ACTA CHEMICA SCANDINAVICA 25 (1971) 2531–2540

Cathepsin B′ in the Thyroid Gland

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Thyroid gland homogenates of several mammalian species were found to hydrolyse the typical trypsin substrate, *N*-benzoyl-DL-arginine β-naphthylamide. The main part of the hydrolytic activity was in the large particle fraction (lysosomes), and the optimal hydrolysis was recorded at pH 6. Sheep thyroid homogenate was fractionated by using ammonium sulphate precipitation, gel filtration on Sephadex G-100, chromatography on DEAE-cellulose and CM-Sephadex A 50. Three enzymic components (isoenzymes) were separated with the following closely similar characteristics: molecular weight was 23 900, pH-optimum at pH 6, BANA was hydrolysed fast, but BAPA not measurably, proteins were hydrolysed slowly, as well as the ester substrates for trypsin. Enzymic activity was strongly dependent on sulphydryl groups, whereas the usual trypsin inhibitors and DFP had no effect. The enzyme was considered the thyroidal cathepsin B′.

Proteolytic enzymes of the thyroid gland have been the subject of special interest, since it was realized that some kind of proteolytic system was required for the release of thyroid hormones from thyroglobulin. Enzymatic breakdown of thyroglobulin was first demonstrated in the isolated follicular colloid by de Robertis,[1] and several clearly defined proteolytic enzymes have since been described and characterized.

An acid lysosomal protease, hydrolysing hemoglobin and thyroglobulin optimally at pH 3.5 – 4.0 has been the subject of several studies (Weiss,[2] McQuillan and Trikojus,[3] Balasubramaniam and Deiss,[4] Kress *et al.*,[5] Herveg *et al.*,[6] Jablonski and McQuillan,[7] *etc.*). Two hemoglobinolytic proteinases with pH optima at pH 3.8 and 5.7 were separated from the sheep thyroid,[8] and the hydrolysis of hemoglobin by the human thyroid extract revealed optima at pH 3.7, 6.5, and 8.5.[9] A neutral proteinase optimally active toward thyroglobulin at pH 8.6 was located in the thyroid mast cells, but not in the acinar cells.[10]

Thorough studies have been carried out on an acidic peptidase, capable of hydrolysing *N*-acetyl-phenylalanine-diiodotyrosine and some other peptides. The enzyme was optimally active at pH 4.0, and dependent on sulphydryl groups.[11,12] A metal-activated (Zn^{2+}, Mn^{2+}, Co^{2+}) peptidase, hydrolysing

cysteinyl-tyrosine and other peptides optimally at pH 5.3 was also clearly characterized.[13,14]

Sulphydryl-dependent enzymes hydrolysing several di- and tripeptides optimally at pH 7.8,[2] and the leucine naphthylamidase ("leucine aminopeptidase"), described histochemically by Talanti and Hopsu,[15] have so far not been thoroughly characterized. Similarly, the sulphydryl-dependent benzoyl-arginine amide hydrolysing enzyme occurring in the thyroid[16] requires further study.

The last mentioned enzyme might represent either cathepsin B[17] or cathepsin B'[18,19]. Since there are no further reports on the presence of trypsin-like proteases in the thyroid tissue, the present study was considered worthwhile.

MATERIAL AND METHODS

Substrates. *N*-Benzoyl-DL-arginine β-naphthylamide (BANA), *N*-benzoyl-DL-arginine *p*-nitroanilide (BAPA), L-arginine β-naphthylamide (ANA), *N*-tosyl and *N*-benzoyl-DL-arginine methyl ester (TAME and BAME), *N*-benzoyl-DL-arginine ethylester (BAEE), and albumin were from Sigma Chem. Co., Ohio. L-Arginine and L-lysine methyl ester (AME and LME) were from Yeda, Israel. Human hemoglobin was from Koch-Light Laboratories, England, and casein from E. Merck AG, Darmstadt, DBR.

