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Expression of gene encoding raw starch digesting α - amylase (Amyl III) in *Escherichia coli*

T Anindyawati

Research Center for Biotechnology, Indonesian Institute of Sciences
Jl. Raya Bogor Km. 46 Cibinong 16911 Indonesia

Email: atrisanti@yahoo.com

Abstract. Amylase generally known as an industrial enzyme that hydrolyzes starch and glycogen. The α -amylase hydrolyzes α -1,4 glycosidic linkage that acts on raw starch. This reaction is very important for direct starch processing for alcohol fermentation and functional oligosaccharide or food additives development. Amyl III gene was isolated from *Aspergillus awamori* KT-11 and inserted into pDONR 201 as entry clone. The recombinant construct was subcloned into pDEST 17 plasmid as the expression vector and into *E. coli* BL21AI cells. The objective of this study was to optimize induction temperatures in the expression process and to know the ability of recombinant Amyl III to hydrolyze raw starch. The expression study of Amyl III was performed using LB medium at 27°C and 37°C. Purification was done using affinity chromatography (His Tag) with 100 mM imidazole. Protein analysis was done using 7.5% of SDS-PAGE gel. Digestion on soluble starch and raw waxy maize starch by Amyl III was performed using TLC plate. The results show that the best protein expression was obtained by incubation at 27°C. Digestion on those starch by using recombinant Amyl III resulted in the production of malto-tetraose, -pentaose, -hexaose and -heptaose.

1. Introduction

Amylases are one of the most important enzymes used in processing industry that hydrolyzes starch and glycogen. Amylases have potential application in several industries which approximately represent 25% of the enzyme market [1]. The efficiency of amylases in various sectors may be achieved by chemical modification of the enzyme or by protein engineering. To improve the potential productivity of amylase there are two ways by classical strain improvement through mutation and selection, and the use of recombinant. There are a lot of potential application in both food and pharmaceutical industries [2] as well as starch-based industries [3]. The starch hydrolytic enzymes comprise about 30% of the world enzyme consumption [4]. Furthermore, it can be used in brewing and fermentation industries for the conversion of starch to fermentable sugars, food industry for modification of food for infants, textile industry for designing textiles, paper industry for sizing, etc. In industrial sectors, application of enzymes mostly from bacterial and fungal sources such as *Aspergillus* sp. [5].

α -Amylases (EC.3.2.1.1 α -1,4-D-glucan glucanohydrolase) catalyze the hydrolysis of α -1,4 glucosidic linkages of starch, glycogen and related polysaccharides to produce α -anomeric form of glucose and oligosaccharides [6]. It can also act directly on raw starch granules below the starch gelatinization temperature. Therefore, the process can reduce the energy compare with conventional process in starch industry [7,8]. α -Amylase (Amyl III) isolated from *A. awamori* KT-11 had a



remarkable raw starch digesting activity. It was also found to be able to hydrolyze maltotriose and maltooligosaccharides to produce maltose and small amount of glucose [9]. On the other hand, there were some differences in the production of oligosaccharides from raw starches by Amyl III. It depends on types of tested starches [10].

The Amyl III has not been exploited for mass production. With the recent development in recombinant DNA technology, it is highly possible to improve the development of Amyl III production. Thus, the use of recombinant gene technology has further improved manufacturing process and enabled the commercialization of enzyme. Recombinant of Amyl III can be expected to be obtained with higher productivity and lower cost.

The purpose of this study was to optimize induction temperatures in the expression process and to know the ability of recombinant Amyl III to hydrolyze raw starch.

2. Materials and Methods

2.1. Chemicals

Soluble starch was purchased from Kishida Chemical Co. Ltd. (Osaka, Japan). Waxy maize starch was from Nihon Shokuhin Kako Co, Ltd. (Shizuoka, Japan). Glucose, maltose, maltotriose and maltotetraose were purchased from Hayashibara Biochemical Laboratories (Okayama, Japan). Maltopentaose, -hexaose, -heptaose were from Nihon Shokuhin Kako Co., Ltd. (Shizuoka, Japan). The molecular weight markers were purchased from Bio-Rad (Richmond, USA).

2.2. Microorganism, medium, and buffer

E. coli BL21AI (Novagen, USA) was used as a host for transformation and over expression studies. Luria Bertani (LB) medium consist of (g/L) polypepton 10, yeast extract 5, NaCl 10 adjust at pH 7.0 and 2 μ L ampicillin (100 mg/ mL). Lysis buffer containing 50 mM potassium phosphate pH 7.8, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100 and 10 mM imidazole. The mixture adjust to pH 7.8 up to 100 mL and stored at 4°C.

