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PROTEOLYTIC ENZYMES IN THE SKIN*

III. Studies on the extractability, stability and modifier characteristics of the caseinolytic enzymes in the rat skin

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Beloff and Peters (1) observed in 1945 that an extract of the rat skin prepared in distilled water or in dilute buffer showed no proteolytic activity toward casein, while an extract prepared in 5 % potassium chloride in neutral phosphate buffer hydrolysed casein readily. With minor modifications this method of extraction of skin proteolytic enzymes has since been often used. Martin and Axelrod (12) reinvestigated the extractability of some proteolytic and esterolytic enzymes from the rat skin and stressed the importance of the high ionic concentration in the extraction medium as well as in the assay medium.

In these experiments, as well as in others, the effectiveness of the extraction procedure has been usually estimated on the basis of activity determinations carried out at neutral pH only, *i.e.* the pH which has been suggested to be optimal for the hydrolysis of casein by the skin proteinases. It is known, however, that the skin of many mammalian species contains several proteolytic enzymes with pH-optima ranging from the low acidic to the high alkaline area (8, 10). Casein is known to be hydrolysed in the rat skin homogenate by at least four separate enzymes (10), and it is quite likely that the optimal extraction of each one of them requires different conditions. The acidity, the molarity, the temperature, the presence of any specific ion or other substances in the extraction medium as well as the length of the homogenization and extraction time could effect the final result. Equally, each one of the enzymes might be inactivated or activated by special conditions employed in preparative steps as well as in later measures of enzyme purification procedures, *e.g.* acid precipitation, heat denaturation, freezing and thawing, dialysis, and storing of the preparations. With these facts in mind an effort was made to find the optimal conditions of extraction and handling of the enzymic activities demonstrable in the rat skin homogenate. In addition, the relation of the enzymic activities to some common modifiers for proteolytic enzymes was studied, in order to get a rough idea of the most essential requirements of the enzymes, such as possible dependence on sulphydryl groups or metal ions. Information on these problems is of importance when efficient procedures for the purification of the individual enzymes are planed.

Materials and Methods

Skin samples

Altogether twenty male adult Long-Evans rats, weight 260–320 g, kept in standard laboratory conditions, were killed by de-

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capitation. The whole trunk skin was removed after shaving with an animal clipper. The skin was cleaned from the subcutaneous fat, muscle tissue and fascia and minced with scissors. The usual yield from one rat was 15 g of minced tissue.

Homogenization and extraction

The scheme given below is that used as a standard procedure. In studies on the effectiveness of the individual steps of the procedure, several parameters were varied one by one in the limits given in the brackets, keeping the other parameters at standard procedure level.

The minced tissue was homogenized for 1 min (0.5–2.5 min) with an Ultra-Turrax homogenizer in ten volumes (w/v) of 10 mM phosphate buffer pH 8.0 (pH 6.0–9.0), containing 8 per cent (0–10 per cent) of potassium chloride. The homogenization was carried out in a metal container kept in crushed ice during the procedure. During the homogenization the container was moved up and down keeping the cutting rotator in the homogenate for 1.5 s and above the homogenate surface for 1.5 s, intermittently. The crude homogenate was allowed to stand at + 4°C (+ 4–40°C) for 2 h (0–20 h) and was centrifuged at 10,000 g for 20 min at + 4°C. The sediment and the flotate were discarded and the opalescent supernatant was used for further studies, and it is referred to as the (centrifuged) homogenate.

Acetone treatment

In one series of experiments acetone precipitation was carried out as follows: One hundred ml of the crude homogenate was slowly added to ten volumes of dry acetone at − 15°C with rapid stirring. The suspension was allowed to stand at − 20°C for 50 min, the precipitate was collected by filtration through a filter paper (Schleiher & Schull nr 589), and transferred to an exicator over concentrated sulphuric acid. The dry precipitate was powdered mechanically. The yield was about 8 g per 10 g of starting minced skin sample. For the extraction of the enzymes 0.8 g acetone powder was suspended into 10 mM phosphate buffer pH 8.0 and let to stand at + 4°C for 24 hours. The suspension was centrifuged at 10,000 × g for 10 min and the supernatant was collected.

Desalting

Salt-free homogenate was needed for some experiments. Desalting was carried out using gel filtration on Sephadex G-25 (Pharmacia, Uppsala). A sample of 10 ml was applied into a column (2.3 × 120 cm) packed with the Sephadex gel pretreated according to the manufacturer's instructions. 10 mM phosphate buffer pH 8.0 was used for elution, and fractions of 10 ml were collected at a flow rate of 1 ml/min. The concentration of proteins in the fractions was estimated by reading the optical density at 280 nm and the concentration of chloride ions was determined according to the method of Shoenfield and Lewellen (14). The fractions containing protein but devoid of chloride were pooled.

