CHAPTER III

RESULTS

3.1 Partial purification of CGTase

CGTase enzyme was obtained from cultivation of *Paenibacillus* sp. A11 in Horikoshi's medium at 37°C with continuous shaking at 250 rpm for 72 hours. After cultivation, cells were removed by centrifugation and supernatant with crude CGTase was collected. Crude CGTase was partially purified by adsorption on corn starch, according to the method described by Rutchtorn (1993). The enzyme was then eluted from corn starch with 0.2 M maltose. The purification fold and % recovery of CGTase obtained at each step are shown in Table 8. The % recovery of CGTase was 97% of the total activity with 47 folds increase in the specific activity which were expressed in terms of β-CD forming activity per mg protein. The enzyme was then concentrated by ultrafiltration and dialyzed against 50 mM acetate buffer containing 10 mM $CaCl_2$, pH 6.0. The final enzyme had a specific activity of 119 U/mg of protein. The enzyme obtained was sufficiently purified by the use of starch adsorption as a sole purification step. This partially purified CGTase was then used as a source of enzyme for immobilization studies.

3.2 Optimization of the immobilization procedure

In this study *Paenibacillus* sp. A11 CGTase was covalently immobilized on solid support using γ-aminopropyltriethoxysilane (APTS) to activate the surface of support which, after reaction with glutaraldehyde (GA), is able to link the enzyme through the carbonyl derivative. Schematic diagram showing an approach used in the immobilization of CGTase is shown in Figure 8. To obtain high immobilization

Table 8. Purification of CGTase from *Paenibacillus* sp. A11

Purification Step	Volume (ml)	Total* Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	5250	8505	4000	2.13	1	100
Starch Adsorption	700	8244	82.4	100	47	97
Ultrafiltration	75	5383	45.2	119	56	63

* β-cyclodextrin-forming activity

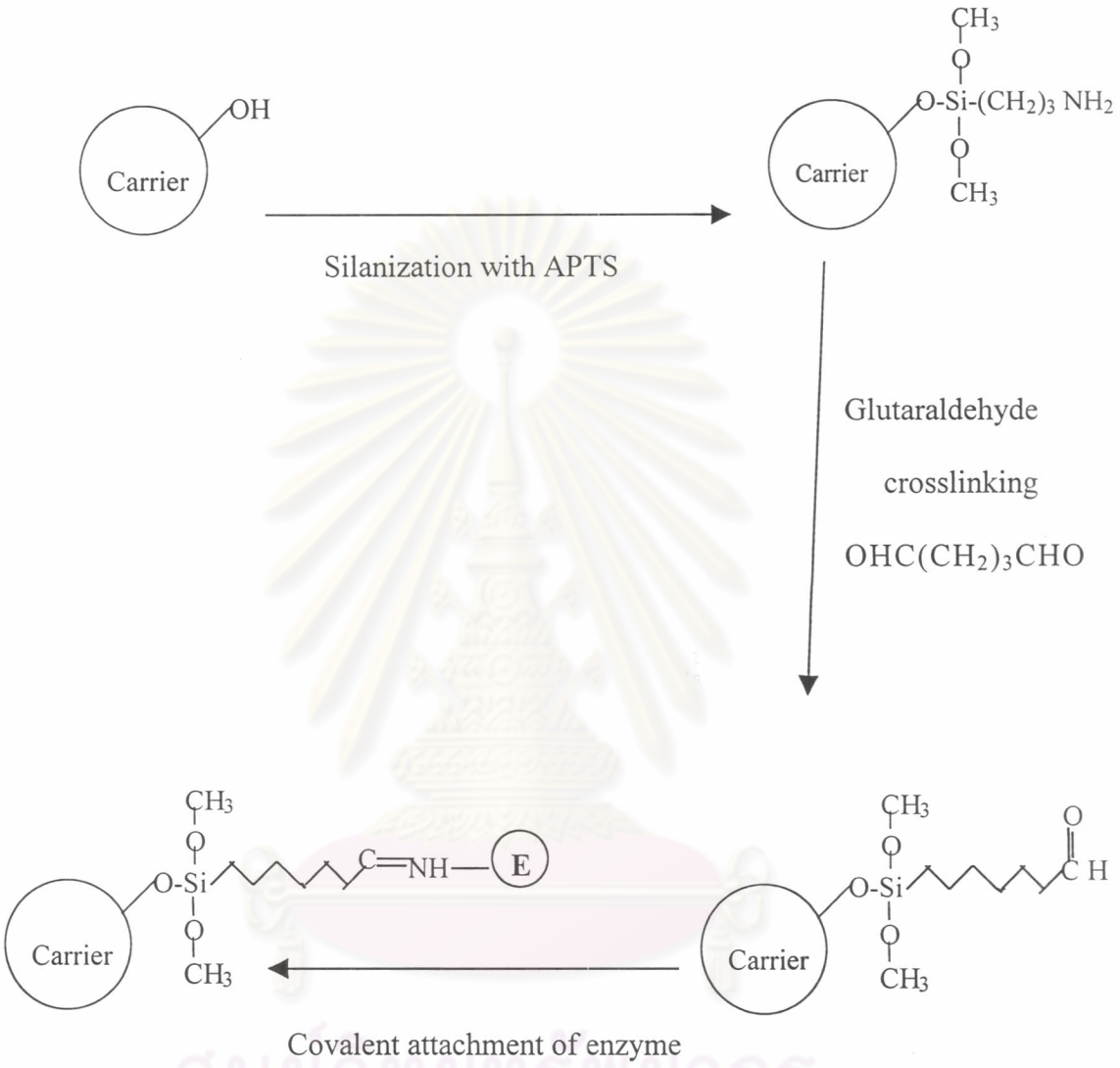


Figure 8. Schematic diagram showing an approach of covalent attachment of CGTase to inorganic carriers. E = CGTase.

efficiency, several reaction parameters involved in support activation and enzyme-coupling steps including, type of support, APTS and GA concentration, coupling time and enzyme loading were optimized.

3.2.1. Selection of a suitable support

The partially purified *Paenibacillus* sp. A11 CGTase was immobilized on several support materials to find a suitable support for CGTase immobilization. Four types of support including alumina, silica, activated carbon and chitosan were tested. Each support was silanized with 2% (v/v) APTS (except chitosan since it already contains the amino groups on the surface) followed by the activation with 0.25% (v/v) GA. After that the support was incubated with 10 U of CGTase for 12 h at 4°C as described in section 2.7. The adsorption of CGTase on these supports was also investigated. The supports, without activation with APTS and GA, were incubated with enzyme solution as in the same conditions used for covalent coupling. After that the immobilized and adsorbed samples were washed thoroughly and assayed for their activities as mentioned. As can be seen in Figure 9, for covalent coupling, the highest immobilization yields among four supports tested, calculated on the % retention of the original activity, was observed with the CGTase immobilized on alumina with a 16.5% immobilization yield. Silica produced 15.6% of immobilization yield whereas the activated carbon and chitosan proved to be poor supports which produced only 2.7% and 6.1% of immobilization yields, respectively. In the case of adsorption, the highest immobilization yield was obtained on silica (20.6%) while other supports, alumina, activated carbon and chitosan showed little CGTase adsorption of 0.37%, 3.8% and 0.40% respectively. In order to achieve high covalent binding and low physical adsorption of enzyme, alumina was selected as a suitable support for the covalent immobilization of CGTase.

3.2.2. Effect of APTS concentration

To investigate the influence of APTS concentration on the amount of CGTase immobilized on alumina (which was selected as a carrier material for CGTase immobilization), the support was then treated with different concentration of APTS varying from 1.0 to 10% (v/v) before activation with 0.25% (v/v) GA. The activated support was then incubated with the enzyme as mentioned previously before the immobilized activities were assayed. It can clearly be seen in Figure 10 that at 2% (v/v) of APTS used, the amount of CGTase immobilized on alumina was found to be maximal with 26% of the immobilization yield. At other concentrations of APTS used, the amount of immobilized CGTase was indifferent with immobilization yield of approximately 19-21%. Therefore, the suitable APTS concentration for silanization of alumina was 2% (v/v).

3.2.3. Effect of glutaraldehyde concentration

Once the optimal concentration of APTS was achieved, another factor that has an effect on the immobilization yield was then investigated. The alumina was silanized with 2% (v/v) of APTS, followed by the addition of different concentration of GA, ranging from 0.1 to 2.5% (v/v), in 0.1 M phosphate buffer, pH 7.0 to activate the support surface. The enzyme was then added and incubated for 12 h at 4°C. This was followed by excessive wash prior to the assay of activities of the immobilized CGTase. As shown in Figure 11, the immobilization yield increased up to the maximal value of 26% when 1% (v/v) of GA concentration was used. However, when GA concentration was higher, the immobilization yield tended to decrease slightly. Therefore 1% (v/v) of GA concentration was selected for the activation of the support.

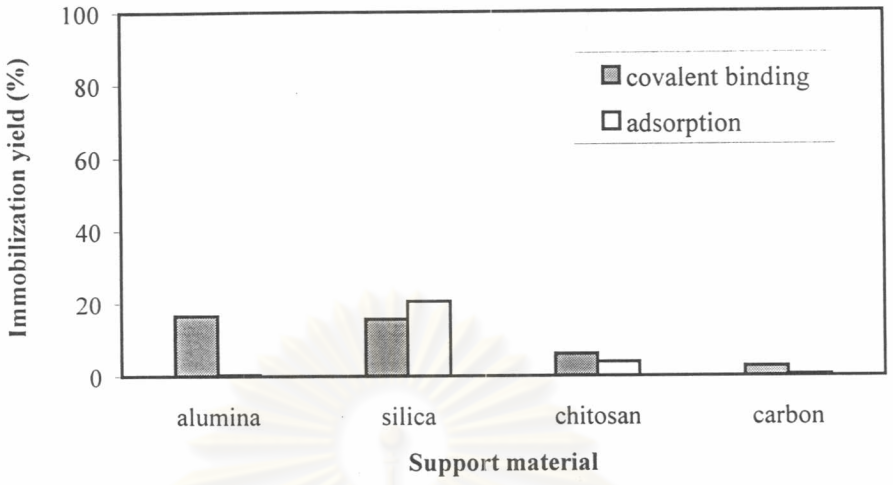


Figure 9. Immobilization of CGTase using different support materials.
One gram of support materials was silanized in 2% (v/v) APTS, activated with 0.25% (v/v) GA and incubated with CGTase solution (10 U) at 4°C for 12 h. Results shown were average values of duplicate experiments.

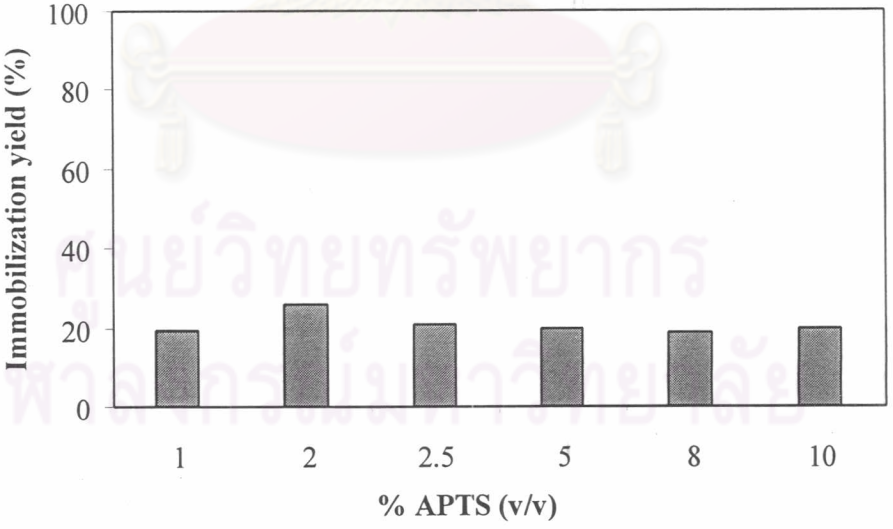


Figure 10. Influence of APTS concentration on the amount of CGTase immobilized on alumina. The alumina support (1 g) was silanized with different concentration of APTS, activated with 0.25% (v/v) GA and incubated with CGTase solution (10 U) at 4°C for 12 h. Results shown were average values of duplicate experiments.

3.2.4. Effect of enzyme concentration

Further optimization of the immobilization procedure for a higher immobilization yield was performed by varying concentrations of the enzyme. The effect of enzyme concentration on the efficiency of immobilization is shown in Figure 12. The CGTase concentrations applied to alumina were varied from 2 to 20 U/g of alumina. As the amount of CGTase added per gram of alumina increased, the activity of immobilized CGTase increased and reached equilibrium when the enzyme of 14 U per gram carrier was added. Further increase of enzyme concentration gave poorer immobilization yield, probably due to saturation of the given quantity of support. The immobilization yield, however, was inversely proportional to the amount of CGTase added, and the immobilization yield at equilibrium point was 36%. Although the coupling rate was much greater when the amount of applied CGTase was lower than 6 U/g carrier, the activity of enzyme per gram of the carrier was significantly reduced due to the fact that the absolute amount of the enzyme immobilized was less. Therefore, the enzyme / support ratio of 14 U/g was used in the immobilization process in further experiments.

3.2.5. Effect of coupling time

The choice of suitable coupling time to efficiently immobilize CGTase on alumina was evaluated for different time intervals at 4°C. The activated alumina was contacted with CGTase solution (14 U/g support) at 4°C for 2-12 hours. The results are illustrated in Figure 13. It was concluded that a coupling time of 6 hours between the enzyme and support, the maximum immobilization yield was achieved (31.2%). A prolongation of the coupling time did not increase the yield of immobilization.

