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A CHLORIDE-ACTIVATED AMINOPEPTIDASE IN RAT INFLAMMATORY EXUDATE: PROPERTIES AND EVIDENCE OF THE ORIGIN OF THE ENZYME

M. Knuuttila[1], K. Virtanen[2], E. Söderling[2] and K. K. Mäkinen[2]

[1]Institute of Dentistry, University of Kuopio, Kuopio, Finland
[2]Institute of Dentistry, University of Turku, Turku, Finland

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SUMMARY

The activity levels of a chloride-dependent arginine aminopeptidase in an inflammatory exudate collected from sponges implanted in the dorsal region of rats, was found to be connected to the number of leukocytes present. Leukopenia caused a corresponding decrease in the exudate enzyme activity. Disruption of the cells by injecting Triton X-100 into the sponges *in vivo* immediately increased the enzyme activity levels. The enzyme was activated by NaCl; the maximum rate of hydrolysis was measured at 0.2 M NaCl. The enzyme was activated by thiols and strongly inhibited by 10^{-6} M *p*-chloromercuribenzoate. The isoelectric point of the enzyme was at pH 5.1. The properties of the enzyme support the idea of its similarity with an arginine aminopeptidase demonstrated earlier in various inflammatory processes.

Aminopeptidase B (EC 3.4.16.1, APB) has been purified first from rat liver and later from other tissues, tissue fluids and erythrocytes, and it has also been demonstrated in inflammatory lesions (1-5). The activity levels of chloride-activated arginine aminopeptidases and especially APB have been shown to increase during inflammatory processes (3,5,6). The functions suggested for APB in inflammation include a possible inflammatory peptide formation (7). Although the occurrence of APB-like activity has been demonstrated in inflammatory exudates (3), only tentative evidence of its origin has been presented (5). The aim of the present report was thus to study the possible connection between the APB-like enzyme activity of rat inflammatory exudate and the number of leukocytes present.

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MATERIALS AND METHODS

Chemical methods. Aminopeptidase activity was determined in the presence of 0.2 M NaCl and without added salt, using N-L-aminoacyl-2-naphthylamines (-2NA) as substrates (3). Proteins were determined according to Lowry et al. (8). Cyclophosphamide was from Lääke Oy (Turku, Finland). Blue Dextran 2000 and Sephadex G-100 Superfine were from Pharmacia Fine Chemicals (Uppsala, Sweden). Triton® X-100 was obtained from E. Merck AG (Darmstadt, Germany). All other chemicals were of analytical grade and their sources were mentioned earlier (9).

Chromatography. The separation of APB-like enzymes was carried out by fractionating the crude enzyme preparations on Sephadex® G-100 columns (130 x 1.7 cm) with a constant flow-rate of 2 ml/hour (temperature +4 °C; elution buffer 0.01 M β,β-dimethylglutarate, pH 7.2, containing 10^{-3} M dithiothreitol; sample vol. 2 ml and fraction vol. 0.5 ml). This is the standardized method to separate APB-like enzymes (9). Flat bed electrofocusing of the cell-free control and the Triton treated exudate was performed using a LKB 2117-101 Ampholine Electrofocusing Kit for polyacrylamide gel together with the LKB 2117-301 Multiphore basic unit (9).

Collection and treatment of leukocytes and exudate. Two sterilized pieces (1x1x3 cm) of viscous sponge (Kongsfors Fabriken A/S, Oslo, Norway), impregnated with 3 ml of 0.9 % NaCl, were implanted subcutaneously in the dorsal region of Long Evans rats anaesthetized with ether. The inflammatory exudate of the sponges removed after 16 hours, was immediately collected and the number of leukocytes was counted in a Bürker chamber (10). The cells were subsequently removed on a Millipore filter (pore size 0.45 μ). 2 ml of the clear filtrate (cell-free exudate, total volume about 4 ml) was applied on a Sephadex G-100 Superfine column.

The rest of the cell-free exudate was dialyzed against 5 l deionized water for 12 hours at +4 °C for the determination of enzyme activity and protein concentration. The number of leukocytes, and the rate of the hydrolysis of N-L-arginyl-2NA in the presence of 0.2 M NaCl and without added salt, were determined in the exudate at different times after inserting the sponges.

The number of leukocytes in three male and three female rats was reduced by injecting intraperitoneally 18 mg/kg cyclophosphamide on three successive days. Leukocytes in the blood were counted daily before and after the injections (Fig. 1). The sponges were implanted subcutaneously into leukopenic rats on the fourth day after the first injection of cyclophosphamide. The exudate from the sponges removed after 16 hours was treated as mentioned above. The chromatographic fractions eluted with about 120 ml elution buffer, obtained from the control exudate, were pooled for further characterization. The effect of NaCl, p-chloromercuribenzoate and thiols on the rate of the hydrolysis of arginyl-2NA, as well as the substrate specificity with different aminoacyl-2-naphthylamines, were studied.

