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Analysis of cell wall mutants of *Saccharomyces cerevisiae* by pyrolysis mass spectrometry

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SUMMARY

The main constituents of the cell wall of *Saccharomyces cerevisiae*, i.e. β-1,3-glucan, β-1,6-glucan and mannan, could be distinguished by pyrolysis ammonia chemical ionization mass spectrometry or Py(CI)MS. Cell walls extracted with alkali, which removes the mannan fraction and part of the glucan from the cell wall, yielded mass spectra that differed clearly from nonextracted cell walls. Multivariate analysis of these spectra showed a shift from 'mannan-like' towards 'glucan-like' cell walls. Known cell wall mutants, such as *mnn*9 and *kre*1, were recognized by this technique. Py(CI)MS in combination with multivariate analysis of the spectra is therefore a useful tool for identifying and characterizing cell wall mutants in yeast.

Key-words: killer resistance mutants, mannosylation mutants, chitin, glucan, mannan, *kre*1, *mnn*9.

INTRODUCTION

Pyrolysis mass spectrometry (PyMS) is a rapid analysis technique which provides mass spectrometric information on the monomeric and oligomeric composition of the sample. PyMS has been applied on a wide range of different samples ranging from plant cell walls (Boon 1989), plant tissues infected by fungi (Niemann *et al.* 1990), to synthetic polymers (Luijk *et al.* 1991). Here PyMS and subsequent multivariate analysis have been successfully used for the characterization of chitin (Van der Kaaden *et al.* 1984a), and for differentiation of polyhexoses according to linkage type (Hoogerbrugge *et al.* 1983; Van der Kaaden *et al.* 1984b; Reinhold 1987). Because the cell wall of *Saccharomyces cerevisiae* consists of almost 95% carbohydrate and only about 5% protein (Ballou 1982), these techniques might be useful to detect differences in the cell wall composition of mutant strains.

The carbohydrate portion of the cell wall of *S. cerevisiae* consists of equal amounts of glucan and mannan and about 2% chitin (Fleet 1991). About 40% of the glucan is

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alkali-soluble and consists of a β-1,3-glucan with 10% of β-1,6-linkages (Fleet & Manners 1976). The alkali-insoluble glucan consists of two types of glucose polymers. The most abundant one is a branched β-1,3-glucan containing 3% β-1,6-glucan (Manners *et al.* 1973a). The other alkali-insoluble glucan is a branched β-1,6-glucan that contains 20% β-1,3-glucan (Manners *et al.* 1973b). The amount of alkali-insoluble β-1,6-glucan is reduced in the wall of the *kre*1 mutant (Boone *et al.* 1990).

Mannan is covalently attached to protein and contains α-1,2-, α-1,3 and α-1,6-linkages (Ballou 1982). Extraction of cell walls with SDS removes about 80% of the cell wall protein and a minor part of the cell wall mannose, which is mainly *O*-glycosidically linked to SDS-extractable proteins (Valentin *et al.* 1984; Sanz *et al.* 1989). The remaining mannoproteins represent a minor part of the cell wall protein, but the bulk of the cell wall mannose. Their mannose is predominantly found in *N*-linked side-chains of the protein (Valentin *et al.* 1984; Pastor *et al.* 1984; Zueco *et al.* 1986). The *mnn*9 mutant lacks the so-called outer-chain of the *N*-linked mannan and, therefore, the amount of mannan in its wall is strongly reduced (Ballou 1982). The mannoproteins can be completely removed from yeast walls by extraction with alkali (Mol & Wessels 1987), which, in addition, solubilizes 40% of the glucan (Fleet & Manners 1976).

Isolated, SDS-extracted walls of *kre*1, *mnn*9, and wild-type cells were analysed by pyrolysis ammonia chemical ionization mass spectrometry (Py(CI)MS). The wall material that remained after alkali extraction of these walls was also analysed by Py(CI)MS. Multivariate analysis of the Py(CI)MS confirmed the expected differences in the composition of the walls, thereby demonstrating that Py(CI)MS can be used to *rapidly* detect and characterize cell wall mutants in yeast.

