5. Preliminary study of β-1,3-1,4-glucanase purification

5.1 Pervaporation

The method of pervaporation with dialysis tubing was conducted with a comparison of the effect of evaporation temperature at 37˚C and 55˚C on enzyme recovery. The enzyme was evaporated until the volume was decreased from 100 ml to 12.5 ml and then remaining activity was assayed. The results are shown in Table 13. Ninety percent of the activity was recovered at both temperatures. However, only 6 h was used when concentrated at 55˚C, while longer time of 9 h was needed for the enzyme concentration at 37˚C. Therefore, pervaporation at 55˚C was used for further purification. This step could be used for only concentrated the enzyme but disadvantage for the purification.

Table 13 Effect of the temperature on enzyme pervaporation

Enzyme	Volume (ml)	Activity (U/ml)	Total activity (U)	Recovery (%)
Crude	100	2.22	222.00	100
Evaporated at 37˚C	12.5	15.96	199.50	89.78
Evaporated at 55˚C	12.5	15.95	199.38	89.71

5.2 Gel-filtration

5.2.1 Sephacryl S200

To find out the molecular weight of β-1,3-1,4-glucanase, a Sephacryl S200 column (2.5 x 37.5 cm) equilibrated with 50 mM citrate phosphate buffer pH 5.5 was used. The void volume of 93.5 ml was determined by using blue dextran (Figure 21).

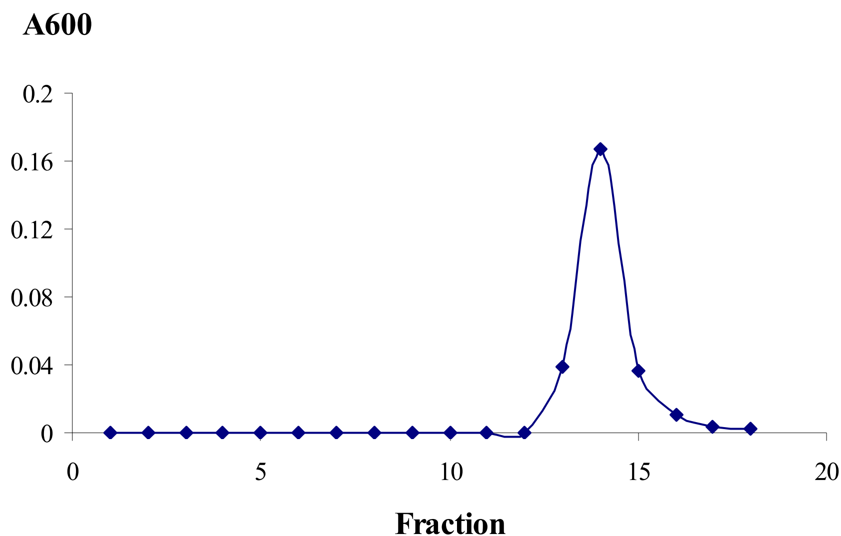

Figure 21 Sephacryl S200 chromatography of blue dextran (2.5 x 37.5 cm column, equilibrated with 50 mM citrate phosphate buffer pH 5.5, with flow rate 14.6 ml/h).

Standard curve of molecular weight of the proteins was determined from the K_{av} (Table 14 and Figure 22). The K_{av} value was calculated from the relationship parameters of below equation:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

V_e: the elution volume

V_t: the bed volume = 1841.52 ml

V_o: the void volume = 93.5 ml

Table 14 Log MW, V_e and K_{av} of standard proteins from Sephacryl S200 chromatography

Protein	MW	Fraction (max.A280 value)	log MW	V_e	K_{av}
Ribonuclease A	13,700	23	4.14	154.1	0.0345
Chrymotrypsinogen A	25,000	22	4.40	147.4	0.0307
Ovalbumin	45,000	19	4.65	127.3	0.0192
BSA	67,000	18	4.83	120.6	0.0153
Aldolase	158,000	16	5.20	107.2	0.0077

