AMINOPEPTIDASE B-LIKE ENZYMES IN LEUKOCYTES

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1. Introduction

Proteolytic enzymes, released during the inflammatory process, have been demonstrated in a variety of cells, i.e., mast cells, PMN leukocytes, lymphocytes and macrophages [1–5]. A specific aminopeptidase, aminopeptidase B (APB, EC 3.4.11.6) purified first from rat liver [6] and later from other tissues and tissue fluids of both human and animal origin [7–11], has shown to be associated with inflammation [9]. Rat liver aminopeptidase B (true APB) differs from other mammalian aminopeptidases (1) in regard to its ability to hydrolyze only *N*-L-arginyl- and *N*-L-lysyl-2-naphthylamines and (2) in its activation by 0.9% NaCl. The latter property has been proposed to indicate an extracellular function for the enzyme during the inflammatory process [12].

The presence of APB-like enzymes in leukocytes has, however, not been reported earlier, although a preceding study [13] indicated that there may be a connection between the APB-like enzyme activity of rat inflammatory exudate and the number of leukocytes present. The aim of the present study was thus to separate and characterize the APB-like enzymes of leukocytes obtained from rat inflammatory exudates. The characteristics of these enzymes compared to those of the APB-like enzyme found in rat inflammatory exudate is also discussed.

2. Materials and methods

2.1. *Materials*

The *N*-L-aminoacyl-2-napthylamines were purchased from Schwarz/Mann, New York, NY and Koch-Light Laboratories, England. Dithiothreitol, 3,3-dimethylglutaric acid and *p*-chloromercuribenzoate were obtained from Calbiochem (City, Ca. USA). Blue Dextran 2000, Sephadex G-100 Superfine, Sephadex G-50 (coarse) and Dextran T500 were from Pharmacia Fine Chemicals (Uppsala, Sweden). Isopaque was from Medica (Helsinki, Finland) and heparin from Orion (Helsinki, Finland). All other reagents were obtained from E. Merck AG (Darmstadt, FRG).

2.2. *Collection and separation of leukocytes from inflammatory exudate*

The collection of the inflammatory exudate from viscous cellulose sponges implanted under the backskin of male and female Long Evans rats, weighing 300–350 g, has been described earlier [14]. The exudate was collected from sponges removed about 16 h after their insertion. The leukocytes and the erythrocytes were counted in a Bürker chamber. For identification of different cell-types, portions of the cell-suspension were centrifuged onto glass slides (Shandon cytocentrifuge) and stained with May-Grünewald-Giemsa solution. To obtain the total inflammatory cell fraction, 4 ml inflammatory exudate containing 5000–12 000 inflammatory cells/mm^3 was centrifuged (200 × *g*, 10 min, 20°C) and washed twice with 10 ml saline at 20°C. Erythrocytes were removed with hemolysis [15].

The separation of PMN and mononuclear leukocytes was made by modifying slightly the method of Böyum [16]. The inflammatory exudate was collected in heparinized test tubes (50 U heparin/10 ml exudate). After collection the exudate was filtered through cheese-cloth and diluted 1:1 with 7% serum albumin solution (w/v, albumin in saline). Four ml of this

mixture was layered on 4 ml of standard Isopaque-Ficoll solution in a centrifuge tube as described by Böyum [16] and centrifuged (400 × *g*, 35 min, +20°C, swing-out). The pellets containing the PMN leukocytes and the interphases containing the mononuclear cells of the exudate were collected and washed twice with saline at 20°C. Before further procedures the cells of these fractions were counted and identified.

2.3. *Separation and characterization of APB-like activity*

The disruption of the cells was performed in 0.01 M β,β-dimethylglutarate buffer, pH 7.2, containing 10^{-3} M dithiothreitol, either by sonicating slightly (amplitude 3, 30 s, end diameter of the probe 3 mm) using a MSE disintegrator at +4°C, or with 0.25% Triton® X-100 (60 min, +4°C). The membrane-free supernatant fluid (crude enzyme preparation) obtained after centrifugation (1700 × *g*, 15 min, +4°C) was stored at +4°C.