MATERIALS AND METHODS

Yeast strains and growth

Saccharomyces cerevisiae strain X2180-1A (*MAT***a**; referred to as wild type) was obtained from the Yeast Genetic Stock Center, Berkeley, California, USA. The strain LB-347-1C (*MAT*α *mnn*9) was provided by Dr L. Ballou, University of California, Berkeley, CA, USA, and is isogenic to the wild type used. The strain 463-1B (*MAT*α *kre*1) was a gift from Dr H. Bussey, McGill University, Montreal, Canada. All strains were grown at 28°C in YPD medium (1% (w/v) yeast extract, Gibco; 1% (w/v) bactopeptone, Difco; 3% glucose). Cells were harvested in the stationary phase.

Preparation of the walls for pyrolysis mass spectrometry

Cell walls were isolated as described by De Nobel *et al.* (1990). Isolated walls (20 mg fresh weight ml^{-1}) were boiled in 2% SDS for 5 min. After extraction, the walls were washed five times with distilled water. A part of the SDS-extracted walls was then extracted with 3% NaOH according to Boone *et al.* (1990). After alkali extraction, the walls were again washed five times with distilled water. The extracted walls were lyophilized and stored at −20°C prior to pyrolysis mass spectrometry.

Pyrolysis ammonia chemical ionization mass spectrometry (Py(CI)MS)

A Jeol DX303 double focusing MS was used for analysis of the carbohydrate standards and the isolated cell walls. A suspension of the material in distilled water was applied to a platinum–rhodium wire attached on top of an insertion probe. The sample was dried

and the probe was inserted into the ion source (180°C) and heated resistively by applying an increasing current up to 0·8 A at a rate of 1 A min^{-1} (13·5°C/s). Desorbed fragments were ionized with ammonia as reagent gas (250 eV; pressure in the source: 20 Pa). The mass range was set from 60 up to 1000 a.m.u. Ionized fragments were accelerated with an accelerating voltage of 2·2 kV. The scan cycle time was 1 s and the maximum run time was 70 s. Data were collected using a Jeol DA 5000 data system. The mass spectra were summarized over 40 scans. Samples of the SDS-extracted walls, the alkali-extracted walls, and the standards, laminarin (β-1,3-glucan), pustulan (β-1,6-glucan), yeast mannan, and crab-shell chitin, were analysed fourfold.

Data analysis on the mass spectra

The spectra were normalized to total ion intensity. Next, discriminant analysis by double-stage principal component analysis (PCA) of the Py(CI)MS spectra was performed according to Hoogerbrugge *et al.* (1983). Basically, the method involves a recombination of the original variables (mass intensities) into new variables (discriminant functions, D) on the basis of the covariance matrix computed for the data set. The procedure is carried out under the prerequisite that the discriminant functions represent that part of variance of the data set, for which the ratio 'between group/within group' variance is as large as possible (Van der Kaaden *et al.* 1984b); a group is defined as the set of four (or five) replicate analyses. The calculations were carried out in a VAX11/785 system with a modified ARTHUR package (Hoogerbrugge *et al.* 1983).

Carbohydrate samples

Mannan (*S. cerevisiae*) and chitin (crab-shells) were purchased from Sigma. Laminarin (β-1,3-glucan; *Laminaria* sp.) was from Fluka, and pustulan (β-1,6-glucan; *Umbilicaria papullosa*) was from Calbiochem. Prior to use, these samples were washed three times at 4°C with 80% (v/v) ethanol. The samples were lyophilized and then stored at −20°C.

RESULTS AND DISCUSSION

Pyrolysis electron impact mass spectrometry (Py(EI)MS) revealed no clear differences between the cell walls of different strains of *Saccharomyces cerevisiae* (not shown). Since ionization of carbohydrates by EI is relatively destructive, the 'softer' ammonia chemical ionization was tried. In source pyrolysis was used to avoid the loss of oligomer information due to condensation on the wall of the transfer line of the inlet. Under these conditions, Pouwels & Boon (1990) and Lomax *et al.* (1991a,b,c) have obtained excellent PyMS spectra of polysaccharides. Because chitin, β-1,3-glucan, β-1,6-glucan, and mannan are the carbohydrate constituents of the yeast cell wall (Ballou 1982), these were first compared. In the upper panel of Fig. 1, the total ion current (TIC) of pyrolysis of β-1,3-glucan is presented. In the lower panels, mass traces of oligomeric ions of β-1,3-glucan are given. These show that the production of monomers (m/z 180) maximized between scans 32 and 40. Dimers (m/z 342), trimers (m/z 504) and tetramers (m/z 666) were formed between scans 35 and 40. Similar results were obtained with the other carbohydrate and cell wall samples (not shown). The mass spectra in Figs 2 and 3 were taken at the position of maximal oligomer production, i.e. from scans 35 to 40. Bearing in mind the fact that ammonia CI is accompanied by the formation of ammonia adducts resulting in $(M+18)^+$ ions, our mass spectra of chitin were qualitatively similar to those obtained by Van der Kaaden *et al.* (1984a). Our most abundant mass peak, m/z