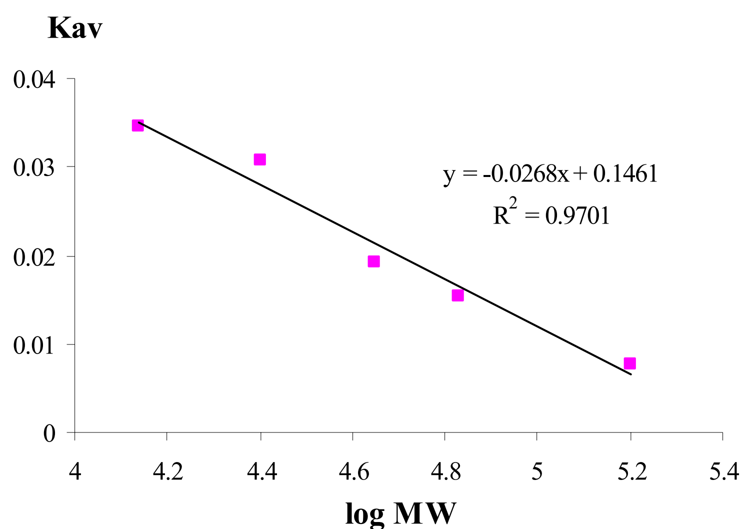

Figure 22 Standard curve of the proteins from Sephacryl S200 chromatography

One ml of crude enzyme, which concentrated to 8-fold from the supernatant by pervaporation, was loaded onto the Sephacryl S200 column. The active fractions were pooled from 25 through 30, assayed for β-1,3-1,4-glucanase activity and protein concentration measured (Figure 23). A protein and activity profile of purification from the Sephacryl S200 gel filtration chromatography was shown in Table 15.

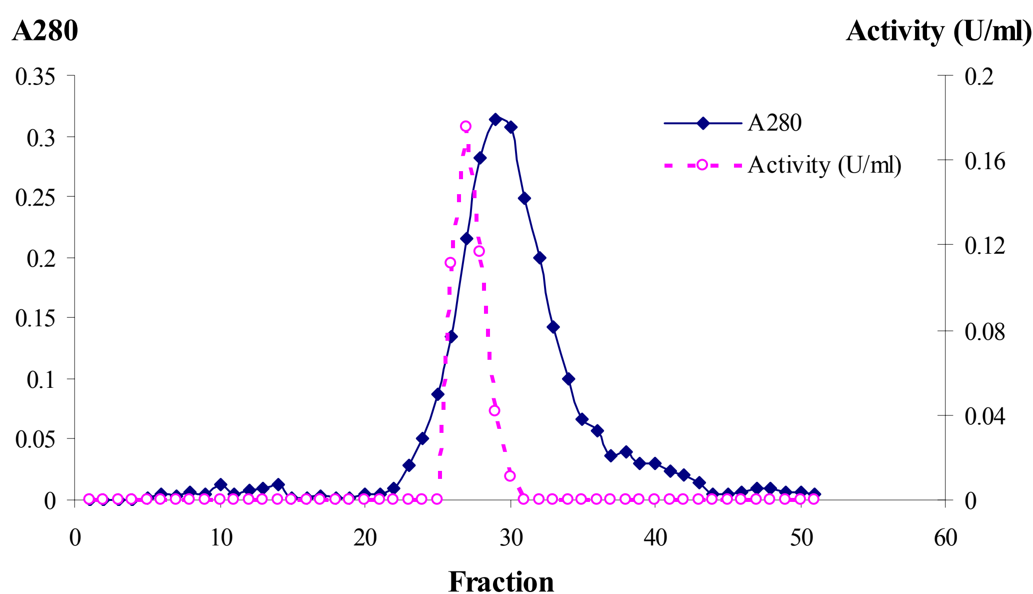

Figure 23 Sephacryl S200 chromatography of β-1,3-1,4-glucanase. (2.5 x 37.5 cm column, equilibrated with 50 mM citrate phosphate buffer pH 5.5, with flow rate 14.6 ml/h)

Table 15 Sephacryl S200 chromatography of β-1,3-1,4-glucanase

Enzyme	Volume (ml)	Total act. (U)	Total Protein (mg)	Spf. act. (U/mg)	purification (fold)	Recovery (%)
Crude enzyme	100	222	2.19	1.01	1	100
Pervaporated enz.	12.5	199.38	18.3	0.87	0.86	89.78
Sephacryl S200	502.5	173.51	0.36	0.97	0.96	78.16

The molecular weight determination of β-1,3-1,4-glucanase by using K_{av} value from Sephacryl S200 chromatography is approximately 4,013 Da, but it is very low and much different from other β-1,3-1,4-glucanases have been reported approximately 24-90 kDa. That might be from the 8-fold concentrated enzyme by using pervaporation caused a high viscosity and affected to more elution volume than it should be. Thus very low molecular weight is obtained from calculation. Sephacryl S200 chromatography is not appropriate technique for molecular weight determination of concentrated crude enzyme from *B. subtilis* GN156.

