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Nerve Growth Factor Biosynthesis: Isolation and Characterization of a Guinea Pig Prostate Kallikrein

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Guinea pig prostate contains one major soluble esteropeptidase activity. The protein has been purified and characterized and found to be a glycoprotein comprised of a single polypeptide chain. The molecular weight of the deglycosylated protein is approximately 26,000. The esteropeptidase has a similar K_m for lysine and arginine synthetic substrates, although the V_{max} for arginine is much greater than that for lysine. Amino-terminal sequence analysis has also revealed a marked degree of homology to mouse γ-nerve growth factor (NGF) and the kallikrein family of serine proteases. In contrast to γ-NGF, however, the guinea pig enzyme does not appear to form stable complexes with β-NGF.

Key words: guinea pig, kallikrein, nerve growth-factor

Nerve growth factor from mature male mouse submandibular glands is isolated as a high-molecular-weight complex (7S) comprised of three distinct polypeptides [1]. The hormonally active β subunit is a dimer and is associated with two subunits each of α- and γ-NGF. γ-NGF is an arginine esteropeptidase [2,3] and appears to form a stable complex with β-NGF by interaction with the C-terminal arginine of the β subunit [4]. cDNA sequencing has revealed that α-NGF has approximately 80% homology with the γ subunit; however, important substitutions at the amino terminus and within the active site have rendered this protein enzymatically inactive [5].

Despite the number of studies on the structure and function of β-NGF, little is as yet known of the mechanism and control of the biosynthesis of the protein. It is apparent, from the c-DNA sequence, that mouse β-NGF is synthesized as a large precursor with flanking residues on both sides of the mature protein sequence [6,7].

Abbreviations used: NGF, nerve growth factor; DFP, diisopropyl phosphofluoridate; TFMS, trifluoromethanesulfonic acid; TAME, N^{α}-p-tosyl-L-arginine methyl ester; TLME, N^{α}-p-tosyl-L-lysine methyl ester; BAPNA, benzylarginylphenylnitroanilide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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The C-terminal extension is comprised of only two amino acids; the N-terminal prepropeptide (approximately 14,000 MW [7]) contains three regions of pairs of basic residues constituting possible sites of processing. The homology of γ-NGF to kallikreins (ie, a subset of serine proteases that have a high degree of substrate specificity and have been attributed a role in the maturation of growth factors [8]) has led to speculation that γ-NGF is involved in the processing of the β-NGF precursor. The 7S NGF entity is then considered to represent, at least in part, a stable enzyme-substrate complex [9,10].

One approach to ascertain the role of γ-NGF in the biosynthesis of β-NGF is to study the structure of β-NGF in tissues other than the mouse submandibular gland and to determine whether or not arginine esteropeptidases, analogous to γ-NGF, are present and form stable complexes with β-NGF. It has previously been shown that guinea pig prostates are also a relatively rich source of NGF [11]. This tissue has been analyzed in the current study for the presence and interaction of arginine esteropeptidases with guinea pig β-NGF.

MATERIALS AND METHODS

Guinea pig prostates were obtained as frozen tissue from Bio-Labs (Minneapolis, MN). Sephadex G-100-120, G-75-40, and DEAE-Sephadex were from Sigma Chemical Co. (St. Louis, MO). [1,3-^{3}H]diisopropyl phosphofluoridate (4 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Trifluoromethanesulfonic acid and anisole were from Aldrich Chemical Company (Milwaukee, WI) and were used without further purification. N^{α}-p-tosyl-L-arginine methyl ester, N^{α}-p-tosyl-L-lysine methyl ester, benzoylarginylphenylnitroanilide, and casein were from Sigma. γ-NGF was prepared from male mouse submandibular glands according to the method of Jeng et al [12].

