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Isolation and Characterization of a Novel Extracellular Metalloprotease from *Bacillus subtilis*

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We have isolated and characterized two minor extracellular proteases from culture supernatants of a strain of *Bacillus subtilis* containing deletion mutations of the genes for the extracellular proteases subtilisin (*apr*) and neutral protease (*npr*) and a minor extracellular protease (*epr*) as well as intracellular serine protease-I (*isp-1*). Characterization studies have revealed that one of these enzymes is the previously described protease bacillopeptidase F. The second enzyme, the subject of this report, is a novel metalloprotease, which we designate Mpr. Mpr is a unique metalloprotease that has been purified to apparent homogeneity by using both conventional and high-performance liquid chromatography procedures. Mpr has a molecular mass of ~28 kilodaltons on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a basic isoelectric point of 8.7. The enzyme showed maximal activity against azocoll at pH 7.5 and 50°C. Mpr was inhibited by dithiothreitol and a combination of β-mercaptoethanol and EDTA. Activity was moderately inhibited by β-mercaptoethanol and EDTA alone as well as by cysteine and citrate and only marginally by phosphoramidon 1,10-phenanthroline and *N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine. Mpr was not inhibited by phenylmethylsulfonyl fluoride. In addition, Mpr showed esterolytic but not collagenolytic activities. Our studies suggest that Mpr is a secreted metalloprotease containing cysteine residues that are required for maximal activity.

Sporulation in *Bacillus subtilis* is a tightly regulated developmental process involving alterations in protein synthesis and intermediary metabolism (5, 6, 20). At the end of exponential growth and paralleling the onset of sporulation, there is increased production and secretion of extracellular proteases.

Two prominent proteases produced during the onset of sporulation are the alkaline serine protease or subtilisin (12, 22) and neutral (metallo-) protease (23–25). The combined activities of these two enzymes account for 96 to 98% of the total protease activity present in culture supernatants of wild-type sporulating cells (8). Efforts to clone and delete the genes coding for subtilisin (8, 21) and neutral protease (28) have made possible the study of minor proteases (1, 13, 14) that contribute to the residual activity present in double mutants of *B. subtilis* incapable of producing either protease. For example, work in our laboratory has identified and resulted in the cloning of a minor extracellular protease gene (*epr*; 17) from a strain of *B. subtilis* deleted for the subtilisin (*apr*) and neutral protease (*npr*) genes.

Utilizing a strain of *B. subtilis* in which null mutations had been created in the *apr*, *npr*, and *epr* genes, as well as in the gene *isp-1* that encodes the major intracellular serine protease ISP-I (9), we have been able to identify two distinct proteases that contribute to the observed proteolytic activity present in these cultures. Isolation and characterization of one of these enzymes indicated that it is the previously characterized bacillopeptidase F (14). The cloning, sequence analysis, and deletion of this gene are the subjects of another report (A. Sloma, G. A. Rufo, Jr., C. F. Rudolph, B. J. Sullivan, K. A. Theriault, and J. Pero, submitted for publication).

In this report, we discuss the isolation and characterization of Mpr, an extracellular metalloprotease from *B. subtilis* that has not been previously identified.

MATERIALS AND METHODS

Chemicals. Morpholinoethanesulfonic acid (MES), morpholinopropanesulfonic acid (MOPS), EDTA, phenylmethylsulfonyl fluoride (PMSF), cysteine, citrate, and 1,10-phenanthroline were purchased from Sigma Chemical Co. All electrophoresis chemicals and mercaptoethanol were purchased from Bio-Rad Laboratories. *N*-[*N*-(L-3-*trans*-Carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine (E-64), phosphoramidon, and 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg were from Boehringer Mannheim Biochemicals, and *N*-(2,4-dinitrophenyl)-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg was from Calbiochem-Behring.

