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Autolytic System of *Staphylococcus simulans* 22: Influence of Cationic Peptides on Activity of *N*-Acetylmuramoyl-L-Alanine Amidase

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Pep 5 and nisin are cationic peptide antibiotics which in addition to their membrane-disruptive action induce autolysis in staphylococci. To investigate the mechanism of lysis induction, the influence of the peptides on the activity of the *N*-acetylmuramoyl-L-alanine amidase of *Staphylococcus simulans* 22 was studied. In experiments with isolated cell walls at low ionic strength, the amidase activity was stimulated by the addition of Pep 5 and nisin, as well as by polylysine, streptomycin, and mono- and divalent cations. The concentrations necessary for activation depended on the nature of the cation and ranged from 5 μM for poly-L-lysine (n = 17) to 150 mM for Na^+ at a cell wall concentration of 100 μg of cell walls per ml. No effect was observed if the cell walls were devoid of polyanionic constituents. Kinetic data suggested that the amidase bound to the teichoic and teichuronic acids of the cell wall and was thereby inhibited. Cationic molecules reversed this inhibition, most likely by displacing the enzyme from the polyanions. If the concentrations of the larger peptides were high in relation to cell wall concentration, the activation turned into inhibition, presumably by interfering with the access of the enzyme to its substrate. These experiments demonstrate that the activity of the amidase is modulated by basic peptides in vitro and help to explain how Pep 5 and nisin may cause lysis of treated cells.

Pep 5 and nisin are small cationic peptide antibiotics which are produced by *Staphylococcus epidermidis* 5 and *Streptococcus lactis*, respectively. Both were reported to consist of mixtures of closely related, structurally similar peptides (M_r, 3,500; isoelectric point, >10.5) and to contain the rare thioether amino acid lanthionine (10, 24). Pep 5 adsorbs to gram-positive bacteria via ionic interaction with negatively charged cell wall polymers (teichoic, teichuronic, and lipoteichoic acids [LTA]) (25). The primary target of Pep 5 and nisin is the cytoplasmic membrane, which becomes permeable for small molecules such as K^+, amino acids, and ATP (19, 20, 23). The concomitant breakdown of the membrane potential is the likely reason for cessation of biosynthesis. Additionally, lysis of treated cells sets in rapidly and initiates a substantial amount of wall hydrolysis (2). Wall destruction occurs primarily in the area of the septum, as revealed by electron microscopy (3).

To elucidate the induction mechanism of lysis, we tried to imitate the effect of Pep 5 on membrane potential by incubating cells in the presence of carbonyl cyanide *m*-chlorophenylhydrazone or valinomycin, which induce lysis in *Bacillus subtilis* (11). In contrast, we could not observe a similar effect in the Pep 5 indicator organism *Staphylococcus simulans* 22 (formerly *Staphylococcus cohnii* 22) (2). However, autolysin activity could be demonstrated in the supernatant of a cell suspension of *S. simulans* treated with Pep 5, including at least two different autolysins which hydrolyzed the glycan chain and peptide bridges of the murein. The strongly basic peptide Pep 5 was shown to associate with the polyanionic LTA (2, 25) which have been discussed as potential regulators of autolysin activity (8, 18). The autolysins of *S. simulans* were inhibited in the presence of LTA and were reactivated by addition of a sufficient quantity of Pep 5. Unexpectedly, the activity of the partially purified autolysins was also stimulated by Pep 5 (2) in the absence of LTA.

To investigate this phenomenon in view of a possible regulatory mechanism, we isolated two autolysins of *S. simulans* 22 and report on the interaction among *N*-acetylmuramoyl-L-alanine amidase, cell walls, and basic peptides. It is demonstrated that addition of small quantities of Pep 5, nisin, or poly-L-lysine at low ionic strength led to a stimulation of enzyme activity if teichoic or teichuronic acids were present in the cell walls.

MATERIALS AND METHODS

Strains and growth media. The Pep 5-producing strain *S. epidermidis* 5 and the indicator strain *S. simulans* 22 (formerly *S. cohnii* 22) are clinical isolates. *Micrococcus luteus* ATCC 4698 was purchased from the American Type Culture Collection, Rockville, Md.

All strains were maintained routinely on blood agar plates. For preparation of purified cell walls or autolysin, strains were grown at 36°C in 20-liter batches of tryptone soya broth (Oxoid Ltd., Basingstoke, England) under vigorous aeration.

Purification of Pep 5. Purification and activity testing of Pep 5 have been described previously (22). Pep 5 was additionally subjected to RP C18 high-pressure liquid chromatography (HPLC); only the most prominent peak, P_5, was used for activity measurements of the amidase (24).

