Proteolytic Activity in Human Thyroid Extracts

I. Purification and Properties of Some Proteinases

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1. In human thyroid extracts, hydrolysis of bovine haemoglobin occurs at pH values 3.75, 6.5, and 8.5. In the presence of cysteine the acid activity is slightly increased; the neutral hydrolysis disappeared, and the activity at pH 8.5 was considerably decreased.

2. The hydrolysis of casein shows three optima: at pH 2.75, 5.5, and 8.5. Cysteine has no influence on the proteolytic activity.

3. Hydrolysis of bovine albumin gives a complex peak with optima at pH 3.5 and 4.5. According to the definition by Press, Porter and Cebra, the hydrolysis is caused by enzymes of cathepsin D type.

4. By means of gel filtration, the proteases can be separated from thyroglobulin with a 50-fold increase in specific activity.

5. Iodine completely inhibits the haemoglobin-splitting activity at pH 3.75 and 8.75. In the presence of cysteine and calcium chloride, ammonium acetate strongly enhances the hydrolysis of haemoglobin.

A clear description of the normal thyroid was given in 1656 by Thomas Wharton, who also gave the gland its name. In 1896 Baumann discovered that the thyroid contains considerable amounts of iodine. Most of the iodine is bound in the gland as a soluble iodinated protein, which was identified as a globulin by Oswald and others. Oswald obtained this globulin called thyroglobulin by extraction of the vesicles in the thyroid with a solution of 0.9% sodium chloride, followed by precipitation with half-saturated ammonium sulphate solution. A study of the precipitation of thyroglobulin by salting-out reagents has been made by Derrien, Michel and Roche. Thyroglobulin has a molecular weight of about 700,000, as estimated by Heidelberger, Svedberg and Pedersen and by Shulman, Rose and Witebsky, or about 690,000 as estimated by Edelhoch. Since the cells surrounding the thyroid follicles are not normally permeable to such large protein molecules, a decomposition of the thyroglobulin must occur before the hormone can enter the blood stream. Salter and Lerman were able to obtain thyroid hormone activity in vitro with extracts of thyroid glands. Gersh and Caspersson, on the basis of histochemical studies, suggested the possibility that enzymes in the thyroid gland.
hydrolyse thyroglobulin into polypeptides or peptones which are capable of rapid transfer across the cell membrane. Lerman,\textsuperscript{10} using immunological methods, was not able to demonstrate the presence of thyroglobulin in the circulating blood even in cases of thyrotoxicosis, thereby providing indirect support for this theory. De Robertis \textsuperscript{11} in 1941 found that a proteolytic enzyme which could digest a gelatin substrate was present in the colloid from single follicles. Dziemian \textsuperscript{12} determined the proteolytic activity of thyroid glycerol extracts using edestine at pH 4.0 as substrate and showed that rats treated with thyroid stimulating hormone (TSH) had higher protease activity than normal animals. De Robertis and Nowinsky \textsuperscript{13} investigated the proteolytic activity of normal and pathological human thyroid tissue using edestine as substrate at pH 4.0. They found great difference in the values from normal glands and from goitrous ones. Several studies have been made on thyroid protease from different animals with gelatin or more often with haemoglobin as substrate.\textsuperscript{14–17}

Studies on purified thyroid protease have been made by precipitation with ammonium sulphate \textsuperscript{18} or acetone \textsuperscript{19} or potassium phosphate;\textsuperscript{20} and by cellulose column chromatography.\textsuperscript{21} In the latter investigation on sheep thyroid two distinct proteases were found with haemoglobin as substrate, having pH optima at 3.8 and 5.7, respectively. Shapland \textsuperscript{22} recently isolated a protease from human thyroids purified by differential centrifugation and column chromatography of the mitochondrial fraction. Lundblad and Immers \textsuperscript{23} found cathepsin B in extracts from human thyroid tissue, and were able to separate it from a protease, which acts at pH 3.75, by means of gel filtration. The hydrolysis of thyroglobulin has been studied by means of common spectrophotometrical methods \textsuperscript{24–27} and by isotopic methods using \textsuperscript{131}I-labelled thyroglobulin.\textsuperscript{28–34} Watson and Trikojus,\textsuperscript{35} Irie\textsuperscript{36} and others have studied the effect of thyrotropin and other substances on the hydrolysis of thyroglobulin. Immunological methods for detection of antibodies have been employed by Witebsky,\textsuperscript{37} Roitt and Doniach,\textsuperscript{38,39} Doniach,\textsuperscript{40} Perelmutter,\textsuperscript{41} and by Jonsson, Fagreus and Espmark.\textsuperscript{42} Antibodies against thyroid protease have been investigated by Pollari, Menozzi, Bozzano and Cacioppo \textsuperscript{43} and by Shapland.\textsuperscript{22} The present study is concerned with the hydrolysis of haemoglobin, casein, and albumin by thyroid extracts and fractions at different pH values and the influence of various agents upon the hydrolysis. By means of gel filtration on Sephadex (as described by Porath, Flodin and Gelotte \textsuperscript{44–47}) and fractionation with ammonium sulphate, the proteases in the human thyroid extracts have been partly separated.

