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Selective Extraction of Polymers from Cell Walls of Gram-Positive Bacteria

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Brief heating of *Bacillus Licheniformis* cell walls at 100°C in aqueous buffers of pH 3.0–4.0 removes some polymers but not others from the mucopeptides. For example, relatively undegraded teichuronic acid can be extracted at 100°C in 20min at pH 3.0 whereas the teichoic acids are not removed. Similar specificity can be shown with walls from three other species of micro-organism.

Cell walls of Gram-positive bacteria consist of mucopeptides to which other polymers are attached by a limited number of covalent bonds. For example, cell walls of *Bacillus licheniformis* N.C.T.C. 6346 isolated from micro-organisms grown in batch cultures contain teichoic acids made of glycerol, phosphate, glucose and galactose and a teichuronic acid made of *N*-acetylgalactosamine and glucuronic acid (Janczura *et al.*, 1961; Hughes & Thurman, 1970). *Micrococcus lysodeikticus* walls contain another teichuronic acid composed of glucose and aminomannuronic acid (Perkins, 1963). Hughes *et al.* (1968) and Hughes (1970) deduced that the teichuronic acid in the walls of *B. licheniformis* is linked through a phosphodiester bond between the reducing *N*-acetylgalactosamine end of the polysaccharide and the C-6 hydroxyl group of the muramic acid of the glycan strands in the mucopeptide. A similar deduction has been made for the linkage of the polysaccharides to mucopeptides of *Lactobacillus casei* (Hall & Knox, 1965) and in the walls of *Bacillus stearothermophilus* (Grant & Wicken, 1968). Linkage through muramic acid phosphate has been established unequivocally for the *N*-acetylglucosamine 1-phosphate polymer and the mucopeptide in a strain of *Staphylococcus lactis* I3 (Button *et al.*, 1966).

The teichoic acids can be removed from the walls of a variety of species of bacteria by treatment with 0.1M-NaOH solution acting at 35°C in the absence of oxygen (Hughes & Tanner, 1968; Archibald *et al.*, 1969). Teichuronic acid, however, is not removed by this treatment of *B. licheniformis* walls. Both teichuronic acid and the teichoic acids, however, are solubilized by extraction with either trichloroacetic acid or 0.1M-HCl. The present communication describes a gentle method for the preferential removal of teichuronic acid rather than teichoic acid from walls of *B. licheniformis*. Evidence is presented that the method may have wider application to the preferential removal of polysaccharides from the walls of micro-organisms.

Experimental

B. licheniformis N.C.T.C. 6346. Cell walls (5mg/ml) were extracted with glycine–HCl buffer, *I* 0.05, at different pH values in sealed ampoules immersed in a boiling-water bath. The insoluble cell walls were then sedimented by centrifuging and washed with water, and the supernatants were pooled. Samples were taken from both the pooled supernatants and the residues, which were then analysed for organic phosphorus (Ames & Dubin, 1960), glucuronic acid (Dische, 1947) and total carbohydrates (as glucose) by the anthrone reaction (Mockrash, 1954). Samples for the quantitative determinations of galactosamine and glycerol were hydrolysed at 100°C for 4h with 4M-HCl and the acid was subsequently removed by evaporation *in vacuo* over NaOH pellets. Before glycerol determinations (Wieland, 1963) the organic phosphates remaining in the acid hydrolysates were hydrolysed with alkaline phosphomonoesterase acting in ammonium carbonate buffer, pH 9.0. Galactosamine in the original hydrolysates was separated and determined in a Beckman automatic amino acid analyser by Miss S. Lathwell of this Institute. At pH 3.0 about 90% of both the uronic acid and the galactosamine were solubilized in 20min, together with only 6% of the total phosphorus and 3% of the glycerol (Fig. 1). The number-average chain-length values for the extracted teichuronic acid as determined by chromogen formation and by treatment with NaB^3H_4 (Hughes & Thurman, 1970) were 34 and 27 respectively by the two methods. Thus no extensive degradation of the polysaccharide had occurred. More prolonged heating at pH 3.0 extracted teichoic acid also (Fig. 2).

Micrococcus lysodeikticus N.C.T.C. 2665. After 20min heating at pH 3.0 glucose to 14% of the cell-wall dry weight was extracted together with 30% of the small amount of phosphorus present. The number-average chain length of the extracted material was obtained from the ratio of sorbitol to glucose present after $NaBH_4$ reduction and determined by

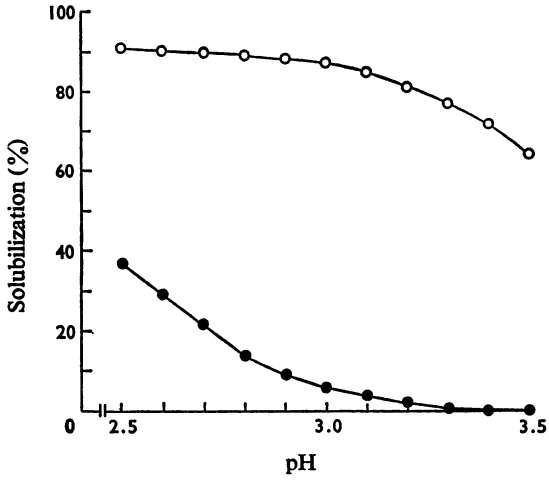

Fig. 1. *Effect of pH on the extraction of polymers from cell walls of B. licheniformis*

