CHAPTER IV

DISCUSSION

Ascorbic acid (AA) is susceptible to chemical and enzymatic oxidation in solution under aerobic conditions. The susceptibility of AA to oxidation limits its usage in the fields of pharmaceuticals, cosmetics and foods (Cort, 1982; Elliott, 1999). Hence, the enhancement of oxidative stability of AA is practically beneficial in industrial applications. 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) is the most promising AA derivative, because this vitamin C derivative shows enhanced stability against oxidative degradation in aqueous solution and exhibits vitamin C activity *in vivo* after its enzymatic degradation to free AA by α-glucosidase (Yamamoto, Suga, e*t al.*, 1990, 1992 and Kumano *et al.*, 1998). AA-2G has been synthesized from AA and maltose or other oligosaccharides by the transglucosylation enzymes such as mammalian and rice seed α-glucosidase (Muto, Nakamura *et al.*, 1990; Muto, Suga *et al.*, 1990 and Yamamoto *et al.*, 1990) and CGTase (Aga *et al.*, 1991; Jun *et al.*, 2001). Among various enzymes, CGTase seems to be most effective in terms of the reaction specificity and efficiency. As the CGTase catalyzes the stereoselective transglucosylation of AA to AA-2G, it is of great potential interest of large-scale preparation of AA-2G. The use of immobilized enzyme is one of the most effective ways to facilitate the industrial production of compounds via enzyme-catalyzed reaction. Hence, the attempt of this research is to prepare immobilized CGTase from *Paenibacillus* sp. A11 and test the feasibility of using immobilized CGTase for AA-2G production.

4.1 Purification of CGTase

One factor that affects the immobilization efficiency was the purity of an enzyme. The use of enzyme solution with a low degree of purity may limit the specific activity of the immobilized enzyme preparation (Messing, 1975). In enzyme immobilization using a crude enzyme, the other components besides enzyme such as the other proteins, peptides, and low molecular weight materials can be coupled to the support. The coupled materials may place the attached enzyme in a microenvironment very different from that of the coupled purified enzyme.

Different separation techniques were evaluated for CGTase purification from supernatant (Depinto and Campbell, 1968; Bender *et al.*, 1982). One common step in the purification procedures reported by the different laboratories was the use of starch adsorption, considering that the interaction between starch and the CGTase involves not only adsorption but also substrate enzyme affinity type binding (Pongsawasdi and Yagisawa, 1988). Immunoaffinity chromatography or ammonium sulfate precipitation and chromatography on DEAE-cellulose column were usually performed to further purify the enzyme (Kim, 1996; Laloknam, 1997).

CGTase from *Paenibacillus* sp. A11, isolated from South-East Asian soil by Pongsawasdi and Yagisawa (1987), was used as an enzyme source for immobilization. The CGTase produced was extracellular enzyme with β-CD as the major product (Techaiyakul, 1991). The enzyme was purified and characterized by our research group in the Department of Biochemistry (Techaiyakul, 1991; Rojtinnakorn, 1994 and Kim, 1996). In this work, the enzyme was partially purified by adsorption to corn starch followed by elution with the buffer containing maltose. Maltose which has higher affinity than starch will compete for binding to CGTase thus the enzyme can easily be separated and present in soluble fraction. After CGTase

was eluted and concentrated by ultrafiltration, it was partially purified 56 folds with 63 % yields. The result in high purification fold indicated that starch adsorption step could get rid of many other proteins. Therefore, this method seems to be sufficient for CGTase preparation used in immobilization experiments. Other investigators (Nakamura and Horikoshi, 1977; Rutchtorn, 1993 and Kuttiarcheewa, 1994) also described the uses of starch adsorption technique for CGTase preparation prior to the immobilization of the enzyme.

