STUDY ON AMYLOGLUCOSIDASE OF A NEWLY ISOLATED *Saccharomycopsis* sp. TJ-1 FROM THE INDONESIAN FERMENTED FOOD (*TAPE*)

by

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ABSTRACT

SDS-PAGE analysis showed that fermentation broth of *Saccharomycopsis* sp TJ-1 contained only one distinct band of protein at approximately 67 kd and one smear band at around 19 kd. Gel filtration studies showed that there were 3 peaks at A280, but only the last two peaks showing glucoamylase (AMG) activity. Observation using native gradient PAGE on an active fractions of gel filtration showed that there was only one band of protein and showed a flourescent spot under UV light when the gel was exposed to 4-methyl-belliferyl aa-D glucoside in 10 mM phosphate citrate buffer. This lead to a preliminary conclusion, that *Saccharomycopsis* sp. TJ-1 contains only 1 type of AMG. The N-terminal amino acid sequence of purified product is AYP SFEAYSNYKV DXTDLET (Ala-Tyr-Pro-Ser-Phe-Glu-Ala-Tyr-Ser-Asn-Tyr-Lys-Val-Asp-X-Thr-Asp-Asp-Leu-Glu-Thr) corresponse to the amino acid sequence of AMG gene GLA1 from *E. fibuligera* KZ at the sequence position between 28 and 47.

RINGKASAN

Analisis SDS-PAGE memperlihatkan bahwa cairan fermentasi *Saccharomycopsis* sp TJ-1 mengandung hanya satu pita protein jelas dengan berat molekul sekitar 67 kd dan satu pita protein yang samar-samar dengan berat molekul sekitar 19 kd. Studi hasil filtrasi gel memperlihatkan adanya tiga puncak pada pengamatan A280, namun hanya dua puncak yang terakhir yang menunjukkan adanya aktivitas glukoamilas (AMG). Observasi menggunakan "native gradient PAGE" terhadap fraksi aktif hasil filtrasi gel memperlihatkan bahwa hanya ada satu pita protein dan memperlihatkan floresensi di bawah sinar UV ketika direaksikan dengan larutan "4-methyl-belliferyl aa-D glucoside" dalam 10 mM bufer fosfat. Dari hasil ini dapat diambil kesimpulan sementara bahwa *Saccharomycopsis* sp. TJ-1 mengandung hanya 1 jenis AMG. Untaian asam amino N-terminal dari produk murni AMG adalah AYP SFEAYSNYKV DXTDLET (Ala-Tyr-Pro-Ser-Phe-Glu-Ala-Tyr-Ser-Asn-Tyr-Lys-Val-Asp-X-Thr-Asp-Asp-Leu-Glu-Thr) yang identik dengan untaian asam amino gen AMG GLA1 dari *E. fibuligera* KZ pada posisi antara 28 dan 47.

INTRODUCTION

Indonesian traditional fermented food is a rich source of microbes. Sukara and his co-worker (1992) screened microbial isolates from traditional fermented food of Indonesia for glucoamylase activity. A yeast, *Saccharomycopsis* sp. TJ-1 was found to have an excellent glucoamylase activity. This particular yeast e.g. *S. fibuligera* has been used to produce traditional fermented foods in South-East Asia. It was used in the production of sugar syrups from starch in Thailand as Look Pang, in Indonesia as Ragi, and in the Philippines as Busbad.

Saccharomycopsis sp Tj-1 was proved to be the best glucoamylase producer among various yeast strains tested (Sukara *et al.*, 1992). The use of this particular strain for glucoamylase production has been attempted. Studies on the effect of various carbon sources for the induction of glucoamylase production, effect of initial pH, temperature condition, and rate of aeration have been investigated (Fuad, 1996). Glucoamylase enzyme of this particular isolate, however, is not very well known.

MATERIALS AND METHODS

1. Microorganism

The *Saccharomycopsis* sp. TJ-1 used was an isolate obtained from the Section of Culture Collection of the Division of Microbial and Genetic Engineering of the Research and Development Center for Biotechnology LIPI. It was maintained on PDA slopes at 4° C after 2 days growth at 30° C. The culture was sub-cultured monthly.

