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PARTIAL PURIFICATION AND PROPERTIES OF A PEPTIDASE FROM THYROID GLANDS

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SUMMARY

1. A peptidase which hydrolyses *N*-acetyl-L-phenylalanyl-L-tyrosine at an optimum pH of 4.1 has been purified more than 500-fold from saline extracts of pig-thyroid glands. The procedure involves removal of thyroglobulin by acid precipitation followed by acetone fractionation and chromatography on DEAE-cellulose.

2. Specificity studies on a variety of peptides at three pH values (pH 4.1, 5.3 and 7.2) indicated a requirement for at least one aromatic amino acid or leucine with the sole exception of L-methionyl-L-methionine which was hydrolysed to a slight extent.

3. A comparison of the optimum pH of hydrolysis of the most susceptible substrates, *N*-acetyl-L-phenylalanyl-L-tyrosine, L-tryptophyl-L-leucine, glycyl-L-phenylalanyl-L-phenylalanine and glycyl-L-leucyl-L-tyrosine, showed slight differences in pH optima but all were within the range pH 4.1–4.9.

INTRODUCTION

Proteolytic activity in thyroid follicles was first demonstrated by DE ROBERTIS[1] using a micromanipulative procedure. Subsequent workers partially purified a protease, active at acid pH (ref. 2), and showed that such preparations, in addition to their action on proteins, were able to hydrolyse the pepsin substrates[3], *N*-acetyl-L-phenylalanyl-L-tyrosine (APAT) and its iodinated derivative, *N*-acetyl-L-phenylalanyl-L-diiodotyrosine[4]. Further purification of the protease by acetone fractionation established that the activity against APAT was due to a contaminating peptidase. A second peptidase was detected in fractions highest in proteolytic activity[5,6]. This enzyme, provisionally termed "cysteinyltyrosinase" (L-cysteinyl-L-tyrosine hydrolase) was purified and shown to be metal dependent and to hydrolyze a wide range of peptides[7].

Abbreviations: Ac-, acetyl; Z-, benzyloxycarbonyl; APAT, *N*-acetyl-L-phenylalanyl-L-tyrosine.

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In the present communication, the purification and properties of the peptidase, APAT hydrolase, are reported.

MATERIALS AND METHODS

All reagents used were of A.R. grade. All solvents were purified by distillation according to standard procedures.

N-Ac-L-Phe-L-Tyr, L-Cys-L-Tyr and L-Phe-L-Tyr were synthesised by V. M. TRIKOJUS and T. A. A. DOPHEIDE of this Department; L-Glu-L-Tyr, L-Leu-L-Asn, L-Leu-L-Gln, L-Tyr-L-Leu and *O*-Ac-L-Tyr-L-Leu were provided by C.S.I.R.O. Division of Protein Chemistry. Other peptides were obtained commercially: Gly-Gly, Gly-L-Trp, Gly-L-Tyr, L-Leu-Gly-Gly and L-Leu-L-Tyr (Roche Products, Ltd., England); DL-Leu-Gly-DL-Phe (Nutritional Biochemicals Corporation, U.S.A.); L-Leu-Gly, L-Leu-L-Leu, L-Leu-L-Phe and L-Leu-L-Trp (Theodor Schuchardt, Germany); L-His-L-Phe·HCl, L-His-L-Tyr, L-Phe-L-Phe, L-Pro-L-Phe, L-Trp-L-Leu, L-Trp-L-Phe, L-Trp-L-Trp, L-Trp-L-Tyr, L-Tyr-L-Phe, L-Tyr-L-Tyr and L-Val-L-Phe (Yeda, Israel). All other peptides were obtained from Mann Research Laboratories, U.S.A.

DEAE-cellulose was from Serva: capacity 0.69 mequiv/g.

