ACTA CHEMICA SCANDINAVICA 20 (1966) 2169–2178

A Chymotrypsin-like Protease from the Rat Submandibular Gland

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A chymotrypsin-like protease was obtained from the rat submandibular gland by precipitation at pH 5.2, ammonium sulphate fractionation, gel filtration on Sephadex G-200 and DEAE-cellulose chromatography. The degree of purity was increased 270 times. By starch gel electrophoresis at pH 6.0 of the final preparation two enzymatically active protein components moving to the anode were separated. The purified enzyme had a molecular weight of 19 300, and a pH optimum at 7.8 with different substrates. It readily hydrolyzed human hemoglobin, N-CBZ-L-phenylalanine *p*-nitroanilide, N-CBZ-L-tyrosine hydrazide, acetyl-L-tyrosine ethyl ester, acetyl-L-phenylalanine ethyl ester and benzoyl-DL-phenylalanine β-naphthyl ester. Arylamides of unsubstituted aromatic amino acids were hydrolyzed more slowly. Liberation of tyrosine from poly-L-tyrosine and ammonia from glycyl-DL-phenylalanine amide and glycyl-L-tyrosine amide was also demonstrated. The hydrolytic activity of the enzyme was stimulated by Ca^{2+} and was not affected by a number of modifying agents.

In our earlier reports in this series enzymes from rat salivary glands capable of hydrolyzing benzoyl-DL-arginyl-β-naphthylamide and *p*-nitroanilide, substrates for trypsin-like enzymes, have been discussed.[1–4] In addition, the partial separation of an organophosphorus resistant enzyme capable of hydrolyzing naphthol-AS acetate, 5-bromoindoxyl acetate, as well as naphthol-AS hydrocinnamoate and benzoyl-L-phenylalanine β-naphthol ester was reported.[2] These substrates are readily hydrolyzed by bovine chymotrypsin. This fact suggests that the enzymic activity in question might be due to a protease with a substrate specificity like that of chymotrypsin. This work presents the further purification and characterization of the material responsible for the enzymic activity described earlier.

MATERIAL AND METHODS

Substrates

N^{α}-Benzoyl-DL-arginine β-naphthylamide (BANA), N^{α}-benzoyl-DL-arginine *p*-nitroanilide (BAPA), L-leucine β-naphthylamide hydrochloride (Leu-β-NA), L-tyrosine β-naphthylamide hydrochloride (Tyr-β-NA), L-phenylalanine-β-naphthylamide hydrochloride (Phe-β-NA), naphthol-AS acetate, 5-bromoindoxyl acetate and benzoyl-DL-phenylalanine β-naphthyl ester (BPANE) were obtained from Sigma Chem. Co. Part of the naphthol-AS hydrocinnamoate was synthesized using the Schotten-Bauman reaction as reported earlier,[5] and the remainder was gifted by Drs. Glenner and Lagunoff.

p-Nitrophenyl-acetate and N-CBZ-DL-phenylalanine *p*-nitroanilide (CBZ-Phe-*p*-Nitro-An) were obtained from Yeda, Rehovoth, Israel.

Poly-L-tyrosine, glycyl-L-phenylalanine amide, glycyl-L-tyrosine amide, glycyl-DL-phenylalanine, glycyl-L-tyrosine, benzoyl-L-arginine amide (BAA), N-CBZ-glycyl-L-tyrosine, L-leucyl-glycine, glycyl-glycine and N^{α}-benzoyl-L-arginine were commercial preparations from Sigma Chem. Co. and Yeda.

The substituted amino acid esters *p*-toluenesulfonyl-L-arginine methyl ester (TAME), benzoyl-L-arginine methyl ester (BAME), glycyl-glycine methyl ester, acetyl-L-tyrosine ethyl ester (ATEE), and acetyl-L-phenylalanine ethyl ester (APEE) were obtained from Sigma Chem. Co.

N-CBZ-L-tyrosine hydrazide was supplied by Yeda and human hemoglobin (Hb) was a gift from Kabi AB, Stockholm, Sweden.

