ELSEVIER

Carbohydrate Research 337 (2002) 1217–1227

CARBOHYDRATE RESEARCH

www.elsevier.com/locate/carres

Application of mass spectrometry to determine the activity and specificity of pectin lyase A

Kudzai E. Mutenda,[a] Roman Körner,[b] Tove M. I. E. Christensen,[c] Jørn Mikkelsen,[c] Peter Roepstorff[a,*]

[a]*Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230, Odense M, Denmark*
[b]*Department of Cell Biology, Max-Plank Institute for Biochemistry, Am Klopferspitz 18a, D-82152 Martinsried, Germany*
[c]*Danisco Biotechnology, Langebrogade 1, DK-1001 Copenhagen, Denmark*

Received 13 November 2001; received in revised form 26 April 2002; accepted 20 May 2002

Abstract

Electrospray ionization (ESI) with quadrupole ion-trap mass spectrometry was used to assess the activity and specificity of the enzyme pectin lyase A (PLA) (EC 4.2.2.10) on model pectins with varying degrees and patterns of methyl esterification. PLA is a pectinase which cleaves α-(1→4)-glycosidic linkages in pectin by a trans-elimination process. Using pectins with different degrees and patterns of methyl esterification, there was a significant variation in the activity rate of PLA. The enzymatic products generated at various time intervals were structurally analyzed by mass spectrometry to determine the specificity of PLA. Although the preferred substrate for PLA is fully methyl esterified polygalacturonate, cleavage was also observed with a non-methyl esterified galacturonic acid residue on either the non-reducing end or the reducing end. The current study shows that although PLA prefers fully methyl esterified substrates it can also accept partially esterified ones. It also demonstrates the suitability of ESI ion-trap mass spectrometry in determining enzyme specificities.

Keywords: Electrospray ionization; Quadrupole ion-trap mass spectrometry; Collision-induced dissociation; Pectin; Pectin lyase; Methyl esterification

1. Introduction

The use of mass spectrometry (MS) in studying the activity and specificity of glycolytic enzymes is fast gaining momentum.[1–4] The technique provides valuable structural information of the enzyme catalysis products, making it possible to determine the enzyme specificity. The technique of electrospray ionization (ESI) with ion-trap mass spectrometry has recently been used for the structural analysis of carbohydrates.[5–11] Ion-trap mass spectrometers allow multiple consecutive isolation and fragmentation steps of selected ions, for up to ten steps making it possible to do comprehensive structural analysis.

Structural analysis of polysaccharides by mass spectrometry has several challenges. The major challenge is encountered in their high degree of complexity as well as heterogeneity. The combined use of mass spectrometric analysis and enzyme digestion has made a considerable contribution to the structural characterization of polysaccharides in general and pectins in particular.[1–4,12–14] Pectins are anionic polysaccharides consisting of α-(1→4)-linked galacturonic acid homopolymer known as the smooth region and L-rhamnose, D-galacturonic acid repeating units with neutral sugar side chains (hairy region).[15] The galacturonic acid residues can either be methyl esterified, acetylated or both. In nature, pectins play mainly a structural role in plant cell walls. Industrially, pectins find use in food applications due their ability to act as gels, stabilizers, binders and thickeners.

For most of the industrial applications of pectins, the degree and pattern of methyl esterification is important. Esterification of pectins is therefore subject to ongoing research. The elucidation of methyl ester distribution in pectins has benefited substantially from the use of

* Corresponding author
E-mail address: roe@bmb.sdu.dk (P. Roepstorff).

0008-6215/02/$ - see front matter
PII: S0008-6215(02)00127-1

enzymes. Recent studies of pectin enzymatic digests by MALDI-TOF and ESI MS[1–3] have proved useful, not only in determining the degree of polymerization (DP) and degree of methyl esterification (DE) of the pectin fragments, but also in determining the precise location of the esterified residues.

Enzymes that modify pectins are important in plant development, plant pathology and in various kinds of industry.[16] Pectin lyase A (PLA) (EC 4.2.2.10) is one of the important pectinases that modify pectins. PLA is known to cleave the α-(1→4)-glycosidic linkage in galacturonosyl residues that are highly methylated, and the cleavage site is believed to be always methylated.[17] The cleavage is through a trans-elimination process, giving products with unsaturated $C_4 = C_5$ at the non-reducing end.

The isolation and characterization of pectolytic enzymes from plants, fungi and bacteria is well documented.[15,18–20] However, there has been very little detail in the determination of the specificity of these pectolytic enzymes. The aim of this study was to implement mass spectrometry in determining the specificity and activity of PLA. Time–course digests of model pectins, with varying degrees and distribution of methyl esterification, with PLA from *Aspergillus niger* were analyzed by ESI MS in a quadrupole ion-trap mass spectrometer.

Table 1
Activity of PLA with model pectin samples of varying degrees and patterns of methyl ester distribution

Pectin sample	DE (%)[a]	Activity (μmol min^{-1})
[b]URS	81	34
[c]P76	76	35
P70	70	31
P66	66	29
P60	60	27
P41	41	25
[d]F76	76	29
F69	69	22
F58	58	17
F43	43	4
F31	31	0
F11	11	0
FA09	9	0
[e]B71	71	20
B64	64	16
B43	43	0
B34	34	0

[a] DE, degree of methyl esterification.[4]
[b] URS, UltraRapid Set pectin.
[c] P pectins, de-esterified by plant pectin methyl esterase.
[d] F pectins, de-esterified by fungal pectin methyl esterase.
[e] B pectins, de-esterified by base hydrolysis.

The analysis was carried out in both the negative and positive-ion modes. Differentiation of fragment ions possessing the reducing end from those possessing the non-reducing end was made possible by labeling with ^{18}O.[3,6,8,21] A combination of the results obtained by MS and tandem MS, together with ^{18}O labeling, is assessed with focus on the requirements of PLA for methyl esterified substrates.

2. Results and discussion

PLA activity.—The pectin samples used in this study are listed in Table 1. As shown in Table 1, the pectin P76 had the highest activity and the lowest activity was obtained with F43. The P pectins are obtained by de-esterifying with plant pectin methylesterase which gives blockwise demethylation. The F and the B pectins are de-esterified by fungal methylesterase and base hydrolysis respectively, which gives random de-methylation.[4] The pectins B43, B34, F31, F11 and FA09 all gave zero activity. This may mean that the enzyme does not accept these pectins as substrates because the degree of methylation is too low and the distribution of methyl groups random. It may also be that they are accepted as poor substrates, but given the time in which the assay was done activity would not have been observed.