Analytical methods. Protein was determined as described by Lowry et al. (14), with bovine serum albumin as a standard. Phosphate determination was performed by the method of Fiske-Subbarow modified by Chen et al. (4) after hydrolysis in 0.1 ml of $HClO_4$ (70%) at 165°C for 2.5 h. D-alanine was estimated after hydrolysis of D-alanine esters in 0.1 M sodium hydroxide at 37°C for 3 h by the method of Graßl (9). Free amino groups and reducing sugars in cell wall

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hydrolysates were determined with 2,4-dinitrofluorobenzene and by a modified Park-Johnson procedure, respectively (6).

Preparation of cell walls. Purified trypsinized cell walls were prepared from *S. simulans* 22 and *M. luteus* as previously described (25). Teichoic acids were extracted by hydrolysis with 70% HF for 3 h at 0°C (13). HF was neutralized by addition of 6 N KOH, and the cell walls were extensively washed with distilled water. They tended to aggregate and had to be resuspended by thorough ultrasonic vibration (Bransonic 12, Branson Sonic Power Co., Danbury, Conn.) after each washing.

To prepare cell walls in which substitution of D-alanine ester was maintained in teichoic acids, the cells were broken in an MSK homogenizer with 0.1 M citrate-sodium hydroxide (pH 3). They were washed in the same buffer and stirred for 2.5 h in the presence of 2% sodium dodecyl sulfate (SDS) at 40°C. After several washings with citrate buffer, they were dialyzed against sodium acetate buffer (pH 4.75), washed several times in the same buffer, and stored at −20°C (further referred to as SDS-walls). All cell walls were homogenized with glass beads before use. They were assayed for purity by hydrolysis and reversed-phase HPLC of amino acids and amino sugars after derivatization with *o*-phthaldialdehyde (24).

Purification of autolysins. *S. simulans* 22 was grown aerobically in tryptone soya broth containing 2 ml of silicon antifoam emulsion. The cells (70 to 80 g [wet weight]) were harvested by centrifugation after 14 h of growth (A_{600}, 3), chilled on ice, and washed once in 50 mM Tris hydrochloride (pH 7) containing 20 μM phenylmethylsulfonyl fluoride.

The autolysins were extracted by suspending the cells in 50 mM Tris hydrochloride (pH 7) containing 20 μM phenylmethylsulfonyl fluoride, 2.5 M NaCl, and 5.7×10^{-3} U of carboxylic-ester hydrolase (EC 3.1.1.1) (Sigma Chemical Co., Munich, Federal Republic of Germany). The cells were collected by centrifugation (25 min, 22,100 × *g*) at 10°C, and the extraction was repeated. The supernatant was dialyzed twice against 10 liters of Tris hydrochloride (pH 7) at 6°C. After dialysis, insoluble material was removed by centrifugation (20 min, 22,100 × *g*) if necessary. All column chromatographies were executed at 2°C.

Ecteola cellulose anion-exchange chromatography. The dialysate then was loaded onto an Ecteola cellulose column (200 ml) equilibrated in 20 mM Tris hydrochloride (pH 7) containing 10 mM NaCl. The column was washed with 2 to 3 bed volumes of the same buffer; for elution a linear gradient of NaCl (10 to 860 mM) in a 20 mM Tris hydrochloride buffer (pH 7) was used. All fractions were monitored for activity and organic phosphate to detect contamination of the enzyme preparation with LTA. Active fractions containing no organic phosphate were pooled, desalted, and concentrated by ultrafiltration (YM10 membrane; Amicon Corp., Lexington, Mass.) with 50 mM sodium acetate (pH 6) containing 1 mM $MgCl_2$ and 0.5 M NaCl.

Peptidoglycan affinity chromatography. The peptidoglycan affinity gel was prepared by coupling a lysate of purified cell walls of *S. simulans* 22 to CNBr-activated Sepharose 4B. The cell walls had been trypsinized, treated with NaOH (0.5 N, 3 h, 37°C) to reduce teichoic acid content, and digested with lysozyme (26).

The sample was applied to a column of Sepharose 4B peptidoglycan resin (9 ml) equilibrated in 50 mM sodium acetate (pH 6) containing 1 mM $MgCl_2$ and 0.5 M NaCl. The column was washed with 2 to 3 bed volumes of the following buffers: 50 mM sodium acetate buffer (pH 6)–1 mM $MgCl_2$–0.5 M NaCl; 50 mM sodium acetate buffer (pH 6)–1 mM $MgCl_2$–3 M NaCl; 0.1 M glycine-sodium hydroxide (pH 7)–1 mM $MgCl_2$. The activity was eluted by a combined gradient of salt and pH with the use of 0.1 M glycine-sodium hydroxide (pH 7) and 0.1 M sodium hydroxide (pH 10.6)–1.8 M NaCl. All fractions (1 ml) were collected in tubes already containing 2 ml of 0.1 M sodium acetate buffer (pH 5.5)–0.2 M NaCl, and active fractions were pooled. As removal of salt at this stage resulted in considerable loss of activity, the enzyme was stabilized by the addition of 0.1 mg of bovine serum albumin per ml, and the sample was desalted by ultrafiltration with 50 mM sodium acetate (pH 6)–1 mM $MgCl_2$.

