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Further characterization of a high molecular weight glycoprotein antigen from the yeast *Saccharomyces cerevisiae*

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A high molecular weight glycoprotein antigen was isolated by size exclusion chromatography on Sepharose 4B from an extract of the yeast *Saccharomyces cerevisiae*. The glycoprotein antigen Sc 500 was shown to be identical to the antigen termed gp200 previously isolated (Heelan *et al.*, 1991). The MW of Sc 500 was determined to be about 500 kDa by size exclusion chromatography on Superose 6 and 460 kDa ± 20k Da by size-exclusion chromatography/multi-angle laser light scattering (SEC/MALLS). Sc 500 contained 90% mannose and traces of *N*-acetylglucosamine. The amino acid composition revealed that serine and threonine were the most abundant amino acids of the protein part. By alkaline borohydride treatment some, but not all bonds between protein and carbohydrate were broken. This indicates that the main type of linkage between protein and carbohydrate is *O*-glycosidic and that a minor type is of *N*-glycosidic nature. Methylation analysis revealed that the mannose residues were connected by 1 → 2 and 1 → 3 linkages with 1 → 2, 1 → 6 linked branch points.

Purified Sc 500 was subjected to a series of chemical and enzymatic modifications followed by studies of antibody binding activity. Treatments with both periodate and alkaline sodium borohydride reduced the human serum IgA, IgG and monoclonal IgM antibody binding activity of Sc 500 whereas trypsin and pronase did not affect its ability to bind these antibodies. The mannosidase Manα1 → 2,3,6Man reduced the IgM binding to Sc 500 while the other enzymes included in this experiment (Manα 1→ 2Man, Manβ1 → 4GlcNAc and PNGase F) had no effect on the antibody binding. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Main *et al.* (1988) first described the presence of specific IgG and IgA antibodies in the serum of patients with Crohn's disease. Using the enzyme-linked immunosorbent assay (ELISA) technique with a crude extract of *Saccharomyces cerevisiae* (*S. cerevisiae*) as solid phase antigen, this group could be distinguished from healthy controls and patients with ulcerative colitis. In the case of antibodies of the IgG isotype, this observation held true for 11 of 12 strains of *S. cerevisiae* examined; furthermore, there was no serological cross-reactivity with *Candida albicans* (McKenzie *et al.*, 1990, 1992). Barnes *et al.* (1990) confirmed and extended these findings suggesting that IgA anti-Sacc antibody, though less sensitive, was a more specific marker for Crohn's disease; in addition, there was no correlation between the presence of anti-Sacc antibodies and those reacting with *Escherichia coli* or other dietary antigens. Preliminary attempts to identify the relevant antigen, based on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immuno-blotting with antibody positive sera, suggested that the predominant antigen was a high molecular weight (200 kDa) soluble glycoprotein, termed gp200 (Heelan *et al.*, 1991). More recently it has been shown that Sacc glycoprotein antigen (gp200) induces *in vitro* human lymphocyte proliferative responses and NK-mediated cytotoxity, thus providing evidence of antigen-specific cellular immune sensitization in man to *S. cerevisiae* (Darroch *et al.*, 1994). Components of the yeast *Saccharomyces cerevisiae* is known to sensitize brewers and bakers and to be

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the causative agents of atopic dermatitis. A high MW mannan has been shown to be heat stable and to be preserved in the conditions simulating the gastrointestinal tract (Kortekangas-Savolainen *et al.*, 1993a, b).

The aim of this study was to gain further insight into the structure and antigenicity of this high MW glycoprotein which may be important for the commercial production of diagnostics. We report here the isolation and partial characterization of Sc 500, a high MW *S. cerevisiae* antigen shown to have the same activity as gp200. The capacity for binding different types of antibodies is determined by ELISA both before and after treatment of the antigen with different chemicals and enzymes.

MATERIALS AND METHODS

Preparation of crude extract

Sainsbury's dried yeast (30 g) which is equivalent to 50 g fresh yeast was heated under agitation with 500 ml 0.15 M NaCl at 100°C for 1 h. (Darrock *et al.*, 1994). The suspension was cooled, clarified by centrifugation and dialysed under magnetic stirring against distilled water (MW cut off 12–14 kDa, 24 h, 4°C). The extract was filtered through a double layer of glass fibre filters followed by lyophilization. The yield was 2.3 g (7.6%).

Isolation of Sc 500

The crude extract was desalted by gel filtration on PD-10 columns (Pharmacia LKB Biotechnology) and lyophilized before gelfiltration on a Sepharose 4B column (2.5 × 90 cm, Pharmacia Fine Chemicals). The column was eluted with PBS buffer pH 7.5 containing 1.3 M guanidinium chloride (Merck). The flow rate was 10 ml/h. 90 fractions of 5 ml were collected. The void volume was measured by Blue Dextran 2000 (Pharmacia). Eight portions of ca. 100 mg desalted crude extract were dissolved in 3 ml PBS/guanidinium chloride buffer, filtrated through a 0.8 µm filter and applied on the column. The protein profile was determined by reading the absorbance at 280 nm (undiluted fraction). The carbohydrate profile was determined by performing the phenol-sulphuric acid test by the method previously described (Dubois *et al.*, 1956). To 100 µl sample was added 200 µl 4% phenol and 1 ml concentrated sulphuric acid. The absorbance was read at 489 nm after 30 min. Every other fraction were diluted 1:40 and tested for serum IgG antibody binding components by the direct ELISA method as described below.

Three pools of the fractions were made. Pool A: fraction 21–40, pool B: fraction 41–60 and pool C: fraction 70–90. Pool B was chosen for further characterization, and was concentrated by the Ultrasette® (Filtron Inc.) MW cut off 100 kDa until a final volume of ca. 50 ml was reached, dialysed against distilled water (4°C, 72 h, MW cut off 12–14 kDa) and lyophilized. The yield was 84.1 mg.

Size exclusion chromatography on superose 6 of Sc 500

The molecular weight of Sc 500 was estimated by gel filtration of pool B material on a column of Superose 6 HR 10/30 (Pharmacia LKB Biotechnology). The column was eluted with distilled water with flow rate 250 µl/min. Fractions of 250 µl were collected. The protein and carbohydrate profile was obtained by measuring absorbance of fractions diluted 1:5 at 280 nm and of the phenol-sulphuric acid test performed on 25 µl undiluted fraction as described for carbohydrate profile by isolation of Sc 500. The fractions were diluted 1:100 and tested for serum IgG antibody binding components by direct ELISA as described below.