Even β-1,3-1,4-glucanase could be recovered up to 78 % by using Sephacryl S200 chromatography, but a low purification of 0.96-fold compared to the crude enzyme showed that Sephacryl S200 chromatography was not efficient technique for the β-1,3-1,4-glucanase purification.

5.2.2 Seharose 6B

To purify the enzyme with gel-filtration chromatography, Sepharose 6B (2.5 x 32 cm) which can separate proteins in the molecular weight range of 10^4 to 4 x 10^6, was used. One ml of crude enzyme, which concentrated to 8-fold from the supernatant by pervaporation, was loaded onto the Sepharose 6B chromatographt. But from the chromatogram (Figure 24), β-1,3-1,4-glucanase could not be purified with this gel either.

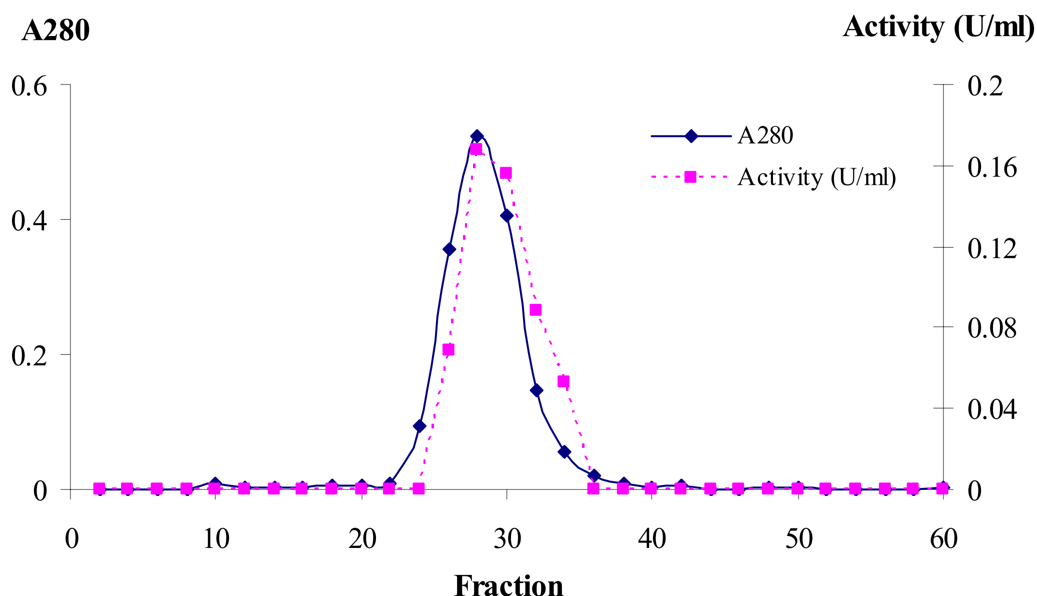

Figure 24 Sepharose 6B chromatography of β-1,3-1,4-glucanase (2.5 x 32 cm column, equilibrated with 50 mM citrate phosphate buffer pH 5.5, with flow rate 25 ml/h).

6. β-1,3-1,4-glucanase purification

6.1 Optimum conditions for affinity chromatography

Affinity chromatography is a type of adsorption chromatography in which the bed material has biological affinity to the specific substance. In the case of enzyme purification, the substrate for the enzyme is usually used as a binding ligand. Hence, to purify β-1,3-1,4-glucanase, barley β-glucan was used as the ligand and was coupled to Epoxy-activated Sepharose 6B. To prevent substrate hydrolysis, optimization conditions of binding and reactivation were studied.

To prevent substrate hydrolysis during purification, the pH at which the enzyme was inactive, was needed. Mixtures of crude enzyme and β-glucan were examined at different pH value from 3.0 – 11.0. After 18 h incubation at 4˚C, the lowest hydrolysis was shown at pH 3.0 with activity of 0.0001 U/ml (Figure 25), corresponding to the results reported previously by Akiyama *et al.* (1996) that at pH

3.0 and 3.5, purified rice β-1,3-1,4-glucanase was completely inactive. Thus, pH 3.0 was appropriate for use during the enzyme-ligand binding process in affinity chromatography.

