PURIFICATION AND CHARACTERIZATION OF TRANSGLUTAMINASE
FROM LOCAL Streptomyces sp. TTA 02 SDS 14

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Abstract

Streptomyces sp. TTA 02 SDS 14 is a transglutaminase producing bacteria which previously had been screened along with more than one hundred isolates. This research aimed to purify and characterize transglutaminase from this strain. Transglutaminase was purified from crude enzyme by ultrafiltration, Q-Sepharose ion exchange chromatography and Sepacryl S200 size exclusion chromatography sequentially, obtaining yield and purification fold of 1.36% and 27 folds, respectively. The molecular weight of the purified transglutaminase was 72 kDa detected by zymogram gel electrophoresis. The optimum temperature and pH were 50°C and 6. The transglutaminase was stable at 45°C and could be activated in the presence of 5 mM and 10 mM of Na⁺, K⁺, Li⁺, Ca²⁺, Mg²⁺, BPB (4-bromo-phenacyl bromide), and IAA (iodo acetamide acid), but the activity was inhibited by the presence of Cu²⁺, Zn²⁺, and PMSF (phenyl methyl sulfonyl fluoride).

Keywords: Streptomyces sp., transglutaminase, purification, characterization, zymogram

1. Introduction

Transglutaminase (EC 2.3.2.13; protein-glutamine-γ-glutamyltransferase) catalyzes an acyl transfer reaction using peptide-bond glutamine residues as acyl donors and several primary amines as acceptors. When the ε-amino groups of the protein-bond lysine residues are present as acyl receptors, this enzyme is capable of forming intra and intermolecular ε-(γ-Glu)-Lys isopeptide bonds (Mahmood, 2013).

Transglutaminases are found in mammalian tissues, plasma, fish, and plants (Pasternack et al., 1998). Unlike TGase from mammalian which are Ca²⁺ dependent, TGase produced by microorganisms (MTGase) are not. Ando et al. (1989) was the first who reported that strains from the genus Streptovercillium, which were screened from several thousand microorganisms, had the ability to produce transglutaminase. The use of microorganisms for the transglutaminase production has several advantages, such as easily manufactured on a large scale, relatively short production time and could be continuously produced at a relatively low cost.

Microbial transglutaminase is generally found in actinomycetes, such as Streptovercillium or genus Streptomyces. The enzyme obtained from microbial fermentation has been applied in the treatment of food of different origins. Extracellular transglutaminase was successfully purified from cultural filtrate of Streptovercillium mobaraensis (Schmidt, Adolf, & Fuchsbaier, 2008), Streptovercillium sp. (Ando et al., 1989), and Streptovercillium ladakanum (Ho, Leu Hsieh, & Jiang, 2000; Tsai, Lin & Jiang, 1996). Intracellular transglutaminase was also found in Bacillus subtilis (Ramanujam & Hageman, 1990) and Physarum polycephalum (Klein, Guzman, & Kuehn, 1992).

Transglutaminase has great potential for food protein modification. The enzyme is commonly used in the food industry as a crosslinking agent (Zhang, Zhu, & Chen, 2009). Some applications of transglutaminase in fish processing are to increase the hardness of fish paste as well as to restructure fish products (Tellez-Luis, Uresti, Ramirez, & Vazquez, 2002) and to improve gell strength and elasticity of fish products (Srianta, 2000). The use of
transglutaminase in fish meat processing could add the values of low economic fish by transforming them into processed products with high sale value.

The invention of new local transglutaminase producing microorganisms will create opportunities for domestic industry to produce transglutaminase using low price local raw material, which eventually will generate competitive transglutaminases to those currently supplied as imported products. The use of transglutaminase produced by domestic industry will reduce the production cost thus resulting products with affordable price. The experiments related to screening, searching of MTGase genes and medium optimization using local isolate, Streptomyces sp. TTA 02 SDS 14 previously (Fawzya et al., 2016; Seprianto et al., 2016) had been done. This paper reports the results of purification and activity of transglutaminase produced by Streptomyces sp. TTA 02 SDS 14 as well as characteristics of the purified enzyme.

2. Material and Methods

2.1. Microorganism

Streptomyces sp. TTA 02 SDS 14 previously collected by Research Center for Biotechnology Indonesian Institute of Sciences (LIPI) was used in this study. The strain was isolated from savana soil of Tambora Mount, West Nusa Tenggara, Indonesia.