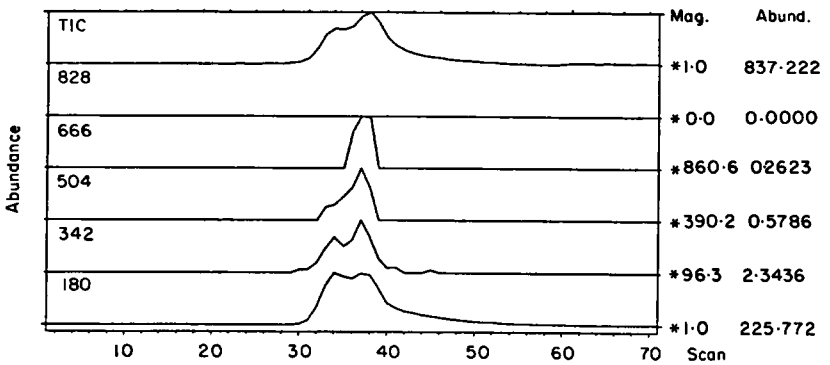

Fig. 1. Time-course of the pyrolysis of β(1-3)-glucan. The mass chromatograms of the mono-, di-, tri-, tetra- and pentamer (not detectable) of the anhydrohexose and the total ion current (TIC) are presented. The scan cycle time was one second.

77 (Fig. 2a), corresponded with their most abundant peak, m/z 59, which was identified as acetamide (C_2H_5NO). Other characteristic mass peaks were m/z 94, 102, 119, 126, 136, 143, 186 and 204. Finally, anhydro-2-acetamido-2-deoxyhexose ($C_8H_{13}NO_5$; m/z 221) was present in relatively large amounts (Fig. 2a), indicating that the conditions chosen for pyrolysis were optimal for the characterization of chitin. At higher masses, mass peaks for chitin dimers (m/z 424) and trimers (m/z 627) were observed.

Both the β-1,3-glucan and β-1,6-glucan mass spectra as well as that obtained for mannan (Fig. 2b–d) contained mass peaks m/z 144, 162, 180 and 342. These peaks are characteristic for anhydro(oligo)-saccharides and related products with ring cleavage fragments. Peaks of m/z 180, 342, 504 and 666 indicate the presence of the anhydro-hexose mono-, di-, tri- and tetramer (Arisz *et al.* 1990; Lomax *et al.* 1991a,c). Anhydro-oligomers with attached ring cleavage fragments of 42 or 60 Da were also present, in particular in the β-1,6-glucan (Fig. 2c): mass peaks m/z 222, 384 and 546 and mass peaks m/z 240, 402 and 564 (Arisz *et al.* 1990). According to Arisz *et al.* (1993), the relatively higher abundance of the anhydro-oligomer+60 fragments in the β-1,6-glucan is due to blocking of the transglycosidation dissociation pathway, because the C_6 atom is involved in the binding to another monomeric unit. In the case of β-1,6-glucan, the anhydro-oligomer+60 fragments presumably consist of the C_5 and the C_6 of the former ring attached via the C_6 to an adjacent ring as was found for α-1,6-glucan (Boon 1992). The dissociation mechanism for the formation of the anhydro-oligosaccharide+42 fragments found in β-1,6- and β-1,3-glucan is still unclear. Tas *et al.* (1989) have proposed that the formation of this series is a dehydrated form of the anhydro-oligomer+60 series. In addition, deoxyhexose-specific peaks, such as m/z 146 and 164 (Kerkenaar *et al.* 1987), were detected. The glucan and mannan spectra revealed no specific mass peaks for either one of the polyhexoses analysed. However, some peak ratios, such as m/z 144 over m/z 162, seemed to depend on the kind of polyhexose used.

