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PURIFICATION AND PROPERTIES OF A PEPTIDASE FROM THYROID GLANDS

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SUMMARY

1. The peptidase, *N*-acetyl-L-phenylalanyl-L-tyrosine (APAT) hydrolase, has been purified 1250-fold from saline extracts of pig thyroid glands. This preparation was free from cysteinyltyrosine hydrolase and acid protease.

2. The purified enzyme was active with *N*-acetyl-L-phenylalanyl-L-3,5-diiodotyrosine as substrate although activity was less than with the uniodinated peptide. There was no detectable hydrolysis of either the A or B chain of insulin.

3. APAT hydrolase was partially inhibited at pH levels above approx. 7.0 by citrate and iodide (10^{-2} M) and was completely inhibited by TPCK ($2.5 \cdot 10^{-4}$ M) and *p*-chloromercuribenzoate (10^{-5} M). *N*-Ethyl-maleimide (10^{-3} M) and iodoacetate (10^{-2} M) inhibited only weakly and diisopropylfluorophosphate had no effect.

4. APAT hydrolase activity was detected in extracts of thyroid glands of six animal species although there was considerable variation between species both in its amount and pH optimum. This activity was also demonstrated to a varying degree in several rat tissues, activity in thyroid gland extracts being exceeded only by that in kidney extracts.

INTRODUCTION

MENZIES AND McQUILLAN[1] reported the partial purification from saline extracts of pig thyroid glands of a peptidase which hydrolysed *N*-acetyl-L-phenylalanyl-L-tyrosine at an optimum pH of 4.1. Specificity studies with this enzyme, APAT hydrolase, indicated a requirement for at least one aromatic amino acid or leucine, the most susceptible substrates being *N*-acetyl-L-phenylalanyl-L-tyrosine (APAT), L-tryptophyl-L-leucine, glycyl-L-phenylalanyl-L-phenylalanine and glycyl-L-leucyl-L-tyrosine. This enzyme preparation was still slightly contaminated with the metal-dependent peptidase, cysteinyltyrosine hydrolase[2] and with the thyroid proteinase active at acid pH, acid protease[3–6]. In the present communication the removal of these contaminants by a modified procedure for purification is reported, together

Abbreviations: Z-, benzyloxycarbonyl; APAT, *N*-acetyl-L-phenylalanyl-L-tyrosine; APADIT, *N*-acetyl-L-phenylalanyl-L-3,5-diiodotyrosine; PCMB, *p*-chloromercuribenzoate; TPCK, L-tosylamido-2-phenylethyl chloromethyl ketone.

with additional studies on the properties of APAT hydrolase. Comparisons of the "APAT hydrolase" activity of extracts of the thyroids of other species of animals and of the distribution of this activity in various tissues of the rat are also included.

MATERIALS AND METHODS

Reagents

All reagents were of A.R. quality. All solvents were purified by distillation according to standard procedures. APAT, *N*-acetyl-L-phenylalanyl-L-3,5-diiodotyrosine (APADIT) and L-Phe–L-Tyr were synthesized by V. M. Trikojus and T. A. A. Dopheide of this Department.

Bovine serum albumin and insulin were obtained from the Commonwealth Serum Laboratories. The A and B chains of insulin were separated by T. A. A. Dopheide using the method of CRAIG *et al.*[7].

Peptides obtained commercially included: Z-L-Glu–L-Tyr, L-Gly–L-Phe–L-Phe, 3,5-diiodotyrosine (Mann Laboratories, U.S.A.); L-Trp–L-Leu (Yeda Chemicals, Israel); Z-L-Phe–L-Tyr (Cyclo Chemical Corporation) and L-Leu–L-Tyr (Roche Products Limited, England).

Inhibitors tested were: *N*-ethyl maleimide (Nutritional Biochem. Corp., U.S.A.); *p*-chloromercuribenzoate (PCMB) (Light Co. Ltd.); iodoacetate (British Drug Houses); L-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) (Cyclo Chemical Corporation) and DFP (supplied by Dr. L. Austin of Monash University, Melbourne).

DEAE-Sephadex A 50 was from Serva: capacity 3.5 ± 0.5 mequiv/g and Sephadex G-100 from Pharmacia (Uppsala).

Enzyme assays

Acid protease, APAT and cysteinyltyrosine (leucyltyrosine) hydrolases were assayed as described by MENZIES AND MCQUILLAN[1] who also define the units used in this work.

