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α-*N*-Benzoylarginine-2-naphthylamide Hydrolase (Cathepsin B1 ?) from Rat Skin. II. Purification of the Enzyme and Demonstration of Two Inhibitors in the Skin

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A thiol-activated, α-*N*-benzoylarginine-2-naphthylamide (BANA) hydrolyzing enzyme was purified from rat skin by ammonium sulfate precipitation, gel filtration, and DEAE cellulose chromatography. In DEAE cellulose chromatography the enzyme was fractionated into two multiple forms (preparations I and II). The activity of undiluted preparation II, but not that of preparation I, was increased, when the enzyme was preincubated at pH 4, at 55 °C. Simultaneously, the isoelectric point of preparation II was shifted to that of preparation I, i.e., from 6.2 to 7.5. Activated preparation II behaved in DEAE cellulose chromatography as preparation I. Molecular weights of the enzymes of both preparations were 27 000, and pH optima were at pH 5.8 and 7.0, for BANA and leucine-2-napthylamide (Leu-NA), respectively. The BANA and Leu-NA hydrolyzing enzymes could not be separated by gel filtration, DEAE, CM, or Amberlite IRC-50 chromatography, isoelectric focusing, or analytical polyacrylamide gel electrophoresis. Two inhibitors of BANA hydrolase were demonstrated by gel filtration in the salt precipitated skin extract. The activities of the BANA hydrolase preparations did not increase linearly with increasing enzyme concentration, with the exceptions of activities of preparation I and acid-activated preparation II. The role of the inhibitors in the nonlinearity of the activity/enzyme concentration curves is discussed.

In a previous report some properties of α-*N*-benzoylarginine-2-naphthylamide (BANA) hydrolyzing enzyme from crude extract of rat skin was presented.[1] The enzyme resembled cathepsin B1 purified from other tissues in that it hydrolyzed BANA optimally at pH 5.8 in the presence of thiol activators (dithiothreitol, EDTA, and KCN). The activity/enzyme concentration curve of the extract that was obtained by plotting the BANA hydrolase activity of the extract against enzyme concentration, was unlinear. This suggested the presence of some inhibitor in the skin extract. In the present report the purification of the skin BANA hydrolase is described and evidence for the existence of two inhibitors of the enzyme in the rat skin is presented.

MATERIALS AND METHODS

Reagents. α-*N*-Benzoyl-DL-arginine-2-naphthylamide hydrochloride (BANA) and pepsinogen were from Fluka A. G., α-*N*-benzoyl-L-arginine amide hydrochloride hydrate (BAA) and papain (for biochemical purposes) were from Merck A. G., glycyl-L-arginine-2-naphthylamide (Gly-Arg-NA) was from Fox Chem. Co., and dithiothreitol (DTT) from Calbiochem. L-Leucine-2-naphthylamide (Leu-NA), L-arginine methylester (Arg-ME), ficin (crude), bromelain (grade II), bovine trypsin (type III), bovine ribonuclease A (type I A), soybean trypsin inhibitor (SBTI, type II S), bovine serum albumin (crystallized) and hemoglobin (Hb, type II) were from Sigma Chem. Co. A cathepsin C preparation was partially purified by Sephadex G-200 gel filtration from the rat skin extract, as an enzyme hydrolyzing Gly-Arg-NA, at pH 5.0, in the presence of thiol activators.

Enzyme assays. The hydrolysis of BANA, Leu-NA, Hb, and BAA was estimated as described previously.[1] Arg-ME was assayed as described for benzoylarginine ethyl ester, except that Britton-Robinson buffer, pH 5.8, containing 0.6 mM DTT, 3 mM EDTA, and 3 mM KCN was used.[1] The hydrolysis of Gly-Arg-NA was assayed in a similar way as that of BANA, but the pH of the buffer was 5.0. Pepsinogen was

assayed by the method of Herriot [2] and ribonuclease according to McDonald.[3]

Inhibition of BANA hydrolase by rat skin inhibitors was determined by mixing 0.1 ml of enzyme (purified preparation AI, see "Results"), 0.1 ml of Britton-Robinson buffer, pH 5.8,[1] containing 0.6 mM DTT, 3 mM EDTA, and 3 mM KCN. To this mixture 0.1 ml of inhibitor solution was added and the solution was allowed to stand at 37 °C for 10 min. Finally, 0.1 ml of 5 mM BANA in 20 % (v/v) methanol was added and the reaction mixture was incubated for 30 min at 37 °C. After incubation, 0.4 ml of 4-dimethylaminobenzaldehyde reagent was added, solutions were allowed to stand for 30 min at room temperature, and the optical density was measured at 450 nm.[1] Optical density values were substracted from those of controls, which contained water instead of inhibitor. The inhibition of cathepsin C was assayed in a similar manner with Gly-Arg-NA as a substrate, at pH 5.0. Inhibition of papain, ficin, and bromelain (0.1 mg/ml) were tested with BANA using Gomori's tris-maleate buffer,[4] pH 7.6, containing 1.2 mM DTT, 3 mM KCN, and 3 mM EDTA. The inhibition of trypsin was assayed as described by Junnila *et al.*[5]

Protein determination. The method of Lowry [6] was used for pooled samples, but during the chromatographic procedures the absorbance at 280 nm was measured with an Isco UA-2 monitor.

