CHAPTER IV

DISCUSSION

4.1 The optimum culturing condition for high glucansucrase production

Glucansucrase (EC 2.4.1,GS) is a family of the enzyme glucosyltransferase consisting of subfamilies of dextransucrase, mutansucrase, alternansucrase and reuteransucrase (Hijum *et al.*, 2006). The glucosyltransferase is one of the most studied groups of enzyme, and there is increasing interest in its application for the synthesis of novel sugars and oligosaccharides (Lopez-Munguia *et al.*, 1992).

Microbial sources for GS enzyme, such as *Leuconostoc mesenteroides*, *Lactobacillus sp.* and *Streptococcus sp.* From previous have been reported, by many investigators.

Bacillus licheniformis TH4-2 was screened from Thailand 's soil and used for the production of thermoactive levansucrase (Ammar *et al.*, 2002). In this study, the focus is on glucansucrase activity of *Bacillus licheniformis* TH4-2. The culturing condition was optimized for high production of glucansucrase. Optimization of carbon source, cultivation temperature and cultivation time was performed. The effect of carbon source on growth and glucansucrase production was followed. The result showed that, soluble starch from cassava could be used to replace soluble starch from potato. This could minimize the cost of culturing since soluble potato starch must be imported while cassava starch is a cheap agriculture material in the country. In

addition, when used starch from cassava, the GS activity was highest, two and eight times higher than when used soluble starch from potato and sucrose, repectively. When varied the concentration of carbon source , 5 % (w/v) soluble starch from cassava gave highest GS production and it was two times higher than when 2 % or 10 % was used. In previous have been report, glucose as a carbon source was used for glucansucrase production from *Leuconostoc mesenteroides* (Moon *et al.*, 2007; Seo *et al.*, 2005)

Temperature is one of the most important factors that influence growth rate and enzyme production of the bacteria. For *Bacillus licheniformis* TH4-2, growth was not much different at 45 °C or 50 °C but GS activity was only observed at 45 °C, no activity at all was detected at 50 °C. In previous have been report, large-scale production of glucansucrase (dextransucrase) involves only *Leuconostoc mesenteroides* strains. Their optimal growth temperature is 25 – 30 °C (William and Wilkins, 1980).

4.2 Purification of glucansucrase

We found that, GS from *Bacillus licheniformis* TH4-2 is an extracellular enzyme. The enzyme was secreted out of the cell into the medium and the crude GS was purified by ammonium sulfate precipitation. This method based on the solubility differences in salt is frequently used to separate protein in early stage of purification protocols. Ammonium sulfate is always the salt of choice because it combines many useful features such as salting out effectiveness, pH versatility, high solubility, low heat of solution and also low price (Bollag *et al.*, 1996). The result showed that 30-60% saturated ammonium sulfate could precipitate most of the enzyme. In this step, 67 % of protein was removed, however, 10 % of enzyme activity was lost.

Most purification protocols involve some forms of chromatography, which has generally accepted as an essential tool in protein purification. In this work, DEAE-cellulose, an anion exchanger was used. The column matrix is a synthetic polymer containing bound cationic group. The enzyme was eluted from the column with stepwise of 0.1-0.3 M NaCl indicated that the net charge of the enzyme is negative at pH 6.0. In this step, approximately 95 % of the protein was removed, however, 27 % of enzyme activity was lost as compared to the previous step of ammonium sulfate precipitation .

In this work, Sephadex G-100 column was used in the last step of purification. The result showed that, 86 % of other contaminated proteins was removed and 58 % GS activity was still remained as compared to the step of DEAE-cellulose column. The success of enzyme purification using Sephadex G-100 column was judged by the homogeniety of GS on non-denaturing electrophoresis gel. The activity staining showed the single red band based on the reduction of TTC to keto sugars (Gabriel and Wang, 1968), the result of which confirmed that the protein band we purified was a sucrase.