Protein determinations

Protein was estimated by one of three methods: (a) determination of dry weight, (b) the biuret method of ELLMAN[8] with reference to a standard curve prepared with bovine serum albumin, (c) absorbance at 280 nm, $A_{280\ nm}$ (1-cm path length), as an approximation to protein concentration in eluates from chromatography columns.

Chromatography

Column chromatography was carried out using (a) DEAE-Sephadex A 50. Initially, linear gradients were produced following the methods of PARR[9] and BOCK AND LING[10]. When concentrations for optimum separation of APAT hydrolase were established, stepwise elution was substituted. (b) Sephadex G-100. The resin was suspended in 0.075 M NaCl and the fines removed by decantation. The suspended resin was then autoclaved at 8 lb/sq. inch for 20 min.

All column chromatography was carried out at 4°.

Paper chromatography was developed at 20°. Samples (10 μl) from incubation mixtures were applied to the paper, together with markers consisting of the appro-

priate amino acids in amounts calculated to be equivalent to 100% hydrolysis of the peptide. The chromatograms were developed by the descending method with the solvent system butanol–acetic acid–water (4:1:1, v/v/v). The spots were detected with ninhydrin.

Paper electrophoresis

Samples were dissolved in pyridine–water (4:1, v/v) and portions run on Whatman No. 3 filter paper in pyridine–acetic acid–water (10:1:89, v/v/v), pH 6.5, 1600 V (40 mA) for 2.5 h using a Pherograph Original-Frankfurt apparatus (L. Hormuth, Wiesloch, Germany).

Analytical ultracentrifugation

This was performed in a Spinco Model E ultracentrifuge employing Schlieren optics and an angular velocity of 59 780 rev./min. The temperature was controlled and recorded with the R.T.I.C. unit.

EXPERIMENTAL

Purification procedure

Pig thyroids were obtained frozen from the abattoirs and the procedure of MENZIES AND MCQUILLAN[1] followed for the initial stages of homogenization, extraction and acid-precipitation. Following dialysis of the latter supernatant, NaCl was added to a concentration of 0.05 M and then acetone to a concentration of 10%. After centrifuging (1500 × *g*, 20 min) the precipitate was discarded and acetone added to the supernatant to a concentration of 40%. The solution was again centrifuged (1500 × *g*, 20 min) and the precipitate dissolved in water, dialysed and freeze–dried.

The 10–40% acetone fraction was dissolved in Tris–HCl buffer (pH 7.2), applied to a DEAE-Sephadex. A 50 column previously equilibrated in the same buffer

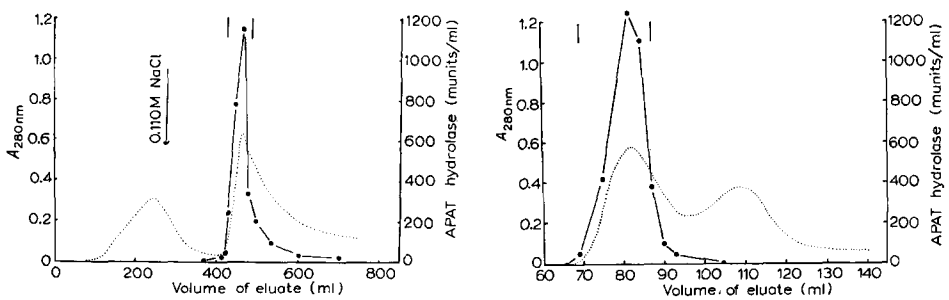

Fig. 1. Chromatography of partially purified APAT hydrolase on DEAE-Sephadex A 50: 10–40% acetone fraction (418 mg) in Tris–HCl buffer (0.02 M, pH 7.2, containing 0.060 M NaCl) applied to column (50 cm × 2.7 cm) and eluted with NaCl as indicated. Flow rate, 24 ml/h; fraction volume, 10 ml; · · · · ·, $A_{280\ nm}$ (1-cm path length); ———, APAT hydrolase (munits/ml); vertical bars, freeze–dried fraction.

