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Lysis of Modified Walls from *Lactobacillus fermentum*

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The *N* and *O* substitution in wall peptidoglycan from *Lactobacillus fermentum* was studied in relation to growth phase, as well as the lytic activities and the effect of trypsin on them. The *N*-nonsubstituted sites were determined by dinitrophenylation techniques. The results indicate that an extensive substitution at the *O* groups takes place as cells go into the stationary growth phase, concomitant with a decrease in their lysozyme sensitivity. *N*-nonsubstituted residues, mainly glucosamine, occurred in both exponential-phase and stationary-phase walls but not in the corresponding peptidoglycans. Small amounts of *N*-nonsubstituted muramic acid were detected in walls and peptidoglycan from cells in the stationary growth phase only. *N* acetylation of isolated walls did not increase their lysozyme sensitivity but rather decreased it. Autolysis of walls was completely inhibited by the chemical modifications used. Trypsin stimulates the lysozyme sensitivity of native walls but has no effect on walls that had been *O* deacetylated and *N* acetylated. It is suggested that the effect of trypsin is due to its action as an esterase removing the *O* acetylation in lysozyme-resistant walls.

Washed cells or isolated walls of *Lactobacillus fermentum* taken from the late stationary phase of growth are almost completely resistant to lysozyme, whereas exponentially growing cells show varying degrees of lysozyme sensitivity with a peak during the late exponential phase (12). Resistance of other organisms to lysozyme has so far been accounted for by the occurrence of *N*-nonsubstituted glucosamine residues (2), the presence of *O*-acetyl groups (5), a high degree of peptide cross-linking (16), the occurrence of free amino groups (14), or the attachment to the peptidoglycan of teichoic acid (9). Results of our earlier studies indicated, however, that the walls of *L. fermentum* do not contain any significant amounts of teichoic acid (17). To determine whether substitution at the *O* groups or deacetylation at the *N* groups was responsible for the variation of lysozyme sensitivity with growth phase, we studied the effect of such modifications of isolated stationary- or exponential-phase walls on their response to lysozyme. The occurrence of *N*-nonsubstituted sugars and amino acids in stationary- and exponential-phase walls and peptidoglycan was examined by means of dinitrophenylation techniques and chromatography. We reported earlier that trypsin and other proteinases significantly increase the lysozyme sensitivity of *L. fermentum* (4, 11, 12). The results of the present investigation indicate that this action of trypsin consists of the removal of *O* substituents from the cell wall.

MATERIALS AND METHODS

Reagents. Egg white lysozyme (three times crystallized), trypsin (twice crystallized), muramic acid, glucosamine-hydrochloride, and *N*-acetylglucosamine were from Sigma Chemical Co., St. Louis, Mo. D-[U-^{14}C]glucosamine-hydrochloride was purchased from the Radiochemical Centre Ltd., Amersham, England. 2,4-Dinitrofluorobenzene, 2,4-dinitrophenylamine, and 2,4-dinitrophenol (DNP) came from Merck, Darmstadt, Germany. All other chemicals were from Kebo AB, Stockholm, Sweden, and all were of reagent grade. DNP-glucosamine and its labeled counterpart, DNP-muramic acid, and the DNP derivatives of alanine, lysine, glutamic, and aspartic acids, used as reference compounds, were prepared in our laboratory from the corresponding amino acids and amino sugars.

Organism, growth media, conditions of culture, and cell harvesting. *L. fermentum* 36 (ATCC 9338) was maintained, stored, and cultivated as described earlier (13). Rogosa medium (6) was used throughout the investigation. Cultures (1.5 liters) were grown in 2-liter Erlenmeyer flasks equipped with side arms for sampling. Growth was followed by optical density measurements. To obtain exponentially growing cells, we diluted the cell suspension used as inoculum so that the exponential phase of growth began the following morning. A suspension with an absorbancy at 540 nm (A_{540}) of 0.7, diluted 10^6 and added at a λ% (vol/vol) level, was usually adequate to produce this effect after 18 h of incubation. Stationary-phase cells were harvested, after 36 to 48 h, from cultures inoculated to an A_{540} of 0.7 (λ%, vol/vol) without further dilution. The A_{540} of stationary-phase cultures was usually 1.7 to 2.0. All cultures were grown statically at 37 C. Cells were harvested in a refrigerated Sorvall centrifuge (5,000 × *g*, 15 min). They were

washed, at 2 to 4 C, once in 0.9% sodium chloride and then twice in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-chloride containing 0.01 M $MgCl_2$ (pH 7.5) to prevent autolysis. The pellets were suspended in deionized water to give thick suspensions and then frozen in small plastic containers. They were sometimes stored at −20 C until disrupted.