Assay of APAT hydrolase

0.03 ml of a solution of APAT in 0.1 M NaOH (containing 2 μmoles of APAT) and 0.32 ml of sodium acetate buffer (0.5 M, pH 4.1) were incubated at 37° with 0.05 ml of the enzyme solution. 100-μl samples were removed at zero time and, as a routine, to check linearity, after both 30 and 60 min (in later work, after 20 and 40 min) incubation and delivered onto small pieces of chromatography paper (previously washed with the alkaline borate buffer in methanol described by CONNELL, DIXON AND HANES[8] to remove traces of ammonia) in constriction tubes calibrated at 10 ml, precooled in an ice-bath. 2 ml of the quantitative ninhydrin reagent of CONNELL, DIXON AND HANES[8] was added to the tubes which were then covered and heated in a boiling-water bath for 20 min. After cooling the tubes, aqueous ethanol (1:1, v/v) was added to 10 ml and the $A^{1\,cm}_{570\,m\mu}$ values were read in a spectrophotometer (Unicam, Model SP 500).

When eluates from columns were assayed, the method was slightly modified in that the volume of enzyme was increased to 0.1 ml and that of the buffer decreased to 0.27 ml. The 30-min sample was omitted.

It was found to be unnecessary to include in the assay control tubes containing enzyme and buffer without substrate when relatively pure APAT hydrolase fractions were assayed. With cruder preparations there was an increase in ninhydrin-reacting material in the control tubes and this increment in extinction was subtracted from corresponding values for enzyme-substrate mixtures before the enzyme activity was calculated. In addition, estimations of the blank value of the paper and of a standard solution of tyrosine were routinely made.

One unit of APAT hydrolase is defined as that amount which, when incubated with APAT (2 μmoles; final concentration 5 mM) at pH 4.1 hydrolyses 1 μmole of the peptide in 1 min.

Assay of leucyltyrosine hydrolase (cysteinyltyrosine hydrolase)

Since the commercially available L-Leu–L-Tyr, as well as L-Cys–L-Tyr, was an effective substrate for the metal-dependent thyroid peptidase[7], the enzyme was assayed using the former as substrate at the optimum pH 5.0.

0.05 ml of the enzyme solution was preincubated for 30 min at 37° with 0.05 ml of sodium acetate buffer (0.1 M, pH 5.0) containing 0.6 mM Zn^{2+} (the acetate salt). 0.3 ml of a solution of 1.33 mM L-Leu–L-Tyr in sodium acetate buffer (0.05 M, pH 5.0) containing 0.3 mM Zn^{2+} was then added giving final concentrations of: L-Leu–L-Tyr, 1 mM; acetate buffer, 0.05 M; Zn^{2+}, 0.3 mM; total volume, 0.4 ml. 100-μl samples were taken at zero time and after 30 and 60 min incubation. The samples were delivered onto paper and treated with ninhydrin as in the APAT hydrolase assay.

Control tubes containing enzyme and buffer without substrate were included only when relatively crude preparations were assayed.

When eluates from columns were tested, 0.1 ml of the fraction was preincubated with 0.1 ml of the concentrated buffer and 0.2 ml of a solution of 2 mM L-Leu–L-Tyr in the dilute buffer was then added. Only zero time and 60-min samples were taken.

One unit of leucyltyrosine hydrolase is defined as that amount which, following preincubation with Zn^{2+} (0.6 mM) and incubation at pH 5.0 with L-Leu–L-Tyr (final concentration 1 mM) in the presence of Zn^{2+} (0.3 mM), hydrolyses 1 μmole of the peptide in 1 min.

Assay of acid protease

This was carried out with haemoglobin as substrate at pH 3.6 by the method of ANSON[9] with slight modifications. The extinction, at 280 mμ, of the filtrate obtained after addition of trichloroacetic acid was converted to mmoles of tyrosine by reference to a standard curve.

One unit of protease is defined as the amount of enzyme required to liberate the equivalent of 0.1 μmole of tyrosine in 1 min. Enzyme solutions containing more than 0.7 units/ml were diluted prior to assay.

Protein determinations

Protein was estimated by one of three methods: (i) Determination of dry weight. (ii) The biuret method of ELLMAN[10] with reference to a standard curve prepared with bovine serum albumin (Commonwealth Serum Laboratories, Melbourne, Batch 001). (iii) Extinction at 280 mμ ($A^{1\,\mathrm{cm}}_{280\,\mathrm{m}\mu}$) as an approximation to protein concentration in eluates from chromatography columns.

