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A fibrinolytic enzyme from a marine green alga, *Codium latum*

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Abstract

A fibrinolytic enzyme was isolated from a marine green alga, *Codium latum*, and designated *C. latum* protease (CLP). It also had fibrinogenolytic activity, hydrolyzing Aα, Bβ and γ chains with preference in this order. As CLP hydrolyzed oxidized insulin B chain at position Arg^{22}–Gly^{23}, and the peptide map of lysozyme digested with CLP was similar to that with trypsin, CLP would be expected to have a high substrate specificity, similar to that of trypsin. Protease activity peaked at pH 10, and was completely inhibited by diisopropyl fluorophosphate (DFP). Therefore, we conclude that CLP is a trypsin-like serine protease.

Keywords: *Codium latum*; Fibrinogenolytic activity; Fibrinolytic activity; Marine alga; Molecular weight; Optimum pH; Protease activity; Purification; Serine protease

1. Introduction

Marine algae contain various biologically active substances including antibacterial and antitumour agents (Hoppe, 1979; Ito & Hori, 1989; Norris & Fenical, 1985; Shiomi & Hori, 1990). Some algal polysaccharides also act as anticoagulants (McLellan & Jurd, 1992) in blood clotting and fibrinolysis processes. Other substances, such as marine algal lectins and fucosterol in brown algae, also affect blood clotting and fibrinolysis. The former induce or inhibit human platelet aggregation (Matsubara, Sumi & Hori, 1996), and the latter induce production of plasminogen activator in endothelial cells (Shimonaka, Hagiwara, Kojima & Inada, 1984). However, few studies have been conducted on fibrinolytic enzymes in marine algae. In this study, we screened for the presence of such enzymes in marine green algae of several species of genus *Codium* and found fibrinolytic activity in their extracts. From *C. intricatum*, two fibrinolytic enzymes, *C. intricatum* protease (CIP)-I and -II, were isolated and characterized (Matsubara, Sumi, Hori & Miyazawa, 1998). Recently, we found strong fibrinolytic activity in the extract of the green alga, *Codium latum*. The isolation and characterization of a fibrinolytic enzyme from *C. latum* are discussed in this report.

2. Results and discussion

During the course of our continuing efforts to investigate fibrinolytic activities in marine algae, a high level of its activity in *C. latum* was detected. Accordingly the purification of a *C. latum* fibrinolytic protease was carried out using various column chromatographic steps (Fig. 1(A,B)). To detect protease activity, the chromogenic substrate S-2444 was used throughout purification being the most sensitive of the 3 chromogenic substrates available (S-2238, S-2251 and S-2444). After anion-exchange chromatography on a Mono Q (Pharmacia Biotech) column, the protease activity fraction showed one major band with a few

