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A *Saccharomyces* mannoprotein that protects wine from protein haze

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A *Saccharomyces cerevisiae* yeast mannoprotein which protected wine from protein haze spoilage was isolated from a red wine and purified by a combination of anion exchange, Concanavalin A, cation exchange and gel permeation chromatography. This mannoprotein corresponded to the highest molecular weight colloids in wine and was present at a trace level in the wine. Its apparent molecular weight when compared with pullulan standards was 420 kDa. The carbohydrate part represented 70% of the mannoprotein and consisted of 98% mannose and 2% glucose. Methylation data suggested that there were 2- and 3-linked mannose chains, some present as side chains on a 6-linked mannose backbone. Some of the mannose chains were *N*-linked to the protein and 60% were *O*-linked. The protein part represented 30% of the mannoprotein and was dominated by serine, glycine, threonine and alanine.

INTRODUCTION

The clarity of white wine is a property that is essential to its quality, but clarity can be marred by the presence of grape proteins. These naturally occurring proteins are inherently unstable in wine and aggregate to form amorphous sediments or unattractive suspended hazes (Bayly & Berg, 1967; Paetzold *et al.*, 1990; Waters *et al.*, 1991). To ensure stability, the proteins are removed from wine before bottling. This is generally achieved by reducing the protein concentration through fining with bentonite (Hsu & Heatherbell, 1987). Unfortunately, this procedure also reduces wine quality (Miller *et al.*, 1985) and results in the costly loss of wine as lees. Other methods of protein removal, such as peptidase degradation, have so far proved unsuccessful (Waters *et al.*, 1992), and it appears that novel methods need to be developed if the use of bentonite is to be discontinued.

In a recent study of wine haze (Waters *et al.*, 1993), a polysaccharide fraction that appeared to have originated from the yeast cell wall was isolated from white wine. This fraction protected wines from protein haze by reducing the haze particle size and thus offers an alternative way of dealing with the problem of protein haze. This paper describes the isolation and purification of this haze protective polysaccharide and its structural characterization as a *Saccharomyces cerevisiae* yeast mannoprotein.

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MATERIALS AND METHODS

Purification of the yeast mannoprotein from red wine

The red wine was the same as previously described (Belleville *et al.*, 1991), prepared from mature Carignan Noir grapes harvested in 1989 at the INRA-Pech Rouge/Narbonne Experimental Station and inoculated with Fermivin (Strain 7013 INRA Narbonne), a commercial wine-making *Saccharomyces cerevisiae* strain supplied by Gist-Brocades, France. The wine (50 litres) was concentrated 16-fold by ultrafiltration on a Carbosep M5 membrane (cut off 20 kDa, Tech-Sep, France) and depigmented through Sephadex LH-20 (Pharmacia, Sweden). The colloids were then precipitated by the addition of five volumes of cold 95% etha-

nol containing 60 mM HCl and washed with 80% and 95% ethanol.

The ethanol-precipitated colloids were loaded on a DEAE-Sephacel (Pharmacia, Sweden) column (5 × 28 cm) equilibrated in 50 mM acetate buffer (pH 4·8), and fractionated by a NaCl concentration step gradient as described by Pellerin and Brillouet (1992). The fraction eluted by 150 mM NaCl was then loaded at 150 ml/h on a Concanavalin A-Ultrogel (Sepracor-IBF, France) column (5 × 9 cm) equilibrated in 50 mM acetate buffer (pH 5·6) containing 150 mM NaCl, and 1 mM each of $CaCl_2$, $MgCl_2$ and $MnCl_2$ (starting buffer). The bound mannans were eluted by changing the buffer to elution buffer (starting buffer containing 100 mM methyl-α-D-mannoside).

The dialysed mannans were loaded at 120 ml/h on a S-Sepharose Fast Flow (Pharmacia, Sweden) column (2·5 × 3·2 cm) equilibrated in 20 mM citrate buffer (pH 3·0), and fractionated by a NaCl concentration step gradient of 0, 50 and 1000 mM NaCl in the equilibration buffer. Before each successive step was made, the A_{280} nm of the eluant was allowed to return to baseline. The high molecular size component in the 50 mM NaCl fraction was purified by gel permeation chromatography on a Sephacryl S-400 (Pharmacia, Sweden) column (1·6 × 100 cm) equilibrated in 20 mM acetate buffer (pH 4·8) containing 200 mM NaCl at 90 ml/h.

