Acta Dermatovener (Stockholm) 50: 412–418, 1970

PROTEOLYTIC ENZYMES IN THE SKIN, IV

Demonstration of Additional (Acidic) Proteinases in Mammalian Skin, and Basic Data on the Chromatographic Separation of the Total Proteinase Complex of the Rat Skin

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Abstract. Acidic hemoglobin-splitting enzymes with pH-optima at 3.5 and 5.0, were demonstrated in rat, guinea-pig, cat, hog and human skin. These enzymes, in contrast to the neutral and alkaline casein hydrolysing enzymes present in the skin of these species, were fully extracted at low ionic strength. The hemoglobinolytic enzymes were separated into two fractions of different molecular size in gel filtration on Sephadex G-100 of rat skin homogenate.

The caseinolytic enzymes of the rat skin were separated into three distinct peaks in the gel chromatographic procedure giving one fraction of high molecular weight enzymes (pH-optima 7.5 and 8.5), another fraction with enzyme(s) of medium molecular size (pH-optimum 5.8), and a third fraction with enzyme proteins salted out in the column during the chromatographic run.

Enzymes hydrolysing BANA were separated into two fractions, the one with higher molecular weight showing a pH-optimum at 7.8, and the other one being optimally active at pH 5.8. Both of the enzymes were highly dependent on sulfhydryl groups for activity.

Enzymes hydrolysing ester substrates ATEE and TEE were separated into three main fractions, the first one hydrolysing ATEE, only, the second one(s) hydrolysing both ATEE and TEE, and the third one hydrolysing TEE, only. These enzyme peaks were only partly coincident with proteinolytic peaks.

In earlier reports of this series of studies on the skin proteolytic enzymes the current status of knowledge on these enzymes was reviewed (2), and the apparent dearth of information was pointed out. This prompted a study of the proteolytic enzyme pattern in the skin of various species of experimental animals, and man (3). These studies revealed the presence of up to three alkaline casein splitting enzymes, one alkaline BANA hydrolysing enzyme, and 2–3 enzymes hydrolysing ATEE and TEE in the mammalian skin. The enzyme pattern was found to vary greatly from one species to another. A constant finding in all species was, on the other hand, an enzyme(s) active toward casein at about pH 6.0, and another enzyme(s) active toward BANA at the same pH. A close resemblance between the rat and the human skin proteinase pattern was pointed out. Studies on the basic characteristics of the caseinolytic enzyme complex in the rat skin homogenate with special reference to the optimal conditions for the extraction and handling of the different enzymic components of this complex have also been reported (4).

Reference to the presence in the rat skin of proteinases splitting hemoglobin at acidic pH was made in an earlier paper (4). In the first part of this paper we shall report basic data on these hemoglobinolytic acidic proteinases in the skin of the rat and of other mammalian species. The results of an inital fractionation of the total proteinase complex of the rat skin by using gel chromatography are reported in the second part of this paper.

MATERIAL AND METHODS

Skin samples and extraction procedures

Thirty male adult Long-Evans rats, weight 260–320 g, were used. Skin samples were obtained and treated as reported earlier (3). The homogenization of the samples and extraction of the enzymes followed the standard scheme outlined in an earlier publication (4). In addition, the supernate obtained from the centrifugation at 10 000 g was subjected to ultracentrifugation at 80 000 g at 0°C for 90 min, using on MSE ultracentrifuge. Thereafter the supernatant was passed through a Millipore® filter (pore size 0.8 μm).

