[Chem. Pharm. Bull. 36( 8 )3168—3172(1988)]

# Application of Mild Acetolysis to Confirm the Comb-like Structure of Cell Wall Mannan from *Pichia pastoris* IFO 0948 Strain

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(Received January 11, 1988)

In order to confirm the chemical structure of the mannan of *Pichia pastoris* IFO 0948 strain, which was assumed to possess a highly branched comb-like structure, a sequential degradation procedure involving partial acid hydrolysis followed by acetolysis under mild conditions was employed. This procedure can widely be adopted as a method for determining the structural shape of fungal mannans possessing a highly branched structure, either comb-like or tree-like, without application of enzymolytic degradation.

**Keywords**——cell wall mannan; *Pichia pastoris*; acetolysis; acid-hydrolysis; comb-like structure polysaccharide; proton magnetic resonance; β-linkage mannan

## Introduction

Recently, Kobayashi *et al.*[1] proposed that the chemical structure of the mannan of *Pichia pastoris* IFO 0948 strain contained large amounts of β-1,2-linkage based on the results of acetolysis under mild conditions using an acetolysis medium consisting of a 100 : 100 : 1 (v/v) mixture of $(CH_3CO)_2O$, $CH_3COOH$, and $H_2SO_4$. It was concluded that this mannan had a so-called comb-like structure composed of a core moiety which consisted solely of α-1,6-linked mannopyranose residues, and many branches containing both α-1,2- and β-1,2-linked mannopyranose residues connected to the C-2 position of the mannopyranose residues of the core moiety through an α-linkage. In another paper, Kobayashi *et al.*[2] examined the usefulness of the mild acetolysis procedure for investigating β-1,2-linkage-containing yeast mannans, employing phosphomannan–protein complex of *Citeromyces matritensis* IFO 0651 strain. In the present study, as a further use of this mild acetolysis procedure, we attempted to establish an analytical technique for confirming the correctness of the proposed structure of the *P. pastoris* mannan, *i.e.*, whether it was of comb-like form or tree-like form. Although Gorin *et al.*[3] provided evidence for the presence of a core moiety consisting solely of α-1,6-linkage in the mannan of *P. pastoris* CBS 2764 strain by another approach based on sequential degradation of the parent mannan by acid degrdation followed by enzymolysis with the *Arthrobacter* GJM-1 α-mannosidase developed by Jones and Ballou,[4] application of the mild acetolysis method developed by Kobayashi *et al.*[1] can be considered appropriate for determining the lengths of the branches of the parent mannan containing both α-1,2- and β-1,2-linkages by cleaving the α-1,6-linkage preferentially. In this paper, we describe the results of a structural analysis of the mannan of *P. pastoris* IFO 0948 strain undertaken by the sequential degradation procedure, in order to demonstrate the usefulness of this technique in the molecular shape analysis of various mannose-containing fungal polysaccharides as a facile method without employing enzymolysis.

## Experimental

**General**——Specific rotations were measured using an Applied Electric automatic polarimeter ($c=1.0$, $l=1.0$, water).

The proton nuclear magnetic resonance ($^1$H-NMR) spectra of the H-1 region of mannans and manno-oligosaccharides were recorded using a JEOL JNM-FX 100 spectrometer in $D_2O$ solution at 70 °C in accordance with the procedures of Gorin and Spencer.[5]

**Materials**——The *P. pastoris* IFO 0948 strain mannan was the same specimen as that used in the previous study.[1] The column packing for gel-filtration chromatography, Bio-Gel P-2 (-400 mesh), with a fractionation range of 100 to 1800 dalton (Da), was purchased from Bio-Rad Laboratories, Richmond, California, U.S.A.

