumption of glucose (●) and sinigrin (◆) are shown.

All three fungal strains were capable of growth in sinigrin-glucose medium, in which mycelial growth was observed within 24 h. Total consumption of glucose was observed at 36 h (Figure 3.9, 3.10 and 3.11). Sinigrin consumption was demonstrated effectively by the *Aspergillus*. In such case, sinigrin was completely degraded within 72 h (Figure 3.11). During the first 48 h, no significant degradation of sinigrin was shown by the *Rhizopus* and *Mucor*. Even when the incubation was extended up to 72 h, the *Rhizopus* and *Mucor* degraded sinigrin in a series of 12.7 and 14.5 %, respectively (Figure 3.9 and 3.10).

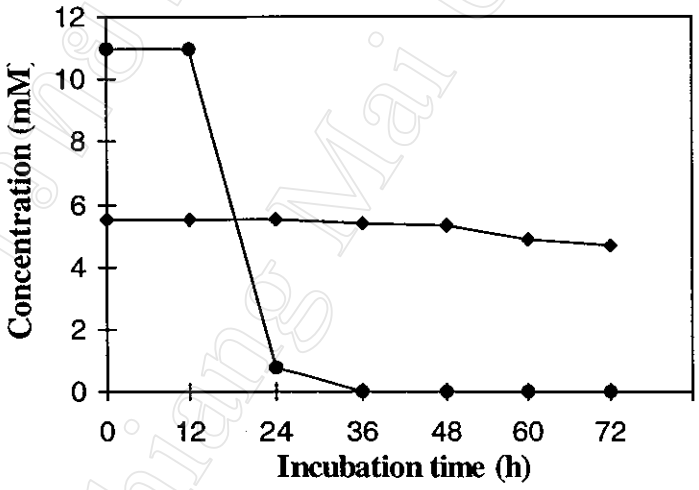

Figure 3.9 Growth of *Rhizopus* sp. in sinigrin-glucose medium. Consumptions of sinigrin (◆) and glucose (●) are shown.

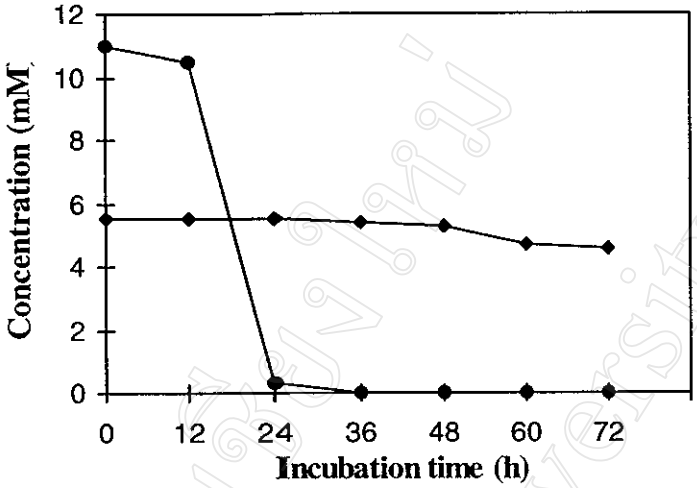

Figure 3.10 Growth of *Mucor* sp. in sinigrin-glucose medium. Consumptions of sinigrin(◆) and glucose (●) are shown.

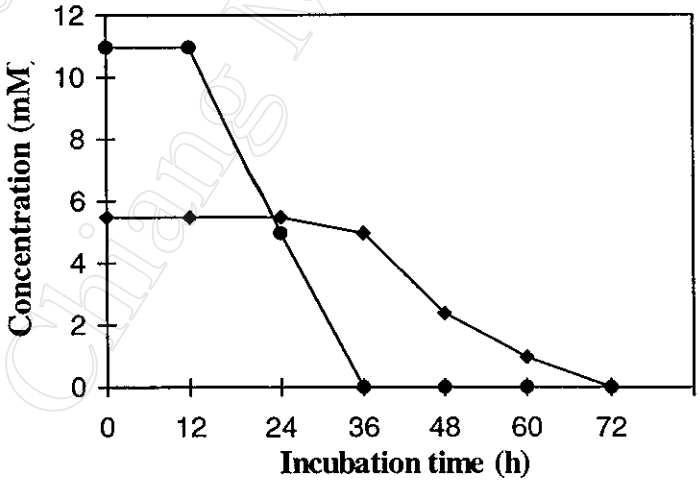

Figure 3.11 Growth of *Aspergillus* sp. in sinigrin-glucose medium. Consumptions of sinigrin(◆) and glucose (●) are shown.

In order to detect myrosinase activity, cultured filtrates and microbial cells of the bacterial culture were collected at 16, 24 and 32 h. While those samples of fungal cultures were collected at 36, 48 and 72 h. No myrosinase activity was detected in all cultured filtrate samples, as determined by the spectrophotometric method. In order to examine myrosinase activity in cell-free extracts, bacterial cells were subjected for sonication (as described in 2.3.11). In cases of fungi, whole cells of each strain were processed for disruption (as described in 2.3.7). It was found that no activity of myrosinase was detected in cell-free extract samples of the bacterium, *Rhizopus* and *Mucor*. While, the activity of approximately 0.2 U was detected in cell-free extracts of the *Aspergillus*.

3.1.3) Scanning electron micrographs of *Aspergillus* sp.

Scanning electron micrographs of the *Aspergillus* revealed that the conidial head (phialide) formed as an uniseriate sterigmata with average size between 35 and 40 microns (Figure 3.12). Conidiophore was finely roughened. Subglobose vesicles had diameters of 12-15 microns. Conidia exhibited as globous shape with approximate diameters between 3 and 3.5 microns (Figure 3.12). These distinguished characteristics belonged to a *flavus* group [119]. The strain was later assigned to be *Aspergillus* sp. NR-4201.

Figure 3.12 Scanning electron micrographs of *Aspergillus* sp. NR-4201. Phialide head with an uniseriate sterigmata (above; x1,900 magnifications) and globous conidia (bottom; x6,000 magnifications) are shown.

3.1.4) Pre-culture of *Aspergillus* sp. NR-4201

From the preceding experiments, *Aspergillus* sp. NR-4201 was an only strain exhibited potentially to degrade sinigrin. The degradation process was probably associated with intracellular myrosinase activity. Consequently, the strain was selected for extensive study of glucosinolate degradation and enzyme production in liquid culture. For this objective, the fungus should be pre-cultured properly in various induction media such as glucose medium (G-medium), sinigrin-glucose medium (SG-medium) and sinigrin medium (S-medium).

After inoculating with spore suspension and incubating at 28 °C, amounts of sinigrin and glucose in cultured filtrates, and myrosinase activity in fungal mycelium were measured. Growth in G-medium, SG-0.5/10.5, SG-1/10, SG-2.5/7.5 and SG-5.5/5.5 medium was observed within 24 h. This was indicated by the formation of fungal cell pellet. While the germination in S-medium was shown at 32 h. The fungus preferred glucose as its carbon source to sinigrin. Total glucose consumption in G-medium was achieved within 36 h (Figure 3.13a), whereas, the consumption of sinigrin in S-medium was not complete within 48 h (Figure 3.13b). However, growth in SG-0.5/10.5, 1/10, 2.5/7.5 and 5.5/5.5 medium, resulted in total degradation of sinigrin within 28, 28, 32 and 48 h, respectively (Figure 3.13c-f). Myrosinase activity was not produced in fungal mycelium when the culture was performed in sinigrin-free medium (Figure 3.13a). Activities of about 0.07-0.2 U were detected in cell-free extracts of the *Aspergillus* which were cultured in sinigrin containing medium (S- and SG-medium). However, the culture in SG-0.5/10.5 medium contained significantly lower myrosinase activity than those cultures in other SG-medium (Figure 3.13c-f). It was found that sporulation occured at 32 h in the cultures of SG-2.5/7.5 and 5.5/5.5

and S-11 medium, but not in SG-0.5/10.5 and 1/10 medium in the same incubation period. In summary, the pre-culture in SG-1/10 medium for 32 h was the most suitable condition, which agreed to the results reported by Smiths *et al.* [43].

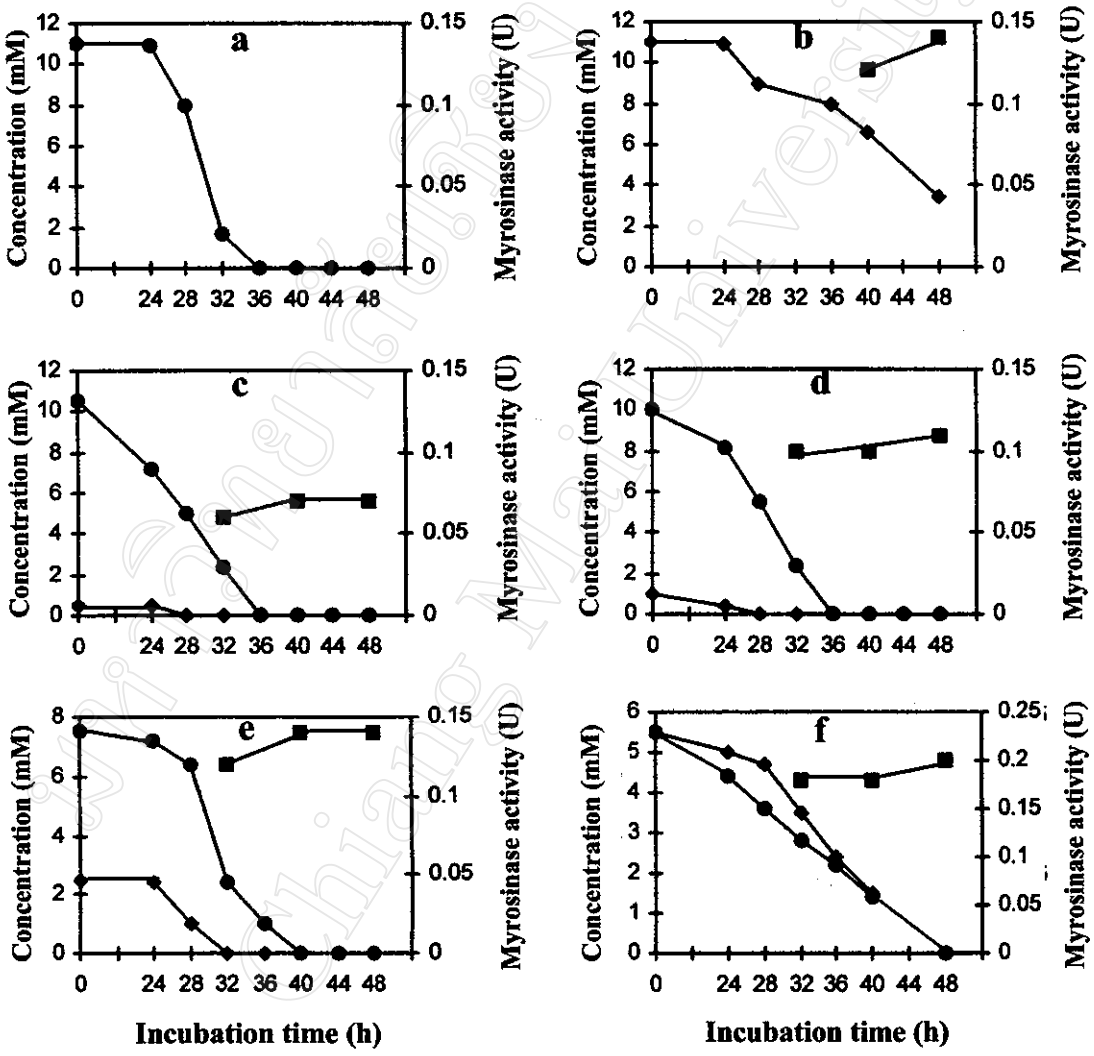

Figure 3.13 Pre-culture of *Aspergillus* sp. NR-4201 in G-medium (a), S-medium (b), SG-0.5/10.5 (c), SG-1/10 (d), SG-2.5/7.5 (e) and SG-5.5/5.5 medium (f). Consumptions of glucose (●) and sinigrin (◆) and the occurrence of intracellular myrosinase activity (■) are shown.

3.1.5) Sinigrin Degradation by *Aspergillus* sp. NR-4201 in liquid culture

In this study, induced and non-induced cells of the *Aspergillus,* pre-cultured as described above and in section 2.3.6, were incubated with sinigrin medium (resting cell experiments) or sinigrin-glucose medium (growing cell experiments) at 28 °C. The amounts of sinigrin and glucose degraded, and sinigrin hydrolysis product(s) formed in cultured filtrates were measured. Myrosinase activity contained in each fungal mycelial sample was also determined.

a) Resting cell experiments

Sinigrin consumption started immediately when induced cells were incubated with sinigrin (Figure 3.14). During sinigrin consumption period, the activity of intracellular myrosinase increased rapidly from 0.1 U to 0.28 U within 3 h and remained constant for several hours (Figure 3.14a). Incubation of the non-induced cells with sinigrin resulted in progressive expression of intracellular myrosinase up to maximum levels of 0.2 U within 18 h. (Figure 3.14b). The induced and non-induced mycelia produced total degradation of sinigrin at 15 and 36 h, respectively. Allylcyanide was an only sinigrin hydrolytic product detected in cultured filtrates (Figure 3.15). However, its accumulation was found to be delayed (Figure 3.14). The maximum levels of allylcyanide formed were approximately 38 and 40 % of the initial sinigrin concentrations, repectively, by the non-induced and induced cells.

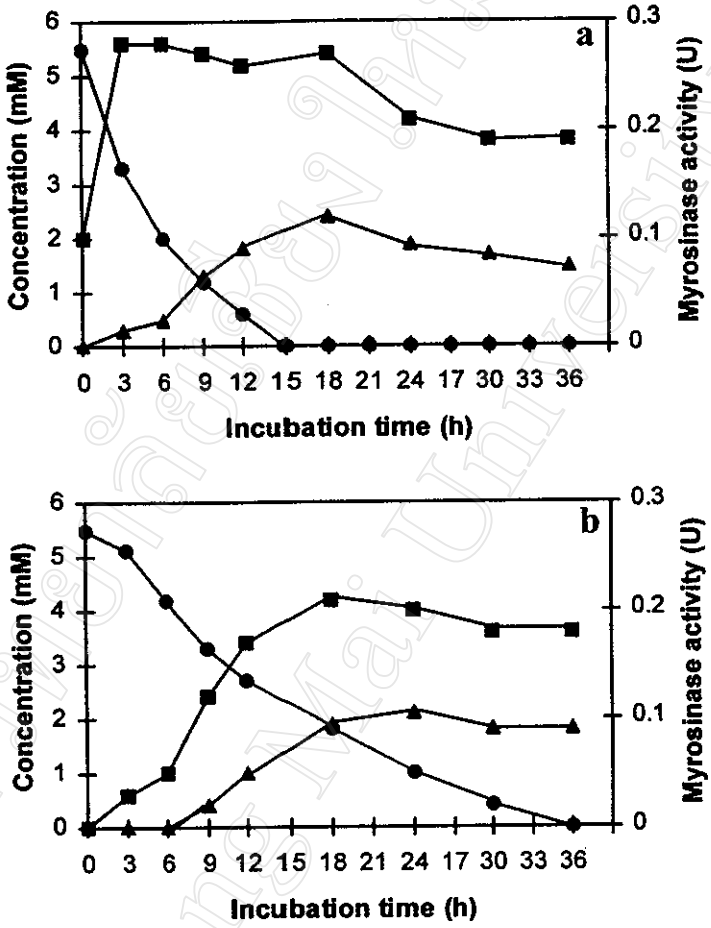

Figure 3.14 Liquid culture of induced (a) and non-induced cells (b) of *Aspergillus* sp. NR-4201 in sinigrin medium. Degradation of sinigrin (◆), formation of allylcyanide (▲) and occurrence of intracellular myrosinase activity (■) are shown.

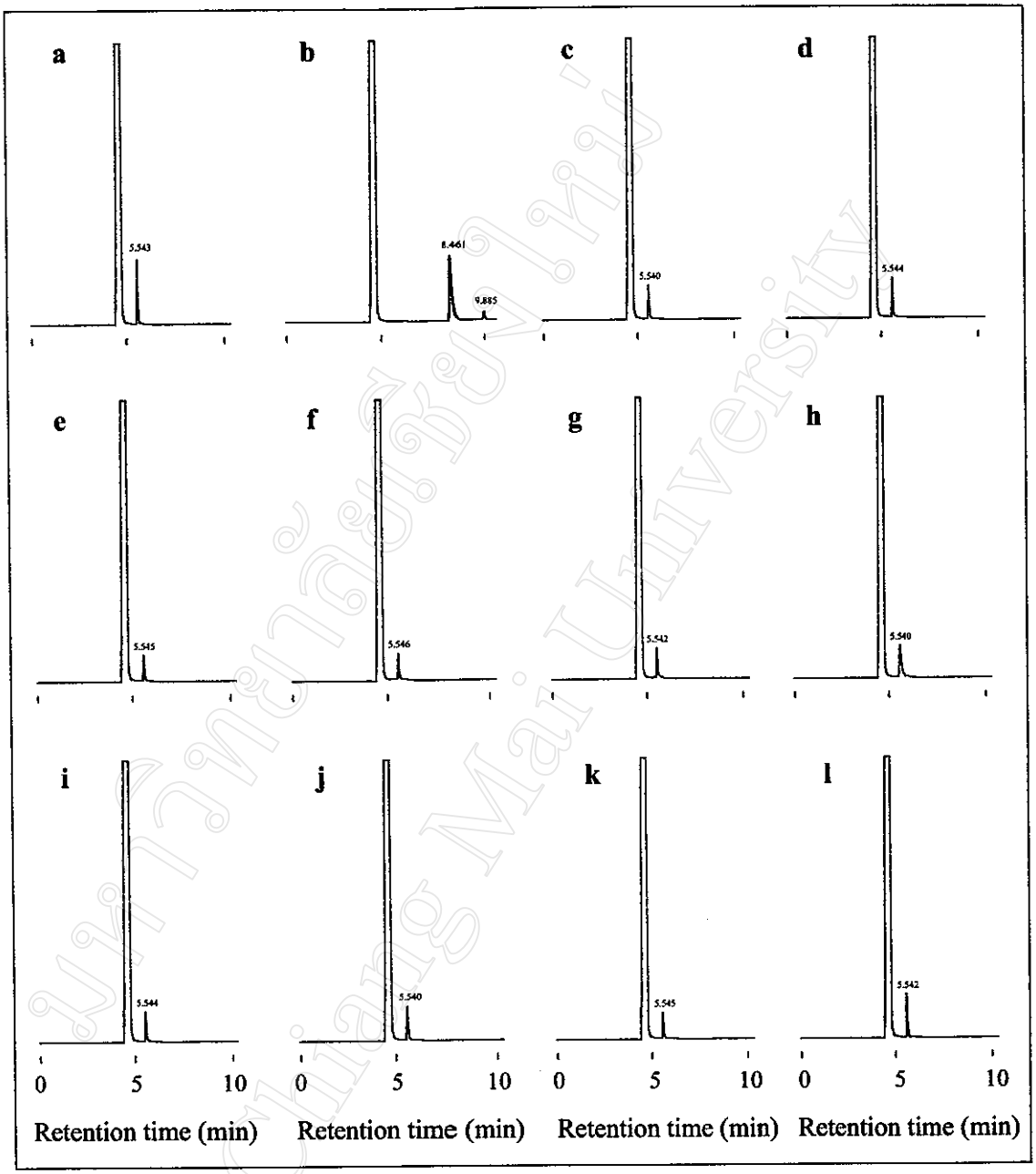

Figure 3.15 Gas chromatogram of sinigrin (or glucosinolate) breakdown products in cultured filtrates of *Aspergillus* sp. NR-4201. Standard allylcyanide : 5 mM (a), standard allylisothiocyanate : 5 mM (b), induced cells in S-medium at 12 h (c) and 15 h (d), non-induced cells in S-medium at 24 h (e), induced cells in SG-medium at 15 h (f) and 18 h (g), non-induced cells in SG-medium at 24 h (h), induced cells in mustard extract medium at 18 h (i), non-induced cells in mustard extract medium at 24 h (j), one-step culture at 36 h (k) and 44 h (l).

b) Growing cell experiments

In growing cell experiments, glucose was completely consumed by the non-induced cells within 6 h (Figure 3.16b). During the first 3 h of incubation, no degradation of sinigrin was observed and myrosinase activity was not detected in the mycelium. After glucose was exhausted, myrosinase was produced and sinigrin was subsequently degraded. However, only 80 % of sinigrin were consumed within the incubation period of 36 h. Sinigrin and glucose were competitively consumed by induced cells (Figure 3.16a). However, the cells used up glucose faster than sinigrin. Maximum enzyme activity was reached within 6 h and the total degradation of sinigrin was achieved within 24 h. It was the same in the resting cell experiments that allylcyanide was formed in cultured filtrates. Maximum accumulation in non-induced and induced cultures were 30 and 35 % of the initial sinigrin concentrations, respectively.