Cation-exchange chromatography on CM Sepharose CL-6B. The enzyme extract was loaded onto a CM Sepharose CL-6B column (31 ml) equilibrated in 50 mM sodium acetate (pH 6)–1 mM $MgCl_2$. The column was washed with the same buffer and eluted with a linear gradient of NaCl (0 to 1 M) in the same buffer. Active fractions of the amidase and glucosaminidase plus amidase were pooled, concentrated by ultrafiltration, and diluted with 0.8 M sodium acetate (pH 6). In this buffer the purified amidase could be stored at −20°C for several months without considerable loss of activity.

Estimation of molecular weight. SDS-polyacrylamide gel electrophoresis was performed by the method of Weber et al. (27) in 0.8-mm horizontal slab gels containing 15% polyacrylamide, 0.38% diallyltartardiamide or bisacrylamide, and 0.1% SDS. Protein was precipitated by the addition of small samples of 20% trichloroacetic acid, collected by centrifugation, and taken up in 20 μl of sample buffer containing 2% SDS. Gels were stained with Coomassie blue or (for carbohydrates) with periodic acid and Schiff's reagent (15).

Gel filtration was performed on a Sephacryl S200 column (0.8 by 46.5 cm) in 0.1 M potassium phosphate buffer (pH 7)–1 mM $MgCl_2$. The column was calibrated with ferritin (M_r, 440,000), aldolase (M_r, 158,000), ovalbumin (M_r, 45,000), and cytochrome *c* (M_r, 12,384) as molecular reference proteins.

Isoelectric focusing. Isoelectric focusing was carried out on PAG plates (LKB, Bromma, Sweden) (pH 3.5 to 9.5) at 4°C for 1.5 h according to the directions of the manufacturer. The amidase was desalted by ultrafiltration (YM 10 membrane) in 0.1% glycine and then concentrated by evaporation. The plates were stained with silver stain (Bio-Rad, Munich, Federal Republic of Germany).

Specificity of enzymes. (i) Amidase. The amidase activity was identified after dinitrophenylation of the N-terminal amino acid in the reaction product, hydrolysis, and subsequent thin-layer chromatography as described by Ghuysen et al. (6), as well as after Edman degradation (1) of digested cell walls and identification of the terminal amino acid as a phenylthiohydantoin derivative. Phenylthiohydantoin amino acids were separated by reversed-phase HPLC on a C18 column (5 μm; Bischoff, Leonberg, Federal Republic of Germany) with 10 mM sodium phosphate (pH 6.5) (A) and methanol–3% (vol/vol) tetrahydrofuran (B) at 45°C and a flow rate of 1.5 ml/min (15 to 45% in 36 min). Amino acids were detected at 254 nm.

(ii) Glycosidase. To specify the glycosidase activity, a digest of cell walls was dialyzed against distilled water, and only those fragments that had passed the dialysis tube (M_r cut off, 10,000) were used for determinations. The fragments were reduced with freshly prepared 0.1 M $NaBH_4$ (6). The reaction products were hydrolyzed (16 h, 110°C, 4 N HCl) and separated after derivatization with *o*-phthaldialdehyde

TABLE 1. Purification of *N*-acetylmuramoyl-L-alanine amidase of *S. simulans* 22

Purification steps	Total activity (U)[a]	Protein (mg)	Sp act (U/mg of protein)	Yield (%)	Purification (fold)
Salt extract	28,900	186	155	100	
Dialyzed extract	27,400	123	223	95	1.4
Anion-exchange chromatography[b]	18,100	22	834	63	5.4
Peptidoglycan affinity chromatography[b]	8,900	3.5	2,600	31	16.8
Cation-exchange chromatography[b]	5,290	0.53	9,980	18	64.4

[a] Separation of the *N*-acetylmuramoyl-L-alanine amidase from the β-*N*-acetylglucosaminidase was achieved during cation-exchange chromatography. Therefore, the activity of glucosaminidase may have interfered with the activity determination of the amidase in the first purification steps. However, it was noted throughout the purification that glucosamindase was probably not active on cell walls of *S. simulans* 22 in the absence of amidase when the optical test was conducted.

[b] Pooled fractions.

on an HPLC reversed-phase C18 column. *O*-Phthaldialdehyde derivatization and chromatographical details have already been published (24).