The mass spectra of walls from different strains (Fig. 3) and of the alkali-insoluble fraction of the walls (not shown) revealed only mass peaks that had also been found in the separate carbohydrate standards. None of the peaks was specific for one of the strains but the spectra showed considerable variations in the peak ratios (Fig. 3). Hence, discriminant analysis was conducted on the data set of scan 30 until 60 using principal components analysis according to Hoogerbrugge *et al.* (1983). Discriminant analysis

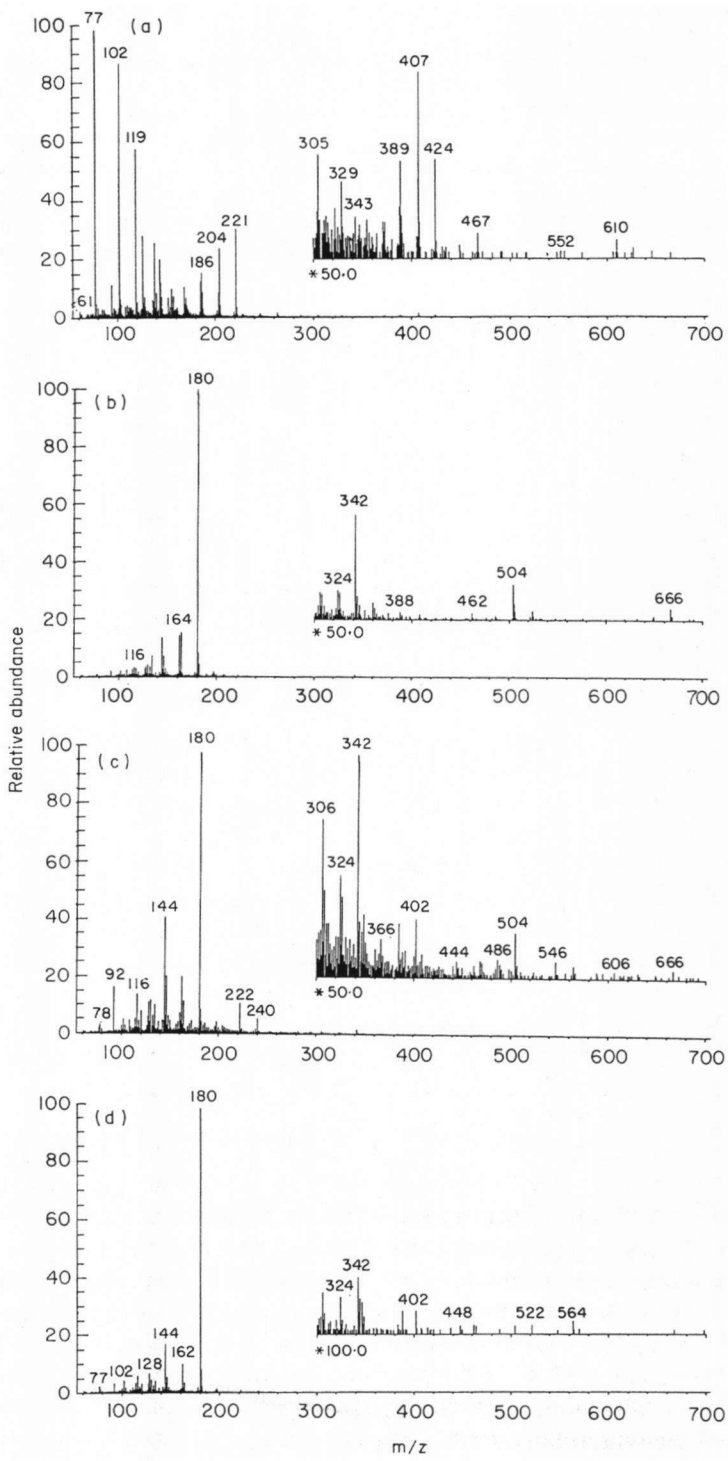

Fig. 2. Pyrolysis (CI) mass spectra of carbohydrate samples: (a) chitin; (b) β-1,3-glucan; (c) β-1,6-glucan; (d) mannan.