3.1.6) Glucosinolate degradation by *Aspergillus* sp. NR-4201 in liquid culture

a) Two-steps culture

In this study, induced and non-induced cells of the *Aspergillus*, pre-cultured as described in 2.3.6, were incubated with mustard extract medium at 28 °C. The results were rather different from those cultures in sinigrin or sinigrin-glucose medium. Glucose was produced in cultured filtrates at an early stage of incubation by either induced and non-induced cells. Highest glucose levels were reached at 6 h, and were totally exhausted within 12 h (Figure 3.17). During glucose production period, glucosinolates were consumed by the cells. Myrosinase activity of the induced

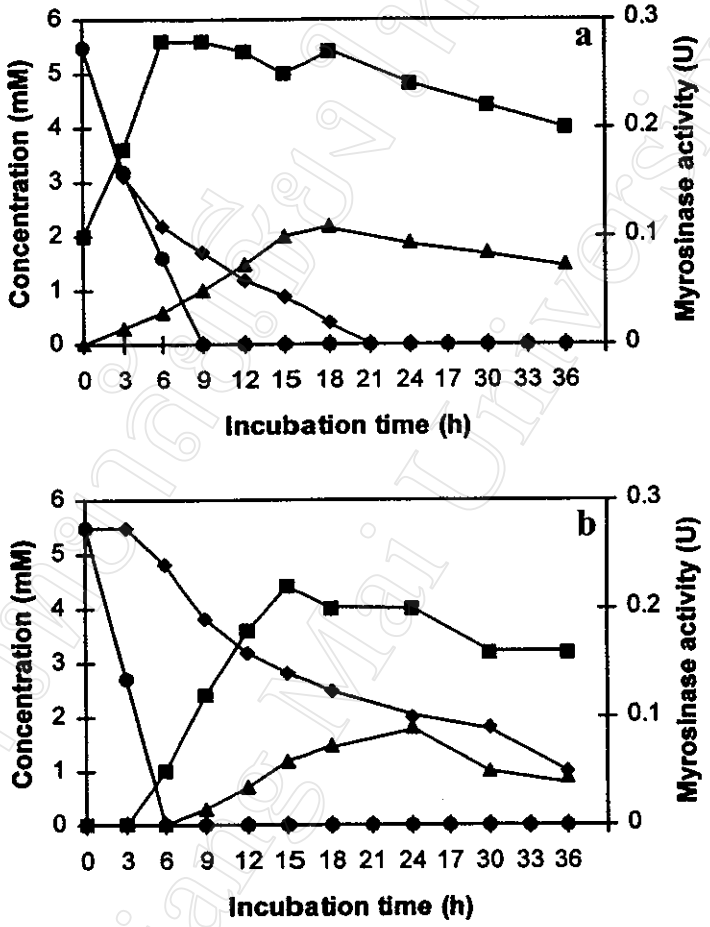

Figure 3.16 Liquid culture of induced (a) and non-induced cells (b) of *Aspergillus* sp. NR-4201 in sinigrin-glucose medium. Degradation of sinigrin (◆), consumption of glucose (●), formation of allylcyanide (▲) and occurrence of intracellular myrosinase activity (■) are shown.

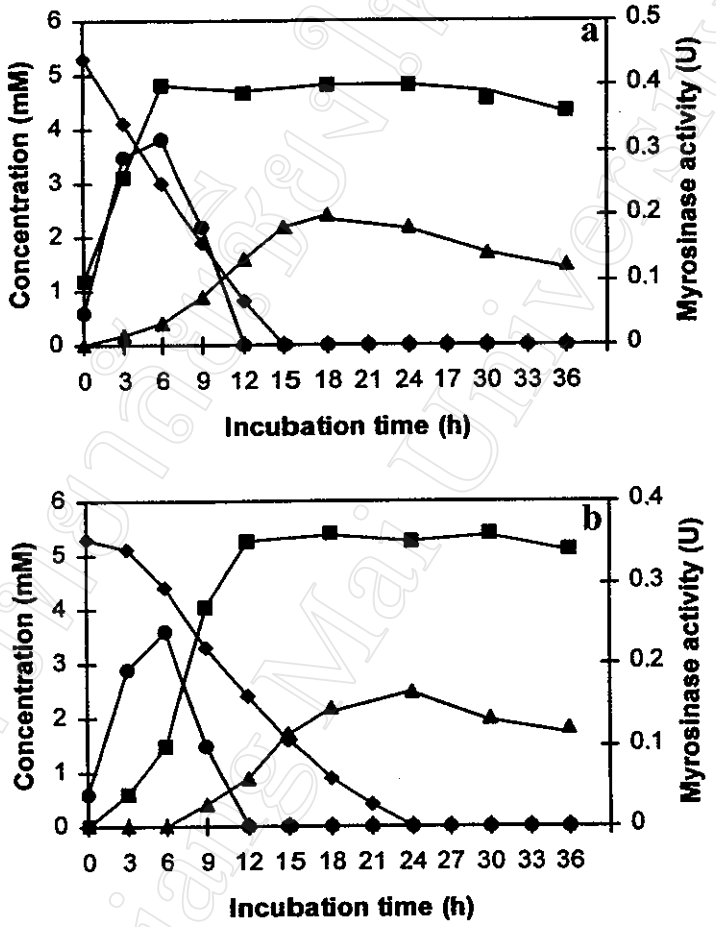

Figure 3.17 Liquid culture of induced (a) and non-induced cells (b) of *Aspergillus* sp. NR-4201 in mustard extract medium. Degradation of glucosinolates (◆), production and consumption of glucose (●), formation of allylcyanide (▲), and occurrence of intracellular myrosinase activity (■) are shown.

mycelium enhanced rapidly from 0.1 U to maximum levels of about 0.4 U within 6 h and glucosinolates were completely degraded at 15 h. In case of the non-induced cells, enzyme activity increased slowly (Figure 3.17b), as occured in sinigrin-glucose medium. The maximum levels reached at 12 h and glucosinolates were totally degraded within 24 h. Accumulation of allylcyanide was appeared highest at 58 and 54 % of the initial glucosinolate concentrations, respectively, by the induced and non-induced cells.

b) One-step culture

Germination study of the *Aspergillus* by one-step culture was performed by spore-inoculating in mustard extract medium and incubated at 28 °C, as described in 2.3.7. Clusters of fungal cells were formed within 16 h. Endogenous production of glucose started at 12 h. Maximum glucose level was reached at 24 h which was about three times higher than those two-step cultures. (Figure 3.18). During glucose production period, no degradation of glucosinolates was found. After 24 h, fungal cells consumed glucose and glucosinolates competitively. Glucose was exhausted completely at 32 h wherase glucosinolates were totally degraded at 36 h. During glucosinolate consumption, the activity of the intracellular myrosinase was produced subsequently and reached maximum levels (0.5 U) at 40 h (Figure 3.18). No significant change of the activity was observed, even when cultivation was extended up to 72 h. It was like the two-steps culture that allylcyanide was the only glucosinolate hydrolytic product detected in culture filtrates. Maximum allylcyanide level was formed at approximately 75 % of the initial glucosinolate concentration.

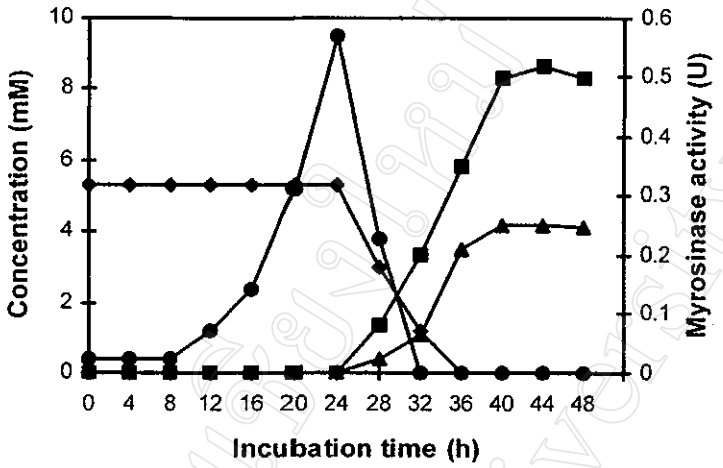

Figure 3.18 Liquid culture of *Aspergillus* sp. NR-4201 in mustard extract medium by one-step culture. Degradation of glucosinolates (◆), production and consumption of glucose (●), formation of allylcyanide (▲) and occurrence of intracellular myrosinase activity (■) are shown.

3.2) PRODUCTION OF INTRACELLULAR MYROSINASE FROM *Aspergillus* sp. NR-4201

From the preceding experiments, *Aspergillus* sp. NR-4201 was capable of growing and producing myrosinase in mustard extract medium. The one-step culture potentially yielded higher enzyme activity comparing to the two-step culture. Therefore, this cultivation method was reasonable for enzyme production purpose. In this study, cultured condition for the production of myrosinase was optimized.

3.2.1) Effect of pH of cultured medium on myrosinase production

The fungus was cultured in mustard extract medium at pH 5.6, 6.5 or 7.2. All were maintained at incubation temperature of 30 °C, glucosinolate concentration of 5.5 mM, inoculum concentration of 10^6 spore/ml (400 µl) and spore age of two weeks (as described in 2.4.1). Amounts of glucose and glucosinolate in cultured filtrates were measured. Fungal mycelium was harvested and washed properly, prior to being measured for dry weight. Another mycelial sample collected at the same time, was disrupted to obtain cell-free extract. Myrosinase activity in each cell-free extract sample was determined by the coupled-enzyme assay.

Growth profiles of the *Aspergillus* in the mustard extract medium at different pH were illustrated in Figure 3.19. It was in the same trend as the preceding one-step culture that glucose was produced in cultured filtrates at early cultivation stage. Fungal cell pellet was formed within 16 h, and then fungal cells used up glucose and glucosinolates competitively. Total glucosinolate degradation in the culture at pH 6.5 (Figure 3.19b) and pH 7.2 (Figure 3.19c) was complete at 32 h, whereas, it was prolonged up to 36 h in the culture at pH 5.6 (Figure 3.19a). Enzyme activity produced up to maximum levels at 44 h, which corresponded to biomass formation. The activity remained at the highest levels for at least 72 h if the reciprocal shaking still operated (data not shown). However, the activity of enzyme produced at pH 6.5 was found being the highest (Figure 3.19b and Figure 20), whereas, the lowest activity was shown in the culture at pH 5.6 (Figure 3.19a and Figure 20).

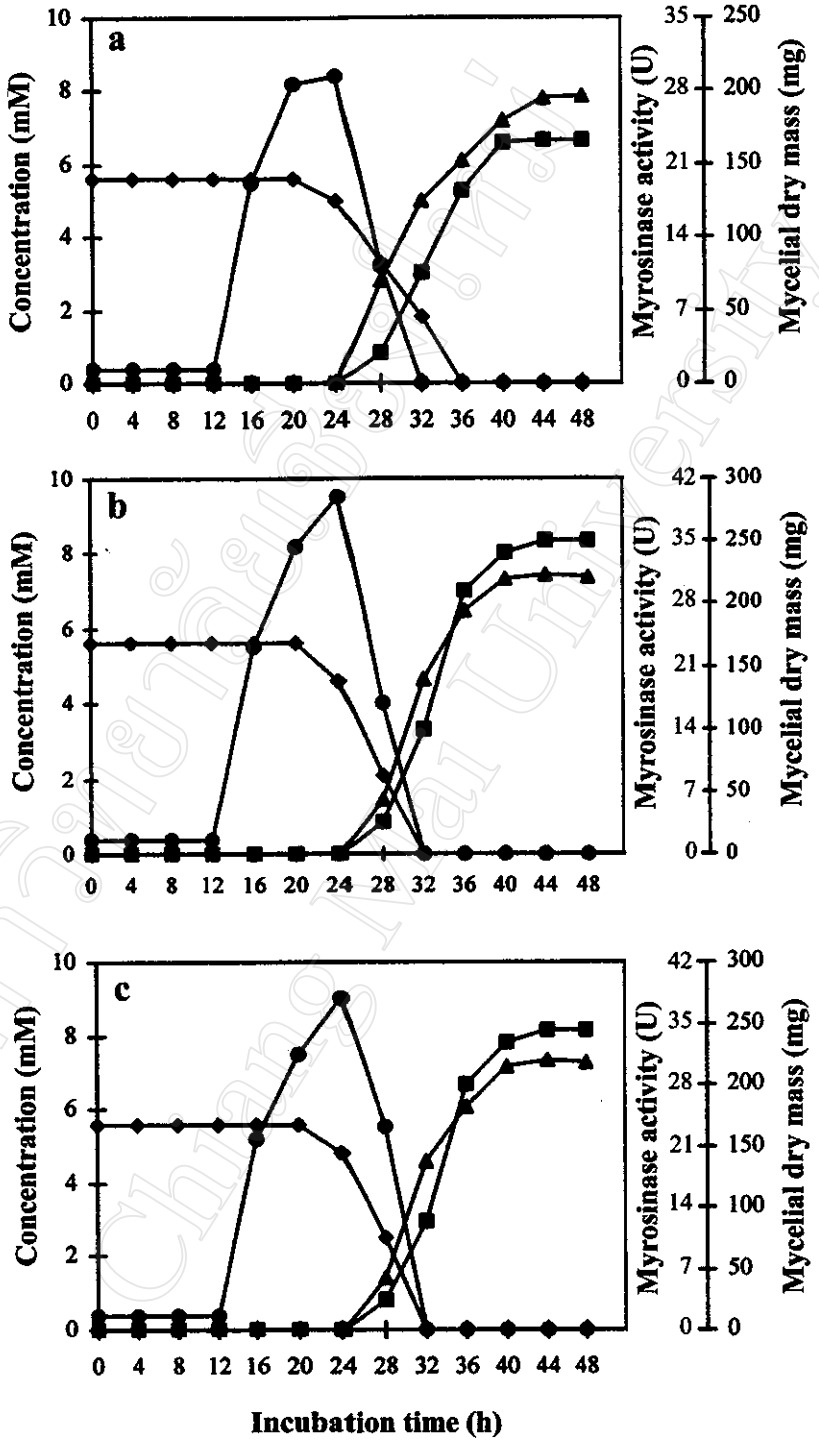

Figure 3.19 Growth profiles of *Aspergillus* sp. NR-4201 in mustard extract medium at pH 5.6 (a), 6.5 (b) and 7.2 (c). Degradation of glucosinolates (◆), producction and consumption of glucose (●), biomass formation (▲) and myrosinase production (■) are shown.

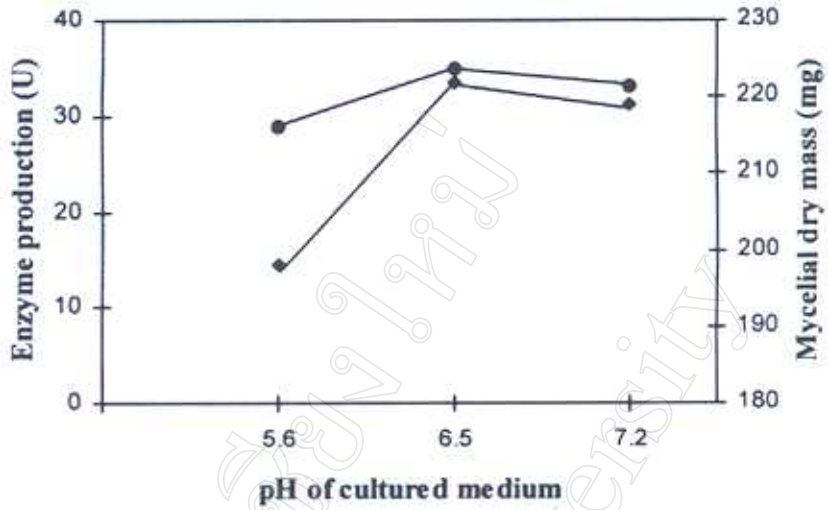

Figure 3.20 Relationship between pH of cultured medium and myrosinase production by *Aspergillus* sp. NR-4201. Enzyme production (●) and fungal biomass (◆) were obtained at 48 h after cultivation.