2. Preparation of starter

A loop of culture from PDA slope was aseptically transferred to a test tube containing 5 ml modified Futatsugi medium consisted of 5 g/l dextrin, 5 g/l yeast extract, 0.5 g/l sodium nitrate, 1.0 g/l dihydrogen potassium phosphate, 0.05 g/l potassium chloride, and 0.05 g/l magnesium sulfate penta hydrate. The culture was incubated at 28°C on a test tube shaker at vigorous shaking for 48 h.

3. Fermentation (production of enzyme)

A 2 l conical flask containing 1 l modified Futatsugi medium (Sukara *et al.*, 1992) was inoculated aseptically with 5 ml of 2 days old starter culture. It was incubated on an incubator shaker at 28° C at 150 stroke per min for 72 h.

4. Preparation of phosphate citrate buffer

To obtaine buffer at 500 mM and pH 5.6 for enzyme assay, a 21 ml of 1M citric acid was mixed with 29 ml of 2M Na_2HPO_4 and filled up to 100 ml. To obtaine buffer at 500 mM and pH 6.0 for dialysis, a 89.5 ml 1 M citric acid was mixed with 160.5 ml 2M Na_2HPO_4 and filled up to 500 ml. The buffer was diluted to 10mM before used.

5. Preparation of culture filtrate

To obtaine culture filtrate, fermentation broth was centrifuged at 4.500 x g for 10 min. A clear supernatant was collected and cell biomass was discarded.

6. Protein determination

Unless otherwise stated, protein was measured using Lowry method. The amount of 1 ml mixture of Folin reagent A (2% of Na_2O_3 in 0.1 M NaOH) and Folin B (1% Rochelle salt and 0.5% $CuSO_4.5H_2O$) with the ratio of 50:1 was added to 0.1 ml of sample. The mixture was incubated at 37° C for 10 min. It was then followed by the addition of 0.1 phenol reagent and mixed. The mixture was incubated again at 37° C for 20 min. The color developed was measured at 610 nm. The protein value was calculated against standard BSA (mg/ml protein = A610x1.12).

7. Assay of glucoamylase

The amount of 0.1 ml enzyme sample was added to a test tube containing 2%(w/v) of dextrin in 0.9 ml buffer phosphate citrate pH 5.6. Enzyme reaction was carried out at 37° C for 1 h. Glucose released was measured with glucose oxidase peroxidase (glucose kit).

8. Purification of glucoamylase

All procedures were done at a temperature under 4°C. The cell free broth of *Saccharomycopsis* sp TJ-1 harvested from 5000 ml of culture were used as the starting material for the study of glucoamylase. Unless otherwise indicated, 10 mM phosphate citrate buffer pH 6.0 was used throughout the study.

Step 1. Solid ammonium sulfate was added to the cell free broth to the final 80% saturation. The pH was adjusted during the addition of ammonium sulfate to 7.0 with 5% ammonia. The mixture was left overnight. The precipitate formed was collected by centrifugation at 4° C and 6.200 x g for 30 min and then dissolved in buffer and dialyzed overnight against buffer. Solid ammonium sulfate was again added to 30% saturation and pH was also adjusted as above. The precipitate (protein contaminant) was removed by centrifugation and the supernatant was collected and again dialyzed overnight against buffer at 4° C.

Step 2. Gel filtration. The enzyme solution was filtered through a celulofine GC-700-m of Seikagaku-kogyo, Japan. To prepare sample, the enzyme was again concentrated by ammonium sulfate precipitation and the pellet was collected on minimal buffer and applied to column which was equilibrated previously by buffer. The enzyme was eluted with the buffer and the active fractions were collected.

