Indian Journal of Biochemistry & Biophysics
Vol. 12, December 1975, pp. 321-325

Purification and Properties of a Neutral Proteinase Isolated from Goat Skin

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Received 28 *January* 1975; *accepted* 28 *August* 1975

A neutral proteinase isolated from goat skin was purified and its physico-chemical properties were studied. The six different treatments when given successively to the original enzyme extract resulted in a 122-fold purification retaining 59% of the original activity. The purified enzyme was found to be homogeneous and capable of hydrolysing casein, egg albumin and haemoglobin although to different degrees. Of the skin proteins studied, albumin, globulin and mucoid were readily hydrolysed by the proteinase, whereas collagen and elastin remained unaffected. The enzyme activity was optimum between *p*H 7.5 and 8.0 in case of almost all protein substrates. The enzyme was found to be quite stable at a *p*H range of 6.5 to 8.5. The enzyme was fairly thermolabile and was found to lose its activity when heated at a temperature above 60°C and exposed to ultraviolet rays. The enzyme was inactivated by thiol group, metal chelating agents and heavy metal ions.

A critical review[1] of the literature on skin proteolytic enzymes reveals that neutral proteinases, active in the neutral or slightly alkaline *p*H range, are present in different skin extracts. But they differ in respect of their properties and activities[2]. Very few attempts have been made to isolate and purify the neutral proteinases from skins. Lazarus and Barrett[3] have recently reported the purification and properties of a neutral proteinase isolated from rabbit skin. This enzyme is capable of degrading skin proteins and inducing inflammatory response. The proteolytic enzyme system in goat skin has not been studied so far. Recent studies[4] have shown that the proteolytic enzyme in goat skin increases considerably on incubation for few days in moist condition after a prior treatment with suitable preservatives, e.g. sodium pentachlorophenate or zinc dimethyldithiocarbamate and results in the unhairing of goat skin. It has also been observed[5] that unhairing action is quicker at *p*H range of 7.0 to 8.0. These observations apparently suggest that a neutral enzyme active at *p*H range of 7.0 to 8.0 might be responsible for the unhairing of skin. With this view in mind, an attempt has been made to isolate and purify the neutral proteinase from goat skin and to study the physico-chemical properties of the purified enzyme.

Materials and Methods

Preparation of enzyme extract from skin — Fresh goat skin pieces were first washed in plain water and treated with a preservative solution (sodium pentachlorophenate 2% and water 400% both on skin weight) for a period of 24 hr. The skin pieces were then taken out of the preservative bath, excess water was allowed to drain away and they were then incubated inside a moist chamber maintained at room temperature (30° ± 2°C) for a period of 72 hr. The proteolytic enzyme was extracted from skin with distilled water at the time of unhairing when the enzymic activity was maximum.

Estimation of protein — The protein at different stages of purification of the enzyme extract was estimated according to the method of Lowry *et al.*[6] as standardised by Dhar and Bose[7] except that crystalline bovine serum albumin was used as reference protein in place of crystallised trypsin.

Estimation of proteolytic activity — For the quantitative estimation of the proteolytic activity of the enzyme extract, Anson's[8] colorimetric method of estimating the liberated tyrosine during proteolysis as adopted by Bose *et al.*[9] was followed. A suitable aliquot (2 ml) of enzyme solution was added to 2 ml of 2.5% solution of a protein (wt/vol.) taken in veronal buffer and incubated at 45°C for a period of 30 min. An aliquot (8 ml) of 5% TCA was added, warmed on the water-bath and filtered. The filtrate was analysed for tyrosine content. A control experiment was always run in an identical manner except that TCA was first added to the protein solution and then the enzyme extract was added to the mixture.

Unit and specific activity — A unit of proteolytic activity was defined as the amount (mg) of tyrosine liberated from 4 ml reaction mixture under experimental conditions mentioned above, and the specific activity of the enzyme solution was calculated as the number of enzyme units per mg of enzyme protein.

Preparation of gel — Calcium phosphate gel was prepared according to the method of Keilin and Hartree[10].