Fig. 2. Chromatography of partially purified APAT hydrolase on Sephadex G-100: DEAE-Sephadex fraction (17 mg) in NaCl solution (0.075 M, pH 6.5) applied to column (40 cm × 3cm) and eluted with 0.075 M NaCl. Flow rate, 6 ml/h; fraction volume, 3 ml; · · · · ·, $A_{280\ nm}$ (1-cm path length); ———, APAT hydrolase (munits/ml); vertical bars, freeze–dried fraction.

TABLE I

SUMMARY OF THE PURIFICATION OF APAT HYDROLASE FROM PIG THYROID GLANDS

Stage of purification	*Activity (munits/mg)*	*Yield (%)*	*Purification factor (protein basis)*
Saline extract	1.74	(100)	(1)*
10–40% acetone fraction	127	68	73
Chromatography on DEAE-Sephadex A 50	1170	21	680
Chromatography on Sephadex G-100	2170	15	1250

* 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.

and the enzyme eluted by stepwise increments in ionic strength. The results of a typical experiment are shown in Fig. 1. The fraction eluted with 0.110 M NaCl was collected as indicated, dialysed, freeze-dried and assayed for APAT hydrolase, cysteinyltyrosine hydrolase and acid protease activity. Neither acid protease nor cysteinyltyrosine hydrolase activity could be detected in the APAT hydrolase peak after this chromatographic step nor could these enzymes be detected when a more concentrated solution was prepared from the freeze-dried powder.

The freeze-dried fraction obtained from chromatography on DEAE-Sephadex was dissolved in 0.075 M NaCl and applied to a Sephadex G-100 column equilibrated with the same solution, The elution curve is shown in Fig. 2. The fraction indicated was dialysed and freeze-dried and assayed for APAT hydrolase activity. The yields

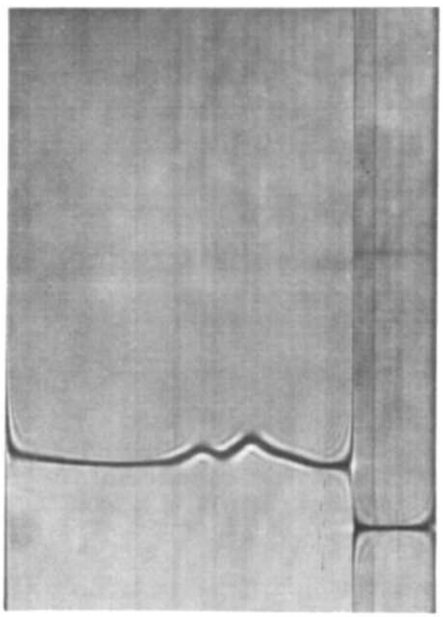

Fig. 3. Sedimentation velocity pattern of APAT hydrolase (Sephadex G-100 fraction, 2170 munits/mg; enzyme concentration, 3.9 mg/0.8 ml) in acetate buffer, pH 5.0 and ionic strength 0.10, at 20°. The angular velocity was 59 780 rev./min and the pattern was recorded at a bar angle of 60°. Sedimentation is from right to left.

and purification factors at several stages during the complete procedure are shown in Table I.

Although, on examination in the analytical ultracentrifuge, two components (*plus* a minor component) were observed in the Sephadex G-100 fraction (see Fig. 3), neither acid protease (4 h incubation) nor cysteinyltyrosine hydrolase activity (up to 20 h incubation) could be detected. This preparation was therefore used for further study.

Properties of APAT hydrolase

(1) Substrate specificity

(a) Peptides. MENZIES AND MCQUILLAN[1] studied a wide range of peptides with their partially purified preparation of APAT hydrolase. These peptide substrates were re-examined over the same pH range (4.1, 5.3 and 7.2) with the more highly purified preparation. In agreement with their results, APAT, Gly–L-Phe–L-Phe and L-Trp–L-Leu were the best substrates. Z-L-Phe–L-Tyr was found qualitatively to be a good substrate, apparently comparable with APAT, but as it was relatively insoluble at pH 4–5, it was unsuitable for use in a quantitative assay. No activity was detected using L-Phe–L-Tyr and Z-L-Glu–L-Tyr as substrates, indicating that the very low activity towards these peptides observed in the earlier work may have been due to contaminants.

In view of the possible biological importance of the hydrolysis of peptide bonds adjacent to iodinated amino acids, the action of the enzyme on the iodinated derivative, APADIT, was studied. The enzyme was assayed under the usual conditions except that, owing to the low solubility of APADIT, the final substrate concentration was reduced to 2.5 mM. The enzyme did hydrolyse APADIT (releasing 3,5-diiodotyrosine), but at a much slower rate compared to the hydrolysis of APAT, the activity at pH 4.1 being 7.4% and, at pH 5.3, 10.4% of that towards APAT at pH 4.1.