A more substantial amount (4·5 mg) of the yeast mannoprotein was obtained by a preparative scale purification from 600 l of Carignan Noir wine.

High performance size-exclusion chromatography

Molecular size distribution was studied by high performance size-exclusion chromatography (HP-SEC), as reported by Pellerin and Brillouet (1992), on two serial Shodex OHpak KB-803 and KB-805 columns (Showa Denko, Japan, 0·8 × 30 cm) with an OHpak KB-800P guard column (0·6 × 5 cm) equilibrated in 100 mM $LiNO_3$. The eluant was monitored with an Erma-ERC 7512 refractive index detector (Erma, Japan) and at 280 nm by a Waters 440 absorbance detector (Waters Millipore, USA) in combination with Baseline 810 software (Waters Millipore, USA). Molecular weights were estimated with a calibration curve of the two serial columns established at 25°C with a pullulan calibration kit (Showa Denko).

Analytical methods

The amino acid composition of the haze protective factor (400 μg) was determined after hydrolysis by 6 M HCl for 16 h at 120°C under nitrogen. Neutral monosaccharide composition was determined, after hydrolysis of the mannoprotein (500 μg) with 2 M trifluoroacetic acid (120°C, 75 min) (Albersheim *et al.*, 1967), by gas chromatography (isothermal 210°C on DB-225 (J & W Scientific, USA) of the alditol acetate derivatives (Harris *et al.*, 1984).

The mannoprotein (500 μg) was methylated using sodium methyl sulphinyl carbanion and methyl iodide in dimethyl sulphoxide according to Hakomori (1964) and then hydrolysed in 2 M trifluoroacetic acid. The resultant partially methylated sugars were derivatized into their alditol acetates and analysed, as described by Brillouet *et al.* (1989) on two fused-silica (DB-1 and DB-225) capillary columns (J & W Scientific, USA), areas being corrected by response factors (Sweet *et al.*, 1975).

Micromethod for haze protective activity

To determine the effect of polysaccharide additions on the haze potential of proteins, the micromethod described by Waters *et al.* (1991) was modified in the following manner. Aqueous solutions of polysaccharide fractions (0–70 μl with Milli-Q water) were added to an ultrafiltered (YM-10 membrane, 10 kDa nominal cut off, Amicon Corporation, Danvers, USA) Chardonnay wine (1055 μl) supplemented with bovine serum albumin (BSA) to give a final concentration of 125 μg BSA/ml wine. After sparging with argon, samples were sealed and heated for 6 h at 80°C, then left for 16 h at 4°C. The induced haze was measured by the absorbance at 540 nm. Values were corrected by subtraction of A_{540} for a control (no BSA added before heat testing).

β-elimination

The haze protective mannoprotein (1 mg) was incubated in aqueous 0·1 M NaOH (1 ml) containing 1·0 M $NaBH_4$ at 20°C for 24 h. The excess of BH_4^- was destroyed by the careful addition of glacial acetic acid until pH 5 was reached (about 100 μl). Polymeric and oligomeric products in the acid-adjusted sample were separated at 30 ml/h on a Bio-Gel P-2 (BioRad, USA) column (1 × 45 cm) equilibrated in 0·05% acetic acid. The sugar content of fractions taken during chromatography was estimated by the phenol-sulphuric acid method of Dubois *et al.* (1956).

Endo-β-*N*-acetylglucosaminidase H (Endo H) digestion

An aqueous solution of the haze protective mannoprotein (5 μl, 26 mg/ml) was diluted into 50 mM acetate buffer containing 0·1% sodium dodecyl sulphate (pH 5·5, 18·5 μl). The solution was boiled for 5 min, Endo H (2·5 μl, Boehringer Mannheim Biochemica, Germany) was added after cooling, and the solution was incubated at 40°C for 20 h. After hydrolysis the solution was evaporated under a stream of nitrogen and then redissolved in SDS PAGE sample preparation buffer (62·5 mM tris-HCl, pH 6·8 containing 10% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate and 10%

(v/v) 2-mercaptoethanol; 20 μl). The control sample consisted of the aqueous mannoprotein solution (5 μl, 26 mg/ml) which had been evaporated under a stream of nitrogen and then redissolved in SDS PAGE sample preparation buffer. Discontinuous SDS PAGE was performed according to the method of Laemmli (1970) with a 4% stacking and a 7·5% separating gel. Proteins were stained with Coomassie Brilliant Blue R-250 and carbohydrates with the periodic acid-Schiff's reagent (PAS) stain procedure of Allen *et al.* (1976).