Cells which had migrated into subcutaneously implanted sponges during 16 hours were disrupted by injecting 1 % Triton X-100 into the sponges. This solution was injected into one side and the control solution (0.9 % NaCl) was injected into the other side of the rat. Three successive 0.5 ml injections at intervals of 30 min were carried out. The sponges were removed 30 min following the last injection and the exudates

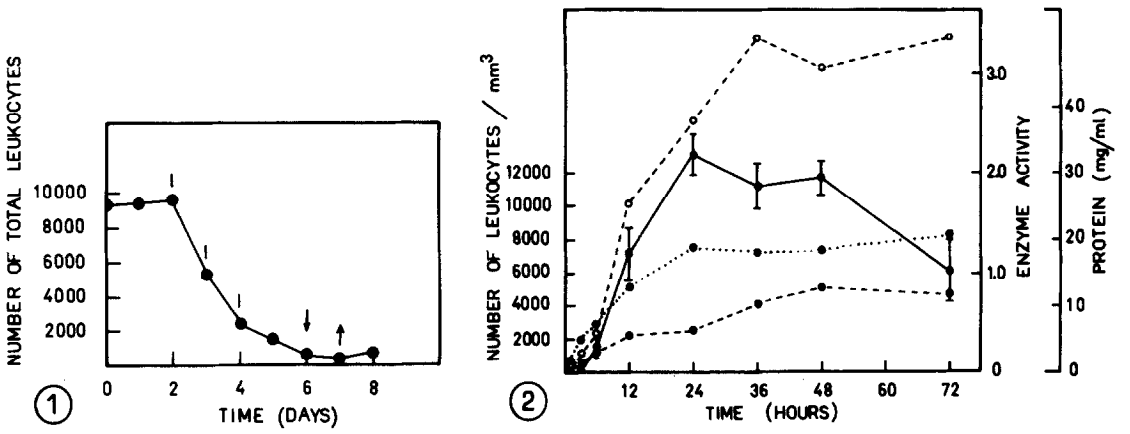


Figure 1. Effect of three successive daily injections (marked with |) of cyclophosphamide on the number of leukocytes per mm^3 blood, determined daily (●-●). The sponges were implanted subcutaneously four days from the first injection (↓) and they were removed 16 hours later (↑).

Figure 2. The correlation between the number of leukocytes (o-o) present in the exudate and the enzyme activity (in 10^{-7} x moles x min^{-1}) toward arginyl-2NA tested without added NaCl (●---●) and in the presence of 0.2 M NaCl (o---o). Protein concentration (●···●) is in mg/ml.

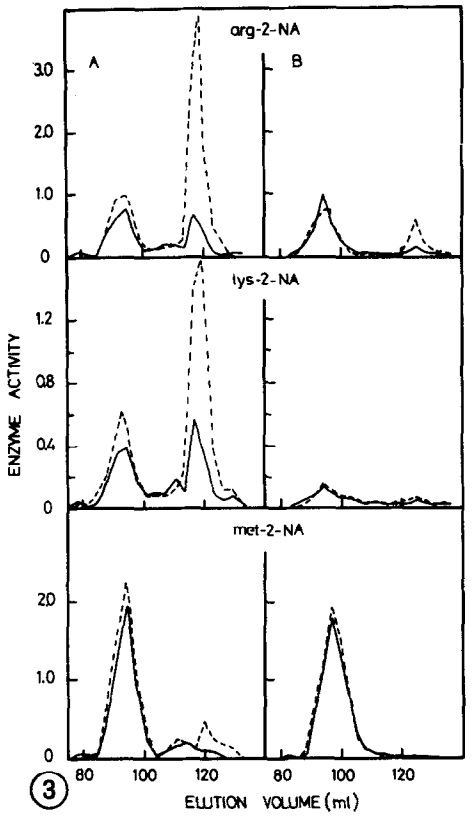
were collected. The leukocytes were counted and the cell-free exudates were fractionated on a Sephadex G-100 Superfine column as mentioned above.

RESULTS

1. Connection between the activity levels of APB and the number of leukocytes in the exudate

Time dependence. Chloride-activated arginine aminopeptidase activity in the exudate was found to parallel to the number of leukocytes (Fig. 2).