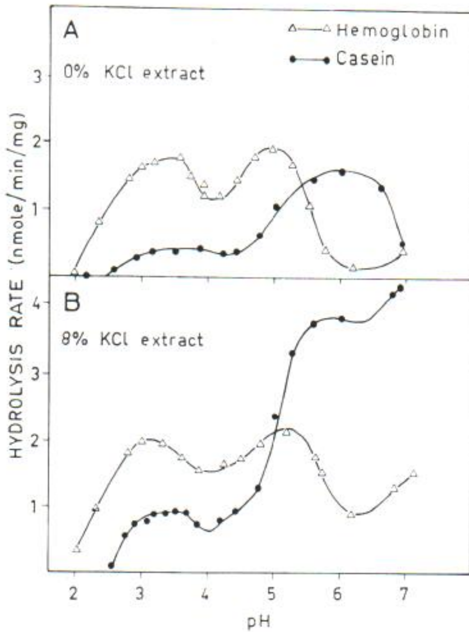

Fig. 1. Hydrolysis of hemoglobin and casein by the rat skin homogenate in the acidic pH-range. (A) An extract made in 10 mM phosphate buffer (8% KCl added prior to assay), (B) an extract made in 10 mM phosphate buffer containing 8% KCl. Incubation time 120 min.

Substrates, modifier substances and assay methods

Stock solutions of Casein nach Hammarsten (E. Merck, AG, Darmstadt), denatured hemoglobin (Nutr. Biochem. Corp., Ohio), N^{α}-benzoyl-*DL*-arginine 2-naphthylamide (BANA), *L*-tyrosine ethyl ester HCl (TEE), and *N*-acetyl-*L*-tyrosine ethyl ester (ATEE) (all from Sigma Chemical Company, St. Louis) were prepared as reported earlier (2, 3).

In studies on the effects of modifier substances on the enzymic activities, stock solutions in water of Lima bean trypsin inhibitor (LBTI, Sigma Chemical Company), *p*-chloromercuric benzoate (*p*CMB, Calbiochem, Los Angeles), mercapthoethanol (MCE, Fluka AG, West Germany) and ethylene tetra-acetic acid (EDTA, E. Merck) were prepared.

Incubation mixtures and assay procedures were the same as used in previous investigations (3, 4), except that incubation volumes were one-fifth of those used in earlier studies. Furthermore, *p*-dimethylamino benzaldehyde (100 mg in 10 ml of methanol and 10 ml of 1.0 M acetate HCl buffer pH 1.4) was used instead of diazonium salt for colour development in assays with BANA as substrate. The incubation times were varied as noted in the text. In assays in the acidic pH-range 0.2 M lactate-acetate buffer (pH 1.0–7.0) was used, while the universal buffer of Britton & Robinson (pH 3.0–12.0) (cf. 3) was used in neutral and alkaline assays. The actual pH of the incubation mixture was always measured using a pH-meter equipped with microelectrodes.

In studies on the effects of modifier substances 100 μl of the 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.

Protein determinations

The distribution of proteins in the chromatographic fractions was estimated by measuring the optical density at 280 nm in a Beckman DB spectrophotometer.

Gel filtration

Sephadex G-100 (Pharmacia, Uppsala) was pretreated according to the manufacturer's instructions and packed into a column of 10×100 cm. The column was equilibrated with 10 mM Tris-HCl buffer pH 7.0. A sample of 350 ml of the Millipore filtrate was introduced into the column and Tris-buffer was used for elution. The run was carried out at 4°C and fractions of 15 ml were collected. Both sample application and elution was performed by using a peristaltic pump producing an upward flow in the column.

Membrane ultrafiltration

For the concentration of the enzyme preparations obtained in the gel chromatography membrane ultrafiltration on Diaflo® membrane UM-10 (Amicon N.V., Holland) was used. This membrane retains molecules of a molecular weight over 10 000, while smaller molecules pass through.