Isolation of Esterase

Approximately 100 guinea pig prostates were used in each preparation, and all procedures were carried out at 4°C. The glands were thawed, minced, and homogenized in a Waring blender with 2 ml cold H_2O/gm tissue. The suspension was centrifuged at 20,000g for 20 min. Streptomycin sulfate (115 mg/ml in 0.1 M Tris Cl, pH 7.5) was added slowly to the supernatant to give a final concentration of 56 mg/gm initial tissue. The sample was incubated at 4°C for 45 min, and the precipitated protein was subsequently removed by centrifugation at 20,000g for 20 min.

The lyophilized supernatant fraction was redissolved in approximately 30 ml of 50 mM NH_4HCO_3, pH 7.8, centrifuged to remove any insoluble material, and then chromatographed on a column (7 × 80 cm) of Sephadex G-100. The protein was eluted at 75 ml/hr with 50 mM NH_4HCO_3, pH 7.8. The eluant was monitored for protein by absorbance at 280 nm and for esterase activity towards 2 mM TAME as described below. Column fractions were also analyzed by SDS-PAGE.

The esterase pool was lyophilized and subsequently dissolved in and dialyzed extensively against 50 mM Tris Cl, pH 7.5. The sample was applied to a column (1.5 × 20 cm) of DEAE cellulose. The column was initially washed with 120 ml 50 mM Tris Cl, pH 7.5, followed by a 400 ml linear gradient of 0–0.25 M NaCl in 50 mM Tris Cl, pH 7.5. The esterase-containing fractions were pooled, dialysed against 10 mM NH_4HCO_3 to remove salt, and then concentrated by lyophilization. Minor

high-molecular-weight contaminants in the esterase sample were removed by gel filtration of the protein on a column (2.7 × 100 cm) of Sephadex G-75. The protein was eluted with 50 mM NH_4HCO_3, pH 7.8.

Esterase Activity

Esterase activity was determined by measuring the rate of methanol release from TAME and TLME according to the method described by Colman and Bagdasarian [13]. Activity towards BAPNA was measured as the increase in absorbance at 412 nm resulting from the hydrolysis of 1.0 ml of a 0.1 mM solution of BAPNA in 1 M Tris Cl, pH 8.0, containing 25 μM EDTA at 24°C [12]. Proteolytic activity towards casein was assayed by a modification of the procedure described by Bothwell et al [14]. A 2% solution of casein in 0.1 M Tris Cl, pH 8.0, was heat denatured by boiling for 30 min. The casein solution was cooled at 4°C for several hours and the insoluble material subsequently removed by centrifugation. Reaction mixtures were comprised of 100 μl of casein, plus enzyme, made up to a final volume of 200 μl. The samples were incubated at 37°C for 1 hr, after which the reaction was stopped by the addition of 1 ml of cold 12.5% trichloroacetic acid. The precipitated protein was removed by centrifugation at 10,000g for 15 min, and the extent of casein digestion was monitored by measuring the absorbance of the supernatants at 280 nm.

Active Site Labeling With Diisopropyl Phosphofluoridate

Samples of the crude extract and purified esterase were reacted with [1,3-^{3}H]DFP. The reaction was carried out in 0.1 M Tris Cl containing 0.1 mM EDTA and at a protein concentration of 0.15 mg/ml. [^{3}H]DFP (10 μl) was added to the reaction mixture and the sample incubated at room temperature. After 1 hr, an additional 5 μl [^{3}H]DFP was added and the incubation continued for a further 1 hr. The protein was separated from unbound reagent by concentrating and washing in a Centricon microconcentrator (Amicon) with a 10,000 molecular weight cut-off. The samples were solubilized in 1% SDS and electrophoresed in 15% polycrylamide gels containing 0.1% SDS. After staining with Coomassie blue, the gels were treated with Enlightning (New England Nuclear, Boston, MA) and autoradiographed.

Deglycosylation

Esterase was treated with trifluoromethanesulfonic acid according to the procedure of Edge et al [15]. The reaction was carried out at 0°C for 1 hr, after which the reagents were removed by ether extraction and the protein recovered after dialysis of the aqueous phase against 5% CH_3COOH for 16–24 hr.

Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis was carried out in 15% gels containing 0.1% SDS according to the procedure of Laemmli [16].

Amino Acid Sequence Analysis

The amino terminal sequence was determined by automated Edman degradation in an Applied Biosystems gas phase protein sequencer. Phenylthiohydantoins were identified by reverse-phase high-pressure liquid chromatography using an Altex Ultrasphere ODS column [3].

RESULTS

Purification of Esterase Activity

Crude homogenates of guinea prostate contain arginine esteropeptidase activity, as evidenced by hydrolysis of the synthetic substrate TAME. Fractionation of the extract on a column of Sephadex G-100 yields a single peak of esterase activity (Fig. 1A). SDS PAGE of the column fractions reveals that, although the initial elution of β-NGF occurs prior to the peak of esterase activity, there is a considerable proportion of β-NGF in the esterase pool (Fig. 2). The esterase activity can be further fractionated on DEAE cellulose (Fig. 1B). At pH 7.5, the esterase binds firmly to the resin and is subsequently eluted with approximately 0.15 M NaCl. Final purification of the esterase is achieved by gel filtration on a column of Sephadex G-75, as is shown in Figure 1C.

It is significant that, during purification of the esterase on DEAE cellulose, β-NGF, which occurred in the initial G-100 esterase pool, fails to bind to the resin and elutes in the flow through and wash from the column (Fig. 2). No esteropeptidase activity was detected when these NGF-containing fractions were assayed for the ability to hydrolyse TAME at pH 7.5. However, because the activity of γ-NGF is markedly reduced in the mouse 7S NGF complex, assays were also carried out at pH 9.5 (at which the 7S complex is dissociated [17]) and at pH 7.5 in the presence of 2 M urea (which increases the activity of γ-NGF in the 7S complex [18]). Under no condition was any esteropeptidase activity observed in the guinea pig NGF samples, suggesting the absence of any γ-like protein in the fractions. Under the same reaction conditions, the rate of hydrolysis of TAME by the purified esterase was comparable at pH 7.5 and pH 9.5, whereas the addition of 2 M urea to the assay at pH 7.5 resulted in an approximately 50% decrease in esteropeptidase activity.

Characterization of Guinea Pig Esterase

The purified esterase migrates as a single, although somewhat diffuse, band on SPS PAGE (Fig. 2). Comparison with the electrophoretic pattern of the crude tissue homogenate reveals that the esterase is one of the major protein components in the extract. The esterase has an apparent molecular weight of 35,000 as judged by SDS gel electrophoresis; the molecular weight estimated from the elution volume on Sephadex G-75 is approximately 37,000. A considerable proportion of the mass of the protein, however, appears to be due to covalently associated carbohydrate. Incubation of the esterase with TFMS, which removes peripheral carbohydrate chains from the protein, yields one major band of approximately 26,000 as judged by SDS PAGE. Two minor bands of 28,000 and 30,000 were also detected together with a higher-molecular-weight band of 42,000; these apparently represent aggregation products arising from the deglycosylation reaction. The 26,000 MW protein appears to be a stable species and persisted during incubation with TFMS for up to 10 hr at 0°C. N-terminal amino acid analysis revealed no significant peptide bond cleavage under these conditions, although prolonged reaction times and/or higher reaction temperatures resulted in the appearance of multiple low-molecular-weight bands after electrophoresis of the protein on polyacrylamide gels.