**Partial Acid Hydrolysis of *P. pastoris* IFO 0948 Strain Mannan**——This was carried out in accordance with the previous description of Peat *et al.*[6] with some modifications as follows. Mannan, 200 mg, was dissolved in 0.6 N $H_2SO_4$, 20 ml, and the solution was heated in a boiling water bath. Aliquots of the solution, each 5 ml, were collected from the reaction mixture at four different reaction periods, 0.75, 1.5, 3, and 4.5 h, successively. Each solution was neutralized with 4 N NaOH, concentrated *in vacuo*, and applied to a column of Bio-Gel P-2 (2.5 × 100 cm). Elution was performed with water (2.5 ml/min), and aliquots of the eluates of the void-volume regions, 10 μl, were assayed for their polysaccharide content by the phenol–sulfuric acid method.[7] Eluates containing carbohydrate were combined, concentrated *in vacuo* to a small volume, and lyophilized. These four partial acid-hydrolyzates of different reaction periods were designated as fractions I, II, III, and IV, respectively.

**Acetolysis of *P. pastoris* IFO 0948 Strain Mannan and Its Acid Hydrolyzates, Frs. I, II, III, and IV**——This was carried out by exactly the same procedure as that described in the previous study of Kobayashi *et al.*[1]

**Calculation of the Average Chain Lengths of Branching Moieties of the Mannan and Its Acid Degradation Products**——The average chain lengths of the branching moieties of the intact mannan, frs. I, II, III, and IV were designated as $X$, $X'$, $X''$, $X'''$, and $X''''$, respectively. Their values were calculated using the following formulas (1—3), in accordance with the report of Matsumoto *et al.*[8]:

$$X=(A\times 1)+(B\times 2)+(C\times 3)+(D\times 4)+(E\times 5)/A+B+C+D+E \quad (1)$$

$$X'-X'''=(A\times 1)+(B\times 2)+(C\times 3)+(D\times 4)/A+B+C+D \quad (2)$$

$$X''''=(A\times 1)+(B\times 2)+(C\times 3)/A+B+C, \quad (3)$$

where $A$, $B$, $C$, $D$, and $E$ represent the molar proportions of mannose (M), mannobiose ($M_2$), mannotriose ($M_3$), mannotetraose ($M_4$), and mannopentaose ($M_5$), and the integral numbers, 1, 2, 3, 4, 5, indicate the degrees of polymerization of the mannose and four manno-oligosaccharides, $A$, $B$, $C$, $D$, and $E$, respectively.

## Results and Discussion

It is well accepted that many fungi elaborate mannan(s) or mannose-containing polysaccharide(s) as cell wall constituents, and that these polysaccharides possess a linear core moiety consisting solely of α-1,6-linked mannopyranose residues, to most of which are attached one branching moiety each, so giving rise to a comb-like structure. In order to provide corrobative evidence for the comb-like structure of bakers' yeast mannan, Peat *et al.*[6] undertook a partial acid hydrolysis study which yielded a series of manno-oligosaccharides from biose to tetraose consisting entirely of α-1,6-linkages. Later, Stewart *et al.*[9] carried out short-term acetolysis of bakers' yeast mannan which yielded mannopentaose, mannohexaose, and mannoheptaose each containing two consecutive α-1,6-linkages, while no evidence was obtained for the presence of manno-oligosaccharides containing only one α-1,6-linkage in the same acetolyzate. Subsequently, Jones and Ballou developed the use of *Arthrobacter* GJM-1 α-mannosidase,[4] and obtained confirmatory evidence for the correctness of the comb-like structure of bakers' yeast mannan based on the findings of a degradation study employing this enzyme.[10] Gorin *et al.*[3] also published the results of a series of sequential degradation studies on several mannans and galactomannans of fungal origin including that of a strain of *P. pastoris* by undertaking sequential degradation with *Arthrobacter* GJM-1 α-mannosidase and 0.33 N $H_2SO_4$. The data obtained indicated that all the polysaccharides gave corresponding degradation products exhibiting the distinct chemical shift of α-1,6-linkage in their $^1$H-NMR patterns, so providing evidence for the presence of a common core moiety consisting solely of

α-1,6-linked mannopyranose residues in the mannans and mannose-containing polysaccharides of various fungi. As an exception, however, Suzuki and Fukazawa[11] proposed another type of branched structure, the so-called tree-like one, for the mannan of a *Candida albicans* strain based on the results of a structural study of higher manno-oligosaccharides isolated by short-term acetolysis of this mannan. In the present study, we carried out a sequential degradation study involving partial acid hydrolysis and acetolysis under mild conditions.

