ISOLATION AND IDENTIFICATION OF A TARGET PROTEIN FOR Z-Leu-Leu-Leu-H, A NEURITE OUTGROWTH FACTOR, FROM BOVINE BRAIN

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ABSTRACT

Benzyloxy carbonyl-Leu-Leu-Leuinal (Z-Leu-Leu-Leu-H), a leupeptin derivative, stimulates neurite outgrowth of PC12 cells in a manner different from NGF and also inhibits thiol-protease such as calpain. In this study, we aimed at isolation and identification of target protein(s) for this drug by means of a newly designed affinity resin, to which Z-Leu-Leu-Leu-OH was coupled at the C-terminal carboxyl group of the peptide. Our affinity chromatography system showed a unique protein retained and eluted in a Ca²⁺-dependent manner. This protein was purified from bovine brain extract by DEAE ion-exchange chromatography and reversed phase chromatography on the basis of its affinity character. The amino acid sequencing of peptides obtained under various degradation conditions of native or pyridylethylated protein has revealed that the target protein agrees with S-100β subunit in 86% of its amino acid sequence.

Neurite outgrowth of PC12 cells or other cultured cells is induced by inhibiting protease activity (4, 16). Out of various protease inhibitors (leupeptin, leupeptin analogue, pepstatin etc.) tested for PC12 cells (19), only Ac-Leu-Leu-Leu-Nle-H, a leupeptin analogue, induced neurite outgrowth at a low concentration.

Ito et al. have synthesized several derivatives of Ac-Leu-Leu-Nle-H, and examined the effect of their chemical structures on neurite outgrowth (8). Benzyloxy carbonyl-Leu-Leu-Leucinal (Z-Leu-Leu-Leu-H) has been found to be 50 times more active than Ac-Leu-Leu-Nle-H, but loses almost all activity by the substitution of C-terminal aldehyde. It has so far been known that Z-Leu-Leu-Leu-H inhibits thiol-protease such as calpain and proteasome (24), DNA polymerase (22), and enhances morphogenetic processes of osteoblast (21).

Saito et al. have isolated clathrin from PC12 cell, using an affinity resin, to which Leu-Leu-Leu-H was immobilized on the N-terminal amino group of the peptide (18, 20, 21).

On the assumption that the moiety of Z-Leu-Leu-Leu- is required for the recognition of protein by Z-Leu-Leu-Leu-H, we prepared a newly designed affinity resin, to which Z-Leu-Leu-Leu-OH is immobilized on the C-terminal carboxyl group of the peptide. Using the affinity resin as a probe, we attempted to isolate and identify a target protein for Z-Leu-Leu-Leu-H.

MATERIALS AND METHODS

Materials

Z-Leu-Leu-Leu-OH was synthesized and purified according to the method of Ito et al. (8), and was identified by mass spectroscopy. Bovine brain was obtained from Sibaura Organ (Tokyo). The follow-
ing chemicals were obtained from sources indicated: water-soluble carbodiimide (WSC)·HCl, Kokusan (Tokyo); Affi-Gel 102, Bio-Rad (U.S.A.); BrCN, Wako Pure Chemical (Osaka); arginylendopeptidase, Takara Biochemical (Otsu); TPCK treated trypsin, Sigma Chemical (U.S.A.); marker protein kit, Bio-Rad (U.S.A.); DEAE-5PW column (21.5 × 150 mm), Tosoh (Tokyo); and Puressil C18 column (4.6 φ × 150 mm), Waters (Tokyo).

Preparation of Bovine Brain Extract

Bovine brain extract was prepared according to the method of Isobe et al. (6) with modifications by Kato et al. (9). Briefly, bovine homogenate obtained in 50% ammonium sulfate solution/1 mM ethylenediamine tetraacetic acid (EDTA) was precipitated at pH 4.0 by the addition of ammonium sulfate to final 90% saturation. The resultant precipitate was dialyzed against 20 mM phosphate buffer (pH 7.5). After centrifugation, the supernatant was used for affinity chromatography and ion-exchange chromatography.

Affinity Chromatography

Preparation of an affinity ligand Affi-Gel 102 resin (1 ml) was packed in a polypropylene column (5φ × 150 mm), and washed with dimethylsulfoxide (DMSO) (10 ml). Z-Leu-Leu-Leu-OH (95.5 μmol) and WSC·HCl (9.5 μmol) in a small amount of DMSO was added to the column. After stirring for 24 h with a mild mixer, it was washed with ethanol (20 ml).

