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The Second Peptidoglycan Hydrolase of *Streptococcus faecium* ATCC 9790 Covalently Binds Penicillin

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A second peptidoglycan hydrolase (muramidase-2) of *Streptococcus faecium* ATCC 9790 (*Enterococcus hirae*) has been purified to apparent homogeneity. The enzyme has been shown to be a β-1,4-*N*-acetylmuramoylhydrolase (muramidase; EC 3.2.1.17) and to differ in substrate specificity from a previously isolated muramidase. Purified enzyme appears as two protein staining bands with molecular masses of 125 and 75 kilodaltons (kDa) on polyacrylamide gels after sodium dodecyl sulfate electrophoresis. Elution and renaturation of protein bands from sodium dodecyl sulfate-polyacrylamide gels showed that both proteins have muramidase-2 activity. Both proteins have been shown to bind radioactive benzylpenicillin and have the same electrophoretic mobilities as penicillin-binding proteins 1 and 5 present in membrane preparations of this organism, respectively. Incubation of a [^{14}C]penicillin G-labeled 125-kDa form of the enzyme with crude alkaline extracts from *S. faecium* (which did not contain added proteinase inhibitors) showed the endogenous conversion of the radiolabeled 125-kDa form to the radiolabeled 75-kDa form of the enzyme.

Streptococcus faecium ATCC 9790 (*Enterococcus hirae* [10]) was previously shown to possess two distinct peptidoglycan hydrolases, which differ from each other in substrate specificity, molecular mass, and, very probably, mechanism of hydrolysis (19). Muramidase-1 was isolated and purified to homogeneity by affinity chromatography on concanavalin A-Sepharose and was shown to occur in a latent 130-kilodalton (kDa) form that can be proteolytically converted to an active 87-kDa form (20, 24). Muramidase-1 was also shown to contain covalently attached monomeric and oligomeric glucose (20) and monomeric 5-mercaptouridine monophosphate (8). In addition, muramidase-1 was shown to processively hydrolyze linear-soluble, un-cross-linked peptidoglycan chains (1).

A second peptidoglycan hydrolase (muramidase-2) has been partially purified from culture supernatants by affinity binding to, and elution from, sodium dodecyl sulfate (SDS)-treated cell walls of *Micrococcus luteus* and has been shown to be a 75-kDa protein (19). It was shown to differ from muramidase-1 in substrate specificity, dissolving the cell walls of *M. luteus* or the acid-insoluble peptidoglycan fraction of *S. faecium* walls but having little ability to dissolve *S. faecium* cell walls. Additional data suggest that muramidase-1 and muramidase-2 are separate gene products.

We have purified muramidase-2 from the insoluble pellet of disrupted *S. faecium* cells. Muramidase-2 was extracted with 0.02 N NaOH at 0°C, bound to the acid-insoluble peptidoglycan fraction of *S. faecium*, and reextracted with alkali. SDS-polyacrylamide gel electrophoresis (PAGE) of fractions highly enriched for muramidase-2 activity showed the presence of two protein bands, at 125 and 75 kDa. Upon elution and renaturation from SDS gels, both protein bands were shown to possess muramidase-2 activity. The two proteins appeared to be biochemically and immunochemically related and may represent different forms of the enzyme. Both proteins bind radioactive penicillin and have the same SDS-PAGE mobilities as penicillin-binding proteins (PBPs) 1 and 5 of *S. faecium*. In addition, incubation of the 125-kDa protein, to which [^{14}C]penicillin G (pen G) was prebound, with crude alkaline extracts from *S. faecium* showed that the 125-kDa protein could be processed to the 75-kDa protein which retained bound [^{14}C]pen G.

MATERIALS AND METHODS

Bacterial growth. Large-scale cultures of *S. faecium* were grown and kindly provided by E. I. du Pont de Nemours & Co., Inc., Wilmington, Del. The bacteria were grown in a complex medium composed of the following (in grams per liter) in 200-liter fermentations: ardamine Z (10); N-Z amine B (10); Na_2HPO_4 (25.65); NaH_2PO_4 (16.45); glucose (20). Cells were grown at 35°C, 200 rpm, with the pH maintained at 6.5 to 6.8 with 10 N NaOH. The fermentations were maintained until a cell concentration of 1.7 mg/ml (dry weight) was reached, at which point they were chilled and harvested by continuous-flow centrifugation at 4°C. Cells were provided to us as frozen pastes (−70°C).

Preparation of disrupted cells. All manipulations for the preparation of disrupted cells and purification of muramidase-2 were carried out at 0 to 4°C, and all solutions contained 0.1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 5 mM nitrilotriacetic acid, 1 μg of pepstatin A per ml, and 1 μg of antipain per ml, unless otherwise stated. Routinely, frozen cell pastes were lyophilized and 100-g (dry weight) portions were suspended in 400 ml of ice-cold 10 mM sodium phosphate (pH 7.0) containing 50 μg of DNase, 50 μg of RNase per ml, and 1 mM $MgSO_4$. The cell suspension was then disrupted in a Ribi Cell Fractionator (Ivan Sorvall, Inc., Norwalk, Conn.) at 40,000 lb/in^2 and at 5 to 10°C. The broken cell preparation was left on ice for 30 min and then centrifuged (20,000 × *g*, 30 min, 4°C). The pellet was suspended in 400 ml of 10 mM sodium phosphate, pH 7.0, and distributed into 20-ml volumes which were stored at −70°C.

