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α-*N*-Benzoylarginine-2-naphthylamide Hydrolase (Cathepsin B1?) from Rat Skin. I. Preliminary Experiments with Skin Extract

M. JÄRVINEN and V. K. HOPSU-HAVU

Department of Anatomy, University of Oulu, and Department of Dermatology, University of Turku, Finland

The cathepsin B1-like, α-*N*-benzoyl-DL-arginine-2-naphthylamide (BANA) hydrolyzing activity of rat skin extract was studied. The enzyme was extracted into low ionic strength buffers, and was activated by dithiothreitol, EDTA and KCN. Incubation of the extract at acidic pH resulted in a 3.6-fold increase in its BANA hydrolase activity. The presence of inhibitor(s) of BANA hydrolase in the skin extract was indicated by the non-linearity of the activity/enzyme concentration curve and by the increase in total activity of the enzyme by Sephadex G-100 gel chromatography, which also caused separation of several other skin proteases.

Otto [1] observed that the purified cathepsin B preparation of Greenbaum and Fruton [2] was a mixture of two enzymes, cathepsins B1 and B2. Cathepsin B1 is a powerful thiol-activated proteinase that also hydrolyzes synthetic substrates like benzoylarginine amide, naphthylamide, 4-nitroanilide and ethylester.[3–5] Cathepsin B2 hydrolyzes histones, some other basic proteins and benzoylarginine amide, but not the corresponding naphthylamide.[4,6]

Cathepsin B1 has been purified and characterized from beef spleen,[1,3–5,7] calf liver,[8,9] rabbit liver,[10] rat liver,[11] human liver,[12] human placenta,[13] and sheep thyroid gland.[14] The enzyme preparations so far obtained differ markedly in some respects; *e.g.* most of them hydrolyze benzoylarginine 4-nitroanilide, while some of them do not.[11,14] Recently some preparations have been shown to hydrolyze native collagen [7,15] and cartilage protein polysaccharides in co-operation with cathepisn D.[16,17] Some of them also inactivate various glycolytic enzymes [4,18–20] and exhibit kininogenase activity.[21]

The presence of cathepsin B1-like enzymes in various organs of rat [22] as well as in human and rat skin has been shown,[23,24] but a detailed study of the skin enzyme has not been made. The interesting properties of the enzymes that suggest their possible role in inflammatory processes, have initiated our closer investigation of the skin enzyme.

This paper deals with some properties of a crude cathepsin B1-like benzoylarginine-2-naphthylamide hydrolase from rat skin as a starting point for purification and characterization of the enzyme.

MATERIALS AND METHODS

Extract of the skin. Adult Long-Evans rats were used. The rats were rendered unconscious by a sudden replacement of air with nitrogen, decapitated and allowed to bleed. The trunk skin was shaved, removed and cleared of fascia and fat. The skin was cut with scissors into 3 × 3 cm pieces and minced with a meat grinder.

Ten grams of minced rat skin was suspended in 100 ml of ice-cold 10 mM sodium phosphate buffer, pH 6.0, containing 10 g/l (0.134 M)KCl, 0.2 mM dithiothreitol and 1 mM EDTA. The suspension was homogenized for 1 min in an Ultra Turrax T-45 homogenizer with constant cooling in an ice-salt bath (−5 °C). The homogenate was allowed to stand for 2 h at +4 °C, and centrifuged for 1 h at +4 °C, at 25 000 *g*, in a MSE HS-18 centrifuge. The opalescent supernatant was filtered by suction through a Millipore type AP-25 prefilter and AAWP filter (pore size 0.8 μm). The filtered solution is

referred to as the extract. The sediment and flotate after centrifugation at 25 000 *g* and the Millipore filters together with the retained material were rehomogenized to the original volume of the homogenate and their BANA hydrolyzing activities were determined as described for the homogenate.