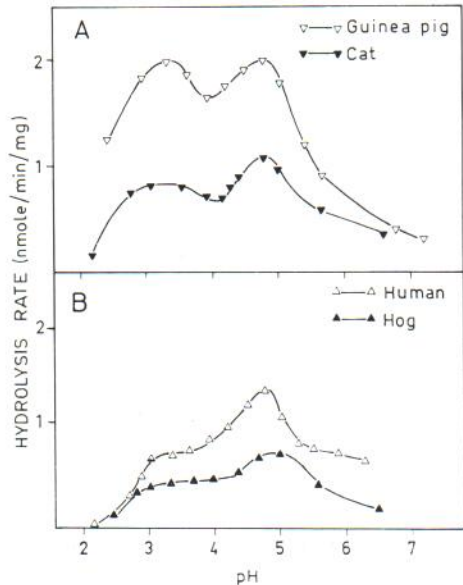

Fig. 2. Hydrolysis by the homogenates of guinea-pig, cat, human and hog skin of hemoglobin in the acidic pH-range. Incubation time 120 min.

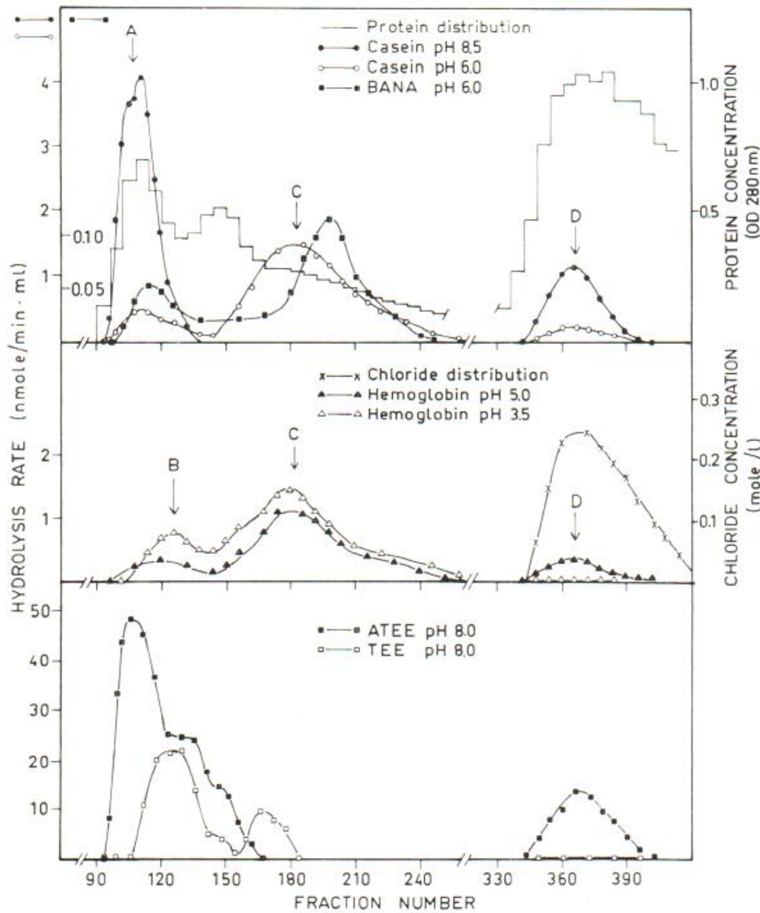

Fig. 3. Sephadex G-100 gel chromatography of the rat skin homogenate. Distribution of proteins, chlorides and of enzymes hydrolysing casein at pH 8.5 (4 h) and at pH 6.0 (10 h), BANA at pH 6.0 (20 h), hemoglobin at pH 5.0 (20 h) and at pH 3.5 (20 h), ATEE at pH 8.0 (2 h) and TEE at pH 8.0 (2 h). The figures in the brackets give the incubation times.

RESULTS

A. Hemoglobinolytic Proteinases in the Skin of Rat and Four Other Mammalian Species

Rat skin

The hydrolysis of protein substrates at pH-range 2–7 by a zero per cent KCl extract of the rat skin is shown in Fig. 1, upper part. A distinct optimum at pH 6.0 was obtained with casein as substrate, while a minimal rate of hydrolysis was recorded at more acidic pH. With hemoglobin as substrate, on the other hand, very low activity was recorded at pH 6.0, while a considerable hydrolysis of this substrate was recorded at lower pH-values, with two distinct optima at about pH 3.5 and 5.0.