Purification procedure. Extraction of the enzymes from the skin was performed as described earlier.[1]

Precipitation with ammonium sulfate. The extract (735 ml) was saturated to 50 % with solid ammonium sulfate at 0 °C and centrifuged for 10 min at 16 000 *g*, at + 4 °C. The precipitate was discarded, and the supernatant was saturated to 80 % with ammonium sulfate. After centrifugation, the precipitate was dissolved in 50 ml of 10 mM sodium phosphate buffer, pH 7.5.

Treatment at pH 4. The concentrated enzyme solution (56 ml) was adjusted to pH 4.0 by adding 0.5 M HCl solution at 0 °C. The solution was centrifuged at + 1 °C, at 16 000 *g*, for 10 min, the inactive sediment was discarded, and the supernatant was adjusted to pH 6.0 with 0.5 M NaOH.

Sephadex G-100 gel chromatography. The acid treated enzyme solution (57 ml) was applied to a 5 × 90 cm Sephadex ® G-100 column at + 4 °C. The column was equilibrated with a 10 mM sodium phosphate buffer, pH 7.5. Fractions of 8 ml were collected at a flow rate of 60 ml/h (Fig. 1), Two protein peaks, responsible for the hydrolysis of BANA, were fractionated. The first one was eluted at V_0 and was discarded. The second peak, of greater activity, was pooled (fractions Nos. 104 – 116) and was subjected to a DEAE cellulose chromatography.

First DEAE cellulose chromatography. A 2.6 × 28 cm column of DEAE cellulose (Whatman DE-32, pretreated following the instructions of the manufacturer) was equilibrated with a 10 mM sodium phosphate buffer, pH 7.5. The pooled enzyme preparation from the Sephadex G-100 chromatography (100 ml) was applied to the column and unabsorbed proteins were eluted with 100 ml of the buffer. The flow rate was 60 ml/h and the fraction volume 5 ml. Absorbed proteins were eluted using a linear salt gradient in the buffer (0 – 0.5 M NaCl, total volume of the gradient was 800 ml). The BANA hydrolase was fractionated into two main peaks (Fig. 2). The first was eluted with the equilibrating buffer and the second with a weak (48 mM) sodium chloride solution. The first peak (fractions Nos. 19 – 31) and the second peak (fractions Nos. 47 – 57) were pooled separately and called preparations I and II, respectively.

Treatment of preparations I and II at pH 4, at 55 °C, increased the BANA hydrolase activity of undiluted preparation II, but not the activity of preparation I (Fig. 3). The fact that preparation I was not further activated suggested that it might be an activation form of preparation II. Following this, one half of preparation II was adjusted to pH 4.0 at + 4 °C by adding 0.5 M HCl solution and was incubated for 20 min at 55 °C. Both acid treated and nontreated portions of the enzyme were dialyzed in 10 mM sodium phosphate buffer, pH 7.5, at + 4 °C, and subjected to a rechromatography on DEAE cellulose.

Second DEAE cellulose chromatography. Two identical columns of DEAE cellulose with dimensions of 2.6 × 25 cm were equilibrated with 10 mM sodium phosphate buffer, pH 7.5. The acid treated and nontreated portions of preparation II were adjusted to the same volume (30 ml) with the buffer and were applied simultaneously using different channels of a peristaltic pump. Unabsorbed proteins were eluted with 50 ml of the buffer (per column). A linear salt gradient (0 – 0.4 M NaCl, total volume 600 ml) was formed in the same mixer and was divided between the two columns *via* different channels of the pump. The flow rates were 30 ml/h and the fraction volumes 4.5 ml. The chromatography of the nontreated preparation II was called DEAE-N and that of the acid treated preparation II DEAE-A (Fig. 4). Elution profiles from the DEAE-N chromatography were similar to those from the first DEAE chromatography. A small peak (NI) was eluted with the equilibrating buffer and a more active peak (NII) at the 0.046 M NaCl concentration. In the DEAE-A chromatography the unabsorbed peak (AI) was much more active than that eluted with NaCl solution (AII). The second peak from the DEAE-N chromatography (fractions Nos. 27 – 33) and the first peak from the DEAE-A chromatography (fractions Nos. 8 – 18) were pooled separately and called preparations NII and AI,

Table 1. Summary of the purification procedure of rat skin BANA hydrolase. The activity values are extrapolated to infinite dilution. Values in parentheses represent the activities of undiluted enzyme preparations.