[^{3}H]DFP, an inhibitor that modifies the active site of serine proteases, is readily incorporated into guinea pig esterase. Examination of the labeling pattern of the crude tissue homogenate (Fig. 3) reveals that the 36,000 MW esterase is the predominant

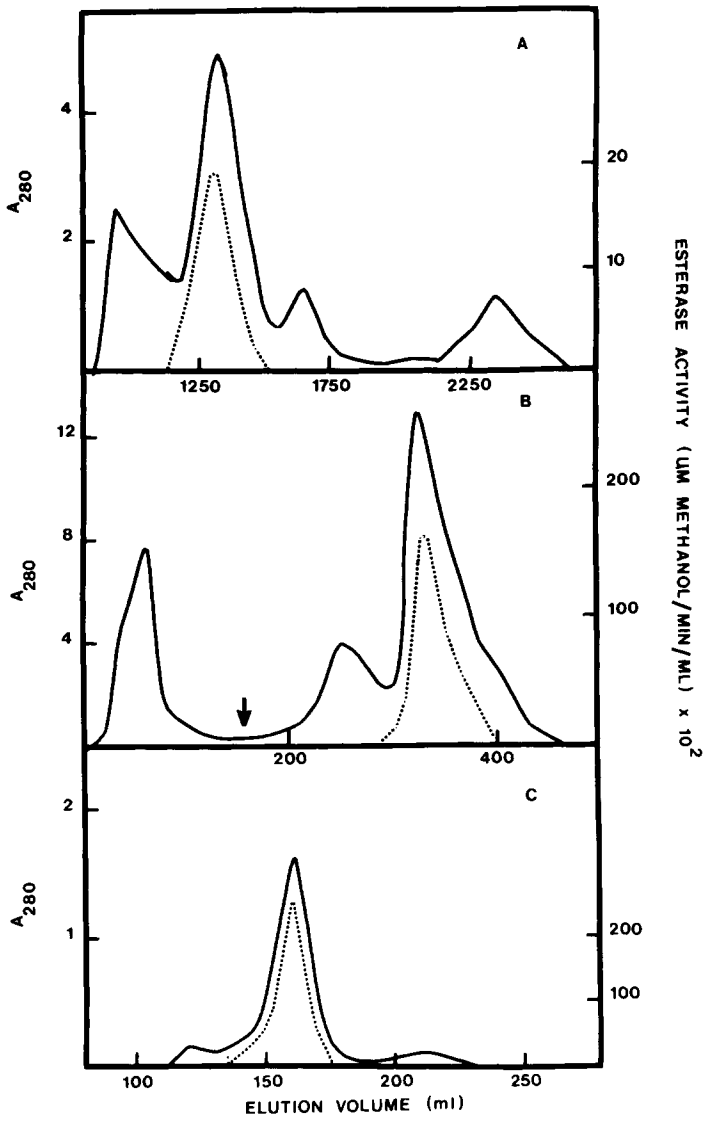

Fig. 1. Purification of guinea pig esteropeptidase. A) gel filtration of the crude tissue extract on a column (7 × 80 cm) of Sephadex G-100. The protein was eluted with 50 mM NH_4HCO_3, pH 7.8, and the eluant was monitored for protein by absorbance at 280 nm and for esterase activity by the rate of hydrolysis of TAME. B) DEAE chromatography of the partially purified esterase. The resin was initially equilibrated with 50 mM Tris Cl, pH 7.5, and after application of the sample the column was washed with a further 120 ml of equilibration buffer. The bound proteins were subsequently eluted with a 400 ml linear gradient of 0–0.25 M NaCl in 50 mM Tris Cl, pH 7.5. The arrow indicates the commencement of the gradient. C) elution profile obtained from gel filtration on Sephadex G-75 of the esterase pool from the DEAE column. The protein was eluted with 50 mM NH_4HCO_3, pH 7.8. Absorbance at 280 nm (——), esterase activity (. . . .).