4.2 Optimization of the immobilization procedure

In previous study, Kuttiarcheewa (1994) demonstrated the successful utilization of immobilized CGTase on alumina for the continuous production of CDs. The CGTase covalently coupled on alumina showed high operational stability. Therefore, the covalent binding of enzyme to solid supports seems to be the appropriate method to prepare immobilized CGTase for the production of AA-2G. The immobilization procedure used in this research involved the silanization of the carrier surface and covalent coupling of enzyme molecule onto the support by the use of bifuctional agent glutaraldehyde. The schematic diagram shown in Figure 35 illustrates the different steps involved in carrier activation and immobilization of CGTase. γ-Aminopropyltriethoxysilane (APTS) was used as silanization agent to introduce amino groups onto the surface of inorganic carrier (step 1) and the resulting alkyamine derivative was activated by glutaraldehyde (step 2). Subsequently, the enzyme molecule was covalently attached to the carbonyl group of glutaraldehyde via a Schiff's base linkage (step 3) (Chibata, 1978). The depicted formula for glutaraldehyde means the monomer or the partially polymerized di-aldehyde. This method has the advantage of providing strong covalent bonds throughout the

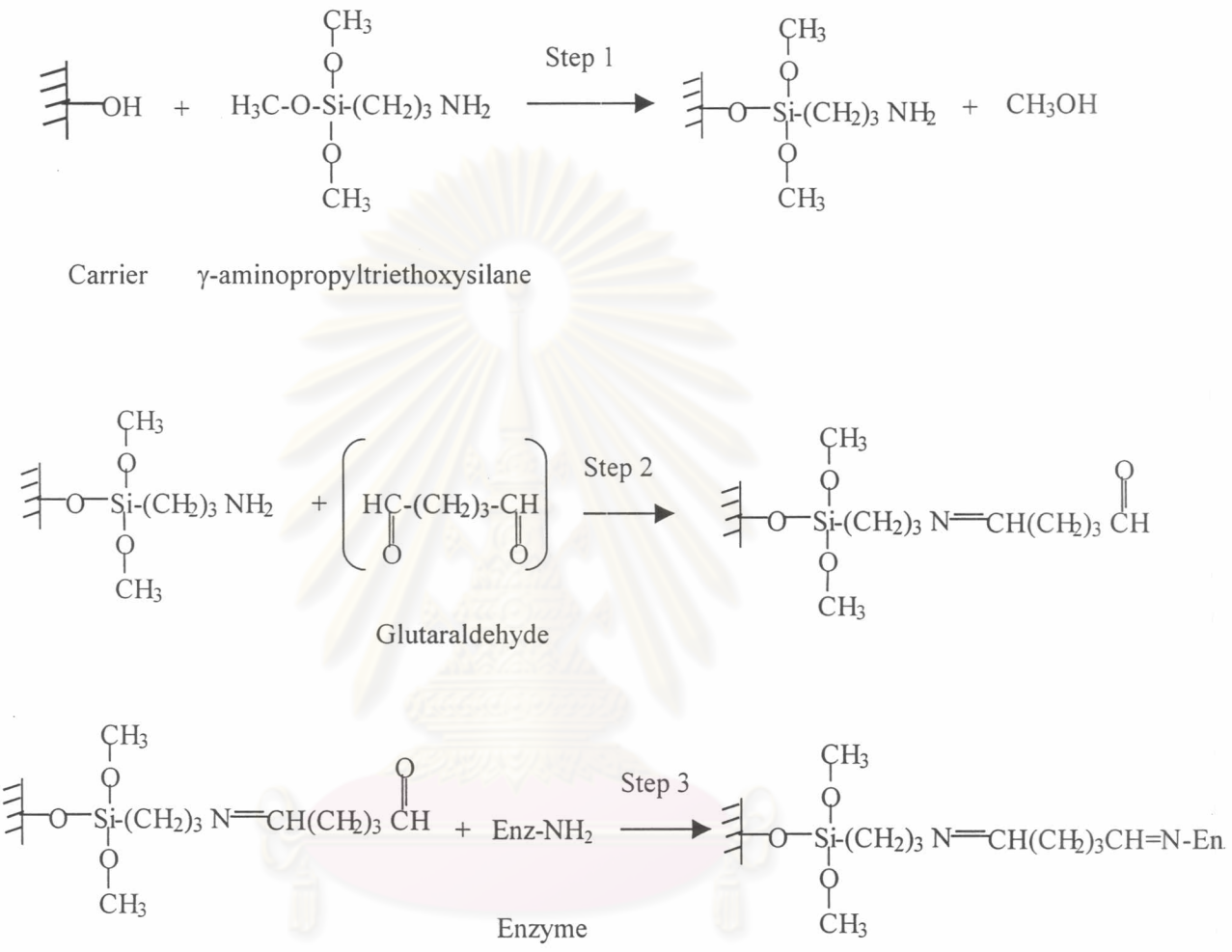

Figure 35. Carrier activation and covalent coupling to CGTase: (1), silanization of carrier surface; (2) activation of silanized carrier; (3) covalent immobilization.

conjugated product that do not lose enzyme into the surrounding solution, even in the presence of substrate and high ionic strength solutions (Kenedy and Cabral, 1987).

In this work, CGTase from *Paenibacillus* sp. A11 was covalently immobilized on various organic and inorganic supports including silica, alumina, activated carbon and chitosan to investigate the suitable support for enzyme immobilization. In the case of inorganic support, the surfaces of these supports have been modified by the silanization to provide moieties suitable for covalent attachment. A prime concern is that these materials should be cleaned and fully activated with respect to their functional groups prior to the immobilization process. Cleaning of the solid support from oils, dirt, detergent, etc., to generate reactive hydroxyl groups on the surface is critical for effective immobilization of enzyme. If the dirty supports were to be employed for covalent coupling, the loading of the silane agents would be extremely low and perhaps loosely bonded (Messing, 1978). Hence, the inorganic supports used in this study (silica and alumina) were soaked with 5% (v/v) HNO_3 (45 min, 100°C) and thoroughly washed with water prior to the incubation with APTS. Among various supports examined, high