Purification step	Volume ml	Protein mg	Activity nmol/min mg	Activity nmol/min	Purific. coefficient	Relative yield %
Extract	735	2250	1.18 (0.22)	2616 (485)	1	100
Ammonium sulfate fractionation	56	795	1.90 (0.07)	1510 (56)	1.6	58
Treatment at pH 4	57	467	3.3	1541	2.8	59
Sephadex G-100	100	56.1	22.8 (6.4)	1279 (361)	19	49
1. DEAE cellulose						
Preparation I	51	1.27	44.8	56.9	38	2
Preparation II	57	3.02	224.5 (63.6)	678.3 (192)	190	26
2. DEAE cellulose						
Preparation AI	48	0.58	400.8	233	339	9
Preparation NII	30	0.93	291.9 (85.2)	272 (79)	247	10

respectively. The purification procedure is summarized in Table 1.

CM cellulose chromatography. A 2.6 × 25 cm column of CM cellulose (Whatman CM-32, pretreated following the instructions of the manufacturer) was equilibrated with a 10 mM sodium citrate buffer, pH 4.8. The sample (25 ml of pooled BANA hydrolase from a Sephadex G-100 chromatography) was dialyzed in the same buffer and the elution was performed using a linear gradient of NaCl in the buffer (0 – 0.5 M NaCl, total volume 800 ml). The flow rate was 60 ml/h and the fraction volume 4.5 ml.

Amberlite IRC-50 chromatography. A 2.6 × 30 cm column of Amberlite® IRC-50 (BDH, standard grade) was equilibrated with 90 mM sodium citrate buffer, pH 5.3. The sample (25 ml of pooled BANA hydrolase from a Sephadex G-100 chromatography) was dialyzed in the same buffer and the chromatographic run was performed as described by Otto.[7]

Isoelectric focusing. Sucrose gradient in a LKB Ampholine® 8100 column was used.[8] The ampholine concentration was 1 % and the pH range 3 – 10. The potential difference was 300 V during the first 3 h and 800 V during the next 45 h.

Polyacrylamide gel electrophoresis. Electrophoresis was performed according to Smith *et al.*[9] using a 7.5 % gel in a tris-borate buffer, pH 8.6. Gels were stained with Coomassie Brilliant Blue (Brilliant Blue G-250, Sigma Chem. Co.).[10] In the determination of the activities of BANA and Leu-NA hydrolyzing enzymes, the gels were sliced to pieces of 2 mm. Proteins were extracted from the pieces with 0.3 ml of Britton-Robinson buffers, pH 5.8 or 7.0, containing thiol activators,[1] for BANA and Leu-NA, respectively. Extractions were performed for 4 h at +4 °C. Then 0.2 ml of extract was taken and 0.1 ml of substrate solution added, the mixture was then incubated for 2 h at 37 °C, and the degree of hydrolysis was determined by adding 0.4 ml of 4-dimethylaminobenzaldehyde reagent.[1]

Molecular sizes were determined by gel chromatography on a Sephadex G-100 column according to Andrews.[11] The dimensions of the column were 1.5 × 90 cm and a 10 M sodium phosphate buffer, pH 7.5, containing 0.1 M NaCl, was used at a flow rate of 5 ml/h, collecting fraction volumes of 2 ml. The column was standardized with 1 mg of Blue Dextran (Pharmacia Fine Chemicals, M. W. 2 000 000), 2 mg of bovine serum albumin (M. W. 68 000), 0.2 mg of pepsinogen (M. W. 40 400), 0.2 mg of soybean trypsin inhibitor (SBTI, M. W. 23 700), and 0.1 mg of ribonuclease A (M. W. 13 700). The standards and the BANA hydrolase preparations were applied to the column consecutively in 2 ml of the buffer; the BANA hydrolase was placed between pepsinogen and SBTI.

RESULTS

Purification of the enzyme. The extraction of BANA hydrolase from rat skin was performed as described earlier.[1] About 72 % of the total activity of the homogenate was recovered in the extract. The specific activity of the extract was increased about five-fold, when the extract was diluted and the activity extrapolated

to infinite dilution (see Table 1). This suggested presence of dissociable inhibitors in the extract.

