Purification and characterization of a clostripain-like protease from a recombinant *Clostridium perfringens* culture

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Clostridium perfringens produces a homologue of clostripain (Clo), the arginine-specific endopeptidase of *Clostridium histolyticum*. To determine the biochemical and biological properties of the *C. perfringens* homologue (Clp), it was purified from the culture supernatant of a recombinant *C. perfringens* strain by cation-exchange chromatography and ultrafiltration. Analysis by SDS-PAGE, N-terminal amino acid sequencing and TOF mass spectrometry revealed that Clp consists of two polypeptides comprising heavy (38 kDa) and light (16 kDa or 15 kDa) chains, and that the two light chains differ in the N-terminal cleavage site. This difference in the light chain did not affect the enzymic activity toward *N*-benzoyl-L-arginine *p*-nitroanilide (Bz-L-arginine pNA), as demonstrated by assaying culture supernatants differing in the relative ratio of the two light chains. Although the purified Clp preferentially degraded Bz-DL-arginine pNA rather than Bz-DL-lysine pNA, it degraded the latter more efficiently than did Clo. Clp showed 2.3-fold higher caseinolytic activity than Clo, as expected from the difference in substrate specificity. Clp caused an increase in vascular permeability when injected intradermally into mice, implying a possible role of Clp in the pathogenesis of clostridial myonecrosis.

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INTRODUCTION

Clostripain (Clo, EC 3.4.22.8) is a cysteine endopeptidase produced by *Clostridium histolyticum*, one of the histolytic clostridia causing fulminant clostridial myonecrosis (Ullmann & Bordusa, 2004; De *et al.*, 2003). It is secreted as an inactive precursor form, which is converted to a heterodimeric active form through autocatalytic removal of a propeptide and a linker peptide (Dargatz *et al.*, 1993; Ullmann & Bordusa, 2004). Recently, it was shown that Clo facilitates the apoptosis of neutrophils, as do gingipains R, the major virulence factors of periodontopathogenic *Porphyromonas gingivalis* (Guzik *et al.*, 2007; Sheets *et al.*, 2008; Guzik & Potempa, 2008). Other pathogenic functions implicated for gingipains R seem also to be exerted by Clo, since these arginine-specific endopeptidases are closely related to each other both phylogenetically and structurally (Chen *et al.*, 1998; Barrett & Rawlings, 2001; Labrou & Rigden, 2004).

Clostripain-like protease (Clp) is produced by *Clostridium perfringens*, the most common causative bacterium of clostridial myonecrosis (De *et al.*, 2003). The expression of Clp is regulated by the two-component system VirR/VirS, like that of other virulence factors (Shimizu *et al.*, 2002a). Since Clp is highly homologous to Clo (Labrou & Rigden, 2004), it seems possible that Clp exhibits functional similarity with Clo and also probably with gingipains R. Elucidation of the pathogenic roles of Clo and Clp would provide useful information for more comprehensive understanding of the pathogenicity of histolytic clostridia, so-called flesh-eating bacteria (Shimizu *et al.*, 2002b).

Preparation of high-grade Clp and Clo is a prerequisite for assessing their *in vivo* toxicity for experimental animals and *in vitro* cytotoxicity. Culture broth filtrates of *C. histolyticum* contain various proteases, e.g. collagenase, gelatinase, clostripain, elastase, aminopeptidase, proteinases and non-specific peptidases (Jóźwiak *et al.*, 2005). Therefore, the possibility cannot be excluded that even highly purified Clo preparations may be contaminated by such proteases. In contrast, no proteases other than Clp, collagenase and

Abbreviations: Ac, acyl; Bz, *N*-benzoyl; Clo, clostripain; Clp, clostripain-like protease of *C. perfringens*; ME, methyl ester; pNA, *p*-nitroanilide; Tos, tosyl.

gelatinase have been detected in culture filtrates of *C. perfringens* (Matsushita *et al.*, 1994; Tanaka *et al.*, 2008). Although a purification method for the identification of Clp has been reported (Shimizu *et al.*, 2002a), it is not well suited for the large- or medium-scale Clp purification required for biochemical and pathological studies. Thus, we have attempted to develop a method for the preparation of recombinant Clp. In this paper we describe a method involving our *C. perfringens* host–vector system (Takamizawa *et al.*, 2004; Tanaka *et al.*, 2008), which allowed us to purify 1.1 mg of recombinant Clp from 100 ml of culture supernatant. We have analysed the biochemical properties of the purified Clp and also examined the *in vivo* effect of Clp by injecting it intradermally in mice.

