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Structure of the Mannan from *Saccharomyces* Strain FH4C, a Mutant Constitutive for Invertase Biosynthesis. II. Protein Moiety and Components of the Carbohydrate–Peptide Bonds†

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ABSTRACT: The cell wall mannan of *Saccharomyces* mutant FH4C has been extracted and separated by ion-exchange chromatography into one acidic and two neutral fractions. The protein moieties of these preparations are rich in serine and threonine which carry oligomannosides of DP 1–4 that are released by dilute alkali. These comprise ~11% of the total mannan and their order of abundance is $M_2 > M_1 \approx M_3 > M_4$. The oligosaccharides are susceptible to enzymolysis by a bacterial α-mannosidase with resultant enrichment of the monosaccharide fraction; however, the mannose–*O*-Ser (Thr) bond is not attacked. The amino acid composition of FH4C mannan protein is similar to those of other mannan peptides. Asp, Glu, Ala, Ser, and Thr comprise >65% of the total protein; over 70% of each of the latter two acids possess attached oligomannosides. FH4C mannan contains 0.5–0.8% of glucosamine. Mannans deproteinated with hot alkali lack this sugar, suggesting that it became exposed and subsequently destroyed during the proteolysis. Thus, glucosamine may represent the reducing terminus of individual mannan chains and may join these to mannan peptide by *N*-glycosidic bonds to asparagine. The molar excess of aspartic acid in all mannans examined strengthens the latter possibility. The mannose : glucosamine ratio of FH4C mannan indicates that individual mannan chains have a molecular weight range of 19,000–37,000. Mannan peptide appears to cross-link these mannan chains to form macromolecules >300,000.

Yeasts have cell walls comprised predominantly of glucan and mannan. The former is a sparsely branched polymer of glucose monomers joined by β-1,3 linkages; some β-1,6 bonds are also present. In contrast, mannan is a highly branched α-linked mannose polymer containing 1,2-, 1,3-, and 1,6-mannosidic bonds (50:15:35 in the usual baker's yeast).

Mannan contains small amounts of protein (4–8% by weight; Eddy, 1958; Sentandreu and Northcote, 1968) which is postulated (Thieme and Ballou, 1972) to cross-link mannan chains, thereby increasing the rigidity of the cell wall and providing a matrix for extracytoplasmic enzymes, *e.g.*, acid phosphatase or invertase.

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Yeast cell wall and mannan–proteins contain a full complement of amino acids and small amounts of glucosamine. Sentandreu and Northcote (1968) presented evidence that, as in other glycoproteins (Fletcher *et al.*, 1963; Marks *et al.*, 1963; Spiro, 1970; Yosizawa *et al.*, 1966), glucosamine links mannan chains to yeast cell wall protein *via* glucosaminyl-asparagine *N*-glycosidic bonds. They also demonstrated *O*-glycosidic bonds between mannose oligosaccharides and serine and threonine residues in mannan protein, a finding recently confirmed by other investigators (Tkacz, 1971; Thieme and Ballou, 1971; Cawley *et al.*, 1972).

We have examined the carbohydrate–protein linkages in the mannan from *Saccharomyces* mutant FH4C and characterized the mannan *O*-glycosides. The amino acid compositions of FH4C mannans were compared with those of other yeast cell wall proteins. Data on the molecular weight of mannan and of the polymannose side chains are also presented.

Materials and Methods

The growth and harvesting of *Saccharomyces* mutant FH4C, and the preparation of *Arthrobacter* α-mannosidase, FH4C bulk mannan, its neutral and acidic components, and mannan core have been described (Colonna and Lampen, 1974).

Alkali Mannan. Bulk mannan (1.38 g) was hydrolyzed at 90–95° for 10.5 hr in 1 l. of 3.5 M NaOH. The hydrolysate was neutralized with glacial acetic acid and exhaustively dialyzed. The retentate was concentrated with Aquacide II (Calbiochem), filtered, then lyophilized to yield 0.85 g of alkali mannan.