The cell walls (5.0mg/ml) were extracted with glycine–HCl buffer, *I* 0.05, at the pH values indicated at 100°C for 20min. The percentage solubilizations of uronic acid (○) and organic phosphorus (●) were then measured. Experimental details are given in the text.

g.l.c. This method makes use of the structural observation on this teichuronic acid that the *N*-acetylmannuronic acid residues are internal to those of glucose (Hase & Matsushima, 1970, 1971). The ratio of sorbitol to glucose rose only from 1:17 after 10min to 1:15 after 30min extraction, suggesting little progressive degradation of the polysaccharide. As would be expected from the indicated chain length, the material did not pass through cellophan tubing during dialysis against water.

Bacillus subtilis 168 *trp* 2. At least two identified polymers are known to be joined to the mucopeptide in the walls of this micro-organism (Duckworth *et al.*, 1972). Successive extractions of the walls at two slightly different pH values enabled the separation of two polymers. Treatment at 100°C and pH4.0 with citrate or acetate buffer (0.1 M) for 20min extracted about 90% of the galactosamine present in the walls along with glucose and phosphorus. The molar proportions of these components were 1.0:1.0:1.0–1.2. Less than 5% of the original galactosamine in the wall remained in the insoluble residue, and no glycerol was present in the soluble extract. The material extracted was only partly diffusible on dialysis and is likely to be the galactosamine–glucose phosphate polymer recognized by Duckworth *et al.* (1972). Some degradation is likely to have occurred. Re-extraction of the walls at pH3.0 for 30min at 100°C

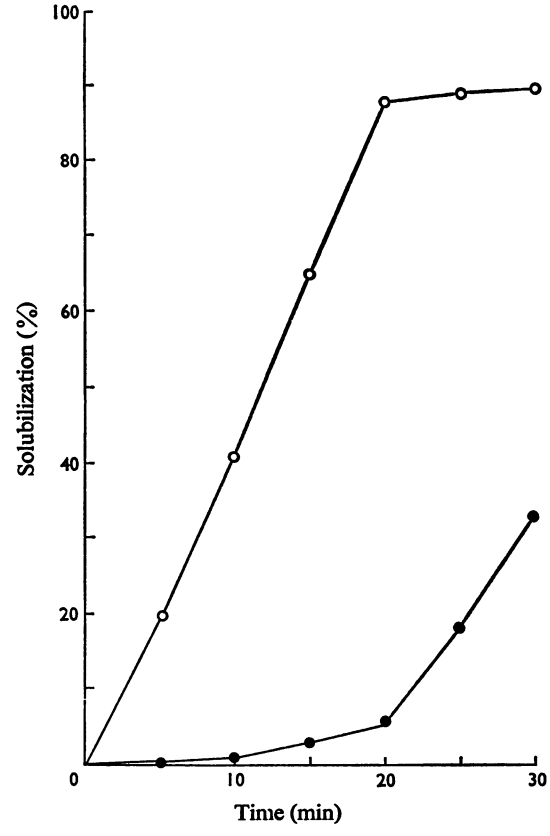

Fig. 2. *Effect of time on the extraction of polymers from cell walls of B. licheniformis at pH*3.0

The cell walls (5.0mg/ml) were extracted with glycine–HCl buffer, pH3.0 and *I* 0.05, at 100°C for the times indicated. The percentage solubilizations of uronic acid (○) and organic phosphorus (●) were then measured. Experimental details are given in the text.

solubilized a teichoic acid containing phosphorus, glycerol and glucose in the molar proportions 1.0:0.98:1.0. Only traces of galactosamine were present in this extract.

Streptococcus faecium A.C.T.C. 9790. Preliminary results on the extraction of walls from this micro-organism have shown that extraction at pH3.0 for 25min at 100°C solubilizes 90% of the galactosamine and 75% of the phosphorus, but only 56% of the rhamnose, determined by the method of Dische & Shettles (1948). The molar proportion of rhamnose to phosphorus left in the walls after this extraction is 8.2:1. Thus it seems possible that a degree of polymer separation from these walls is also possible by mild acidic extraction.

Discussion

The above results show that, by aqueous extraction of bacterial walls at carefully controlled pH, considerable selective extraction of the polymers is possible. In two instances it would appear that the bond, probably a diester phosphate that links polysaccharides to the mucopeptide, is considerably more labile to pH values of about 3.0 than is that which links the polyol phosphate teichoic acids.

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