Cell walls. The frozen suspensions were transferred to an X press (17) kept at −25 C, and disrupted by four successive pressings through the orifice. The disrupted suspension was fractionated in Sorvall refrigerated centrifuge, essentially as described earlier (13, 17). Cell walls were collected at 18,000 × *g*. Tris-chloride (0.1 M; pH 7.5) containing 0.01 M $MgCl_2$ was used throughout the fractionation. The purified cell walls were sometimes stored as frozen pellets until used. Peptidoglycan was prepared as described previously (17).

Lysis by lysozyme. Suspensions of walls or peptidoglycan were prepared in calibrated test tubes, which were suitable for use in a Coleman spectrophotometer, by using 0.1 M Tris-chloride (pH 7.5) containing 0.01 M $MgCl_2$ (10-ml total volume). They were usually adjusted to an A_{420} of 0.7 to 1.0 (we tried to obtain the same initial optical density in each experimental series). Lysozyme was added at a level of 400 μg/ml; trypsin (when indicated) was used at a level of 200 μg/ml. Incubation was in a water bath at 37 C with gentle shaking to keep the contents in suspension.

Modified cell walls. For *N* acetylation of walls or peptidoglycan, wet pellets were suspended in 1 ml of saturated $NaHCO_3$ and mixed with 1 ml of a freshly prepared 5% solution of acetic acid anhydride. The suspensions in 5-ml stoppered containers were gently stirred at 4 C overnight. The preparations were then centrifuged at 30,000 × *g* for 30 min and washed three times in 0.1 M Tris-chloride (pH 7.5) containing 0.01 M $MgCl_2$. The completeness of the *N* acetylation was tested by subjecting a portion of the preparation to dinitrophenylation, hydrolysis, and chromatography as described below.

Removal of substitution at O sites (deacetylation). Deacetylation was carried out by incubating wet pellets of walls, approximately 1 ml, with 10 ml of 0.01 M NaOH at 37 C for 60 min. The suspensions were then centrifuged and washed as above or, in some experiments, with deionized water instead of buffer. Some portions of these deacetylated walls were used directly for incubation with lysozyme; others were used after subsequent *N* acetylation as described above. In certain experiments the native walls were first *N* acetylated and then deacetylated to remove *O* substituents. The results were essentially similar.

Analytical procedures. *N*-nonsubstituted amino sugars and free terminal amino groups of amino acids were determined by dinitrophenylation techniques. In the finally adopted procedure, the wet wall pellets, approximately 0.2 ml, were suspended in 0.5 ml of 4% trimethylamine and then mixed with 1 ml of 2.5% 2,4-dinitrofluorobenzene in ethyl alcohol. The suspensions were incubated in stoppered test tubes, protected from light, at 30 C for 17 h with gentle shaking. Concentrated HCl, 0.75 ml, was then added, and the mixture was extracted three times with ether to remove excess reagent. The water phase was freed from ether by evaporation at 60 C, and the residue was hydrolyzed in a sealed tube at 95 C for 6 h. The hydrolysates were extracted three times with ether, and the combined ether extracts were evaporated to dryness at 37 C. The water phase was centrifuged and the supernatant was freeze dried. The dry ether extract was dissolved in 0.2 ml of 0.05 M NH_4OH, and 10-μl portions were subjected to two-dimensional chromatography on paper or on thin-layer silica plates. The dinitrophenylated residues were identified by using DNP derivatives of authentic specimens as reference compounds. The dry, water-phase residue was dissolved in 0.2 ml of deionized water, and 2-μl portions were used for chromatography as above, with 0.5% ninhydrin in acetone to detect the separated spots of amino acids and amino sugars. In separate runs, the reducing sugars were determined with alkaline silver nitrate. In addition, the location on the chromatograms of glucosamine and its DNP derivative was established by using ^{14}C-labeled glucosamine and scanning in a Packard chromatogram scanner or by liquid scintillation counting of eluted spots. Quantitative estimations were also based on spectrophotometric measurements, at A_{575}, of the eluted ninhydrin spots. The paper chromatograms were usually easier to evaluate than the thin-layer plates. The results reported here are based on paper chromatograms.

