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FEBS Letters 344 (1994) 229–233

FEBS 14003

FEBS LETTERS

Purification and characterization of an endogenous inhibitor specific to the Z-Leu-Leu-Leu-MCA degrading activity in proteasome and its identification as heat-shock protein 90

Satoshi Tsubuki*, Yumiko Saito, Seiichi Kawashima

Department of Molecular Biology, The Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113, Japan

Received 11 March 1994; revised version received 7 April 1994

Abstract

We previously identified a benzyloxycarbonyl(Z)-Leu-Leu-Leu-4-methylcoumaryl-7-amide (ZLLL-MCA) degrading activity in proteasome as a candidate for the regulator of neurite outgrowth. As its counterpart, we purified a protein from bovine brain that specifically inhibits the ZLLL-MCA degrading activity in proteasome. This protein is heat stable and has no effect on the other catalytic activities in proteasome, or on the activities of trypsin, chymotrypsin, or m- and μ-calpains either. The molar ratio of inhibitor-to-proteasome that inhibits 50% of the ZLLL-MCA degrading activity of proteasome is 1:1. The inhibitory mechanism of the inhibitor against proteasome is non-competitive. Finally, the inhibitor was identified as heat-shock protein 90 (HSP90) by partial amino acid sequencing and immunodetection. The results suggest that HSP90 initiates neurite outgrowth through the inhibition of the ZLLL-MCA degrading activity in proteasome.

Key words: Proteasome; Proteasome inhibitor; Heat-shock protein 90; Neurite outgrowth

1. Introduction

A tripeptide aldehyde protease inhibitor, benzyloxycarbonyl(Z)-Leu-Leu-leucinal (ZLLLal), initiates neurite outgrowth in PC12 cells at an optimal concentration of 30 nM [1]. This result suggests the existence of a protease that regulates neurite formation in PC12 cells. Thus we attempted to identify this target protease in bovine brain using Z-Leu-Leu-Leu-4-methylcoumaryl-7-amide (ZLLL-MCA), in which the aldehyde moiety of ZLLLal was changed to 45-methylcoumaryl-7-amide to serve as a substrate for the protease. As a result, we purified a proteasome with a molecular mass of about 660 kDa as a ZLLL-MCA degrading protease, and suggested the possibility that the proteasome is involved in the regulation of neurite formation in PC12 cells [2].

Proteasome, a multicatalytic protease, has been identified in many species and plays an essential role in the ATP/ubiquitin-dependent pathway [3–5]. It has a high molecular mass (about 700 kDa), is composed of 13–15 distinct subunits of similar size (21–31 kDa), and contains three or four different peptidase activities [6–10]. It is believed that proteasome is related to multiple cellular phenomena because it plays an essential role in the ATP/ubiquitin-dependent pathway. If the ZLLL-MCA degrading activity in proteasome is involved in the regulation of neurite formation in PC12 cells, there should exist an endogenous inhibitor that inhibits ZLLL-MCA degrading activity. The fact that the ZLLL-MCA degrading activity increased dramatically during the purification of proteasome [2] encouraged us to isolate a specific endogenous inhibitor of a ZLLL-MCA degrading activity in proteasome.

*Corresponding author. Fax: (81) (3) 5685-6609.

Abbreviations: Z, benzyloxycarbonyl; ZLLLal, benzyloxycarbonyl-Leu-Leu-leucinal; MCA, 4-methylcoumaryl-7-amide; ZLLL-MCA, benzyloxycarbonyl-Leu-Leu-4-methylcoumaryl-7-amide; SucLLVY-MCA, succinyl-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide; Boc-VLK-MCA, tertiary butyloxycarbonyl-Val-Leu-Lys-4-methylcoumaryl-7-amide; ZLLE-NA, benzyloxycarbonyl-Leu-Leu-Glu-β-naphthylamide; buffer A, 20 mM Tris-HCl, pH 7.0, containing 10% glycerol; buffer B, 20 mM Tris-HCl, pH 7.5, containing 5 mM EDTA and 10% glycerol; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; HSP, heat-shock protein.

2. Materials and methods

2.1. Materials

Reagents and chemicals were purchased from the following manufacturers: succinyl(Suc)-Leu-Leu-Val-Tyr-MCA (SucLLVY-MCA) and tertiary-butyloxycarbonyl(Boc)-Val-Leu-Lys-MCA (BocVLK-MCA) from Peptide Institute Inc. (Japan); Z-Leu-Leu-Glu-β-naphthylamide (ZLLE-NA) from Sigma Chemical Co. ZLLL-MCA was synthesized according to the azide method [11] and the dicyclohexylcarbodiimide method [12]. Proteasome was purified by a previous method [2].