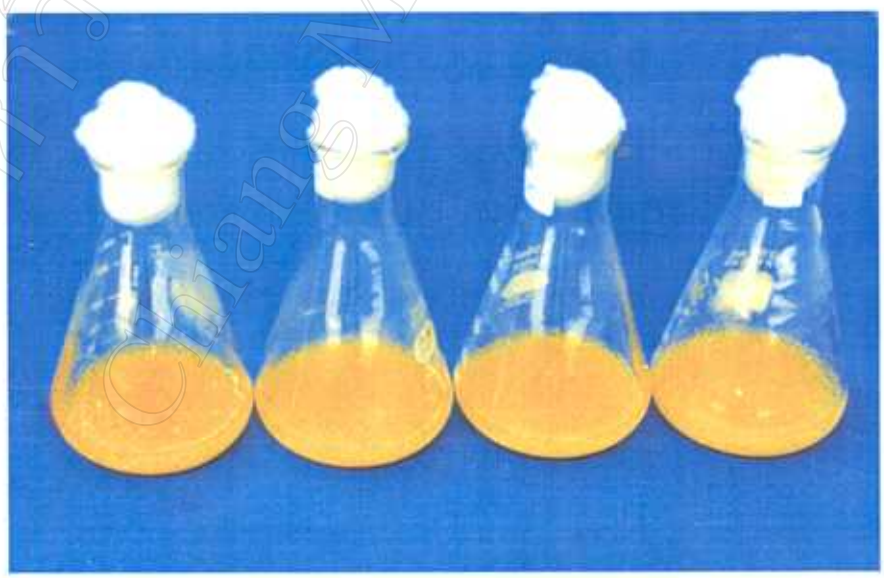

Figure 3.21 Fungal mycelium of *Aspergillus* sp. NR-4201 grown in mustard extract medium. The 40-ml culture was carried at pH 6.5, 5.5 mM glucosinolates, 30 °C, for 48 h without any supplements.

3.2.2) Effect of glucoinolate concentration on myrosinase production

To investigate the effect of glucosinolate concentration on myrosinase production, different concentrations of glucosinolates (2.8, 5.5, 8.3 and 11 mM) were studied (as described in 2.4.2). Other variables kept constant were incubation temperature of 30 °C, pH of 6.5, inoculum concentration of 10^6 spore/ml (400 µl) and spore age of 2 weeks.

As investigated at glucosinolate concentrations of 2.8, 5.5 and 8.3 mM, glucosinolates were totally degraded within 28, 32 and 40 h, respectively (Figure 3.22). Fungal biomass formed and enzyme activity produced in the culture with 5.5 mM glucosinolates were approximately two times higher than that was achieved in the culture with 2.8 mM glucosinolates (Figure 3.22 and 3.23). Biomass formation in the culture at 8.3 mM glucosinolats was 1.6 times higher than with 5.5 mM. However, the activity of enzyme production was only 1.2 times higher. Therefore, mustard extract medium containing 5.5 mM glucosinolates was optimal for myrosinase production.

3.2.3) Effect of incubation temperature on myrosinase production

To assess the effect of incubation temperature on enzyme production, cultures were carried out at 28, 30 or 37 °C. In such cases, other variables were kept constant, *i.e.* pH 6.5, glucosinolate concentration of 5.5 mM, inoculum concentration of 10^6 spore/ml and spore age of two weeks.

Growth profiles of the culture at 28 °C were not different from the culture at 30 °C. However, growth at 37 °C resulted in lowered of lag-phase times, 22 h, compared with 26 h of the culture at 30 °C (Figure 3.24). Glucose and glucosinolates

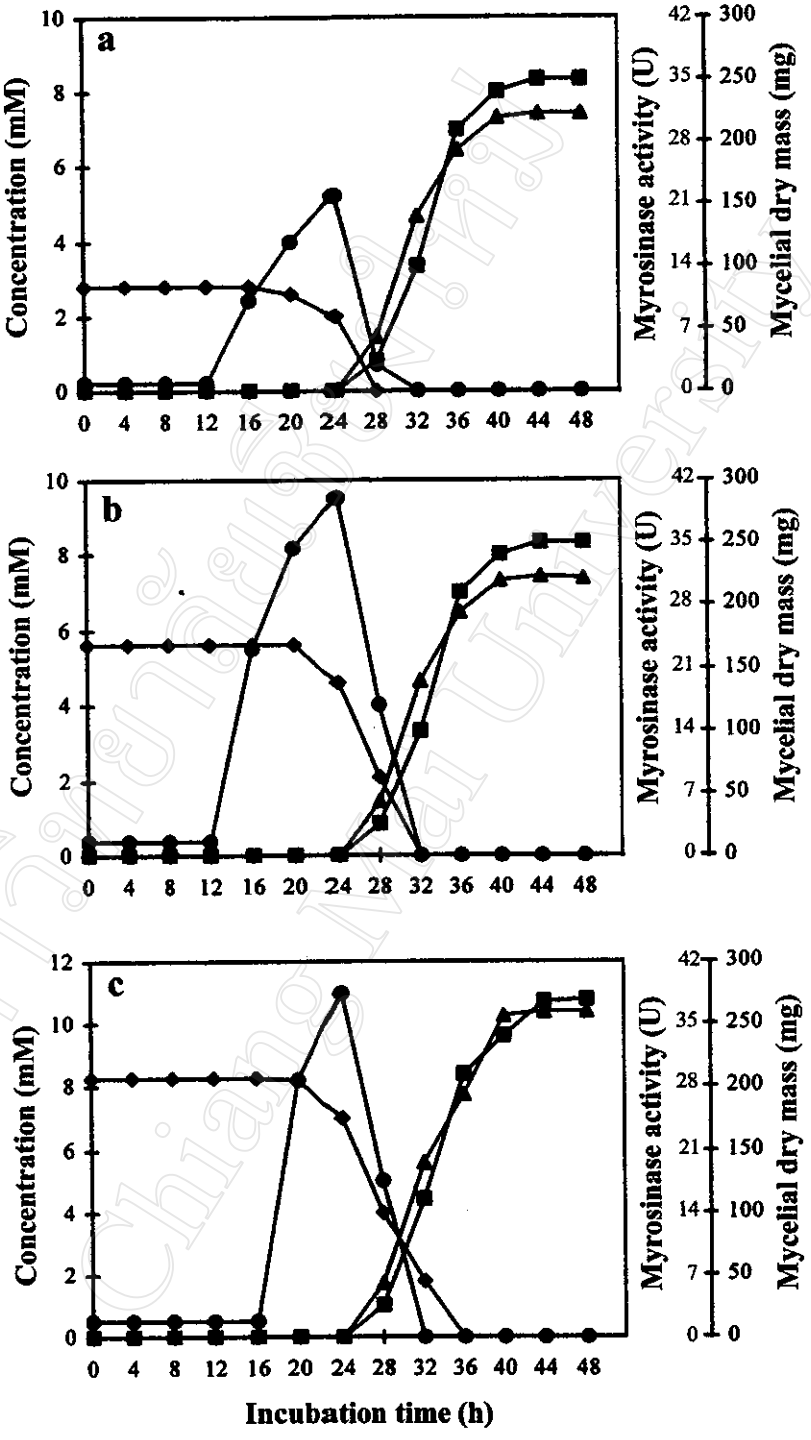

Figure 3.22 Growth profiles of *Aspergillus* sp. NR-4201 in mustard extract medium at glucosinolate concentrations of 2.8 mM (a), 5.5 mM (b) and 8.3 mM (c). Degradation of glucosinolates (◆), production and consumption of glucose (●), biomass formation (▲) and myrosinase production (■) are shown.

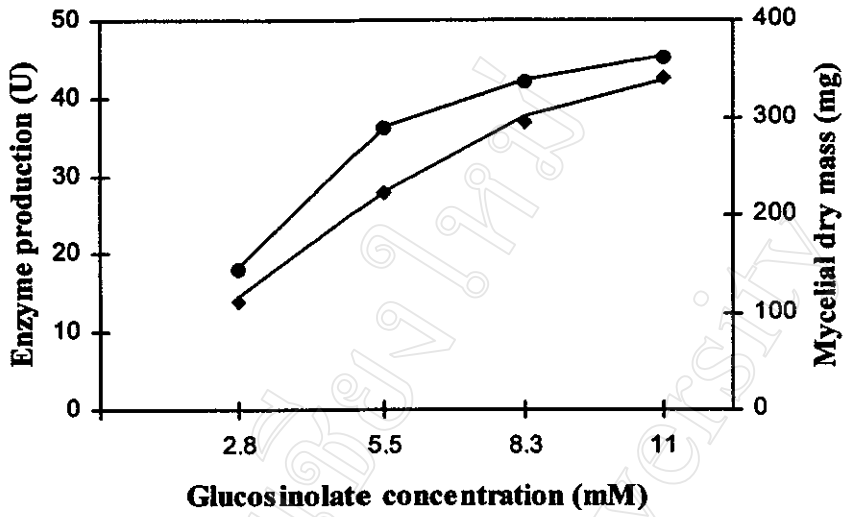

Figure 3.23 Relationship between glucosinolate concentration and myrosinase production by *Aspergillus* sp. NR-4201. Enzyme production (●) and fungal biomass (◆) were obtained at 48 h after cultivation.

were completely consumed at 28 h. In such case, enzyme production and biomass formation were not significantly different from those cultures at 30 °C. In addition, it was found that little growth was observed if the cultivation was performed at 40 °C.

3.2.4) Effect of inoculum size and spore age on myrosinase production

In order to investigate the effect of inoculum size on myrosinase production, 400 µl of inoculum at varying concentrations of 10^5, 10^6, 5×10^6 or 10^7 spores/ml were used. The cultures were performed at pH 6.5, glucosinolate concentra-tion of 5.5 mM, incubation temperature of 30 °C and spore age of two weeks. Enzyme activity and fungal biomass produced at inoculum concentrations of 10^6, 5×10^6 and 10^7 spores/ml were not different (Figure 3.25). However, the culture at inoculum concentration of 10^5

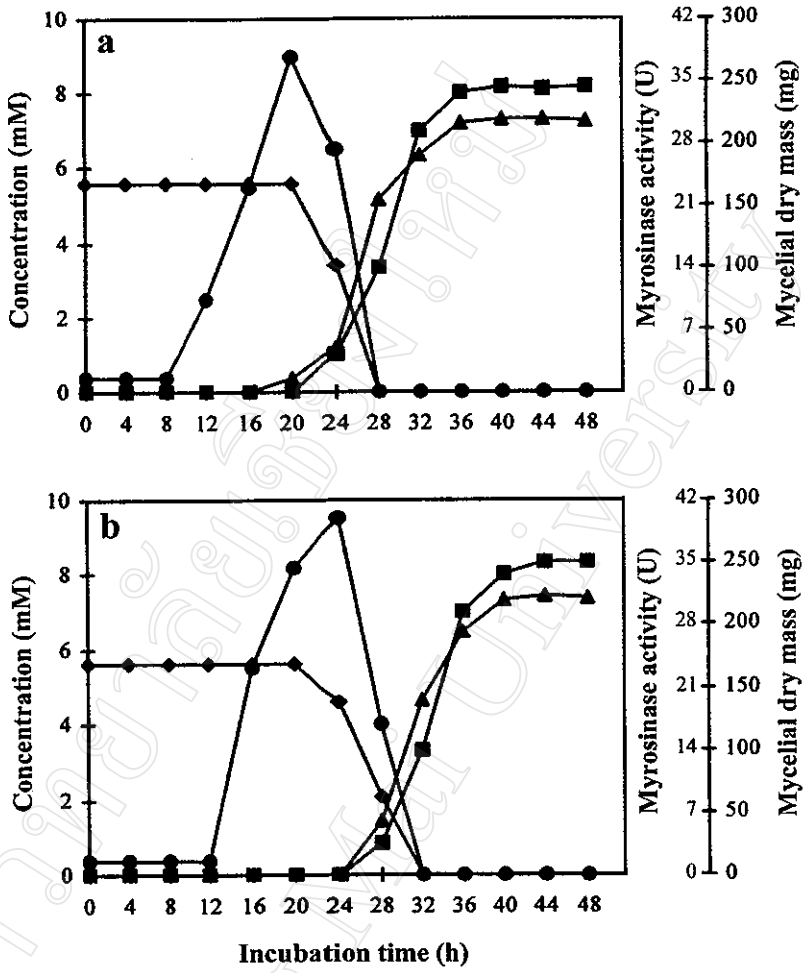

Figure 3.24 Growth profiles of *Aspergillus* sp. NR-4201 in mustard extract medium at 37 °C (a) and at 30 °C (b). Degradation of glucosinolates (◆), production and consumption of glucose (●), biomass formation (▲) and myrosinase production (■) are shown.

spores/ml resulted in the decrease of enzyme activity and fungal biomass (Figure 3.25).

Concerning with spore age, similar enzyme production and fungal biomass formation were obtained in cultures with inoculum made from spores grown in PDA for 1, 2, 3 or 4 weeks (data not shown).

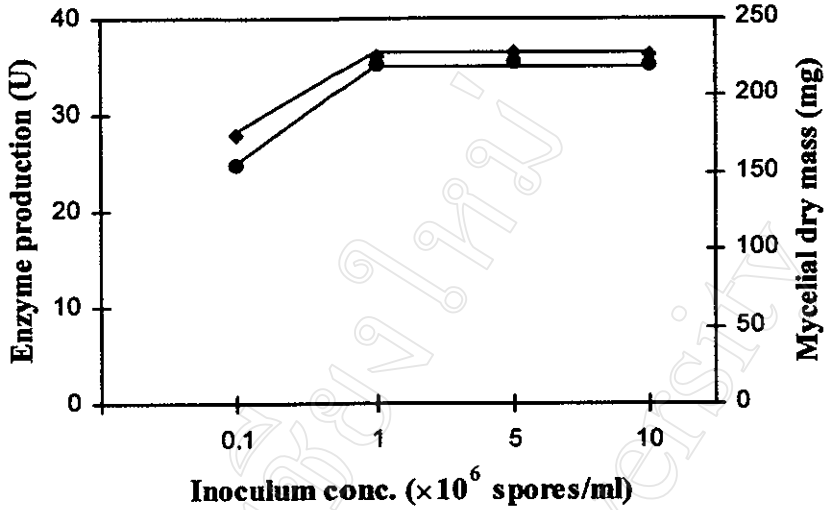

Figure 3.25 Relationship between inoculum concentration and myrosinase production by *Aspergillus* sp. NR-4201. Enzyme production (●) and fungal biomass (◆) were obtained at 48 h after cultivation.

3.2.5) Effect of L-ascorbic acid or inorganic salt supplement on myrosinase production

To assess the effect of L-ascorbic acid and glucose supplement on myrosinase production, each compound was added separately into mustard extract medium to final concentrations of 1, 2.5, 5 or 10 mM. It was shown that both L-ascorbic acid and glucose affected directly on fungal growth. At 5 mM of supplemented L-ascorbic acid, glucosinolates were not completely degraded within 48 h (Figure 3.26). Fungal biomass increased proportionally to the amounts of L-ascorbic acid supplement. However, the activity of enzyme production was decreased (Figure 3.26 and 3.27). Similar effect was also demonstrated by the supplement with glucose (data not shown).

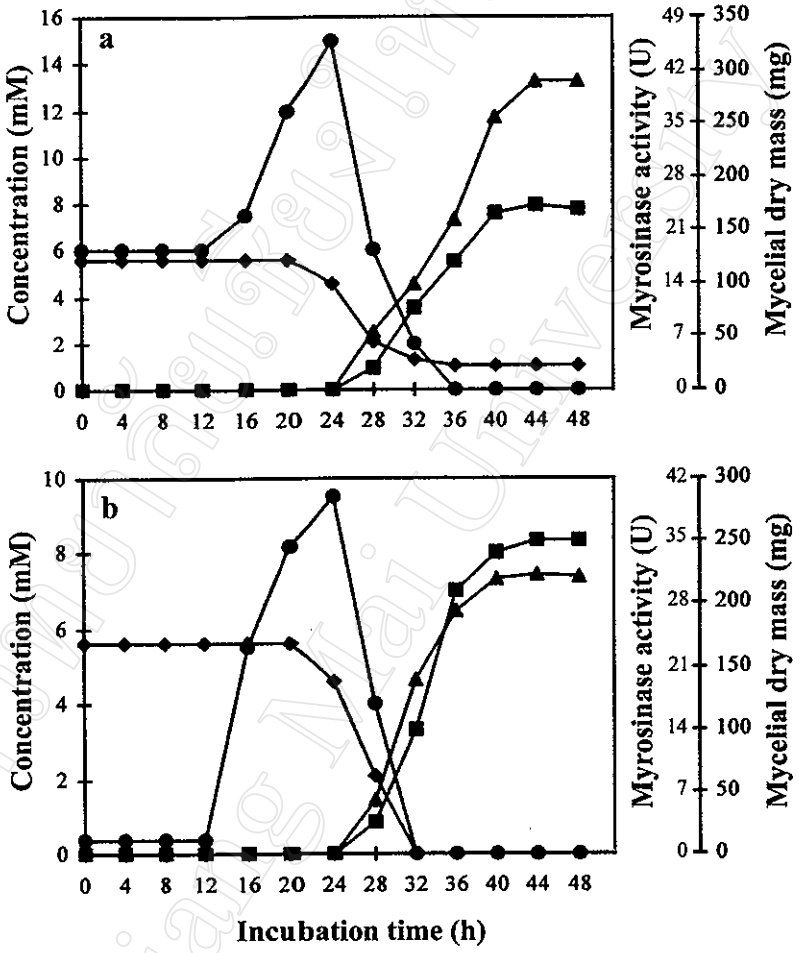

Figure 3.26 Growth profiles of *Aspergillus* sp. NR-4201 in mustard extract medium with 5 mM L-ascorbic acid (a) or without any supplements (b). Degradation of glucosinolates (◆), production and consumption of glucose (●), biomass formation (▲) and myrosinase production (■) are shown.

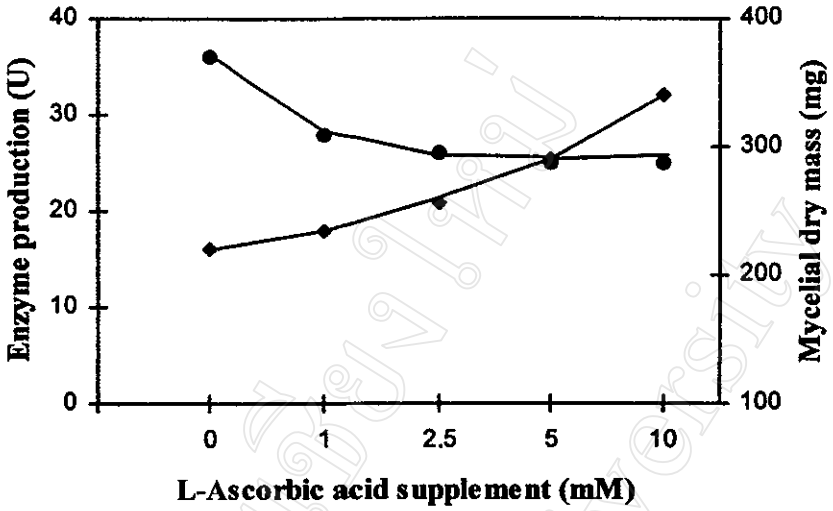

Figure 3.27 Relationship between L-ascorbic acid supplement and myrosinase production by *Aspergillus* sp. NR-4201. Enzyme production (●) and fungal biomass (◆) were obtained at 48 h after cultivation.

