Properties of the Polysaccharide and Mucopeptide Components of the Cell Wall of *Lactobacillus casei*

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1. The polysaccharide and mucopeptide components of the cell wall of *Lactobacillus casei* have been separated by mild conditions of acid hydrolysis. 2. Removal of the polysaccharide renders the mucopeptide susceptible to lysozyme. 3. The mucopeptide and polysaccharide components have been analysed and the results compared with those obtained previously. 4. The polysaccharides responsible for group specificity have a terminal reducing *N*-acetylgalactosamine residue substituted on C₆ by the adjacent sugar; estimation of this component gave an indication of the molecular weight of the polysaccharides. 5. Evidence has been obtained for the presence of rhamnosyl-(1→3)-*N*-acetylgalactosamine among the products of acid hydrolysis of the group B polysaccharide.

The results described in the preceding paper (Knox & Hall, 1965b) indicate that conditions of mild acid hydrolysis ruptured a covalent linkage between the polysaccharide and mucopeptide components of the cell wall of strains of *Lactobacillus casei*. The present paper describes the preparative separation of the polysaccharide and mucopeptide, by using these mild conditions, and subsequent investigation of their properties. As there is evidence for a phosphodiester linkage between the two polymers, the identity of a phosphorylated residue was sought.

MATERIALS AND METHODS

Organisms. Cultures of *Lactobacillus* were obtained from the National Collection of Type Cultures, Colindale, London (designated NCTC), and the National Institute for Research in Dairying, Reading (designated NIRD). These organisms were *L. casei* NIRD strain RO 94, *L. helveticus* (*L. casei* var. *rhamnosus*) NCTC 6375 of serological group C and *L. casei* NIRD H 831 of serological group B (Knox, 1963; Knox & Hall, 1965b); an additional strain, strain 55 of serological group B (Knox & Brandsen, 1962), was also included.

Cell-wall components. The procedures for preparing cell wall and the subsequent isolation of soluble fractions have been described previously (Knox, 1963; Knox & Hall, 1965b).

Carbohydrates. *O*-β-D-Glucopyranosyl-(1→3)-*N*-acetyl-D-galactosamine was isolated from the products of mild acid hydrolysis of the cell wall of *L. casei* NIRD RO 94 (Knox & Hall, 1965a). Dr W. M. Watkins, Lister Institute of Preventive Medicine, London, kindly provided samples of the α- and β-anomers of D-galactopyranosyl-(1→6)-*N*-acetyl-D-glucosamine; Dr H. Heymann, Ciba Pharmaceutical Co., New Jersey, U.S.A., kindly provided samples of the α- and β-anomers of methyl L-rhamnopyranoside; muramic acid

was synthesized and kindly supplied by Dr M. D. Montague, Department of Biochemistry, University of Sydney, Sydney, Australia.

Nucleotide. ATP (disodium salt) was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Enzymes. Hexokinase was obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Egg-white lysozyme (B grade) was purchased from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. Fluorescein-labelled lysozyme was prepared by conjugating lysozyme with fluorescein isothiocyanate at pH 7.6 and 2° by the antibody-conjugation method described by Goldstein, Slizys & Chase (1961); Gould, Georgala & Hitchins (1963) showed that rhodamine-labelled lysozyme would form a fluorescent complex with lysozyme-susceptible cells.

Fluorescent-microscopy equipment. A Leitz Ortholux microscope with an Osram HBO-200 high-pressure mercury-vapour burner was used. The filter system consisted of a KG1 (2 mm.) heat-absorbing filter, UG1 (2–4 mm.) plus BG38 (4 mm.) filters for ultraviolet and BG12 (5–8 mm.) for ultraviolet-blue excitation, with the corresponding Leitz ultraviolet- or ultraviolet-blue-absorbing secondary filters. A bright-field condenser (Leitz no. 72v, N.A. 1.40) was employed.

Column chromatography. DEAE-Sephadex (Pharmacia, Uppsala, Sweden) was prepared for use as described by Knox & Hall (1965b). DEAE-cellulose (Whatman DE-50) was prepared for use by washing with 0.4M-K₂B₄O₇; the potassium salt was substituted for the sodium salt used by Neukom, Deuel, Heri & Kündig (1960), as its greater solubility enabled higher concentrations of borate to be used. Eluates containing borate were collected into tubes containing appropriate amounts of 1.5N-H₂SO₄ for neutralization (e.g. 0.1 ml./4 ml. of 0.02M-borate), thus minimizing alkaline degradation of *N*-acetylhexosamine; for the same reason chromatography was performed at 2°. These precautions were shown to be effective as chromogen (an alkaline degradation product of *N*-acetylated hexosamines) was not detected in eluates collected in the manner de-

scribed. It was, however, present in eluates that were not neutralized and were left at room temperature.

The rate of elution of carbohydrate-containing materials from columns was followed with the primary cysteine- H_2SO_4 reaction for hexose (Dische, 1953) and the method of Gibbons (1955) for rhamnose.