From Table 1 it may appear as if the pectin P76 (DE 76%) is a better substrate for PLA than URS (DE 81%) although it has a slightly lower degree of methylation. Theoretically, URS should give the highest activity under these conditions. A difference of 1 unit in the activity however, may not be significant and may be attributed to a slightly different distribution of methyl ester groups or smooth and hairy regions in the two samples.

Analysis of PLA cleavage products.—Figs. 1 and 2 show the ESI MS spectra obtained in negative and positive-ion modes after 15 min, 1 h and 6 h digestion of the pectin P60 with PLA. In the negative-ion mode the pectin digests are detected in their de-protonated form and, under the conditions used, galacturonic acid (GalA) oligomers of up to 12mers were observed. More partially methyl esterified fragments were observable in the higher mass to charge (m/z) region. Comparison of the spectra in Figs. 1 and 2 shows that fully methyl esterified pectin fragments are not detected if analysis is performed in the negative-ion mode, whereas ions from these pectin fragments are abundant in the positive-ion mode as ammonium, sodium and/or potassium adduct ions. The pectin fragments do not have functional groups that readily protonate and therefore can only be detected as their cationized species in the positive-ion mode. The reason for the absence of ions corresponding to the fully methyl esterified oligomers in negative

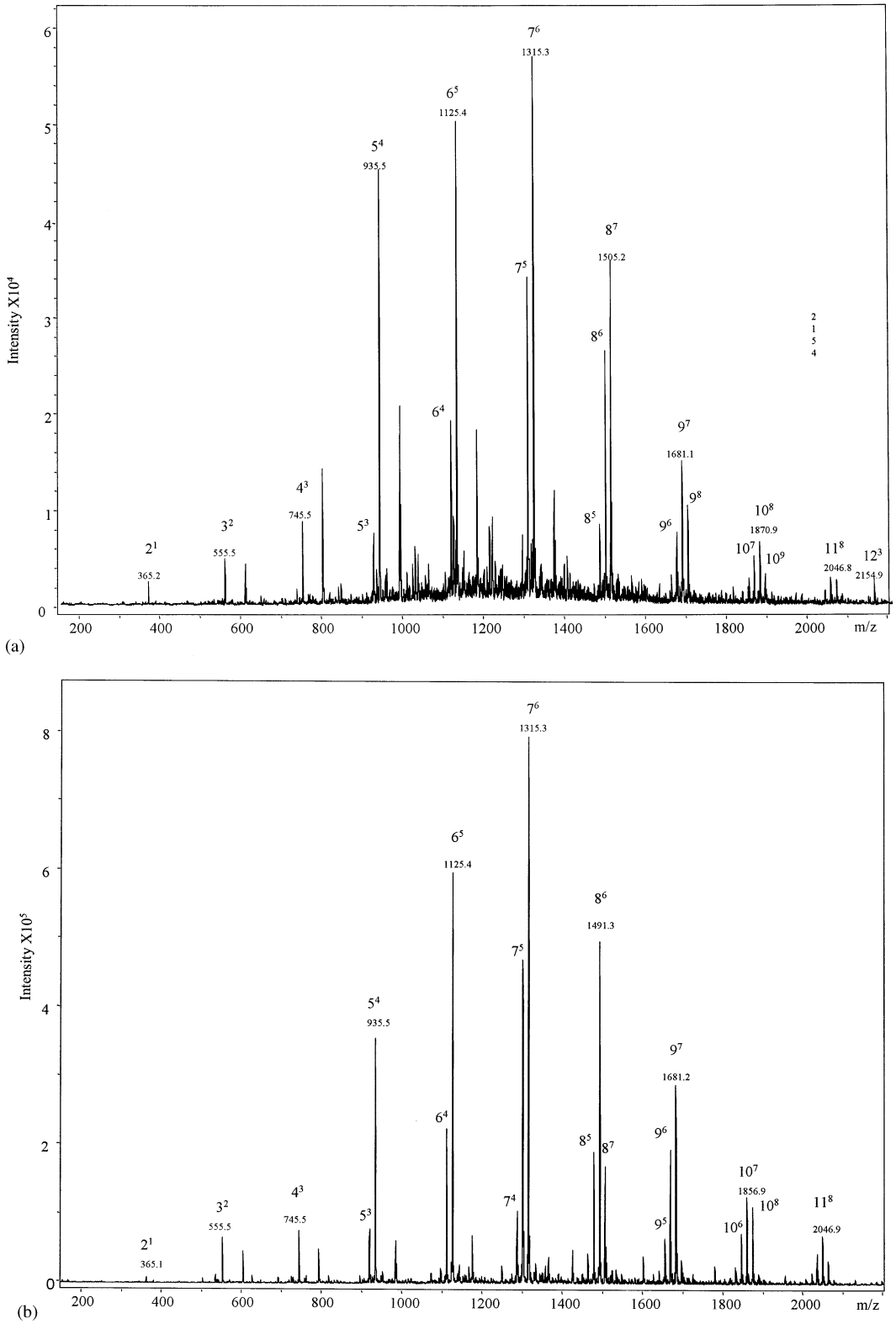

Fig. 1. Negative-ion mode ESI MS spectrum of a P60 digest. The pectin P60 was digested with 0.2 U of PLA for (a) 15 min (b) 1 h and (c) 6 h. The numbers above the m/z peak values represent the size (DP) of the generated fragment ion and the number of methyl ester groups (superscript) on the oligomer.

mode is that they do not ionize as efficiently as oligomers with at least one free carboxylic acid. Thus, after 15 min of digestion, the most abundant peaks in negative-ion mode (Fig. 1a) were the ones corresponding to pectin fragments containing one non-methyl esterified GalA residue, whereas the fully methyl es-