Substrates, buffers and other reagents. α-*N*-Benzoylarginine-DL-2-naphthylamide hydrochloride (BANA, Sigma Chem. Co.) and L-leucine-2-naphthylamide (Leu-NA, Sigma Chem. Co.) were dissolved in methanol and diluted with water to give final concentrations of 5 mM of substrate and 20 % (v/v) of methanol. α-*N*-Benzoyl-L-arginine-4-nitroanilide hydrochloride (BAPA, Fluka AG) was used as a 1 mM solution in 20 % (v/v) methanol. Methanol (6.7 % in the reaction mixture) that was used to increase solubilities of BANA, Leu-NA, and BAPA had no effect on hydrolysis rates of these substrates. α-*N*-Benzoyl-L-arginine ethylester hydrochloride (BAEE, Merck AG) was dissolved in water (10 mM) just prior to use. α-*N*-Benzoyl-L-arginine amide hydrochloride hydrate (BAA, Merck AG) was dissolved in water (60 mM) and used within two days. Bovine Hemoglobin (Hb, Sigma Chem. Co., type II) was dissolved in water (20 g/l) and used within two days.

The universal buffer of Britton and Robinson,[25] with thiol activators, was modified for the determination of hydrolysis rates of BANA, Leu-NA, BAPA, and BAA. The buffers were prepared by titrating a mixed acid solution containing 80 mM acetic, phosphoric, and boric acids with 0.4 M NaOH to give the desired pH's of 5.8 for BANA, BAPA, and BAA, and 7.0 for Leu-NA, and then adding 0.6 mM dithiothreitol (DTT, Calbiochem), 3 mM EDTA (disodium salt, Merck AG) and 3 mM KCN. The BAEE hydrolyzing enzyme was assayed in Britton-Robinson buffer, pH 6.0, without added activators.

The hemoglobin hydrolyzing activity was assayed in a lactate-acetate buffer, which was made by titrating a solution of 0.4 M sodium lactate and sodium acetate with 5.0 M HCl to the desired pH (4.3 for routine assays).

Enzyme assays. To a test tube 0.1 ml of enzyme solution and 0.1 ml of suitable buffer were pipetted and the mixture was allowed to stand for 10 min at room temperature. The tubes were transferred to a water bath (37 °C), reactions were started by adding 0.1 ml of substrate solution and the mixtures were incubated for 15 min – 4 h depending on enzyme activities. The pH's of reaction mixtures were measured during the incubations. All assays were performed in duplicate.

Naphthylamine, liberated from BANA and Leu-NA, was assayed by stopping the enzyme reaction with 0.4 ml of 4-dimethylaminobenzaldehyde reagent.[26] The reagent was made by dissolving 250 mg of 4-dimethylaminobenzaldehyde in 25 ml of methanol, and adding 25 ml of an acetate-HCl buffer, pH 1.4. The buffer was made by titrating 1 M sodium acetate to pH 1.4 with concentrated HCl. The solutions were allowed to stand for 30 min at room temperature and their absorbance was measured with a spectrophotometer at 450 nm against a blank containing water instead of the enzyme solution. The stopping reagent caused precipitation of some proteins when crude enzyme preparations were used. The turbidity caused by the precipitate was removed by centrifugation (10 min at 1000 *g*), or corrected against a blank, to which 4-dimethylaminobenzaldehyde reagent was added immediately after the enzyme solution. A standard curve was prepared by pipetting 2-naphthylamine solutions (0 – 15 nmol in 20 % methanol) to the reaction mixture instead of the enzyme solution. The BANA hydrolase activity of the skin homogenate was assayed by using 0.5 ml of the homogenate, buffer and substrate, incubating in a shaking water bath (37 °C) and stopping the reaction with 2 ml of 4-dimethylaminobenzaldehyde reagent. The colour solution was filtered through Whatman No. 1 paper and the absorbance of the filtrate was measured at 450 nm.

The hydrolysis of hemoglobin was assayed by estimating the solubilized tyrosine and tryptophan containing peptides by the Folin-Ciocalteau reagent.[27] The enzyme reaction was stopped by adding 0.4 ml of 0.61 M trichloroacetic acid to the reaction mixture and centrifuging (1000 *g*, 10 min), after standing at room temperature for 15 min. To 0.4 ml of the supernatant, 2 ml of 0.1 M NaOH containing 20 g/l Na_2CO_3 was added. Finally, 0.2 ml of Folin-Ciocalteau reagent (Merck AG, diluted 1:1 with water) was added with rapid mixing. The solution was allowed to stand for 30 min and the absorbance was recorded at 750 nm against a blank of water instead of enzyme solution. The absorbance caused by the enzyme solution was subtracted by using a blank containing water instead of the substrate. A standard curve was prepared with 0 – 0.25 μmol tyrosine instead of the enzyme solution, and hemoglobin hydrolyzing enzyme activities were expressed in tyrosine equivalents (nmol) solubilized in one minute.