2.2. Crude Enzyme Preparation

The TTA 02 SDS 14 strain was cultivated in petri dishes containing media as reported by Bahrim, lançu, Butu & Negoita (2010) (peptone water1.5%, MgSO₄·7H₂O 0.1%, KH₂PO₄ 0.2%, Na₃HPO₄ 0.5%, soybean flour 2%, potato flour 2%, glucose 1.5% and agar 2%) for 6 days at 25 °C. One cylinders (1 cm) of isolate from solid medium were inoculated into a 50 mL erlenmeyer flask containing 20 mL of liquid media adjusted to pH 6.0 and incubated at 25 °C 125 rpm for 6 days in an orbital shaker before being used as starter. Ten % (v/v) of starter was inoculated into 50 mL erlenmeyer flask with 20 mL of production medium. Sixty inoculation flasks were prepared to obtain 1 L of crude enzyme. The culture was harvested after 5 days incubation and then centrifugated at 9,000 x g (Beckman Coulter™) for 30 min at 4 °C. The supernatant was collected and stored for further analysis.

2.3. Purification of Transglutaminase

The supernatant obtained was concentrated using 10,000 NMWC ultrafiltration (Watson Marlow 323). The concentrated transglutaminase was then applied on a 1.6 cm x 10 cm Q-Sepharose™ Fast Flow column (Pharmacia Biotech) previously equilibrated with 0.1 M citrate buffer (pH 6.0). The fraction samples were eluted with 1 M sodium chloride in similar buffer at a flow rate of 2.0 mL/min, and the active fractions were then collected. The active fraction obtained from the Q-Sepharose™ Fast Flow column was then applied to 1 cm x 60 cm, High Resolution, Sepacryl™ S-200 column (Pharmacia Biotech) equilibrated with 0.1 M citrate buffer (pH 6.0) at flow rate of 0.5 mL/min. The active fractions were collected and then measured for their transglutaminase activity, protein concentration and zymogram before finally characterized.

2.4. Characterization of Transglutaminase

2.4.1. Optimum pH

The enzyme activity was measured at different pH value ranging from 4-9 using 0.2 M citrate buffer (pH 4-6), 0.2 M phosphate buffer (pH 6-8), 0.2 M Tris-HCl buffer (pH 8-9) (El-Hofi, Ismail, Nour, & Ibrahim, 2014). Incubation temperature of enzyme and substrate was carried out at 50°C for 60 min which was optimum temperature of crude enzyme as reported by Prestisia (2016).

2.4.2. Optimum temperature

Tubes containing the reaction mixture and enzyme extract were incubated at different temperatures of 25, 30, 35, 37, 40, 45, 50, 55, and 60 °C at the optimum pH for 60 min. The enzyme activity was then assayed at each temperature to determine its transglutaminase optimal temperature.

2.4.3. Transglutaminase thermal stability

Thermal stability of obtained transglutaminase was determined by pre-incubating the enzyme at two temperatures (45 and 50 °C) for 15, 30, 45, 60, 90, 120, and 240 min, and the samples were placed on ice immediately before measuring the enzyme activity.

2.4.4. Effect of metal ions and inhibitors

The effects of 5 mM and 10 mM metal ion of Na⁺, K⁺, Li⁺, Cu²⁺, Ca²⁺, Mg²⁺, Zn²⁺, and Fe²⁺ in the chloride salt solution on enzyme activity was studied (Lin, Hsieh, Lai, Chao, & Chu, 2008) as well as those of 5 mM and 10 mM of EDTA (ethylene diamine tetraacetic acid), PMSF (phenyl methyl sulfonlit fluoride), BPB (4-bromo-phenacyl bromide), and the IAA (iodoacetamide acid) (Suzuki et al., 2000). The activity was measured under standard assay condition and the relative activity was calculated as the percentage of...
activity remaining after incubation with various reagents for 60 min at 50 °C. The relative activity assayed in the absence of additives and without incubation was taken as 100%.