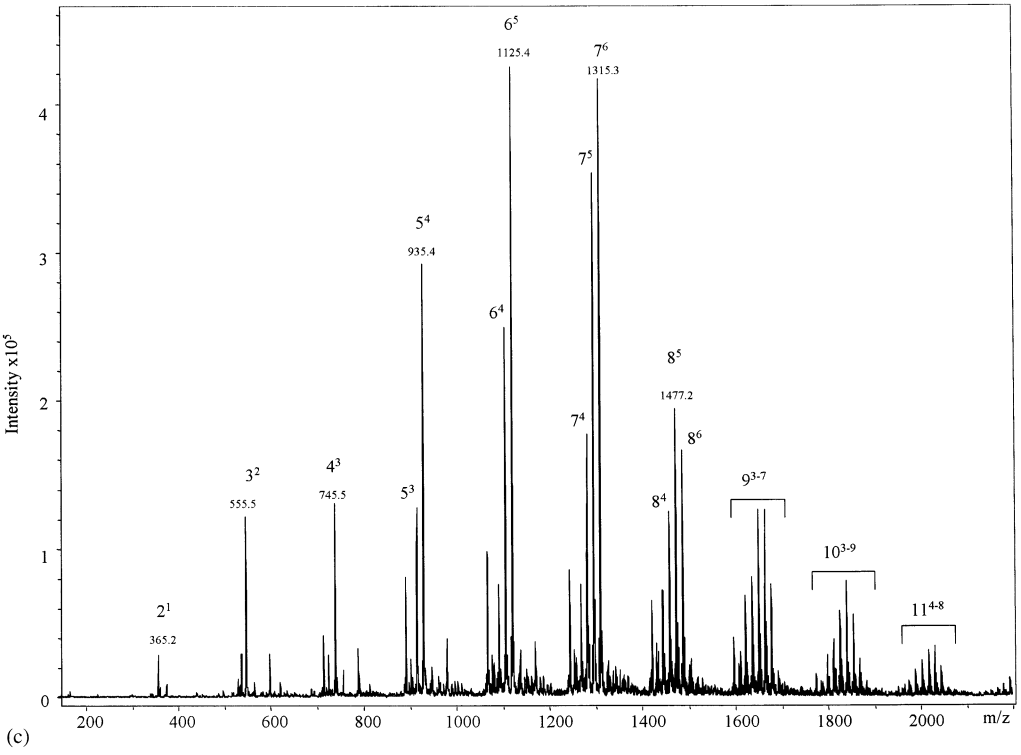

Fig. 1. (*Continued*)

terified fragment ions are abundant in the positive-ion mode (Fig. 2a). However, for pectin fragments of degree of polymerization (DP) of five and higher, the most abundant peaks do not correspond to the fully methylated oligomers. After 6 h of digestion, pectin fragments with lower degrees of methyl esterification (and DP 8 or higher) start to appear (Fig. 1).

The fragmentation pattern in Fig. 3a (MS^2) shows the generation of C and/or Z ions. The C ions retain the glycosidic oxygen atom while the Z ions do not. Since the parent ion is unsaturated, the C and Z ions generated are isobaric. Therefore, it is not possible to deduce from the fragmentation pattern if the ions produced are either C or Z. The fragmentation pattern shows a sequential loss of methyl esterified GalA residues (m/z, 190) from the parent ion. Further isolation and fragmentation (MS^3) of the tetramer fragment ion with three methyl ester groups and the trimer fragment ion with two methyl ester groups (MS^4) gives the series of $Z_3 + 2CH_3$ ($C_3 + 2CH_3$) and $Z_2 + CH_3$ ($C_2 + CH_3$) ions in Figs. 3b and 3c, respectively, that show that the non-esterified GalA residue may either be on the reducing or non-reducing end. The combined fragmentation pattern in the tandem MS is shown in the insert in Fig. 3a.

The positive-ion mode MS^2 spectrum after isolation of the pentamer ion with four methyl groups shows the same pattern (Fig. 4). The loss of one residue from the pentamer ion with four methyl groups to give a tetramer ion with four methyl corresponds to a loss of 176 m/z. This may either be the equivalent of a C_1 or Z_1 ion. Generation of $Z_4 + 4CH_3$ ($C_4 + 4CH_3$) ions indicates that either the reducing or the non-reducing end has a non-esterified GalA residue. Also the sequential generation of $Z_3 + 3CH_3$ ($C_3 + 3CH_3$) and $Z_2 + 2CH_3$ ($C_2 + 2CH_3$) ions means that the non-esterified GalA residue is located at either the reducing or non-reducing end. A rather low abundant $Y_3 + 3CH_3$ ions indicate that the non-methylated GalA residue is at the non-reducing end.

Thus, tandem MS (Fig. 3) in the negative-ion mode shows that the non-esterified GalA residue is located at either the reducing end or the non-reducing end. A low abundance of $Y_3 + 3CH_3$ ions observed in the positive-ion mode (Fig. 4) indicates that it must be at the non-reducing end. Thus, the combination of MS/MS spectra in the negative and positive-ion modes in this case allowed assignment of the non-esterified residue.

By labeling the products with ^{18}O it was possible to distinguish at which end the methyl esterified or the non-esterified residue was located (Figs. 5 and 6). The fragment ion with the reducing end residue has a 2 Da mass increase compared to the fragment ion with the non-reducing end due to the incorporation of ^{18}O. In Fig. 5a the labeled pentamer ion with four methyl ester groups was isolated and subsequent fragmentation (MS^2) in the negative-ion mode showed that the generated ions were C ions. This implies that the non-es-