**EXPERIMENTAL**

_Materials._ The human thyroid tissue for extraction was generally from non-toxic goitre patients; and in a few instances from post mortem cases. The material was promptly frozen and stored at \(-22^\circ\text{C}\). Before the extraction the material was freed from fat and connective tissue in the frozen state; cut into thin slices and suspended in 3 ml of 0.9 % NaCl containing 1 % butanol per gram of tissue. The mixture was stirred for 20 h at \(+4^\circ\text{C}\), filtered through 140-mesh nylon cloth, and centrifuged at 6000 \(\times g\) for 20 min at \(2^\circ\text{C}\).
Assay of proteolytic activity

Haemoglobin. Urea-denatured bovine haemoglobin (Enzyme Substrate Powder, Armour, England) was used. Its enzymatic activity was estimated by the method of Anson.44 Haemoglobin (4 g) was added to 36 g of urea and 8 ml of 1 M NaOH in a total volume of 100 ml. The solution, whose pH was 7.5, was kept 1 h at 37°C with 0.01 % mercaptoethanol, divided into portions, frozen and stored at −22°C. The incubation mixture contained 2.0 ml of 0.4 M buffer, 1.0 ml of 4 % haemoglobin, and 1.0 ml of diluted enzyme solution (0.50 ml enzyme solution and 0.50 ml of water). The mixture was kept at 35.5°C for 1 h (if not otherwise stated) in a shake incubator. The incubation was stopped with 5 ml of 0.3 M trichloroacetic acid (TCA) (0.6 M TCA for samples with pH over 8.5); the mixture was kept for 30 min at 35.5°C, filtered, and the liberated tyrosine in the filtrate was estimated at 280 μg. Blanks were run with the enzyme added after incubation. In some experiments a 2 % haemoglobin solution containing 0.02 M CaCl₂ was used (according to McDonald and Kunitz 45).

Casein. 4 g “Casein nach Hammarsköld” (E. Merck, Darmstadt), 4 ml 1 M NaOH, and 1 ml 1 % mercaptoethanol (Schmitt-Jourdan, France) was made up to 100 ml. The proteolytic activity was determined according to Kunitz.46 The incubation mixture contained 2.0 ml of 0.4 M buffer, 1.0 ml of 4 % casein and 1.0 ml of enzyme solution. The mixture was incubated at 37°C for 1 to 4 h and treated as described for haemoglobin. Blanks were run in parallel.

Albumin. Bovine albumin powder (Fraction V from bovine plasma, Armour, England) was treated according to Press, Porter and Cebra,47 albumin (1.7 % in 0.33 M citric acid, pH 1.8) at 37°C for 1 h. The pH was then adjusted with 1 M NaOH and water was added to a final albumin concentration of 0.85 %. Cysteine was added to a molarity of 0.01. For assay, 2.5 ml of substrate and 1.0 ml of enzyme solution were mixed and incubated at 37°C for 3 h. The reaction was terminated by the addition of 5 ml of 0.6—1.2 M TCA. After 1 h at 37°C the mixture was filtered and the amount of tyrosine estimated at 280 μg.

Fractionation and concentration. Ammonium sulphate fractionation was made at pH 7 in the cold (+ 2°C). Precipitates were collected by centrifugation. Concentration of extracts and pooled fractions after gel filtration was made in Visking tubings with dry saccharose in the cold.

