Purification of Tonin by Affinity Chromatography

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SUMMARY Tonin has been purified from rat submaxillary glands. The purification procedure included affinity chromatography on Sepharose 4B coupled to antitonin followed by DEAE chromatography and gel filtration on Sephadex G-100. Homogeneity of the purified enzyme was confirmed by Sephadex G-100 gel filtration, disc electrophoresis, and isoelectric focusing on polyacrylamide gel, immunodiffusion, and immunoelectrophoresis. The tonin was purified 11.5-fold, with 35% recovery. The purified tonin has full enzymatic or immunological activity. (Hypertension 3: 81-86, 1981)

KEY WORDS • Tonin • affinity chromatography • angiotensin II • purification

Tonin is a proteolytic enzyme that can generate angiotensin II from angiotensinogen, from the synthetic tetradecapeptide renin substrate, or from angiotensin I.1,2 Tonin has been shown to differ from angiotensin I-converting enzyme, renin, or isorenins, and appears to play an important role in the local generation of angiotensin II in tissues.3 This enzyme has been purified from submaxillary glands of rats4 and is classified as a selective endopeptidase of the serine protease family.5

Affinity chromatography has been shown by many authors6,7 to be useful for the rapid and simple purification of enzymes and other proteins. This study describes the purification of tonin from rat submaxillary glands by affinity chromatography using antitonin coupled to Sepharose 4B as an immunoadsorbent and 125I-tonin as a tracer. A simple procedure is presented that yields tonin with intact enzymatic activity and immunological properties.

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Materials and Methods

Chemicals

Sephadex G-100 and cyanogen bromide-activated Sepharose 4B were obtained from Pharmacia Fine Chemicals. DEAE-cellulose (DE-52) was purchased from Whatman Biochemicals Ltd. Cyanogum 41 was purchased from Fisher Scientific Co., Fairlawn, New Jersey, and Ampholine from LKB Produkter, Bromma, Sweden. All other chemicals used were reagent grade commercial products.

Homogenates of Submaxillary Glands

All purification steps were carried out at 4°C. Submaxillary glands (6.3 g) from 10 male rats, each weighing more than 400 g, of the Sprague-Dawley strain were homogenized in 40 ml of 0.25 M sucrose solution, pH 7.0, containing 1 mM EDTA. The homogenate was centrifuged for 10 minutes at 17,000 g. The supernatant was centrifuged for 90 minutes at 105,000 g in a preparative ultracentrifuge. The supernatant obtained by ultracentrifugation was exhaustively dialyzed against 0.02 M Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl and 0.02% sodium azide. The final volume was adjusted to 30 ml, and this solution was used as a starting material. Homogenates were stored at -20°C until time of processing.

Preparation of Antiserum

In rabbits, antiserum to purified tonin was produced, and 50 μg of the tonin was emulsified in 1 ml of physiological saline with an equal volume of complete Freund's adjuvant, and injected at several
sites in the back of a rabbit. One day later, 0.5 ml of *Bordetella pertussis* vaccine was injected intramuscularly. Every month, 50 μg of tonin in complete Freund's adjuvant was administered. Tonin appeared to be a very antigenic protein as all immunized animals produced antibodies of a very high titer. The titer of these antibodies was measured in a radioimmunoassay system. The final dilution of antibodies used in the radioimmunoassay was 1:500,000 to 1:1,000,000.

A pool of four such antisera was used for preparation of an antitonin-Sepharose column. The binding capacity of antitonin was not affected by ammonium sulphate precipitation and dialysis. The specificity of antitonin was verified using different available proteins and peptides. The antibodies against purified tonin did not cross-react significantly (less than 0.1%) with either rat renin, renin substrate, trypsin, chymotrypsin, subtilisin, angiotensin I, or angiotensin II. However, the possibility that cross-reacting proteins and peptides. The antibodies against purified tonin did not cross-react significantly (less than 0.1%) with either rat renin, renin substrate, trypsin, chymotrypsin, subtilisin, angiotensin I, or angiotensin II. However, the possibility that cross-reacting substances other than those we tested may exist cannot be excluded.