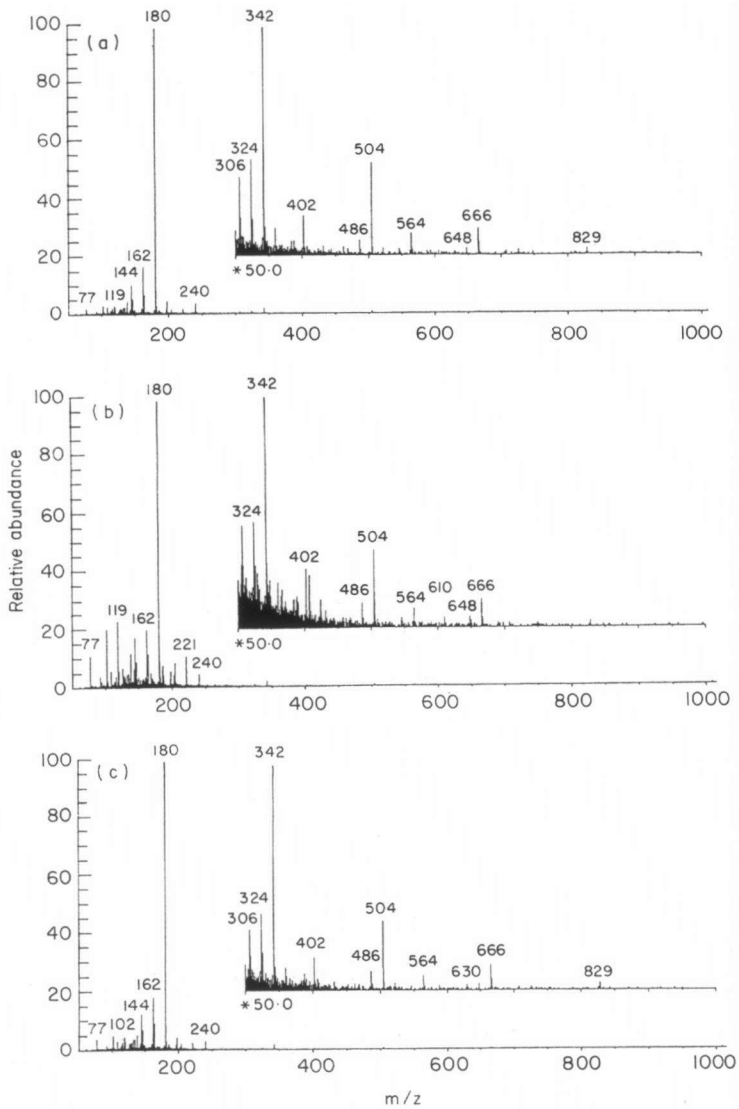

Fig. 3. Pyrolysis (CI) mas spectra of cell walls: (a) wild type; (b) *kre*1; (c) *mnn*9.

applied to pyrolysis mass spectra, is a multivariate analysis technique which uses linear combinations of masses taken from these spectra. These linear combinations of masses are called discriminant functions and help to separate different groups of samples, such as micro-organisms in this particular case. Group separations are achieved by maximizing the ratio of among-group variance to within-group variance. Often, discriminant functions also reveal the underlying biochemical composition of samples. Different compounds have different mass spectra and therefore will point in a specific direction in a multidimensional discriminant analysis space. In Figs 4, 5 and 6 schematic representations are given of two discriminant functions using this mathematical approach. Chitin could be readily distinguished from the polyhexoses by the presence of several

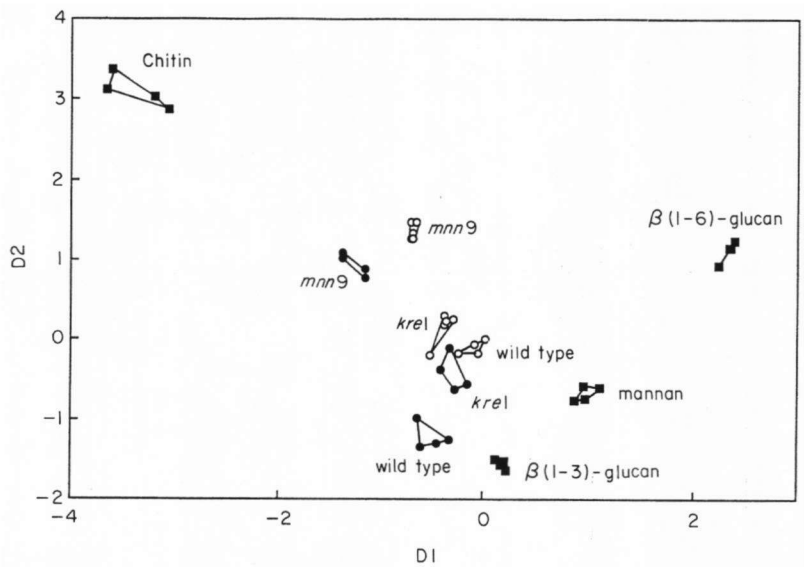

Fig. 4. Score plot of the first two discriminant functions, D1 and D2, of the pyrolysis (CI) mass spectra. The replicate analyses have been connected by solid lines. Solid circles, walls before extraction with alkali; open circles, walls after extraction with alkali.

