High Molecular Weight Transglutaminase Inhibitor Produced by a Microorganism (Streptomyces lavendulae Y-200)

Koji Ikura,† Kazuo Minami, Chiemi Otomo, Hiroyuki Hashimoto, Shunji Natsuka, Kohei Oda, and Katsuyoshi Nakanishi

Department of Applied Biology, Kyoto Institute of Technology, Matsugasaki, Kyoto 606-8585, Japan

Received August 9, 1999; Accepted September 21, 1999

Transglutaminases catalyze the cross-linking and amine incorporation of proteins, and are implicated in various biological phenomena such as blood clotting, wound healing, apoptosis, and cell differentiation. *Streptomyces lavendulae* Y-200, isolated from soil, produced a substance that inhibited transglutaminases. The inhibitory substance was purified from the cultured medium by procedures of acid precipitation, deoxyribonuclease treatment, and gel filtration chromatography. The partially purified sample was dark brown. The inhibitory activity was stable under acidic, alkaline, and high temperature conditions, and resistant to the treatment with proteinases such as trypsin and Pronase. The molecular weight of the inhibitory substance was estimated to be between 10^4 and 10^5 from its permeability through ultrafilter membranes. The acid hydrolysate of the inhibitory substance contained amino acids and sugars. The inhibitory substance inhibited both calcium-dependent and calcium-independent transglutaminases in a competitive manner with a glutamine substrate. The extent of inhibition caused by the calcium-dependent transglutaminase increased with increasing calcium concentration. The results obtained here may help identify a novel regulatory substance of transglutaminase in biological systems.

Key words: transglutaminase; transglutaminase inhibitor

Transglutaminases (EC 2.3.2.13) catalyze the formation of an amide bond between the γ-carboxamide group of peptide-bound glutamine residues and primary amine groups of either protein-bound lysine or free amines, resulting in protein/protein cross-links or protein/amine conjugates.1 These enzymes are widely distributed in tissues and fluids of animals and classified into at least five groups based on biochemical properties and encoding genes: plasma-, tissue-, keratinocyte-, epidermis-, and prostate-types. Several are implicated in diverse biological functions such as blood clotting, wound healing, keratinization of epidermis, and stiffening of the erythrocyte membrane (for reviews, see Refs. 2–5). Transglutaminases also seem to be involved in the regulation of cellular growth,6,7 differentiation,8,9 and apoptosis.10,11 Recently, a tissue-type transglutaminase of rat liver was shown to be a new class of GTP-binding proteins that participate in receptor signaling.12 The idea that transglutaminase might be involved in the pathology of neurodegenerative diseases such as Alzheimer disease and polyglutamine diseases has also been suggested.13–15

Hitherto some studies concerning transglutaminase inhibitors have been done to understand the physiological roles and reaction mechanism of transglutaminase, and to develop pharmacological agents for diseases assumed to be related to over-expression of transglutaminase such as acne,16 psoriasis,17 cataracts,18 and immunological disorders.19 In the transglutaminase-catalyzed reaction, an acyl-enzyme intermediate is first formed between an SH group of the active site cysteine residue and a glutamine of substrate peptide (acyl donor), and this is followed by the formation of a ternary complex of the acyl-enzyme intermediate and an amine substrate (acyl acceptor), and the acyl-transfer reaction to the amine substrate.20 Cystamine,2 alkyl isocyanates,21 peptidyl halohydroisoxazoles,22 and 2-[2-oxopropyl]thio] imidazolium derivatives23 inhibited transglutaminases by blocking the active site SH group of transglutaminase. Monodansylcadaverine,24 α-difluoromethylornithine,25 and phenylthiourea derivatives26 have been used as competitive inhibitors of the amine substrates. Among physiological substances, GTP,27 ATP,28 and zinc ion2 inhibit the tissue-type transglutaminase and they are thought to regulate the intracellular enzyme activity together with the calcium ions as activator concertedly. Interestingly there are reports29,30 suggesting that tissue-type transglutaminase does not work in the cells correspondingly to its induction and its activity may be repressed by unknown regulatory

† To whom correspondence should be addressed. Koji Ikura, Fax: +81-75-724-7535; E-mail: ikura@ipc.kit.ac.jp
mechanism(s).