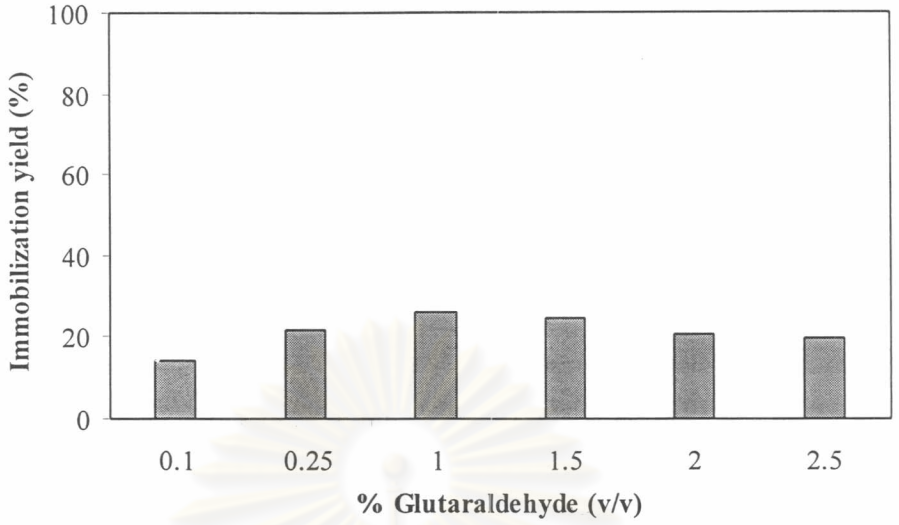


Figure 11. Influence of glutaraldehyde concentration on the amount of CGTase immobilized on alumina. The alumina support (1 g) was silanized in 2% (v/v) APTS, activated with different concentration of GA in 0.1 M phosphate buffer (pH 7.0) and incubated with CGTase solution (10 U) at 4°C for 12 h. Results shown were average values of duplicate experiments.

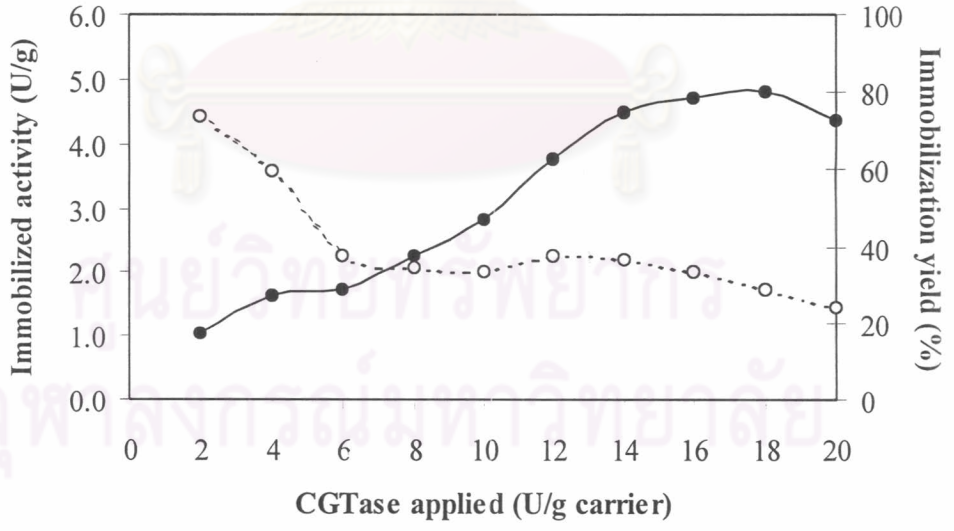


Figure 12. Effect of the amount of CGTase applied on the exhibited activity (●) and immobilization yield (○). The alumina support (1 g) was silanized with 2% (v/v) APTS, activated with 1% (v/v) GA in 0.1 M phosphate buffer (pH 7.0) and incubated with CGTase solution (2-20 U) at 4°C for 12 h. Result shown were average values of duplicate experiments.

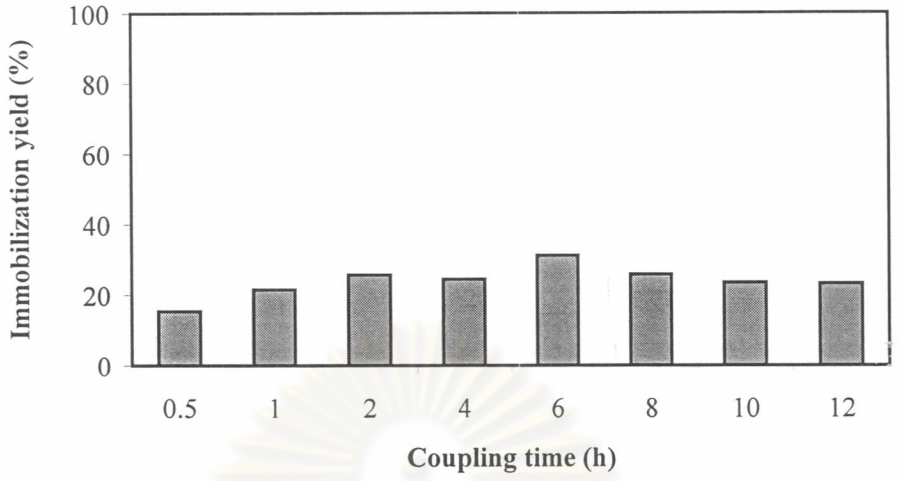


Figure 13. Effect of coupling time on the amount of CGTase immobilized alumina. The alumina support (1 g) was silanized with 2% (v/v) APTS, activated with 1% (v/v) GA and incubated with CGTase solution (14 U) at 4°C at different incubation times. Results shown were average values of duplicate experiments.

Therefore, a coupling time of 6 hours was selected to be used in subsequent studies of CGTase immobilization.

The summary of optimal conditions for covalent immobilization of CGTase is shown in Figure 14. Under these optimal conditions, the activity of immobilized enzyme was 4.36 U/g alumina with 31.2% of immobilization yields.

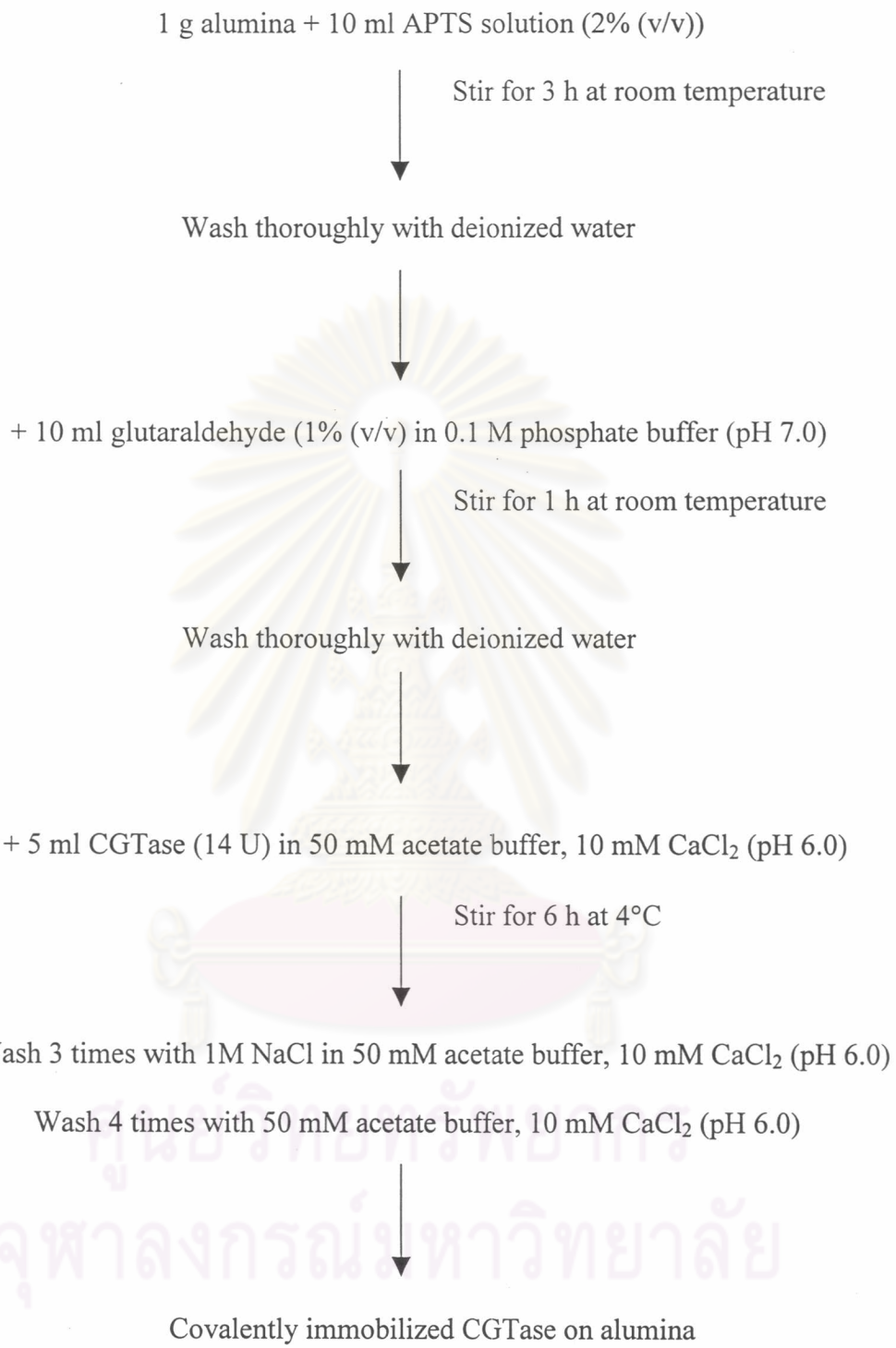


Figure 14. The optimal conditions for CGTase immobilization on alumina by covalent method

3.3 Characterization of the free and immobilized CGTase

The procedure of enzyme immobilization on insoluble supports had a variety of effects on the protein conformation as well as on the state of ionization and dissociation of the enzyme and its environment, and it is commonly led to changes in properties of the enzyme. The catalytic properties such as pH optimum, pH stability, optimum temperature, thermal stability and storage stability of the immobilized CGTase were then studied and compared to those of the free enzyme. All the activities were assayed as CD-forming activity.

3.3.1. pH optimum and pH stability

The effect of pH on the free and immobilized CGTase activities was investigated as mentioned in section 2.12.1. The rate of reaction of both enzyme preparations was measured with substrate solution at various pH values (4.0-11.0) and calculated for the relative activity expressed in percentage. The enzyme immobilized on alumina exhibited a shift about 1 unit towards the alkaline when compared to the free enzyme (Figure 15). The pH optimums of the free and immobilized CGTase were 6.0 and 7.0, respectively.

The stability of immobilized CGTase as a function of pH was also investigated and compared to that of the free enzyme (Figure 16). Both enzyme preparations were exposed to different pH values (4.0-11.0) at room temperature for 60 minutes before the enzyme activity was determined under standard condition (phosphate buffer, pH 6.0 at 60°C) (section 2.9). Both free and immobilized CGTase were stable over the wide range of pH values (5.0-9.0), then immobilization did not affect the pH stability of the enzyme.

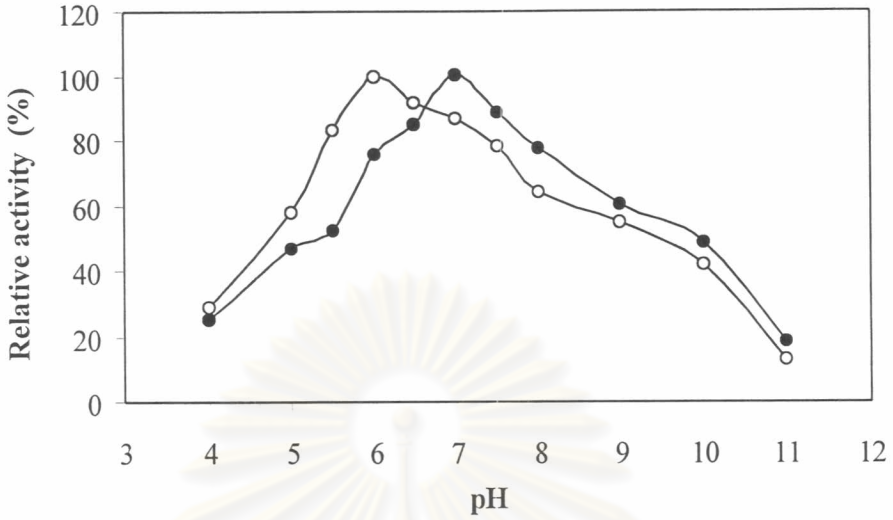


Figure 15. Effect of pH on the activity of free (○) and alumina immobilized CGTase (●). The reaction was performed at different pH values at 60°C. The buffers (50 mM) used were acetate buffer (pH 4.0-5.5), phosphate buffer (pH 6.0-7.5), Tris-HCl (pH 8.0-9.0) and Glycine-NaOH (pH 10-11). Results shown were average values of triplicate experiments.

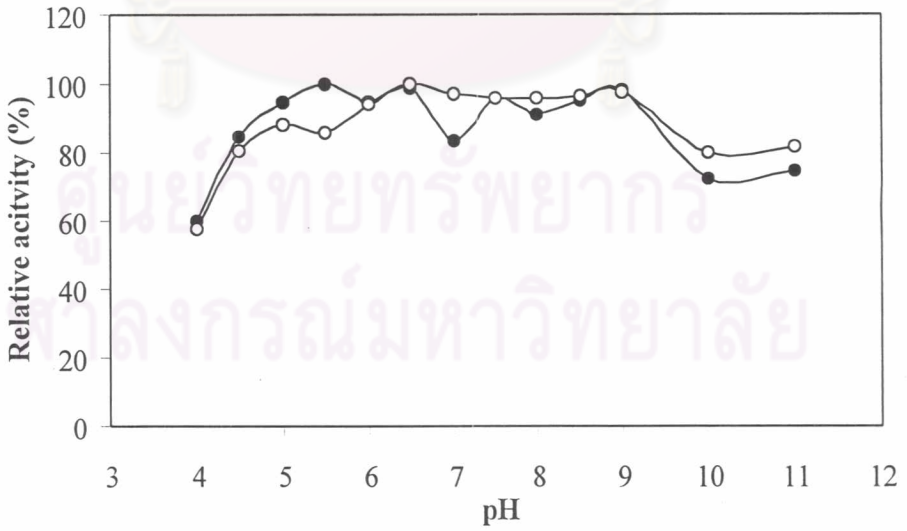


Figure 16. pH stability of free (○) and alumina immobilized CGTase (●). Enzyme samples were preincubated at different pH values at 25°C for 60 minutes and the residual activity was assayed under standard condition (pH 6.0). Results shown were average values of triplicate experiments.