Paper chromatography. Paper chromatography was carried out by an ascending technique with Whatman no. 1 paper. The solvent systems used are as listed below, with the chromatograms being dried before each change. For DNP derivatives, the solvent in the first dimension was *n*-butyl alcohol–pyridine–water (6:4:3, vol/vol/vol; 4 h); that in the second dimension was 1.5 M potassium phosphate (pH 6.0; 1.5 h). For the hydrolysates of the water phase, the solvent in the first dimension was *n*-butyl alcohol–acetic acid–water (3:1:1, vol/vol/vol; 6 h); the chromatogram was then dried and run again (6 h) in the same solvent. For the second dimension, *n*-butyl alcohol–pyridine–water (6:4:3, vol/vol/vol; 4 h) was used.

RESULTS

Effect of lysozyme on chemically modified walls from exponential- and stationary-phase cells. Figures 1 and 2 show the lysis by lysozyme of *N*-acetylated and *O*-deacetylated walls from stationary- and midexponential-phase cells, respectively. It is seen that in neither case does the *N* acetylation alone increase the sensitivity of native walls to lysozyme. Instead, a certain decrease of this sensitivity is observed with the stationary-phase walls (Fig. 1) and also during the initial stages of incubation of the exponential-phase walls. The exponential-phase walls become somewhat more sensitive to lysozyme upon prolonged incubation (Fig. 2).

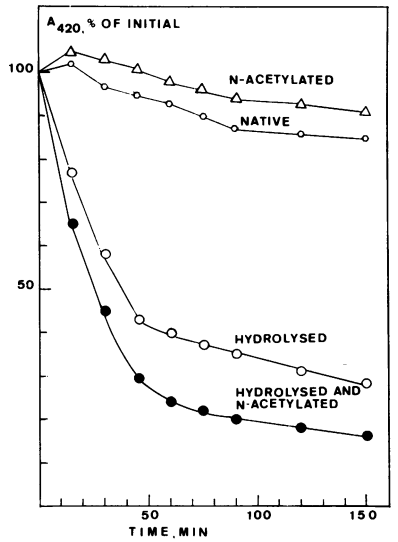

FIG. 1. *Effect of lysozyme on native and chemically modified walls from stationary-phase cells of L. fermentum. Incubation was at 35 C in 0.1 M Tris-chloride-0.01 M $MgCl_2$, pH 7.5; lysozyme, 400 μg/ml. In this and subsequent figures "hydrolyzed" = O deacetylated.*

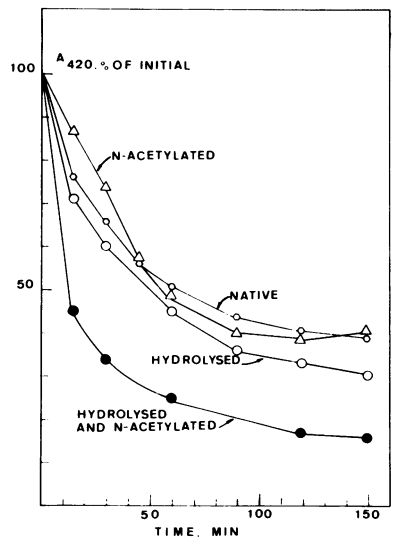

FIG. 2. *Effect of lysozyme on native and chemically modified walls from exponential-phase cells of L. fermentum. Conditions as in Fig. 1.*

The effect of *O* deacetylation is much more pronounced. Such modification drastically increases the lysozyme sensitivity of the stationary-phase walls, which are normally rather resistant (Fig. 1). Also, the exponential-phase walls become more sensitive to lysozyme after *O* deacetylation (Fig. 2), although this effect is quantitatively less pronounced than in the case of the stationary-phase walls (cf. Fig 1).

A subsequent *N* acetylation of the *O*-deacetylated walls further increases the lysozyme sensitivity of both stationary- and exponential-phase walls (cf. Fig. 1 and 2).

Autolysis of chemically modified walls. *N* acetylation, *O*-deacetylation, or *O* deacetylation followed by *N* acetylation completely inhibited the autolytic activity of exponential-phase walls and further increased the resistance to autolysis of stationary-phase walls (Fig. 3A and B).