Substrates, modifiers and other chemicals

Casein acc. to Hammarsten (E. Merck AG, Darmstadt) was dissolved in 10 mM phosphate buffer pH 8.0 (1 g/100 ml) and denatured by boiling for 10 min. This stock solution was stored at + 4°C, for no longer than 5 days. A stock solution of bovine serum albumin (Poviet Producten N.V., Amsterdam) was prepared in the same way. Denatured hemoglobin (Nutr. Biochem. Corp., Ohio) was dissolved in 10 mM phosphate buffer pH 8.0 (2 g/100 ml) and used freshly.

Trypsin inhibitor from lima bean, Trypsin inhibitor from soybean, and Trypsin inhibitor from ovomucoid all were from Sigma Chemical Company, St. Louis.

Human blood was obtained by venepuncture and rat blood by puncturing the heart of an ether anestesized rat. The blood samples were drained into one fifth volume of 3 per cent sodium citrate and centrifuged to remove the blood cells. The plasma was diluted with distilled water 1 : 4 and used as a modifier.

The universal buffer of Britton and

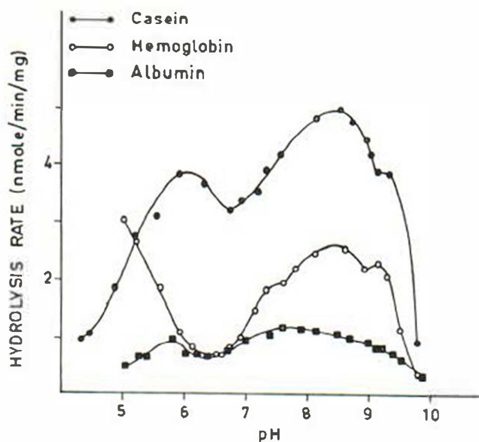

Fig. 1. The hydrolysis of casein, hemoglobin and albumin by the rat skin homogenate. Assay conditions as given in 'Materials and Methods'.

Robinson (3) was used throughout the study.

Assay procedures

To measure the rate of the hydrolysis of the protein substrates a mixture consisting of 0.5 ml of buffer of suitable pH, 0.5 ml of substrate stock solution and 0.5 ml of enzyme solution was incubated in a water bath at 37°C. In studies on the effects of the modifier substances an additional 0.2 ml of modifier stock solution of suitable concentration was included and the substrate was added after a preincubation of 10 min. In controls the modifier stock solution was replaced by water. After incubation, usually 60 min, 0.5 ml of 5 per cent trichloroacetic acid was added and the mixture was centrifuged at 5000 xg for 15 min. The soluble peptides in the supernatant were estimated according to the method of Folin and Ciocalteu (5). A standard curve was prepared from tyrosine in identical conditions. The effect of the spontaneous hydrolysis of the protein substrates was eliminated by using blank assays in which the enzyme was replaced by buffer. The effect of soluble peptides present in the enzyme preparations was eliminated by using controls in which the enzyme was added just prior to the acidification. The enzyme assays in all experiments were carried out at a wide pH-range, usually from pH 5 to pH 10. The actual pH of each incubation mixture was determined in the middle of the incubation period using a pH-meter equiped with microelectrodes.

The proteolytic activity is usually expressed as the amount of tyrosine liberated in a unite of time per mg of homogenate protein (nmole/min/mg). The protein concentration of the homogenate samples was assayed according to the method of Lowry *et al.* (11). Bovine serum albumin (Poviet Producten N.V., Amsterdam) was used as reference protein.

Results

Comparison of casein, hemoglobin and albumin as substrate

It was shown earlier that with casein as substrate a complex pattern of proteolytic activity at pH-range 5–10 can be demonstrated in the rat skin homogenate (10). Four separate possible pH-optima were repeatedly encountered, *i.e.* roughly at pH 6.0, 7.5, 8.5 and 9.0–9.5. Since, however, other proteins, *e.g.* hemoglobin and albumin are often used as substrates for proteolytic enzymes and since it is known that different proteins are hydrolysed at different rates by different enzymes, a comparison was made with casein, hemoglobin and albumin as substrate for the rat skin proteinases through pH 5–10.