Paper electrophoresis. Electrophoresis of carbohydrates was performed in 0.05M- $\text{Na}_2\text{B}_4\text{O}_7$ (Frahn & Mills, 1959) as described by Knox & Hall (1965a). Hexosamines and hexosamine phosphates were separated by electrophoresis in pyridine-acetic acid buffer, pH 3.5, on Whatman no. 1 paper. A potential difference of 1000 v was applied over a distance of 50 cm. for 2-3 hr. Hexosamines were detected with alkaline acetylacetone followed by Ehrlich's reagent (Partridge, 1948), and phosphorus-containing compounds by the method of Hanes & Isherwood (1949).

Analytical methods. Components were estimated by the methods described by Knox & Hall (1965b). Hydrolysis before the estimation of hexosamines was performed for 1 hr. at 100° in 4N-HCl. Johansen, Marshall & Neuberger (1960) concluded that 4N-HCl was the most suitable for hydrolysing hexosamine-containing polymers, and preliminary experiments with cell-wall preparations indicated that the maximum value was reached in 1 hr.

N-Acetylhexosamine substituted on $\text{C}_{(3)}$ by the adjacent sugar was estimated by the method developed by Knox & Hall (1965b), with *O*- β -D-glucosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosamine as the standard.

Amino acid analyses. Dr C. M. Mauritzen, School of Biochemistry, University of Melbourne, Melbourne, Australia, kindly carried out amino acid analyses, using a Beckman 120B automatic amino acid analyser.

Ultracentrifugal analyses. Samples for ultracentrifugal analysis were dissolved in phosphate buffer, pH 7.0 and 1.0-1, to give a final concentration of 1.0%. Examination of the materials in the Spinco model E analytical ultracentrifuge was kindly performed by Mr G. Jones, Department of Biochemistry, University of Sydney.

Immunological methods. Serological properties of cell-wall components were examined by the methods described by Knox (1963).

RESULTS

Separation of polysaccharide and mucopeptide components

L. casei (var. *casei*) NIRD RO94. Cell wall (2.0 g.) was heated for 20 min. at 60° in 200 ml. of 0.1N-sulphuric acid with stirring. After cooling, the suspension was centrifuged and the supernatant removed. The residue was suspended in acid and reheated as before. This procedure was repeated to yield six soluble extracts that were pooled and designated S1. Heating the residue for two 2 hr. periods yielded a second pooled extract designated S2; fractions S1 and S2 were dialysed and dried from the frozen state. The residue was finally heated in acid for 16 hr., washed until free from acid and dried from the frozen state. Analyses (Table 1) showed that the residue contained the expected components of the mucopeptide but less than 0.5% of hexose.

To determine the recovery of hexoses in the preparation of polysaccharide and mucopeptide, 50 mg. of wall was heated at 60° in 2 ml. of 0.1N-sulphuric acid for five 1 hr. periods. The supernatant solutions were pooled and dialysed. The residue was then reheated for 16 hr. and washed until free from acid. Hexose values were determined for the indiffusible and diffusible fractions, the material extracted by overnight heating (including washings) and the residue; results were expressed as percentages of the total hexose in the starting material. The total

Table 1. *Composition of the cell wall and cell-wall fractions from strains of L. casei*

Results are expressed as percentages of the dry weight of the fraction. Procedures for the preparation of the fractions are given in the text. —, Analyses not performed.

Strain	Fraction	Phosphorus	Rhamnose	Glucose	Galactose	Glucosamine	Galactosamine	Muramic acid	Total nitrogen
NIRD RO94	Wall	0.20	1.8	23.0	15.0	18.5	10.5	6.7	6.6
	Mucopeptide	0.38	0	0.4*	—	17.2	1.0	19.2	11.9
	Polysaccharide S1	0.02	3.8	32.5	25.0	21.2	13.8	0.2	2.3
NCTC 6375	Wall	0.20	28.0	20.0	14.0	4.1	3.3	3.5	3.7
	Mucopeptide	0.35	1.7	0.5*	—	12.0	—	15.2	11.1
	Polysaccharide S1	0.02	38.5	29.0	24.0	11.5†	—	—	—
	Polysaccharide S1/A	—	63.0	29.0	20.0	0.4†	—	—	—
	Polysaccharide S1/B	—	1.0	30.0	22.0	13.0	15.0	—	—
NIRD H 831	Wall	0.72	22.0	3.5	5.6	8.5	5.9	4.9	8.5
	Mucopeptide	0.38	1.0	0.5	—	10.0	—	11.2	12.9
	Polysaccharide S1	—	44.0	6.0	11.5	14.7	9.0	—	3.0
55	Polysaccharide S/A	—	43.5	9.0	12.0	12.5	10.0	—	—
	Polysaccharide S/B	—	28.0	25.0	23.0	11.2	7.8	—	—

* Total hexose by the primary cysteine- H_2SO_4 reaction.