3.3.2. Optimum temperature

The optimum temperature of the free and immobilized CGTase was investigated by incubating the reaction mixture in 50 mM phosphate buffer pH 6.0 at various temperatures ranging from 30°C to 90°C as described in section 2.12.3. As shown in Figure 17, both soluble and immobilized enzymes showed the same profile with the optimum temperature of 60°C. At higher temperatures (70°C), the immobilized enzyme showed slightly higher activity than that of the soluble form.

3.3.3. Energy of activation

The temperature data in the range of 30-60°C from Figure 17 were plotted in the form of Arrhenius plots (Figure 18). The slope of a plot of log relative activity against 1/T is related to the activation energy (E_a) for the molecule by the relationship: slope = E_a/2.303R, where R is gas constant. The plot for both free and immobilized enzymes were linear and the values of activation energy, calculated by Arrhenius equation (section 2.12.2) were equal to 2.50 kcal/mol and 3.62 kcal/mol for the free and immobilized CGTase, respectively (Table 9).

3.3.4. Thermal stability

The results of thermal stability of the free and immobilized CGTase are shown in Figure 19A. Both enzyme preparations were preincubated in 50 mM acetate buffer containing 10 mM $CaCl_2$ pH 6.0 at various temperatures (30-90°C) and the residual activities were assayed under standard conditions which was at 60°C. The enzyme activity prior to incubation was defined as 100%. As shown in Figure 19, the immobilized CGTase could retain its full activity at temperature up to 50°C, whereas the free enzyme retained only up to 30°C. Both enzyme preparations lost about half of their activities at 70°C and at 90°C both forms of enzymes lost all their activities.

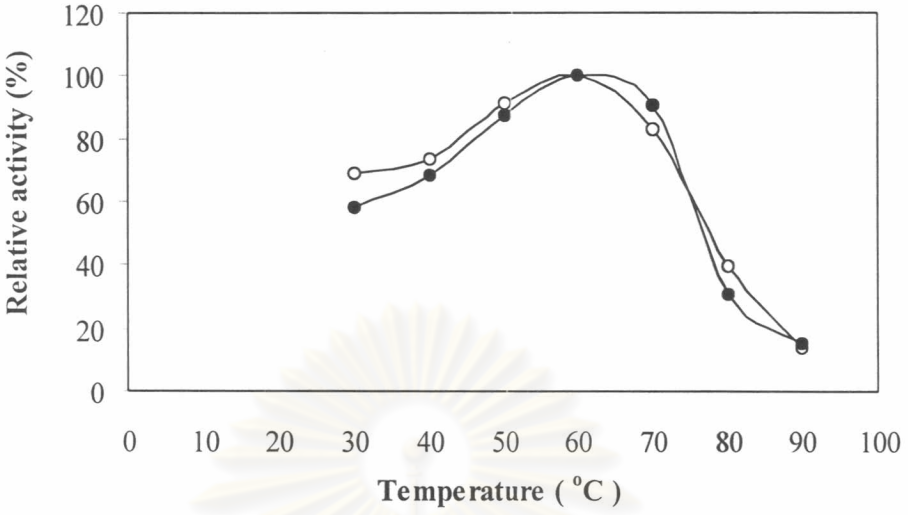


Figure 17. Effect of temperature on the activity of free (○) and alumina immobilized CGTase (●). The reaction was performed in 50 mM phosphate buffer (pH 6.0) at various temperatures. Results shown were average values of triplicate experiments.

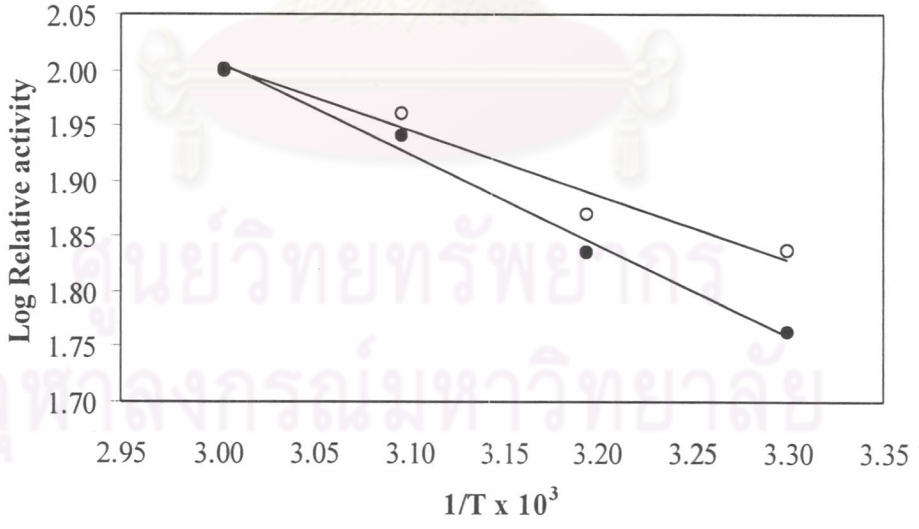


Figure 18. Arrhenius plots of free (○) and alumina immobilized CGTase (●).

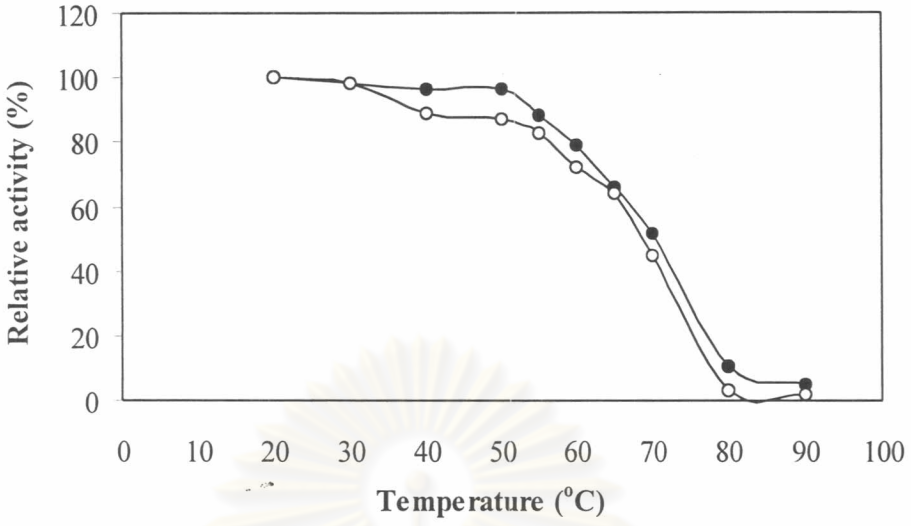


Figure 19. Effect of temperature on the stability of free (○) and alumina immobilized CGTase (●). Enzyme samples were preincubated in 50 mM acetate buffer containing 10 mM $CaCl_2$, pH 6.0 for 20 min at various temperatures and then were assayed for residual activities at 60 °C. Results shown were average values of triplicate experiments.

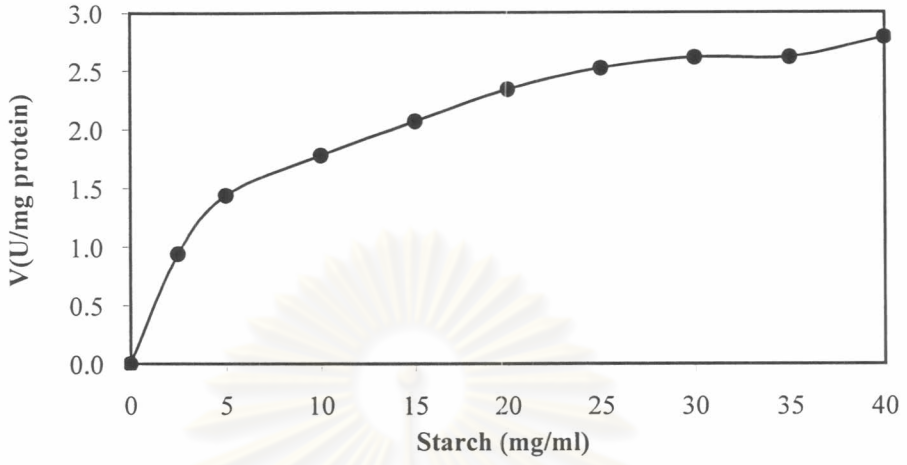
3.3.5. Kinetic parameters

The effect of substrate concentration on β-CD synthesis by immobilized CGTase was investigated and compared to that of the free form. The rate of β-CD formation was measured based upon the phenolphthalein method described in section 2.9. For the immobilized CGTase, the plot of the rate of β-CD formation versus starch concentration was hyperbolic, suggesting Michalelis-Menten kinetics (Figure 20, 21A). This point was confirmed by the linearity of the Lineweaver-Burk plot (Figure 20, 21B). The apparent K_m values (determined from Lineweaver-Burk plot) of free and immobilized CGTase were found to be 0.59 ± 0.25 and 5.62 ± 0.20 mg/ml, respectively. The V_{max} value of the free CGTase was 9.69 ± 0.38 U/mg protein and 5.82 ± 0.13 U/mg protein for the immobilized CGTase. Immobilized CGTase exhibited V_{max} value about 1.7 times lower than that of the free CGTase. Michaelis constant of immobilized CGTase was found to be 10 times higher than that of free CGTase.

3.3.6 Storage stability

Figure 22 showed the result of storage stability experiments of the free and immobilized CGTase on alumina. Both enzymes were stored in 50 mM acetate buffer containing 10 mM $CaCl_2$, pH 6.0 at 4°C and at room temperature for 2 months. The samples were withdrawn every 5 days for the determination of the residual activities as described in section 2.12.6. At 4°C both free and immobilized enzymes were apparently stable after 20 days of storage without any loss of enzyme activity. When the enzymes were stored at room temperature a 19% loss was seen after 40 days for the free enzyme whereas the immobilized enzyme could retain its activity approximately 91%. After 60 days of storage at room temperature, the immobilized

(A)



(B)

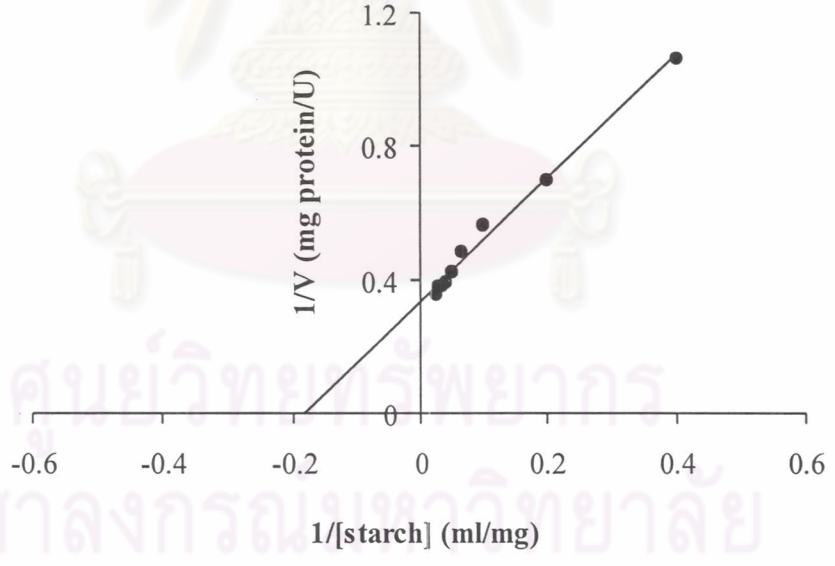
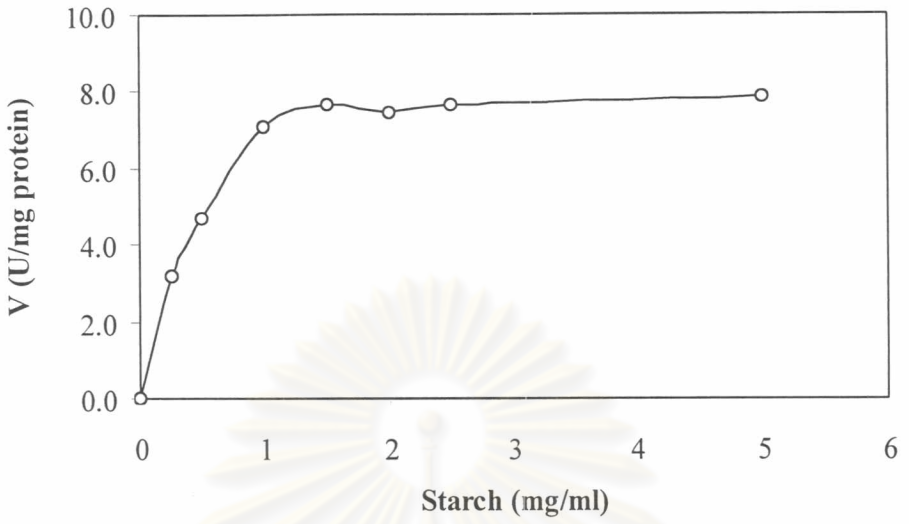


Figure 20 Effect of starch concentration on the initial reaction rate of the immobilized CGTase (A) and the corresponding Lineweaver-Burk plot (B).