immobilization activities were obtained for CGTase immobilized on alumina (16.5%) and silica (15.6%) (Figure 9). However, silica did not seem to be an appropriate carrier, because the enzyme was also shown to physically adsorb well onto it. This may result in protein desorption from the carriers' surface during operation of the immobilized enzyme system. Therefore, alumina was chosen as the support for covalent immobilization of CGTase. Alumina offers several advantages include high mechanical strength, thermalstability, resistance to organic solvents and microbial attack, easy handling, excellent shelf-life and easy regenerate ability by simple pyrolysis followed by acid treatment (Messing, 1975). The above

characteristics of alumina make them a suitable support for the preparation of immobilized enzyme to be used in various industries.

The efficiency of CGTase immobilization on the alumina was affected by various parameters such as the amount of reactive groups on support surface, the contact time and the concentration of enzyme per support ratio. The amount of the reactive groups on the support surface involved in the coupling of enzyme onto it was the first factor to be considered. The degree of functionalization of support surface is related to the amount of alkylamino groups incorporated during incubation of the alumina with APTS. The results showed that it was enough to use low concentration of APTS (2% (v/v)) to achieve the highest immobilization yield on alumina (Figure 10).

Glutaraldehyde (GA) was employed as the crosslinking agent to attach the enzyme molecule onto the silanized surface of alumina. The optimum GA concentration was at the level of 1% (v/v), and the yield of immobilization decreased with the enhancement of GA concentration (Figure 11). This finding can be explained by assuming that the increase in GA concentration creates more bonds per enzyme molecule and, consequently, may cause molecular structure deformation. Also, at higher GA concentration the probability for covalent modifications close to the active site increases leading to enzyme inactivation. The GA concentration used in the coupling of enzyme onto alumina surface in this study (1% (v/v)) was 4 times higher than that reported by Kuttiarcheewa (1994), whose work was on the immobilization of *Paenibacillus* sp. A11 on alumina by covalent method. In this previous study, GA concentration was kept constant at 0.25% (v/v) and only APTS concentrations were varied. Their optimum concentration was to use 1% APTS and 0.25% GA to activate the support. However, the concentration of APTS and GA that have been reported are

normally in the range of 2-10% and 0.25-5%, respectively, depending on the enzyme and support employed (Puvanakrishnan and Bose, 1980; Wehtje *et al.*, 1988 and Yang and Su, 1989).