Assay of autolysin activity. The activity of the amidase was routinely measured at 37°C in a reaction mixture containing 10 to 250 μg of purified cell walls in 20 mM sodium acetate buffer (pH 6) in a total volume of 1 ml. The reaction was started by the addition of enzyme (0.64 μg of protein) and monitored by recording the optical density at 600 nm. An A_{600} of 0.3 was not exceeded. Reaction rates were calculated from initial velocity measurements, and for Lineweaver-Burk plots, a statistical evaluation (linear regression analysis) was undertaken. One unit of enzyme was defined as the amount of protein that decreased the A_{600} by 0.001/min with a suspension of purified cell walls of *S. simulans* 22 in 20 mM sodium acetate buffer (pH 6). As purified cell walls are an insoluble substrate, all kinetic data are given in milligrams per milliliter.

It was noticed that addition of cations to purified cell walls resulted in an increase of optical density depending on type and concentration of the polycation and the cell walls. Therefore, the shift in optical density was determined for all peptides, and streptomycin and substrate concentrations were corrected accordingly.

Enzyme activity was also assayed with cell walls which had been coupled to fluorescamine (16). The reaction was stopped after 2 min of incubation by the addition of 5 μl of 20% SDS. Residual cell walls were removed by centrifugation, and fluorescence in the supernatant (0.7 ml) was measured after dilution with 1.3 ml of 0.5 M sodium borate buffer (pH 8.5) by using a Shimadzu spectrofluorometer RF-540 with the excitation wavelength set at 390 nm and emission wavelength set at 475 nm. The pH optimum of the amidase was determined in 40 mM sodium acetate buffers (pH 4.0 to 6.5) and 40 mM 3-(*N*-morpholino)-propanesulfonic acid–sodium hydroxide buffers (pH 6.0 to 8.0).

Materials. Biochemicals were obtained from Sigma Chemical Co., Deisenhofen, Federal Republic of Germany. Ecteola cellulose, lysyl-lysine, poly-L-lysine (n = 5), and streptomycin sulfate were purchased from Serva, Heidelberg, Federal Republic of Germany. CM Sepharose CL6B, CNBr-activated Sepharose 4B, and Sephacryl S200 were obtained from Pharmacia AB, Uppsala, Sweden. Nisin was purchased from Koch&Light, Colnbrook, England. Hydrogen fluoride, 72 to 75%, p.a., was a gift from Bayer, Leverkusen, Federal Republic of Germany. Chemicals for SDS-polyacrylamide gel electrophoresis were supplied by Bio-Rad Laboratories, Richmond, Calif.

RESULTS

Purification of autolysins of *S. simulans* 22. A purification protocol is shown in Table 1. The enzymes were extracted from the cells by treatment with 2.5 M NaCl. Despite the high isoelectric point of the amidase, the enzyme adsorbed to an anion exchange resin. This interaction is probably mediated by a complex of the enzyme with polyanions such as LTA, which was found in enzyme-containing fractions.

The autolysins bound specifically to the Sepharose 4B peptidoglycan resin and could only be eluted by alteration of pH and ionic strength. The cation-exchange chromatography served to separate the autolysins from each other (Fig. 1).

The activity was eluted in two peaks. The enzyme that produced free amino groups in a digest of cell walls was associated with peak 1. In SDS-polyacrylamide gel electrophoresis, this enzyme migrated at least in two bands (M_rs, ~48,000 and ~50,000), and in some preparation batches, up to six close bands were discernible (M_rs, ~48,000 to ~56,000) (Fig. 2). The higher-molecular-weight bands only occurred when freshly prepared affinity gels were used. Attempts to separate the bands on an HPLC column by reversed-phase and ion-exchange chromatography or chromatofocusing were unsuccessful. It seems possible that repeating units of the peptidoglycan were bound to the enzyme which could not be removed by SDS treatment, as the enzyme could be stained with Schiff's reagent after treatment with periodic acid, which is specific for carbohydrates.

Peak 2 contained an enzyme that hydrolyzed the glycan chain of the murein, liberating free reducing groups. It also migrated in several bands after peptidoglycan affinity chromatography and displayed an M_r of ~57,000 (Fig. 2).