We have also been aiming to establish a method for Clo purification that is more efficient than previously reported ones. Despite the difficulty in the preparation of pure Clo and its limited supply, it is often used for sequence analysis (Ullmann & Bordusa, 2004) and processing of recombinant peptides (Kim *et al.*, 2007; Liu *et al.* 2007; Park *et al.*, 1998). Furthermore, its unique property in the reverse reaction has been utilized for the syntheses of isosteres (Günther *et al.*, 2000) and biomimetic molecules (Yoo & Kirshenbaum, 2005). Attempts to produce recombinant Clo in heterologous hosts failed to give satisfactory yields, probably due to problems associated with codon bias and secretion (Kim *et al.*, 2007; Witte *et al.*, 1994). Thus, we have addressed the question of whether recombinant Clp can be used as an alternative enzyme; we also discuss the applicability of our method to the purification of recombinant Clo.

METHODS

Bacterial strains, plasmids and culture conditions. *C. perfringens* strain 13 (Shimizu *et al.*, 2002b) was used as the host strain. Plasmid pFFC (Tanaka *et al.*, 2008) used throughout this study is a derivative of pFF (Kaji *et al.*, 2003), in which a DNA fragment of the Clp gene (*clp*) spanning nucleotide positions 1032749 to 1034366 on the *C. perfringens* strain 13 genome (Shimizu *et al.*, 2002b) was cloned into the *Bam*HI/*Kpn*I site of pFF. *C. perfringens* strain 13 carrying pFFC stored as a glycerol stock at -80°C was grown on a Gifu anaerobic medium (GAM) agar plate (Nissui) containing 20 μg erythromycin ml^{-1} . A colony was precultured overnight in tryptone–yeast extract–glucose (TYG; Kaji *et al.*, 2003) broth medium containing 20 μg erythromycin ml^{-1} (TYGE). The 20 ml preculture was centrifuged at 3000 *g* for 10 min at room temperature. The bacterial pellet was washed once with 40 ml PBS by centrifugation in the same manner. The pellet was resuspended in 2 ml fresh TYGE medium, and then a 1 ml aliquot was inoculated into 100 ml of the medium. The culture was grown at 37°C for 2.5–4 h.

Enzyme and protein assays. Chromatographically pure Clo (Sigma-Aldrich) was dissolved in TG buffer [20% (v/v) glycerol, 50 mM Tris/HCl, pH 8.0] containing 0.4 M NaCl and stored at -80°C . The purified Clp preparation (described below) was stored in the same manner. Prior to assaying, Clo and Clp were activated as follows: 0.1 vol. 10-fold concentrated activation buffer (500 mM Tris/HCl, pH 7.0, 50 mM CaCl_2 , 50 mM DTT) was added to aliquots of

the enzyme stock solution, followed by incubation on ice for 2 h. Proteolytic activity was assayed using azocasein (Sigma-Aldrich) and reaction buffer (50 mM Tris/HCl, pH 7.5, 0.2 M NaCl, 5 mM CaCl_2 , 5 mM DTT), as described previously (Tamai *et al.*, 2008). One unit of caseinolytic activity was defined as the amount of enzyme that caused an increase in A_{400} of 1 min^{-1} under the assay conditions used. The caseinolytic activity was also examined by detection of the halo formed on a skim milk agar plate. A 15 μl sample was added to a well in skim milk agar [1% agarose, 1.5% skim milk, TBS buffer (20 mM Tris/HCl, pH 7.5, 0.15 M NaCl)]. After incubation at 37°C for 5 h, the transparent zone formed around the well was photographed.