Effect of cyclophosphamide. The effect of cyclophosphamide on the number of total leukocytes in blood is shown in Fig. 1. The lowest values were obtained 4-6 days after the first injec-



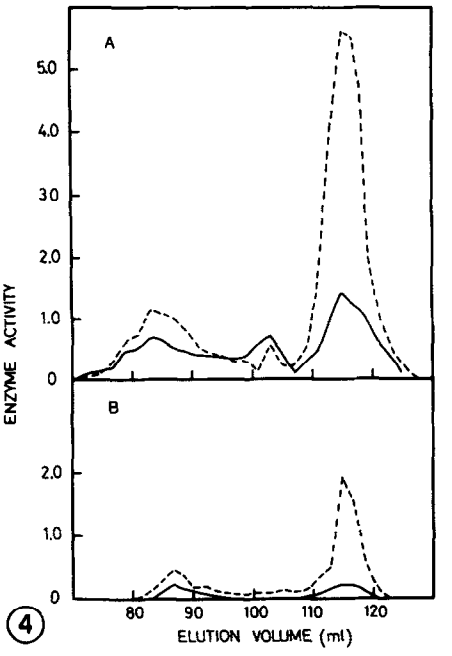


Figure 3. Gel permeation chromatography (descending) of the inflammatory exudate from sponges implanted subcutaneously in healthy (A) or leukopenic (B) rats. Sample volume was 2 ml. Enzyme activities (in 10^{-7} x moles x min^{-1}) were measured with arginyl-, lysyl- and methionyl-2NA in the presence of 0.2 M NaCl (---) and without added salt (——).

Figure 4. Gel permeation chromatography (descending) of the inflammatory exudates sucked from subcutaneously implanted sponges after treatment with Triton X-100 (A) or saline (B). Sample volume was 2 ml. The enzyme activity (in 10^{-7} x moles x min^{-1}) toward arginyl-2NA was tested in the presence of 0.2 M NaCl (---) and without added salt (——).

tion, the reduction being about 87 %. The number of leukocytes in the control and leukopenic exudates was 6700±400 and 150±63, respectively. Exudate protein concentrations of leukopenic

rats were nearly equal to those of control rats.

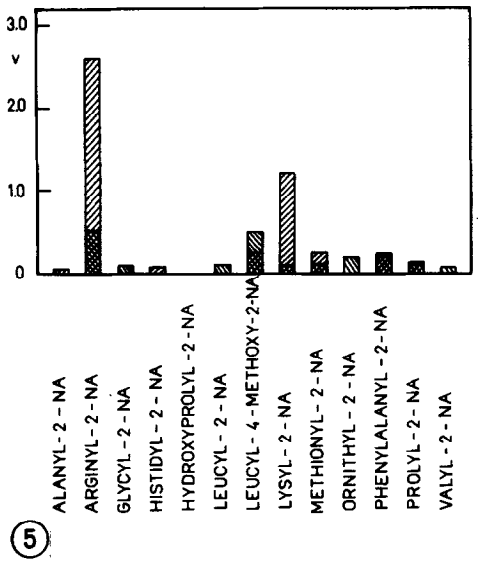
Gel permeation chromatography revealed two enzyme peaks both in the control and the leukopenic exudates (Fig. 3). The enzyme of the control samples eluted at 85-100 ml and it was slightly activated by 0.2 M Cl^- ions, whereas the enzyme of the corresponding peak of the leukopenic sample was not affected by Cl^- ions. However, the activity of the second enzyme peak, eluted at 118-130 ml, was clearly chloride-dependent in both samples, when arginyl-2NA was used as the substrate. The differences between the control and leukopenic exudates were chiefly the same when lysyl-2NA was used as the substrate. The number of leukocytes seemed to be connected with the activity levels of chloride-activated arginine aminopeptidases in the exudate.

Lysis of leukocytes with Triton X-100. Triton X-100 caused a very effective lysis of the cells *in vivo*. The control exudates contained 4200±800 and the test exudates 490±190 leukocytes, respectively. An increase of 60 % in the protein concentrations and about a fourfold increase in the enzyme activities (in 10^{-7} x moles x min^{-1}), determined both in the presence of 0.2 M NaCl and without added salt, was obtained in Triton-treated samples compared with the controls. However, the specific activities did not differ significantly.

Chromatography of the above mentioned exudate after Triton X-100 treatment revealed three enzymes hydrolyzing arginyl-2NA. The one with the highest molecular weight was slightly and the one with the lowest molecular weight was to a great extent activated by Cl^- ions (Fig. 4A). Corresponding smaller enzyme peaks were found in the chromatogram obtained from the control sample (Fig. 4B). The lysis of leukocytes in the exudates *in vivo* caused a marked increase in the activity levels of the enzyme with the lowest molecular weight. Triton X-100 in the concentration used did not affect the rate of the hydrolysis of arginyl-2NA catalyzed by the chloride-activated aminopeptidases of the exudate.