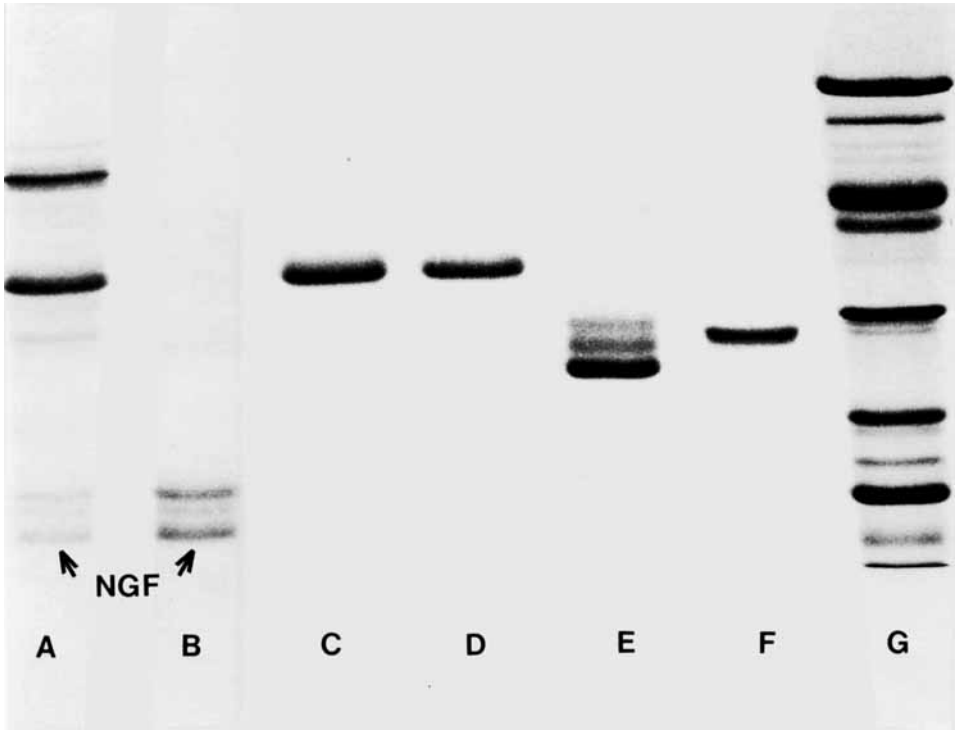

Fig. 2. SDS polyacrylamide gel electrophoresis of guinea pig kallikrein samples. Samples were initially denatured with 1% SDS and 0.1 mM DTT and subsequently electrophoresed in 15% polyacrylamide gels containing 1% SDS. A) esterase pool from Sephadex G-100 chromatography of the crude guinea pig extract; B) proteins that eluted in the flow through and wash from the DEAE column; C) partially purified esterase that eluted at approximately 0.15 M NaCl from the DEAE column; D) purified esterase; E) esterase after deglycosylation with TFMS; F) mouse γ-NGF; G) molecular weight markers comprised of phosphorylase B (92.5 K), bovine serum albumin (66 K), ovalbumin (45 K), carbonic anhydrase (31 K), soybean trypsin inhibitor (21.5 K), and lysozyme (14 K).

species labeled. Although there is a minor band of radioactivity corresponding to a molecular weight of approximately 90,000, no [^{3}H]DFP was incorporated into proteins of molecular weight equivalent to that of mouse γ-NGF.

Analysis of the kinetics of esterase activity towards TAME and TLME is shown in Figure 4. The Km for both the arginine and lysine methyl ester is approximately 8 mM, which is very similar to the affinity exhibited by mouse γ-NGF for TAME and TLME under the same reaction conditions. Both enzymes also reveal a considerably higher V_{max} for TAME than TLME. However, the esterase activity of γ-NGF is much greater than that of the guinea pig esterase. The V_{max} for TAME hydrolysis by γ-NGF, extrapolated from Figure 4, is 27.8×10^{-2} μM/min compared to 5.3×10^{-2} μM/min for the guinea pig enzyme.

