Purification and Characteristics of a Novel Transglutaminase Derived from Microorganisms

Hiroyasu Ando, Masae Adachi, Koichi Umeda, Akira Matsuura, Masahiko Nonaka,* Ryosuke Uchio,* Haruo Tanaka* and Masao Motoki*

Central Research Laboratories, Amano Pharmaceutical Co., Ltd., Kantotsubo, Nishiharu-cho, Nishikasugai-gun, Aichi 481, Japan

*Central Research Laboratories, Ajinomoto Co., Inc., Suzuki-cho, Kawasaki-ku, Kawasaki 210, Japan

Received March 24, 1989

A microorganism producing transglutaminase was screened as an indication of hydroxamate-forming activity. The microbial transglutaminase was purified from the culture filtrate of the strain, S-8112, which was supposed to belong to the genus Streptoverticillium. The molecular weight of the purified enzyme was found to be about 40,000 on SDS-polyacrylamide gel electrophoresis, the isoelectric point 8.9 and the optimal pH of the reaction 6–7. The present enzyme requires no calcium ions for its activity. Thus, it clearly differs from known transglutaminases derived from mammalian organs, which have been defined as calcium-dependent enzymes.

Transglutaminase (EC 2.3.2.13) is a calcium-dependent enzyme that catalyzes the acyl transfer reaction between γ-carboxyamid groups of glutamine residues in proteins as well as peptides and various primary amines. When the ε-amino groups of lysine residues in proteins act as an acyl-acceptor, ε-(γ-Glu)Lys bonds (iso peptide bonds) are formed both intra- and inter-molecularly. If there is no primary amine in the reaction system, water becomes the acyl-acceptor, and the γ-carboxyamide groups of glutamine residues are deamidated, becoming glutamic acid residues.

All transglutaminases hitherto reported were derived from mammals. For example, they are widely distributed in various organs, such as liver and blood, of mammals, and their enzymatic properties have been studied. In recent years, it has been attempted, through the use of a reaction catalyzed by transglutaminase, to gelatinize various food proteins through the formation of cross-links to immobilize the coenzyme, NAD+, which was combined with protein, and to introduce lysine, amino acid esters and lysylpeptides into food protein covalently to improve its nutritive value and its functional properties.

The transglutaminase used in those studies was derived from the liver of guinea pigs, so little is known about the possibility of industrial application. Thus, the authors screened for microorganisms that produce transglutaminase for the purpose of mass production. As a result, it was found that several kinds of microorganisms show transglutaminase activity.

Described in this report are the methods for the culture and purification of a microorganism that produces the enzyme, as well as the enzymatic properties.

Materials and Methods

Screening method. A medium (pH 7.0) composed of 2.0% Polypepton, 2.0% soluble starch (Lustergen; Nippon Starch Chemical Co., Ltd.), 0.2% dipotassium hydrogen phosphate, 0.1% magnesium sulphate, 0.2% yeast extract and 0.05%, foam-extinguisher (Adekanol; Asahi Denka Kogyo K.K.) was cultured for 3 days at 30°C and then filtered, and then the enzyme activity was measured by a colorimetric hydroxamate procedure with N-carbo-
benzoxyl-L-glutaminyl-glycine. One unit causes the formation of one micromole of hydroxamic acid per min. A calibration curve was prepared using L-glutamic acid-γ-monohydroxamic acid.

**Culture conditions.** The microorganism screened was inoculated into 100 ml of medium (pH 7.0, 0.2% Polypepton, 0.5% glucose, 0.2% diopotassium phosphate, 0.1% magnesium sulphate) in 500 ml Sakaguchi flasks and cultured for 48 hr at 30°C under aeration with agitation. The culture fluid of the species obtained was added to 201 of fresh medium (pH 7.0) composed of 2.0% Polypepton, 2.0% Lustergen, 0.2% diopotassium hydrogen phosphate, 0.1% magnesium sulphate, 0.2% yeast extract and 0.05% Adekanol, and then cultured for 3 days at 30°C under aeration (10 l/min) with agitation (250 rpm).