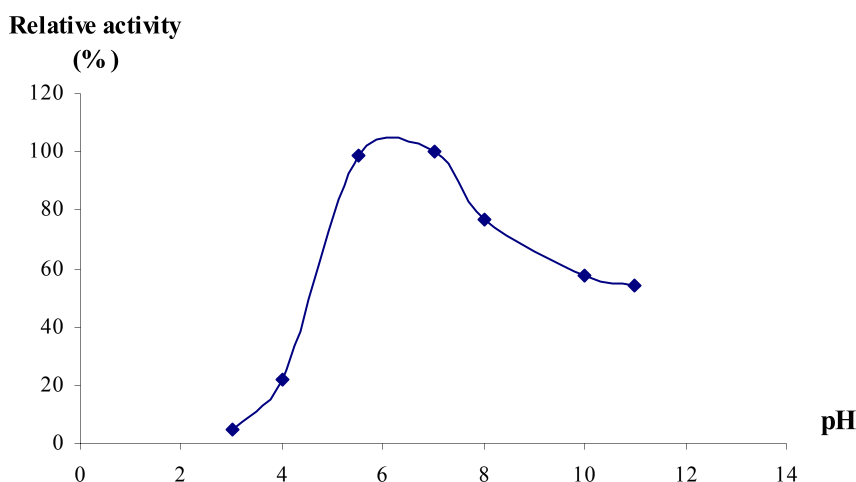

Figure 25 Effect of pH on crude β-1,3-1,4-glucanase activity at 4˚C for 18 h (50 mM citrate phosphate buffer pH 3-5.5, 50 mM phosphate buffer pH 7-9, 50 mM glycine-NaOH buffer pH 10-11).

Not only did pH inactivate β-1,3-1,4-glucanase activity, but the effect of pH on enzyme stability had to be examined. The results showed that the enzyme was stable to various pH values from 3-11 at 4˚C for 24 h (Figure 26). A pH 3.0 buffer was therefore chosen to equilibrate β-glucan-Epoxy-activated Sepharose 6B coupled material for affinity chromatography in the next step.

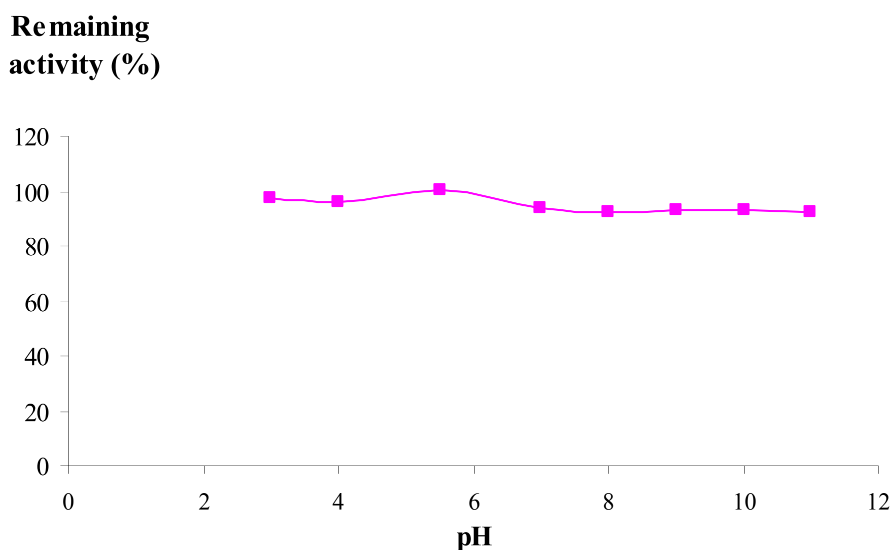

Figure 26 Effect of pH on β-1,3-1,4-glucanase stability at 4°C for 24 h. (50 mM citrate phosphate buffer pH 3-5.5, 50 mM phosphate buffer pH 7-9, 50 mM glycine-NaOH buffer pH 10-11) The remaining activity was performed at pH 6, 50°C for 20 min.