Modifiers. Lima bean and ovomucoid trypsin inhibitors, iodoacetamide (IAA), *N*-ethyl-maleimide (NEMI), and β-mercaptoethanol (BME) were from Sigma Chem. Co., Ohio. E-600 (Mintacol) and Trasylol® were from Bayer, Leverkusen, DBR, *p*-chloromercuribenzoate (pCMB) from Calbiochem, California, di-isopropylphosphofluoridate (DFP) (Collyre au D.F.P.) from Boots Pure Drug Co., England, tetra-*N*-butylammonium iodide from British Drug Houses, England, tetra-*N*-methylammonium iodide from Fluka AG, Schweiz. The other chemicals were of analytical grade from various commercial sources.

Determination of enzyme activity. Michaelis veronal-acetate buffer (0.1 mol/l) was used at pH-range 2.0 – 9.2, and acetate-HCl buffer (0.1 mol/l) at pH-range 1.0 – 5.0.

For the proteinase assay, a modification of Anson's method[20] was used, by measuring the hydrolysis products at 280 nm after precipitation of proteins, or using Folin-Ciocalteau's reagent.[21] The hydrolysis of BANA and other naphthylamides was measured by determining the amount of liberated naphthylamine with *p*-dimethylaminobenzaldehyde at pH 2.5.[22] The concentration of BANA was 0.5 mmol/l if not otherwise mentioned. The hydrolysis of methyl and ethyl esters was measured by determining the amount of non-hydrolysed ester substrate with Hestrin's method.[23] The incubation medium contained 0.4 ml of buffer, 0.2 ml of substrate stock-solution (in aq. dest.), and 0.2 ml of enzyme solution.

The hydrolysis of BAPA was determined by measuring the absorption of liberated *p*-nitroaniline at 383 nm.[24]

A 15 min preincubation was used in studies with modifiers. During the purification procedure and in studies with purified enzyme preparations, the incubation medium always contained EDTA (0.5 mmol/l) and cysteine (2.0 mmol/l), if not otherwise indicated.

Proteins. The method of Lowry *et al.*[25] was used, or the absorption at 280 nm was measured.

Tissue homogenate. The thyroid glands of sheep, cow, rat, and guinea pig were stored in a deep freeze. They were usually homogenized in distilled water with Ultra-Turrax. The unbroken tissue debris was removed by centrifugation at 1000 *g* for 15 min (International High-speed Refrigerated Centrifuge, model HR-1). The supernatant was used for further studies.

Centrifugal fractionation of the homogenate. The distribution of the enzymes in tissue particles was studied with fresh sheep thyroid (250 mg). The tissue was homogenized gently with a teflon homogenizer in isotonic saline, and centrifuged at 1000 *g* for 15 min, to remove the debris. The supernatant was centrifuged at 32 000 *g* for 1 h, to obtain a

fraction containing lysosomes and mitochondria. The pH of the supernatant was adjusted to 5.2 in an ice bath, and the precipitation was centrifuged at 12 000 *g* for 15 min. The sediment was dissolved in a volume of water equal to the volume of the original homogenate, and neutralized. It was called the microsomal fraction. The hydrolysis rate of BANA was determined in all these preparations.

Chromatography and gel filtration. DEAE-cellulose (Whatman, DE-11), Sephadex G-100 and G-200 (particle size 40 – 120 μm) and CM-Sephadex A-50 Medium (Pharmacia, Uppsala, Sweden) were used according to the manufacturer's orders at +4°C. Details are given below.

Determination of the molecular weight. Gel filtration in a Sephadex G-200 column of 1.5 × 110 cm was used. The eluent was 10 mmol/l Tris-HCl, pH 6.9. In the first run, 2 mg Blue Dextran, 10 mg γ-globulin (Bovine, fraction III, Calbiochem, Calif.) M.W. 150 000, 10 mg albumin (Sigma Chem. Co., Ohio) M.W. 70 000, and 2 mg trypsin (2 × cryst. Type I from bovine pancreases, Sigma Chem. Co., Ohio) M.W. 23 800 in 2.9 ml of aq. dest. were applied simultaneously. In an other similar run, trypsin was replaced by 6.0 ml of the purified enzyme preparation C, concentrated with dry Sephadex G-50.