† Total hexosamine.

recovery was 95%, made up as follows: indiffusible, 85%; diffusible, 3.5%; overnight extract, 6%; residue, 0.4%.

Polysaccharide has also been prepared from the purified soluble fraction III of strain NIRD RO 94 (Knox & Hall, 1965b). A solution of 120mg. of fraction III in 12ml. of 0.1N-sulphuric acid was heated at 60° for 1 hr. After dialysis, the indiffusible material was concentrated *in vacuo* to 2ml. and applied to a column (30cm. $\times$ 1.4cm.) of DEAE-Sephadex A-50 previously equilibrated with 0.05M-tris-hydrochloric acid buffer, pH 7.6. Polysaccharide was eluted with 0.05M-tris-hydrochloric acid buffer and apparently undegraded fraction III with 0.05M-tris-hydrochloric acid buffer containing sodium chloride (0.05M). Heating in acid for 1 hr. was expected to release half of the polysaccharide from the polysaccharide-mucopeptide complex; the yield of polysaccharide (44mg.) was consistent with this assumption. The polysaccharide was similar in composition to that obtained by heating the cell wall of this strain under the same conditions.

Material that was diffusible after heating fraction III in acid was neutralized with sodium hydroxide, concentrated *in vacuo* and applied to a column (44cm. $\times$ 0.5cm.) of Sephadex G-25. Eluates (1ml.) were tested for hexose, total phosphorus and sulphate. Most of the hexose (97%) was eluted in advance of sulphate, whereas approx. 40% of the phosphorus-containing material was eluted in the fractions containing sulphate.

L. casei var. *rhamnosus* NCTC 6375. Cell wall (4.0g.) was heated at 60° in 0.1N-sulphuric acid as described for strain NIRD RO 94. Extracts from the series of 20min. heatings (fraction S1) and 2hr. heatings (fraction S2) were dialysed and dried from the frozen state. The residue after the completed period of heating was washed with water until free from acid and dried from the frozen state.

Fraction S1 contained 38.5% of rhamnose and 29% of glucose (complete analyses in Table 1), and attempts were made to separate the two polysaccharide components expected to be present. To a solution of fraction S1 (500mg.) was added ammonium sulphate, a precipitate being formed at 70% saturation. After centrifuging, the supernatant and a solution of the precipitate were dialysed and dried from the frozen state. The precipitate (300mg.; fraction S1/A) contained 63% of rhamnose and 29% of glucose (Table 1), whereas the material in the supernatant (180mg.) contained 12% of rhamnose and 25% of glucose.

A further portion of fraction S1 (200mg.) was applied to a column (30cm. $\times$ 1.4cm.) of DEAE-cellulose, previously washed with 0.4M-potassium tetraborate, and material eluted with increasing concentrations of potassium tetraborate at 2°. Fractions were collected into tubes containing

sufficient amounts of 1.5N-sulphuric acid to neutralize the eluate and so minimize alkaline degradation of *N*-acetylhexosamine. The material eluted with 0.02M-borate (60mg.; fraction S1/B) contained 1% of rhamnose and 30% of glucose (Table 1), whereas all fractions eluted with borate concentrations from 0.08M to 0.2M contained 43–44% of rhamnose and 26–27% of glucose.

L. casei NIRD H 831. Polysaccharide was extracted from the cell wall by the procedure described above. Rhamnose is a characteristic component of the cell wall of this strain, which belongs to serological group B, and the polysaccharide preparation (fraction S1) contained 44% of rhamnose (Table 1).

L. casei strain 55. A preparation (500mg.) of fraction II was heated in 0.1N-sulphuric acid at 60° for 4 hr. The yield of indiffusible material (fraction 55/S) was 380mg. A portion (200mg.) was applied to a column of DEAE-cellulose (borate form) as described above. Two fractions were obtained, one of which (fraction 55/S/A) was not adsorbed, the other (fraction 55/S/B) being eluted with 0.02M-borate. Analysis of the preparations indicated that a separation of the polysaccharide components had been achieved, the molar ratios of rhamnose to glucose for the starting material and fractions A and B being 1.6:1, 5.4:1 and 1.2:1 respectively. The analyses for fractions A and B are given in Table 1.

Properties of the mucopeptide

Chemical analyses. Analyses of preparations for hexoses, hexosamines, total nitrogen and total phosphorus are included in Table 1. The results of amino acid and hexosamine analyses on the cell wall and mucopeptide from strain NIRD RO 94 and for the mucopeptide from strain NIRD H 831 are given in Table 2. Except for histidine (1.8%), the mucopeptide from strain NIRD RO 94 did not contain detectable amounts of other amino acids. However, the mucopeptide from strain NIRD H 831 was apparently contaminated with protein, as it contained a wide variety of additional amino acids accounting for 12.8% of the material.