DEAE-cellulose column chromatography — DEAE-cellulose was packed into a column of 80 × 4·5 cm. The column was equilibrated with 0.01 *M* phosphate buffer (*p*H 6.5). Enzyme powder (900 mg) was

Abbreviations used : BAEE, *N*-α-benzoyl-L-arginine ethyl ester HCl; BAPNA, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide HCl; BAA, N-α-benzoyl-L-arginine amide HCl; ATEE, *N*-α-acetyl-L-tyrosine ethyl ester Monohydrate, DFP, di-isopropyl-fluorophosphate; DEAE-cellulose, diethylaminoethyl cellulose; TCA, trichloroacetic acid; BANA, *N*-α-benzoyl-DL-arginine-β-naphthylamide, HCl.

dissolved in 50 ml of 0.01 *M* phosphate buffer and applied to the column. One hundred fractions were collected by straight elution with 0.01 *M* phosphate buffer (*p*H 6.5) and 60 fractions by gradient elution with 0.01 *M* to 1.0 *M* phosphate buffer. Afterwards, 1.0 *M* phosphate buffer was passed through the column. Fractions of 10 ml were collected at the flow rate of 120 ml/hr.

Agar gel electrophoresis — Agar gel electrophoresis was carried out according to the method of Giri[11] in order to ascertain the protein homogeneity. A thin layer of agar gel was allowed to be formed on a glass slide (7.5 cm × 2.5 cm × 0.1 cm) and a sample of protein was subjected to electrophoresis at a constant voltage of 150 volts and 25°C for 3 hr using the buffer of *p*H 8.6.

Starch gel electrophoresis — Starch gel electrophoresis was done by the method of Smithies[12,13] in order to find out protein homogeneity. The protein sample applied at the centre of the gel (6 cm × 20 cm × 1 cm) was subjected to electrophoresis at a constant voltage of 200 volts for 16 hr using the buffer of *p*H 8.6.

Preparation of skin proteins — Collagen was prepared by a modified method of Bowes and Kenten[14]. Elastin was prepared by the method of Lansing *et al.*[15]. Albumin, globulin and mucoids were prepared by the method of Bose *et al.*[16].

Ultraviolet irradiation — A Towers UV lamp was used for ultraviolet irradiation. The enzyme solutions (7 mg/100 ml) were taken in uncovered petri dishes and placed at a distance of 10 cm from the source of irradiation in an air-conditioned (25°C) dark room. After different periods of exposure, the volume of the enzyme solution was brought to the original level by adding bi-distilled water and the proteolytic activity of the enzyme was estimated as described earlier.

Inhibition studies — Five millilitre portions of purified enzyme solution (14 mg/100 ml) were mixed with 5 ml portions of inhibitor solution (0.05 *M*) whereby the concentration of the inhibitor in pre-incubation system becomes 0.025 *M*. The enzyme-inhibitor mixture was incubated at 37°C for 3 hr at the end of which the proteolytic activity of the mixture was determined as described before. As *p*-chloromercuribenzoic acid was not fully soluble in water, it was dissolved in 0.1 *N* NaOH solution and then made to the required volume.

Results and Discussion

Purification of the enzyme — The following purification procedure was worked out as a result of the preliminary experiments. All steps were carried out at room temperature (about 30°C) except acetone precipitation. The preliminary experiments showed that the enzyme was active at *p*H 7.5–8.0.

Step 1 : centrifugation — Crude extract was centrifused at 4000 rpm for 30 min and the centrifugate was collected.

Step 2 : ammonium sulphate precipitation — Ammonium sulphate which was required for half saturation of enzyme extract was gradually added to the centrifugate which had been adjusted to *p*H 7.5 under stirring. When the requisite amount of salt was added, the mixture was stirred well and centrifused after a few min. The precipitate was collected, reconstituted and dialysed against bi-distilled water at refrigerator temperature for 48 hr.

Step 3 : acetone precipitation — The dialysed enzyme solution (*p*H 7.5) was precipitated using two and half volumes of pure redistilled acetone (after cooling the solvent and the enzyme solution to −10°C and +4°C respectively). The enzyme solution was added drop by drop to the acetone under constant stirring. The precipitated enzyme was removed by centrifugation, dried under vacuum and reconstituted to a suitable volume with bi-distilled water.

Step 4 : adsorption of impurities on calcium phosphate gel — To the extract obtained after acetone precipitation was added dilute calcium phosphate gel. The mixture was adjusted to *p*H 10.5, shaken for 10 min in a mechanical shaker and centrifuged, and the centrifugate was collected.