The results are presented in Fig. 1. It was found that casein is the most sensitive substrate when the enzymic hydrolysis is measured as the liberation of tyrosine using the methods of this work. The general form of the pH-curve obtained with albumin as substrate was roughly identical to that obtained with casein, but the hydrolysis rate was much lower, about one fourth of that of casein. The pH-curve obtained with hemoglobin as substrate was roughly of the same form at the neutral and alkaline range, the rate of hydrolysis being about half of that obtained with casein. The form of the pH-curve obtained at acidic pH-values differed, however, markedly from that obtained with casein as substrate. No optimum was obtained at pH 6 and the

hydrolysis rate was very low. Higher hydrolysis rates were obtained at lower pH-values. This is due to the presence of proteolytic enzymes active toward this substrate at pH-values more acidic than pH 5 (4).

On the basis of these findings casein was used as substrate in all further experiments of this study.

Extractability of the proteolytic activities

1. *Homogenization*. Homogenization of the skin tissue is difficult because of its toughness owing to its high collagen content. No comparative data on the effectiveness of various homogenization procedures are available in the literature. Preliminary studies with different homogenisators (Potter-Elvehjem, Bühler, Waring Blendor, and Ultra-Turrax) showed that a superior disintegration of the tissue was obtained with the Ultra-Turrax. To determine the length of the homogenization period necessary for obtaining an optimal yield of proteolytic activity the homogenization time was varied from 0.5 to 2.5 min.

When extending the homogenization time from 0.5 min up to 1–1.5 min an increasing caseinolytic activity over the whole pH-range (tested between pH 5–10) was obtained. A further increase in the homogenization time gave a clearly lower yield probably because of inactivation of the enzymes, even while the temperature of the homogenate, measured immediatedly after the homogenization, was below 17°C.

For further work homogenization with an Ultra-Turrax homogenizator for 1 minute was considered suitable.

2. *Salt concentration*. Since the salt concentration of the extraction medium effects the extraction of proteolytic enzymes active at neutral pH (1, 12) it was studied whether the same is true for all the proteinases in rat skin. Skin samples were homogenized in 10 mM phosphate buffer, pH 8.0 containing 0, 2, 4, 6, 8, or 10 per cent (w/v) potassium chloride. After homogenization the crude homogenates were allowed to stand for 2 h at + 4°C, and were centrifuged. Potassium chloride was added to obtain a final concentration of 10 per cent in all of the samples and the proteolytic activity was assayed 90 min later.

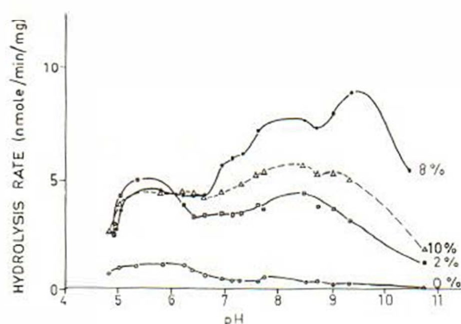

Fig. 2. The influence of salt concentration on the extractability of the caseinolytic enzymes in the rat skin. Assay conditions as given in 'Materials and Methods'. The percentage values give the concentrations of potassium chloride used in the different extraction procedures.

The results obtained at four of the salt concentrations are presented in Fig. 2. Only minimal proteolytic activity was present in the extract with buffer containing no added potassium chloride. A concentration of 2 per cent of potassium chloride in the buffer extracted effectively the proteolytic activity at pH 5–6. A further increase in the salt concentration neither increased nor decreased the yield of this activity. On the other hand, the yield of the enzymes active at alkaline pH was constantly increased with increasing salt concentration up to 8 per cent KCl in the extraction medium. Extraction at a salt concentration of 10 per cent, however, gave a lower yield of these activities, especially of the most alkaline one.

On the basis of these findings it can be concluded that an extraction medium with 8 per cent of KCl is suitable when high extraction yield of the proteolytic enzymes is desired.

3. *Length of extraction*. It has been customary to let the disintegrated skin tissue or acetone powder to stand for variable periods in the extraction buffer (homogenization buffer) for extraction of the enzymes. The length of the extraction period employed varies considerably, from 30–40 min (1, 9, 15,) to 3–4 h (6, 7, 12). Only few authors offer any reasons for their

choice of time (1, 12), and no systematic data on this subject have been reported.

A batch of crude homogenate (200 ml) was prepared and allowed to stand at + 4°C. Samples of 10 ml were withdrawn immediately and after 1, 2, 3, 4, 5, 10 and 20 h. Each sample was centrifuged at once and stored at + 4°C. The caseinolytic activity at pH 5–10 was measured in the samples simultaneously, after the withdrawal of the last sample.

The caseinolytic activity in the samples taken at zero time was high, even while an increase in yield took place during the extraction up to 2–3 hours. This increase was, however, quite variable, being in repeated experiments 10–70 per cent of the zero time value. The activity in the samples extracted for more than 4 h remained constant. These data point out that optimal activity is obtained by using an extraction period of 2–4 hours, even while considerable yield can be obtained without any prolonged extraction measures.