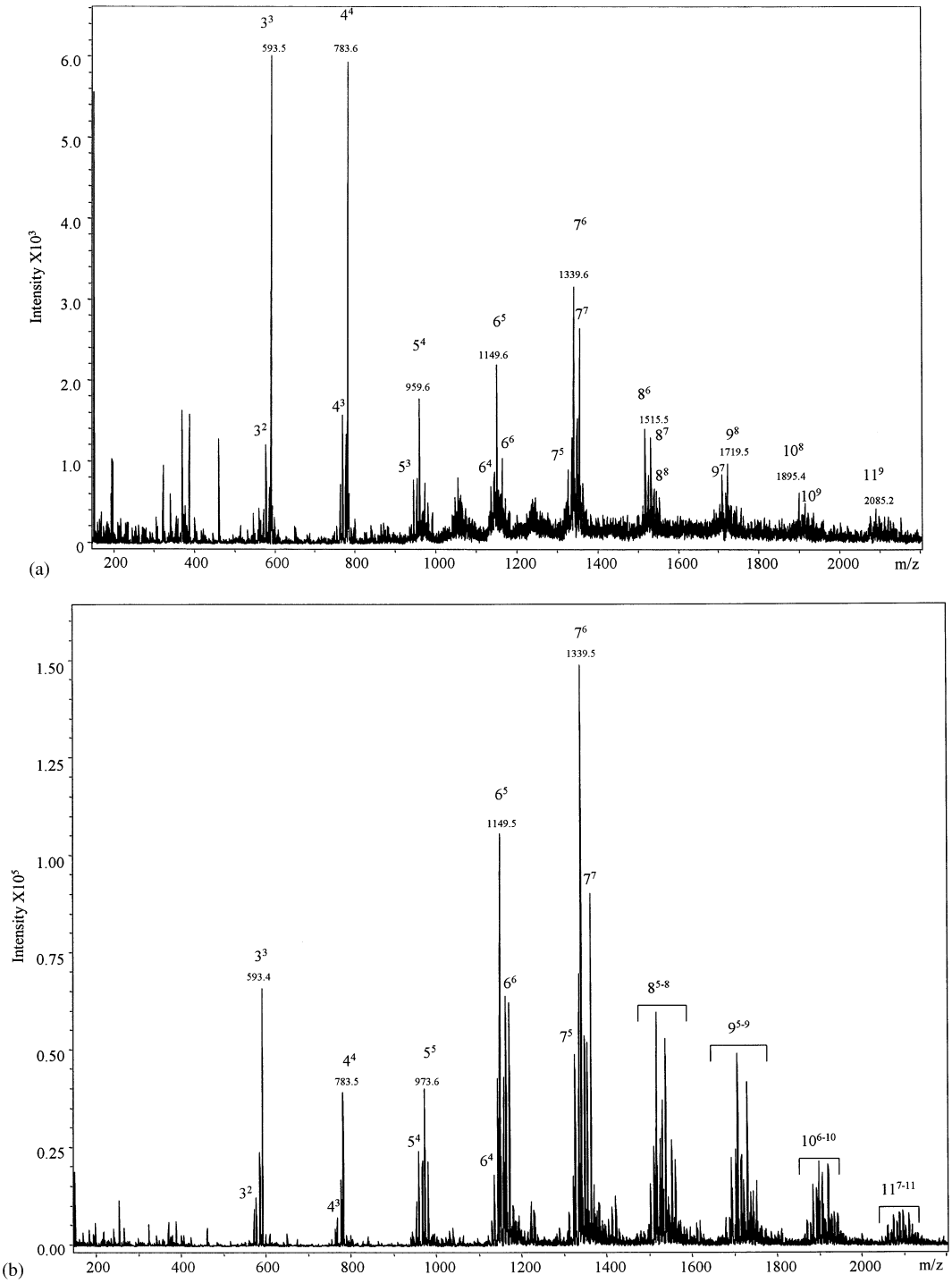

Fig. 2. Positive-ion mode ESI MS spectrum of a P60 digest. The pectin P60 was digested with 0.2 U of PLA for (a) 15 min (b) 1 h and (c) 6 h. The numbers above the m/z peak values represent the size (DP) of the generated fragment ion and the number of methyl ester groups (superscript) on the oligomer.

terified GalA residue is located at the non-reducing end. In Fig. 5b, a trimer ion with two methyl ester groups fragments to $Z_2 + CH_3$ ions and Z_1 and to $C_2 + CH_3$ ions and C_1. The generation of Z_1 ions implies a non-esterified GalA residue at the reducing end in the parent ion. The presence of C_1 ions implies a non-esterified GalA residue at the non-reducing end. Since the parent ion is a trimer with two methyl groups it must represent a mixture of two components, some with a non-esterified GalA residue at the reducing end and others with a non-esterified GalA residue at the non-reducing end, but not in both positions in one molecule. In Fig. 6, the labeled tetramer ion with three methyl ester groups was fragmented in the positive-ion mode

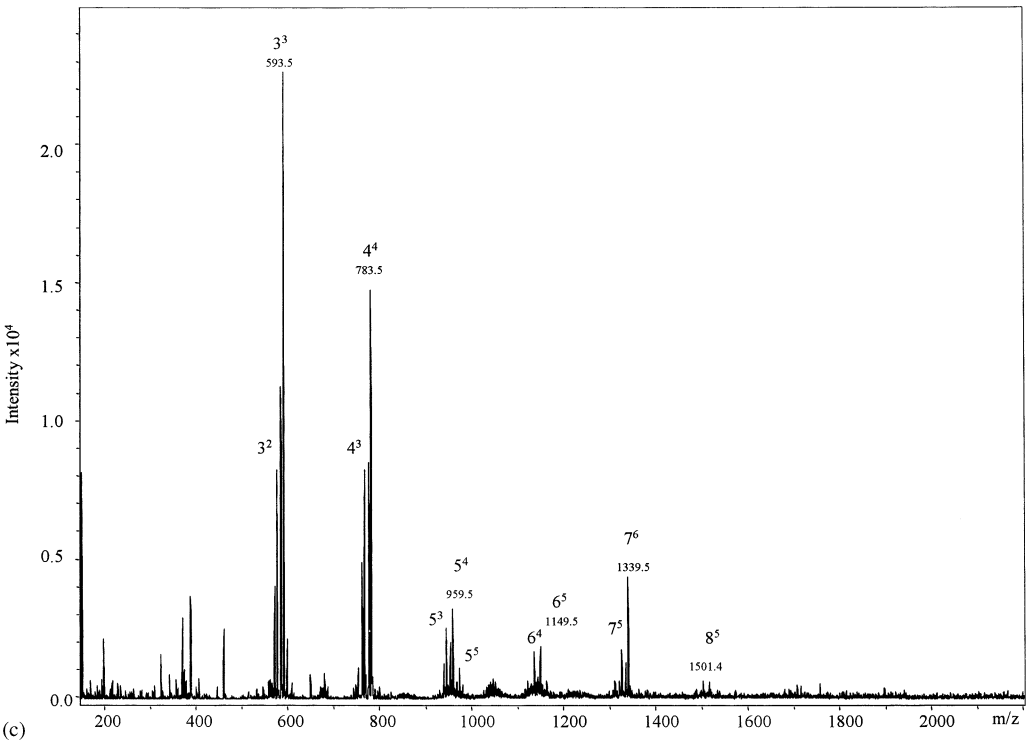

Fig. 2. (*Continued*)

and the generation of $Y_3 + 2CH_3$ and $B_3 + 3CH_3$ ions proves the presence of a non-esterified GalA residue at the reducing end. The detection of $Y_2 + 2CH_3$ and $B_3 + 2CH_3$ also proves the presence of a non-esterified GalA residue at the non-reducing end in the parent ion. The combined MS and MS/MS data show that the pectin digests contained products with a non-methyl esterified GalA residue at either the reducing end or the non-reducing end.