The column was calibrated with the following dextrans: Dextran 70, Fr 11 4 (MW 98.4 kDa). Dextran T 250 lot 8374, (MW 233 kDa) and Dextran T500 lot 9307 (MW 475 kDa) kindly provided by Pharmacia. The void volume was determined by Blue Dextran 2000 (Pharmacia).

Size-exclusion chromatography/multi-angle laser light scattering (SEC/MALLS)

To verify the MW estimated by size exclusion chromatography, the sample (2 mg/ml phosphate in chloride buffer, I = 0.1, pH 6.8) was analyzed by SEC/MALLS) essentially as described by Wyatt (1992). The chromatographic system consisted of a programmable HPLC pump (Model 510, Waters, Millipore, Watford, UK) fitted with a 100 µl loop. Macromolecules were separated using two HemaBio columns (HemaBio linear and HemaBio 40) connected in series and protected by a guard column (PSS, Mainz) and the eluting solution was analyzed on-line using a DAWN-DSP light scattering photometer (Wyatt Technology, Santa Barbara) and an Optilab 903 Interferometric refractometer (Wyatt Technology). The flow rate was 0.8 ml/min. and experiments were performed at abient temperature (ca. 20°C).

Carbohydrate composition of Sc 500

The sample (ca. 250 µg Sc 500) was methanolyzed (4 M HCl in MeOH, 80°C, 20–24 h) and the resulting methylglycosides were trimethylsilylated (Reinhold, 1972). Gas chromatography was performed on a Carlo Erba 4200 gas chromatograph equipped with a flame ionisation detection system, a splitless injector (6:1), and a fused silica capillary column (30 m × 0.32 mm).

Helium was used as carrier gas at a rate of ca. 3 ml/min.

The gas chromatography was done with a column temperature gradient starting at 140°C at injection, followed by an increase of 1°C/min to 170°C and 6°C/min from 170°C to 250°C. GC-MS was also performed on this material using the same conditions as referred to below.

Amino acid composition and protein content analysis

Sc 500 was hydrolysed under vacuum in 6 M HCl at 110°C for 24 h. The HCl was removed under reduced pressure, and the amino acid composition was determined with a Biotronic LC 5000 automatic amino acid analyzer. These results were used for calculation of total protein present.

Methylation analysis

The polysaccharides were methylated (Harris *et al.*, 1984; Kvernheim, 1987) using the lithiumsalt of methylsulfinyl carbanion (Blakeney & Stone, 1985). The partially methylated alditol acetates were analysed by GC-MS (Barsett *et al.*, 1991).

SDS-page

Homogeneous SDS-PAGE gels were moulded in a Protean™ mini-dual slab cell apparatus (Bio-Rad) with 6.0% C and 2.67% T stacking gel on top of a 8% C and 2.67% separation gel. Electrophoresis was performed at 200 V for 1 h with discontinuous buffer system (Neville, 1971). The samples were prepared for SDS-PAGE by heating for 5 min at 100°C with SDS and 2-mercaptoethanol. Gels were stained for proteins with Coomassie brilliant blue R-250, silver nitrate as recommended for ExcelGel™ precast gels (Instruction Manual 80-1310-00, Pharmacia) or stained for carbohydrate detection with the periodic acid-Schiff reagent (PAS) (Zacharius *et al.*, 1969). Details are given in legends to Fig. 2.

Origin of human sera and murine monoclonal IgM antibody

Human sera were collected from patients with Crohn's disease and were anti-*S. cerevisiae* antibody positive for either IgG or IgA or both by ELISA (Barnes *et al.*, 1990). Murine monoclonal IgM antibody (anti-*S. cerevisiae* gp200 Mab 92-276/018 lot 091192 IGP Sel) was prepared and characterized (Bröker *et al.*, 1994).

Direct ELISA of fractions obtained from size exclusion chromatography on Sepharose 4B and superose 6

The fractions from the Sepharose 4B column were diluted 1:40 with PBS (pH 7.5). The fractions obtained from chromatography on Superose 6 were diluted first 1:5 followed by a 1:40 dilution.

The wells (Nunc Maxisorp 96 well) were coated in duplicate with 100 µl diluted fraction for 16 h at 4°C and were blocked by incubation with 0.5% BSA (Sigma) in PBS pH 7.5 for 4 h. 100 µl human serum containing IgG antibodies towards *S. cerevisiae* antigens diluted 1:500 in PBS (pH 7.5) containing 0.5% BSA was added followed by incubation for 16 h at 4°C. The wells were washed twice with 200 µl PBS before incubation for 4 h at 20°C with 100 µl alkaline phosphatase conjugated affinity purified rabbit immunoglobulin to human IgG γ chains (Dakopatts A/S) diluted 1:1000 with PBS (pH 7.5). The wells were washed three times with PBS (pH 7.5) before addition of 100 µl of substrate solution containing 1 mg/ml ρ-nitrophenyl phosphate (Sigma 104 phosphate substrate, Sigma) in TRIS buffer (pH 10). The absorbance was measured at 405 nm in a Bio-Rad Microplate reader after 30 min.

Reactivity of Sc 500 to human serum IgA, IgG and murine monoclonal IgM determined by ELISA

The wells (Nunc Maxisorp 96 well) were coated with Sc 500 by incubation with 100 µl (10 ng Sc 500/ml PBS) for 16 h at 4°C. The wells were blocked by incubation with 0.5% BSA (Sigma) in PBS pH 7.5 for 4 h. Two solutions were added in duplicate (i) 50 µl solution with modified Sc 500 and (ii) 50 µl of human serum containing antibodies (IgA or IgG) towards *S. cerevisiae* antigens diluted 1:200 or murine monoclonal IgM against gp200 diluted 1:500. Both antigen and antibody were dissolved in PBS (pH 7.5) containing 0.5% BSA. The mixture was incubated for 16 h at 4°C, the first hour on a shaker.

