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PROTEOLYTIC ENZYMES IN THE SKIN

V. *Extraction and Column Chromatographic Separation of the Human Skin Proteinases*

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Abstract. Extractability of the human skin proteolytic enzymes hydrolysing casein, hemoglobin and BANA was found to be dependent on the ionic strength of the extraction medium. The human skin was found to contain a BAPA-hydrolysing salt-extractable enzyme not present in the skin of rat, pig and guinea-pig. The enzymes of the human skin were separated by gel chromatography on Sephadex G-100 into a high and a low molecular weight group. The former contained neutral and alkaline caseinolytic enzymes, an enzyme hydrolysing hemoglobin optimally at pH 5.0, an enzyme hydrolysing BANA optimally at pH 6.0 and the BAPA hydrolysing enzyme (pH-optimum 7.8). The low molecular weight enzyme group contained an enzyme hydrolysing casein optimally at pH 6.0, another enzyme hydrolysing hemoglobin optimally at pH 3.5 and a SH-dependent enzyme hydrolysing BANA optimally at pH 6.0. The pattern and chromatographic behaviour of human and rat skin proteinases are compared.

The skin homogenates of several mammalian species have been shown to hydrolyse the protein substrates casein and hemoglobin, as well as BANA, a synthetic substrate for trypsin (4, 6). The total enzyme pattern of the rat's skin was found to resemble closely that of human skin. The extractability and chromatographic behaviour of these hydrolytic enzymes in the rat skin have already been investigated (5, 6). In this paper the extractability of the proteolytic enzymes which hydrolyse these substrates in the human skin are explored. In addition, an enzyme hydrolysing the trypsin substrate BAPA is shown to be present in human skin. Preliminary results of the chromatographic separation of the proteolytic enzymes of human skin are also reported.

MATERIAL AND METHODS

Skin samples

Samples of human skin were obtained in connection with mammectomies (Surgical Clinic, Turku University Hospital). The skin was immediately removed and frozen to −20° for 1–7 days. Homogenization was carried out in 10 mM phosphate buffer pH 8.0 containing 8% KCl, as reported earlier (5). The homogenate was centrifuged in an International refrigerated centrifuge at 10 000 *g* for 20 min at +2°C. The supernatant was recentrifuged in a MSE Superspeed 50 ultracentrifuge at 100 000 *g* for 2 hours. The supernatant was used for further analysis after filtration through a Millipore® filter (pore size 0.8 μm).

Substrates and chemicals

Casein nach Hammarsten (E. Merck AG, Darmstadt, BRD) 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, not longer than 5 days.

Denatured hemoglobin (Nutr. Biochem Corp., Ohio, USA) was dissolved in 10 mM phosphate buffer pH 8.0 (2 g/100 ml) and used freshly.

N-α-benzoyl-*DL*-arginine-β-naphthylamideHCl (BANA; Sigma Chem. Comp., St. Louis, USA) was dissolved in distilled water (5 mM) by heating to 70°C and used freshly.

N-α-benzoyl-*DL*-arginine-*p*-nitroanilideHCl (BAPA; Sigma) was dissolved in distilled water (1 mM) by heating at 70°C for 10 min and used freshly.

Ethyl-di-amino-tetra-acetic acid (EDTA, Titriplex® III; E. Merck AG), 2-mercaptoethanol (Fluka AG, Buchs SG, Switzerland), *p*-chloromercuribenzoate (pCMB; Fluka AG) and E-600 (Mintacol, *p*-nitrophenol-diethyl-phosphoric acid, (Bayer, Leverkusen, BRD) were used as aqueous solutions.