Chromatography

Column chromatography was carried out on DEAE-cellulose at 4°. In preliminary studies, linear gradients were produced following the methods of PARR[11] and BOCK AND LING[12]. When concentrations for optimum separation of APAT hydrolase were established, a procedure of stepwise elution was substituted.

Paper chromatography was carried out at 25°. For qualitative estimation of peptidase activity, 10-μl samples of incubation mixtures (see text) were spotted onto Whatman No. 1 paper. Marker spots of the appropriate amino acids were also applied in amounts calculated to be equivalent to 100% hydrolysis of the peptide. Descending chromatograms were developed for approx. 15 h with *n*-butanol–acetic acid–water

(4:1:1, v/v/v). Chromatograms were dried and then sprayed with ninhydrin solution and heated at 60°. When incubations contained APAT, a duplicate chromatogram was sprayed with 10% aqueous Na_2CO_3, dried and sprayed with diazotized sulphanilic acid to detect APAT and tyrosine (a modification of the method of GROSS AND LEBLOND[13]).

EXPERIMENTAL

Purification procedure

Pig thyroids were obtained frozen from the abattoirs and when required were thawed and dissected free from extraneous tissue. All subsequent steps in the preparation of the enzyme were carried out at 0° or 2°, except during acetone fractionation when temperatures were maintained at −5° to −10°. The glands were homogenised with 3 volumes of 0.9% NaCl for 2 min in a blendor (M.S.E. Ato-Mix), toluene (1 ml per 100 g glands) added and the homogenate left overnight at 2°. After centrifuging (2000 × *g*, 20 min), the residue was washed with one-tenth volume of saline and the combined supernatant *plus* washing filtered through cotton wool. The extract was adjusted to pH 3.5 by the very slow addition of HCl and the suspension centrifuged (2000 × *g*, 20 min). The precipitate after washing with one-tenth volume of saline (pH 3.5) was discarded and the supernatant and washing combined and dialysed.

NaCl was added to the dialysed solution to a concentration of 0.05 M and then acetone to 40%. After centrifuging (1500 × *g*, 20 min), the precipitate was dissolved in water, dialysed and freeze-dried. The supernatant contains the bulk of the acid

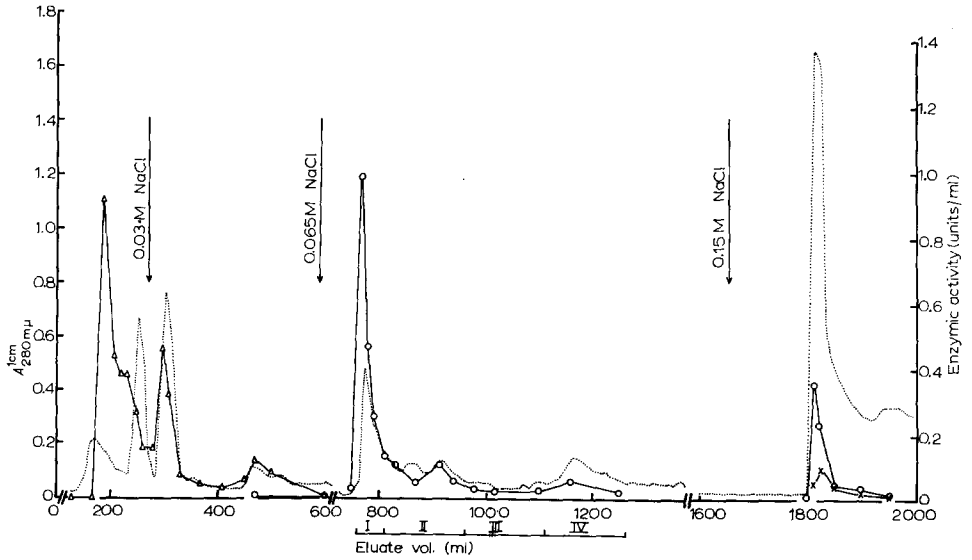


Fig. 1. Chromatography of partially purified APAT hydrolase on DEAE-cellulose: 40% acetone fraction (1 g) in Tris–HCl buffer (0.02 M, pH 7.2) applied to column (64 cm × 2.3 cm) and eluted stepwise with increasing concentrations of NaCl as indicated. Flow rate, 15 ml/h; fraction volume, 10 ml. ....., $A^{1cm}_{280 m\mu}$; ○—○, APAT hydrolase (units/ml); △—△, protease (units/ml); ×—×, leucyltyrosine hydrolase (units/ml).

