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# THYROID ACID PROTEINASE

## PROPERTIES AND INACTIVATION BY DIAZOACETYL-NORLEUCINE METHYL ESTER

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SUMMARY

A method is described for the purification of thyroid acid proteinase from extracts of pig thyroid glands. Weight-average molecular weight determinations are used to show that the enzyme undergoes a pH- and temperature-dependent dimerization reaction in 0.10 ionic strength buffers, involving monomers of molecular weight 21 000. The extent of dimerization is maximal at the isoelectric point of the enzyme (pH 7.5) and decreases as the net charge borne by the protein increases. Also, at pH 7.5 an increase of temperature from 1–25° favours dimer formation. The amino acid composition of the proteinase is reported, and it is shown that glycine is the only major component evident in N-terminal analysis. The enzyme is active against haemoglobin as substrate (37° and pH 3.6) and is inhibited to the extent of 90–100% by the pepsin inhibitor, diazoacetyl-DL-norleucine methyl ester. As with pepsin, only one dicarboxylic acid residue appears to be involved in reaction with the inhibitor. Studies with radioactively labelled thyroglobulin (alone and in thyroid extracts) as substrate indicate that the inhibitor is partially effective in preventing proteolysis by acid proteinase of its natural substrate.

## INTRODUCTION

A system of one or more peptide-bond hydrolases is clearly involved in the thyroid gland in the release of the iodinated amino acids from their bound form, thyroglobulin[1]. In particular, thyroid acid proteinase appears to be implicated in the initial cleavage of the protein while peptidases may act synergistically in furthering the release of iodinated tyrosines from intermediates[1–4].

In the present communication the purification of thyroid acid proteinase, its properties and its inactivation by the pepsin inhibitor, diazoacetyl-norleucine methyl ester[5] are described. While these investigations were in progress a paper by KRESS,

Abbreviation: Z-, benzyloxycarbonyl.

PEANASKY AND KLITGAARD[6] appeared on the purification of this enzyme with an account of some of its properties. In certain particulars our findings are at variance with those of the authors mentioned.

## MATERIALS AND METHODS

All chemicals were of analytical grade. Solvents were distilled before use. DEAE-cellulose was from Serva (capacity 0.84; batch No. 26063). Diazoacetyl-DL-norleucine methyl ester was a gift from Dr. T. A. A. DOPHEIDE. *N*-Acetyl-L-phenylalanyl-L-tyrosine and L-cysteinyl-L-tyrosine were prepared by published methods. *N*-Acetyl-L-phenylalanyl ethyl ester, L-phenylalanyl-L-phenylalanylamide, Z–Gly–L-leucine and Z-glycylamide were from Mann Biochem. *p*-Bromophenacyl bromide (from British Drug Houses), β-phenylpropionate (Merck), *N*-tosyl-L-phenylalanyl-chloromethyl ketone (Cyclo Chem. Corp.) and *N*-tosyl-L-lysyl-chloromethyl ketone (Calbiochem.) were supplied as indicated. [$^{35}$S]Phenylisothiocyanate (Radiochemical Centre, Amersham; 287 μC/μmole) was from a sample kindly supplied by Professor A. DOYLE.

### *Enzyme assays*

*N*-Acetyl-L-phenylalanyl-L-tyrosine hydrolase, L-cysteinyl-L-tyrosine hydrolase and acid proteinase activities were determined as described previously[7], a unit of proteinase activity being defined as the amount of enzyme required to liberate the equivalent of 0.1 μmole of tyrosine per min (haemoglobin substrate; 37°, pH 3.6).

### *Physical methods*

Sedimentation velocity experiments were 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, specific values being reported in the text. Each sample was dialysed in the cold for 24 h against the appropriate buffer and approx. 1 h was allowed for temperature equilibration before sedimentation was commenced. The weight-average sedimentation coefficients, $\bar{s}$, were calculated from the rate of movement of the square root of the second moment of the entire Schlieren pattern[8]. Total concentrations were determined refractometrically using the area under the peak, corrected for radial dilution[9] and by assuming that the specific refractive increment of the protein[10] was $1.8 \cdot 10^{-3}$ dl·g$^{-1}$.

The Archibald method as outlined by KLAINER AND KEGELES[11] was used to evaluate weight-average molecular weights from experiments employing protein concentrations $\geqslant 0.6\%$. Only values for the observed refractive index gradient at the meniscus, $(dn/dx)_m$, were employed to calculate the required ratio, $(dn/dx)_m/x_m c_m \omega^2$ (where $x_m$ is the distance from the centre of rotation, $c_m$ is the concentration of solute at the meniscus in appropriate refractometric units and $\omega$ is the angular velocity).