6.2 Purification of β-1,3-1,4-glucanase by Epoxy-activated Sepharose 6B

Epoxy-activated Sepharose 6B, which was coupled to β-glucan, was equilibrated with 5 mM citrate buffer pH 3.0. Fifty ml enzyme (173 U, 124 mg protein) was loaded onto the column, unbound proteins were washed with 2 bed volumes of 5 mM citrate buffer pH 3.0 and then the enzyme was eluted with a linear gradient of 0–0.25 M sodium chloride in the same buffer. As shown in Figure 27, two peaks of enzyme activities were obtained, fractions 7-10 were pooled and named AJ1, while fractions 18-28 were pooled and named AJ2. This purification process was performed 20 times to collecte enough J1 and J2 for further characterization. The purification is summarized in Table 16. The enzymes AJ1 and AJ2 were purified 118 and 823-fold for β-1,3-1,4-glucanase, respectively.

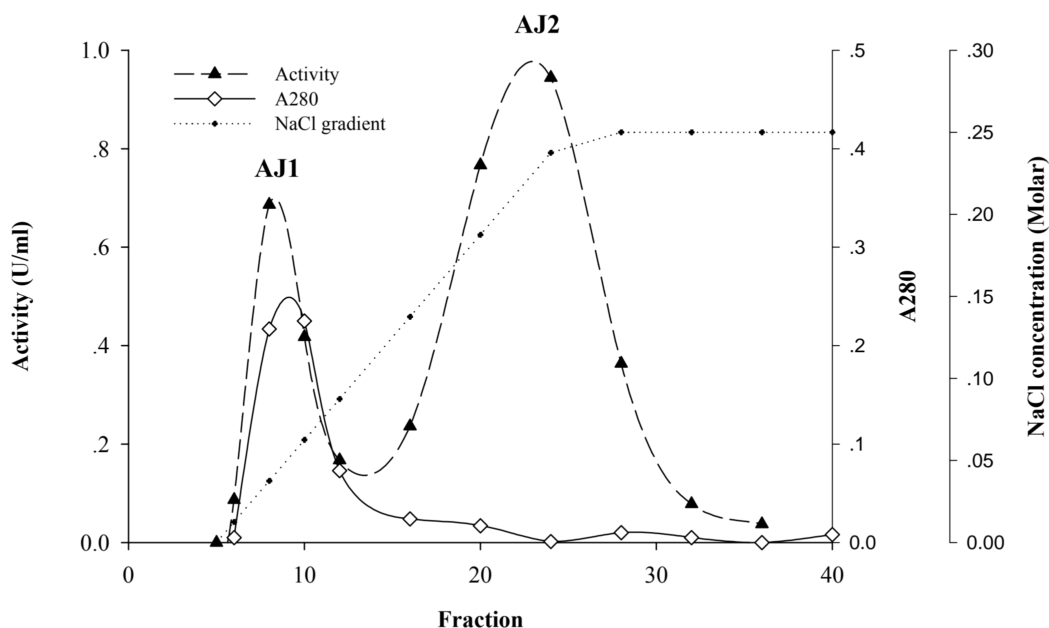

Figure 27 Affinity chromatography of β-1,3-1,4-glucanases on Epoxy-activated Sepharose 6B column (2.5 x 10 cm column, equilibrated with 5 mM citrate phosphate buffer pH 5.5, with flow rate 4 ml/min, eluted with a linear gradient of 0–0.25 M sodium chloride in the same buffer).

Table 16 Purification of β-1,3-1,4-glucanases from *B. subtilis* GN156 by Epoxy-activated Sepharose 6B column

Enzyme	Volume (ml)	Total Activity (U)	Total protein (mg)	Specific activity (U/mg)	purification (fold)	Yield (%)
Crude	1,000	3463.10	2486.10	1.44	1	100.00
Affinity						
AJ1	880	661.90	3.91	169.34	117.66	19.11
AJ2	950	324.11	0.27	1184.61	823.10	9.36

The two β-1,3-1,4-glucanases, AJ1 and AJ2 were subjected to SDS-PAGE to determine their purity. As shown in Figure 28, A J1 from affinity chromatography showed two major bands with several minor bands, with 2 bands (b) around middle of the gel which were proven to be due to the sample buffer of SDS-PAGE. AJ2 from affinity chromatography showed five major bands and a few minor bands.