In order to study the effect of some inorganic salts on myrosinase production, $MgCl_2$, $CaCl_2$, $MnCl_2$ and $CoCl_2$ were supplemented in the mustard extract medium to a final concentration of 1 or 5 mM each. All these salts at specified concentrations showed neither stimulatory nor inhibitory effect on myrosinase production, compared to control experiment with no salt supplement (data not shown).

In conclusion, optimal condition of this one-step culture to produce myrosinase was operated in mustard extract medium at pH 6.5, glucosinolate concentration of 5.5 mM, at 30 °C, for 48 h without any supplements.

3.2.6) Preservation of mycelium containing myrosinase

Fungal mycelium from each culture flask grown in mustard extract medium at optimal condition for 48 h, were harvested, washed and maintained at 4 °C or -40 °C (as described in 2.4.6). Mycelial samples were then subjected for disruption to determine for remaining activity. It was found that enzyme levels did not changed if the mycelium was preserved at 4 °C for 24 h (Figure 3.28). About 15 % of the activity lost, when the mycelial sample was kept at the same condition for 48 h. Preservation at -40 °C for 24 and 48 h, resulted in the loss of 50 and 65 %, respectively (Figure 3.28).

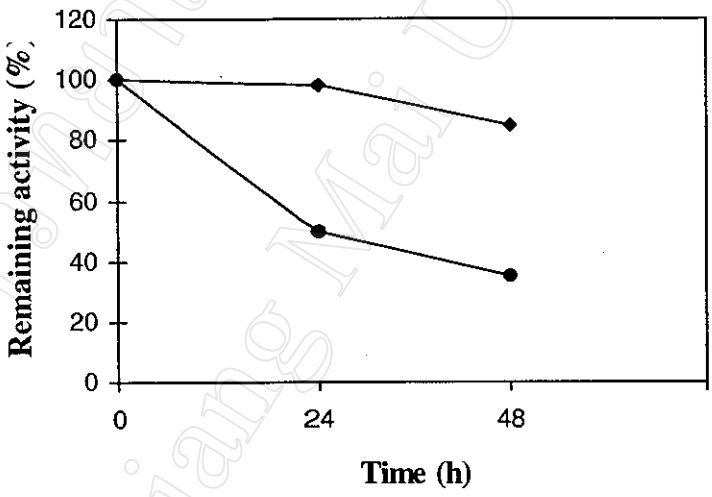

Figure 3.28 Stability of myrosinase activity in fungal mycelium. Mycelial samples were maintained at 4 °C (◆) or -40 °C (●).

3.2.7) Stability of crude myrosinase extract

Cell-free extracts of the *Aspergillus* in 10, 40 and 100 mM of sodium phosphate buffer pH 6.0, 7.0 or 7.4) were maintained at room temperature or at 4 °C.

Anti-microbial agents such as thimerosal or sodium azide were included to a final concentration of 0.02 % each. Then, samples were assayed for the remained activity.

Stabilities of the enzyme in 10-100 mM sodium phosphate buffer pH 6.0, 7.0 and 7.4 were not different (data not shown). However, enzyme activity was gradually decreased when stored at room temperature (about 30 °C). In such case, about 90 % of the activity were destroyed in the first 12 h (Figure 3.29). However, no significant decrease of the activity was observed within two days if it was maintained at 4°C without adding any preservatives (Figure 3.29). In the presence of 0.02 % of sodium azide, most activity could be preserved up to 5 days. However, the activity was totally disappeared when thimerosal was added to a final concentration of 0.02 %.

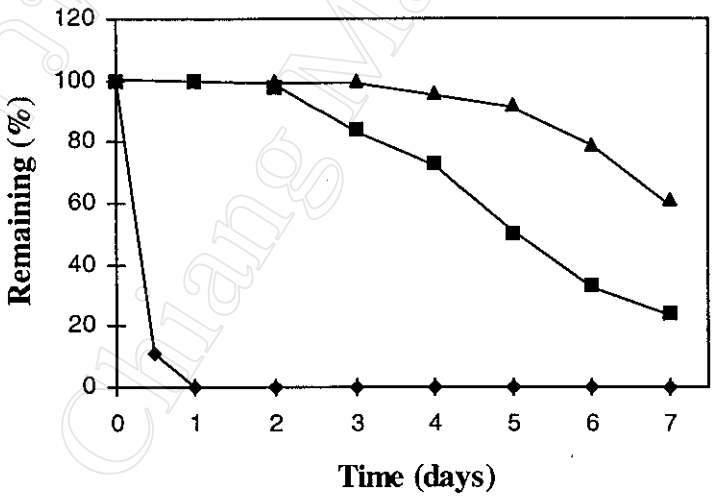

Figure 3.29 Stability of crude *Aspergillus* myrosinase. Enzyme samples in 40 mM sodium phosphate buffer, pH 7.0 were maintained at room temperature (◆) or at 4°C which contained with (▲) or without 0.02 % sodium azide(■).

3.3) PURIFICATION OF *Aspergillus* MYROSINASE

3.3.1) Ammonium sulfate fractionation

Crude enzyme sotution obtained from 12 culture flasks (40-ml each) was subjected for ammonium sufate fractionation (as described in 2.5.1). It was found that myrosinase was dominantly precipitated in a fraction between 50 and 60 % saturation of ammonium sulfate. After dialysis against 40 mM sodium phosphate buffer, pH 7.0 followed by centrifugation (10000×g, 20 min, 4 °C), specific activity of this enzyme fraction was elevated from 0.65 to 3.34 U/mg protein and the yield was 49 % (Table 3.2).

Table 3.2 Summaries of ammonium sulfate fractionation of crude *Aspergillus* myrosinase

Fraction	Volume (ml)	Protein content (mg/ml)	Enzyme activity (U/ml)	Total activity (U)	Spec. activity (U/mg protein)	Yield %
Crude	40	2.7	1.8	72	0.65	100
0-40 %*	3.5	7.8	0	0	0	0
40-50 %*	2.8	3.1	4.2	12	1.28	16
50-60 %*	6.1	1.7	5.7	35	3.34	49
60-70 %*	1.9	2.9	1.6	3	0.55	4

* referred to % saturation of ammonium sulfate

3.3.2) Preliminary study of separating conditions of *Aspergillus* myrosinase by small DEAE Sephadex A-25 columns

A set of DEAE Sephadex A-25 columns (1-ml packed gel in a 3-ml syringe) were equilibrated with 5 ml of 20 mM of sodium phosphate buffer, at desired pH (6.0, 6.5, 7.0 and 7.4), as described in 2.5.3b. Each column was loaded with 1 ml of enzyme solution (50-60 % ammomnium sulfate precipitated fraction), which was contained in 20 mM sodium phosphate buffer at the specified pH. Elution was performed by using 5 ml of the corresponding buffer, in which concentrations of such buffer were increased in a series of 20, 40, 75 and 125 mM, stepwisely. Each eluate fraction was then measured for protein content and enzyme activity.

Results of DEAE Sephadex A-25 chromatography (stepwise elution) of *Aspergillus* myrosinase are summarized in Table 3.3. After washing with the equilibrating buffer (20 mM sodium phosphate buffer), total binding capability of myrosinase was shown in the pH 6.5-, pH 7.0- and pH 7.4-columns, whereas, only 27 % (3.5 U) of myrosinase bound the pH 6-column. Elution with 40 mM sodium phosphate, enzyme activity was slightly detected in eluate fraction from the pH 6.5-column, not in those fractions from the columns of pH 7.0 and 7.4. When columns were consecutively eluted with 75 mM sodium phosphate, eluate fractions from the pH 6.5- and 7.0-columns contained 73 (10 U) and 76 (10.4 U) % of enzyme activity, respectively. When elution was performed with 125 mM sodium phosphate, the eluate from the pH-7.4 column contained 73 % (10 U) of enzyme activity. However, the enzyme fraction from the pH 7.0-column eluted with 40-75 mM sodium phosphate buffer exhibited the highest specific activity, comparing to the other two fractions (Table 3.3).

Table 3.3 Summaries of the separation of *Aspergillus* myrosinase by small columns of DEAE Sephadex A-25

Eluate fraction		Volume (ml)	Protein content (mg/ml)	Enz. activity (U/ml)	Total activity (U)	Spec. activity (U/mg protein)	yield (%)
Applied sample		1	4	13.6	13.6	3.4	100
6.0	20 mM	5	0.5	2	10	3.3	73
	40 mM	5	0.2	0.7	3.5	3.5	27
	75 mM	5.1	<0.05	trace	-	-	-
	125 mM	5.2	<0.05	0	-	-	-
6.5	20 mM	5.2	n.d.	trace.	-	-	-
	40 mM	5	n.d.	0.5	-	2.5	18
	75 mM	5	0.2	2	10	10	73
	125 mM	5	0.1	0	-	-	-
7.0	20 mM	5.2	n.d.	0	-	-	-
	40 mM	5	n.d.	0	-	-	-
	75 mM	5.2	0.1	2	10.4	20	76
	125 mM	5.1	n.d.	trace	-	-	-
7.4	20 mM	5	n.d.	0	-	-	-
	40 mM	5.2	n.d.	0	-	-	-
	75 mM	5	<0.05	0.5	2.5	-	18
	125 mM	5	0.3	2	10	7	73

n.d. : not done

Above results indicated that the myrosinase was considerably separated between 40 and 75 mM sodium phosphate buffer, pH 7.0. Therefore, this separating condition was introduced for use in the large-scale separation.

3.3.3) DEAE Sephadex A-25 chromatography (I)

A DEAE Sephadex A-25 column (2.6×15 cm) pre-equilibrated with 40 mM sodium phosphate buffer, pH 7.0 (as described in 2.5.3) was loaded with enzyme solution (50-60 % ammonium sulfate precipitated fraction). The column was washed with the equilibrating buffer at a flowrate of 2 ml/min. After washing with 500 ml of the buffer, absorbance values at 280 nm of eluates from the column were nearly zero. Subsequently, the column was eluted with a linear gradient of 40-100 mM sodium phosphate buffer, pH 7.0 (600 ml). Ten-ml fractions were collected, which were then measured for protein content and myrosinase activity. Elution profiles of myrosinase separation by DEAE Sephadex A-25 chromatography are illustrated in Figure 3.30. It was shown that myrosinase active fractions were present in the second peak (fraction no. 65-76), corresponding to 60-75 mM sodium phosphate gradient. These active fractions were combined and concentrated, using ultrafiltration (molecular weight cut-off 50 kDa).

After ultrafiltration process, no enzyme activity was present in the filtrate. This suggested that molecular weight of the myrosinase was over 50 kDa. The enzyme solution obtained after ultrafiltration showed a specific activity of 46.8 U/mg protein and yield of 26 % (Table 3.4). SDS-PAGE analysis of this concentrated fraction revealed that three major protein bands were shown (Figure 3.33).

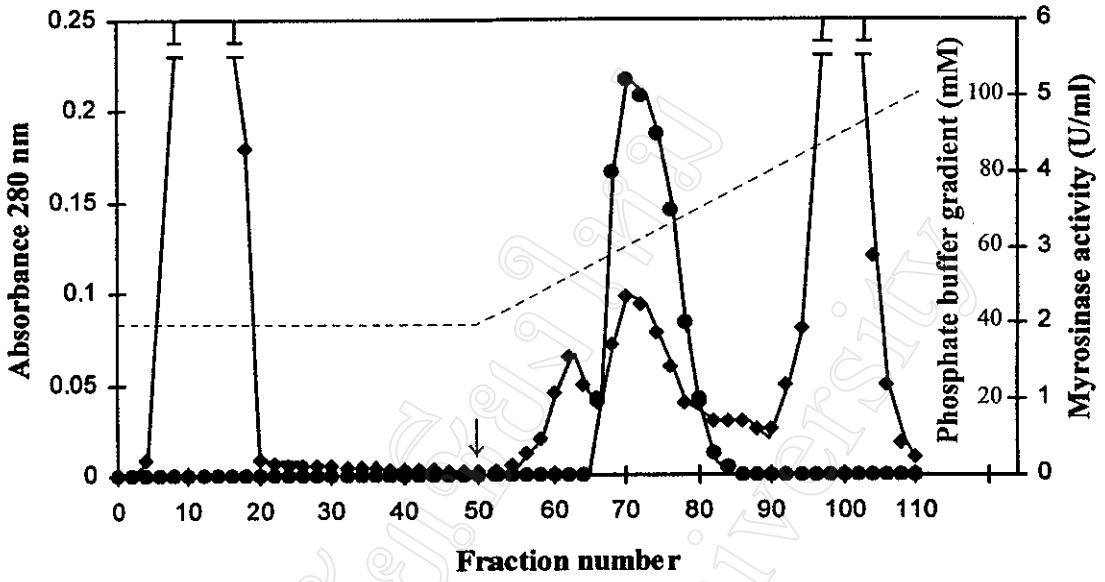

Figure 3.30 Elution profiles of DEAE Sephadex A-25 chromatography (I) of the *Aspergillus* myrosinase. Protein content (◆) and myrosinase activity (●) are shown. An arrow indicates starting of gradient elution (dash line).

3.3.4) DEAE Sephadex A-25 chromatography (II)

A DEAE Sephadex A-25 column (1.0×15 cm) equilibrated with 50 mM sodium phosphate buffer, pH 7.0 (as described in 2.5.5), was applied with the concentrated enzyme fraction from DEAE Sephadex A-25 chromatography (I) followed by ultrafiltration (activity of 55 U/ml in 2 ml of 50 mM sodium phosphate buffer, pH 7.0). The column was washed with the equilibrating buffer for at least 5 times of total column volumes, operating at a flow rate of 1 ml/min. Then, elution was performed by using a concave gradient of 50-90 mM sodium phosphate buffer, pH 7.0 (225 ml), and five-ml fractions were collected. Elution profiles of myrosinase purification from the DEAE Sephadex A-25 chromatography (II) are shown in Figure

3.31. It was found that myrosinase active fractions were present in the first peak (fractions no. 14-22). Then, these fractions were combined and concentrated, using ultrafiltration. The concentrated enzyme solution exhibited a specific activity of 55.3 U/mg protein. SDS-PAGE analysis revealed that one major band correponded to 94 kDa, and one minor band corresponded to 55 kDa were present (Figure 3.33).

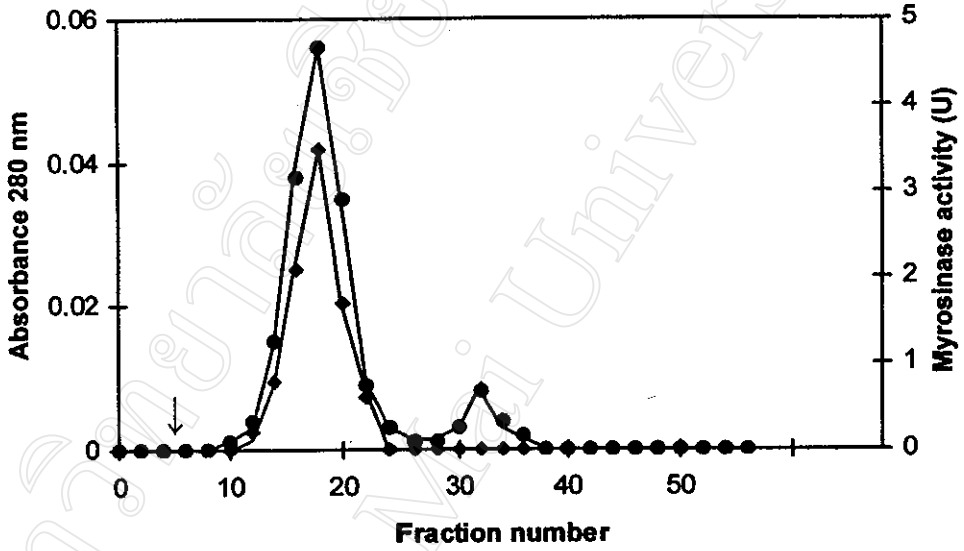

Figure 3.31 Elution profiles of DEAE Sephadex A-25 chromatography (II) of the *Aspergillus* myrosinase. Protein content (●) and myrosinase activity (◆) are shown. An arrow indicates starting of gradient elution.

3.3.5) Sephadex G-100 gel-filtration

A Sephadex G-100 column (1.6×100 cm) equilibrated with 40 mM sodium phosphate buffer, pH 7.0 was loaded with 0.4 ml of concentrated enzyme solution (activity of about 120 U/ml) from the DEAE Sephadex A-25 chromatography (II). A flow rate was operated at 15 ml/h and one-ml fractions were collected. Elution profiles

of the Sephadex G-100 chromatography of myrosinase were illustrated in Figure 3.32. It was shown that the myrosinase protein was eluted at the first peak (fractions no. 83-88). These concentrated fractions (specific activity of 65.9 U/mg protein) exhibited one major band on SDS-PAGE (Figure 3.33), whose the molecular weight was calculated to be 94 kDa (Figure 3.34). Purification of the *Aspergillus* myrosinase is summarized in Table 3.4.

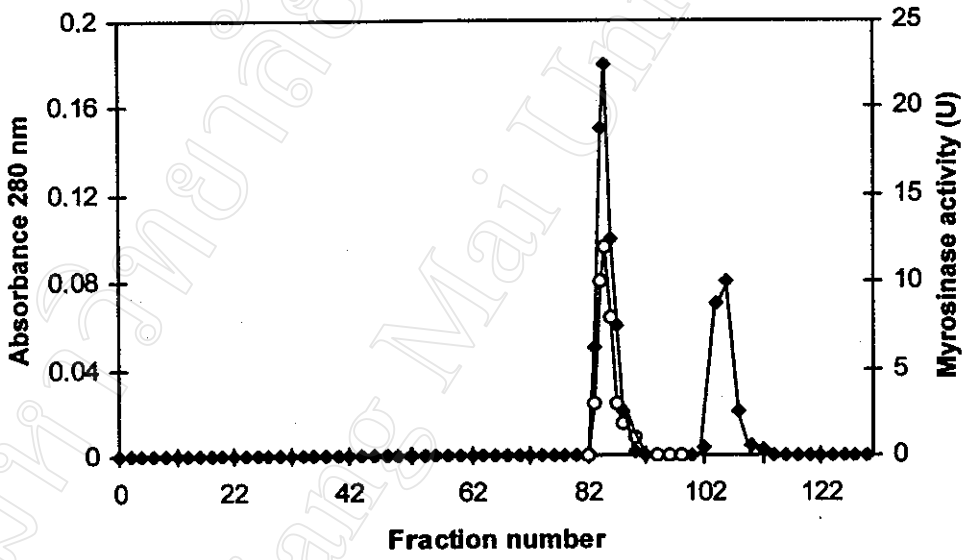

Figure 3.32 Elution profiles of Sephadex G-100 chromatography of the *Aspergillus* myrosinase. Protein content (◆) and myrosinase activity (○) are shown.