RESULTS

Studies with the homogenate. Hydrolysis rate of BANA in 0.1 mol/l Tris-HCl buffer (pH 7.0) was about equal (0.2 – 0.5 nmol/min/mg) in the thyroids of sheep, guinea pig, cow, rat, and rabbit, while it was much lower (0.05 nmol/min/mg) in the hog. About 1/8 – 1/10 of the total activity remained in the sediment, when the once frozen thyroids were homogenized in water.

In all the species mentioned, the hydrolysis of BANA was found at the broad pH-range of 5.0 – 7.5 (Fig. 1). The maximum activity was found at pH 7.0 – 7.2 in guinea pig and cow, but at pH 6.5 – 6.6 in sheep. The hydrolysis was very sensitive to pCMB, Cu^{2+}, and Co^{2+} (0.25 mmol/l) in all these species. During the storage at +2°C, the activity decreased to 1/10 of the original in two days. The activity could be completely regenerated with cysteine + EDTA (1 mmol/l). The activity was found to be stable in frozen homogenate. In the presence of cysteine (1 mmol/l), markedly lower hydrolytic activities were

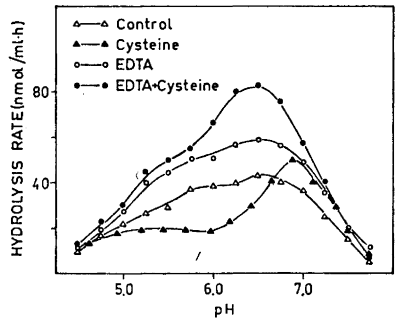

Fig. 1. The effect of pH and of some modifiers on the hydrolysis of BANA by the sheep thyroid homogenate supernatant. Incubation medium: 1.0 ml 0.1 mol/l of Michaelis veronal-acetate buffer, 0.5 ml supernatant (0.1 mg protein/ml), and 0.5 ml substrate solution 1.0 mmol/l. All the modifier concentrations, 1 mmol/l. Incubation time, 2 h.

recorded at the lower pH values in sheep, guinea pig, and cow (Fig. 1). Such an effect was not found with thioacetic acid (0.5 mmol/l).

The buffer used had a profound effect on the activity. The guinea pig thyroid homogenate at pH 7.0 showed the best activities in Michaelis veronal-

acetate buffer, followed by Tris-HCl, Tris-maleate, and phosphate in ratios 100:70:50:30.

The pH 7.0 activity was clearly more stable at pH 4.0 – 5.0 than at pH 8.0 or above.

Distribution of the enzymic activity in centrifugal fractions. The distribution of the enzymic activity in the centrifugal tissue fractions is shown in Table 1. The main part of the activity, hydrolysing BANA, was found in the lysosomal fraction. A thorough homogenization liberated more enzyme into the supernatant fraction, as did the homogenization in distilled water. If a once frozen tissue was homogenized in distilled water, about 85 % of the enzyme was found in the supernatant.

Table 1. Distribution of the enzymes hydrolyzing BANA in tissue fractions. The hydrolysis was tested in 0.1 mol/l Michaelis veronal-acetate buffer, pH 6.0, in the presence of EDTA (0.5 mmol/l) and cysteine (2.0 mmol/l).

Large particle (lysosomal) fraction (sediment of 32 000 *g* centrifugation, 1 h)	80.0 %
Microsomal fraction (precipitate at pH 5.2)	5.4 %
Supernatant (after precipitation at pH 5.2)	14.6 %

Purification of the enzyme activity. The purification procedure is summarized in Table 2. Sheep thyroid tissue (35 g) was homogenized in distilled water, centrifuged at 1000 *g* for 15 min, and the pH of the supernatant was adjusted with 0.1 mol/l HCl to pH 5.2. Ammonium sulphate was added up to 35 % saturation, and the precipitate was separated 30 min later by centrifugation at 25 000 *g* for 45 min. Ammonium sulphate was added to the supernatant up to 90 % saturation, and the precipitate was separated as before. The supernatant was discarded. The sediment was dissolved in 50 ml of distilled water,

Table 2. Summary of the purification procedure.