The detection of a non-esterified GalA residue at the non-reducing end of oligomers in the enzyme digests was unexpected and indicates a non stringent requirement of PLA for fully methyl esterified GalA residues at both the reducing and the non-reducing end of the cleavage site.

Comparison of analysis in the positive and negative-ion modes show that more information is obtained in the positive-ion mode. MS analysis in the positive-ion mode detects all the pectin fragment ions, i.e., partially and fully esterified ones, whereas in the negative-ion mode only the partially esterified ones are detected. In MS/MS analysis, fragmentation in the positive-ion mode generally generates B, C, Y and Z ions. Based on the B and Y ions produced, it is possible to distinguish ions containing the reducing and non-reducing end without the need for labeling. Fragmentation of other pectin fragments, especially when lowly methylated, requires labeling to distinguish between C and Z ions. Fragmentation in the negative-ion mode generates mainly C and Z ions and some $^{0,2}A$ ions which also do not allow distinction between the reducing and the non-reducing end. Thus, labeling is always required to make distinction possible. In general, the spectra obtained in the negative-ion mode exhibit considerably better signal/noise ratio than those obtained in the positive-ion mode. Interpretation of the spectra generated in the positive-ion mode is more complex and the sensitivity less (about one order of magnitude) than in the negative-ion mode.

3. Conclusion

We have been able to demonstrate the specificity of PLA using ESI ion-trap mass spectrometry for analyzing the enzyme catalysis products. The technique is suitable for the determination of enzyme specificities. The enzymatic digests do not require extensive purification or isolation of the products formed, just a desalting step. This makes the method ideal for analyzing complex mixtures of enzymatic digests in one step. Although the preference of PLA is for fully methyl esterified residues, it also accepts partially esterified substrates. The fact that not all species of the pectin lyase digests could be observed by analyzing them either in the negative- or positive-ion mode alone means that a combination of both modes is more informative that just one of them. From this study, ESI ion-trap mass spectrometry has proved to be an excellent tool in the determination of the specificity of PLA with model pectin molecules.