Partial acid hydrolyzates of *P. pastoris* IFO 0948 strain mannan were obtained by heating the parent mannan in 0.6 N $H_2SO_4$ at 100 °C for 0.75, 1.5, 3, and 4.5 h. The results shown below in Fig. 2(A) and (B) indicate that the parent mannan underwent acid degradation in such a manner that the rate of reaction was relatively high and proceeded promptly in the earlier stage (0 to 1.5 h). The rate decreased in the later stage (3.0 to 4.5 h), as shown in Fig. 2(D) and (E), releasing mannose as the sole reaction product of low molecular weight. These observations suggest that, if the structure of the parent mannan is postulated to be of comb-like form, the α-1,2- and β-1,2-linkages composing the branching moieties underwent elimination at an almost identical rate. Also, the possibility of a tree-like structure can be ruled out because of the absence of any peak of lower manno-oligosaccharides containing α-1,6-linkage together with α-1,2- and β-1,2-linkages eluted in the diffusable region of each elution profile of the four acid degradation products. The mannan of *P. pastoris* IFO 0948 strain and its partial acid hydrolyzates, frs. I to IV, revealed a change of specific rotation value from +13.1° in the intact mannan to +51.8° in fr. IV, in agreement with the results of the present acid hydrolysis/gel chromatographic study, so excluding any possibility of a tree-like structure for this *P. pastoris* mannan, in which α-1,6-linkages serve as the connecting points of the branches and core moiety.

Figure 1 shows the $^1$H-NMR patterns of the acid-degraded mannans designated as frs. I, II, III, and IV, together with that of the parent mannan. The change in $^1$H-NMR patterns from the intact mannan to the degradation products seems to resemble that observed by Gorin *et al.*[3] in another *P. pastoris* mannan; *i.e.*, the patterns of the parent mannan and fr. I are quite different, while those of frs. III and IV are closely identical, and in the latter two a decrease in the chemical shift corresponding to β-1,2-linkage, 4.85 to 4.87 ppm, is evident in

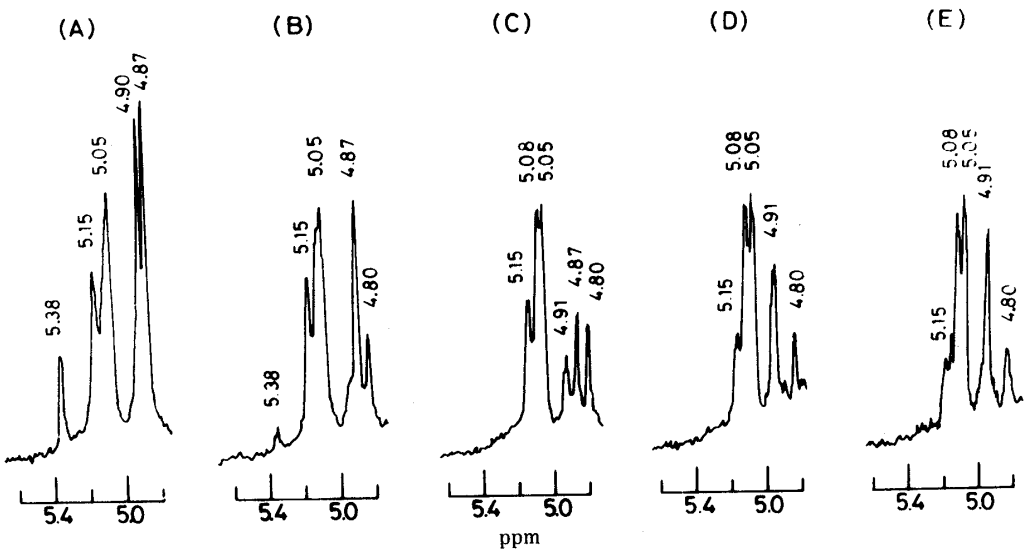


Fig. 1. $^1$H-NMR Spectra of the Intact Mannan of *P. pastoris* IFO 0948 Strain and Its Acid-hydrolyzates

(A) Intact mannan, (B) fr. I, (C) fr. II, (D) fr. III, and (E) fr. IV.