Purification stage	Volume (ml)	Amount of protein (mg)	Specific activity (nmol/mg min)	Units (nmol/min)
Homogenate (aqueous)	220	3470	5.88	20403
pH 5.2 supernatant	205	2460	6.37	15700
$(NH_4)_2SO_4$ fractionation (35 – 90 %)	50	480	8.90	4270
Sephadex G-100, pooled preparation	151	86.5	20.1	1740
DEAE-cellulose, pooled preparation	115	4.88	29.4	143
CM-Sephadex, pooled preparation A	35	0.49	28.9	14.2
Preparation B	40	0.32	49.5	15.8
Preparation C	60	0.48	100.6	50.8

and dialysed for 24 h against water, containing EDTA and cysteine (0.1 mol/l). The preparation was applied to a column of Sephadex G-100 (3 × 75 cm) and eluted with 10 mmol/l Tris-HCl, pH 6.9, containing EDTA and cysteine (1 mmol/l). The result is seen in Fig. 2. Fractions 35 – 48 were pooled, and the proteins precipitated between 35 – 90 % ammonium sulphate satura-

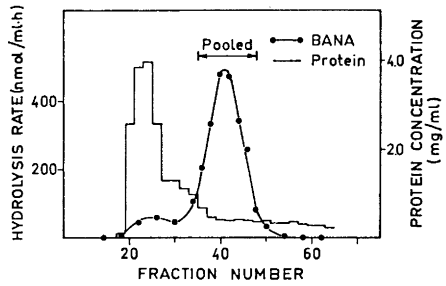

Fig. 2. Gel filtration of the ammonium sulphate (35 – 90 %) precipitate of sheep thyroid homogenate on Sephadex G-100. Incubation time for BANA hydrolysis, 1 h. Details are given in the text.

tions were collected as before. The precipitate was dissolved in 10 ml of distilled water, dialysed as before, and applied to a column of DEAE-cellulose (3.5 × 25 cm). The eluent was 15 mmol/l Tris-HCl, pH 6.9, containing EDTA and cysteine (1 mmol/l) and a linear gradient of NaCl 0 – 0.4 mol/l. Flow rate was 60 ml/h, and fractions of 6.0 ml were collected. The result is seen in Fig. 3.

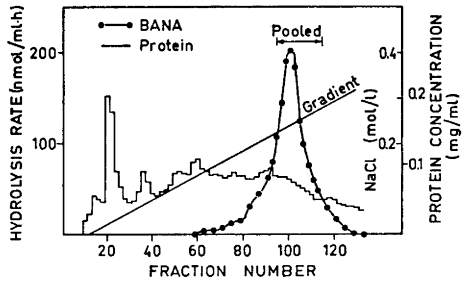

Fig. 3. DEAE-cellulose chromatography of the pooled fractions (35 – 48) after Sephadex G-100 gel filtration. Incubation time for BANA hydrolysis, 1 h.

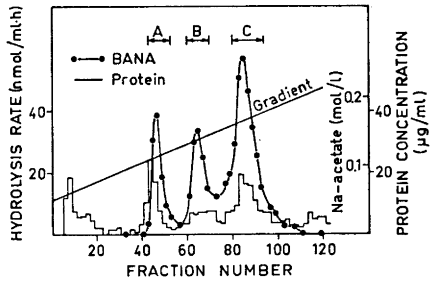

Fig. 4. CM-Sephadex chromatography of the pooled fractions after DEAE-cellulose chromatography. Incubation time for BANA hydrolysis 4 h. Proteins were determined by measuring the absorption at 280 nm.

Fractions 93 – 115 were pooled (115 ml); the proteins precipitated between 35 – 90 % ammonium sulphate concentration were collected and deionized in a column (2 × 40 cm) of Sephadex G-25 with 40 mmol/l acetate buffer, pH 5.5, as eluent.