(b) A and B chains of oxidized insulin. The A and B chains of oxidized insulin were tested as possible substrates for APAT hydrolase at two pH levels, 4.5 and 5.3.

The B chain of oxidized insulin (2 mg) was dissolved in 0.02 M sodium acetate buffer (0.20 ml) at the appropriate pH and the solutions were equilibrated at 37° in a shaker bath. They were both faintly cloudy.

The A chain (2 mg) was dissolved in 0.10 ml of water and 0.10 ml of the appropriate double strength sodium acetate buffer (0.04 M) was added. The solutions were then equilibrated at 37° in a shaker bath, but were also faintly cloudy.

At zero time, 0.2 ml of a solution of APAT hydrolase (30 munits) was added. At zero time, 2 h and 18 h, 0.1 ml was removed and freeze–dried. These freeze–dried samples were dissolved in 12 μl pyridine–water (1:4, v/v) and 5 μl applied to Whatman No. 3 filter paper and examined by paper electrophoresis as described in MATERIALS AND METHODS.

The enzyme was found to have no detectable action on either the A or B chain of oxidized insulin.

(2) Inhibition

The enzyme was not inhibited by pre-incubation with thyroxine for 30 min at pH 4.1 at concentrations up to 10^{-4} M, but 40% inhibition was observed with 0.01 M iodide and 100% with 0.1 M iodide under these conditions.

TABLE II

INHIBITION OF APAT HYDROLASE WITH INCREASING pH

All pre-incubation solutions were 0.05 M except for Tris at pH 7.20, the concentration of which was 0.02 M (buffer used in column chromatography). Enzyme concentration during pre-incubation was 0.28 mg/ml (220 munits/ml). Samples (0.05 ml) of the enzyme in the pre-incubation buffer were added to 0.32 ml of 0.5 M sodium acetate buffer (pH 4.1) *plus* APAT to give a final substrate concentration of 5 mM and assayed as in MATERIALS AND METHODS.

Buffer		*Residual activity*		
	pH	*4 h at 37°**	*4 h at 0°**	*9 h at 0°**
Acetate	4.1	(100)	(100)	(100)
Phosphate	6.82	73	100	100
	7.23	39	100	100
	7.77	18	100	100
Tris	7.20	54	100	100
	7.87	42	82	79
	8.25	20	61	44
	8.78	2	34	18

* Indicates conditions of pre-incubation.

Citrate (0.01 M) slightly inhibited the enzyme, the inhibition approaching 40% at 0.1 M citrate.

(a) Effect of pre-incubation at different pH values. Preliminary experiments involving pre-incubation with phosphate buffer above pH 7.0 resulted in a partial inactivation of APAT hydrolase. To test whether this effect was due to the phosphate ion or the pH of the solution, both Tris and phosphate buffers were used and the pH, time and temperature of pre-incubation were varied.

Sodium acetate buffer, pH 4.1 (as in the normal enzyme assay), was used in the control.

The experimental conditions and results are given in Table II. Results indicate that, with both Tris and phosphate buffers, inhibition increases with increasing pH, temperature and time of preincubation, but is greater with phosphate compared to Tris at the corresponding pH.

(b) Inhibition with group-modifying agents. (i) Iodoacetate, PCMB and N-ethylmaleimide: The effect, on the activity of the enzyme, of pre-incubation with these agents which affect predominantly the –SH groups of proteins is shown in Table III. Under the conditions stated, inhibition by iodoacetate and *N*-ethylmaleimide was only slight, but PCMB caused complete inhibition. *(ii) Diisopropylfluorophosphate*: DFP was dissolved in isopropanol to give a concentration of 0.02 M. This DFP solution (0.02 ml) was added to 0.20 ml of enzyme solution (0.13 mg; 280 milli-units) *plus* 0.20 ml of 0.008 M phosphate buffer (pH 6.5) and pre-incubated at 25° or 37°. Control tubes contained no DFP. Samples (0.025 ml) were taken at zero, 30, 120 and 240 min when pre-incubated at 25° and after 30 min when pre-incubated at 37° and assayed as indicated in MATERIALS AND METHODS.

