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TONIN, AN ESTEROPROTEASE FROM RAT SUBMAXILLARY GLANDS

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Tonin is an enzyme found in the rat submaxillary glands which liberates angiotensin II from angiotensinogen, the Skeggs tetradecapeptide renin substrate, and angiotensin I. Tonin hydrolyzes benzoyl-arginine ethyl ester, benzoyl-arginine methyl ester, tosyl-arginine methyl ester, benzoyl-arginine *p*-nitroanilide and other small synthetic substrates at an optimum pH of 9.0. Tonin shows, however, a great specificity with respect to angiotensin I. Tonin is inhibited by diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride at high concentrations ($>10^{-2}$ M) and by soybean trypsin inhibitor and aprotinin. Tonin is thus an esteroprotease of the class of the serine proteases with trypsin- and chymotrypsin-like activity. Tonin belongs to the same family of enzymes as glandular kallikrein and the γ subunit of the nerve growth factor.

Introduction

Tonin is an enzyme found in the submaxillary glands of rats. It hydrolyzes angiotensin I with liberation of a histidyl-leucine dipeptide to produce angiotensin II, the most potent vasoactive polypeptide hormone [1,2]. Tonin has been purified to homogeneity by conventional and immunoaffinity techniques [3,4] and has been crystallized [5]. It has a molecular weight of 30 000 [3] and an amino acid composition and N-terminal amino acid sequence that are similar to those of serine proteases [6]. The substrate specificity of tonin indicates that it is not a renin-like enzyme or an angiotensin-converting enzyme. Tonin generates exclusively angiotensin II from angiotensinogen, the Skeggs tetradecapeptide substrate of renin and angiotensin I, and it is not inhibited by pepstatin, EDTA, SQ 20,881 or SQ 14,225 [2,7]. Rat plasma contains a potent inhibitor of tonin, which has been identified as a protein with a molecular weight of 350 000 [8].

We now present new data on the substrate specificity of tonin and on inhibitors of this enzyme which demonstrate that it is a serine protease with trypsin- and chymotrypsin-like activity, depending on the type of substrate.

Materials and Methods

Chemicals

Ac-Phe-OEt, azoalbumin, azocasein, Bz-Arg-OEt, Bz-Arg-OMe, Bz-Pro-Phe-Arg-pNA, Cbz-Gly-Pro-Arg-pNA, Bz-Gly-His-Leu, ovomucoid from egg white, phenylmethylsulfonyl fluoride, Suc-Phe-pNA and Tos-Arg-OMe were purchased from Sigma Chemical Co. (St. Louis, MO); Ac-Tyr-OEt, Bz-Arg-NH_2, Bz-Arg-pNA, Bz-Tyr-OEt, Bz-Tyr-pNA and diisopropyl fluorophosphate from Aldrich Chemical Co. (Milwaukee, WI); azocollagen, Nα-Tos-Lys-chloromethane and Nα-Tos-Phe-chloromethane from Calbiochem (San Diego, CA); lima bean trypsin inhibitor and soybean trypsin inhibitor from Nutritional Biochemicals Corp. (Cleveland, OH); bovine α-chymotrypsin and bovine trypsin from Miles Laboratories (Elkhart, IN); aprotinin (Trasylol) from Boehringer Ingelheim (Burlington, Ontario); (Ile^5)-angiotensin I from Peninsula Laboratory (San Carlos, CA); (des-

Asp[1], Ile[5])-angiotensin I was kindly provided by Dr. P.W. Schiller of the Clinical Research Institute of Montreal (Montreal, Quebec); Cbz-His-Pro-Phe-His-Leu and Cbz-Pro-Phe-His-Leu by Dr. H. Immer of Ayerst Laboratories (Ville St-Laurent, Quebec); (Ile[5], des-Leu[10])-angiotensin I was synthetized by Schiller et al. [9].

DEAE-Sephacel and Sephadex IEF were obtained from Pharmacia Fine Chemicals (Dorval, Quebec); DEAE-Bio-Gel A from Bio-Rad Laboratories (Mississauga, Ontario); phospho-cellulose from Sigma Chemical (St. Louis, MO).