**Method of purification.** After incubation, the culture fluid was filtered by centrifugation at 3,000 rpm. The filtrate thus obtained was concentrated with a UF membrane (A11 1010; Asahi Chemical Industry Co.) and then applied on a column of Amberlite CG-50 that had previously been equilibrated with 0.05 M sodium phosphate buffer (pH 6.5). The column was washed with the same buffer and then the active fractions were collected, elution being performed with a gradient of 0.05 ~ 0.5 M sodium phosphate buffer. After the present fractions had been diluted, the chromatography was repeated under the same conditions. They were then diluted, to reduce the conductivity to below 10 mS, and passed through a column of Blue Sepharose (Pharmacia Co.). Transglutaminase was adsorbed during this operation. After impurities had been washed out with 0.05 M sodium phosphate buffer (pH 7.0), high activity fractions were collected, with elution with a gradient of 0 ~ 1.0 M sodium chloride buffer.

Furthermore, transglutaminase derived from the liver of guinea pigs used as a control was prepared according to the method of Connellan et al. Its specific activity was 4.2 units/mg.

**Electrophoresis.** Polyacrylamide gel electrophoresis (PAGE) was carried out on a slab gel in the presence of SDS, as previously described. The isoelectric points were obtained by isoelectric focusing using Pharmalyte pH 3 ~ 10 (Pharmacia Co.).

**Chemicals.** CBZ-L-glutaminylglycine, CBZ-L-glutaminylglycine ethyl ester and CBZ-L-glutamine were obtained from Kakusen Chemical Works, Inc. CBZ-L-glutaminyl-L-glutaminylglycine, CBZ-L-glycylglycyl-L-glutaminylglycine, CBZ-L-asparaginyl-glycine and (S)-glycyl-t-glutaminylglycine were prepared by the reference procedures.

Other reagents were purchased from Wako Pure Chemical Industries, Ltd., unless otherwise specified.

### Results and Discussion

**Screening**

About 5,000 strains were isolated from soil collected from a variety of locations, and hydroxamate-forming activity was investigated, strong enzyme activity being found in an actinomycete strain that seemed to be *Streptoverticillium* S-8112. The enzyme activity in the culture filtrate of strain S-8112 amounted to 2.5 units/ml. In this study, the enzyme in this culture fluid was investigated as described below. Additionally, there was hardly any detectable transglutaminase activity in the culture fluids of other microorganisms except for *Streptoverticillium* sp.

**Culture**

As shown in Fig. 1, after 10 hr incubation,

**Fig. 1. Culture of Streptoverticillium S-8112 Isolated from Soil.**

○, hydroxamate-forming activity (units/ml); □, dry cell weight (mg/10 ml); Δ, absorbance at 490 nm, expressed as the sugar content determined by the phenol-sulfuric acid method.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (Units)</th>
<th>Specific activity</th>
<th>Recovery of activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>280,800</td>
<td>35,100</td>
<td>0.13</td>
</tr>
<tr>
<td>Ultrafiltration</td>
<td>52,938</td>
<td>32,292</td>
<td>0.61</td>
</tr>
<tr>
<td>Amberlite CG-50</td>
<td>1,533</td>
<td>25,625</td>
<td>16.7</td>
</tr>
<tr>
<td>Second Amberlite</td>
<td>902</td>
<td>19,300</td>
<td>21.4</td>
</tr>
<tr>
<td>CG-50</td>
<td>657</td>
<td>14,865</td>
<td>22.6</td>
</tr>
</tbody>
</table>

Table 1. **Purification of the Transglutaminase from the Culture Filtrate**

---

H. ANDO et al.
production of the enzyme began outside the cell wall of the microorganism, the highest level being reached after 70 hr. The growth and enzyme production of the strain were not interlinked.

**Purification of the enzyme**

The results of purification at each step are shown in Table I. For purification of the enzyme being investigated (called BTGase hereafter), chromatography on Amberlite CG-50 was most effective, it was purified 174 times. The enzyme gave a single band on SDS-polyacrylamide gel electrophoresis (Fig. 2).