Peptide-*N*-glycosidase F (PNGase F) digestion

Aqueous solutions of the haze protective mannoprotein (3 × 50 μl, 2 mg/ml) were evaporated under a stream of nitrogen. Two were redissolved in 250 mM phosphate buffer containing 10 mM each of EDTA and 2-mercaptoethanol (pH 7·5, 50 μl). To one of these was added PNGase F (0·8 U, Boehringer Mannheim Biochemica, Germany); the other aliquot was the control. The third aliquot was redissolved in 250 mM phosphate buffer containing 10 mM each of EDTA and 2-mercaptoethanol and 0·5% (w/v) sodium dodecyl sulphate (pH 7·5, 10 μl) and then boiled for 5 min to denature the mannoprotein. After cooling, the sample was diluted fourfold with 250 mM phosphate buffer containing 10 mM each of EDTA and 2-mercaptoethanol and 7·5% (w/v) Triton X-100 (pH 7·5, 40 μl) and then PNGase F (0·8 U) was added. All three aliquots were incubated at 40°C for 16 h. After hydrolysis the solutions were evaporated under a stream of nitrogen, redissolved in SDS PAGE sample preparation buffer (40 μl) and then analysed by discontinuous SDS PAGE as described above.

RESULTS

Isolation and purification of the wine haze protective factor

Colloids from the Carignan Noir wine that could be precipitated by ethanol showed no haze protective effect but, on the contrary, they contributed strongly to the haze. The yields of the purification steps and the haze protective activity shown by each fraction are given in Table 1 and the high performance size-exclusion chromatograms of the fractions are shown in Fig. 1. The first step in the purification scheme was to fractionate these colloids by anion exchange chromatography on DEAE-Sephacel. This allowed the separation of neutral polysaccharides (43% of the total) from four acidic fractions. The fractionation profile, the relative proportions, and high performance size-exclusion chromatographic analysis of the different fractions of DEAE-Sephacel have been given elsewhere (Pellerin & Brillouet, 1992).

The fraction eluted from the DEAE-Sephacel column by 150 mM NaCl represented 9·9% of the ethanol precipitated colloids of the Carignan Noir wine (Table 1) and showed a peak with a broad molecular size range and a discrete shoulder at the high molecular size end (Fig. 1). This shoulder, which represented the mannans in the fraction, was collected after chromatography on Concanavalin A-Ultrogel and exhibited weak haze protective activity (Table 1).

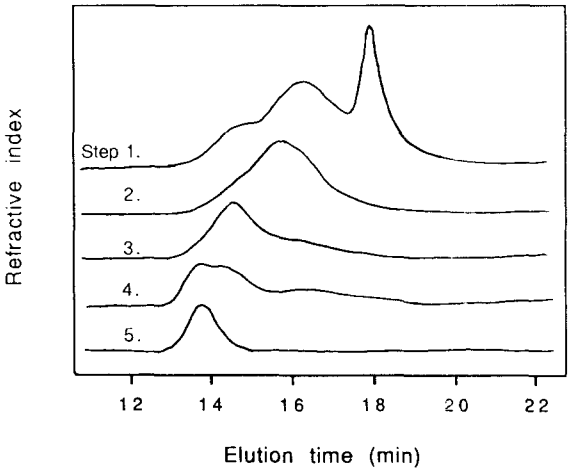

Fig. 1. High performance size-exclusion chromatography of fractions given by the purification protocol. Details of the purification steps are given in Table 1.

Table 1. Purification of the haze protective factor from 50 l of wine

Step	Recovery of polysaccharide[a] (mg)	Yield (%)	Concentration required to reduce haze to 50% of the level given by BSA alone (μg/ml)
1. Ethanol precipitation	16830	100	∞[b]
2. DEAE pH 4·8	1666	9·9	nd[c]
3. Concanavalin A	300	1·8	550
4. S-Sepharose pH 3·0	6	0·036	90
5. Sephacryl S-400	1·2	0·007	65

[a] Measured by phenol-sulphuric acid assay (Dubois *et al.*, 1956).
[b] Increased haze.
[c] Not determined.

The mannan fraction was then further separated by cation exchange chromatography on S-Sepharose at pH 3. Most (98%) of the mannans did not bind to this column. However, it was the small amount which did bind that exhibited haze protective activity.

