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Unusual chromatographic behaviour and one-step purification of a novel membrane proteinase from *Bacillus cereus*

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Abstract

Cell envelopes of *Bacillus cereus* contain a casein-cleaving membrane proteinase (CCMP) and an insulin-cleaving membrane proteinase (ICMP), which differ in their substrate and inhibitor specificity from all *Bacillus* proteinases described previously. They remained localized in the cytoplasmic membrane after treatment with lysozyme and mutanolysin and they are strongly attached to the membrane compared with other known membrane proteinases. Only high a concentration of the zwitterionic detergent sulfobetain SB-12 enabled an effective solubilization of both membrane proteinases. The usual conventional purification methods, such as chromatofocusing, ion-exchange chromatography and hydrophobic interaction chromatography in the presence of detergent concentrations beyond their critical micelle concentration, could not be applied to the purification, because the solubilized membrane proteinases bound strongly and irreversibly to the chromatographic matrix. In the search for other purification methods, we used a tentacle ion-exchanger (EMD trimethylaminoethyl-Fractogel) to reduce the hydrophobic interactions between the proteinases and the matrix. All contaminating proteins could be removed by a first gradient of sodium chloride without elution of CCMP; a second gradient with isopropanol and a decreasing salt concentration resulted in an efficiently purified CCMP. The ICMP was irreversibly denaturated.

Purified CCMP is a member of the metalloproteinase family with a pH optimum in the neutral range and a temperature optimum of 40°C, whose properties differ from the serine-type membrane proteinase of *Bacillus subtilis* described by Shimizu et al. [Agric. Biol. Chem., 47 (1983) 1775]. It consists of two subunits in sodiumdodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions (M_r 53 000 and 65 000); however, the molecular mass of the purified enzyme could not be determined by size exclusion or SDS–PAGE, because the purified enzyme aggregated at the top of the gel matrix. CCMP solubilized before the purification process, could be eluted in the presence of 0.1% octylphenol-poly(ethyleneglycol ether)$_{9-10}$ (Triton X-100) in two peaks of M_r 56 000 and 128 000, respectively.

We discuss this special chromatographic behaviour of the CCMP from *Bacillus cereus*, with regard to the strong hydrophobic interactions of the enzyme with the chromatographic matrix and additional self-aggregation, which could only be dissolved by solvents such as isopropanol.

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1. Introduction

Membrane proteins can be released from membranes by treatment with detergents, which surround the hydrophobic regions of the membrane proteins and mediate their solubility in aqueous media [2–4]. At high detergent/membrane protein concentration ratios, the detergent molecules replace the phospholipid molecules from the protein surface and protein–detergent micelles are formed [5–7]. In this solubilized state, the membrane proteins can be subjected to various purification methods in the presence of detergent above its critical micellar concentration (CMC). For example, solubilized leader peptidase from *E. coli* was purified by hydrophobic interaction chromatography on phenyl-sepharose in the presence of the non-ionic detergent Triton X-100 [8–11]. The neutral endopeptidase from kidney microvilli could be purified by ion-exchange chromatography in its detergent form [12–14]. Generally, the purification of membrane proteins is more difficult than that of soluble proteins, because mixed micelles between phospholipids and different membrane proteins and detergent molecules can be generated during the solubilization process and the interactions between the membrane proteins and the chromatographic matrix are customarily decreased in the presence of detergent. Therefore many membrane proteins were first purified in their protease form without their hydrophobic anchor [15,16]. Additionally, integral membrane proteins, such as receptor or transport proteins are often markedly dependent on membrane phospholipids, which form the typical environment for these proteins in the membrane. Removal or dilution of the phospholipids during and after the solubilization, may result in a significant reduction in protein stability and subsequently in the loss of biological activity [17–20].

We were especially interested in the purification of the membrane proteinases from *Bacillus cereus* in their detergent form. The behaviour of the solubilized proteinases from *B. cereus* during various purification procedures was very unusual and different from that of other membrane proteins in their amphipathic forms, as described in the previous literature. In the present article we examine the interactions of the membrane proteinases from *B. cereus* with different gel matrices and describe a new way to dissolve this unusually strong binding of membrane proteins with the chromatographic matrix.

2. Experimental

2.1. Chemicals

Sulfobetain SB-12, Triton X-100, SDS, trypsin, lysozyme from chicken egg white, marker proteins for size exclusion chromatography (catalase, aldolase, bovine albumin, ovalbumin, dinitrophenyl alanine) and the synthetic peptide substrates Ala-pNA, Glu-pNA, Bz-D,L-Arg-pNA, Suc-Ala-Ala-Val-pNA, Suc-Ala_3-pNA, Glp-Phe-Leu-pNA and Fa-Gly-Leu-NH_2 (FAGLA) were obtained from Serva (Heidelberg, Germany). The detergents sodium cholate, sodium deoxycholate, Brij 35 and Triton X-100 were purchased from Ferak (Berlin, Germany). CHAPSO, octylthioglucoside, EMD TMAE-Fractogel-650(S) and marker proteins for SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (myoglobin, soya-bean trypsin inhibitor, carboanhydrase, ovalbumin, bovine serum albumin, glutamate dehydrogenase, ovotransferrin, phosphorylase b, β-galactosidase, myosin) were obtained from Merck (Darmstadt, Germany). DEAE-Sephacel and Sephacryl S-200 were purchased from Pharmacia (Freiburg, Germany).