4. *Extraction temperature*. Extraction of the proteolytic enzymes has been carried out at a wide variety of temperatures, ranging from 1°C (12), through room temperature (1, 6, 15) to 38°C (1, 9). We wanted to study systematically whether the extraction temperature effects the yield of any of the enzymic components catalysing caseinolysis. After the usual homogenization samples of the crude homogenate were allowed to stand at + 4, + 10, + 20, + 30, or + 40°C for 30 min after which they were centrifuged and the proteolytic activity was measured at pH 5–10.

It was found that the caseinolytic activity obtained was, throughout the pH-range, the better the lower the extraction temperature. The activity obtained at 40°C was about 25 per cent lower than that obtained at + 4°C. On the basis of this finding it is evident that a low temperature of extraction is to be preferred.

5. *pH of extraction*. Extraction procedures have usually been carried out at pH 7.0–7.5 (1, 7, 10, 12). Since, however, the isoelectric point of enzyme proteins may vary markedly and the extractability depends upon the isoelectric point, it is possible that different enzyme proteins are extracted differently depending on the pH. Therefore, samples of crude homogenate were extracted at pH 6, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0. Before assaying, all of the samples were brought to pH 7.5 and assayed with casein as substrate at a pH-range of 5–10. The pH-profiles obtained for the hydrolysis were identical, even while the proteolytic activity at any pH was somewhat (10–20 %) lower in the extracts made at more acid pH. This suggests that the pH of extraction has no selective effect but evidently alkaline medium gives better over-all yields.

6. *Acetone treatment*. Both acetone powder and minced fresh tissue has been used as the starting material for the extraction of the proteolytic enzymes in the skin. A better yield of caseinolytic activity (measured at pH 7–7.5) from fresh skin than from acetone powder has been reported by some investigators (1), while the opposite has been claimed by others (12).

Crude homogenate was divided in two parts, one of which was extracted directly in the usual way, while the other one was made acetone powder and extracted according to the scheme given in Material and Methods. Both of the extracts were tested with casein as substrate. It was checked that the concentration of chloride ions was the same in both of the enzyme solutions.

The proteolytic activity in the acetone powder extract was considerably (30–50 %) lower than in the extract prepared from fresh skin tissue as shown in Fig. 3. This difference was demonstrable throughout the pH-range but no selective lack of any of the components of the caseinolytic complex could be clearly demonstrated.

7. *Freezing-thawing and Triton-X-100*. These procedures are known to disrupt tissue particles and to effect the solubilization of several tissue components, wherefore it was considered to be of value to test their effect on the extractability of the skin proteinases. Triton X-100 (0.1 %) was added to a batch of crude homogenate immediatedly after the homogenization. Another batch was freezed and thawed three

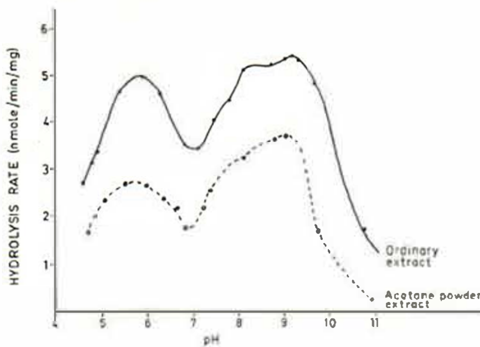

Fig. 3. Comparison of the caseinolytic activity extractable from aceton-powderized rat skin tissue and from a comparable amount of fresh rat skin tissue. Enzyme assay conditions as given in 'Materials and Methods'.

times, while a third batch served as control. All the samples were simultaneously centrifuged and assayed for proteolytic activity (pH 5–10) with casein as substrate. No increase nor decrease of the proteolytic activity could be observed after any of the treatments. In separate experiments on centrifuged homogenate it was ascertained that Triton X-100 did not affect the assay, and that neither one of the procedures effected the stability of the enzymes.

8. *All-over effectiveness of the extraction.* It was considered to be of importance to establish to what degree proteolytic activity still remained with the solid phase after the employment of our standard extraction procedure, and particularly, whether certain activities would be found mainly or solely in the solid phase. Beloff and Peters (1) state that after their extraction procedure about 50 per cent of the proteolytic activity (as measured at neutrality, only) still remained with the solid phase.