Elution method The affinity column was equilibrated with 20 mM phosphate buffer (pH 7.5), and eluted with various buffer solutions in the presence or absence of calcium ion at a flow rate of 1 ml/min. A_{280}nm was measured for each fraction (1 ml).

a) Elution condition A Bovine brain extract (1.0 ml) was applied to the affinity column, and eluted with buffer solutions (1.0 ml × 5) in the following order: 20 mM phosphate buffer (pH 7.5)/1.0 mM CaCl₂; 20 mM phosphate buffer (pH 7.5)/1.0 mM CaCl₂/0.2 M NaCl; 20 mM phosphate buffer (pH 7.5)/1.0 mM EDTA; 20 mM phosphate buffer/6.0 M urea; 20 mM phosphate buffer (pH 7.5)/0.1% SDS.

b) Elution condition B Bovine brain extract (1.0 ml) mixed with 10 mM CaCl₂ (0.1 ml), was applied to the affinity column, and eluted with the buffer solutions (1.0 ml × 5) in the following order: 20 mM phosphate buffer (pH 7.5)/1.0 mM CaCl₂; 20 mM phosphate buffer (pH 7.5)/1.0 mM CaCl₂/0.2 M NaCl; 20 mM phosphate buffer (pH 7.5)/1.0 mM EDTA; 20 mM phosphate buffer/6.0 M urea; 20 mM phosphate buffer (pH 7.5)/0.1% SDS.

Ion-Exchange Chromatography of Bovine Brain Extract

Bovine brain extract (5 ml) was applied to HPLC on DEAE-5PW column (21.5 × 150 mm). Separation of the extract was done using an SPD-6AV detector and LC-6A HPLC pump. The elution was carried out by a linear gradient of 0.2–0.3 M NaCl in 20 mM phosphate buffer (pH 7.5) for 60 min at a flow rate of 5 ml/min, and monitored at A_{220}nm.

Reversed-Phase Chromatography of Protein and Peptide

Protein or peptide samples were applied to Puressil C18 column (4.6 φ × 150 mm). Purification of samples were done using an SPD-10AV detector and LC-10AD HPLC pump. The elution was carried out by a linear gradient of 45–55% acetonitrile for 30 min or 0–60% acetonitrile containing 0.1% trifluoroacetic acid for 30 or 90 min at a flow rate of 1 ml/min, and monitored at A_{210}nm.

Amino Acid Analysis

Protein or peptide samples were hydrolyzed in vacuo at 110°C for 24 h in 6 N hydrochloric acid containing 1% phenol. The hydrolyzates were dried, and derivatized according to the method of Cohen et al. (1), and analyzed by an automatic amino acid analyzer (PICO-TAG™, Waters).

Pyridylethylolation of Protein

Protein sample was pyridylethylolated according to the standard method (17). The reaction was allowed to proceed at room temperature for 6 h. Pyridylethylolated sample was applied to HPLC, eluted by a linear gradient for 90 min, and monitored at 210 nm or 255 nm. The modified protein was eluted at about 80 min.

Cleavage and Digestion of Target Protein

Cyanogen bromide cleavage The cleavage was
carried out essentially according to the method of Gross (5), with modifications by Kuboki and Mechanic (12). Protein sample (100 μg) was treated for 24 h with 1% cyanogen bromide/70% formic acid (100 μl). Degraded protein was recovered by lyophilization of the reaction mixture after the addition of distilled water (400 μl).

Arginylendopeptidase digestion of BrCN peptide
The digestion was carried out according to the standard method (15). Peptide (100 μg) was incubated at 37°C for 3 h with arginylendopeptidase (7.9 μl of 1.18 mg/ml) in 200 μl of 50 mM phosphate buffer (pH 8.0).

Tryptic digestion Native or pyridylethylated protein (200 μg) was dissolved in 100 μl of 20 mM phosphate buffer (pH 7.5), and incubated with trypsin (10 μl or 2.5 μl of 0.8 mg/ml solution) at 37°C for 24 h or 2 h. After the incubation, 6 N hydrochloric acid (20 μl) was added to the mixture (10).

**Protein Sequence Analysis**

Automated, repetitive Edman degradations (2, 3) of peptide samples were performed with an Applied Biosystem 491 protein sequencer. All procedures, including identification of phenylthiohydantoïn (PTH) derivatives, were done using a 785A UV detector and 140C HPLC pump.

**SDS-Polyacrylamide Gel Electrophoresis**
Electrophoresis was performed in a 12.5% polyacrylamide gel by Laemmli’s method (13).

**RESULTS**

**Affinity Chromatography and SDS Polyacrylamide Gel of a Target Protein for Z-Leu-Leu-Leu-H**
Affinity chromatography was devised using our newly prepared affinity resin, to which Z-Leu-Leu-Leu-OH was immobilized on the C-terminal car-
bovine brain extract was applied to the affinity column, and eluted as mentioned above. Affinity fractions obtained were dialyzed against distilled water, lyophilized and subjected to SDS-polyacrylamide gel electrophoresis.