specific mass peaks (m/z 77, 102, 119, 204 and 221; cf. Fig. 2a and 2b–d). It accounted, therefore, also for most of the variation when the samples were compared by discriminant analysis. For better discrimination between the polyhexoses, the data were analysed with the chitin test sample absent from the training set.

In Fig. 4, the scores of the first two discriminant functions have been plotted. The results show that the carbohydrate test samples can be distinguished by this procedure. The walls of the different yeast strains can also be distinguished. Although the chitin test sample was absent from the training set, chitin-specific mass peaks are still the largest source of variation between the different samples because of the presence of chitin in the cell wall. This can be concluded from the shift of the chitin-containing cell walls from the glucan and mannan test samples into the $D1^-$, $D2^+$ direction (Fig. 4). On the basis of the discriminant spectra, general conclusions can be drawn about the mass peaks of the pyrolysis products responsible for the differentiation. Comparison of the discriminant spectra of the $D1^-$, $D2^+$ direction shows characteristic mass peaks m/z 77, 94, 102, 119, 126, 136, 143, 186, 204 and 221 (Fig. 5). These peaks strongly point to the presence of chitin-like pyrolysis products. When the chitin test sample is plotted in the discriminant plot, it can be seen that the $D1^-$, $D2^+$ direction is indeed characterized by chitin fragments (Fig. 4).

Beta-1,6-glucan, which is localized in the $D1^+$, $D2^+$ direction (Fig. 4), is characterized by mass peaks m/z 61, 78, 92, 116, 120, 172 and 222 (Fig. 5). Bearing in mind the mass shifts due to ammonia adduct formation, this is in agreement with the data by Van der Kaaden *et al.* (1984) who have shown that in pyrolysis electron impact mass spectrometry some of these peaks are also preferentially formed from β-1,6-glucan.

Although the distinction between β-1,3- and β-1,6-glucan is quite clear in the score plot on the first two discriminant functions, that between β-1,3-glucan and mannan is less obvious. Both hexoses are in the $D1^+$, $D2^-$ direction (Fig. 4), which is