The flotate and precipitate, usually discarded, were collected and resuspenced in extraction buffer by a short (10 s) homogenization procedure, and tested for caseinolytic activity (pH 5–10). The pH-curve obtained from the flotate was similar to that obtained from the supernatant, the activities being, however, very low (roughly 1/20 of the activity in the supernatant). This suggests that all of the main components of the caseinolytic activity were present to a low degree in the flotate. The pH-curve from the precipitate, on the other hand, showed very little activity at the acid pH (about 1/20 of that found in the supernatant), but a fair share of the alkaline components (roughly 1/4 of the activity in the supernatant) was demonstrable. Thus, about 25 per cent of the caseinolytic activity demonstrable at the neutral and alkaline pH and about 10 per cent of the acidic activity will be left in the solid phase when employing our standard scheme of homogenization and extraction. On the other hand no additional subunit of caseinolytic activity—not demonstrable in the homogenate supernatant—could be detected in the solid phase.

The stability of the caseinolytic activity in the homogenate

1. *Acid treatment.* Precipitation of microsomal particles and of inert proteins at pH 5.0–5.2 is an often used step in purification procedures for enzymes. Any one of the components of the proteolytic activity might be precipitated in this procedure, either in an active or in an inactive form. This was tested by adding 1 N HCl to a batch of centrifuged homogenate (20 ml) bringing the pH to 5.0. The homogenate was allowed to stand in ice water for 5 min, and centrifuged in a refrigerated centrifuge at 5000 xg for 5 min. Thereafter, the pH was brought up to 7.5 with 1 N

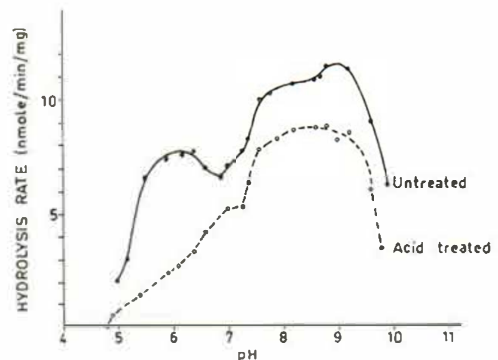

Fig. 4. The effect of acid treatment at pH 5.0 on the caseinolytic enzyme complex of the rat skin homogenate. Details on the experiment are given in the text. Enzyme assay as given in 'Materials and Methods'.

NaOH, and the proteolytic activity in the supernatant was determined with casein as substrate (pH 5–10). As seen in Fig. 4 the main part of the acidic caseinolytic activity, the peak at around pH 6, was lost while the main part of the alkaline caseinolytic activity was retained in the solution. To test whether the acidic proteolytic activity was precipitated in an active form the precipitate was dissolved in the original volume of the extraction buffer and assayed for caseinolytic activity at pH 5.8. Half of the activity lost from the solution was recovered from the precipitate in an active form. Precipitation at pH 4.0 produced a practically complete loss of the acid activity peak from the solution, but the amount of activity recoverable from the precipitate was not increased. These experiments prove that preferentially the enzyme(s) active at acid pH is precipitated and also inactivated by acid treatment.

2. *Heat treatment.* To clarify the heat stability of the proteolytic enzymes, samples (10 ml) of centrifuged homogenate were kept in a water bath at temperatures of 50, 55, 60, 65, 70, 75 and 80°C for ten minutes whereafter the proteolytic activity was assayed (pH 5–10). The control samples were kept at +4°C. As shown in Fig. 5 the activity decreased throughout the pH-range with increasing temperature. The enzymic activity of pH 6 was clearly more thermolabile than the other caseinolytic components, the treatment at 60°C causing a complete loss of activity. At 70°C no proteolytic activity was left at any pH.

3. *Dialysis.* A decrease in ionic strength produced by dialysis decreases the solubility of proteins and can thus cause specific precipitation of some enzyme proteins. This has been used to separate rabbit skin proteolytic enzymes (7). Dialysis is also an often used preparative step for further purification measures of enzymes and therefore the behaviour of the caseinolytic activity during dialysis was studied.

The centrifuged homogenate (50 ml) was dialysed at +4°C for 12 h against 100 volumes of distilled water, changed at 6 hours. The sample was centrifuged and the sediment was suspenced in the original volume of water. Potassium chloride was added both to the supernatant and to the suspenced sediment to obtain a salt concentration of 8 per cent, and the preparations were assayed for caseinolytic activity. The original proteolytic activity was totally recovered. The acidic activity was found almost exclusively in the supernatant, while the neutral and alkaline activity was distributed to an equal degree in the precipitate and in the supernatant. Thus, a partial fractionation of the caseinolytic activities could be produced by dialysis.