Assays of the hydrolysis

Measurements of the degree of hydrolysis of the naphthol and naphthylamide substrates were based on the azo coupling reaction of the liberated naphthol or naphthylamine with fast garnet GBC (Verona Dyestuffs).[1]

The extent of hydrolysis of 5-bromoindoxyl acetate was measured spectrophotometrically using the blue indigo colour of the liberated indoxyl.[2]

The rate of hydrolysis of *p*-nitrophenyl compounds was followed by measuring the yellow colour of *p*-nitrophenol spectrophotometrically at 410 mμ.[6] The same principle was used for measuring the amount of hydrolysis of the various *p*-nitroanilides.[7]

The extent of hydrolysis of acetyl-L-tyrosine ethyl ester and acetyl-L-phenylalanine ethyl ester was followed by the method of Kabacoff.[8] That of the various methyl esters was based on the use of chromotropic acid to estimate the liberated methanol.[9]

The extent of hydrolysis of the hydrazide substrate was followed by determining the liberated hydrazine using Ehrlich's reagent.[10]

Measurements of the degree of hydrolysis of Hb were made using a modification of Anson's method.[4]

All the measurements were carried out at pH 7.0, except for the nitrophenols in which case pH 8.0 was used, unless otherwise stated.

Hydrolysis of the peptides was demonstrated qualitatively using paper chromatography. The solvent phase was 90 % ethanol and staining was carried out with 0.5 % ninhydrin in acetone:pyridine:water (30:5:5).

Proteins were determined according to the method of Lowry [11] using bovine serum albumin as a standard.

Cellulose chromatography. DEAE-cellulose (Diethylaminoethyl cellulose, Powder DE 50, Whatman, W.R., Balston, Ltd., England) was washed twice with 0.5 M NaOH and HCl, treated with 5 mM Tris-HCl buffer and packed in cooled columns of various diameters. Elution was performed using various linear salt gradients at a constant temperature of + 4°C. Fractions were collected with LKB Radi-Rac fraction collector.

Gel-filtration. Samples of Sephadex G-100 and G-200 (Pharmacia, Uppsala, Sweden) were allowed to stand in buffer for 4 days before packing in columns of various diameters. Elution was performed using 5.0 mM Tris-HCl buffer pH 5.0 at a temperature of + 4°C.

Starch gel electrophoresis. A procedure essentially similar to that described by Hunter and Markert [12] was used. For details see Vanha-Perttula and Hopsu.[13]

Determination of the molecular weight. The molecular weight of the enzyme preparation was estimated using gel filtration with a column of Sephadex G-100 (2 × 150 cm). Eluent (2.0 ml) containing albumin (20 mg), trypsin (100 μg), chymotrypsin-like protease (110 μg) and a few crystals of blue dextran were placed on the column. The eluent was 0.1 M NaCl in 10 mM Tris-HCl, pH 7.0, flow rate 0.3 ml/min and fraction volume 4.9 ml.

The void volume of the column was determined by ascertaining the position of the blue dextran peak. The activities of trypsin and chymotrypsin-like proteases were determined with BANA and BPANE, respectively, in order to locate the enzymes exactly. Proteins were determined with Lowry's method.

Table 1. Summary of the purification procedure for submandibular chymotrypsin-like protease.

Purification stage	Volume (ml)	Amount of protein (mg)	Hydrolytic activity * (mμmole/mg) min)	Purification coefficient
Homogenate	105	6950	3.8	1.0
pH 5.2 supernatant	82	3240	7.3	1.9
$(NH_4)_2SO_4$ fractionation 35–60 %	20.0	1510	15.2	4.1
Sephadex G-100 pooled preparation	59	135	138	38
DEAE-cellulose pooled preparation	77	11	1030	275

* Hydrolytic activity presents the hydrolysis rate of BPANE in the presence of E-600 (10^{-5} M) during various stages of purification.