A major influence on the efficiency of CGTase immobilized on alumina was CGTase concentration exposed to the unit of support surface during immobilization process. It was found that the retaining activity of immobilized CGTase increased linearly up to 14 U/g support with the increment of enzyme concentration (Figure 12). At the enzyme concentration per alumina ratios above 14 U/g support, the immobilized enzyme activity did not increase due to the reactive groups on the support appear to be saturated. Moreover, high enzyme loading on the support generally leads to low immobilization yield due to steric hindrance preventing access of the substrate to the active sites of the enzyme and/or diffusional effect of the substrate to the immobilized enzyme molecule. Other enzymes were shown to be covalently linked to solid support by using identical procedure of immobilization, but the ratio of enzyme to support required and the amount of enzyme immobilized varied depending upon the enzyme and support used (Weetall, 1976).

The coupling time is the additional parameter that was investigated to efficiently immobilize CGTase on alumina. It was found that the retaining activities of the immobilized enzymes were notably dependent on the immobilization time. As shown in Figure 13, the coupling time in the range of 2-12 hours did not show much difference in the immobilization yield, except at 6 hours incubation which gave the highest immobilization yield of 31.2%. In some cases, a long incubation time may result in much higher operational stability of the immobilized enzyme due to increased multipoint attachment (Martin *et al.*, 2003). However, in this study it was

best to incubate the activated support with CGTase for 6 hours in order to achieve high immobilization yield on alumina.

According to the CGTase immobilization under the optimum conditions (Figure 14), the activity yield of the immobilized CGTase on alumina was calculated to be 31.2% which was 4.36 U/g carrier. Prior to the present investigation, Kuttiarcheewa (1994) had reported that the immobilization of *Paenibacillus* sp. A11 CGTase on alumina yielded the recovered activity of 70%. The difference in activity yield could be attributed to different method used in enzyme activity measurement and different source of alumina. Moreover, to confirm that the low activity of the immobilized enzyme observed was not due to low enzyme attachment, the amount of protein bound to the support was determined. This was done by subtracting the amount of unbound protein found in the filtrate and washings from the amount of CGTase used in the immobilization process. It was found that most of the protein added was bound to the support since hardly any CGTase was detected in the filtrate and washings (data not shown).

The loss of enzyme activity after immobilization can be caused by the modifications in the conformation of CGTase, which may prevent the proper conformational changes required for catalysis or the covalent coupling of certain lysine residue implicated in catalytic machinery of CGTase. Martin *et al.* (2002) reported that some of the lysines in the starch-binding site (Lys 547 and Lys 549) displayed the highest accessibly to the solvent, and these residues were possible targets for enzyme immobilization.

Table 10 summarizes previous reports on covalent immobilization of CGTases. As shown in Table 10, Most of the recovered activity of CGTase are lower than 10% except CGTase from *Bacillus* sp., *Bacillus macerans* and *Paenibacillus*

Table 10. Covalent immobilization of CGTases reported in the literature

Support	Organism	Recovered activity (%)	Activity (U/g support)	Reference
Akrilex C	*Bacillus macerans*	3.4-6.3	n.r.	Ivony *et al.*, 1983
Styrene-based resin	*Bacillus stearothermophilus*	≤ 0.8	≤ 2	Sakai *et al.*, 1991
Silochrome	Bacillus sp.	37-49	6	Abelyan and Afrikyan, 1992
Trisoperl	*Bacillus macerans*	25	0.7	Steighard and Kleine, 1993
PVC	*Paenibacillus macerans*	27-45	63-154	Abdel-Naby, 1999
Controlled pore silica	*Thermoanaerobacter* sp.	0.7	58	Martin *et al.*, 2002
CNBr-Sepharose	*Thermoanaerobacter* sp.	4.4	4U/ml gel	Martin *et al.*, 2002
EAH-Sepharose	*Thermoanaerobacter* sp.	2.4	2 U/ml gel	Martin *et al.*, 2002
Eupergit C	*Thermoanaerobacter* sp.	10.2	147	Martin *et al.*, 2003

n.r. = not reported

macerans which show a comparable recovered activity to that of our CGTase immobilized on alumina (31.2%). However, our immobilized activity per gram support (4.36 U/g carrier) was found to be comparable to most of the preparations except CGTase from *Paenibacillus macerans* and *Thermoanaerobacter* sp. This could be due to the difference in the sources of enzyme and the types of support used in the immobilization process.