Two equilibrium ultracentrifugation experiments, employing Rayleigh interference optics, were conducted by the meniscus depletion method described by YPHANTIS[12]. In each experiment a standard 12-mm-thick, filled-epoxy double-sector centrepiece was employed: Kel-F polymer oil was used as the inert base fluid and equal weights of solution (protein concn. 0.1%) and solvent were employed to give 3-mm columns in each channel. The runs were performed at 25° and 1°, the selected

angular velocities being 35 600 rev./min and 37 020 rev./min, respectively. After 9 h the distributions of concentration in each run proved to be independent of time. The method of measuring the experimental records has been outlined in detail previously[12,13]: in this work, a Nikon Shadowgraph (Model 6C) fitted with a projection screen and accurate to $2 \cdot 10^{-4}$ cm was used. The apparent weight-average molecular weights reported in the text were calculated from the slope of the tangent to the curve, obtained by plotting ln $c$ *vs.* $x^2/2$, at a point corresponding to a total protein concentration $c$ (where $c$ is the total protein concentration in terms of fringe displacement at a position $x$ in the equilibrium distribution from the centre of rotation). Points corresponding to net fringe displacements of less than 100 $\mu$ are subject to considerable error (*cf.* Fig. 10 of YPHANTIS[12]) and were not employed in molecular weight calculations. A lower limit of 0.01% for $c$ is thereby imposed.

Polyacrylamide gel electrophoresis was performed by the method described by CARNEGIE, LAMOUREUX AND BENCINA[14], the gel matrix being formed from a solution containing acrylamide (10%) and *N,N'*-methylenebisacrylamide (0.3%). The duration of all experiments was 3 h (with 20 V/cm and 25–30 mA).

## RESULTS

### *Purification of pig thyroid acid proteinase*

The method, like that of KRESS *et al.*[6], is based on the observations of LAVER AND TRIKOJUS[15,16] and MCQUILLAN, MATHEWS AND TRIKOJUS[17] that an acid-precipitation step removed the major thyroid protein, thyroglobulin, from extracts containing the proteinase, while subsequent acetone fractionation substantially raised the purification factor. MENZIES[2] added a final chromatographic step with DEAE-cellulose to give a product containing 53–60 units/mg protein. The first 3 steps in our present procedure are similar to those of KRESS *et al.*[6] and are therefore not presented. However, before lyophilizing at the end of Step 3, dialysis against distilled water was continued until the dialysate was free of $Cl^-$.

Step 4: The lyophilized product from Step 3 was dissolved in 0.2% saline (pH 7.0) to give a 1% protein solution which was cooled in ice salt during the slow addition, with stirring, of acetone (chilled to $-10°$) to 30%. After 1–1.5 h at $-10°$ the suspension was centrifuged at $-10°$ (Fraction 1). The process was continued with the supernatant to give Fraction 2 (30–40% acetone) and Fraction 3 (40–55% acetone). All fractions were dialysed until free from chloride, assayed and lyophilized. Fraction 3, which contained about 75% of the proteinase units before fractionation, usually assayed at 33 units/mg protein or above. Before fractionating the main bulk of the aqueous solution a test sample was first fractionated, since on one occasion, the active fraction (3) was located in the 45–60% acetone range.

Step 5: Combined Fractions 3 (about 200 mg) were applied to a column of DEAE-cellulose (40 cm × 1.5 cm) equilibrated at 2° with 0.02 M Tris–HCl (pH 7.2) and elution continued with the same buffer. The flow rate of the column was restricted to 12 ml/h and 1.2-ml fractions were collected: an elution profile obtained by rerunning the most active fractions in the proteinase peak is shown in Fig. 1, in which both $A_{280\ m\mu}$ values and enzymic activity are reported. The most active fractions (No. 33–40 in Fig. 1) were pooled, dialysed and lyophilized. The specific activity of this material usually dropped from 133–173 units/mg of protein to 117–133 units/mg

on lyophilization. Such preparations were devoid of *N*-acetyl-L-phenylalanyl-L-tyrosine hydrolase, L-cysteinyl-L-tyrosine hydrolase or esterase (substrate:acetyl-L-tyrosine ethyl ester) activities. Unlike KRESS *et al.*[6] we found it unnecessary to apply further column chromatography with CM-cellulose followed by Sephadex G-75. The failure of these authors to obtain adequate resolution with DEAE-cellulose probably stems mainly from their use of low activity material (*i.e.*, that obtained at Step 3), other factors being a higher flow rate and the larger fractions collected.