TABLE I

RECOVERY AND PURIFICATION OF APAT HYDROLASE FOLLOWING CHROMATOGRAPHY OF 40% ACETONE FRACTION, PREP. IV, ON DEAE-CELLULOSE

Fractions pooled as shown in Fig. 1. Assayed (see MATERIALS AND METHODS) after dialysis and freeze-drying.

Properties	*Initial material*	*Fractions from column*			
		I	*II*	*III*	*IV*
Weight (mg)	1000	15.8	24.2	23.0	23.5
APAT hydrolase					
% yield	(100)	16.0	7.5	1.6	1.9
milli-units/mg soluble protein	87	880	269	62	72
purification factor	(1.0)	10.1	3.1	0.7	0.8
Acid protease					
milli-units/mg soluble protein	120	96	43	16	21
Leucyltyrosine hydrolase					
milli-units/mg soluble protein	26.7	trace	4.8	5.8	4.2

protease and leucyltyrosine hydrolase and may be further fractionated with acetone for the purification of these enzymes.

A solution of the 40% acetone fraction dissolved in Tris–HCl buffer (pH 7.2) was applied to a DEAE-cellulose column previously equilibrated with the buffer and eluted with stepwise increments in ionic strength. The elution pattern is shown in Fig. 1. The fractions eluted by 0.065 M NaCl were pooled as indicated, dialysed, freeze-dried and assayed for APAT hydrolase, acid protease and leucyltyrosine hydrolase. Results for a typical preparation, Preparation IV, are shown in Table I and yields and purification factors at several stages during the complete procedure are given in Table II.

Inactivation of leucyltyrosine hydrolase

The preparation used in all experiments subsequently reported was a sample containing 0.82 APAT hydrolase units/mg. It was slightly contaminated with acid protease (0.12 units/mg). Leucyltyrosine hydrolase was too low for quantitative

TABLE II

SUMMARY OF THE PURIFICATION OF APAT HYDROLASE FROM PIG-THYROID TISSUE

Stage of purification	*% yield*	*Purification factors**	
		Protein basis	*Protease basis*
Crude saline extract	(100)	(1.0)**	(1.0)
Supernatant after acid precipitation	77	11**	1.7
40% acetone fraction	75	52**	48
Chromatography on DEAE-cellulose	12	528	600

* Considerable purification was also obtained on the basis of leucyltyrosine hydrolase activity but the degree could not be estimated quantitatively (see text).

** Protein content determined by the biuret method. In the other cases the weight of the freeze-dried fraction was taken to be equivalent to the protein content.

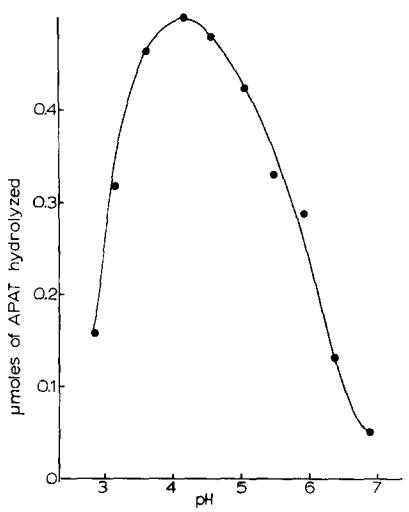
determination but, in a qualitative test, when the enzyme in final concentration of 0.5 mg/ml was incubated with L-Leu–L-Tyr for 2 h at 37°, very slight hydrolysis of the peptide was detected on chromatography of the incubation mixture. VALLEE *et al.*[14] found that Zn^{2+} can be removed from carboxypeptidase by dialysis against buffers of pH below 5.5. Since leucyltyrosine hydrolase is a metal-dependent enzyme[7], the APAT hydrolase preparation was dialysed against sodium acetate buffer (0.05 M, pH 4, containing 10 mM EDTA) for 72 h. Following this treatment no hydrolysis of L-Leu–L-Tyr could be detected under conditions similar to those reported above. Activity of the preparation against APAT was unaffected. Enzyme samples used in subsequent work were always pretreated by this procedure.

pH-activity curve

Fig. 2 indicates that the optimum pH of the enzyme is 4.1 although there is little change in activity over the range pH 4–4.4.