An extract prepared in 8% KCl contained a higher caseinolytic activity at neutral and alkaline pH (Fig. 1, low part), which is in accordance with our earlier findings (4). In contrast to this, the yield of the acidic hemoglobinolytic enzymes was not affected by the presence of a higher ionic concentration.

Human, guinea-pig, cat, and hog skin

The pH curve obtained in tests on the rate of hydrolysis of hemoglobin at pH 2–7 by the homogenates of human, guinea-pig, rat and hog skin are shown in Fig. 2. Similar pH optima, i.e. at about pH 3.5 and about pH 5.0, were found in all of these mammalian species.

B. Fractionation of the Rat Skin Proteinase Complex by Gel Chromatography

Hydrolysis of casein by the chromatographic fractions

The distribution in the chromatographic fractions of proteins and of the enzymes hydrolysing casein at alkaline and acidic pH is shown in Fig. 3, upper part. Proteins were eluted out as three main peaks corresponding to the molecular size of globulins, albumin and peptides. The hydrolysis of casein revealed three separate peaks in the chromatogram, designated peaks A, C and D.

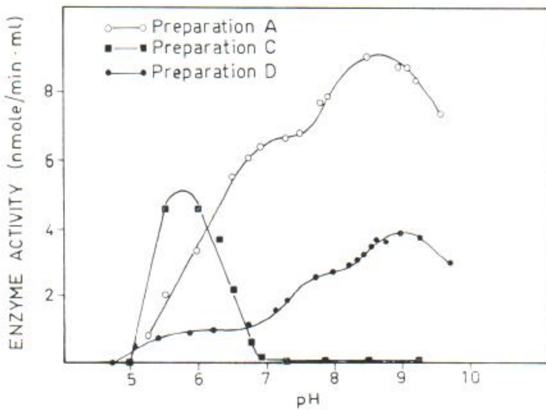

Fig. 4. pH-Dependence of the hydrolysis of casein by the pooled and 10-fold concentrated enzyme preparations A, C, and D obtained in the gel chromatography depicted in Fig. 3. Incubation time 120 min.

The enzymic activity towards casein in the fractions of peaks A and D was high at alkaline pH and low at acid pH. The hydrolyis of casein by the fractions of peak C could be demonstrated only at acid pH.

Fractions 90–120 were combined and the preparation obtained was designated pool A, combination of fractions 160–210 yielded pool C, and combination of fractions 350–380 yielded pool D. The pooled preparations were concentrated 10-fold by using membrane ultrafiltration. The pH-dependence of the enzymic hydrolysis of casein by the enzyme preparations was tested and the results are presented in Fig. 4. The pH-curve obtained with pool A as enzyme showed two separate optima, one at about pH 7.5 and the other one at around pH 8.5. Only one optimum, at about pH 6, was obtained with pool C as enzyme. The hydrolyis of casein by pool D was low at acid pH and most marked at about pH 9.0.

The addition of MCE (1 mM), EDTA (1 mM), or *p*CMB (0.03 mM) did not significantly affect the enzymic activity toward casein in any of the pooled preparations, nor was it sensitive to increased neutral salt concentration (KCl 0–1.0 M).

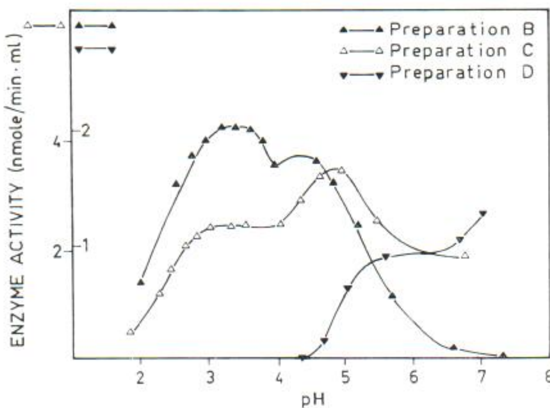

Fig. 5. pH-Dependence of the hydrolysis of hemoglobin by the pooled and 10-fold concentrated enzyme preparations B, C, and D obtained in the gel chromatography depicted in Fig. 3. Incubation time 2 h for preparation C and 5 h for preparations B and D.