Mutanolysin (4200 units/mg) and Fa-Leu-Gly-Pro-Ala-NH_2 (FALGPA) were from Sigma (Deisenhofen, Germany). RNase, DNase I from bovine pancreas and the protease inhibitors Pefabloc, PMSF, pCMB, phosphoramidon, leupeptin, antipain and elastatinal were manufactured by Boehringer (Mannheim, Germany). All other chemicals were of analytical grade.

2.2. Organism and culture conditions

B. cereus is included in our strain collection and was characterized by Dr. Verbarg (German

Collection for Microorganisms, Braunschweig). The bacteria were grown in a medium consisting of 20 g/l yeast extract (Difco) in a phosphate buffer medium [21]. The cultivation was carried out in a fermenter Biostat S (Fa. Braun, Melsungen, Germany) with a 7-l working volume at 37°C, a pH regulated at 7.0 and an aeration rate of 7 l/min until the end of the logarithmic phase (6–8 h cultivation time).

2.3. Cell disintegration

Freshly harvested cells were washed three times with an excess of sodium chloride solution (5 g/l) and diluted in Tris–HCl buffer (pH 7.5, 0.05 *M*, buffer A) to a dry weight of about 100 mg/ml. After adding the same volume of glass beads (diameter 0.10–011 mm), the cells were disintegrated by stirring in an ice bath. The cell homogenate was separated from the glass beads and the few intact cells by centrifugation (4000 *g*, 15 min), and the glass beads were washed three times. The combined supernatants were submitted to ultracentrifugation (90 000 *g*, 60 min) and separated into the particulate (crude cell envelope) and the soluble fraction (cytosol). The crude cell envelope fraction was resuspended in buffer A, washed again under the same conditions and stored at −20°C.

2.4. Protease-releasing experiments with lysozyme, mutanolysin, nuclease and trypsin

Washed cell envelopes (10 ml) were homogenized and diluted in 25 ml Tris–HCl buffer (0.05 *M*, pH 7.5, buffer A). First a 5-ml sample was taken as blank value. Then 10 000 units mutanolysin were added to the remaining cell envelope suspension. The suspension was shaken on a rotatory shaker at 37°C and samples (5 ml) were taken every 15 min over a 75-min period. All samples were collected in an ice bath and centrifuged (60 000 *g*, 1 h). Sediments were suspended in 5 ml buffer A. The lysozyme (20 mg) and nuclease treatment (10 mg RNase, 10 mg DNase) were carried out in the same way in buffer A.

For trypsin treatment, cell envelopes (8 ml) were diluted twice in buffer A and trypsin solution (1.2 mg in 6 ml buffer A) was added. The suspension was shaken and samples (3 ml) were taken at timed intervals of 10 min (0–60 min). After the different incubation times, 1 ml Pefabloc (2 mg/ml) was added to inhibit the trypsin action. All samples were centrifuged under the conditions described above, to separate the protease-solubilized enzyme and the activities of CCMP and ICMP in sediments and supernatants were determined.

2.5. Solubilization

Washed crude cell envelopes were suspended in Tris–HCl buffer (0.05 *M*, pH 7.5), mixed with an equal volume of detergent in different concentrations and shaken (150 rpm) for 1 h at room temperature. Solubilized protein in the supernatant was separated from the insoluble sediment by ultracentrifugation (90 000 *g*, 60 min). The sediments were dissolved and homogenized in buffer with the same detergent concentration as in the corresponding supernatants.

For the solubilization procedure prior to purification of the enzymes, detergent–buffer (8% w/v sulfobetain SB-12 in buffer A) and the same volume of washed cell-envelopes were mixed and treated as described above.

In the supernatant and in the sediment, the proteolytic activities (azocasein assay, [^{125}I]insulin assay) and the protein concentration (BCA method) were determined. The solubilizate was then stored at −20°C.

2.6. Determination of proteolytic activities

Proteolytic activities were determined with azocasein and insulin, by measuring the generation of TCA-soluble coloured or radioactive peptides in the TCA supernatants. For the determination of the insulin-cleaving proteinase, ^{125}I-labeled insulin (100 000 dpm/assay) was incubated with the enzyme-containing fractions and the radioactive, TCA-soluble peptides generated by the proteolytic action were measured in a γ-counter (LB-2104, Berthold) as described before [22]. Cleavage of azocasein was tested by

the method of Langner et al. [23] and expressed in proteolytic units (PU, 1 PU = 1 nmol azocasein cleaved per second at 37°C). Hydrolysis of synthetic *p*-nitranilide (pNA) substrates (0.5 m*M*) was photometrically determined at 405 nm in an end-point assay. After incubation at 37°C for various periods of time, protein was precipitated by the addition of TCA (5% final concentration) and sedimented by centrifugation. The clear supernatants were readjusted by sodium hydroxide to neutral pH. Cleavage of the thermolysin substrate Fa-Gly-Leu-NH_2 and of the collagenase substrate Fa-Leu-Gly-Pro-Ala-NH_2 was determined at 345 nm in a continuous spectrophotometric assay [24].