In the separation by Sephadex G-100 column, GS peak was eluted closed to the void volume (Fig 3.6) which was not in agreement with the size of this enzyme determined by SDS-PAGE (Fig3.9). Since GS was reported to be a glycoprotein (Ghazi *et al.*, 2006), retardation in a Sephadex column is expected. Thus, calibration for native molecular weight was not determined. The last broad A_{280} peak in the column profile came out after $K_2Cr_2O_7$ was surprising, however, this peak was proved by Bradford method not to be a protein.

It can be concluded that GS form *Bacillus licheniformis* TH4-2 was purified to 112 fold with 28 % yield from cell free extract by the 3 steps of purification in this study.

From other works, GS from *Leuconostoc mesenteroides* B-1299CB, an extracellular enzyme. The supernatant was concentrated using membrane filtration (MW 30 kDa cut-off). The concentrated enzyme was purified by DEAE-Cellulose (Moon *et al.*, 2007; Kim and Kim, 1999). *Streptococcus sobrinus* was able to produce extracellular glucansucrase. The supernatant was concentrate by ultrafiltration. The concentrated enzyme was purified by three volumn of -20°C ethanol were mixed into the solution and the precipitates subjected to hydroxyapatite chromatography and FPLC Gel filtration (Lee *et al.*, 1997). *Leuconostoc mesenteroides* NRRL B-1299 was able to produce intracellular dextransucrase. The cells has been purified by adsorption on hydroxyapatite, followed by chromatographies on DEAE- cellulose and DEAE-Sephadex (Kobayashi and Matsuda, 1975). *Streptococcus bovis* 148 was able to produce extracellular dextransucrase. The supernatant was concentrated and salted out with ammonium sulfate at 80% saturation. The crude enzyme was applied to chromatography on Butyl-Toyopearl 650S and DEAE-Sephacel column (Takagi *et al.*, 1994).

4.3 Characterization of purified glucansucrase

The molecular weight of the enzyme was estimated to be 64 kDa by its mobility in SDS-PAGE compared with those of standard proteins. From previous report, the molecular weight of bacterial glucansucrase was reported to be in the range of 64-185 kDa (Gonzalez *et al.*, 1999). The molecular weight of purified dextransucrase from

Leuconostoc mesenteroidea NRRL B -1299 was 69 kDa (Kobayashi and Matsuda, 1975) and the molecular weight of purified dextransucrase from *Leuconostoc mesenteroidea* NRRL B -512F was 65 kDa (Kobayashi and Matsuda, 1980). Thus, the molecular weight of GS from our study was in the range of those enzymes previously reported.

This GS showed highest activity at pH6.0 with broad optimum activity profile from pH 6.0 to pH 7.0. This was similar to GS from *Streptococcus mutans* FA1 (Scales *et al.*, 1975). From previous reports, most of GS had optimum pH between 5.2-6.8 (Kubik *et al.*, 2004 ; Robyt and Kitaoka, 1998).

The optimum temperature of GS from *Bacillus licheniformis* TH4-2 was 45 °C for the hydrolysis reaction. The activity dropped about 30 % and 70 % at 50 °C and 55 °C, respectively. This was similar to GS from *Leuconostoc mesenteroides* NRRL B -1299 (Kobayashi and Matsuda, 1975). However, from previous reports, most of GS had optimum temperature between 25-30°C (Dols *et al.*, 1998; Kubik *et al.*, 2004 ; Moon *et al.*, 2006).

The Michaelis constant (K_m) and the maximum velocity (V_{max}) for sucrose hydrolysis activity were calculated, the K_m of glucansucrase was 38.14 mM for sucrose and the V_{max} was 0.042 umole/min. However, The K_m values of glucansucrase from different bacteria were reported to have different values such as the enzyme from *Leuconostoc mesenteroidea* NRRL B -1299 (10.7 mM) (Kobayashi and Matsuda, 1975) and from *Streptococcus mutans* FA1 (55 mM) (Scales *et al.*, 1975).