Enrichment and purification of muramidase-2. Routinely, 40 ml of the insoluble residue of disrupted cells (equivalent to approximately 2.5 g of cell wall [dry weight]) was used for extraction. The samples were thawed on ice, and the suspension was centrifuged at 28,000 × *g* for 15 min at 4°C. The

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pellet was suspended and washed in 200 ml of 10 mM sodium phosphate (pH 8.0), pelleted, and washed with 200 ml of 10 mM glycine-NaOH (pH 10; pH was adjusted to 10 with 2.5 N NaOH after the addition of the proteinase inhibitors). Enzyme activity was extracted by resuspending the insoluble residue of the disrupted cells in 200 ml of ice-cold distilled water containing the proteinase inhibitors and then, immediately before centrifugation (28,000 × *g*, 15 min, 4°C), adding 1.6 ml of 2.5 N NaOH (final concentration, 0.02 N; final pH, 11.0 to 12.0). Immediately after centrifugation, the supernatant was decanted and neutralized to pH 7.0 with 0.5 M sodium acetate, pH 5.4. The insoluble residue was reextracted, and after neutralization, the extracts were combined and maintained at 0°C. Sonicated *S. faecium* peptidoglycan (100 mg in 10 ml of 10 mM sodium phosphate, pH 7.0) was added to the combined extracts, and the mixture was allowed to stand at 0°C for 30 min with occasional mixing. After binding, the peptidoglycan-enzyme complex was pelleted (28,000 × *g*, 15 min, 4°C), suspended in 15 ml of 10 mM sodium phosphate (pH 8.0), and recentrifuged. The insoluble *S. faecium* peptidoglycan-protein complex was then washed with 15 ml of 10 mM glycine-NaOH, pH 10 (pH was adjusted to 10 with 2.5 N NaOH). The pellet was suspended in 5 ml of ice-cold distilled water, and the enzyme was extracted by the addition of 2.5 N NaOH to a final concentration of 0.01 N NaOH immediately prior to centrifugation. Immediately after centrifugation, the supernatant was decanted and neutralized with 0.5 M sodium acetate, pH 5.4. Alkaline extraction was normally carried out twice, but the neutralized extracts were not combined. All fractions were assayed for muramidase-2 activity throughout purification.

Preparation of *S. faecium* peptidoglycan. *S. faecium* peptidoglycan was prepared from the insoluble residue of alkaline-extracted, disrupted *S. faecium* cells (as described above). The insoluble pellets (100 mg/ml) were treated with 0.5 μg of DNase per ml, 0.5 μg of RNase per ml, and 1.0 μg of pronase per ml in 10 mM sodium phosphate, pH 7.0, containing 1 mM $MgCl_2$, for 4 h at 37°C. The walls were pelleted and washed twice with ice-cold distilled water (20,000 × *g*, 15 min, 4°C). The washed cell walls were then suspended in 2% SDS and incubated at 4°C for 12 h with continuous stirring. The walls were then sedimented and washed with ice-cold distilled water until there was no foaming upon shaking (a minimum of six times). The SDS-washed cell walls were then treated with 0.2 N HCl at 60°C for 16 h (19). The suspension was then neutralized with 2.5 N NaOH and centrifuged (20,000 × *g*, 15 min, 4°C), and the pellet was washed five times with ice-cold water.

Assay of enzymatic activity. Muramidase-2 activity was assayed by its ability to hydrolyze either of two different substrates, *S. faecium* peptidoglycan or SDS-washed *M. luteus* cell walls, both of which are poorly or are not dissolved by muramidase-1 (19). The standard assay consisted of 0.67 mg of either substrate per ml plus the sample in a final volume of 1.5 ml of 10 mM sodium phosphate, pH 7.0. Dissolution of substrate was monitored at 450 nm on a Gilford 300 spectrophotometer. One enzymatic unit was defined as the amount of enzyme that results in a decrease in optical density of 0.001 per min.

Penicillin-binding assay. The ability of proteins to bind penicillin was determined by using [^{14}C]pen G (56 mCi/mmol of potassium salt; Amersham Corp., Arlington Heights, Ill.) or [^{3}H]pen G (62.5 mCi/mg of *N*-ethylpiperidine salt; kindly provided by Merck & Co., Inc., Rahway, N.J.) at saturating concentrations (5). Routinely, 50 μl of sample was mixed with 20 μl of [^{14}C]- or [^{3}H]pen G (40 μg/ml, final concentration) and incubated at 37°C for various times, up to 90 min. The reaction was then quenched with 5 μl of cold pen G (100 mg/ml), followed by the addition of an equal volume of SDS sample buffer (0.125 M Tris hydrochloride [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and heating at 100°C for 5 min. PBPs were visualized by fluorography (5) of SDS-polyacrylamide gels (as described below).