Buffers

Universal buffer of Britton and Robinson pH 3.0–12.0 (3) and lactate-acetate buffer 1.0–7.0 were used for studies of pH optima. Phosphate buffer pH 8.0 was used for

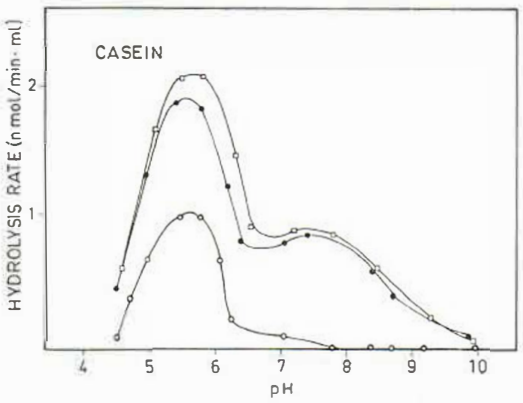

Fig. 1. The effect of salt concentration on the extractability of the caseinolytic enzymes of human skin. The percentage values in the figure are the concentrations of potassium chloride used in different extraction procedures. ○—○, 0% KCl; ●—●, 2% KCl; □—□, 8% KCl.

homogenizations, and Tris-HCl buffer 10 mM pH 7.0 for chromatographies.

Assay methods

For the measurement of hydrolysis of the substrates the incubation medium consisted of 0.1 ml of buffer solution, 0.1 ml of substrate and 0.1 ml of enzyme solution. Incubation was carried out in a water bath at 37°C.

Assay procedures for casein, hemoglobin and BANA were the same as used in previous investigations (4, 5, 6). With BAPA as substrate incubation time was 1–2 hours. Deproteinization was carried out by adding 0.1 ml of 10% zinc sulphate, the mixture was centrifuged, 0.2 ml of Na-acetate buffer pH 4.2 was added to 0.2 ml of the supernatant and the optical density was measured at 383 nm. A standard curve for the hydrolysis was prepared using *p*-nitroaniline. The enzymic activity is expressed as the rate of *p*-nitro-aniline liberation (nmol/min ml).

Modifier tests were carried out by mixing the modifier in the buffer and preincubating the enzyme in this solution. Mercaptoethanol and EDTA were used in 1 mM, E-600 in 0.1 mM and pCMB in 0.03 mM concentration. Preincubation time was 15 min except for E-600 which was preincubated for 1 hour.

The actual pH of the incubation mixtures was measured in the middle of the incubation period with a pH-meter equipped with micro-electrodes. The contribution of spontaneous hydrolysis of the substrates was eliminated by using controls with boiled enzyme.

Protein determinations

The protein concentrations of the homogenates and chromatographic fractions were measured according to Lowry et al. (3). Bovine serum albumin was used as standard.

Chromatography

Sephadex G-100 (Pharmacia, Uppsala, Sweden) was pretreated according to the manufacturers' instructions and packed into a column 2.5 × 100 cm or 10 × 100 cm. The columns were equilibrated and the elution was performed with 10 mM Tris-HCl buffer, pH 7.0. Sample sizes were 20 ml for the small column and 300 ml for the large column. The fraction sizes were 4.5 and 15 ml, respectively. Peristaltic pumps were used for application and elution.

Membrane ultrafiltration

Membrane ultrafiltration on Diaflo® membrane UM-10 (Amicon N.V., Holland) was used for the concentration (5–10 fold) of the enzyme preparations obtained at gel chromatography. This membrane retains molecules of a molecular weight of over 10 000.

RESULTS

A. *Extractability of the enzymes*

Casein hydrolysing enzymes of the human skin were strongly dependent on the ionic strength for optimal extraction, as shown in Fig. 1. An extract obtained in a buffer of low ionic strength showed an activity at pH 5–6, with no hydrolysis at more alkaline pH. An optimal extraction of both acidic and alkaline enzymes was obtained with an extraction buffer containing 2% KCl.

Hemoglobin hydrolysing enzymes of the human skin were readily extracted in a buffer of low ionic strength (Fig. 2.). They showed pH-optima at 3.5 and 5.0. An increase of the KCl concentration in the extraction buffer from zero to 8% roughly doubled the yield of the enzyme active at pH 5.0, while the effect on the enzyme(s) active at pH 3.5 was considerably smaller.