Purification of tonin

The conventional technique of Demassieux et al. [3] for the purification of tonin was modified in order to increase its rapidity. All purification steps were performed at 4°C. The submaxillary glands from 100 Sprague-Dawley male rats were homogenized with a Virtis 45 apparatus at maximum speed in 0.01 M potassium phosphate buffer, pH 6.8/0.25 M sucrose/0.1% Tween 20 (5 ml/g tissue). The slurry was centrifuged at 30000 × *g* for 20 min and the supernatant dialyzed for 24 h against 0.02 M Tris-HCl, pH 8.0/0.1% Tween 20. The clear homogenate was applied to a DEAE-Bio-Gel A column (2.6 × 96 cm) previously equilibrated with the dialyzing buffer. Tonin was eluted at 15 ml/h with a linear sodium chloride gradient. Tonin was present in fractions collected at a sodium concentration of 0.03 M.

The second purification step consisted of a chromatography on a phospho-cellulose column (2.6 × 60 cm). The fractions containing angiotensin I-hydrolyzing activity were pooled, concentrated with Aquacide II, dialyzed against 0.2 M K_2HPO_4/0.1 M citric acid (McIlvaine's buffer) [10], at pH 5.5, diluted 16-fold with distilled water, and applied to the column. Tonin was eluted by means of a gradient consisting of 250 ml of 16-fold-diluted McIlvaine's buffer, pH 5.5, and 250 ml of undiluted McIlvaine's buffer, pH 6.8, at a flow rate of 15 ml/h. The active fractions were pooled, dialyzed against 40-fold-diluted McIlvaine's buffer, pH 8.0, and applied to a DEAE-Sephacel column (1.6 × 36 cm) equilibrated with the dialyzing buffer. A linear gradient of 200 ml of 40-fold-diluted McIlvaine's buffer, pH 8.0, and 200 ml of 40-fold-diluted McIlvaine's buffer, pH 2.2, containing 0.03 M NaCl was used for the elution of tonin. The fractions with angiotensin I-hydrolyzing activity were pooled for the final step which consisted of preparative isoelectric focusing on Sephadex IEF. The technique used was essentially that published by LKB-Produkter (Bromma, Sweden) with a pH gradient of 5–7.5 [11]. The sample to be focused contained 200 mg protein.

Tonin obtained after these four purification steps was homogeneous according to various criteria (isoelectric focusing, polyacrylamide gel electrophoresis and gel filtration) and as compared to tonin purified by other techniques [3,4]. The specific activity at 37°C of pure tonin is about 2000 nmol angiotensin II liberated/min per mg protein.

Measurement of tonin

Tonin activity was measured at pH 6.8 by a fluorometric automated method which allows the detection of histidyl-leucine liberated by tonin from angiotensin I. This method, described by Boucher et al. [1], was slightly modified by the use of a second proportioning pump to allow simultaneous entry and mixing of the sample with 80 μg angiotensin I.

Hydrolysis of synthetic substrates

Hydrolysis of all substrates was measured at 37°C. Hydrolysis of Bz-Arg-pNA, Bz-Tyr-pNA, Bz-Pro-Phe-Arg-pNA, Cbz-Gly-Pro-Arg-pNA and Suc-Phe-pNA was followed spectrophotometrically by the liberation of *p*-nitroanilide at a wavelength of 405 nm ($\epsilon = 9900\ M^{-1} \cdot cm^{-1}$). Hydrolysis of Bz-Arg-OEt, Bz-Arg-OMe and Bz-Arg-NH_2 was measured by the increase of absorbance at 253 nm caused by the liberation of benzoyl-arginine ($\epsilon = 1150\ M^{-1} \cdot cm^{-1}$). Liberation of tosyl-arginine from Tos-Arg-OMe was followed at 247 nm ($\epsilon = 540\ M^{-1} \cdot cm^{-1}$). The incubation medium for these substrates consisted of 0.7 ml of 0.1 M Tris-glycine, pH 8.5, 0.1 ml of 5 mM substrate, 0.1 ml of enzyme (trypsin, α-chymotrypsin or tonin, 0.1–400 μg) and 0.1 ml of H_2O or 0.25 M $CaCl_2$ for trypsin or α-chymotrypsin.

Hydrolysis of substrates containing the ethyl ester group, such as Bz-Tyr-OEt, Ac-Tyr-OEt and Ac-Phe-OEt, was measured by incubating the ethanol liberated with NAD-alcohol dehydrogenase. The incubation medium consisted of 0.5 ml 0.1 M Tris-glycine, pH 8.5/0.1 ml enzyme/0.1 ml 5 mM substrate/0.1 ml H_2O or 0.25 M $CaCl_2$/0.1 ml 2 mM

NAD/0.1 ml 15 mg/ml alcohol dehydrogenase. NADH formation was followed at 340 nm ($\epsilon = 6220$ $M^{-1} \cdot cm^{-1}$).