Finally, the lower molecular size components in this fraction were eliminated by gel permeation chromatography. The final polysaccharide represented only 0·007% of the ethanol-precipitated polysaccharides from wine but exhibited excellent haze protective activity (Table 1).

High performance size-exclusion chromatography

From the high performance size-exclusion chromatographic analysis (Fig. 1) of the fractions obtained during the purification, it is evident that the purified haze protective factor corresponded to colloids with the highest molecular weight in wine and that it is present in trace amounts. Its apparent molecular weight, when compared with that of the pullulan standards, was 420 kDa. The UV-absorbing peak and the peak observed with refractometry (Fig. 2) co-eluted, suggesting an association between protein and polysaccharide.

Similar analysis as that described in the preceding two sections was performed on the haze protective factor isolated on a preparative scale from 600 l of wine. The haze protective factor isolated in this large-scale purification was shown to have identical properties to that described in the sections above in addition to an identical neutral sugar composition. All the following data were obtained from the haze protective factor isolated from 600 l of wine.

Composition of the haze protective factor

Analysis (Table 2) revealed that the haze protective factor was a mannoprotein, containing 71·2% of neutral sugars, predominately mannose (97·4%). Some (2·4%) glucose was also present, and traces of hexosamines.

The protein content of the haze protective factor was high (28·8%). There was a high concentration of the aliphatic amino acids and, in particular, the hydroxylated amino acids serine and threonine. A total of 3·2% of the amino acids in the mannoprotein were lysine and histidine. These amino acids have a positive charge at pH 3·0. The estimated residue number of each amino acid in the mannoprotein was calculated and is given in Table 2.

Methylation analysis

Terminal mannose and 2- and 3-linked mannose were the main structural features, followed by 2,6-linked mannose (Table 3). These structural features are in good agreement with the model proposed by Ballou

Table 2. Composition of the haze protective factor

	% Dry weight	Molar %	Calculated number of residues[a]
Neutral sugars			
mannose		97·4	
glucose		2·4	
glucosamine		0·2	
Total	47·4 (71·2)[b]		
Protein[c]			
serine		28·2	391
glycine		17·2	364
threonine		11·8	141
alanine		10·2	174
glutamic acid/glutamine		6·6	62
valine		5·3	65
aspartic acid/asparagine		3·9	41
isoleucine		3·2	34
leucine		2·8	30
proline		2·6	32
lysine		1·8	17
phenylalanine		1·7	14
tyrosine		1·8	13
histidine		1·4	13
Total	19·2 (28·8)		

[a]Calculated from the relative proportion of protein and a molecular mass of 420 000 for the haze protective factor.
[b]Values in parentheses are the relative proportions.
[c]Traces of methionine and arginine were also detected; tryptophan and cysteine were not detected.

(1982) in which the carbohydrate moiety of mannoproteins from *Saccharomyces* can be present in two forms. One is attached to the protein at asparagine residues and consists of a long 6-linked backbone, highly substituted on position 2 with 2- and 3-linked mannose side chains. The other form is attached to serine and threonine residues and is composed of short 2- and 3-linked mannose chains.

No glucose methyl ethers were detected in the chromatograms except perhaps 6-linked glucose, but its presence could not be confirmed since it was not distinguishable from 6-linked mannose (same relative retention time and mass spectra).

β-elimination

The purified haze protective mannoprotein was subjected to β-elimination and the products separated by gel permeation chromatography. The lower M_r components accounted for 61% of the total sugars. Thus it appears that approximately 60% of the mannose residues belong to oligosaccharide chains which are *O*-glycosidically linked to the protein. This result is consistent with the high content of hydroxylated amino acids in the protein.

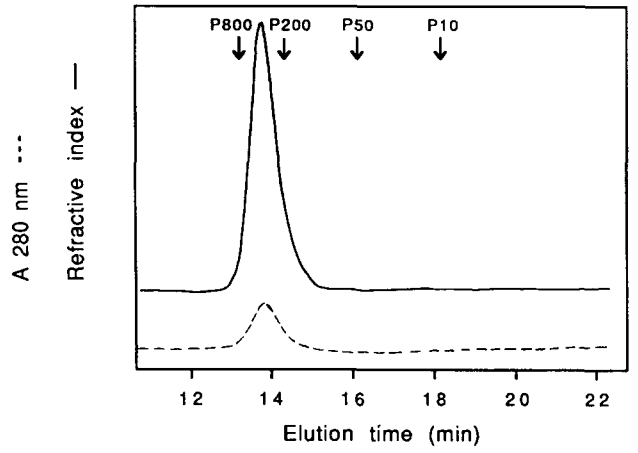

Fig. 2. High performance size-exclusion chromatography of active mannoprotein. The position of several pullulan standards is indicated and their M_r values are as follows: P800 is 853 kDa, P200 is 186 kDa, P50 is 48 kDa and P10 is 12·2 kDa.