RESULTS

Preparation of the enzyme

The whole purification procedure was carried out at + 4°C and the results are summarized in Table 1. The submandibular glands were removed from 42 adult male rats (200–280 g). The glands were dissected and homogenized in distilled water at a concentration of 100 mg/ml. The homogenate was centrifuged at 3000 rpm for 10 min. The supernatant was collected and adjusted to pH 5.2 with 0.1 N HCl and allowed to stand in ice water for 1 h. The precipitate was removed by centrifugation at 3000 rpm for 20 min. Ammonium sulphate was added to the supernatant to 35 % saturation and the precipitate was removed 6 h later by centrifugation at 3000 rpm for 1 h. Ammonium sulphate was added to the supernatant to 60 % saturation. The precipitate was collected by centrifugation as in the earlier step and dissolved in 20 ml of distilled water. The preparation was subjected to gel filtration in a column (120 × 2.0 cm) of Sephadex G-200. The eluent was 0.1 M Tris-HCl, pH 7.0 and fractions of 3.5 ml volume were collected. The distribution among the fractions of the enzymes having hydrolytic activity towards BANA and BPANE in the presence of E-600 (10^{-5} M) and the distribution of proteins was tested using suitable enzyme dilutions (about 1:20). The result can be seen in Fig. 1. Numerous peaks, which can be partially separated from each other, contained enzyme

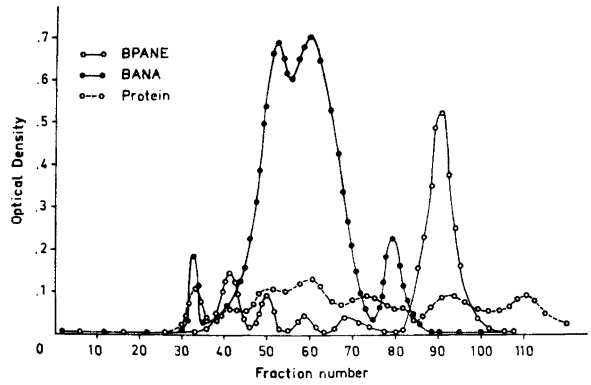

Fig. 1. Sephadex G-200 superfine chromatography of the preparation following ammonium sulphate fractionation. Column 2 × 120 cm, eluent 0.1 M Tris-HCl pH 7.0, elution rate 0.1 ml/min. Dilution for protein and activity assays 1:20. Activity demonstrated with BPANE and BANA in the presence of E-600 (10^{-5} M) at pH 7.0.

material capable of hydrolyzing BANA. The fraction containing the enzyme active to BPANE but resistant to E-600 was eluted later than the fractions containing the BANA-hydrolyzing activity. Fractions 89—99 were pooled for further purification. The activity of the E-600 resistant fraction in the pooled sample was 138 mμmole/mg protein/min.

The preparation was concentrated with the aid of carbowax and dialyzed against 10 mM Tris-HCl buffer, pH 6.2, for 12 h and applicated to a column of DEAE-cellulose (40 × 2 cm). Elution was carried out with a linear gradient

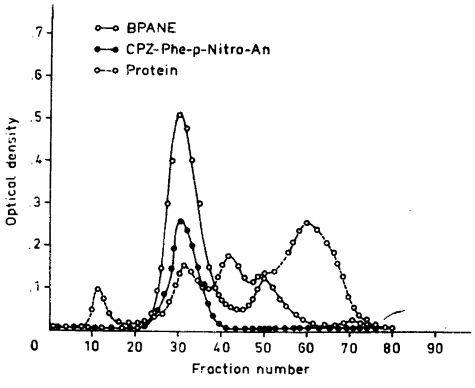

Fig. 2. DEAE-cellulose chromatography of the pooled preparation obtained from Sephadex G-200 chromatography. Column 2 × 40 cm, flow rate 0.8 ml/min. Continuous gradient from 5 mM to 0.75 M NaCl buffer 10 mM pH 7.0. Dilution 1:6 was used, for the assays of hydrolysis rates.

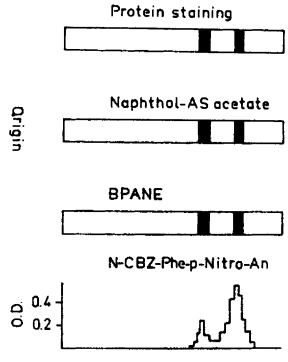

Fig. 3. Starch gel electrophoresis of the purified preparation. Sample 0.3 ml containing 35 μg protein, running time 6 h at 12 V/cm. Proteins and activity demonstrated as given in the text.