4.4 Synthesis and detection of prebiotic oligosaccharide products

From previous studies on OS synthesis have been reported. Synthesis of glucosyl melibiose using dextransucrase from *Leuconostoc mesenteroidea* NRRL B -512F was performed and the product was identified as Gul α1-4 Gal α1-6 Glu. Another study on the synthesis of glucosyl melibiose using alternansucrase from *Leuconostoc mesenteroidea* NRRL B -21297 was performed and the product was identified as Glu α1-3 Gal α1-6 Glu (Fu *et al.*, 1990). In addition, other OS products were obtained when used different saccharides as glucosyl acceptor. Such as, raffinose (Gal α1-6 Glu β1-2 Fruc) the product was identified as Glu α1-3 Gal α1-6 Glu β1-2 Fruc and Glu α1-4Gal α1-6 Glu β1-2 Fruc (Neely *et al.*, 1959). For gentiobiose (Glu β1-6 Glu), the product was identified as Glu α1-6 Glu β1-6 Glu. In the production of novel OS from sucrose donor and cellobiose acceptor by alternansucrase, the product was identified as Glu α1-2 Glu β1-4Glu (compound A), Glu α1-6 Glu β1-4 Glu (compound B), Glu α1-6 Gul α1-6 Glu β1-4 Glu (compound D) and Glu α1-3 Glu α1-6 Glu α1-6 Glu β1-4Glu (compound C). Compound B, C and D were never have been reported (Arguello *et al.*, 2000).

In this study, we found that after incubation of glucansucrase (GS) with sucrose donor and various saccharides as glucosyl acceptor, the results showed that GS from *Bacillus licheniformis* TH4-2 was able to transfer the glucose moiety of sucrose donor to other saccharides as glucosyl acceptor. However, G1 could not act as acceptor. From previous report, the GS showed broad acceptor specificity and disaccharides were more favorable glucosyl acceptors than monosaccharides (Park *et al.*, 2003). From this study, G2 to G7, lactose, melibiose, cellobiose, raffinose and palatinose were able to act as acceptors and their transferred acceptor products were observed on

TLC plate. The expected oligosaccharide (OS) products were detected by TLC, this chromatography was used to basically detect the synthesized OS products. In our system, various sugar used as donor, acceptor and OS products are polar compounds, then adsorbed with siliga gel. They slightly moved from the origin by the non-polar mobile phase (n-propanol : ethyl acetate : water, 7:1:2,v/v). Among tested sugars, melibiose was one of the best glucosyl acceptor judging from clearly observable and resovable product spots, and melibiose is interesting because the structure was consisted of (Gal α1-6 Glu). Though the synthesis of glucosyl melibiose using GS from *Leuconostoc mesenteroidea* had been reported and structures of products were identified as Glu α1-4 Gal α1-6 Glu and Glu α1-3 Gal α1-6 Glu (Fu *et al.*, 1990). However, from our preliminary identification of our products from melibiose acceptor, the product was resistant to glucoamylase, thus it might be a novel OS. Since glucoamylase catalyze the hydrolysis of Glu α1-4 Glu glycosidic lingkage, the structure of product from melibiose acceptor might be glucosyl melibiose with α1-6 glycosidic linked which has never been reported. Thus, we decided to use melibiose as acceptor for glucosyl transfer by GS from *Bacillus licheniformis* TH 4-2 in this work.

From TLC chromatogram (Fig 3.14), the one spot of product from melibiose acceptor was detected. While the reaction products analyzed by HPLC showed the peak of products from melibiose acceptor at Rt 8.3min (named product A), and the peak of product from sucrose, not from melibiose acceptor at Rt 7.1 min (named product X). It might be possible that, product X at Rt 7.1 min had Rf equal to melibiose acceptor on TLC chroatogram, thus the spot of product X overlapped with spot of melibiose acceptor.