*Physical properties*

The apparent weight-average molecular weights, $(M_w)_{app}$, found with the approach to equilibrium and equilibrium ultracentrifugations are, together with the experimental conditions, summarized in Table I. At pH 3.7, values of the ratio

TABLE I

WEIGHT-AVERAGE MOLECULAR WEIGHT OF ACID PROTEINASE IN 0.10 IONIC STRENGTH UNIUNIVALENT BUFFERS

| *pH* | *Concn. (g/100 ml)* | $(M_w)_{app}$ | |
|---|---|---|---|
| | | *25°* | *1°* |
| 3.7 | 1.19 | 21 000 | 21 000 |
| 7.5 | 1.61 | 42 000 | 37 000 |
| 7.5 | 1.19 | 40 000 | 33 000 |
| 7.5 | 0.60 | 39 000 | |
| 7.5 | 0.05 | 38 000 | 32 000 |
| 7.5 | 0.01 | 36 000 | 30 000 |

$(dn/dx)_m/x_m c_m \omega^2$ found in Archibald experiments at 25° and 1° proved to be independent of time, suggesting that the material was homogeneous. Moreover, two further evaluations of the molecular weight under these conditions at daily intervals yielded values of 21 000 ± 1000, showing that no detectable autolysis had occurred. If the species existing at pH 3.7 is termed monomer, it appears that an increase of the pH to a value of 7.5 favours dimer formation. Two observations from Table I support the hypothesis.

Firstly, at pH 7.5 and 25° the weight-average molecular weight decreases with the concentration of protein. Under these conditions, the lowest value of 36 000 for $(M_w)_{app}$ reported in Table I was determined by the procedure of YPHANTIS[12]: it corresponds to a total proteinase concentration of 0.01%, the lower limit for accurate molecular weight determination by this method. In this experiment, the plot of ln $c$ *vs.* $x^2/2$ exhibited upward curvature, such that the slopes of tangents to the curve (and hence $(M_w)_{app}$ values) increased with increasing protein concentration. The value of $(M_w)_{app}$ obtained at $c = 0.05\%$ (the upper limit set by optical resolution of the observed fringe pattern) was 38 000. The data describing the concentration dependence of weight-average molecular weights obtained at pH 7.5 and 25° were used in Eqn. 7 of NICHOL, BETHUNE, KEGELES AND HESS[18] to give an apparent association equilibrium constant of 60 ± 10 l/g, characterizing the reaction between monomer (21 000) and dimer (42 000).

Secondly, at pH 7.5 a decrease in temperature to 1° resulted in a decrease in the weight-average molecular weights at each concentration studied. Again, the plot of ln $c$ *vs.* $x^2/2$ found in the YPHANTIS[12] experiment exhibited an upward curvature and yielded $(M_w)_{app}$ values (within observable limits) of 30 000 and 32 000 at protein concentrations of 0.01% and 0.05%, respectively. The data found at 1° are approximately fitted by an apparent dimerization constant of $5 \pm 4$ l/g. The association reaction to form dimer is evidently characterized by a positive enthalpy change, even though the apparent values of the equilibrium constants are of insufficient accuracy to provide a reliable estimate of the magnitude of the change.

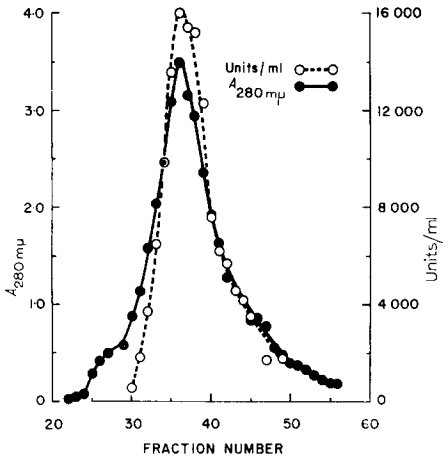
Since the extent of association of the proteinase was shown to depend on pH (Table I), it was of interest to examine both the electrophoretic mobility and weight-average sedimentation coefficient as a function of pH. Firstly, a series of polyacrylamide gel electrophoresis experiments was conducted, in which the sample was introduced at the origin in the same buffer used to equilibrate the gel. The compositions of the buffers are given in Table II, together with the migration results found upon application of the electric potential gradient for 3 h. In each experiment single bands were observed, and from their migration behaviour (Table II) it may be concluded that the isoelectric point of the proteinase is approximately 7.5 in the