For reasons of solubility, some substrates (Ac-Tyr-OET, Ac-Phe-OEt, Bz-Tyr-OEt and Bz-Tyr-pNA) were dissolved in 50% methanol or acetone.

Hydrolysis of azoproteins

Proteolytic activity of tonin was measured with azoproteins as substrates at 37°C. The incubation medium consisted of 3 ml 2% azoprotein (azocasein or azoalbumin dissolved in 0.1 M NaCl)/1.47 ml 0.1 M Tris-glycine, pH 8.5, (containing 0.075 M $CaCl_2$ in the case of trypsin and α-chymotrypsin)/0.03 ml enzyme (tonin, trypsin or α-chymotrypsin, up to 50 μg). At various intervals, 1 ml reaction mixture was added to 1 ml 10 g/dl trichloroacetic acid. After 30 min of standing, the solution was filtered to remove the precipitated proteins and the amount of azo groups hydrolyzed was determined spectrophotometrically at a wavelength of 365 nm [12]. For azocollagen, which is insoluble, the reaction mixture was constantly agitated. Aliquots were taken at different times and filtered immediately through glass wool. The absorbance of the filtrate was measured at 580 nm [13].

Hydrolysis of angiotensin I and its derivatives

Liberation of histidyl-leucine from (Ile^5)-angiotensin I, (des-Asp^1, Ile^5)-angiotensin I, Cbz-His-Pro-Phe-His-Leu, Cbz-Pro-Phe-His-Leu and Bz-Gly-His-Leu was measured by the fluorimetric assay already described, except that the incubation period was set manually. For the substrate (Ile^5, des-Leu^{10})-angiotensin I, the histidyl group liberated was measured by the same assay except that the entry of HCl to the automated system was blocked with consequent increase in fluorescence of the final product [14].

For all these substrates, the incubation mixture consisted of 0.1 ml substrate (40 nmol)/0.85 ml 0.05 M sodium borate/0.1 M potassium phosphate, pH 7.0/0.05 ml enzyme (tonin or α-chymotrypsin, 0.1–200 μg). After incubation at 37°C, up to 60 min, 2.0 ml borate-phosphate buffer was added and the histidyl-leucine or histidine liberated was quantified immediately by the automated assay.

Optimum pH

The optimum pH of tonin on synthetic substrates was determined in 0.1 M citrate-phosphate buffer and in 0.1 M Tris-glycine buffer.

Inhibitors

The action of some protease inhibitors, such as aprotinin, soybean trypsin inhibitor, ovomucoid and lima bean trypsin inhibitor, on tonin was quantified by the fluorimetric assay in the presence of 1 μg tonin, with angiotensin I as substrate.

Inhibitory effects of diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, Tos-Lys-chloromethane and Tos-Phe-chloromethane on tonin were determined by the fluorimetric assay with angiotensin I as substrate and by the spectrophotometric assay with Bz-Arg-pNA. Various concentrations of diisopropyl fluorophosphate or phenylmethylsulfonyl fluoride, dissolved in isopropanol, and Tos-Lys-chloromethane or Tos-Phe-chloromethane, dissolved in methanol were added to the assay buffers containing 1 μg tonin, at pH 7.0, for the fluorimetric assay, and at pH 9.0 for buffers containing 100 μg tonin for the spectrophotometric assay. The residual activity after 4 h incubation at 22°C was measured as described. Bz-Arg-pNA, for trypsin, and Suc-Phe-pNA for α-chymotrypsin were used as substrates to verify the inhibitory effects of these compounds at pH 9.0 on trypsin and α-chymotrypsin.

Results

The methods of purification described yield pure tonin with a purification factor of 15 and a recovery of 55%. Comparison of the specific activity of pure tonin with its initial specific activity and to the weight of the whole gland shows that tonin represents about 8% of the protein content of the homogenates, and it can be calculated that 1 g submaxillary glands contain about 4 mg tonin.

Actions of tonin, trypsin and α-chymotrypsin on various synthetic substrates are shown in Table I. Tonin has a trypsin-like activity, since it hydrolyzes the majority of substrates cleaved by trypsin. Bz-Pro-Phe-Arg-pNA, a good substrate for kallikrein [15], is slowly hydrolyzed by tonin. In general, tonin shows more esterolytic than amidolytic activity. Substrates containing tyrosine or phenylalanine residues, which are easily hydrolyzed by α-chymotrypsin, are not appreciably hydrolyzed by tonin.