BANA hydrolysing enzymes of the human skin were also readily extracted in a buffer of low

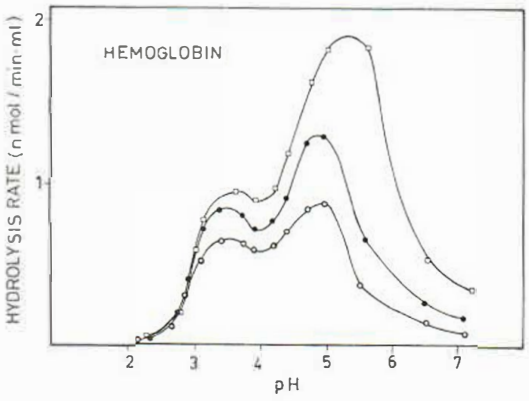

Fig. 2. The effect of salt concentration on the extractability of the hemoglobinolytic enzymes of human skin. Symbols as in Fig. 1.

ionic strength (Fig. 3). They showed pH-optima at 5–6 and 7–8. The activity at the more acidic pH was approximately doubled by an increase in KCl concentration of the extraction buffer to 8 %.

BAPA hydrolysing activity was not present in the human skin extract of low ionic strength (Fig. 4). The presence of salt in the extraction medium, however, yielded a strong enzyme activity with optimum at about pH 7.5.

Since hydrolysis of BAPA by the homogenate of rat skin is known not to take place (unpublished data), it was of special interest to look for it in the guinea-pig, cat and pig skin homogenate. No such activity was found in the skin homogenates of these species, except for a low activity in cat skin.

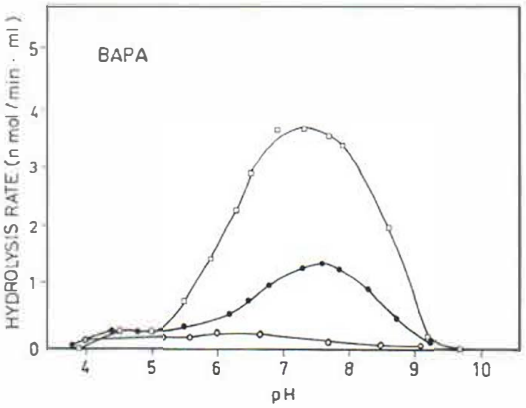

Fig. 4. The effect of salt concentration on the extractability of BAPA-hydrolysing enzymes of human skin. Symbols as in Fig. 1.

B. *Gel chromatographic fractionation*

Casein hydrolysing enzymes. The distribution of proteins and of enzymes hydrolysing casein is shown in Fig. 5, upper part. Proteins were eluted as three main peaks, the last one representing peptides and precipitated protein moving with the salt front (6). Caseinolytic activity was recorded as two peaks, the first one containing enzyme(s) active preferentially at alkaline pH, and the second one containing enzyme(s) active mainly at acidic pH. The pH-curve of casein hydrolysis by a pooled preparation of the first enzyme peak (fractions 40–50) is shown in Fig. 6. The broad pH-curve suggests the presence of more than one caseinolytic enzyme. These caseinolytic activities were unaffected by above-mentioned enzyme modifiers.

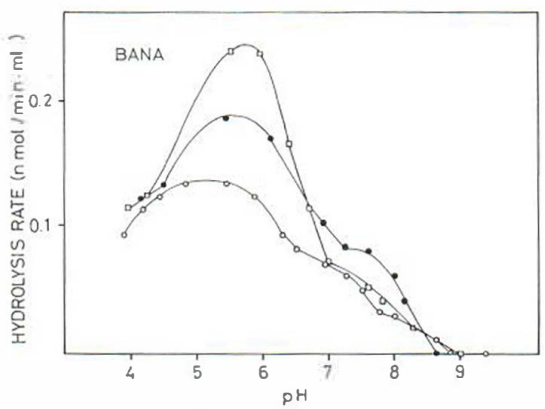

Fig. 3. The effect of salt concentration on the extractability of BANA-hydrolysing enzymes of human skin. Symbols as in Fig. 1.