For inhibition assays, the protease-containing fractions were preincubated for 30 min at room temperature; then the usual assay with azocasein or [^{125}I]insulin was carried out.

2.7. Protein determination

In the presence of detergents interfering with the Lowry method, the BCA method [25] was used for the determination of the protein content, according to the manufacturer's instructions (BioRad, Munich, Germany).

2.8. Lipid extraction

A general lipid extraction procedure with chloroform–methanol with some modifications was applied for the isolation of lipids from *B. cereus* [26]. Methanol–chloroform (2:1, v/v) was added to a suspension of crude cell envelopes containing 40–50 mg bacterial dry weight/ml, in a ratio of 3.75 ml per ml cell suspension. The mixture was shaken (120 rpm) in an Erlenmeyer flask for several hours at room temperature. After filtration, the supernatant was decanted and the residue was resuspended in 4.75 ml of methanol–chloroform–water (2:1:0.8, v/v). The mixture was then shaken and filtered. Amounts of 2.5 ml each of chloroform and water were added to the combined supernatants, and the mixture was separated in a separatory funnel. The lower chloroform phase was dried in a rotary evaporator (30–35°C). The lipid residue was dissolved in a small amount of diethyl ether, subdivided into small portions, evaporated to dryness and stored under nitrogen at −20°C.

2.9. Determination of the molecular masses

The purity and the molecular mass of CCMP were assessed by SDS–PAGE in a Tris–glycine buffer system [27] using various gel concentrations. The enzyme samples were mixed with 1% SDS, 2% mercaptoethanol and sodium-EDTA to prevent autolysis of the protease during heat denaturation (3 min, 100°C). The gels were stained according to a silver-staining method [28].

The molecular mass of CCMP was also determined by size exclusion with Sephacryl S-200, adding different detergents and also the phospholipids of *B. cereus* to the elution buffer to examine the aggregation behaviour of CCMP [buffer A with 0.2 *M* NaCl and with or without detergents and phospholipids–0.1% (w/v) Triton X-100, 0.2% (w/v) sulfobetain SB-12, and 0.1 mg/ml *B. cereus* phospholipid].

3. Results

The membrane proteinases of *B. cereus* were difficult to solubilize. High concentrations of Triton X-100, Brij 35, octylthioglucoside and related non-ionic detergents were ineffective in releasing the enzymes from the cell envelopes. Treatment with mutanolysin and lysozyme had no significant effect–a clear sign that the proteases are not cell wall constituents (Table 1). Only the zwitterionic detergent sulfobetain SB-12 was able to release both an insulin-cleaving (ICMP, data not shown) and a casein-cleaving proteinase (CCMP) from the cell envelopes (Fig. 1).

These enzymes retained their full activity in the solubilized state for days without the addition of phospholipids during the solubilization process. Dilution of the solubilizate caused enzyme activation with decreasing detergent concentration, especially for ICMP (Fig. 2). Only at a sulfobetain SB-12 concentration in the

Table 1
Solubilization of the membrane-bound proteinases by various reagents and detergents

Treatment	CCMP (%)	ICMP (%)
Nuclease	8.5	7.2
Lysozyme (75 min)	9.0	7.5
Mutanolysin (75 min)	8.8	7.9
Trypsin (60 min)	11.0	8.0
Triton X-100 (1% w/v)	16.0	7.3
1 *M* NaCl after Triton X-100 (2% w/v)	15.8	10.5
Brij 35 (1% w/v)	10.9	12.6
Octylthioglucoside (0.5% w/v)	6.5	10.5
CHAPSO (1% w/v)	7.8	3.7
SDS (0.2%)	10.8	32.6
Sodium deoxycholate (1% w/v)	35.0	27.6
Sulfobetain SB-12 (4% w/v)	79.2	88.4

Cell envelopes were prepared by stirring with glass beads. The solubilization extent is expressed in percentage of the sum of proteolytic activities in the supernatants and sediments after the treatment.

[^{125}I]insulin assay below its CMC of 0.12 g/100 ml [29] could this proteinase be measured.

3.1. Enzyme purification

All attempts to purify the enzymes by usual chromatographic methods were unsuccessful. At neutral pH in the presence of various detergents the proteinases were bound well on DEAE-Sephacel, but neither 2 *M* sodium chloride nor increased detergent concentrations (up to 4% w/v sulfobetain SB-12) nor decreasing the pH (acetate buffer pH 3.6) were able to elute the enzymes. Both proteinases (ICMP and CCMP) remained bound to the top of the column and were finally inactivated by the acidic pH. The solubilized proteinases were bound to a chromatofocusing column of polybuffer exchanger PBE-94 and again could not be eluted. Hydrophobic interaction chromatography on phenyl-sepharose and also on the less hydrophobic butyl-sepharose resulted in irreversible binding of the proteinases. Only the use of HA-Ultrogel and subsequent size-exclusion chromatography on Sephadex G-150 in the presence of 0.2% (w/v) sulfobetain led to a partially purified casein-cleaving membrane proteinase (unpublished results). The tentacle ion-exchanger EMD TMAE-Fractogel was selected for the purification procedure, to reduce the interactions between the membrane proteinases and the gel matrix and to prevent possible deformation of the enzymes during ionic binding to the matrix.