SDS-PAGE. The method used was similar to that described by Laemmli (21). The stacking and separating gels were 4 and 10%, with pHs of 6.8 and 8.8, respectively. Gels were subjected to electrophoresis at 10 mA constant current for approximately 16 h under a continuous SDS-Tris-glycine buffering system (25 mM Tris, 192 mM glycine [pH 8.3], 0.1% SDS). Proteins were visualized by incubation with Coomassie blue G-250 (0.07% in 3.5% perchloric acid) for 16 h at 42°C, followed by destaining with 5% acetic acid for 1 h and storage in H_2O (16). SDS gels containing low amounts of protein were stained with silver by the procedure specified by Bio-Rad Laboratories, Richmond, Calif. Molecular masses were determined on the basis of migration of molecular mass standards (Pharmacia, Inc., Piscataway, N.J.). Samples were prepared by the addition of an equal volume of SDS-sample buffer followed by exposure at 100°C for 5 min.

Renaturation of muramidase activity from SDS-polyacrylamide gels. Protein bands isolated from SDS-polyacrylamide gels were renatured by a method similar to that of Hager and Burgess (15). Duplicate samples were separated on 10% SDS-polyacrylamide gels. After electrophoresis, one lane was stained with Coomassie blue, the adjacent lane was cut into 2-mm slices with a scalpel blade, and each slice was placed into separate Eppendorf tubes. To each tube, 200 μl of extraction buffer (2 mM β-mercaptoethanol, 0.1 mg of bovine serum albumin per ml, 0.1 mM EDTA, 0.1% SDS in 10 mM Tris hydrochloride [pH 7.4]) was added, and the gel piece was crushed with a silicanized glass rod. After 2 h at room temperature with occasional shaking, each tube received an additional 200 μl of extraction buffer and the samples were placed at 4°C overnight. The tubes were then centrifuged (14,000 × *g*, 10 min, 4°C), and the supernatants were separately collected. The pellets were reextracted with 200 μl of extraction buffer for 2 h at 4°C and centrifuged, and the individual supernatants were collected and pooled with the initial extracts. The extracts were then evaporated to dryness in a vacuum concentrator (Savant Instruments, Inc., Hicksville, N.Y.) and dissolved in 100 μl of H_2O (at 4°C). Protein was precipitated at −20°C for 1 h after the addition of 1 ml of acetone (−20°C). Precipitated protein was collected by centrifugation (14,000 × *g*, 15 min, 4°C), washed once with 1 ml of acetone (−20°C), and allowed to air dry at room temperature. Precipitates were then dissolved (and unfolded) in 45 μl of 6 N guanidine hydrochloride at room temperature (1 h). Samples (15 μl) of the denatured protein were then renatured by dilution into a final volume of 1.5 ml of 10 mM sodium phosphate, pH 7, containing 1.0 mg of *S. faecium* peptidoglycan and assayed for enzymatic activity.

Enzymatic specificity. The enzymatic specificity of muramidase-2 was determined by hydrolysis of SDS-washed *M. luteus* cell walls and analysis of the hydrolytic products. *M. luteus* cell walls (500 μg) were incubated at 37°C with 50 U of muramidase-2 (specific activity, 750 U/mg of protein) in 1.5 ml of 10 mM sodium phosphate, pH 7.0, until the suspension was clear (approximately 16 h). The incubation mixture was then centrifuged, and the supernatant was brought to dryness in a vacuum concentrator. NaB^3H_4 (0.1 M) (10 mCi/mmol; Amersham Corp., Arlington Heights, Ill.) in 0.01 N NaOH (500 μl) was added, and the wall hydrolysate was

incubated at 4°C for 6 h. After destruction of excess reagent by acidification, the reduced wall hydrolysate was hydrolyzed with 6 N HCl (100°C, 4 h). The HCl was removed in vacuo, and the hydrolysate was dissolved in 0.1 M pyridine-acetate, pH 2.8, and fractionated on a Dowex 50W ×2 column (1.0 × 20 cm) equilibrated in 0.1 M pyridine-acetate, pH 2.8 (24). The column was eluted with the same buffer (60 ml) followed by 0.133 M pyridine-acetate, pH 3.85 (60 ml). Samples (50 μl) of fractions (2 ml) were monitored for radioactivity. Standards were prepared by NaB^3H_4 reduction of *N*-acetylmuramic acid, *N*-acetylglucosamine, muramic acid and glucosamine by the above mentioned procedure and used to calibrate the column. Radioactive peaks were pooled and further identified by ascending paper chromatography on Whatman No. 1 paper with a solvent system of butanol:acetic acid:water (3:1:1 [vol/vol/vol]; 1).