of NaCl from 10 mM to 0.75 M and fractions of 7 ml were collected. The distribution of the proteins and the fractions having activities towards BPANE and CBZ-Phe-*p*-Nitro-An were determined. The results can be seen in Fig. 2. Several protein components were separated. Material from two separate fractions was found to hydrolyze BPANE. The larger peak (around fraction 30) was identical with that capable of hydrolyzing CBZ-Phe-*p*-Nitro-An. Hydrolysis of Hb and naphthol-AS acetate was demonstrated as occurring in the fractions of the same peak (not given in the figure). A very low hydrolytic activity towards BANA was found in a broad area. Fractions 28—38 were pooled. Considerable purification had been achieved since the activity toward BPANE was now 1030 mμmole/mg protein/min. The preparation was diluted to 100 ml and now contained 110 μg protein/ml. This preparation was used in the further characterization of the active constituent. If it is estimated that about 50 % of the E-600-resistant activity toward BPANE in the homogenate is due to the purified enzyme, then a purification of 275 times was attained.

Test of purity

Using starch gel electrophoresis two protein components were separated, both moving to the anode, as can be seen in Fig. 3. Both components hydrolyzed BPANE and naphthol-AS-acetate. However, the hydrolyzing effect of the faster moving component could be seen in zymograms after a much shorter time of incubation. Gel slabs were cut into pieces transversely at distances of 0.3 cm and homogenized in two milliliters of water. Thereafter, N-CBZ-Phe-*p*-Nitro-An activity was demonstrated as usual. It was found that enzyme material in both bands hydrolyzed this anilide substrate. The faster moving component had about three times the greater activity. No staining was observed using BANA as substrate.

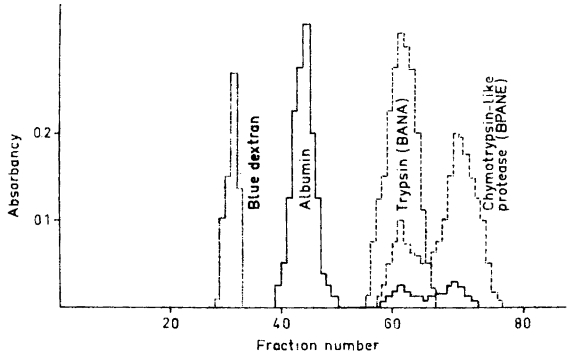

Fig. 4. Distribution of blue dextran, albumin, trypsin, and chymotrypsin-like protease in Sephadex G-100. Column 2 × 160 cm, particle size 40—120 μ. The presence of protein demonstrated by Lowry's method (solid line), trypsin with BANA at pH 8.0 Tris-HCl buffer 0.1 M, chymotrypsin-like protease as in Fig. 1.

Molecular weight

The distribution of albumin and the enzymes after gel filtration can be seen in Fig. 4. Whitaker's plot [14] of the data in Fig. 4 is seen in Fig. 5. The molecular weight of the enzyme has been calculated to be 19 300. A molecular weight of 70 000 [15] was used for albumin and 24 000 [14] for trypsin.

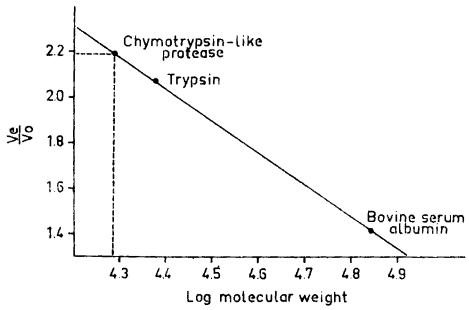

Fig. 5. Whitaker's plot of the data presented in Fig. 4. for calculation of the molecular weight.

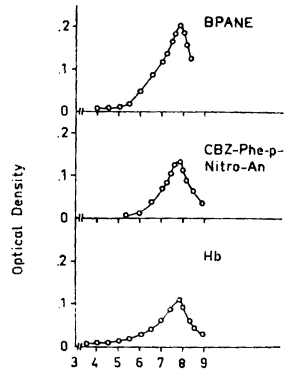

Fig. 6. Dependence of hydrolysis on pH. Enzyme assay is 5 μg, incubation time 1 h for Hb and for N-CBZ-Phe-*p*-Nitro-An, 5 min for BPANE. Mc Ilvain's buffer pH 3.0—8.0 and NaOH-glycine buffer pH 8.0—10.0.