The wells were washed twice with 200 µl PBS (pH 7.5) before incubation for 4 h at 20°C with either 100 µl alkaline phosphatase conjugated affinity purified rabbit immunoglobulins to IgA α chains (Dakopatts A/S), rabbit immunoglobulins to human IgG γ chains (Dakopatts A/S) diluted 1:1000 with PBS (pH 7.5) containing 0.5% BSA or monoclonal goat anti-mouse IgM (Boehringer Mannheim Biochemicals) diluted 1:8000. The wells were washed three times with PBS (pH 7.5) before addition of 100 µl of substrate solution containing 1 mg/ml *p*-nitrophenyl phosphate (Sigma 104 phosphate substrate, Sigma) in TRIS buffer (pH 10). The absorbance was measured at 405 nm in a Bio-Rad Microplate reader after 30 min.

Inhibition of IgA, IgG and IgM antibody binding to Sc 500 by modified Sc 500

To vials with 127 µg Sc 500 (1–8) were added the following modifiers:

(1) α-mannosidase (Manα1 →2 Man) EC 3.2.1.34, 10 μ units dissolved in 50 μl 100 mM sodium acetate buffer, pH 5.0.
(2) β-mannosidase (Manβ1 → 4GlcNAc) EC 3.2.1.25, 0.2 units dissolved in 50 μl 10 mM sodium citrate/phosphate pH 4.0.
(3) α-mannosidase (Manα1 → 2,3,6Man) EC 3.2.1.24, 2 units dissolved in 50 μl 10 mM sodium acetate pH 5.0 containing 2 mM Zn^{2+}
(4) 20 μl 20 mM sodium phosphate buffer, pH 7.5 containing 50 mM EDTA and 0.02% sodium azide (20 μl concentrate, 5 μl 10% SDS, 5 μl 2-mercaptoethanol and 70 μl distilled water) and 2.7 units Peptide-*N*-Glycosidase F (PNGase F) EC 3.2.2.18 dissolved in 50 μl buffer.

The enzymes 1–4 were obtained from Oxford Glycosystems. These vials were incubated for 24 h at 37°C.

(5) 100 μl TPCK treated trypsin Type XIII: from bovine pancreas, EC 3.4.21.4, (Sigma) 0.1 mg/ml in PBS pH 7.5, incubated for 24 h at 37°C.
(6) 100 μl pronase (R) 45,000 P.U.-K/B-grade (Calbiochem) 0.1 mg/ml in PBS pH 7.5, incubated for 24 h at 37°C.
(7) 0.125 M NaOH/1 M $NaBH_4$, incubated at 50°C for 6 h.
(8) 10 mM sodium metaperiodate, incubated at 20°C for 1 h.
(9) Control, dissolved in distilled water. No modifier added, incubated for 24 h at 37°C.

Inhibition of binding of human serum IgA, IgG or murine monoclonal IgM to native Sc 500 were determined in 3 parallel experiments by the enzyme competitive binding assay as described above.

RESULTS

The crude extract was fractionated by gel filtration on Sepharose 4B. Three pools (A–C) of the fractions were made (Fig. 1).

The crude extract and the pools obtained by Sepharose 4B chromatography were analysed by SDS-PAGE (Fig. 2). The crude extract contained multiple proteins with MW less than 50 kDa which were stained with the silver stain (lane 2). The PAS stain of crude extract (lane 6) revealed the presence of a high MW glycoprotein. This component was not visualized by the silver stain (lane 2) or with the Coomassie brilliant blue staining technique (results not shown). By fractionation on the Sepharose 4B column, the high MW carbohydrate component was eluted in fractions 22–60 and appeared as a red band after PAS-staining of the SDS-PAGE gel of both pool A and pool B (Fig. 2, lane 7 and 8 respectively). This component was not visualized by the silver stain (Fig. 2, lane 3 and 4) nor by silver stain of pool C (Fig. 2, lane 9).

The low MW crude extract material was eluted in the C pool fractions (Fig. 2, lane 5).

The MW of Sc 500 was estimated by gel filtration of pool B on Superose 6. The antigen was eluted as one broad peak as shown by the phenol-sulphuric acid test and by measuring the absorbance at 280 nm. The material appeared to be immunologically homogeneous by measuring the IgG binding activity (ELISA). The average apparent MW of Sc 500 was estimated to be about 500 kDa relative to dextrans (Fig. 1b).

The size of Sc 500 was also determined by SEC/MALLS method previously described. The M_w was 460 ± 20 kDa, the M_n was 340 kDa ± 20 kDa and the M_z 940 kDa ± 90 kDa. These results are in agreement with the results obtained by the gel filtration experiment.

The protein (amino acid analysis) to carbohydrate ratio by weight of Sc 500 is 10:90. The carbohydrate part consists of mannose residues and traces of *N*-acetylglucosamine (Table 1). The amount of *N*-acetylglucosamine was so small that the only method to verify the presence of *N*-acetylglucosamine was selected ion monitoring by GC-MS of the TMS-derivatives of the methylglycosides obtained after methanolysis of the antigen. When located, the mass spectrum obtained was identical to that obtained for methyl 2-acetamido-2-deoxy-tri-*O*-(trimethylsilyl)-*D*-glucoside (McLafferty & Stauffer, 1989). Retention time compared to standards showed that the aminosugar was *N*-acetyl-glucosamine.

Calculated on the basis of a molecular weight of 500 kDa, a total of 440 amino acid residues per molecule with a very high content of serine and threonine were found by amino acid analysis (Table 1).

Methylation analysis revealed that the mannose residues were connected by 1 → 2, 1 → 3, small amounts of 1 → 6 linkages in addition to 1 → 2, 1 → 6 linked units as branch points. Terminal units were also present (Table 2).

Purified Sc 500 was treated with trypsin, pronase, NaOH/$NaBH_4$, sodium metaperiodate (Fig. 3) or mannosidases (Manα1 → 2Man, Manβ1 → 4GlcNAc, Manα1 → 2,3,6Man or PNGase F (Fig. 3) followed by assay of antibody binding activity. Both periodate and sodium borohydride treatment reduced IgA, IgG and IgM antibody binding activity to Sc 500. The proteases and PNGase F did not affect the antibody binding significantly. The mannosidase Manα1 → 2,3,6Man reduced the IgM binding ability of Sc 500. This effect was studied further by use of more enzyme; two vials initially followed by one vial after 24 h. (Fig. 6). By this treatment regime the IgM binding activity was clearly reduced.