Bacterial strains. *B. subtilis* GP227 (Δ*apr* Δ*npr* Δ*isp-1* Δ*epr metC amyE* [*sacQ**]) is derived from *B. subtilis* 168 strain IS75. *sacQ** (18) is a modified form of the *sacQ* gene (10), whose product enhances production of several extracellular enzymes in *Bacillus* spp., including neutral and alkaline proteases and levansucrase. The product of *sacQ** is more effective than that of *sacQ* in stimulating production of certain extracellular enzymes, including the residual proteases.

Purification scheme for Mpr. Cells were removed from culture supernatants of *B. subtilis* GP227 grown in MRS medium (Difco Laboratories) by centrifugation at 10,000 × *g* for 20 min by using a GS-3 (Ivan Sorvall, Inc.) rotor. The cell-free supernatant was then concentrated 5- to 10-fold by using a CH_2PR concentration system (Amicon Corp.) equipped with a S1Y10 spiral cartridge. In-place dialysis was performed against 50 mM MES, pH 6.8. The concentrated, dialyzed supernatant was fractionated over DEAE-Sephacel (7 by 23 cm) equilibrated with 50 mM MES, pH 6.8. The flowthrough fractions containing Mpr activity were pooled and adjusted to pH 7.5. The pool was then applied directly to

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carboxymethyl (CM)-Sepharose CL-6B (7 by 23 cm) equilibrated with 50 mM MOPS, pH 7.5. Mpr was eluted from the column in stepwise fashion by using 50 mM MOPS–0.5 M KCl, pH 7.5. The active pool was concentrated by using a stirred cell (Amicon) equipped with a YM5 membrane and dialyzed (Spectra/Por 4; Spectrum Medical Industries, Inc.) overnight against 50 mM MES, pH 6.8. The remaining steps in the purification scheme were carried out by using high-performance liquid chromatography (HPLC) techniques.

The CM pool was refractionated over a weak cation exchange column (4.6 by 250 mm; Genenchem, San Francisco, Calif.) equilibrated with 50 mM MES, pH 6.8. A linear gradient to 0.5 M NaCl was used to elute activity. Finally, the weak cation exchange pool containing Mpr was size fractionated over a TSK-125 gel filtration column (7.5 by 300 mm; Bio-Rad) equilibrated with 50 mM MES–0.4 M NaCl–5 mM $CaCl_2$ (pH 6.8). To obtain a highly purified preparation of Mpr, the active TSK pool was fractionated a second time over the same column. This pool was concentrated by using Centricon 10 (Amicon) and analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE. Electrophoresis was carried out by the methods of Laemmli (11). Slab gels (0.75 mm) containing 10% acrylamide with a 1.5-cm stacking gel consisting of 5% acrylamide were typically electrophoresed at 4 mA per gel overnight. Visualization of proteins was accomplished by using Fast Stain according to the instructions of the supplier (Zoion Research Inc., Allston, Mass.).

Molecular weight determination of Mpr. The protease molecular weight was determined by two methods: (i) SDS-PAGE, comparing its migration with those of standard low-molecular-weight proteins (Bio-Rad), and (ii) HPLC gel filtration, comparing the elution position with those of standard proteins (Bio-Rad gel filtration calibration kit).

Protease activity measurements. (i) Azocoll method. Protease activity was routinely determined by using azocoll (Sigma; Calbiochem) as a substrate. Tubes containing 10 mg of azocoll and appropriate volumes of 50 mM Tris hydrochloride–5 mM $CaCl_2$ (pH 8.0) were preincubated for 30 min at 55°C with constant shaking. Inhibitors were preincubated with the enzyme for 30 min at room temperature unless stated otherwise. After preincubation, designated amounts of enzyme and fresh inhibitor were added to the tubes containing the substrate and buffer. The reaction was carried out at 55°C for 1 h. A control without enzyme was run with each set of reactions. At the end of incubation, tubes were centrifuged to remove unhydrolyzed azocoll, and the A_{520} of the resulting supernatant was determined. Plots of absorbance versus the amount of protein were linear up to an absorbance reading of 2.0. One unit of activity was arbitrarily designated as the amount of protein which yields an A_{520} of 0.5. Standard assays on *B. subtilis* GP227 crude supernatants and early purification steps included 2 mM PMSF to eliminate interference by the serine protease(s).

