High-Molecular-Mass Multicatalytic Proteinase Complexes Produced by the Nitrogen-Fixing Actinomycete Frankia Strain BR

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A major-high-molecular mass proteinase and seven latent minor proteinases were found in cell extracts and in concentrates of culture medium from Frankia sp. strain BR after nondenaturing electrophoresis in mixed gelatin-polyacrylamide gels. All of these complexes showed multicatalytic properties. Their molecular masses and their sedimentation coefficients varied from 1,300 kDa (28S) to 270 kDa (12S). The electroeluted 1,300-kDa proteinase complex dissociated into 11 low-molecular-mass proteinases (40 to 19 kDa) after sodium dodecyl sulfate activation at 30°C and electrophoresis under denaturing conditions. All of these electroeluted proteinases hydrolyzed N-carbobenzoxy-Pro-Ala-Gly-Pro-4-methoxy-1-naphthylamide, N-Val-Leu-Arg-4-methoxy-β-naphthylamide, N-Val-Val-Pro-Arg-4-methyl-7-coumarylamide, and Boc-Val-Pro-Arg-4-methyl-7-coumarylamide, whereas Boc-Leu-Leu-Val-Tyr-4-methyl-7-coumarylamide was cleaved only by the six lower-molecular-mass proteinases (27.5 to 19 kDa). Examination by electron microscopy of uranyl acetate-stained, electroeluted 1,300- and 650-kDa intracellular and extracellular proteinase complexes showed ring-shaped and cylindrical particles (10 to 11 nm in diameter, 15 to 16 nm long) similar to those of eukaryotic prosomes and proteasomes. Polyclonal antibodies raised against rat skeletal muscle proteasomes cross-reacted with all of the high-molecular-mass proteinase complexes and, after denaturation of the electroeluted 1,300-kDa band, with polypeptides of 35 to 38, 65, and 90 kDa. Electrophoresis of the activated cell extracts under denaturing conditions revealed 11 to 17 gelatinases from 40 to 19 kDa, including the 11 proteinases of the 1,300-kDa proteinase complex. The inhibition pattern of these proteinases is complex. Thiol-reactive compounds and 1-10-phenanthroline strongly inhibited all of the proteinases, but inhibitors against serine-type proteinases were also effective for most of them.

Frankia actinomycetes fix atmospheric nitrogen in vitro or in symbiotic association with the root systems of a variety of nonleguminous plants denominated actinorhizal plants (4). Under the static conditions currently employed, this filamentous bacterium grows slowly and nonexponentially. The cultures are morphologically and biochemically heterogeneous (3), and the growth curves show irregularities suggesting cycles of protein synthesis and protein degradation (9). We recently succeeded in growing Frankia sp. strain BR and others exponentially under appropriate conditions with stirring (37). In these optimal conditions, cells grew as homogeneous microcolonies without irregularities during exponential growth, but a proteolytic phase was always observed starting shortly after growth arrest. Because of this and because of the general importance of proteinases in eukaryotic and prokaryotic cells (5, 33), we have undertaken a general survey of the proteolytic system in Frankia sp. strain BR.

Information on Frankia proteases is scarce. Only a global proteolytic activity (19) and a single intracellular aminopeptidase (17) have been reported for some strains. In a previous study, we identified six intracellular aminopeptidase activities that are sensitive to NaCl, CoSO₄, and ZnSO₄ salts (2). These aminopeptidases are secreted during the exponential growth phase in stirred cultures (27). In this paper we report the existence and some characteristics of a variety of Frankia proteinases, including a family of high-molecular-mass multicatalytic proteinase complexes (HMPC) detected in both intracellular and extracellular spaces. Our results indicate that the 28S HMPC shows similarities with megapain (20) or ubiquitin-conjugate-degrading enzyme (UCDEN) (46) complexes, whereas the 19S HMPC shows features with the 19S to 20S eukaryotic and prokaryotic proteasomes (6, 30, 32, 39), which are supposed to play an important role in protein turnover and also to regulate the translation of mRNA (prosomes) in eukaryotic cells (35).

MATERIALS AND METHODS

Bacterial strain and culture conditions. Frankia sp. strain BR (ORS 020608), isolated from the root nodules of Casuarina equisetifolia (27), was used in this study. Bacteria were grown at 28°C in BAP-PCM (37), a mineral medium containing NH₄Cl and propionate as the only nitrogen and carbon sources, buffered with 10 mM morpholineethanesulfonic acid-Tris buffer (pH 6.8) and supplemented with soybean phosphatidylcholine (2 mg/liter). Frankia hyphae were stored in liquid QMod medium (24) or routinely subcultured every 3 days in magnetically stirred cultures (37). Cells were collected by centrifugation (1,700 × g, 15 min, 4°C), resuspended in BAP-PCM medium, homogenized by repetitive passages through a 0.7-mm needle, and used as an inoculum at a final concentration of 1 μg of protein (Bradford microassay as recommended by BioRad) per ml of culture medium.

Preparation of cellular extracts and extracellular concentrates. Cells from 5-day-old stirred cultures were harvested by centrifugation (1,700 × g, 15 min, 4°C) and washed twice in cold lysis buffer (20 mM Tris-HCl [pH 7.5], 100 mM KCl, 1 mM dithiothreitol [DTT], 10% glycerol). The pelleted cells...
were sonicated 10 consecutive times over a CaCl$_2$-ice mixture ($-15^\circ$C) at 20 W for 1 min. Cell extracts were obtained after ultracentrifugation at 150,000 $\times$ g for 30 min (4°C). Extracellular concentrates from 3-day-old cell-free medium were obtained by a rapid DEAE adsorption, salt elution, and Bio-Gel P2DG desalination method as described previously (27). The protein concentrations in cell extracts and extracellular concentrates were determined using a biuret-chinonic acid assay as recommended by the manufacturer (Pierce Chemical Co.) with pure crystalline bovine serum albumin (BSA) as a standard.