Arginyl-endopeptidase activity was assayed using benzoyl-L-arginine *p*-nitroanilide (Bz-L-arginine pNA; Peptide Institute), benzoyl-DL-arginine *p*-nitroanilide (Bz-DL-arginine pNA; Wako) and tosyl-L-arginine methyl ester (Tos-L-arginine ME; Peptide Institute). Lysyl-endopeptidase activity was assayed using benzoyl-DL-lysine *p*-nitroanilide (Bz-DL-lysine pNA; Wako), tosyl-L-lysine methyl ester (Tos-lysine ME; Peptide Institute) and acyl-L-lysine *p*-nitroanilide (Ac-L-lysine pNA; Bachem). The activities toward the substrates containing *p*-nitroaniline were determined as described by Witte *et al.* (1994) with slight modifications. Briefly, the assay mixture (0.95 ml) comprising 0.25 M substrate, 50 mM Tris/HCl (pH 7.5), 5 mM DTT and 5 mM CaCl_2 was incubated at 25°C . The reaction was initiated by the addition of 50 μl of appropriately diluted enzyme solution. After a specific incubation time at 25°C , the reaction was stopped by adding 0.29 ml 50% (v/v) acetic acid and then the A_{405} was measured using an absorption coefficient of $9620 \text{ M}^{-1} \text{ cm}^{-1}$ for *p*-nitroaniline (Chohnan *et al.*, 2004). One unit of hydrolytic activity toward each synthetic substrate was defined as the amount of the enzyme that hydrolysed 1 μmol substrate min^{-1} at 25°C . The esterase activities toward Tos-L-arginine ME and Tos-L-lysine ME were determined as described by Witte *et al.* (1994) and Mitchell & Harrington (1970). Protein concentrations were measured using Pierce bicinchoninic acid (BCA) protein assay reagent (Pierce) or Bradford protein assay reagent (Bio-Rad) with BSA as the standard. The protein concentrations of the culture supernatants and other samples containing reducing agents were determined using a BCA reducing agent compatible protein assay kit (Pierce) to prevent interference by thiol reagents, as described previously (Tamai *et al.*, 2008).

pH optimum determination and enzyme kinetic assays. The optimal pHs of Clp and Clo activities were determined using 0.25 mM Bz-L-arginine pNA and 2 mM Ac-L-lysine pNA at various pH values between 4.5 and 8.5 in TA buffer (50 mM Tris/acetic acid, 5 mM DTT, 5 mM CaCl_2). Kinetic parameters for the hydrolysis of these substrates by Clp and Clo were determined in TA buffer, pH 6.5. Enzyme activity was assayed with Bz-L-arginine (final concentration of 0.125–4 mM) and 0.072 μg Clp or 0.114 μg Clo, and also with Ac-L-lysine pNA (final concentration 0.5–12 mM) and 1.1 μg Clp or 16.6 μg Clo. The initial velocity of hydrolysis (V) was calculated from the increase in the A_{405} during the first 20 min of incubation since it increased linearly with the incubation time under the conditions used. K_m and $V_{\max}$ values were calculated by linear regression analysis of Lineweaver–Burk plots. Values of k_{cat} (turnover number; $V_{\max} [\text{E}]^{-1}$) and $k_{\text{cat}} K_m^{-1}$ (catalytic efficiency) were calculated based on K_m , $V_{\max}$, and $[\text{E}]$ (enzyme concentration) values.

SDS-PAGE. Protein samples were subjected to SDS-PAGE on 14% polyacrylamide gels and then stained with Coomassie brilliant blue R as described previously (Tanaka *et al.*, 2008). The amounts of the two different light chains of Clp were determined by measuring the intensities of the bands corresponding to the chains on an SDS-polyacrylamide gel with Image Gauge software (Fuji Photo Film), as described previously (Tanaka *et al.*, 2008). The relative amounts in each sample were normalized as to the amount of the heavy chain determined for the same sample.

N-terminal amino acid sequencing and protein identification by mass spectrometry. The N-terminal amino acid sequences of the light and heavy chains of Clp, and the truncated form of the Clp heavy chain, were determined on an automatic protein sequencer (ABI 492; Perkin-Elmer), after being transferred to a PVDF membrane as described previously (Matsushita *et al.*, 1998). To determine the cleavage site between the light and heavy chains of Clp, the purified Clp was subjected to determination of the molecular sizes of the two chains by MALDI-TOF/MS analysis with a Voyager-DE STR (Applied Biosystems).

Purification of Clp. Culture supernatants were prepared from 100 ml cultures of *C. perfringens* 13/pFFC as described above. After the supernatants had been passed through a 0.45 µm pore-size filter, they were subjected to chromatography on a 5 ml SP Sepharose cation-exchange column (HiTrap SP XL, GE Healthcare) equilibrated with TG buffer. The culture supernatants (100 ml) were loaded at a flow rate of 4 ml min⁻¹. After loading, the column was washed with the same buffer until the A_{280} was <0.05. The bound proteins were eluted from the column with a linear gradient of 0–1 M NaCl built in 20 ml TG buffer. The amount of Clp in eluates was measured by SDS-PAGE. Clp-containing fractions were collected, and then subjected to ultrafiltration in a Centricon YM-10 (molecular mass cutoff 10 000 Da) to remove contaminating low-molecular-mass substances. After diluting the retained fraction with TG buffer containing 0.4 M NaCl, the same ultrafiltration was repeated three times to remove contaminants.