DISCUSSION

gp200 is a glycoprotein antigen isolated from *S. cerevisiae* by gel filtration on Sepharose CL 6B column (Darroch *et al.*, 1994). gp200 was eluted in the void

volume of this column. This fraction showed a single band of apparent MW 200 kDa after SDS-PAGE and staining with Coomassie brilliant blue and PAS. In the present work, the crude extract has been prepared as previously described (Darroch *et al.*, 1994) and a Sepharose 4B column was used for the purification.

The heterogeneous crude extract carbohydrates were eluted from the void volume to fraction 60, all with antibody binding activity. Fraction 41-60 were pooled and freeze dried. This pool, named B gave one single peak from fraction 29 to fraction 35 by gel filtration on Superose 6 which corresponds to the MW range from 620 to 360 kDa (middle value 490 kDa). The material was eluted as one symmetrical protein and carbohydrate containing peak having IgG-binding activity (Fig. 1b). The Superose 6 column was calibrated with dextrans. Dextrans were chosen as calibration substances instead of proteins because of the high carbohydrate content of Sc 500.

The size of Sc 500 was also investigated by the SEC/MALLS method. The results were in agreement with the results obtained by gel filtration strongly indicating that the MW of the isolated antigen is about 500 kDa.

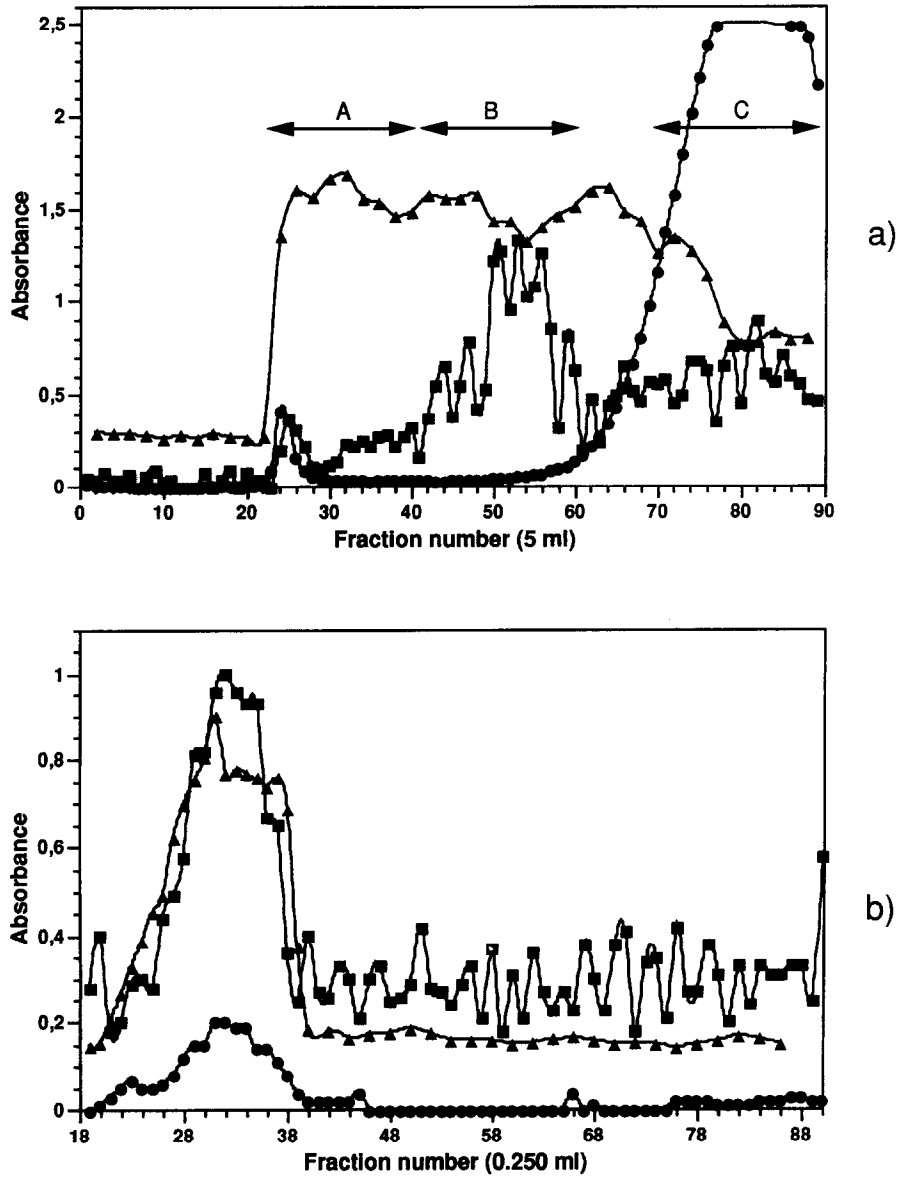

Fig. 1. a) Gel filtration of the crude extract on Sepharose 4B. The void volume (Blue Dextran) 110 ml (fraction 21). Three pools were prepared: A: fraction 22–40, B: fraction 41–60 and C: fraction 70–89 based on results obtained from: absorbance at 489 nm (phenol-sulphuric acid test) (■), absorbance at 280 nm (•) and IgG binding (ELISA) (▲). Fraction B showed high IgG-binding activity and the content of a high MW component (SDS-PAGE, Fig. 1). b) Size exclusion chromatography of fraction B on Superose 6 HR 10/30 of Sc 500. The column was calibrated with dextrans with the following MW. Peak max is given in parentheses: 98 kDa (fraction 49), 233 kDa (fraction 40), 475 kDa (fraction 32) and void volume, Blue Dextran (fraction 25). Absorbance at 489 nm (phenol-sulphuric acid test (■), absorbance at 280 nm (•) and IgG binding (ELISA) (▲). Sc 500 was eluted as a broad peak with MW in the range from 360–620 kDa.