In the ammonium sulfate fractionation, 9 % of the total BANA hydrolase activity and 42 % of the total proteins of the extract was recovered in the precipitate in 50 % saturation. The precipitate in 80 % saturation contained 58 % of the enzyme activity and 35 % of the total proteins of the extract. The specific activity was increased 1.6 times, as compared to the activity of the extract. The main part of inhibitor activity of the extract was precipitated together with BANA hydrolase (see Fig. 8), and a marked increase of specific activity was noticed after dilution of the enzyme solution. Dialysis of the enzyme solution in 10 mM sodium phosphate buffer, pH 7.5, increased specific activity of undiluted enzyme 2.2 times, but had no effect on BANA hydrolase activity when the enzyme was highly diluted. Dialysis was not used in the purification procedure, because it did not increase the specific activity of pooled BANA hydrolase after Sephadex G-100 gel chromatography.

Treatment of the ammonium sulfate fractionated enzyme solution at pH 4, at 0 °C, caused precipitation of some inactive proteins, and after centrifugation, increased the specific activity of the BANA hydrolase slightly. The specific activity of the enzyme was increased 2.8 times and the yield was 59 %, as compared to the activity of the extract.

In Sephadex G-100 gel chromatography two protein peaks, responsible for the hydrolysis of BANA, were found (Fig. 1). The first one was eluted at V_0 and was discarded. The second peak, of greater activity, hydrolyzed BANA, Leu-NA, and Arg-ME. The maximal activity obtained with the above substrates was found in the fraction No. 106 ($V_e/V_0 = 1.66$). The highly active Leu-NA hydrolyzing enzyme which in the preliminary Sephadex G-100 chromatography [1] was eluted in V_0 had been eliminated during ammonium sulfate and acid precipitations. The principal BAA hydrolyzing activity (cathepsin B2) was separated from the BANA hydrolyzing enzyme, but a smaller peak was found in the same elution volume as the BANA hydrolase (fraction No. 106). The hemoglobin hydrolyzing enzyme (cathepsin D) was incompletely separated from the BANA hydrolase. When the fractions of BANA hydrolase activity

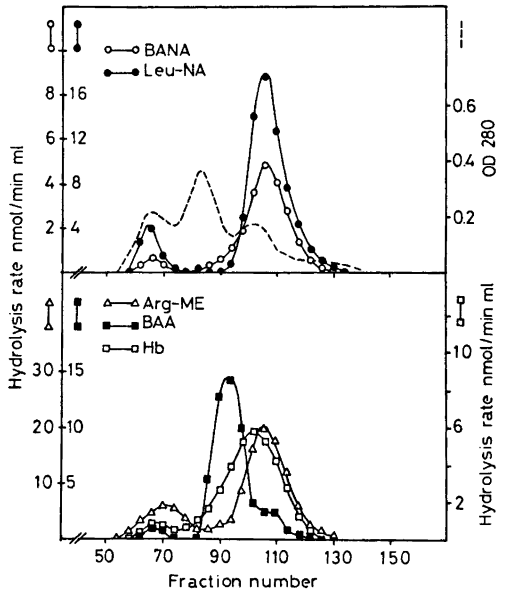

Fig. 1. Sephadex G-100 chromatography of the acid treated ammonium sulfate fraction (50–80 % saturated). Details are in the text.

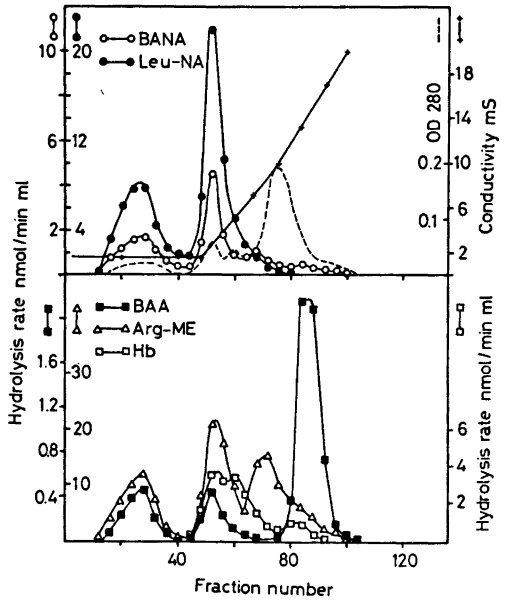

Fig. 2. First DEAE cellulose chromatography of the pooled preparation from the Sephadex G-100 chromatography. Details are in the text.

were pooled, the majority of the hemoglobin hydrolase activity was included. This pooled preparation gave a purification coefficient of 19 and a yield of 49 %, as compared to the activity of the extract. The majority of BANA hydrolase inhibiting activity was separated from the enzyme during the gel chromatography (see Fig. 8) but the specific activity of BANA hydrolase in the pooled preparation was still increased by diluting the enzyme.