(ii) Esterase method. Esterase activity was measured at room temperature at pH 8.0 by using *N-tert*-butoxy-carbonyl-L-glutamic acid-α-phenyl ester (Sigma) as the substrate, according to the method of Drapeau (3). One unit of activity was designated as 1 μmol of substrate hydrolyzed per min per μg of protein.

Protein determination. Protein was determined according to the standard Bio-Rad protein assay, as outlined by the supplier, by using gamma globulin as a standard.

Isoelectric point determination. The isoelectric point of Mpr was determined by using chromatofocusing. Purified Mpr was loaded onto a column (1 by 48 cm) of Polybuffer Exchanger 118 (Pharmacia) equilibrated with 25 mM triethylamine HCl, pH 10.5. The column was developed with 500 ml of a 1:45 dilution of Pharmalyte, pH 8 to 10.5, adjusted to pH 7.0 with HCl. Fractions were assayed for protease activity by using the azocoll method.

TABLE 1. Purification of Mpr from *B. subtilis* GP227 culture supernatant[a]

Step	Protein (mg)	Protease activity		Purification (fold)	% Recovery
		Specific (U/mg)[b]	Total (U)		
Concentrated dialyzed crude supernatant	15,840	3	47,520	1	100
DEAE-Sephacel	2,898	15	43,470	5	91
CM-Sepharose	108	46	4,968	15	10
HPLC weak cation exchange	0.350	16,667	5,833	5,747	13
HPLC TSK-1	0.084	20,000	1,680	6,897	4
HPLC TSK-2	0.080	20,000	1,640	6,897	4

[a] Details of each purification step and protease measurements are described in Materials and Methods.

[b] One unit was defined as the amount of protein which yields an A_{520} of 0.5 in the standard azocoll assay measured in the presence of 2 mM PMSF.

RESULTS

Purification of Mpr. The purification scheme for Mpr is summarized in Table 1. Azocoll assays were used routinely to monitor Mpr and were conducted in the presence of 2 mM PMSF to eliminate possible interference in the assay by the presence of bacillopeptidase F, a serine protease which is inhibited by PMSF. Fractionation of crude supernatants over DEAE-Sephacel gave a recovery of 95% of the applied activity while eliminating over 80% of the applied protein. Liquid chromatography over CM-Sepharose resulted in poor recoveries of activity; however, this step enhanced the specific activity of the pooled DEAE fractions by threefold and reduced or eliminated the amber coloration. In addition, CM-Sepharose was effective in removing any contaminating bacillopeptidase F present in the DEAE pool. Resolution of cation exchange chromatography was dramatically enhanced by employing an HPLC weak cation exchange column developed with a linear salt gradient. This particular step enhanced the specific activity of the CM pool over 360-fold while incurring no loss of activity. Purification of Mpr was completed by gel filtration, and a double fractionation over TSK-125 assured recovery of pure enzyme. As indicated in Table 1, Mpr was purified over 6,900-fold and was calculated to represent only 0.01% of the total protein in culture fluids of *B. subtilis* GP227.

Purity of the final product was assessed by gel filtration and SDS-PAGE. Fig. 1A is a typical chromatogram of the final Mpr pool eluted over the TSK-125 column. The 280-nm trace revealed no heterogeneity of the Mpr peak, which eluted at a position corresponding to ~39 kilodaltons (kDa) (Fig. 1B). SDS-PAGE (Fig. 2) confirmed the purity of Mpr, which was seen to migrate as a single 28-kDa species. Also shown is a sample of bacillopeptidase F, which was isolated from the same cultures as Mpr. The discrepancy in the determined molecular mass for Mpr between gel filtration and SDS-PAGE may have been the result of conformational differences between the native enzyme and the reduced, denatured form of the protein. The two methods of molecu-