We have been searching for natural transglutaminase inhibitors produced by microorganisms to find a clue to the regulatory mechanisms of transglutaminase and to provide inhibitory substances useful for experimental studies of transglutaminase and for pharmaceutical applications related to the transglutaminase actions. Here we report that a microorganism (Streptomyces sp.) isolated from soil produced a high molecular weight substance that inhibits transglutaminases. The substance inhibited the enzyme in a competitive manner with a glutamate substance.

Materials and Methods

Materials. Guinea pig liver transglutaminase was purified on an immunoadsorbent column with a monoclonal antibody as described previously. Recombinant human keratinocyte- and mouse epidermis-transglutaminases were a gift from Drs. K. Hitomi and M. Maki (Nagoya University, Japan). Microbial transglutaminase from Streptovermicilli

um was a gift from Ajinomoto Co., Inc. (Japan). β-Casein was prepared from cow's milk by the method of Hupp et al. Other materials were obtained commercially from the sources indicated: N-Carbobenzoxy-L-glutaminylglycine and leupeptin (Peptide Institute Inc., Osaka, Japan); [2,5-3H]histamine (Amer sham International Plc., Amer sham, UK); deoxyribonuclease I, TPCK-treated trypsin, human thrombin, and hirudin (Sigma, St. Louis, MO); Pronase (Calbiochem, San Diego, CA); 4-amidophenylmethanesulfonyl fluoride (Wako Pure Chemical Industries, Osaka, Japan); Ultrafree-C3, a plastic microtube with an ultrafiltration membrane having a definite pore size (Millipore Japan, Tokyo, Japan); human Factor XIII, plasma-type protransglutaminase (Hexit Japan, Tokyo, Japan); Toyopearl HW-65F (Tosoh, Tokyo, Japan).

Culture conditions. A seed culture (0.8 ml) was inoculated into 80 ml of a medium (pH 7.0) containing 1% glucose, 1% Polypeptone, 1% meat extract, and 0.3% NaCl, and grown at 30°C with shaking in a 500-ml flask. After 5 days of growth, the culture fluid was harvested by centrifugation for the isolation of the inhibitory substance.

Transglutaminase assay and inhibitory activity. The transglutaminase activity was measured by two methods. One was the colorimetric method using N-carbobenzoxy-L-glutaminylglycine and hydroxylamine as the substances with some modifications; that is 0.15 M Tris-HCl buffer (pH 7.5) was used instead of 0.2 M Tris-acetate buffer (pH 6.0) and 8 mM dithiothreitol was added. The total volume of the reaction mixture was 150 μl. The other was the filter paper method using dimethylcasein and [2,5-3H]histamine as the substrates. The assay mixture contained, in a total volume of 100 μl, 40 mM Tris-HCl (pH 7.5), 5 mM CaCl2, 20 mM dithiothreitol, 5 mg/ml dimethylcasein, 1 mM [2,5-3H]histamine (2.7 μCi), and an enzyme sample. The reaction was started by the addition of the enzyme sample at 37°C. The reaction mixtures (20 μl each) were spotted at intervals onto filter paper discs (Whatman No. 3MM) treated with 5% trichloroacetic acid in acetone. The discs were washed as described elsewhere, and the radioactivity on each disc was counted in 5 ml of toluene-based scintillation fluid by a liquid scintillation analyzer (Packard Model 1500CA). One unit of transglutaminase activity was defined as the amount of activity that catalyzes the formation of 1 micromole of peptide-bound γ-glutamyl hydroxamate per minute in the colorimetric assay. The polymerization of β-casein through the cross-linking catalyzed by transglutaminase was monitored by analyzing the products by SDS-PAGE. The reaction mixture contained 0.1 M Tris-HCl (pH 7.5), 3.0 mM CaCl2, 10 mM dithiothreitol, 3 mg/ml β-casein, and 10 μg/ml guinea pig liver transglutaminase. Factor XIII, a plasma-type protransglutaminase, was activated by thrombin as follows; the mixture containing Factor XIII (10 mg/ml), human thrombin (5 NIH units/ml), 0.1 mM NaCl, 40 mM CaCl2, 1 mM EDTA, 0.1 mM dithiothreitol, and 50 mM Tris-HCl (pH 7.5) was incubated at 37°C for 10 min, and the activating reaction was stopped by the addition of hirudin (10 NIH units/ml). Recombinant epidermis-type protransglutaminase was activated by proteolysis with dispase as described by Kim et al. with incubation at 30°C for 20 min at a proteinase level of 0.06 unit/ml.