Step 5 : second acetone precipitation — The above centrifugate was adjusted to *p*H 7.5, treated with 2½ volumes of cold acetone as mentioned in Step 3, centrifuged and the precipitate was dried in a vacuum desiccator.

Step 6 : chromatography on DEAE-cellulose — Acetone precipitated powder was dissolved in phosphate buffer (*p*H 6.5) and subjected to DEAE-cellulose column chromatography in four instalments. The protein concentration in each fraction was measured using Beckman spectrophotometer (DU) at 280 mμ and proteolytic activity was estimated according to the method mentioned earlier. The results obtained are presented in Fig. 1. Fractions collected in tubes 16-38 (which represented the protein peak) were pooled and dialysed against distilled-water for 48 hr. They were then concentrated by vacuum distillation. Finally, the enzyme was precipitated with cold acetone, centrifused and dried in vacuum desiccator.

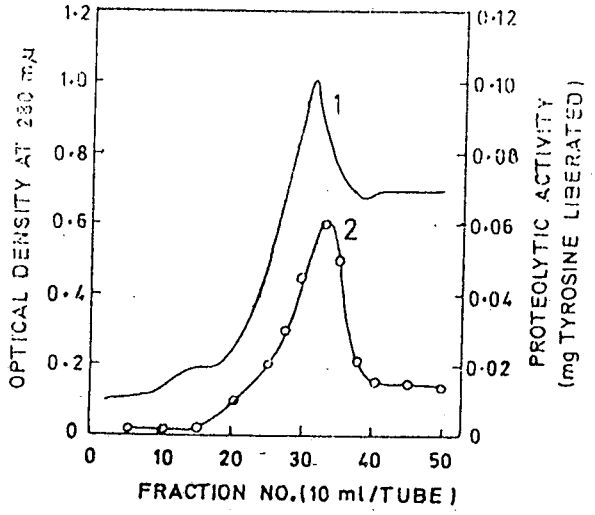

Fig. 1.— Elution pattern of the enzyme on DEAE-cellulose column chromatography with 1.0*M* phosphate buffer (*p*H 6.5) [1, optical density at 280 mμ; 2, proteolytic activity].

After every step of purification, proteolytic activity and the enzyme protein contents were estimated. It is apparent from the results presented in Table 1 that after final purification a 122-fold purity of the enzyme is achieved with 59% enzyme recovery. The dried enzyme powder obtained after final purification was collected and stored in refrigerator for further studies. The purified enzyme was found to be homogeneous by both agar gel electrophoresis and starch gel electrophoresis.

Effect of substrate concentration on enzyme activity — Aliquots (2 ml) of egg albumin solution containing varying amounts of protein (10-110 mg) in veronal buffer (*p*H 7.5) were incubated with 2 ml portions of enzyme solution (7 mg/100 ml) for 30 min at 45°C. The proteolytic activity in each case was estimated as before. It was observed that a linear relationship existed between the substrate concentration and the proteolytic activity when egg albumin was used upto the concentration of 12.5 mg per ml of digestion mixture.

Effect of pH on the enzyme activity using different protein substrates — Proteolytic activity of the enzyme was determined by using casein, haemoglobin and egg albumin (all E. Merck quality) as substrates at different *p*H levels. Haemoglobin and egg albumin were dissolved in veronal buffer whereas casein was first dissolved in 0.1*N* NaOH solution and then adjusted to various *p*H values with veronal buffer. Proteolytic activity was estimated as described earlier. The results obtained are recorded in Fig. 2.

Purified enzyme shows a broad *p*H dependence with optimum activity at *p*H 7.5 against egg albumin and casein and *p*H 7.9 against haemoglobin. Lazarus and Barrett[3] reported that partially purified neutral proteinase from rabbit skin showed maximum activity at *p*H 7.5 when tested on casein.

Proteolytic activity of the enzyme on different skin and hide proteins, e.g. albumin, globulin, mucoid, elastin and collagen, was also studied at different *p*H (5.0, 7.5 and 9.0) for a period of 30 min at 45°C. Enzymatic hydrolysis of albumin, globulin and mucoid was carried out with Anson's colorimetric method as described earlier. Due to low content of tyrosine and tryptophane in collagen and elastin, the extent of hydrolysis of these proteins was determined by estimating the non-protein nitrogen using micro-kjeldahl method under the assay condition described earlier.