2.2. Purification of an endogenous protein inhibitor of proteasome

In the purification of proteasome as a ZLLL-MCA degrading protease from bovine brain, the ZLLL-MCA degrading activity in pro-

SSDI 0014-5793(94)00388-C

teasome increased after phenyl-Sepharose column chromatography [2]. This result suggests that a specific endogenous protein inhibitor is separated from the proteasome fraction after phenyl-Sepharose column chromatography. The proteins were separated according to a previous procedure [2] up to the phenyl-Sepharose column chromatography step. From there, the 50% ethylene glycol eluent was collected and dialyzed against buffer A (20 mM Tris-HCl, pH 7.0, containing 10% glycerol). The dialyzed material was applied to a Mono Q column (0.5 × 5 cm), equilibrated with buffer B (20 mM Tris-HCl, pH 7.5, containing 5 mM EDTA and 10% glycerol) containing 0.1 M NaCl, and the adsorbed material was eluted with 50 ml of a gradient of 0.1–1 M NaCl in buffer B. The fractions with inhibitory activity were pooled and dialyzed against buffer A. This dialyzed material was used as the purified inhibitor.

2.3. Electrophoretic techniques

Electrophoresis under non-denaturating conditions was performed in slab gels at a constant (5%) polyacrylamide concentration. The slab gels were run in 7 mM Tris-glycine, pH 8.3, at 5 mA for 3 h at room temperature. Protein was detected by silver staining. Electrophoresis under denaturing conditions in the presence of SDS was performed in 12.5% polyacrylamide gels [13]. Electrophoresis was performed at 15 mA for 90 min at room temperature. The gels were stained with 0.1% Coomassie brilliant blue-50% methanol-10% acetic acid and destained by diffusion in 5% methanol-7.5% acetic acid.

2.4. Assay for proteasome inhibition

The fluorescent substrate degrading activities were determined by measuring the fluorescence of the liberated fluorescent materials. The reaction mixture (1 ml) contained 100 mM Tris-acetate, pH 7, enzyme, inhibitor and 25 μM substrate dissolved in dimethylsulfoxide. After incubation at 37°C for 15 min, the reaction was stopped by adding 10% SDS (100 μl) and 0.1 M Tris-acetate (900 μl), pH 9. The fluorescence of the reaction products was measured (MCA substrates, excitation 380 nm/emission 440 nm; NA substrate, excitation 335 nm/emission 410 nm).

2.5. Assays for the inhibition of trypsin, chymotrypsin, m- and μ-calpains

Trypsin, chymotrypsin, m- and μ-calpain activities were measured by previously described methods [14–16]. To determine the inhibitory activities of the inhibitor, the inhibitor in various concentrations was included in the assay mixtures.

2.6. Amino acid sequence determination of the endogenous protein inhibitor

After SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified inhibitor, the protein was electroblotted onto a polyvinylidene difluoride (PVDF) membrane and then the PVDF membrane pieces with the protein attached were subjected directly to an Applied Biosystems 470A sequencer equipped with an on-line 120A PTH analyzer to determine the sequence around the N-terminus [17]. To get internal sequences, the protein inhibitor in SDS-polyacrylamide gels was digested by lysylendopeptidase and the peptides were sequenced after purification on HPLC [18].

3. Results

3.1. Purification of an endogenous protein that specifically inhibits the ZLLL-MCA degrading activity of proteasome

The elution patterns of the inhibitor from the phenyl-Sepharose and Mono Q columns are shown in Figs. 1 and 2, respectively. As shown in Fig. 1, the inhibitor fractions (the bar fractions) were separated from the proteasome fractions (the fractions eluted with a decreasing linear gradient of 1–0 M NaCl in buffer B). When the dialyzed inhibitor fractions were chromatographed on