The inhibition rate (%) of the inhibitory substance was calculated by using the equation (A - I) / A × 100, in which A is the enzyme activity measured in the absence of the inhibitory substance and I is the activity measured in the presence of the inhibitory substance. One inhibition unit (IU) was defined as the amount of the inhibitory activity that inhibits 6×10^{-3} unit of guinea pig liver transglutaminase to 50% in the colorimetric assay conditions, since the inhibition rate correlated linearly to the amount of the inhibitory substance up to 50% inhibition. The specific activity of the inhibitory substance was expressed per mg of the dried substance.

Electrophoresis. SDS-PAGE with 3% stacking and 10% separating gels was done by the method of Laemmli. The gel was stained with a solution containing 0.1% Coomassie brilliant blue, 50% methanol, and 10% acetic acid, and destained in a solution containing 7% acetic acid and 25% ethanol. Agarose gel electrophoresis with 1% gel was done in the buffer solution containing 40 mM Tris-acetate,
pH 8.0, and 1 mM EDTA. The gel was stained in the same buffer containing 0.5 μg/ml ethidium bromide.

**Amino acid analysis.** The inhibitory substance was hydrolyzed with a vapor of 6 M HCl containing 1% phenol at 110°C for 24 h under evacuated conditions by using a Pico-Tag Workstation (Waters). The amino acid composition of the hydrolysate was analyzed with an amino acid analyzer (Hitachi L-8500A).

**Analysis of sugar components.** The sugar components of the inhibitory substance were analyzed by the pyridylamination method with some modifications. Briefly, the sample (10 μg) was hydrolyzed with a vapor of a mixture of 4 M HCl and 4 M trifluoroacetic acid (1:1) at 100°C for 4 h under evacuated conditions, and the free amino groups of the hydrolysates were acetylated by adding 50 μl of a mixture of methanol, pyridine, and water (6:3:2) and 2 μl of acetic anhydride. The N-acetylated hydrolysates were pyridylaminated by using a GlycoTAG system (Takara) and analyzed by HPLC with a Pal-pak Type A column. The buffer used was a mixture of nine parts of 0.7 M boric acid adjusted to pH 9 with potassium hydroxide and one part of acetonitrile.

**Results**

**Isolation and identification of the microorganism**

One of 1100 microorganism strains isolated from soil was found to produce a substance in a medium, which inhibited the transglutaminase activity in the colorimetric assay method. The microorganism, coded Y-200, was identified as *Streptomyces lavendulae* by taxonomic analyses in the National Collections of Industrial and Marine Bacteria Limited (Scotland, UK).

**Production of the inhibitory substance**

The inhibitory activity in the culture medium of *Streptomyces lavendulae* Y-200 was measured during its cultivation. Since the inhibitory substance produced during the culture did not pass through the cellulose membrane of the dialysis bag, the inhibitory activity in the cultured medium was assayed after the medium had been dialyzed against 10 mM Tris-HCl, pH 7.2. The inhibitory activity reached the highest level after 5 or 6 days of culture (Fig. 1A). The cultured medium after 5 days of culture inhibited guinea pig liver transglutaminase in a dose-dependent manner (Fig. 1B).

**Purification of the inhibitory substance**

The inhibitory substance was purified from the cultured medium of *Streptomyces lavendulae* Y-200 after 5 days of culture by procedures of acid precipitation, deoxyribonuclease treatment/acid precipitation, and gel filtration chromatography: The pH of the cultured medium (100 ml) was adjusted to 3.0 by the addition of 1 M HCl, put on ice for 1 h, and the formed precipitate was collected by centrifugation at 5500 × g for 15 min. The precipitate was dissolved in 20 ml of 10 mM Tris-HCl (pH 7.2) and dialyzed against the same buffer. To the dialyzed solution, 2.4 ml of 1 M Tris-HCl (pH 7.2), 1.2 ml of 0.1 M MgSO4, and deoxyribonuclease I (480 units) were added and the total volume was adjusted to 24 ml with water. The mixture was incubated at 25°C for 3 h. This deoxyribonuclease treatment was effective to remove DNA in the sample without loss of the inhibitory activity (data of agarose gel electrophoresis, which is not shown, and Table 1). The pH of the