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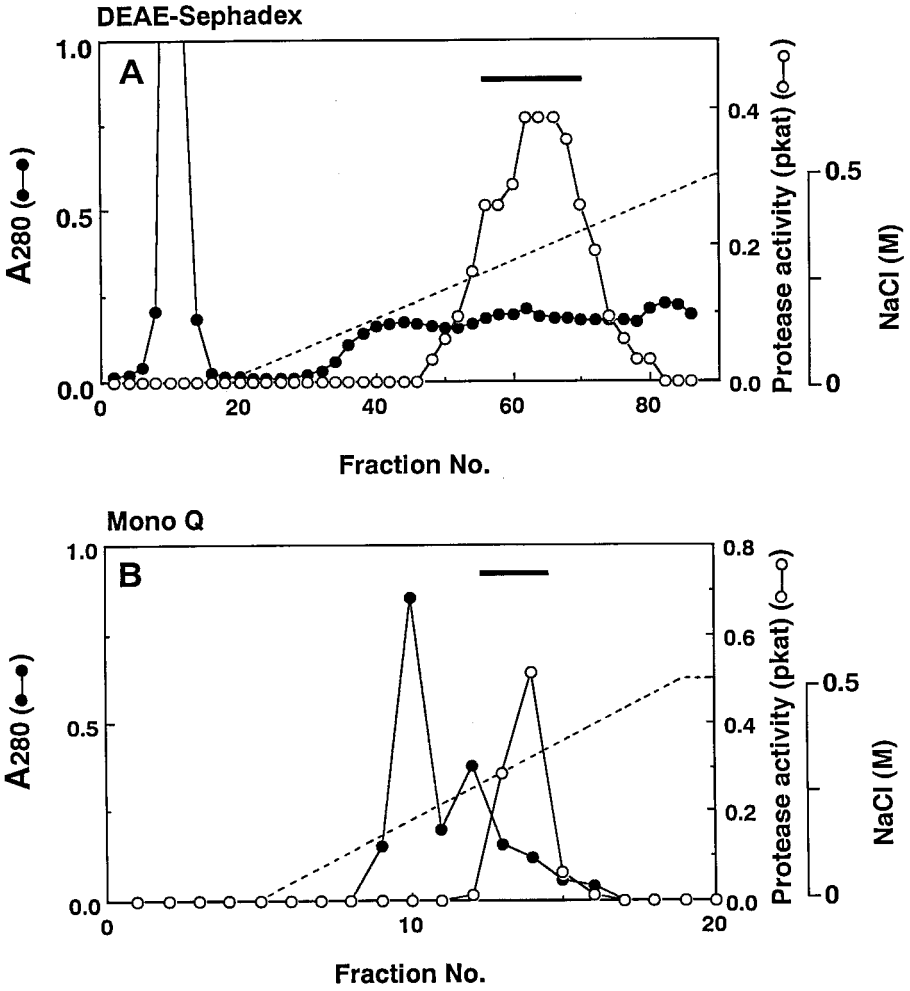

Fig. 1. Purification of *C. latum* protease by DEAE-Sephadex (A), Mono Q (B) and Sephasil C_{18} (C) column. Only one peak showed protease activity (C). Five μg of purified protease (CLP), stained with Coomassie Brilliant Blue R250, showed single band on SDS-PAGE (D), which was performed by the method of Schägger and von Jagow (1987). SDS-PAGE sample buffer used was 50 mM Tris–HCl buffer, pH 6.8 containing 4% SDS, 2% 2-mercaptoethanol and 12% glycerol.

faint bands on SDS-PAGE (data not shown). Gel permeation chromatography failed to eliminate these faint bands. We purified the protease by reversed-phase chromatography using a Sephasil C_{18} (Pharmacia Biotech) column and designated it as *C. latum* protease, CLP (Fig. 1(C,D)). Purified CLP had ca. 60-fold higher specific activity than fibrinolytic proteases (CIPs) isolated from *C. intricatum* (Matsubara et al., 1998). TFA (0.1%) and acetonitrile (ca. 30%) did not inhibit the proteolytic activity of CLP, since the eluted CLP was immediately diluted in 0.1 M phosphate buffer, pH 7.4 to avoid denaturation for protease assay. The purification procedure for CLP is summarized in Table 1.

The fibrinolytic and fibrinogenolytic properties of CLP were examined. The fibrinolytic area caused by purified CLP (2 μg) when applied to fibrin plate was 314 mm^2. This level of activity was comparable to that

Table 1
Purification procedure for CLP

Step	Protein (mg)	Total activity (pkat)	Specific activity (pkat/mg)	Purification fold	Yield (%)
70%$(NH_4)_2SO_4$	250	376	1.50	1	100
DEAE ion-exchange	4.4	190	43.2	29	51
Mono Q	0.18	42	233	155	11
Sephasil C_{18}	0.04	24	600	400	6