4.5 Optimization of transglucosylation reaction

In order to produce higher yield of OS product, the synthesis condition was optimized. Parameters optimized were ; concentration of melibiose acceptor, sucrose donor, enzyme (unit/ml), pH, temperature and incubation time. After optimization, the result showed the optimum condition for OS production was 15% (w/v) melibiose (acceptor), 5% (w/v) sucrose (donor) , GS concentration of 5U/ml at pH 6.0 , 45°C for 24 hours. Two products were obtained from melibiose acceptor, main product (product A at Rt 8.3 min) and minor product (product B at Rt 10.3 min) with the yields of 17.2% and 3.3% , respectively.

In the first step for optimization, when incubating 0.5 unit/ml of glucansucrase with 5 % (w/v) sucrose donor and various concentrations of melibiose 0-22.5 % (w/v) in 20 mM acetate buffer pH 6.0 at 45 °C for 24 hours, we found that, at 0% melibiose two products were found at Rt 7.1 min (product X) and Rt 8.9 min (product Y). Thus, these two products were expect to be from sucrose donor. Previous report about the substrate specificity of the GS in term of "self- transfer of glucose moiety" is used when a sucrose substrate acts simultaneously as both glucosyl donor and acceptor. The enzyme catayzes glucosyl transfer to produce the transferred product from sucrose such as isomalto oligosaccharides (IMO) (Tanrisevan and Dogan, 2002). After that, when increased melibiose concentration to 12.5% (w/v), product X and Y were disappeared. The resulte showed, in contrast with the yield of product A at Rt 8.3 min which was increased when increased melibiose concentration from 1-22.5% (w/v). These might be possible that, at high concentration of acceptor, the transglucosylation reaction from sucrose donor to acceptor was induced.

For the effect of pH on transglucosylation reaction, previous have been reports showed that most GS has high activity at pH 5.0-7.0. No activity is observed below pH 4.0 and above pH 8.0. In this study we found that, GS showed highest activity for the OS synthesis at pH 6.0 and showed broad optimum activity profile from pH 6.0 to pH 7.0 This was similar to GS from *streptococcus mutans* FA1 (Scales *et al.*, 1975) and *Leuconostoc mesenteroides* (Kubik *et al.*, 2004 ; Robyt and Kitaoka, 1998).

For the effect of temperature on transglucosylation reaction, most GS has the rather uniform range of optimal temperature for glucan and OS formation (25°C -30°C) (Tanrseven and Dogan, 2001; Morales *et al.*, 2000). One striking feature is that the synthesis of Glucan and OS by glucan occurs at low temperatures or at room temperature , though the apparent activity determined in terms of fructose-releasing activity in the hydrolysis reaction is fairly small at lower temperature (Song and Rhee, 1993).

In this study we found that, GS showed highest activity for the hydrolysis activity and OS synthesis at the temperature of 45 °C. However, the yield of products obtained when incubation was performed at 40°C-50°C was not significantly different.

4.6 Larger scale preparation and isolation of glucosyl melibiose

To prepare higher amount of glucosyl melibiose products for characterization, 10 ml reaction mixtures were performed. The glucosyl melibiose products were isolated from the mixture of reaction products using the Sephadex LH-20 column. Sephadex LH-20 is prepared by hydroxyl propylation of Sephadex G 25, a bead-

formed dextran medium, and has been specifically developed for gel filtration of natural products, such as sugars steroids, terpenoids, lipids and low molecular weight peptides, in organic solvents (GE Healthcare, 2006). The components in the reaction mixture were separated due to their different properties in size and polarity. From Sephadex LH -20 column profile of reaction products (Figure 3.24), the fractions eluted with 70% n-propanol were from fractions 1-150. The fractions in the range of 97-145 containing reducing sugars as Somagyi-Nelson's method revealed two peaks (Figure 3.24). However, this method cannot identify type of sugars in the reaction mix ture because many sugars had reducing property. Thus, the fractions in the range of 97-145 were identification by HPLC. The result show that, three main peaks (I, II, III) were observed ; they were identified as product A , melibiose acceptor, glucose and fructose, respectively. Glucose and fructose could not be separated by the HPLC column in the condition used. In addition, no peak of sucrose donor was observed because sucrose was hydrolyzed by invertase and became glucose and fructose before applied to the column and then melibiose acceptor was pooled and concentrated and kept for reuse.