Immunoaffinity chromatography. A mouse monoclonal antibody {MAb; A12F7F10 (immunoglobulin G1 [IgG1])} specific for muramidase-2 (D. L. Dolinger, Ph.D. thesis, Temple University School of Medicine, Philadelphia, Pa., 1988) was purified from mouse ascitic fluid by affinity chromatography on protein A-Sepharose 4B (9) and concentrated to 1 mg/ml. A 1-mg portion of purified MAb A12F7F10 (IgG1) (titer, 1:32,000 as assayed against crude muramidase-2 bound to *S. faecium* peptidoglycan) was coupled to 1 g of activated CH-Sepharose 4B by the procedure specified by Pharmacia Fine Chemicals. To affinity-purify muramidase-2, preparations of muramidase-2 (fraction 6 or 7; 1 mg of protein) were allowed to interact with the affinity column (500 μl) for 4 h at 4°C. The mixture was then poured into a column, and the column was washed successively with 2 ml of 0.1% Triton X-100 in 10 mM sodium phosphate, pH 7.0, and 0.5 M NaCl in 10 mM sodium phosphate, pH 7.0. To elute bound material, the column was washed with 200 mM NH_4OH (1 ml) and the eluant was immediately neutralized.

Analytic techniques. Protein was determined by binding to Coomassie blue G-250 (3; Bio-Rad, Rockville Centre, N.Y.).

RESULTS

Purification of muramidase-2. Preliminary studies indicated that treatment of intact cells with 0.01 N NaOH at 0°C efficiently extracted muramidase-1 activity but was able to only partially extract muramidase-2 activity. The alkali extracts contained, in addition, all six of the PBPs normally found in membrane preparations of *S. faecium*. To increase yields of muramidase-2, *S. faecium* cells were disrupted, the insoluble crude cell wall and membrane containing residue were successively washed with 10 mM sodium phosphate (pH 8.0) and 10 mM glycine-NaOH (pH 10), and muramidase-2 activity was extracted with 0.02 N NaOH. A large number of proteins (Fig. 1, lane 1) and approximately 10% of the sum of extracted muramidase-2 activity (Table 1, fraction 1) were found in the pH 8.0 wash of disrupted cells. The pH 10.0 wash of disrupted cells also removed a large number of proteins (Fig. 1, lane 2). Only about 2% of the extractable muramidase-2 was found in this fraction (Table 1, fraction 2). In other experiments, detectable levels of muramidase-2 activity were not found in either the pH 8.0 or 10.0 washes.

At pH 11.0 and above, 88% of the sum of extracted muramidase-2 activity present in the insoluble residue of broken cells (Table 1, fraction 3), 46% of the sum of extracted protein, and high yields of PBPs 1 and 5 were extracted. Low levels of muramidase-1 were also extracted at pH 11.0, but the majority of the muramidase-1 activity (80%) was extracted at pH 12.0 (data not shown).

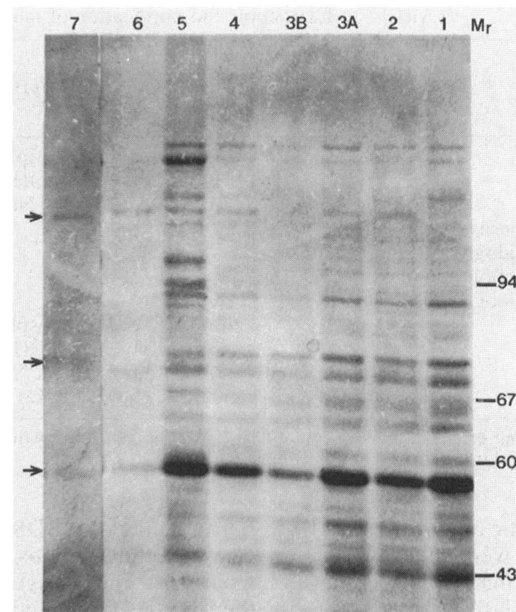

FIG. 1. Protein profile of the fractions from the extraction and purification of muramidase-2. A 50-μl portion of each fraction as listed in Table 1 was used for SDS-PAGE analysis. The lanes correspond to the fraction numbers shown in Table 1. Arrows indicate the locations of the 125-, 75-, and 55-kDa bands.

Of the 520 mg of protein extracted at pH 11.0, only 7% (37 mg of protein) bound to the *S. faecium* peptidoglycan. However, all of the muramidase-2 activity in the extract was bound (Table 1, fraction 3B). Binding to the peptidoglycan was found to be time dependent. With no incubation period, only 50% of the total muramidase-2 was bound to 100 mg of peptidoglycan. Incubation for 15 min at 0°C resulted in 70% of the activity being bound, and after 30 min at 0°C, all of the muramidase-2 activity was bound to the peptidoglycan. The peptidoglycan-protein complex was sequentially washed at pHs 8.0 and 10.0. Elution of the complex at pH 11 to 12 resulted in the recovery of 87% (1,220 U) of the bound muramidase-2 (Table 1, fraction 7; Fig. 1, lane 7). This fraction contained only 0.55 mg of protein and had a specific activity of 2,219 U/mg of protein. However, of the 34 mg of protein bound to the peptidoglycan, only 36% was accountable by the successive washes and extractions. Additional alkaline extractions of the peptidoglycan-protein complex at pH 11 to 12 removed additional protein and accounted for 46% of the total protein bound (data not shown). The ratio of extraction volume to bound protein was important. Extraction with 2 ml resulted in the formation of an aggregate of extracted proteins. Such aggregates contained muramidase-2 activity which could be detected after dissolution in 6 M guanidine hydrochloride and renaturation, as well as high levels of protein staining bands at molecular masses 125, 75, and 55 kDa (data not shown).