Table 3.4 Summaries of *Aspergillus* myrosinase purification

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme	648	421	0.65	100	1
Ammonium sulfate	65	217	3.34	52	5
DEAE Sephadex A-25 (I) and ultrafiltration	2.35	110	46.80	26	72
DEAE-Sephadex A-25 (II)	0.87	123	56.32	12	87
Sephadex G-100	0.44	29	65.90	7	101

3.3.6) SDS-PAGE of the *Aspergillus* myrosinase purification

Relationship between relative mobility and log molecular weights of standard protein markers (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lactalbumin) in SDS-PAGE are shown in Table 3.5 and Figure 3.33. In this case, the molecular weight of the *Aspergillus* myrosinase was calculated to be 94 kDa.

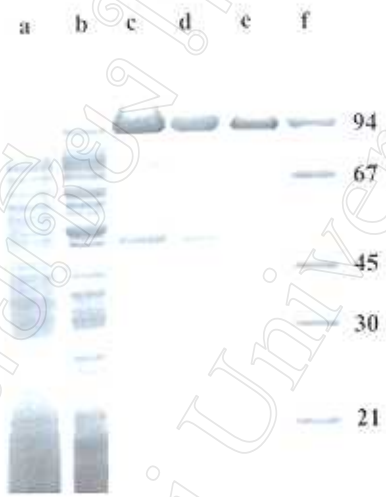

Figure 3.33 SDS-PAGE of the *Aspergillus* myrosinase. Crude enzyme (a), 50-60 % ammonium sulfate fraction (b), active fractions after DEAE Sephadex A-25 chromatograpgy-I and ultrafiltration (c), active fractions after DEAE Sephadex A-25 chromatography-II (d), highly purified enzyme after Sephadex G-100 chromatography (e; 2 µg) and molecular weight protein markers in kDa (f).

Table 3.5 Relative mobility and log molecular weight of standard protein markers by SDS-PAGE.

Protein marker	Molecular weight (Da)	Log molecular weight	Relative mobility
Phosphorylase b	94,000	4.97	0.185
Bovine serum albumin	67,000	4.82	0.245
Ovalbumin	43,000	4.63	0.478
Carbonic anhydrase	30,000	4.47	0.644
Trypsin inhibitor	20,100	4.30	0.855
Lactalbumin	14,400	4.16	0.980
Aspergillus myrosinase	-	-	0.185

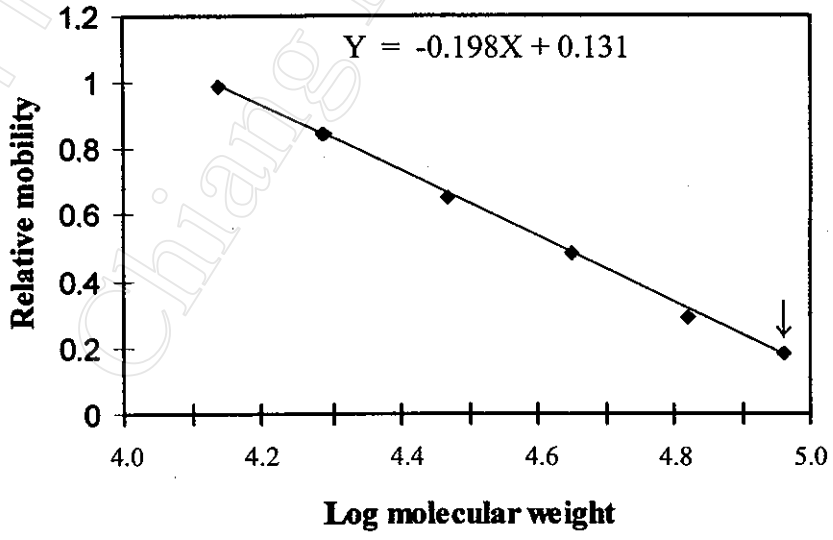

Figure 3.34 Calibration curve for the determination of molecular weight by SDS-PAGE. An arrow indicates relative mobility of the purified *Aspergillus* myrosinase.

3.3.7) Sephadex G-200 gel-filtration

To assess the native molecular weight of the purified *Aspergillus* myrosinase, it was achieved with Sephadex G-200 chromatography. Separating condition was operated at 12.5 ml/h and one-ml fraction were collected. Even after calibration with blue dextran 2000 and potassium dichromate, void volume of the column was 77 ml, whereas the total liquid volume was 187 ml. Elution profiles of standard protein markers are shown in Figure 3.35. The *Aspergillus* enzyme was eluted from the column at fraction number 137-143 (peak at fraction number 140), corresponded to the retention volume of 63 ml, in which the native molecular mass was calculated to be 90 kDa (Table 3.6 and Figure 3.36).

Table 3.6 Distribution coefficient and log molecular weight of standard protein markers by Sephadex G-200 chromatography.

Protein marker	Molecular weight (Da)	Log molecular weight	Retention volume (ml)	K_d
Alcohol dehydrogenase	150,000	5.18	28	0.26
Bovine serum albumin	67,000	4.82	68	0.62
Ovalbumin	43,000	4.63	78	0.71
Chymotrypsinogen	26,000	4.41	97	0.88
Aspergillus myrosinase	-	-	63	0.57

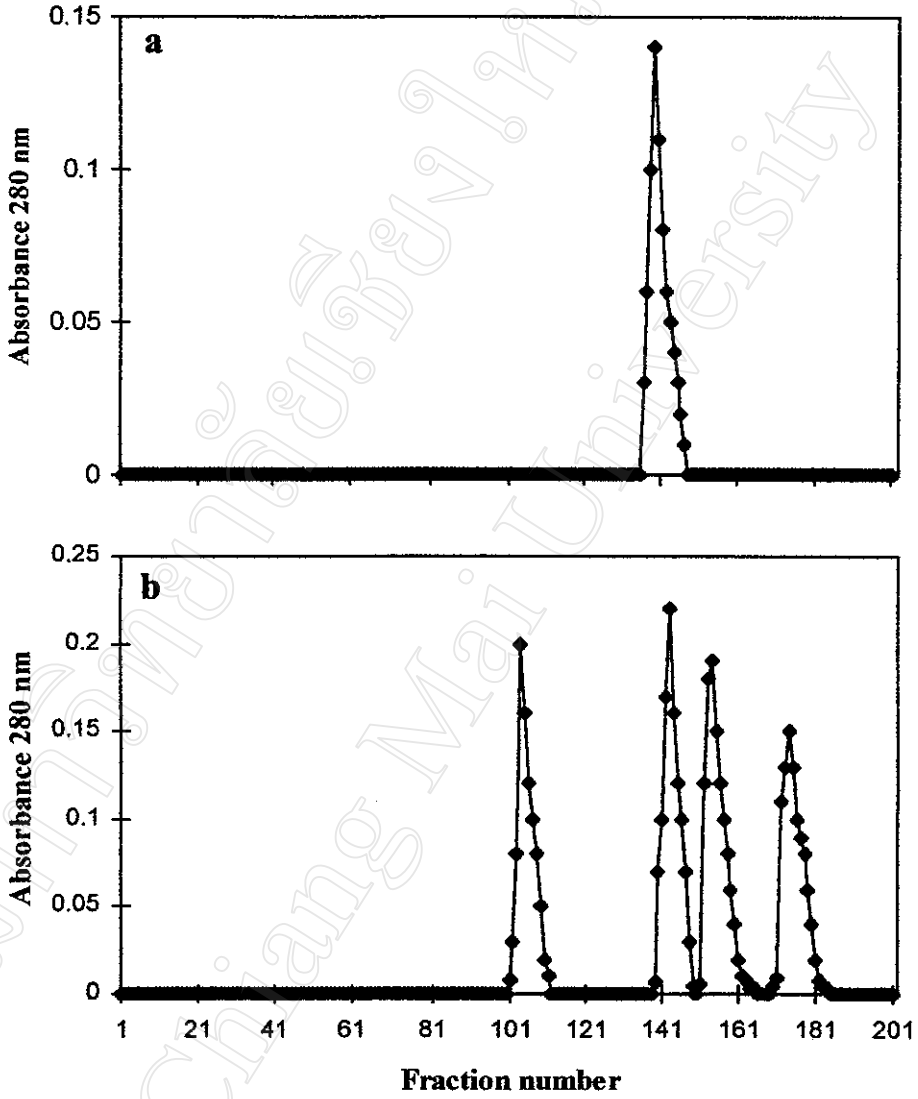

Figure 3.35 Elution profiles of Sephadex G-200 chromatography of the purified *Aspergillus* myrosinase (a) and molecular weight protein markers (alcohol dehydrogenase, bovine serum albumin, ovalbumin and chymotrypsinogen,(b)).

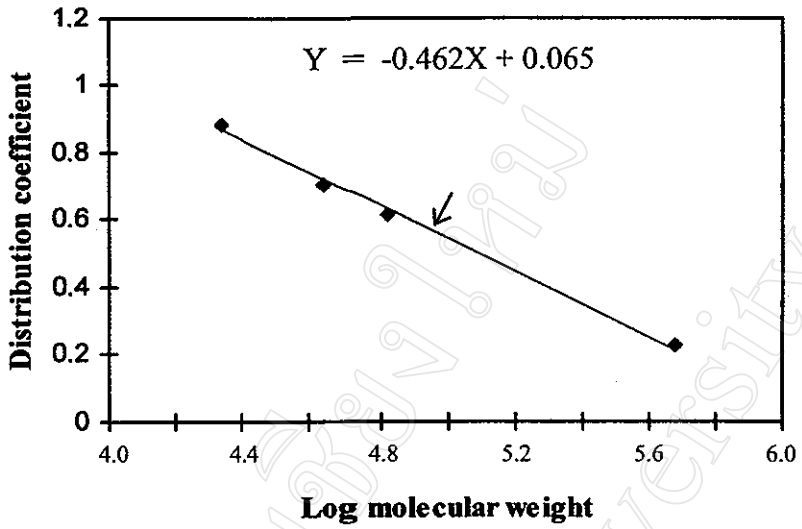

Figure 3.36 Calibration curve for the determination of molecular weight by Sephadex G-200 chromatography. An arrow indicates distribution coefficient of the purified *Aspergillus* myrosinase which corresponded to a molecular weight of 90 kDa.

3.4) CHARACTERIZATION OF THE PURIFIED *Aspergillus* MYROSINASE

Enzyme properties of the purified *Aspergillus* myrosinase *e.g.* pH and temperature optima, effect of some inorganic salts or organic compounds on enzyme activity, kinetic constants and enzyme inhibition, were assessed by the spectrophotometric method. While, the coupled-enzyme method was traditionally employed for activity assay, as described in section 2.6.1. Normally, the assayed system comprising of 1.0 ml buffered substrate and 10 µl enzyme solution were carried out at 25 °C according to the zero-ordered reaction. The determination of enzyme activity was done in triplicate.

3.4.1) Extinction coefficient for sinigrin

In this study, ε_{227nm} for sinigrin was determined to be 7546 $M^{-1}cm^{-1}$.

3.4.2) Effect of enzyme concentration on activity assay of myrosinase

To assess the effect of enzyme concentration on activity assay, aliquots of enzyme solution in 10 mM sodium phosphate buffer, pH 7.4 (activity between 1 and 4 U/ml, determined by HK/G-6-PD method at 25 °C) were used. A constant reaction rate ($\Delta A/\Delta t$) was monitored continuously for at least 5 min.

By the spectrophotometric method, enzyme activity up to 3 U/ml gave constant reaction rates agreeing with the zero-ordered reaction (Figure 3.37). Reaction rates of enzyme activity above 3.0 U/ml were not constant, due to substrate depletion. In case of the coupled-enzyme method, enzyme concentrations up to only 2 U/ml gave constant reaction rates which followed the zero-ordered reaction (Figure 3.38).

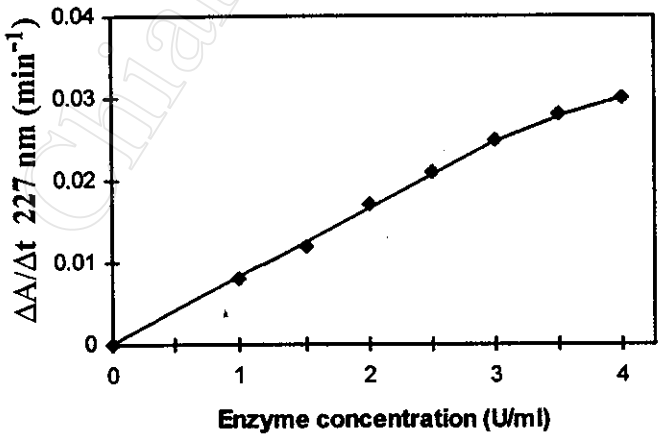

Figure 3.37 Effect of enzyme concentration on the determination of activity by spectrophotometric assay

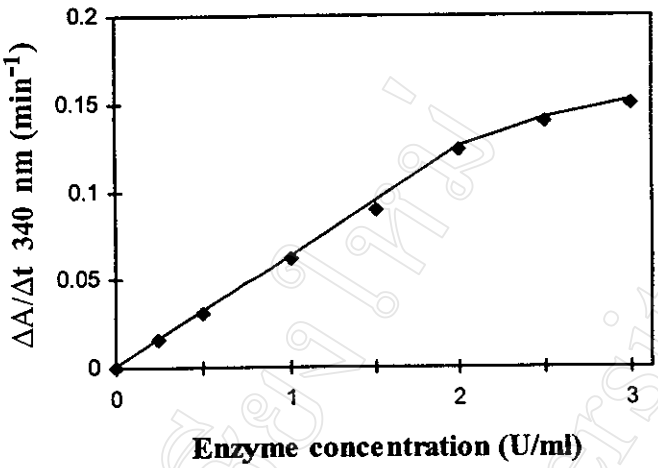

Figure 3.38 Effect of enzyme concentration on the determination of myrosinase activity by coupled-enzyme assay.

3.4.3) Optimum pH

To assess optimum pH, myrosinase activity was determined by the spectrophotometric method as precedingly described. Buffered substrates with varying pH from 4 to 9 (4-6 : sodium boric acid-phosphate buffer, 6-8 : sodium phosphate buffer and 8-9 : tris buffer) were used. From the result, sinigrin hydrolyzing-activity was able to function at pH range from 5.6 to 9. Enzyme activity assessed at pH 5.6 and 9 was about 5 and 20 %, respectively. However, the enzyme was mostly active at a pH 7.4 (Figure 3.39).

3.4.4) Optimum temperature

Optimum temperature of the *Aspergillus* enzyme was determined by the spectrophotometric method at varying temperatures from 25 to 37 °C. At temperatures between 27 and 29 °C, constant reaction rates were observed for about 2-3 min and then decreased dramatically. While a gradual decrease of reaction rates were shown at

temperature above 29 °C. In this case, a reaction rate for each temperature used to plot graph was an average among the first 5 min. Such temperature vs. activity plot of the *Aspergillus* myrosinase was illustrated in Figure 3.40. Enzyme activity was optimal at 28-29 °C.

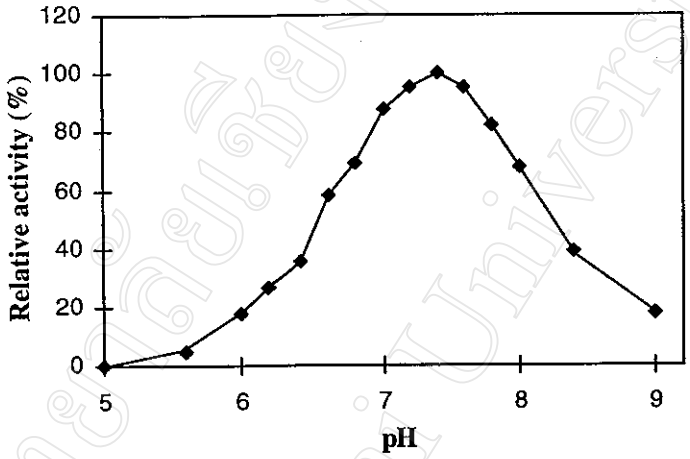

Figure 3.39 pH activity plots of the purified *Aspergillus* myrosinase.

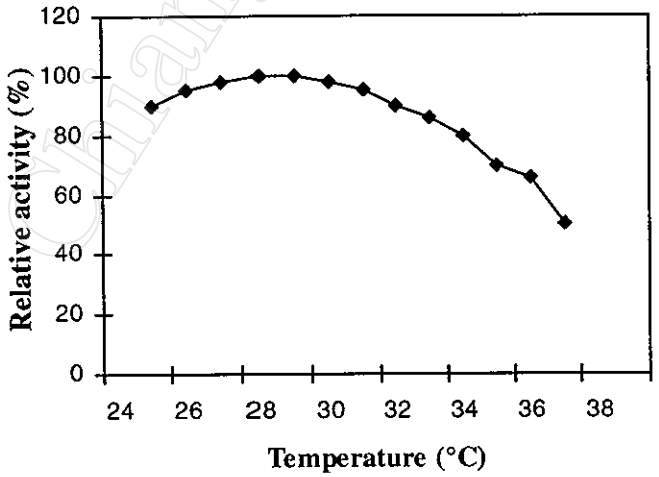

Figure 3.40 Temperature activity plots of the purified *Aspergillus* myrosinase.

3.4.5) pH stability

To determine pH stability, the purified enzyme (10 U/ml) in various 10 mM buffers at designed pH (4-6 : sodium boric acid-phosphate buffer, 6-8 : sodium phosphate buffer and 8-9 : tris buffer) were kept at 4 °C for 24 h (as described 2.6.5). Then, the enzyme samples were diluted 5-times with 100 mM sodium phosphate buffer, pH 7.4, prior to being measured for remaining activity. It was shown that the enzyme was mostly stable at pH range between 6 and 8 (Figure 3.41). When enzyme samples were maintained at pH 4, 5 and 9 for 24 h at 4 °C, 76, 28 and 65 % of the activity were destroyed, respectively.

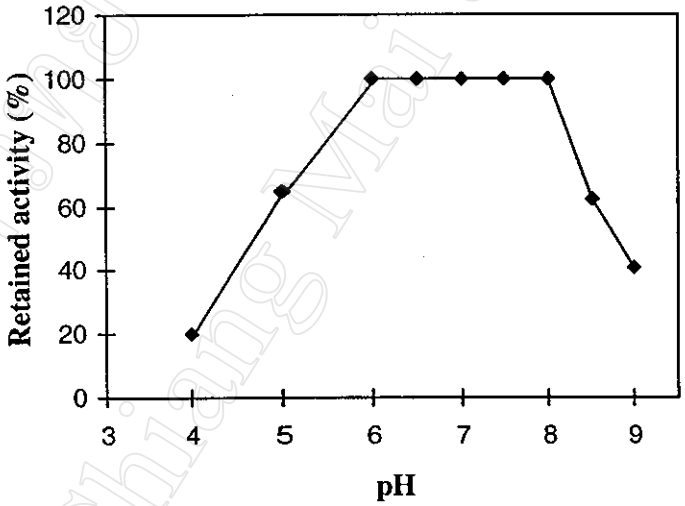

Figure 3.41 pH stability plots of the purified *Aspergillus* myrosinase.

