Volume 255, number 2, 237-240 FEB 07610 September 1989

Investigation of neutral endopeptidases (EC 3.4.24.11) and of neutral proteinases (EC 3.4.24.4) using a new sensitive two-stage enzymatic reaction

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Received 25 June 1989; revised version received 3 August 1989

A sensitive two-stage enzymatic reaction for mammalian and bacterial metalloendopeptidases has been developed using the substrate 3-carboxypropanoyl-alanyl-alanyl-leucine-4-nitroanilide supplemented with *Streptomyces griseus* aminopeptidase. Neutral endopeptidase EC 3.4.24.11 from bovine kidney hydrolyzes the substrate (pH 7.5, 25°C) with a catalytic efficiency ($k_{cat} = 1.2 \times 10^2$ s^{-1}, $K_m = 0.15$ mM) of the highest ever reported for the enzyme acting on synthetic chromophoric and fluorogenic substrates. Thermolysin hydrolyzes the substrate at a faster rate ($k_{cat} = 1.2 \times 10^3$ s^{-1}) but the overall efficiency is diminished by a higher K_m (4.2 mM). Suspensions of human neutrophil cells and culture filtrates of *Bacillus cereus* have been assayed sensitively for their neutral endopeptidase and neutral proteinase activities, respectively. The assay provides a convenient tool for the kinetic investigation of neutral endopeptidases and neutral proteinases and for assessing their function in biological systems.

Neutral endopeptidase; Neutral proteinase; Enkephalinase; Thermolysin; Common acute lymphoblastic leukemia antigen

1. INTRODUCTION

Neutral endopeptidase or enkephalinase (EC 3.4.24.11), a membrane-bound zinc metalloenzyme first isolated from rabbit kidney [1], is an ectoenzyme present on the surface of many cell types and is widely distributed in mammalian tissues, including brain tissue [2,3]. It shares basic elements of specificity and mechanistic properties with the well-characterized bacterial neutral proteinase, thermolysin (EC 3.4.24.4) [4-6]. The enzyme is implicated in the metabolism and regulation of a variety of peptides, e.g. the enkephalins, substance P and atrial natriuretic factor (review [7]). Recently, a near identity in amino acid sequence between human endopeptidase EC 3.4.24.11 and the common acute lymphoblastic leukemia antigen (CALLA, CD 10) has been established [8,9], raising the possibility that it may be involved in yet other functions related to cell differentiation and proliferation.

Here we have used a new method for the kinetic investigation of mammalian and bacterial metalloendopeptidases, e.g. endopeptidase EC 3.4.24.11 from bovine kidney and thermolysin, utilizing a sensitive two-stage enzymatic reaction [6,10]. The method allows very sensitive detection of neutral endopeptidase on mammalian cell surfaces and of neutral proteinase in bacterial cultures, thereby, assisting in the exploration of these important groups of enzymes.

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2. EXPERIMENTAL

2.1. *Substrate*

3-Carboxypropanoyl-alanyl-alanyl-leucine-4-nitroanilide (Suc-Ala-Ala-Leu-NH-Np) was prepared by stepwise addition of two Ala residues to leucine 4-nitroanilide (Sigma) via reaction

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

with the *N*-hydroxysuccinimide ester of *t*-butoxycarbonyl-alanine and deblocking of the *t*-butoxycarbonyl groups with trifluoroacetic acid, followed by succinylation with succinic anhydride. The product was recrystallized from 20% ethanol, overall yield 32%; m.p. 212–214°C; TLC (Silica-gel plates coated with fluorescent dye, Kieselgel $60F_{254}$, 0.2 mm thick, Merck) gave a single spot: $R_f = 0.86$ (*n*-butanol/acetic acid/pyridine/water = 4:1:1:2); HPLC (C_8 column, 4.6 × 250 mm (5 μm), protected with a C_8 guard column, Supelco) showed >98% purity (4.1 mM phosphoric acid/triethylamine, pH 3.3, in acetonitrile, 60:40); analysis calculated for $C_{22}H_{31}N_5O_8$, C = 53.54; H = 6.33; N = 14.19; found: C = 53.48; H = 6.25; N = 13.87.