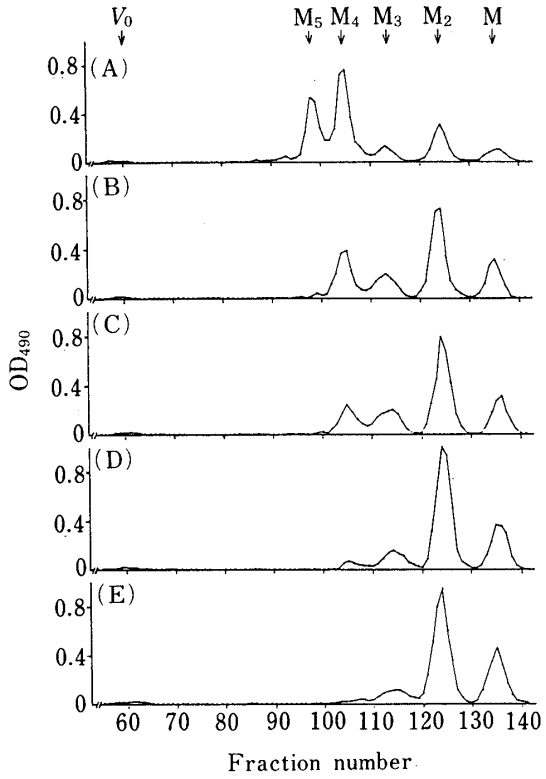


Fig. 2. Elution Patterns of Oligosaccharide Mixtures Obtained from the Intact Mannan of *P. pastoris* IFO 0948 Strain and Its Acid-hydrolyzates by Acetolysis with a 100 : 100 : 1 (v/v) Mixture of $(CH_3CO)_2O$, $CH_3COOH$, and $H_2SO_4$ at 40 °C for 36 h on a Column of Bio-Gel P-2, 2.5 × 100 cm

(A) Intact mannan, (B) fr. I, (C) fr. II, (D) fr. III, and (E) fr. IV. $M_5$, $M_4$, $M_3$, $M_2$, and M indicate mannopentaose, mannotetraose, mannotriose, mannobiose, and mannose, respectively. $V_0$ refers to the void volume.

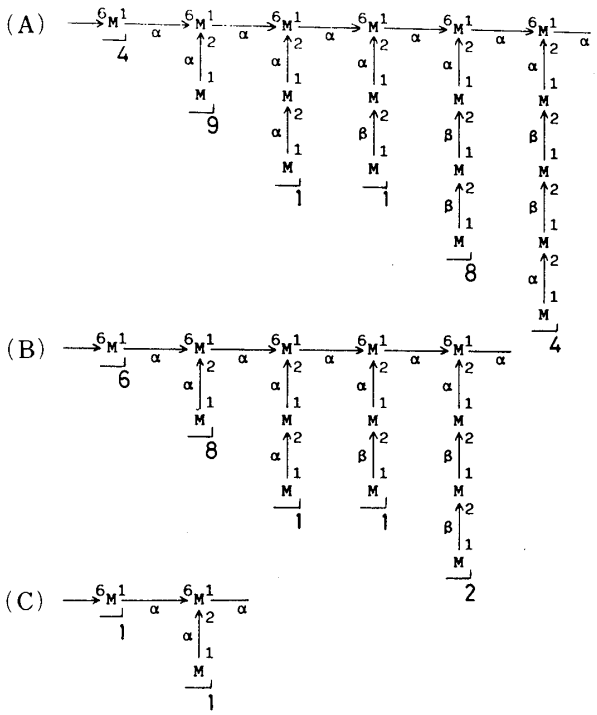


Fig. 3. Possible Structures for the *P. pastoris* IFO 0948 Strain Intact Mannan (A), Fr. I (B), and Fr. IV (C)