3.4.6) Temperature stability

In the study of temperature stability, enzyme solution (2 U/ml) in 40 mM sodium phosphate buffer, pH 7.0 were maintained at different temperatures for 15 min (as described in 2.6.6). Then, samples were determined for remained activity by the

coupled-enzyme method. It was demonstrated that the enzyme was stable up to 25 °C with no significant decrease of enzyme activity (Figure 3.42). At temperature above 25 °C, the activity was gradually destroyed.

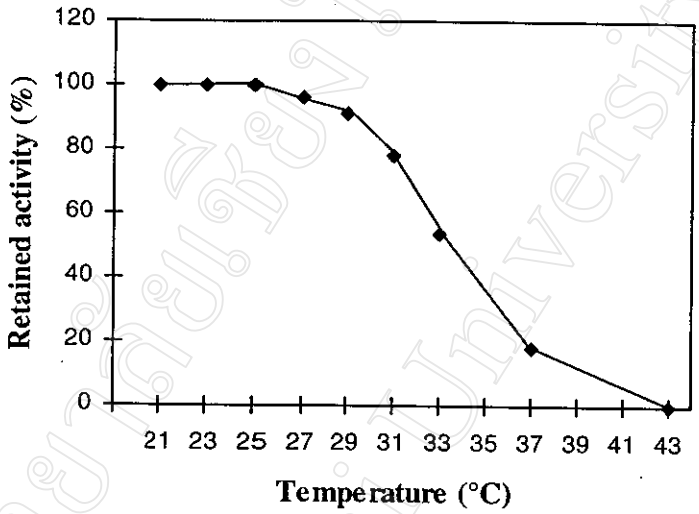

Figure 3.42 Temperature stability plots of the *Aspergillus* myrosinase

3.4.7) Preservation of the purified *Aspergillus* myrosinase

Enzyme solutions (10 U/ml each, in 10 mM sodium phosphate buffer, pH 6, 7 or 7.4) contained in screw-capped vials were kept at 4 °C. Sodium azide was added to final concentration of 0.02 % (w/v). Samples were taken periodically to determine for the remaining activity. At pH 7.0 and pH 7.4, the enzyme was stable up to one week (Figure 3.43, data at pH 7.4 was not shown). Slight decrease in enzyme activity was observed at pH 6 in the same preserving period. Almost activity could be retained up to three weeks with the presence of 0.02 % sodium azide (Figure 3.43).

In case of freeze-dry preservation, 80 % of the activity was still retained after the freeze-dry powder was kept at -40 °C for three weeks. It was observed, however,

that some precipitates appeared after reconstitution with 50 mM sodium phosphate buffer, pH 7.0.

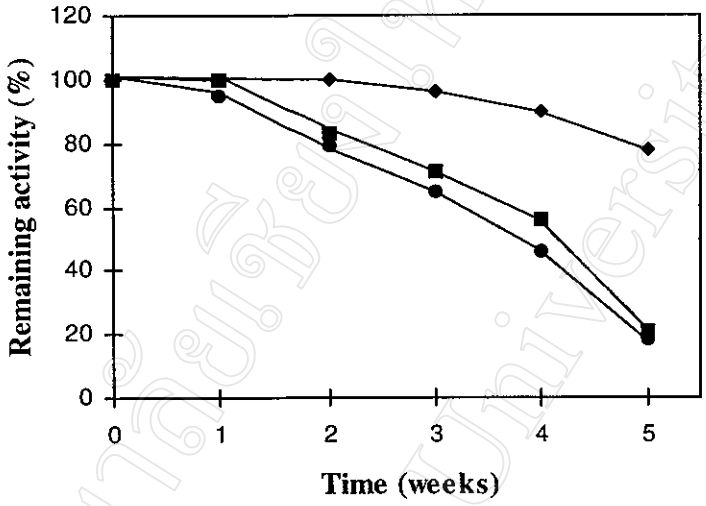

Figure 3.43 Stability of the purified *Aspergillus* myrosinase at 4 °C in 10 mM sodium phosphate buffer, pH 6 (●), 10 mM sodium phosphate buffer, pH 7 (■) and 10 mM sodium phosphate buffer, pH 7 with 0.02 % sodium azide (◆).

3.4.8) Effect of some inorganic salts and organic compounds on enzyme actvity

To investigate the effect of some inorganic salts and organic compounds on myrosinase activity, the spectrophotometric method was performed. One ml of 33 mM sodium phosphate buffer, pH 7.4 containing 1 mM of each inorganic salts or organic compounds (as listed in Table 3.7) were incubated in a cuvette at 25 °C for 3-5 min. Then, 10 µl of the purified enzyme (3 U/ml) were added and mixed gently for exactly 5 sec. Subsequently, 10 µl of 10 mM sinigrin in deionized water were added. After mixing, the reaction rate at 227 nm was measured. In cases of some metal ions that

Table 3.7 Effect of some inorganic salts and organic compounds on enzyme activity of the purified *Aspergillus* myrosinase.

Compounds	Relative activity (%)
None	100
L-Ascorbic acid	100
L-Cysteine	33
L-Cystine	100
Sodium bisulfate	100
Sodium bisulfite	100
Sodium thiosulfate	0
$AgNO_3$	0*
$Al_2 (SO_4)_3$	0*
$CaCl_2$	100
$CoCl_2$	53*
$FeSO_4$	100*
$FeCl_3$	0*
Hg(I)Cl	0*
$Hg(II)Cl_2$	0*
$MgCl_2$	100
MoO_3	0
$NiSO_4$	21*
$PbSO_4$	0*
$SnSO_4$	0*
$ZnCl_2$	7*

*Assay in deionized water

formed precipitates in phosphate buffer (Ag^{+}, Al^{3+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Mo^{3+}, Ni^{2+}, Pb^{2+}, Sn^{2+} and Zn^{2+}), the assay was performed in deionized water. Relative activity could be compared with enzyme activity in deionized water without any additives.

At a final concentration of 1 mM, no metal ion was found to activate enzyme activity. In contrast, the addition of Ag^{+}, Co^{2+}, Hg^{+}, Hg^{2+}, Ni^{2+}, Mn^{2+}, Sn^{2+}, Zn^{2+}, Fe^{3+} and Al^{3+} at a final concentration of 1 mM caused 100, 47, 100, 100, 79, 100, 100, 93, 100 and 100 % inhibition of enzyme activity, respectively (Table 3.7). Whereas, Ca^{2+}, Fe^{2+} and Mg^{2+} at the same concentration exhibited no inhibitory effect. Sodium bisulfite and sodium bisulfate also showed no inhibition on activity of the *Aspergillus* enzyme. However, the activity was totally inhibited by sodium thiosulfate.

Myrosinase activity was reduced by almost 77 % in the presence of 1 mM L-cysteine, whereas, the activity was not inhibited by L-cystine at the same concentration (Table 3.7). L-ascorbic acid at all concentrations tested (0.5, 1 and 2 mM), did not show either activation or inhibition effects on sinigrin-hydrolyzing activity, as determined by the spectrophotometric and coupled-enzyme assay.

3.4.9) Substrate specificity

For the assessment of substrate specificity, various kinds of p-nitrophenyl-O-glycosides were used at a concentration of 2 mM each. Reaction rate was measured according to the liberation of p-nitrophenol released at 430 nm (as described in 2.6.9). Activity was reported relative to the activity towards pNPG (Table 3.8). Non p-nitrophenyl glycosides were performed by end-point method. Quantities of 500 µl of each glycoside (1% contained in 50 mM sodium phosphate buffer, pH 7.4) and 50 µl of the purified enzyme (0.3 U) were incubated at 25°C for 2 h. The amount of

reducing sugar released was determined by dinitrosalicylic method, as described in 2.2.9.

The *Aspergillus* myrosinase showed strong affinity towards pNPG. However, no activity was shown towards other glycosides such as p-nitrophenyl-β-D-arabinoside, p-nitrophenyl-β-D-galactoside, p-nitrophenyl-β-D-xyloside, p-nitrophenyl-β-D-maltoside, cellobiose, starch, digitonin, stevioside, amikacin and gentamicin (Table 3.8)

Table 3.8 Substrate specificity of the purified *Aspergillus* myrosinase

Substrates	Relative activity (%)
p-nitrophenyl-β-D-glucoside	100*
p-nitrophenyl-β-D-arabinoside	0*
p-nitrophenyl-β-D-galactoside	0*
p-nitrophenyl-β-D-xyloside	0*
p-nitrophenyl-β-D-maltoside	0*
Cellobiose	0†
Starch	0†
Digitonin	0†
Stevioside	0†
Amikacin	0†
Gentamicin	0†

* : determined by the spectrophotometric method at 430 nm.

† : determined by the end-point method

3.4.10) Kinetic constants

Kinetic constants for sinigrin-hydrolyzing activity of the purified *Aspergillus* myrosinase were determined either by the spectrophotometric method (25-150 μM sinigrin) or coupled-enzyme method (0.1-5.0 mM sinigrin), depending on sinigrin concentration. In case of β-glucosidase activity, it was performed by the spectrophotometric method using different pNPG concentrations (0.25-5.0 mM).

By the spectrophotometric assay, the K_m and V_{max} values of 0.18 mM and 18.86 μmol/min/mg protein were obtained (Figure 3.44). While those values of 0.65 mM and 69.0 μmol/min/mg protein were achieved by the coupled-enzyme method (Figure 3.45). In case of the pNPG substrate, the K_m and V_{max} values were calculated to be 2.8 mM and 21.46 μmol/min/mg protein (Figure 3.46).

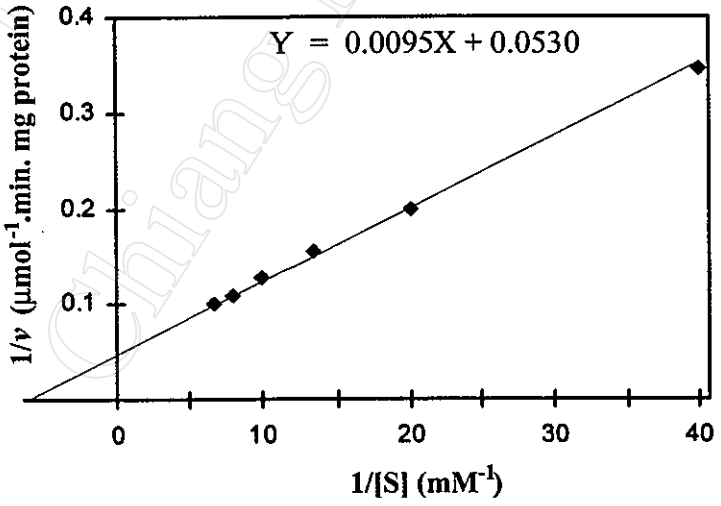

Figure 3.44 Lineweaver-Burk plots of sinigrin-hydrolyzing activity of the purified *Aspergillus* myrosinase. Enzyme activity was determined by the spectrophotometric method at 227 nm.

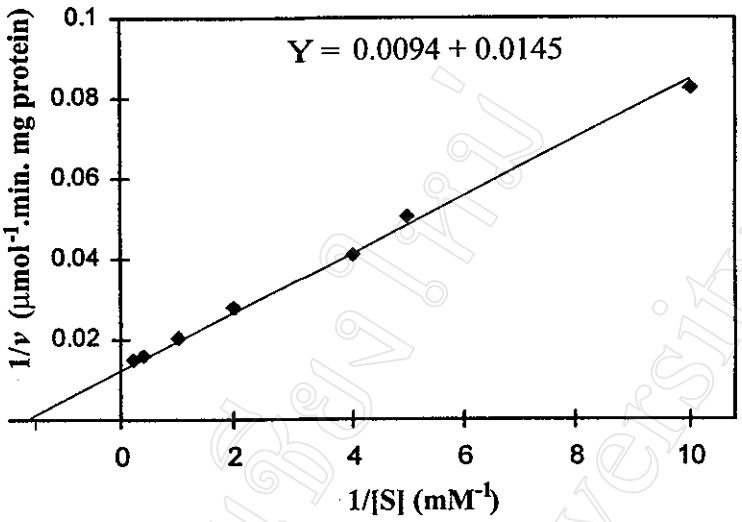

Figure 3.45 Lineweaver-Burk plots sinigrin-hydrolyzing activity of the purified *Aspergillus* myrosinase. Enzyme activity was determined by the coupled-enzyme method.

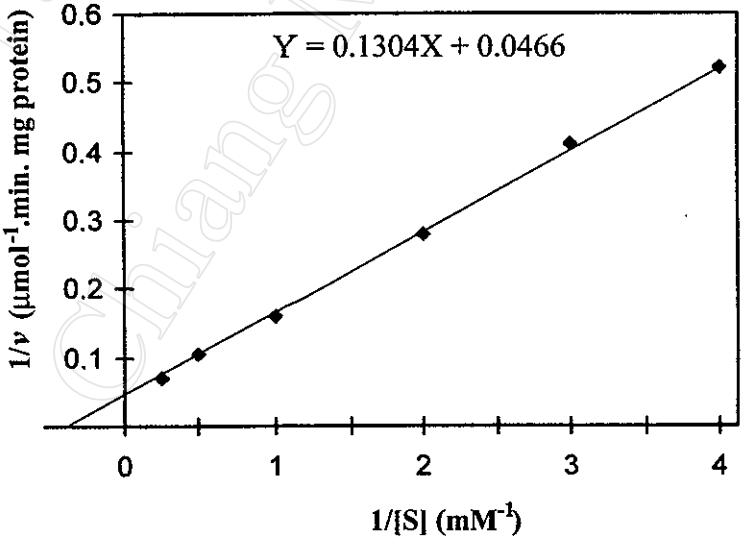

Figure 3.46 Lineweaver-Burk plots of pNPG-hydrolyzing activity of the purified *Aspergillus* myrosinase. Enzyme activity was determined by the spectrophotometric method at 430 nm.

3.4.11) Inhibition of sinigrin hydrolysis by pNPG

The purified enzyme in phosphate was mixed gently with pNPG inhibitor at concentrations of 0, 50, 100 or 150 μM for exactly 5 sec. Subsequently, sinigrin substrate was added to final concentrations of 25, 50, 75, 100, 125, 150 μM (as described in 2.6.10). Sinigrin hydrolysis was monitored at 227 nm for at least 5 min. Double-reciprocal plots of the inhibition of sinigrin hydrolysis by pNPG were illustrated in Figure 3.47. It was shown that V_{max} value did not changed at any concentrations of pNPG added. Apparent K_m value at 50, 100 and 150 mM pNPG inhibitor were calculated to be 0.33, 0.56 and 0.77 mM, respectively, comparing to 0.18 mM without the inhibitor. In this case, pNPG displayed as a competitive inhibitor for sinigrin-hydrolyzing activity of the *Aspergillus* myrosinase.

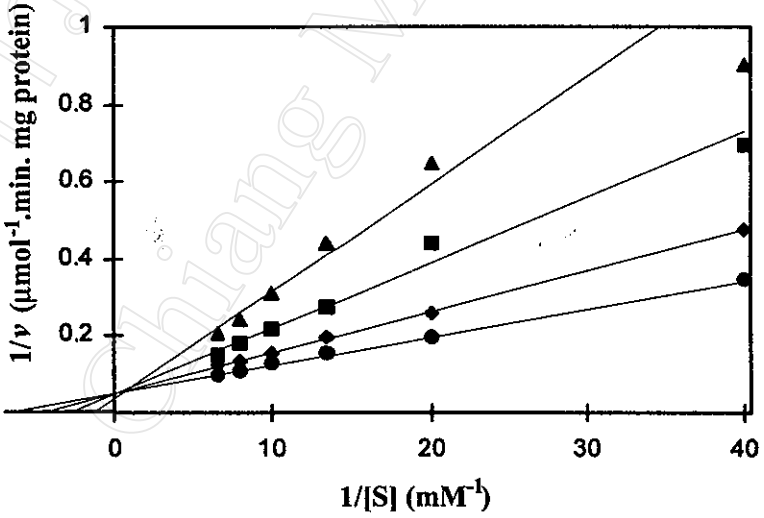

Figure 3.47 Double-reciprocal plots of sinigrin-hydrolyzing activity of the *Aspergillus* myrosinase without inhibitor (●), with 50 μM pNPG (◆), with 100 μM pNPG (■) and with 150 μM pNPG (▲). Enzyme activity was determined by the spectrophotometric method at 227 nm.

3.4.12) Inhibition of pNPG hydrolysis by sinigrin

To study the inhibitory effect of pNPG hydrolysis by sinigrin, different concentrations of sinigrin inhibitor (0, 200, 400 or 600 μM) were mixed gently with the enzyme for 5 sec before adding pNPG substrate to final concentrations of 0.25, 0.5, 1, 2, 2.5 or 5 mM, as described in section 2.6.12. The hydrolysis of pNPG was monitored spectrophotometrically at 430 nm. The double-reciprocal plots of the inhibition of pNPG hydrolysis by sinigrin were illustrated in Figure 3.48. It was shown that sinigrin exhibited as a competitive inhibitor for pNPG-hydrolyzing activity of the *Aspergillus* myrosinase. At inhibitor concentrations of 200, 400 and 600 μM, apparent K_m values were calculated to be 2.97, 3.33 and 5.0 mM, respectively, comparing to 2.8 mM without the inhibitor.

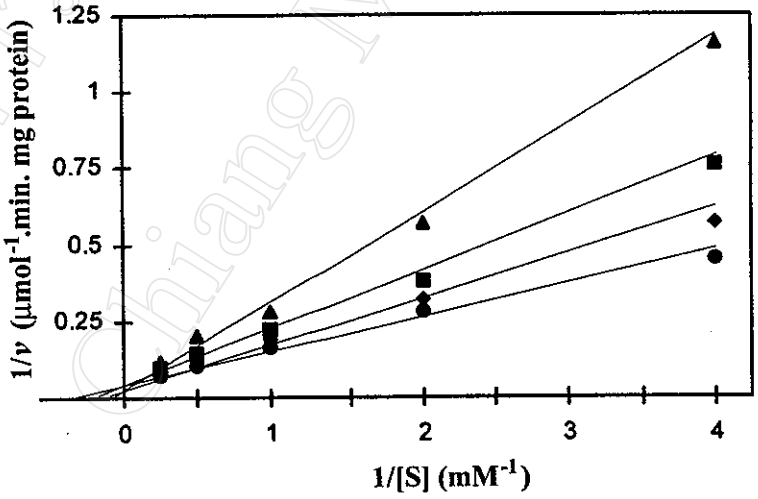

Figure 3.48 Double-reciprocal plots of pNPG-hydrolyzing activity of the *Aspergillus* myrosinase; without inhibitor (●), with 200 μM sinigrin (◆), with 400 μM sinigrin (■) and with 600 μM sinigrin (▲). Enzyme activity was determined by the spectrophotometric method at 430 nm.