Action of lysozyme on mucopeptide

Suspensions (1mg./ml.) of cell-wall and mucopeptide preparations from strain NIRD RO 94 were incubated at 37° with an equal volume of lysozyme (50 μ g./ml.) in 0.1M-ammonium acetate. Evidence for enzyme action was obtained by measuring the extinction at 600m μ after appropriate intervals. For the mucopeptide preparation the extinction decreased by 75% in 5 hr., whereas there was no change for the cell-wall preparation. A similar result was obtained with the cell wall and mucopeptide from strain NIRD H 831.

Table 2. *Amino acid and hexosamine components of cell-wall products from strains of L. casei*

In the left-hand portion of the Table results are expressed as percentages of the dry weight of the fraction. In the right-hand portion the relative molar proportions have been calculated with respect to glutamic acid.

Component	Percentage of component present in preparation			Relative molar proportions of components in preparation		
	Strain RO94 cell wall	Strain RO94 mucopeptide	Strain H831 mucopeptide	Strain RO94 cell wall	Strain RO94 mucopeptide	Strain H831 mucopeptide
Glutamic acid	4.2	14.1	8.4	1.00	1.00	1.00
Aspartic acid	3.0	7.7	6.8	0.82	0.60	0.89
Lysine	3.8	10.2	7.8	0.92	0.73	0.92
Alanine	4.0	11.6	7.5	1.60	1.36	1.51
Muramic acid	6.7	19.2	11.2	0.80	0.80	0.79
Glucosamine	18.5	17.2	10.0	3.40	1.00	0.98

Evidence that removal of the polysaccharide chain renders the mucopeptide susceptible to lysozyme has also been provided by studies with fluorescein-conjugated lysozyme. One drop of suspensions (1 mg./ml.) of cell wall and mucopeptide from strain NIRD RO 94 was allowed to dry at room temperature on a microscope slide and then covered with 1 drop of the conjugated enzyme. After standing in a moist atmosphere for 30 min. the slides were rinsed several times with 0.9% sodium chloride buffered at pH 7.0 and examined through a Leitz Ortholux microscope. The cell wall gave a very faint fluorescence compared with a very bright fluorescence for the mucopeptide. Whole cells of strain NIRD RO 94 gave no detectable fluorescence when examined by this means, whereas preliminary heating at 60° for 2 hr. in 0.1 N-sulphuric acid resulted in a distinct fluorescence being obtained.

Preliminary experiments have been carried out on the products of lysozyme action on mucopeptide. Mucopeptide from strain NIRD RO 94 (200 mg. suspended in 100 ml. of water) was incubated at 37° for 16 hr. with 5 mg. of lysozyme; complete dissolution resulted. After dialysis, the indiffusible material, in 0.01 M-tris-hydrochloric acid buffer, pH 7.6, was applied to a column (12 cm. × 1.0 cm.) of DEAE-cellulose previously equilibrated with the same buffer. Elution was effected with a sodium chloride gradient in 0.01 M-tris-hydrochloric acid buffer to a maximum concentration of 0.36 M-sodium chloride. Fractions (5 ml.) were collected and every eight fractions were pooled and dialysed before analysis. Products containing hexosamine (9 mg. as glucosamine), but insignificant amounts of organic phosphorus, were eluted at sodium chloride concentrations below 0.09 M. Products containing hexosamine (14 mg.) and organic phosphorus were eluted at sodium chloride concentrations between 0.09 M (tube 17) and 0.22 M (tube 48). The amount of organic phosphate in the material applied to the column represented 75% of the phosphorus in the muco-

peptide, and the phosphorus-rich fraction contained 90% of this total. The molar ratio of muramic acid to phosphorus in this fraction was 2.8:1.0 compared with 6.2:1.0 for the mucopeptide.

Phosphorylated components of the mucopeptide

The analyses of mucopeptide in conjunction with previous studies on the action of phosphomonoesterase on cell-wall products (Knox & Hall, 1965b) indicates the presence of one or more phosphorylated component(s) in the mucopeptide. Mucopeptide was therefore further investigated in an attempt to detect a phosphorylated compound. The most suitable fraction for detecting the compound proved to be the diffusible material obtained on heating fraction III from strain NIRD RO 94 for 1 hr. at 60° in 0.1 N-sulphuric acid. As described above, this material was applied to a column of Sephadex G-25 and eluted with water; the phosphorus-containing fractions were pooled and hydrolysed for 3 hr. at 100° in 6 N-hydrochloric acid. After removal of hydrochloric acid by repeated evaporation *in vacuo*, the material was applied to Whatman no. 1 paper and components were separated by electrophoresis in pyridine-acetic acid buffer, pH 3.5, for 2.5 hr. at a potential difference of 1000 v. Duplicate strips were sprayed to detect hexosamine and phosphorus. A compound containing hexosamine and phosphorus was detected moving towards the anode (M_{ATP} 0.57). The material remaining at the origin also gave a reaction for both hexosamine and phosphorus, and a number of non-phosphorylated hexosamine-containing compounds were detected moving towards the cathode.