TABLE 1 — PURIFICATION OF THE NEUTRAL PROTEINASE

Step	Volume	Total enzyme activity (units)	Total protein (mg)	Sp. activity	Yield %
Crude enzyme after centrifugation	3500 ml	336	28700	0.012	100
Ammonium sulphate precipitation	950 ml	330	6270	0.052	98
Acetone precipitation	230 ml	328	5740	0.057	98
Adsorption of impurities on calcium phosphate gel	484 ml	265	4240	0.062	79
Second acetone precipitation	3.445 g	260	2239	0.116	77
DEAE-Cellulose	—	201	137	1.467	59

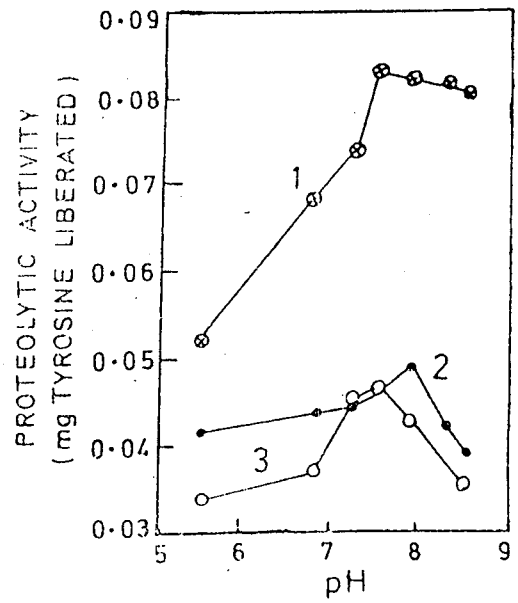

Fig. 2 — Hydrolytic action of the enzyme on different protein substrates at various *p*H levels [1, casein; 2, haemoglobin; 3, egg albumin].

The results presented in Fig. 3 show that the proteinase is most active on albumin at *p*H 8.0 and on mucoid at *p*H 7.6. In the case of globulin, no optimum *p*H value has been noted, but enzyme activity is found to be comparatively higher at *p*H ranging from 7.5 to 9.0. The proteinase is unable to hydrolyse collagen or elastin. Lazarus and Barrett[3] also observed that partially purified rabbit skin proteinase was able to hydrolyse casein, albumin and haemoglobin but not collagen. It is thus apparent from the above observations that goat skin neutral proteinase is able to hydrolyse various natural proteins and the enzyme is most active at the *p*H range of 7.5 to 8.0. It has been recognised earlier[16-19] that removal or breakdown of interfibrillary proteins in skin, e.g. albumin, globulin and mucoid, helps unhairing action. It is thus possible that neutral proteinase present in goat skin is responsible for unhairing under suitable environmental conditions.

Hydrolytic action of the enzyme on synthetic substrates — Hydrolytic action of goat skin neutral proteinase was studied on BAEE, BAA, BANA, BAPNA and ATEE, following the paper chromatographic method of Tsuru and Yamamoto[20]. The peptide (1 mg) was dissolved in 0.1 ml of veronal buffer (*p*H 7.5) containing 50 μg of the enzyme and the mixture was incubated for 24 hr at 37°C under toluene. After incubation, the mixture was subjected to paper chromatography.

BAEE and BAA were hydrolysed readily and ATEE slowly by goat skin neutral proteinase while BANA and BAPNA were not attacked by the enzyme.