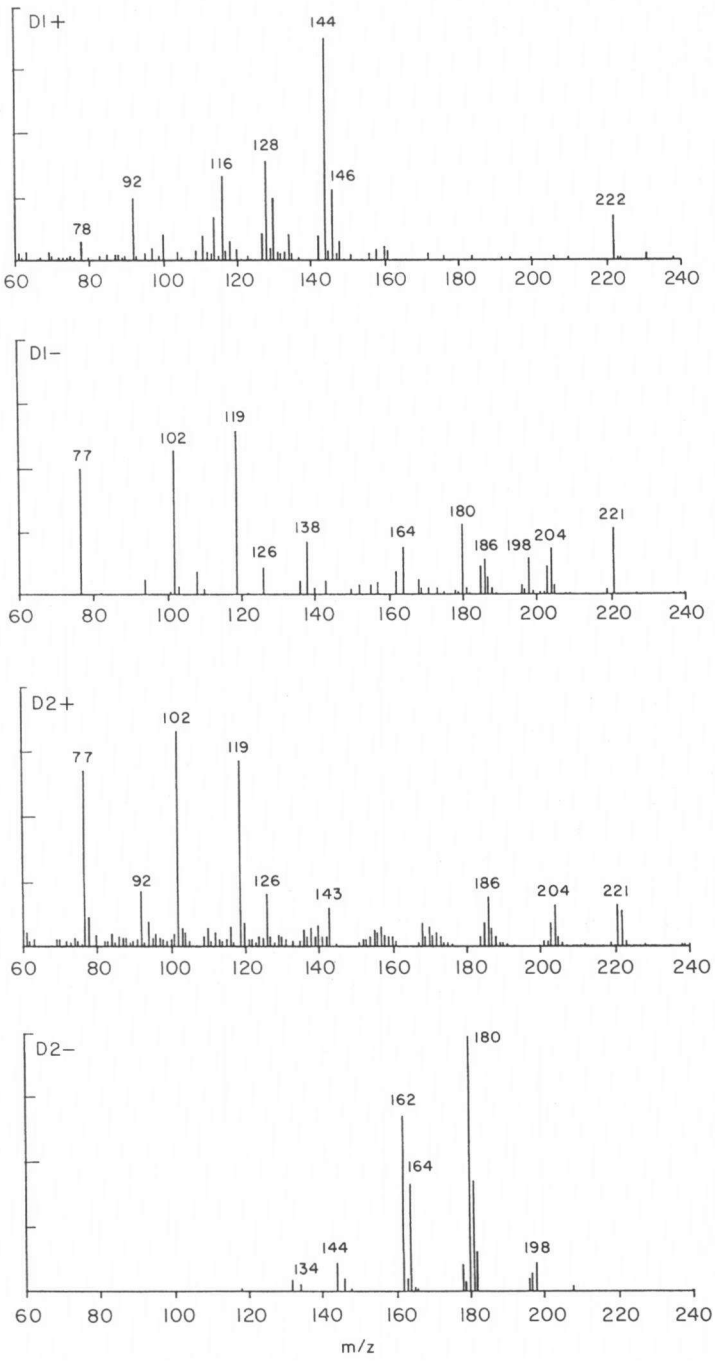

Fig. 5. The discriminant spectra that represent the set of ions characteristic for discrimination in the directions: $D1^+$, $D1^-$, $D2^+$ and $D2^-$, from top to bottom, respectively.

characterized by mass peaks m/z 134, 144 and 146 (Fig. 5). M/z 134 points to the presence of alkali cations in the sample (Scheijen & Boon 1989) and m/z 146 indicates a contamination with fucose. However, plotting the first (D1) against the third (D3) discriminant function revealed a clear discrimination between mannan and β-1,3-glucan (Fig. 6). Comparison of the three discriminant spectra (Figs 5 and 6) indicates that β-1,3-glucan is characterized by mass peaks m/z 134 and 146 and mannan by mass peak m/z 144 tri-anhydrohexose). The presence of the latter mass peak might be related to the high degree of branching in mannan.

The score plot on D1 and D3 also shows very clearly that alkali extraction of the cell walls, independent of the strain used, resulted in a shift from 'mannan-like' towards 'glucan-like' cell walls (Fig. 6). This is consistent with the observation that mannan is completely removed by alkali extraction whereas glucan is only partially extracted from the cell walls (Fleet & Manners 1976; Mol & Wessels 1987).

Furthermore, both plots (Figs 4 and 6) show clear differences between the walls of the strains used. Both plots show that the *mnn*9 walls differ more from the mannan standard than the walls of the wild-type cells. This is consistent with the observation that *mnn*9 cells contain significantly reduced amounts of mannan in their walls compared with wild-type cells (Ballou 1982). The walls of the wild type and *kre*1 cells can also be distinguished (Figs 4 and 6). Before alkali extractions, the *kre*1 walls are shifted towards the β-1,6-glucan standard compared to the wild-type walls; this seems inconsistent with the reduced amount of this glucan in *kre*1 cells (Boone *et al.* 1990). However, comparison of the positions of the cell wall samples in the discriminant plots reveals that they are plotted in an area that is enclosed by chitin, mannan, and β-1,3-glucan indicating that the β-1,6-glucan in the cell wall does not strongly affect their position (Figs 4 and 6). Therefore, the relative position of *kre*1 walls should not be related to β-1,6-glucan but to the other standards. From Fig. 4 it can be seen that *kre*1 walls, compared with wild-type walls, shift from the polyhexoses towards the chitin standard, which is consistent with the reduced amount of β-1,6-glucan in *kre*1 cells.