(A)



(B)

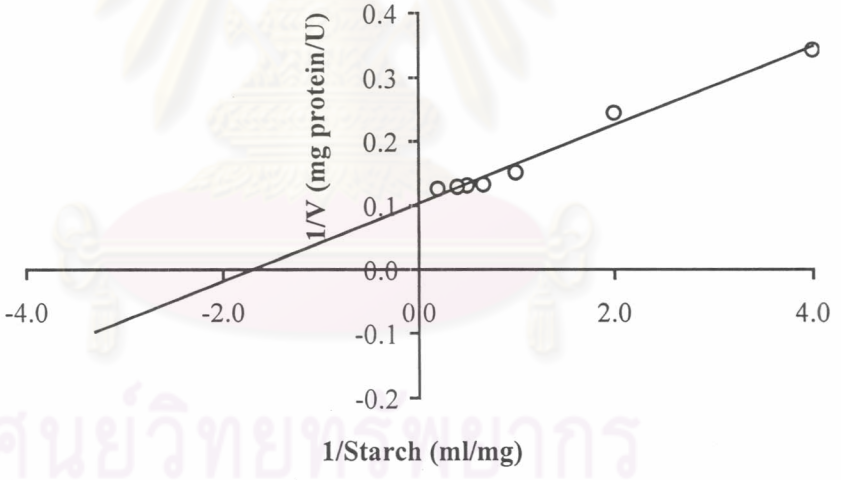
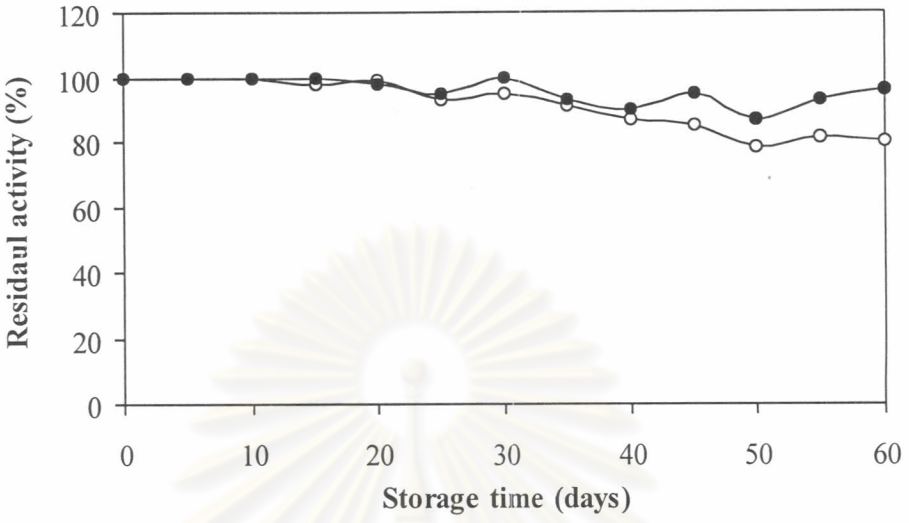


Figure 21. Effect of starch concentration on the initial reaction rate of the free CGTase (A) and the corresponding Lineweaver-Burk plot (B).

(A)



(B)

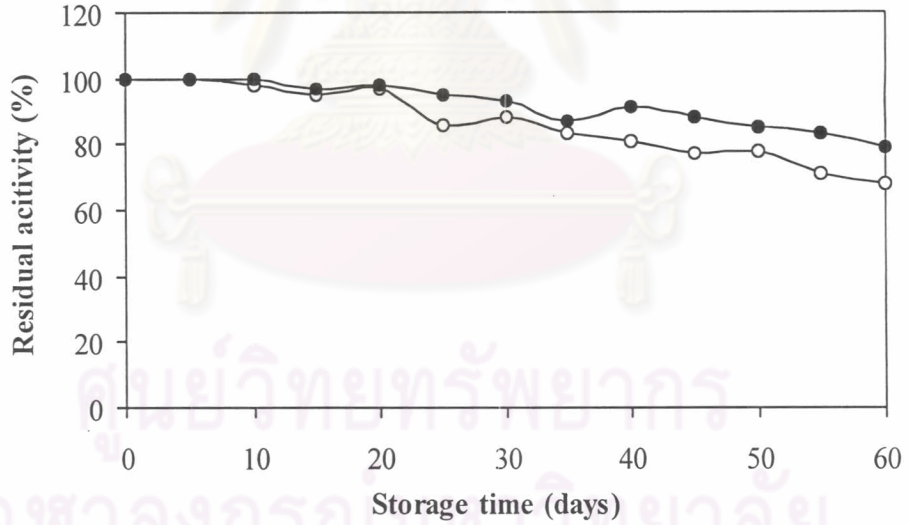


Figure 22. Storage stability at 4°C (A) and room temperature (B) of the free (○) and alumina immobilized CGTase (●). Enzyme samples were stored in 50 mM acetate buffer containing 10 mM $CaCl_2$ (pH 6.0) and then residual activity was assayed at time intervals.

Table 9. Summary of some properties of the free and immobilized CGTase on alumina.

Parameters	Free CGTase	Immobilized CGTase
pH optimum	6.0	7.0
pH stability (25°C 60 min)	5.0-9.0	5.0-9.0
Temperature optimum [°C]	60	60
Thermal stability (20 min) [°C]	≤ 40	≤ 50
Residual CGTase activity after 2 months Storage at 4°C, 25°C [%]	80 68	96 79
K_m [mg/ml starch]	0.50 ± 0.25	5.62 ± 0.20
V_{max} [U/mg protein]	9.69 ± 1.70	5.82 ± 0.13
E_a [kcal/mol]	2.50	3.62

CGTase retained 96 % and 79 % of its original activity when stored at 4°C and 25°C in acetate buffer (pH 6.0), respectively. For soluble enzyme, it could retain its activity for only 80% and 68% when the enzyme was stored at 4°C and 25°C, respectively after 60 days of storage.

3.4 Synthesis of 2-O-α-D-glucopyranosyl-L-ascorbic acid by *Paenibacillus* sp. A11 CGTase

The transglycosylation to synthesize ascorbic acid derivatives by CGTase has previously reported (Aga *et al.*, 1991; Tanaka *et al.*, 1991 and Jun *et al.*, 2001). In the presence of CDs or other glycosyl donors and AA as an acceptor, CGTase was found to transfer glycosyl residues from the donors to AA. The main product formed by this reaction was identified as 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) (Tanaka *et al.*, 1991). In this study, the production of AA-2G by soluble CGTase purified from *Paenibacillus* sp. A11 was first examined. After that the immobilized CGTase was then studied for the possibility of AA-2G production.

3.4.1 Soluble CGTase

Investigation of the AA-2G formation catalyzed by *Paenibacillus* sp. A11 CGTase was performed under the conditions previously described by Wongsangwattana (2000) since the same source of enzyme was used. Soluble CGTase (42 units/g β-CD) was incubated with a mixture containing 2% (w/v) β-CD, 0.5% (w/v) AANa and 0.02% (w/v) thiourea at 40°C, pH 6.0 for 24 hours in the dark. After this time, the reaction was terminated by the addition of 1.06% metaphosphoric acid and analyzed by HPLC. In order to be assured that the peaks of products

observed in the HPLC profiles were transglycosylation products by the action of CGTase, the reaction mixture without CGTase was used as control.

HPLC analysis of the reaction mixture was performed on a C_{18} column (4.6 x 150 mm) and 0.1 M KH_2PO_4-H_3PO_4 (pH 2.0) was used as eluent at a flow rate of 0.7 ml/min. The chromatograms of standard AA, AA-2G, reaction mixture with and without CGTase are shown in Figure 23 A-D, respectively. The retention time (t_R) of standard AA and AA-2G were around 3.9 and 4.5, respectively (Figure 23A-B). In HPLC profiles of reaction mixture containing CGTase (Figure 23C), it was found that the peak at t_R of 4.402 minutes, observed just after the peak of AA, had a similar t_R as that of the standard AA-2G, indication that this could be AA-2G. However, when compared to HPLC profiles of the control experiment, the peak at the t_R around 4.4 minutes was also observed with lower peak area (Figure 23 D). This could be stated that the unknown product (in the control sample) and AA-2G were eluted at a similar retention time and it is difficult to distinguish them under this HPLC condition. In addition, five more peaks which could be other transglycosylation products (t_R 5.665, 6.913, 7.989, 8.863 and 9.889 minutes) were only observed in the reaction mixture containing the enzyme. The amounts of these products decreased with increased retention time.

In an attempt to identify the peak of AA-2G in the reaction mixture, HPLC analysis was done on a longer C_{18}-column (4.6 x 250 mm) and eluted with the same buffer at a flow rate of 0.7 ml/min. Also, the effect of substrate concentration on the AA-2G formation by soluble CGTase was investigated. Various concentrations of β-CD and AA were incubated with 49 units of CGTase in 1 ml of 50 mM acetate buffer containing 10 mM $CaCl_2$, pH 6.0 at 40°C for 24 h. The resultant reaction mixtures were analyzed by HPLC. Separation of AA-2G and unknown product was achieved.

Figure 24 shows HPLC analysis of the reaction mixtures with and without CGTase (4% AA and 4% β-CD). As shown in Figure 24D, the peaks at t_R of 5.005 and 6.893 minutes were identified as thiourea and AA, respectively. The peak at t_R of 7.997 minutes in Figure 24C coincided with the retention time of standard AA-2G which was not observed in the control (when no CGTase was added) (Figure 24D). As a result, the peak at t_R of 7.997 minutes could be AA-2G. For other transglycosylation products, only two peaks at t_R of 11.020 and 14.296 minutes were observed within a 15-minute record of this HPLC condition.

For other AA and β-CD concentration used, HPLC profiles were obtained as those mentioned previously and the amount of AA-2G formed was calculated on the basis of its standard curve of peak area. For the production yield, it was calculated as in section 2.14. As shown in Figure 25A, the amount of AA-2G formed increased when AA concentrations increased. Moreover, the production of AA-2G was doubled when the concentration of β-CD increased from 2% to 4% and the concentration of AA was more than 3%. At 4% β-CD, it can be seen that the production yield of AA-2G increased gradually and reached equilibrium at 4% AA. Above that the production yield did not increase (Figure 25B). Thus, the concentration of β-CD and AA at 4% were then selected for the production of AA-2G by immobilized CGTase.

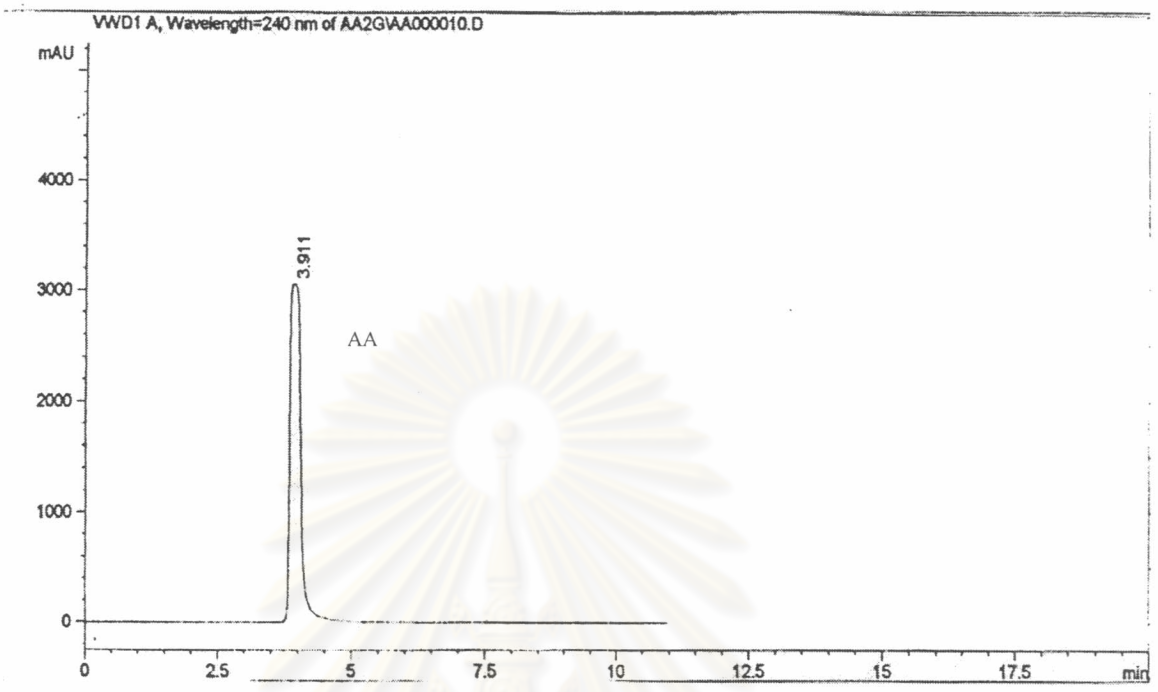
3.4.2 Immobilized CGTase

The transglycosylation of AA to form AA-2G catalyzed by CGTase immobilized on alumina was also investigated. The immobilized CGTase (60 g wet weight, 280 units) was incubated with the mixture containing 4% (w/v) AA, 4% (w/v) β-CD and 0.2% (w/v) thiourea at 40°C, pH 6.0, for 24 hours. Typical HPLC chromatograms of immobilized CGTase catalyzed reaction (Figure 26) had the same