Electrophoregrams for bovine brain extract, and citrate buffer and EDTA fractions are shown in Fig. 1. EDTA fraction obtained under the elution condition B, gave one main (indicated by arrow) and several minor protein bands. This main protein was of a low molecular weight, and retained to the affinity column in the presence of calcium ion, and eluted with EDTA. The protein was not retained to the column in the absence of calcium ion, and not detected in citrate buffer fraction eluted under the condition A. This protein is thought to be one of the target proteins for Z-Leu-Leu-Leu-H. The amino acid analysis of the protein after the collection on a maxyyield showed a high content of Asx and Glx. As the concentration by affinity chromatography was not successful, another chromatography system was needed to identify this protein.

Purification of the Target Protein for Z-Leu-Leu-Leu-H by Means of DEAE-5PW and C18 Column Chromatography

Bovine brain extract was subjected to ion-exchange chromatography on DEAE-5PW. A typical chromatographic pattern is shown in Fig. 2. Each fraction was applied to the affinity column described above. The fraction underlined in Fig. 2, was retained to the column in the presence of calcium ion, and eluted with EDTA. For the further purification of this fraction, reversed-phase HPLC on a C18 column was performed. A typical chromatographic pattern is shown in Fig. 3. The protein in question was eluted at the underlined position (Fig. 3) as a single, symmetrical peak at a concentration of acetonitrile higher than 45%. On SDS polyacrylamide gel electrophoresis, the protein gave a single band which closely corresponded to the band indicated by arrow in Fig. 1. The amino acid compositions of a purified target protein for Z-Leu-Leu-Leu-H and S-100β were determined. Phe and Tyr were not detected. Asx, Ser, Glx, Gly, His, Arg, Ala, Thr and Pro were contained in a purified target protein for Z-Leu-Leu-Leu-H.

Table 1 Comparison of Amino Acid Composition between a Purified Target Protein for Z-Leu-Leu-Leu-H and S-100β

| Amino Acid | Purified Target Protein for Z-Leu-Leu-Leu-H | S-100β
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<tr>
<td></td>
<td>mol%</td>
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</tr>
<tr>
<td>Asx</td>
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<td>9</td>
</tr>
<tr>
<td>Ser</td>
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</tr>
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<td>Glx</td>
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<td>4</td>
</tr>
<tr>
<td>His</td>
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<td>5</td>
</tr>
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<tr>
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<tr>
<td>Tyr</td>
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</tr>
<tr>
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<td>2</td>
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</tr>
<tr>
<td>Phe</td>
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</tr>
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</table>

* Amino acid residues were calculated based on the primary structure of S-100β (7).
acid composition of this protein is shown in Table 1, which displays a striking similarity with that of S-100β subunit. This protein was isolated in a yield of about 1–2 mg from 500 g of bovine brain.

**Identification of the Target Protein for Z-Leu-Leu-Leu-H as S-100β Subunit**

Sequential Edman degradation of the intact target protein released no detectable PTH amino acid derivatives, indicating that the protein has a blocked amino terminus. This protein was first cleaved with cyanogen bromide. As shown in Fig. 4, the cleaved protein was separated into three fractions, CB1, CB2 and CB3, by reversed-phase chromatography. Fraction CB2 was subjected to amino acid sequence analysis, which indicated that this fraction was composed of two peptides (CB2-1, CB2-2) in an equimolar amount. Each peptide was consistent with ETLDSDGDGEFDQ (residues 58–71) and ITTAXHEFFEHE (residues 80–90) of bovine brain S-100β subunit, except for cysteine at the residues 68 and 84.

The arginylendopeptidase digest of fraction CB3 was separated into two fractions RE1 and RE2 by reversed-phase chromatography. Amino acid sequence analysis of fraction RE2 resulted in KSELKELNENSLXFLLEIKEK (residues 29–48) of bovine brain S-100β, except for histidine at the residue 42.