2.2. *Enzymes*

Streptomyces griseus aminopeptidase was purified to homogeneity from pronase E (Sigma) by heat treatment followed by gel-filtration and anion-exchange chromatography [11]. Endopeptidase EC 3.4.24.11 from bovine kidney was purified by Triton X-100 extraction, followed by DEAE-Sepharose Fast Flow, concanavalin A-Sepharose, Q-Sepharose and hydroxyapatite chromatographic procedures (Indig, F.E. and Blumberg, S., unpublished). Thermolysin (Sigma) was recrystallized essentially as described [12]. Human neutrophils from donor blood were kindly supplied by Dr Ina Fabian, Tel Aviv University. Culture filtrates of *Bacillus cereus* (X-3 DSM 3101, German Collection of Microorganisms, Braunschweig, FRG) containing the neutral proteinase were obtained by overnight cultivation in a rich medium [13], supplemented with calcium chloride.

2.3. *Assay methods*

Neutral endopeptidase and neutral proteinase activities were assayed by the two-stage assay using the synthetic substrate Suc-Ala-Ala-Leu-NH-Np, supplemented with *Streptomyces griseus* aminopeptidase, usually 6–7 μg/ml. Assays were performed in 0.01 M NaCl/0.05 M Tris-HCl, pH 7.5, at 25°C, unless otherwise stated, with or without 0.01 M $CaCl_2$. Assay volume was 1 ml or 2.5 ml when using a Varian Techtron spectrophotometer or 0.2 ml at 23°C when performed with microplates (Nunc Immuno Plate II F) using a Kinetic Microplate Reader (Molecular Devices Corp.). Activity was measured by following the increase in absorbance at 405 nm, due to the release of 4-nitroaniline, $E_{405} = 10600\ M^{-1} \cdot cm^{-1}$ [14]. The molar concentration of thermolysin was estimated by using $A_{280}^{1\%} = 17.65$ [15] and a molecular mass of 34.6 kDa [16]; the molar concentration of bovine kidney neutral endopeptidase by protein determination [17] and a molecular mass of 90 kDa, deduced from SDS-PAGE [18].

3. RESULTS AND DISCUSSION

Based on well-established principles of subsite interactions between hydrolytic enzymes and their oligomeric substrates and inhibitors [19–21], we have synthesized the substrate Suc-Ala-Ala-Leu-NH-Np. Both endopeptidase EC 3.4.24.11 from bovine kidney and thermolysin cleave the Ala-Leu bond of the compound at a very fast rate (table 1). The Leu residue of the substrate interacts with the major hydrophobic specificity sites of the enzymes (S_1'), whereas the two Ala residues interact with additional subsites (S_1, S_2), thereby contributing to catalysis and/or binding. Even the 4-nitroaniline leaving group probably interacts with the enzyme at subsite S_2'. Previously reported assays of endopeptidase EC 3.4.24.11 usually utilized a hydrophobic Phe residue [6,9,10], but Leu seems preferable in reaching higher k_{cat} values [6]. The Leu residue is also preferable for the present two-stage assay utilizing *Streptomyces* aminopeptidase, since this enzyme very rapidly hydrolyzes the product of the endopeptidase attack, Leu-NH-Np [11]. The rapid hydrolysis coupled with K_m values in the 1 mM range and overall k_{cat}/K_m values in the $10^6\ M^{-1} \cdot s^{-1}$ range (table 1), render the substrate most suitable for detailed kinetic investigations of the enzymes and for detection of the EC 3.4.24.11 and EC 3.4.24.4 groups of enzymes in mammalian tissues and in bacterial extracellular fluids, respectively.

Using microplates for enzyme detection, purified bovine kidney endopeptidase EC 3.4.24.11 was readily assayed at below 1×10^{-15} mol enzyme and the assay is linear over a wide range of enzyme con-

Table 1

Kinetic parameters for bovine kidney neutral endopeptidase EC 3.4.24.11 and for thermolysin hydrolysis of Suc-Ala-Ala-Leu-NH-Np

Enzyme	(S) Range (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1}s^{-1} \times 10^{-6}$)
Endopeptidase EC 3.4.24.11	0.04–4.0	1.2×10^2	0.15	0.80
Thermolysin	0.4 –4.0	1.2×10^3	4.2	0.29

Reactions were carried out in the presence of 10 mM $CaCl_2$ at pH 7.5, 25°C. 2.2×10^{-9} M and 3.0×10^{-10} M of endopeptidase EC 3.4.24.11 and thermolysin, respectively, were used. Values of k_{cat} and K_m were determined by Lineweaver–Burk plots