No inhibition of APAT hydrolase was observed under these conditions.

(iii) L-*Tosylamido-2-phenylethyl chloromethyl ketone*: Inhibition by TPCK was

TABLE III

INHIBITION OF APAT HYDROLASE BY IODOACETATE, PCMB AND *N*-ETHYLMALEIMIDE

Pre-incubation solution: enzyme (0.02 mg; 43.5 munits) in 0.3 ml of 0.02 M Tris buffer (pH 7.2) containing the inhibitor at the listed concentrations was pre-incubated for 30 min at 37°. Samples (0.10 ml) of pre-incubation solution were added to 0.27 ml of 0.5 M sodium acetate buffer (pH 4.1) *plus* APAT to give a final substrate concentration of 5 mM and solutions were assayed as in MATERIALS AND METHODS.

Inhibitor	*Concentration of inhibitor (M)**	*Residual activity (%)*
None	—	(100)
Iodoacetate	10^{-3}	100
	10^{-2}	80
PCMB	10^{-5}	0
N-Ethylmaleimide	10^{-3}	90

* Indicates concentration during pre-incubation.

tested at three different pH values (4.1, 6.04 and 7.0) under the experimental conditions shown in Fig. 4. Although inactivation occurred at all three pH levels, this was most rapid at pH 6.04.

Since, in the specificity studies, it was verified that APAT hydrolase was highly active with APAT, L-Gly–L-Phe–L-Phe and L-Trp–L-Leu as substrates, the inhibition of the enzyme by TPCK after pre-incubation at pH 6.04 was compared using each of these three peptides. The activity against each substrate was reduced to zero after 8 h incubation.

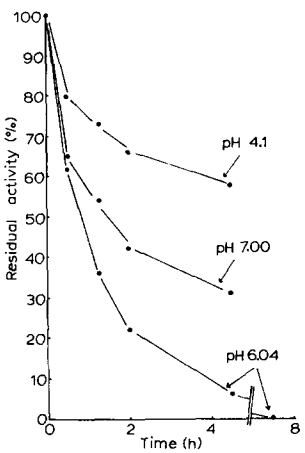

Fig. 4. Influence of pH on the rate of inactivation of APAT hydrolase by TPCK. Experimental conditions: APAT hydrolase (0.12 mg; 260 munits) was pre-incubated at 37° in 0.4 ml of buffer at the required pH, together with 0.01 ml of TPCK (to give a final concentration of $2.5 \cdot 10^{-4}$ M) in methanol. Buffers used: pH 4.1, 0.5 M sodium acetate; pH 6.04, 0.05 M phosphate buffer; pH 7.00, 0.05 M phosphate buffer. Control tubes contained methanol (0.01 ml) without TPCK. Samples (0.025 ml) were taken from the pre-incubation solutions at the times indicated and assayed as in MATERIALS AND METHODS, except that the volume of 0.5 M sodium acetate buffer was increased to 0.345 ml.

TABLE IV

ACTIVITY AND pH OPTIMUM OF APAT HYDROLASE IN THE THYROID GLANDS OF DIFFERENT ANIMAL SPECIES

Enzyme preparation: Fresh thyroid tissue was homogenized in 3 vol. of 0.9% NaCl, pH 6.5 (2 × 30-sec intervals at 12 000 rev./min). An homogenizer of the Potter–Elvehjem type was used for rat and guinea pig thyroids. After extraction overnight at 2° the homogenate was centrifuged at 2000 × *g* for 20 min. The precipitate was washed with 0.1 vol. of 0.9% NaCl, recentrifuged and the combined supernatants adjusted to 1% NaCl with solid NaCl. HCl (2 M) was added slowly to pH 3.55 and the suspension centrifuged at 2000 × *g* for 20 min. The supernatant was dialysed overnight and then assayed for APAT hydrolase activity over the pH range 3.6 to 7.0.

Species	*pH optimum*	*Activity* (munits/g wet wt. tissue)*
Beef	5.2–5.6	240
Pig	4.1	330
Human	4.1	130
Sheep	5.4–5.6	45
Rat	5.5–5.8	920
Guinea pig	4.9–5.4	310

* Refers to activity at optimum pH.