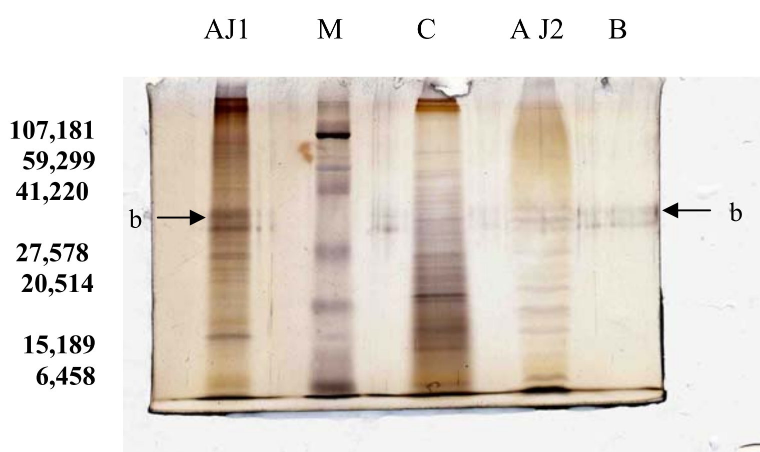

Figure 28 SDS-PAGE of β-1,3-1,4-glucanase preparation samples from each purification step; M: prestained marker, C: crude enzyme, AJ1: pooled of active fractions 7-10 from affinity chromatography, AJ2: pooled of active fractions 18-28 from affinity chromatography, B: sample buffer, b: two bands from sample buffer effect

6.3 Purification of β-1,3-1,4-glucanase by ultrafiltration

The enzymes AJ1 and AJ2 were further purified by using ultrafiltration. With molecular weight cut off 50,000 dalton filter membrane, activity of AJ1 was detected from filtrate and named J1. On the other hand, activity of activity AJ2 was detected from retentrate and named pJ2.

The J1 and pJ2 β-1,3-1,4-glucanase showed specific activity of 82.45 U/mg with a 0.65 % recovery and 555.11 U/mg with a 2.96 % recovery, respectively. The results are summarized in Table 17.

Table 17 Purification of β-1,3-1,4-glucanases from *B. subtilis* GN156

Enzyme	Volume (ml)	Total Activity (U)	Total protein (mg)	Specific activity (U/mg)	purification (fold)	Yield (%)
Crude	1,000	3463.10	2486.10	1.44	1	100.00
Affinity						
AJ1	880	661.90	3.91	169.34	117.66	19.11
AJ2	950	324.11	0.27	1184.61	823.10	9.36
UF						
J1	132	22.62	0.27	82.45	57.29	0.65
pJ2	78	28.06	0.05	555.11	385.71	2.96

J1 activity was detected from the filtrate fraction of ultrafiltration. Its molecular weight was determined on SDS-PAGE and a single band approximately 25 kDa, analyzed by GeneTools version 3.06.04 (Syngene, USA), was shown in Figure 29, and standard molecular weight curve was shown as Appendix Figure A3.

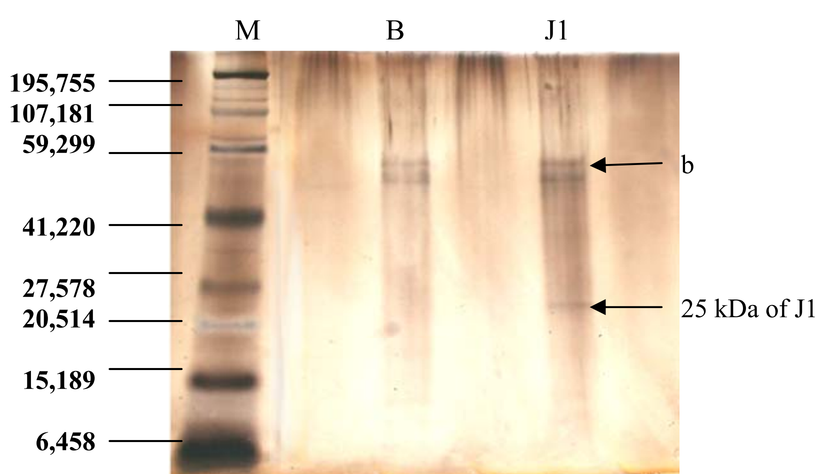

Figure 29 SDS-PAGE of the purified J1 β-1,3-1,4-glucanase; M: prestained marker, B: sample buffer, J1: purified J1, b: couple bands from sample buffer effect.