The pH-curve of casein hydrolysis, by a pooled preparation of the second enzyme peak (fractions 60–70), is shown in Fig. 6. An optimal rate of hydrolysis was recorded at pH 5–6 and hydrolysis at the neutral and alkaline pH was negligible. Hydrolysis of casein by this preparation was not effected by the above sulfhydryl compounds or reagents, metal chelator or organophosphorus compound.

Hemoglobin hydrolysing enzymes. Distribution of the enzymes hydrolysing hemoglobin is shown in Fig. 5, lower part. Two hydrolysis peaks were recorded, coinciding with the two caseinolytic activity peaks. Hemoglobinolytic activity in the first enzyme peak was optimal at pH 5.0 and minimal at pH 3.5 (Fig. 7) while the reverse was true for the second hemoglobinolytic enzyme peak.

BANA hydrolysing enzymes. As seen in Fig. 5, lower part, two hydrolysis peaks were obtained with BANA as substrate. The first activity peak coincided with the first caseinolytic peak. It showed a pH-optimum at about 6.0 (Fig. 8) and was affected slightly by pCMB or mercaptoethanol. The second peak, on the other hand, was eluted somewhat later than the second caseinolytic enzyme peak. This enzyme(s), too, showed a pH-optimum at about 6 (Fig. 8), but it was strongly dependent on sulfhydryl groups for activity since the activity was approximately fourfold in the presence of 1 mM mercaptoethanol and totally inhibited in the presence of 0.03 mM pCMB. EDTA and E-600 did not affect the function of either enzyme.

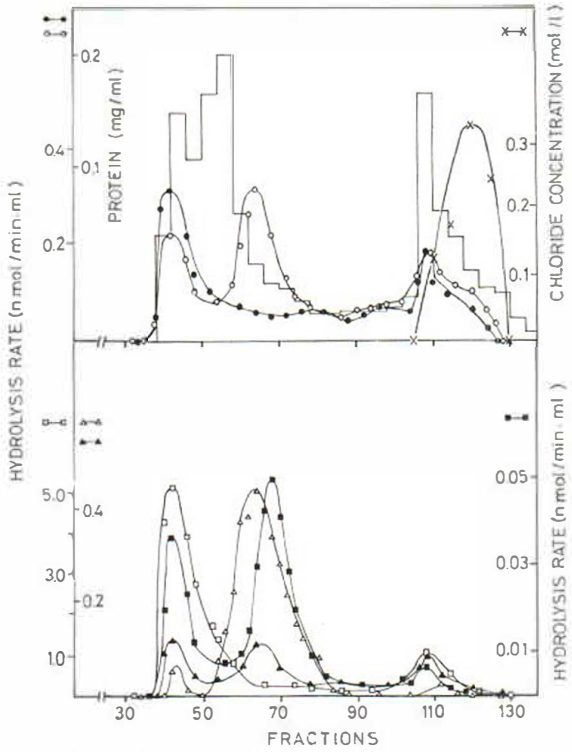

Fig. 5. Fractionation of the human skin extract by gel chromatography on Sephadex G-100 column. Distribution of enzymes is shown by the names of the substrates and pH values of the assays. —, protein distribution; × —×, chloride distribution; ○—○, casein pH 6.4; ●—●, casein pH 7.5; ▲—△, Hb ph 3.9; ▲—▲, Hb pH 5.5; ■—■, BANA pH 5.3; □—□, BAPA pH 7.8.

BAPA hydrolysing enzymes. One highly active enzyme peak hydrolysing this substrate was recorded in the chromatogram coincident with the first caseinolytic enzyme peak. pH-Optimum for the enzyme was recorded at pH 7.8, as seen in Fig. 9. The enzyme activity was not significantly affected by pCMB, mercaptoethanol, EDTA or E-600.

The enzyme peak eluted late in the chromatogram (fractions 100–120) containing hydrolytic activity towards BAPA as well as towards casein, hemoglobin and BANA was shown to represent enzymes from the earlier part of the chromatogram, salted out during the chromatographic run (cf. 6).