In vivo study. Male ddY retired mice (38–43 g in weight) were purchased from Japan SLC. All experimental procedures were performed under the guidelines for the care and use of animals as established by the Kagawa University Animal Facility. Purified Clp, which had been stored in TG buffer containing 0.4 M NaCl, was mixed with an equal volume of 50 mM Tris/HCl (pH 7.0) containing 4 mM DTT and 4 mM CaCl₂, followed by incubation on ice for 2 h to activate Clp. The solution was further mixed with an equal volume of 100 mM NaCl. The resulting solution was divided into two portions: one was heated at 96 °C for 10 min and the other was kept on ice until use. An intravascular permeability test was carried out as described previously with slight modifications (Jin *et al.*, 1996). Briefly, a 36 µl sample of each solution containing 10 µg Clp was injected into the dorsal skin of a mouse. After 5 min, the mouse was injected intravenously with 0.3 ml 0.5 % Evans blue in 145 mM NaCl. The mouse was sacrificed by cervical dislocation at 2 h postinjection, and then the dorsal skin was removed for observation of the blue spot caused by extravasation of the dye.

RESULTS

Purification of Clp from *C. perfringens* 13/pFFC

The production of Clp by *C. perfringens* 13/pFFC was examined under various culture conditions. When the bacterium was grown in TYGE medium, Clp was produced most abundantly in the late-exponential growth phase, but autocatalytic degradation of the enzyme also occurred (data not shown). The light chain of Clp comprised a mixture of 16 and 15 kDa polypeptides, which corresponded to the major form of the light chain in 2.5 and 4 h cultures, respectively. To rule out the possibility that this heterogeneity in the light chain might affect the activity of Clp, the relative ratios of the two light chains in the two cultures were determined by densitometry of the bands

after SDS-PAGE and compared to the levels of the enzyme activity in the two cultures (Fig. 1). The two cultures showed a marked difference in the relative ratio of the light chain components. On the other hand, they differed only slightly in the amount of the heavy chain, the difference being almost the same as that in the enzyme activity (Fig. 1). Therefore, it can be concluded that the difference in the light chains does not affect the enzyme activity. A 32 kDa band detected for the 4 h culture seemed to represent a truncated form of the heavy chain, which would be unable to constitute an active form (see below). This band increased with the incubation time (data not shown). Therefore, washed bacterial cells were inoculated at a high density and grown only for 2.5 h for purification of Clp, as described in Methods.

Fig. 1. Comparison of the light chains and enzyme activity of Clp between 2.5 and 4 h cultures. Supernatants were prepared from 2.5 and 4 h cultures. After concentration by TCA precipitation, a 300 µl portion of each supernatant was analysed in triplicate by SDS-PAGE. The band intensities of the heavy and light chains of Clp were determined by densitometry as described in Methods. The enzymic activity toward Bz-L-arginine pNA was assayed for each culture supernatant. Data are means ± SD of triplicate determinations. Lanes: 1, 2.5 h culture; 2, 4 h culture. The mobilities of molecular mass markers are shown on the right. The locations of the 38 kDa heavy chain of Clp and its truncated form are indicated by filled and open arrowheads, respectively. The locations of the 16 and 15 kDa light chains of Clp are indicated by filled and open arrows, respectively. The intensity determined for the 38, 16 and 15 kDa bands is shown on the left of each band. The sum of the 16 and 15 kDa band intensities is in bold. The enzymic activities of 2.5 h and 4 h cultures toward Bz-L-arginine pNA are expressed as U (ml culture)⁻¹. The figures in parentheses are the relative ratios of the band intensity and enzyme activity for the two cultures.

When the culture supernatants were applied to an SP Sepharose column equilibrated with TG buffer, only Clp and low-molecular-mass (<10 000 Da) peptides bound to the column (Fig. 2). The fractions eluted with TG buffer containing 0.4–0.7 M NaCl were collected and subjected to SDS-PAGE analysis. Only Clp components (38, 32, 16 and 15 kDa polypeptides) and low-molecular-mass peptides were detected (Fig. 2). The latter peptides were completely removed by repeated ultrafiltration (three times) with TG buffer containing 0.4 M NaCl (Fig. 2). The final yield, specific activity and recovery of the purified Clp were 1.2 mg (100 ml culture medium)⁻¹, 13.1 U (mg protein)⁻¹ and 56 %, respectively (Table 1).