One might argue that the differences found for the three yeast strains as reflected in their relative positions in the discriminant plot (Fig. 4) might be strongly affected by biological variation or by lack of reproducibility in the isolation procedure of cell walls. Therefore, the following control experiment was carried out. The different strains were now cultured in triplicate and their walls were isolated and extracted with SDS as in the former experiment. The isolated cell walls were then examined in triplicate by Py(CI)MS, resulting in nine pyrograms for each strain. Multivariate analysis of these data was conducted with the independently isolated cell walls of each strain considered as separate groups. Although the cell walls of each strain were isolated independently and were considered as separate groups in multivariate analysis, the three strains still appeared as three, and not as nine, separated groups in the discriminant plot (not shown). In other words, the relative positions in this plot of the strains used were reproducible for separately isolated cell walls. The walls of *mnn*9, *kre*1 and wild-type cells were as in the former experiment positioned in an area that is enclosed by chitin, mannan, and β-1,3-glucan, and both mutants, compared with wild-type walls, were again shifted from the polyhexoses towards the chitin standard, indicating that in this method the presence of chitin dominates the samples. Taken together, the results demonstrate that cell wall mutants can be easily distinguished from wild-type cells by ammonia chemical ionization.

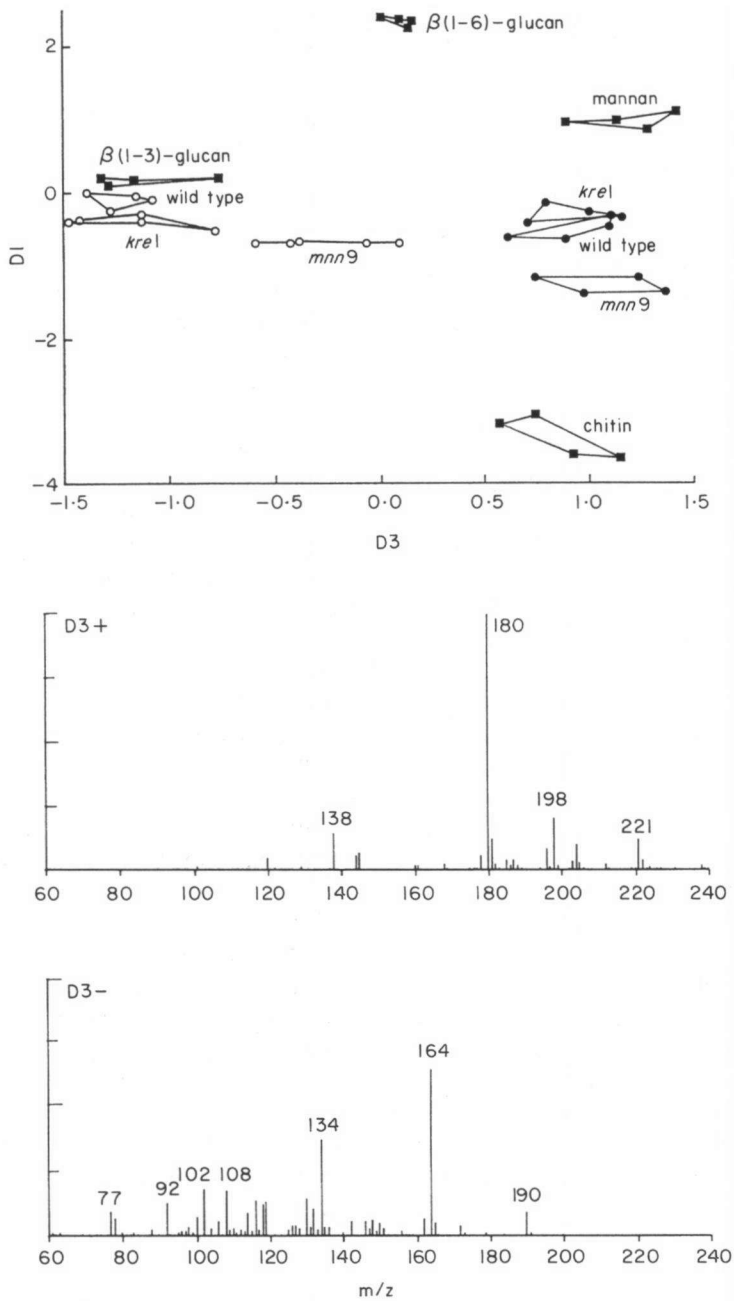

Fig. 6. Score plot on the first, D1, and the third, D3, discriminant functions of the pyrolysis (CI) mass spectra (top figure). The replicate analyses have been connected by solid lines. Solid circles, walls before extraction with alkali; open circles, walls after extraction with alkali. Below the score plot, the discriminant spectra are given; these spectra represent the set of ions characteristic for $D3^+$ and $D3^-$, respectively.

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