2.3. Protein analysis

The analytical method of sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Weber and Osborn [11]. The marker protein kit was high molecular weight that contains myosin (20 kDa), β -galactosidase (115,2 kDa), rabbit muscle phosphorylase b (97,4 kDa), bovine serum albumin (66,2 kDa) and ovalbumin (42,6 kDa). Proteins were stained with Coomassie Brilliant Blue R-250.

2.4. Protein analysis expression

Glycerol stock of Amyl III was added on LB medium consisting 2 μ L of ampicillin. The culture was incubated at 37°C overnight by shaking, added with 20 ml of 20% arabinose and kept it at 27°C for 3 hours. Then, the mixture was centrifuged at 13.000 rpm for 30 seconds and was added with 200 μ L lysis buffer. The process was continued by freeze (-190°C) and thaw (42°C) method for five times followed by centrifuged at 4°C, 12.000 rpm for 5 minutes. The induction process was done at 27°C and 37°C in water.

2.5. Purification

The purification process was done using affinity chromatography (HisTag) Chelating HP1 with a concentration of 100 mM imidazole [12].

2.6. Analysis of products

Each fraction (fraction no. 9-16) was added on 0.5% soluble starch and 2% raw waxy starch (1:1). The reaction mixture was incubated at 37°C for 48 hours. The enzymatic digest was identified qualitatively by thin layer chromatography (TLC) using a silica plate (20 x 20 cm) in a solvent system of *n*-butanol

: acetic acid : water (3:3:2) in room temperature. After dipped in 50% sulfuric acid : 1% orcinol, the sugars on the plate were detected by heating in an oven at 100°C for 10 minutes.

3. Results and Discussion

Previously, an α -amylase gene (Amyl III) was isolated from *A. awamori* KT-11. The primers used in this experiment were T7 promoter 5'-TAA TAC GAC TCA CTA TAG GG-3' (forward primer) and T7 terminator 5'-TAG TTA TTG CTC AGC GGT GG-3' (reverse primer). Figure 1 shows agarose gel electrophoresis of the reaction products obtained after simultaneous PCR process (≥ 1900 bp). Those DNA fragments were similar to the sequence of Amyl III which consist of a 1902 bp open reading frame encoding 634 amino acids [6]. The gene was inserted into *pDONR* as the entry clone followed by subcloning into *pDEST 17* as the expression vector. To further study the expression of this gene, the DNA was cloned into *pDEST 17* expression vector and expressed in *E. coli* BL21AI cells. For the purpose of screening, this vector contains ampicillin resistant gene (*bla*). Satoh *et al.* [13] had reported that Amy I and Amy II genes from *Streptococcus bovis* 148 had been constructed in *E. coli* MC 1061 expression system using *pBA101* for Amy I and *pBA109* for Amy II gene. Those genes were analyzed with restriction endonucleases. In another similar research, Jeang *et al.* [14] had cloned raw starch digesting amylase gene of a *Cytophaga* sp. with *pARMH* vector in *E. coli* expression system. The recombinant amylase possessed the properties of the authentic enzyme.

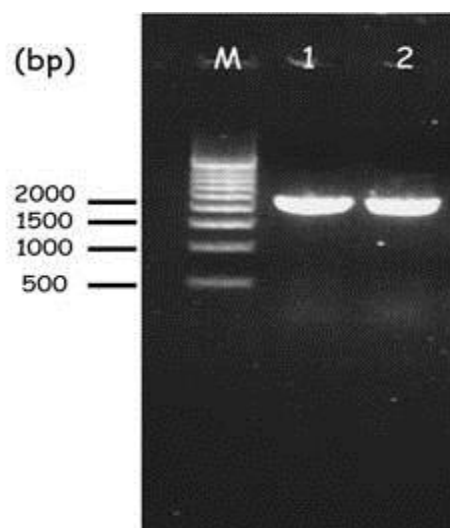


Figure 1. PCR product of Amyl III. M: Marker, Lane 1 and 2: two independent clones of Amyl III genes.

Gene expression of Amyl III in *E. coli* was performed by induction using arabinose 0.2% with variation induction temperature at 27°C and 37°C. The induction process at 27°C was found to be better than that of 37°C as presented in Figure 2. It is true that incubation at 37°C is mostly used for the protein expression study. However, the different protein may have its specific temperature to be optimum at its expression. In addition, since in this study induction at 27°C is better than that of 37°C, it is also highly possible that expression at 37°C may promote protein degradation. The molecular weight of the recombinant Amyl III was 90 kDa almost same to the original Amyl III of *A. awamori* KT-11 as determined by SDS-PAGE [6].