Alkali mannan core was prepared by the procedure used for making bulk mannan core (Colonna and Lampen, 1974). A yield of 334 mg of alkali mannan core was obtained by exhaustive enzymolysis of alkali mannan (0.82 g) with 3.8 units of α-mannosidase.

β-Eliminated Mannan. Removal of serine- and threonine-linked *O*-glycosides from mannan was accomplished by alkaline β elimination. Bulk mannan (141 mg) was dissolved in 300 ml of 0.5 M NaOH and incubated at 4°. After 55 hr, the solution was adjusted to pH 6.3 with glacial acetic acid, then dialyzed against distilled water. The retained β-eliminated mannan (125 mg) was recovered by lyophilization.

Methods for the determination of carbohydrate, protein, and reducing sugar, and the procedure for paper chromatography of sugars have been described (Colonna and Lampen, 1974). Solvent system 2 consists of 1-butanol–ethanol–water (3:1:1). Glucosamine was detected as its *N*-acetyl derivative by the procedure of Levvy and McAllan (1959).

β elimination of serine and threonine *O*-glycosides was estimated from the increase in optical density at 241 mμ which resulted on exposure of mannan to dilute alkali. The reaction was carried out at room temperature in a final volume of 1.5 ml in quartz cuvets (1-cm path length). Readings were made on either a Beckman DU-2 spectrophotometer or a Beckman DB spectrophotometer equipped with a Sargent recorder. The reaction mixture contained 1–1.5 ml of freshly prepared NaOH (0.5 N) plus 0.05–0.50 ml of mannan solution (0.22–0.53 mg carbohydrate). On addition of mannan, the initial OD was read against an appropriate NaOH blank; additional readings were made until no further increase in extinction was apparent (200 min).

Amino Acid Analysis. Mannans were hydrolyzed *in vacuo* at 110° with 6 N HCl. After 24 hr, the acid was removed by repeated lyophilization over NaOH pellets and the hydrolysates analyzed on a Durrum 500 amino acid analyzer.

TABLE I: Extent of β Elimination in FH4C Mannans.

Mannan Preparation	Mannose:Phosphorus Molar Ratio	Mannose:Protein Weight Ratio	Change in OD_{241}/mg of Carbohydrate	Change in OD_{241}/mg of Mannan Protein
Neutral mannan 1	94.5	30	0.23	7.22
Neutral mannan 2	75.1	28.8	0.38	10.55
Bulk mannan	50.7	14.7	0.84	12.54
(two determinations)			0.77	11.32
Acidic mannan	35.5	10.5	1.24	13.17

Results

β Elimination of FH4C Mannans. In the accompanying paper (Colonna and Lampen, 1974), it was shown that FH4C bulk mannan can be resolved on DEAE-cellulose into three mannan subspecies which differ primarily in their phosphate content. When bulk mannan and its neutral and acidic components (designated neutral mannans 1 and 2 and acidic mannan) were exposed to dilute alkali, a progressive increase in optical density at 241 mμ was observed, attributable to the formation of the dehydro derivatives of serine and/or threonine. Table I shows that, in addition to ionic properties, the mannan subspecies differ in the number of bonds susceptible to alkaline elimination. Mannan core, the residue derived from bulk mannan by exhaustive hydrolysis with a bacterial α-mannosidase (Colonna and Lampen, 1974) was also capable of undergoing the elimination reaction (not shown). These observations confirm the initial report by Tkacz (1971) that, like other yeast mannans (Sentandreu and Northcote, 1968; Thieme and Ballou, 1971; Cawley *et al.*, 1972) the mannan from mutant FH4C has *O*-glycosidic bonds between residues of carbohydrate and the hydroxyamino acids of mannan protein. Furthermore, these linkages are either inert to cleavage by α-mannosidase or protected therefrom by steric barriers.