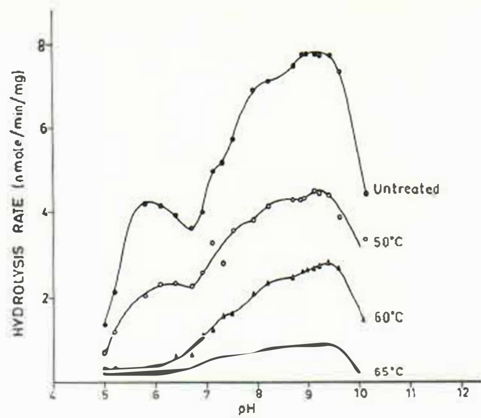

Fig. 5. The effect of heat on the caseinolytic enzyme complex of the rat skin. Details on the experiment are found in the text. Enzyme assay as given in 'Materials and Methods'.

4. *Storage.* Since the enzyme preparation is to be stored, because of practical reasons, at various temperatures during a purification procedure, it was important to find out whether the proteinolytic profile is changed during storage. Therefore, centrifuged homogenate samples (100 ml) were stored at −40°, +5° and +25°C for up to 3 weeks. Small aliquots (5 ml) were withdrawn from the samples at regular intervals and tested for proteinolytic activity with casein as substrate at pH 6, 7.5, 8.6 and 9.4.

The enzymic activity remained constant during the whole period at −40°C and decreased only 10–20 per cent during storage at +25°C. On the other hand, the activity was decreased considerably, 50–70 per cent in 3–5 days, at +25°C.

Modifier characteristics

1. *Potassium chloride*. The existence of an optimal salt concentration for the enzyme activity, as distinct from the optimum for extraction, has been recognized since the work of Beloff and Peters (1). They carried out their enzyme assays at pH 7.0 and little is known on the effect of salt ions on the proteolytic activity at other pH-values.

Centrifuged homogenate (30 ml) was desalted as described in Materials and Methods. The concentration of chloride ions in the preparation obtained was found to be 15 mM. To this preparation, known amounts of potassium chloride were added to obtain in the assay solutions the final concentrations of 5, 140, 275, 410 and 550 mM, and the caseinolytic activity (pH 5–10) was assayed. Fig. 6 presents the effect of the salt concentration on the caseinolytic activity at pH 6.3, 8.4, and 9.2. The data are derived from the pH-curves obtained at the different salt concentrations. It is evident that changes in the salt concentration effect these proteolytic activities and each one in a different way. The activity measured at pH 6.3 was decreased with the increase in the salt concentration, activity at

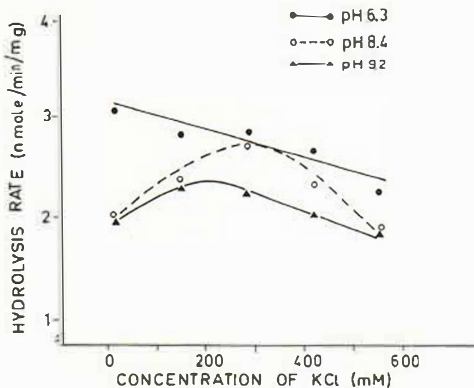

Fig. 6. The effect of the potassium chloride concentration on the hydrolysis of casein by the rat skin caseinolytic enzyme complex. The data presented are plotted from full range pH-curves, and the readings have been taken at those pHs at which distinct optima for the hydrolysis of casein are found. Further details on the experiment are given in the text. Enzyme assays as given in 'Materials and Methods'.

8.4 was optimal at a concentration about 200 mM while the activity at pH 9.2 was optimal at a concentration of about 300 mM.

This suggests that for an optimal assay of the caseinolytic activity at each one of these pH areas different concentrations of salts are to be used.

2. *Heavy metal ions and cysteine*. Earlier data have shown that a part of the skin proteolytic activity is inhibited by heavy metals, like $HgCl_2$ (13). To find out whether any of the caseinolytic components demonstrable as an optimum in the pH-curve is especially sensitive to heavy metal ions, a test was made with mercuric and copper ions. It was found that a concentration of 0.1 mM of $HgCl_2$ or of $CuCl_2$ in the assay system for the caseinolytic activity caused no significant changes in the form of the pH-curve between pH 5–10 with only a slight (10–20 %) over-all depression of the activities. On the other hand, a concentration of 1 mM of either one of the metals caused marked inhibitory effects as seen in Fig. 7. Both of the ions caused roughly a 50 per cent inhibition of the acidic caseinolytic component. The copper salt caused an even more marked inhibition on the alkaline side of neutrality, and a complete inhibition of the alkaline activities was accomplished by mercuric ion. It is thus evident, that even while some of the proteolytic enzymes in the rat skin are sensitive to fairly high concentrations of these heavy ions, no excessive sensitivity, which could hamper the common purification procedures, could be detected.