The solubilizate containing 4% (w/v) sulfobetain SB-12 was dialyzed overnight against excess buffer A containing 2 m*M* $CaCl_2$ and 0.2% SB-12 (buffer B). Sulfobetain SB-12 is easy to remove by dialysis because of its high CMC [29]. A column of Fractogel EMD TMAE-650(S) (150 × 10 mm) connected to a fast protein liquid chromatographic (FPLC) apparatus (Pharmacia, Uppsala, Sweden) was preequilibrated with buffer B. After loading the dialyzed solubilizate, the column was washed with buffer B (2 ml/min) until the optical density at 280 nm was reduced to its initial value. The gradients consisted of different concentrations of NaCl and isopropanol.

The best purification (Table 2) was obtained when the first gradient was ranged from 100% buffer B and 0% buffer C (buffer B with 1 *M* NaCl) to 0% buffer B and 100% buffer C in 60 min (2 ml/min) with subsequent washing for 15 min with buffer C. The second gradient consisted of 100% buffer C and 0% buffer D (buffer B with 0.5 *M* NaCl and 50% (v/v) isopropanol) to 0% buffer C and 100% buffer D in 90 min (2 ml/min) (Fig. 3).

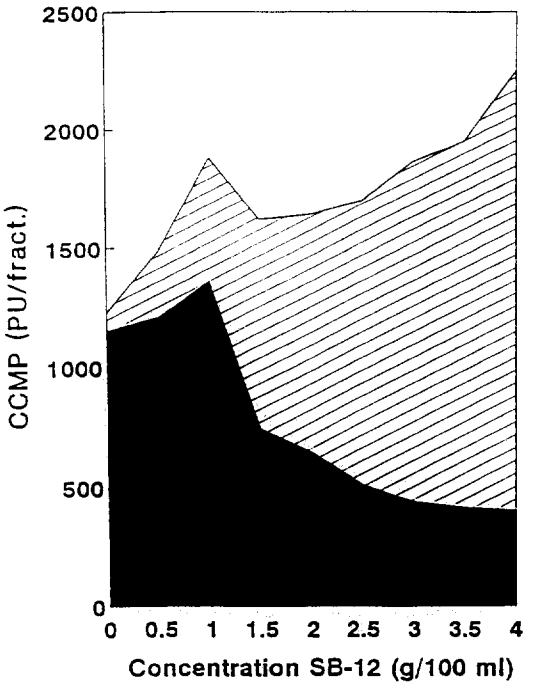

Fig. 1. Solubilization of the CCMP from *B. cereus* with sulfobetain SB-12. Washed cell envelopes were suspended in Tris–HCl buffer (pH 7.5, 0.05 *M*, buffer A) and mixed with an equal volume of the detergent sulfobetain SB-12 in the same buffer. The suspension was shaken for 60 min at room temperature and separated by centrifugation at 90 000 *g* for 60 min. Proteolytic activities in supernatants and sediments were determined after dialysis of the fractions against buffer A with 0.2% sulfobetain SB-12.

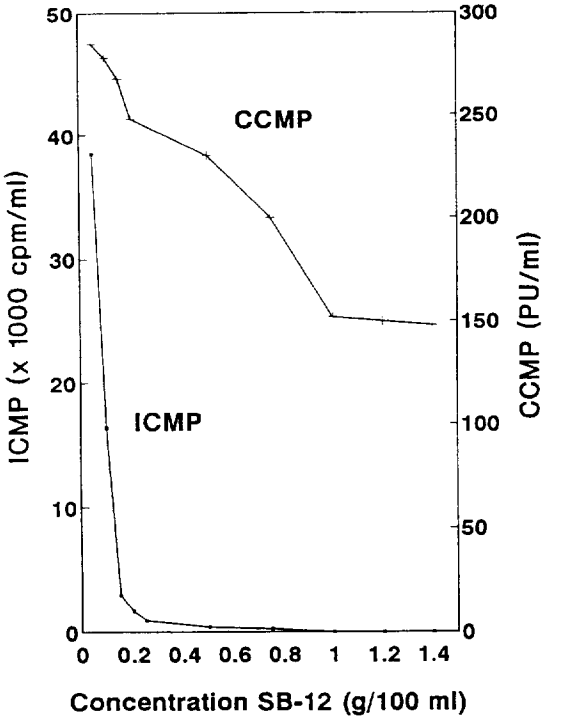

Fig. 2. Dependence of the proteolytic activity on the detergent concentration in the proteinase assay. Supernatant from sulfobetain SB-12 solubilization with high proteolytic activity was diluted with buffer A to a defined detergent concentration in the proteolytic assay and the proteolytic activity was determined in the usual way with azocasein and [^{125}I]insulin.

A higher sodium chloride concentration in the first gradient (up to 2 *M* NaCl) caused formation of small salt crystals in the buffer containing isopropanol during the second gradient and the chromatographic matrix was damaged by the pressure occurring in the column. Only a salt concentration of 1.0 *M* is soluble in a buffer with 50% (v/v) isopropanol. An increased salt concentration (above 0.5 *M*) in buffer D led to a stronger attachment of the proteinase to the chromatographic matrix, and the enzyme was eluted at a higher isopropanol concentration in a less purified state.