9. SDS-PAGE

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in the mixture containing 3.4 ml of 30% acrylamide, 2.1 ml buffer, 0.008 ml TEMED and 2.75 ml water and 0.25 ml of 10% ammonium persulfate with the stacking gel containing 0.54 ml 30% acrylamide, 1.04 ml buffer, 0.003 ml TEMED, 2.54 ml water and 0.125 ml 10% ammonium persulfate at constant current of 75 V on stacking gel and increased to 150 V at separation gel. The amount of sample for this particular purpose was 10 mml. The same amount of loading buffer (a mixture of 4 ml of 0.5 M Tris HCl pH 6.8, 4 ml of 20% SDS, 4 ml of 100% glycerol, and 4 ml BPB in distilled water and 4 ml of 1 M DTT in 10 mM Na-acetate pH 5.2) was mixed with the sample. The mixture has been exposed to the boiling water for 5 min before it was applied to the gel.

10. Native gradient PAGE

Separating gel preparation. First step. Prepare solution A (10.75 g of Tris, 5.04 g boric acid and 0.93 g of Na2EDTA and filled up with 1 l of water and adjust pH to 8.3 with HCl), B (Acrylamide 57.6 g, bis acrylamide 2.4 g and filled up to 100 ml with buffer A), C (Acrylamide 7.68 g, bisacrylamide 0.32 g filled up to 100 ml with buffer A), D (3-dimethyl amino propionitrile 0.3 ml and filled up to 100 ml with buffer A), E (APS solution 0.03 g filled up to 10 ml with buffer A). Second step. Prepare F (4% acrylamide gel containing 4 ml C, 2 ml D, and 2 ml of E), G (30% acrylamide gel containing 4 ml B, 2 ml of D and 2 ml of E).

Preparation of stacking gel. First step. Prepared H (1 N HCl 48 ml, Tris 5.98 g and TEMED 0.46 ml and filled up with 100 ml with distilled water), I (acrylamide 10 g and Bis acrylamide 2.5 g and filled up to 100 ml with distilled water), J (Riboflavin 4 mg and filled up to 100 ml with distilled water). Second step. Prepared stacking gel by adding 1 ml of buffer H and 2 ml of buffer I and 2 ml of H_2O and 1 ml of J.

Preparation of loading buffer. Amount of 10.90 g of Tris, 4.95 g of Boric acid and 0.93 g of Na_2EDTA and filled up to 1 l wit distilled water.

Preparation of sample. Amount of 20 ul sample was added by 20 ul of 2M sucrose

in buffer A and 2 ul of BPB.

Native gradient gel chromato-graphy was carried out at 4° C with constant voltage of 70 Volt at stacking gel and 150 volt at separating gel. The protein band was stand with comassie blue. To observe the glucoamylase activity of protein band, the gel containing protein band was soaked in 30 ml of 10 mM phosphate citrate buffer pH 5.6 containing 20 mg 4-methylum belliferyl aa-D glucoside. The mixture was incubated at 37° C for at least 1 h. Florescent indicating an active protein band was observed under UV light.

11. N-Terminal amino acid sequence of glucoamylase

The purified enzyme (1 nmol) was electrophoreses by SDS-PAGE and blotted onto immobilion P-Sq (millipore) membrane and washed with 10 mM Na-borate buffer pH 8.0, 25 mM NaCl for 5 minutes. It was stained in 0.1% (w/v) of ponceau S in AcOH and distained by 1% AcOH. The N-terminal amino acid sequence was analyzed by automated Edman degradation using Applied Biosystems model 476A.

RESULTS

As it was depicted in Table 1, the total amount of 4.490 ml of cell free broth was obtained from a five littre fermentation medium. It was containing 1.39mg protein per ml with glucoamylase activity of 13.402 unit and the specific activity of 9.642. The enzyme was than collected through precipitation technique with ammonium sulfate at saturation between 30 and 80% at 6.400 g and resuspended in phosphate buffer pH 6.0 and dialyzed against the same buffer at 4°C overnight. The total amount of enzyme solution was 42.50 ml with the enzyme activity of 56.111 unit and the specific activity of 94.527 or 9.8 fold increase. The recovery noted was only approximately 3.9%.

To obtained a good picture on the degree of purity of the glucoamylase in cultural broth, the cell free broth was run on SDS-PAGE. As it was depicted in Figure 1, the broth containing only one distinct and one smear protein bands. The molecular weight of a distinct band was approximately 67 kd while the smear band was 19 kd. To further identify an active protein two strategies e.g. gel filtration and native gradient PAGE were carried out.