Considering J2, its activity was detected from retentrate of ultrafiltration with molecular weight cut off 50 kDa. Purity of pJ2 was determined by using SDS-PAGE and four protein bands were approximately 40, 32, 25 and 18 kDa, which analyzed by GeneTools version 3.06.04 (Syngene, USA) was shown in Figure 30.

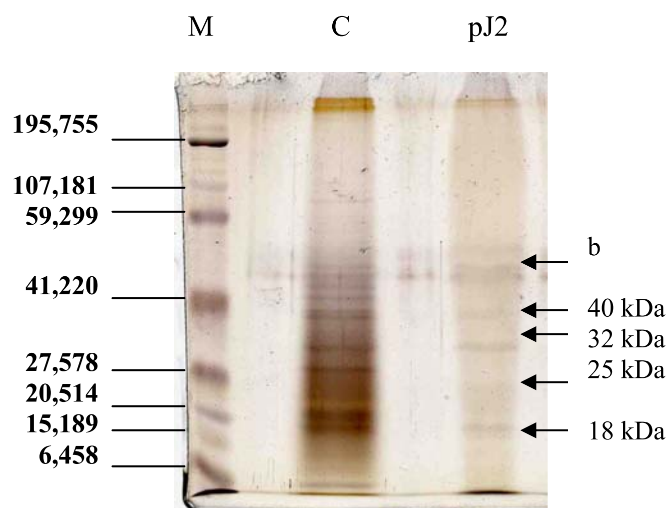

Figure 30 SDS-PAGE of β-1,3-1,4-glucanase preparation samples from each purification step; M: prestained marker, C: crude enzyme, pJ2: retentrate J2 from UF50 kDa, b: couple bands from sample buffer effect

Therefore, purity of pJ2 was further studied on native PAGE. It was found that only two bands of high molecular weight (HMW) and low molecular weight (LMW) were shown as in Figure 31. The low molecular weight band (LMW) of pJ2 was in the same position as the single protein of J1 by GeneTools version 3.06.04 analysis (Syngene, USA). Thus, it could be concluded that pJ2 was mixed proteins of J1 and HMW named J2. While only J2 composed of three protein subunits of 40, 32 and 18 kDa. Summation of these three subunits is approximately 90 kDa.

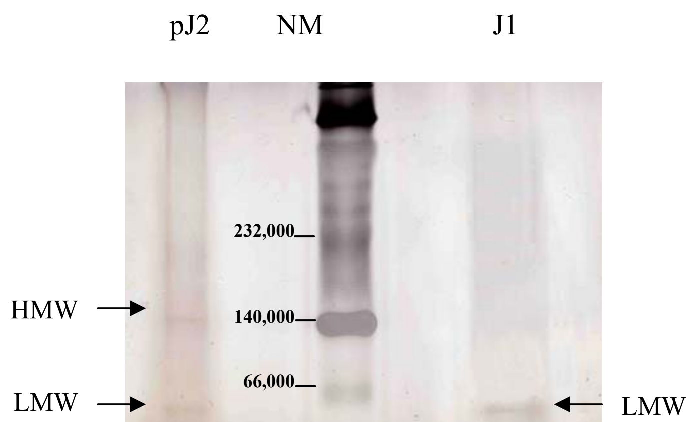

Figure 31 Native - PAGE of purified β-1,3-1,4-glucanase; J1: purified J1, J2: partial purified J2, HMW: high molecular weight band, HMW: high molecular weight band, NM: Native marker.

To confirm the purity of J1 and pJ2, pI values were determined using isoelectricfocusing. The pI value of 3.5 was found from J1 and two pI values of 3.5 and 3.6 were found from pJ2, which were analyzed by GeneTools version 3.06.04 program (Syngene, USA), as shown in Figure 32. It was clear that the pJ2 was a mixture of two proteins. Standard pI value was shown as Appendix Figure A4.

