Purification of Tonin by Chromatography Using Soybean Trypsin Inhibitor Coupled CH-Sepharose 4B

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Tonin was purified from rat submaxillary gland homogenates by affinity chromatography on soybean trypsin inhibitor coupled CH-Sepharose 4B and two additional steps of conventional chromatography. The use of affinity chromatography by soybean trypsin inhibitor coupled CH-Sepharose 4B permits a new approach in the purification of tonin, since it can completely separate in one step troublesome contamination of the enzymes which showed tosyl-L-arginine methyl ester hydrochloride esterase activity.

Tonin is purified 11-fold to a homogeneous state on polyacrylamide gel electrophoresis at a yield of 21%.

**TONIN**, a new enzyme discovered by Boucher et al., has been shown to be able to generate angiotensin II not only from angiotensin I, but also directly from a synthetic tetradecapeptide renin substrate and from a natural renin substrate.¹⁻³

This enzyme has been purified from rat submaxillary glands and was classified as a selective endopeptidase of the serine protease of the trysin-chymotrypsin family.⁴ The first purification procedure consisted of many steps which included differential centrifugation, ammonium sulfate precipitation, gel filtration on Sephadex G-150 and ion exchange chromatographies on DEAE cellulose, phosholcellulose, SP-Sephadex C-25 and SP-Sephadex C-50.⁵

To simplify the purification process, we successfully used an immunoadsorbent for purification.⁶ Immunoadsorption, one of the special use of affinity chromatography, enabled us to purify tonin in a simple step. However, immunoadsorbent affinity chromatography has drawbacks in preparing the specific anti-tonin antibody and in needing a large-sized column.

In this paper, we report purification of tonin from rat submaxillary glands by affinity chromatography using a soybean trypsin inhibitor coupled CH-Sepharose 4B column, followed by DEAE cellulose and gel filtration on Sephadex G-100.

**MATERIALS AND METHODS**

**Chemicals**

Sephadex G-100, activated CH-Sepharose 4B were purchased from Pharmacia Fine Chemicals, Sweden, DE-52 from Whatman Biochemical Ltd., U.S.A. and soybean trypsin inhibitor from Millipore Corporation, U.S.A. [Ile⁵]-Angiotensin I, L-histidyl-L-leucine (His-Leu) and tosyl-L-arginine methyl ester hydrochloride (TAME) were obtained from Protein Research Foundation, Osaka, Japan, and O-phthalaldehyde from Katayama Chemicals, Osaka, Japan. All other chemicals used were commercial products of reagent grades.

**Preparation of Homogenates of Rat Submaxillary Glands**

Submaxillary glands obtained from 10 male rats of Sprague-Dawley strain weighing about 300 grams were homogenized in 30 ml of 0.25 M sucrose solution, pH 7.0, containing 1 mM EDTA.
The inhibitory effect of soybean trypsin inhibitor was conducted in the same experimental procedure as that of tonin activity except addition of the inhibitor. The reaction was initiated by adding 40 μl of tonin solution in the incubation buffer and 40 μl of soybean trypsin inhibitor solution in the same buffer with 20 μl of angiotensin I solution (5 mg/ml) so that each reaction mixture had a final concentration of 1 μg of tonin, 100 μg of angiotensin I and 25, 50, 200, 400, 2,000 μg of soybean trypsin inhibitor per ml. The inhibitory activity was expressed as % of inhibition compared to tonin activity without the inhibitor.

Others

Protein concentration was determined according to Lowry et al. using BSA as a standard. Polyacrylamide gel electrophoresis was carried out according to Davis with slight modification of gel concentration to give 10% polyacrylamide.
gel. Gels were stained with Coomassie Brilliant Blue G-250.10

The rate of hydrolysis of p-tosyl-L-arginine methyl ester (TAME esterase activity) was measured by the increase in absorbance at 247 nm, based upon the method of Hammel.11

RESULTS
Inhibitory Effects of Soybean Trypsin Inhibitor

Inhibitory effect of tonin activity by soybean trypsin inhibitor was investigated. When angiotensin I (100 μg) was used as a substrate, a concentration of 125 μg/ml of soybean trypsin inhibitor was found to produce 50% inhibition of activity of tonin (1 μg/ml) (Fig. 1).

Affinity Chromatography of Tonin
Preliminary tests for affinity chromatography
of soybean trypsin inhibitor coupled CH-Sepharose 4B (STI-CH-Sepharose 4B) were carried out at different pH conditions. One and half ml of submaxillary gland homogenates were dialyzed against appropriate buffers at different pHs and applied to STI-CH-Sepharose 4B column (2.0 x 7.0 cm) which was previously equilibrated with each appropriate buffer.

The column was washed with 6 column volume of each initial buffer until absorbance at 280 nm was constant.

Adsorbed proteins were eluted with either 0.05 M glycine-NaOH buffer, pH 9.5 or 0.2 M glycine-HCl buffer, pH 2.5. Aliquots of each fraction were assayed to determine tonin activity.

When chromatography was achieved at pH 4.5 and pH 9.5, tonin activity was eluted in the early non-adsorbed peak. However, when chromatography was carried out between pH 5.3 and pH 7.5, fraction containing tonin activity were retarded from the early protein peak and at pH 5.3, distinct segregation from the early non-adsorbed fraction which corresponded void volume was shown (Fig. 2).