Gel filtration. Sephadex G-100 or G-200 (AB Pharmacia, Uppsala, Sweden) was added to water and equilibrated with the solvent and poured into columns. The effluent was collected in 3—5 ml fractions and their optical density at 280 and 280 μg measured in a Beckman spectrophotometer. The protein content in the extracts and samples was determined according to the method by Lowry, Rosebrough, Farr and Randall.48 The protein in the fractions was calculated by means of a nomogram as described by Warburg and Christian.49

RESULTS

I. Investigation of unpurified thyroid extracts. The hydrolysis of haemoglobin at different pH’s with and without the addition of cysteine is shown in Fig. 1. The extract was made from the thyroid tissue of an atoxic goitre patient. The pH optima are around 3.75, 6.5, and 8.75. There is a slight activation in the presence of cysteine in acid solution. Proteolytic activity disappeared in the presence of cysteine at pH 6.5, and at pH 8.5 the activity decreased. Fig. 2 shows a hydrolysis curve of haemoglobin without cysteine of an extract from normal post mortem thyroid tissue. The extract was made with two volumes of 0.9 % NaCl per gram of tissue. The same pH optima are found as in Fig. 1. All experiments were made with 0.50 ml of extract at 35.5°C with an incubation-time of 1 h.

The hydrolysis of casein at different pH's is shown in Fig. 3. The extract was the same as that of the previous experiment. The hydrolysis rate is lower. The incubation temperature was 37°C. Three distinct optima were found: at pH 2.75, 5.5, and 8.5.

A study of the hydrolysis of albumin in the presence of cysteine is shown in Fig. 4. Albumin with cysteine was chosen as the substrate in order to test the presence of cathepsin D, after the method of Press, Porter, and Cebra. An extract of atoxic goitre tissue from operative patients was used. There appears to be albumin hydrolysis at pH 3.5 and 4.5. The activity was destroyed
Table 1. Hydrolysis of albumin by a thyroid extract. Assay mixture: 0.50 ml extract + 0.50 ml water + 2.5 ml 0.85% albumin. Incubation 3 h at 37°C.

<table>
<thead>
<tr>
<th>pH</th>
<th>Cysteine</th>
<th>µg tyrosine</th>
<th>% activation</th>
<th>Extract heated at 60°C for 40 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>+</td>
<td>42.6</td>
<td>50</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>28.4</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>4.5</td>
<td>+</td>
<td>36.4</td>
<td>87</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.9</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

at 60°C after 40 min at pH 7, which indicates that the enzyme is of the cathepsin D type. The results of this investigation are shown in Table 1.

The shape of a typical curve, as for example Fig. 4, appears to indicate the presence of two enzymes, but of the cathepsin D type, according to the results shown in Table 1. The different degree of activation at pH 3.5 and 4.5 are easier to understand in terms of two enzymes.

The influence of substrate concentration and of time on the hydrolysis of casein are shown in Figs. 5 and 6. A concentration of casein higher than 1% in the incubation mixture, gives only a small increase in the rate of hydrolysis. The rate of casein hydrolysis is constant for about 90 min, after which a slight decrease occurs. In both experiments, an unpurified extract from thyroid tissue taken post mortem was used.

As can be seen in Table 2, the presence of iodine completely stops hydrolysis at both pH 3.75 and 8.75. Cysteine increases the activity a few per cent at

![Fig. 5. Influence of substrate concentration on the hydrolysis rate of casein by a thyroid extract. One ml extract, 1.0 ml casein and 2.0 ml buffer were mixed and incubated at 37°C for 1 h at pH 2.85.](image)

![Fig. 6. Hydrolysis of casein by a thyroid extract. Incubation at pH 2.85 with a final concentration of 1% casein.](image)

*Acta Chem. Scand. 20 (1966) No. 3*
Table 2. Influence of some agents on the hydrolysis of haemoglobin by an extract from an atoxic goitre. The incubation mixture consisted of 0.50 ml extract, 0.50 ml agent, 2.0 ml buffer and 1.0 ml 4.0 % haemoglobin. Incubation: 60 min at 35.5°C. Concentration of reagents in mixture $10^{-4}$ M.