**Determination of latent low-molecular mass PF activities by cold or SDS activation.** Fresh cell extracts exhibited only weak proteolytic activity when assayed against azocoll, azocasein, and various synthetic substrates. Also, low-molecular mass Frankia proteins (PF) were detected after sodium dodecyl sulfate (SDS)-gelatin-polyacrylamide gel electrophoresis (PAGE). However, after the cell extracts were stored at $-80^\circ$C for 2 months (cold activation), proteolytic activity was clearly measurable and a variety of PF were revealed. Proteins in fresh cell extracts were also rapidly activated by incubating them (3 mg of protein per ml at 30°C for 10 min) in 2% SDS–20 mM Tris-HCl (pH 7.5)–100 mM NaCl (SDS activation). Then the mixture was poured into a G-25 fine Sephadex column built in a 1-ml plastic syringe. The resin was preequilibrated in lysis buffer overnight at room temperature and packed in the column by centrifugation (200 $\times$ g, 5 min, 25°C; RC2B, swinging-bucket HS4 rotor). Samples in lysis buffer were recovered by centrifugation as described above, cooled over ice, and used immediately for electrophoresis.

**Electrophoretic procedures and in-gel inhibition tests.** Vertical slab SDS-gelatin-PAGE was carried out as described previously (27) with 280- by 140- by 1.5-mm gels. Before loading, samples were incubated at 37°C for 15 min with denaturing buffer without $\beta$-mercaptoethanol or DTT (2.5% SDS, 1% sucrose, 10% glycerol, 0.5% [vol/vol] saturated bromophenol blue). Electrophoresis under nondenaturing conditions was performed at 10 mA (constant current) for 40 h with samples containing 200 $\mu$g of protein from fresh cell extracts or 100 $\mu$g of protein from extracellular concentrates. Resolving and stacking gels contained 5% acrylamide (0.33% bisacrylamide) and 3.5% acrylamide (0.23% bisacrylamide), respectively.

Irreversible inhibitors were tested by preincubating the cold-activated cell extracts for 30 min at 30°C before SDS-gelatin-PAGE. After electrophoresis, proteinsases were revealed as described previously (27), except that DTT, which activated all of the PF, was omitted. Reversible inhibitors were tested after electrophoresis by adding them during the revealing step (27) in the absence of DTT.

**Electroelution of proteases.** After denaturing or nondenaturing gelatin-PAGE, proteases were revealed by incubating a section of the gel at 37°C. The appropriate strips of the unprocessed gel (maintained at 4°C) containing the proteinsases were electroeluted overnight (3 W, 7 to 8 mA, 4°C) in 0.05 M Tris–0.384 M glycine (pH 8.3)–1 mM DTT in an ISCO model 1750 sample concentrator. Electroeluted fractions were equilibrated with 20 mM Tris-HCl (pH 7.5)–25 mM NaCl-10% glycerol and concentrated with Centricon microconcentrators (Amicon Corp., Danvers, Mass.). We used a Centricon 10 microconcentrator for denatured proteases and a Centricon 100 microconcentrator for native proteinase.

**Assay of proteolytic activities.** For 4-methyl-7-coumarylamide (MCA) peptide derivatives, the assay mixture (0.2-ml final volume) containing 0.05 mM substrate (from a 10 mM stock solution in dry dimethyl sulfoxide) and 1 $\mu$g of electroeluted protein was incubated in 20 mM Tris-HCl (pH 8.0)–1 mM DTT–0.05% Triton X-100 for 3 h at 37°C. The reaction was arrested by adding 1.8 ml of cold 100 mM acetic acid (pH 3.0). For the assay of $\beta$-naphthylamide (NA) or 4-methoxy-$\beta$-naphthylamide (MNA) peptide derivatives, 0.5 mM substrate was used under the same conditions. Released methylcoumarylamide or (methoxy)naphthylamine was detected fluorometrically (fluorescence spectrophotometer, model MPF-44 A; Perkin Elmer) at a 380-nm excitation wavelength and a 460-nm emission wavelength or at a 340-nm excitation wavelength and a 425-nm emission wavelength, respectively.

The HMPC showed identical migration and were well separated by electrophoresis in the presence or absence of gelatin. Thus, their proteolytic activities were determined in gels after migration in polyacrylamide gels without gelatin. The gels were cut into 3.5-mm-wide strips. For the $[^3]$H gelatin assay, each gel slice was incubated at 37°C for 16 h in 0.2 ml of 100 mM glycine-NaOH (pH 8.3)–0.05% Triton X-100–1 mM DTT containing 50 kcpm of [acetyl]$^{14}$H gelatin (1.5 kcpm per $\mu$g of protein). The reaction was stopped by adding 0.2 ml of 16% (wt/vol) cold trichloroacetic acid. After 10 min on ice and centrifugation, 0.1 ml of the trichloroacetic acid-soluble supernatant was mixed with 5 ml of Aquasol for counting. For the synthetic peptide-MCA assay, incubation was in 0.2 ml of 50 mM Tris-HCl (pH 8.0)–1 mM DTT–0.05% Triton X-100 containing the substrate at 50 $\mu$M. The hydrolysis of synthetic peptides was arrested with 1.8 ml of cold 100 mM acetic acid (pH 3.0) and the fluorescence was measured as described above.