TABLE I

HYDROLYSIS OF VARIOUS SYNTHETIC SUBSTRATES BY TONIN, α-CHYMOTRYPSIN AND TRYPSIN AT pH 8.5

Substrates were incubated at 37°C in a final volume of 1 ml in the presence of tonin, α-chromotrypsin or trypsin. The incubation medium contains 0.025 M $CaCl_2$ for trypsin and α-chymotrypsin. Tyrosine and phenylalanine substrates were dissolved in 50% methanol or acetone. Hydrolysis rate represents the initial velocity of the reaction. n.d., no detectable hydrolysis

Substrate	Hydrolysis rate (nmol/min per mg)		
	α-chymotrypsin	trypsin	tonin
Bz-Arg-NH_2 [a]	n.d.	130	n.d.
Bz-Arg-OMe [a]	n.d.	39600	32700
Bz-Arg-OEt [a]	210	38500	48500
Bz-Arg-pNA [b]	n.d.	830	70
Tos-Arg-OMe [c]	160	159300	29000
Bz-Pro-Phe-Arg-pNA [b]	n.d.	23000	60
Cbz-Gly-Pro-Arg-pNA [b]	n.d.	101000	9600
Ac-Tyr-OEt [d]	200	30	4
Bz-Tyr-OEt [d]	230	70	10
Bz-Tyr-pNA [b]	980	2	7
Ac-Phe-OEt [d]	200	25	1
Suc-Phe-pNA [b]	24	<1	<1

[a] Measured by liberation of benzoyl-arginine at 253 nm.
[b] Measured by liberation of *p*-nitroanilide at 405 nm.
[c] Measured by liberation of tosyl-arginine at 247 nm.
[d] Measured by liberation of ethanol after coupling to the NAD-alcohol dehydrogenase at 340 nm.

The proteolytic activity of tonin was verified on azoproteins (Table II). Tonin had lower activity on azocasein and azocollagen than trypsin or α-chymotrypsin. It was, however, more active on azoalbumin.

The substrate specificity of tonin was further investigated on some derivatives of angiotensin I (Table III). Tonin acts on these substrates by hydrolyzing the Phe-His bond (or Gly-His bond). Tonin cleaves the Phe-His bond of angiotensin I and (des-Asp^1)-angiotensin I, as does α-chymotrypsin, to produce angiotensin II and angiotensin III. Elimination of the first amino acids in Cbz-His-Pro-Phe-His-Leu and Cbz-Pro-Phe-His-Leu or of the last amino acid in (des-Leu^{10})-angiotensin I dramatically reduces the rate of hydrolysis of the Phe-His bond by tonin. Bz-Gly-His-Leu, a good substrate for angiotensin-converting enzyme [16], is not cleaved by tonin. The integrity of the structure of angiotensin I for the action of α-chymotrypsin is far less important, since the majority of angiotensin I fragments are hydro-

TABLE II

HYDROLYSIS OF AZOPROTEINS BY TONIN, α-CHYMOTRYPSIN AND TRYPSIN AT pH 8.5

Azoproteins (2%), in solution in the case of azocasein and azoalbumin or in suspension for azocollagen, were incubated at 37°C in a total volume of 4.5 ml in the presence of the enzyme. At various intervals, up to 15 min, 1.0 ml incubation medium was added to 1.0 ml 10 g/dl trichloroacetic acid or filtered. The absorbance of the supernatant was measured at 365 nm for azocasein and azoalbumin or at 580 nm for azocollagen. Hydrolysis rate represents the initial velocity of the reaction

Substrate	Hydrolysis rate (ΔA/min per mg)		
	α-chymotrypsin	trypsin	tonin
Azocasein	9.2	8.6	2.2
Azoalbumin	2.6	5.4	10.7
Azocollagen	6.2	56.0	1.5

TABLE III

HYDROLYSIS OF ANGIOTENSIN I AND SOME DERIVATIVES BY α-CHYMOTRYPSIN AND TONIN AT pH 7.0

Substrates (40 nmol) were incubated at 37°C in a total volume of 1 ml in the presence of α-chymotrypsin or tonin. After various periods of incubation, up to 60 min, the mixture was diluted to 3 ml with phosphate-borate buffer (pH 7.0) and His-Leu or His liberated were analyzed by the fluorimetric automated method. Hydrolysis rate represents the initial velocity of the reaction. n.d., no detectable hydrolysis

Substrate	Hydrolysis rate (nmol/min per mg)	
	α-chymotrypsin	tonin
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu [a]	1970	1600
–Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu [b]	1660	1930
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His– [c]	<1	1.1
Cbz-His-Pro-Phe-His-Leu	3190	0.3
Cbz-Pro-Phe-His-Leu	36	n.d.
Bz-Gly-His-Leu	<1	n.d.