The sensitivity of CCMP for isopropanol was tested and the enzyme was found to be as sensitive as chymotrypsin. ICMP was inactivated at low isopropanol concentrations (Fig. 4).

3.2. Detergent and phospholipid dependence of CCMP

The membrane proteinases retain their activity during solubilization without the addition of phospholipids even at high detergent concentrations and remain active for days at 4°C in this solubilized state. A 50-fold dilution of the solubilized membrane proteases in detergent-free buffer activated the proteolytic activity; this activation is due to the dilution of the reversible inactivating detergent sulfobetain SB-12. This indicates that the resulting lack of phospholipids had no inactivating effect (Fig. 2). Addition of *B. cereus* phospholipids to the solubilizate did not increase the activity of the enzymes during the subsequent dilution. The strong interaction of the enzymes with the chromatographic matrix

Table 2
Solubilization and purification of CCMP from *B. cereus*

Purification step	Protein (mg/fraction)	Proteolytic activity (PU/fraction)	Recovery (%)	Purification factor
Washed cell envelopes	19	352.0	100	1
Solubilizate with 4% sulfobetain SB-12	12	522.1	148	2.4
Dialyzate against 0.2% sulfobetain SB-12	10	246.0	70	1.1
Eluate from TMAE-Fractogel	0.07	102.5	29	63

during FPLC on EMD TMAE-Fractogel can not be decreased by the addition of phospholipids to the elution buffer together with high salt concentrations. In the presence of Triton X-100 (0.1%), strong dilution of the phospholipid content of the solubilizate during size exclusion was possible without precipitation or inactivation of the solubilized enzyme.

By contrast, purified CCMP after FPLC on TMAE-Fractogel could not be eluted from the same column under identical conditions, because of strong self-aggregation of the purified hydrophobic enzyme.

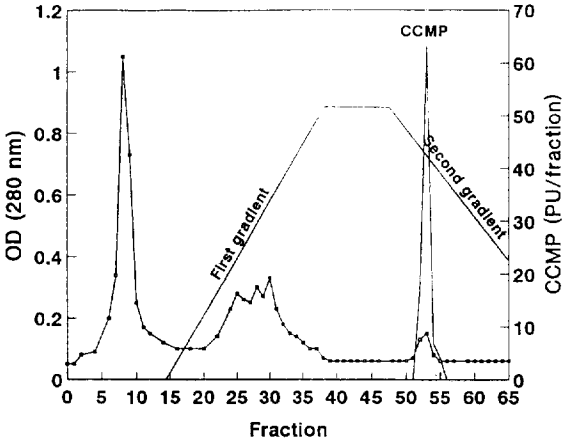

Fig. 3. Purification of CCMP by ion-exchange chromatography on a column of EMD TMAE-Fractogel 650(S) (150 × 10 mm). The column was equilibrated and prewashed with Tris–HCl buffer (pH 7.5, 0.05 *M*, buffer B) containing 2 m*M* $CaCl_2$ and 0.2% sulfobetain SB-12. Elution was performed by two gradients, a first gradient from 100% buffer B and 0% buffer C (buffer B with 1 *M* NaCl) to 0% buffer B and 100% buffer C and a second gradient from 100% buffer C and 0% buffer D (buffer B with 0.5 *M* NaCl and 50% (v/v) isopropanol) to 0% buffer C and 100% buffer D with a flow-rate of 2 ml/min.

3.3. *Inhibition behaviour and substrate specificity of the purified enzyme*

The purified CCMP shows the typical inhibition pattern of a metalloprotease when reacted with sulfhydryl reagents like pCMB (Table 3), but the enzyme is different from the thermolysin-like enzymes in its behaviour with phosphoramidon. Neither the *p*-nitranilide substrates of aminopeptidases, subtilisins, trypsin-like and chymotrypsin-like proteases tested, nor the typical thermolysin substrate FAGLA and the collagenase substrate FALGPA, were cleaved. In its inhibition behaviour and activity with synthetic peptide substrates the purified CCMP

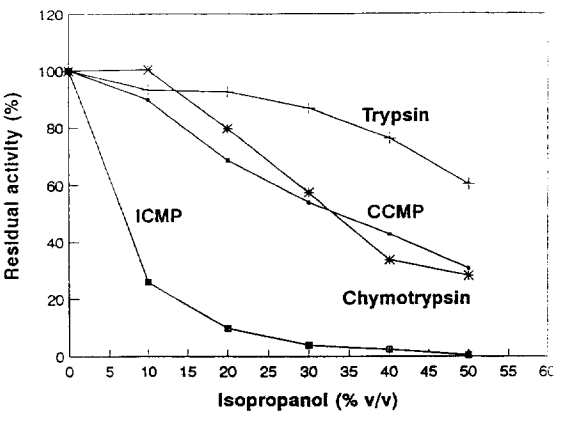

Fig. 4. Influence of isopropanol on the activity of CCMP and ICMP of *B. cereus*, trypsin and chymotrypsin.