Effect of substrate concentration

As illustrated in Fig. 3, although a true plateau was not obtained, the curve flattened considerably above a final APAT concentration of 4 mM. Due to the low solubility of the peptide, 7.5 mM was the highest concentration possible under our conditions and this was used in further assays.



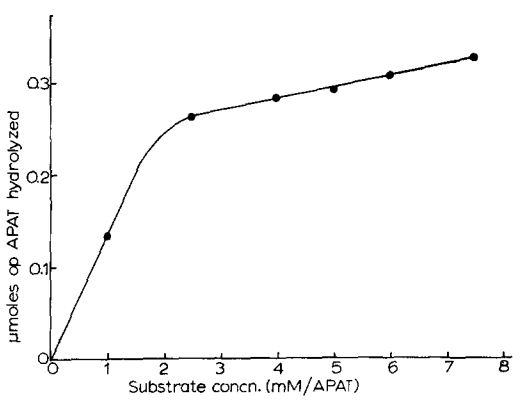


Fig. 2. pH–activity curve. Enzyme, 0.025 units/ml; APAT, 5 mM. Incubation: 60 min, 37°. Buffers used: pH 2.8–5.5, sodium acetate (0.5 M); pH 6.0–7.0, sodium phosphate (0.5 M).

Fig. 3. Effect of substrate concentration. Enzyme, 0.025 units/ml; APAT, 1.0–7.5 mM. Incubation 40 min, 37°.

Substrate specificity — qualitative

The action of the enzyme against various peptides was tested at 3 pH values, the extent of hydrolysis being estimated visually following chromatography of the incubation mixtures and detection of ninhydrin-reacting components. Experimental details and results are given in Table III.

TABLE III

SUBSTRATE SPECIFICITY OF APAT HYDROLASE DETERMINED QUALITATIVELY

Final concentrations of components of incubation mixtures: peptide, 5 mM; enzyme, 0.25 mg/ml (0.2 APAT hydrolase units/ml); buffer, 0.5 M sodium acetate containing 15 mM EDTA (pH 4.1 or 5.3) or 0.5 M Tris–HCl containing 15 mM EDTA (pH 7.2). Controls: peptide + buffer. Incubation: 2 h, 37°. Chromatography: as in MATERIALS AND METHODS. Degree of hydrolysis estimated visually from intensities of ninhydrin spots. ++++ = 100% hydrolysis. The following peptides were also tested with negative results: L-Ala–L-Ala, L-Ala–Gly, L-Cys–L-Tyr, L-Glu–L-Tyr, L-Gln–L-Asn, Gly–L-Asn, Gly–Gly, Gly–L-Leu, Gly–L-Phe, Z-Gly–L-Phe, Gly–L-Pro, Gly–L-Trp, Gly–L-Tyr, L-His–L-Phe·HCl, L-His–L-Tyr, L-Leu–L-Ala, L-Leu-amide, L-Leu–Gly, L-Leu–Gly–Gly, DL-Leu–Gly–DL-Phe, L-Leu–L-Tyr, L-Lys–Gly, L-Phe–Gly, L-Pro–L-Phe, L-Val–Gly, L-Val–L-Phe, L-Val–L-Val.