DISCUSSION

As reported earlier (4), the total proteinase patterns of rat and human skin, as deduced from

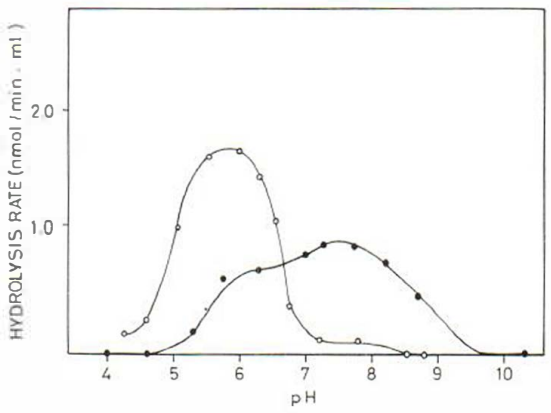

Fig. 6. pH-Curves for the hydrolysis of casein by the preparations obtained by combining the fractions of the first and second caseinolytic peaks in Fig. 5. ●—●, casein, first peak; ○—○, casein, second peak.

studies on skin homogenates, are very similar. Extractability of some of the enzymes in the skin of the rat is known to depend on the ionic strength of the extraction medium (2, 5, 6, 10). Our present data indicate that the same is true for human skin proteolytic enzymes which hydrolyse casein, hemoglobin and BANA.

Studies with human skin homogenate have revealed the presence of a strong enzyme activity hydrolysing BAPA at pH 7.8. It was not present in the other species studied (or only present as inactive zymogens, data to be presented). This enzyme is particularly strongly dependent on high KCl concentration for an optimal extraction.

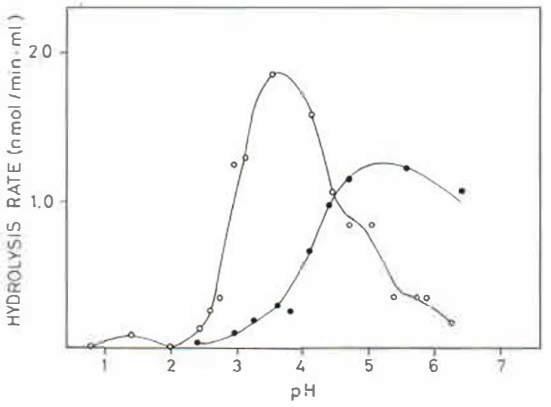

Fig. 7. pH-Curves for the hydrolysis of hemoglobin by the preparations obtained by combining the fractions of the first and second caseinolytic peaks in Fig. 5. ●—●, Hb, first peak; ○—○, Hb, second peak.

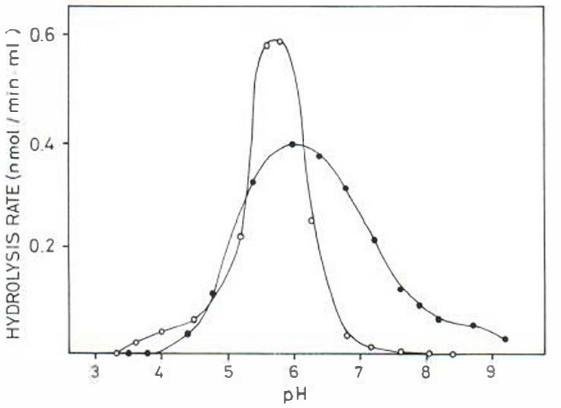

Fig. 8. pH-Curves for the hydrolysis of BANA by the preparations obtained by combining the fractions of the first and second caseinolytic peaks in Fig. 5. ●—●, BANA, first peak; ○—○, BANA, second peak.