Mode of autolytic enzyme action. To identify the cleavage site of the smaller enzyme, the amino groups released from murein were labeled with 2,4-dinitrofluorobenzene (6). After hydrolysis and thin-layer chromatography, alanine was identified as the N-terminal amino acid. An *N*-acetylmuramoyl-L-alanine amidase releases L-alanine, and a peptidase liberates D-alanine. To distinguish between amidase and peptidase, the digest was subjected to Edman degradation,

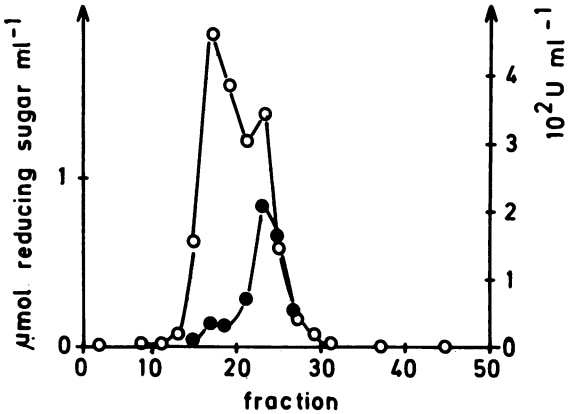

FIG. 1. Separation of the autolysins of *S. simulans* 22 on CM Sepharose CL-6B. The enzymes were eluted by a gradient of 0 to 1 M NaCl. Symbols: ○, total enzyme activity tested with *M. luteus* cell walls as substrate; ●, release of reducing sugars from the cell walls.

which again yielded alanine in cycle 1. In cycle 2, no amino acid was released, which agrees with D-isoglutamine as the next amino acid. In cell walls of staphylococci, D-isoglutamine is linked by its γ-carboxyl group to lysine, and only its α-amino group reacts with phenylisothiocyanate (5). To verify this result, murein obtained after cycle 1 of Edman degradation was again labeled with 2,4-dinitrofluorobenzene, and glutamine (i.e., glutamate after acidic hydrolysis) was identified as second amino acid. This proved that the enzyme acts as *N*-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28), which cleaves the bond between *N*-acetylmuramic acid and D-alanine.

The second enzyme liberated reducing groups. To specify whether a glucosaminidase or a muramidase was involved, the location of the reducing group in *N*-acetylglucosamine or *N*-acetylmuramic acid had to be determined. Small fragments from peptidoglycan digests, mostly disaccharides, were reduced with sodium borohydride and characterized after hydrolysis and derivatization with *o*-phthaldialdehyde by chromatography on an HPLC reversed-phase C18 column. Unreduced samples were shown to contain glucosamine and muramic acid in a ratio of 1:1. After reduction, the glucosamine peak disappeared almost completely and was replaced by a new peak of glucosaminitol, whereas the amount of muramic acid remained unchanged. This demonstrated that *N*-acetylglucosamine carried the reducing group in the disaccharide that was produced by the glycosidase and thus characterized the enzyme as a β-*N*-acetylglucosaminidase.

Properties of *N*-acetylmuramoyl-L-alanine amidase. The molecular weight of this enzyme as determined by SDS-polyacrylamide gel electrophoresis was ~48,000. A value of ~44,000 was calculated after gel filtration on Sephacryl 200 under nondenaturing conditions, proving that the enzyme consisted of one peptide chain only.

When the isoelectric point was determined by isoelectric focusing on PAG plates (pH 3.5 to 9.5), the enzyme focused close to the cathode strip. Therefore, it must be concluded that the isoelectric point of the amidase is higher than 9.5. The amidase was active on *S. simulans* 22 cell walls, as well as on purified cell walls of *M. luteus* ATCC 4698. The amidase reached its maximum activity between pHs 5.5 and 6.5.

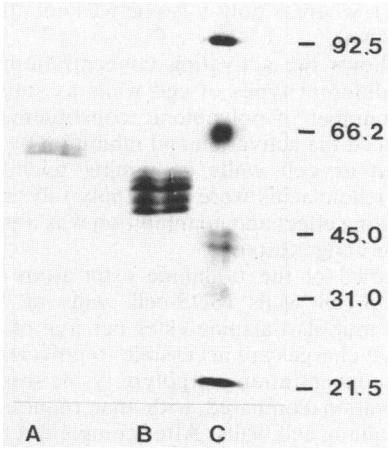

FIG. 2. SDS-polyacrylamide gel electrophoresis of the autolysins of *S. simulans* 22 in a 15% polyacrylamide gel. Lanes: A, β-*N*-acetylglucosaminidase (4 μg of protein); B, *N*-acetylmuramoyl-L-alanine amidase (25 μg of protein); C, M_rs of standard peptides given in thousands.