The hydrolysis of BAEE was determined by measuring unhydrolyzed substrate according to the method of Hestrin.[28] After incubation, the reaction was stopped by adding 0.2 ml of cold, alkaline hydroxylamine solution, which was freshly made by mixing equal amounts of 2 M hydroxylamine hydrochloride and 3.5 M NaOH, to the reaction mixture. After 15 min, 0.1 ml of 4 M HCl was added, followed later by 0.1 ml of 0.7 M $FeCl_3$. The colour developed was measured after 20 min, at 540 nm, against a blank containing water instead of BAEE. These values were subtracted from corresponding blank values containing water instead of the enzyme solution. A standard curve was constructed from increasing concentrations of BAEE.

The hydrolysis of BAA was estimated by measuring the ammonia liberated during the reaction by a modified phenol-hypochlorite reaction.[29] After incubation, the reaction was stopped by adding 1 ml of phenol-nitroprusside (17 g/l phenol and 50 mg/l sodium nitroprusside) and 1 ml of alkaline hypochlorite solution (5 g of NaOH and 5 ml of 10 % sodium hypochlorite solution was dissolved in water and diluted to 1 l) to the reaction mixture. The colour was developed at 55 °C for 10 min, and after cooling, measured at 625 nm against a blank containing water in place of the enzyme solution. In every assay standards were run containing 0.05 and 0.1 mmol ammonium sulfate in place of the enzyme solution.

The hydrolysis of BAPA was assayed by measuring the 4-nitroaniline formed. The reaction was stopped by adding 0.2 ml of zink sulfate solution (100 g/l), the solution was centrifuged for 10 min at 1000 *g* and the absorbance of the supernatant measured at 383 nm. A standard curve was prepared by replacing the enzyme solution with 4-nitroaniline (0 – 10 nmol) dissolved in 20 % methanol.

The protein concentration was determined according to Lowry *et al.*[30] with bovine serum albumin (Sigma Chem. Co.) as a standard. The proteins from chromatographic separations were estimated by absorbance at 280 nm with an Isco UA-2 monitor.

Gel chromatography. A 5 × 70 cm column of Sephadex G-100 was equilibrated with 10 mM sodium phosphate buffer, pH 7.5. The sample, 60 ml of extract, was applied by a peristaltic pump and elution was carried out with the equilibrating buffer at +4 °C. The flow rate was 60 ml/h and the fraction volume 10 ml.

RESULTS

Extraction. The effect of pH on the extraction of the enzyme was studied by extracting minced skin with 10 mM phosphate buffers, at pH 8.0 and 6.0, or with 10 mM citrate buffer, pH 4.0, in the presence of 0.2 mM DTT and 1 mM EDTA. A similar set of extracts was prepared with the same buffers, but containing KCl (10 g/l) in order to study the effects of salt concentration on the extraction. BANA-hydrolase activities of the extracts, when assayed at pH 5.8, are given in Fig. 1. Acidic pH increased both the specific and total activities of the extracts. The addition of salt increased the total activity, but the extraction of inactive proteins was increased to the same proportion. Although acidic extraction at pH 4.0 gave the best activity, 10 mM sodium phosphate buffer, pH 6, with 1 % (w/v) KCl was used in later extractions. The reason for avoiding the acidic

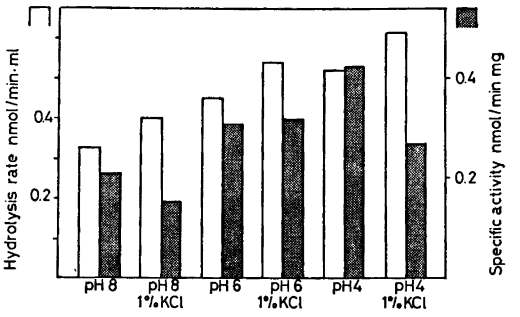

Fig. 1. Effect of pH and KCl (1 %) on extraction of rat skin BANA hydrolase. Phosphate buffers (10 mM) were used at pH 8 and 6, and citrate buffer (10 mM) at pH 4. Buffers contained 0.2 mM DTT and 1 mM EDTA.