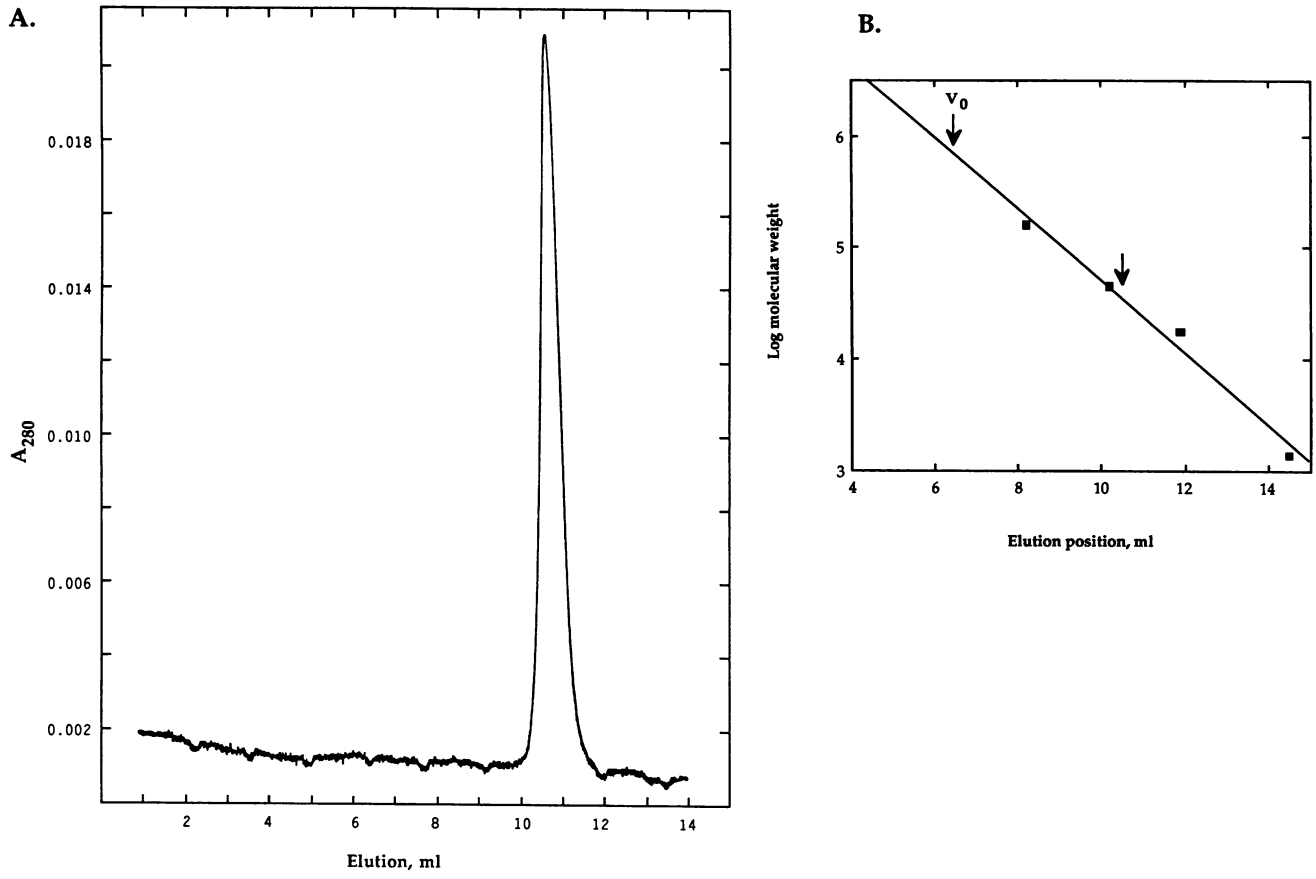

FIG. 1. (A) TSK-125 analysis of purified Mpr. Purified Mpr (4.5 μg) was chromatographed over a TSK-125 gel filtration column monitored at 280 nm (0.02 absorbance units, full scale) as described in Materials and Methods. (B) Comparison of the elution position of Mpr to molecular weight standards on a TSK-125 column. The unlabeled arrow indicates the relative position of Mpr. Standards: bovine gamma globulin, 158 kDa; ovalbumin, 44 kDa; myoglobulin, 17 kDa; vitamin B_{12}, 1.4 kDa. V_0, voided volume.

lar mass determination agreed closely enough, however, to suggest that Mpr exists as a single polypeptide chain devoid of quaternary structure.