Distribution of APAT hydrolase activity

(1) Animal species

MENZIES[11] found the level of APAT hydrolase activity in extracts of human thyroid glands to be similar to that of pig, but was unable to detect this activity at pH 4.1 in saline extracts of beef and sheep thyroid glands. Since the distribution of this enzyme may be relevant to its physiological importance, studies were extended to include a comparison of activity and pH optimum of this enzyme in extracts of thyroid glands of six animal species. The results are given in Table IV. It was found necessary to include the acid-precipitation step in the preparation of the enzyme extract, otherwise activity was low, particularly for beef and human thyroid glands. The very low activity detected in sheep thyroid extracts was possibly due to the difficulty found in obtaining an extract due to the formation of a gel on centrifuging

TABLE V

ACTIVITY OF APAT HYDROLASE IN RAT TISSUES

Enzyme preparation as in Table IV. For preparation of homogenates of pituitary, adrenal and thyroid glands an homogenizer of the Potter–Elvehjem type was used. pH of assay 5.7.

Tissue	*Activity (munits/g wet wt.)*
Liver	205
Kidney	1600*
Spleen	166
Pituitary	142
Adrenal	148
Thyroid	920

* A slight spot of phenylalanine was detected when the incubation mixture was examined chromatographically.

after acid-precipitation. This was not avoided by variation of pH or salt content, although the lower the pH, the more gel was formed.

(2) Rat tissues

Results obtained with different rat tissues are shown in Table V. Optimum pH was determined for kidney, spleen and liver extracts and was found to be 5.7, in agreement with that for rat thyroid. Chromatographic examination of the incubation tubes revealed a slight phenylalanine spot (in addition to the tyrosine spot) with the kidney extract indicating that this tissue could hydrolyse the acetyl–Phe bond. However, it was estimated that production of phenylalanine would contribute no more than 15% to the total activity detected.

DISCUSSION

Modification of the method of MENZIES AND MCQUILLAN[1] for the purification of APAT hydrolase has resulted in a 1250-fold purification on a protein basis and the removal of contaminating acid protease and cysteinyltyrosine hydrolase. Although two major components were demonstrated with the ultracentrifuge, the activity towards other substrates earlier found to be moderately or highly susceptible—in particular, towards the two other most susceptible substrates, Gly–L-Phe–L-Phe and L-Trp–L-Leu—increased in parallel with the increase in activity towards APAT. In addition, the activity of the preparation towards all three substrates was totally inhibited by TPCK, supporting the assumption that APAT hydrolase is the enzyme responsible for the hydrolysis of these three peptides. Inhibition by TPCK indicated that a histidine residue is present at the active centre[12], while the lack of effect of DFP on the hydrolysis of APAT under the conditions used suggested that a serine residue is not an important component at this site. From the total inhibition of APAT hydrolysis with PCMB it appeared that APAT hydrolase is dependent on an –SH group (or groups) for activity. However, since iodoacetate and *N*-ethylmaleimide were ineffective under our conditions, it is possible that inhibition by PCMB may be due, not to blocking of an essential –SH group, but to steric interference with the approach of substrate (compare SINGER[13]).

The role of APAT hydrolase in thyroid metabolism is not yet definitely established, but we have shown that this enzymic activity is present in the thyroid glands of all species tested although there was considerable variation in its amount and pH optimum. APAT hydrolase activity is not exclusive to the thyroid since it was detected at various levels in extracts of liver, kidney, spleen and pituitary and adrenal glands of the rat. This finding was not unexpected since APAT is also a substrate for pepsin[14]. However, activity in thyroid extracts was exceeded only by that in kidney extracts. Whether this activity in the various tissues is due to the characterized APAT hydrolase is not known, but it is of interest that the pH optimum for this activity in rat kidney, spleen and liver extracts is similar to that in thyroid.

APAT hydrolase has no detectable effect on intact thyroglobulin[11,15], but will hydrolyse peptide fragments obtained by proteolysis of ^{131}I-labelled thyroglobulin by acid protease to release, in particular, the iodotyrosines[15,16]. Our enzyme preparation hydrolysed APADIT, releasing 3,5-diiodotyrosine. It is reasonable to assume that this action is due to APAT hydrolase, particularly as both APAT and APADIT are

also substrates for pepsin[14]. It is of interest that we were unable to detect any action on the A and B chains of insulin even though both of these polypeptides contain peptide bonds which specificity studies indicate are susceptible, in smaller molecules, to hydrolysis by the enzyme.

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