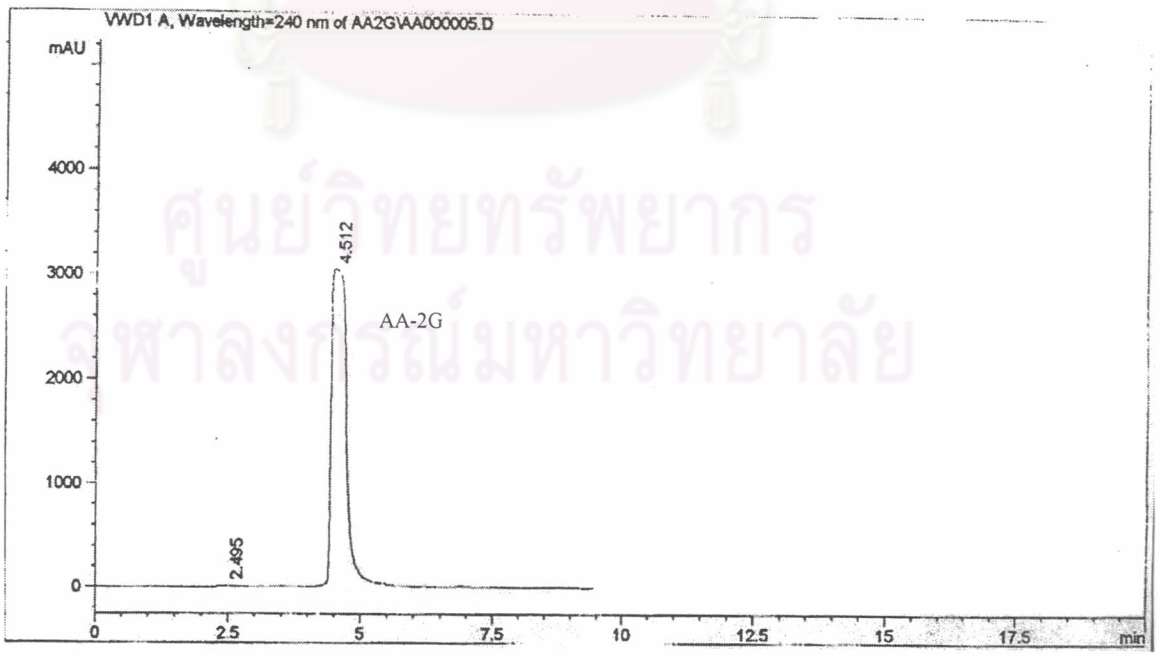
pattern as in the case of soluble enzyme. To improve the resolution of the compound peaks, the C_{18}-column was eluted with the mobile phase at a flow rate of 0.3 ml/min. The peak at t_R of 17.621 minutes was observed just after a peak of AA (t_R = 15.301 minutes) and was thought to be AA-2G (Figure 26C) by comparing the retention time of standard AA and AA-2G (Figure 26A). However, the peak at t_r around 18 minutes was observed in both control and sample containing immobilized CGTase. In addition, immobilized CGTase may have produced another transglycosylation product of AA at t_R of 23.705 minutes which was not observed in the control experiment.

Thus, to investigate that the peak at t_R of 17.653 and 23.705 minutes were the transglycosylation products of AA, the reaction mixture was then treated with glucoamylase (Figure 27). After the addition of glucoamylase (20 U/ml) to the reaction mixtures and overnight incubation at 40°C, the peaks at t_R of 23.705 minutes disappeared and only the peak at t_R 17.621 increased. Thus, the peak at t_R of 17.621 and 23.705 minutes was confirmed to possibly be AA-2G and AA-$2G_2$, respectively. The other peak observed apart form AA, AA-2G and AA-$2G_2$ in the reaction mixture containing CGTase was not identified and could be further studied. From these results, it was possible to use the immobilized CGTase to catalyze the formation of AA-2G in the presence of β-CD and AA.

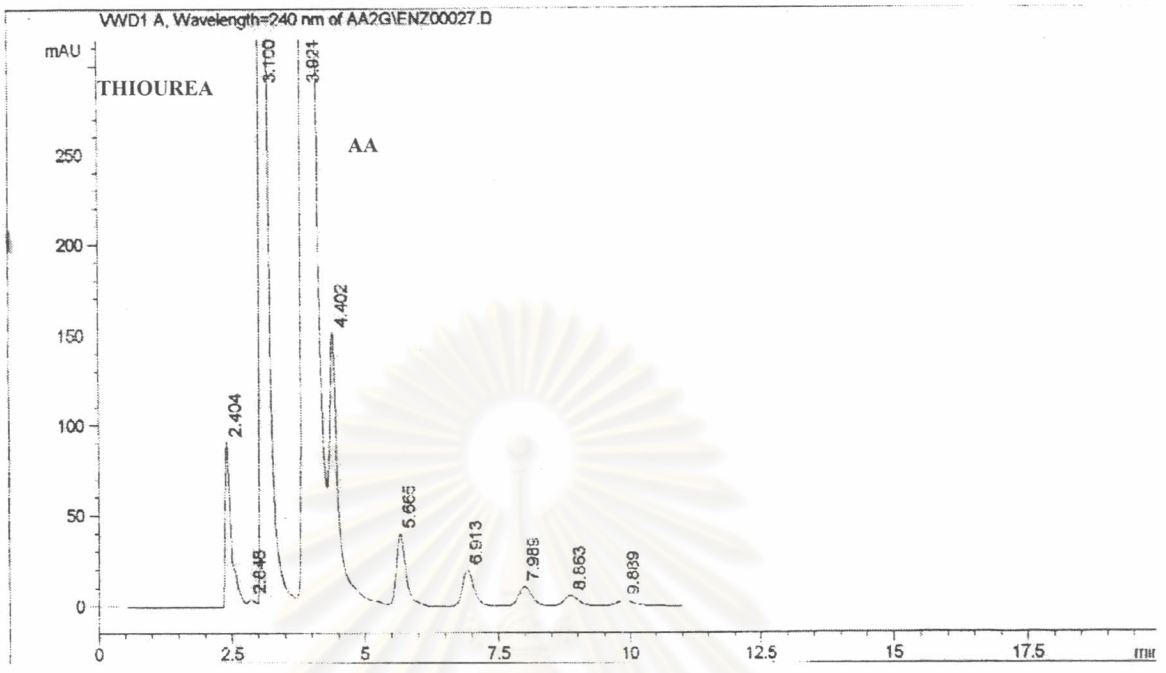
Figure 23. HPLC chromatograms of AA-2-oligosaccharides (AA-2G_n) formation with CGTase from *Paenibacillus* sp. A11. A LUNA C_{18} reverse-phase column (4.6 x 150mm) was used and eluted with 0.1 M KH_2PO_4-H_3PO_4 (pH 2.0) at a flow rate of 0.7 ml/min. The absorbance was measured at 240 nm. The reaction mixture consisted of 0.5% (w/v) AANa, 2% (w/v) β-CD, and 0.02% thiourea were incubated with 42 U/g β-CD of CGTase at 40°C, pH 6.0, for 24 h. The numbers in the figure represent the retention times of the peaks.



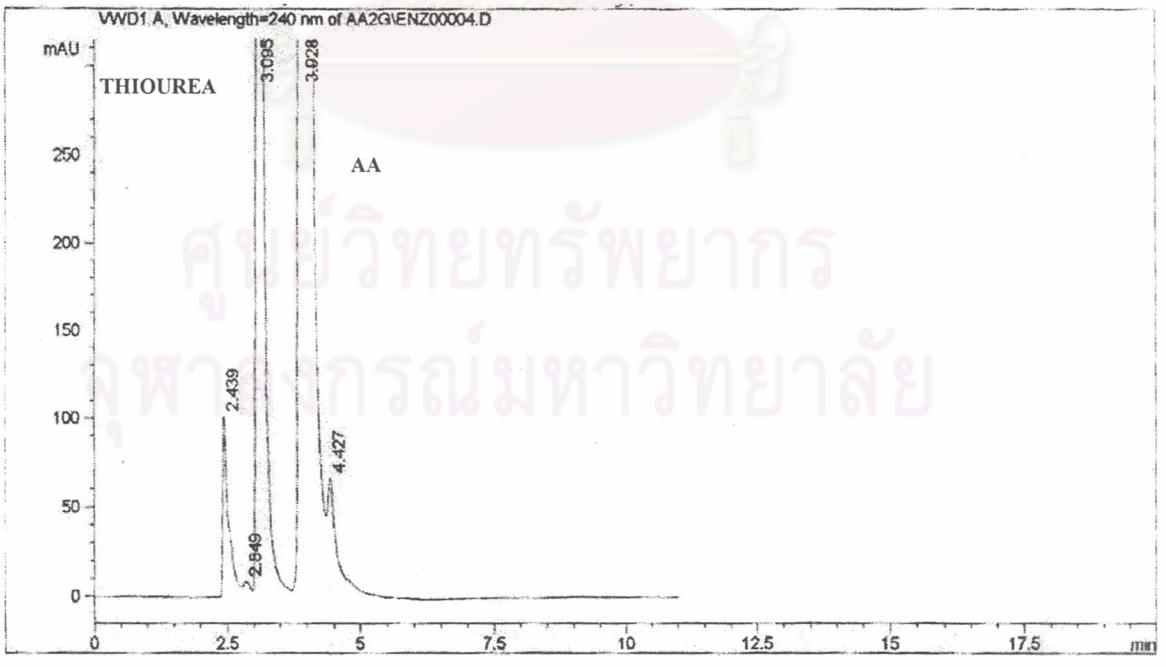
(A) standard AA



(B) standard AA-2G

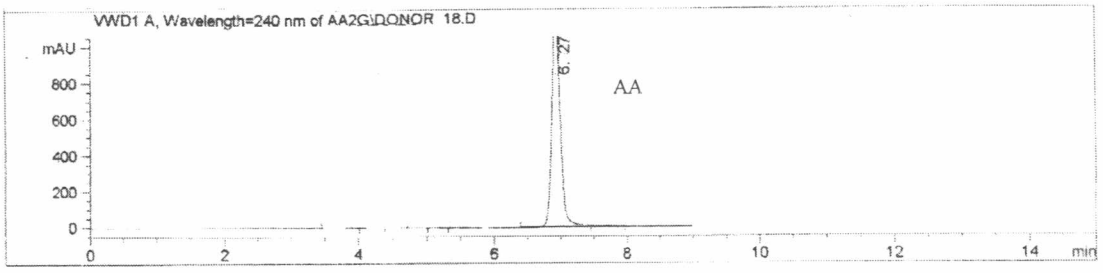


(C) reaction mixture incubated with CGTase

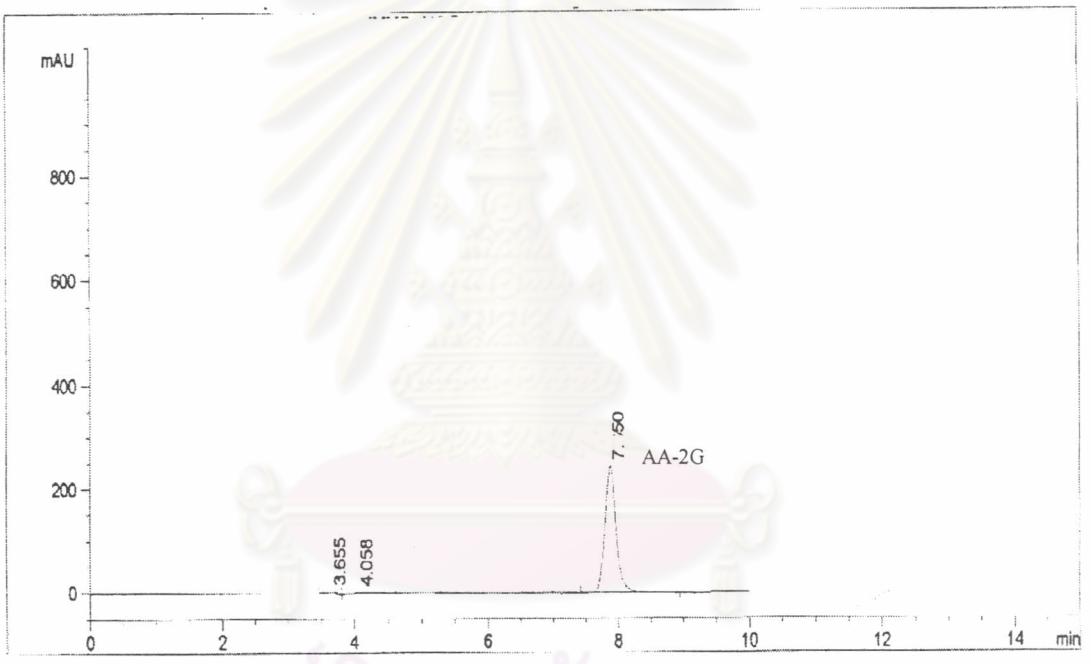


(D) control (reaction mixture without CGTase)

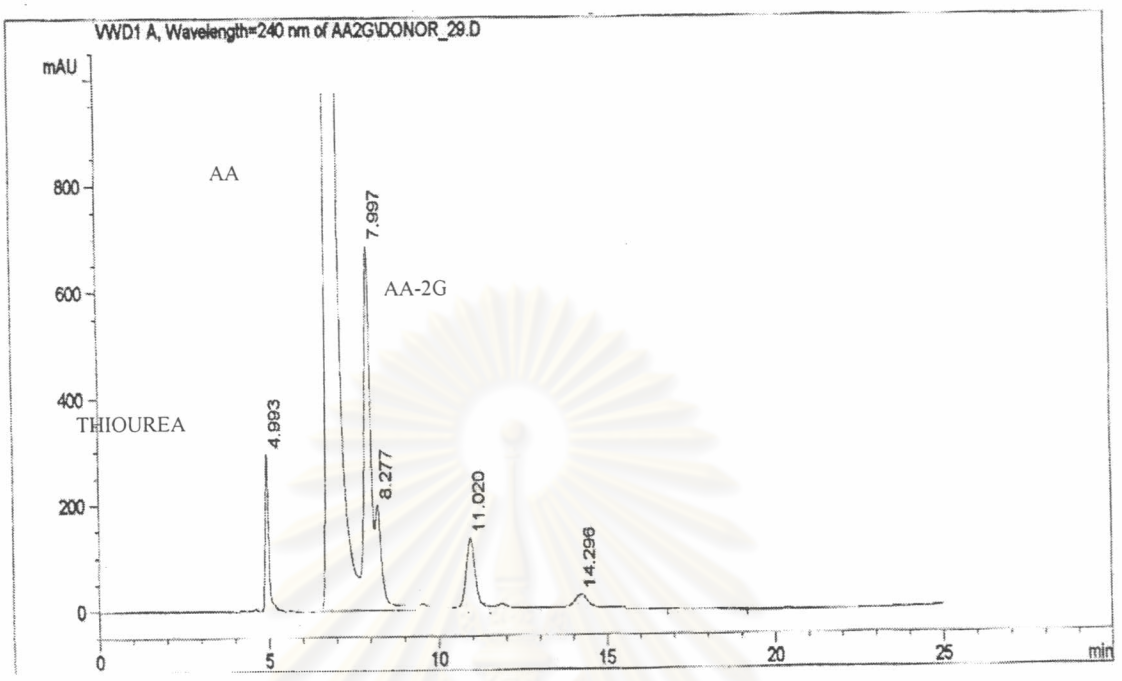
Figure 24. HPLC chromatograms of AA-2-oligosaccharides (AA-2G_n) formation with soluble CGTase from *Paenibacillus* sp. A11. A LUNA C_{18} reverse-phase column (4.6 x 250mm) was used and eluted with 0.1 M KH_2PO_4-H_3PO_4 (pH 2.0) at a flow rate of 0.7 ml/min. The absorbance was measured at 240 nm. The reaction mixture consisted of 4% (w/v) β-CD, 4% (w/v) AA and 0.2% (w/v) thiourea were incubated with 350 U/g β-CD of CGTase was incubated at 40°C, pH 6.0, for 24 h. The numbers in the figure represent the retention times of the peaks.