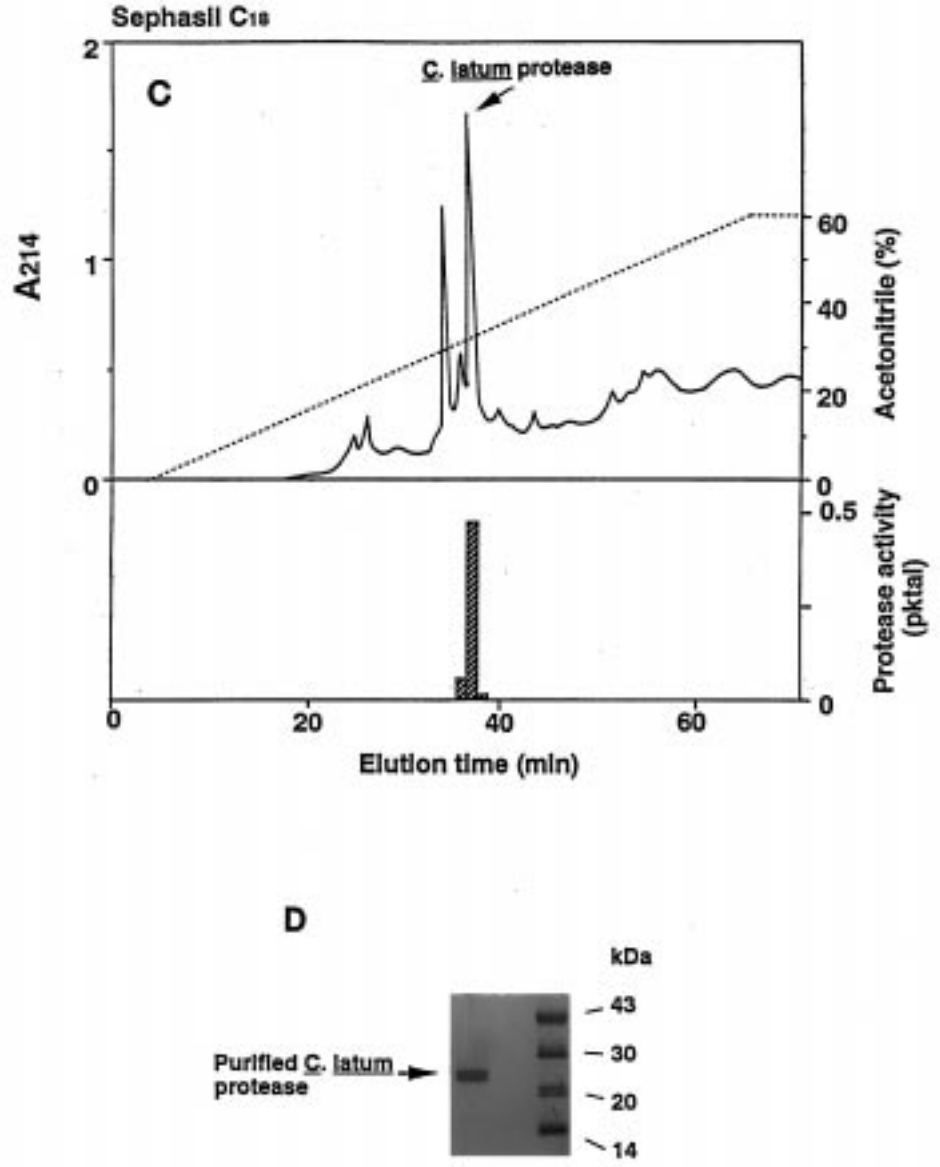

Fig. 1 (*continued*)

of powerful fibrinolytic enzymes found in snake venom (Daoud, Tu & El-Asmar, 1986; Datta, Dong, Witt & Tu, 1995; Ouyang, Hwang & Huang, 1983; Willis & Tu, 1988). The hydrolysis of fibrin by CLP was analyzed by SDS-PAGE (Fig. 2). CLP rapidly hydrolyzed the α chain, and more slowly the β and γ–γ chains. The hydrolysis pattern by CLP was similar to that caused by plasmin, suggesting that CLP has a narrow substrate specificity like plasmin. CLP showed fibrinogenolytic activity, and the CLP-mediated degradation products of fibrinogen were analyzed by SDS-PAGE (Fig. 3). As shown in Fig. 3, CLP rapidly hydrolyzed the Aα chain, and then the Bβ chain. The Aα chain was completely hydrolyzed within 0.5 h, and the Bβ chain was completely degraded within 2 h. After prolonged incubation (3 h), the γ chain was also completely degraded. These results showed that CLP does not have any high specificity for specific chains of fibrinogen. On the other hand, many snake venom fibrinolytic enzymes have high specificity for the Aα or the Bβ chain of fibrinogen (Markland, 1991). The activity of CLP was similar to that of CIPs (Matsubara et al., 1998), though more efficient. Fibrin formation was not observed, thus CLP did not have thrombin-like activity.