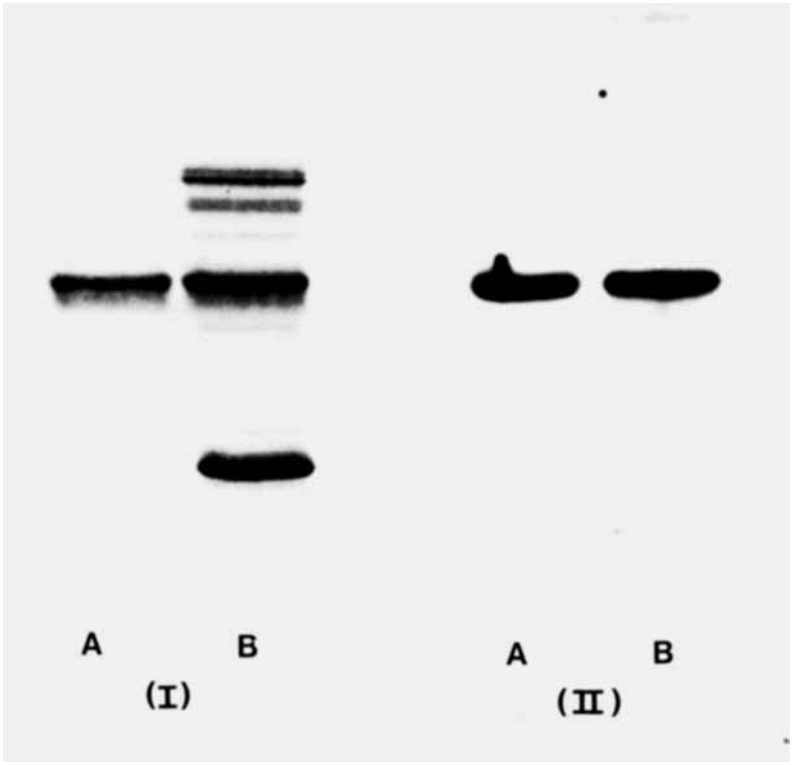

Fig. 3. Active site labeling of esterase with diisopropyl phosphofluoridate. Samples of purified esterase (A) and crude tissue extract (B) were incubated with [^{3}H]DFP and subsequently electrophoresed on 15% polyacrylamide gels containing 1% SDS. I) Coomassie blue staining pattern; II) autoradiogram of the [^{3}H]labeled proteins.

In contrast to γ-NGF, which readily hydrolyses BAPNA [19], the guinea pig enzyme displays no detectable activity towards this substrate even at enzyme concentrations as high as 2 mg/ml. Guinea pig esterase also exhibits limited protease activity, defined as the amount of TCA-soluble material (A_{280}) formed during incubation with casein at 37°C. Guinea pig esterase exhibited an activity less than 1% that of trypsin (0.018 and 3.88 A_{280} units/mg/min, respectively), whereas the rate of hydrolysis of casein by mouse γ-NGF was approximately 0.25 A_{280} units/mg/min.

The sequence of the first 25 amino acid residues of guinea pig esterase as determined by automated Edman degradation is shown in Table I, where it is aligned with the N-terminal sequence of mouse γ-NGF [3], rat pancreatic kallikrein [20], and rat trypsin [21]. The N-terminus of guinea pig esterase is comprised of two β-branched amino acids followed by a pair of glycine residues and is homologous with the consensus N-terminal sequence for the kallikrein family of serine proteases. The guinea pig enzyme shares 40% and 48% identity with the 25 N-terminal residues of the mouse and rat proteins, respectively, compared to less than 30% homology with rat trypsin. Where differences in the sequences of the kallikreins are observed, they