Characterization of the Mannan O-glycosides. Bulk mannan (55.7 mg dry weight) and mannan core (53.7 mg dry weight, equivalent to approximately 103 mg of bulk mannan) were each dissolved in 2 ml of distilled water and the resulting solutions were measured for absorbance at 241 mμ. Each was made 0.5 N in NaOH and incubated at 0–4°. After 48.5 hr, both samples showed a marked increase in ultraviolet absorbance and contained free reducing groups, indicating that alkaline cleavage had occurred (Table II). Each preparation was dialyzed against dilute acetic acid and the diffusible fractions were desalted with AG 50W-X2 (H^+ form) and then lyoph-

TABLE II: β Elimination of Bulk Mannan and Mannan Core in Dilute Alkali.

Mannan Preparation	Change in OD_{241}/mg of Original Carbohydrate	Reducing Power (Mannose Equiv)[a]
Bulk mannan	0.93	5.3
Mannan core	1.40	18.8

[a] One mannose equivalent = reducing power of 1 μmol of D-mannose.

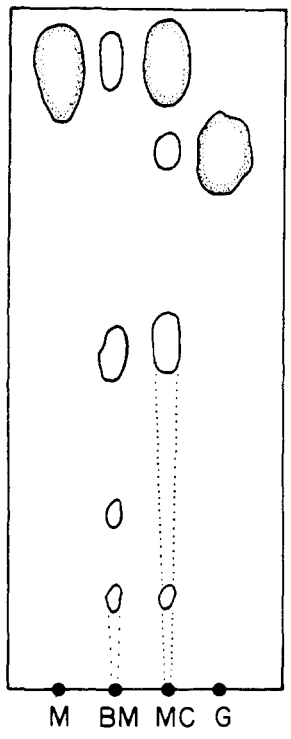

FIGURE 1: Resolution of oligosaccharides released by alkaline β elimination of bulk mannan and mannan core. Chromatogram was developed at room temperature for 48 hr with solvent system 2, then stained with alkaline silver nitrate (Materials and Methods).

ilized. When chromatographed on paper in solvent system 2, the mannan sugars were resolved into four distinct components which included mannose and three other sugars with mobilities suggestive of di-, tri-, and tetrasaccharides (Figure 1); similarly, the carbohydrate from mannan core contained mannose and sugars corresponding to two of the oligosaccharides found in bulk mannan. The trisaccharide spot was absent; however, a trace of glucose was observed, no doubt generated by epimerization of mannose during the elimination reaction. Glucose was not detected in the mannan sugars.

The size, number, and staining intensities of the sugar spots indicated that both qualitative and quantitative differences existed among the mannosides eliminated from mannan and mannan core. Accordingly, each oligosaccharide mixture was fractionated on a G-15 Sephadex column (1.3 × 105 cm) previously calibrated with mono-, di-, and trisaccharides. In addition to sugars which emerged at the positions of the calibration standards, each mixture contained a larger fragment which eluted as a tetrasaccharide (Figure 2). By summing the peak areas, the distributions of the oligosaccharides were determined for both mannan and mannan core. These data (Table III) confirm the results of paper chromatography. Thus, mannobiose was the predominant sugar released from mannan; lesser (but nearly equivalent) amounts of mannose and mannotriose were also present while mannotetraose was least abundant. In contrast, mannose was the major elimination product from mannan core, while the higher saccharides were reduced 50–60% from their levels in the intact mannan. Since the core was prepared by enzymolysis of bulk mannan by α-mannosidase, it is clear that the oligosaccharides are susceptible, at least in part, to this enzyme. Thus, mannose is enriched in the *O*-glycosides of the core at the expense of the higher oligomannosides and remains as a "stub" following enzymolysis of the latter.