The proteolytic activity of CLP was further examined using the digestion products of the insulin B chain and peptide map of lysozyme. Oxidized insulin B chain was digested by CLP, and the resulting peptides were isolated by reversed-phase chromatography. Only two peptide peaks appeared after 6 h of incubation (data not shown). Thus, CLP cleaved the insulin B

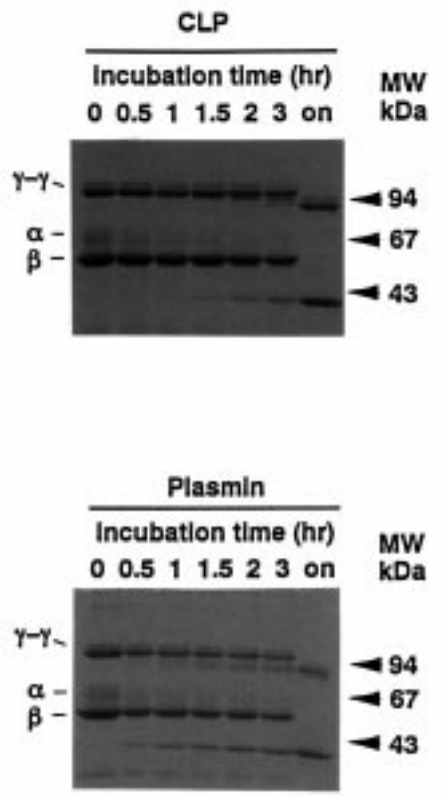

Fig. 2. Analysis of the pattern of fibrinolysis by CLP or plasmin. Fibrin was incubated with CLP or plasmin for various time indicated (on; overnight), and resulting peptides were resolved by SDS-PAGE using a 7.5% gel. SDS-PAGE sample buffer used was 50 mM Tris–HCl, pH 6.8 containing 5 M urea, 2% SDS, 2% 2-mercaptoethanol and 20% glycerol. Each lane contained 12.5 μg of fibrin.

chain at only one site. The amino acid sequences of these two peptides were Phe[1]–Val–Asn–Gln–His- - -Arg[22] and Gly[23]–Phe–Phe–Tyr–Thr- - -Ala[30], indicating CLP to only hydrolyze the peptide bond between Arg[22] and Gly[23]. However, CLP did not hydrolyze the C-terminal peptide bond, –Lys[29]–Ala[30]. The peptide bond between Lys–Ala would not be cleaved effectively if (1) CLP possessed different affinity for arginine and lysine residues, (2) if structural differences between both basic amino acids affect enzyme/substrate recognition, or if (3) structural constraints of a single amino acid at the C-terminal end imposed suboptimal binding conditions at the substrate recognition site. Similar peptide map patterns were obtained when trypsin and CLP digested lysozyme (Fig. 4). CLP hydrolyzed the chromogenic substrates, X–X–Arg–pNA and X–X–Lys–pNA. CLP, like trypsin, thus exhibits narrow substrate specificity for arginine and lysine residues and accordingly should be useful for the restricted cleavage of proteins.