Isoelectric point of Mpr. The isoelectric point for Mpr was determined by chromatofocusing to be 8.7. This value was consistent with the chromatographic data and the findings that Mpr failed to bind pellicular anion exchange resin (weak anion exchange HPLC resin) at pH 8.0 and showed no electrophoretic mobility in the absence of SDS and heat treatment (data not shown).

Temperature and pH optimum of Mpr. Purified Mpr in the standard azocoll assay was optimally active at 50°C (data not shown). The enzyme was moderately thermophilic (58% activity remaining at 70°C, 30% activity remaining at 85°C) and displayed little activity at ambient temperatures. The pH optimum of Mpr was 7.5 (data not shown), and activity was half-maximal at pH 9.5. Because little or no activity was detected at pH values below 5, Mpr probably does not belong to the acidophilic or aspartic acid protease family.

Inhibition pattern of Mpr. To categorize Mpr with regard to protease type, several compounds were tested as potential inhibitors of Mpr activity. Typically, the inhibitor studies were conducted by using the standard azocoll assay; however, when possible, the inhibitors were also tested using the *N-tert*-butoxy-carbonyl-L-glutamic acid-α-phenyl ester esterase assay. PMSF, up to concentrations as high as 10 mM, failed to inhibit Mpr esterase or protease activity; thus Mpr is not a serine protease (Table 2). This result allowed us to discriminate bacillopeptidase F (a PMSF-sensitive serine protease) from Mpr in cultures of *B. subtilis* GP227. EDTA was only marginally inhibitory in the esterase assay and moderately inhibitory in the azocoll assay, whereas dithiothreitol (DTT) was shown to effectively inhibit both activities of Mpr. Similarly, cysteine was shown to be inhibitory, albeit at elevated concentrations (data not shown). Citrate showed moderate inhibition, while compounds such as phosphoramidon 1,10-phenanthroline and E-64 were only marginally effective (data not shown).

Perhaps of greater significance were the results obtained with mercaptoethanol in the presence and absence of EDTA. While the mercaptan and EDTA were moderately inhibitory alone, EDTA in combination with mercaptoethanol produced an additive and significant inhibitory effect upon Mpr activity. Collectively, these data suggest that Mpr is a metalloprotease that displays unique structural properties that tend to favor association of the metal-enzyme complex even in the presence of metal chelating agents.

The inhibition of Mpr by DTT and cysteine and the relatively high activity of Mpr against azocoll (Mpr was 10-fold more active versus azocoll than was bacillopeptidase F; data not shown) suggested that Mpr could be a collagenaselike enzyme (15). Mpr was, therefore, tested for its ability to hydrolyze insoluble, undenatured collagen as well as two synthetic collagenase substrates, 4-phenylazobenzy-

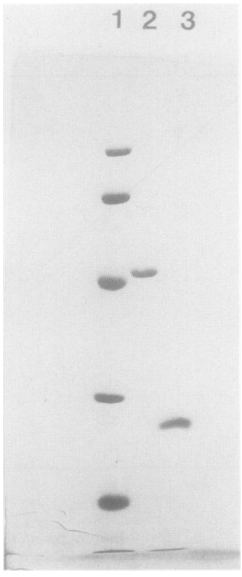

FIG. 2. SDS-PAGE analysis of Mpr. Details of the analysis are given in Materials and Methods. Lane 1, Molecular weight standards (phosphorylase *b* [92.5 kDa], bovine serum albumin [66.2 kDa], ovalbumin [45 kDa], carbonic anhydrase [31 kDa], and soybean trypsin inhibitor [21.5 kDa]); lane 2, 5 μg of bacillopeptidase F (47 kDa); lane 3, 6 μg of Mpr (28 kDa).

loxycarbonyl-Pro-Leu-Gly-Pro-D-Arg and *N*-(2,4-dinitrophenyl)-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg. In each case, Mpr failed to show any activity against these substrates (data not shown) and therefore is considered not to be a collagenase.