The gamma-globulin fraction was obtained by ammonium sulphate precipitation at 0–5°C. The pooled antiserum from rabbits (63 ml) was slowly adjusted to 35% of saturation by addition of 13.17 g of finely powdered ammonium sulphate. After being stirred for 30 minutes, the suspension was centrifuged at 10,000 rpm for 10 minutes. The precipitate was resuspended in 20 ml of 0.02 M sodium phosphate buffer, pH 7.5, containing 0.15 M NaCl, and dialyzed exhaustively against 2 liters of the same buffer for 48 hours with two changes of buffer. The gamma-globulin fraction was concentrated in cellophane tubing under reduced pressure (200 mm Hg) to a volume of 25 ml and stored at 4°C. The resultant protein concentration was 22.4 mg/ml.

Before coupling to Sepharose 4B, this gamma-globulin solution was dialyzed overnight against 1 liter of 0.1 M sodium bicarbonate buffer, pH 8.3, containing 0.5 M sodium chloride.

**Coupling of Antitonin to Sepharose 4B**

Immunoadsorbent was prepared by coupling the gamma-globulin fraction of antiserum to cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions.

Then 20 g of CNBr-activated Sepharose 4B was allowed to swell in and be washed with 4 liters of 1 mM HCl. The slurry was suspended in an equal volume (70 ml) of 0.1 M sodium bicarbonate buffer, pH 8.3, containing 0.5 M sodium chloride, and 25 ml of the antiserum concentrate (i.e., 560 mg protein) was added. The tube was gently mixed end to end at 4°C for 24 hours with the aid of a rotary mixer. Then 14 ml of 1 M monoethanolamine, pH 9.0, was added and allowed to react with residual active sites for 2 hours at room temperature. The resin was repeatedly washed with 200 ml of 0.05 M Tris/HCl, pH 7.5, containing 0.5 M NaCl, 200 ml of 0.1 M sodium acetate buffer, pH 4.0, and 200 ml of 3 M KSCN in 0.01 M Tris/HCl, pH 7.5, containing 0.1 M NaCl. The Sepharose was finally equilibrated with 0.05 M Tris/HCl, pH 7.5, containing 0.5 M NaCl and 0.02% NaN₃, and stored at 4°C.

The amount of coupling was determined by measurement of the absorbance of the supernatant before and after the reaction. About 95% of the antibody was coupled to Sepharose 4B. The capacity of the antitonin-Sepharose 4B column was studied using different quantities of submaxillary gland homogenate containing radioactive tonin. When 3 ml or less of submaxillary gland homogenate was applied to the column, more than 92% of radioactivity was adsorbed.

**Other Methods**

Purification of tonin was monitored by polyacrylamide gel electrophoresis, according to a modification of the method of Davis. Size of the electrophoretic column was 0.5 × 9.0 cm. Concentrations of 10% and 1.5% polyacrylamide were used for the running gel and concentration gel respectively. Then 20 to 50 μg of protein in 0.1 M phosphate buffer, pH 7.4, was applied on each gel, and electrophoresis was run at 3 mA per column for 120 minutes at pH 8.3. The gels were stained with Coomassie blue R-250.

Isoelectric focusing was performed on a 115 × 250 × 2 mm polyacrylamide gel slab with an LKB 2117 Multiphor. The gel was composed of 20 ml of 18.06% of Cyanogum 41, 0.6 ml each of Ampholine pH 4-6, pH 5-7, and pH 7-9, 1.2 ml of Ampholine pH 3.5-10, and 36.6 ml of distilled water containing 7.5 g of sucrose. The polymerization is achieved by ultraviolet light after the addition of 0.4 ml of a solution of 0.01% riboflavin containing 0.1% N, N'-tetramethyl-ethylendiamine. The plate was focused at a maximum power of 30 W for 2 hours at a temperature of 4°C controlled by a bath circulator and with 1 M H₃PO₄ and 1 M NaOH as electrode solutions. After completion, a part of the gel was washed with 1 liter of 10% trichloroacetic acid for 2 hours and the proteins colored by the method of Blakesley and Boezi. The other part of the gel was cut into 1.5 × 1 cm pieces, which were eluted in 1 ml of distilled water for the measurement of the pH gradient.