As the only hexosamines known to be components of the mucopeptide are glucosamine and muramic acid, attempts were made to prepare their phosphorylated derivatives by incubation with hexokinase and ATP for 1 hr. (Leloir & Cardini, 1956). Examination of the products of enzyme action on

glucosamine by paper electrophoresis showed a phosphorylated hexosamine, presumably glucosamine 6-phosphate, at the origin. Attempts to obtain phosphorylated muramic acid were unsuccessful despite increase in both enzyme and substrate concentrations and the time of incubation.

Properties of the polysaccharide

Carbohydrate components. Analyses of the polysaccharide preparations are compared in Table 1 with those for cell-wall and mucopeptide preparations.

Chromogen formation from *N*-acetylhexosamine. A series of tubes containing a solution of polysaccharide (1mg./ml. of fraction S1) from strain NIRD RO 94 in 0.5M-phosphate buffer, pH 7.0, was heated at 100° and the rate of chromogen formation followed. The maximum was reached in 45min.; when the amount of chromogen was plotted against time, the curve was indistinguishable from that obtained for *O*- β -D-glucosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosamine (Knox & Hall, 1965b).

Each of the polysaccharide preparations listed in Table 1 was heated in 0.05M-phosphate buffer, pH 7.0, for 45min.; all except fraction S1/A from strain NCTC 6375 formed chromogen. By comparing the amount of chromogen formed by the polysaccharide in 45min. with the amount formed by the disaccharide under the same conditions, an estimate has been made of the average molecular weight of the preparations (Table 3); these values are dependent on the assumption that each polysaccharide chain contains a terminal reactive *N*-acetylgalactosamine residue.

The rate of release of reactive *N*-acetylhexosamine on acid hydrolysis of fraction S1 from strain NIRD RO 94 has been determined. A series of tubes con-

Fig. 1. Comparison of rates of release of 'reactive *N*-acetylhexosamine' from 1mg. of cell wall (○) and 1mg. of polysaccharide S1 (●) from *L. casei* NIRD RO 94 on heating in 0.1N-H₂SO₄ at 60° for appropriate intervals. 'Reactive *N*-acetylhexosamine' is defined as *N*-acetylhexosamine that forms chromogen on heating at pH 7.0 and 100° for 45 min. Details are given in the text.

taining 1mg. of material in 0.2ml. of 0.1N-sulphuric acid was heated at 60° for appropriate intervals and C₍₈₎-substituted *N*-acetylhexosamine estimated. The results are compared in Fig. 1 with those obtained on heating 1mg. amounts of cell wall in 0.1N-sulphuric acid.

Evidence for a terminal reducing *N*-acetylgalactosamine. The possibility that *N*-acetylgalactosamine is a terminal reducing sugar in the polysaccharide preparations has been investigated. Portions (2.5mg.) of fraction S1 from strain NIRD RO 94 in 1ml. of 0.033M-phosphate buffer, pH 7.0, were heated at 100° for 1hr. Hydrochloric acid was added to give a final concentration of 4N and the solution heated at 100° for 1hr.; galactosamine was then estimated on a portion of the solution. The galactosamine content of the material was 11.8% compared with 13.8% for material that had not been subjected to the preliminary heating in phosphate buffer. Heating fraction III from strain NIRD RO 94 (which does not contain a free terminal *N*-acetylhexosamine residue) at pH 7.0 caused no change in the galactosamine content.

The destruction of galactosamine by heating at pH 7.0 was also observed with two other polysaccharide preparations. For fraction S1/B from strain NCTC 6375 the galactosamine content decreased from 13.8 to 11.6%, and for fraction S1 from NIRD

Table 3. *Estimated average molecular weights of the polysaccharide components isolated from the wall of strains of L. casei*

The values were calculated from the relative amounts of *N*-acetylhexosamine chromogen formed by the polysaccharides and *O*- β -D-glucosyl-*N*-acetyl-D-galactosamine.

Strain	Fraction	Estimated average mol.wt.
NIRD RO 94	S1	11500
	S2	11500
NCTC 6375	S1/A	(No chromogen formed)
	S1/B	11000
NIRD H 831	S1	12500
55	S/A	10000
	S/B	17000

Fig. 2. Ultracentrifuge patterns of polysaccharides S1 from strain NIRD RO94 (upper pattern) and S1/A from strain NCTC 6375 (lower pattern). The concentration of polysaccharide was 1.0% in phosphate buffer, pH 7.0 and I 0.1; the centrifugal force was 260 000g and the time 100 min.

H 831 from 9.0 to 7.7%. If it is assumed that all the polysaccharide chains contain a terminal reducing *N*-acetylglucosamine residue that can be destroyed by heating at pH 7.0, then the average molecular weight of the preparation can be estimated. The approximate values for the preparations from strains NIRD RO 94, NCTC 6375 and NIRD H 831 are 12 500, 8000 and 13 000 respectively.