**Immunoblotting.** After nondenaturing PAGE, the resolved proteins were electroblotted at 25 V constant voltage (4°C) as described previously (43) for 4 h (nondenaturing gels) or overnight (denaturing gels) with 0.2-$\mu$m-pore-size nitrocellulose sheets. The SDS was omitted when native proteins were transferred. Thereafter, nitrocellulose membranes were washed with 20 mM Tris-HCl (pH 7.5)–500 mM NaCl–0.02% NaN$_3$ (TBS) for 15 min and blocked with 3% BSA in TBS for 30 min. Then they were incubated overnight at 4°C with anti-rat skeletal muscle proteasome polyclonal antibodies (whole antisera 108 and 109) diluted at 1:150 in TBS containing 1% BSA 0.05% Tween 20. After two 15-min washes in TBS, the nitrocellulose membranes were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:700 dilution in TBS containing 1% BSA–0.05% Tween 20) for 2 h at room temperature. Finally, they were washed twice with TBS, rapidly rinsed with distilled water, and stained by the 4-chloronaphthol reaction (18) in 50 mM Tris-HCl (pH 7.5) for 15 min.

**Antibodies.** Proteasome was isolated from rat skeletal muscle as described previously by Dahlmann et al. (7). Purified enzyme (0.5 mg) was emulsified in an equal volume of complete Freund adjuvant dissolved in 0.9% NaCl and intradermically injected into rabbits. An intravenous booster injection of 0.1 mg of purified enzyme dissolved in 0.9% NaCl was given 4 weeks later. One week after that, the blood was collected and the serum was separated. The antibodies 108 and 109 (a generous gift of B. Dahlmann, Diabetes Forschungsinstitute, Düsseldorf, Germany) were generated from two different proteasome preparations. Immunoblot controls done with preimmune sera were negative.

**Reagents.** The substrates N-carbobenzoxy (N-CBZ)-Gly-Gly-Arg-MNA, glutaryl (Glu)-Ala-Ala-Ala-MNA, N-CBZ-Pro-Ala-Gly-Pro-MNA, succinyl (Suc)-Gly-Pro-NA, N-CBZ-
Ala-NA, and N-CBZ-Pro-NA were from Bachem; butyloxy-carbonyl (Boc)-Val-Pro-Arg-MCA was from Peptide Institute Inc. (Osaka, Japan); d-Val-Leu-Arg-MNA was from Serva; and Suc-Leu-Leu-Val-Tyr-MCA, N-CBZ-Ala-Ala-Lys-MNA, N-CBZ-Leu-NA, and N-CBZ-Phe-NA were from Sigma Chemical Co. [14C]gelatin was obtained as described previously (27).

RESULTS

Latency and electrophoretic analysis of high- and low-molecular-mass proteinases. Fresh Frankia extracts exhibited a low proteolytic activity when assayed against azocoll and [acetyl-14C]gelatin, two collagen-rich substrates. A variety of p-nitroanilide NH$_2$-blocked peptide derivatives and also [methyl-3H]casein were poorly hydrolyzed or not hydrolyzed. However, after cold activation, a clear proteolytic activity could be detected against azocoll and [acetyl-14C]gelatin. Furthermore, the addition of 0.1% Triton X-100 to fresh cell extracts also increased the [acetyl-14C]gelatin-hydrolyzing activity. Nondenaturing gelatin-PAGE of fresh cell extracts revealed only one major high-molecular-mass proteinase (Fig. 1A) and no low-molecular-mass proteinases. In sedimentation experiments, this electroeluted proteinase was found as a single component sedimenting at 28S, corresponding to a molecular mass of about 1,300 kDa (see below). This proteinase represented 1% (i.e., 550 µg of protein per liter of culture medium) of the total soluble protein content. After electroelution, concentration with Centricon 100, SDS activation, and SDS-gelatin-PAGE, the 1,300-kDa proteinase dissociated into 11 low-molecular-mass PFs from 19 to 40 kDa (Fig. 1B). Therefore, the 1,300-kDa proteinase appeared to be a high-molecular-mass multimeric proteinase complex. Seven other high-molecular-mass proteinase complexes from 270 to 1,150 kDa (see below) were present as latent gelatinolytic activities in fresh cell extracts, since they were detected in gels after PAGE in nondenaturing conditions only after cold activation (data not shown). In contrast, their activities against synthetic peptide substrates could be measured in gels after native PAGE of fresh cell extracts (see below). The addition of 2% SDS to the cold-activated cell extracts and analysis by nondenatur-

FIG. 1. Electrophoretic analysis of the major high-molecular-mass (1,300-kDa) proteinase. (A) Native gelatin-PAGE of fresh cell extracts (200 µg of protein). (B) SDS-gelatin-PAGE of the electroeluted, concentrated, and SDS-activated 1,300-kDa proteinase (50 µg of protein). The weak PF bands are indicated by an asterisk. The molecular mass of the complex was obtained after sucrose gradient sedimentation (see Fig. 8).

ing gelatin-PAGE revealed only low-molecular-mass proteinases. In addition, no other proteinase was visualized in cold-activated cell extracts. Surprisingly, the same family of high-molecular-mass proteinase complexes was detected in concentrates from Frankia growth medium. On day 5, the electroeluted extracellular 1,300-kDa proteinase represented 1.5% (i.e., 85 µg per liter of culture medium) of the total extracellular protein. Separate evidence indicated that their presence in the extracellular space was not due to cell lysis, which started on the seventh growth day under the conditions used (27).