In the first DEAE cellulose chromatography the BANA hydrolase from the Sephadex G-100 gel chromatography was fractionated into two main peaks (Fig. 2). The enzymes in both peaks hydrolyzed BANA, Leu-NA, and Arg-ME, and the second contained an enzyme that hydrolyzed hemoglobin. The main BAA hydrolyzing enzyme (cathepsin B2) was eluted at a high salt concentration (fraction No. 84). Arg-ME was also hydrolyzed by an enzyme (fraction No. 72) that was separate from the major BANA hydrolyzing enzymes. The pooled enzyme of the first BANA hydrolase peak was called preparation I and the enzyme of the second peak preparation II, respectively. Preparation I had a 38-fold increase in specific activity and preparation II a 190 fold increase, in comparison with the specific activity of the extract. The relative yields were 2 % for preparation I and 26 % for preparation II, in comparison to the total activity of the extract. The specific activity of preparation I was independent on the enzyme concentration, but the specific activity of preparation II was increased when the enzyme concentration was lowered by dilution.

Treatment of preparation II at pH 4. At all stages of purification, lowering the pH to 4.0 and preincubation of the enzyme at 55 °C increased the activities of undiluted enzymes. When preparations I and II were preincubated at pH 4, at 55 °C, only the activity of preparation II was increased (Fig. 3). Its BANA and Leu-NA hydrolyzing activities were increased in the same proportion, while the hemoglobin hydrolyzing enzyme was slightly inactivated. The fact that the acid treatment did not increase the BANA hydrolase activity of preparation I suggested that preparation I might be an activated form of preparation II. In order to study if the chromatographic behavior of preparation II is changed after acid treatment to

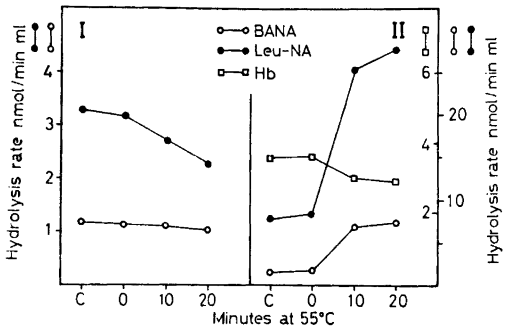

Fig. 3. Effect of treatment at pH 4, at 55 °C, on the activity of undiluted preparations I (left) and II (right) obtained after the first DEAE chromatography. C = control (nontreated enzymes).

resemble the behaviour of preparation I, one half of preparation II was treated at pH 4, at 55 °C. Both acid treated and nontreated portions were subjected to an additional chromatography on DEAE cellulose.

Second DEAE cellulose chromatography. The chromatograms obtained from nontreated

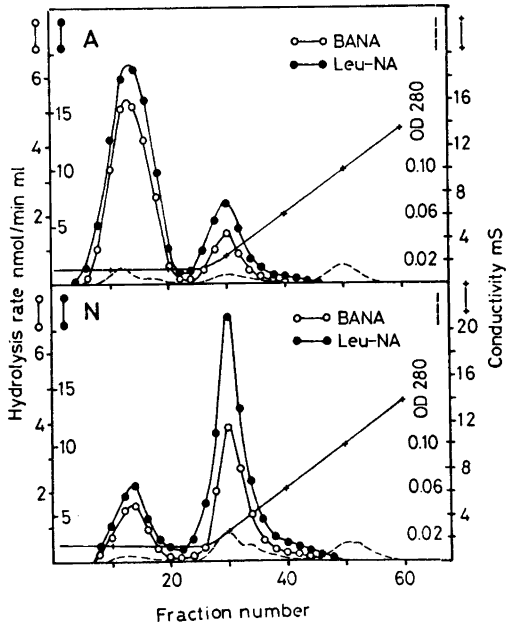

Fig. 4. Second DEAE cellulose chromatography. In run A the sample was the acid treated preparation II and run N was of nontreated preparation II, from the first DEAE chromatography. Details are in the text.

(DEAE-N) and acid treated (DEAE-A) portions of preparation II are in Fig. 4. The elution profiles from the DEAE-N chromatography were similar to those of the first DEAE chromatography. A small BANA hydrolase peak (NI) corresponding to preparation I was eluted with the equilibrating buffer and a more active peak (NII) corresponding to preparation II at the 46 mM NaCl concentration. In the DEAE-A chromatography the unabsorbed peak (AI) was much more active than that eluted with NaCl solution (AII). The increase of the unabsorbed, preparation I-like peak after the acid treatment of preparation II suggests that preparations I, NI, and AI are derivatives of preparation II.

