Purification of thyroid protease by acetone fractionation

In previous studies in this Department[1,2,3] of the properties of the proteolytic enzyme of the thyroid gland, preparations of the enzyme were used with an activity of the order of 40 units*/mg. These were obtained by $(NH_4)_2SO_4$ precipitation and subsequent aqueous methanol-NaCl fractionation of hog thyroid extracts. The following method provides substantial improvements in the yield and purity of the enzyme.

Procedure

Hog thyroids (1000 g) freed of superficial fat and connective tissue were blended with 0.9% NaCl (3 l), toluene was added, the suspension allowed to stand for 15 hours at 0° C and then centrifuged. The residue was discarded, a second extraction being avoided in order to reduce the volume of acetone required. The supernatant (Extract I containing approx. $3 \cdot 10^5$ protease units and 2 units/mg protein), after filtration through cotton wool, was stirred and acetone added gradually to 80% (v/v) concentration, the temperature being maintained at −5° C. After filtration at 0° C and washing with cold 80% acetone the precipitate was blended with cold water (2500 ml) in which it mostly dissolved, NaCl (25 g) was added, the extract stirred, and the pH slowly adjusted to 3.5 with 2 *N* HCl. The copious precipitate was removed by centrifugation and discarded; the light brown supernatant was then stirred while acetone was added at 10° to 80% concentration. After several hours in the cold room the precipitate was collected by centrifugation, stirred with cold water (150 ml) and centrifuged; the supernatant was added to that obtained by washing the residue with 20% acetone (40 ml) and recentrifuging. The clear combined supernatants (Extract II), which still contained 55% or more of the protease activity of Extract I, were brought to 80% acetone concentration at 0° C and the precipitate removed by centrifugation and dissolved in cold water (50 ml). The solution was centrifuged to remove a small amount of insoluble material. The supernatant (Extract III) which contained 40% or more of the activity of Extract I (and about 150 units/mg protein) was either dried in the frozen state or immediately fractionated with acetone. This was carried out with stirring at 0° C, the fractional precipitates being dissolved in water, dialysed, and dried in the frozen state. Results in two typical preparations are set out in Table I which also includes details of the acetone fractionation of Extract III. As can be seen from this table about 35% of the total activity of Extract I can be isolated in Fraction III of Extract III with an average activity of 300 units/mg protein compared with the value of the original extract of approx. 2 units/mg.

TABLE I

FRACTIONATION OF HOG THYROIDS FOR PROTEASE ACTIVITY

Prepn. No.	*Wt thyroids (g)*	*Extract I (total units)*	*Extract II (total units)*	*Extract III (total units)*
12	712	236,400	—	131,000
13	1,354	377,000	209,000	145,000

Fractionation of Extract III

Prepn. No.	*Volume of extract (ml)*	*Fraction I (30% acetone) (units)*	*Fraction II (40% acetone) (units)*	*Fraction III (55% acetone) (units)*	*Fraction IV (80% acetone) (units)*
12	38	4,300 (41/mg)	46,000 (112/mg)	78,000 (330/mg)*	2,800 (18/mg)
13	40	2,800 (48/mg)	11,200 (64/mg)	129,000 (275/mg)	1,800 (21/mg)

* After prolonged dialysis the activity of this fraction increased to 424 units/mg.

Properties

The most active fractions were obtained as faintly brown powders readily soluble in water. When submitted to electrophoresis at least three major and one minor components were revealed.

* One unit is the amount of enzyme required to liberate 10^{-4} m-equiv. tyrosine in 30 minutes at 37° (haemoglobin substrate; pH 3.5); compare ANSON[4].

A 1.8% solution of Fraction III (Preparation 12 – Table I) was examined in a Perkin-Elmer Portable Electrophoreter Model 38 at pH 5.9 (0.02 *M* phosphate; 0.15 *M* NaCl), with 90 V and 14 mA applied during 6 hours. At the conclusion of the experiment fractions corresponding to the position of the zones on the photographs were removed, dialysed and assayed for protease activity. The results are given in Table II.

TABLE II

ELECTROPHORESIS OF PURIFIED PROTEASE

(Fraction III, Preparation 12; 424 units/mg)

*Zone**	*1*	*2*	*3*	*4*
Protease units/mg	34	370	480	1,150

* Zone 4 was stationary. Zone 1 migrated furthest towards the anode.

As can be seen from Table II electrophoresis at pH 5.9 does provide a further purification of the enzyme; it can also be assumed from the results that the enzyme has an isoelectric point at about 5.9 in view of the immobility of the most active fraction at the pH used.

Thyroid peptidases

In a previous report[3] from this laboratory thyroid protease preparations were shown to possess peptidase activity at pH 3.2 (within the optimal pH range for protease activity) and to exhibit a specificity similar to that of pepsin. WEISS[5] found that thyroid extracts hydrolysed a range of peptides at pH 7.8 but no tests of this property at acid pH were reported by him. He also studied the intracellular distribution in beef thyroids of protease and peptidase activities at pH 4.0 and 7.8 respectively and found that the peptidases were associated with the submicroscopic particles (mitochondrial supernatant) while the protease appeared in the nuclear and mitochondrial fractions.