Ultracentrifugal analyses. Analyses were performed on polysaccharide preparations S1 from strain NIRD RO 94 and S1/A from strain NCTC 6375 (Fig. 2). Both preparations were relatively monodisperse and their sedimentation indicated no apparent heterogeneity. The sedimentation coefficient, S , for the preparation from strain NIRD RO 94 was 1.5s, a value that is not inconsistent with the molecular weight estimated by end-group analysis. For the preparation S1/A from strain NCTC 6375, the sedimentation coefficient was 2.4s.

Identification of hexosamine-containing disaccharides among the products of acid hydrolysis of the cell-wall polysaccharide from L. casei strain NIRD H 831

The studies on the polysaccharide component of the cell wall of *L. casei* strain NIRD H 831 (serological group B) indicated the presence of *N*-acetylhexosamine residues substituted on C_6 by the adjacent sugar. As the identity of the adjacent sugar was not known the products of mild acid hydrolysis of the polysaccharide have been examined. The material (2.0g. of fraction II) was dissolved in 200 ml. of 0.1N-sulphuric acid and heated under reflux in a boiling-water bath for 2 hr. The products of acid hydrolysis were then separated on a column of charcoal-Celite 535 followed by preparative paper chromatography as described by Knox & Hall

(1965a). The hydrolysate contained a series of oligosaccharides with a terminal reducing *N*-acetylhexosamine residue (as indicated by chromogen formation), and evidence for the structure of two of the disaccharides has been obtained. The methods employed were those used by Knox & Hall (1965a).

O- α -D-Galactopyranosyl-(1 $\rightarrow$ 6)-*N*-acetyl-D-glucosamine. The disaccharide moved as a single component on chromatography in ethyl acetate-pyridine-water (10:4:3, by vol.) (R_{Gl} 0.49) and on electrophoresis in borate (M_R 0.35). Acid hydrolysis followed by chromatography in butan-1-ol-pyridine-water (6:4:3, by vol.) indicated the presence of galactose and glucosamine; the presence of D-galactose was shown by oxidation with D-galactose oxidase (Avigad, Amaral, Asensio & Horecker, 1962). The molar ratio of galactose to glucosamine was 1.00:0.98. From estimations of the component sugars, the approximate yield of the disaccharide was 20 mg.

When a solution of the disaccharide in 0.05N-sodium carbonate was heated at 100° for 4 min. (Aminoff, Morgan & Watkins, 1952), the yield of chromogen was 103% of that expected from the *N*-acetylglucosamine content. The yield of chromogen suggested that the disaccharide contained a terminal *N*-acetylglucosamine residue substituted on C_6 (Jeanloz & Trémège, 1956), and the preparation was therefore compared with authentic samples of the α - and β -anomers by chromatography in pentan-1-ol-dioxan-water-acetic acid (43:34:23:0.6, by vol.); the R_{Gl} values for the α - and β -anomers and the disaccharide were 0.68, 0.61 and 0.67 respectively. Further evidence for an α -linkage was provided by the high optical rotation ($[\alpha]_D^{20} + 122 \pm 2^\circ$, c 0.5 in water, compared with $+118^\circ$ reported by Watkins, 1958), and the partial hydrolysis by α -galactosidase from *Streptococcus bovis*.

L-Rhamnosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosamine. In ethyl acetate-pyridine-water, the fastest-moving component (disaccharide I) had R_{Gl} 0.90. Electrophoresis in borate showed the presence of two components, the minor one (M_R 0.26) being designated IA, and the major one (M_R 0.35) designated IB. Attempts to separate the two components by paper chromatography were unsuccessful. Acid hydrolysis of disaccharide I followed by chromatography in butan-1-ol-pyridine-water indicated the presence of rhamnose, glucosamine and galactosamine; from estimations of the component sugars the approximate yield of disaccharide was 2.5 mg.

The preparation (1.5 mg.) was applied as a band to a sheet of Whatman no. 3MM paper. After electrophoresis in borate for 3 hr. at 800–900 v, the bands corresponding to the two components were eluted at 5° with 0.01N-hydrochloric acid. These precautions were insufficient to prevent alkaline

degradation of the disaccharide, as each eluate contained chromogen. Undegraded disaccharide was obtained by applying each eluate to a column of charcoal-Celite 535, and eluting with water until the washings were neutral and then with 10% (v/v) ethanol. Estimates of the amounts of the disaccharide were obtained by determining components in one-fifth of the usual volumes for quantitative analysis. These results indicated a recovery of 100 μ g. of the slower-moving component (IA) and 250 μ g. of the faster-moving component (IB). Chromatography of acid hydrolysates showed that component IB contained rhamnose and galactosamine whereas component IA still contained rhamnose, glucosamine and galactosamine. Both preparations readily formed chromogen at pH 7.0 and 100°, the maximum being reached in 45 min. The amount of chromogen formed by component IB was 11 times that expected from the calculated content of *N*-acetylgalactosamine and, further, when the disaccharide was heated at 65° in 0.1 *N*-pyridine for 30 min. the products obtained were rhamnose and free *N*-acetylhexosamine chromogen. These properties are similar to those described for *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosamine by Knox & Hall (1965a), and suggest that disaccharide IB is rhamnosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosamine.