Table 3. Methylation analysis of the haze protective factor

Methyl ether	Linkage	Molar %
2, 3, 4, 6 Mannose[a]	terminal	30
3, 4, 6 Mannose	1 → 2	25
2, 4, 6 Mannose	1 → 3	22
2, 3, 6 Mannose	1 → 4	2
3, 6 Mannose	1 → 2, 4	1
3, 4 Mannose	1 → 2, 6	18
2, 3, 4 Mannose/2, 3, 4 glucose[b]	1 → 6	2

[a]2, 3, 4, 6 Mannose = 2, 3, 4, 6-tetra-*O*-methyl-1,5-di-*O*-acetyl mannitol, etc.
[b]Not resolved on DB-1 and DB-225 columns.

SDS PAGE analysis

Analysis of the haze protective mannoprotein by SDS PAGE showed that it had migrated a few millimetres into the stacking gel (Fig. 3), confirming its high molecular weight previously observed in HP-SEC. It was only stained with the periodic acid-Schiff's reagent (PAS) stain for carbohydrate and did not respond to the protein stain, Coomassie Brilliant Blue. The failure of yeast cell wall structural mannoproteins to respond to Coomassie and to enter the gel during electrophoresis has been reported (Frevert & Ballou, 1985).

Enzymatic de-*N*-glycosylation

To determine if the purified, haze protective mannoprotein had mannose-containing chains *N*-linked to protein via asparagine residues, the mannoprotein was first denatured with SDS, then treated with enzymes that remove these chains and the products subjected to SDS PAGE analysis. *N*-linked mannose chains in yeast are attached to the amide nitrogen of asparagine via a di-*N*-acetylchitobiosyl unit (Ballou, 1982). Endo-β-*N*-

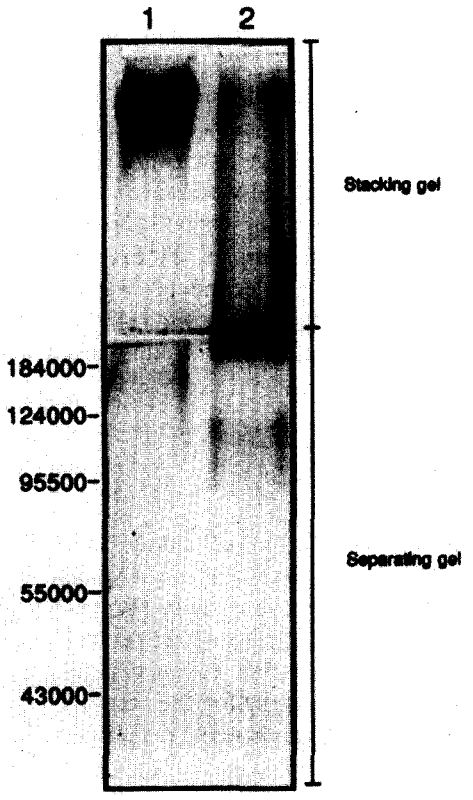

Fig. 3. SDS PAGE patterns of active mannoprotein before (lane 1) and after (lane 2) Endo H digestion. The position of several standard proteins are indicated.

acetylglucosaminidase H (Endo H), an enzyme commonly used to de-*N*-glycosylate glycoproteins (Trimble & Maley, 1984), releases the chains by hydrolysing the bond between the two *N*-acetylglucosamines of the linkage unit. Thus it leaves the protein with one *N*-acetyl glucosamine *N*-glycosidically attached to asparagine.