<table>
<thead>
<tr>
<th>pH at incubation</th>
<th>Agent</th>
<th>Activity µg tyrosine</th>
<th>Rel. act.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.75</td>
<td>H$_2$O</td>
<td>125</td>
<td>100</td>
</tr>
<tr>
<td>3.75</td>
<td>Cysteine</td>
<td>129</td>
<td>103</td>
</tr>
<tr>
<td>3.75</td>
<td>I$_2$-KI</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3.75</td>
<td>EDTA</td>
<td>115</td>
<td>91</td>
</tr>
<tr>
<td>3.75</td>
<td>Ca$^{++}$</td>
<td>131</td>
<td>105</td>
</tr>
<tr>
<td>8.75</td>
<td>H$_2$O</td>
<td>37</td>
<td>100</td>
</tr>
<tr>
<td>8.75</td>
<td>Cysteine</td>
<td>26</td>
<td>70</td>
</tr>
<tr>
<td>8.75</td>
<td>I$_2$-KI</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8.75</td>
<td>EDTA</td>
<td>27</td>
<td>74</td>
</tr>
<tr>
<td>8.75</td>
<td>Ca$^{++}$</td>
<td>15</td>
<td>41</td>
</tr>
</tbody>
</table>

pH 3.75 but decreases the activity about 30 % at pH 8.75. EDTA has an inactivating effect in both media. It is interesting that calcium has a slight activating effect at acid pH’s but a strong inhibiting effect at pH 8.75, possibly as a result of the blocking of an activating ion.

II. Purification of thyroid extracts by gel filtration and investigation of the proteolytic activity. The distribution of material from a thyroid extract, and the localization of a haemoglobin-splitting acid protease after gel filtration on G-100 are shown in Fig. 7. The separation of thyroglobulin, (which is the first peak) from the other components is not satisfactory, but for the acid protease at pH 4.3 a rather good separation is obtained after fraction 100.

![Fig. 7. Gel filtration of an extract from thyroids of atoxic goitre on Sephadex G-100.](image)
The proteolytic assay was first performed with a haemoglobin substrate containing 0.02 M calcium chloride, according to the method of McDonald and Kunitz.\(^4\) The hydrolysis was very slow, as may be seen in the figure. When a calcium chloride-free substrate was chosen, a much stronger hydrolysis activity was obtained, as the upper curve demonstrates. Apparently calcium has a strong inhibiting effect on the acid protease. This is quite remarkable, since, with a calcium-free substrate (Table 2), the calcium ion has no effect at pH 3.75, whereas the protease activity at pH 8.75 is inactivated to about 65\%.

The protease activity on a calcium-free haemoglobin substrate was also tested at pH 8.8: only a faint activity in fractions around No. 126 was obtained.

During the work with an acid protease from sea urchin eggs,\(^4\) it was found that ammonium acetate had a strong activating effect. The effect of this substance was therefore studied in the present investigation. The values from one of the experiments are shown in Fig. 8. Cysteine gives about a 50\% increase in activity, ammonium acetate gives a slight inhibition, and both substances together result in more than 300\% activation. In order to elucidate the mechanism of activation, sodium acetate was tried, and the influence of the order of addition of the agents was studied. As can be seen in Fig. 9, sodium acetate gives only a slight activation, cysteine about a 50\% activation, whereas both cysteine and ammonium acetate gave 200\% activation when added to the substrate, and 300\% activation when added to the enzyme.

*Fig. 8. Activation of a purified thyroid protease. Material: fraction 103 (from Fig. 7). Agents in final conc.: I: H\(_2\)O; II: 0.01 M cysteine; III: 0.017 M ammonium acetate; IV: 0.01 M cysteine + 0.017 M ammonium acetate. Incubation: 0.2 ml agent + 2.30 ml substrate (2.0\% haemoglobin with 0.02 M CaCl\(_2\)) + 0.50 ml enzyme solution were mixed and shaken for 17 h at pH 4.7 and 35.5°C.*

*Fig. 9. Activation of a purified thyroid protease. Material: the pooled fractions 104 and 105 (from Fig. 7). Agents in final conc.: I: 0.015 M sodium acetate; II: 0.01 M cysteine; III: 0.015 M ammonium acetate + 0.01 M cysteine. Explanation: S = substrate, A = agent, E = enzyme. Incubation: 0.50 ml enzyme solution + 0.10 ml agent + 2.4 ml substrate (2.0 ml haemoglobin with 0.02 M CaCl\(_2\)) were mixed and shaken for 17 h at pH 4.6 and 35.5°C.*
Fig. 10. Gel filtration of a thyroid extract on Sephadex G-200 and determination of the proteolytic activity. The extract was applied to a column of Sephadex G-200 in 0.1 M Na-phosphate, pH 7.4 with 1 M NaCl and 1% butanol, and collected in 3.4 ml fractions. Flow rate 5 ml/h at 5°C. Protein content of extract 19 mg/ml. The hydrolysis of casein at pH 3.0, 5.25, and 8.35 were run for 4 h at 37°C. Column: 2.2 × 145 cm.