---

**Fig. 1.** Inhibitory Activity in the Culture Medium.

(A) Inhibitory activity in the medium during the cultivation of *Streptomyces lavendulae* Y-200. The volume of the culture medium was 80 ml. (B) Dose-dependency of the inhibition by the culture medium. Various amounts of cultured medium after 5 days of culture were added to the assay mixture. The inhibitory activity was assayed by the colorimetric method.
The nuclease-treated solution was adjusted to 3.0 by the addition of 1 M HCl and the precipitate formed was collected by centrifugation as described above. The precipitate was dissolved in 10 ml of 10 mM Tris-HCl (pH 7.2) containing 0.1 M NaCl (buffer A) and dialyzed against the same buffer. The dialyzed solution was centrifuged at 15,000 × g for 20 min to remove insoluble materials. The clear supernatant (10 ml) was added to a column (2.0 × 125 cm) containing Toyopearl HW-65F equilibrated with buffer A, and the column was developed with the same buffer. Figure 2 shows an elution profile showing the inhibitory activity and dark-brown color (shown as absorbance at 500 nm) of the eluted fractions. A peak of the inhibitory activity overlapped that for the dark-brown color and the eluted position of these peaks was at the peaks of bovine serum albumin (Mₐ: 67000) and chymotrypsinogen A (Mₐ: 25000) (data not shown). These results suggested that the inhibitory substance is a high molecular weight substance with a dark-brown color. The active fractions were pooled.

Results of the purification are summarized in Table 1. The specific activity of the inhibitory substance increased 6.8-fold after the purification procedure. When the purified sample was put on SDS-PAGE, the dark-brown sample smeared the polyacrylamide gel in the region of a molecular mass of 10~45 kDa (data not shown). The smeared region was stained with Coomassie brilliant blue.

![Elution Profile from Toyopearl HW-65F Column](image)

**Fig. 2.** Elution Profile from Toyopearl HW-65F Column. Elution fractions were 10 ml. The flow rate was 20 ml/h. The horizontal line indicates the active fractions pooled after elution. Detailed conditions are described in the text. (●) Inhibitory activity; (△) Absorbance at 500 nm.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total activity (IU)</th>
<th>Specific activity (IU/mg²)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured medium supernatant¹</td>
<td>3800</td>
<td>9</td>
<td>100</td>
</tr>
<tr>
<td>Acid precipitation</td>
<td>3700</td>
<td>42</td>
<td>97</td>
</tr>
<tr>
<td>DNase treatment/acid</td>
<td>3220</td>
<td>58</td>
<td>85</td>
</tr>
<tr>
<td>precipitation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel filtration</td>
<td>2350</td>
<td>61</td>
<td>62</td>
</tr>
</tbody>
</table>

¹ Cultured medium (100 ml) after 5 days of culture was used as starting material.

² Weight of the sample lyophilized after being desalted.
Properties of the inhibitory substance

Stability. The stability of the inhibitory activity of the purified sample was tested under various temperatures and pHs. The inhibitory activity was stable at temperatures of 25 and 60°C (Fig. 3A). After incubation at 95°C for 30 min, the inhibitory activity of the substance increased to 125% of the unheated control. The inhibitory activity was stable in a wide range of pH from 2.0 to 10.0 (Fig. 3B). Under acidic conditions, the solubility of the inhibitory substance decreased. At pH 4.0, about 15% of the inhibitory activity was found in the insoluble fraction and the residual activity was present in the soluble fraction. At pHs lower than 3.0, the inhibitory substance precipitated completely without loss of the inhibitory activity.

Resistance to proteinases and deoxyribonuclease. The purified inhibitory substance (200 µg) was mixed with trypsin (2 µg) or Pronase (2 µg) in 0.3 ml of 0.1 M Tris-HCl, pH 7.5, and incubated at 37°C for 1.5 h. The proteinase reactions were stopped by heating the reaction mixtures in boiling water for 30 min and the subsequent addition of 3 µl of proteinase inhibitor solution containing 10 mM leupeptin and 10 mM 4-aminophenylmethanesulfonyl fluoride. The inhibitory activity of the substance did not change even after these proteinase treatments. As described in the section of purification above, the deoxyribonuclease treatment did not affect the inhibitory activity of this substance.