Few low-molecular-mass PFs were detectable after SDS-gelatin-PAGE of fresh cell extracts, but up to 17 gelatinases from 19 to 40 kDa were revealed when the cell extracts were cold activated (Fig. 2A and 3A). The latency of PF was overcome by SDS activation, after which 11 to 13 gelatinases

FIG. 2. SDS-gelatin-PAGE analysis of cold-activated cell extracts (100 µg of protein). (A) Electrophoretic pattern of low-molecular-mass PF. (B) Electrophoretic pattern of intermediate-molecular-mass Frankia proteinases. These activities are denoted only by their apparent molecular masses. The poorly defined gelatinolytic activity present on top of the resolving gel is indicating by an asterisk. We used phosphorylase b (92.5 kDa), bovine serum albumin (66 kDa), chicken egg albumin (45 kDa), l-lactic dehydrogenase (36.5 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), and cytochrome c (12.4 kDa) to determine the molecular mass of the PF.

FIG. 3. Comparison of two activation procedures. Gels G1 and G2 show electrophoretic patterns of PF present in cell extracts (100 µg of protein) before and after activation, respectively. (A) Storage at –80°C for 2 months (cold activation); (B) SDS treatment followed by filtration on G-25 Sephadex column (SDS activation).
were clearly detected (Fig. 3B). This latency was not observed for PF from extracellular concentrates (27). The molecular masses of the 11 PF observed after SDS activation and SDS-gelatin-PAGE of the electroeluted intracellular 1,300-kDa complex were the same as those of the 11 major PF activities revealed in cell extracts after any activation treatment. However, dissociation of the 1,300-kDa intracellular protease by SDS activation, unlike that of the 1,300-kDa extracellular protease, was usually incomplete, as indicated by the presence of a poorly defined gelatinolytic activity on top of the resolving gel. This activity was also visualized in cell extracts (Fig. 2B). In some cases, other gelatinolytic activities of intermediate molecular mass (45, 50, 60, and 65 kDa) were also seen in the SDS-activated electroeluted 1,300-kDa protease and in cell extracts (Fig. 2B).

**Morphological characteristics of high-molecular-mass proteases.** Electron microscopic examination of the electroeluted 1,300-kDa protease after negative staining with 2% uranyl acetate in the presence of 50 mM CaCl₂ revealed hollow-ring, raspberrylike, and cylindrical particles. These shapes were found for both intracellular and extracellular 1,300-kDa particles (Fig. 4A and D). Electron microscopic examination of the electroeluted, intracellular 650-kDa protease showed the same type of particles (size and shape) as those observed for the 1,300-kDa protease (data not shown). Although some variation in size and shape was observed, ring-shaped structures with a 10- to 11-nm mean diameter with a dense central hollow about 5 nm in diameter predominated in CaCl₂-treated samples, but some cylindrical particles and raspberrylike structures like those described previously for prosomes (36) were seen. Higher-magnification views showed the hollow circles to be composed of globular subunits that varied in number and size; 6 to 11 globular subunits about 1.8 to 2.7 nm in diameter and often separated by slits were observed. In some cases, globules were also seen inside the ring (Fig. 4B and E). Cylindrical structures appeared to be composed of four to five stacked disks 15 to 16 nm long and 10 to 11 nm in diameter (Fig. 4C and F); longer cylinders were sometimes observed. These findings suggest that the hollow rings may correspond to overviews of single stack units from cylindrical structures or may represent more complex standing cylindrical structures. As mentioned above, the electron microscopic image of the 28S (1,300-kDa) complex was indistinguishable from that of 19S (650-kDa) complex and was nearly identical in shape and size to eukaryotic 19S to 20S proteasomes and prosomes (1, 14, 22). However, the morphological similarity of these two complexes may result artifically from cell breakage (sonication), electrophoresis, electrolution, and staining steps. Such particles were not observed when the electroeluted 380-kDa band was similarly analyzed by electron microscopy.

**Immunological cross-reaction of Frankia proteases against antibodies to eukaryotic proteasomes.** After nondenaturating PAGE of fresh cell extracts and extracellular concentrates, the immunological cross-reactivity of Frankia high-molecular-mass protease complexes was tested against two anti-rat skeletal muscle proteasome polyclonal antibodies (whole sera 108 and 109). As shown in Fig. 5A and B, up to eight cross-reacting bands were visualized. The eight bands corresponded in electrophoretic mobility to the eight high-molecular-mass protease bands detected gels after gelatin-PAGE of cold-activated cell extracts. Furthermore, they exhibited 3H-gelatin-hydrolyzing activity and cleaved some synthetic peptide substrates that are known to be hydrolyzed by eukaryotic proteasomes (see below). Polyclonal antibody 108 cross-reacted preferentially with the 470- and 380-kDa intracellular bands (Fig. 5A, lane 1) but only faintly with the corresponding extracellular bands (Fig. 5A, lane 2). However, polyclonal antibody 109 cross-reacted strongly with the intracellular and extracellular 650-kDa bands and weakly with the 890- and 800-kDa bands but only very faintly with the 1,300-kDa intracellular and extracellular bands. The reasons for these differences have not been determined. An ill-defined intracellular 890- to 800-kDa band was also observed. In a separate experiment, polypeptides of intermediate and low molecular mass, obtained after SDS dissociation or SDS-/β-mercaptoethanol denaturation (23) of the electroeluted 1,300-kDa complex, were also revealed (Fig. 6). Antibody 108 cross-reacted strongly with two ill-defined bands of 35 to 38 kDa and weakly with a 90-kDa band (Fig. 6A). faint bands of 40, 45, 50, and 65 kDa were also detected. A different immunological pattern was obtained with antibody 109 (Fig. 6B). A strong band of about 65 kDa and five faint bands of 35, 45, 50, 60, and 90 kDa were revealed. Thus, it appears that at least one (1,300-kDa) and probably the four intermediate molecular mass proteasomes (45, 50, 60, and 65 kDa), and one polyepitopic component (90 kDa; see Discussion) of the 1,300-kDa complex share common epitopes with rat skeletal muscle proteasomes.