2. Characterization of APB from the exudate

The rates of the hydrolysis of 13 various aminoacyl-2-



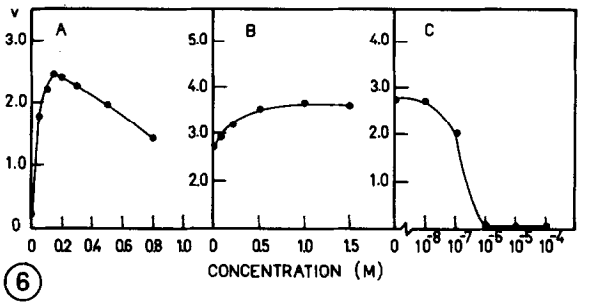


Figure 5. Rates (v, in 10^{-4} x M x min^{-1}) of the hydrolysis of various aminoacyl-2NA catalyzed by the APB-like enzyme from the inflammatory exudate in the presence of 0.2 M NaCl (////) and without added salt (\\\\).

Figure 6. Effect of the concentration of NaCl (A), dithiothreitol (B) and p-chloromercuribenzoate (C) on the rate (v, in 10^{-4} x M x min^{-1}) of the hydrolysis of arginyl-2NA catalyzed by the APB-like enzyme from inflammatory exudate. In the case of dithiothreitol and p-chloromercuribenzoate the reactions were performed in the presence of 0.2 M NaCl.

naphthylamines, catalyzed by the enzyme from the untreated sample (fractions 117-122; Fig. 3), are shown in Fig. 5. The highest rates of the hydrolysis were observed with arginyl-2NA and lysyl-2NA in the presence of 0.2 M NaCl. The ratio of these rates was about 2:1. The chloride activation was also most effective with these two substrates.

The rate versus the concentration of NaCl curve passed through a maximum at 0.2 M concentration (Fig. 6A). Thiols slightly increased the rate of the hydrolysis of arginyl-2NA (Fig. 6B). However, p-chloromercuribenzoate inhibited the hydrolysis by about 30 % at a concentration of 10^{-7} M. Total loss of activity was found with 10^{-6} M concentration (Fig. 6C).

Chloride-activated arginine aminopeptidase activity,

located at an isoelectric point of 5.1, was found in flat bet isoelectric focusing of both the control and the Triton-treated samples. The chromatograms were qualitatively identical, but the Triton-treated sample caused higher rates of the hydrolysis of arginyl-2NA.

DISCUSSION

The present results showed that chloride-dependent arginine aminopeptidase activity was present in the inflammatory exudates obtained from subcutaneously implanted sponges in the rat. This enzyme was found to be similar to the APB-like enzymes demonstrated earlier in other inflammatory exudates (3,5). The following characteristics supported this assumption: 1) Elution properties in molecular permeation chromatography, 2) isoelectric point, 3) substrate specificity, 4) chloride activation, 5) inhibition and activation characteristics with p-chloromercuribenzoate and dithiothreitol, respectively, and 6) the stability of the enzyme during storage at +2°C. The properties of the enzyme were quite similar to those of APB obtained from rat liver (11,12), human erythrocytes (4) and human serum (13), although slight variation was found depending on the origin of the enzymes. The inhibition and activation characteristics suggest that SH-groups are essential for the activity of the enzyme.

The activity levels of APB have been shown to increase during inflammatory processes (4-6,14). This increase in the present experimental model was found to be connected with the increase of the number of leukocytes and mononuclear phagocytes of the exudate. The correlation between the number of the cells present and the levels of APB-like enzyme activity in the exudate was confirmed with leukopenic rats. Cyclophosphamide is known to depress hemopoiesis and to reduce both the number of neutrophils, lymphocytes and erythrocytes (15). However, the reduction in the number of the red blood cells was quite small as compared to that of leukocytes in blood (15). Because of the low number of erythrocytes in the present exudate material, their contribution to the changes of this chloride-dependent aminopeptidase was assumed to be insignificant.

Disruption of the cells in the subcutaneously planted sponges with Triton X-100 caused an immediate increase in the

activity of the peptidase studied. It has been shown that the activity levels of the APB-like enzyme in rat erythrocytes are low as compared to the corresponding enzyme activities found in the leukocytes and mononuclear phagocytes of the exudate (9). Therefore, the increase in the activity levels in the exudate was most likely caused by the lysis of the last mentioned cell group.

Although high APB-like activity has been found in different inflammatory fluids and lesions, the present results gave new information about the possible origin of this enzyme. These findings suggest that APB could be one of the hydrolytic enzymes released into extracellular space during the inflammatory process. The activation of this neutral peptidase by physiological concentrations of Cl^- ions and the preservation qualities indicate an extracellular function of the present enzyme.

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