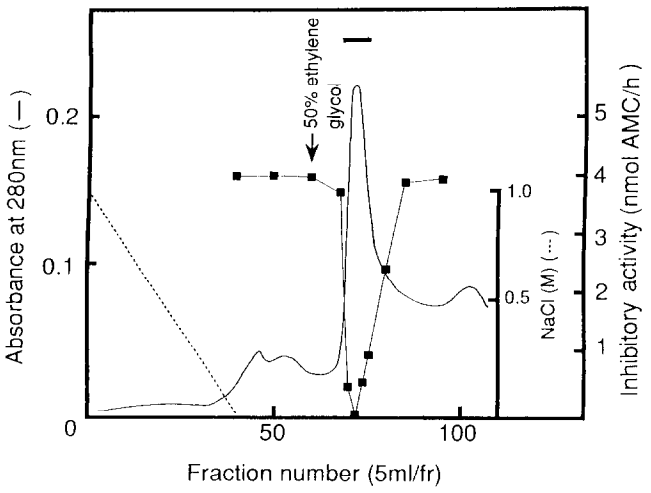

Fig. 1. Phenyl-Sepharose column chromatography. (■) Inhibitory activity toward ZLLL-MCA degrading proteasome. The fractions (50 μl) were heated at 70°C for 10 min and added to the assay mixture. The released fluorescent reaction products in nmol per 1 h using 2 μg proteasome are shown. The bar indicates the fractions pooled.

the Mono Q column, the inhibitor was eluted at 0.25 M NaCl. About 2 mg of the inhibitor was obtained.

3.2. Electrophoretic properties of the purified inhibitor

As shown in Fig. 3A, the purified inhibitor had a molecular mass of about 170 kDa on native-PAGE (5% polyacrylamide). On SDS-PAGE (12.5% polyacrylamide), the purified inhibitor gave a single protein band (about 85 kDa) (Fig. 3B). This result suggests that the inhibitor is a homodimer of 85 kDa subunits.

3.3. Inhibition of proteasome and other proteases by the purified inhibitor

The effects of the purified inhibitor on the catalytic activities in proteasome were examined. As shown in Fig. 4, the ZLLL-MCA degrading activity in proteasome was inhibited strongly, while the well-known SucLLVY-MCA (chymotrypsin-like), BocVLK-MCA (trypsin-like) and ZLLE-NA (V8 protease-like) degrading activities were hardly affected. Fifty percent inhibition of the ZLLL-MCA degrading activity was observed at a molar ratio of inhibitor-to-proteasome of 1:1, assuming the molecular weights of the inhibitor and proteasome to be 170 and 660 kDa, respectively. The inhibitor had no effect on the activities of trypsin, chymotrypsin, or m- and μ-calpains, even when present at a 10-fold molar excess to these proteases. The inhibitory mechanism of the inhibitor against the ZLLL-MCA degrading activity in proteasome was determined to be non-competitive by Lineweaver–Burk plot analysis (Fig. 5).