Separation of OS products by chromatographic technigue is allowed as OS has higher molecular weight than sucrose , fructose and glucose , they can be separated by gel-filtration chromatography e.g Bio-Gel P2 (Ammar *et al.*, 2002). In another report, sucrose transfer products were isolated by alkali-degradation followed by charcoal column chromatography using 20%(v/v) ethanol, then purified by ion exchange and Biogel P-2 gel permeation chromatography (Young Lee *et at.*, 2003).

4.7 Characterization of Product

The molecular weight of the product A at Rt 8.3 min was estimated to be 504 daltons by ESI-TOF mass spectrometry with [M+Na]+ at m/z of 527 (Figure 3.25). This corresponded to the size of a trisaccharide containing melibiose (Gal α1-6 Glu), and one molecule of glucose attached. For the structural elucidation by NMR, the result suggested that, glucosyl residue was connected to melibiose acceptor by α-1, 6 linkage.

4.8 Determination of biological activity of product

Growth of *Lactobacillus acidophilus*, a probiotic organism, was monitored in MRS medium supplemented with various carbon sources at a concentration of 2 % as described in section 2.16.1. The result showed that *Lactobacillus acidophilus* grew maximally on glucose (positive control), with overnight culture reaching A_{600} level of more than 6.0 (Fig 3.30A). In MRS medium supplemented with melibiose, FOS (commercial) and product A, this probiotic organism could grow better than the negative control with A_{600} reaching 1.0-1.2 at 24 hours. The lowest growth (overnight cultures reaching A_{600} level less than 0.6) was observed in the medium with no C source added (negative control) and the medium when raffinose was added.

The result indicates that product A can support significant growth of *Lactobacillus acidophilus* but at a lower rate than melibiose and FOS (commercial). Raffinose, on the contrary, did not support growth of this bacteria. FOS and raffinose are prebiotics, while melibiose acceptor is chassified as digestible sugar. For our trisaccharide product, it should be considered as prebiotic according to the structure and linkage.

The study on identification of suitable prebiotics for synergistic combinations with representative probiotics from the lactobacilli and bifidobacterium genera was reported (Su *et al.*, 2007). Eleven different types of commercially available carbohydrates and compounds were screened in basal media for their effects on the growth of the probiotic cultures *Lactobacillus acidophilus* LAFTIs L10 (L10), *Bifidobacterium animalis lactis* LAFTIs B94 (B94) and *Lactobacillus casei* LAFTIs L26 (L26). The results showed that all three probiotic strains were able to utilize a range of carbohydrates. Maximal growth of L10 was observed in basal medium supplemented with soybean oligosaccharide (SOS), followed by fructooligosaccharide (FOS) and inulin, while maximal growth of B94 was observed in basal medium supplemented with SOS, followed by raffinose, FOS, β -glucan hydrolysate, inulin and Fibregum. The strain L26 was shown to exhibit maximal growth in basal medium supplemented with FOS followed by inulin, SOS, β-glucan hydrolysate and concentrate.

In conclusion, synthesis of glucosylmelibiose was obtained and optimized, via catalysis of the enzyme glucansucrase. However, identification of the enzyme has not been conclusive since previous in formation shows no GS gene in *Bacillus*. The enzyme we purified and used in this study may be α-glucosidase, of which a novel type that can catalyse glucosyl transfer from sucrose donor has been recently reported in *Archachatina ventricosa* (Soro *et al.*, 2007), *Bacillus subtilis* (Schonert *et al.*, 1999). This has to be proved in our further study. However, the oligosaccharides product synthesized in this work was likely to be Glu α1-6 Gal α1-6 Glu, a novel prebiotic.