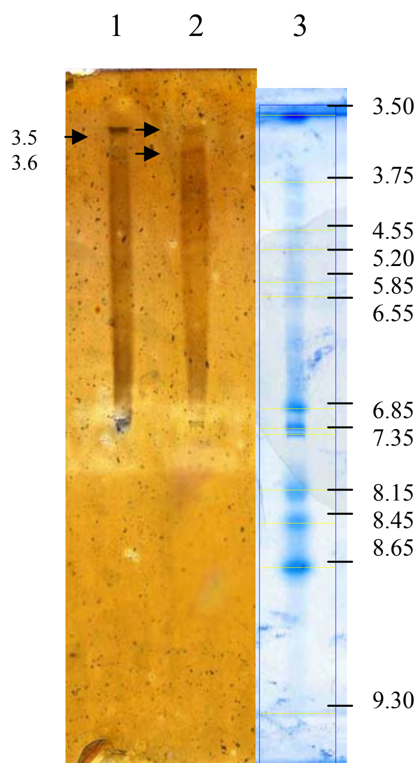

Figure 32 Isoelectric focusing lectrophoresis of purified J1 and partial purified J2; Lane 1: J1, Lane 2: pJ2, Lane 3: Broad pI calibration kit, ⟶ : point of pI bands at 3.5 and 3.6

In conclusion, *B. subtilis* GN156 contains 2 types of β-1,3-1,4-glucanases, J1 and J2, were obtained from cell-free culture fluid. J1 was a single protein of 25 kDa with pI of 3.5, while J2 might compose of three-subunit protein of 40, 32 and 18 kDa with single pI of 3.6. Two β-1,3-1,4-glucanases have been previously reported from *B. subtilis* HL-25 (Yuuki *et al*., 1989) but they have an identical molecular weight of 24 kDa with different pIs of 8.55 and 8.75. Furthermore, two β-1,3-1,4-glucanases were also found in the extract of germinating barley (Woodward and Fincher, 1982), they were 28 and 33 kDa with pI of 8.5 and greater than 10, respectively. Moreover, unique β-1,3-1,4-glucanase, which an equal molecular weight of 25 kDa was also found from *B. brevis* gene (Louw *et al*., 1993), while difference molecular weight of 35, 29 and 34 kDa has been found from *Clostridium thermocellum* gene (*licB*) (Schimming *et al*., 1991), *Streptococcus bovis* JB1 (Ekinci *et al*., 1997) or even in rice *Oryza sativa* L. (Akiyama *et al*., 1996), respectively.

Considering pI of β-1,3-1,4-glucanases were generally basic of 7.5-9.1 (Planas, 2000), except β-1,3-1,4-glucanase from hybrid gene (HA512-M) of *B. meacerans* and *B. amyloliquefaciens* showed pI of 5.6 (Olsen *et al.*, 1991). While the lowest pI of 4.9 which has been reported, was found from rice β-1,3-1,4-glucanase (Akiyama *et al.*, 1996). β-1,3-1,4-glucanases β-1,3-1,4-glucanases J1 and J2 from *B. subtilis* GN156 exhibited molecular weight of 25 and approximately 90 kDa with low pI of 3.5 and 3.6, which differ from the other β-1,3-1,4-glucanases have been found.

7. Characterization of J1

7.1 pH effect on J1

The optimum pH of β-1,3-1,4-glucanases was determined by conducting the activity assays on β-glucan at 50° C at various pH values from 3-10. The highest activity of J1 was shown at pH 6 (Figure 33). Correspond to the pH optimum of *Bacillus* β-1,3-1,4-glucanases which are around neutral pH of 6-7.5 (Planas, 2000), except for the enzymes of *B. brevis* which showed a higher optimum pH of 8-10 (Louw *et al.*, 1993). The optimum pH of non-*Bacillus* β-1,3-1,4-glucanases are in the range of 5–9, *Streptococcus bovis* JB1, pH 6.5 (Ekinci *et al.,* 1997) and *Clostridium thermocellum*, pH 8-9 (Schimming *et al.*, 1991).

Furthermore, crude and J1 were much difference in pH stability. It was found that J1 was stable at a pH range from 6-9 at 4°C for 2 h (Figure 33) with remaining activity of 50 %. While the crude enzyme was stable in the pH range of 3-11 at 4°C for 24 h with remaining activity of more than 90 % (Figure 3). It might be possible that the stability of enzymes depend on their concentrations, when the concentration of protein is very diluted, enzyme instability can occur (Pornbanlualap, 1997).