3.4. Amino acid sequence determination of the purified inhibitor

The N-terminal 14 amino acid residues of the inhibitor

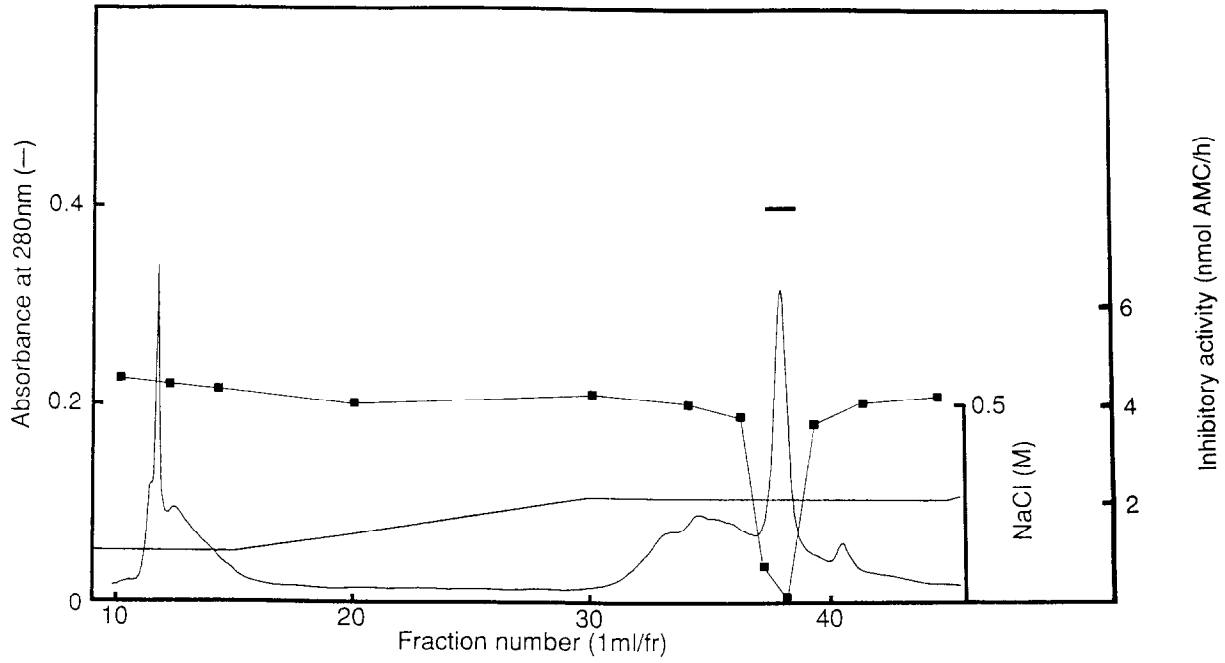

Fig. 2. Mono Q column chromatography of the protein fraction from phenyl-Sepharose column chromatography. (■) Inhibitory activity towards ZLLL-MCA degrading proteasome. The fractions (50 μl) were heated at 70°C for 10 min and added to the assay mixture. The released fluorescent reaction products in nmol per 1 h using 2 μg proteasome are shown. The bar indicates the fractions pooled.

and 9–13 amino acid residues of six peptides generated by lysylendopeptidase digestion were sequenced. This analysis showed the inhibitor to be analogous to human and murine HSP90. The sequences obtained were compared with those of HSP90 from human [19] and mouse [20] (Fig. 6). Homology as high as 94% was observed for human HSP90 (alternative name HSP90α); and 92% for murine HSP90 (alternative name HSP86). Moreover, the inhibitor was positive on immunoblotting using anti-rabbit HSP90 polyclonal antibody (Fig. 7). Its existence as a homodimer is also consitent with the characteristics of HSP90. Thus, we conclude that the inhibitor is, in fact, HSP90.

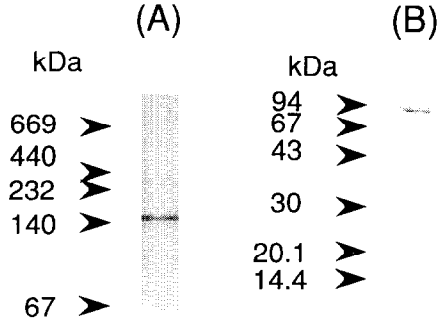

Fig. 3. Native-PAGE (A) and SDS-PAGE (B) of the purified endogenous inhibitor. Samples containing 1 μg inhibitor were used. Molecular mass standards are as indicated: (A) thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa); (B) phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

4. Discussion

From bovine brain, we purified a protein that specifically inhibits the ZLLL-MCA degrading activity in proteasome. This protein was identified as HSP90 by partial amino acid sequencing and immunodetection. Other characteristics of the inhibitor support this conclusion.

Although HSP90 acts as a chaperon, it is suggested that the inhibition of the ZLLL-MCA degrading activity in proteasome is due to HSP90–proteasome binding, not to HSP90–substrate binding. The reasons are (i) HSP90

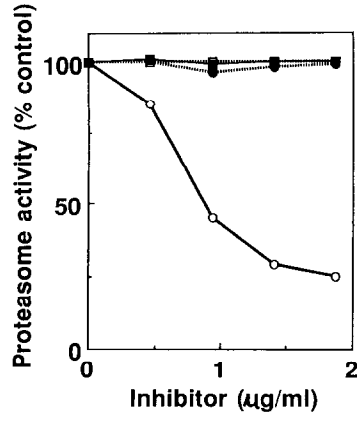

Fig. 4. The effect of the endogenous inhibitor on proteasome. (○) ZLLL-MCA degrading activity; (●) SucLLVY-MCA degrading activity; (□) BocVLY-MCA degrading activity; (■) ZLLE-NA degrading activity. Activity of proteasome (3.3 μg) in the absence of inhibitor was considered to be 100%.