DISCUSSION

This report describes the isolation and characterization of a novel extracellular protease from a strain of *B. subtilis* (GP227) bearing null mutations in the protease genes *apr*, *npr*, *isp-1*, and *epr* (17). The apparent total extracellular protease activity (with azocoll as the substrate) present in supernatant fluids of cultures of *B. subtilis* GP227 can be ascribed to two distinct activities: those of bacillopeptidase F (14) and Mpr.

Ion exchange and gel filtration chromatography was used to obtain Mpr in its pure form as judged by a constant specific activity (Table 1) and SDS-PAGE analysis (Fig. 2). Mpr is a single polypeptide species of ~28 kDa having a basic pI (8.6 to 8.8).

TABLE 2. Inhibition pattern of purified Mpr[a]

Inhibitor	Concn (mM)	% Inhibition	
		Esterase	Azocoll
None		0	0
PMSF	2	0	0
	5		0
	10	0	0
DTT	1	85	94
	2	85	96
	5	92	90
Mercaptoethanol	10		45
	20		49
EDTA	25		45
	50	16	
Mercaptoethanol + EDTA	10		
	25		91

[a] 1.5 U of purified Mpr was assayed in duplicate by using either the esterase or the azocoll assay, as described in Materials and Methods.

Endoproteases generally fall into four major categories: the serine proteases, which require an essential serine residue for activity and are thus inhibited by hydroxyl-reactive organofluorides such as diisopropylfluorophosphate and PMSF; the acid or aspartic proteases, which typically exhibit unusually low pH optima; the cysteine or sulfhydryl proteases, which are stimulated by low levels of thiol compounds (i.e., DTT and cysteine); and the metalloproteases, which are usually inhibited by divalent metal ion chelators such as EDTA and 1,10-phenanthroline (26). Mpr cannot be an aspartic protease because it has a pH optimum of 7.5. Likewise, the insensitivity of Mpr to PMSF inhibition suggests that our purified protein is not a serine protease. Inhibition rather than stimulation of Mpr by DTT and by mercaptoethanol indicates that Mpr is probably not a cysteine protease. Furthermore, mercaptans such as DTT can inhibit metalloproteases by forming covalent bonds with metals (2, 7). Inhibition of Mpr by DTT suggests that Mpr is a metalloprotease rather than a cysteine protease. The moderate inhibition of Mpr activity by EDTA alone and the marginal inhibition by 1,10-phenanthroline suggest that the metal-binding site on the enzyme has a sufficiently high stability constant to render the metal inaccessible to chelating agents. It is noteworthy that others have reported examples of chelating agents being unable to remove the metal ion from some metalloenzymes (4, 27).

We have recently cloned and sequenced the gene that encodes Mpr (19). Interestingly, DNA sequence information reveals the existence of four cysteine residues in Mpr that are spaced exactly the same as disulfide bond-linked cysteine residue pairs 30–46 and 184–214 in porcine elastase (EC 3.4.21.11) (16). It is interesting to speculate that the inhibition of Mpr activity by DTT is a result of the ability of the mercaptan to reduce, and thus disrupt, disulfide-linked cysteine residues while simultaneously chelating the conformation-protected metal ligand. Support for this speculation is given by the data showing marginal inhibition by EDTA alone but quantitative inhibition by the chelator in the presence of the disulfide-reducing agent mercaptoethanol. Future studies are being designed to evaluate possible involvement of disulfide bridges in the structural and catalytic integrity of Mpr.

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