4.3 Properties of the immobilized CGTase on alumina

The examination of the changes of enzymatic properties caused by immobilization of the enzyme provides useful information not only for the application of immobilized system but also for the elucidation of structure function relationships and mechanism of enzyme reaction. The procedure of enzyme immobilization on insoluble carriers has a variety of effects on the protein conformation as well as on the state of ionization and dissociation of the enzyme and its environment (Chibata, 1978).

As enzymes consist of protein, the catalytic activity is markedly affected by environmental conditions, especially the pH of aqueous media. The optimum pH of the enzyme after immobilization can be changed or unchanged. The change in optimal pH may depend on the charge in the enzyme molecule or in the support which has profound influence on pH activity behavior of an immobilized enzyme due to creation of microenvironment. The optimal pH of the CGTase immobilized on alumina exhibited a shift of 1.0 pH unit toward the alkaline values as compared to the free enzyme (from 6.0 to 7.0) (Figure 15). The optimal pH for both enzymes were coincided with that reported by Kuttiarcheewa (1994). Yang and Su (1989) described

differences between pH optimum for native and CGTase covalently coupled to chitosan that the optimum pH was shifted from 8.0 to 8.5. The change in optimum pH is usually explained by an alteration of the microenvironment of the enzyme due to immobilization or support. A possible explanation for this pH change should be that alumina behaves as a polyanion, producing an uneven distribution of hydrogen and hydroxyl ions between the area closed to the carrier surface and the remainder of the solution (Trevan, 1980). The positive charges clustering close to the immobilized enzyme resulted in a decrease of actual pH so that the apparent pH optimum of the immobilized CGTase became higher than that of the free enzyme.

The pH stability of the immobilized CGTase tested did not increase beyond that of the free form (Figure 16). Both enzymes were stable over a wide pH range of 5.0 to 9.0 upon incubation at room temperature for 60 minutes. The wide range in pH stability of the immobilized enzyme offers a valuable characteristic for use in industrial application. This influence of the pH on the CGTase bound alumina was corresponded to that observed by Kuttiarcheewa (1994). Moreover, an increase in pH stability after immobilization had been observed with CGTase immobilized on PVC (Abdel-Naby, 1999). Different pH profiles of the immobilized CGTase have been obtained depending on the type of carrier chosen and on the immobilization method applied (Ivony *et al.*, 1983; Lee *et al.*, 1991 and Abelyan and Afrikyan, 1992).

The catalytic activity of enzymes is also dependent on temperature, as in the case of ordinary chemical catalysts, but the activity is lost at temperatures above a certain limit due to the denaturation of enzyme protein (Chibata, 1978). The optimum temperature for CD-forming activity was at 60°C for both free and immobilized CGTase (Figure 17). The immobilization process with respect to the free enzyme introduced no major differences, only slight protective effect in the immobilized

enzyme was detected at temperature over 60°C. Kuttiarcheewa (1994) reported the optimum temperature for starch-degrading activity of immobilized CGTase on alumina shifted from 60°C to 55°C after immobilization. The difference in optimum temperature of the two immobilized CGTase could be due to the different reaction mechanisms of CGTase catalyzed reactions (Tachibana, 1999).

This behavior is extended to the energy of activation obtained (Figure 18), 3.62 kcal/mol for immobilized and 2.50 kcal/mol for the free CGTase. The higher value of the activation energy obtained for the immobilized CGTase indicated that the applied immobilization procedure introduced changes in the structure of the enzyme molecule which impeded the enzyme catalyzed reaction. The increases in activation energy were also reported previously for other immobilized CGTase (Lee *at el.*, 1991; Abdel-Naby, 1999).