Table 3
Influence of different inhibitors and metal ions on purified CCMP from *B. cereus*

Concentration (final concentration in the assay)	Inhibition (%)
o-Phenanthroline (1 m*M*)	100
EDTA (1 m*M*)	100
pCMB (1 m*M*)	75
Benzamidine hydrochloride (5 m*M*)	0
PMSF (1 m*M*)	24
Pefabloc (1 m*M*)	24
Phosphoramidon (10 μM)	0
Elastatinal (10 μM)	0
Chymostatin (10 μM)	0
Leupeptin (10 μM)	0
Antipain (10 μM)	0
Ca^{2+} (1 m*M*)	0
Mg^{2+} (1 m*M*)	0
Mn^{2+} (1 m*M*)	24
Co^{2+} (1 m*M*)	71
Zn^{2+} (1 m*M*)	74

The proteinase was preincubated with different inhibitors and metal ions for 30 min at room temperature.

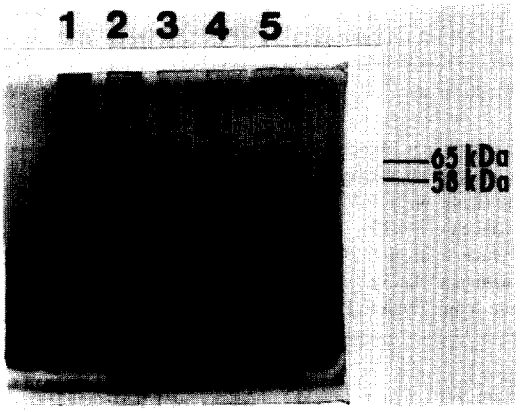

Fig. 5. SDS–PAGE with silver-staining. Different electrophoretic mobility of the purified CCMP depending on the treatment before the SDS–PAGE. Lane 1: calibration proteins, lane 2: solubilizate after dialysis (with 5% ME and 1% SDS boiled for 5 min at 100°C), lane 3: purified CCMP (without ME and SDS, without boiling), lane 4: purified CCMP (without ME, boiled with 1% SDS for 5 min at 100°C, lane 5: purified CCMP (boiled for 5 min with 5% ME and 1% SDS).

differs from all extracellular and endocellular *Bacillus* proteases described so far.

3.4. Determination of the molecular mass

The crude solubilizate (after solubilization with sulfobetain SB-12) was subjected to the size-exclusion procedure under various conditions (addition of *B. cereus* lipid, addition of 2 mmol Ca^{2+}, content of 0.2% sulfobetain SB-12, or 0.1% Triton X-100 in the elution buffer). Two peaks with azocaseinolytic activity (corresponding to molecular masses of 56 000 and 128 000, respectively, Fig. 7) were eluted in similar positions, but with a different percentage partition between them and with different amounts of activity, depending on the buffer used (Fig. 6a–c). In the presence of phospholipids in the elution buffer, the two peaks with azocaseinolytic activity were unified by the formation of mixed phospholipid–detergent–protein micelles (Fig. 6d). The elution behaviour of the purified enzyme was markedly changed; aggregation of the enzyme led to a complex which could not be eluted with detergent buffer.

The purified CCMP did not enter 7% SDS–PAGE gels, not even after boiling for 5 min with 1% SDS (data not shown). By the addition of mercaptoethanol, two diffuse protein bands of M_r 53 000 and 65 000 could be detected for the purified enzyme after silver-staining (Fig. 5, lane 5), corresponding well in their molecular masses to the results of the size-exclusion chromatography (Fig. 7).

4. Discussion

Bacillus species are known to secrete several proteases during their transition to the stationary phase. Besides three relatively major proteases (alkaline serine proteases–subtilisins, neutral metalloproteases–thermolysin-like proteases and bacillopeptidase F–a serine protease with high esterolytic activity) there are also several minor extracellular proteases, which could be demonstrated only after constructing deletion mutants for the major extracellular proteases (for a review see Ref. [30]). Intracellular serine proteases (ISP) with high structural similarities

