Characterization of the "Microprotease" from *Bacillus cereus*. A Zinc Neutral Endoprotease†

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ABSTRACT: The neutral protease isolated from *Bacillus cereus* (BRL-70) has been purified by affinity chromatography and characterized. The enzyme exhibits a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, has a molecular weight of 34 000 by ultracentrifugation, and contains one enzymatically essential zinc atom per 34 000 g. These data together with the amino acid composition, response to metal substitution, chemical modification, and substrate specificity all indicate that this protease is monomeric and is a typical bacterial neutral metalloprotease.

Proteases of microbial origin are commonly classified based on the presumed nature of active site residues, i.e., seryl, thiol, metal, or acid proteases (Morihara, 1974). A number of the metal-dependent neutral endoproteases have been purified from various bacteria and identified as zinc metalloenzymes. Examples include thermolysin from *B. thermoproteolyticus* (Latt et al., 1969), the *B. subtilis* neutral protease (McConn et al., 1964; Keay, 1969), and a neutral protease of *B. cereus* strain NCTC 945 (Feder et al., 1971), among others. In addition to the functional zinc atom, this class of enzymes frequently requires structural Ca^{2+} atoms.

Schenk and Bjorksten (1974) have recently isolated an endoprotease from *B. cereus* and reported that the enzyme is composed of subunits as small as 2700 daltons and that calcium is necessary for stability. Such a low subunit molecular weight is most unusual and would present novel experimental opportunities. Hence, we have purified the commercially available material to homogeneity and subjected it to extensive characterization. However, based on ultracentrifugation, electrophoretic behavior, amino acid composition, metal content, specificity, and response to specific chemical modification, we find this enzyme to be a monomer with a molecular weight of 34 000 with characteristics typical of other microbial zinc neutral endoproteases.

Methods and Materials

"Microprotease," isolated from *B. cereus* strain BRL-70 (lot x5N016), was obtained from Worthington Biochemicals. L-Phenylalanyl-L-phenylalanine methyl ester hydrochloride (mp 198–199 °C from methanol) was prepared by standard procedures involving coupling *Z*-L-Phe to L-phenylalanine methyl ester using DCC[1] followed by removal of the carbobenzoxy group by catalytic hydrogenation on 10% Pd/carbon in methanol at atmospheric pressure. *N*-Diphenylphosphoryl-L-phenylalanyl-L-phenylalanine methyl ester was prepared by the procedure of Zervas and Katsoyannis (1955). Thus, to 0.8 g (2.5 mmol) of L-phenylalanyl-L-phenylalanine methyl ester hydrochloride dissolved in 10 mL of pyridine at 0 °C was added 0.75 g (2.8 mmol) of diphenyl phosphorochloridate (Aldrich). After 2 h, the reaction was poured into ice water. The water suspension was adjusted to pH 1 and extracted with ethyl acetate. The ethyl acetate was washed twice with 1 M HCl, then with water, dried over magnesium sulfate, and evaporated to an oil. The oil was dissolved in ethanol, 10 mL, to which water was added to the cloud point. Upon cooling the product crystallized to give 0.4 g of white needles, mp 106–107 °C. This phosphorylated peptide does not inhibit "microprotease" activity nor the activity of the related neutral protease, thermolysin. However, hydrolysis of the peptide in base for 45 min in 75% dioxane, conditions which remove a single phenyl group from the phosphate ester (Zervas and Katsoyannis, 1955), as well as the methyl ester, affords a very potent inhibitor of both enzymes. This product, *N*-monophenylphosphoryl-L-phenylalanyl-L-phenylalanine, was used as a ligand for the affinity chromatography resin. The resin was prepared by dissolving 56 mg of *N*-diphenylphosphoryl-L-phenylalanyl-L-phenylalanine methyl ester in 15 mL of dioxane to which was added 5 mL of 1 M sodium hydroxide. After 45 min at room temperature, the reaction was adjusted to pH 5.6 with 1 M HCl and the mixture added to the 25 mL of packed Affi-Gel 102 gel (Bio-Rad Laboratories) previously washed with 4 volumes of water. Immediately, 110 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride dissolved in 1 mL of water was added. The pH of the reaction was maintained between 5.4 and 6.0 and allowed to proceed for 48 h at room temperature. The resulting resin, washed and equilibrated with 50 mM Tris–10 mM $CaCl_2$–0.1 M NaCl (pH 7.0), was packed in a 1 × 15 cm column. A sample of enzyme (53 mg) was dissolved in 1 mL of the same buffer and applied to the column. After washing with approximately 10 column volumes of starting buffer, the pH was raised to 9.0 (50 mM ammediol–10 mM $CaCl_2$–0.1 M NaCl) and the activity eluted as a symmetrical peak. In all subsequent studies, the peak active fractions were used.