To perform a larger scale application, the newly prepared STI-CH-Sepharose 4B column (2.0 x 14.0 cm) was equilibrated with 0.05 M sodium acetate buffer, pH 5.3 containing 0.02% sodium azide and 6 ml of submaxillary gland homogenate equilibrated with the same buffer was applied to the column. The column was washed with about 8 column volumes of the initial buffer until the eluent had a constant adsorbance at 280 nm. The adsorbed proteins were eluted with 0.02 M glycine-NaOH buffer, pH 9.5 and successively with 0.2 M glycine-HCl buffer, pH 2.5. Fractions containing tonin activity were retarded and separated from the early
Fig. 4. Ion exchange chromatography on DEAE-cellulose (DE-52). The column (1.5 x 12 cm) was equilibrated with 0.02 M tris-HCl buffer, pH 8.0 containing 1 mM EDTA and eluted with 500 ml of eluate in a linear gradient of NaCl from 0 to 0.15 M. Each fraction contained 5.0 ml.

Fig. 5. Gel filtration on Sephadex G-100. The column (1.9 x 85 cm) was equilibrated and eluted with 0.1 M potassium phosphate buffer, pH 7.0 containing 0.02% NaN₃. Fractions of 2.7 ml were collected.
TABLE I PURIFICATION OF TONIN FROM RAT SUBMAXILLARY GLANDS

<table>
<thead>
<tr>
<th></th>
<th>Total Protein (mg)</th>
<th>Total Activity (μmole His-Leu/min)</th>
<th>Specific Activity (μmole His-Leu/min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMG Homogenate</td>
<td>244.7</td>
<td>163.9</td>
<td>0.67</td>
</tr>
<tr>
<td>SMG Homogenate (pH 5.3)</td>
<td>164.3</td>
<td>129.5</td>
<td>0.79</td>
</tr>
<tr>
<td>after STI-CH Sepharose</td>
<td>14.2</td>
<td>65.2</td>
<td>4.59</td>
</tr>
<tr>
<td>after DE-52</td>
<td>6.7</td>
<td>34.8</td>
<td>5.19</td>
</tr>
<tr>
<td>after Sephadex G-100</td>
<td>4.6</td>
<td>34.1</td>
<td>7.41</td>
</tr>
</tbody>
</table>

non-adsorbed protein peak.
Both adsorbed fractions detached by 0.05 M glycine-NaOH buffer, pH 9.5 and 0.2 M glycine-HCl buffer, PH 2.5 showed TAME esterase activity (Fig. 3). Thus fractions containing tonin activity was separated from the large quantity of contaminants in this step.

Ion Exchange Chromatography and Gel Filtration
Ion exchange chromatography on DE-52 and gel filtration on Sephadex G-100 were conducted according to previous study. The pooled and concentrated solution from 5 runs of the affinity chromatography was equilibrated with 0.02 M tris-HCl buffer, pH 8.0 containing 0.1 M EDTA and applied to a DE-52 column (1.5 x 12 cm) previously equilibrated with the same buffer. The column was eluted with 500 ml of eluent in a linear gradient of sodium chloride from 0 to 0.15 M (Fig. 4).
Fractions containing tonin activity were pooled, concentrated and applied to the Sephadex G-100 column previously equilibrated with 0.1 M potassium phosphate buffer, pH 7.0, containing 0.02% sodium azide. The tonin activity was shown as a symmetrical peak which was superimposed with the elution pattern of protein (Fig. 5).
Tonin preparation after Sephadex G-100 showed one band on a polyacrylamide gel electrophoresis (Fig. 6). Table I summarizes the results of purification of tonin from rat submaxillary gland homogenates.

Starting with 244 mg of submaxillary gland

Fig.6. Polyacrylamide gel electrophoretic pattern of the final product (15 μg) after Sephadex G-100. Electrophoresis was carried out in 10% gel of polyacrylamide at pH 8.3.
homogenates, 4.6 mg of purified tonin was obtained at a yield of 21%.

DISCUSSION

Purification of tonin was attempted by using soybean trypsin inhibitor coupled CH-Sepharose 4B.

We confirmed soluble soybean trypsin inhibitor had a weak inhibitory activity on tonin when angiotensin I was used as a substrate as Boucher et al. had reported. This weak inhibitory activity seemed to be advantageous for affinity chromatography by using immobilized soybean trypsin inhibitor. As a result of loose interaction of tonin with soybean trypsin inhibitor coupled CH-Sepharose 4B, the tonin containing fractions were retarded and segregated from the early protein peak which corresponded to void volume and also separated from completely adsorbed fractions. After affinity chromatography on STIC-CH-Sepharose 4B column, all fractions were subjected to determine the TAME esterase activity. Completely adsorbed fraction detached by buffer solutions pH 9.5 or pH 2.5 showed both activity but non-adsorbed fractions and tonin containing fractions did not show any activity. This result suggests that tonin has a different substrate specificity from trypsin or kallikrein, because the substrate used was specific substrates for trypsin and kallikrein.

Thus, the use of affinity chromatography enabled us to separate the tonin containing fractions from a large quantity of contaminants which included the enzymes belonging to the same serine protease family in relatively mild condition.

In addition, since soybean trypsin inhibitor is commercially available, a larger scale application of this method seems to be possible for purification of tonin from various biological materials.

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REFERENCES