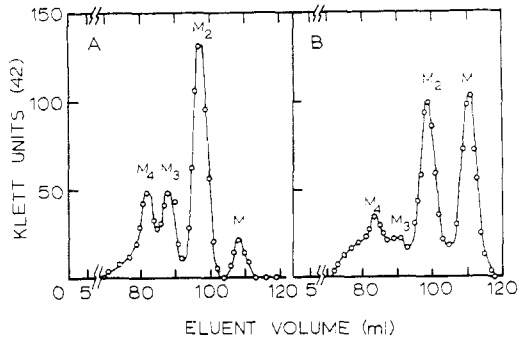

FIGURE 2: Sephadex G-15 elution profile of β-eliminated sugars from (A) bulk mannan and (B) mannan core. The eluent was monitored for carbohydrate (○) with phenol and H_2SO_4 (Materials and Methods). M = mannose; subscript denotes degree of polymerization of oligosaccharide.

It is noteworthy that, following alkaline elimination, most of the carbohydrate (~89% in mannan, ~84% in mannan core) remains associated with the nondialyzable mannan-peptide fraction. This same observation was made with a baker's yeast mannan–peptide by Sentandreu and Northcote (1968) and is consistent with their conclusion that only a small part of the total mannan is joined to mannan protein by bonds to serine and threonine while most of the carbohydrate is attached to protein by a second, alkali-stable bond.

Amino Acid Composition of FH4C Mannan Protein. Bulk mannan and two of its component fractions (neutral mannan 2 and acidic mannan) have very similar amino acid compositions (Table IV). This indicates that the protein moieties do not contribute significantly to the different ionic properties of the individual subfractions; rather, the differences are largely due to structural variations in their respective carbohydrate moieties, *e.g.*, the content of mannan-phosphate (Colonna and Lampen, 1974) and the extent of glycosylation of the hydroxyamino acids (Table I).

The amino acid composition of mannan core is nearly identical with that of intact bulk mannan; hence, the mannan protein was not degraded during exposure to the partially purified *Arthrobacter* α-mannosidase.

Mannan which has undergone complete β elimination has an amino acid composition which is in excellent agreement with those of the other mannans examined; however, its content of serine and threonine is much lower owing to their destruction (~71% each) during elimination of the mannan *O*-glycosides. Thus, at least 71% of both the serine and threonine residues of mannan protein are glycosylated.

Table V presents the amino acid compositions of FH4C bulk mannan and cell walls (Montenecourt, 1968) and a

TABLE III: Distribution of Oligosaccharides Released from Mannan and Mannan Core by Alkaline β Elimination.

Prepn Exposed to Dilute Alkali	% of Original Carbohydrate Released by Alkali	Oligosaccharides Released[a]			
		Mannose	Mannobiose	Mannotriose	Mannotetraose
Bulk mannan	11.3	15.8	58.4	14.5	11.4
Mannan core	16.0	60.3	29.5	5.8	4.4

[a] Mol %.

TABLE IV: Amino Acid Compositions of FH4C Mannans.

Amino Acid	Mol of Amino Acid/Mol of Aspartic Acid				
	Neutral Mannan 2	Acidic Mannan	Mannan Core	Bulk Mannan	β-Eliminated Bulk Mannan
Aspartic acid	1.00	1.00	1.00	1.00	1.00
Threonine[a]	1.77	2.67	2.58	2.24	0.66
Serine[b]	1.97	1.94	2.04	1.86	0.54
Glutamic acid	0.67	0.99	0.89	0.85	0.89
Proline	0.71	0.54	0.62	0.98	1.00
Glycine	0.54	0.45	0.49	0.47	0.46
Alanine	1.24	1.07	1.09	1.10	1.10
Valine	0.71	0.92	0.78	0.84	0.83
Methionine				0.02	0.02
Isoleucine	0.26	0.39	0.31	0.34	0.32
Leucine	0.28	0.45	0.30	0.39	0.38
Tyrosine	0.06	0.15	0.12	0.12	0.14
Phenylalanine	0.05	0.02	0.10	0.14	0.15
Histidine	0.13	0.09	0.07	0.11	0.05
Lysine	0.31	0.26	0.23	0.29	0.06
Arginine	0.04	0.10	0.02	0.06	0.04
Glucosamine[c]	0.73	0.25	0.55	0.43	0.30