The separation of APB-like enzymes was carried out by fractionating the crude enzyme preparations on Sephadex® G-100 130 × 1.7 cm, constant flow-rate 2 ml/h, 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 [17]. The pooled fractions after molecular permeation chromatography were studied for chloride activation, substrate specificity and the effect of *p*-chloromercuribenzoate (*p*CMB) and dithiothreitol. These parameters, in addition to the fractionation qualities on Sephadex-gel, have been used to elucidate the identity between the true APB and the enzyme the properties of which are compared with those of APB [7,18].

Flat-bed electrofocusing of the samples was performed using an LKB 2117-101 Ampholine Electrofocusing Kit for polyacrylamide gel together with the LKB 2117-301 Multiphore basic unit. Ampholine PAG plates (pH 3.5–10.0) contained 2.4% (w/v) of Ampholine carrier ampholytes. The featured gel concentration of T was 5% and degree of cross-linkage of C was 3%. The staining of proteins was carried out with Coomassie Brilliant Blue (Schwarz Mann, New York, NY, USA). For determination of enzyme activity the plates were cut in slices (0.15 × 1.5 cm).

2.4. *Assay of enzyme activity*

Aminopeptidase activity was assayed using *N*-L-aminoacyl-2-naphthylamines (-2NA) as described earlier [9]. The addition of 0.2 mol NaCl/liter to the reaction mixtures reveals the involvement of the APB-like enzymes in column chromatography [17].

3. Results

3.1. *Fractionation of the leukocyte preparations*

The inflammatory exudate of rat obtained 16 h after inserting the sponges was found to obtain approximately 80% PMN and 20% mononuclear cells (macrophages, monocytes and lymphocytes). Erythrocytes represented 25% total cell-population. Chloride-dependent arginine aminopeptidase activity of the crude enzyme preparations counted /cell was about the same for both PMN and mononuclear cells (table 1). The activity of the enzyme preparations did not change during several weeks of storage at +4°C.

Figure 1 shows the molecular permeation chromatography of the crude enzyme preparations from disrupted total inflammatory cells, and separately from

Table 1
The total activity (in *v*)/cell of chloride-activated arginine aminopeptidases from various cell-types present in rat inflammatory exudate

Cell-type	*v* (μmol × min^{-1})	
	Without added NaCl	In 0.2 M NaCl
PMN leukocytes	1.0–2.0 × 10^{-9}	4.0–5.0 × 10^{-9}
Mononuclear cells	0.5–2.0 × 10^{-9}	1.0–5.0 × 10^{-9}
Erythrocytes	2.0–3.0 × 10^{-11}	4.5–5.5 × 10^{-11}

Substrate: 0.167 mM *N*-L-arginyl-2-NA. Tested in 0.05 M phosphate buffer, pH 7.2. Range of 3 to 6 experiments.