Peptide	*Degree of hydrolysis at pH*			*Peptide*	*Degree of hydrolysis at pH*		
	4.1	*5.3*	*7.2*		*4.1*	*5.3*	*7.2*
N-Ac–L-Phe*–L-Tyr(APAT)	++++	++++	±	L-Met*–L-Met	+	±	–
				L-Tyr*–L-Phe	±	+	–
Gly–L-Leu*–L-Tyr	+++ +	++++	–	L-Trp*–L-Trp	+	–	–
Gly–L-Phe*–L-Phe	++++	++++	–++	L-Tyr*–L-Tyr	±	–	–
L-Trp*–L-Leu	++++	++++	+	L-Leu*–L-Gln	±	±	–
L-Leu*–L-Leu	+++	++	+	L-Leu*–L-Trp	±	+	–
O-Ac–L-Tyr*–L-Leu	++ +	++ +	±	L-Phe*–L-Phe	±	–	–
L-Tyr*–L-Leu	+ +	+ +	±	L-Phe*–L-Tyr	±	–	–
L-Trp*–L-Phe	+++	++	±	Z–L-Glu*–L-Phe	±	–	–
L-Trp*–L-Tyr	++	++	±	Z–L-Glu*–L-Tyr	±	–	–
L-Leu*–L-Asn	+	+	–				
L-Leu*–L-Phe	+	+	–				

* An asterisk in the peptide abbreviation denotes those bonds hydrolysed. In the case of *O*-Ac–L-Tyr–L-Leu the major hydrolysis products were L-leucine and *O*-Ac–L-Tyr but at pH 4.1 and 5.3 traces of L-tyrosine were also detected in the hydrolysate.

All peptides which were hydrolysed to the extent of 50% or more at pH 4.1 and pH 5.3 under the conditions of this experiment contained either 2 aromatic amino acids, 2 leucine molecules or leucine and an aromatic amino acid. Other peptides which showed slight hydrolysis contained at least one aromatic amino acid or leucine with the sole exception of L-Met–L-Met.

The activity of the peptidase at pH 4.1 against four of the more susceptible peptides was compared semi-quantitatively with that against APAT. Activity against L-Trp–L-Phe was of the order of 0.1, against L-Tyr–L-Leu slightly less and against L-Leu–L-Leu and L-Trp–L-Tyr 0.05 of that against APAT.

Substrate specificity — quantitative

The activity of the peptidase at pH 4.1 against three of the most susceptible substrates, Gly–L-Leu–L-Tyr, Gly–L-Phe–L-Phe and L-Trp–L-Leu was compared with that against APAT by a method similar to the usual assay. Results are given in Table IV. Although, because of their high extinction coefficients, the other three peptides were used in lower concentration than APAT, the degree of hydrolysis increased linearly with time over the 40-min incubation period, implying substrate saturation. The results show that, at this pH, the enzyme is most active against APAT but has

TABLE IV

COMPARISON OF ACTIVITY OF APAT HYDROLASE AGAINST SEVERAL PEPTIDES

Assay: Components: 0.03 ml of peptide in 0.1 M NaOH; 0.32 ml sodium acetate buffer (0.5 M, pH 4.1); 0.05 ml enzyme solution. Incubation: 40 min, 37°.

Peptide	*Final peptide concn. (mM)*	*Final enzyme concn. (mg/ml)*	*μmoles hydrolysed per mg enzyme per h*
APAT	7.5	0.025	49
L-Trp–L-Leu	2.5	0.025	44
Gly–L-Phe–L-Phe	1.0	0.016	38
Gly–L-Phe–L-Phe	1.5	0.025	40
Gly–L-Leu–L-Tyr	1.0	0.025	*
Gly–L-Leu–L-Tyr	1.0	0.125	7.7

* Not detectable.

considerable activity against L-Trp–L-Leu, Gly–L-Phe–L-Phe and much less against Gly–L-Leu–L-Tyr.

pH-activity curve against different substrates

The optimum pH of the enzyme activity against APAT, L-Trp–L-Leu, Gly–L-Phe–L-Phe and Gly–L-Leu–L-Tyr was compared. The curves (see Fig. 4) show a slight difference in pH optima but all were within the range pH 4.1–4.9.