The enzymatically active fractions were applied on a column of CM-Sephadex (1.4 × 30 cm), stabilized with the buffer mentioned. Elution was carried out with acetate buffer, pH 5.5, using a linear gradient of acetate

(0.04 – 0.2 mol/l), a flow rate of 25 ml/h, and a fraction volume of 4.0 ml. The result is seen in Fig. 4. Fractions 43 – 53 (preparation A), 60 – 70 (preparation B), and 80 – 94 (preparation C) were pooled. These were used in further characterization studies.

Characteristics of the enzyme preparations

Molecular weight. Molecular weight of the preparation C was determined with a Sephadex G-200 column, and the result is seen in Fig. 5. Preparation C was eluted from the column with the same elution volume as bovine trypsin.

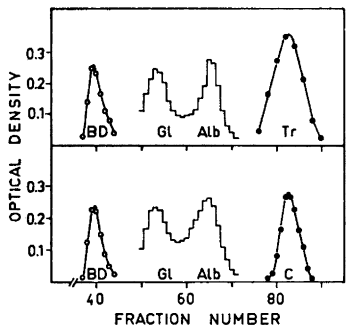

Fig. 5. Distribution of the purified enzyme preparation C and standard proteins on Sephadex G-200 gel filtration. Enzyme preparation C was assayed with BANA, incubation time 6 h. Trypsin was assayed with BAPA, incubation time 30 min, other proteins were determined by measuring the absorption at 280 nm, and Blue Dextran, respectively, at 618 nm. Tr = trypsin.

Whitaker's plot [26] of the data gave a molecular weight of 23 900. It is very likely that the molecular weights of preparations A and B are close to this, since the preparations A, B, and C were not separated by chromatography on Sephadex G-100 earlier in the purification procedure. Additionally, no separation was obtained in separate experiments, using a column of Sephadex G-200.

Dependence on pH. The maximum hydrolysis rates of BANA by all the preparations (A, B, and C) were found roughly at pH 6.0, when tested in 0.1 mol/l veronal-acetate buffer (Fig. 6).

Heat stability. Samples of preparations A, B, and C were incubated in 0.1 mol/l Michaelis buffer, pH 6.0 (in the presence of EDTA and cysteine, 1

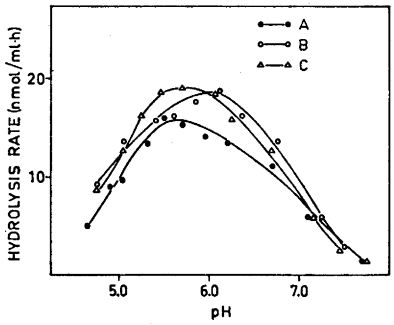

Fig. 6. The effect of pH on the hydrolysis of BANA by the three purified preparations A, B, and C. Incubation time 8 h. The hydrolysis values for preparation C were divided by two. Other experimental conditions as given in the text.

mmol/l), for 10 min at various temperatures. The enzyme activity with BANA was determined, and the results are seen in Fig. 7. All preparations were destroyed at temperatures over 50°C.

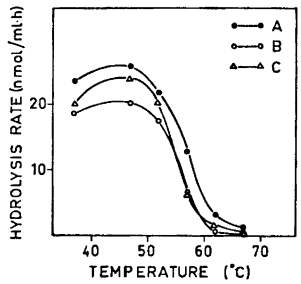

Fig. 7. The heat stability of the three purified preparations A, B, and C. Enzymes were tested with BANA as substrate, and incubation time was 8 h. The hydrolysis values for preparation C were divided by two.

Table 3. The rates of hydrolysis of various substrates by preparations A, B, and C at pH 6.0 (Michaelis veronal-acetate buffer) in the presence of EDTA (0.5 mmol/l) and cysteine (2.0 mmol/l).