Disaccharide I was an effective inhibitor of the precipitin reaction between fraction II from strain H831 (10 μ g.) and homologous antiserum (0.2 ml.), 0.5 μ M giving 35% inhibition; by comparison 10 μ M of the α - and β -anomers of methyl-L-rhamnopyranoside gave 25 and 11% inhibition respectively. These results provide evidence for an α -L-rhamnoside linkage in disaccharide I. Insufficient disaccharide IB was available for this test.

Serological properties of the mucopeptide and polysaccharide components

Polysaccharide components from organisms of both serological groups B and C gave a precipitate with homologous antiserum.

Cell-wall and mucopeptide preparations from strain NIRD RO 94 were tested for their ability to adsorb antibodies from homologous antiserum. Preparations of cell wall (5 mg.) and mucopeptide (5 mg.) were shaken with 0.5 ml. of antiserum for 1 hr. at room temperature. After centrifuging, the antiserum was removed, added to a further 5 mg. of the appropriate preparation and shaken for 1 hr. The antisera were then tested for their ability to form a precipitate with fraction II from strain NIRD RO 94. No precipitate was formed with antiserum adsorbed with cell wall, whereas a precipitate was formed with antiserum adsorbed with mucopeptide.

DISCUSSION

The observation that the linkage between the polysaccharide and mucopeptide components of the cell wall of *L. casei* can be hydrolysed under mild conditions of acid hydrolysis (Knox & Hall, 1965b) has afforded a convenient means of separating the components with minimal hydrolysis of linkages within the polymers. Krause & McCarty (1961, 1962) observed that mild acid hydrolysis released only part of the polysaccharide component of the cell wall of strains of streptococci belonging to serological groups A and C. A more complete separation of the polysaccharide and mucopeptide components was achieved by heating in formamide, although this procedure resulted in considerable loss of carbohydrate.

After separation, both the polysaccharide and mucopeptide components were analysed for their constituents, and the results compared with those for the intact cell wall. The amino acid constituents of the mucopeptide are glutamic acid, aspartic acid, lysine and alanine, the relative molar proportions for both cell-wall and mucopeptide preparations being similar to those found for a laboratory strain of *L. casei* by Knox & Brandsen (1962). Further, for each of the preparations of mucopeptide, the relative molar proportions glutamic acid:glucosamine:muramic acid are approximately 1.0:1.0:0.8, values that are similar to those reported by Czerkawski, Perkins & Rogers (1963) for *Micrococcus lysodeikticus*.

After removal of polysaccharide the mucopeptide is susceptible to lysozyme (cf. Krause & McCarty, 1961, 1962). As shown by Gould *et al.* (1963), coupling of lysozyme with a fluorescent dye provides a satisfactory means of detecting susceptibility to lysozyme. The reason for the lack of lysozyme action on the intact cell wall is not known, but it may be due to steric hindrance by the polysaccharide chains.

The products of lysozyme action included indiffusible phosphorylated mucopeptides, and phosphorus was also found to be a component of purified cell-wall fractions (fraction III) obtained by the action of the *Streptomyces* enzyme. Ågren & de Verdier (1958) isolated muramic acid phosphate from the products of acid hydrolysis of *L. casei*, and Liu & Gotschlich (1963) detected both muramic acid phosphate and galactosamine 6-phosphate among the products of hydrolysis of pneumococcal C polysaccharide. For detecting possible phosphorylated hexosamines in the cell-wall fractions of *L. casei*, fraction III from strain NIRD RO 94 was considered suitable as the molar ratio of muramic acid to phosphorus was 1.5:1.0 compared with 6.3:1.0 for mucopeptide from the same strain. A phosphorylated hexosamine, which moved towards the anode

on electrophoresis at pH 3.5, was detected among the products of hydrolysis of fraction III. It could be distinguished from glucosamine 6-phosphate, which did not move from the origin; the only other hexosamine known to be a component of the mucopeptide is muramic acid, but muramic acid phosphate was not available for comparison. As the procedure employed for detecting phosphorylated products did not separate glucosamine 6-phosphate from non-migrating products of partial acid hydrolysis, the possibility of its presence cannot be disregarded. The tentative identification of a phosphorylated residue in the mucopeptide lends added support to the concept of a phosphodiester linkage between mucopeptide and polysaccharide components of the cell wall (Knox & Hall, 1965b).