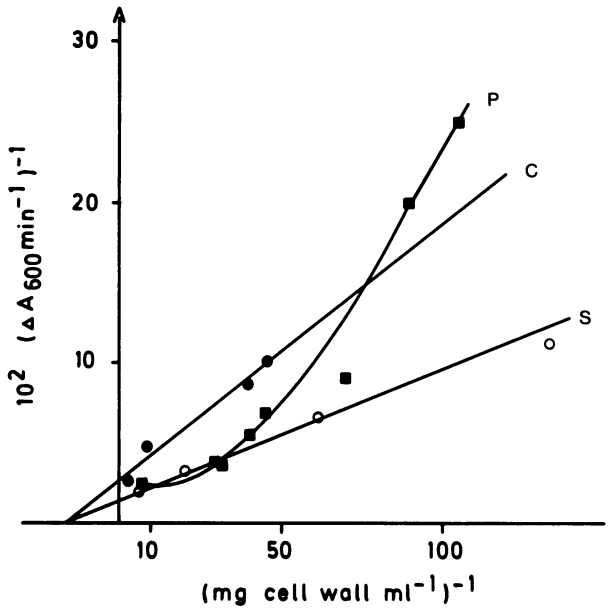

FIG. 3. Influence of polycations on the activity of amidase. Shown is a double-reciprocal plot of SDS-cell wall hydrolysis. C, Control (20 mM sodium acetate, pH 6); S, addition of 0.1 mM streptomycin; P, addition of 0.1 mM poly-L-lysine (n = 5).

Kinetics of amidase. To investigate whether the stimulation of autolysis by nisin and Pep 5 can be deduced from an effect of the basic peptides on the activity of the amidase, we studied the influence of inorganic and organic cations on the kinetics of this enzyme. The dependence of the reaction rate on substrate concentration corresponded to Michaelis-Menten kinetics. An apparent K_m of 36.8 μg of cell walls per ml and a corresponding V_{max} of 4.79 mg of cell walls of *S. simulans* 22 per ml per min were determined for enzyme activity in 20 mM sodium acetate (pH 6.0).

Influence of mono- and divalent cations. Addition of monovalent cations (Na^+, K^+) to the assay mixture in 20 mM sodium acetate (pH 6.0) resulted in a mixed type of activation of the amidase; i.e., K_m was lowered and V_{max} increased. A broad range of maximum activity was obtained between 100 and 150 mM KCl or NaCl.

Divalent cations activated the enzyme in the same way as monovalent cations did, with maximum enzyme activity between 20 and 40 mM $MgCl_2$. The amidase had no essential requirement for divalent cations because it was not inhibited by the addition of EDTA. If ionic strength of the reaction mixture was raised beyond the range of maximum activity, the K_m of the enzyme was decreased, whereas V_{max} remained at its maximum value.

Influence of polycations. Addition of small organic cations (M_r, 300 to 500) with increasing numbers of positive charges, such as lysyl-lysine (5 mM) and streptomycin (0.1 mM), resulted only in a higher V_{max}, whereas K_m remained unchanged. Double-reciprocal plots were linear, as shown for streptomycin in Fig. 3. However, when larger polycations such as poly-L-lysine (n = 5 or n = 17), nisin, and Pep 5 were tested, the pattern became more complex, as indicated by the curved double-reciprocal plot for poly-L-lysine (n = 5) in Fig. 3. Activation of the amidase occurred only in a certain range of polycation concentration, and inhibition of the enzyme was observed when this range was exceeded. The

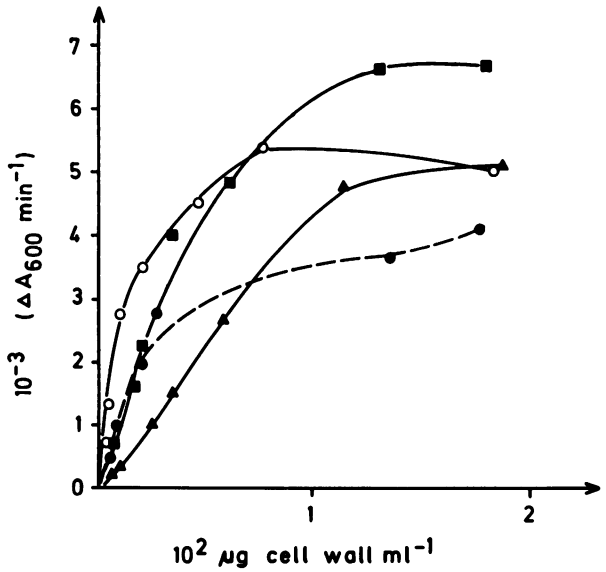

FIG. 4. Influence of different concentrations of poly-L-lysine (n = 17) on the activity of the amidase. Amidase activity was plotted versus cell wall concentration in the presence of 2 (○), 5 (■), and 10 (▲) µM poly-L-lysine (n = 17); the control (●) is also indicated. Assays were performed in 20 mM sodium acetate with SDS-cell walls of *S. simulans* 22 as the substrate.

modulation of enzyme activity by large polycations was affected by several factors, such as concentration and chemical properties of the peptide, nature of the polyanionic constituents of the substrate cell wall, and ionic strength of the assay.