Table 1. Amino acid and carbohydrate composition of Sc 500

	Residues per 100 residues	Residues per molecule Sc 500	Weight %
Asparagine/Aspartic acid	9	37	
Threonine	23	102	
Serine	21	93	
Glutamine/Glutamic acid	7	31	
Glycine	6	25	
Alanine	6	28	
Valine	6	27	
Isoleucine	2	10	
Leucine	3	13	
Tyrosine	2	9	
Phenylalanine	2	9	
Histidine	1	5	
Lysine	2	10	
Arginine	1	2	
Proline	5	22	
Methionine	4	17	
Total protein	100	440	10
Mannose		2790	90
N-acetylglucosamine		trace	trace

Table 2. Methylated alditol acetates identified after methylation analysis of Sc 500

Alditol acetate corresponding to	Primary fragments	Relative peak area (%)	Type of linkage
2,3,4,6 tetra-*O*-methylmannose	45, 118, 161, 162, 205	25	T-man
3,4,6 tri-*O*-methylmannose	45, 161, 190	24	1 → 2 Man
2,4,6 tri-*O*-methylmannose	45, 118, 161, 234	25	1 → 3 Man
2,3,4 tri-*O*-methylmannose	118, 162, 189, 233	3	1 → 6 Man
3,4 di-*O*-methylmannose	189, 190	23	1 → 2, 1 → 6 Man

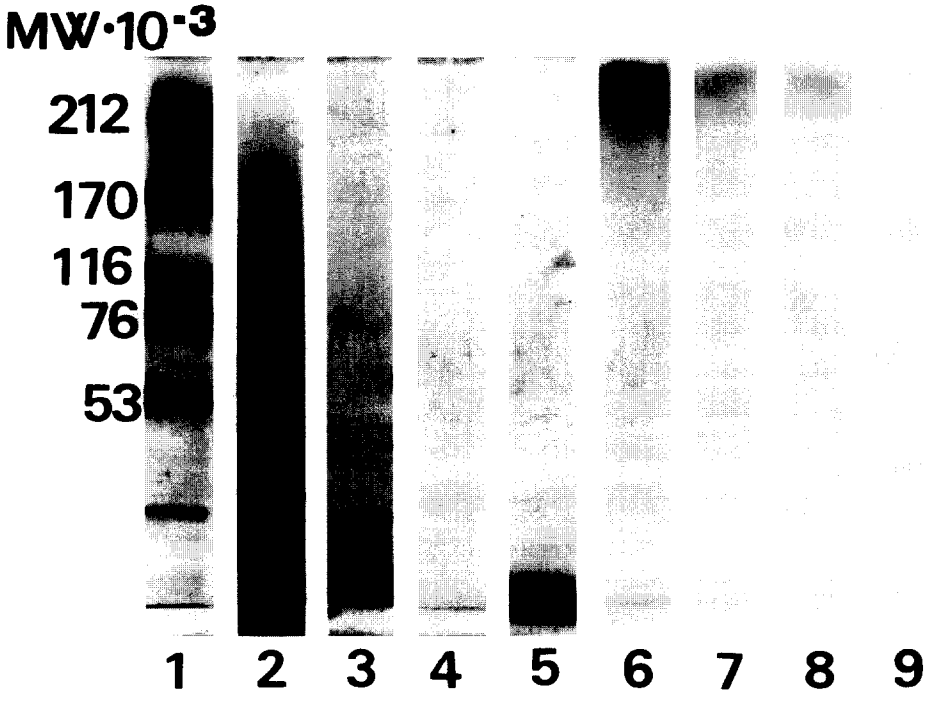

Fig. 2. SDS PAGE on crude extract and the fractions obtained by size exclusion chromatography on Sepharose 4B. Lane 1–5 is stained with the silver method. Lane 6–9 is stained with the periodic acid-schiff stain. Lane 1: MW standard proteins, Lane 2 and 6: 280 μg crude extract, lane 3 and 7: 30 μg pool A, lane 4 and 8: 30 μg pool B and lane 5 and 9: 30 μg pool C.

Comparative experiments showed that Sc 500 and gp200 had apparently the same MW both by gel filtration on the Superose 6 column and by SDS-PAGE (results not shown). Preliminary methylation on gp200 gave the same linkage pattern as found for Sc 500.

Pool B showed a single band with apparent MW of 200 kDa or higher after SDS-PAGE followed by PAS staining. In the present work, 8% polyacrylamide separation gels were used. Sc 500 is a very large molecule and therefore gels with large pores should be used. The antigen moves very slowly in a 8% gel and at the end of electrophoresis it was located only a few millimeters from the top of the separation gel. The standard curve is very steep in this area which makes it difficult to do a precise MW determination. The only conclusion it is possible to draw from the SDS-PAGE experiment is that Sc 500 has an apparent MW of 200 kDa or higher relative to the protein standards used. An 8% polyacrylamide SDS-PAGE gel used in the present work was more suitable for Sc 500 than the 12% gel previously used (Heelan *et al.*, 1991), but the gel concentration should be further reduced to 4–6% for optimal results. This gel is very soft and difficult to handle. Staining may lead to breakage during the many incubation steps of the PAS procedure.

Alternatively gels composed of a mixture of acrylamide and agarose could have been used. Such gels have been found to be suited for electrophoresis of other high molecular weight glycoproteins (Holden *et al.*, 1971).

The protein content of Sc 500 is not very high, but high enough to ensure electrophoretic mobility. By Coomassie brillant blue staining after SDS-PAGE, Sc