Substrate	Incubation time (h)	Concentration (mmol/l)	Hydrolysis rate (nmol/mg min) A	B	C
Albumin (bovine serum)	10	3.3 g/l	0.13	0.58	0.48
Casein (denatured)	10	3.3 g/l	0.34	1.1	1.1
Hemoglobin (human, denatured)	10	3.3 g/l	0.08	0.58	0.58
BANA (benzoyl-arginine naphthylamide)	6	1.0	28.9	49.5	100.6
BAPA (benzoyl-arginine *p*-nitroanilide)	24	0.5	0	0	0
ANA (arginine naphthyl-amide)	24	0.25	0	0	0
BAEE (benzoyl-arginine ethyl ester)	10	5.0	63.9	142	162
BAME (benzoyl-arginine methyl ester)	10	5.0	103	114	256
TAME (tosyl-arginine methyl ester)	10	5.0	11	26	19
AME (arginine methyl ester)	10	10.0	92	81	96
LME (lysine methyl ester)	10	10.0	91	140	126

Substrate specificity. The hydrolysis rates of several substrates by preparations A, B, and C are given in Table 3. BANA was hydrolysed by the preparations at very different rates, in an approximate ratio of 3:5:10. This suggested differences in the purity of the preparations. The dependence of BANA hydrolysis on substrate concentration is given in Fig. 8. Maximum rate of hydrolysis by all enzyme preparations was obtained at the 2 mmol/l substrate concentration. BAPA was hydrolysed minimally, if at all. Proteins (albumin, casein and hemoglobin) were hydrolysed very slowly.

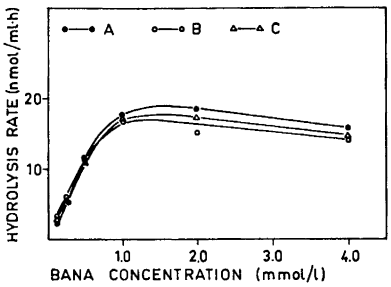

Fig. 8. The effect of substrate concentration on the hydrolysis of BANA by the three purified preparations A, B, and C. Incubation time, 8 h. The hydrolysis values for preparation C were divided by two.

No hydrolysis of ANA could be measured. The esters of unsubstituted basic amino acids were hydrolysed almost to the same degree as the substituted ester substrates. Among the *N*-substituted arginine esters, benzoyl-derivatives were hydrolysed clearly faster than TAME.

Effect of modifier substances. The effects of several modifier substances are presented in Table 4.

Table 4. Effect of some enzyme modifiers on the hydrolysis of BANA by the three enzyme preparations A, B, and C. Results are given as relative values, and the hydrolysis of BANA in the presence of EDTA and cysteine was marked as 100. Incubation time 6 h, substrate concentration 1 mmol/l.

Modifier	Concentration (mmol/l)	Preparation		
		A	B	C
EDTA	0.5	0	0	0
Cysteine	2.0	6	9	4
EDTA + BME	2.0	100	64	100
EDTA + thioacetic acid	0.5	40	33	26
EDTA + cysteine	2.0	100	100	100
EDTA + cysteine + pCMB	0.1	59	44	50
EDTA + cysteine + IAA	2.0	1	1	0
EDTA + cysteine + NEMI	2.0	1	2	0
Lima bean trypsin inhib.	0.2 mg/ml	113	116	128
Ovomucoid trypsin inhib.	0.2 mg/ml	122	120	126
Trasylol	50 IU/assay	103	110	105
Tetra-*N*-butylammonium-iodide	5	72	72	60
Tetra-*N*-methylammonium-iodide	5	85	83	81
DFP	10^{-3}	72	62	58
E-600	10^{-3}	74	68	92
ε-Aminocaproic acid	2.0	85	78	87

The enzymes in all preparations are sensitive to SH-reagents, *e.g.* (IAA, NEMI and pCMB). Furthermore, the enzymic activities could not be demonstrated during or after the purification procedure in the absence of EDTA and cysteine. In the presence of cysteine (1 mmol/l) alone, only 5 – 10 % of the total activity could be demonstrated, and no activity in the presence of

EDTA (0.5 mmol/l) alone. The presence of 0.01 mmol/l EDTA in addition to cysteine (2 mmol/l) gave about 95 % of the total activity. The effect of various cysteine concentrations on the BANA hydrolysis is seen in Fig. 9.