2.5. Transglutaminase Assay

The transglutaminase activity was determined by hydroxamate formation with the specific substrate N-Carbobenzoxy-L-Glutaminylglycine (Z-Gln-Gly) (Sigma, Aldrich) according to Folk and Cole (1966), with some modifications. The reaction mixture, containing 100 μL of enzyme, 200 μL of 0.1 M citrate buffer pH 6.0, 25 μL of 2.0 M Hydroxylamine hydrochloride (Sigma, Aldrich), 25 μL of 0.1 M L-Glutathione, reduced form (Sigma, Aldrich), and 75 μL of 0.1 M N-Carbobenzoxy-L-Glutaminylglycine (Z-Gln-Gly) (Sigma, Aldrich), was incubated at 50 °C for 60 min and stopped by adding an equal volume (425 μL) of 15% trichloracetic acid containing 5% FeCl₃. The absorbance was measured at 525 nm (Thermo Scientific). One unit of transglutaminase activity was defined as the amount causing the formation of 1 μmol of hydroxamic acid per minute at 50 °C. A calibration curve was prepared using L-glutamic acid γ-monohydroxamate.

2.6. Protein Assay

Protein concentration was measured according to Lowry method (Bollag & Edelstein, 1991) with bovine serum albumin (BSA) as a standard.

Electrophoresis Zymogram was carried out to determine the molecular weight of the transglutaminase. Electrophoresis was performed using a 10% separating gel with 1% substrat and 4% stacking gel. Prestained Protein Ladder (Base, Singapore) was used to estimate the molecular mass of transglutaminase. To identify the transglutaminase band, after running the electrophoresis, the gel was renaturated in 1% Triton X-100 at 4 °C for 2 hours and then incubated with citrate buffer pH 6.0 at temperature of 45 °C for 8 hours, followed by staining with FeCl₃ 5% in the mini rocker shaker for 2 hours.

3. Results and Discussion

3.1. Purification of Transglutaminase

After 5 days fermentation, 1L of crude enzyme was harvested with specific activity of 0.36 U/mg. After ultrafiltration the specific activity of 100 ml enzyme increased up to 13.15% with 1.14 fold purity. The increased specific activity of the transglutaminase by ultrafiltration was also reported by Ando et al. (1989) where specific activity of crude extract of transglutaminase from Streptoverticillium S-8112 was 0.13 U/mg and after concentrated with ultrafiltration, the specific activity reached 0.61 U/mg. In line with these results, Gerber, Jucknischke, Putzien, & Fuchsbaier (1994) reported that specific activity of transglutaminase produced by Streptoverticillium moharaense increased from 0.28 U/mg to 9.0 U/mg after being concentrated with ultrafiltration. The activity of 40 ml ion exchange fraction was 0.85 U/mg with yield of 3.83% and 2.36 fold purity (Figure 1 and Table 1). Further purification was performed by gel filtration chromatography using Sepacryl S-200 column (Figure 2). The result showed that the activity of 120 ml active fraction was 9.78 U/mg. This technique could increase the yield and purification fold up to 1.36% and 27 fold, respectively. Macedo, Sette, and Sato (2011) reported that purification of the transglutaminase of Streptomyces sp. using Sephadex G-75 column increase yield and fold up to 17.7% and 5 fold, respectively, while with the same technique, Cui, Du, Zhang, Liu, and Chen (2007) obtained higher result with yield and purification fold were 21.1% and 30 folds respectively.

Table 1. Purification of the transglutaminase from Streptomyces sp. TTA 02 SDS 14

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total volume (mL)</th>
<th>Total protein (mg)</th>
<th>Enzyme activity (U mL⁻¹)</th>
<th>Total activity (Unit)</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification fold (x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Enzyme</td>
<td>1000</td>
<td>2171.81</td>
<td>0.774</td>
<td>774.00</td>
<td>0.36</td>
<td>100.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>100</td>
<td>247.80</td>
<td>1.018</td>
<td>101.80</td>
<td>0.41</td>
<td>13.15</td>
<td>1.14</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>40</td>
<td>35.00</td>
<td>0.741</td>
<td>29.64</td>
<td>0.85</td>
<td>3.83</td>
<td>2.36</td>
</tr>
<tr>
<td>Sepacryl S-200</td>
<td>120</td>
<td>1.08</td>
<td>0.088</td>
<td>10.56</td>
<td>9.78</td>
<td>1.36</td>
<td>27.17</td>
</tr>
</tbody>
</table>

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Purification summary of transglutaminase produced by *Streptomyces sp.* TTA 02 SDS 14 is showed in Table 1. Figure 1 and Figure 2 showed profile of transglutaminase of *Streptomyces sp.* TTA 02 SDS 14 fractionated using Q-Sepharose column and Sepacryl S-200 column, respectively.