Thermal stability of the immobilized enzyme was one of the most important criteria for its long-term and commercial applications. The immobilized CGTase on alumina showed a slight increase in thermal stability when compared to the free enzyme (Figure 19A). The immobilized CGTase retained its full activity up to 50 °C, whereas the free form retained only up to 40°C. Generally, enhancement of thermal stability on immobilization has been observed in many enzymes, but no correlation between thermal stability and immobilization method has yet been established. This characteristic depends on source of enzyme and immobilization process applied (Chibata, 1978).

As the conformational changes of the enzyme protein may occur upon immobilization and the affinity between enzyme and substrate may be changed, the investigation of kinetic parameters of the immobilized enzyme is very important. The kinetic constants for immobilized enzyme are usually different from those of

native form of enzyme which may be due to interaction of several factors like conformational changes, steric hindrance and diffusional effects (Chibata, 1978). The effect of substrate concentration on immobilized enzyme activity was plotted by the method of Lineweaver-Burk for estimation of Michaelis constant (K_m) and maximum reaction rate (V_{max}) (Figure 21-22). The K_m values for the cyclization activity using starch as substrate were determined to be 0.59 ± 0.25 mg/ml for the free enzyme and 5.62 ± 0.20 mg/ml for the immobilized enzyme. The apparent K_m of the immobilized enzyme was 10 times higher than that of the free enzyme for starch. The large increase in K_m in immobilized enzymes (reflects the reduction in affinity for the substrate) is mainly ascribable to the conformational changes which the protein undergoes following immobilization on the support and/or the difficulty of the substrate to diffuse toward the catalytic site (Chibata, 1978). The V_{max} value shows the reaction efficiency of enzyme to decompose the complex to products, hence this value can be used in the comparison of enzyme efficiency. From the result, it was found that the V_{max} value of immobilized CGTase (5.82 ± 0.13 U/ mg protein) was lower than that of the free form (9.69 ± 0.38 U/ mg protein) (Table 9). An increase in K_m value and a decrease in V_{max} of CGTases after immobilization process have been reported with CGTase covalently immobilized on chitosan (Yang and Su, 1989), and with CGTase attached to PVC (Abdel-Naby, 1999). It has been reported that the catalytic activity of the immobilized enzyme may be improved by the presence of its substrate which is believed to protect the active site of the enzyme during the immobilization process (Abdel-Naby, 1999). However, this needs to be further studied.

The storage stability of the immobilized enzyme preparations is an important additional factor that determines their usefulness. Hence, the stability of the

immobilized CGTase at 4°C and 25°C was studied (Figure 22). When the alumina-bound enzyme was stored at 4°C, it was found to be stable without any activity loss for more than 30 days and lost only 21% of its initial activity after 2 months. The result indicated that immobilization of CGTase on alumina increased the stability of enzyme preparation, probably resulting from stiffening of the protein tertiary structure.

4.4 Synthesis of AA-2G by *Paenibacillus* sp. A11 CGTase

CGTase is a unique enzyme that catalyzes the conversion of starch to CD by intramolecular transglycosylation. In the presence of a suitable acceptor (such as glucose or sucrose), glycosyl residues are transferred from starch or CD to the acceptor and form an α-1,4-glycosidic linkage (Kitahata, 1978). CGTase also catalyzes transglycosylation to various compounds other than saccharides, such as naringin (Kometani *et al.*, 1995) and hesperidin (Kometani *et al.*, 1994). From above interesting characteristic, this enzyme is very useful for functional modification of natural bioactive compounds to improve their physiochemical properties (Sato *et al.*, 1991; Shibuya *et al.*, 1993 and Kometani *et al.*, 1994).

Kitahata *et al.* (1978) reported that the structure of an effective acceptor of transglycosylation by CGTase is the pyranose structure having the same configuration of the free C2- and C3- and C4-hydroxyl groups as D-glucopyranose. In this sense, the structure of AA is quite different from the structures of effective acceptors they mentioned.