M denotes a mannopyranose residue. The numbers given below the brackets indicate the approximate number of side chains, although their sequences are not specified.

comparison with that of the intact mannan. However, it seems difficult to obtain any precise information concerning minimization of the branching moieties of the parent mannan, because of the complexity of these spectra. Acetolytic degradation of frs. I, II, and III under mild conditions to yield manno-oligosaccharide mixtures corresponding to the same lengths of branches of the parent mannan, was therefore considered more feasible for this purpose. Figure 2(A)—(E) shows elution profiles of acetolyzates of the parent mannan and its acid hydrolyzates, frs. I, II, III, and IV, on a column of Bio-Gel P-2. Clearly, the peak corresponding to the longest branching moiety, mannopentaose ($M_5$), detected in large amounts in the acetolyzate of the parent mannan disappeared after only 0.75 h of acid hydrolysis. The decrease in the branching moiety corresponding to one of the higher oligomannosyl residues, mannotetraose ($M_4$), was also significant; *i.e.*, the amounts of $M_4$ in the acetolyzates of both fr. III and IV were negligible. Thus, the $M_2$, $M_3$, and $M_4$ in each of the acetolyzates were identified as Man*p*α1-2Man, a mixture of Man*p*α1→2Man*p*α1→2Man and Man*p*β1→2Man*p*α1→2Man, and Man*p*β1→2Man*p*β1→2Man*p*α1→2Man, respectively, by $^1$H-NMR analysis in accordance with the report of Kobayashi *et al.*[1] Table I summarizes the molar ratios of manno-oligosaccharides and mannose produced by the acetolysis of the intact mannan and four acid-degraded mannans. The values of the average chain lengths for

TABLE I. Molar Ratios of Oligosaccharides and Mannose Produced from Mannan of *P. pastoris* IFO 0948 Strain and Its Acid-hydrolyzed Fractions by Acetolysis with a 100:100:1 (v/v) Mixture of $(CH_3CO)_2O$, $CH_3COOH$, and $H_2SO_4$ at 40 °C for 36 h

| Fraction[a] | $M_5$ | $M_4$ | $M_3$ | $M_2$ | M[b] | Av. chain length |
|---|---|---|---|---|---|---|
| (Intact) | 1.12 | 2.23 | 0.60 | 2.37 | 1.00 | 3.01 |
| I | Trace | 0.40 | 0.32 | 1.32 | 1.00 | 2.04 |
| II | Trace | 0.23 | 0.36 | 1.38 | 1.00 | 1.94 |
| III | — | 0.06 | 0.18 | 1.29 | 1.00 | 1.72 |
| IV | — | Trace | 0.16 | 1.09 | 1.00 | 1.63 |

*a*) Acid-hydrolyzed mannan obtained with 0.6 N $H_2SO_4$ at 100 °C for 45 min (fr. I), 1.5 h (fr. II), 3.0 h (fr. III), and 4.5 h (fr. IV). *b*) The molar ratios are expressed with M as unity.

each of these mannans, *X* to *X''''*, are also included. It is evident that the average chain length of the branching moieties of the intact mannan, 3.01, became reduced with elongation of the partial acid hydrolysis period, giving rise to modification products possessing shorter branches in an inversely proportional manner to the reaction period. It seems reasonable to conclude therefore that the intact *P. pastoris* IFO 0948 strain mannan possesses a comb-like structure, and that any possibility of the presence of tree-like structure can be ruled out.

Figure 3 depicts possible structures for the intact mannan of *P. pastoris* IFO 0948 strain and its partial acid degradation products, frs. I and IV, summarizing the present findings indicating that the parent mannan had a comb-like structure. It is considered that the present sequential degradation procedure without any enzymolytic process can be widely applied in the structural analysis of mannans and mannose-containing polysaccharides of fungal origin as well as other polysaccharides possessing α-1.6-linkages, and that this sequential degradation procedure is much more facile and accurate than either methylation or Smith degradation as the method for determining the average chain length of branching moieties.

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