Equal amounts of BANA hydrolase were applied to DEAE-N and DEAE-A columns and approximately the same amounts of proteins should be obtained by summation of proteins in BANA hydrolase preparations from each charomatography. In DEAE-N chromatography the sum of proteins in preparations NI and NII was 1.23 mg and in DEAE-A chromatography the sum of proteins in preparations AI and AII was 1.01 mg. These results are in agreement with the hypothesis that preparations AI and NI are formed from preparation II, but loss of some protein as a consequence of the acid treatment is noticed.

The enzyme preparations NI, NII, AI, and AII hydrolyzed BANA, Leu-NA, BAA, and Arg-ME, but hemoglobin was hydrolyzed only by an enzyme in preparations NII and AII. This suggests that the hemoglobin hydrolyzing enzyme is quite distinct from the BANA hydrolase. The specific activity of the diluted BANA hydrolase preparation NII was 292 nmol/min mg and the activity of preparation AI 401 nmol/min mg, which gave purification coefficients of 247 and 339, respectively, as compared to the activity of the extract. The relative yield of preparation NII was 10 % and that of preparation AI 9 %, as compared to the total activity of the extract. The activity of preparations NI and AI increased linearly with enzyme concentration, while activities of preparations NII and AII did not. The complete purification procedure is summarized in Table 1.

Additional purification efforts. At all stages of purification those fractions hydrolyzing BANA also hydrolyzed Leu-NA to an even greater extent (×4.5). This is interesting, since cathepsin B1 preparations that hydrolyse Leu-NA are usually regarded as impure. The separation of Leu-NA and BANA hydrolyzing activities of pooled preparations from Sephadex G-100 chromatography was attempted by CM cellulose chromatography, Amberlite IRC-50 chromatography,[7] isoelectric focusing in a sucrose gradient,[8] and analytical polyacrylamide gel electrophoresis.[9] Two peaks of enzyme activity were demonstrated using CM cellulose chromatography, gel electrophoresis, and isoelectric focusing and they corresponded to preparations I and II of the DEAE chromatography. Both peaks obtained by these methods hydrolyzed BANA and Leu-NA. In isoelectric focusing the peak with a pI of 6.2 corresponded to preparation II and that with a pI of 7.5 to preparation I. The peak with a pI of 6.2 was activated at acidic pH treatment and its activity did not increase linearly with enzyme concentration. In Amberlite IRC-50 chromatography, which according to Otto [7,12] separates cathepsin B1 and the Leu-NA hydrolyzing enzymes of beef spleen, BANA and Leu-NA hydrolyzing enzymes were eluted together with the equilibrating buffer (90 mM sodium citrate buffer, pH 5.3).

No purification method attempted was able to separate the BANA and Leu-NA hydrolyzing activities.

Molecular weight. The molecular weights of the purified preparations were estimated using a calibrated Sephadex G-100 column. The results obtained with preparation NII are given in Fig. 5. The same approximate molecular

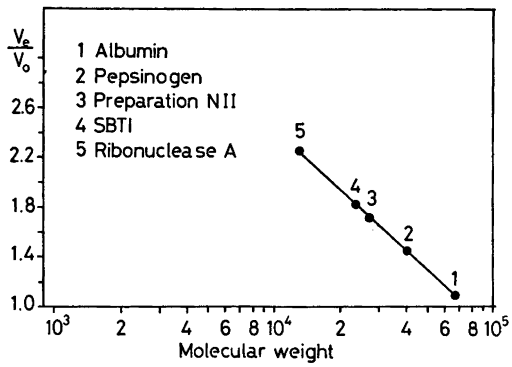

Fig. 5. Estimation of the molecular weight of preparation NII after gel filtration on Sephadex G-100. Details are in the text.

