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Purification and characterization of a ubenimex (Bestatin)-sensitive aminopeptidase B-like enzyme from K562 human chronic myeloid leukemia cells

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Abstract

A ubenimex-sensitive aminopeptidase B-like enzyme was purified from the non-membrane-bound fraction of K562 cells by a series of chromatographic procedures and slab-gel electrophoresis. The apparent molecular mass of the enzyme was estimated to be 73 kDa by SDS-PAGE. The aminopeptidase activity was activated by chloride ions and inhibited by Zn^{2+}, Cu^{2+}, Cd^{2+}, and *p*-chloromercuribenzoic acid. Ubenimex was a potent inhibitor of this aminopeptidase in the nanomolar range. The sequence of the N-terminus of the protein was not determined. Partial amino acid sequencing revealed that the N-terminus of this aminopeptidase B-like enzyme was blocked by acylation. The partial sequences of the two fragments produced by CNBr cleavage and an acylamino acid-releasing reaction showed this enzyme to be a new aminopeptidase.

Key words: Aminopeptidase B-like enzyme; K562 cell; Amino acid sequence

1. Introduction

Aminopeptidase B (APase B; EC 3.4.11.6), an exopeptidase widely distributed in mammalian tissues, catalyzes the hydrolysis of arginyl and lysyl residues from the amino-terminus of polypeptide chains [1–4]. Although APase B was first purified from rat liver in 1966 [5], the physiological functions of this enzyme have yet to be elucidated. In our laboratory, the presence of a ubenimex-sensitive APase B-like enzyme was indicated by the fact that ubenimex inhibited proliferation of K562 human chronic myeloid leukemia cells. Ubenimex, a dipeptide found in the culture filtrate of *Streptomyces olivoreticuli*, is a potent inhibitor of aminopeptidase N (APase N) and APase B [6]. Recently, it was shown that the myeloid cell surface glycoprotein, CD13, was identical to APase N [7], and that APase N limited neutrophili inflammatory responses and promoted monocyte antigen processing [8]. Previous studies have revealed that ubenimex has immunomodulatory [9–12] and host-mediated anti-tumor activities [13].

These results suggest that immunomodulatory activities are related to cell surface aminopeptidases [14]. However, the proliferation of K562 cells was inhibited by ubenimex and arphamenin B, a potent inhibitor of APase B, but not by an anti-CD13 monoclonal antibody. Further, ubenimex effectively inhibited APase B. Therefore, a ubenimex-sensitive APase B-like enzyme in K562 cells was speculated to act for APase N of other myeloid cells, because expression of APase N was very low in K562 cells [15].

In this report, we describe the purification and partial amino acid sequencing of a ubenimex-sensitive APase B-like enzyme from the non-membrane-bound fraction of K562 cells.

2. Materials and methods

2.1. Materials

Ubenimex (Bestatin) and leupeptin were prepared by Nippon Kayaku Co. AMC, Arg-MCA, Leu-MCA, Ala-MCA, Phe-MCA, E-64, and antipain were purchased from The Peptide Institute. Mono Q and EAH–Sepharose were from Pharmacia Fine Chemicals. Biofine HIC-PH was from Japan Spectroscopic Co. Immobilon-P was from Millipore Co. Acylamino acid-releasing enzyme was from Takara Shuzo Co. Trypsin (sequencing grade) was from Boehringer-Mannheim Biochemica. Isolated peptides were sequenced using an Applied Biosystems model 470A Protein Sequencer equipped with a model 120A on-line PTH analyzer.

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Abbreviations: AMC, 7-amino-4-methyl-coumarin; AUFS, absorbance unit full scale; CD, cluster of differentiation; CNBr, cyanogen bromide; DMSO, dimethyl slufoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis (β-aminoethylether)-*N,N,N′,N′*-tetra acetic acid; kDa, kilodaltons; MCA, 4-methylcoumaryl-7-amide; PCMB, *p*-chloromercuribenzoic acid; PMSF, phenylmethylsulfonyl fluoride; PTH, phenylthiohydantoin; SDS-PAGE, sodium dodecyl polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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2.2. Cells

K562 cells were maintained in RPMI1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin in a humidified 5% CO_2 atmosphere at 37°C.