Hydrolysis of hemoglobin by the chromatographic fractions

The distribution of the enzymes hydrolysing hemoglobin at pH 3.5 and pH 5.0 is shown in Fig. 3, middle part. Three peaks of enzymic activity were recorded, the first one (designated peak B) being eluted out somewhat later than the caseinolytic peak A, the second one being located in the same fractions as the caseinolytic peak C, and the third one located in the same fractions as the caseinolytic peak D. Fractions 120–140 were combined and concentrated 10-fold to give enzyme preparation B. The pH-optima obtained for the preparations B, C and D are given in Fig. 5. Two of the preparations (B and C) showed pH-optima at about pH 3.5 and 5.0, while in one of them hydrolysis was recorded in the less acid pH-range, only. Tests with MCE, EDTA, *p*CMB and LBTI (3 mg/ml) revealed no significant effect of these substances on the enzymic activities toward hemoglobin. An inhibitory effect was, however, recorded when the concentration of KCl in the incubation medium was increased (Fig. 6).

Hydrolysis of the naphthylamide substrate by the chromatographic fractions

The hydrolysis of BANA by the fractions of the chromatographic run is presented in Fig. 3, upper part. Two peaks were recorded, one being located in the same fractions as the caseinolytic peak A, while the second one was located in somewhat later fractions than the proteinase peak C. The pH-optimum for the hydrolysis of BANA by the first enzyme preparation was 7.8, while the optimum for the second enzyme preparation was at 5.8 (Fig. 7). Both enzymes were strongly dependent on sulfhydryl groups for their activity; they were inactivated by sulfhydryl blocking agents

(*p*CMB 0.03 mM) and activated by mercaptoethanol (1 mM). The latter effect was potentiated by EDTA (1 mM) (Fig. 8). LBTI and high KCl concentrations did not affect these enzymes.

Hydrolysis of the ester substrates by the chromatographic fractions

The hydrolysis of the esters ATEE and TEE by the fractions of the Sephadex chromatogram is presented in Fig. 3, lower part. The chromatogram suggested the presence of one ATEE-hydrolysing enzyme coincident with the caseinolytic peak A. Both ATEE and TEE were, on the other hand, hydrolysed in one (or possibly two) esterolytic peak(s) located in the same fractions as the hemoglobinolytic peak B. An additional peak of esterolysis, demonstrable with TEE but not with ATEE as substrate, was recorded coincident with the first part of the proteinase peak C. Hydrolysis of ATEE, but not of TEE, was recorded in the fractions of proteinase peak D.

Clearcut optima for these enzymes were hard to obtain because of the overlapping of the enzyme peaks. However, the enzymes active toward ATEE, only, appeared to be optimally active somewhere around pH 8.0–9.0, and the enzyme hydrolysing TEE only was optimally active at about pH 7.0–8.0.