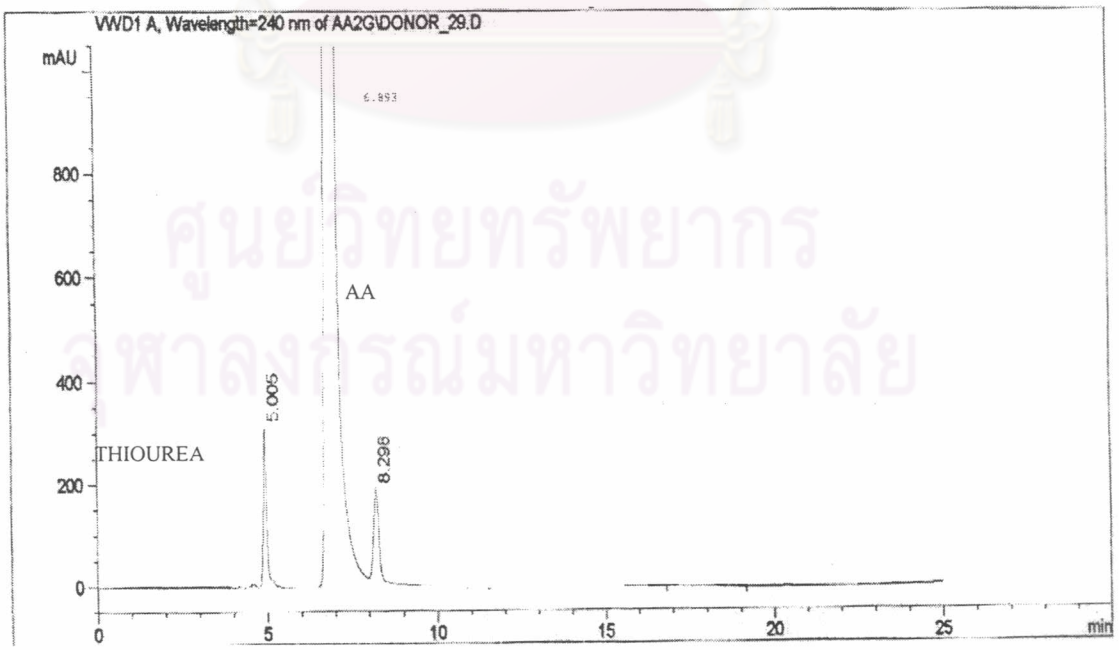
(A) standard AA



(B) standard AA-2G

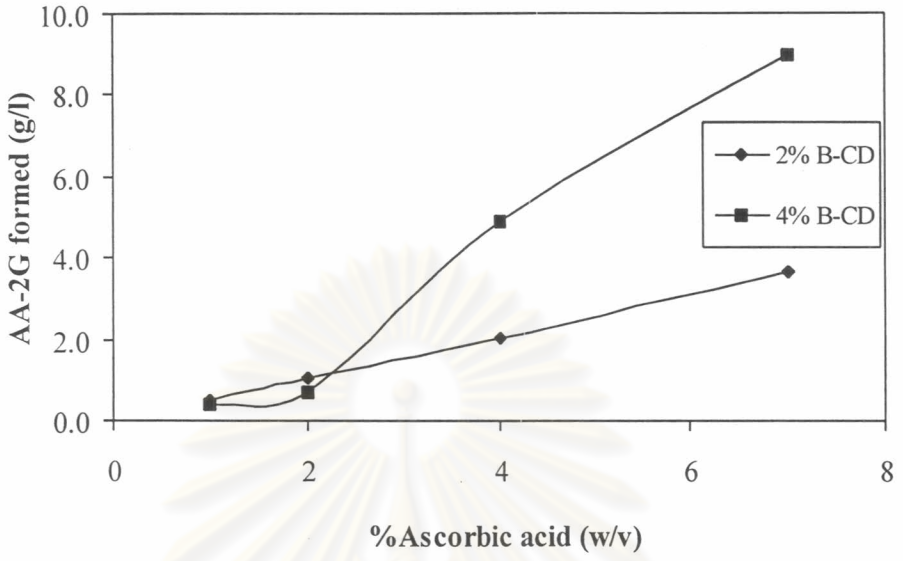


(C) reaction mixture incubated with CGTase



(D) control (reaction mixture without CGTase)

(A)



(B)

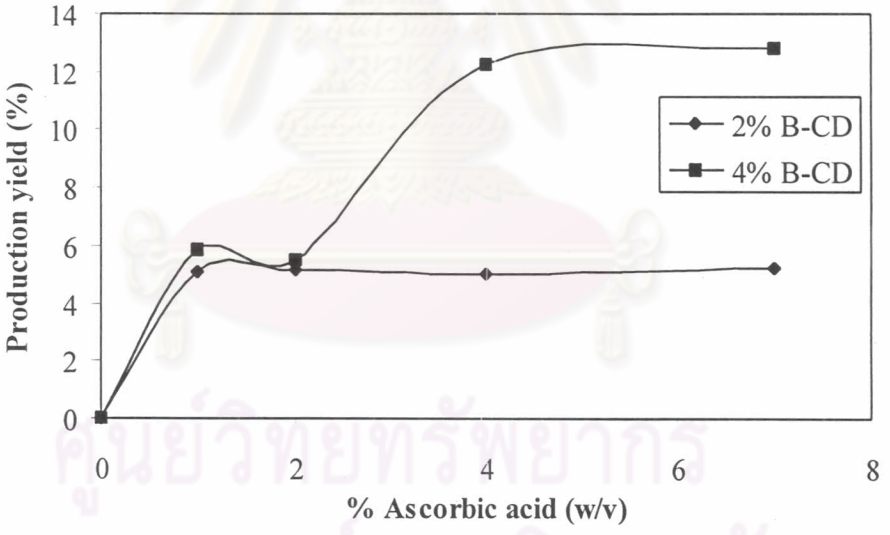
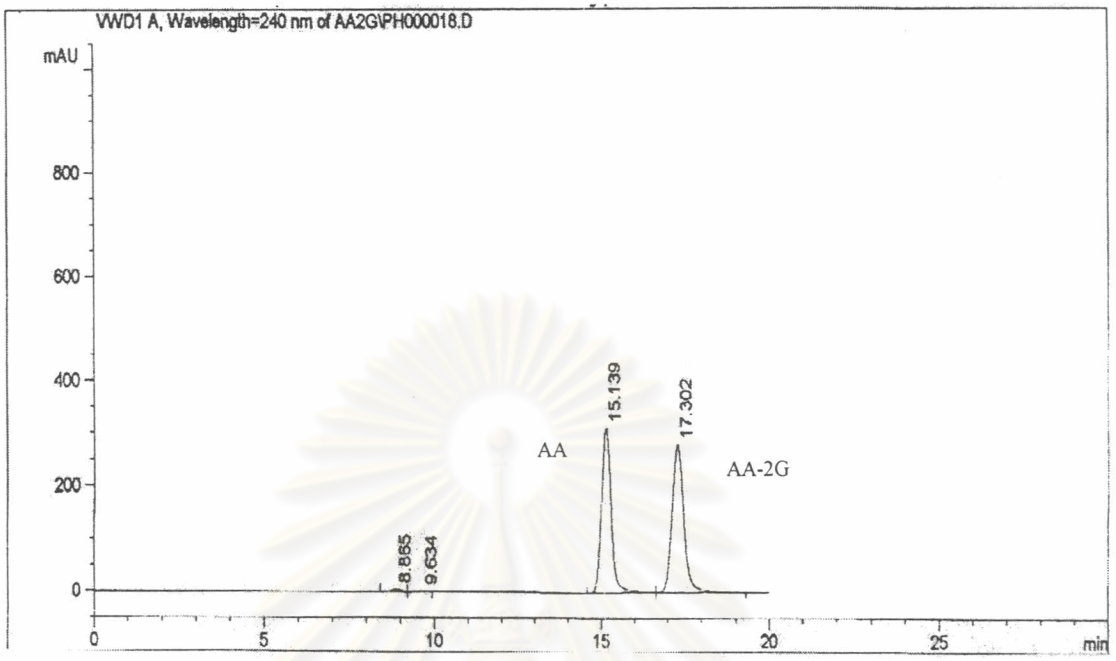
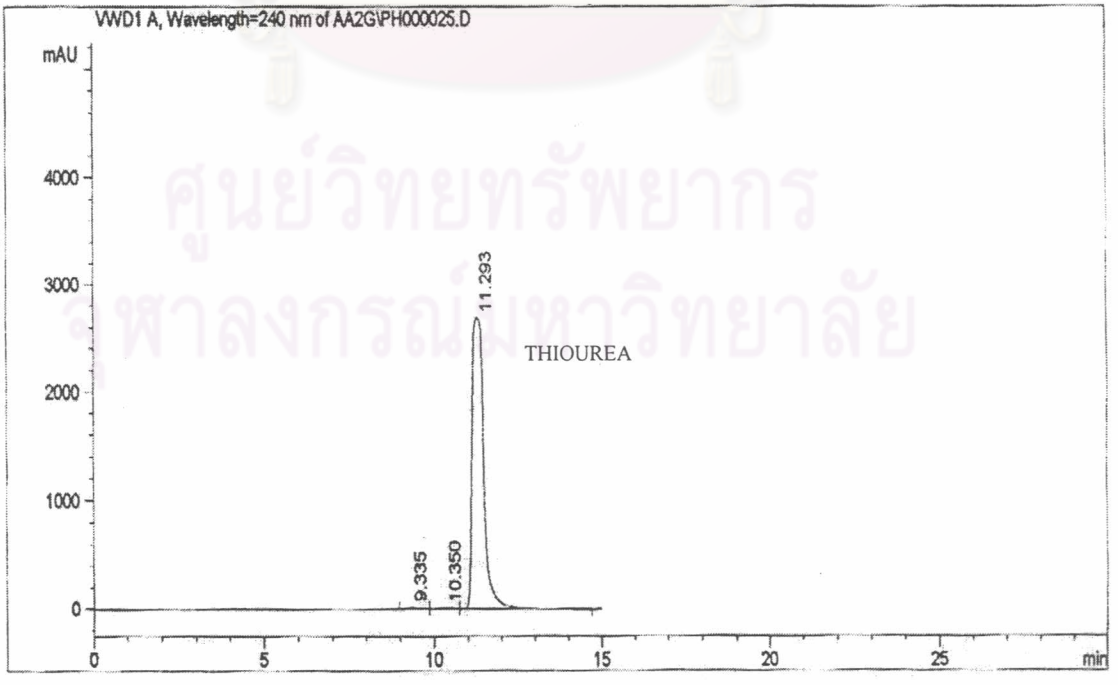


Figure 25. Effect of substrate concentration on the amount of AA-2G formed (A) and production yield (B). Soluble CGTase (280 units) was incubated with different concentrations of β-CD and ascorbic acid in 1 ml of 50 mM acetate buffer containing 10 mM $CaCl_2$, pH 6.0. Production yield was calculated as described in section 2.14.

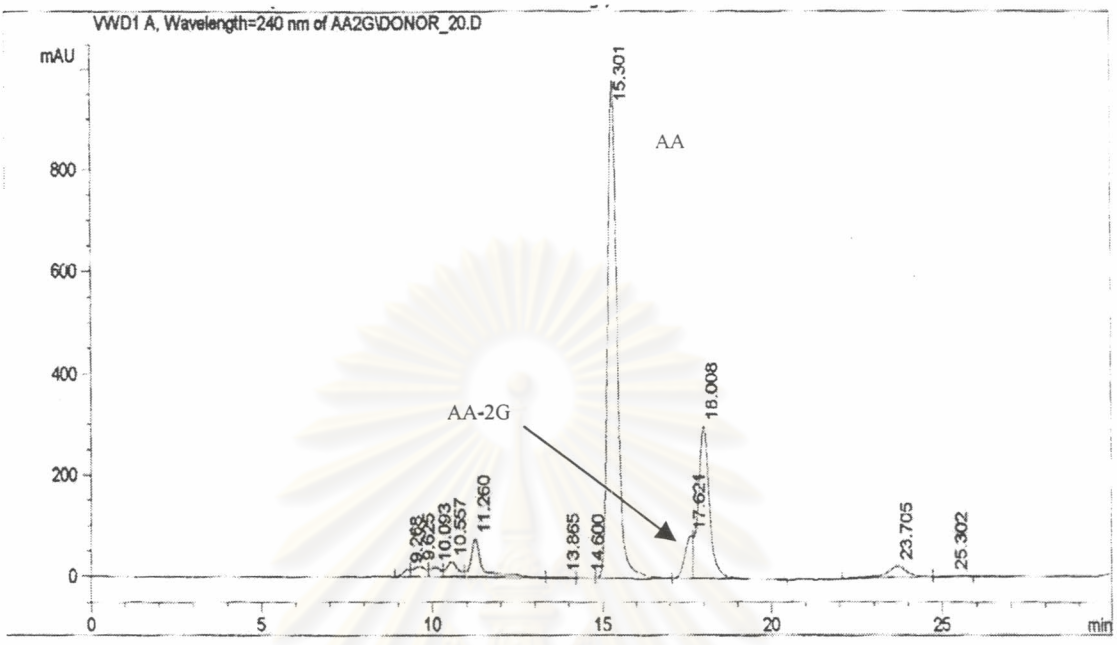
Figure 26. HPLC chromatograms of AA-2-oligosaccharides (AA-2G_n) formation with immobilized CGTase from *Paenibacillus* sp. A11. A LUNA C_{18} reverse-phase column (4.6 x 250mm) was used and eluted with 0.1 M KH_2PO_4-H_3PO_4 (pH 2.0) at a flow rate of 0.3 ml/min. The absorbance was measured at 240 nm. The reaction mixture consisted of 4% (w/v) β-CD, 4% (w/v) AA and 0.2% (w/v) thiourea were incubated with 350 U/g β-CD of immobilized CGTase (60 g wet weight) at 40°C, pH 6.0, for 24 h. The numbers in the figure represent the retention times of the peaks.