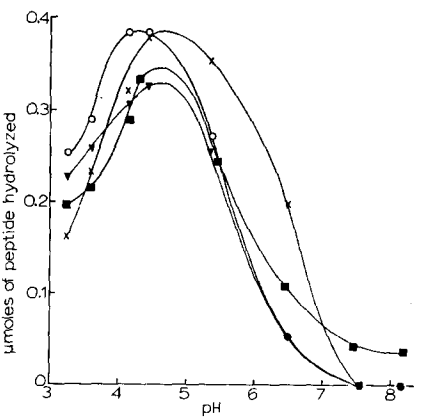


Fig. 4. pH–activity curve using different substrates. Enzyme, 0.02 units/ml for APAT, L-Trp–L-Leu and Gly–L-Phe–L-Phe; 0.13 units/ml for Gly–L-Leu–L-Tyr; substrate concentration: APAT, 7.5 mM; L-Trp–L-Leu, 2.5 mM; Gly–L-Phe–L-Phe and Gly–L-Leu–L-Tyr, 1.5 mM. Incubation: 40 min, 37°. Buffers used: pH 3.3–5.4, sodium acetate (0.5 M containing 15 mM EDTA); pH 6.5–8.2, sodium phosphate (0.5 M containing 15 mM EDTA). ○—○, APAT; ■—■, L-Trp–L-Leu; ×—×, Gly–L-Phe–L-Phe; ▼—▼, Gly–L-Leu–L-Tyr.

DISCUSSION

Although the method developed for the purification of APAT hydrolase resulted in a purification of the enzyme of more than 500 and 600-fold on a protein and acid

protease basis, respectively, compared with the initial activity in saline extracts of pig-thyroid glands, the material obtained was still slightly contaminated with protease and leucyltyrosine hydrolase. However, the latter activity was inhibited by treatment with EDTA at acid pH and therefore did not affect the experiments on the specificity of APAT hydrolase. It is most unlikely that these studies were affected by acid protease contamination since incubation of purified protease with APAT (under the same conditions as for the qualitative determination of the substrate specificity of APAT hydrolase) at a final concentration equivalent to 1700 times that contaminating the purified peptidase resulted in only slight hydrolysis of the peptide.

APAT hydrolase has a rather narrow range of susceptible substrates, acting on peptides containing an aromatic amino acid or leucine. In addition, L-Met–L-Met was hydrolysed although to a lesser extent. Thus APAT hydrolase appears to show preference for peptide bonds involving amino acids with large, non-polar side chains.

As a rule, the peptidase shows greatest activity against compounds containing two adjacent aromatic amino acids or an aromatic amino acid and leucine when the α-amino group of the amino acid supplying the carboxyl moiety of the bond is substituted *e.g.* as against APAT, Gly–L-Phe–L-Phe and Gly–L-Leu–L-Tyr compared with L-Phe–L-Tyr, L-Phe–L-Phe and L-Leu–L-Tyr. The exception is L-Trp–L-Leu which is a highly susceptible substrate. It is of interest that L-Leu–L-Tyr, but not Gly–L-Leu–L-Tyr, is hydrolysed by thyroid cysteinyltyrosine hydrolase[7].

In view of the action of pepsin on APAT a qualitative comparison was made of the activity of these two enzymes towards several substrates. Crystalline pepsin at pH 2, in amount producing 100% hydrolysis of APAT (1000 proteolytic units), caused only slight hydrolysis of Gly–L-Phe–L-Phe and had no detectable action against Gly–L-Leu–L-Tyr, L-Trp–L-Leu, *O*-Ac–L-Tyr–L-Leu, L-Tyr–L-Leu and L-Leu–L-Leu. A rough comparison of activity (on a weight basis) of pepsin and APAT hydrolase indicated that the latter enzyme is over 40 times more active against APAT than is pepsin.

The intrathyroidal location of APAT hydrolase is discussed in an accompanying paper[15]. The role of this enzyme in thyroid metabolism is not as yet definitely established. An earlier report from this laboratory[16] indicated that, whereas thyroid acid protease degraded ^{131}I-labelled thyroglobulin releasing monoiodotyrosine, diiodotyrosine and thyroxine, APAT hydrolase alone released very little radioactivity. However, when protease and APAT hydrolase together were incubated with thyroglobulin, the amounts of free monoiodotyrosine and diiodotyrosine particularly were increased, suggesting that the two enzymes may act synergistically in the hydrolysis of thyroglobulin. Subsequent experiments with purer enzyme preparations[17] confirmed these results.

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