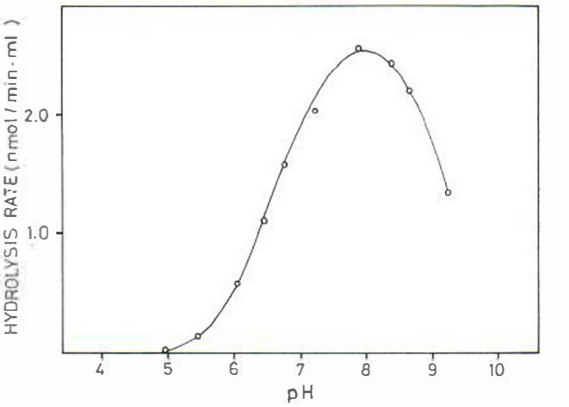

Fig. 9. pH-Curves for the hydrolysis of BAPA by the preparation obtained by combining the fractions of the first caseinolytic peak in Fig. 5. ○—○, BAPA, first peak.

Gel chromatography, in which separation of protein is based on differences in the molecular size, caused a division of the proteolytic enzymes of the human skin into two main groups. The enzymes present in the high molecular weight fractions (fractions 40–50) were active towards casein, hemoglobin, BANA and BAPA, while the enzymes in the lower molecular weight fractions were active towards casein, hemoglobin and BANA. The chromatogram resembles that obtained from rat skin extract (6). In particular, the locations of the two caseinolytic enzyme peaks are quite similar to those obtained in identical runs with rat skin homogenate (suggesting similar molecular size).

A comparison of the main features of the chromatograms of rat and human skin proteases is presented in Table I. Both species showed an enzyme activity hydrolysing BANA in the void volume fractions, but the human enzyme has a more acidic pH-optimum (pH 6.0 as compared with pH 7.8) and it is less dependent on sulfhydryl groups for activity. The potent BAPA hydrolysing enzyme, present only in the human skin, with a fairly high pH-optimum (7, 8), is apparently a separate enzyme which hydrolyses BANA minimally (cf. the low alkaline hydrolysis of BANA

Table I. *Schematic comparison of the proteinases in human and rat skin chromatograms*

Fractions	Human skin	Rat skin
Fractions of the first caseinolytic peak (void volume fractions)	Caseinolysis (neutral and alkaline) BANA-hydrolysis (pH 6.0, not SH-dependent) BAPA-hydrolysis (pH 7.8, not SH-dependent) Hemoglobinolysis (pH 5.0)	Caseinolysis (neutral and alkaline) BANA-hydrolysis (pH 7.8, SH-dependent) --- ---
Intermediate fractions	---	Hemoglobinolysis (pH 3.5 and 5.0)
Fractions of the second caseinolytic peak	Caseinolysis (pH 5.8) Hemoglobinolysis (pH 3.5)	Caseinolysis (pH 5.8) Hemoglobinolysis (pH 3.5 and 5.0)
Later fractions	BANA-hydrolysis (pH 5.8, SH-dependent)	BANA-hydrolysis (pH 5.8, SH-dependent)

in Figs. 3 and 8). It is probably this enzyme, which was measured by Zaruba et al. (14) in fetal skin, but after an unfavourable extraction (water extract) and at a less suitable pH (5.5). In addition, both of the species appeared to have a low molecular weight, sulfhydryl-dependent BANA-hydrolysing enzyme. This enzyme represents cathepsin B′, an intracellular protease isolated from bovine spleen by Otto and co-workers (11, 12). This enzyme is to be distinguished from cathepsin B, which hydrolyses benzoyl-arginyl-amide (BAA) but not BANA or BAPA.

The skin of both species contains a low molecular weight acidic hemoglobinolytic enzyme(s) which coincides with the second caseinolytic peak of the chromatograms. Both species also contain an acidic hemoglobinolytic enzyme(s) of higher molecular weight, the human enzyme(s) being eluted in the void volume fractions while the rat enzyme(s) is eluted somewhat later. The chromatographic separation of these acidic hemoglobinolytic enzymes, one optimally active at pH 5.0 and the other at pH 3.5, confirms earlier findings made on human skin homogenate (6, 7). These enzymes are representatives of group D and E cathepsin (1, 8, 13).

These findings confirm the concept of an overall similarity of the protease complexes of the human and rat skin, even though certain clear-cut differences exist. A thorough analysis of the skin proteases of both of these species, as well as of others, is a prerequisite for the interpretation and integration of data obtained in experimental laboratory studies and observations made on normal and diseased human skin.

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