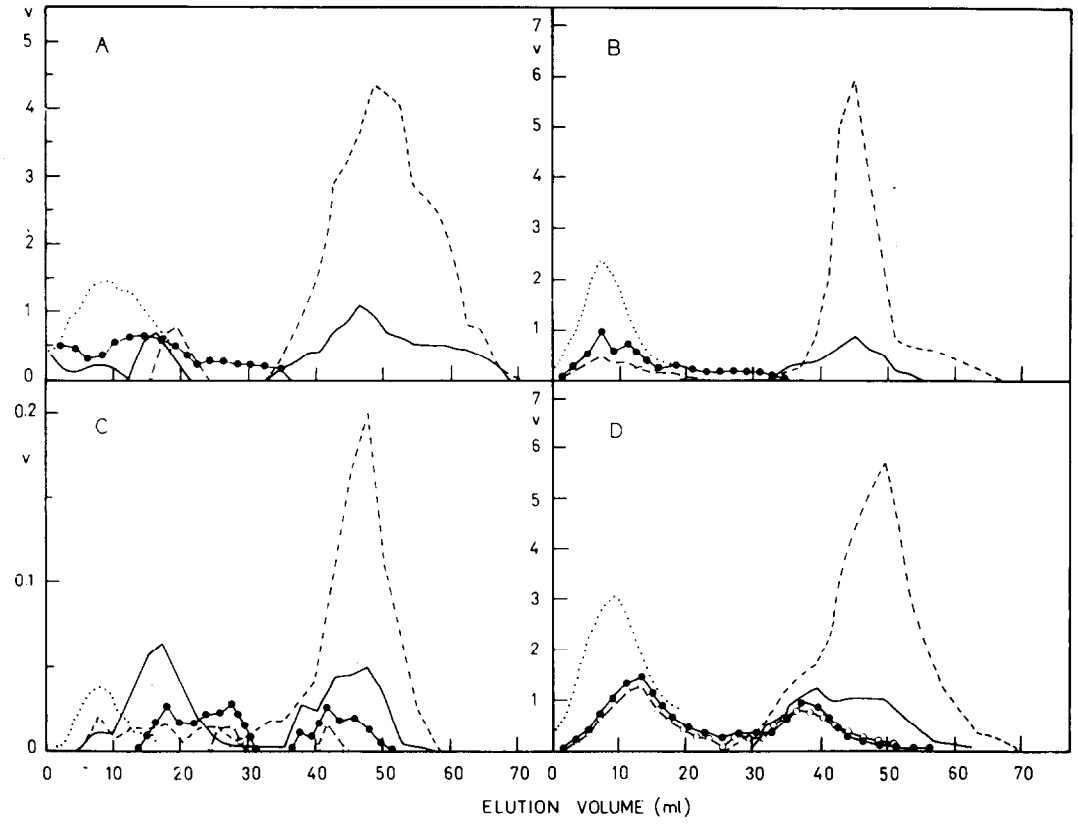

Fig.1. Molecular permeation chromatography of aminopeptidases from the inflammatory cells of the exudate (A) v given in $10^{-4} \times M\ min^{-1}$. PMN (B), v given in $10^{-4} \times M$ min. Mononuclear cells (C) v given in $10^{-5} \times M\ min^{-1}$. The cells were disrupted either by sonication (A,B,C) or with Triton® X-100 (D). Sample: 2 ml crude enzyme solution mixed with 0.3 ml Blue Dextran solution. (. . . .) Blue Dextran. The rate of the hydrolysis of *N*-L-arginyl-2-NA tested in the presence of 0.2 M NaCl (- - - -) and without added salt (——). Rate of hydrolysis of *N*-L-methionyl-2-NA tested in the presence of 0.2 M NaCl (•-•-•) and without added salt (o-o-o).

PMN and mononuclear leukocytes. Figure 2 shows the chromatogram of rat blood erythrocytes. An APB-like enzyme, revealed by 0.2 M NaCl, was present in the chromatograms of all above-mentioned preparations. The fractionation qualities of the APB-like enzyme in molecular permeation chromatography closely resembled those described for true APB [17].

The chromatograms revealed that the same yield of the APB-like enzyme was obtained both by slight

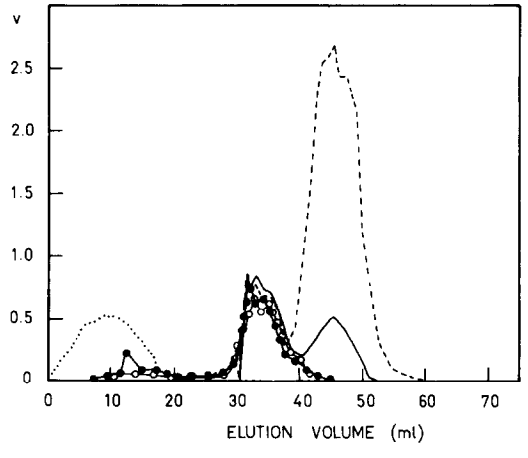

Fig.2. Molecular permeation chromatography of aminopeptidases from rat erythrocytes disrupted by sonication (v is given in $10^{-5} \times M\ min^{-1}$). Other details as for fig.1.

sonication and Triton® X-100 treatment (fig.1). This suggests that the enzyme is soluble. Freezing and thawing could not be used in disruption of the inflammatory cells because this method destroyed the APB-like enzyme activity. Hypotonic lysis of the cells with deionized water resulted in low yields of the chloride-activated arginine aminopeptidase as compared to sonication or treatment with Triton® X-100. The lower yields may be due either to true decrease in the amounts of the chloride-activated arginine aminopeptidase, or to the decreasing effect of deionized water on the enzyme activity.