**Multicatalytic properties of high- and low-molecular-mass Frankia proteasomes.** The proteolytic characteristics of high-molecular-mass protease complexes immunologically related to rat skeletal muscle proteasomes were studied in gel slices after nondenaturing electrophoresis of fresh cell extracts and extracellular concentrates. As shown in Fig. 5, they exhibited multicatalytic activities. Therefore, we proposed the general abbreviation HMPC to designate these complexes. Thus, [acetyl-14C]gelatin was strongly degraded by the 1,300-kDa protease but weakly by all the other proteasomes (Fig. 5C). All of these intracellular and extracellular HMPC presented an essentially identical proteolytic profile. Suc-Leu-Leu-Val-Tyr-MCA has been described as a good substrate for proteasomes from archaeabacteria (6) and a variety of eukaryotic cells (10, 14, 20, 21, 41). Among the Frankia HMPC, this substrate was hydrolyzed strongly only by the 650-kDa protease and weakly by the 890- and 800-kDa proteasomes. Boc-Val-Pro-Arg-MCA (20) was also cleaved by the majority of the HMPC but preferentially by the 270-kDa protease. In a separate sucrose gradient sedimentation experiment (see below), the N-CBZ-Pro-Ala-Gly-Pro-MNA substrate was cleaved essentially by the 1,300-kDa (28S) and 270-kDa (12S) proteases.

When the 1,300-kDa protease was electroeluted from nondenaturating gelatin-polyacrylamide gels, activated with SDS, and submitted to SDS-gelatin-PAGE, a variety of gelatin-hydrolyzing activities of low molecular mass (PF) were revealed (Fig. 1B). Electrolution of these PF and analysis of their specificities against synthetic peptide substrates are shown in Table 1. All of the PFs hydrolyzed N-CBZ-Pro-Ala-Gly-Pro-MNA, a collagenase substrate; PF 27.5 and PF 24 showed the highest sensitivity. d-Val-Leu-Arg-MNA (26) was also a general substrate cleaved by all of the PF and preferentially by PF 21. In contrast, the Boc-Val-Pro-Arg-MCA peptide was a poor substrate for all the PF but was preferentially hydrolyzed by PF 24. Interestingly, Suc-Leu-Leu-Val-Tyr-MCA was recognized only by the lowest-molecular-mass proteasomes (PF 27.5, 24, 22.5, 21, 19.5, and 19); PF 27.5 was the most active. Thus, as a general property, PF preferentially cleaved fluorogenic peptides composed of at least three amino acids and with a
FIG. 4. Electron micrographs of the electroeluted 1,300-kDa proteinase from cell extracts and extracellular concentrates. After electroelution, samples were washed and concentrated in 20 mM Tris-HCl (pH 7.5)-25 mM NaCl-10% glycerol by using a Centricon 100 microconcentrator. For electron microscopic observations, 30 µg of the electroeluted proteinase was preincubated with 50 mM CaCl₂ for 15 min in ice and then negatively stained with 2% uranyl acetate (pH 4.0). The carbon film-coated grids (400 mesh) were then examined with a Philips model EM 410 electron microscope at a magnification of ×40,000. (A, B, C) Intracellular particles; (D, E, F) extracellular particles; (A, D) survey electron micrographs showing ring-shaped and cylindrical structures. The cylindrical shapes are indicated by arrows (bar, 100 nm). Higher-magnification views show the rings (B and E) and cylindrical particles (C and F) (bar, 20 nm).

basic, aromatic, or neutral amino acid residue at the P1 position (34); this is also a property of eukaryotic proteasomes (30, 32).

Effects of inhibitors and various compounds on low-molecular-mass proteinases. The effects of various inhibitors and compounds on the degradation of gelatin by PF were studied after SDS-gelatin-PAGE of cold-activated cell extracts (Fig. 7). The general serine proteinase inhibitors phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate (2 mM; data not shown) showed a weak inhibitory effect. Toxyl-L-lysine
The molecular mass of the immunoblots and concentrates obtained after native (see Fig. 8).