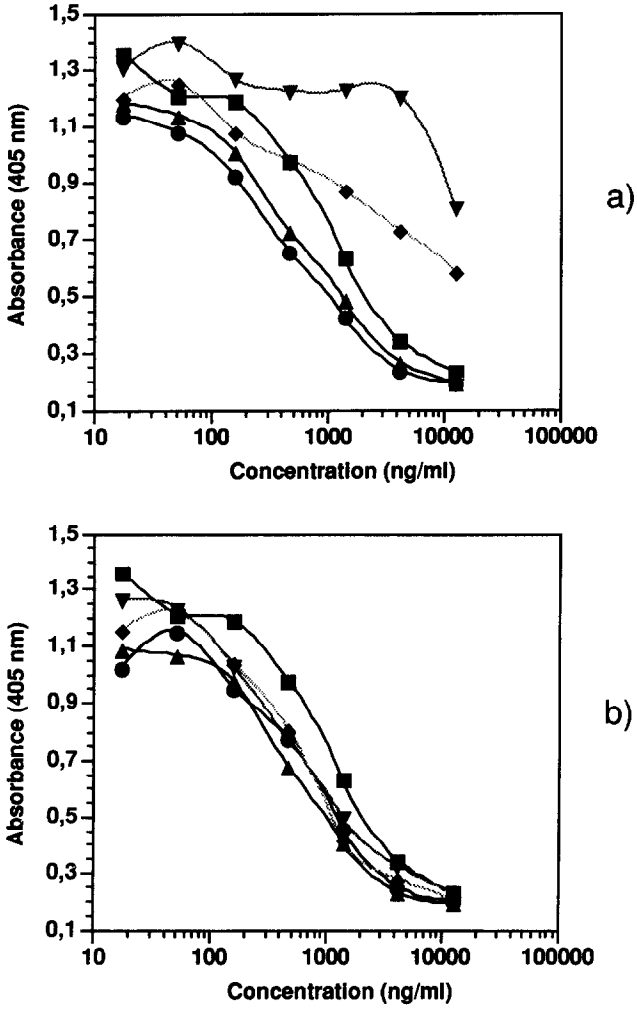

Fig. 3. Inhibition of binding of human serum IgA antibody to Sc 500 by Sc 500 or modified Sc 500. a) Control of native Sc 500 (■), Pronase 0.1 mg (●) trypsin 0.1 mg (▲), 0.125 M NaOH/1M $NaBH_4$, 6 h, 50°C (◆) and 10 mM sodium meta periodate, 1 h, 20°C (▼). b) Control (■), α-mannosidase (Manα1 → 2 Man) (●), β-mannosidase (Manβ1 → 4 GlcNAc) (▲), α-mannosidase (Manα1 → 2,3,6Man) (◆) and Peptide-*N*-Glycosidase F (▼).

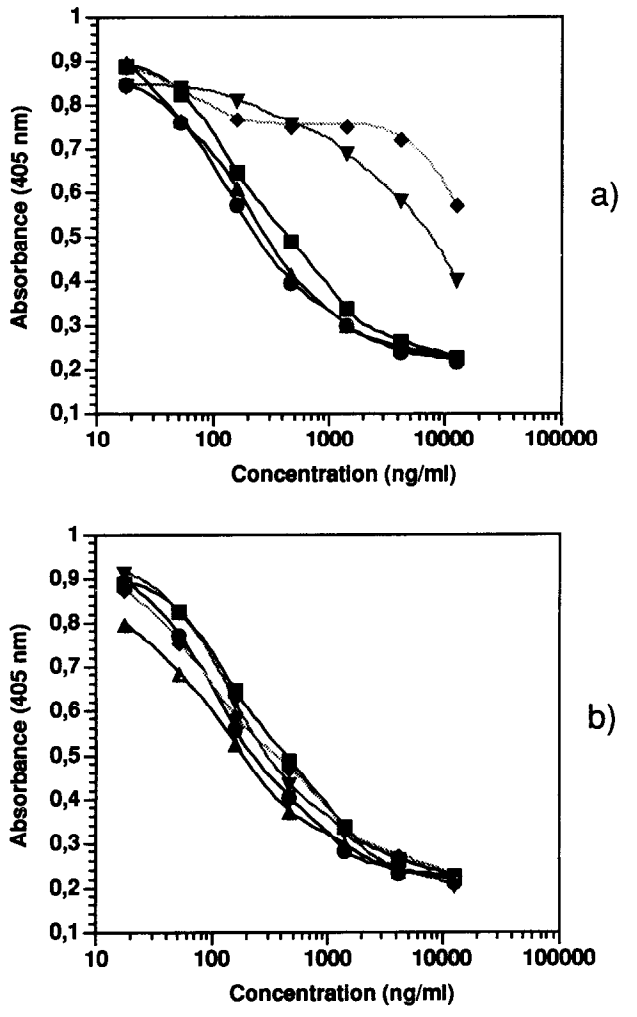

Fig. 4. Inhibition of binding of human serum IgG antibody to Sc 500 by Sc 500 or modified Sc 500. a) Control of native Sc 500 (■), Pronase 0.1 mg (●) trypsin 0.1 mg (▲), 0.125 M NaOH/1M $NaBH_4$, 6 h, 50°C (◆) and 10 mM sodium meta periodate, 1 h, 20°C (▼). b) Control (■), α-mannosidase (Manα1 → 2 Man) (●), β-mannosidase (Manβ1 → 4 GlcNAc) (▲), α-mannosidase (Manα1 → 2,3,6Man) (◆) and Peptide-*N*-Glycosidase F (▼).

500 was not visible. The reason why Sc 500 is not stained by Coomassie, may be that the protein part of the molecule is surrounded by mannose chains which prevent binding of Coomassie brilliant blue to the polypeptide. This may also explain the high thermic stability of Sc 500 which allow boiling for 1 h as a step in the isolation procedure.

The glycoprotein antigen Sc 500 consists of 90% mannose and 10% protein. By methanolysis followed by GC-MS of the TMS-derivates, traces of *N*-acetylglucosamine were identified. High content of threonine and serine found by amino acid analysis suggests that *O*-glycosidic bonds are involved in the binding between protein and carbohydrate. By alkaline borohydride treatment of Sc 500 followed by fractionation on the Superose 6 column, a low MW protein part was cleaved off. This experiment indicates that the majority of linkages between protein and carbohydrate are *O*-glycosidic, and that a minor number are *N*-glycosidic. Representative structures illustrating the nature of carbohydrate components in *S. cerevisiae* mannan include both short oligosaccharides attached to residues of serine and threonine in the protein and large polysaccharide units linked to asparagine residues (Ballou, 1976 p. 97). This means that by alkaline borohydride treatment, followed by fractionation on a gel filtration column, it should be possible to isolate and collect the liberated oligosaccharides for structure analysis. Further studies are in progress including this. The methylation data show that the mannan is highly branched, i.e. one branch point for every 4 monomer (Table 2). Based on these data the following three possible structures of mannan can be proposed:

(1) a chain of 1 → 2 linked mannose units where every other unit is branched on C-6. The branches are on average composed of disaccharide units being 1 → 3 linked.
(2) a chain of alternating 1 → 2 and 1 → 3 linked units in the ratio 2:1, where every other 1 → 2 linked units have one mannose unit linked on C-6.
(3) a core of 1 → 6 linked units, the majority having branches on C-2 consisting of 1 → 2 and 1 → 3 linked side chains.