The purity of tonin was demonstrated by Ouchterlony's double immunodiffusion and immunoelectrophoresis. For the immunodiffusion, the concentration of agarose gel was 1% in 0.02 M Tris/HCl buffer at pH 7.4, containing 0.15 M NaCl. Tonin and antisera were placed in the appropriate wells. After formation of the precipitin line (24 hours at 4°C), the agarose gel was soaked in 0.02 M Tris/HCl buffer at pH 7.4, 0.15 M NaCl for 48 hours with two changes, and in distilled water for 1 hour. The plates were dried with filter paper and stained for the protein by Coomassie blue G-250. Immunoelectrophoresis was performed on 1% agarose gel in 0.075 M Veronal buffer at pH 7.4 in an LKB 2117 Multiphor. Electrophoresis took place for 2 hours at 150 V at 4°C, and was followed by diffusion against...
antsin antiserum for 24 hours. After precipitin lines had formed, the plates were washed and stained as described for immunodiffusion.

Protein concentration was measured according to the method of Benadoun and Weinstein with bovine serum albumin as a standard, which was a modification of a standard Lowry protein assay.

Tonin activity was measured by a fluorometric method that uses angiotensin I as a substrate. Tonin was iodinated and I-Tonin was purified by the method of Gutkowska et al. Kinetic studies were performed by radioimmunoassay of angiotensin II and fluorometric determination of His-Leu using angiotensin I as a substrate. Samples of 2 μg of tonin were incubated with five different concentrations of substrate for 2 minutes at 37°C. Three separate measurements were done for each of the five substrate concentrations, and Km was determined by means of a Lineweaver-Burk plot. No significant difference was observed between both methods, angiotensin II and His-Leu, of measurement of tonin activity on angiotensin I.

Results

Affinity Chromatography on Antitonin-Sepharose 4B

First, 3 ml of submaxillary gland homogenate was mixed with an equal volume of 0.05 M Tris/HCl buffer, pH 7.5, containing 0.5 M NaCl (buffer A) and about 15,000 cpm of I-Tonin. This solution was then applied to an antitonin-Sepharose 4B column (1.6 X 40 cm) which has been equilibrated with 0.05 M Tris/HCl buffer, pH 7.5, containing 0.5 M NaCl and 0.02% Tween 20 (buffer B). The column was washed with 90 ml of buffer B and successively washed with buffer A until the eluate had a constant absorbance at 280 nm.

Tonin was eluted with 3 M KSCN in 0.01 M Tris buffer, pH 7.5, containing 0.1 M NaCl. Minor and major peaks of radioactivity (fig. 1) coincided in position with protein peaks absorbing at 280 nm. Fractions 56 to 63 (40 ml) (fig. 1) were pooled and immediately dialyzed against 1 liter of 0.02 M Tris/HCl buffer, pH 8.0, and concentrated to 3 ml in cellophane tubing under reduced pressure. The concentrated samples were kept at 4°C until the next step. The immunoadsorbent column was thoroughly washed with buffer A and equilibrated with buffer B for reuse. The procedure gave the same elution pattern at least 10 times.

The adsorbing capacity of the affinity column (1.6 X 40 cm) was 3.7 mg of tonin, or 0.051 mg of tonin per 1 ml of gel. Recovery of tonin from the Sepharose-antitonin column was 67%. This value was obtained by measurement of tonin activity and by measurement of the radioactive tonin used as tracer.

DEAE-Cellulose Chromatography

The pooled and concentrated solution (2 ml) from the affinity column was equilibrated with 0.02 M Tris/HCl buffer, pH 8.0, containing 1 mM EDTA and applied to a DEAE-cellulose column (DE-52, 1.5 X 12 cm) previously equilibrated with the same buffer. This
column was eluted with 500 ml of eluent in a linear gradient (0–0.15 M NaCl in the same buffer) at a flow rate of 15 ml/hour, and 5 ml fractions were collected.

The major peak corresponded to the peak of radioactivity (fig. 2). Fractions 46–50 (25 ml) (fig. 2) were pooled and concentrated to a volume of 2 ml in a cellophane tube under reduced pressure. The concentrated sample was dialyzed against 1 liter of 0.02 M Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl and 0.02% NaN₃ and kept at 4°C until the next step.

In this step, 14.8 mg of proteins were obtained with overall tonin purification of 11-fold and an overall tonin recovery of 37%.