Characteristics of the enzyme preparation

Effect of pH. The pH dependence of the hydrolysis of some substrates is presented in Fig. 6. The pH optimum for the hydrolysis of three types of substrate (naphthol ester, substituted amino acid anilide and a protein) BPANE, CBZ-Phe-*p*-Nitro-An, and Hb was the same, *i.e.*, 7.8.

Hydrolysis of various substrates. Table 2 gives the rates of the hydrolysis of the various substrates tested. ATEE, APEE, and BPANE and naphthol-AS hydrocinnamoate were the most rapidly hydrolysed. Fairly good hydrolysis rates were also found using naphthol-AS acetate, *p*-nitrophenyl acetate or 5-bromoindoxyl acetate as substrate. All of these are known to be hydrolyzed readily by bovine chymotrypsin and some are widely used to assay chymotryptic activity.

The typical trypsin substrates BAME and TAME were also hydrolyzed but at a low rate even when very high substrate concentrations were used.

Of the *p*-nitroanilides tested N-CBZ-Phe-*p*-Nitro-An was hydrolyzed to the greatest extent. The unsubstituted amino acid naphthylamides were hydrolyzed slowly as were the substituted trypsin substrates BANA and BAPA, whereas the hydrolysis of Hb was marked.

Table 2. Hydrolysis rates of various substrates by the enzyme preparation. 0.1 M Michaelis' barbital buffer, pH 7.0. For details see Material and methods.

Substrate	Concentration	Hydrolysis rate (mμmole/mg/min)
Esters:		
APEE	5.0 mM	1250
ATEE	5.0 mM	2133
BPANE	0.1 mM	1030
Naphthol AS acetate	0.25 mM	167
Naphthol AS hydrocinnamoate	0.05 mM	571
p-Nitrophenyl acetate	0.1 mM	75
5-Bromoindoxyl acetate	0.25 mM	51
BAME	10 mM	125
TAME	10 mM	45
Glycyl-glycine methyl ester	10 mM	2.0
Naphthylamides:		
BANA	0.25 mM	2
Glut-Phe-NA	0.25 mM	12.5
LNA	0.25 mM	0.83
Phe-NA	0.25 mM	1.1
Tyr-NA	0.25 mM	2.2
Anilides:		
BAPA	0.25 mM	2.2
CBZ-Phe-*p*-Nitro-An	0.1 mM	15.3
Hydrazide:		
N-CBZ-tyrosine hydrazide	3.0 mM	21.6
Protein:		
Hb (amount of tyrosine liberated (μmole/mg enzyme protein)	1.0 %	1.0

Semiquantitative information on the hydrolysis of many other peptide substrates was obtained using paper chromatography. The substrates were incubated with enzyme (5 μg/ml) at pH 7.0 for 4 h at a concentration of 1 mM. After incubation 25 μl were applied to paper and chromatographed. The substrates used were poly-L-tyrosine, glycyl-L-phenylalanine amide, glycyl-L-tyrosine amide, N-CBC-glycyl-L-tyrosine, glycyl-L-tyrosine, glycyl-glycine, leucyl-glycine, and N-benzoyl-L-arginine amide. Tyrosine was liberated from poly-L-tyrosine and ammonia from glycyl-L-phenylalanine amide and glycyl-L-tyrosine amide. No liberation of glycine from any of the substrates was found. This demonstrates that probably no aminopeptidases are present as contaminants in the enzyme preparation and consequently hydrolysis of the amino acid naphthylamides is due solely to the protease.

Effect of various modifiers. The effect of various additives on the hydrolysis of BPANE, CBZ-Phe-*p*-Nitro-An, and Hb are presented in Table 3. Hydrolysis of these three typical substrates was affected in the same way by all of the addi-

Table 3. Effect of various modifiers on the hydrolysis of BPANE, CBC-Phe-*p*-Nitro-An and Hb by the enzyme preparation. 0.1 M Tris-HCl buffer pH 7.0. Preincubation 15 min before adding the substrate. The numbers express the percentage change.