Characterization of Clp

Two Clo subunits, a 43 kDa heavy chain and a 15.4 kDa light chain, are generated on autocatalytic removal of the propeptide and linker nonapeptide from a single precursor polypeptide (Ullmann & Bordusa, 2004). Pairwise alignments performed using the EMBOSS-Align program with the Needleman–Wunsch global alignment algorithm

(<http://www.ebi.ac.uk/emboss/align>) revealed that Clp is highly homologous to Clo, and also that the amino acid residues in the catalytic site and those predicted to be responsible for binding of Ca²⁺ are well conserved in Clp (Fig. 3). However, there is some sequence divergence in amino acid residues near the cleavage sites of the propeptide and linker peptide between the two homologues. To determine the autoprocessing sites of Clp, purified Clp was subjected to N-terminal amino acid sequencing and TOF mass spectrometry. The N-terminal amino acid sequences of the 15 and 16 kDa light chains on a SDS-polyacrylamide gel were determined to be EAEKTEGDKK and TEGDKKLTVM, respectively. The N-terminal amino acid sequence of the Clp heavy chain was AI(C)(W)DDSN, this being identical to that reported for the Clo heavy chain (Fig. 3). This indicates that Clp is cleaved after K189, and suggests that the putative linker peptide is an undecapeptide, DDKDRASTVVK, which is generated by cleavage after K178. If this is the case, the molecular masses of the two different light Clp chains are theoretically 15 396 Da and 14 939 Da. TOF mass spectrometry of the purified Clp gave three major peaks, the materials in two of which were estimated to have molecular masses of 15 374 Da and 14 916 Da. These are in good agreement with the theoretical values described above, strengthening the validity of the assumption. The polypeptide detected as the other major peak was determined to have a molecular mass of 38 195 Da, which coincided with the 38 203 Da calculated for the Clp heavy chain extending from A190 to Y522 (Fig. 3). The N-terminal amino acid sequence of the 32 kDa truncated heavy chain of Clp was determined to be GDNGEVD (Fig. 3). This processing results in the loss of two amino acid residues constituting the catalytic site and also four of the six amino acid residues at the two putative Ca²⁺-binding sites (Fig. 3).

Determination of the enzymic properties of Clp

Two catalytic amino acid residues (H176 and C231) and the P1 substrate-specificity-determining residue (D229) proposed for Clo are conserved in Clp, suggesting that Clp exhibits strict specificity for Arg-Xaa peptidyl bonds. To assess this possibility, we examined the hydrolytic activity of the purified Clp toward Bz-DL-arginine pNA and Bz-DL-lysine pNA, and compared it to that of Clo. The specific activity of Clp toward Bz-DL-arginine pNA was $4.28 \pm 0.03 \mu\text{mol min}^{-1} \text{mg}^{-1}$ (mean $\pm$ SD), comparable to that of Clo, $5.10 \pm 0.06 \mu\text{mol min}^{-1} \text{mg}^{-1}$. In contrast, the specific activities toward Bz-DL-lysine pNA of Clp and Clo were $(1.72 \pm 0.01) \times 10^{-1}$ and $(7.00 \pm 0.35) \times 10^{-3} \mu\text{mol min}^{-1}$, respectively. Thus, we assumed that Clp hydrolyses Lys-Xaa peptidyl bonds less efficiently than Arg-Xaa ones but it hydrolyses them more efficiently than Clo. To verify this assumption, we attempted to determine pH optima and kinetic parameters of the enzymic activities of Clp and Clo for synthetic substrates containing only L-amino acids and not D-isomers. Phosphate buffer generated precipitates when the Ca²⁺ concentration exceeded 1 mM. Bistris and PIPES buffers caused an increase in A₂₄₇

Fig. 2. SDS-PAGE analyses of Clp preparations during purification and of commercially available purified Clo. All samples before and after SP Sepharose chromatography and after ultrafiltration, and reference commercial Clo, were subjected to denaturing SDS-PAGE on 14 % polyacrylamide gels. Lanes: 1, culture supernatant; 2, SP Sepharose flow-through fraction; 3, SP Sepharose eluted fraction; 4, retentate after ultrafiltration; 5, commercial Clo (Sigma-Aldrich); 6, molecular size markers. Lanes 1 and 2 contained TCA precipitates of 300 μl portions of culture supernatants and the SP Sepharose flow-through fraction, respectively. Lanes 3–5 contained 3 μg protein (determined by Bradford's method). The filled and open arrowheads denote the 38 kDa heavy chain of Clp and its truncated form, respectively. The filled, open and hatched arrows indicate the 16 and 15 kDa light chains of Clp, and low-molecular-mass peptides, respectively.