Evidence for the rapid rate of hydrolysis of the linkage between the polysaccharide and mucopeptide components of the cell wall arose from the observation that heating preparations in dilute acid resulted in the release of products containing a terminal reactive *N*-acetylhexosamine residue (Knox & Hall, 1965b). Studies on the isolated polysaccharides have confirmed that those responsible for group specificity contain this terminal residue. Further, acid hydrolysis of the polysaccharide preparations releases additional reactive *N*-acetylhexosamine residues at a rate comparable with the second, slower, stage of release of reactive *N*-acetylhexosamine from cell wall. A series of oligosaccharides arising from partial acid hydrolysis of cell-wall products from group C organisms have been identified (Knox & Hall, 1965a); most of these contain a terminal reactive *N*-acetylhexosamine residue, exemplified by *O*- β -D-glucopyranosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosamine. Partial acid hydrolysis of cell-wall products from a group B organism (NIRD H 831) also gives rise to a series of oligosaccharides containing a terminal reactive *N*-acetylhexosamine residue; in this instance it is adduced that L-rhamnosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-galactosamine is present. The products of hydrolysis of the group B polysaccharide also include *O*- α -D-galactopyranosyl-(1 $\rightarrow$ 6)-*N*-acetyl-D-glucosamine; the group C polysaccharide contains the corresponding β -anomer.

The amount of chromogen formed by heating the polysaccharide preparations at pH 7.0 for 45 min. provided an estimate of the molecular weights of the preparations; a second estimate was provided by determining the amount of galactosamine destroyed under these conditions. Results of the two methods were comparable and indicated that the average molecular weights of most of the preparations were between 10000 and 12500. The polysaccharide from strain NIRD RO 94 was also examined in the ultracentrifuge, and the sedimentation coefficient is not inconsistent with the molecular

weight estimated by end-group analysis. The rhamnose-containing polysaccharide from *L. casei* var. *rhamnosus* does not contain a terminal reactive *N*-acetylhexosamine residue, so that end-group analysis could not be performed. However, the sedimentation coefficient for this preparation indicates that the molecular weight is higher than that for the other preparations.

The cell wall of *L. casei* var. *rhamnosus* contains two polysaccharide constituents, one being responsible for group C specificity, the other being found only in those strains classified as var. *rhamnosus* (Knox, 1963; Glastonbury & Knox, 1963). The group-specific polysaccharide contains glucose, galactose, glucosamine and galactosamine, and forms chromogen on heating at pH 7.0, whereas the polysaccharide specific for var. *rhamnosus* contains rhamnose, glucose and galactose, but no hexosamine. Each of the polysaccharides is liberated from cell wall under mild conditions of acid hydrolysis (Knox & Hall, 1965b), and it has been shown that they can be separated by ammonium sulphate fractionation in conjunction with chromatography on DEAE-cellulose. This confirms previous studies on the soluble products obtained on digestion of cell wall with a *Streptomyces* enzyme preparation (Knox, 1963) in which it was shown that polysaccharide-mucopeptide complexes containing these two polysaccharides could be separated by ammonium sulphate fractionation.

Group B specificity depends on a rhamnose-containing polysaccharide, α -L-rhamnopyranoside being an effective inhibitor. Strains of group B organisms contain various amounts of glucose and galactose as cell-wall components (Glastonbury & Knox, 1963). More detailed studies on laboratory strain 55 (Knox & Brandsen, 1962) indicated that two different components were present, since products of differing molar ratios of rhamnose to glucose (0.9:1.0 and 5.0:1.0) could be obtained on fractionation of an autolysate of cell wall. Chromatography of the polysaccharide preparation from this strain on DEAE-cellulose has confirmed the previous observations, the molar ratios of rhamnose to glucose for the two fractions being 1.2:1.0 and 5.4:1.0.

Mild conditions of acid hydrolysis were used by Lancefield (1933) to prepare serologically active extracts, and have been used for the serological classification of streptococci and lactobacilli (Sharpe 1955). The preceding (Knox & Hall, 1965b) and present studies indicate that the conditions used for preparing these extracts would release polysaccharides. This is confirmed by the observation that it is these components that are responsible for serological specificity of lactobacilli of groups B and C (Knox, 1963; Knox & Hall, 1965a). For some groups of lactobacilli, serological classification depends on the presence of wall teichoic acids (Baddiley

& Davison, 1961), and these components would also be released under mild conditions (Armstrong, Baddiley, Buchanan, Carss & Greenberg, 1958). However, such conditions would also release any intracellular teichoic acids, and could cause the degradation of serologically active material, particularly oligosaccharides (Wicken & Baddiley, 1963).

The results of this and previous studies indicate that the cell wall of *L. casei* contains polysaccharide chains joined through phosphodiester linkages to a mucopeptide. These polysaccharides enable strains of *L. casei* to be divided into two main groups on the basis of their serological properties. Further, more than one type of polysaccharide may be present, as shown by strains of *L. casei* var. *rhamnosus*. Sims (1964) described a strain of this variety that forms a capsule, and studies on this strain (Knox & Hall, 1964) have indicated that the capsule is composed of the rhamnose-rich polysaccharide normally found as a component of the cell wall. This strain apparently lacks the ability to complete the synthesis of cell wall by attaching the rhamnose-rich polysaccharide to the mucopeptide.

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