pH in extraction was that acidic pH seemed to increase activity of purified BANA hydrolase preparations (details to be described). Avoiding treatment with acidic pH until the enzyme was purified, made it possible to study the activation phenomenon closer. The extraction procedure described in "Materials and Methods" gave a recovery of 72 % for BANA hydrolase, as compared to the total activity of the skin homogenate. About 18 % of BANA hydrolase was retained in the sediment after centrifugation of the homogenate at 25 000 *g* for 1 h and about 0.4 % was found in the floating lipid material. The material retained on Millipore filters contained less than 0.1 % of the total BANA hydrolase activity of the homogenate.

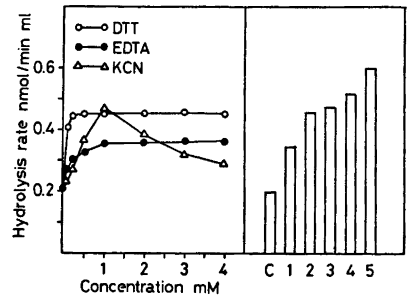

Fig. 2. Activation of BANA hydrolase of rat skin extract with DTT, EDTA, and KCN. On the left: Effect of activator concentration. On the right: Additional effects of activators. C = control with no activators, 1 = 1 mM EDTA, 2 = 0.2 mM DTT, 3 = 1 mM KCN, 4 = 1 mM EDTA + 0.2 mM DTT, 5 = 1 mM EDTA + 0.2 mM DTT + 1 mM KCN.

Enzymatic characteristics of the extract. The BANA hydrolase of the extract behaved like a typical thiol-activated enzyme (Fig. 2). DTT, EDTA, and KCN all increased the hydrolysis rate of BANA. In the case of KCN a distinct optimal concentration (1 mM) was found. At this concentration KCN activated the enzyme even better than did DTT or EDTA. In the cases of DTT and EDTA the activity of the enzyme reached a plateau at 0.2 and 1.0 mM concentration, respectively. Maximal activation of the enzyme was obtained when all activators, DTT, EDTA and KCN, were simultaneously present at the concentrations mentioned above.

The pH-optima of the skin extract in Britton-Robinson buffer were pH 5.8 for BANA, and pH 5.5 for BAA (Fig. 3). An upward bend in the hydrolysis rates of BANA and BAA was noticed between pH 6 and 8. The hydrolysis of both

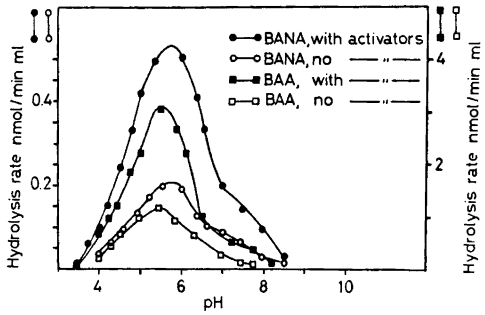

Fig. 3. Effects of pH and thiol activators on BANA and BAA hydrolase activities of rat skin extract in Britton-Robinson buffer. Activators were 1 mM EDTA, 0.2 mM DTT and 1 mM KCN.

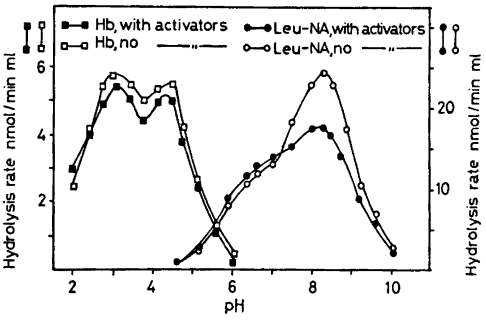

Fig. 4. Effects of pH and thiol activators on hemoglobin and Leu-NA hydrolase activities. The hydrolysis of hemoglobin was assayed in a lactate-acetate buffer. Other conditions were as in Fig. 3.