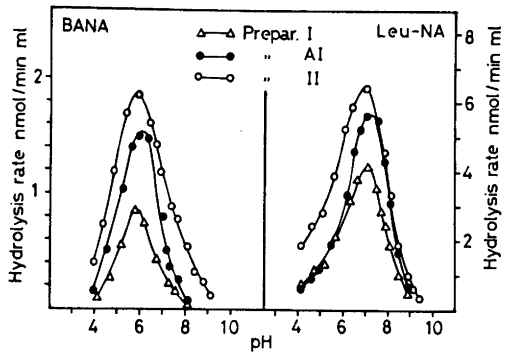

Fig. 6. Effect of pH on the hydrolysis of BANA and Leu-NA using the purified preparations I, AI, and NII. The buffer was Britton-Robinson universal buffer.[1]

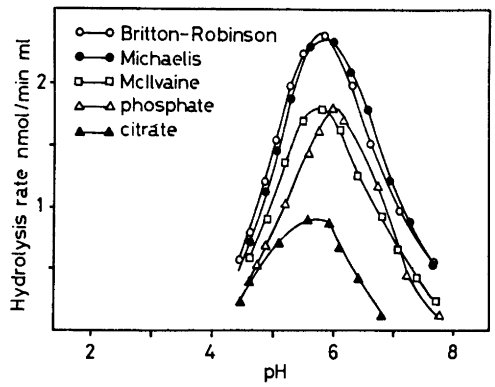

Fig. 7. Effect of pH on the BANA hydrolase activity of preparation II in various buffers. Britton-Robinson buffer=acetate-phosphate-borate,[1] Michalis' buffer = veronal-acetate,[4] McIlvaine buffer = citrate-phosphate buffer.[4] Sørensen's citrate [4] and phosphate [4] buffers were also used.

weight of 27 000 was obtained for all preparations.

Optimal pH. BANA was hydrolyzed optimally at pH 5.8 and Leu-NA at pH 7.0 by the purified preparations I, AI, and NII (Fig. 6). The hydrolysis rate of BANA in various buffers is presented in Fig. 7. Britton-Robinson buffer [1] and Michaelis' veronal-acetate buffer [4] gave the best activities. Sørensen's phosphate buffer [4] and McIlvaine's citrate-phosphate buffer [4] gave activities that were 20 % lower than the activities in Britton-Robinson buffer. In the Sørensen's citrate buffer [4] the activity was 60 % less than in the Britton-Robinson buffer.

Demonstration of inhibitors. The activity of

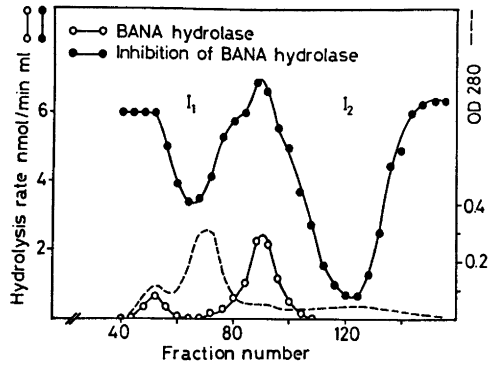

Fig. 8. Demonstration of inhibitors by Sephadex G-100 gel chromatography in the acid treated ammonium sulfate fraction. A sample of 60 ml was applied to a 5 × 70 cm column at 4 °C and the elution was performed with a 10 mM sodium phosphate buffer, pH 7.5. Fractions of 9 ml were collected at a flow rate of 60 ml/h.

BANA hydrolase from various purification stages did not increase linearly with increasing enzyme concentrations, excepting activities of the DEAE cellulose preparations I, NI, and AI. The presence of inhibitors that might have caused the nonlinearity of the activity/enzyme concentration curves was studied by fractionating the various pools obtained during the purification procedure by Sephadex G-100 gel chromatography and measuring the inhibitory capacity of the chromatographic fractions on BANA hydrolase. Two inhibitors were clearly demonstrated in the extract and acid treated ammonium sulfate fraction, but pooled BANA hydrolase preparations from the Sephadex G-100 and DEAE chromatographies did not contain measurable amounts of inhibitor activity. In Fig. 8, the fractionation of inhibitors in the acid treated ammonium sulfate fraction is presented. The first inhibitor eluted in fractions Nos. 62 – 80 was called I_1 and the second inhibitor in fractions Nos. 102 – 128 was called I_2. The inhibitory activity of I_2 was greater than that of I_1. They were also found to inhibit the thiol proteinases papain, ficin, bromelain, and cathepsin C, but not trypsin. Either inhibitor may be as a minor contaminant of the BANA hydrolase in preparations II and NII. Disappearance of the nonlinearity of the activity/enzyme concentration curve during acid treatment may account for irreversible dissociation of these inhibitors.