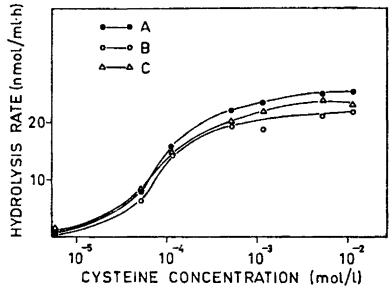

Fig. 9. The effect of cysteine concentration on the hydrolysis of BANA by three purified preparations A, B, and C. EDTA concentration 0.5 mmol/l. Incubation time, 8 h. The hydrolysis values for preparation C were divided by two.

The natural trypsin inhibitors caused no inhibition, and *N*-butyl- and *N*-methylammonium iodide inhibited only at high concentrations. The organophosphorus compounds tested caused slight inhibition of all enzyme preparations.

Among the bivalent metal ions, Mg^{2+} and Ca^{2+} (2 mmol/l) caused some, while Mn^{2+}, Cu^{2+}, Zn^{2+}, and Pb^{2+} caused a practically complete inhibition. It was evident that no great differencies could be seen between the separate enzyme preparations.

DISCUSSION

The data reveal that the typical trypsin substrate, benzoyl-arginine β-naphthylamide, is hydrolysed by the thyroid tissue of several mammalian species. Only minor differencies in the enzymic activity, as well as in the pH-optimum, were observed. The sheep thyroid was chosen as subject for a more detailed study, due to its relatively high activity and to the easy access to reasonable amounts of this tissue.

The purification procedure employed resulted in the separation of three enzymic components with closely similar characteristics: molecular size, substrate specificity, pH-dependence, heat stability, and modifier characteristics. The three components could be separated only on the basis of differencies in ionic characteristics in exchange chromatography. It appears reasonable to classify them as isoenzymes.

The fact that only about 0.5 % of the original enzyme activity was left at the end of the purification procedure, is a sign of great lability of the enzymes The studies with the purified enzyme preparations also indicated the lability of the enzymes, and the requirement of effective sulphydryl compounds during the storage, as well as in the enzyme assays, was found to be an absolute necessity. The dependence of the activity on sulphydryl groups was further proved by the observations, that even inactive enzyme preparations could be reactivated by adding cysteine and EDTA, and that sulphydryl blocking agents caused a complete inactivation.

The purified enzyme could be characterized as a lysosomal, acidic and strongly sulphydryl-dependent protease, with a specificity resembling that of trypsin. Its substrate specificity differed, however, from that of trypsin in so far, that all the tested protein and ester substrates were hydrolysed much more slowly than by trypsin. As further difference, benzoylarginine *p*-nitroanilide was not hydrolysed measurably, although benzoylarginine naphthylamide was an excellent substrate for the enzyme. The characteristics of the thyroid enzyme were, on the other hand, closely similar to those of cathepsin B′ from calf spleen:[18,19] molecular weight about 23 900 (cathepsin B′ 25 000), sulphydryl dependence, pH-optimum at 6.0 (cathepsin B′ at pH 6.5), ready hydrolysis of benzoylarginine naphthylamide, low hydrolysis rate of protein and ester substrates, and no effect by usual trypsin inhibitors, nor by DFP. The lack of hydrolysis of benzoyl-arginine *p*-nitroanilide by the thyroid enzyme could, however, be pointed out as a difference between the enzymes; the calf spleen enzyme has been reported to hydrolyse this substrate at a rate of 7 % of that of benzoyl-arginine naphthylamide.[19]

On the basis of the present study, it appears that to the list of proteolytic enzymes in the thyroid lysosomes, an additional catheptic enzyme must be added – cathepsin B′. It remains to be seen, whether this enzyme is directly involved in the liberation of thyroid hormones from thyroglobulin, or whether it takes part in other lytic processes in the metabolism of the follicular cells.

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Received October 30, 1970.