Activity was measured spectrophotometrically at 338 nm using 0.2 mM 2-furanacryloylglycyl-L-leucylamide (FAGLA) at pH 7.5 in 50 mM Tris–10 mM $CaCl_2$–0.1 M NaCl at 25 °C. Reactions were first-order for at least 3 half-lives both at 1 mM substrate monitored at 345 nm, and 0.2 mM substrate indicating that the first-order rate constant k_{obsd} obtained at 0.2 mM substrate is a measure of $k_{cat}[Eo]/K_M$. Preparation of other 2-furanacryloyl blocked peptides used as substrates has been described (Blumberg and Vallee, 1975) and their hy-

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[1] Abbreviations used: FA, 2-furanacrylic acid; FAGLA, 2-furanacryloylglycyl-L-leucylamide; ammediol, 2-amino-2-methyl-1,3-propanediol; DCC, dicyclohexylcarbodiimide; Tris, tris(hydroxymethyl)-aminomethane.

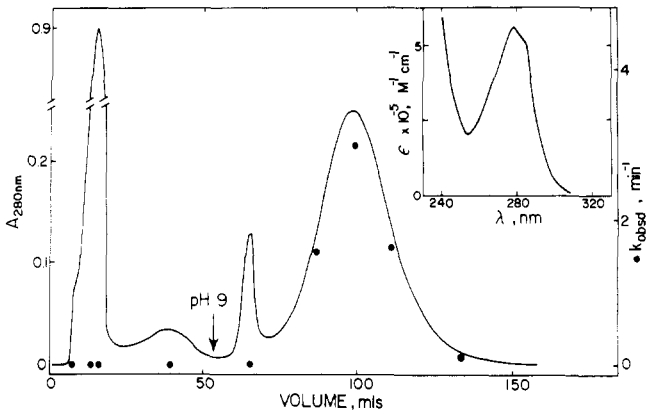

FIGURE 1: Purification of "microprotease" by affinity chromatography on *N*-phenylphosphoryl-Phe-Phe-agarose. Commercial crude enzyme powder (53 mg) was dissolved in 1 mL of 50 mM Tris, pH 7.0, containing 10 mM Ca^{2+} and applied to the column (1 × 15 cm). Elution was initiated with the same buffer and 2-mL aliquots were collected. At the point indicated, the pH of the eluting buffer was raised to 9.0. Activity at selected points of the chromatogram (●) was measured using FAGLA as described under Materials and Methods. Inset: Absorption spectrum of the purified enzyme obtained from the peak fraction of the affinity column.

drolyses were followed in an identical manner with FAGLA, except where noted.

Polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate was performed by the method of Weber et al. (1972). The gels used contained 10% acrylamide and were stained with Coomassie blue.

Amino acid analyses were performed with a Durrum Model D-500 amino acid analyzer. Tryptophan was determined by magnetic circular dichroism (Holmquist and Vallee, 1973). Cysteine was determined as cysteic acid after performic acid oxidation (Bailey, 1962). High speed sedimentation equilibrium experiments were carried out with a Spinco Model E ultracentrifuge in 50 mM Tris–10 mM $CaCl_2$–0.1 M NaCl, pH 7.5. Zinc was determined by atomic absorption spectrometry (Fuwa and Vallee, 1963). Precautions were taken to avoid contamination with adventitious metals (Thiers, 1975). Deionized distilled water was used throughout.

Zinc-free enzyme was prepared by dialysis against *o*-phenanthroline under conditions used previously for thermolysin (Holmquist and Vallee, 1974); it was reconstituted with spectroscopically pure metal salts (Johnson-Matthey). Samples subjected to polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate (Weber et al., 1972) were first incubated in *o*-phenanthroline (2 mM), followed by rapidly mixing with boiling 0.1% sodium dodecyl sulfate also containing *o*-phenanthroline (2 mM). Boiling was continued for 5 min before application to the gel.

Acylation with the *N*-hydroxysuccinimide ester of *N*-acetyl-L-phenylalanine was carried out under conditions previously employed to superactivate thermolysin (Blumberg and Vallee, 1975). The substrate 2-furanacryloylglycyl-L-alanyl-L-leucine (0.2 mM) was used to monitor the activation process.