In the first experiment, the purified haze protective mannoprotein was denatured with SDS and then digested with Endo H. Analysis by SDS PAGE of the hydrolysis products revealed an intense PAS staining band at the beginning of the separating gel (Fig. 3). This band is assumed to be de-*N*-glycosylated mannoprotein. Its position indicated that although it had greater mobility than the native mannoprotein, it was still of high molecular mass. Since it would still contain all of the mannose chains *O*-linked to serine and/or threonine, which was shown above to account for about 60% of the total carbohydrate moiety, its large size was not unexpected. Also present in the Endo H treated mannoprotein was a series of more mobile, faintly PAS staining indistinct bands (Fig. 3).

In an attempt to determine if these faintly staining PAS bands were an artefact, the haze protective mannoprotein was de-*N*-glycosylated with a different enzyme, peptide *N*-glycosidase F (PNGase F). PNGase F differs in its action to Endo H. It hydrolyses the *N*-glycosidic bond between the first *N*-acetylglucosamine residue of the linkage unit and the asparagine residue of the protein. PNGase F treatment, with and without prior denaturation with SDS, gave the same result as seen with Endo H (data not shown). This indicated that enzymatic de-*N*-glycosylation of the active mannoprotein resulted in one large macromolecule and several smaller, minor molecules, which are all mobile in SDS PAGE and react with the carbohydrate specific stain. Due to the paucity of samples, the products released by Endo H and PNGase F treatment could not be further studied. Nevertheless, these results demonstrate that a proportion of the mannose chains are *N*-linked to protein.

DISCUSSION

By using a combination of similar chromatography steps used in a previous study on a yeast-derived fraction with haze protective activity (Waters *et al.*, 1993) but introducing a final step of gel permeation chromatography, it was possible to successfully purify the polysaccharide responsible for the activity.

Analysing the haze protective activity of fractions during the purification allowed the evolution of this activity to be followed. It was not until the majority of wine polysaccharides were eliminated from the fractions containing the mannoprotein that haze protective activity could be expressed. This is in keeping with the low concentration of the mannoprotein in wine (0·007% of ethanol-precipitated polysaccharides) and indeed a low concentration of yeast-derived polysaccharides was not unexpected since the wine used for the isolation had not been produced by a method designed to increase yeast autolysis (e.g. stored on yeast lees). However, the properties of the haze protective factor, in particular its positive charge at pH 3, allowed this mannoprotein to be purified.

The polysaccharide moiety of the haze protective mannoprotein showed structural features that were in good agreement with the general model proposed for yeast mannoproteins (Ballou, 1982) and since plant materials (including grapes), do not contain mannoprotein-like macromolecules (Aspinall, 1980; Saulnier *et al.*, 1988), the wine haze protective factor was unambiguously of yeast origin.

The protein moiety of the purified haze protective mannoprotein, however, contained features significantly different to that of structural cell wall mannoproteins (Frevert & Ballou, 1985). The total amount of protein of the haze protective factor was more than twice that of structural cell wall mannoproteins (30% versus 12%); in addition, their amino acid compositions varied significantly.

A small proportion of the amino acids in the haze protective factor have the potential to carry a positive charge at pH 3·0, whereas structural cell wall mannoproteins contain only traces of these amino acids (Frevert & Ballou, 1985). Their presence in the active mannoprotein may account for its retention on a cation exchange resin at pH 3·0. Although both types of mannoproteins have a high content of the hydroxylated amino acids, the haze protective factor has twice as much serine than threonine, whereas the converse is true for structural cell wall mannoproteins. The active mannoprotein also contains less proline than structural cell wall mannoproteins but more glycine.

The content of measured aspartic acid, and hence potential asparagine, in the haze protective factor was approximately half that of structural cell wall mannoproteins. However, the high total protein content of the active mannoprotein meant that there were potentially 41 asparagine residues, and thus 41 potential *N*-linkage sites, present in the protein. The presence of *N*-linked chains was confirmed by the reduction in molecular size seen after enzymatic de-*N*-glycosylation. There was also a high content of the hydroxylated amino acids, serine and threonine, which are commonly involved in *O*-glycosidic linkages, and a corresponding high level of *O*-linkage observed in the mannoprotein. Similar levels of *O*-linkage have been observed in other studies on yeast mannoproteins (Van Rinsum *et al.*, 1991).

The discovery of this yeast mannoprotein with haze protective activity is significant from a commercial viewpoint, because it offers an alternative and 'natural' solution to the wine protein haze problem. Its char-

acterization, which has been partially completed in this study, will help to locate the macromolecule in the intact yeast and aid us in designing methods for its production so that its haze protective property can be commercially exploited.

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