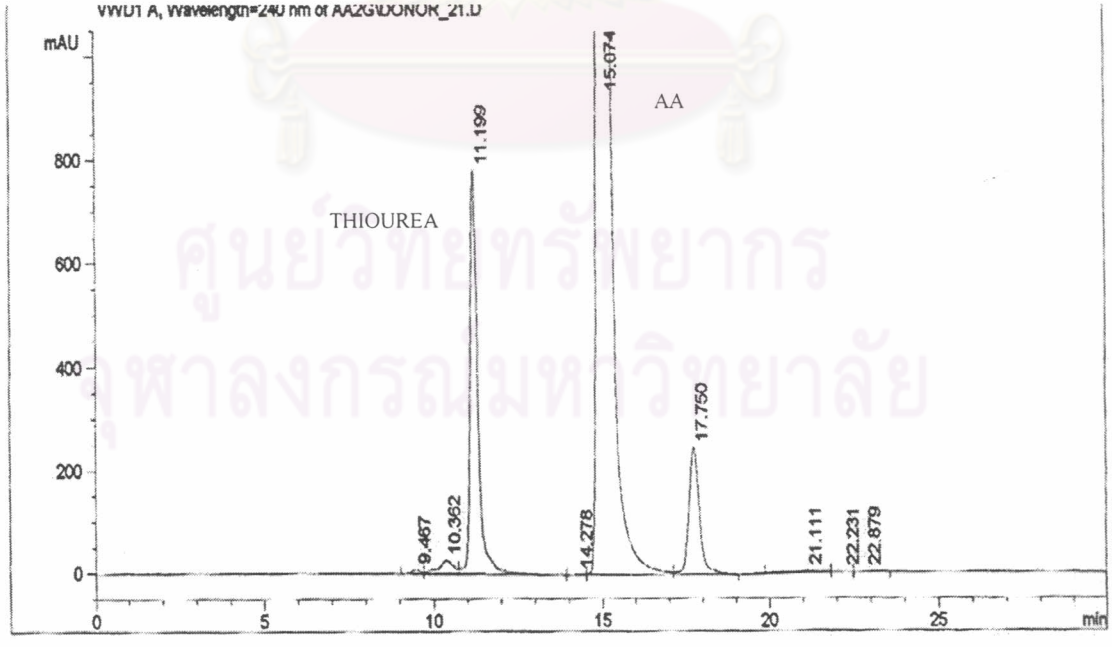
(A) mixture of standard AA and AA-2G



(B) standard thiourea



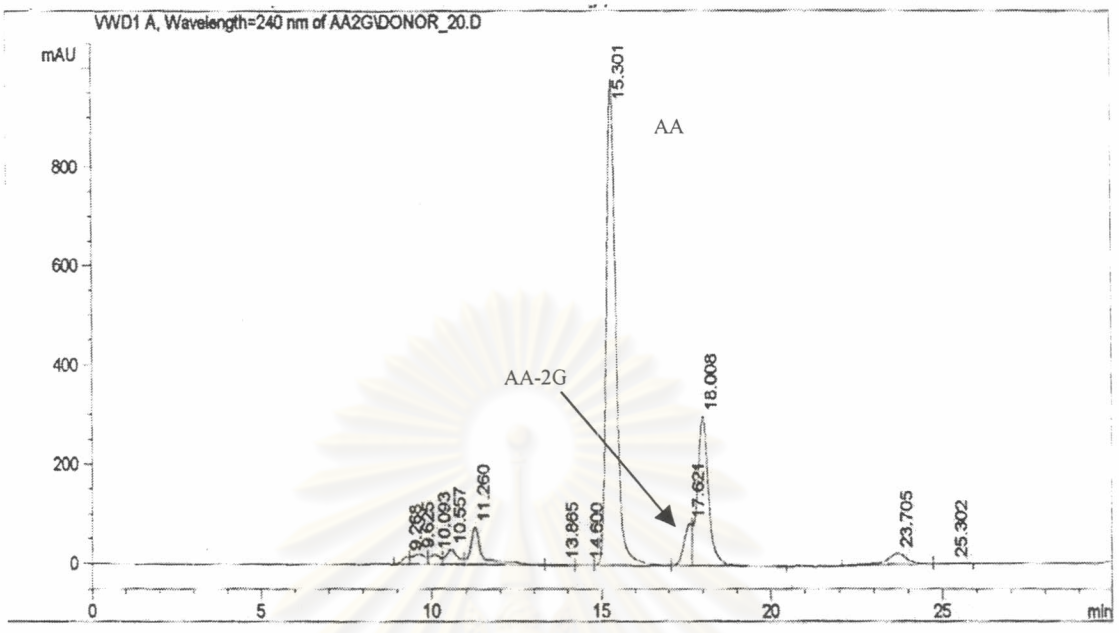
(C) reaction mixture incubated with immobilized CGTase



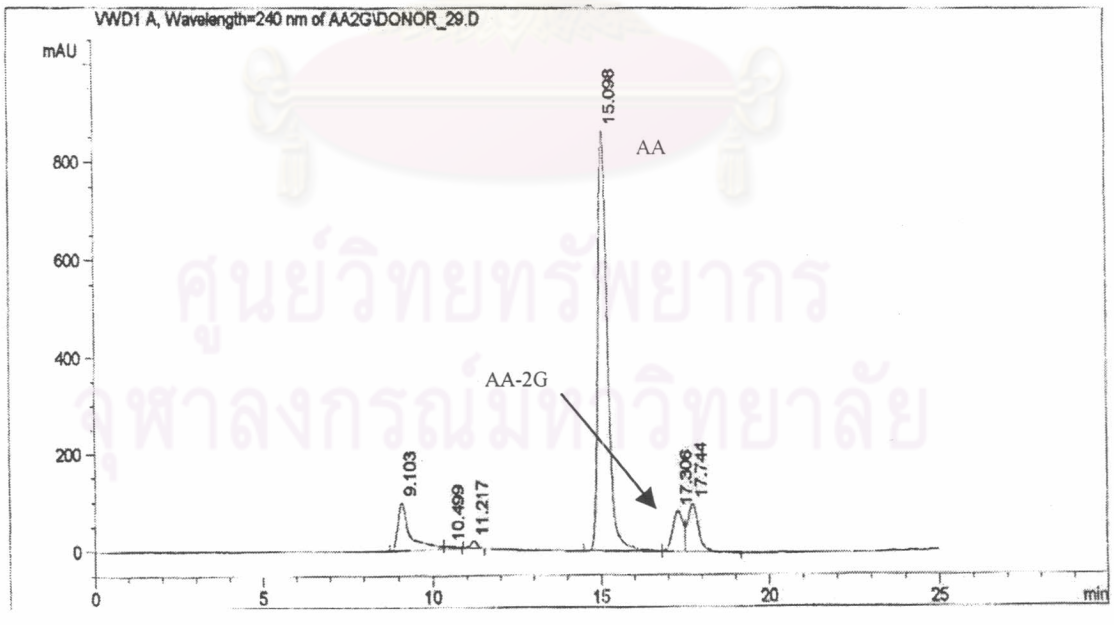
(D) control (reaction mixture without immobilized CGTase)

Figure 27. Effect of glucoamylase on the digestion of AA-2G$_n$.

A LUNA C_{18} reverse-phase column (4.6 x 250mm) was used and eluted with 0.1 M KH_2PO_4-H_3PO_4 (pH 2.0) at a flow rate of 0.3 ml/min. The absorbance was measured at 240 nm. AA-2G formation was done for 24 h. Then glucoamylase (20 U/ml) was added to a reaction mixture and incubated overnight at 40°C. The numbers in the figure represent the retention times of the peaks.



(A) reaction mixture (with CGTase) before treated with glucoamylase



(B) reaction mixture (with CGTase) after treated with glucoamylase

3.5 Determination of suitable conditions for AA-2G production by immobilized CGTase

The optimum conditions for the production of AA-2G by immobilized CGTase need to be identified for the possibility of its mass production. Various parameters such as substrate concentration, pH, temperature and incubation time for the immobilized CGTase reaction were then examined. The reaction mixture was prepared as described in section 2.13 except the change of these reaction parameters. The amount of AA-2G formed from each reaction was measured by HPLC as described in section 2.14 and was calculated on the basis of its standard curve of peak area (Appendix E).

The optimal condition from each parameter was then selected for high production of AA-2G and further study for the reusability of the immobilized enzyme.

3.5.1 Effect of pH

The effect of pH on CGTase-catalyzed coupling reaction from β-CD to AA for AA-2G production was investigated. The immobilized CGTase (60 g wet weight, 280 units) was incubated with the reaction mixture containing 4% (w/v) β-CD, 4% (w/v) AANa and 0.2% (w/v) thiourea at different pH values (pH 5.0, 5.5 and 6.0). As shown in Figure 28, the amount of AA-2G was maximum at the reaction pH of 5.0 (0.746 g/l) with 1.86% of total AA added. At higher reaction pH values (pH 5.5 and 6.0), the yield of AA-2G was decreased. Therefore, the reaction performed at pH 5.0 was chosen as a suitable pH for the production of AA-2G.

3.5.2 Effect of temperature

The effect of incubation temperature on AA-2G formation by immobilized CGTase was examined at 30°C, 40°C and 50°C. The immobilized CGTase and the reaction mixture containing 4% (w/v) β-CD, 4% (w/v) AANa and 0.2% (w/v) thiourea in acetate buffer, pH 6.0 were incubated at indicated temperatures for 24 hours. Figure 29 shows that the highest amount of AA-2G formed was obtained at 40°C (0.432 g/l). Thus, the optimal temperature of 40°C was chosen for AA-2G production from this experiment.

3.5.3 Effect of incubation time

The time-course of AA-2G formation by immobilized CGTase was investigated during 0 to 48 hours. The reaction mixture consisted of 4% (w/v) β-CD and 4% (w/v) AA and 0.2% (w/v) thiourea were incubated with immobilized CGTase (60 g wet weight, 280 units) at 40°C, pH 6.0 for 0 to 48 hours. Although the amount of AA-2G increased as the reaction time was prolonged, the incubation time at 24 hours was chosen due to sufficient AA-2G was achieved and the incubation time used was not too long (Figure 30).

By comparison with the systems of soluble CGTase, by using the enzyme activity equal to that of the immobilized one, it was found that the production of AA-2G using soluble CGTase gave higher production yield than immobilized CGTase (Figure 31).

3.5.4 Effect of AA concentration

In preliminary experiments, it was found that an increase in β-CD concentration had an effect on AA-2G synthesis (Figure 25). Therefore, the level of

β-CD concentration was fixed at 4% (w/v) and the concentration of AA was changed from 0.5% (w/v) to 10% (w/v). The concentration of β-CD higher than 4% has low solubility. The amount of AA-2G formed by immobilized CGTase depended on the amount of the acceptor (AA) (Figure 32). Although the amount of AA-2G formed increased with increasing the concentration of AA, it was found that the yield was not significantly different when the AA concentration increased from 1% (w/v) to 4% (w/v) and at concentration above 4% (w/v), the yield did not increase either.

Thus, to be assured for the optimal conditions for AA-2G production by immobilized CGTase, the concentrations of AA at the level of 1% (w/v), 2% (w/v) and 4% (w/v) were tested under the selected optimal conditions (4% (w/v) β-CD, pH 5.0, 40°C, 24 hours). From the results obtained in Figure 33, the amount of AA-2G formed increased as the concentration of AA increased but the production yield was found to be highest when AA concentration was 2% (w/v).

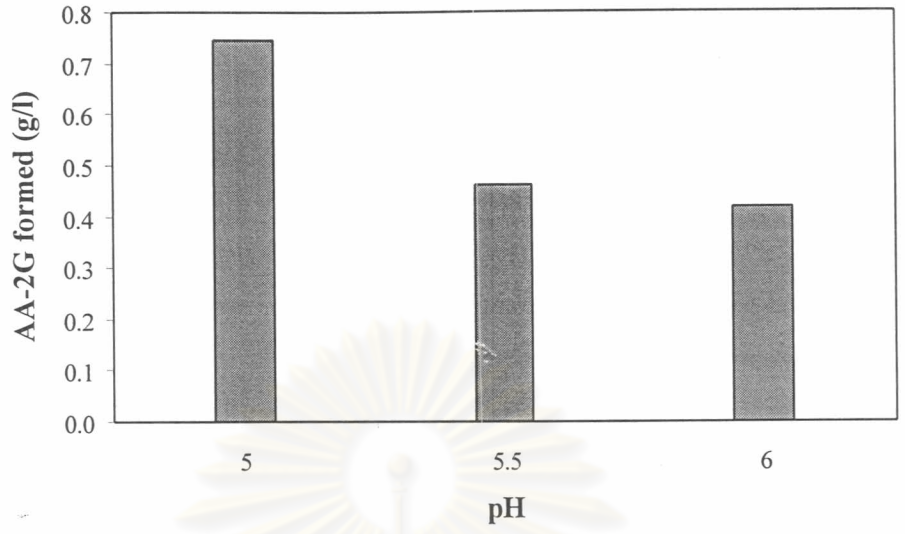


Figure 28. Effect of pH on the AA-2G production by immobilized CGTase.