**Molecular weight and isoelectric point**

The molecular weight of BTGase was about 40,000, as judged from the results of SDS-polyacrylamide gel electrophoresis (Fig. 2) and gel chromatography on a Sephadex G-75 column (data not shown), and it was found to be a monometric enzyme consisting of a single polypeptide chain. This was about 1/2 the molecular weight of the transglutaminase (called GTGase hereafter) derived from the liver of guinea pigs. The isoelectric point, pI, obtained on electrofocusing of the Pharma-

---

**Table II. Influence of Inhibitors on the Transglutaminase Activity**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>BTGase</th>
<th>GTGase</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>111</td>
<td>109</td>
</tr>
<tr>
<td>PCMB</td>
<td>56</td>
<td>2</td>
</tr>
<tr>
<td>NEM</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>MIA</td>
<td>76</td>
<td>3</td>
</tr>
<tr>
<td>PMSF</td>
<td>111</td>
<td>106</td>
</tr>
</tbody>
</table>

Each inhibitor was added at the concentration of 1 mM. After allowing the mixture to stand at 25°C for 30 min, the activity was measured.

*Optimal pH, optimal temperature, pH stability and thermal stability*

When hydroxylamine and CBZ-glutaminylglycine were used as substrates, the optimal pH was about 6.5~7 with the reaction time of 10 min at 37°C, the optimal temperature when the reaction time was 10 min at pH 6.0 was about 50°C, and the stable pH range on treatment for 10 min at 37°C was 5~9. As to thermal stability, 100% activity remained at 40°C on treatment for 10 min at pH 7.0, and 74% activity at 50°C.

**Influence of inhibitors**

Each inhibitor examined was added to the 1 mM, followed by standing for 30 min at 25°C, and then the activity was measured. The results are shown in Table II. For any transglutaminase, the activity was not inhibited by EDTA or PMSF (phenylmethylsulfonylfluoride). It was inhibited, however, by PCMB (parachloromercuribenzoic acid), NEM (N-ethylmaleimide) and MIA (monooiodoacetate), indicating that there was an SH-group participating in the reaction.

**Influence of calcium ions**

The relative activity, when calcium ions were added or not added to the reaction system, is shown in Table III. The enzyme being investigated acts in both the presence and the absence of calcium ions. And the
activity was not inhibited on adding EDTA. Thus this enzyme clearly differs from GTGase, which is defined as a calcium-dependent enzyme, as well as other transglutaminases of mammalian origin.

Influence of various metal ions
After each metal ion had been added to 1 mM and standing for 30 min at 25°C, the activity was measured. Taking the activity in the absence of a metal ion to be 100%, the relative activity with each metal ion is shown in Table IV. It was found that 85% of the activity was inhibited in the presence of Zn²⁺, and 20% in the presence of Cu²⁺. The activity was not noticeably inhibited in the presence of other metal ions in this test.

Substrate specificity
The hydroxamate-forming activity of the transglutaminase toward synthetic peptides is shown in Table V. The present transglutaminase (BTGase) did not utilize CBZ-Gln, CBZ-Asn-Gly or Gly-Gln-Gly as a substrate, like GTGase. However, the substrate specificity of BTGase is lower than that of GTGase as to the reaction behavior toward three peptides, CBZ-Gln-Gly-OET, CBZ-Gln-Gln-Gly and CBZ-Gly-Gln-Gly-Gly. Thus it was found that there are various differences in enzymatic properties between these two transglutaminases, especially regarding thermal stability, molecular weight, isoelectric point and substrate specificity. Also, in both the presence and absence of calcium ions, the present transglutaminase clearly differs in its action. Therefore, it is considered that the present enzyme of microbial origin is of great practical use, because the enzyme can be supplied at a low cost and is easy to purify.

In terms of the present discovery, the uses of transglutaminase in studies on food processing and applications in medical treatment are increased, thus opening the way to practical application.

References
5) K. Ikura, K. Okumura, M. Yoshikawa, R. Sasaki
Purification of Microbial Transglutaminase

7) J. H. Connellan, S. I. Chung, N. K. Whetzel, C. M.