The active fractions were pooled and used for further characterization.

3.2. *Characterization of the partly purified APB-like enzymes*

Only one protein band with an isoelectric point of 4.9–5.1 was found in flat-bed isoelectric focusing of the partly purified APB-like enzyme preparations when the gel was stained for proteins, but no arginine aminopeptidase activity could be detected in the gels slices. The crude enzyme preparations of the inflammatory cell fractions retained their APB-like activity in flat-bed isoelectric focusing and the activity was also located at an isoelectric point of 4.9–5.1 (fig.3).

The following characteristics were similar for the freshly fractionated, chloride-activated arginine aminopeptidase pools of both PMN and mononuclear cells of the exudate:

(i) The effect of NaCl on the rates of hydrolysis of the arginine derivative is presented in fig.4. The highest increase of the rate was obtained at the physiological concentrations of Cl^--ions.

(ii) Dithiothreitol, in the presence of 0.2 M NaCl, slightly increased the rate of the hydrolysis by both enzyme preparations (fig.4).

(iii) APB-like enzymes were strongly inhibited by *p*-chloromercuribenzoate (fig.4).

The freshly fractionated APB-like enzymes of PMN and mononuclear cells of the exudate hydrolyzed predominantly the *N*-L-arginyl- and *N*-L-lysyl-derivatives of 2-naphthylamine. The ratio of the rates of these two substrates was about 2. Both enzyme fractions hydrolyzed also *N*-L-methionyl-2-napthylamine at a considerable rate. The APB-like enzyme from the PMN leukocytes was activated and the enzyme from the