DISCUSSION

The fairly simple purification procedure used revealed that in the rat skin there were at least two cathepsin B1 like enzymes hydrolyzing BANA optimally at pH 5.8. The number of multiple forms of cathepsin B1 in various mammalian tissues varies greatly; one form in rat liver,[13] three forms in sheep thyroid gland,[14] five forms in bovine spleen,[15,16] and six forms in human liver [17] have been noticed. The molecular weight of the rat skin BANA hydrolase preparations was about 27 000. This is of the same magnitude as those obtained for cathepsin B purified from various other tissues.[14–14,16–20]

The rat enzymes hydrolyzed BANA optimally at pH 5.8. This corresponded to the pH-optima of other cathepsin B1 preparations (pH 5.5 – 6.0). An exception is rat liver BANA hydrolase, which has a pH-optimum of 7.0.[13] The pH-optimum for the hydrolysis of Leu-NA by the rat skin BANA hydrolase was at pH 7.0. Snellman,[18] who is the only worker to have reported Leu-NA hydrolysis by cathepsin B1, obtained from calf liver, has reported the same optimum pH of 7.0. Phosphate ions that are known to inhibit bovine spleen and sheep thyroid cathepsin B1 preparations,[12,14,21] also inhibited the rat skin enzyme to a minor degree. In addition, a marked inhibition of the rat skin BANA hydrolase was noticed in a citrate buffer.

The treatment of preparation II at pH 4, at 55 °C, for 20 min transformed the enzyme to a form that is very similar to preparation I. After the acid treatment, the isoelectric point of preparation II was changed from 6.2 to 7.5, which is the pI obtained with preparation I, and the chromatographic behaviour of the enzyme became similar to that of preparation I. The activity of acid-treated preparation II increased linearly with enzyme concentration, as did the activity of preparation I, but not that of preparation II.

The mechanism of activation of preparation II remains unclear. One probable hypothesis is that the hemoglobin hydrolyzing cathepsin D contaminant that has a pH optimum of 4.3, destroyed some acidic inhibitory peptide during incubation at pH 4, at 55 °C. However, the possibility that the BANA hydrolase molecule itself is modified during the acid treatment has not been excluded.

The purified preparations hydrolyzed classical substrates of cathepsin B1, like BANA and BAA. In addition, substrates with a free α-amino group, like Leu-NA and Arg-ME were hydrolyzed even better than BANA. The ratio Leu-NA/BANA was 4.5 and Arg-ME/BANA 6.7, for preparation II. This aminopeptidase-like activity is worth of noticing since pure cathepsin B1 preparations from other tissues do not hydrolyze Leu-NA.[12–14,17,22] The early preparation of Snellman [18] from calf liver was found to exhibit some Leu-NA hydrolyzing activity. This was strongly criticized by Barrett and Poole,[23] McDonald *et al.*,[24] and Otto,[12] and it was later admitted by Sylvén and Snellman [25] to be caused by a contaminating aminopeptidase. In the rat skin, BANA and Leu-NA hydrolyzing activities seemed to be tightly bound together. They were not separated by gel filtration, DEAE, CM, or Amberlite IRC-50 chromatography, isoelectric focusing or analytical polyacrylamide gel electrophoresis. In addition, treatment of preparation II at acidic pH caused similar changes in the BANA and Leu-NA hydrolyzing activites: The activity of undiluted enzyme tested against both substrates increased in the same proportion and the isoelectric point shifted from 6.2 to 7.5; neither activity was retained on the DEAE cellulose and after the acid treatment hydrolysis of both substrates increased linearly with increasing enzyme concentrations.

The nonlinearity of the activity/enzyme concentration curve of the extract suggested the presence of inhibitors of BANA hydrolase in the skin. Two inhibitors were indeed separated from the extract, as well as from the acid treated ammonium sulfate fraction, by Sephadex G-100 chromatography. Approximate molecular weights of these inhibitors were 74 000 for I_1 and 13 000 for I_2. The low molecular weight suggests that I_2 is probably similar to the inhibitor purified by Udaga and Hayashi [26] from rabbit skin with a healing Arthus inflammation. Lazarus and Dingle [27] have also demonstrated the inhibitory activity of rabbit skin extract on human cathepsin B1. Human liver cathepsin B1 is also inhibited by α_2-macroglobulin and IgG, but not with haptoglobin,[28] which according to Snellman and Sylvén inhibits proteolytic activity of calf liver cathepsin B1.[29] Later Snellman and Sylvén

found that mouse haptoglobin itself was not inhibitory but it contained an inhibitory polysaccharide with a molecular weight of 900.[30] This inhibitor may be dissociated from haptoglobin by a treatment with hemoglobin, or low pH. I_1 preparations from rat skin are also known to contain polysaccharides.[31] Relationships between I_1 and haptoglobin remain to be elucidated.

The rat skin inhibitors I_1 and I_2 inhibited thiol proteinases, such as BANA hydrolase, cathepsin C, papain, ficin, and bromelain. Since trypsin was not inhibited, the rat skin inhibitors appear to be group specific and polyvalent against thiol proteinases in a similar manner as inhibitors of serine proteinases.

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