Modifier	Concentration mM	BPANE	CBZ-Phe-*p*-tion Nitro-An	Hb
Metal salts:				
$CaCl_2$	50	+30	+80	+98
$CaCl_2$	20	+20	+73	+86
$CaCl_2$	0.2	+ 5	+ 7	+23
$CdCl_2$	1.0	0	0	0
$CoCl_2$	2.0	+10	+18	+13
$CuCl_2$	0.1	0	0	0
$HgCl_2$	1.0	−12	− 8	0
$MgCl_2$	20	+14	+37	+34
$MnCl_2$	1.0	—	0	0
$Pb(NO_3)_2$	1.0	0	0	0
$ZnCl_2$	1.0	−80	−68	−75
Chelators:				
EDTA	10.0	−21	−30	−25
EDTA	1.0	− 6	−13	−18
EDTA	0.2	0	0	0
KCN	1.0	0	0	0
Na-citrate	1.0	− 7	−10	−10
Sulfhydryls:				
Thioglycolate	1.0	0	0	0
Cysteine	1.0	—	− 6	+ 5
Glutathione	1.0	—	0	0
Sulfhydryl reagents:				
Mercaptoethylamine	1.0	—	0	0
Iodoacetamide	1.0	−18	0	0
Iodoacetic acid	1	−12	0	0
p-Chloromercuribenzoate	0.1	−15	0	− 5
Others:				
Hydrocinnamic acid	10.0	−51	−41	−24
Gly-tyr-amide	10.0	−85	−85	−32
Benzalkoniumchloride	10.0	−80	−87	−95
NaF	1.0	0	0	0
Na-taurocholate	1.0	0	0	0
Tetrabutylammonium iodide	1	0	0	0
Tetramethylammonium iodide	1	0	0	0

tives. Ca^{2+} was found to have a pronounced activating effect, whereas that of Co^{2+} and Mg^{2+} was less. EDTA causes a marked and citrate a lower inhibition. A partial inhibition was found also in the presence of Zn^{2+}, hydrocinnamic acid, benzalkonium chloride as well as of the competitive substrate glycyl-tyrosine amide at a high concentration (10 mM). Esterase inhibitors, CN^-, F^- and taurocholate had no effect. A weak inhibition of the hydrolysis of BPANE was found in the presence of sulfhydryl reagents but no obvious effect was

observed on the hydrolysis of the other substrates. It was not possible to test with the present method whether sulfhydryl compounds could cause activation in the hydrolysis of BPANE but it was found to have no effect on the hydrolysis of the other substrates.

DISCUSSION

The purification procedure employed is fairly simple and resulted in the production of an enzyme preparation free from contaminating proteins detectable by the means used. The two active fractions separated by the starch gel electrophoresis seem to be enzymatically very similar to each other and may represent two molecular forms of the same active protein.

The studies on substrate specificity clearly show that the purified enzyme is a protease with the capacity to hydrolyze also synthetic peptides and esters. The preference of the enzyme for synthetic substrates containing N-substituted aromatic amino acids clearly demonstrates the close resemblance of the substrate specificity to that of bovine pancreatic chymotrypsin. Further similarities with chymotrypsin are the alkaline pH optimum (around pH 8) and the relative lack of effect of a number of enzyme modifying substances. The molecular weight of the enzyme (19 300) was found to be lower than that of chymotrypsin (24 800).[17]

Enzymes with alkaline pH optimum and a substrate specificity resembling that of chymotrypsin have been described earlier as existing in other tissues. Dannenberg and Smith [18] have shown the presence of an enzyme capable of hydrolyzing N-acetyl-L-tyrosine ethyl ester and N-acetyl-L-tyrosine amide in lung tissue. The enzyme was not purified but, so far as can be concluded, its characteristics closely resemble those of the enzyme under discussion. Martin [19] has obtained from rat skin enzymes capable of hydrolyzing amides and esters of substituted aromatic amino acids. Proteinase A seems to resemble very closely the submandibular gland enzyme. Benditt and Arase [20] described a chymotrypsin-like esterase enzyme in rodent mast cells. Curoff [21] purified from rat brain a Ca^{2+}-activated protease hydrolyzing the tyrosine-leucine bond in the β-chain of insulin as does chymotrypsin. However, this enzyme did not hydrolyze synthetic chymotrypsin substrates. Blackwood *et al.*[22] have quite recently synthesized a new chromogenic chymotrypsin substrate, glutaryl-L-phenylalanine β-naphthylamide and have shown, *e.g.*, that mammalian liver without doubt contains more than one chymotrypsin-like enzyme since several peaks can be found in the pH curves of the substrate hydrolysis. Bleck *et al.*[23] demonstrated in the rat kidney an enzyme capable of hydrolyzing N-acetyl-L-tyrosine ethyl ester at an optimum pH 8.0 and Nagel [24] found that kidney tissue homogenate hydrolyzed N-succinyl-L-phenylalanine *p*-nitroanilide optimally at the same pH. The possible comparison of these enzymes with the present protease can only be made after the further characterization of the enzymes in other tissues has been carried out.