By means of gel filtration on Sephadex G-200 instead of Sephadex G-100, a better separation was obtained, as seen in Fig. 10. The zone of proteolytic activity is quite well separated from the high-molecular-weight components 25 S (Svedberg units) globulin and thyroglobulin (19 S) in the fraction interval 45—70. The caseinase activity at pH 3.0 and 5.25 is in the same range with a maximum at fraction 100. The enzyme active at pH 8.35 is much weaker, and has a maximum at fraction 120.

The hydrolysis of haemoglobin over a period of 4 h at pH 4.35 was studied on the thyroglobulin-free fractions 102 and 103. The enzymatic course gives a straight line. The substrate contained calcium chloride in this experiment.

Fig. 11. The hydrolysis of haemoglobin by combined fractions 102 and 103 from the gel filtration shown in Fig. 10. Incubation with shaking at 35.5°C and pH 4.35 of a mixture of 8.0 ml enzyme fraction and 40.0 ml 2% haemoglobin containing 0.02 M CaCl₂.

Fig. 12. Gel filtration of a dialyzed extract from thyroids taken post mortem and assay for proteolytic activity. The raw extract was concentrated 5 times with saccharose and then dialyzed for 17 h at 5°C against 0.1 M Na-phosphate — 1 M NaCl buffer at pH 7.4. 10 ml of this material (146 mg protein/ml) was applied to a column of Sephadex G-200 (2.6 × 135 cm) in 0.1 M Na-phosphate — 1 M NaCl buffer at pH 7.4 with 1% butanol. Flow rate 15 ml/h at 5°C. Hydrolysis of haemoglobin with 0.50 ml and of casein with 1.0 ml fractions was made at 37°C for 4 h.

Some experiments on gel filtration of extracts from thyroids taken post mortem are shown in Figs. 12—14. The column of Sephadex G-200 was the

Fig. 13. Gel filtration of the pooled fractions 83—133 (Fig. 12) and assay of proteolytic activity. The fraction pool was concentrated 5 times with saccharose and then dialyzed for 17 h at + 5°C against 0.1 M Na-phosphate, pH 7.4 and 1 M NaCl. 5 ml of this material was applied to Sephadex G-100 (2.6 × 130 cm) in the same medium as in Fig. 12. The eluate was collected in 3.5 ml fractions. Flow rate 65 ml/h at + 5°C. Hydrolysis of haemoglobin at pH 3.75 was made with 0.50 ml fractions for 6 h and of casein with 1.0 ml fractions for 6 h at pH 2.8 and for 17 h at pH 6.6. The temperature was 37°C at all incubations.

Fig. 14. Gel filtration of a sample of the same dialyzed extract as in Fig. 12 on the same G-200 column. Instead of a 10 ml sample, 5 ml was applied to the column and the eluate collected in 3.6 ml fractions. Hydrolysis was made as in Fig. 12. Flow rate 15 ml/h at +5°C. Proteolytic assay was made as in Fig. 12.

same as before except for a smaller sample size (5 ml). Some of the fractions from the chromatogram shown in Fig. 12 were pooled (83—133) and used for a repeated gel filtration on Sephadex G-100. The results are shown in Fig. 13. The proteolytic activity assayed on haemoglobin as substrate at pH 3.7 and on casein at pH 2.8 and 5.6 is localized in the same interval in all three fraction series, as was the case in the chromatogram illustrated in Fig. 10.