2.3. Preparing Ubenimex–Sepharose 4B

Ubenimex–Sepharose 4B was prepared essentially according to the method described in the brochure issued by Pharmacia Fine Chemicals. In brief, 297 mg of ubenimex was coupled with 25 ml of EAH–Sepharose 4B. Binding of bestatin to Sepharose was checked by inhibition of leucine aminopeptidase hydrolytic activity.

2.4. Enzyme purification

The following steps were all performed at 4°C. K562 cells (1.1×10^9 cells) were homogenized with 40 ml of 20 mM sodium acetate buffer, pH 6.0, containing 50 μM leupeptin, 50 μM antipain, 2 mM PMSF, and 1 mM β-mercaptoethanol in a Teflon homogenizer. The homogenates were centrifuged at 20,000 × *g* for 30 min. The supernatant was adjusted to pH 6.0 and applied to a ubenimex–Sepharose 4B column (diam. 10 × 113 mm), equilibrated with 20 mM sodium acetate buffer, pH 6.0. The column was washed with the same buffer until the absorbance of the eluate at 280 nm had reached 0.05 AUFS. The enzyme was then eluted with a linear gradient of 0–0.5 M NaCl in 20 mM sodium acetate buffer, pH 6.0. Aminopeptidase activity was assayed by measuring AMC liberated from amino acid-MCA after incubation. The solution was incubated at 37°C in 20 mM Tris-HCl buffer, pH 7.2 (total 200 μl) containing 0.1 mM amino acid-MCA. The liberated AMC was measured fluorometrically with a Fluorescence Concentration Analyzer (Baxter). The active fractions that eluted at about 0.23 M NaCl were pooled and dialyzed against 20 mM Tris-HCl buffer, pH 8.0, containing 1 mM β-mercaptoethanol for 12 h. The dialyzed fraction was applied to a Mono Q column (diam. 5 × 50 mm) equilibrated with 20 mM Tris-HCl buffer, pH 8.0, and was eluted with a linear gradient of 0–0.5 M NaCl. The active fractions were eluted about 0.17 M NaCl. The enzyme solution was adjusted to 0.5 M NaCl and 20% ammonium sulfate, and centrifuged at 15,000 × *g* for 20 min. The supernatant was applied to a Biofine HIC-PH column (diam. 7.5 × 75 mm), equilibrated with 20 mM Tris-HCl, pH 8.0, containing 20% ammonium sulfate. The enzyme was eluted at about 4% ammonium sulfate, and was dialyzed against 20 mM Tris-HCl buffer, pH 7.5 (Table 1). To remove contaminants, a slab gel electrophoresis was performed. The enzyme fraction was applied to a slab gel and a voltage of 300 V was run for 2.5 h at 4°C. A 2 mm-wide section of the gel was cut out and was crushed in 20 mM Tris-HCl, pH 7.5. After centrifugation at 10,000 × *g* for 10 min, the supernatant was collected and assayed. The enzyme fractions, which hydrolyzed Arg-MCA and migrated as a single protein band on SDS-PAGE under reducing conditions (Fig. 1), were used for amino acid sequencing.

2.5. Aminopeptidase assay

Aminopeptidase activity was assayed by measuring AMC liberated from amino acid-MCA after incubation. The enzyme solution was incubated for 1 h at 37°C in a Hanks' solution (total, 200 μl) containing 0.1 mM amino acid-MCA dissolved in DMSO. The final concentration of DMSO was 1%. Each reaction was terminated by addition of 100 mM EDTA (50 μl). The liberated AMC was measured fluorometrically with a Fluorescence Concentration Analyzer (Baxter). Aminopeptidase activity was quantified by reference to a standard curve prepared with AMC. Results were expressed as nM of AMC formed per 1 min at 37°C. For the inhibition experiments, Ca^{2+}- and Mg^{2+}-free PBS was used instead of Hanks' solution.