FIG. 6. Immunoblot analysis of Frankia intracellular (I) and extracellular (E) 1,300-kDa proteinase (50 µg of protein) after SDS treatment (lanes 1) or boiling with SDS plus β-mercaptoethanol (lanes 2) with anti-rat skeletal muscle polyclonal antibodies 108 (A) and 109 (B). Clear and faint cross-reactive bands are denoted by arrows and arrowheads, respectively.

chloromethyl ketone and tosyl-L-phenylalanine chloromethyl ketone, respectively, acting as trypsin and chymotrypsin inhibitors, strongly inactivated all of the proteinases; only PF 40, 38, 37.5, and 35 were weakly detected. Leupeptin, a microbial inhibitor of thiol- and serine-type proteinases, blocked the activities of all PF. A similar effect was observed with antipain, a microbial inhibitor of serine-type proteinases; the exception was PF-40, which reacted weakly. In contrast, the chymostatin and elastatin (2 µg/ml; data not shown), two microbial inhibitors of serine-type proteinases, had no apparent effect. No PF activity was revealed in the presence of the thiol-group blocking reagents mercuric acetate, N-ethylmaleimide, and monomodoacetic acid (5 mM; data not shown). In contrast, p-chloromercurphenyl sulfonic acid and epoxysuccinyl-leucyl-agmatine (0.5 mM; data not shown) did not inactivate the gelatinolytic activity of PF. CaCl₂ and HgCl₂ (1 mM; data not shown) abolished the gelatinolytic activity of all of the proteinases except PF 40, which reacted weakly. Different responses were observed with some metal chelators. 1-10-Phenanthroline was a strong inhibitor, whereas ethylene glycol bish(β-aminoethyl ether)tetraacetic acid (EGTA) moderately stimulated most of them. The activating effect of EGTA could be explained by the chelation of inhibitory Ca²⁺ ions. EDTA (10 mM) weakly inhibited all of the PF. Other inhibitory assays (data not shown) indicated that soybean trypsin inhibitor (20 µg/ml), aminobenzamidine (1 mM), aprotinin (10 µg/ml), captopril (1 mM), and pepstatin A (2 µg/ml), an aspartic proteinase inhibitor, were ineffective.

Taken together, our results do not allow easy classification of any of the 11 major PF in a separate group. Although they appear mainly as a thiol class of proteinases, these intracellular PF are also strongly sensitive to various inhibitors characteristic of the serine-type proteinases (with the excep-
tion of PF 40) and to the metal chelator 1,10-phenanthroline. A similar unclear pattern of inhibitory effects has been described for prokaryotic and eukaryotic proteasomes (6, 32, 44). However, because we used crude extracts, the possibility of comigration of other low-molecular-mass endopeptidases with some PF cannot be rigorously discarded.

**Sedimentation analysis of intracellular HMPCs.** With two synthetic peptide substrates, five HMPC were clearly identified in sucrose gradients as sedimenting at 28S, 19S, 16S, 14S, and 12S (Fig. 8). Their molecular masses were estimated to be 1,300, 650, 470, 380, and 270 kDa, respectively. Three other HMPC (25S [1,150 kDa], 22S [890 kDa], and 21S [800 kDa]) were less precisely located because of their poor ability to cleave N-CBZ-Pro-Ala-Gly-Pro-MNA and Suc-Leu-Leu-Val-Tyr-MCA (Fig. 8) as well as Boc-Val-Pro-Arg-MCA and d-Leu-Val-Arg-MNA (data not shown). Interestingly, although N-CBZ-Pro-Ala-Gly-Pro-MNA was strongly cleaved by all of the low-molecular-mass proteinases of the 1,300-kDa complex, it was efficiently hydrolyzed only by the 28S (1,300-kDa) and 12S (270-kDa) complexes and, to a lesser extent, by the 19S (650-kDa) complex. In contrast, Suc-Leu-Leu-Val-Tyr-MCA was preferentially hydrolyzed by the 19S (650-kDa) complex, confirming previous observations after native PAG (see above). The cleaving activities of the 890- and 800-kDa proteinases, which were easily measurable in gels after native PAGE (see above), were just detectable in sucrose density gradients. This indicates that the cleaving efficiency of some HMPC varies with the method of separation. Furthermore, the global proteolytic response obtained after sucrose density gradient centrifugation was lower than that measured after electrophoresis. Interestingly, the gelatinolytic, slow-migrating HMPC obtained after native gelatin-PAGE and electrophoresis also sedimented at 28S (Fig. 8). Electron microscopic analysis of the 28S and 19S peaks (fractions 20 and 48, respectively) showed no clear differences between them; both were composed of the same hollow-ring and cylindrical structures as described above. However, compared with the structures of the corresponding electroeluted 1,300- and 650-kDa proteinases, longer ribbonlike structures were observed in fractions 20 and 48 from the sucrose gradient. None of these shapes was found in the 12S peak.

**DISCUSSION**

We report here for the first time the existence of a family of HMPC in *Frankia* sp. strain BR cell extracts and extracellular concentrates. Electron microscopic examination of the 28S (1,300-kDa) complex showed that it was composed of hollow-ring and cylindrical structures like those described previously for eukaryotic prosomes and proteasomes (1, 14, 22, 36, 39). Similar shapes were observed for the 19S (650-kDa) complex. The possibility that these structures are related to proteasome complexes was supported by their

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<th>Sp act (pmol·min⁻¹·mg⁻¹) of:</th>
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<tr>
<td></td>
<td>PF 40</td>
</tr>
<tr>
<td>N-CBZ-Pro-Ala-Gly-Pro-MNA</td>
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*Subunits were obtained after SDS dissociation (room temperature) of the electroeluted 1,300-kDa proteinase complex and electrophoresis in denaturing conditions (SDS-gelatin-PAGE). Subunits showing gelatinolytic activity were obtained as described in Materials and Methods. The following peptides were not hydrolyzed by the electroeluted 1,300-kDa proteinase complex and its subunits: N-CBZ-Ala-NA, N-CBZ-Leu-NA, N-CBZ-Phe-NA, N-CBZ-Pro-NA, Suc-Gly-Pro-NA, Glut-Ala-Ala-Ala-MNA, N-CBZ-Ala-Ala-Lys-MNA, and N-CBZ-Gly-Gly-Arg-MNA.*