Effect of trypsin. Figure 4 compares the effect of trypsin on lysis by lysozyme of native walls with a corresponding effect on chemically modified walls. It is seen that stationary-phase

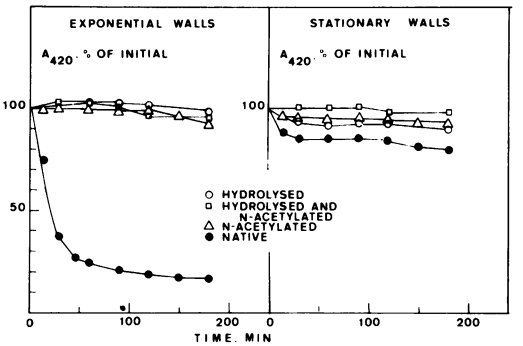

FIG. 3. *Autolysis of native and chemically modified walls of L. fermentum. Incubation was at 35 C in 0.02 M potassium-phosphate, pH 6.0.*

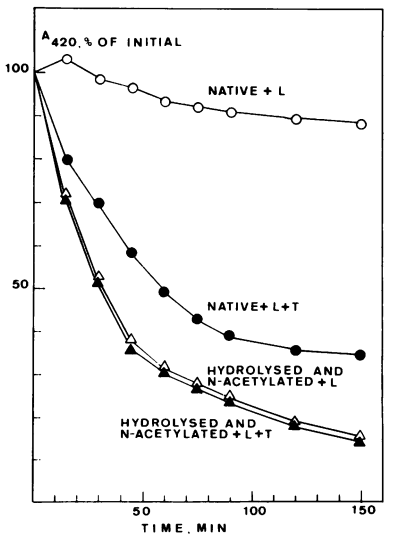

FIG. 4. *Effect of trypsin (T) on lysozyme (L) susceptibility of native and chemically modified walls of L. fermentum. Conditions as in Fig 1; trypsin, 200 μg/ml.*

walls, which have been rendered lysozyme sensitive by *O* deacetylation and subsequent *N* acetylation, do not further increase their sensitivity in the presence of trypsin as is the case with native walls. Similar results were obtained with walls from other growth phases which had a variable degree of lysozyme sensitivity, invariably stimulated by trypsin (12).

Occurrence of N-nonsubstituted residues in walls and peptidoglycan. Table 1 summarizes the results of the dinitrophenylation and chromatography experiments. It is seen that *N*-nonsubstituted glucosamine was present in both stationary- and exponential-phase walls, but not in the corresponding peptidoglycans. Smaller amounts of *N*-nonsubstituted muramic acid were found in walls and peptidoglycan from the stationary-phase only. The amount of *N*-nonsubstituted glucosamine increased with the age of the culture, at least compared with the amount of free amino groups of aspartic acid. Substantial amounts of the latter were present along with free amino groups of alanine in both walls and peptidoglycan from the exponential and stationary phase alike.

DISCUSSION

The results reported here indicate that substitution at *O* sites, presumably *O* acetylation, may be the main factor responsible for the lysozyme resistance of cell walls from *L. fermentum*. Since this resistance is much more pronounced in stationary-phase walls than in exponential-phase walls (12), it can be concluded that an extensive *O* acetylation takes place as cells go into the stationary phase of growth. Such a conclusion is corroborated by the data in Fig. 1 (cf. also Fig. 4), where highly resistant stationary-phase walls are shown to become lysozyme sensitive after mild hydrolysis (0.01 M NaOH), a treatment known to remove the *O*-acetyl groups in peptidoglycan. The association of *O* acetylation with lysozyme resistance was originally suggested by Brumfitt et al. for *Micrococcus lysodeikticus* (5). The contribution of *O*-acetyl groups to lysozyme resistance of cell walls has also been reported for certain other bacteria, e.g., *Bacillus megaterium* (14), *Streptococcus faecalis* (1), and *Staphylococcus aureus* (7). In the case of *B. cereus*, however, no such connection could be found. Instead, *N* nonsubstitution at the glucosamine residues in the peptidoglycan was postulated to be the factor responsible for the lysozyme resistance of this organism (2). Cell walls from several other organisms have been reported to acquire an increased lysozyme sensitivity after *N* acetylation (2, 14, 15). In some cases, this has been attributed to the blocking of free amino groups in the peptide portion of peptidoglycan (14) or in muramic acid.