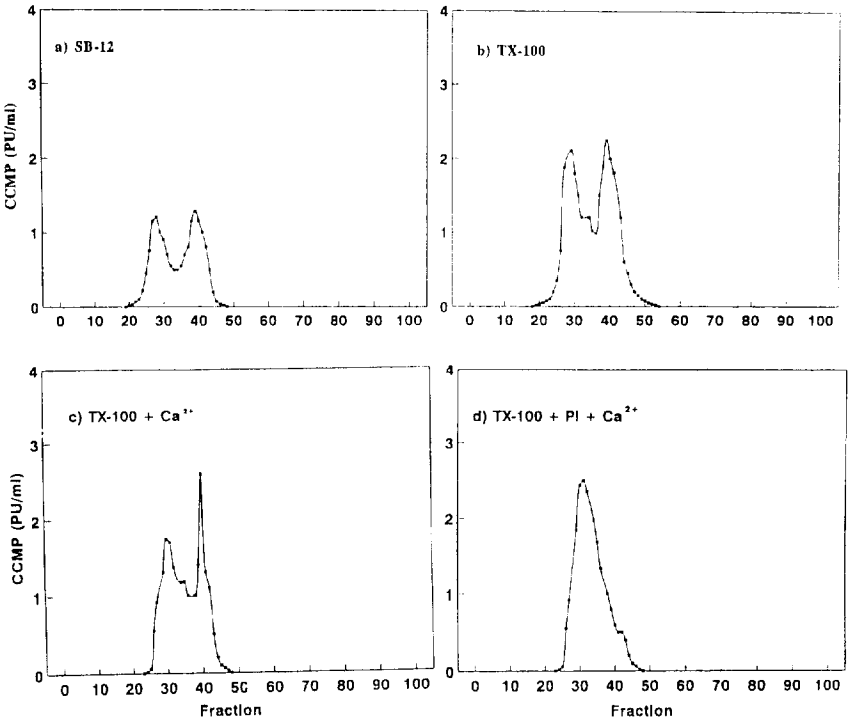

Fig. 6. Elution behaviour of the CCMP from a column of Sephacryl S-200 under the influence of different detergents, phospholipids and metal ions. The same amount of solubilizate was loaded onto the column (800 × 10 mm I.D.) in all instances, the elution was performed at a rate of 1 ml/20 min with a Tris–HCl buffer (pH 7.5, 0.05 *M*, 0.2 *M* NaCl) with the following additions: (a) elution with 0.2% SB-12, (b) elution with 0.1% Triton X-100, (c) elution with 0.1% Triton X-100 and 2 m*M* $CaCl_2$, (d) elution with 0.1% Triton X-100, 2 m*M* $CaCl_2$ and additionally 1 mg *B. cereus* lipids/ml buffer.

to subtilisins seem to play an important role in the sporulation process [31,32].

We could isolate a novel membrane proteinase called CCMP from *B. cereus* with unusually strong hydrophobic properties and a high tendency to self-aggregation. Strongin et al. [33] reported that the membrane proteinase isolated from *B. subtilis* is very similar to the cytoplasmic serine protease. Mäntsäla and Zalkin [34] extracted the cell membrane of *B. subtilis* YY88 with a very low detergent concentration (0.1% Triton X-100) and found that the greater part of the membrane proteinase activities corresponded to the extracellular serine and metalloproteases in their immunological reactivity and substrate specificity [34]. The only membrane proteinase which is distinct from all other reported *Bacillus* proteases in its special behaviour as an amphipathic protein, was purified from *B. subtilis* IFO 3027 [1]. This membrane-bound proteinase could only be solubilized by butanol extraction or a high Triton X-100 concentration in the presence of 1 *M* salt. In buffers of low ionic strength, the enzyme precipitated and could only be purified in the presence of detergent and 0.5 *M* sodium chloride. This solubilization behaviour is different from that of the CCMP from *B. cereus*; Triton X-100, even together with high salt concentrations, was not able to release the membrane proteinases from the cell envelopes (Tables 1, 4). Other differences are the different behaviour with class-specific inhibitors and the substrate specificity (Table 4).

There are several reasons why a protein may bind to the top of a column:

1. The protein is in a particulate state and therefore not able to pass the column matrix.

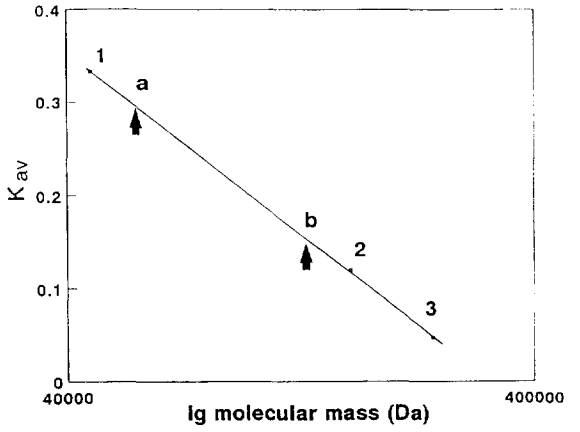

Fig. 7. Size-exclusion chromatography on Sephacryl S-200 (800 × 10 mm). Determination of the molecular mass of the CCMP in the solubilizate in comparison with calibration proteins (1: bovine serum albumin, 2: aldolase, 3: catalase) resulting in two molecular masses (a: 56 000, b: 128 000). Elution of the proteolytic activity was performed at a rate of 1 ml/20 min with a Tris–HCl buffer (pH 7.5, 0.05 *M*) containing 0.1% Triton X-100 and 0.2 *M* NaCl; calibration proteins were eluted with the same buffer, but without detergent addition.

2. A lack of phospholipids due to protein dilution during the chromatographic step causes the precipitation of the protein.
3. As a result of the interaction between protein and chromatographic matrix the conformation of the protein is changed and it binds to the chromatographic matrix.