**FIG. 7.** Effect of various inhibitors and other compounds on the gelatinolytic activity of PF. Cold-activated cell extracts (100 μg of protein) were directly subjected to SDS-gelatin-PAGE or preincubated for 30 min at 30°C in the presence of irreversible inhibitors (gels B, C, D, E) before electrophoresis. The reversible inhibitors were added only at the revealing step. The final concentrations were as follows: A, no additions; B, 0.5 mM phenylmethylsulfonyl fluoride; C, 2 mM p-chloromercuri phenyl sulfonic acid; D, 0.5 mM tosyl-L-lysine chloromethyl ketone; E, 0.5 mM tosyl-L-phenylalanine chloromethyl ketone; F, 1 mM 1,10-phenanthroline; G, 5 mM N-ethylmaleimide; H, 1 mM mersalyl acid; I, 10 mM EGTA; J, 5 mM CaCl₂; K, 2 μg of chymostatin per ml; L, 2 μg of antipain per ml; M, 2 μg of leupeptin per ml.
cross-reactivity with polyclonal antibodies against purified rat skeletal muscle proteasomes. This also indicates that some (probably essential) common antigenic epitopes have been conserved from archaeabacterial (6) and actinomycete proteases during evolution. Furthermore, the 19S proteasome-like complex closely resembles in molecular mass, and multicalytic properties the eukaryotic proteasome or prosome complexes (30, 32, 35, 39).

SDS-gelatin-PAGE analysis of the electroeluted SDS-activated (30°C) 28S (1,300-kDa) protease complex revealed a set of 11 low-molecular-mass proteinases. Their molecular masses, which ranged from 19 to 40 kDa, were similar to those of the 11 major PF in cell extracts. This is reminiscent of the characteristics of eukaryotic proteasomes (32). The majority of PF (and HMPC) were cryptic (latent) in fresh cell extracts; a similar behavior has been reported for eukaryotic proteasomes, which need activation (40). Two activation procedures were useful for Frankia cell extracts: cold activation and 30°C SDS treatment. Some additional gelatinolytic activities observed after cold activation may be proteolytic degradation products from other activities. Alternatively, cold activation and electrophoresis in denaturing conditions may remove some endogenous inhibitors or facilitate dissociation of oligomeric forms. In fact, in separate experiments, we found that the increase of the gelatinolytic activities of PF 22.5, 21, 19.5, and 19 was correlated with the decrease of a thermostable soluble inhibitor when cell extracts were incubated at pH 5.0 (37°C) (unpublished results). The possibility that the cryptic nature of proteinases is due to endogenous inhibitors has also been suggested for eukaryotic proteasomes (13). Oligomeric dissociation may also occur; after SDS activation, the activity of a poorly defined band (Fig. 2B) notably decreased. Also, activating procedures may lead to activation by facilitating a conformational change after oligomeric dissociation.

All 11 low-molecular-mass PF were able to cleave a variety of synthetic substrates such as N-CBZ-Pro-Ala-Gly-Pro-MNA, D-Val-Leu-Arg-MNA, and Boc-Val-Pro-Arg-MCA, but Suc-Leu-Leu-Val-Tyr-MCA was cleaved only by the six lowest-molecular-mass PF. These results indicate a rather low specificity for PF. However, N-CBZ-Gly-Gly-Arg-MNA, which is a substrate for serine-type proteinases, was not cleaved by any of the PF. Also, Glut-Ala-Ala-Ala-MNA, a substrate for elastase, was not recognized by any PF. The fact that each PF showed a particular pattern of specific activities against the four substrates employed here supports the idea that they are different enzymes. Also, it appears that at least three amino acids are needed for a synthetic peptide to be recognized by a substrate and efficiently cleaved by the 1,300-kDa multicatalytic protease complex and by the PF bands. Thus, Suc-Gly-Pro-NA was not split, whereas N-CBZ-Pro-Ala-Gly-Pro-MNA was quite efficiently hydrolyzed. This hypothesis should be tested with more appropriate substrates. Differences in the sensitivity of detection of the cleaved fluorophore may also account for these results.

The fact that a single low-molecular-mass proteinase can cleave a variety of synthetic substrates (collagenase-like, chymotryptsinlike, trypsinlike) is surprising; two or more catalytic sites in a proteinase of low molecular mass are unexpected. One possible explanation is that, as shown by Tanaka et al. using two-dimensional PAGE, every proteinase is, in fact, composed of a subfamily of polypeptides with almost identical molecular masses (40-42). Some of them may be low-molecular-mass proteinases with different specificities. This implies a high degree of monomeric complexity of Frankia proteasomes and probably of eukaryotic proteasomes as well. The presence of artifactually retained low-molecular-mass proteinases in the 1,300-kDa complex is unlikely, because essentially the same numbers of these PF were seen after 30°C SDS dissociation of the 1,300-kDa complex and cold- or SDS-activated whole-cell extracts. Moreover, there may be other subunits in the 1,300-kDa complex that are noncatalytic or that recognize other types of substrates, as indicated by the finding of two different low-molecular-mass caseinolytic but nongelatinolytic activities after SDS-gelatin-PAGE (not shown).