[a,b,c] Correction factors applied, respectively, 0.98, 0.93, and 0.428 (Neumann and Lampen, 1967).

mannan glycopeptide from *S. cerevisiae* cell walls (Sentandreu and Northcote, 1968). The glycopeptide resembles in its composition the cell walls and mannan from mutant FH4C. Furthermore, in all of the mannan and cell wall preparations (Tables IV and V) aspartic acid, glutamic acid, serine, threonine, and alanine predominate, comprising 65–72% of the total protein. These values are consistent with the amino acid compositions of *K. brevis* mannan (Thieme and Ballou, 1972) and walls of *S. carlsbergensis* (Masschelein *et al.*, 1963) and *S. cerevisiae* (Orenstein, 1971) and are indicative of a close structural similarity among yeast cell wall proteins.

Table V also lists the amino acid composition of FH4C external invertase, a glycoenzyme which is found in the yeast cell wall matrix and which consists of approximately equal amounts of mannan and protein (Gascon *et al.*, 1968; Neumann and Lampen, 1967). Unlike FH4C mannan, the amino acids Ser, Thr, Glu, Asp, and Ala account for only ~48% of the total invertase protein. Furthermore, the disparities in the proportions of these acids in mannan and invertase minimize the possibility that bulk mannan isolated from the cell wall is contaminated with mannan from external invertase.

All of the mannans examined contained varying amounts of glucosamine. This sugar has been sugested (Sentandreu and Northcote, 1968; Thieme and Ballou, 1972) to link mannan chains to cell wall protein *via N*-glycosidic bonds with asparagine. The observation that aspartic acid was in excess of the glucosamine in all of the FH4C mannans (Table IV) supports this proposal. Such bonds have been demonstrated in other glycoproteins (Fletcher *et al.*, 1963; Marks *et al.*, 1963; Spiro, 1970; Yosizawa *et al.*, 1966).

Location of Glucosamine. The kinetics of the release of glucosamine from mannan were studied. Bulk mannan (22 mg) was hydrolyzed under nitrogen in a screwed-capped tube with 0.8 N HCl at 108–110°. The hydrolysate was examined at intervals for reducing sugar and hexosamine. Figure 3 shows

TABLE V: Comparison of the Amino Acid Compositions of Mannan, Cell Walls, and External Invertase from *Saccharomyces* Mutant FH4C and a Mannan Glycopeptide from *S. cerevisiae*.

Amino Acid	Mol of Amino Acid/Mol of Aspartic Acid[a]			
	S. cerevisiae Mannan Glycopeptide A_2[b]	FH4C Cell Walls[c]	FH4C Bulk Mannan[d]	FH4C External Invertase[e]
Asp	1.00	1.00	1.00	1.00
Thr	3.22	3.36	2.24	0.47
Ser	1.87	2.43	1.86	0.64
Glu	0.90	1.48	0.85	0.65
Pro	0.91	0.82	0.98	0.37
Gly	0.87	0.78	0.47	0.40
Ala	1.35	1.85	1.10	0.38
Cys		0.11		
Val	0.69	1.16	0.84	0.39
Met		0.03	0.02	0.12
Ile	0.19	0.56	0.34	0.22
Leu	0.26	0.44	0.39	0.47
Tyr	0.10	0.22	0.12	0.37
Phe	0.10	0.15	0.14	0.45
His	trace	0.14	0.11	0.09
Lys	0.26	0.53	0.29	0.34
Arg	trace	0.06	0.06	0.15
$GlcNH_2$	0.46	8.09	0.43	0.21

[a] Recalculated from the original reports by dividing all values by the number of moles of aspartic acid. [b] Sentandreu and Northcote (1968). [c] Montenecourt (1968). [d] Hydrolyzed 24 hr at 110° in 6 N HCl. [e] Gascon *et al.* (1968).

the kinetics of mannan hydrolysis and the progressive release of glucosamine. When hydrolysis was complete, the total amino sugar was 0.66 μmol, corresponding to an initial mannose:glucosamine molar ratio of ~120 (corrected for the 11.3% mannose found in serine and threonine *O*-glycosides; Table III). If a single glucosamine represents the reducing terminus of a polymannose chain, then a polymer with a mannose:glucosamine ratio of 120 would have a molecular weight of 19,000, comparable to Thieme and Ballou's (1972) figures for the size of mannan subunits released by pronase from high molecular weight mannans of *K. brevis*.