Determination of molecular mass of muramidase-2. Upon SDS-PAGE, highly purified muramidase-2 (Table 1, fraction 7) showed three Coomassie blue staining bands at about 125, 75, and 55 kDa (Fig. 1, lane 7). This observation differed from previous data which showed a single band at about 75 kDa for muramidase-2 purified from culture medium by affinity binding to *M. luteus* SDS cell walls (19). To determine the location of muramidase-2 activity on SDS gels, fractions 6 and 7 (Table 1), containing samples of equal

TABLE 1. Extraction and purification of muramidase-2 from the insoluble fraction of disrupted *S. faecium*

Fraction	Wash (pH)	Vol (ml)	Protein (mg/ml)	Muramidase-2 activity		Purification (fold)
				Total (U)[a]	Specific (U/mg of protein)	
1	10 mM sodium phosphate (8.0)	200	1.6	144	0.45	
2	10 mM glycine-NaOH (10)	200	1.5	34	0.11	
3	Addition of 0.02 N NaOH (11)	200	2.6	1,400	2.7	1
A. Supernatant after peptidoglycan binding		210	2.3	0	0	
B. Fraction bound to peptidoglycan			37[b]	1,400	38	14
4	10 mM sodium phosphate (8.0)	15	0.55	0	0	
5	10 mM glycine-NaOH (10)	15	0.27	0	0	
6	(Addition of 0.01 N NaOH) (10-11)	5	0.06	27.2	91	33.5
7	0.01 N NaOH (11-12)	5	0.11	1,220	2,219	821

[a] One unit equals the decrease in optical density of 0.001/min, with *S. faecium* peptidoglycan as substrate.
[b] Total protein bound to *S. faecium* peptidoglycan.

enzymatic activities (150 U), were fractionated on SDS gels, the gels were sliced, and the protein was eluted, unfolded in 6 N guanidine hydrochloride, renatured, and assayed for muramidase-2 activity. Both the purified muramidase-2 (fraction 7 [Table 1]) and enriched muramidase-2 (fraction 6 [Table 1]) fractions showed the presence of muramidase-2 activity in gel slices corresponding to proteins at molecular masses of 125 and 75 kDa (Fig. 2). For both the enriched and highly purified samples, approximately 25% of the enzymatic activity applied to the gels was recovered after renaturation of the gel slices.

The bond hydrolyzed by muramidase-2. Although all previous data (7, 14, 23, 24) were consistent with the hydrolysis of only muramidase-sensitive bonds in the cell wall peptidoglycan of *S. faecium*, it was necessary to establish the hydrolytic specificity of muramidase-2. SDS-washed cell walls of *M. luteus* were incubated with muramidase-2 until wall dissolution was complete. The soluble products were reduced and labeled with NaB^3H_4, acid hydrolyzed, and analyzed by ion-exchange chromatography (26). The only radioactivity product detected behaved the same as standard borohydride-reduced muramic acid (muramicitol) in both an ion-exchange system and in an ascending paper chromatographic system (data not shown). These data indicate that, under the conditions used, muramidase-2 hydrolyzed the bond between *N*-acetylmuramic acid and *N*-acetylglucosamine, producing *N*-acetylmuramic acid termini that retain a reducible anomeric carbon. Thus, muramidase-2 is an *N*-acetylmuramoylhydrolase (EC 3.2.1.17).

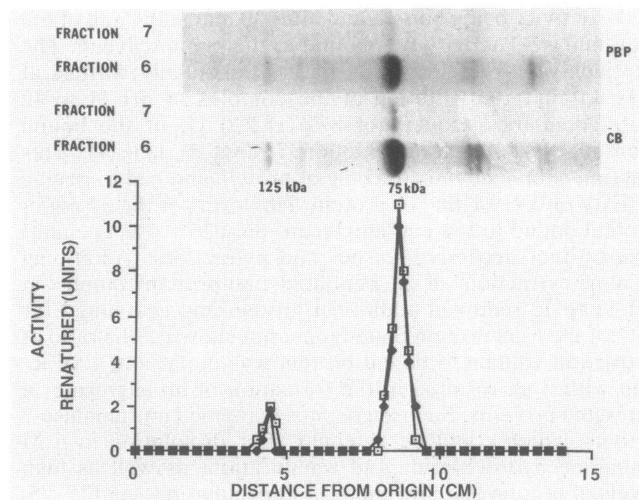

FIG. 2. Determination of the molecular masses of the proteins which bind [^{14}C]pen G and have muramidase-2 activity. To determine which protein bands contain muramidase activity, both purified (fraction 7; 150 U [■]) and partially purified (fraction 6; 150 U [□]) muramidase-2 fractions were subjected to SDS-PAGE and the lanes were separately sliced every 2 mm; the slices were extracted, denatured, and renatured, and enzyme activity was determined. In addition, equal volumes of fractions 6 (150 μg) and 7 (5 μg) were prebound with 4 μg of [^{14}C]pen G (56 mCi/mmol) for 15 min at 37°C, electrophoresed, stained with Coomassie blue (CB), and analyzed for the presence of PBPs.