Results

The commercial protease from *B. cereus* was purified by affinity chromatography on ω-aminoalkylagarose gel (Affi-Gel 102) to which was coupled *N*-phenylphosphoryl-L-phenylalanyl-L-phenylalanine. This column is similar to that of Komiyama et al. (1975), who used the natural endoprotease inhibitor, phosphoramidon, as a ligand in the purification of metalloendopeptidases. The synthetic ligand employed here, *N*-phenylphosphoryl-L-Phe-L-Phe, exhibits a K_i of $\sim 10^{-6}$ M toward the protease and proved to be excellent for the purification of the enzyme.

TABLE I: Amino Acid Composition of *B. Cereus* (BRL-70) Protease.

Amino acid	*B. cereus* protease: Analysis[a]	Nearest integer	*B. megaterium*[b] M A protease	Thermolysin[c]
Asp	48.3	48	44	44
Thr[d]	29.1	29	30	25
Ser[d]	25.1	25	30	26
Glu	18.85	18	22	21
Pro	6.10	6	13	8
Gly	37.3	37	39	36
Ala	33.5	34	32	28
Val	19.50	20	19	22
Met	2.27	2	2	2
Ile	15.30	15	12	18
Leu	18.75	19	21	16
Tyr	23.5	24	23	28
Phe	11.05	11	8	10
His	7.20	7	7	8
Lys	13.80	14	15	11
Arg	8.75	9	9	10
Cys[e]	0.20	0	0	0
Trp[f]	2.87	3		3
Total		321	326	316
MW (Calcd)		34 163		34 400

[a] Molar quantities based on quantitative analysis using an ϵ_{278} of 56 000 M^{-1} cm^{-1}. Average of duplicate 48-h hydrolyses. [b] Data of Keay et al. (1971). [c] Based on amino acid sequence (Titani et al., 1972). [d] Extrapolated to zero time for from 24- and 48-h hydrolyses. [e] Determined as cysteic acid (Bailey, 1962). [f] Determined by magnetic circular dichroism (Holmquist and Vallee, 1973).

A single active fraction emerged after adjusting the eluting buffer to pH 9 (Figure 1). The specific activity of the peak fractions, based on ϵ_{278} = 56 000 M^{-1} cm^{-1} (see below), is 46 × 10^3 M^{-1} s^{-1} and is constant throughout the fractions exhibiting enzymatic activity. Based on the A_{278} and activity, a twofold purification was achieved resulting primarily from removal of the large 280-nm absorbing peak eluting with the void volume of the column.

The absorption spectrum of the purified protein (Figure 1) is typical of a nonconjugated protein containing Trp and Tyr. An A_{280}/A_{260} of 2.5 indicates the absence of significant amounts of nucleic acids.

Yphantis high-speed sedimentation equilibrium centrifugation gave a linear plot of ln *c* vs. r^2 indicating homogeneity and yielding a molecular weight of 33 980. A value of $A_{278}^{1\%}$ of 14.6 was obtained based on refractive index using a synthetic boundary forming cell in the Spinco Model E ultracentrifuge.

The zinc content of the protease measured in triplicate by atomic absorption on two different preparations obtained by affinity chromatography gave an average value of 1.00 ± 0.08 g-atom per mol of enzyme using a molar absorptivity of 56 000 M^{-1} cm^{-1} at 278 nm. This value of ϵ_{278} is close to that obtained by ultracentrifugation, 49 600 M^{-1} cm^{-1}, and was used for determinations of protein concentration.

Polyacrylamide gel electrophoresis on slab gels in the presence of sodium dodecyl sulfate and 2-mercaptoethanol under standard conditions (Weber et al., 1972) was unsatisfactory as a criterion of molecular weight or homogeneity. Although one major band was observed near molecular weight 35 000 up to eight additional bands, presumably products of