Gel filtration as it is summarized in Figure 2 indicated that there are three peaks at A280 with only two last peaks have an enzymatic activity. Protein analysis of the first peak by Lowry method proved that this peak does not contain protein.

The crude enzyme and the protein fractions resulted from the gel filtration was also analyzed by Native gradient PAGE. The product of PAGE was stained by comassie brilliant blue (CBB). In addition to that, it was also stained for its enzyme activity using 4-methylum- belliferyl aa-D glucoside (MBADG) of SIGMA. When a product of PAGE of the crude preparation was exposed to MBADG in 10 mM phosphate citrate buffer pH 5.6 a florescent spots under UV light indicating a protein band having glucoamylase activity was appeared. This lead to a preliminary conclusion, that *Saccharomycopsis* sp. TJ-1 containing 1 glucoamylase enzymes with molecular weight of spproximately 67 kd. In order to identify and characterize the enzyme, native gradient PAGE product of crude enzyme and an active fraction were also stained by CBB. The result was depicted in Figure 3. The crude enzyme resulted in 2 bands protein while an active fraction containing 1 band of protein. The location of protein band at 67 kd correspond to the location of active protein as indicated when the native gradient product was exposed to MBADG.

The N-terminal amino acid sequence of purified product is AYP SFEAYSNYKV DXTDLET (Ala-Tyr-Pro-Ser-Phe-Glu-Ala-Tyr-Ser-Asn-Tyr-Lys-Val-Asp-**X**-Thr-Asp-Asp-Leu-Glu-Thr where **X** could not properly be identified.

CONCLUSION AND DISCUSSION

Fermentation broth of *Saccharomycopsis* sp. TJ-1 contained only one distinct band of protein with the molecular weight of approximately 67 kd and one smear band at around 19 kd. Meanwhile, gel filtration studies showed that there were 3 peaks at A280. But only the last two peaks were showing AMG activity. Observation using native gradient PAGE on crude enzyme and an active fractions resulted from gel filtration showed that there was only one band of protein and produced a flourescent spot under UV light when the gel was exposed to 4-methyl-belliferyl aa-D glucoside in 10 mM phosphate citrate buffer. This lead to a preliminary conclusion, that *Saccharomycopsis* sp. TJ-1 contains only 1 type of AMG. This finding was supported by Hattori (1961), Hattori and Takeuchi (1961), and Fukui and Nikuni (1987) who found that *Saccharomycopsis* species produced only single extracellular glucoamylase. Gogoi and his co worker (1987) reported that *S. fibuligera* ST 2 isolated from yeast cakes used for preparation of beer from rice by the people of Mongolian origin residing in the state of Assam, India has been purified and characterized as aa-amylase. Futasugi and his co-worker (1993), however found two forms of glucoamylase in a 6-d culture of *S. fibuligera* IFO 0111 cultivated under aerobic condition with oxygen transfer rate of 35×10^{-7} g mol/ml/min. The molecular weight of those enzymes are 55 and 56 kd respectively. The isoelectric points, thermal stability, and Km values for maltose are differed between the two enzymes. This is also true for *Rhizopus* sp. (Ueda *et al.*, 1975) and *Aspergillus niger* (Smiley *et al.*, 1971) which have multiple glucoamylases.

The N-terminal amino acid sequence of purified product is AYP SFEAYSNYKV DXTDLET (Ala-Tyr-Pro-Ser-Phe-Glu-Ala-Tyr-Ser-Asn-Tyr-Lys-Val-Asp-X-Thr-Asp-Asp-Leu-Glu-Thr) where X could not properly be identified. This amino acid sequence of the purified glucoamylase *Endomycopsis* sp. TJ-1 corresponse to the amino acid sequence of glucoamylase gene GLA1 from *E. fibuligera* KZ at the sequence position between 28 and 47 (AYP SFEAYSNYKV DRTDLET) (Hostinova *et al.*, 1991).