Penicillin-binding experiments. Extracts of intact cells and of the insoluble fraction of disrupted cells (pH 11 to 12 [0.01 or 0.02 N NaOH]) were shown to contain all six of the PBPs previously observed in membrane preparations of this organism (D. L. Dolinger, I. Said, G. D. Shockman, and L. Daneo-Moore, manuscript in preparation). Incubation of fraction 7 (Table 1) with [^{14}C]pen G showed the presence of PBPs at 125 and 75 kDa as well as at 55 kDa (Fig. 2). The 125- and 75-kDa penicillin-binding activities migrated with the same mobilities as the protein bands that contained muramidase-2 activity (Fig. 2). Both PBPs 1 and 5 of *S. faecium* ATCC 9790 have molecular masses of 125 and 75 kDa in this SDS gel system (5). Fraction 6 contained several additional PBPs, including PBPs at about 200, 90 (PBP 2), 86 (PBP 3), and 80 (PBP 4) kDa, a PBP at 43 kDa (PBP 6), and one at 55 kDa. In fractions 6 and 7, the amounts of penicillin bound were approximately proportional to the amounts of Coomassie blue staining material (Fig. 2).

The concentrations of [^{3}H]pen G required to label each of the PBPs present in fractions 6 and 7 to 50% of maximum labeling after 90 min at 37°C were determined and compared with the values reported by two other laboratories (5, 12) for 50% binding by PBPs present in standard membrane preparations incubated for 15 (5) and 60 (12) min (Table 2). The pen G concentrations required to saturate PBPs 2, 4, and 6 by 50% in our extracts were similar to those reported by Fontana et al. (12) for the same PBPs in membrane preparations. Similarly, high concentrations of pen G were required for 50% saturation of PBP 5, both in membrane preparations (12) and in our extracts, displaying the known low-affinity characteristics of PBP 5. PBP 3 in fraction 6 showed a somewhat lower affinity than the corresponding protein in membrane preparations. The most striking differ-

TABLE 2. Affinity of pen G for binding to PBPs for 50% saturation of the protein

PBP (kDa)	Concn of pen G required for PBP saturation in:			
	Membrane preparations reported by:		Fraction[a]	
	Coyette et al.[b]	Fontana et al.[c]	6	7
1 (125)	0.21	0.33	5.52	6.24
2 (90)	0.10	0.22	0.38	
3 (85)	0.03	0.08	0.58	
4 (80)	0.16	1.20	2.72	
5 (75)	ND[d]	8.00	16.50	11.58
(55)	ND	ND	8.66	4.59
6 (43)	0.45	0.96	1.11	1.88

[a] Binding for 90 min.
[b] Binding for 15 min.
[c] Binding for 60 min.
[d] ND, Not determined.

ence observed was for PBP 1. The concentrations of pen G required for 50% saturation of the 125-kDa protein (PBP 1) in fractions 6 and 7 (5.5 to 6.2 μg/ml) were about 15 to 20 times higher than those (0.2 to 0.3 μg/ml) reported (5, 12) to saturate PBP 1 in membrane preparations and are similar to the corresponding values for the 75- (PBP 5) and 55-kDa proteins. It should be noted here that the proteins in fractions 6 and 7 have been briefly exposed to high pH at 0°C and are no longer in the same hydrophobic environment of the membrane.

The time course of binding of [^{3}H]pen G by proteins present in fractions 6 and 7 was also examined (data not shown). PBPs 1 and 5 and the 55-kDa PBP all bound pen G at a relatively slow linear rate for 60 to 90 min, as has been previously observed (12) for PBP 5 in membrane preparations. In contrast, PBP 6, present in some preparations of fraction 6, bound penicillin rapidly, consistent with previous observations (12) of PBP 6 activity in membrane preparations (data not shown).