[a] (Ile^5)-angiotensin I.
[b] (des-Asp^1, Ile^5)-angiotensin I.
[c] (Ile^5, des-Leu^{10})-angiotensin I.

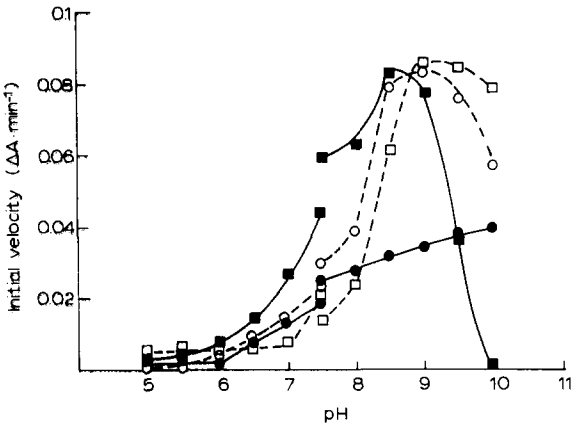

Fig. 1. The pH optimum of tonin acting on four synthetic substrates. Substrates (0.5 mM) were incubated with tonin in a total volume of 1 ml in citrate-phosphate buffer or Tris-glycine buffer. Absorbance was monitored immediately after addition of enzyme at 247 nm for Tos-Arg-OMe (■———■), at 253 nm for Bz-Arg-OMe (□- - - - -□) and Bz-Arg-OEt (○- - - - -○) and at 405 nm for Bz-Arg-pNA (●———●).

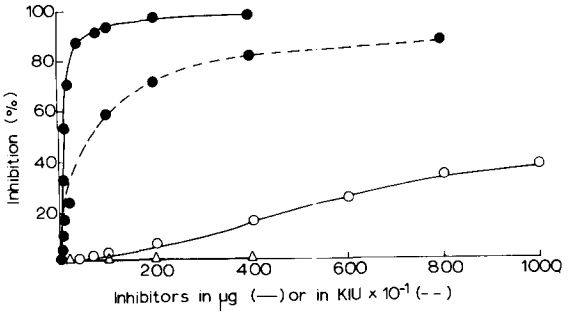

Fig. 2. Inhibition of tonin by protein inhibitors. Various quantities of soybean trypsin inhibitor (●———●), lima bean trypsin inhibitor (○———○), aprotinin (●- - - - -●) or ovomucoid (△———△) were added to 1 μg tonin. The residual enzymatic activity was immediately measured with angiotensin I as substrate by the fluorimetric automated assay.

lyzed. The specificity of action of α-chymotrypsin, however, is not restricted to the Phe-His bond, since it also cleaves the Tyr-Ile bond of angiotensin I [17].

The optimum pH of tonin on some synthetic substrates was determined (Fig. 1). The optimum pH of tonin for Tos-Arg-OMe is 8.5, 9.0 for Bz-Arg-OEt, 9.0–9.5 for Bz-Arg-OMe and above 10.0 for Bz-Arg-pNA. The kinetic parameters for these substrates were also analyzed at pH 9.0 (Table IV). Among them, Bz-Arg-OEt is the best substrate based on the K_{cat} value.

The inhibitory action of some proteins, inhibitors of serine proteases, on the enzymatic activity of tonin was measured (Fig. 2). Only soybean trypsin inhibitor and aprotinin (depending on the lot number) had a potent inhibiting action on tonin. 200 μg soybean trypsin inhibitor were required to inhibit tonin completely. Lima bean trypsin inhibitor and ovomucoid were less effective. The action of chemical inhibitors of serine proteases was also studied. Diisopropyl fluorophosphate and phenylmethylsulfonyl fluoride, which inhibited trypsin and chymotrypsin almost completely at a molar ratio (inhibitor/enzyme) higher than 100, inhibited tonin by 40% only when present at a molar ratio exceeding 10000. Tos-Phe-chloromethane did not appreciably inhibit tonin, although it inhibited α-chymotrypsin at a molar ratio higher than 10. However, Tos-Lys-chloromethane, a specific inhibitor of trypsin-like proteases, inhibited trypsin at a molar ratio greater than 10 and reduced tonin activity by 20% at a molar ratio of 10000.