The 1 → 6 linked mannose residues detected may represent end points of the main chains in model 1 and 2 and unsubstituted residues of the core in model 3.

Structures similar to proposal 3) has been identified in the baker's yeast, in *S. cerevisiae* strain S 288C and in its derivative X 2180 (Ballou, 1976 p. 109) and might be the dominating structure of the *N*-glycosidic bound mannose chains of Sc 500 as well.

Sc 500 was treated with mannosidases as well as

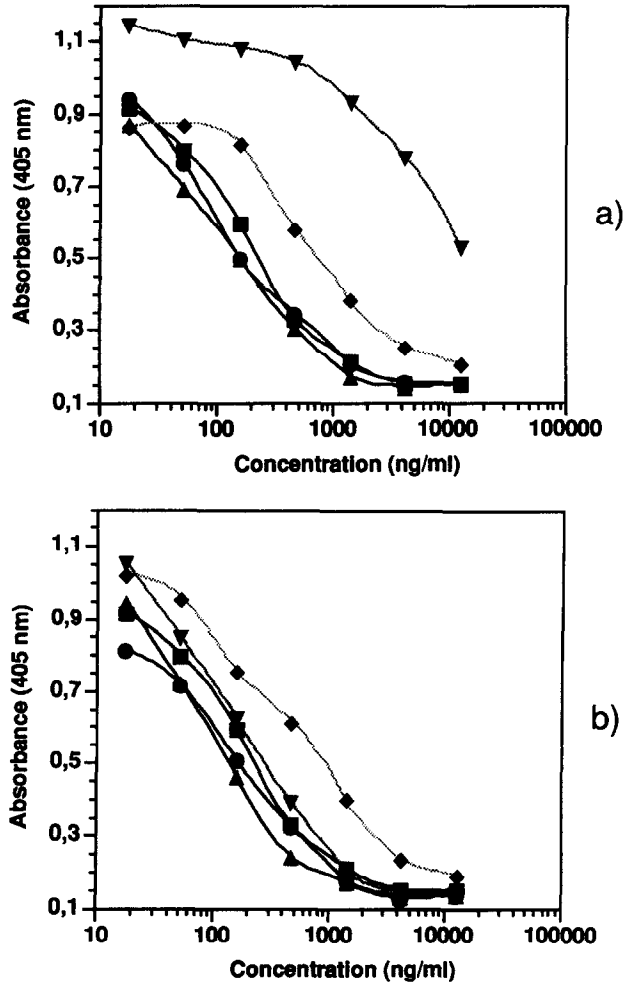

Fig. 5. Inhibition of binding of murine monoclonal IgM antibody to Sc 500 by Sc 500 or modified Sc 500. a) Control of native Sc 500 (■), Pronase 0.1 mg (●) trypsin 0.1 mg (▲), 0.125 M NaOH/1M $NaBH_4$, 6 h, 50°C (◆) and 10 mM sodium meta periodate, 1 h, 20°C (▼). b) Control (■), α-mannosidase (Manα1 → 2 Man) (●), β-mannosidase (Manβ1 → 4GlcNAc) (▲), α-mannosidase (Manα1 → 2,3,6Man) (◆) and Peptide-*N*-Glycosidase F (▼).

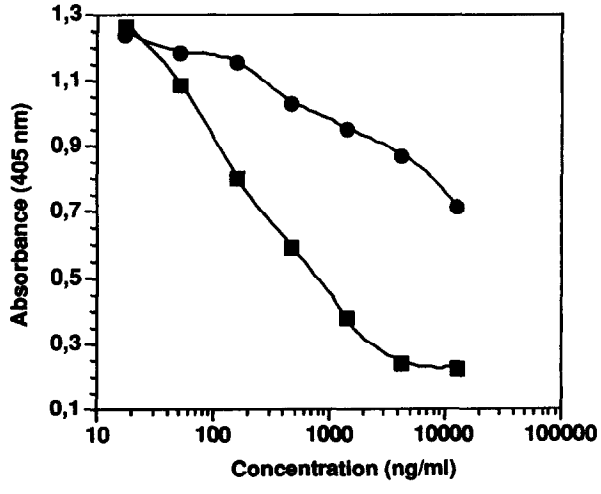

Fig. 6. Inhibition of binding of murine monoclonal IgM to Sc 500 by Sc 500 modified by the α-mannosidase (Manα1 → 2,3,6Man). Control of native Sc 500 (■) and α-mannosidase (Manα1 → 2,3,6Man) 3 vials, 48 h (●).

proteases and selected forms of chemical treatment. Treatment of Sc 500 with trypsin, pronase, PNGase F or the mannosidases (Manα1-2Man and Manβ1-4 GlcNAc) did not reduce the antigenicity of Sc 500 compared to the control. We do not know, however, if these enzymes affected the antigen structurally.

One of the effects of the oligosaccharide moieties of glycoproteins is to protect the polypeptide core against attack by proteolytic enzymes (Varke, 1993). It is therefore reasonable to believe that the the high content of carbohydrate on Sc 500 protects the protein part from being digested by trypsin and pronase (Fig. 3a, Fig. 4a and 5a). The inability of these enzymes to reduce the antibody binding activity of Sc 500 does thus not rule out the possibility that some of the human IgA and IgG antibodies are directed against epitopes on the protein part of the molecule. The large carbohydrate moiety of Sc 500 will, however, probably prevent antibody binding to most of the protein core of the molecule. Such masking of potential peptide epitopes are commonly encountered on heavily glycosylated glycoproteins (Burchell & Taylor-Papadimitriou, 1993).

Treatment with alkaline sodium borohydride lead to a cleavage of some of the bonds between protein and carbohydrate, revealed by change of the gel filtration profile by this treatment. The alkaline borohydride degradation lead to a slight decrease in the antibody binding of human IgG and IgM (mab) and to a somewhat more pronounced reduction in the binding of human IgA antibodies (Figs 4a, 5a and 3a respectively). This indicates that the carbohydrate-protein bonds that are broken by this treatment are of importance for maintaining an antigenically active conformation of the glycoprotein, but as the treatment with alkali did not cleave all the carbohydrate-protein bonds and as testing of the purified released carbohydrate part has not been performed we don't know whether the carbohydrate-protein linkages are essential for the epitopes studied.