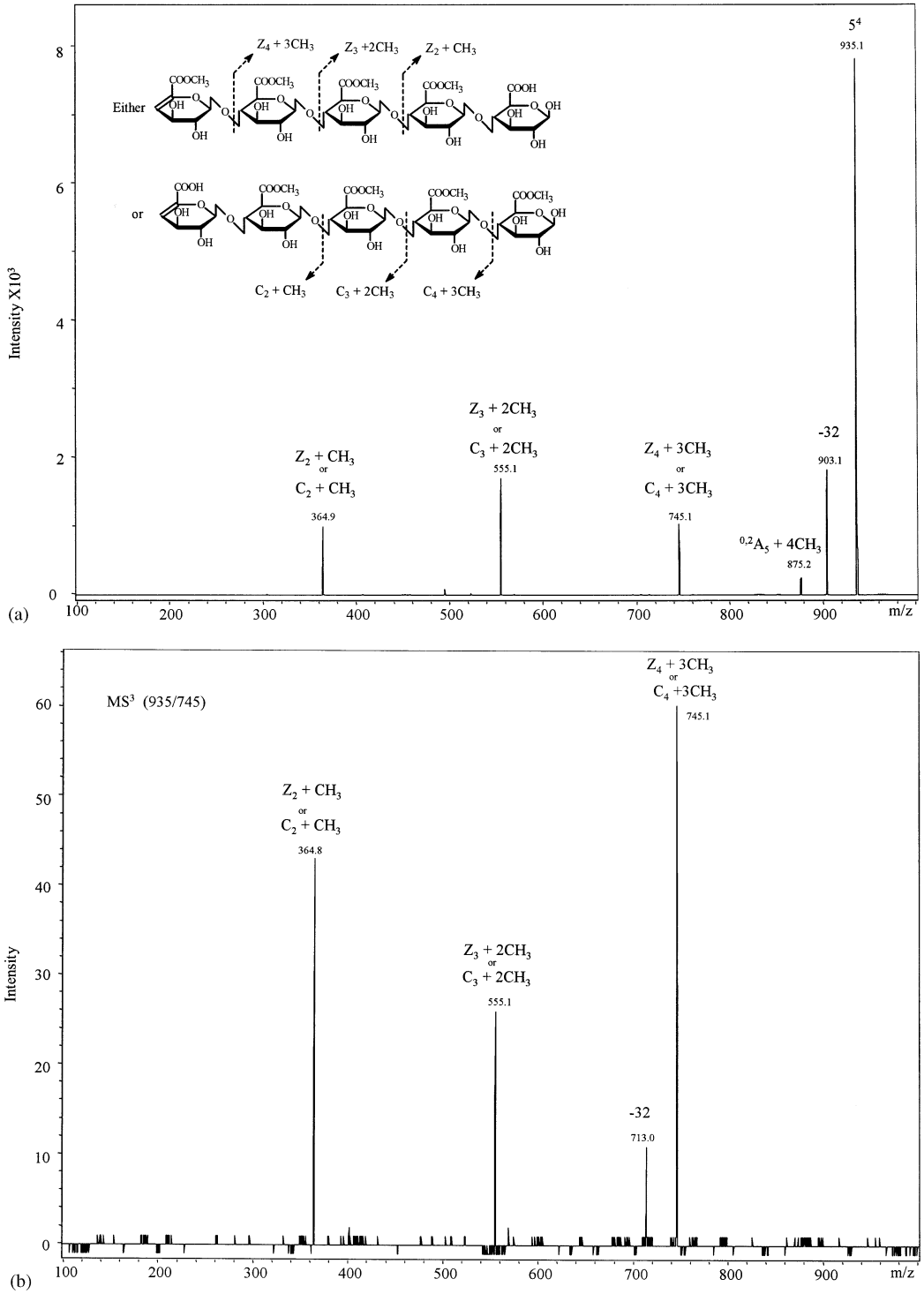

Fig. 3. Negative-ion mode ESI (a) MS^2 spectrum of an ion corresponding to a pentamer pectin fragment with four methyl ester groups, m/z 935.1 (b) MS^3 spectrum of the tetramer fragment ion with three methyl ester groups, m/z, 745.1 and (c) MS^4 spectrum of the trimer fragment ion with two methyl ester groups, m/z, 555.2. The generated ions are named according to Domon and Costello.[22] A, B and C ions retain the non-reducing end while X, Y and Z ions retain the reducing end.

4. Experimental

PLA activity.—To assess the activity of PLA, pectin samples of varying degrees and patterns of methylation were used. The preparation of the model pectins is described in Limberg et al.[4] Activity was followed in 1 mL of 5 mg/mL pectin sample in 50 mM NaOAc buffer pH 5.0 at 40 °C. The reaction was initiated by addition

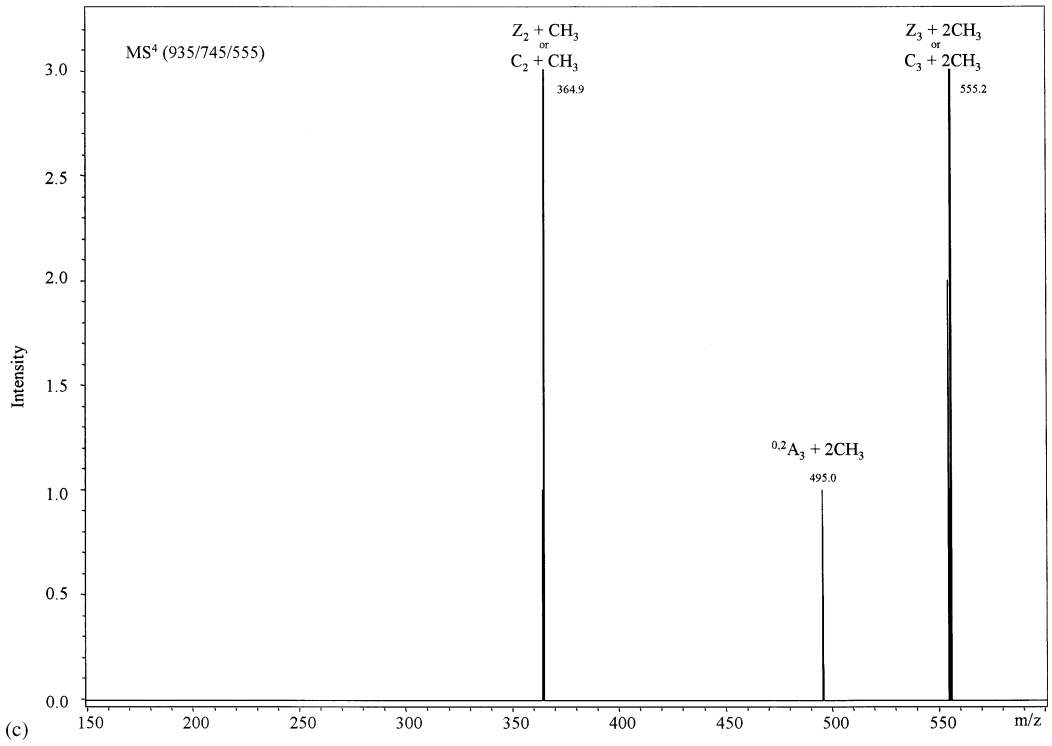

Fig. 3. (*Continued*)

of enzyme. Initial rates of reaction were determined spectrophotometrically by measuring the rate of formation of C=C by its absorbance at 235 nm in a Shimadzu UV-1601 spectrometer over a period of 4 min. The rates were calculated using 5500 M^{-1} cm^{-1} as the molar absorption coefficient of C=C at 235 nm.

PLA specificity.—For specificity determinations, the pectin samples were incubated with PLA for time inter-

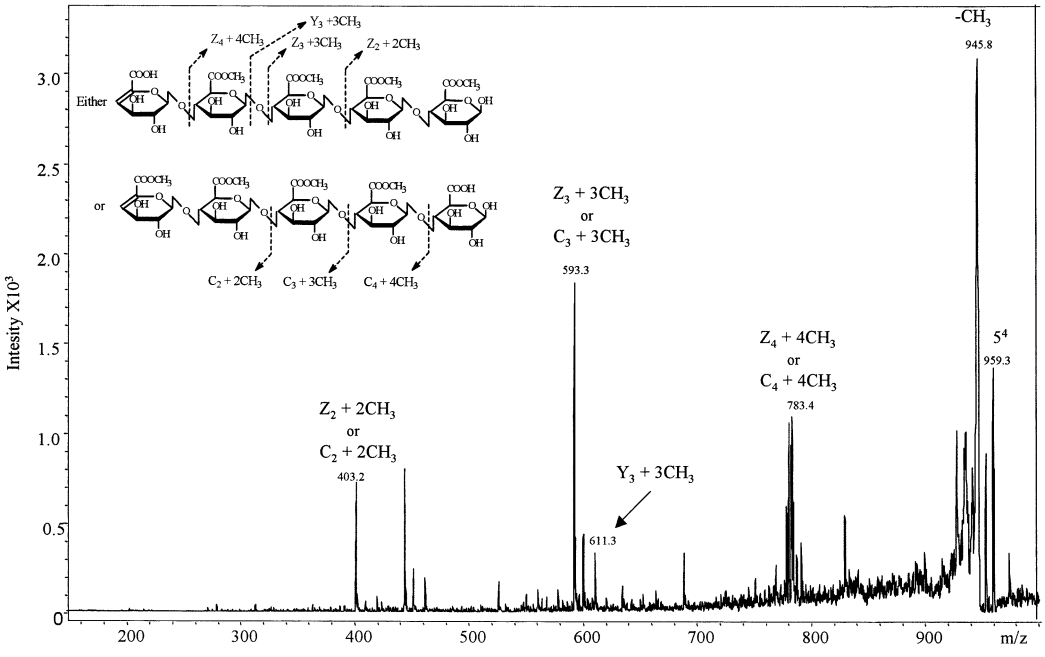

Fig. 4. Positive-ion mode ESI MS^2 spectrum of an ion corresponding to a pentamer pectin fragment with four methyl ester groups, m/z 959.3.