Nevertheless, in 1991, Aga and his co-workers reported that AA-2G was efficiently synthesized from AA and α-CD by regioselective transglucosylation with CGTase from *Bacillus stearothermophilus*. In the presence of an acceptor such as AA,

CGTase first cleaves the ring of α-CD and simultaneously transfers a decyclized α-CD, namely maltohexaose unit, to the C-2 position of AA, resulting in the formation of AA-2G_6. After that CGTase exhibits an exoglucosidase activity on its oligosaccharide moiety of AA-2G_n (Tanaka *et al.*, 1991).

Although, there have been a few reports on AA-2G formation, there has yet been no report on the exploitation of immobilized enzyme to synthesize AA-2G.

The transglycosylation to AA by free CGTase from *Paenibacillus* sp. A11 was first investigated according to previous report (Wongsangwattana, 2000) because the enzyme from the same source was used. In this work, β-CD, a good substrate for coupling activity reported by Tongsima (1998), was used as glycosyl donor. However, transglycosylated products were not observed then. This could be due to the difference in the incubation time used including different elution condition. Aga *et al.* (1991) reported that the transglycosylation to AA was performed in the reaction mixture containing AA and α-CD by incubating the enzyme and substrates for 24 hours. Hence, the incubation time was increased to 24 hours. Under this condition, AA-2G was synthesized and identified, by comparing the retention time with that of the AA-2G standard, from the reaction mixtures containing β-CD and AA by the action of *Paenibacillus* sp. A11 CGTase (Figure 23). Although the peak at the same t_R as AA-2G was also observed in the control experiment where CGTase was not added, the peak area was much lower. Additional five more peaks observed only in the reaction mixture containing CGTase could be other transglycosylation products. These were assumed that maltooligosaccharides are combined with the C-2 hydroxyl group of AA (Tanaka *et al.*, 1991). In addition, in the separation of AA and AA-2G or transglycosylation products, the HPLC analysis was performed on a reversed phase C_{18} column. The separation of the compounds is based on their polarities, in which

several physicochemical phenomena, including hydrophobic interactions, are involved. The stationary phase comprised of octadecyl group (C_{18}) which is highly nonpolar. Therefore, the compound with less polarity was eluted with higher retention time. As shown in HPLC chromatograms (Figure 23 and 24), the AA-2G was eluted after AA due to the fact that its glucose moiety can interact with the stationary phase. In the case of the other transglycosylation products (AA-2G_n), there are more glucose units attached. Thus, it took longer time to elute the AA-2G_n. The HPLC profiles of AA-2G_n production here were consistent with those of *Bacillus stearothermophilus* CGTase (Tanaka *et al.*, 1991), *B. circulans* and *B. macerans* CGTase (Aga *et al.*, 1991) and *Paenibacillus* sp. CGTase (Jun *et al.*, 2001). Nevertheless, an attempt was made to distinguish the A-2G from an unknown product by using a longer column. AA-2G was clearly identified. The unknown compound could be the degradation of AA due to its instability at high temperature and pH. It was also found that the degraded product increased with reaction time (data not shown). However, this product needs to be further identified.

4.5 Synthesis of AA-2G by immobilized *Paenibacillus* sp. A11 CGTase

The HPLC profiles of AA-2G production by immobilized CGTase corresponded to those obtained with soluble enzyme. The unknown peak which had the t_R close to the AA-2G and the peak of another transglycosylation product were also observed (Figure 26). Since the t_R of AA-2G and the unknown compound were so similar, the reaction mixture was then treated with glucoamylase. After treatment, the peak of AA-2G_n disappeared with increasing amount of AA-2G while the unknown compound remained unchanged (Figure 27). These results confirmed that the transglycosylation products were AA-2G and probably AA-2G_2. A series of

transglycosylation products were not observed as with the case of soluble enzyme which could be due to the short HPLC running time.

4.6 Suitable conditions for AA-2G production by immobilized CGTase

The yield of the reaction depends on various factors such as the concentration of donor, acceptor, enzyme, pH, temperature and reaction time.