The immobilized CGTase on alumina (60 g wet, 280 U) was incubated with the mixture containing 4% (w/v) β-CD, 4% (w/v) AA Na and 0.2% (w/v) thiourea at different pH values with shaking at 40°C for 24 h. Results shown were average values of duplicate experiments.

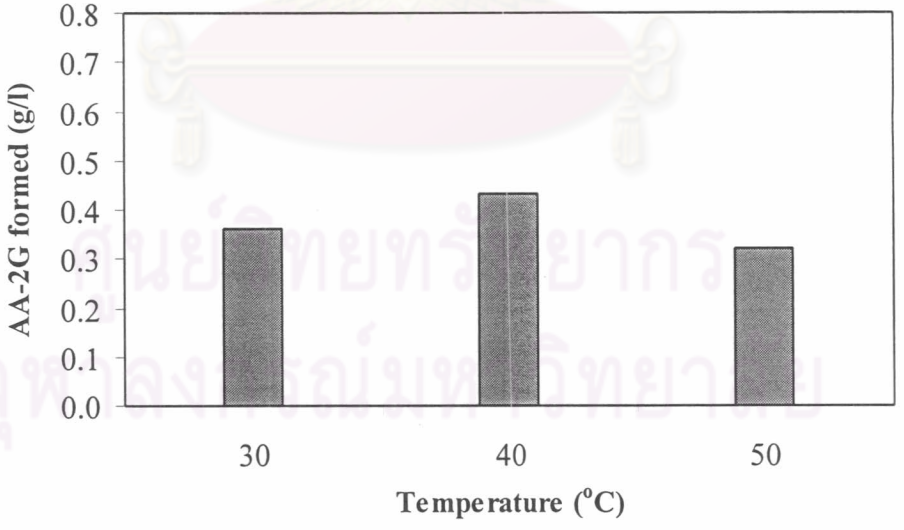


Figure 29. Effect of temperature on the AA-2G production by immobilized CGTase. The immobilized CGTase on alumina (60 g wet, 350 U/g β-CD) was incubated with the mixture containing 4% (w/v) β-CD, 4% (w/v) AA Na and 0.2% (w/v) thiourea at pH 6.0 with shaking at indicated temperatures for 24 h. Results shown were average values of duplicate experiment.

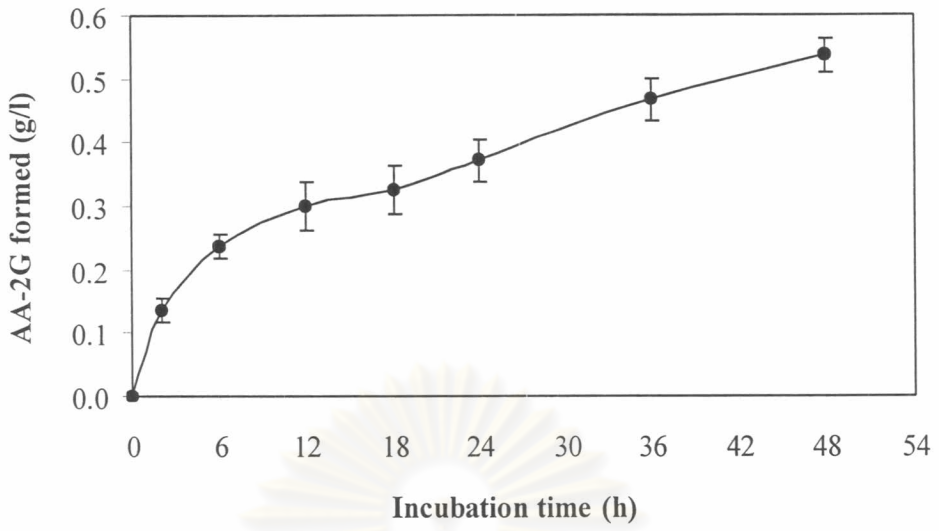


Figure 30. Time-course of AA-2G production with immobilized CGTase.

The immobilized CGTase on alumina (60 g wet, 350 U/g β-CD) was incubated with the mixture containing 4% (w/v) β-CD, 4% (w/v) AA Na and 0.2% (w/v) thiourea at 40°C, pH 6.0. The reaction was terminated by adding 1.06% metaphosphoric acid at the indicated time. Results shown were average values of triplicate experiments.

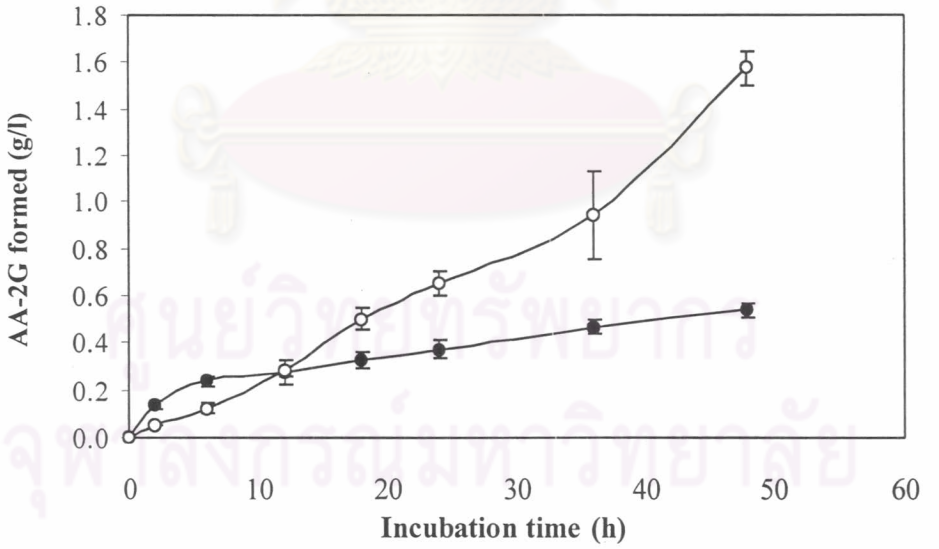


Figure 31. Time courses of AA-2G production by soluble (○) and immobilized CGTase (●). The soluble and immobilized CGTase on alumina (350 U/g β-CD) was incubated with the mixture containing 4% (w/v) β-CD, 4% (w/v) AA Na and 0.2% (w/v) thiourea at 40°C, pH 6.0. The reaction was terminated by adding 1.06% metaphosphoric acid at the indicated time. Results shown were average values of triplicate experiments.

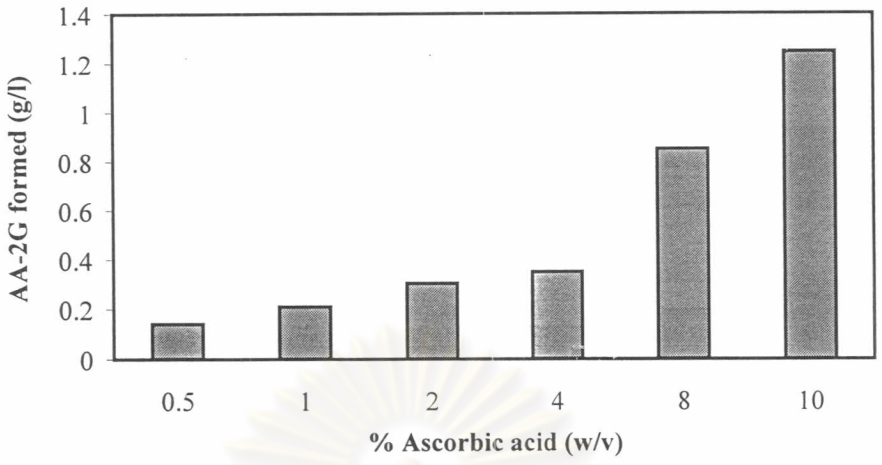


Figure 32. Effect of ascorbic acid concentration on AA-2G production by immobilized CGTase. The immobilized CGTase on alumina (60 g wet, 350 U/g β-CD) was incubated with the mixture containing 4% (w/v) β-CD, 0.5-10% (w/v) AA Na and 0.2% (w/v) thiourea with shaking at 40°C, pH 6.0. Results shown were average values of triplicate experiments.

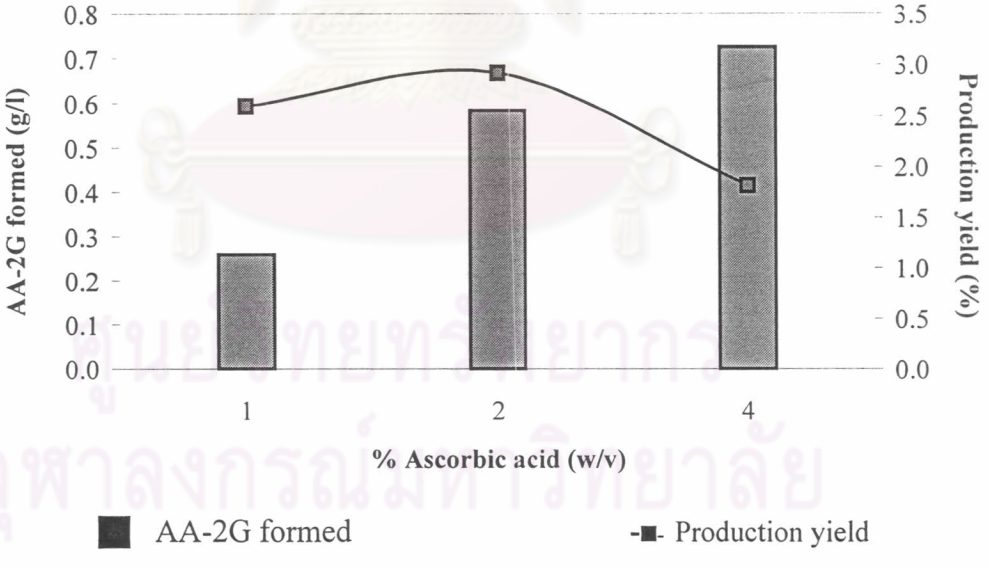


Figure 33. AA-2G production by immobilized CGTase at the optimal conditions.

The immobilized CGTase on alumina (60 g wet, 350 U/g β-CD) was incubated with the mixture containing 4% (w/v) β-CD, 1-4% (w/v) AA Na and 0.2% (w/v) thiourea at pH 5.0 with shaking at 40°C for 24 h.

The optimal conditions for maximum production of AA-2G were then to use 350 units / g β-CD of immobilized CGTase (60 g wet weight), 4% (w/v) β-CD, 2% (w/v) AANa, at pH 5.0 and 24 hours of reaction time (Figure 33). The content of AA-2G obtained under these conditions was 0.570 g/l and 2.85% of the total AA added. When the reaction mixture was hydrolyzed by glucoamylase (20 U/ml), the content of AA-2G increased about 30-40% (Figure 34).

3.6 Batch reusability of immobilized CGTase for AA-2G production

Since one of the advantages of immobilized enzyme is its repeated use, an attempt was then made to ascertain the possibility in the reusability of immobilized CGTase for the production of AA-2G. The reaction was repeated three times. As shown in Figure 34, it was found that the immobilized CGTase produce a good yield of the AA-2G in the successive cycles, with AA-2G production of 0.570, 0.435, and 0.283 g/l, respectively. The activity of first batch was taken as 100%. The decreases in activity on repeated use are given in Figure 34. The alumina-bound CGTase retained 74.4% of its original activity after 3 repeated uses.

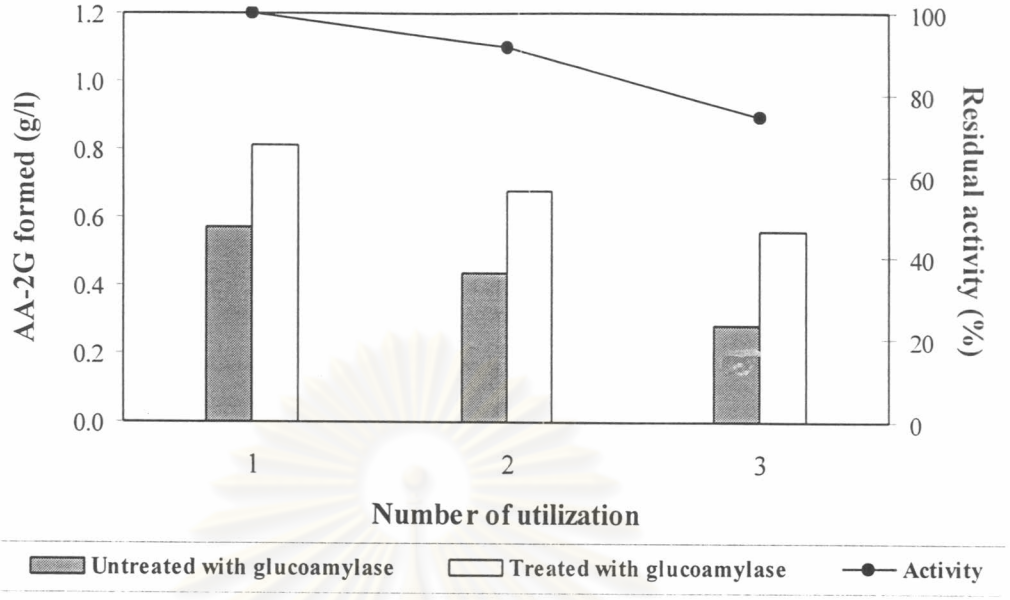


Figure 34. Batch reusability of immobilized CGTase on alumina for AA-2G production. The immobilized CGTase on alumina (60 g wet, 350 U/g β-CD) was incubated with the mixture containing 4% (w/v) β-CD, 2% (w/v) AA Na and 0.2% (w/v) thiourea at pH 5.0 with shaking at 40°C for 24 h.