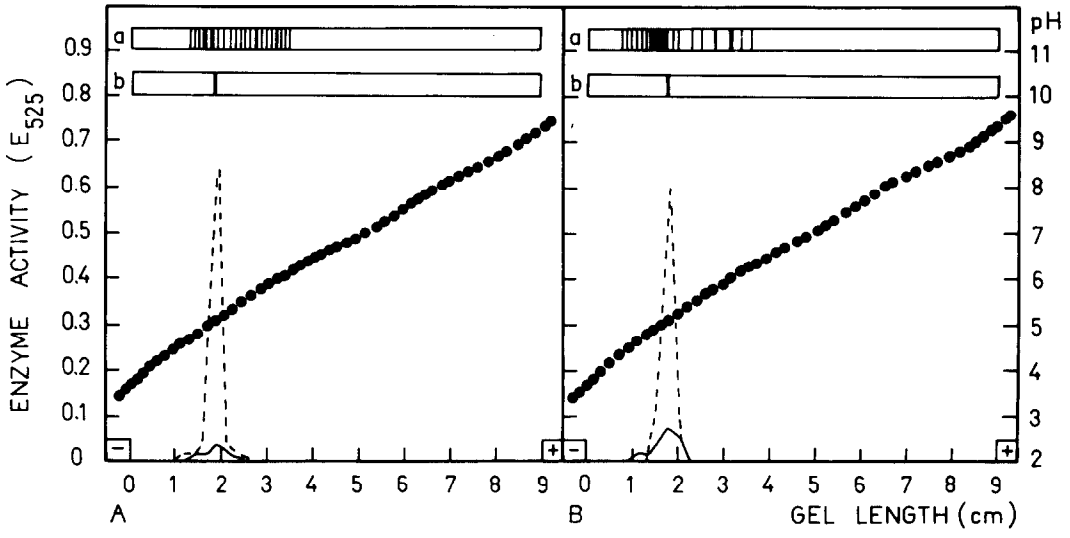

Fig.3. Flat-bed electrofocusing of the crude enzyme preparations of polymorphonuclear (A) and mononuclear (B) inflammatory cells. The pH-range (. . . .) was 3.5–9.5. The enzyme reactions were performed in 0.05 M phosphate buffer, pH 7.2, in the presence of 0.2 M NaCl (- - -) and without it (——). Substrate concentration was 0.167 mM. The gel slices were preincubated in the reaction mixtures overnight (+4°C). Protein staining of the gels: (a) Crude enzyme preparation, (b) partly purified enzyme preparation.

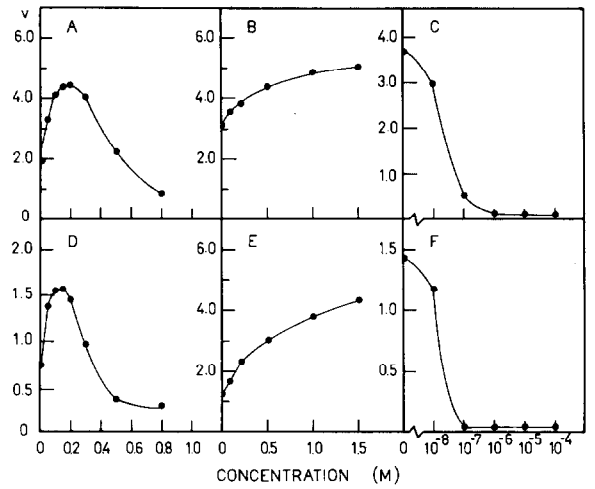

Fig.4. Effect of sodium chloride (A,D), dithiothreitol (B,E) and *p*-chloromercuribenzoate (C,F) on the rate of the hydrolysis of *N*-L-arginyl-2-NA catalyzed by the APB-like enzyme from PMN (A,B,C) *v* given in 10^{-4} × M min^{-1}. Mononuclear cells (D,E,F) *v* given in 10^{-5} × M min^{-1}. The reactions were performed in 0.05 M phosphate buffer, pH 7.2, in the presence of either varying concentrations of sodium chloride (A,D) or in 0.2 M sodium chloride (B,C,E,F).

mononuclear cells was inhibited by 0.2 M NaCl (fig.5).

Storage of the fractionated APB-like enzymes of both PMN and mononuclear cells at +4°C affected the enzymatic properties. Storage decreased the efficacy of chloride activation altering the substrate specifity and it also weakened the inhibition by *p*CMB (fig.6).

4. Discussion

The results of this report confirm that both the PMN and mononuclear cell fractions contained a chloride-activated arginine aminopeptidase. This finding is in agreement with the earlier results which show-