Sensitive sulfhydryl-dependent enzymes may be oxidized during the homogenization procedure or bound by metal ions and, therefore, would not show up in the assays unless reactivated by reducing agents. Therefore, the effect of cysteine, added to the incubation medium at a concentration of 1 mM was tested. A moderate activation (10–20 %) of the caseinolytic components active at pH 6 and at about pH 7.5 was demonstrated.

3. *Natural proteinase inhibitors*. It is known since the works of Beloff (2), that

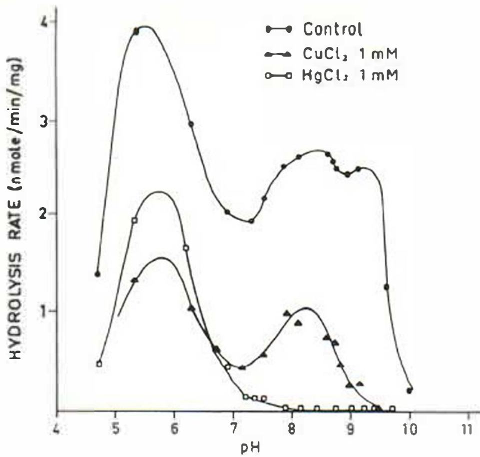

Fig. 7. The effect of some metal salts on the caseinolytic enzyme complex of the rat skin homogenate. Enzyme assay as given in 'Materials and Methods'.

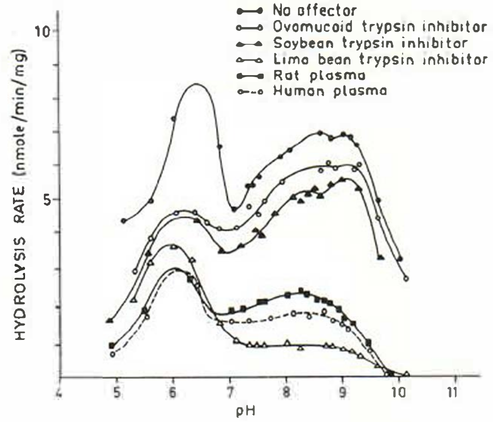

Fig. 8. The effect of some natural proteinase inhibitors on the caseinolytic enzyme complex of the rat skin homogenate. Enzyme assay as given in 'Materials and Methods'.

serum inhibits a part of the skin proteolytic complex, and Martin and Axelrod showed that some natural trypsin inhibitors have an inhibitory effect (for references see 8) on certain components of this complex. However, these data are fragmentary and no uniform picture is easily obtainable. Therefore, the effect of several natural inhibitors on the hydrolysis of casein by rat skin homogenate was tested throughout pH 5–10. The data are presented in Fig. 8. As can be seen, the enzymatic caseinolysis at neutral and alkaline pH was not much affected by ovomucoid and soybean trypsin inhibitors, while marked inhibition was obtained with lima bean trypsin inhibitor as well as with rat and human plasma. The acidic enzymatic caseinolysis was about halved by all of the inhibitors tested.

Discussion

From earlier studies it was known that the pattern of proteolytic enzymes in the rat skin is quite complicated. Since the activities of the individual enzymes overlap extensively at each pH-value it is self-evident that all enzymologic studies performed on rat skin proteinases at the homogenate level can give only summary data of enzyme function. In addition, each of the enzymic activities may be modified by other components of the homogenate, *e.g.* by the binding of beneficial or harmful agents, by inert proteins present in the homogenate, etc. A certain amount of basic information can, however, readily be obtained from studies on tissue homogenates. Since systematic basic information on the whole proteolytic enzyme complex of the rat skin is not found in the literature, this kind of study was considered necessary.

Since it is known that tissue proteinases hydrolyse various proteins at a widely different rates, it is evident that proteinase assays with one single protein as substrate are only non-specific measurements of the total proteolytic activity in the tissue. With one protein as substrate, some proteinases may not be measured at all while the relative contribution of some other enzymes to the total proteolysis may be accentuated. An example was obtained with hemoglobin as substrate for the rat skin proteinases, since the enzyme(s) active at pH 6, revealed as a distinct peak of optimum with casein and albumin as substrate, was not demonstrable with hemoglobin. On the other hand, proteolytic activity at pH lower than 5 was very low toward casein and albumin but marked toward hemoglobin. On the alkaline side of neutrality the en-

zyme pattern demonstrated with all of these substrates was similar, but because of the markedly higher sensitivity of the assay with casein, this is to be preferred as substrate. Most of the earlier works on rat skin proteinases have been carried out at a neutral or slightly alkaline pH and therefore the usual choice of casein as a substrate is to be considered advantageous.