TABLE II

THE DEPENDENCE ON pH OF ELECTROPHORETIC MOBILITIES AND WEIGHT-AVERAGE SEDIMENTATION COEFFICIENTS OF ACID PROTEINASE IN 0.10 IONIC STRENGTH BUFFERS

| *Buffer composition* | *Concn. (M)* | *pH* | *Distance of migration* (cm)* | *s (S)* |
|---|---|---|---|---|
| NaCl | 0.08 | | | |
| Sodium acetate | 0.02 | 3.7 | — | 2.9 |
| Acetic acid | 0.18 | | | |
| NaCl | 0.03 | | | |
| Sodium acetate | 0.07 | 5.0 | 2.0 | 3.2 |
| Acetic acid | 0.03 | | | |
| NaCl | 0.08 | | | |
| Sodium cacodylate | 0.02 | 6.0 | 1.5 | 3.4 |
| Cacodylic acid | 0.02 | | | |
| NaCl | 0.05 | | | |
| Sodium cacodylate | 0.05 | 6.7 | 0.7 | — |
| Cacodylic acid | 0.01 | | | |
| NaCl | 0.09 | | | |
| Sodium diethyl barbiturate | 0.01 | 7.5 | 0 | 3.8 |
| Diethylbarbituric acid | 0.02 | | | |
| NaCl | 0.08 | | | |
| Sodium diethyl barbiturate | 0.02 | 8.0 | −0.3 | 3.4 |
| Diethylbarbituric acid | 0.01 | | | |
| Tris–HCl | 0.1 | 8.7 | 0.8 | — |

* In each electrophoresis experiment the potential gradient was applied for 3 h. Migration toward the anode is denoted as negative.



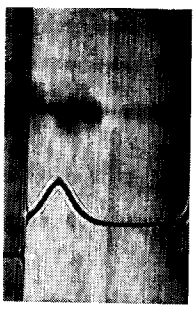



Fig. 1. Chromatography of partially purified pig thyroid acid proteinase on DEAE-cellulose: the column (40 cm × 1.5 cm) was equilibrated with Tris–HCl buffer (0.02 M, pH 7.2) and the sample was eluted at a flow rate of 12 ml/h with the same buffer and collected in 1.2-ml fractions. Enzyme activity on the ordinate scale is × 30 that defined in the experimental section.

Fig. 2. Sedimentation velocity pattern of pig thyroid acid proteinase (1%) in diethylbarbiturate buffer, pH 7.5 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 70°, 1 h after start. Sedimentation is from left to right.

specified environment. Secondly, sedimentation velocity analyses were performed at 20°: a typical result obtained with a 1% proteinase solution in diethylbarbiturate buffer of pH 7.5 and ionic strength 0.10 is shown in Fig. 2. In a series of experiments employing 0.5% proteinase solutions, weight-average sedimentation coefficients, $\bar{s}$, referring to the specified environment and 20°, were evaluated from the single peaks

TABLE III

AMINO ACID COMPOSITION OF PIG THYROID ACID PROTEINASE

The specific activity of the enzyme employed was 170 units/mg protein after chromatography on DEAE-cellulose and 157 units/mg protein after lyophilization. Values for the number of residues are based on the results of two separate analyses (22 h hydrolysis time) with corrections applied for tyrosine, threonine and serine (results of duplicates in parentheses). Glucosamine was separately determined and corrected for losses by extrapolation to zero time.

| *Residue* | *Residues per monomer unit (mol. wt. 21 000)* | *Residue* | *Residues per monomer unit (mol. wt. 21 000)* |
|---|---|---|---|
| Lysine | 11 (10.5, 10.6) | Alanine | 10 (10.0, 10.0) |
| Histidine | 3 (3.1, 3.2) | Half-cystine | 4 (4.1, 3.9) |
| Ammonia | 19 (18.5, 19.8) | Valine | 15 (15.1, 15.1) |
| Arginine | 5 (4.7, 4.5) | Methionine | 4 (4.0, 3.9) |
| Aspartic acid | 17 (16.8, 16.9) | Isoleucine | 11 (11.3, 11.3) |
| Threonine | 12 (12.1, 12.1) | Leucine | 15 (15.2, 15.5) |
| Serine | 14 (13.8, 13.7) | Tyrosine | 9 (9.2, 9.1) |
| Glutamic acid | 16 (15.8, 15.8) | Phenylalanine | 7 (6.5, 6.7) |
| Proline | 11 (11.3, 10.7) | Tryptophan | 3 (3.3, 3.3) |
| Glycine | 21 (21.3, 21.3) | Glucosamine | 2 (1.8, 1.8) |

observed in Schlieren patterns and the results are shown in Table II. It is apparent that $\bar{s}$ values tend to decrease systematically as the pH is either raised or lowered from the value of 7.5.