substrates was increased by DTT, EDTA, and KCN. The pH-optima for the hydrolysis of hemoglobin and Leu-NA by the extract are given in Fig. 4. Hemoglobin hydrolase had two pH-optima, pH 3.0 and 4.3, and thiol activators did not effect the hydrolysis rate. Leu-NA was hydrolyzed maximally at pH 8.0, with a lower optimum at pH 7.0. The thiol activating mixture (DTT, EDTA and KCN) inhibited the enzyme maximally at pH 8.0, while activating it slightly at pH 7.0. BAPA was not hydrolyzed by the extract.

The BANA hydrolase activity of the extract was increased, when the pH of the solution was lowered to between 3.0 and 4.0 by adding 0.5 M HCl, and the temperature of the solution was elevated to 55 °C for 20 min (Fig. 5). At the same time, some protein was precipitated, and after centrifugation the specific activity of the undiluted extract was increased 3.6 times. The effect of elevated temperature was to accelerate the rate of activation. At 0 °C, no activation was evident during 2 h, but some protein was

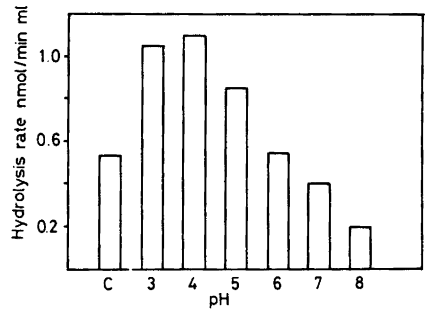

Fig. 5. Effect of preincubation of the extract at 55 °C, for 20 min, at various pH's, on BANA hydrolase activity. C = control, incubated at 0 °C, at pH 6.0.

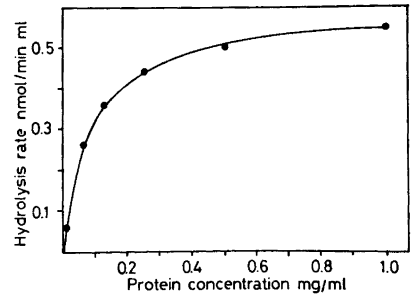

Fig. 6. Effect of enzyme concentration of the assay mixture on BANA hydrolase activity of the extract.

precipitated. At alkaline pH, elevated temperatures destroyed the BANA hydrolase.

The hydrolysis of BANA by the extract increased linearly with time. The effect of enzyme concentration on the hydrolysis rate of BANA was tested by diluting the extract with the homogenization buffer and plotting enzyme activity against protein concentration of the reaction mixture. The activity/enzyme concentration curve was in no way linear (Fig. 6), suggesting the presence of some dissociable inhibitor(s) of BANA hydrolase in the extract. Dialysis of the extract against 10 mM phosphate buffer, pH 7.5, did not abolish the effect of dilution on enzyme activity. This suggests that the possible inhibitor is a high-molecular-weight protease inhibitor. In later experiments undiluted enzyme preparations were used, unless otherwise stated.

Chromatography on Sephadex G-100. Fig. 7. represents a Sephadex G-100 gel chromatography of the skin extract. The BANA hydrolase was eluted as two peaks. The first peak was eluted together with the void volume (V_0, fraction No. 44). The second peak (fraction No. 75) with a lower molecular weight was more active than the first. The main Leu-NA hydrolyzing enzyme and a minor BAA hydrolyzing enzyme were eluted at V_0. The major BAA hydrolase peak (cathepsin B2) was eluted slightly before (fraction No. 68) the low molecular weight

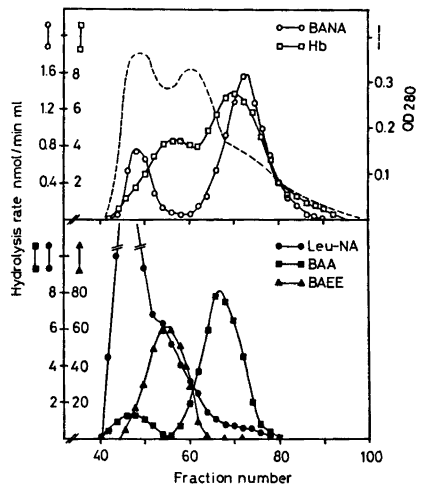

Fig. 7. Sephadex G-100 gel chromatography from rat skin extract. Details of the chromatography are given in "Materials and Methods".