Table 1. Summary of the two-step purification of Clp

Cultures were grown in TYGE medium for 2.5 h after inoculating washed bacterial cells at 10%. Protein amounts were determined with a BCA reducing agent compatible kit (Pierce), to avoid the effect of reducing agents present in samples. Enzyme activity was determined by using Bz-L-arginine pNA as a substrate, and expressed as means $\pm$ SD of three determinations.

	Volume (ml)	Protein (mg)	Specific activity [U (mg protein) ⁻¹]	Total activity (U)	Purification (fold)	Yield (%)
Culture supernatant	100	1240	0.0225	27.9 $\pm$ 0.2	1	100
SP Sepharose (flow-through)	8.05 (100)	4.77 (1210)	3.23 (0.00075)	15.4 $\pm$ 0.12 (0.910 $\pm$ 0.02)	144	55.2
Retentate after ultrafiltration	1.09	1.20	13.1	15.7 $\pm$ 0.08	582	56.0

and hence could not be used for assays with Tos-L-arginine ME and Tos-L-lysine ME. Thus, we used TA buffer to determine pH optima of the enzymes. The esterase activities of Clp and Clo toward Tos-L-arginine ME were high enough but those toward Tos-L-lysine ME were too low to determine their pH optima (data not shown).

We determined the optimal pHs of Clp and Clo for two commercially available L-amino acid pNA derivatives, Bz-L-arginine pNA and Ac-L-lysine pNA, in TA buffer, pH 5.5–8.5 (data not shown). The optimal pH of Clp for both substrates was 6.5. The maximal amidase activity of Clo for the two substrates was observed at pH 6.5–7.0,

Fig. 3. Comparison of the amino acid sequence and processing sites between Clp and Clo. (A) Alignment of the predicted amino acid sequences. The similarity and identity of the two amino acid sequences were determined using the EMBOSS pairwise alignment program (<http://www.ebi.ac.uk/emboss/align>): identity, 58.3%; similarity, 71.9%; gaps, 3.4%. The predicted catalytic amino acid residues are boxed (Labrou & Rigden, 2004). The amino acid residues at Ca²⁺-binding sites positioned near the cleavage and catalytic sites (Labrou & Rigden, 2004) are enclosed in circles and rhombi, respectively. The autocatalytic cleavage sites for removal of the prepeptide, propeptide and linker peptide are indicated by thick arrows. The cleavage site within the heavy chain is indicated by a thin arrow. The linker peptides are underlined. (B) Schematic representation of the precursor and matured forms of Clp and Clo. The prepeptides, propeptides and linker peptides are denoted by dotted, hatched and filled boxes, respectively.

differing from optimal pH values, 7.2 and 7.4–7.8, reported for the esterase activity toward L-arginine methyl ester in phosphate buffer and α -benzoylarginine ethyl ester in Tris/HCl buffer, respectively (Mitchell & Harrington, 1970). Although the reason for this discrepancy is unknown, the pH optima of the amidase activities of Clp and Clo for the pNA substrates were approximately 6.5. Therefore, all kinetic parameters for these substrates were determined using TA buffer, pH 6.5 (Fig. 4). The K_m values for Bz-L-arginine pNA of Clp and Clo were 0.852 and 0.336 mM, respectively, and their V_{max} values were 123.46 and 43.67 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively (Table 2). It cannot be excluded that the difference in V_{max} may be partly due to differences in the purity of the two enzymes. Further purification of Clo might be required to assess its kinetic parameters correctly. However, SDS-PAGE analysis of the Clo sample showed that it was pure enough for substrate specificity analyses (Fig. 2). In the case of Ac-L-lysine pNA, analysis was performed with the substrate at concentrations below the respective tentatively determined K_m value because of its limited solubility and low affinity for the enzymes. Thus, all kinetic parameters for Ac-L-lysine pNA hydrolysis calculated by extrapolating to V_{max} of the two enzymes might be inaccurate (Fig. 4, Table 2). Nonetheless, there was a marked difference in the activities of the two enzymes, especially of Clo, between the two substrates. Therefore, it can be concluded that Clp is an arginine-specific endopeptidase, and that it hydrolyses Lys-Xaa peptidyl bonds less efficiently than Arg-Xaa ones but it hydrolyses them more efficiently than Clo.