Molecular size. The inhibitory activity in the cultured medium and of the purified samples did not pass out through the dialyzing bag made of regenerated cellulose film when they were dialyzed against one thousand times volume of 10 mM Tris-HCl buffer, pH 7.2 or water for 48 h. To roughly measure the molecular weight of the inhibitory substance, the permeability of the inhibitory substance through the ultrafiltration membrane was tested by use of Ultrafree-C3 tubes (Millipore) having different pore size membranes. The purified inhibitory substance did not pass through the membrane limiting the passage of substances with a molecular weight of more than 10^6 and passed through the membrane limiting the passage of substances with that of more than 10^4. These results suggested that the molecular weight of the inhibitory substance is between 10^6 and 10^4.

Amino acid and sugar components. The amino acid and sugar compositions of the inhibitory substance were analyzed (Table 2). The high content of glycine and proline was characteristic in the amino acid composition. The amino acid content was about 30% (wt/wt), though the tryptophan content was not considered for the calculation. All of the seven sugars (GalNac, Xyl, GlcNac, Glc, Man, fucose, and Gal) analyzed here were detected in the inhibitory substance sample.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>(mol%)</th>
<th>Sugar</th>
<th>(mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>8.3</td>
<td>GalNac</td>
<td>2.2</td>
</tr>
<tr>
<td>Thr</td>
<td>4.4</td>
<td>Xylose</td>
<td>38.0</td>
</tr>
<tr>
<td>Ser</td>
<td>4.9</td>
<td>GlcNac</td>
<td>3.3</td>
</tr>
<tr>
<td>Gix</td>
<td>13.0</td>
<td>Glucose</td>
<td>31.5</td>
</tr>
<tr>
<td>Gly</td>
<td>22.6</td>
<td>Mannose</td>
<td>16.4</td>
</tr>
<tr>
<td>Ala</td>
<td>10.2</td>
<td>Fucose</td>
<td>1.3</td>
</tr>
<tr>
<td>Cys</td>
<td>0.6</td>
<td>Galactose</td>
<td>7.3</td>
</tr>
<tr>
<td>Val</td>
<td>4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Met</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ile</td>
<td>2.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leu</td>
<td>4.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tyr</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>3.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>10.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Tryptophan was not measured.
*b Seven sugars indicated were measured.

Inhibitory activity in different assay systems. Since the inhibitory substance was found by measuring its inhibitory activity in the transglutaminase reaction using N-carbobenzyloxy-λ-glutaminylglycine and hydroxylamine as the substrates, we confirmed its inhibitory function in different transglutaminase assay systems such as the incorporation of 3H-labeled histamine into protein and the cross-linking of proteins. The inhibitory substance inhibited both the incorporation of the labeled histamine into dimethyl casein (Fig. 4) and the intermolecular cross-linking of β-casein (Fig. 5) catalyzed by guinea pig liver transglutaminase.

Inhibitory actions. Transglutaminases other than tissue-type liver transglutaminase, such as plasma-, keratinocyte-, and epidermis-types of transglutaminases and calcium-independent microbial transglutaminase, were also inhibited by the inhibitory substance with similar dose-dependency profiles (Fig. 6). On all types of transglutaminase, 50% inhibition occurred at around 0.5 mg/ml of the inhibitory substance. The inhibition of the calcium-independent microbial transglutaminase was not affected significantly by the presence of calcium. The inhibitory substance did not inhibit the activity of a thiol-proteinase papain, which has an amino acid sequence surrounding the active-site cysteine residue similar to that of transglutaminases (data not shown).

To clarify the mode of inhibition of the inhibitory substance, we examined the relationship between the guinea pig liver transglutaminase activity and the substrate concentration in the presence and absence of the inhibitory substance by Lineweaver-Burk plot analysis. The inhibitory substance inhibited the enzyme in a competitive-type inhibitory manner with
Fig. 4. Inhibition of Histamine Incorporation into Dimethyl Casein.

The reaction conditions and the assay method were described in the text. The concentrations of the inhibitory substance added were: (●) none; (○) 0.32 mg/ml; (▲) 0.65 mg/ml, (△) 1.29 mg/ml.

Fig. 5. Inhibition of Cross-linking of β-Casein.