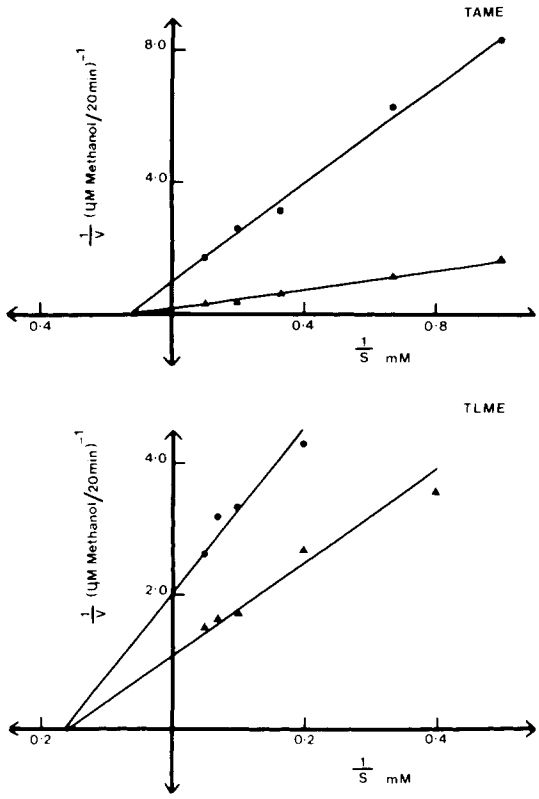

Fig. 4. Enzymatic activity of guinea pig esteropeptidase (●) and mouse γ-NGF (▲) towards tosyl-arginine-methylester (TAME) and tosyl-lysine-methylester (TLME). Reactions were carried out at 37°C in 0.1 M sodium phosphate, pH 7.3. The enzyme concentration was 1.6 μg/ml, except for the analysis of guinea pig esteropeptidase activity towards TLME for which the protein concentration was 6.4 μg/ml.

are, in many cases, conservative substitutions such as an aspartic acid for asparagine found at position 10.

DISCUSSION

Guinea pig prostate appears to contain one major soluble arginine esteropeptidase; only a single "peak" of esterase activity is detected throughout the fractionation of the crude prostate extract, and the purified enzyme corresponds to the major protein in the tissue homogenate labeled by [^{3}H]DFP.

The purified guinea pig esteropeptidase is comprised of a single polypeptide chain with an apparent MW on SDS polyacrylamide gels of approximately 36,000. Carbohydrate contributes significantly (~ 10,000 Da) to the molecular weight as evidenced by the increase in electrophoretic mobility after deglycosylation of the

TABLE I. Amino Terminal Sequence Analysis of Guinea Pig Esteropeptidase as Compared to Mouse γ-NGF [3] and Rat Pancreatic Kallikrein [20] and Rat Trypsin [21]*

	Position																								
Enzyme	1				5					10					15					20					25
Guinea pig esterase	V	I	G	G	Q	E	C	A	R	D	S	H	P	W	Q	A	A	V	Y	Y	Y	S	D	I	K
Mouse γ-NGF	I	V	G	G	F	K	C	E	K	N	S	Q	P	W	H	V	A	V	Y	R	Y	T	Q	Y	L
Rat Pancreatic kallikrein	V	V	G	G	Y	N	C	E	M	N	S	Q	P	W	Q	V	A	V	Y	Y	F	G	E	Y	L
Rat trypsin I	I	V	G	G	Y	T	C	P	E	H	S	V	P	Y	Q	V	S	L	N	S	–	G	Y	H	F

*Sequence analysis of the guinea pig esteropeptidase was performed by automated Edman degradation of the S-carboxymethylated protein. The abbreviations for the amino acids are A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; E, Glu; Q, Gln; G, Gly; H, His; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; Y, Try; V, Val.

protein with TFMS. Consistent with the observed MW of 26,000 for the deglycosylated protein, amino acid sequence analysis indicates that the protein is comprised of 239 amino acids with two potential sites of carbohydrate attachment [Dunbar and Bradshaw, in preparation]. The large amount of bound carbohydrate also presumably underlines the relative early elution of the guinea pig esterase from gel filtration columns by increasing the effective Stokes' radius of the protein.