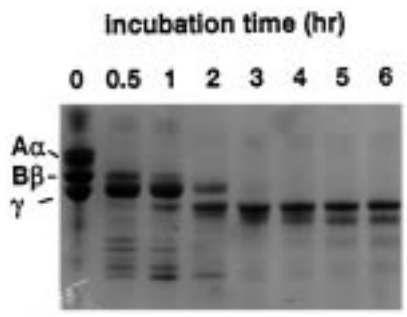

Fig. 3. Analysis of the pattern of fibrinogenolysis by CLP. Fibrinogen was incubated with CLP for various times indicated, and peptides were resolved by SDS-PAGE using a 10% gel. SDS-PAGE sample buffer used was the same as described in Fig. 1. Each lane contained 12.5 μg of fibrinogen.

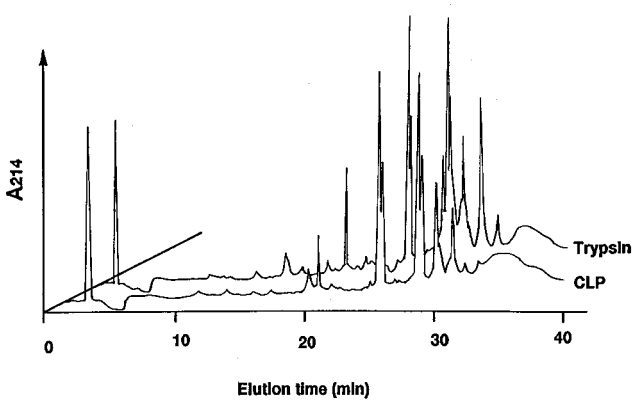

Fig. 4. Comparison of peptide map of lysozyme digested by CLP or trypsin. Lysozyme was digested by CLP or trypsin, and analyzed by reverse-phase chromatography using a Smart System.

The effects of various protease inhibitors on the proteolytic activity of CLP were examined (Table 2). Proteolytic activity was completely inhibited by DFP, a serine protease inhibitor, and almost completely by TLCK. SBTI and aprotinin strongly blocked CLP activity, whereas EDTA and PCMB showed no effect. CLP is thus shown to be a trypsin-like serine protease.

The M_r of CLP was estimated as 23 kDa in SDS-PAGE, and the protease activity of CLP for S-2444 was optimal at pH 10 (Fig. 5). Both of these characteristics are similar to those exhibited by CIPs.

Ten cycles of N-terminal sequencing were done, and 8 residues were identified as follows: Val–Val–Gly–Gly–Asp–Glu–Pro–Pro–. This N-terminal sequence differed from those of CIPs (Matsubara et al., 1998),

Table 2
Effect of protease inhibitiors on CLP activity

Inhibitor	Concentration	Residual activity (%)
DFP	1 mM	0
TLCK	1 mM	1
PCMB	1 mM	109
EDTA	10 mM	106
SBTI	200 μg/ml	32
Aprotinin	10 TIU/ml	20

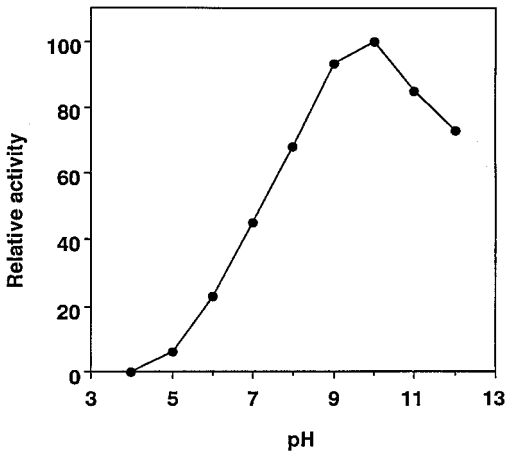

Fig. 5. Effect of pH on protease activity.

although both algal enzymes (CLP and CIPs) belong to the trypsin-like serine protease group. Six N-terminal amino acid residues of CLP were identical to those of RVV-Vα and -Vγ, factor V-activating enzymes isolated from *Vipera russelli* (Russell's viper) venom (Tokunaga et al., 1988) (Fig. 6). CLP had strong fibrinolytic activity and did not form fibrin. RVV-Vα and -Vγ, however, induce coagulation. Elucidation of the complete amino acid sequence of CLP should indicate the differences in enzymatic and physiological action of CLP, RVV-Vs, and other fibrinolytic enzymes.