3.4.13) Inhibition of sinigrin hydrolysis by D-glucose

To assess the effect of glucose on sinigrin-hydrolyzing activity, glucose (0, 5 or 10 mM) was mixed with the enzyme for 5 sec before adding the sinigrin substrate (as described in 2.6.12). Sinigrin hydrolysis was monitored at 227 nm. At inhibitor concentrations of 5 and 10 mM, appearent V_{max} values were calculated to be 7.46 and 6.94 μmol min^{-1} mg $protein^{-1}$, respectively, comparing to 9.26 μmol min^{-1} mg $protein^{-1}$ of the purified enzyme without inhibitor (Figure 3.49). While the K_m value did not changed at any inhibitor concentrations. From these results, D-glucose displayed as a non-competitive inhibitor for the activity of sinigrin hydrolysis of the *Aspergillus* myrosinase.

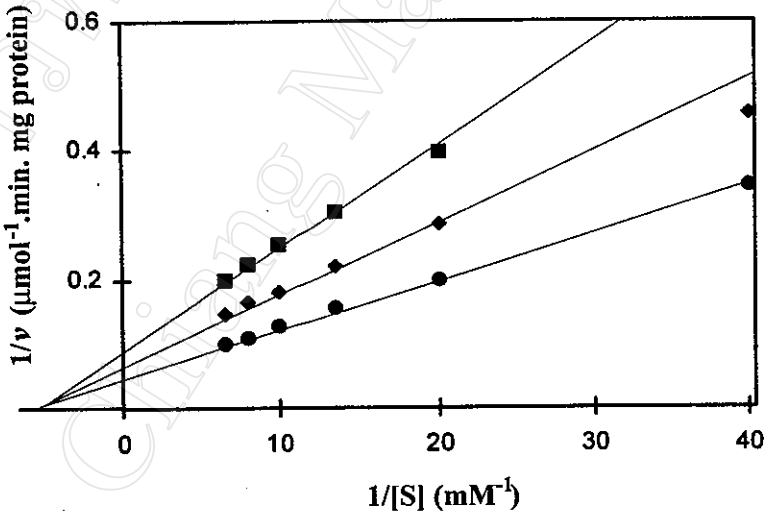

Figure 3.49 Double-reciprocal plots of sinigrin-hydrolyzing activity of the *Aspergillus* myrosinase; without inhibitor (●), with 5 mM D-glucose (◆) and with 10 mM D-glucose (■). Enzyme activity was determined by the spectrophotometric method at 227 nm.

3.5) ANALYSIS OF GLUCOSINOLATE BREAKDOWN PRODUCTS BY GAS CHROMATOGRAPHY

3.5.1) Products from sinigrin degradation by the purified *Aspergillus* myrosinase and *Sinapis alba* myrosinase

To examine product(s) generated from sinigrin hydrolysis, reaction mixtures of 1.0 ml total volume comprising of sinigrin (10 mM) and the purified *Aspergillus* myrosinase (0.3 U) were incubated at 25 °C. The amounts of sinigrin degraded and glucose released were then measured. In hydrolysis at pH 6, 7, 7.4 and 8, sinigrin was completely degraded within 1 h. Whereas, the hydrolysis at pH 5 and 9 for 2 h, resulted in 11 % and 18 % degradation, respectively. No degradation of sinigrin was observed at pH 4 in the same incubation period. In case of the *S. alba* myrosinase, sinigrin degradation at pH 4 to 9 was complete within 1 h.

Gas chromatogram of standard allylcyanide (retention time of 3.73 min) and standard allylisothiocyanate (major and minor peaks at the retention times of 6.00 and 7.63 min) are shown in Figure 3.50. Sinigrin degradation by the *Aspergillus* enzyme led to the production of allylisothiocyanate, occurring as a main hydrolysis product at the pH range of 5-8, as revealed by gas chromatography (Figure 3.51). Whereas, no formation of allylcyanide was observed in any incubation mixtures. In case of the *S. alba* enzyme, allylcyanide was produced dominantly at pH 4 and partially at pH 5 (Figure 3.52). At pH ranging from 6 to 9, the major product was allylisothiocyanate. In addition to the allylisothiocyanate occurring during sinigrin degradation, there was a minor product detected at the retention time of 7.63 min, corresponding to the minor peak of standard allylisothiocyanate (Figure 3.50b). The amount of this compound was approximately 10 % of that of allylisothiocyanate.

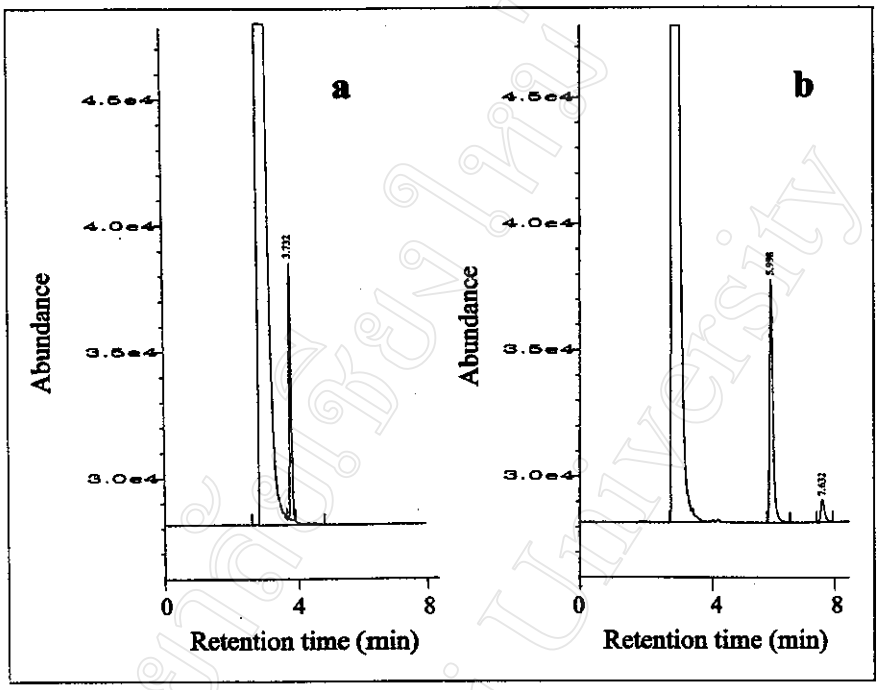

Figure 3.50 Gas chromatogram of standard allylcyanide (a) and allylisothiocyanate (b) by a Hewlett-Packard 5890 series II gas chromatograph (carbowax column).

3.5.2) Products from sinigrin degradation by disrupted fungal cells of the *Aspergillus*

Allylcyanide was not detected in the incubation mixtures of sinigrin and the purified *Aspergillus* enzyme in a wide pH range (4-8). Implying that the *in vivo* production of allylcyanide required some other factors which were possibly present in fungal mycelium. To test this hypothesis, the *Aspergillus* was grown in a 25-ml erlenmeyer flask containing 2.5 ml mustard extract medium for 48 h. Then, fungal

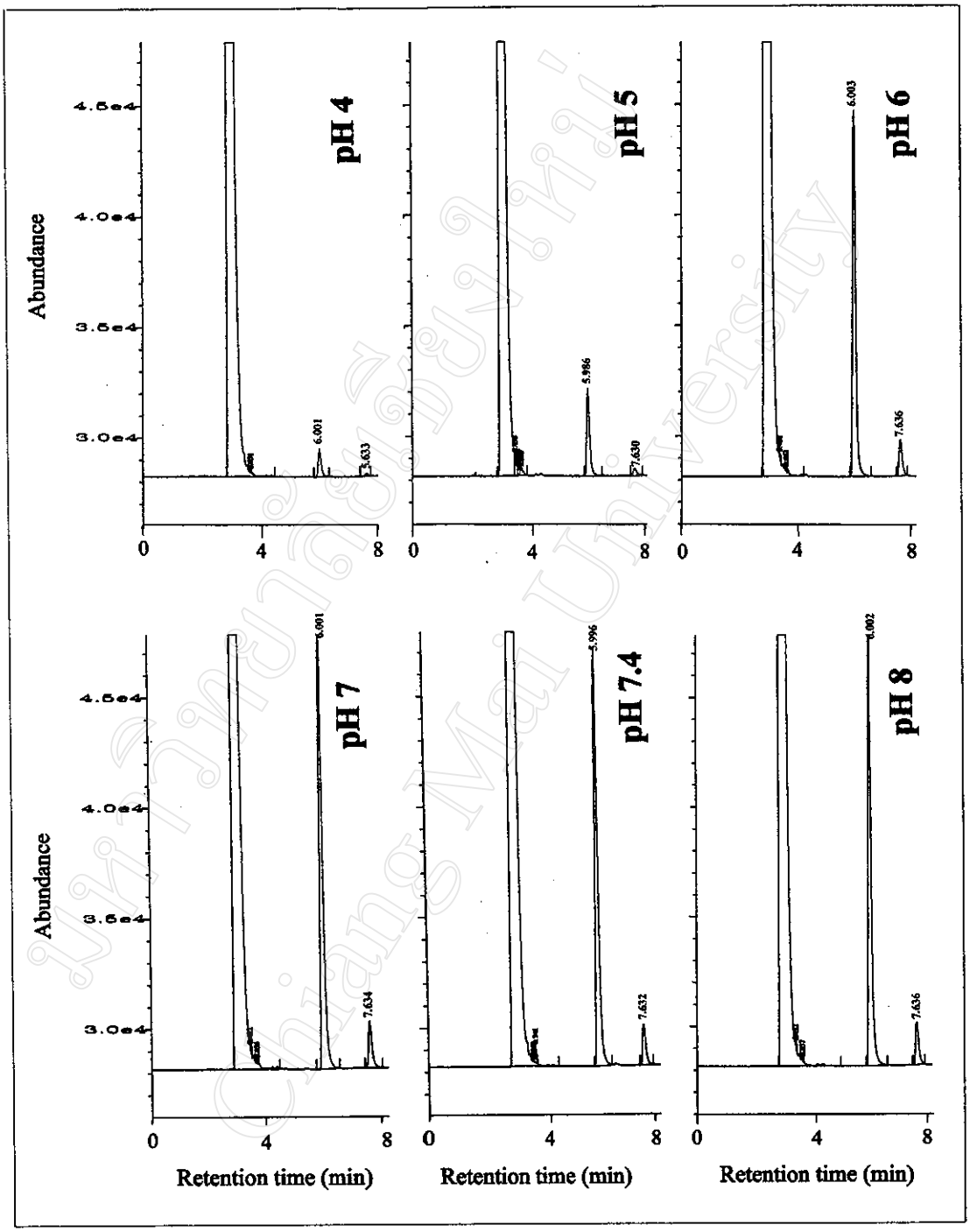

Figure 3.51 Gas chromatogram of breakdown products from sinigrin degradation by the *Aspergillus* myrosinase at pH 4, 5, 6, 7, 7.4 and 8. Chromatography was achieved by a Hewlett-Packard 5890 series II gas chromatograph (carbowax column).

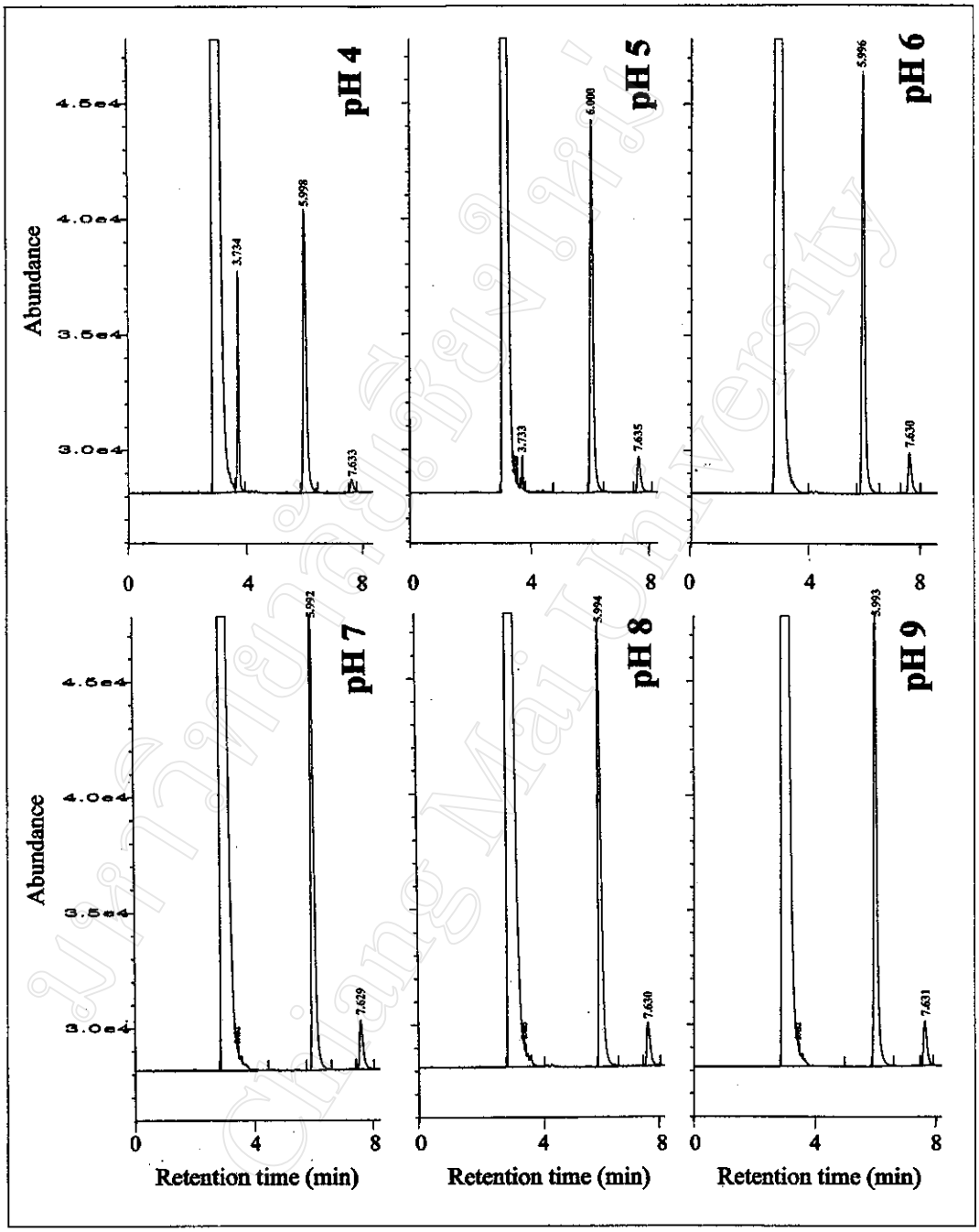

Figure 3.52 Gas chromatogram of breakdown products from sinigrin degradation by *Sinapis alba* myrosinase at pH 4, 5, 6, 7, 8 and 9. Chromatography was achieved by a Hewlett-Packard 5890 series II gas chromatograph (carbowax column).

cells were collected, washed with deionized water and disrupted immediately. The fungal suspension was adjusted to final volume of 1 ml with deionized water.

It was shown that cell-free extracts of the *Aspergillus* in deionized water exhibited pH values between 6.2 and 6.5. Then, 0.5 ml of the suspension were incubated with 0.5 ml of 20 mM sinigrin in deionized water at 25 °C. After incubation for 1 h, sinigrin was completely degraded. From gas chromatograms in Figure 3.53, allylisothiocyanate was the main reaction product formed with the association of a minor compound at the retention time of 7.635 min. Whereas, the formation of allylcyanide was still not observed.

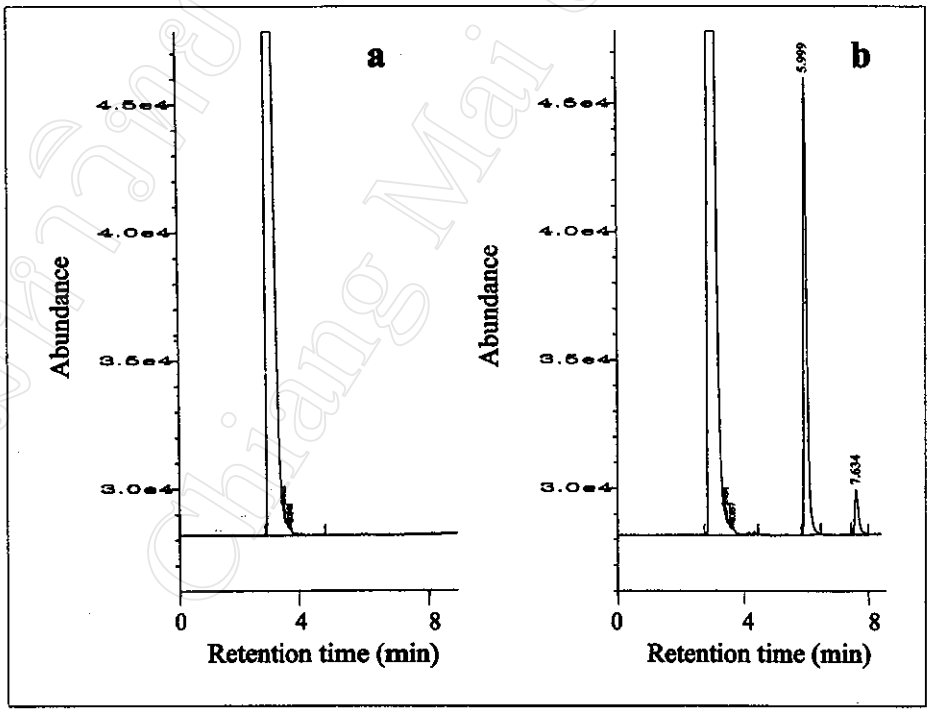

Figure 3.53 Gas chromatogram of disrupted fungal cells of the *Aspergillus* (a) and with sinigrin in deionized water (b). Chromatography was achieved by a Hewlett-Packard 5890 series II gas chromatograph (carbowax column).

Allylcyanide formation was not found even when pH values of incubation mixtures of the disrupted fungal suspension and sinigrin were adjusted to 4, 5, 6, 7 and 8 (data not shown). In this cases, allylisothiocyanate and the minor compound were produced at pH 5-8, as demonstrated with the purified *Aspergillus* enzyme (data not shown). The results of using disrupted fungal suspension as a source of myrosinase ruled out the hypothesis that formation of allylcyanide required cellular factors other than the enzyme.