*Amino acid analysis*

This was carried out in duplicate on a Spinco Model 120B amino acid analyser, the results of the analyses being in close agreement. The mean result, expressed as the number of residues per monomer of molecular weight 21 000 (Table I), is given in Table III. Tryptophan was estimated separately by the spectrophotometric method of EDELHOCH[19]. Glucosamine was also shown to be present: the possibility of the presence of other sugars has not yet been investigated. It may be noted that the amino acid composition of acid proteinase (Table III) differs markedly from that of pepsin[20].

An apparent specific volume of 0.73 for acid proteinase was calculated from the data in Table III, together with the known specific volumes of the residues. This value was used in the determination of molecular weights reported in Table I.

*N-Terminal amino acids*

The enzyme employed possessed a specific activity of 160 units/mg protein after DEAE-cellulose column chromatography, but the activity decreased to 117 units/mg protein following lyophilization. A sample of lyophilized material (1.9 mg) was treated by the EDMAN method[21] with 2.03 mg of phenylisothiocyanate containing 0.16 $\mu$C of the $^{35}$S-labelled derivative. The phenylthiohydantoins were run in solvent systems D, E and F and the papers scanned in a Nuclear Chicago Actigraph II instrument. Qualitative assessment of the results of several experiments consistently showed glycine as the only major component although there were traces of other amino acids.

*Inhibition studies*

Previous findings[6,22] indicated that bivalent metals, diisopropylphosphorofluoridate (DFP), *p*-chloromercuribenzoate and methylmercuriiodide were without effect. Furthermore, using haemoglobin as substrate, we have shown the following compounds also to be non-inhibitory: *N*-tosyl-L-phenylalanyl-chloromethyl ketone, *N*-tosyl-L-lysyl-chloromethyl ketone, iodine[23], $\beta$-phenylpropionic acid[24], the pepsin inhibitor, *p*-bromophenylacylbromide (using both the conditions of ERLANGER *et al.*[25] and GROSS AND MORELL[26]) and the peptides, *N*-acetyl-L-phenylalanyl-L-tyrosine, L-phenylalanyl-L-phenylalanylamide, *N*-acetyl-L-phenylalanylethyl ester, Z-glycyl-L-leucine and Z-glycylamide. However, inhibition to the extent of 90–100% was obtained using the pepsin inhibitor diazoacetyl-DL-norleucine methyl ester[5]. The enzyme sample used in the inhibition experiments had an activity of 117 units/mg protein. The composition of the inhibitor solutions was similar to that described by RAJAGOPALAN *et al.*[5] for pepsin, proportions being adjusted in accord with the differences in molecular weights of the two proteins (proteinase monomer (21 000) and pepsin (32 700)). Haemoglobin was used as substrate. $Cu^{2+}$ was found to be essential. However, diazoacetyl-DL-norleucine methyl ester does not appear to have as high an affinity for the acid proteinase as for pepsin[5] which is inactivated within 10 min at 14° and pH 5. Under these conditions the thyroid enzyme was only 50% inhibited after 2 h. At 20° the

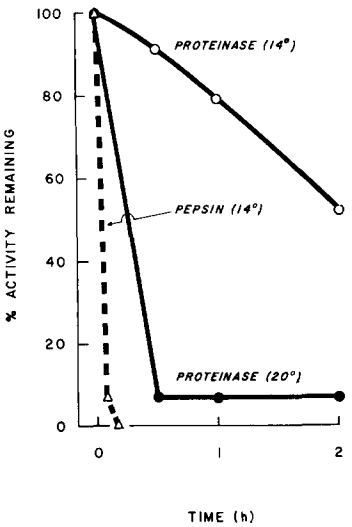



Fig. 3. The effect of time of incubation and temperature on the activity of pig thyroid acid proteinase incubated with the inhibitor, diazoacetyl-DL-norleucine methyl ester .After incubation with the inhibitor, the proteinase was assayed at 37° and pH 3.6, employing haemoglobin as substrate. The broken line, included for comparison, pertains to the studies of RAJAGOPALAN, STEIN AND MOORE[5] with the inhibitor and pepsin.

reaction was complete (90–100%) within 30 min (Fig. 3). The rate was also markedly pH dependent, being substantially decreased at pH 3.6 and 6.0. The enzyme–inhibitor complex was stable towards water and acetate buffer (pH 3.7), since no reactivation occurred after dialysis for 24 h at 0°.