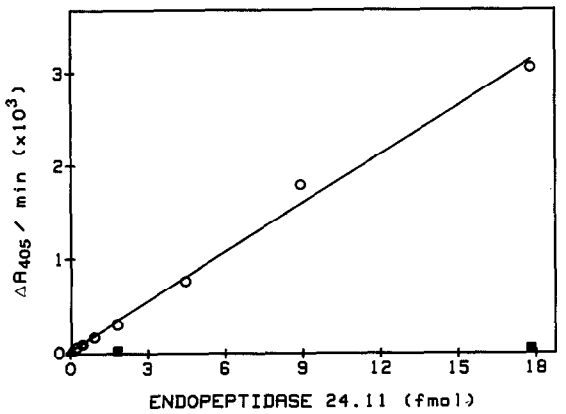

Fig.1. Determination of the enzymatic activity of bovine kidney neutral endopeptidase EC 3.4.24.11, using 0.4 mM Suc-Ala-Ala-Leu-NH-Np at pH 7.5. (○) The increase in absorbance at 405 nm upon addition of enzyme; (■) inhibition of the reaction by 1.8 μM phosphoramidon; (▲) substrate, including the aminopeptidase, alone. Measurements were carried out in microplates continuously during 60-min periods. Each point represents an average of 4–6 wells.

centrations (fig.1). This sensitivity exceeds immunoassay levels of detection and may substitute and/or supplement these assays in studies of CALLA, which is nearly identical to human endopeptidase EC 3.4.24.11 [8,9].

Human neutrophils from a healthy donor (5×10^5 cells/ml) hydrolyze the substrate (0.4 mM, pH 7.5) at a fast rate: approx. 1.3 nmol/min per 10^6 cells. Assuming that the bovine kidney and human neutrophil enzymes have similar activities, the above rate would amount to approx. 1.5×10^5 active endopeptidase EC 3.4.24.11 enzyme molecules per cell. However, detailed comparisons of the activities of purified endopeptidase EC 3.4.24.11 from different species and from different tissues, using a variety of substrates, are still required.

Using the above microplates for enzyme detection, 5×10^3 neutrophils were readily measured for their endopeptidase EC 3.4.24.11 activity.

Like other previously reported substrates for endopeptidase EC 3.4.24.11, the substrate employed may, in principle, be hydrolyzed by chymotrypsin-like enzymes. To assess the contribution of the neutral endopeptidases and neutral proteinases to the observed color development, the specific inhibitor of these groups of enzymes, phosphoramidon, was added [22,23] (table 2). The inhibitor completely abolished the activity of the purified enzymes, and strongly inhibited the enzymatic reaction displayed by the intact neutrophils or by the bacterial culture used (table 2). These experiments demonstrate the specificity of the enzymatic method and its applicability to purified neutral endopeptidases and neutral proteinases as well as to neutral endopeptidases present on intact mammalian cells and to neutral proteinases present in crude bacterial culture filtrates. In addition, the method may be applied to the determination of neutral endopeptidase activity in tissue homogenates, membrane fractions and detergent-solubilized membranes, as we observed during the different steps of purification of the bovine kidney enzyme.

Human neutrophils and leukemic lymphoblasts are high expressors of neutral endopeptidase EC 3.4.24.11, the former cells probably requiring the enzyme to act upon chemotactic peptides [7]. The

Table 2

Inhibition of neutral endopeptidases EC 3.4.24.11 and neutral proteinases EC 3.4.24.4 activities by phosphoramidon

Enzyme	Phosphoramidon (μM)	% Inhibition
Endopeptidase EC 3.4.24.11	3.6	>99
Thermolysin	18.0	>99
Human neutrophils	3.6	>95
B. cereus culture	18.0	~95

Inhibition was measured at pH 7.5, 25°C, in the presence of 10 mM $CaCl_2$, except for the neutrophils that were assayed at 37°C and in the absence of added Ca^{2+}. The substrate, Suc-Ala-Ala-Leu-NH-Np, at 0.4 mM was used. Endopeptidase EC 3.4.24.11 from bovine kidney and thermolysin were used at 2.2×10^{-9} M and 1.5×10^{-9} M, respectively; human neutrophils at 5×10^5 cells/ml and *B. cereus* culture filtrate at 5 μl/ml substrate

sensitivity of the method presented here, however, allows detection of the enzyme on other cell types, expressing the enzyme at a much lower level, and may therefore help to investigate its roles under normal cell function.

Acknowledgements: We are indebted to Dr Ina Fabian for the human neutrophils, to Dr Shlomo Weintroub for use of the kinetic microplate reader and Ms Rene Wide for her secretarial assistance.

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