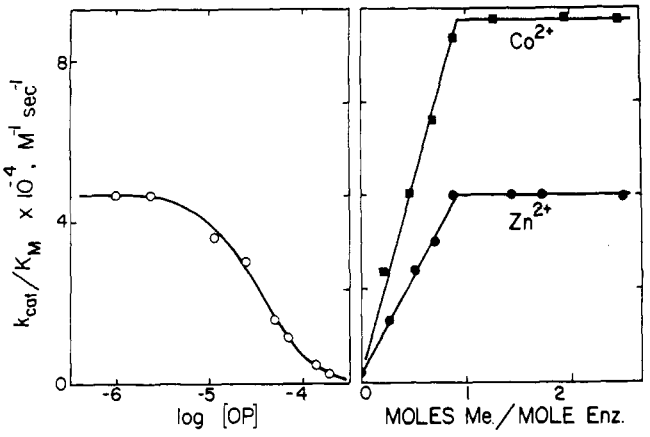

FIGURE 2: (Left): Inhibition of protease activity by *o*-phenanthroline (OP). Aliquots of enzyme, 25 μL, were added to substrate (0.2 mM FAGLA at pH 7.5, 50 mM Tris–10 mM Ca^{2+}–0.1 M NaCl) containing the indicated concentrations of *o*-phenanthroline. (Right): Addition of Zn^{2+} (●) and Co^{2+} (■) to zinc-free protease prepared by dialysis of the native enzyme against *o*-phenanthroline to remove zinc. Assays were initiated by adding the zinc-free enzyme to buffered substrate. After assurance of less than 5% residual activity, as indicated by the initial recorder slope, metal as the sulfate salt in water was added and the rate of hydrolysis monitored. Metal recombination was instantaneous, i.e., less than 5 s, as indicated by first-order behavior over the entire time course of hydrolysis.

autolysis, appeared in significant amounts at positions reflecting lower molecular weights. When the inhibitor, *o*-phenanthroline, was incorporated into the sample preparation steps prior to application to the gel, autolysis was prevented; under these conditions only a single band was observed corresponding to a molecular weight of approximately 35 000.

The amino acid composition of the protease is similar to that of two other zinc-containing bacterial neutral proteases which are shown for comparison (Table I). Typical of the microbial zinc neutral endoproteases (Morihara, 1974) that from *B. cereus* does not contain cysteine and its Met and Trp content is identical with that of thermolysin.

The zinc atom is essential for activity. The chelating agent *o*-phenanthroline completely inhibits activity with a pK_i of 4.5 (Figure 2). Dialysis of the protein against *o*-phenanthroline removes zinc to a value of less than 0.01 g-atom per mol and the resulting apoenzyme exhibits less than 1% activity. However, readdition of stoichiometric amounts of zinc restores full activity (Figure 2). Similarly Co^{2+} (Figure 2) and Mn^{2+} restore activity to 200% and 20% of the native enzyme, respectively.

In order to explore some aspects of specificity, a number of peptides structurally related to FAGLA, the substrate commonly utilized to assay the activity of zinc neutral proteases, were examined (Table II). In all cases the endo-amide bond is cleaved to yield a dipeptide as one of the products, as indicated by the spectral changes accompanying hydrolysis. This enzyme, like other bacterial zinc neutral proteases, clearly prefers hydrophobic amino acids at the scissile bond. This is especially evident when Leu is at the position donating the amino group. Thus, this preference closely parallels that of thermolysin (Table II) as well as that of other neutral proteases (Morihara, 1974).

Upon acylation by *N*-hydroxysuccinimide esters of amino acids and peptides, the proteolytic activity of at least four zinc neutral endoproteases from bacteria increases when expressed as k_{cat}/K_M (Holmquist et al., 1976). Under conditions previously employed for other neutral proteases, treatment of the protease with the *N*-hydroxysuccinimide ester of *N*-acetyl-L-phenylalanine increases its activity toward FA-Gly-Ala-Leu 22-fold, i.e., from 2.25 to 49 × 10^3 M^{-1} s^{-1}. Addition of NH_2OH (1 M, pH 8) to the resultant activated enzyme restores native activity, consistent with deacylation and removal of the *N*-acetyl-L-phenylalanyl group. This behavior is analogous to the results of previous studies with other zinc neutral proteases (Holmquist et al., 1976).

TABLE II: Substrate Specificity of the *B. cereus* (BRL-70) Protease.[a]

Substrate	$k_{cat}/K_M \times 10^{-3}$ (M^{-1} s^{-1})	
	B. cereus protease	Thermolysin
FA-Phe-Gly-Gly	0.23	0.28
FA-Gly-Ala-Gly	0.51	0.13
FA-Gly-Ala-Leu	2.25	6.5
FA-Gly-Leu-NH_2	46	22
FA-Ala-Ala-Ala	75	55
FA-Gly-Leu-Gly	126	83
FA-Phe-Leu-Gly[b]	2010	2300
FA-Gly-Leu-Ala	2070	879

[a] Measured at pH 7.5 in 50 mM Tris–10 mM $CaCl_2$–0.1 M NaCl at 25 °C at a substrate concentration of 0.2 mM. [b] Substrate concentration was 0.02 mM and hydrolysis was monitored at 335 nm.