2.6. Protein sequencing

The purified enzyme fraction was subjected to SDS-PAGE (12.5% polyacrylamide) and separated proteins were electrophoretically transferred to Immobilon-P. The ubenimex-sensitive APase B-like enzyme on Immobilon-P was carefully cut out and subjected to amino-terminal sequence analysis. Several enzyme fragments were generated by limited CNBr proteolysis of the protein on the membrane [16]. Deblocking and subsequent sequence analysis were performed according to the method of Hirano et al. [17]. The enzyme on a membrane was digested with trypsin treated with phenylisothiocyanate, following addition of performic acid to convert the N-terminal phenylthiocarbamyl groups of the peptides to phenylcarbamyl groups by oxidation. The acylamino acid-releasing enzyme (81 μg/ml) was added to the peptide mixture to remove the N-acylated amino acids.

3. Results and discussion

3.1. Purification of a ubenimex-sensitive APase B-like enzyme from the non-membrane-bound fraction of K562 cells

Leu-, Ala-, Arg-, and Phe-MCA hydrolytic activities were examined for membrane and non-membrane bound fractions of K562 cells. The non-membrane-bound fraction had 50- to 140-fold higher activity than the membrane fraction (data not shown). Furthermore, Arg-MCA hydrolytic activity was the highest among the substrates tested and was inhibited by ubenimex most efficiently.

We tried to purify the ubenimex-sensitive aminopeptidase by monitoring Arg-MCa hydrolytic activity. The enzyme exhibited both affinity- and ionic-exchange interactions on a ubenimex–Sepharose column and a Biofine HIC-PH hydrophobic chromatography procedure was also successfully used (Table 1). The aminopeptidase fraction eluted was examined by SDS-PAGE (Fig. 1A). Due to the existence of several extraneous protein bands, we used gel-electrophoresis and eluted the 73 kDa protein with Arg-MCA hydrolytic activity. Finally, the enzyme was purified as a single band of 73 kDa on SDS-PAGE (Fig. 1B). Because the molecular weight of this enzyme was identical on SDS-PAGE under both reducing and non-reducing conditions, it was likely that the ubenimex-sensitive APase B-like enzyme was a single polypeptide. This molecular weight is similar to a 72 kDa

Table 1
Purification of ubenimex-sensitive APase B-like enzyme from K562 cells

Purification step	Total volume (ml)	Total protein (mg)	Activity (nmol/min)[a]	Specific activity (nmol/min/mg)[a]	Recovery (%)
Crude extract	42	76.4	1,197	15.7	100
Ubenimex–Sepharose	8.0	6.05	851	140.7	71
Mono Q	4.5	0.639	229	358.4	19
Biofine HIC-PH	3.8	0.0067	14.5	2,164.2	1.2

[a] Hydrolyzing activity of Arg-MCA.

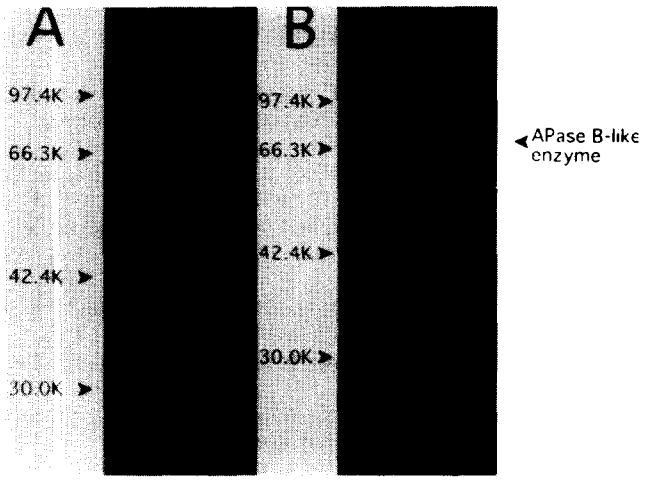

Fig. 1. SDS-PAGE of ubenimex-sensitive APase B-like enzyme. (A) SDS-PAGE of Biofine fraction: left lane, molecular weight marker; right lane, Biofine fraction. (B) SDS-PAGE of active fraction of gel-electrophoresis performed according to the method of Davis [20]: left lane, molecular weight marker; right lane, active fraction.