Considering the above difference in enzyme activity, it seemed probable that Clp would exhibit stronger peptidase

activity toward natural protein substrates than would Clo. Therefore, we examined their hydrolytic activity toward casein. Azocaseinase activities determined in Tris/HCl buffer, pH 7.5, for Clp and Clo were 3.30 ± 0.02 and $1.41 \pm 0.02 \text{ U (mg protein)}^{-1}$, respectively (mean $\pm$ SD); the former is 2.3-fold higher than the latter. This difference in caseinase activity was reproduced clearly on casein-containing agarose plates (Fig. 5), where Clp formed transparent zones dose-dependently, while Clo formed only opaque zones, probably due to incomplete digestion.

Effect of intradermal administration of Clp

The finding that Clo exhibits an apoptosis-inducing effect on neutrophils like that of gingipains R suggested that Clo and its homologue Clp may function as virulence factors similarly to gingipains R. To assess this possibility, we examined whether the purified Clp exhibits vascular permeability enhancement activity, like gingipains R. Evans blue dye extravasation occurred only weakly in the mouse dorsal skin when purified Clp (10 μg) was injected after treatment at 96 °C for 10 min, while extensive dye extravasation was observed in the skin when the same Clp sample without heat treatment was injected (Fig. 6). Thus Clp possesses vascular permeability enhancement activity. Injection of smaller doses of Clp (<10 μg but >2 μg) also gave a positive test result (data not shown).

DISCUSSION

Depending on the host–parasite relationship, pathogens produce various types of proteases: some contribute to

Fig. 4. Lineweaver–Burk plots to determine the kinetic parameters for the hydrolysis of Bz-L-arginine pNA and Ac-L-lysine pNA by Clp and Clo. The inset is a plot of V vs $[S]$ for the same data. (A) Assay was carried out with Bz-L-arginine pNA (final concentration 0.125–4 mM) and Clp (○; $[E]$ of 0.072 $\mu\text{g ml}^{-1}$) or Clo (●; $[E]$ of 0.114 $\mu\text{g ml}^{-1}$). (B) Enzyme activity was assayed with Ac-L-lysine pNA (final concentration 0.5–12 mM) and Clp (○; $[E]$ of 1.1 $\mu\text{g ml}^{-1}$) or Ac-L-lysine pNA (final concentration 1.0–24 mM) and Clo (●; $[E]$ of 16.6 $\mu\text{g ml}^{-1}$). All reactions were carried out at 25 °C in TA buffer, pH 6.5. The data are the means $\pm$ SD of three determinations. The kinetic constants calculated from these data are presented in Table 2.

Table 2. Kinetic parameters of the hydrolysis of Bz-L-arginine pNA and Ac-L-lysine pNA by Clp and Clo

K_m and k_{cat} values were calculated from double-reciprocal plots of the data shown in Fig. 4.

Substrate	V_{max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	$k_{cat} K_m^{-1}$ ($\text{M}^{-1} \text{s}^{-1}$)
Clp				
Bz-L-arginine pNA	123.46	0.852	109.31	128.32
Ac-L-lysine pNA	25.45	25.40	22.53	0.887
Clo				
Bz-L-arginine pNA	43.67	0.336	38.84	115.50
Ac-L-lysine pNA	5.33	69.12	4.74	0.069

nutrient acquisition and others contribute additionally to modulation of the immune system, dysregulation of the inflammatory pathways and disruption of the barrier networks in the host. Such multifunctional proteases exist among clans CA, CD and CE of cysteine proteases, e.g. streptopain, gingipains and ubiquitin-targeting proteases (Chiang-Ni & Wu, 2008; Edelmann & Kessler, 2008; Sheets *et al.*, 2008). Gingipains R and a *C. histolyticum* homologue, Clo, have recently been shown to enhance the 'eat-me' signal of neutrophils and induce their apoptosis (Guzik *et al.*, 2007; Sheets *et al.*, 2008; Guzik & Potempa, 2008). Clo and probably Clp seem likely to exert other pathogenic functions demonstrated for gingipains R. However, the involvement of these clostridial proteases in myonecrosis has remained unknown. As the first step toward assessment of Clp as a virulence factor, we attempted to purify recombinant Clp in a large amount and to determine its biochemical properties. The method described here allows efficient purification of Clp.

Fig. 5. Proteolytic activity of the purified Clp and commercial Clo on agarose plates containing skim milk. Clp and Clo were activated by incubation in the activation buffer on ice for 2 h. The samples were threefold serially diluted with the buffer, and 15 μl aliquots were added to wells in 1% agarose containing 1.5% skim milk, 5 mM DTT and TBS buffer, followed by incubation at 37 °C for 5 h. Wells: 1, 0.074 μg ; 2, 0.22 μg ; 3, 0.67 μg ; 4, 2.0 μg Clp or Clo. Clp showed a larger transparent zone around a well than Clo, which showed a marked opaque halo surrounding a transparent zone.