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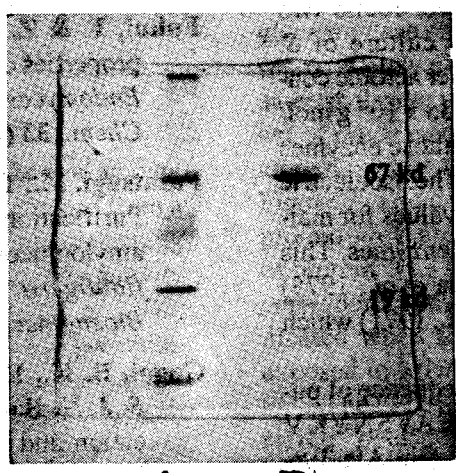
Table 1. Purification of glucoamylase from *Saccharomycopsis* sp. TJ-1

Step	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Enzyme act. (U)	Spec. activ. (U/mg)	Purif. (fold)	Yield (%)
Culture filtrate	4.490	1.3900	6.241	13.402	9.642	1	100
Amm. sulf. 30-80%	42.50	0.5936	25	56111	94.527	9.8	3.9



A B

Figure 1. SDS-PAGE analysis of cultural broth of *Saccharomycopsis* sp. TJ-1. **A** ia a standard low molecular weight protein; **B** is a cultural broth.

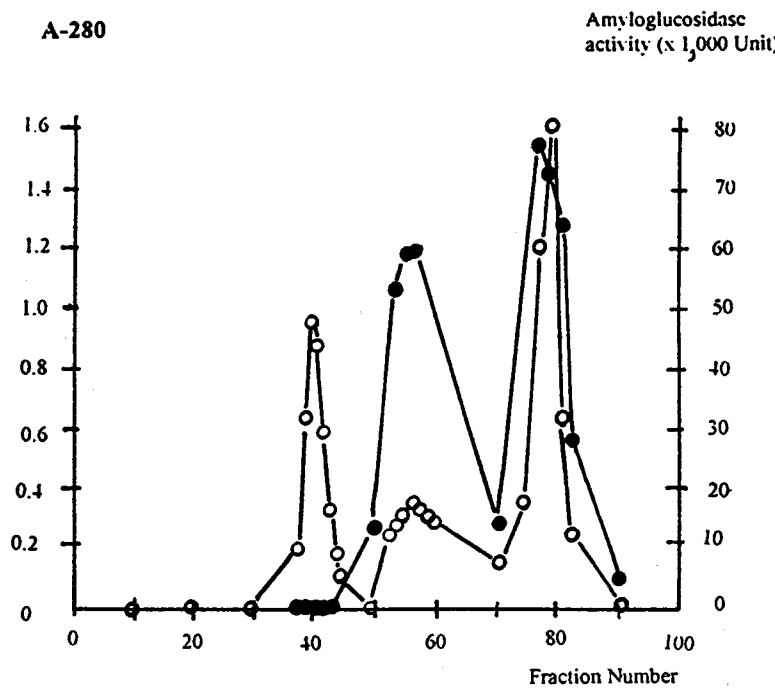


Figure 2. Gel filtration profile of fermentation broth of *Saccharomycopsis* sp. TJ-1 showing A280 peaks (O) and glucoamylase activity (●)

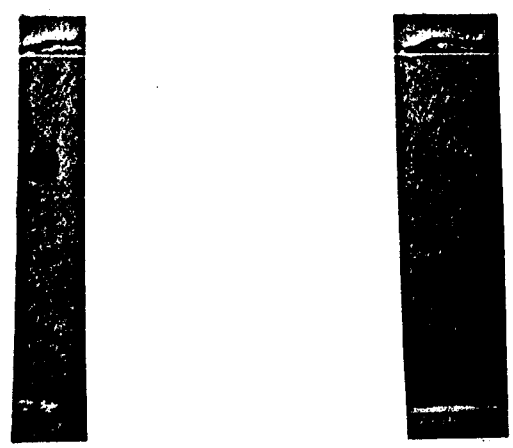


Figure 3. Native gradient PAGE of crude enyme **(A)** and an active fraction resulted from the gel filtration stained by comassie briliant blue (CBB) **(B)**.