The protein content of the applied sample in Fig. 12 was 146 mg/ml and the haemoglobinase activity was equivalent to 243 μg tyrosine per hour per 0.50 ml sample, i.e. 1.66 μg tyrosine/h/mg protein/0.5 ml. After gel filtration on G-200 (Fig. 12) the optimum activity (No. 108) was 116 μg tyrosine per 4 h and 0.50 ml or 25.2 μg tyrosine/h/mg protein/0.5 ml. Thus a 15-fold increase in the specific activity was obtained by the gel filtration. When a 5 ml sample was applied on G-200 (Fig. 14) instead of a 10 ml sample as in Fig. 12, a 16-fold increase in specific activity was obtained. After another gel filtration of the fractions 83—133 (Fig. 12) on G-100 (Fig. 13), the optimum haemoglobinase activity was 64 μg tyrosine per 6 h per 0.5 ml or 89 μg tyrosine/h/mg protein/0.5 ml. Calculated on the original value of the sample (Fig. 12) 1.66, this is a 54-fold increase in specific proteolytic activity.

A thyroid extract from operative patients was fractionated with saturated ammonium sulphate and the precipitate obtained with at 37 %, saturated ammonium sulphate dissolved in 3 volumes of distilled water. This solution was used for a study of the influence of enzyme concentration over a period of time on the hydrolysis of haemoglobin at pH 3.8. The results demonstrated in Figs. 15 and 16 show that the rate of hydrolysis decreases rapidly with increasing concentration of enzyme (Fig. 15), and is linear with time only for a short period.
Fig. 15. Influence of enzyme concentration of the hydrolysis of haemoglobin by a fractionated thyroid extract. The precipitate obtained at a saturation of 37% ammonium sulphate of the extract was dissolved in distilled water to the original volume of the extract. Incubation was made at pH 3.8 and at 37°C with shaking (2 h for I, 30 min for II). The volume of extract was maintained with distilled water.

Fig. 16. Influence of time on the hydrolysis of haemoglobin by the same fractionated thyroid extract as in Fig. 15. Incubation was made at pH 3.8 and 37°C of 0.50 ml extract, 0.50 ml H2O, 2.0 ml buffer, and 1.0 ml 4% haemoglobin.

Fig. 17. Gel filtration of the 0—37% fraction obtained by fractionating an extract from thyroids taken post mortem with ammonium sulphate and assayed for proteolytic activity. The precipitate was dissolved in distilled water to 1/5 of the original volume. 7 ml of this solution was applied on a G-100 column (1.8 × 141 cm) in 0.1 M phosphate buffer pH 7.4 with 0.5 M NaCl and 1% butanol and collected in 3.7 ml fractions. Flow rate 35 ml/h at +5°C. Hydrolysis of haemoglobin with 0.50 ml samples for 6 h and of casein with 1.0 ml samples for 16 h at 37°C.

Fig. 18. Gel filtration of the 37—42% fraction of the same thyroid extract as in Fig. 17 and assay of the proteolytic activity. The precipitate obtained was dissolved in distilled water to 1/5 of the original volume. 25 ml of this solution was applied to a G-200 column (6.0 × 129 cm) in 0.1 M tris-HCl buffer pH 8.0 with 0.5 ml NaCl and 1% butanol and collected in 10 ml fractions. Flow rate 5 ml/h at +5°C. Hydrolysis of haemoglobin and casein was performed as described in Fig. 17.

III. Purification of thyroid extracts by ammonium sulphate fractionation followed by Sephadex gel filtration and investigation of the proteolytic activity. Thyroglobulin is generally prepared from the precipitate obtained after 37—42% saturation of a thyroid extract with ammonium sulphate. The two fractions obtained at 0—37 and 37—42% saturation were treated by gel filtration. The thyroglobulin containing 37—42% fraction was filtered through Sephadex G-200. Sephadex G-100 was chosen for the former fraction in order to obtain a better estimate of the molecular weight of the proteases. The results are shown in Figs. 17 and 18. The proteolytic activity from the former fraction tested on haemoglobin at pH 3.75 and on casein at pH 2.8 and 5.65 occurs in the same interval as can be seen in Fig. 17, and thus seems to be derived from enzyme(s) of the same molecular weight: around 100 000. In the latter fraction after gel filtration on G-200, the enzyme which hydrolyses haemoglobin at pH 3.75 and casein at pH 5.65 occurs in the same fractions, but the shape of the curve indicates the presence of two enzymes. The casein hydrolysis at pH 2.8 is very weak in both chromatograms, occurring in the same fractions as the strongest activity of the haemoglobin hydrolysis. The position of the proteolytic activity in the G-200 chromatogram indicates a high molecular weight of the enzymes.