BANA hydrolase, and the main hemoglobin hydrolyzing enzyme was eluted between the BAA and BANA hydrolases. The BANA hydrolase containing fractions from the Sephadex G-100 chromatography were pooled (fraction Nos. 66 – 78). The specific activity of the pooled enzyme was increased 7.4-fold, *i.e.*, from 0.26 nmol/min × mg to 1.92 nmol/min × mg. The total activity of the pooled preparation was increased 2.3-fold from the original activity of the extract. This increase in total activity suggests removal of some inhibitor(s) during the gel chromatography.

The pooled BANA hydrolase from the Sephadex G-100 gel chromatography increased in activity during incubation at pH 4, at 55 °C, as did the activity in the extract (see Fig. 5). The enzyme was fairly stable, and the activity decreased by about 16 – 20 % in a week in 10 mM sodium phosphate buffer, pH 7.5, at +4 °C. EDTA (1 mM) and DTT (0.2 mM) increased the stability of the enzyme. The frozen enzyme (– 18 °C) was stable for at least three months.

DISCUSSION

The acidic rat skin BANA hydrolase can, like the human skin enzyme,[32] be extracted with low ionic strength buffers. The recovery of the enzyme in acidic extraction was higher than in alkaline extraction, and incubation at acidic pH and elevated temperatures increased the activity of the extract. Corresponding phenomena have also been observed with human liver and bovine spleen cathepsin B1 extracts, where the enzyme activity was similarly increased during autolysis at acidic pH.[7,12] In the present investigation an acidic extraction was avoided in order to be able to study the activation phenomenon more thoroughly. The enzyme preparation, when purified by Sephadex G-100 gel chromatography, was still activated by treatment at pH 4. A possible explanation of the acid activation of the BANA hydrolase is irreversible dissociation of some inhibitor from the enzyme at acidic pH, or autolytic modification of the enzyme by some acidic proteinase.

Rat and human skin BANA hydrolases have been shown to be activated by DTT and other thiols as well as EDTA.[23,24,31] In addition, the rat skin BANA hydrolase is also activated by KCN.

The activation with KCN was maximal at a 1 mM concentration. Otto and Bhakdi [3] observed slight activation of beef spleen cathepsin B1 with KCN, while in some other tissues inhibition with KCN has been reported.[11,12] Besides the hydrolysis of BANA, the hydrolysis of BAA was activated by thiol activators, while the hemoglobin hydrolyzing enzyme was not effected, and the main Leu-NA hydrolyzing enzyme was inhibited by the activation mixture (DTT, EDTA and KCN), at pH 8.0. BANA was hydrolyzed optimally at pH 5.8 and BAA at pH 5.5. In addition, an upward bend in pH curves of both substrates was noticed between pH 6 and 8. This bend is probably caused by the alkaline BANA hydrolyzing enzyme demonstrated earlier from rat skin.[24]

The activity/enzyme concentration curve reached a plateau with increasing enzyme concentrations. This phenomenon suggested the presence of inhibitor(s) of BANA hydrolase in the extract. Sephadex G-100 gel chromatography separated some of these inhibitors from the enzyme, as indicated by an increase in total enzyme activity. The presence of an inhibitor of thiol proteases in rabbit skin has earlier been demonstrated.[33,34] A purified preparation is known to inhibit the so called "Arthus protease" of Udaka and Hayashi [33] as well as papain, and according to Lazarus and Dingle [34] rabbit skin extract inhibits human liver cathepsin B1. The inhibitor(s) in the rat skin may be similar to the one shown by Udaka and Hayashi,[33] and Lazarus and Dingle.[34]

The stability of the rat skin BANA hyrolase is fairly good when partially purified by Sephadex G-100 chromatography. Since the enzyme is stable, and the estimation of its activity is not difficult, it is very suitable for extensive purification and characterization; details of which will be presented in our forthcoming reports.

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