APase B from the cytosol of Jurkat T cells [18]. For characterization of the enzyme, we used the enzyme solution hydrophobic chromatography step. The same preparation was also subjected to SDS-PAGE, then transferred to a PVDF membrane and was used for amino acid sequencing.

3.2. Characterization of enzyme

In the synthetic substrates tested, Arg-MCA was the best substrate and Lys-MCA hydrolytic activity was 75% of Arg-MCa, Leu-, Ala-, Phe-, and Met-MCA were not hydrolyzed. The results show that the purified enzyme preferentially hydrolyzes basic amino acid-MCA substrates. The optimal pH of the enzyme activity was pH 7.0 (data not shown). Table 2 shows the effects of various affectors on the enzyme. Sodium chloride at a concentration of 50 mM increased the hydrolytic activity of Arg-MCA up to 170%, but PCMB and heavy metal ions markedly inhibited the activity. These properties are similar to APase B from other sources reported [1–5]. Benzamidine and PMSF, inhibitors of serine proteases, had little inhibitory effect on the enzyme. 1,10-Phenanthroline, a metal-chelating agent, exhibited 65% inhibition at a concentration of 0.1 mM, whereas EDTA and EGTA had no effect up to 1.0 mM. Since exo-type metalloproteases are inhibited by EDTA and EGTA [19], this enzyme may be a member of exo-type metalloprotease. Of the inhibitors tested, ubenimex is the strongest inhibitor for the purified enzyme with an IC_{50} value of 38 nM. E-64, an inhibitor of cysteine protease, did no inhibit the APase N or B activity. These results showed that the purified enzyme is a metalloprotease exhibiting identical properties to APase B.

Table 2
Effect of various affectors on ubenimex-sensitive APase B-like enzyme

Affectors	Concentration (mM)	Relative activity (%)
Cl^-	50	170.3
PCMB	0.1	0.93
Zn^{2+}	0.1	1.43
Cu^{2+}	0.1	0.0
Cd^{2+}	0.1	5.95
o-Phenanthroline	0.1	44.20
EDTA	1.0	107.72
EGTA	1.0	103.32
Benzamidine	1.0	84.57
PMSF	1.0	70.48
E-64	0.01	94.23
Bestatin	0.001 (IC_{50} 38 nM)	0.0

Table 3
Sequence of ubenimex-sensitive APase B-like enzyme

N-Terminal fragment	Acyl-xxIILNQVVxN
CNBr treatment fragment	xNxFEAYFK

x denotes unidentified amino acid residue.

3.3. Protein sequencing

The N-terminal amino acid sequence of the APase B-like enzyme was analyzed after transfer on to an Immobilon-P membrane. However, no signals were detected, suggesting that the N-terminus of the enzyme is blocked. Treatment with 0.6 N HCl or with vapor phase TFA was not succesful, indicating that the N-terminus is neither modified by formylation nor was it an acetylserine or acetyl-threonine. The APase B-like enzyme with acylamino acid-releasing enzyme deblocked the N-terminus of the enzyme. The first amino acid signal could not be identified because of amino acid contamination, but the sequence from the second amino acid signal was determined (Table 3). The peptide fragment that was generated from the whole protein by CNBr treatment was sequenced, and the result is also shown in Table 3. Homology searches were performed for two partial sequences with the data-bases NBRF-PIR (R37.0) and SWISS-PROT (R25.0). However, the sequences did not correspond to any proteins in these data-bases. Thus, at present, this ubenimex-sensitive APase B-like enzyme is expected to be a new aminopeptidase.

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