A systematic study of the individual variables in the scheme for the homogenization and extraction of the proteolytic enzymes, designed by us on the basis of preliminary experiments, showed that the procedure is to be considered appropriate. It pointed out that a fairly high salt concentration, *i.e.* about 8 per cent of potassium chloride is essential for a successful extraction of all of the components of the caseinolytic enzyme complex active at pH 5–10. This salt concentration is higher than that used in most of the works on skin proteinases, in which the scheme of extraction designed by Beloff and Peters, employing 5 per cent of potassium chloride, has been followed. It is lower than that suggested by Martin and Axelrod, who used 1.34 M (= 10 per cent) potassium chloride. It is apparent, however, that the extracts prepared according to any of the above investigators contain a part of all of the proteinases extractable at 8 per cent of salt concentration, only the yield is lower. The length of the homogenization and of the extraction as well as the pH and temperature of the extraction medium appeared each one to have a certain optimal range, even while all of the modifications used in earlier investigations should give satisfactory results. It was further apparent that the use of any special methods for extraction, such as freezing and thawing, Triton X-100 or acetone precipitation, do not improve the result of the extraction. In fact, the acetone treatment seemed to give yields inferior to those obained with a direct extraction. The all-over effectiveness of the extraction method used in this study was found to be quite good for each of the compoents of the caseinolytic complex.

All the main components of the proteinolytic enzyme complex appeared to be fairly stable during storage and during several physical treatments which are often used as steps in procedures of enzyme purification. This suggests that successful schemes for the purification of each of these enzymes may be designed without any extreme safety measures. Only the enzyme(s) hydrolysing casein at acid pH is considerably less resistant to acid and heat treatment than the other activities. All of the enzymes were found to be fairly unaffected in the homogenate by the few metal ions and cystein tested. More extensive studies on this line were not considered intelligible since it is often found that even very characteristic features of enzymes may be demonstrable only when purified enzyme preparations are used.

The fact that many of the skin proteinases were found to be effectively inhibited by natural enzyme inhibitors renders it quite likely that a part of the proteolytic activity in the homogenate may be in an inactive form and thus not demonstrable by usual assay procedures. This is in agreement with the reports of Beloff (2) as well as of Martin and Axelrod (13) on the presence of proteinase inhibitors in the blood and skin.

Beloff and Peters (1) already showed that there is a certain optimum of the salt concentration in the incubation medium for the enzymatic caseinolysis at neutral pH in the skin homogenate. Martin and Axelrod (13) confirmed this finding, and, in addition, demonstrated that still higher concentrations of salt caused an increasing dissociation of an inhibitor from an enzyme-inhibitor complex. Our findings demonstrate that several components of the caseinolytic complex are affected by the salt concentration in the incubation medium, each one in a different way. The two most alkaline caseinolytic activities, those demonstrable as optima at pH 8.5 and 9.2 in the pH-curve, were activated, the former optimally at about 300 mM and the latter at about 200 mM of potassium chloride. The acidic caseinolytic activity, on the other hand, was slightly inhibited by the addition of salt. Since the effects were, however, relatively low the assays may be

performed throughout the pH-range at one single salt concentration, preferentially at 200–300 mM of potassium chloride.

The results obtained in this study confirm and further expand many of the findings of earlier investigators. At several points they clarify some of the confusion caused by variances and limitations in earlier reports. The results give basic data on the main characteristics of the entire caseinolytic enzyme complex of the rat skin, and provide a basis for further studies, which are to be directed toward the separation of the individual enzymes.

SUMMARY

A scheme for the extraction of the caseinolytic enzymes active at pH 5.0–10.0 in the rat skin was presented, and the effectiveness of the individual steps of the procedure was analysed. The salt concentration of the extraction medium was found to be of decisive importance, 8 per cent of potassium chloride being optimal. Variations in the extraction time, pH, and temperature were of minor importance. Repeated freezing and thawing, Triton X-100, and acetone precipitation offered no advantage. The yield of the extraction was about 80 per cent. The enzyme complex was fairly stable toward storage, heat, acid treatment, dialysis, and metal ions. Distinct differences between the individual sub-units of the caseinolytic complex were, however, observed. Soybean and ovomucoid trypsin inhibitors had little effect on the caseinolytic complex, while the lima bean trypsin inhibitor as well as human and rat plasma caused a marked inhibition, especially on the alkaline side of neutrality. An increase in the ionic strength decreased the enzymatic caseinolysis at acid pH, while it enhanced the caseinolysis at neutral and alkaline pH, optimally at a concentration of 200–300 mM of potassium chloride.

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