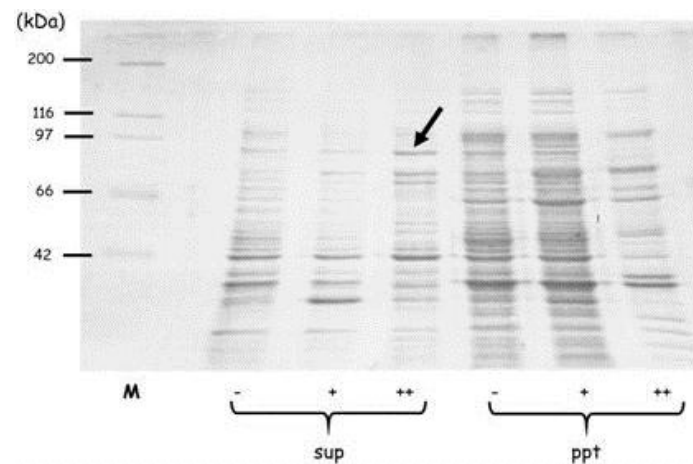


Figure 2. Electrophoresis of Amyl III protein in different time induction. M, marker, +, 37°C; ++, 27°C; -, Control

Regarding raw starch digesting amylase, the enzyme (Amy I) produced by the transformant of *E. coli* MC 1061 (*pBA101*) was adsorbed on raw corn starch completely. This enzyme strongly hydrolyzed not only in soluble starch but also in raw corn starch. In contrast, Amy II produced by the transformant *E. coli* MC 1061 (*pBA109*) was not adsorbed on raw corn starch, although it was able to hydrolyze soluble starch well [12]. The raw starch digestibility of Amy BS-1 from *Bacillus subtilis* strain AS01a in *E. coli* could hydrolyze the raw wheat, potato and rice starches to 61, 58 and 44%, respectively after six hours at 60°C [15].

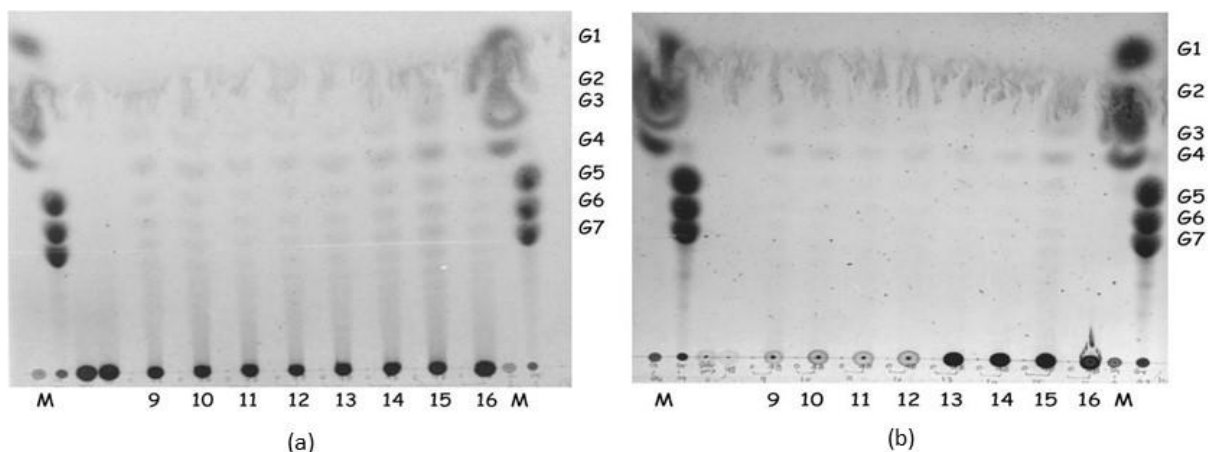


Figure 3. Identification of digest soluble starch (a) and raw waxy starch (b) with recombinant Amyl III. M, marker. G1, glucose; G2, maltose; G3, maltotriose; G4, -tetraose; G5, -pentaose; G6, -hexaose; G7, -heptaose. Lane 9-16, fraction number

Hydrolytic products of the enzyme using soluble starch and waxy maize starch were maltotetraose, -pentaose, -hexaose and heptaose as shown in Figure 3. The product from fraction number 15 was found to be the highest among other fractions. It shows that recombinant Amyl III was able to degrade soluble starch as well as raw waxy starch. The cloned raw starch digesting amylase from *B. circulans* F2 in *E. coli* produced the same oligosaccharides pattern (maltose, -tetraose, -hexaose) as that produced by the original amylase. The cloned gene was capable of digesting raw starch granules such as potato, corn and barley starches [16]. The α -amylase capable of digesting various raw starches are

attractive from the industrial perspective since they reduce the energy consumption, as well as starches from different sources, can directly be used for the hydrolysis by the same enzyme.

4. Conclusion

The best condition for protein expression of Amyl III gene was at 27°C. Digestion on soluble starch and raw waxy maize starch by recombinant Amyl III resulted in the production of malto-tetraose, -pentaose, -hexaose and -heptaose.

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