*Amino acid analysis of inhibited enzyme*

A sample of enzyme (70 units/mg protein) was assayed before and after inhibition, the inhibited enzyme being prepared for analysis by passage over a column of Sephadex G-25 as for inhibited pepsin[5]. The value of approximately 0.39 μmole/100 μmoles for norleucine was found which corresponds to about 0.8 mole per monomer of enzyme of molecular weight 21 000. Thus, as with pepsin, only one dicarboxylic acid residue appears to be involved in reaction with the inhibitor.

*Enzyme and inhibitor with labelled thyroglobulin substrate*

A young female pig was injected with $^{131}I$ (2 mC) and the thyroid removed *post mortem* 48 h later. The labelled thyroglobulin was purified by the method of UI AND TARUTANI[27] with minor modifications. A sample of enzyme was inactivated as follows: 0.25 ml enzyme solution (43 units/ml) was mixed with 0.02 ml cupric acetate (8.9 mM) and 0.05 ml of 0.2 M ammonium acetate, pH 5.0. After equilibrating at 20° for 10 min, 0.025 ml of the inhibitor in methanol (8.69 mM) was added and the solution incubated for 1 h at 20°. It was then lyophilized and redissolved in 0.3 ml of 0.04 M ammonium acetate, pH 3.6. A control enzyme solution to which no inhibitor had been added was treated similarly (Control I). Samples (0.1 ml) of each solution were incubated in duplicate for 16 h at 37° with 0.1 ml of the purified labelled thyroglobulin (2 mg). A sample of thyroglobulin alone was also incubated at the same time

TABLE IV

AREAS OF RADIOACTIVITY PEAKS CORRESPONDING TO IODINATED AMINO ACIDS RELEASED FROM [$^{131}$I]THYROGLOBULIN BY INHIBITED AND NON-INHIBITED THYROID ACID PROTEINASE
16 h, pH 3.6, 37°. Duplicate values in parentheses.

| *Incubation solution* | *Area (cm²)* | | |
|---|---|---|---|
| | *Monoiodotyrosine* | *Diiodotyrosine* | *Thyroxine (+ triiodothyronine* |
| [$^{131}$I]Thyroglobulin + enzyme (inhibited) | 7.0 (6.8; 7.3) | 9.1 (10.3; 8.9) | 4.2 (4.4; 4.0) |
| [$^{131}$I]Thyroglobulin + enzyme (no inhibitor) | 14.9 (15.1; 14.7) | 28.4 (27.8; 29.0) | 12.3 (11.4; 13.2) |
| [$^{131}$I]Thyroglobulin alone | 6.4 (6.6; 6.2) | 7.7 (8.5; 7.0) | 4.6 (4.5; 4.8) |

(Control 2). Chromatograms of the three solutions were prepared using the solvent system: *n*-propanol–propionic acid–water (20:3:15, v/v) and Whatman 3 MM paper and scanned in a Nuclear Chicago Actigraph II instrument. Table IV gives the areas under the peaks corresponding to the iodinated amino acids as measured by a planimeter. The purified thyroglobulin sample when incubated alone showed, unusually, the presence of free iodinated amino acids which had presumably escaped the purification procedure. However, the values are close to those for the inhibited enzyme and in marked contrast to those for the enzyme without added inhibitor (Control 1).

*Effect of inhibitor on the autolysis of thyroid extracts*

A pig thyroid, labelled *in vivo* as previously described, was sliced and stirred for 1.5 h in 3 vol. of 0.14 M NaCl, pH 6.5. After centrifuging, 0.5 ml of the supernatant (in duplicate) was mixed with 0.05 ml buffer (pH 5.0) and 0.05 ml cupric acetate (5.9 mM) and, after equilibration for 10 min at 20°, 0.05 ml inhibitor (7.16 mM) was added and incubation continued at 20° for 1 h. Duplicate samples treated similarly but lacking inhibitor served as controls. Both solutions were lyophilized, 0.05 ml buffer (pH 3.6) was added to each residue and incubation then carried out for 16 h at 37°. After centrifuging, 0.01-ml samples were chromatographed (*n*-propanol–propionic acid–water), the papers scanned and the areas measured as described previously. The results are given in Table V. The thyroxine areas could not be accurately compared but it appears that the inhibitor is at least partly effective in crude thyroid extracts as judged by the iodotyrosine values.