Earlier Hill and Bourne [25] had suggested the presence of a chymotrypsin-like protease in the rat submandibular gland tissue. This was based on evidence from histochemical studies on the hydrolysis of naphthol-AS acetate in the slices of this tissue. The results of the present work verify this assumption and

the present enzyme is very likely one of those indicated by histochemical means in the rat submandibular gland tissue.

Acknowledgement. This work has been aided by grants from *Sigrid Jusélius Foundation*, *The Finnish State Medical Research Council* and *National Institutes of Health Grant* No. TW-00104 to Väinö K. Hopsu-Havu.

REFERENCES

1. Riekkinen, P. J. and Hopsu, V. K. *Ann. Med. Exptl. Biol. Fenniae (Helsinki)* **43** (1965) 6.
2. Riekkinen, P. J. and Hopsu, V. K. *Ann. Med. Exptl. Biol. Fenniae (Helsinki)* **43** (1965) 15.
3. Riekkinen, P. J., Ekfors, T. O. and Hopsu, V. K. *Biochim. Biophys. Acta. In press.*
4. Riekkinen, P. J., Ekfors, T. O., Holmén, T. and Hopsu-Havu, V. K. *Biochim. Biophys. Acta. In press.*
5. Hopsu, V. K. and Glenner, G. G. *J. Histochem. Cytochem.* **11** (1963) 520.
6. Martin, C. J., Golubow, J. and Axelrod, A. E. *J. Biol. Chem.* **234** (1959) 294.
7. Erlanger, B. F., Kokowsky, N. and Cohen, W. *Arch. Biochem. Biophys.* **95** (1961) 271.
8. Kabacoff, B. L., Umbey, M., Wohlman, A. and Avakian, S. *J. Pharm. Sci.* **52** (1963) 1188.
9. Siegelman, A. M., Carlson, A. S. and Robertsohn, T. *Arch Biochem. Biophys.* **97** (1962) 159.
10. Kallos, J., Kahn, D. and Rozok, D. *Can. J. Biochem.* **42** (1964) 235.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
12. Hunter, R. L. and Markert, C. L. *Science* **125** (1957) 1294.
13. Vanha-Perttula, T. P. J. and Hopsu, V. K. *Histochemie* **6** (1966) 34.
14. Whitaker, J. R. *Anal. Chem.* **35** (1963) 1950.
15. Andrews, P. *Biochem. J.* **91** (1964) 222.
16. Nord, F. F. and Bier, M. *Biochem. Biophys. Acta* **12** (1953) 56.
17. Tietze, F. and Neurath, H. *J. Biol. Chem.* **194** (1952) 1.
18. Dannenberg, A. M. and Smith, E. L. *J. Biol. Chem.* **213** (1955) 45.
19. Martin, C. J. and Axelrod, A. E. *J. Biol. Chem.* **224** (1957) 309.
20. Benditt, E. P. and Arase, M. *J. Exptl. Med.* **110** (1959) 451.
21. Curoff, G. *J. Biol. Chem.* **239** (1964) 149.
22. Blackwood, E. C., Erlanger, B. F. and Mandl, I. *Anal. Biochem.* **12** (1965) 128.
23. Blech, W., Hanson, H., Hermann, P. and Kleine, R. *Z. physiol. Chem.* **337** (1964) 211.
24. Nagel, W., Willing, F., Peschke, W. and Schmidt, F. H. *Z. physiol. Chem.* **340** (1965) 1.
25. Hill, C. R. and Bourne, G. H. *Acta Anat.* **20** (1954) 116.

Received April 16, 1966.