The optimum pH for AA-2G production was found to be 5.0 (Figure 28) which was the same as in the case of rat and rice-seed α-glucosidase (Muto *et al.*, 1990; Yamamoto *et al.*, 1990) and *Bacillus stearothermophilus* CGTase (Tanaka *et al.*, 1991). This indicates that it may be due to the dissociation of AA in aqueous solutions. This value was lower than that of β-CD forming activity of CGTase (Figure 15). AA contains two acidic protons with pKa values of 4.25 and 11.79 for the 3- and 2- hydroxyls, respectively (Muto *et al.*, 1990) and its dissociation and stability vary with pH.

The reaction temperature of 40°C was chosen due to maximum amount of AA-2G achieved (Figure 29) and also enzyme stability. At 50°C, the amount of AA-2G formed decreased, possibly because of enzyme inactivation. Another possible explanation is the degradation of AA-2G at high temperature. When operating the reaction at 30°C, the amount of AA-2G was lower which could be explained by low enzyme activity at lower optimum temperature.

The optimal incubation time was found to be 24 hours (Figure 30). The result in Figure 30 showed that the amount of AA-2G formed increased as the incubation time was prolonged. However, longer incubation time may cause further degradation of AA.

For the determination of the effect of donor and acceptor on AA-2G production, the AA concentrations were varied, whereas the β-CD concentration fixed at 4% (w/v) (Figure 25). At concentration higher than 4% β-CD has low solubility. The concentrations of the donor used that have been reported are generally high. Tanaka *et al.* (1991) reported the use of 12.8% α-CD as donor in the transglycosylation to AA by CGTase. The production of AA-2G catalyzed by *Paenibacillus* sp. CGTase was investigated in the reaction mixture containing 7% soluble starch and 3% ascorbic acid (Jun *et al.*, 2001). Although the amount of AA-2G formed increased with increasing concentration of AA, the production yield was not significant different and 2% AA was suitable for AA-2G production.

Under the optimum condition, the immobilized CGTase produce AA-2G with the yield of 2.85% (0.570 g/l) (Figure 33). The production yield obtained by the use of immobilized enzyme was lower that that obtained by soluble CGTase (Figure 31). This could be due to the adsorption of the compound in the reaction mixture onto the immobilized supports which may have blocked its active site. Thus low product was observed. To test whether the compounds were adsorbed onto the immobilized carrier, the immobilized enzyme was then washed after the production of AA-2G. The content in the washing solution was then determined and it was found that AA and AA-2G adsorbed on immobilized CGTase (data not shown). In mass production of AA-2G, glucoamylase was used to hydrolyze other transglycosylation products (Aga *et al.*, 1991). Thus, the yield of AA-2G could be improved by 30-40% (Figure 34).

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

One of the most important criteria for evaluating the possibility of a practical application of the immobilized enzyme is its reusability. The alumina bound CGTase retained 74.4% of its original activity after 3 repeated uses (Figure 34). It was found that the activity of immobilized CGTase drastically lost after being used for three times. This could be attributed to the leakage of enzyme from the immobilized preparation during each operation and/or the adsorption of substrate onto the immobilized enzyme can block the active site of enzyme toward the substrate. This corresponded to the decrease in production yield of AA-2G from each batch.

CGTase can be of great value in various industries for production of CD and various glucosylated compounds. In this study, CGTase was covalently immobilized on alumina for the production of AA-2G. The storage stability of the immobilized enzyme was considerably higher compared to the free enzyme. Despite this advantage, however, the possible industrial applications of the immobilized CGTase on alumina are limited by the fact that the substrate and the product were adsorbed onto it which led to the loss of product. Washing the immobilized enzyme with the reaction buffer after the first cycle of AA-2G production can elute the AA-2G and also AA. AA-2G recovered from the alumina immobilized CGTase was approximately 50% of AA-2G content in reaction mixture (Appendix F). From these preliminary studies, the washings can overcome the adsorption problem. The washing solutions containing AA (after separation of AA-2G) can be reused as the substrate in the next cycle of AA-2G production. Other promising solution to this problem is the alternative use of immobilization support which gives low product adsorption. Further studies need to be conducted with enzyme immobilization on other supports suitable for the production of AA-2G.