Separation of tryptic digest of native protein by reversed-phase chromatography, gave eleven T peptides (T1-1, T1-2, T2, T4-1, T4-2, T5-1, T5-2, T6-1, T6-2, T7, T8) as shown in Fig. 5A. Amino
Fig. 6  Amino acid sequences of peptides obtained under various digestion conditions from a target protein for Z-Leu-Leu-Leu-H. All the peptides obtained in this study was aligned with the amino acid sequence of bovine brain S-100β subunit (7). Arrows indicate amino acid residues determined by automated, repetitive Edman degradation. T: Tryptic digests of native protein. T': Tryptic digests of native protein under a different condition. pT: Tryptic digests of pyridylethylated protein. CB: Cyanogen bromide cleavage products of native protein. RE: Arginylenopeptidase digests of cyanogen bromide fragment. X: Not determined

acid sequence of each peptide corresponds to that of S-100β as follows; T1-1, KSELK (residues 29–33); T1-2, SELK (residues 30–33); T2, EQUEVVDK (residues 49–55); T4-1, ELINNELSH (residues 34–42); T4-2, DVFHQY (residues 12–17); T5-1, IDVHFQY (residues 11–17); T5-2, VAMITTXHE (residues 77–86); T6-1, VMETLDSGDGEQEXDF (residues 56–70); T6-2, VMAITTXHEFFEHE (residues 77–91); T7, AVVALIDVFQYH (residues 6–17) and T8, ELINNELSHFLEEIK (residues 34–48). T1, T4, T5 or T6 was composed of major (-1) and minor (-2) peptides in the same fraction, respectively. T3 peptide corresponded to a degraded fragment of trypsin itself. Tryptic digest of pyridylethylated protein by reversed-phase chromatography, gave pT peptides (pT1–pT5) as shown in Fig. 5B. Amino acid sequence of each peptide corresponds to that of S-100β as follows: pT2, VAMITTXHEFFEHE (residues 77–91); pT3, AVVALIDVFQYH (residues 6–20); pT4, VMETLDSGDGEQEXDF (residues 56–76) and pT5, AVVALIDVFQYH (residues 6–17). pT1 peptide corresponded to a degraded fragment of trypsin itself. T1-1 and T1-2 peptides obtained under a different digestion condition were consistent with AVVALIDVFQYH (residues 6–20) and ELINNELSHFLEEIK (residues 34–48) of S-100β. All the fragments obtained under various conditions are aligned in Fig. 6. The aligned sequence of purified target protein for Z-Leu-Leu-Leu-H coincides with that of bovine brain S-100β subunit (about
A TARGET PROTEIN FOR Z-Leu-Leu-Leu-H

86% of the whole molecule), except for N-terminal five residues and residues 21–28.

DISCUSSION

In this study, we have isolated one of the target proteins for Z-Leu-Leu-Leu-H, and intended to identify it as S-100β (7).

Depending on the substitution of C-terminal aldehyde portion of this agent, the stimulation of its neurite outgrowth activity has been maintained or lost (8). By the substitution of N-terminal protecting group and the third amino acid residue, almost all the activity has been lowered (8, 18). Based on these observations, we have postulated two sites on this peptide molecule; first, a recognition site that constitutes the skeleton, and second, a reactive site on the aldehyde portion responsible to physiological activities.

We have prepared an affinity resin, to which Z-Leu-Leu-Leu-OH was immobilized on the C-terminal carboxyl group without modification of the recognition site. In our affinity chromatography system using this resin, many proteins have been retained. We could detect one characteristic protein, which was retained in the affinity column in the presence of calcium ion, and eluted with EDTA. This protein was detectable, though weakly, on SDS polyacrylamide gel electrophoresis. It became clearly visualized after concentration with DEAE-ion exchange chromatography and reversed phase chromatography. Almost all the physiological activities for Z-Leu-Leu-Leu-H have been reported to relate with calcium ion. When calmodulin or albumin was applied to our affinity column, calmodulin was retained in the column in the presence of calcium ion, but albumin was not retained. However, calmodulin band was not detected on SDS polyacrylamide gel electrophoresis of the EDTA fraction obtained from bovine brain extract by our affinity chromatography, probably because of higher affinity of S-100β compared with calmodulin.

Analysis of the target protein showed the N-terminal residue to be blocked. Peptide sequencing after tryptic digestion and cyanogen bromide cleavage followed by arginyldendopeptidase digestion has revealed that the protein isolated as one of the target proteins for Z-Leu-Leu-Leu-H is almost identical with bovine brain S-100β subunit (7) in its amino acid sequence determined.

S-100β is found in a high level in glial cells (25). Although the physiological roles of S-100β are not clear, it has been shown that the level of two mRNAs (for S-100-like proteins) is increased in PC12 cells by treatment with NGF (14), and that addition of the disulfide-bonding form of S-100β into cultured medium induces neurite extension of chick embryo cerebral cortical neurons (11); neurotrophic activity on central nervous system neurons of proteins in the S-100 family is also reported (11, 25).

Tsubuki et al. have recently isolated proteasome that degrades a Z-Leu-Leu-Leu-related substrate (23). The moiety of Z-Leu-Leu-Leu is necessary for isolation of the target protein(s), and we further attempt to investigate the reactivity of S-100β with Z-Leu-Leu-Leu-H without modification of C-terminal aldehyde portion.

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