3. Experimental

3.1. Materials

Codium latum was collected at the coast of Kagoshima Prefecture, Japan. The alga was immediately washed, frozen and stored at −20°C. Bovine and human fibrinogen, bovine and human thrombin, oxidized insulin B chain, oxidized lysozyme, soybean trypsin inhibitor (SBTI), aprotinin, diisopropyl fluorophosphate (DFP) and 1-chloro-3-tosylamido-7-amino-L-2-heptanone (TLCK) were purchased from Sigma (St. Louis, MO, USA). Para-nitroanilide (pNA) chromogenic substrates, pyro–Glu–Gly–Arg–pNA (S-2444), H–D–Val–Leu–Lys–pNA (S-2251) and H–D–Phe–Pip–Arg–pNA (S-2238) were obtained from Chromogenix (Mölndal, Sweden). Trifluoroacetic acid (TFA) and *p*-(chloromercuri) benzoic acid (PCMB) were from Wako Pure Chem. Co. (Osaka, Japan). DEAE-Sephadex 25-A was from Pharmacia Biotech (Uppsala, Sweden). Other reagents were special grade as commercially available.

	1 5	
CLP	Val-Val-Gly-Gly-Asp-Glu	Pro-Pro-
RVV-Vα	Val-Val-Gly-Gly-Asp-Glu	Cys-Asn-
RVV-Vγ	Val-Val-Gly-Gly-Asp-Glu	Cys-Asn-

Fig. 6. Comparison of N-terminal amino acid sequences of CLP, RVV-Vα, and RVV-Vγ. The box delineates identical amino acid sequences to that of CLP.

3.2. Purification of protease

Algal sample (ca. 2.8 kg) was homogenized in 2800 ml of 20 mM phosphate buffer (PB), pH 7.0 containing 0.15 M NaCl and 0.02% NaN_3, and kept at 4°C with stirring overnight. The homogenate was centrifuged at 8000 × *g* for 30 min at 4°C, and the pellet was extracted once more in a similar manner. To both supernatants, ammonium sulfate was added to 70% saturation. The salted-out proteins were collected by centrifugation (8000 × *g*). The obtained ppts. were combined, dissolved in distilled water, and dialyzed against 20 mM Tris–HCl buffer, pH 8.0. After removing the insoluble materials by centrifugation, the crude preparation was applied to a DEAE-Sephadex A-25 column (6.0 × 3.0 cm) equilibrated in the dialysis buffer. Elution was carried out at a flow rate of 35 ml/h with a linear gradient of 0–0.5 M NaCl in the same buffer. Fractions of 4 ml each were collected and measured for absorbance at 280 nm and protease activity with a chromogenic substrate S-2444. The fractions showing high protease activity were combined, dialyzed against 20 mM Tris–HCl buffer, pH 7.8 and loaded on a Mono Q column (50 × 5 mm) (Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer, using a FPLC system (Pharmacia Biotech). Elution at a flow rate of 0.8 ml/min with a linear gradient of 0–0.5 M NaCl in the same buffer was performed. Absorbance was monitored at 280 nm and protease activity assays were performed as described below (Matsubara et al., 1998). The protease fractions were applied directly to a Sephasil C_{18} column (100 × 2.1 mm) in a Smart System (Pharmacia Biotech) and eluted with a 0–60% gradient of acetonitrile/0.1% TFA. All peaks showing absorbance at 214 nm were collected and measured for protease activity.

3.3. Protease activity

Protease activity was determined using chromogenic substrates as described previously (Matsubara et al., 1998). Briefly, 20 μl of each sample solution was added to 100 μl of 0.1 M PB, pH 7.4, and to this solution 30 μl of 3 mM chromogenic substrate was added. Absorbance of released pNA at 405 nm was measured with a microplate reader, Model 450 (Bio-Rad,

Richmond, CA). The amount of released pNA was calculated from the change in absorbance (ΔA405/min). One Katal (kat) of enzyme activity was defined as 1 mol/s of pNA formed.