To obtain further evidence that [^{14}C]pen G was bound to the proteins that correspond to muramidase-2 activity, purified muramidase-2 (300 μg, 630 U) was incubated with [^{14}C]pen G (8 μg) for 1 h at 37°C, and the presumably labeled muramidase-2 was then bound to and eluted from an affinity column consisting of a MAb (A12F7F10), shown to be specific for muramidase-2, covalently linked to CH Sepharose 4B. Samples of material that was not specifically bound to the column (and that, therefore, was washed off the column with 0.1% Triton X-100 and 0.5 M NaCl) and of the material that was bound to the MAb and specifically eluted with 0.2 M NH_4OH were counted. Approximately 85% of the ^{14}C applied (32,700 cpm) was bound to the column, and the ^{14}C not bound to the column did not precipitate with cold 5% trichloroacetic acid and, therefore, was considered to be free [^{14}C]pen G. Approximately 76% (24,000 cpm) of the applied counts were recovered in the 0.2 M NH_4OH eluate, whereas only about 1.5% (500 cpm) of the ^{14}C applied to the column was recovered in the Triton X-100–0.5 M NaCl wash. Figure 3 shows the SDS-PAGE analysis of the fraction eluted with 0.2 M NH_4OH (lane A) and the combined fractions eluted with 0.1% Triton X-100 and 0.5 M NaCl (lane B). Proteins at molecular masses 125, 75, and 55 kDa both bind [^{14}C]pen G and are specifically bound to the MAb column. Since the 55-kDa band was not seen in the initial preparation applied to the MAb column, the data were interpreted as indicating that the newly appearing band at 55 kDa was derived from the 75- or 125-kDa form of the enzyme by proteolysis or exposure to 0.2 M NH_4OH. Data presented elsewhere (Dolinger et al., manuscript in preparation) suggest that the 55-kDa polypeptide is a stable proteolytic fragment that retains PBP activity but not muramidase activity.

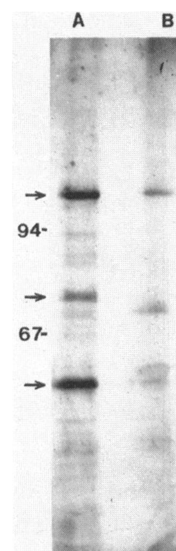

FIG. 3. SDS-PAGE analysis of the proteins bound to and eluted from a MAb (A12F7F10) affinity column specific for muramidase-2. Samples were handled as described in the text. Lane A is the silver-stained profile of proteins which were specifically bound to and eluted from the column with 0.2 M NH_4OH. Lane B is the profile of the proteins which were not specifically bound to the affinity column and which were eluted with 0.1% Triton X-100 and 0.5 M NaCl. Arrows indicate the location of the 125-, 75-, and 55-kDa bands.

The NH_4OH-eluted fraction was concentrated, applied to an anion-exchange column (Mono Q fast protein liquid chromatography system; Pharmacia), and eluted with a linear gradient of 0 to 0.5 M NaCl. Mono Q fractionation of the NH_4OH eluant (Fig. 4) clearly showed elution of mura-

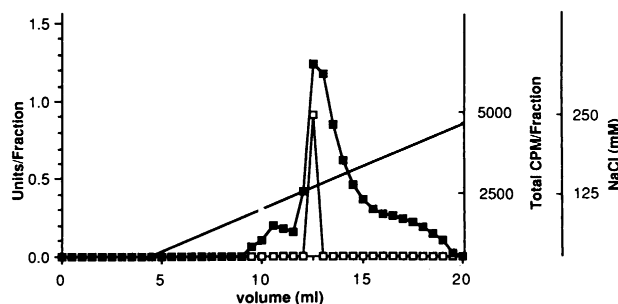

FIG. 4. Profile of proteins specifically bound to and eluted from the anti-muramidase-2 column fractionated over an anion-exchange column. A concentrated sample (200 μl) of the proteins specifically bound to and eluted from the anti-muramidase-2 column with 0.2 M NH_4OH was fractionated over a Mono Q column. The protein was loaded in 10 mM sodium phosphate, pH 7.0, and eluted with a linear gradient of NaCl (0 to 250 mM). Fractions (0.5 ml) were collected, 100-μl samples were assayed for radioactivity, and the remainder of the fraction was used to determine enzyme activity, utilizing *S. faecium* peptidoglycan as the substrate. □, Units per fraction; ■, counts per minute (CPM) per fraction.

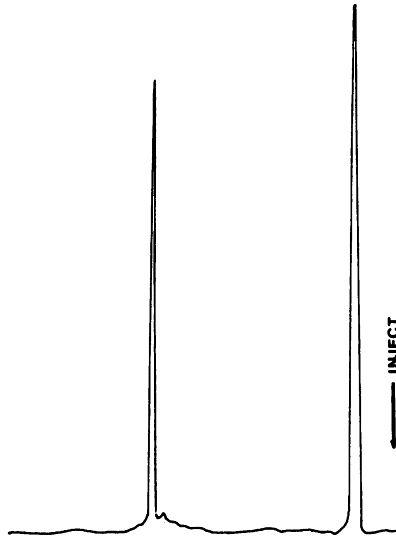

FIG. 5. Reverse-phase chromatography of fraction 7 (Table 1). Fraction 7 (100 μg, 500 μl) was chromatographed on a Pro RPC column (5 × 100 mm; Pharmacia) in 0.1% trifluoroacetic acid in water. Protein was then eluted with a linear gradient of 0 to 100% of 0.1% trifluoroacetic acid in acetonitrile. Elution was monitored at 280 nm.