A sample of purified thyroid protease prepared as described above (Preparation 13, Extract III, Fraction III (Table I)) was tested for peptidase activity at pH 7.8. The enzyme 0.4 ml (containing 250 protease units in *M*/50 veronal buffer, pH 7.8) was mixed with 0.2 ml of an *M*/20 solution of glycyl-L-leucine in buffer and 0.4 ml buffer added. The solution was incubated at 37° C for 1 hour together with controls: (a) no peptide, (b) no enzyme. Chromatographic examination on paper (solvent system: propanol-water; 80:20) showed that approximately 25% hydrolysis of the peptide had been effected. However, a 0.4 ml sample of a dialysed saline extract of fresh hog thyroids equivalent to 10 protease units brought about complete hydrolysis of the peptide under comparable conditions. Two other acetone fractions (I and IV) of Extract III (Preparation 13) were also assayed on the peptide and very low or no activity was found. Consequently it seems that the purification procedure reported above preferentially concentrates the protease at the expense of the peptidase (active at pH 7.8). It is perhaps possible that peptidase activators were removed in the process; however, in one experiment, the addition of Mn^{2+} (WEISS[5]) as 0.004 *M* $MnSO_4$ was not found to enhance the slight peptidase activity of the purified protease.

TABLE III

CELLULAR DISTRIBUTION OF PEPTIDASE (pH 7.8) AND PROTEASE (pH 3.5) ACTIVITIES IN HOMOGENATES OF HOG THYROIDS (200 g)

Fraction	*Volume (ml) of extract or eluate (0.15 M KCl)*	*Protease units*	*Peptidase activity**
I Mitochondria and nuclei combined	200	8,100	About 1/5 hydrolysis
II Supernatant from I	610	13,800	Complete hydrolysis
IIa Microsomes	36	2,800	Complete hydrolysis
IIb Microsomal supernatant	370	5,500	Complete hydrolysis

* 1 ml of dialysed extract or eluate mixed with 1 ml buffer (*M*/50 veronal) and adjusted to pH 7.8 with NaOH; 0.4 ml incubated with 0.1 ml glycyl-L-leucine (*M*/20) for 60 minutes at 37° C and the digests then examined chromatographically (propanol-water; 80:20).

In view of WEISS's claims[5] as to the intracellular distribution of protease and peptidase his procedure[5,6] was repeated, using hog thyroids, to ascertain whether the particulate fractions provided better starting materials for the purification of the enzymes. In addition, the microsomal fraction was sedimented from the nuclear and mitochondrial supernatant by centrifuging for 1 hour at 60,000 *g* (mid point of cup). The results of the enzyme assays are given in Table III.

These results confirm essentially the findings of WEISS as regards the distribution of peptidase; in the case of the protease activity, however, this was present to a greater extent in the supernatant from the combined nuclear and mitochondrial fraction. It should be pointed out that such results may bear no relationship to the enzymic distribution *in situ*; the implication may be that during the blending and centrifuging of the cellular material the two enzymes have become selectively adsorbed on the large aggregate surfaces of the particles from which they can then be eluted. No explanation can be offered for the discrepancy between the protease activity value of Fraction II and the combined values of Fractions IIa and IIb (Table III).

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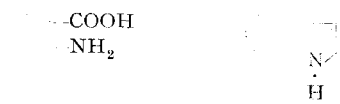
The participation of ribose derivatives in the conversion of anthranilic acid to indole by extracts of *Escherichia coli**

Although the mechanism of tryptophan formation from indole is understood in both *Escherichia coli*[1] and *Neurospora crassa*[2–5], comparatively little is known about the conversion of anthranilic acid to indole in these organisms. The available evidence indicates that the amino nitrogen of anthranilic acid is retained during indole formation (shown with *Neurospora*[6] while the carboxyl carbon is lost (in both *E. coli*[7] and *Neurospora*[8]). Loss of the carboxyl carbon of anthranilic acid necessitates the addition of two carbon atoms to anthranilic acid to form indole. The present experiments with extracts of a mutant strain of *E. coli* demonstrate that these two carbon atoms may be derived from ribose or ribose derivatives.



anthranilic acid indole

Extracts were prepared from a tryptophan auxotroph of the K-12 strain of *E. coli*. The tryptophan requirement of this mutant is also satisfied by anthranilic acid or indole. For the experiments reported here, this strain was grown on a minimal medium[9] supplemented with anthranilic acid. The cells were collected by centrifugation, washed, and subjected to sonic oscillation. The sonic extracts were centrifuged for 20 minutes at 60,000 × *g* in a Spinco ultracentrifuge. The clear supernatant solutions were dialyzed against 0.02 *M* phosphate buffer at pH 7.8 before use.

Dialyzed extracts of the mutant, as can be seen in Table I, readily convert anthranilic acid to indole in the presence of the proper supplements. Both ribose and ribose-5-phosphate (R-5-P) serve as excellent sources of the two carbon atoms needed for indole synthesis from anthranilic acid[10]. However, neither compound is effective in the absence of ATP. The results of a preincubation experiment with potato apyrase[11] (Table II) also indicate that ATP is required for indole synthesis when R-5-P is employed as carbon donor. ATP apparently is not required when 5-phosphoriboxyl-

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