The activating effect depended on the concentration of the basic peptide per milligram of cell wall. If the concentration of some peptides was too high in relation to cell wall concentration, the amidase activity was inhibited. This is demonstrated in Fig. 4 for poly-L-lysine (n = 17) at 2, 5, and 10 µM concentrations. It is shown that at a fixed high concentration of the polycation (e.g., 10 µM in Fig. 4), the amidase activity was inhibited at low substrate concentrations (less than 80 µg of cell wall per ml) and activated at high substrate concentrations (more than 80 µg of cell wall per ml). If the concentration ratio between cell wall and basic peptide was shifted to high cell wall concentrations and low concentrations of basic peptide, the influence of the basic peptide approached zero at high cell wall concentrations.

These differences in activity were not only detected by monitoring the optical density but were also reflected in the release of free amino groups and in experiments with fluorescamine-labeled cell walls (data not shown). The different activating potency of several peptides is indicated in Table 2, where the minimum concentrations necessary for activation and inhibition, as well as the concentration for maximum activation, are given.

Two properties of the peptides determined their influence on the amidase activity. (i) Activation was dependent on the number of monomeric units of the basic peptide. A comparison of lysyl-lysine and poly-L-lysine (n = 5 and n = 17, respectively) demonstrates that for the longer peptides, lower concentrations had to be used. (ii) Activation was dependent on the number of positive charges of the peptide. This is shown by a comparison of poly-L-lysine (n = 17 [17 positive charges]), Pep 5 (8 positive charges), and nisin (3 positive charges). The more positive charges a peptide carried, the lower were the concentrations necessary for maximum activation and inhibition. Furthermore, the inhibitory effect of nisin was not detectable in the concentration range studied, whereas it was relatively strong for poly-L-lysine (n = 17). This correlates well with in vivo experiments on intact cells: nisin was more effective in inducing lysis than Pep 5 was (2), whereas poly-L-lysine was not effective at all (data not shown).

Table 3 shows the activating concentrations of poly-L-lysine with different types of cell walls as substrates. The nature and number of polyanionic constituents in the cell walls influenced the activation and inhibition by the cations: when *M. luteus* cell walls containing teichuronic acids instead of teichoic acids were used, poly-L-lysine showed a strong activating effect and no inhibition was observed in the concentration range tested.

After removal of the D-alanine ester from the teichoic acids of SDS-cell walls (SDS-cell walls of *S. simulans* contain 0.36 mol of D-alanine ester per mol of phosphate), more negative charges are accessible to polycations. Therefore, higher concentrations of poly-L-lysine were necessary for full activation compared with that required for intact alanine-containing cell walls. After complete removal of the teichoic acids from the cell walls by treatment with 70% HF, no activation or inhibition by basic peptides could be demonstrated. This showed that the effect of the peptides is mediated via the polyanionic components of the cell wall.

Finally, the ionic strength of the assay was also important.

TABLE 2. Influence of polycations on activity of amidase

Polycation[a]	Amt (µmol/mg of cell wall) of polycation necessary for[b]:		
	Activation	Maximum activation	Inhibition
Streptomycin	0.5–11	0.5–11	None
Poly-L-lysine			
n = 2	20–1,100	20–1,100	None
n = 5	0.5	3	7.7
n = 17	0.025	0.05	0.1
Pep 5	0.05	0.8	1.9
Nisin	0.05	2.7	None

[a] The substrate was *S. simulans* 22 cell walls containing D-alanine ester.
[b] Obtained from double-reciprocal plots (e.g., Fig. 3). The concentration ranges tested were 0.5 to 11 µmol/mg of cell wall for streptomycin and 20 to 1,100 µmol/mg of cell wall for poly-L-lysine (n = 2). Double-reciprocal plots are linear in these ranges of concentration. For nisin, the range tested was 0.005 to 2.7 µmol/mg of cell wall.

TABLE 3. Effect of poly-L-lysine (n = 17) with different cell walls as substrates

Type of cell wall	Amt (µmol/mg of cell wall) of poly-L-lysine necessary for[a]:		
	Activation	Maximum activation	Inhibition
M. luteus	0.025	1.0	None[b]
S. simulans 22 without D-alanine ester	0.02	0.3	0.5
S. simulans 22 with D-alanine ester[c]	0.025	0.05	0.1
Cell walls without teichoic acids	0	0	0

[a] Obtained from double-reciprocal plots (e.g., Fig. 3).
[b] Concentration range tested was 0.005 to 1.0 µmol/mg of cell wall.
[c] SDS-wall.

All measurements were performed in 20 mM sodium acetate buffer. If 200 mM Na^+ was added, no activation by basic peptides could be detected.

DISCUSSION

Autolysins are potentially dangerous enzymes to the cell and have to be strictly regulated to avoid damage to the cell envelope resulting in lysis of the protoplast and cell death. The experiments described in this paper were performed to explain the induction of autolysis by the basic peptides Pep 5 and nisin. The question was raised whether these peptides could influence the activity of the autolysins directly or whether autolysis has to be regarded as a secondary effect of membrane damage and subsequent deenergization.