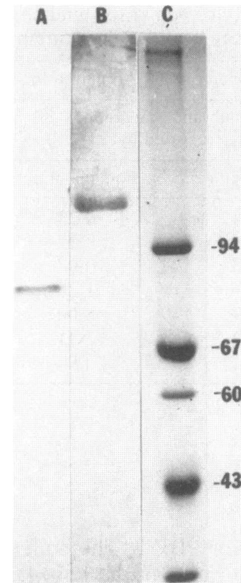

FIG. 6. SDS-PAGE analysis of the 125-kDa and 75-kDa forms of muramidase-2 separated by reverse-phase chromatography (described in the legend to Fig. 5). Lane A, The first peak eluted from the reverse-phase column; lane B, the second peak eluted from the reverse-phase column; lane C, molecular mass standards. Lanes were stained with Coomassie blue.

midase-2 activity at 12.5 ml, which was accompanied by elution of the majority of the ^{14}C applied to the column. The 12.5-ml peak was collected, concentrated, and analyzed by SDS-PAGE. Fluorography of this gel showed the presence of three ^{14}C-labeled proteins of 125, 75, and 55 kDa.

Relationship between the two forms of muramidase-2. Although indirect data suggested that the 125-kDa protein was a precursor of the 75-kDa protein, to obtain evidence of a direct relationship between the two proteins the 125-kDa protein was separated from the 75-kDa protein by reverse-phase chromatography (Fig. 5 and 6). The fraction containing the purified 125-kDa protein was incubated with [^{14}C]pen G (5.2 μCi, 40 μg/ml) for 60 min at 37°C. The protein-pen G complex was then incubated with 5 μg of protein of fraction 3 (Table 1) prepared in the absence of proteinase inhibitors. At intervals, the reaction was stopped (100°C, 5 min) and analyzed by reverse-phase chromatography. After 1 h at 37°C, 30% of the 125-kDa form was lost and replaced by the 75-kDa form (Table 3). After 2 h, 90% of the radioactivity and protein were found as the 75-kDa form of the protein.

DISCUSSION

A second peptidoglycan hydrolase of *S. faecium* was obtained in a highly purified form from mechanically disrupted cells (Fig. 1; Table 1) and shown to be an *N*-acetylmuramoylglycan hydrolase. Highly purified enzyme preparations contained two polypeptides (125 and 75 kDa), both possessing muramidase activity and both able to specifically bind radioactive pen G (Fig. 2 and 4). The molecular masses of these two polypeptides correspond to those previously found for PBPs 1 and 5 in standard membrane preparations of *S. faecium* (5, 12).

Evidence that the 75-kDa form is derived from the 125-kDa form and retains its ability to bind pen G was obtained (Table 3). Since muramidase-2 activity is not affected (neither inhibited nor stimulated) by pen G, we assume that both forms of the enzyme possess separate sites for muramidase activity and pen G-binding activity. The 55-kDa pen G-binding protein could be a further derivative that retains only the pen G-binding site. The 75-kDa form of the enzyme is present in the culture medium (19), and preliminary data suggest that this form may have *trans*glycosidase activity (J. F. Barrett, personal communication). Thus, muramidase-2 might have both *trans*peptidase and *trans*glycosidase activities, similar to those of the high-molecular-weight PBPs of *Escherichia coli* (17, 18, 22), and thus be involved with wall biosynthesis as well as hydrolysis.

Muramidase-2 activity could work in concert with and potentiate the processive hydrolytic action of muramidase-1 (1), which requires binding of the enzyme to nonreducing ends of glycan chains (1). Muramidase-2 hydrolysis in the midst of glycan chains would increase the number of binding sites (and effective substrate concentration) for muramidase-1. Extracellular (75-kDa) muramidase-2 may function in facilitating cell separation, consistent with one aspect of the phenotype of a thermosensitive mutant of *S. faecium*, Lyt-14 (25). Lyt-14 appears to possess a defect in muramidase-2 activity at 42°C and, at the nonpermissive temperature

TABLE 3. Relationship between the 125- and 75-kDa proteins

Sample and incubation time	Counts per minute of protein at:	
	125 kDa	75 kDa
Protein-pen G complex		
Initial (time zero)	17,400	617
1 h	11,280	4,780
2 h	1,949	19,044
Control		
Initial (time zero)	21,350	318
2 h	20,870	335

(42°C), grows in long chains. However, since we were unable to detect cell wall turnover in this organism (2), most of the external wall surface appears to be, in some unknown way, protected from the action of extracellular muramidase-2.

A correlation between the level of pen G resistance and the amount of PBP 5 seen on fluorograms has been observed in penicillin-resistant laboratory and clinical isolates of *S. faecium* (6, 12, 27). Data indicating that spontaneous penicillin-resistant mutants of *S. faecium*, which were shown to contain increased levels of PBP 5 (12), autolyzed more rapidly than the parent strain were interpreted to suggest that PBP 5 "...possesses both peptidoglycan polymerizing and depolymerizing activities..." (11). Canepari et al. (4) proposed that PBP 5 alone is sufficient for cell growth in cells that divide at a slow rate. However, at a normal growth rate (doubling time of approximately 35 min), PBP 5 does not appear to be essential for cell growth (13) and can compensate for only the partial inactivation of the other essential PBPs.

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