Protein purification was successfully performed as evidenced by the results of electrophoresis on zymogram. A single band of enzyme activity was seen on polyacrylamide gel electrophoresis containing MTGase substrate. The molecular weight of the enzyme was estimated about 72 kDa (Figure 3). This result shows that the molecular weight of MTGase produced by *Streptomyces sp.* TTA 02 SDS 14 was much heavier than those from *Bacillus subtilis* (29 kDa) (Suzuki et al., 2000), *Streptomyces hygroscopicus* (38 kDa) (Cui et al., 2007), *Streptoverticillium* sp. S 8112 (40 kDa) (Ando et al., 1989), *Streptomyces platensis* YK-2 (45 kDa) (Ko & Kim, 2009), *Streptomyces* sp. (45 kDa) (Macedo et al., 2011), *Bacillus circulans* (45 kDa) (Soares, Assmann, & Ayub, 2003). However, it is still smaller than those from liver of pig guinea (90 kDa) (Folk & Cole, 1966) and *Oreochromis niloticus* (85 kDa) (Worratao & Yongsawatdigul, 2005).

### 3.2. Effect of pH on Enzyme Activity

The effect of pH on transglutaminase activity was determined using the reaction mixtures as described
Figure 3. Zymogram of transglutaminase from *Streptomyces* sp. TTA 02 SDS 14; (M) Standard Marker, (A) Crude Enzyme, (B) Ultrafiltration, (C) Q-Sepharose, (D) Sepacryl S-200.

Figure 4. Effect of pH on the activity of the purified transglutaminase from *Streptomyces* sp. TTA 02 SDS 14.

Previously at pH 4-9 and at 50 °C. The enzyme exhibited optimum activity in phosphate buffer at pH 6 (Figure 4). The optimum pH of this enzyme was nearly the same as that from *Streptoverticillium* S-8112 with a optimum pH of 6-7 (Ando et al., 1989), *Streptoverticillium ladakanum* of 6 (Tsai et al., 1996), *Streptomyces* sp. of 6-6.5 (Macedo et al., 2011), while *Streptomyces hygroscopicus* has optimum pH of 6-7 (Cui et al., 2007). It was different from *Bacillus subtilis* transglutaminase which has an optimal pH value of 8.2 (Suzuki et al., 2000). The enzyme from mammals and fishes has a optimum pH of 8.0 while soybean transglutaminase has an optimal pH value of 7.6 (Gerber et al., 1994; Worratao & Yongsawatdigul, 2005). This result confirmed that optimum pHs of MTGase produced by *Streptomyces* sp. were lower than that produced by *Bacillus* sp. which were not significantly different with TGase isolated from mammals.
Figure 5. Effect of temperature on the activity of the purified transglutaminase from *Streptomyces* sp. TTA 02 SDS 14.

Figure 6. Thermal stability of the purified transglutaminase produced by *Streptomyces* sp. TTA 02 SDS 14.

Table 2. Effect of metal ions on the transglutaminase produced by *Streptomyces* sp. TTA 02 SDS 14

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Na⁺</td>
<td>102.23 112.49</td>
</tr>
<tr>
<td>K⁺</td>
<td>96.37 115.91</td>
</tr>
<tr>
<td>Li⁺</td>
<td>102.13 107.05</td>
</tr>
<tr>
<td>Cu⁺</td>
<td>29.24 5.13</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>96.89 115.24</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>104.25 109.95</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>19.96 18.2</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>94.4 98.65</td>
</tr>
</tbody>
</table>

3.3. Effect of Temperature on Enzyme Activity

The enzyme exhibited optimum activity at 50 °C for the catalytic reaction (Figure 5). Comparing with other transglutaminase from different sources, the optimal temperature of this enzyme was almost similar with that from *Streptoverticillium* S-8112 (Ando et al.,
Table 3. Effect of inhibitors on the transglutaminase produced by *Streptomyces* sp. TTA 02 SDS 14

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>109.21 124.62</td>
</tr>
<tr>
<td>PMSF</td>
<td>46.24   30.55</td>
</tr>
<tr>
<td>BPB</td>
<td>104.61  101.13</td>
</tr>
<tr>
<td>IAA</td>
<td>293.98  206.58</td>
</tr>
</tbody>
</table>

1989) and *Streptoverticillium ladakanum* (Tsai et al., 1996), which have optimal temperature of 50 °C. However, it was different from that of *Rosmarinus officinalis* L. (El-Hofi et al., 2014) and *Bacillus circulans* (Soares et al., 2003) transglutaminases, which have optimal temperature of 55 and 60 °C, respectively. The enzyme activity increased gradually with temperature up to 50 °C, while it declined sharply at temperature over 50 °C.