Two autolytic enzymes of *S. simulans* 22 were isolated: *N*-acetylmuramoyl-L-alanine amidase and β-*N*-acetylglucosaminidase. Lytic glucosaminidase (M_r, 45,000), purified from the culture supernatant of *S. simulans*, has also been described by Valisena et al. (26).

The experiments with the purified amidase showed that basic peptides had a stimulating effect on autolysin activity which could be easily converted into inhibition. For these effects it was essential that ionic strength was low and the cell walls contained polyanionic constituents.

Activation of the autolytic system of *Staphylococcus aureus* by the cationic proteins lysozyme and ribonuclease has also been reported (28). Ginsburg and Lahav (7) proposed that staphylococcal lysis was promoted in the presence of low concentrations (10^{-9} to 10^{-7} M) and inhibited in the presence of higher concentrations (10^{-6} to 10^{-5} M) of polycations. Activation by cationic peptides has also been previously described for the galactosyltransferase from rat mammary golgi membranes (17). The authors of that study showed that the peptides meet a requirement for cations of the enzyme itself.

In view of the results described here, the explanation for the modulation of amidase activity is quite different. At low ionic strength, the amidase exhibits an affinity for polyanionic compounds, as previously demonstrated by its binding to LTA (2). Therefore, the amidase should also bind to the teichoic or teichuronic acids of the cell walls and thereby be inhibited. Binding presumably occurs via ionic interaction for which the high isoelectric point of the enzyme is a prerequisite. There are reports on the inhibition of staphylococcal autolysis by anionic polyelectrolytes (8, 29). The endo-β-*N*-acetylglucosaminidase of *B. subtilis* is also inhibited by LTA (18).

Basic peptides also associate with teichoic acids. This has been shown for Pep 5 (25), dihydrostreptomycin (12), and polylysine (data not shown). High ionic strength prevents binding of the peptides to polyanionic compounds (25). As the addition of a sufficient amount of Pep 5 can reverse the binding of the amidase to LTA (2), so mono- and divalent cations, small cationic molecules, and basic peptides are also probably able to prevent binding of the enzyme to or to displace it from teichoic and teichuronic acids, thereby causing activation. The concentration necessary for optimal activity ranged from 150 mM Na^+ to 5 μM poly-L-lysine (n = 17) at a cell wall concentration of 100 μg of cell walls per ml. This demonstrates that the activating property of a cation does not simply depend on the number of positive charges but rather reflects the affinity of the cations for the negatively charged polymers of the cell wall. Thus, the teichoic acids act as noncompetitive inhibitors. Therefore, the activity of the enzyme may be modulated by the interaction between the basic peptide and the inhibitors (teichoic and teichuronic acids) rather than by the interaction of the basic peptide with the enzyme itself. The inhibitory effect of basic peptides was not seen with smaller molecules (mono- and divalent cations, streptomycin, and lysyl-lysine) and became visible only when larger molecules were used. This could indicate a steric hindrance; at a fixed high concentration of the basic peptide, a part of the substrate may not be accessible to the enzyme.

The experiments reported here demonstrate that the activity of the amidase is modulated by the basic peptides in vitro. However, to evaluate the physiological significance of the activation mechanism described here, the following facts have to be taken into consideration. (i) The concentration ranges at which the membrane-active peptides Pep 5 and nisin activated the amidase are limited. (ii) In experiments with intact cells, the peptide-induced autolysis is enhanced after energization of the cells with glucose (2). As the membrane-disruptive action of the peptides is potential dependent, this treatment also increases membrane depolarization (20). (iii) Activation occurred only at low ionic strength and could also be achieved by an increase in ionic strength. Thus, any membrane leakage caused by Pep 5 or nisin would rapidly increase ionic strength in the adjacent cell wall areas, perhaps activating the amidase. On the other hand, treatment of cells with valinomycin, which should cause K^+ leakage and thereby display an effect similar to that of Pep 5 or nisin, did not stimulate autolysis (2). Therefore, it seems likely that the membrane-disruptive effect, as well as the binding of basic peptides to teichoic and teichuronic acids, is essential for the effective induction of autolysis observed earlier (2).

Cationic peptides have been isolated from a variety of mammalian and insect body fluids and seem to constitute a part of the unspecific host defense system (21). Furthermore, the activation of *S. aureus* autolysins has been shown to occur in the presence of lysosomal cationic protein from macrophages (8). It would be an interesting phenomenon if degradation of invading bacteria by cellular defense mechanisms were supported by activation of the endogenous bacterial autolytic system (8).

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