The reaction conditions were described in the text. Reactions were stopped at intervals by mixing portions of the reaction mixture with SDS-PAGE sample buffer. Migration was from top to bottom. The arrow indicates bands of monomer β-casein. P1 indicates bands of polymerized casein that could not enter the stacking gel, and P2 indicates bands of polymerized casein that could not enter the separating gel. Concentrations of the inhibitory substance in the reaction systems: (++) 1.6 mg/ml and (++) 6.5 mg/ml. Lane M, molecular mass marker proteins.

Fig. 6. Inhibition of Different Types of Transglutaminase.

Activities were assayed by the filter paper method described in the text in the presence of various concentrations of the inhibitory substance. (●) guinea pig liver transglutaminase; (○) human keratinocyte transglutaminase; (▲) mouse epidermal transglutaminase; (△) human Factor XIII; (■) microbial transglutaminase; (□) microbial transglutaminase (in the absence of CaCl₂).

Fig. 7. Lineweaver-Burk Plots in the Presence and Absence of the Inhibitory Substance.

Guinea pig liver transglutaminase was assayed by the colorimetric method by using N-carbobenzoxy-L-glutaminylglycine (at concentrations indicated) and hydroxylamine (at 100 mM) as the substrates. Initial velocity (v) is expressed as the increase in absorbance at 525 nm per 60 min. Molar concentration of the inhibitory substance was calculated on the basis of its tentative molecular weight 5 × 10⁶: (●) none, (○) 0.6 μM, (▲) 1.2 μM, (△) 2.4 μM, (■) 3.6 μM, and (□) 5.4 μM.
respect to glutamine substrate (Fig. 7). Dixon plot analysis also showed the competitive-type inhibition with the glutamine substrate and apparent $K_i$ value of 1.3 $\mu$M. The inhibitory substance also inhibited the calcium-independent microbial transglutaminase competitively with the glutamine substrate ($K_i = 2.6$ $\mu$M).

The effect of the calcium concentration on the inhibition was tested with the calcium-dependent liver transglutaminase. As shown in Fig. 8, the extent of the inhibition by the inhibitory substance increased as the concentration of CaCl$_2$ was increased from 0.4 to 1.0 mM.

**Discussion**

The reports concerning naturally occurring transglutaminase inhibitors have been limited. Here we reported one microorganism (*Streptomyces laven-dulae* Y-200) isolated from soil that produces a dark-brown high molecular weight substance that inhibits transglutaminases. The purified inhibitory substance seems to be heterogeneous in molecular size and contain a peptide(s) and a sugar chain(s) as components. From findings that some strains of *Streptomyces* produce melanin-like pigments$^{39}$ and that the inhibitory substance and standard melanin samples (synthetic and *Sepia officinalis* melanins) show similar spectra in Fourier-transform infrared spectrometry (data not shown), we presume that the high molecular weight inhibitory substance with a dark-brown color may be a melanin-like substance. Melanins are usually insoluble in water,$^{40}$ but the inhibitory substance found here was water-soluble.

This implies that the inhibitory substance contains water-soluble glycoproteins, like various natural melanin samples that often contain proteins called melanoproteins.$^{41}$ More structural analyses are required to identify the inhibitory substance. The findings that the inhibitory activity of the inhibitory substance is stable under acidic, alkaline, and high temperature conditions, and resistant to the treatment with proteinases suggest that the inhibitory activity is based on a component other than the possible protein and sugar components.

The inhibitory substance seems to interfere competitively with the binding of a glutamine substrate to the enzyme in the catalytic process. Whether or not the inhibitory substance directly interacts with the active site cysteine residue of transglutaminase is not clear. In the case of the calcium-dependent transglutaminase, calcium increased the rate of inhibition by the inhibitory substance. This indicates that the binding of calcium to the enzyme may cause a structural change which is favorable for the inhibition by the inhibitory substance.

It is thought that transglutaminase activities in vertebrates are regulated by transcription controls, limited proteolysis, and allosteric effectors such as calcium and GTP.$^{35}$ The precise identification of the inhibitory substance reported here may lead to the discovery of novel regulatory substances of vertebrate transglutaminase. The extent of inhibition of the inhibitory substance decreased in the presence of a high concentration of NaCl (data not shown). This might be related to the finding that bovine endothelial cells contain a particulate fraction-residing inhibitor of transglutaminase which interacts via ionic interaction with the enzyme.$^{42}$

**References**


7) Birckbichler, P. J., Orr, G. R., Patterson, M. K.,