3.4. Effect of Thermal Stability on Enzyme Activity

The thermal stability of the purified transglutaminase was found at 45 °C and the enzyme retained 69% of the activity at 45 °C after 2 hours of incubation in phosphate buffer pH 6, whereas the enzyme retained 50% after 180 min of incubation (Figure 6). The thermal stability of this enzyme was nearly the same as that from *Streptomyces* sp. i.e. at 45 °C (Macedo et al., 2011; Mahmood, 2013).

3.5. Effect of Different Metal Ions and Inhibitors

The relative activity of transglutaminase was investigated in the presence of several metal ions and inhibitors, which were added at different concentrations of prepared enzyme and left for 60 min at 50 °C (Table 2 and 3). The purified transglutaminase was strongly inhibited by the presence of 5 mM and 10 mM of Cu²⁺ and Zn²⁺. However, Na⁺, K⁺, Li⁺, Ca²⁺, and Mg²⁺ could increase its activity although not significant (Table 2). The presence of metal ions may induce the conformational change of the enzyme which then promotes or reduces its activity depending on amino acid residues displayed in the catalytic centre (Kieliszek & Misiewicz, 2014). This result is similar to that of *Streptoverticillium mobaraense*, which was strongly inhibited only by Zn²⁺, moderately by Pb²⁺ yet unaffected by Cu²⁺ (Ando et al., 1989). Cui et al. (2007) reported that transglutaminase of *Streptomyces hygroscopicus* could be inhibited by Zn²⁺, Cu²⁺, Hg²⁺, Pb⁺⁺, and Fe⁺⁺. Cu²⁺ and Zn²⁺ known to preferably react with thiol groups. The strong inhibition of the enzyme by these ions might suggest this enzyme has a thiol group in its active site, similar to transglutaminases from both tissues and other microorganisms (Ando et al., 1989; Ho et al., 2000; Soares et al., 2003).

The purified transglutaminase was not inhibited by EDTA (Table 3), which was completely different from those of calcium dependent transglutaminase from animal tissues or organs (Worratao & Yongsawatdigul, 2005) and similar to those from microorganism sources (Ando et al., 1989; Ho et al., 2000). Microbial transglutaminase is not influenced by Ca²⁺, so the presence of metal chelating compounds such as EDTA could not inhibit enzyme activity (Lin et al., 2008). The activity of the purified transglutaminase was inhibited partially by PMSF indicating that active site contained serin (Susanti, 2003; Zilda, 2013). Tsai et al. (1996) reported that transglutaminase produced by *Streptoverticillium ladakanum* is strongly inhibited by PMSF but not affected by EDTA. The investigation showed that purified transglutaminase was not inhibited by BPB. It means that the histidine which was blocked by BPB did not have role in active site of the enzyme (Tauber, Imbri, & Opatz, 2014). The results showed that addition of IAA increased the activity up to 206%. It may due to the bound between IAA and histidine at active side cause a better conformational of the enzyme which then can increase the activity.

4. Conclusion

This paper reports the purification and characterization of transglutaminase of *Streptomyces* sp. TTA 02 SDS 14 isolated from savana soil of Tambora mount, West Nusa Tenggara Province, Indonesia. The transglutaminase was purified after being concentrated using ultrafiltration. The purification was conducted using both ion exchange chromatography Q-Sepharose and gel filtration chromatography Sepacryl S-200 so that molecular weight of the enzyme could be estimated around 72
kDa. The obtained enzyme showed optimum activity in phosphate buffer (pH 6.0), at 50 °C. It was stable at 45 °C for 2-h incubation. The results showed that the pure transglutaminase could be easily activated by the presence of 5 mM Na+, Li+, Mg2+, but inhibited by Cu+, Zn2+, and PMSF. These characters make enzyme as a good applicant in food industry. However, additional work are required to increase the yield during extraction and purification for commercialization, especially in medical field.

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