The covalent incorporation of [^{3}H]DFP by guinea pig esterase is indicative that the enzyme belongs to the serine protease family of esteropeptidases, which is exemplified by trypsin. However, in contrast to trypsin, guinea pig esterase discriminates between esters of lysine and arginine, with a strong preference for arginine methyl ester substrates. Furthermore, the hydrolysis of casein by guinea pig esterase is negligible, suggesting that the enzyme possesses a high degree of substrate specificity. The lack of general protease activity and the preferential cleavage of the C-terminal bond of arginine are properties that distinguish the esteropeptidase from trypsin and are also characteristic of the kallikrein family of serine proteases. Guinea pig esterase, like many of the glandular kallikreins (eg, pig pancreatic kallikrein and plasma kallikreins [22]) fails to hydrolyze BAPNA. However, the ability to cleave substituted p-nitroanilides appears to vary considerably; a number of kallikreins, such as rat pancreatic kallikrein and mouse γ-NGF, are capable of hydrolyzing BAPNA at rates comparable to that of trypsin [22].

The amino terminal sequence of guinea pig esterase reveals significant homology to the kallikrein family and much less sequence identity to trypsin. On the basis of the sequence homology, the substrate specificity and catalytic properties, the guinea pig esterase purified in the current study is designated as a kallikrein.

The function of guinea pig prostate kallikrein, like that of the majority of kallikreins, remains to be determined. Despite the structural and catalytic similarities to mouse γ-NGF, evidence suggests that this guinea pig kallikrein does not interact and form stable complexes with β-NGF. By analogy with mouse 7S NGF, for which it has been shown that the association between the subunits is greatest at pH 7.0 [17], and based on the assumption that guinea pig NGF is involved in similar interactions, all steps in the fractionation of the crude guinea pig extract were carried out at near neutral pH. During the initial Sephadex G-100 gel filtration step, guinea pig γ-NGF

did elute as a high-molecular-weight ($\sim$60,000) complex, which overlapped with the elution of the kallikrein. However, the two species were clearly separated during DEAE chromatography at pH 7.5, implying that β-NGF and guinea pig kallikrein do not form a stable complex similar to that of mouse 7S NGF. Furthermore, there was no detectable esterase activity coeluting with NGF from the DEAE column, and [^{3}H]DFP failed to significantly label any species other than the kallikrein in the crude guinea pig extract. (Incubations with DFP were carried out in the presence of EDTA to decrease any metal-stabilized protein-protein interactions, such as the zinc stabilization of 7S NGF [23], which could inhibit DFP accessibility to potential binding sites.) It appears unlikely then that β-NGF in the guinea pig prostate is complexed with a kallikreinlike protein. It is of interest that NGF synthesized by mouse fibroblast cells in culture has also been found in a high-molecular-weight complex that by radioimmunoassay was devoid of any γ-NGF cross-reactive material [24].

The presence of a kallikrein in guinea pig prostates that is homologous to γ-NGF but that fails to bind to guinea pig β-NGF raises questions about the physiological role of these kallikreins in the processing of β-NGF. It has previously been shown that there is extensive homology between the N-terminal sequences of guinea pig and mouse β-NGF [25]. It has yet to be determined whether or not this identity also extends to the C-terminal residues, which have been shown by cDNA sequencing [6,7] to be Arg-Arg-Gly and Arg-Arg-Ala in mouse and human β-NGF, respectively. It has been speculated that mouse γ-NGF removes the Arg-Gly dipeptide from mouse β-NGF to generate a C-terminal arginine, through which it then interacts to form a stable complex. Thus, it will be of interest to compare the C-terminal residues deduced from cDNA and protein sequencing of guinea pig β-NGF. The presence of this dipeptide (and therefore no C-terminal arginine residue) would be consistent with lack of interaction of guinea pig β-NGF with this kallikrein (or any other γ-like subunit) and preclude complex formation. It would also suggest that this cleavage at the C-terminus is not an essential event in the processing of the β-NGF precursor in any species.

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