TABLE V

EFFECT OF INHIBITOR ON THE AUTOLYSIS OF THYROID EXTRACTS .AREAS OF IODOTYROSINE PEAKS RELEASED IN THE PRESENCE AND ABSENCE OF INHIBITOR
16 h, pH 3.6, 37°. Duplicate values in parentheses.

| *Incubation solution* | *Area (cm²)* | |
|---|---|---|
| | *Monoiodotyrosine* | *Diiodotyrosine* |
| Extract + inhibitor | 9.7 (10.0; 9.5) | 9.1 (9.6; 8.7) |
| Extract control | 19.0 (18.2; 19.9) | 15.6 (15.0; 16.2) |

## DISCUSSION

The basic finding which emerges from the sedimentation studies is that pig thyroid acid proteinase undergoes a pH- and temperature-dependent dimerization reaction, involving monomers of molecular weight 21 000 (Table I). It is clear from Tables I and II that the maximum values of the weight-average quantities, $(M_w)_{app}$ and $\bar{s}$, were observed at pH 7.5 in 0.10 ionic strength diethylbarbiturate buffer: in this environment, the protein was shown by polyacrylamide gel electrophoresis to bear no net charge. Also under these conditions the relative proportion of monomer was favoured not only by decreasing the protein concentration but also by decreasing the temperature (Table I). Similar temperature effects have been observed with other protein systems where hydrophobic interactions were implicated in polymer formation[28–30]. The systematic decrease of $\bar{s}$ values observed on decreasing the pH below the isoelectric point, pH 7.5 (Table II), suggests that hydrophobic bonding between monomer units becomes increasingly more difficult as the net positive charge borne by the proteinase increases, until at pH 3.7 essentially only monomer exists (Table I).

KRESS *et al.*[6] have shown that pig thyroid acid proteinase in buffer of pH 5.5 and ionic strength approximately 0.18 when subjected to sedimentation velocity reveals a major peak, asymmetric and characterized by a sedimentation coefficient, measured from the maximum ordinate, of 3.2 S. The value is in general agreement with the weight-average sedimentation coefficients reported in Table II, although the environmental conditions differed. However, the weight-average molecular weight of 47 600–52 000 reported by KRESS *et al.*[6] at pH 5.5 and at an unspecified temperature is greater than any value observed in this study (Table I). Since the extent of hydrophobic bond formation is favoured by an increase in ionic strength[31], the possibility exists that in the former study, conducted in only one environment at a relatively high ionic strength, small amounts of polymers larger than the dimer (42 000) existed, even though the results of the previous workers were interpreted on the basis of the behaviour of a single non-associating solute.

The sedimentation velocity pattern shown in Fig. 2 is regarded in the light of the above evidence as a reaction boundary[32,33] involving refractive index gradients of both monomer and dimer in equilibrium. Similar spread, asymmetric and unimodal reaction boundaries have been observed with other rapidly polymerizing protein systems[29,34–36] and have been predicted theoretically for monomer–dimer systems[32,33]. There exists, however, evidence that the present method of preparation leads essentially to a single solute, albeit capable of self-association. Firstly, sedimentation velocity analysis revealed no material with a sedimentation coefficient markedly different from that of the major component and, at pH 3.7 where monomer is favoured, the ratio $(dn/dx)_m/x_m c_m \omega^2$ found in Archibald experiments was independent of time. Secondly, end-group analysis revealed only trace amounts of N-terminal residues other than glycine. Thirdly, migration in polyacrylamide gel electrophoresis revealed essentially only single reaction zones.

Although the amino acid analyses and physical properties of pepsin[20,37] and thyroid acid proteinase show that the two enzymes are entirely different proteins, it is of interest that both are inhibited by diazoacetyl-norleucine methyl ester. Moreover, it has been found (unpublished data) that adrenal acid proteinase[38] is also inactivated by the same compound and therefore it is a question of interest whether other tissue cathepsins would react similarly.