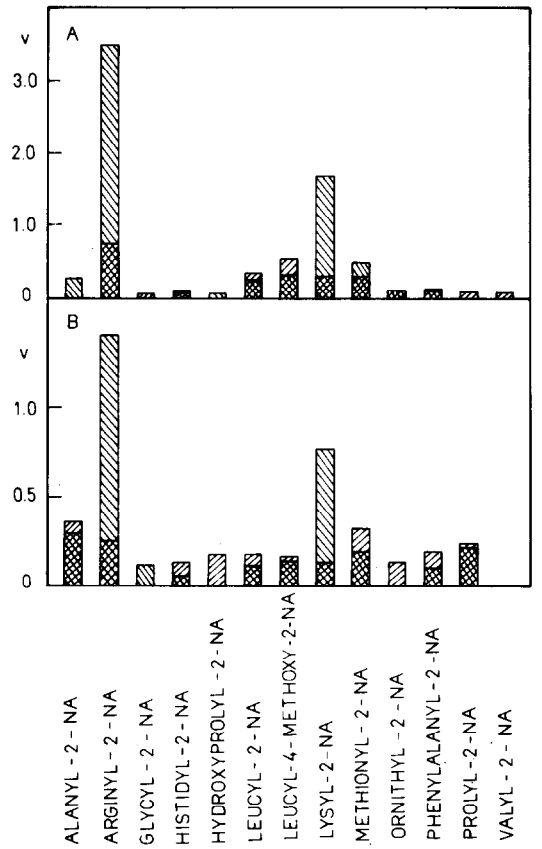

Fig.5. Rates of the hydrolysis of various *N*-L-aminoacyl-2-NAs catalyzed by the APB-like enzymes from PMN (A), *v* given in 10^{-4} × M min^{-1}. Mononuclear cells (B) *v* given in 10^{-5} × M min^{-1}. The reactions were performed in 0.05 M phosphate buffer, pH 7.2, in the presence of 0.2 M NaCl (\\\\) and without added salt (////). Substrate concentration was 0.167 mM.

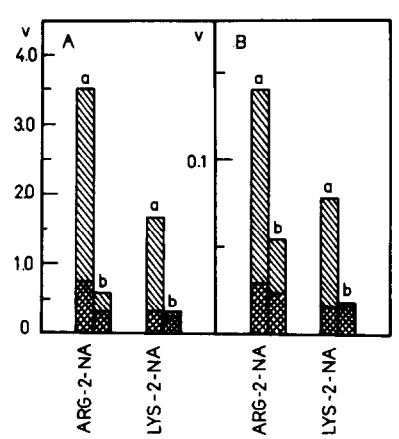

Fig.6. Effect of storage on enzyme activity. The rate of the hydrolysis of *N*-L-arginyl-2-NA, catalyzed by the APB-like enzyme from PMN (A) *v* given in 10^{-4} × M min^{-1}. Mononuclear cells (B) *v* given in 10^{-5} × M min^{-1}, was tested after two-week storage of the enzymes at +4°C. The reactions were performed in the presence of 0.2 M NaCl (\\\\) and without added salt (////). Other details as for fig.5.

ed that the arginine aminopeptidase activity of the inflammatory exudate during the course of inflammation parallelled with the number of leukocytes present [13]. It would thus seem the chloride-activated arginine aminopeptidase has a physiological role in early as well as later stages of inflammation in contrast to many other inflammatory neutral protease [19].

The characteristics of the partly purified enzymes of both PMN and mononuclear inflammatory cells were very similar even when considering the heterogenity of the mononuclear cell-fraction as well as the differences in maturation stages of the inflammatory cells. These two matters may, however, explain the slight differences in substrate specificities between the enzymes. On the other hand, a comparison of the freshly purified arginine aminopeptidases from the inflammatory cells with the APB-like enzyme found in the inflammatory exudate showed the following similarities:

(1) Elution properties in molecular permeation chromatography

(2) Isoelectric point

(3) Substrate specifity

(4) Chloride activation

(5) Inhibition and activation characteristics with *p*CMB dithiothreitol, respectively.

These observations confirm the earlier [13] suggestion according to which the bulk of the chloride-activated APB-like enzyme found in the inflammatory exudate in rat is originated from the leukocytes of the exudate.

In contrast to the partly purified enzyme of exudate, the enzymes obtained by molecular permeation chromatography from the leukocytes lost their activity during storage at +4°C. The poor stability of the leukocyte enzyme restricted its purification. Preliminary experiments performed with blood leukocytes of rats showed that the APB-like enzyme lost its activity already in molecular permeation chromatography. These findings suggest that the chloride-activated arginine aminopeptidase may undergo some structural alterations which are associated with its appearance in the exudate. This kind of observations have not been reported earlier for neutral proteases purified from inflammatory cells [20,21].

The role of the APB-like enzyme of leukocytes remains to be elucidated. Neutral proteolytic enzymes from leukocytes are known to produce vasoactive peptides [22] and chemotactic factors [22], activate leukocytes [23] and degrade tissue [24,25]. Most of these endopeptidases seem to have more than one function in inflammation. APB, as a specific exopeptidase, may have a specific function as compared to many other proteolytic enzymes involved in inflammation. As suggested previously [12], the chloride-dependence of the APB action may indicate an extracellular function for the enzyme.

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