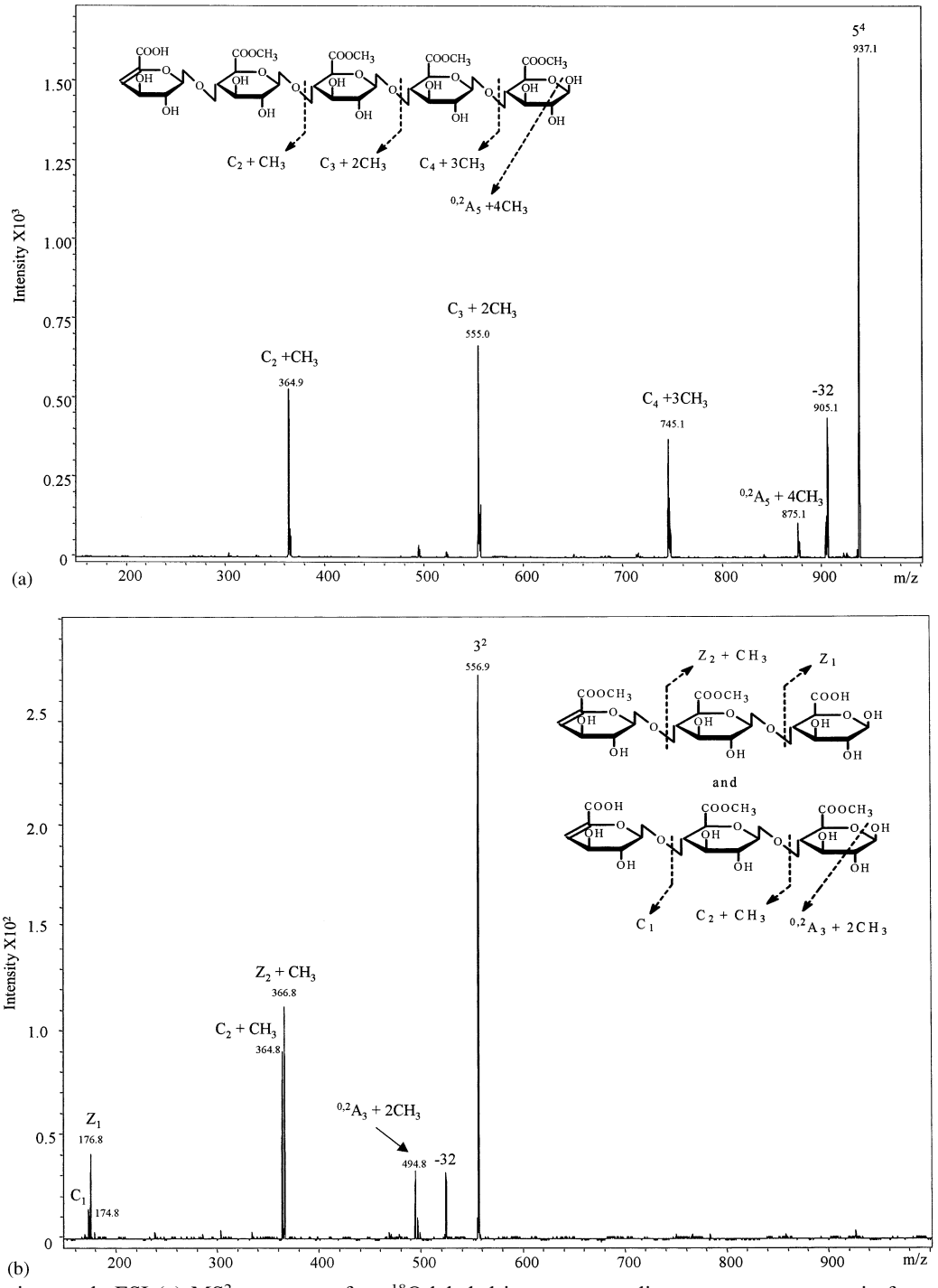

Fig. 5. Negative-ion mode ESI (a) MS^2 spectrum of an ^{18}O-labeled ion corresponding to a pentamer pectin fragment with four methyl ester groups, m/z 937.1 and (b) MS^2 spectrum of an ^{18}O-labeled ion corresponding to a trimer pectin fragment with two methyl ester groups, m/z, 557.

vals ranging from 0 to 48 h. Samples taken out at different times were incubated for 5 min in a boiling waterbath to stop the enzyme reaction. The digests were desalted over cation-exchange resin, Dowex 50W X8, miniature columns before analyzing the products by ESI MS, as described in Körner et al.[1]

Mass spectrometry.—ESI MS spectra were acquired on an Esquire-LC quadrupole ion-trap mass spectrometer (Bruker Daltonik, Bremen, Germany). Analysis was done both in the positive and negative-ion mode at a fundamental radio frequency of 781 kHz. A standard scan range of 50 to 2200 m/z was used at a scan speed of 13000 m/z s^{-1}.