From the sucrose gradient analysis, it is notable that, although the N-CBZ-Pro-Ala-Gly-Pro-MNA substrate was efficiently cleaved by all of the PF, it was hydrolyzed strongly only by the 1,300- and 270-kDa HMPC and faintly by the 650-kDa HMPC. The other HMPC did not cleave this substrate. Similarly, Suc-Leu-Leu-Val-Tyr-MCA was a good substrate for the 650-, 470-, 380-, and 270-kDa HMPC but was not split by the others. Topological reasons may explain this apparent paradox; also, allosteric, hydrophobic, saline, or other interactions may explain the differences in proteolytic specificity among PF and between the HMPC complexes and their PF subunits. Similarly, topological considerations may explain the variations observed in the intensity of the HMPC immunoblots in our experiments.

Our immunological and enzymological results suggest that the different HMPC may originate by different multimeric arrangements of the 11 PF and probably some other units. Alternatively, HMPC may be artifactually derived from the 28S (1,300-kDa) complex (which we propose to designate as megaproteinase) during cell breakage by sonication or by other dissociating events. Indeed, the megaproteinase may be formed by assembling some lower complexes members of the HMPC family. The fact that the eukaryotic 26S (1,000- to 1,500-kDa) complex (29), megapain (20), and UCDEN (46) complexes have been found to be built in such a way (11, 12, 16, 29) argues in favor of this idea (see below). Also, protein subunits other than proteinases may be present in Frankia.
HMPC. SDS-PAGE (23) analysis of proteins of the electroeluted megaproteinase showed that, besides the 11 polypeptides corresponding to molecular mass to the 11 major PF, a set of strongly silver-stained polypeptides from 45 to 105 kDa was also detected (data not shown). Although some protein contaminants may be presented in the electroeluted fraction, this finding is in good agreement with recent reports indicating that the 26S complex (29) and megapain (20) contained 9 to 15 protein factors of similar molecular mass in addition to the proteolytic proteasome subunits.

Although the physiological function and the nature of endogeneous substrates of the Frankia megaproteinase and the 19S proteasomelike complex remain to be determined, a variety of roles have been suggested (30). One may be the transport and regulation of mRNA translation as proposed for prosomes (35, 38). Prosomes discovered in 1984 (36) are now considered identical to proteasomes (1, 14) that had been described separately (32). Thus, the existence of small untranslated RNA molecules in purified Frankia proteasomes is an interesting question with reference to the probable prosomal nature of some HMPC. On the other hand, such large multicatalytic proteinase complexes may be essential to prokaryotic systems as subcompartments for regulated, localized proteinase action and for rapid degradation of proteins. Furthermore, the molecular mass, size, and subunit composition parameters of the megaproteinase appear to correspond to those of the eukaryotic 26S, megapain, and UCDEN complexes. These complexes preferentially degrade ubiquitinated proteins in vitro through an ATP-dependent reaction (13, 46) as well as some nonubiquitinated proteins and synthetic peptide substrates by a reaction that does not require ATP (13, 20). However, using synthetic peptide substrates, we did not find any ATP dependence for proteinase action. Whether ubiquitin is present in Frankia has not been determined, although it is considered improbable, because ubiquitin is characteristic of only eukaryotic cells (15).

The fact that the megaproteinase and the 19S proteasomelike complex were detected in extracellular concentrates of Frankia growth medium opens the question of their biological significance. Extracellular proteinase activities could be useful to provide amino acids for saprophytic growth, but it is known that Frankia sp. can grow in vitro with only propionic acid as a carbon source in the absence of any soluble nitrogen source because of its capacity to fix atmospheric nitrogen (28). Another intriguing possibility is that the extracellular megaproteinase and 19S proteasomelike complex participate in the infection process by depolymerizing some cell wall proteins. Results indicating that pectolytic and proteolytic activities are needed for phytopathogenicity of Xanthomonas campestris pv. campestris (8) are in line with this idea. In fact, structural studies of a variety of plant cell wall proteins called extensins (45) show them to be composed of repeating structures rich in proline and hydroxyproline separated by sequences such as Hyp-Val-Tyr-Lys, Pro-Thr-Pro-Val-Tyr, and His-Thr-Pro-Val-Tyr-Lys, which closely resemble the sequences of the synthetic peptides and gelatin that are cleaved by the Frankia megaproteinase and the 19S proteasomelike complex. Further studies will be required to determine the function of extracellular HMPC and the process of their secretion as whole complexes or as individual polypeptides. It is to note that a high-molecular-mass membrane-bound proteinase denominated MCP was recently found in human erythrocytes and that its properties are almost identical to those of the intracellular erythrocyte MCP (21).

Our results strongly suggest that the HMPC are important components of the Frankia proteolytic system and confirm the idea that the megaproteinase, the 19S proteasomelike complex, and perhaps all of the other HMPC were acquired early in evolution, as indicated by their known presence in archaeabacteria (6), their probable presence in Escherichia coli (44), and their immunological cross-reactivity with antieukaryotic proteasome antibodies.

The finding of such a variety of HMPC reported here is a step toward a understanding of the proteolytic events in Frankia sp. It will be necessary to further purify these molecules, to determine precisely their relationship with the reported proteasome, prosome, megapain, or UCDEN complexes, to define their proteolytic pathway, and to examine in detail their physiological regulation.

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