3.5.3) Products from glucosinolate degradation (*Brassica juncea* seeds) by crude *Aspergillus* myrosinase

To assess the potential of allylisothiocyanate production, 1-ml incubation mixtures of mustard extract (equivalently to 10 mM glucosinolates) and crude *Aspergillus* enzyme (0.3 U) were incubated at 25 °C. Glucosinolates were completely hydrolyzed at pH 7 and 7.4 within 1 h. As shown in gas chromatograms, There were two glucosinolate hydrolytic products detected in those incubation mixtures at the retention time of 6.00 and 7.63 min (Figure 3.54). The main product was allylisothiocyanate, the same that of sinigrin degradation. While, the second one corresponded to the minor product occured in sinigrin case. The concentration of allylisothiocyanate formed at pH 7 and 7.4 were measured to be 7.8 and 7.6 mM, respectively (Figure 3.55). While contents of the minor product were 0.77 and 0.76 mM, respectively.

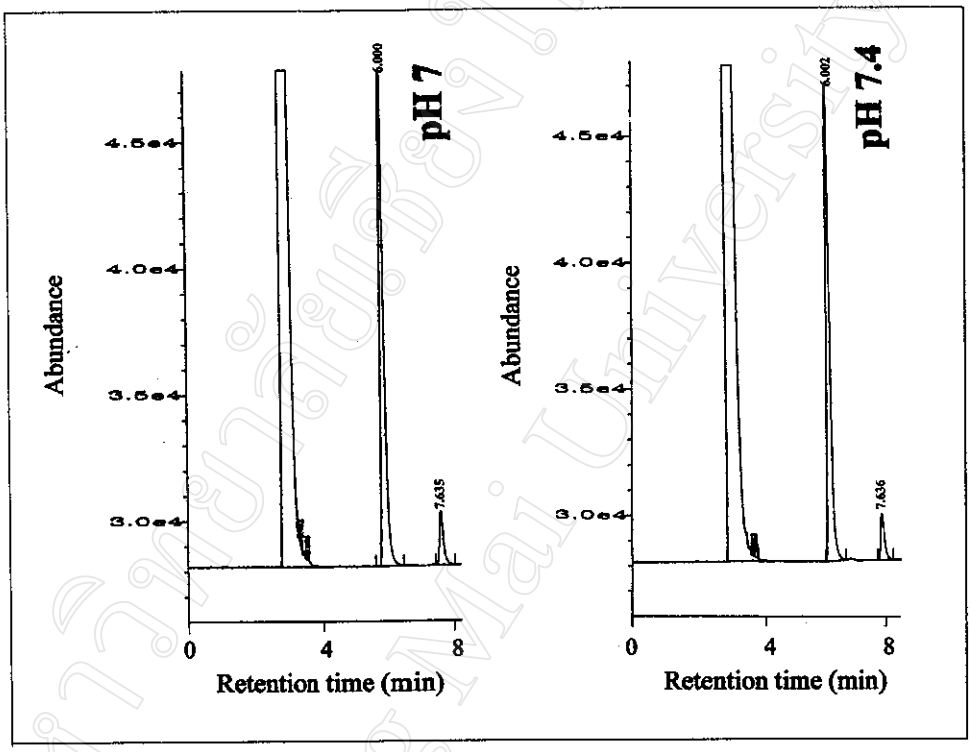

Figure 3.54 Gas chromatogram of breakdown products from glucosinolate degradation (*Brasica juncea*) by crude *Aspergillus* myrosinase at pH 7 and 7.4. Chromatography was achieved by a Hewlett-Packard 5890 series II gas chromatograph (carbowax column).

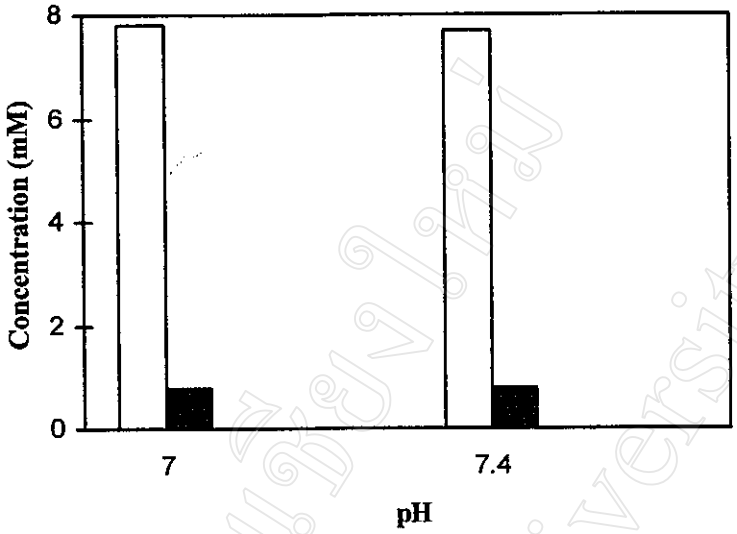

Figure 3.55 Contents of glucosinolate breakdown compounds, allylisothiocyanate (□) and minor product (■).

3.5.4) GC-MS analysis of sinigrin breakdown products

Breakdown products generated from sinigrin hydrolysis were analyzed by a Hewlett-Packard 6890 series GC-MS ((5 %)-diphenyl-(95 %)-dimethylpolysiloxane column). The minor compound was shown at 3.48 min while the major compound was at 3.74 min (Figure 3.56). Mass spectrum (electron impact) of the main product were resemble to reference spectrum of allylisothiocyanate (92 % similarity ; Figure 3.57). In case of the minor compound, its mass spectral pattern was also similar to that of the allylisothiocyanate (89 % similarity ; Figure 3.58). Total mass of this compound was shown at the m/z of 99, the same as that of allylisothiocyanate.

When these breakdown products were separated by polydimethylsiloxane column, the minor and the major compound were shown at the retention times of 4.34 and 4.89 min, respectively (Figure 3.59). MS analysis employing with the chemical

impact revealed that those spectral patterns of the minor and major compounds were quite similar (Figure 3.60).

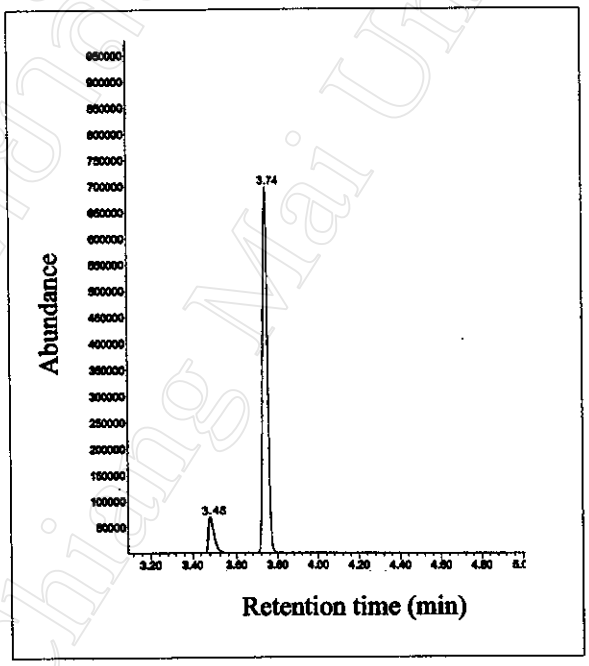

Figure 3.56 Gas chromatogram of sinigrin breakdown products by a Hewlett-Packard 6890 series gas chromatograph ((5 %)-diphenyl-(95 %)-dimethylpolysiloxane column).

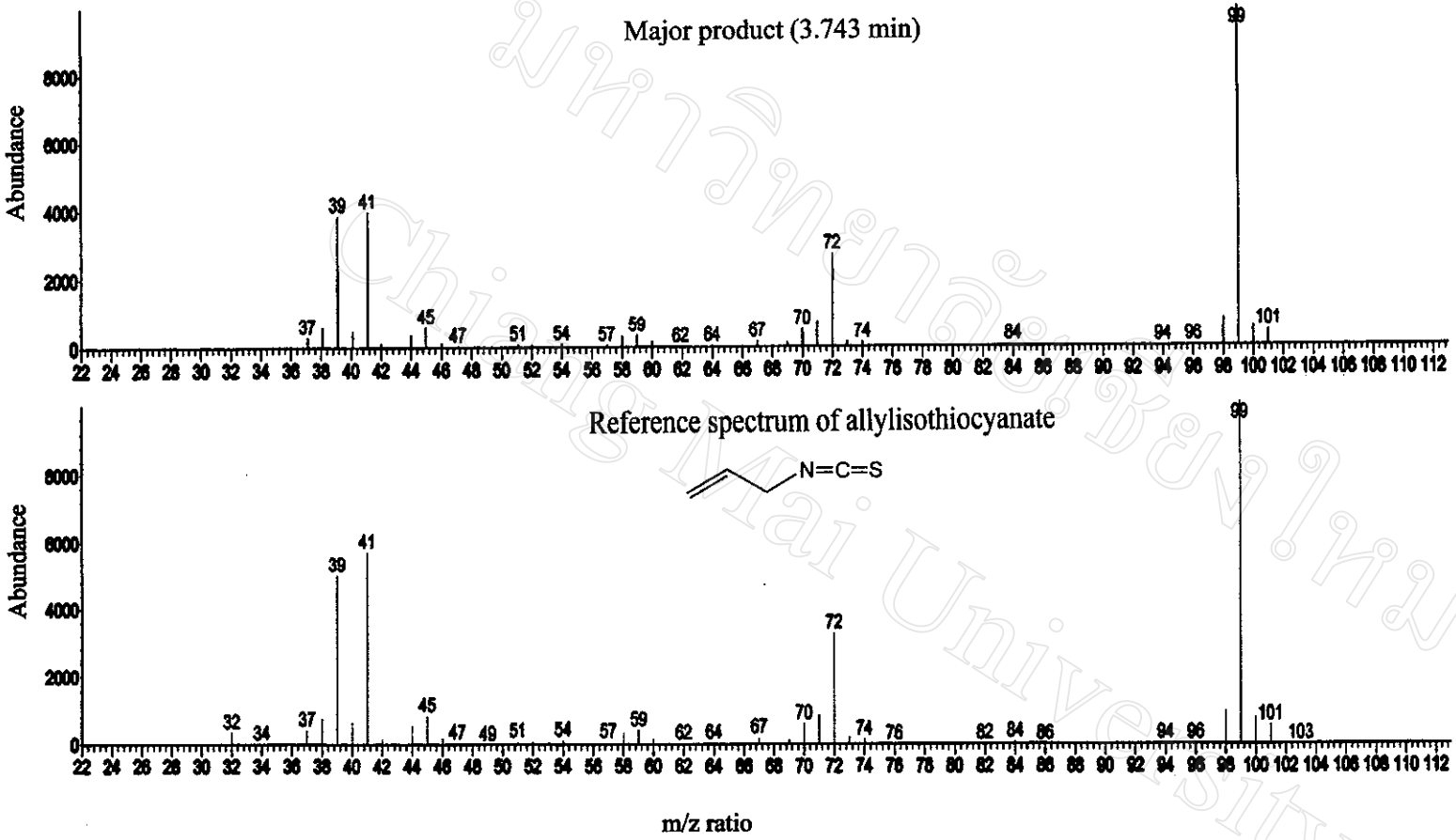

Figure 3.57 Mass spectrum (electron impact based) of major product from the degradation of sinigrin (above) and reference spectra of allylisothiocyanate (bottom).

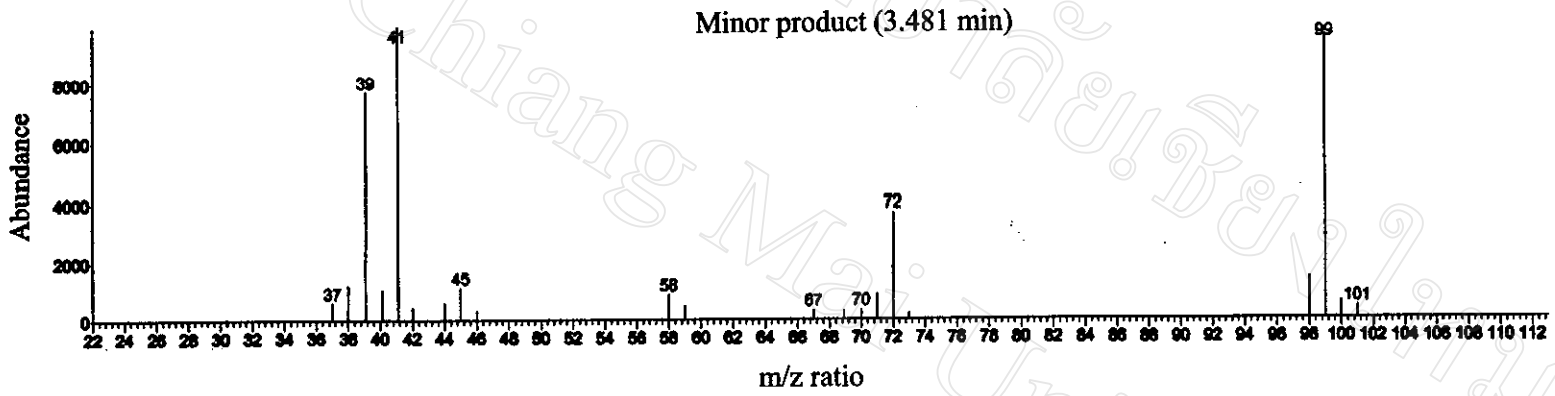

Figure 3.58 Mass spectrum (electron impact based) of minor product from the degradation of sinigrin.

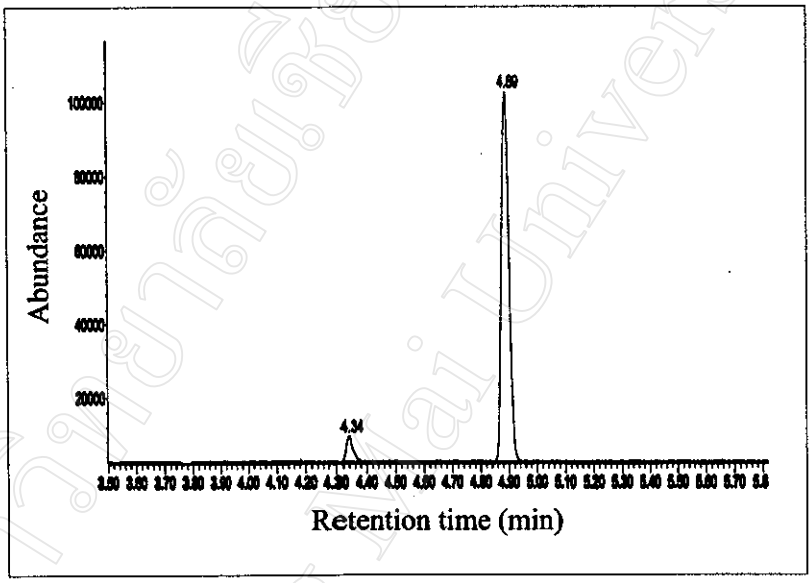

Figure 3.59 Gas chromatogram of sinigrin breakdown products by a Hewlett-Packard 6890 series gas chromatograph (polydimethylsiloxane column).

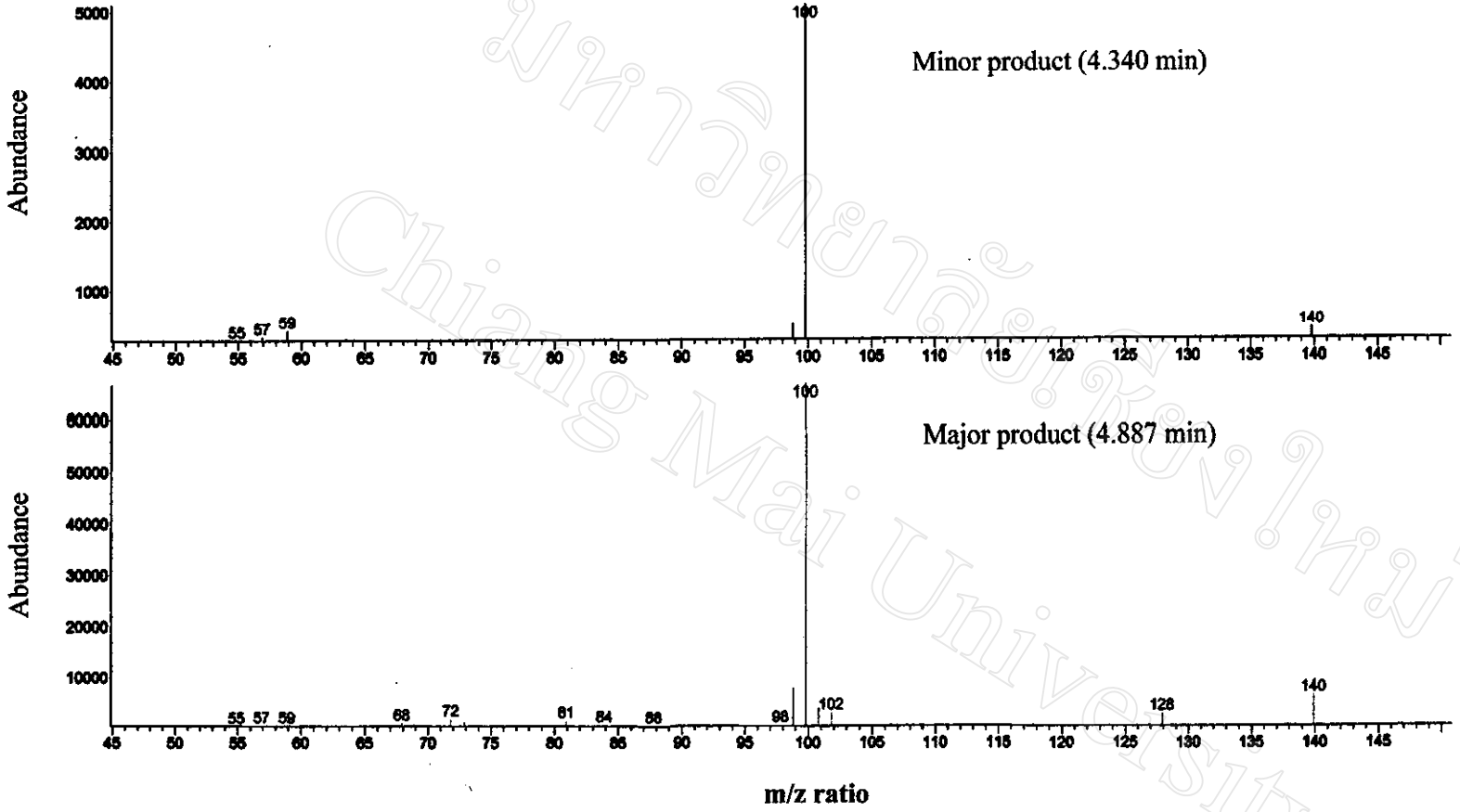

Figure 3.60 Mass spectrum (chemical impact based) of minor (above) and major products (bottom) from the degradation of sinigrin.