Discussion

The protease isolated from *B. cereus*, strain BRL-70, known by the trivial name "microprotease" (Schenk and Bjorksten, 1974), is in fact a zinc-containing neutral endoprotease similar to such enzymes as those from *B. thermoproteolyticus* (Latt et al., 1969), *B. subtilis* (McConn et al., 1964), and *B. megaterium* (Keay et al., 1971), among others. Characteristically the molecular weights of this class of protease vary from 30 000 to 40 000; they are monomeric, contain calcium to stabilize structure and zinc for catalytic activity, are inhibited by *o*-phenanthroline, lack cysteine, and prefer hydrophobic amino acids adjacent to the site of substrate hydrolysis. Additionally, acylation with amino acid *N*-hydroxysuccinimide esters potentiates their catalytic activity. The present results indicate that this protease shares all of these features and, thus, is the analogous enzyme from *B. cereus*, strain BRL-70.

The name "microprotease" for this neutral protease stems from initial reports of its isolation (Schenk and Bjorksten, 1974). Evidence based on ultrafiltration, gel chromatography and sodium dodecyl sulfate gel electrophoresis suggested that the protein is an oligomer consisting of subunits as small as 2700 daltons, a deduction which the present data do not confirm.

A molecular weight near 35 000 was found consistently by ultracentrifugation, quantitative amino acid analysis, stoichiometry of zinc content, and by gel electrophoresis in the presence of sodium dodecyl sulfate. Based on this last method, initial studies indicated many low molecular weight species under standard conditions (Weber et al., 1971, method 1). However, when precautions were taken to prevent autolysis, i.e., the addition of *o*-phenanthroline to the protein prior to electrophoresis, such heterogeneity became minimal. Autocatalytic digestion has been cited in the past as a source of anomalous molecular weights of proteolytic enzymes based on gel filtration experiments (Voordouw et al., 1974). It is not surprising that this highly active protease should undergo autolysis in sodium dodecyl sulfate. In fact, Cleveland et al. (1977) have suggested proteolysis in the presence of sodium dodecyl sulfate as a useful technique for peptide mapping.

A zinc neutral protease has been isolated from *B. cereus*

NCTC 945 cultures by Feder et al. (1971). Based on the zinc content, the molecular weight was thought to be 63 000, though the molecular weight was not determined. The zinc-free enzyme was inactive and Zn^{2+}, Co^{2+}, and Mn^{2+} restored 100, 230, and 26% of native activity, respectively. Its specific activity is twice that of the "microprotease" when the two are assayed under identical conditions. Such similarities minimally suggest a close relationship and optimally perhaps identity of the two proteins.

Thus, the *B. cereus* protease isolated from strain BRL-70 (Worthington Biochemical) is a monomeric protein of molecular weight 34 000, and there is no evidence of a polymeric structure. It is a zinc metalloenzyme with properties characteristic of bacterial neutral endoproteases. Hence, it is suggested that future reference to this protein should avoid the misnomer "microprotease." The convenient source and ease of purification of this protease described here provide an additional system to explore energy transfer measurements between metals at specific protein sites (Horrocks et al., 1975). Studies along these lines including Tb^{3+} incorporation and spectral studies of cobalt enzyme are in progress.

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Pulse Fluorimetry Study of Octopine Dehydrogenase–Reduced Nicotinamide Adenine Dinucleotide Complexes†

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ABSTRACT: We measured the transient fluorescence of NADH bound to octopine dehydrogenase in the binary octopine dehydrogenase–NADH complex and in the ternary complexes containing D-octopine, L-allooctopine, L-arginine, D-arginine, or 5-guanidinovaleric acid. The fluorescence decay in all these complexes is biexponential. This is explained by the presence of several conformations of the single NADH binding site. In addition, transient anisotropy measurements show that the nicotinamide moiety is rigidly bound to the enzyme.

Octopine dehydrogenase from muscles of *Pecten maximus* L. catalyzes reversibly the dehydrogenation of D-octopine to give L-arginine and pyruvate according to Scheme I (Thoai and Robin, 1961; Thoai et al., 1969). The enzyme is a monomer of molecular weight 38 000, with a single polypeptide chain and a single active site (Huc et al., 1971; Olomucki et al., 1972; Thomé-Beau and Olomucki, 1973).

The results of kinetic studies are consistent with a bi-ter sequential mechanism, in which NAD^+ [1] binds first to the enzyme followed by D-octopine and the products are released in the order L-arginine, pyruvate, and NADH (Doublet and Olomucki, 1975). The activity of ODH seems to be submitted to two types of regulation: a regulatory mechanism of memory

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[1] Abbreviations used are: ODH, octopine dehydrogenase; GDH, beef liver glutamate dehydrogenase; LADH, liver alcohol dehydrogenase; TPB, 1,1,4,4-tetraphenyl-1,3-butadiene; DTT, dithiothreitol; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; NADPH, NADH phosphate; EDTA, (ethylenedinitrilo)tetraacetic acid.