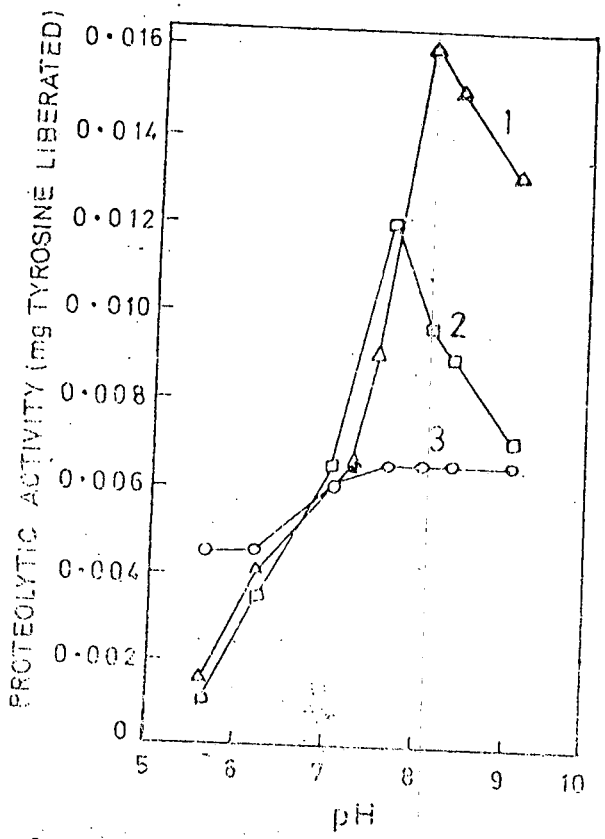

Fig. 3 — Hydrolysis of skin proteins by enzyme at different *p*H levels [1, albumin; 2, mucoid; 3, globulin].

It has been reported that BAEE is hydrolysed by human skin extracts from epidermis[21]. BAA is found to be unaffected by rat[22] and rabbit[23] skin extracts. Skin extracts from different animals[2] are also able to hydrolyse ATEE. BAPNA remains unaffected by rabbit skin neutral proteinase[3], but BANA is hydrolysed by various skin extracts[2] except hog skin.

Effect of pH and temperature on enzyme stability — In order to study the *p*H stability of the enzyme, 5 ml portions of the enzyme solution (14 mg/100 ml) were mixed with 5 ml portions of veronal buffer of different *p*H (4.3 to 8.5). The enzyme buffer mixtures were then kept at 18°C for 24 hr. To determine the thermal stability of the enzyme, 5 ml portions of the proteinase solution (7 mg/100 ml, *p*H 7.5) were heated in glass stoppered bottles at different temperatures for 1 hr and then rapidly cooled to room temperature. In both the cases, the residual enzyme activity was calculated with reference to initial activity. The enzyme was more stable at *p*H range of 6.5 to 8.5 and at temperatures below 60°C. About 84% of enzyme activity was lost at 75°C.

Effect of ultraviolet rays — The enzyme solution was exposed to UV rays for different periods, e.g. 1, 5, 10, 20 and 30 min. Results obtained indicated that the enzyme was rapidly inactivated by UV rays within one min (9%) and 50% of the enzyme activity was lost after 30 min of exposure.

Table 2 — Effect of Various Inhibitors on the Activity of Neutral Proteinase

Inhibitors	Per cent inhibition
Control	0.0
Ascorbic acid	4.8
Sodium cyanide	28.5
Sodium thio-sulphate	0.0
Sodium bisulphite	71.4
Ovomucoid	0.0
DFP	11.0
Maleic acid	100.0
Iodine	100.0
EDTA	100.0
Para-chloromercuribenzoic acid	100.0
Silver nitrate	100.0
Nickel chloride	76.2
Mercuric chloride	100.0
Copper sulphate	66.7
Ferric chloride	100.0
8-Hydroxyquinoline	100.0

Effect of enzyme inhibitors — Effect of various inhibitors on the enzyme activity has been shown in Table 2. Oxidising agents like maleic acid and iodine, metal chelating agents like EDTA and 8-hydroxyquinoline, heavy metal salts like silver nitrate, mercuric chloride and ferric chloride, and thiol group reagent, parachloromercuri-benzoic acid show 100% inhibition of the enzyme at 0.025 *M* concentration. Reducing agents show either no or slight inhibitory effect. Ovomucoid, a trypsin inhibitor, has no action, and DFP, a serine proteinase inhibitor, possesses slight inhibitory action. The inhibitors causing 100% inhibition have also been tried at a lower concentration (0.005 *M*) and all of them showed considerably strong inhibitory action.

According to Lazarus and Barrett[3], rabbit skin neutral proteinase was not inhibited by EDTA and thiol group blocking reagents. These observations tend to show that possibly partially purified rabbit skin proteinase and the purified goat skin neutral proteinase are not identical. Due to the paucity of data on purified skin proteinases, it is difficult to compare their properties and to find out whether the neutral proteinases isolated from different animals are the same or differ from one an other.

Acknowledgement

One of the authors (MS) is indebted to the Council of Scientific and Industrial Research, New Delhi for the award of a senior research fellowship.

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