Discussion

These results show that tonin is a serine protease, since it hydrolyzes small synthetic substrates and is

TABLE IV

MICHAELIS-MENTEN PARAMETERS FOR THE HYDROLYSIS OF SYNTHETIC SUBSTRATES BY TONIN AT pH 9.0

Michaelis-Menten parameters were calculated from Lineweaver-Burk plots. Substrates (0.05–2 mM) were incubated at pH 9.0, at 37°C in the presence of tonin in a total volume of 1 ml, and the hydrolysis rate was monitored immediately at 247 nm for Tos-Arg-OMe, at 253 nm for Bz-Arg-OMe and Bz-Arg-OEt, and at 405 nm for Bz-Arg-pNA after addition of 5.0, 1.25, 1.0 or 100 μg tonin, respectively

Substrate	K_m (mM)	V (nmol/min)	K_{cat} (s^{-1})
Bz-Arg-pNA	0.49	17.7	0.09
Tos-Arg-OMe	1.20	448.3	0.49
Bz-Arg-OEt	2.73	545.0	2.73
Bz-Arg-OMe	5.13	1035.0	2.03

inhibited by diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride and by soybean trypsin inhibitor and since Seidah et al. [6] have found that the N-terminal amino acid sequence of tonin is similar to that of other serine proteases. Although tonin displays tryptic activity but no activity on synthetic chymotrypsin substrates, it exerts exclusively a chymotrypsin-like activity on the Phe-His bond of angiotensin I and (des-Asp^1)-angiotensin I. Progressive removal of N-terminal amino acids from angiotensin I abolishes completely the hydrolysis of the Phe-His bond. Schiller et al. [9] have demonstrated that the rate of hydrolysis of tonin on (des-Asp^1, des-Arg^2)-angiotensin I was 10-times lower than on angiotensin I or (des-Asp^1)-angiotensin I. The integrity of angiotensin I thus appears very important for the binding of these substrates to tonin. These results clearly demonstrate that tonin is highly specific in its action on angiotensin I. Tonin also possesses a high activity toward the tetradecapeptide substrate of renin and angiotensinogen (Refs. 2 and 18, Gutkowska, J. and Corvol, P., unpublished data). Tonin is thus able to generate angiotensin II and angiotensin III directly, two polypeptide hormones which share important physiological actions, from natural biological material.

Although, the specificity of its action on angiotensin I is high, tonin demonstrates a trypsin-like activity. Seidah et al. [19] and Chrétien et al. [20] have reported that tonin hydrolyzes β-LPH, ACTH and substance P. This action on some Phe and Arg bonds corresponds thus to a trypsin- and a chymotrypsin-like activity.

According to all these results, tonin may be considered a member of the family of esteroproteases. Many esteroproteases have been described and purified from the submaxillary glands of rats and mice [21–27]. In particular, Riekkinen et al. [24] have purified an alkaline esteroprotease, salivain, with a molecular weight of 30000 and an isoelectric point of about 6.0 and which possesses proteolytic activity and a high activity at pH 9.0 toward Bz-Arg-OEt and Bz-Arg-OMe. This enzyme was also inhibited by diisopropyl fluorophosphate and aprotinin but not by lima bean trypsin inhibitor or ovomucoid. All these data indicate that tonin and salivain may be identical.

Recent results of Thomas et al. [28] on the complete amino acid sequence of the γ subunit of mouse nerve growth factor, an esteroprotease with a molecular weight of 30000, and those of Lazure et al. (Lazure, C., Seidah, N.G., Thibault, G., Boucher, R., Genest, J. and Chrétien, M., unpublished data) which have extended the sequencing of rat tonin [6] to 95 of 272 residues, revealed a 75% homology between the two molecules. The sequence Val-Leu-Thr-Ala-Ala-Gly-Tyr, found in all serine proteases, has been found to be completely preserved in tonin.

Moreover, one of the esteroproteases found in the rat submaxillary glands has been identified as glandular kallikrein [29,30]. This enzyme possesses esterolytic and amidolytic activities and has a molecular weight of about 30000. Thus, rat submaxillary glands apparently contain tonin, which may generate a vasopressor peptide, and kallikrein, which liberates Lys-bradykinin, a vasodilator peptide. An equivalent situation is found in mouse submaxillary glands, which contain isorenin and glandular kallikrein [31].

It seems thus that tonin, an esteroprotease which generates angiotensin II, belongs to the same family of esteroproteases as glandular kallikrein and the γ subunit of nerve growth factor.

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