3.4. Fibrinolytic activity

Fibrinolytic activity was determined by a slightly modified method of Astrup and Müllertz (1952) as follows. Ten ml of 0.4% human fibrinogen in 0.1 M PB, pH 7.4, was poured into a 10 cm petri dish and then clotted by the addition of 0.2 ml of human thrombin (100 NIH U/ml) in the same buffer. The clot was allowed to stand for 1 h at room temperature. Twenty microliters of purified protease solution (0.1 mg/ml) was carefully placed on the plate. The plate was incubated for 18 h at 37°C and the diameter of the lytic circle formed was measured. In this fibrin plate method, a clear transparent region is observed where fibrin is hydrolyzed, and its diameter is proportional to the potency of fibrinolytic activity. Fibrinolytic activity was expressed in terms of the lytic area.

In addition, fibrin degradation analysis was performed by a slightly modified method of Datta et al. (1995) as follows: One hundred microliters of 1% human fibrinogen solution in 50 mM Tris–HCl buffer, pH 7.8 containing 0.1 M NaCl was mixed with 100 μl of thrombin solution (10 NIH U/ml) in the same buffer. The clot was allowed to stand for 1 h at room temperature. Eighty microliters of CLP solution (20 μg/ml) was placed on the clot surface and incubated at 37°C for various time intervals. Plasmin (0.1 U/ml) was used as positive control. The reaction was stopped by the addition of 280 μl of denaturing solution (10 M urea, 4% SDS, and 4% 2-mercaptoethanol). The resulting peptide products were analyzed by SDS-PAGE on 7.5% gel (Laemmli, 1970).

3.5. Fibrinogenolytic activity

Fibrinogenolytic activity was measured as described previously (Matsubara et al., 1998). Briefly, 100 μl of 1% solution of human fibrinogen in 50 mM Tris–HCl buffer, pH 7.8 containing 0.1 M NaCl was incubated with 10 μg of a purified algal protease at 37°C. At intervals, a portion of the reaction solution was withdrawn, and analyzed by SDS-PAGE according to the method of Schägger and von Jagow (1987).

3.6. Proteolytic property and peptide map

Insulin B chain was digested by the purified algal protease. Six hundred microliters of insulin B chain solution (1 mg/ml) in 0.1 M PB, pH 7.4 was mixed with 10 μg of purified protease, and the reaction solution was incubated at 37°C for 6 h. The resulting peptides were isolated by reversed-phase chromatography using a Smart System (Pharmacia Biotech). Sequencing of isolated peptides were performed on a protein sequencer, Model 476A (ABI, Foster City, CA).

CLP peptide mapping using lysozyme was compared with corresponding tryptic peptides. Fifty micrograms of lysozyme was digested with 1 μg of trypsin or the purified algal protease in 0.1 M PB, pH 7.4 at 37°C for 6 h. The peptide mapping was performed by reverse-phase chromatography on a Sephasil C_{18} column (100 × 2.1 mm) using a Smart System (Pharmacia Biotech).

3.7. Other biochemical characteristics of protease

The *Mr* of CLP was estimated by SDS-PAGE. The effects of several protease inhibitors (DFP, TLCK, EDTA, PCMB, SBTI and aprotinin) were examined as follows: Purified protease was incubated in 0.1 M PB, pH 7.4 with the addition of a protease inhibitor at 37°C for 10 min, and the inhibition of protease activity was determined using S-2444 as a substrate. Effect of pH was also examined as previously described (Matsubara et al., 1998).

3.8. N-terminal sequence of CLP

Purified CLP (ca. 1 μg) was used for amino acid sequence analysis on a Model 476A protein sequencer (ABI, Foster City, CA).

3.9. Protein concentration

Protein concentrations were determined by using a BCA or a Micro BCA protein assay reagent Kit (Pierce, Rockford, IL). Bovine serum albumin was used as a protein standard.

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