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REFERENCES

1 M. T. McQUILLAN AND V. M. TRIKOJUS, in A. GOTTSCHALK, *Glycoproteins, Their Composition, Structure and Function*, Elsevier, Amsterdam, 1966, p. 516.
2 C. A. MENZIES, M. Sc. Thesis, University of Melbourne, 1963.
3 T. A. A. DOPHEIDE, Ph. D. Thesis, University of Melbourne, 1965.
4 C. A. MENZIES, T. A. A. DOPHEIDE, M. T. McQUILLAN AND V. M. TRIKOJUS, *Biochim. Biophys. Acta*, in the press.
5 T. G. RAJAGOPALAN, W. H. STEIN AND S. MOORE, *J. Biol. Chem.*, 241 (1966) 4295.
6 L. F. KRESS, R. J. PEANASKY AND H. M. KLITGAARD, *Biochim. Biophys. Acta*, 113 (1966) 375.
7 C. A. MENZIES AND M. T. McQUILLAN, *Biochim. Biophys. Acta*, 132 (1967) 444.
8 R. J. GOLDBERG, *J. Phys. Chem.*, 57 (1953) 194.
9 T. SVEDBERG AND K. O. PEDERSEN, *The Ultracentrifuge*, Oxford Univ. Press, London and New York (Johnson Reprint Corp., New York,) 1940.
10 G. E. PERLMANN AND L. G. LONGSWORTH, *J. Am. Chem. Soc.*, 70 (1948) 2719.
11 S. M. KLAINER AND G. KEGELES, *J. Phys. Chem.*, 59 (1955) 952.
12 D. A. YPHANTIS, *Biochemistry*, 3 (1964) 297.
13 L. W. NICHOL and A. B. ROY, *Biochemistry*, 4 (1965) 386.
14 P. R. CARNEGIE, G. LAMOUREUX AND B. BENCINA, *Nature*, 214 (1967) 407.
15 W. G. LAVER AND V. M. TRIKOJUS, *Biochim. Biophys. Acta*, 16 (1955) 592.
16 W. G. LAVER AND V. M. TRIKOJUS, *Biochim. Biophys. Acta*, 20 (1956) 444.
17 M. T. McQUILLAN, J. D. MATHEWS AND V. M. TRIKOJUS, *Nature*, 192 (1961) 333.
18 L. W. NICHOL, J. L. BETHUNE, G. KEGELES AND E. L. HESS, in H. NEURATH, *The Proteins*, 2nd ed., Vol. 2, Academic Press, New York, 1964, p. 305.
19 H. EDELHOCH, *Biochemistry*, 6 (1967) 1948.
20 T. G. RAJAGOPALAN, S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 241 (1966) 4940.
21 P. EDMAN AND J. SJÖQUIST, *Acta Chem. Scand.*, 10 (1956) 1507.
22 M. T. McQUILLAN AND V. M. TRIKOJUS, *Australian J. Biol. Sci.*, 6 (1953) 617.
23 G. LUNDBLAD, M. BERNBÄCK AND C. WIDEMANN, *Acta Chem. Scand.*, 20 (1966) 675.
24 P. S. SARFARE, G. KEGELES AND S. J. KWON-RHEE, *Biochemistry*, 5 (1966) 1389.
25 B. F. ERLANGER, S. M. VRATSANOS, N. WASSERMANN AND A. G. COOPER, *J. Biol. Chem.*, 240 (1965) PC 3447.
26 E. GROSS AND J. L. MORELL, *J. Biol. Chem.*, 241 (1966) 3638.
27 N. UI AND O. TARUTANI, *J. Biochem.*, 50 (1961) 508.
28 M. MURAYAMA, *Federation Proc.*, 15 (1956) 318.
29 J. L. BETHUNE, *Biochemistry*, 4 (1965) 2691.
30 L. W. NICHOL AND A. B. ROY, *Biochemistry*, 5 (1966) 1379.
31 W. KAUZMANN, *Advan. Protein Chem.*, 14 (1959) 1.
32 G. A. GILBERT, *Proc. Roy. Soc. London, Ser. A*, 250 (1959) 377.
33 L. W. NICHOL AND A. G. OGSTON, *Proc. Roy. Soc.*, B ,163 (1965) 343.
34 M. S. N. RAO AND G. KEGELES, *J. Am. Chem. Soc.*, 80 (1958) 5724.
35 R. TOWNEND, R. J. WINTERBOTTOM AND S. N. TIMASHEFF, *J. Am. Chem. Soc.*, 82 (1960) 3161.
36 D. J. WINZOR, *Biochim. Biophys. Acta*, 74 (1964) 144.
37 R. C. WILLIAMS AND T. G. RAJAGOPALAN, *J. Biol. Chem.*, 241 (1966) 4951.
38 P. E. E. TODD AND V. M. TRIKOJUS, *Biochim. Biophys. Acta*, 45 (1960) 234.