Treatment with sodium periodate reduced antibody binding of human IgA and IgG and IgM (mab) to Sc 500. Similar observations have been made by treatment of gp200 blotted to nitrocellulose membrane with periodate (IgG and IgA) (Heelan *et al.*, 1991). This treatment oxidizes carbon atoms carrying vicinal hydroxyl groups and will specifically oxidize terminal, 1,6- and 1,2,6-linked mannopyranosyl residues, but not 1,3-linked residues. The experiment shows therefore that the terminal, 1,2-, 1,6- linked and branchpoint mannopyranosyl residues all can be essential for the epitopes recognized by all the antibodies studied.

The broad spectrum α-mannosidase which cleaves the $1 \rightarrow 2$, $1 \rightarrow 3$ and $1 \rightarrow 6$ mannose linkages reduced IgM mab-, but not serum IgA- or IgG-antibody binding ability of Sc 500. This effect was more pronounced by use of longer incubation time and higher enzyme to antigen ratio. This suggests that the monoclonal IgM antibody binds to an epitope in the carbohydrate moiety of the antigen which is modified by this enzyme. This mannosidase will be a very important tool for our further work, as it makes it possible to strip off part of the carbohydrates.

Further studies to verify the structure of the polymer and the structure requirements for the IgM binding activity are in progress.

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REFERENCES

Ballou, C. (1976). Structure and biosynthesis of the mannan component of the yeast cell envelope. *Adv. Microbiol. Physiology*, **14,** 97 & 109.

Barnes, R.M.R., Allan, S., Taylor-Robinson, C.H., Finn, R. & Johnson, P.M. (1990). Serum antibodies reactive with *Saccaromyces cerevisiae* in inflammatory bowel disease: Is IgA antibody a marker for Crohn's Disease?. *Int. Arch. Allergy Appl. Immunol.*, **92,** 9–15.

Barsett, H., Haraldstad, K.-A. & Paulsen, B.S. (1991). Separation and structural investigations of polysaccharides present in the seeds of Ulmus glabra Huds. *Acta Pharm. Nord*, **3,** 87–90.

Blakeney, A.B. & Stone, B.A. (1985). Methylation of carbohydrates with lithium methylsulphinyl carbanion. *Carbohydr. Res.*, **140,** 319–324.

Bröker, M., Harthus, H.-P. & Barnes, R.M.R. (1994). A murine monoclonal antibody directed against a yeast cell wall glycoprotein antigen of the yeast genus *Saccharomyces*. *FEMS Microbiol. Letts.*, **118,** 297–304.

Burchell, J. & Taylor-Papadimitriou, J. (1993). Effect of modification of carbohydrate side chains on the reactivity of antibodies with core-protein epitopes on the MUC 1 gene product. *Epith. Cell Biol.*, **2,** 155–162.

Darroch, C.J., Christmas, S.E. & Barnes, R.M.R. (1994). *In vitro* human lymphocyte proliferative responses to a glycoprotein of the yeast *Saccharomyces cerevisiae*. *Immunology*, **81,** 247–252.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, **28,** 350–356.

Harris, P.J., Henry, R.J., Blakeney, A.B. & Stone, B.A. (1984). An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydr. Res.*, **127,** 59–73.

Heelan, B.T., Allan, S. & Barnes, R.M.R. (1991). Identification of a 200-kDa glycoprotein antigen of *Saccaromyces cerevisiae*. *Immunol. Lett.*, **28,** 181–186.

Holden, K.G., Yim, N.C.F., Giggs, L.J. & Weisbach, J.A. (1971). Gel electrophoresis of mucous glycoproteins. I. Effect of gel porosity. *Biochemistry,* **10,** 3105–3109.

Kortekangas-Savolainen, O., Kalimo, K., Lammintausta, K. & Savolainen, J. (1993a). IgE-binding components of baker's yeast (*Saccharomyces cerevisiae*) recognized by immunoblotting analysis. Simultaneous IgE binding to mannan and 46-48 kD allergen of *Saccharomyces cerevisiae* and *Candida albicans*. *Clin. and Exp. Allergy,* **23,** 179–184.

Kortekangas-Savolainen, O.Savolainen, J.Einarsson, R. (1993b). Gastrointestinal stability of baker's yeast allergens: an *in vitro* study. *Clin. and Exp. Allergy,* **23,** 587–590.

Kvernheim, A.L. (1987). Methylation analysis of polysaccharides with butyllithium in dimethyl sulfoxide. *Acta Chem. Scand.,* **41,** 150–152.

Main, J., McKenzie, H., Yeaman, G.R., Kerr, M.A., Robson, D., Pennington, C.R. & Parratt, D. (1988). Antibody to *Saccharomyces cerevisiae* (baker's yeast) in Crohn's disease. *Br. Med. J.,* **297,** 1105–1106.

McKenzie, H., Main, J., Pennington, C.R. & Parratt, D. (1990). Antibody to selected strains of *Saccharomyces cerevisiae* (baker's and brewer's yeast) and *Candida albicans* in Crohn's disease. *Gut,* **31,** 536–538.

McKenzie, H., Parratt, J. & Pennington, C.R. (1992). Antigenic heterogeneity of strains of *Saccharomyces cerevisiae* and *Candida albicans* recognized by serum antibodies from patients with Crohn's disease. *FEMS Microbiol. Immunol.,* **89,** 219–224.

McLafferty, F.W. & Stauffer, D.B. (1989). The Wiley/NBS Registry of Mass Spectral Data Vol 5. John Wiley & Sons, USA, p. 5126.

Neville, D.M. Jr. (1971). Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. *J. Biol. Chem.,* **246,** 6328–6334.

Reinhold, V.N. (1972). Gas-liquid chromatography analysis of constituent carbohydrates in glycoproteins. *Methods Enzymol.,* **25,** 244–249.

Varke, A. (1993). Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology,* **3,** 97–130.

Wyatt, P.J. (1992). *Laser Light Scattering in Biochemistry*, Chap 3, eds S.E. Harding, D.B. Sattelle & V.A. Bloomfield. Royal Soc. Chem., Chambridge, UK. .

Zacharius, R.M., Zell, T.E., Morrison, J.H. & Woodlock, J.J. (1969). Glycoprotein staining following electrophoresis on acrylamide gels. *Anal. Biochem.,* **30,** 148–152.