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 trypsin-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, phosphocellulose, 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 anti-

body 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-phthaldialdehyde 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

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Fig.1. Inhibitory effect of soybean trypsin inhibitor on tonin activity. Tonin (1 μ g/ml) and angiotensin I (100 μ g/ml) were incubated in the presence of varying amounts of soybean trypsin inhibitor (25, 50, 200, 400, 2000 μ g per ml).

by Polytron homogenizer tipped with 10 S blade. The homogenate was centrifuged at 35,000 G for 45 minutes. The supernatants were used as starting materials.

Preparation of Soybean Trypsin Inhibitor Coupled CH-Sepharose 4B

Coupling of soybean trypsin inhibitor to CH-Sepharose 4B was performed according to the manufacturer's instruction. Fifteen grams of activated CH-Sepharose 4B were swollen and washed with 3,000 ml of 1 mM HCl. The slurry was suspended in a volume equal to it (40 ml) of 0.1 M sodium carbonate buffer, pH 8.3 containing 0.5 M NaCl, and 6 ml of soybean trypsin inhibitor solution (200 mg) was added. Coupling was achieved at 4°C with gentle agitation. After 20 hours, 8 ml of 2 M ethanolamine was added to block the residual active sites at room temperature for 2 hours. The gels were washed with 0.05 M tris-HCl, pH 7.5 containing 0.5 M NaCl, 0.05 M glycine-NaOH, pH 9.5 and 0.2 M glycine-HCl, pH 2.5. It was finally washed with 0.05 M tris-HCl, pH 7.5 containing 0.02% sodium azide and stored at 4°C until use.

Assay of Tonin Activity

Tonin activity was manually measured by a

fluorometric method with angiotensin I as a substrate according to Piquilloud et al.⁷ and Boucher et al.¹, with modification.

The incubation buffer used for measurement of tonin activity consisted of 150 ml of borate-phosphate buffer, pH 7.0, using 0.05 M sodium borate and 0.1 M monopotassium phosphate solutions mixed with 1 ml of DFP (2% in isopropanol), 1 ml of dipyrindyl (20% in methanol) and 2 ml of 0.1 M EDTA. Determination of tonin activity was performed by the measurement of histidyl leucine (His-Leu) liberated from angiotensin I. The concentration of His-Leu was estimated by comparison of the generated fluorescence with that of standard solutions. Eighty μ l of enzyme preparation in incubation buffer was added to 20 μ l of angiotensin I solution (5 mg/ml in 25% methanol). After 10 minute incubation at 37°C, the reaction was stopped by placing the tubes in an ice bath and by adding 2.9 ml of borate-phosphate buffer, pH 7.0. Four tenth ml of 2 M NaOH and 0.1 ml of o-phthal-dialdehyde solution (0.1% in methanol) were added. After 4 minutes, two tenths ml of 6 M HCl was finally added. The fluorescence was measured 90 minutes after addition of 6 M HCl with Hitachi Spectrophotometer (Model MPF-4) at the wave lengths of λ_{ex} = 370 nm and λ_{fl} = 500 nm. Tonin concentration was expressed micromoles of His-Leu released from angiotensin I.

Inhibition of Tonin Activity by Soybean Trypsin Inhibitor

The inhibitory effect of soybean trypsin inhibitor on tonin activity was conducted in the same experimental procedure as that of tonin activity except addition of the inhibitor. The reaction was initiated by adding 40 μ l of tonin solution in the incubation buffer and 40 μ l of soybean trypsin inhibitor solution in the same buffer with 20 μ l of angiotensin I solution (5 mg/ml) so that each reaction mixture had a final concentration of 1 μ g of tonin, 100 μ g of angiotensin I and 25, 50, 200, 400, 2,000 μ g 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.⁸ Polyacrylamide gel electrophoresis was carried out according to Davis⁹ with slight modification of gel concentration to give 10% polyacrylamide

Fig.2. Chromatography of submaxillary gland homogenates on soybean trypsin inhibitor coupled CH-Sepharose 4B at various pHs. 1.5 ml of submaxillary gland homogenates was dialyzed against various buffers, pHs ranging from 9.5 to 4.5 and applied on soybean trypsin inhibitor coupled CH-Sepharose 4B column (2.0 × 7.0 cm) previously equilibrated with each appropriate buffers. Each tube contained 5.3 ml.

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

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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

Fig.3. Chromatography of submaxillary gland homogenates on soybean trypsin inhibitor coupled CH-Sepharose 4B. The column (2.0 × 14.0 cm) was equilibrated with 0.05 M sodium acetate buffer, pH 5.3 containing 0.02% sodium azide. Adsorbed fractions were detached with 0.05 M glycine-NaOH buffer, pH 9.5 and successively 0.2 M glycine-HCl buffer, pH 2.5. Tonin activity and TAME esterase activity were determined on each fraction. Each tube contained 6.4 ml.

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 × 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 × 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 absorbance 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 (1.5×12 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 (1.9×85 cm) was equilibrated and eluted with 0.1 M potassium phosphate buffer, pH 7.0 containing 0.02% NaN_3 . Fractions of 2.7 ml were collected.

TABLE I PURIFICATION OF TONIN FROM RAT SUBMAXILLARY GLANDS

	Total Protein (mg)	Total Activity (μ mole His-Leu/min)	Specific Activity (μ mole His-Leu/min/ml)
SMG Homogenate	244.7	163.9	0.67
SMG Homogenate (pH 5.3)	164.3	129.5	0.79
after STI-CH Sepharose	14.2	65.2	4.59
after DE-52	6.7	34.8	5.19
after Sephadex G-100	4.6	34.1	7.41

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 study⁶. 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 × 12 cm) previously equilibrated with the same buffer. This 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 μ g) 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.¹ 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 STI-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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