Several additional FH4C mannans were examined for glucosamine. These included bulk mannan, β-eliminated

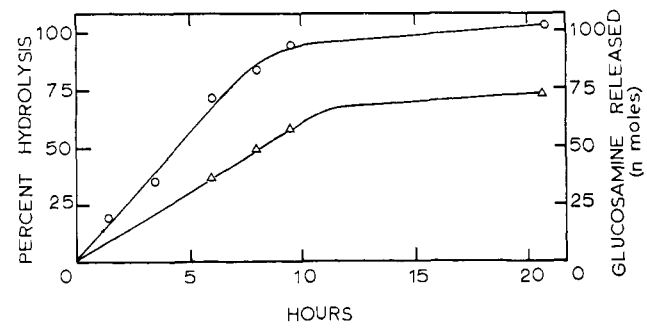

FIGURE 3: Kinetics of hydrolysis of and glucosamine release from FH4C bulk mannan. Hydrolysis conditions were 0.8 N HCl, 108–110°: (○) % hydrolysis of mannan based on release of reducing sugar; (△) glucosamine released.

TABLE VI: Mannose: Glucosamine Ratios and Molecular Weights of FH4C Bulk Mannan, β-Eliminated Mannan, and Mannan Core.

Mannan Preparation	Mannose: Glucosamine Molar Ratio	Calcd Mol Wt[a]	Method of Anal.
Bulk mannan	120[b]	19,000[c]	Levvy and McAllan (1959)
	177[b]	29,000[c]	Levvy and McAllan (1959)
	148[b]	24,000[c]	Amino acid analysis
β-Eliminated mannan	161[b]	26,000	Levvy and McAllan (1959)
	228[b]	37,000	Amino acid analysis
Mannan core[d]	94	15,000[c]	Amino acid analysis

[a] Calculated assuming a molecular weight of 162 for anhydromannose. [b] Replicate determinations. [c] Corrected for mannose present in serine- and threonine-linked oligomannosides (Table III). [d] Hydrolyzed ~52% by *Arthrobacter* α-mannosidase.

mannan, mannan deproteinated by hot alkali, and the core was derived from the latter by hydrolysis with α-mannosidase. After acid hydrolysis, each mannan was neutralized and passed through a cation-exchange resin, from which the retained material was then eluted with 2 N HCl. Only the two eluates obtained from bulk mannan and β-eliminated mannan contained hexosamine, yielding chromogens whose spectra coincided with that of authentic glucosamine (Table VI).

The absence of hexosamine in the mannans treated with hot alkali suggests that the amino sugar molecules were exposed as reducing termini during alkaline proteolysis and subsequently destroyed. This is evidence against an internal location for glucosamine in mannan, and supports instead the proposed terminal position for this sugar as the linking moiety between mannan chains and protein (Gorin, 1971).

Table VI compares the mannose: glucosamine ratios and molecular weights of bulk mannan, mannan core, and β-eliminated mannan as determined by several analytical methods. The lack of precision may reflect the limitations of the assay procedures employed. However, the molecular weight values for β-eliminated and bulk mannan fall within a narrow range and are consistent with published data (Thieme and Ballou, 1972). The figure obtained for mannan core is also acceptable, as the latter was prepared by enzymatic hydrolysis of bulk mannan.