TABLE 1. *DNP derivatives in hydrolysates of dinitrophenylated walls and peptidoglycan of L. fermentum taken from the midexponential (EXP) and stationary (STA) phase of growth*[a]

DNP derivative of:	Walls		Peptidoglycan		A_{370}	
	EXP	STA	EXP	STA	EXP	STA
Glucosamine	+	+	−	−	0.064	0.068
Muramic acid	−	(+)	−	(+)		
Alanine	+	+	+	+		
Glutamic acid	−	−	−	−		
Lysine	−	−	−	−		
Aspartic acid	+	+	+	+	0.110	0.078
Ratio: glucosamine/aspartic acid					0.6	1.1

[a] Separation by two-dimensional paper chromatography as described in the text. The spot of DNP-glucosamine, run as the ^{14}C-labeled compound, and that of DNP-aspartic acid were eluted with 2.5 ml of 0.05 M NH_4OH and measured at 370 nm. The spot of DNP-aspartic acid was far from those of all the other substances and was therefore used as a reference for quantitative estimations of glucosamine.

It appears from our results that *N* acetylation per se does not increase the lysozyme sensitivity of walls or peptidoglycan from *L. fermentum*. However, *N* acetylation of walls that were previously rendered sensitive to lysozyme by removal of *O*-acetyl groups further increases their sensitivity (cf. Fig. 1 or 2). A possible explanation could be that the hydrolysis employed to remove *O*-acetyl groups, however mild, also removes some of the *N*-acetyl groups. The latter have to be replaced for maximum lysozyme sensitivity. It is generally believed that lysozyme has an absolute requirement for acetamido groups of *N*-acetylglucosamine, whereas the importance of those of *N*-acetylmuramic acid is not established (cf. reference 2).

Dinitrophenylation experiments revealed the occurrence of *N*-nonsubstituted glucosamine in cell walls but not in purified peptidoglycan (Table 1). This indicates that the nonacetylated glucosamine is mainly present in the non-peptidoglycan wall portion. This is in agreement with our earlier observation that significant amounts of glucosamine were present in trichloroacetic acid extracts of cell walls from *L. fermentum* (17). The amount of *N*-nonsubstituted glucosamine is larger in stationary- than in exponential-phase walls (Table 1). It thus seems that aging of the culture is accompanied either by an increasing degree of incorporation of "non-peptidoglycan glucosamine"

or by an increased deacetylation of such glucosamine. Small amounts of *N*-nonsubstituted muramic acid were found in both walls and peptidoglycan from the stationary phase of growth, but not in the corresponding preparations from the exponential phase (Table 1). Thus, some *N* deacetylation may indeed take place as cells go into the stationary phase of growth. The occurrence of *N*-non-acetylated muramic acid in lysozyme digests of *M. lysodeikticus* has been reported by Ingram and Salton (8). Mirelman and Sharon have found it in intact cell walls from this organism (10).

We have suggested earlier that an *N*-deacetylating enzyme may form a part of an autolytic enzyme system in *L. fermentum* since peptidoglycan fragments solubilized during autolysis are largely *N* non-acetylated (13). The autolytic system as a whole comes to expression only during early stages of growth and under conditions normally not favoring lysozyme action (13). It is not known, however, whether this also applies to the *N*-deacetylating activity. The present results provide evidence that the degree of *N* non-acetylation in wall peptidoglycan is rather small and not of any major importance for lysozyme resistance. These results thus also strengthen our earlier conclusion that enzymatic *N* deacetylation occurs during autolysis.

Table 1 shows that both walls and peptidoglycan from exponential- and stationary-phase cells alike contain significant amounts of free amino groups in alanine and aspartic acid. This indicates that the peptidoglycan of *L. fermentum* is not completely cross-linked. In some places, the cross-peptide bridges are presumably open at the α-amino group of D-alanine. In other places they are open at the α-amino group of D-aspartic acid. The amount of the latter seems to decrease with the age of the culture (cf. Table 1). Thus, the degree of cross-linking may increase correspondingly.

Both *N* acetylation or *O* deacetylation completely inhibit the autolysis of exponential-phase walls (Fig. 3A). The resistance of stationary-phase walls to autolysis is increased further by such treatments (Fig. 3B). However, when *O*-deacetylated exponential-phase walls were mixed with autolysates of native walls (i.e., with solubilized autolysin), some limited autolysis did occur (data not recorded). This indicates that the treatments involved in chemical wall modifications may be detrimental to the autolytic enzymes.

We reported earlier that trypsin and other proteinases drastically increase the susceptibility of *L. fermentum* to lysosyme (4, 11, 12). A similar phenomenon has also been reported for *L. casei* (3). The present investigation offers a possible explanation of this effect. It can be concluded from Fig. 4 that the effect of trypsin is equivalent to the removal of *O*-acetyl groups by mild alkali hydrolysis. Thus, trypsin may act here as an esterase rather than as a proteinase.

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