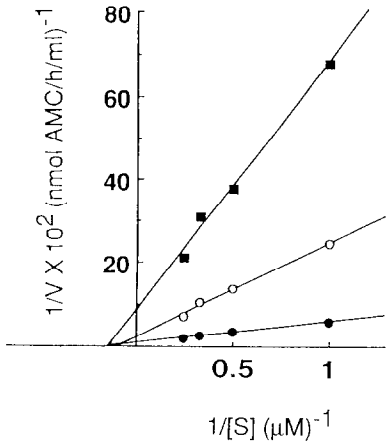

Fig. 5. Lineweaver–Burk plot analysis of the inhibition of proteasome by the endogenous inhibitor. (●) No inhibitor; (○) 0.46 μg inhibitor/assay; (■) 0.91 μg inhibitor/assay.

is not separated from proteasome until phenyl-Sepharose column chromatography (data not shown), (ii) ZLLL-MCA is added to the reaction mixture in far excess to the HSP90, (iii) the inhibitory mechanism of HSP90 against proteasome is non-competitive, and (iv) other substrate degrading activities in proteasome are not inhibited by HSP90.

In cells, heat-shock proteins (the other name is stress proteins) are synthesized in response to various biological stresses such as exposure to non-physiological temperatures [21,22], drugs [23], amino acid analogs [24], and transition metals [25]. Heat-shock proteins are classified into four families, namely, HSP90, HSP70, HSP60, and low molecular weight heat-shock proteins. Among these, HSP90 and HSP70 are the major ones [26,27].

HSP90 has a molecular mass of about 90 kDa and exists as a dimer [28]. The primary structure of HSP90s are highly homologous in many species. It is one of the most abundant cellular proteins found even under non-stress conditions. It has been found to be associated with a number of other intracellular proteins, including actin [28,29], pp60$^{V\text{-}src}$, pp140^{fps}, pp94^{yes} [30,31], and steroid

Inhibitor	PEETQAQDQPMEEE	Peptide 4	LGIHEXSQNRK
Human (2-15)	PEETQTQDQPMEEE	Human (447-457)	LGIHEDSQNRK
Mouse (2-15)	PEETQTQDQPMEEE	Mouse (448-458)	LGIHEDSQNRK
Peptide 1	ESDDKPEIED	Peptide 5	HIYYITGETK
Human (251-260)	ESEDKPEIED	Human (490-499)	HIYYITGETK
Mouse (251-260)	ESDDKPEIED	Mouse (491-500)	HIYFITGETK
Peptide 2	YIDQEELNK	Peptide 6	EGLYLPEDEEEK
Human (284-292)	YIDQEELNK	Human (547-558)	EGLELPEDEEEK
Mouse (285-293)	YIDQEELNK	Mouse (548-559)	EGLELPEDEEEK
Peptide 3	SLTNDWEDXLAV		
Human (315-326)	SLTNDWEDHLAV		
Mouse (316-327)	SLTNDWEEHLAV		

Fig. 6. Amino acid sequence of the inhibitor and its homology to human HSP90 and murine HSP90. X, amino acid residues not identified.

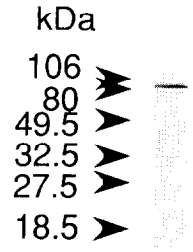

Fig. 7. Immunological detection of the inhibitor by anti-rabbit HSP90 polyclonal antibody. The immunogen of this antibody is the synthetic peptide of the N-terminal amino acids 2–12 in murine HSP90.

hormone receptors [32]. It is suggested that HSP90 plays several important roles in cells, one being to act as a chaperon. On the other hand, proteasome is the catalytic machinery in the ATP-dependent proteolytic system. In this system, some targeting mechanism as a mark for proteins to be degraded is necessary and the involvement of chaperons has been speculated. Ubiquitination by ubiquitin, a kind of stress protein, is the only chaperon integrated into the proteolytic system. Now another stress protein, HSP90, has joined as a new member of the system and is expected to regulate neurite outgrowth in the nervous system as follows.

We began this work based on the fact that a cell-permeable protease inhibitor, ZLLLal, initiates neurite outgrowth in PC12 cells [1]. Next we purified proteasome as the target protease of ZLLLal and found a good correlation between neurite promotion activity and inhibitory activity towards the ZLLL-MCA degrading activity in proteasome of a series of protease inhibitors [2]. If neurite outgrowth is induced by the inhibition of ZLLL-MCA degrading activity, as found for ZLLLal, then there should exist a physiological modulator of ZLLL-MCA degrading activity. We found HSP90 was the inhibitor. In fact, there is a report showing that when heat shock is applied to neuroblastoma cells, HSPs are synthesized and, at the same time, the development of neurites is induced after 3–6 days [33].

Although another endogenous protein that inhibits the substrate degrading activities in proteasome has been isolated, this endogenous inhibitor does not have catalytic site specificity [34]. For this reason, too, it is a very significant finding that HSP90 specifically inhibits the ZLLL-MCA degrading activity in proteasome.

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