Discussion

Glycoproteins containing serine and/or threonine *O*-glycosidic linkages include such examples as ovine submaxillary gland glycoprotein (Carubelli *et al.*, 1965), chrondroitin sulfate (Anderson *et al.*, 1965), and others (Komatsu *et al.*, 1970; Pazur *et al.*, 1971). The carbohydrate residues of these linkages can be either neutral sugars or *N*-acetylhexosamines, *e.g.*, *N*-acetylglucosamine, *N*-acetylgalactosamine, etc. (Spiro, 1970).

O-Glycosidic bonds were first demonstrated in mannan by Sentandreu and Northcote (1968) and more recently by Thieme and Ballou (1971) and by Cawley *et al.* (1972). In this communication, we have shown that the mannan from *Saccharomyces* mutant FH4C contains oligomannosides of DP 1–4 joined *O*-glycosidically to serine and threonine residues of mannan protein; collectively, these sugars account for >11% of the carbohydrate moiety of the mannan molecule. The latter figure is in good agreement with the data of Cawley *et al.* (1972). However, the observation that alkali removes only a small part of the total carbohydrate (Sentandreu and Northcote, 1968; Cawley *et al.*, 1972) suggests that most of the mannan is joined to mannan–peptide *via* a second, alkali-stable bond.

N-Acetylglucosamine could not be detected in the *O*-glycosides released from mannan or mannan core. This does not rule out the possibility that this sugar is present in the form of mixed oligosaccharides (*e.g.*, Man-GlcNAc, Man-Man-GlcNAc, etc.). However, from the chromatogram in Figure 1, a straight line was obtained by plotting the DP of the resolved oligosaccharides *vs.* a function of their individual mobilities (*i.e.*, DP *vs.* log $[1000R_{\text{mannose}}/(1 - R_{\text{mannose}})]$; data not presented), indicating that the sugars are members of a homologous series (Jones and Ballou, 1969). Therefore, it is unlikely that the *O*-glycosides are comprised of sugars other than mannose, a conclusion in agreement with Sentandreu and Northcote (1968) who recovered only mannose in acid hydrolysates of the *O*-glycosides from *S. cerevisiae* mannan.

Like the rest of the mannan molecule, the serine- and threonine-linked *O*-glycosides are susceptible to a bacterial α-mannosidase. Exhaustive hydrolysis (~52%) of the FH4C mannan with the latter enzyme shifted the distribution of these oligomannosides with a resultant enrichment of the monosaccharide fraction. However, since the *O*-glycosides now accounted for a larger percentage (>16%) of the total remaining carbohydrate, it appears that these sugars, in the intact mannan, are less accessible to the α-mannosidase than the mannose residues which comprise the rest of the mannan molecule. This conclusion is strengthened by the observation that the oligosaccharides are not entirely reduced in size to single mannose "stubs" attached to serine and threonine residues. These sugars are probably shielded from the mannosidase, at least in part, either by the rest of the mannan molecule (which in itself is partially resistant; Colonna and Lampen, 1974), or by mannopeptide. The terminal mannose in the Man–*O*-Ser (Thr) bond may also be sterically protected from or, more likely, structurally inert to the α-mannosidase. In the accompanying paper, it was shown that bound phosphate sterically shields the mannan molecule from total enzymatic hydrolysis. It is possible that some of the mannan *O*-glycosides are phosphorylated and were thus able to survive exposure to α-mannosidase. However, paper chromatography of the sugars released by β-elimination indicated that only traces, at most, of sugar phosphates could be present. Also, Cawley *et al.* (1972) reported that alkaline β elimination of *O*-glycosides did not alter the phosphate content of their baker's yeast phosphomannan. Therefore, it is unlikely that the Ser-/Thr-oligomannosides contain significant amounts of phosphate.

FH4C bulk mannan and its three subspecies isolated by DEAE chromatography were all capable of undergoing β elimination in dilute alkali but appear to differ in the number of *O*-glycosidic bonds (Table I). Since the protein moieties of at least three of the mannans have nearly the same amino acid composition (Table IV), the differences are probably due to variations in the number of serine and threonine residues which are glycosylated. Each mannan species may thus represent a different stage in the mannan biosynthetic pathway.