DISCUSSION

Earlier comparative studies on the skin proteinases of several mammalian species have revealed the presence of several caseinolytic enzymes active at neutral or alkaline pH (4). The present studies disclose the presence of acidic proteinases in the skin active preferentially on hemoglobin. While the pattern of caseinolytic enzymes is known to vary from species to species, the acidic hemoglobinolytic proteinase pattern was quite similar in all of the species studied showing pH-optima at about 3.5 and 5.0. In contrast to the caseinolytic enzyme complex of the rat skin, which is

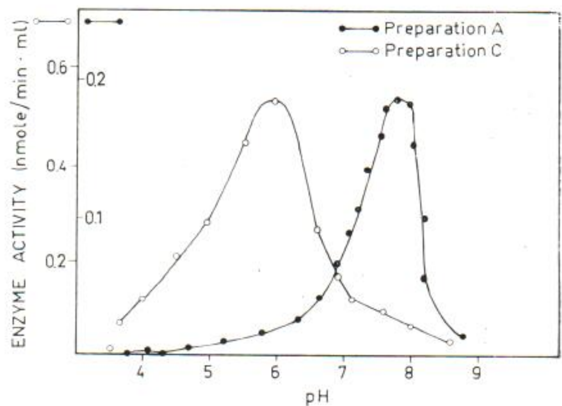

Fig. 7. pH-Dependence of the hydrolysis of BANA by the pooled and 10-fold concentrated enzyme preparations A (incubation time 4 h) and C (incubation time 2 h). Incubation solutions contained 1 mM of MCE and EDTA. A preincubation for 10 min was allowed before the addition of substrate.

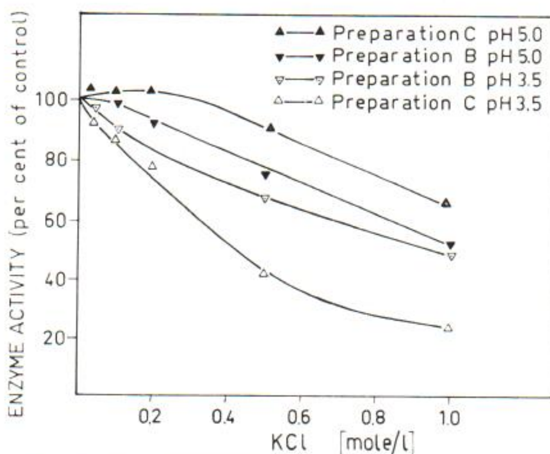

Fig. 6. Effect of increasing concentrations of KCl on the hydrolysis of hemoglobin by the concentrated hemoglobinolytic enzyme preparations B (incubation time 5 h) and C (incubation time 2 h).

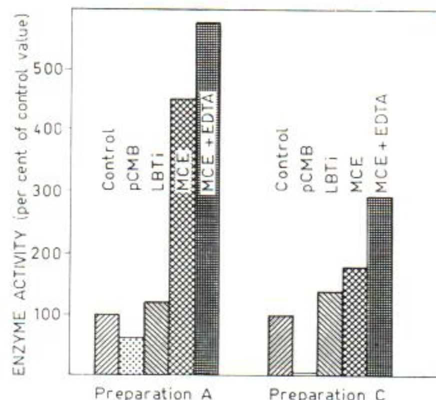

Fig. 8. Effect of *p*CMB (0.025 mM), LBTI (2.5 mg/ml), MCE (1 mM) and MCE + EDTA (1 mM + 1 mM) on the hydrolysis of BANA by the pooled and 10-fold concentrated enzyme preparations A and C. Preincubation time was 10 min and incubation time 4 h for preparation A and 2 h for preparation C. Further details are given in "Materials and Methods".

known to be extracted fully only at quite high an ionic strength, the hemoglobinolytic enzyme complex is well extractable in solutions of low ionic strength.

Gel chromatographic fractionation of rat skin homogenate extract revealed the presence of more than one molecular form of the hemoglobinolytic enzymes. The pH-optima obtained from the partly separated enzyme preparations (B and C) appeared to be the same as those obtained with the whole skin extract. Additional features were independence of sulfhydryl groups and metal ions as well as inhibiton by high ionic concentration. These enzymes are to be considered to beiong to the class of catheptic enzymes of the type cathepsin D and E, earlier described to be present in several mammalian organs (1, 5, 11).