Since repeated preparative ultracentrifugation of the solubilized enzyme did not result in enzyme sedimentation and a separation of the crude enzyme after solubilization by size-exclusion chromatography was also possible, the first possibility can be excluded. In contrast to the membrane proteinases from *B. cereus*, phospholipid-dependent membrane proteins react with rapid and often irreversible inactivation to a dilution of the solubilizate; for example the tetrodotoxin binding component from the electroplax of *Electrophorus electricus* is completely inactivated after a solubilizate dilution in as short

Table 4
Comparison between the CCMP from *B. cereus* and the membrane proteinase from *B. subtilis* IFO 3027 [1]

	CCMP from *B. cereus*	Membrane proteinase from *B. subtilis*
Molecular mass		
SDS–PAGE		62 000
Size exclusion		540 000
pH optimum	6.5–7.5	11
Inhibition by	Residual activity	Residual activity
PMSF (1 m*M*)	100%	0%
EDTA (5 m*M*)	0%	100%
Leupeptin (100 μg/ml)	100%	100%
Antipain (100 μg/ml)	100%	56%
Chymostatin (50 μg/ml)	100%	18%
Cleavage of Suc-(L-Ala)$_2$-pNA per 1 PU	no hydrolysis	$0.17 \cdot 10^{-3}$ μmol/min
Cleavage of Z-(L-Ala)$_2$-L-Leu-pNA per 1 PU	no hydrolysis	$54 \cdot 10^{-3}$ μmol/min
Solubilization by 1 *M* LiCl after Triton X-100 (2% w/v)	solubilized activity 8%	solubilized activity 63.4%

a time as just one hour at room temperature [17]. The transport activity of the branched-chain amino acid transport system of *Lactococcus lactis* was recovered only when solubilization was performed in the presence of acidic phospholipids [35]. Such phospholipid-dependent membrane proteins always need the presence of a minimal phospholipid content (a phospholipid mixture or one defined phospholipid) during each purification step [36–39] independent of their function as an enzyme or a membrane receptor or as a transport protein; often such phospholipid-dependent proteins are integral membrane proteins.

We discuss the strong aggregation of the membrane proteinases from *B. cereus* in ion-exchange chromatography as the binding of protein by ionic interactions and subsequent conformational changes with resulting strong hydrophobic interactions of the enzyme. TMAE-Fractogel is a tentacle gel with long hydrophilic polymer chains with repeating ion-exchange groups, and was chosen to reduce the interactions mentioned above. However, the enzyme showed the same behaviour as on ion-exchange gels without tentacles (DEAE-Sephacel, chromatofocusing ion-exchanger PBE 74). Addition of phospholipids could not reduce the aggregation of the proteinases on the TMAE-Fractogel. Isopropanol used for the elution of the bound CCMP from the anion-exchange column (Fig. 4), also serves in affinity chromatography to solve hydrophobic interactions between the affinity ligands and the bound enzyme. It is used, for example, in the affinity chromatography on bacitracin-sepharose to elute thermolysin [40,41] and in the affinity chromatography with peptidyl methyl ketones as ligands for the elution of papain and thermitase [42]. We postulate that isopropanol solves the hydrophobic interactions of the CCMP with the chromatographic matrix. Both proteinases are strong hydrophobic proteins, as was demonstrated by phase-separation experiments. After salt addition to solubilizates in non-ionic detergents, the membrane proteinases of *B. cereus* are concentrated in the detergent phase [21].

The purified CCMP tends to self-aggregation (Figs. 5, 6). Only after treatment with mercaptoethanol were two discrete protein bands detected in SDS–PAGE after silver-staining. In samples with untreated enzyme or enzyme boiled with SDS, no protein could be detected (Fig. 5).

ICMP was only detectable in column eluents after FPLC on TMAE-Fractogel in very low amounts. Cleavage of intact insulin is a property of only few proteinases–for example, IDE (insulin-destroying enzyme [43–45]); most proteinases are only able to cleave the single insulin chains. It is possible that the insulin cleavage in the membranes and in the solubilizate is the result of a common action of different enzymes or that ICMP is very labile in the purification process. Since pooling of the fractions after chromatography did not result in restoration of the insulin-destroying activity, and ICMP is very sensitive to detergents (Fig. 2) and organic solvents (Fig. 4), we will test various stabilizing agents for the purification of this enzyme and search for other purification possibilities, such as solvent–solvent extraction.

Abbreviations

BCA	bichinchinonic acid,
Bz	benzoyl,
Brij 35	dodecylpoly(oxyethyleneglycol ether)$_{23}$,
CCMP	casein-cleaving membrane proteinase,
CHAPSO	3-(3-cholamidopropyldimethylammonio)-2-hydroxy-propane sulfonate,
CMC	critical micelle concentration,
DEAE	diethylaminoethyl,
Fa	furylacroleyl,
Glp	pyroglutamyl,
ICMP	insulin cleaving membrane proteinase,
Mutanolysin	endo-N-acetyl muramidase from *Staphylococcus globisporus*,
Pefabloc	4-(2-aminoethyl)benzylsulfonyl fluoride,

pCMB	*para*-chloromercuri benzoate,
PMSF	phenylmethanesulfonyl fluoride,
pNA	*para*-nitranilide,
SDS	sodiumdodecyl sulfate,
Suc	succinyl,
Sulfobetain SB-12	N-dodecyl-N,N-trimethyl-3-ammonio-1-propanesulfonate,
TCA	trichloroacetic acid,
TMAE	trimethylaminoethyl,
Triton X-100	octylphenol-poly-(ethyleneglycol ether)$_{9-10}$,
Z	benzyloxycarbonyl.

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