FH4C mannan protein and its neutral and acidic components all contain a full complement of amino acids and show only minor variations in composition (Table IV). The FH4C

mannan proteins resemble those of mannans and cell walls from other yeasts, with aspartic acid, glutamic acid, alanine, serine, and threonine comprising >65% of the total protein. Over 70% of each of the latter two acids were joined to oligomannosides by alkali-labile *O*-glycosidic bonds. Sentandreu and Northcote (1968) observed that 42 and 64%, respectively, of the serine and threonine residues are glycosylated in *S. cerevisiae* mannan; Cawley *et al.* (1972) reported somewhat higher values (70 and 78%, respectively, for serine and threonine) for baker's yeast mannan.

Glucosamine was found in all the FH4C mannan fractions examined (Table IV) and its presence confirmed by gas–liquid chromatography (results not presented). Previous investigators (Sentandreu and Northcote, 1968; Thieme and Ballou, 1972) have suggested that this sugar (probably as its *N*-acetyl derivative) represents the reducing termini of high molecular weight mannan chains and joins these to mannan protein *via* glucosaminylasparagine *N*-glycosidic bonds which, unlike *O*-glycosidic bonds, are stable to dilute alkali. A terminal reducing position for glucosamine is supported by our observation that the latter is absent in mannans deproteinated by hot alkali; presumably, during alkaline proteolysis, the hexosamine would become exposed and subsequently destroyed on prolonged exposure to base. Furthermore, in all the FH4C mannans, aspartic acid occurred in a 1.3- to 4-fold excess, more than satisfying the required stoichiometry for glucosamine–asparagine bonds. The latter may thus represent the second carbohydrate–protein linkage in FH4C yeast mannan.

The presence of both *N*- and *O*-glycosidic bonds in the same glycoprotein is unusual, but not unknown. Examples include fetuin (Spiro, 1970), a human A myeloma globulin (Dawson and Clamp, 1967), and a human chorionic gonadotropin (Bahl, 1969). Oddly enough, FH4C external invertase (mol wt 270,000), which contains 50% mannan (Neumann and Lampen, 1967) contains negligible carbohydrate in the form of serine and threonine *O*-glycosides. Instead, most (perhaps all) of the mannan is joined to invertase protein by alkali-stable *N*-glycosidic bonds between *N*-acetylglucosamine and asparagine.

Thieme and Ballou (1972) demonstrated that pronase digestion of 500,000 molecular weight mannan from *K. brevis* released protease-resistant mannan fragments of 25,000–35,000 which contained 1 mol each of glucosamine and aspartic acid. They concluded that native mannan is comprised of smaller carbohydrate subunits of mol wt 25,000–35,000 cross-linked by peptide to form macromolecules of up to mol wt 500,000. Native bulk mannan from mutant FH4C is heterogeneous with respect to molecular weight, having a size range of from 40,000 to >300,000 (Colonna and Lampen, 1974). However, the mannose: glucosamine ratios indicate that individual mannan chains have a molecular weight range of from 19,000 to 37,000, comparable to the mannan subunits described by Thieme and Ballou (1972).

The glucosamine: aspartic acid ratios for neutral mannan 2, bulk mannan, and acidic mannan are 0.73, 0.43, and 0.25, respectively (Table IV); the corresponding percentages of protein for each mannan are 3.3, 6.1, and 8.1%. In short, the progressive increase in protein content of the three mannans (2.5-fold) is not matched by a parallel increase in glucosamine associated with mannan peptide. Therefore, the differences in size and in the percentages of carbohydrate (96.7, 93.9, and 91.9%, respectively) for individual mannan species may not be due primarily to variations in the overall lengths of their constituent polymannose chains, but instead to the number of these chains attached to mannan peptide through glucosaminylasparagine bonds. Both situations probably occur. However, the latter alternative requires that mannan chains of fairly uniform size be attached to mannan peptide and is thus supported by our molecular weight data and the subunit of Thieme and Ballou (1972).

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