Gel chromatography of rat skin extract made in 8% KCl gave three distinctly separate peaks of caseinolytic activity. The first one (peak A) was eluted in the void volume and thus contains proteins or protein complexes with high molecular weight, i.e. 200 000–300 000 or higher. Proteins contained in the second caseinolytic peak (peak C) are of considerably smaller size, corresponding roughly to molecular weights of 50 000–100 000. The third caseinolytic peak (peak D) was eluted in very late fractions. Molecules with such a marked retardation in a column of Sephadex G-100 are very small, e.g. small peptides and inorganic salts. In fact, chloride analysis showed that the chloride ions applied into the column in the 8% KCl extract sample were eluted in the same fractions as this caseinolytic enzyme(s) (Fig. 3, middle part). The presence of enzymic activity in the chromatographic fractions consisting of molecules of this size is surprising, since enzymes with proteolytic activity must be supposed to be by themselves of the size of proteins. An apparent explanation for this phenomenon is that the retardation of enzymically active protein molecules was not due to low molecular weight but to precipitation of the molecules in the column because of decrease of the ionic strength in the protein front (salting out). The front of neutral salts moving behind the protein front would dissolve the precipitated proteins (salting in) and thus carry the proteins through the column. In fact, separate experiments have proved that this explanation is a correct one (data to be reported).

The form of the pH-curve obtained for the caseinolysis by the pooled preparation A suggests the presence in this preparation of alkaline enzymes with possible pH-optima at about 7.5 and 8.5, while acidic caseinolytic enzyme(s) is present in preparation C, optimally active at pH 5.8. These data are in accordance with the data obtained in studies on rat skin homogenate (3, 4). The form of the pH-curve for caseinolysis obtained from pool D supports the view that this preparation contains the same enzymes as present in the earlier fractions of the chromatogram. Any classification of these caseinolytic enzymes must await a further fractionation and purification of the enzymes of this complex.

The separation of two enzymes active toward BANA, a substrate known to be hydrolysed by trypsin and similar esteropeptidases, is also a finding which is in accordance with our earlier suggestions based on findings on rat skin homogenate. The enzymes appear to differ markedly to their molecular size, but both of them seem to be highly dependent on sulfhydryl groups, and are easily inactivated during the chromatographic procedures. The more acidic one of the enzymes, active optimally at pH 5.8, could be tentatively identified as cathepsin B′, recently described in bovine spleen (9, 10). The more alkaline enzyme, optimally active at pH 7.8, on the other hand, does not appear to have any counterpart among the well known proteinases.

The results obtained with the ester substrates ATEE and TEE, substrates known to be hydrolysed by chymotrypsin and similar esteropeptidases, reveal the partial fractionation of several esterolytic enzymes of a fairly high molecular weight. One of them (peak in fraction 105) is active toward ATEE, another one probably active toward both ATEE and TEE (peak in fraction 130 and another possibly in fraction 140) and a further enzyme (peak in fraction 165) which is active toward TEE, only. The relation of these enzyme activities to the caseinolytic and hemoglobinolytic enzymes present in the same fractions remains to be elucidated. At present it appears, however, to be evident that not all ester hydrolysis is related to proteolysis nor is all proteolysis related to ester hydrolysis demonstrable with ATEE and TEE as substrates. These results are by and large concordant with the data reported by Martin & Axelrod (6, 7, 8), even while a closer comparison is not possible at present.

The results of this report reveal that the complex pattern of rat skin proteolytic enzymes can be divided into clearcut subgroups with definite characteristics by using a single gel chromatographic run. Some of the enzymes can be tentatively classified on basis of the characteristics obtainable at this stage of enzyme separation, while a more elucidative analysis of the other enzymes can be obtained only after further separation and division of the enzyme complex by employing additional chromatographic measures.

ACKNOWLEDGEMENT

Financial support was obtained as grants from the Sigrid Juselius Foundation and from the Finnish Medical Council to the senior author (V. K. H.-H.).

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Received June 18, 1970

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