DISCUSSION

Three different substrates have been used for the proteolytic assay of human thyroid extracts: bovine haemoglobin, casein, and bovine albumin. Of these substrates apparently only the hydrolysis of haemoglobin had been studied previously. The pH optima for the hydrolysis of haemoglobin are at 3.75, 6.5 (weak) and 8.5. Kammer, Peranio and Bruger 16 found optimum hydrolysis of dog’s thyroid protease occurring optimally at pH 3.55. Weiss 17 found hydrolysis of beef thyroid protease best at 4.0. McQuillan, Trikojus and Mathews 18, 26 found that thyroid extract from hog was active around a pH optimum of 3, and that from rats, at pH 3.5. Beckers and de Visscher 55 found maximum activity in human endemic goitre protease at 3.5, and recently Shapland, who used human haemoglobin for the proteolytic assay of thyroid extracts from patients suffering from Hashimoto’s disease reports an optimum at pH 3.5. In all these investigations and in those reviewed in the introduction, only one pH optimum has been found except in the report by Haddad and Rall, 21 who purified thyroid extracts from sheep by means of ion-exchange chromatography, and found two proteases active at pH 3.8 and 5.7 respectively. Hydrolysis of casein reported in the present study gave three pH optima; 2.75, 5.5, and 8.5. The two first may correspond to those noted by Haddad and Rall, 21

The hydrolysis of albumin in the present study seems to indicate two enzymes of cathepsin D type. The un-symmetrical shape of the first peak of the haemoglobin hydrolysis curve would support this assumption. Particularly in the presence of cysteine, the unsymmetrical shape of the haemoglobin hydrolysis curve (Fig. 1) is noticeable. The two cathepsin D enzymes may correspond to the two casein peaks in the acid region of Fig. 3.

The inhibiting effect of iodine on certain enzymes is well known. Hopkins 56 used the oxidizing property of iodine in an assay of thiol groups of proteins. Herriot 57 studied the influence of iodine as an inhibitor for pepsin, and showed

that the inactivating effect was due to iodination, and the formation of diiodothyrosine. Iodine was introduced as a therapeutic agent for the treatment of toxic goitre by Plummer,55 and several clinicians have tried to explain its action on the thyroid gland. De Robertis 11 demonstrated a decrease in proteolytic activity after treatment of patients with iodine for a long period. Llamas 14 found a decrease of 95 % in the proteolytic activity upon treatment with 0.006 M iodine at pH 6.5 using gelatin as substrate. Cathepsin B, also, is inactivated by iodine, as shown by Lundblad and Immers.23

In the present study, 0.01 M iodine completely inhibited the hydrolysis of haemoglobin at pH 3.75 and at pH 8.75. The inhibiting effect on the acid protease (cathepsin D) confirms results of other authors, but it should be kept in mind that also the protease active at pH 8.75 is inactivated.

The gel filtrations of thyroid extracts on Sephadex G-200 show that the fraction interval with proteolytic activity is separated from the thyroglobulin. The component with sedimentation constant 19S constitutes the major peak of high molecular material (Fagreus 49) shown in Fig. 10 and consequently the 25S component must be in the first peak. It is remarkable that the two acid proteases which split casein at pH 2.8—3.0 and at pH 5.25—5.65, respectively, are found in the same fractions in all experiments (Figs. 10, 12, 13, and 14). Therefore the enzymes may have the same molecular weight, or else the active sites which are effective at different pH values belong to the same enzyme molecule. (Or less probably, the enzymes are in some way attached to molecules of the same type).

The position of this proteolytic activity in the fraction series suggests that the molecular weight of the enzyme(s) is less than 200 000 (Figs. 10, 12, 14, and 18) and also less than but near 100 000 (Figs. 7, 13, and 17). The casein-hydrolyzing enzyme activity at pH 8.5 appears later in the fraction series than the acid proteases, but only a weak activity remains after gel filtration (Fig. 10).

The haemoglobin hydrolysis at pH 3.75 (cathepsin D) is much stronger than the casein hydrolysis at any pH, but after gel filtration, haemoglobin-splitting activity has not been found either at pH 6.5 (in the absence of cysteine) or at pH 8.5.

Acknowledgements. The authors wish to thank Professor A. Fagreus for providing the material and Mrs. E. von Zeipel for valuable help with the preparation of the extracts.

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Received November 11, 1965.