To determine the location of the methyl groups in the

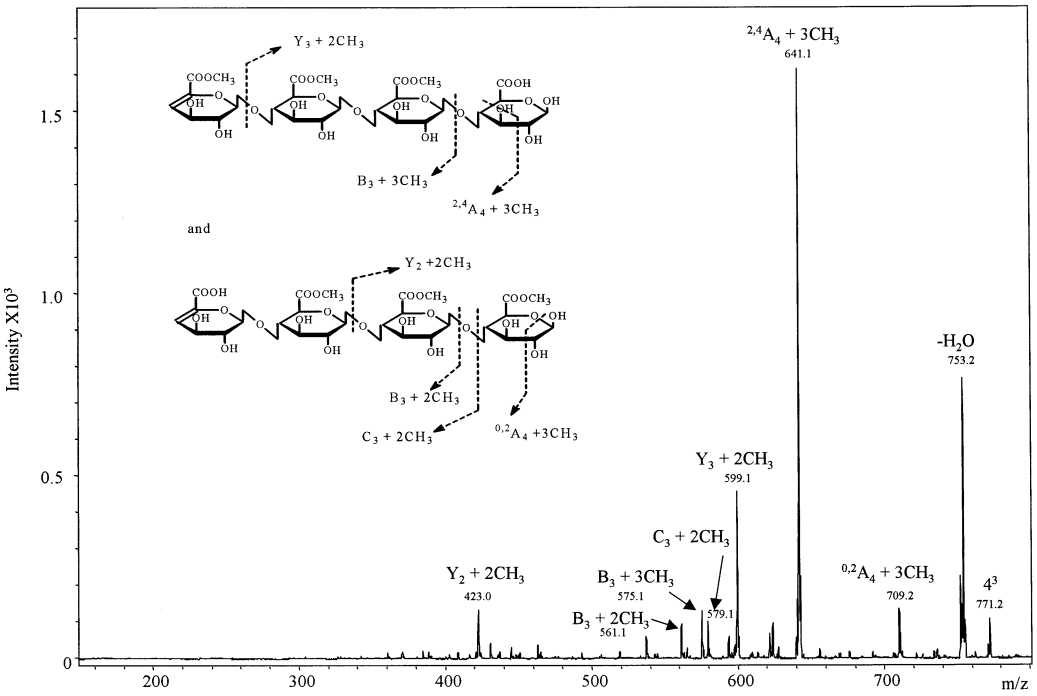

Fig. 6. Positive-ion mode ESI MS^2 spectrum of an ^{18}O-labeled ion corresponding to a tetramer pectin fragment with three methyl ester groups, m/z 772.

digests, ions corresponding to specific oligomer products were isolated and systematically fragmented by collision-induced dissociation. To distinguish oligomer products possessing the reducing end from those possessing the non-reducing end, digests were labeled with ^{18}O as previously described,[3] after which tandem mass spectrometry was carried out. With this technique, the fragment ion with the reducing end residue is 2 Da higher than that with the non-reducing end residue.

Acknowledgements

We wish to thank Dr Jacques Benen, Wageningen Agricultural University, The Netherlands, for providing the PLA used in this project. The project is supported by the Danish Research Council Center Contract Programme (9800940). The work is part of the activities of the Danish Biotechnology Instrument Center.

References

1. Körner R.; Limberg G.; Mikkelsen J. D.; Roepstorff P. *J. Mass Spectrom.* **1998**, *33*, 836–842.
2. Daas P. J. H.; Arisz P. W.; Schols H. A.; De Ruiter G. A.; Voragen A. G. J. *Anal. Biochem.* **1998**, *257*, 195–202.
3. Körner R.; Limberg G.; Christensen T. M. I. E.; Mikkelsen J. D.; Roepstorff P. *Anal. Chem.* **1999**, *71*, 1421–1427.
4. Limberg G.; Körner R.; Buchholt H. C.; Christensen T. M. I. E.; Roepstorff P.; Mikkelsen J. D. *Carbohydr. Res.* **2000**, *327*, 321–332.
5. Reinhold V. N.; Sheeley D. M. *Anal. Biochem.* **1998**, *259*, 28–33.
6. Viseux N.; de Hoffmann E.; Domon B. *Anal. Chem.* **1997**, *69*, 3193–3198.
7. Weiskopf A. S.; Vouros P.; Harvey D. J. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1493–1504.
8. Asam M. R.; Glish G. L. *J. Am. Soc. Mass Spectrom.* **1997**, *8*, 987–995.
9. Bahr U.; Pfenninger A.; Karas M. *Anal. Chem.* **1997**, *69*, 4530–4535.
10. Gaucher S. P.; Leary J. A. *Anal. Chem.* **1998**, *70*, 3009–3014.
11. Xie M.; Giraud D.; Bertheau Y.; Casetta B.; Arpino P. *Rapid Commun. Mass Spectrom.* **1995**, 1572–1575.
12. Schols H. A.; Voragen A. G. J. In *Progress in Biotechnology*, *Pectins and Pectinases*; Visser J.; Voragen A. G. J., Eds.; Elsevier: Amsterdam, 1996; Vol. 14, pp 3–19.
13. Daas P. J. H.; Meyer-Hansen K.; Schols H. A.; De Ruiter G. A.; Voragen A. G. J. *Carbohydr. Res.* **1999**, *318*, 135–145.
14. Daas P. J. H.; Voragen A. G. J.; Schols H. A. *Carbohydr. Res.* **2000**, *326*, 120–129.
15. Voragen A. G. J.; Pilnik W.; Thibault J. F.; Axelos M. A. V.; Renard C. M. G. C. In *Food Polysaccharides and their Application*; Stephen A. M., Ed.; Marcel Dekker: New York, 1995; p 287.
16. Vincken J. P.; van den Broek L. A. M.; van der Lei D. D.; Beldman G.; Voragen A. G. J. *J. Sci. Food Agric.* **1997**, *73*, 407–416.
17. Pilnik W.; Voragen A. G. J. In *Food Enzymology*; Fox P. F., Ed.; Elsevier Applied Science: London, 1991; Vol. 1, pp 303–336.

18. Hayashi K.; Inoue Y.; Shiga M.; Sato S.; Takano R.; Hirayae K.; Hara S. *Phytochemistry* **1997**, *45*, 1359–1362.
19. Mayans O.; Scott M.; Connerton I.; Gravensen T.; Benen J.; Visser J.; Pickersgill R.; Jenkins J. *Structure* **1997**, *5*, 677–689.
20. Vitali J.; Schick B.; Kester H. M. C.; Visser J.; Jurnak F. *Plant Physiol*. **1998**, *116*, 69–80.
21. Hofmeister G. E.; Zhou Z.; Leary J. A. *J. Am. Chem. Soc*. **1991**, *113*, 5964–5970.
22. Domon B.; Costello C. E. *Glycoconjugate J*. **1988**, *5*, 397–409.