Fig. 6. Permeability-enhancing activity of the purified Clp. The purified Clp sample was activated and diluted appropriately for intradermal injection as described in Methods. The Clp samples with and without heat treatment (96 °C, 10 min) were injected into mouse dorsal skin at a dose of 10 μg of Clp, and then Evans blue dye was injected intravenously. PBS was injected as a control. The animals were sacrificed after 2 h, and then the skin was removed and photographed.

The purified Clp is not homogeneous in the light chain components, but is functionally homogeneous, as demonstrated by the assay involving Bz-L-arginine pNA. As cultures were prolonged, the 16 kDa light chain decreased and the 15 kDa one increased. Therefore, it is possible that all the light chain is converted to the 15 kDa form on longer incubation. However, this would also give rise to the problem that the heavy chain is truncated to its inactive 32 kDa form. In order to increase the yield of Clp and also to avoid contamination by the truncated heavy chain, we employed high-dose inoculation and short-time cultivation. Inactivation through autocatalytic processing also occurred during purification under ordinary conditions. We attempted to reversibly inhibit the Clp activity with inhibitors such as EDTA and 4,4'-dithiopyridine disulfide (Potempa *et al.*, 1998), but we failed to fully restore the Clp activity by incubation in the activation buffer (data not shown). Another characteristic feature of Clp is that it is easily denatured into insoluble aggregates unless a high ionic strength buffer (0.15 M or higher NaCl) is used. We therefore used TG buffer containing 0.4 M NaCl to protect the enzyme from autocatalytic degradation and self-aggregation. The purified enzyme was stable in this solution, and retained full activity at least for 3 months at -80 °C. Clo exhibits stricter substrate specificity than Clp and hence it is preferable to use the former as an arginine-specific peptidase. However, our expression system involving a *clp*-disrupted strain of *C. perfringens* (Tanaka *et al.*, 2008) and the purification method described here would be useful for further improvement of the Clo purification method in terms of both quantity and quality.

The subtle difference in substrate specificity between Clp and Clo may be related to the difference in one amino acid residue (E110 of Clo vs S107 of Clp), which is regarded as

one of the three amino acid residues responsible for Ca^{2+} binding near catalytic sites (Fig. 3; Labrou & Rigden, 2004). We are currently attempting to confirm this hypothesis by comparing the substrate specificities of the wild-type Clp and mutant Clp with E107. Another difference which may affect the substrate specificity is a linker peptide: the linker peptides of Clo and Clp are a nonapeptide cleaved at the two arginine residues and an undecapeptide cleaved at the two lysine residues, respectively (Fig. 3). The difference in length of the linker peptide may underlie the difference in substrate specificity. It also seems possible that Clo and Clp differ in a putative chaperonic function of the linker peptide (Witte *et al.*, 1996), since the two lysine residues of Clp may be less efficiently cleaved than the two arginine residues of Clo. Analysis of how mutation of the linker peptide affects the regulation of the enzyme activity in a homologous host would provide clues to answer these questions.

Arginine-specific gingipains R, RgpA and RgpB, and lysine-specific gingipain K contribute synergistically to the pathogenesis of *P. gingivalis* (Potempa *et al.*, 2000; Tam *et al.*, 2009; O'Brien-Simpson *et al.*, 2009). Clp can hydrolyse Bz-DL-lysine pNA slightly but significantly, while Clo can only do so marginally. This seems to cause the difference in caseinolytic activity between the two enzymes, which may affect the pathogenic potency of Clp. To our knowledge, this is the first paper to document the *in vivo* toxicity of Clp, i.e. its vascular permeability enhancement effect. A likely explanation for the effect is that Clp activates prekallikrein and releases bradykinin, as demonstrated for gingipains R (Potempa *et al.*, 2000). The tissue swelling characteristic of clostridial myonecrosis may well be explained by this effect. Platelet aggregation and leukostasis caused by α -toxin induces an anaerobic milieu, a favourable condition for the multiplication of *C. perfringens* (Bryant *et al.*, 2006), while an increase in vascular permeability caused by Clp would ensure an abundant supply of nutrients for *C. perfringens*, which is a fast-growing bacterium. More precise *in vivo* study of Clp should shed light on the molecular mechanism underlying clostridial myonecrosis and might identify potential targets for therapeutic intervention.

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