Gel Filtration on Sephadex G-100

Two ml of the concentrated solution from DEAE-cellulose chromatography was applied on a Sephadex G-100 column (1.5 X 80 cm) equilibrated with 0.02 M Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl and 0.02% NaN₃. A single major symmetrical peak was obtained, although radioactivity was found in many of the fractions (fig. 3). Fractions 28–34 were collected, concentrated to 2.0 ml, and dialyzed against 0.1 M potassium phosphate buffer, pH 6.8, containing 0.02% NaN₃. This preparation of tonin solution at a concentration of 5.0 mg/ml was kept at 4°C and was stable over 2 months.
The tonin preparation after DEAE-cellulose chromatography showed one band on polyacrylamide gel electrophoresis (fig. 4) and had a specific activity almost as high as that of the final preparation (table 1).

The purity of tonin was demonstrated by the appearance of only one band on polyacrylamide gel electrophoresis (fig. 4, column 4) and by isoelectric focusing (fig. 4, column 5). The isoelectric point of tonin is about pH 6.2. The homogeneity was also shown by appearance of only one arc on the immunodiffusion and immunoelectrophoresis (fig. 5A and B).

The Product

Table 1 summarizes the results of the purification of tonin from submaxillary glands of rats by affinity chromatography using immunoadsorbent. From 6.3 g of submaxillary glands from 10 rats, 12.8 mg of pure tonin was obtained, with a yield of 35%. The Michaelis-Menten constant (Km) for angiotensin I calculated from the Lineweaver-Burk plot was 1.97 ± 0.52 × 10⁻⁸M (n = 19), and Vmax was 2.69 ± 0.47 nmoles/min/μg (n = 19). These results were obtained with tonin purified by affinity chromatography as well as with tonin purified by the classical procedure.*

Discussion

Affinity chromatography is extensively used for the purification of proteins and enzymes. Immunoabsorption, an application of affinity chromatography, was achieved by using a specific antibody against highly purified tonin. Immunoabsorption is a simple and effective method for isolating antibodies or antigen, but a major drawback can be the nonspecific adsorption of other proteins.18,19 In our system, nonspecific binding was reduced by the addition of Tween 20 and a high concentration of salt to the washing buffer.18 Desorption can present another problem, since the desorbing reagent is generally an acidic buffer or a concentrated solution of urea, either of which may denature proteins or deactivate enzymes.

Tonin activity is denatured at a pH below 2.8 or in concentrations of urea higher than 4 M.

We found that the chaotropic reagent, 3 M KSCN, could be used to elute tonin at neutral pH and had no effect on the activity of tonin, so long as the eluate was dialyzed less than 2 hours after desorption. The essential processes were, therefore, the use of a detergent to reduce nonspecific adsorption and of 3 M KSCN for desorption of the enzyme. Use of ¹²⁵I-tonin as a tracer enabled us to readily detect tonin peaks without measuring tonin activity in each fraction.

The overall yield with the classical procedure was approximately 70% whereas a lower yield, 35%, was observed with the affinity chromatography.

The enzyme was purified to homogeneity, using commonly accepted criteria of purity that is: 1) gel filtration on Sephadex G-100 (fig. 3) yielded a single

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*Figures 4 and 5 are included in the document, but the text does not reference them specifically.
symmetrical peak of protein; 2) polyacrylamide gel electrophoresis (fig. 4, column 4) demonstrated a single homogenous band; 3) isoelectric focusing on polyacrylamide slab (fig. 4, column 5) also showed a single protein band; 4) in addition, the homogeneity of purified tonin was shown by the appearance of a unique arc on immunoelectrophoresis (fig. 5B) and immunodiffusion (fig. 5A).

The discrepancies that exist between the much higher specific activity for tonin (2000 nmoles of His-Leu liberated/min/mg of protein compared to 18,000 reported earlier) and fold of purification (12-fold as compared to 150-fold reported previously) was due to a decimal error in the calculation of incubation time which escaped the attention of the authors. As is shown on (fig. 4, column 1) polyacrylamide electrophoresis, tonin is one of a major protein components in homogenates of submaxillary glands. Based upon the measurement of tonin activity in crude homogenates of rat’s submaxillary glands and of purified tonin specific activity, we have calculated that tonin represents 7% to 8% of the protein content of supernatant obtained by 105,000 g centrifugation.

Tonin, prepared accordingly to the classical procedure, is consistent with a specific activity of about 2000 nmoles of His-Leu liberated/min/mg of protein and with a 12-fold purification. So large a concentration of tonin in the submaxillary gland remains to be explained.

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References

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