Tandem mass spectrometric analysis of *Aspergillus niger* pectin methyl-esterase: mode of action on fully methyl-esterified oligogalacturonates

Harry C. M. KESTER^{*1}, Jacques A. E. BENEN^{*}, Jaap VISSER^{*2}, Maria Esteban WARREN^{†1}, Ron ORLANDO[†], Carl BERGMANN[†], Didier MAGAUD[‡], Daniel ANKER[‡] and Alain DOUTHEAU[‡]

^{*}Section Molecular Genetics of Industrial Microorganisms, Wageningen Agricultural University, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands, [†]Complex Carbohydrate Research Centre and the Departments of Chemistry and Biochemistry and Molecular Biology, University of Georgia, 220 Riverbend Road, Athens, GA 30602-4712, U.S.A., and [‡]Laboratoire de Chimie Organique, UMR-CNRS 5622, INSA, Bâtiment 403, 20 Avenue Albert Einstein, 69621 Villeurbanne Cedex, France

The substrate specificity and the mode of action of *Aspergillus niger* pectin methyl-esterase (PME) was determined using both fully methyl-esterified oligogalacturonates with degrees of polymerization (DP) 2–6 and chemically synthesized monomethyl trigalacturonates. The enzymic activity on the different substrates and a preliminary characterization of the reaction products were performed by using high-performance anion-exchange chromatography at neutral pH. Electrospray ionization tandem MS (ESI–MS/MS) was used to localize the methyl esters on the ¹⁸O-labelled reaction products during the course of the enzymic

reaction. *A. niger* PME is able to hydrolyse the methyl esters of fully methyl-esterified oligogalacturonates with DP 2, and preferentially hydrolyses the methyl esters located on the internal galacturonate residues, followed by hydrolysis of the methyl esters towards the reducing end. This PME is unable to hydrolyse the methyl ester of the galacturonate moiety at the non-reducing end.

Key words: galacturonates, mass spectrometry (MS), pectin methyl-esterases.

INTRODUCTION

Pectin, a major constituent of the cell walls of higher plants, is a complex heteropolysaccharide composed of segments of ‘smooth’ homogalacturonan regions and ‘hairy’ rhamnogalacturonan regions. During plant ripening and other developmental processes, as well as during attack by plant pathogens, pectin is degraded by an array of enzymes that can either depolymerize the pectin backbone (polygalacturonases, pectin and pectate lyases) or alter the structure of the intact backbone [pectin acetyl-esterases and pectin methyl-esterase (PME)]. PMEs catalyse the hydrolysis of galacturonate (GalA) methyl esters in the backbone, producing pectate, which is vulnerable to the depolymerizing polygalacturonases and pectate lyases [1]. PMEs, produced by numerous plants, bacteria and fungi [2,3], may differ with respect to their pH optima, isoelectric points and the requirement of calcium for activity [2]. Another notable difference is the mode of action by which methyl groups are hydrolysed. Fungal PMEs have been shown in some cases to hydrolyse the methyl esters of pectin in a random fashion, whereas in plant PMEs a ‘blockwise’ de-esterification of pectin has been reported [4,5].

The activity of the PMEs is thought to be restricted to the ‘smooth’ homogalacturonan regions of the pectin molecule, but absolute substrate specificity of fungal PME has not been firmly established. Recently, it was demonstrated that PME from *Aspergillus niger* hydrolysed only 50 % of the methyl esters of apple pectin [6]. Instead of using complex, poorly characterized pectin, well-defined oligogalacturonates may be more suitable substrates in examining the specificity of PME. This approach has been used to study the activities of tomato and fungal pectinases on partially methyl-esterified oligogalacturonates. Although the results revealed clear differences between the activities of the two enzymes [3], only preliminary conclusions regarding the substrate specificity could be drawn, since the

reaction products were not fully characterized. Determining the substrate specificity of PME would require activity measurements on a series of fully characterized oligogalacturonates, with subsequent analysis of the reaction products.

MS is becoming an important tool in the analysis of the substrate specificity of pectin-degrading enzymes. Information on substrate specificity of the pectolytic enzymes, polygalacturonase and pectin lyase, has been obtained using MS techniques [7–9]. Products resulting from the digestion of partially methyl-esterified pectin by endopolygalacturonase were identified by matrix-assisted laser-desorption ionization–MS (MALDI–MS) analysis, in conjunction with high-performance anion-exchange chromatography (HPAEC) [7]. Although this method effectively determined the number of methyl esters on the products, it cannot be used to distinguish between methyl ester isomers in a mixture. More recently, tandem MS using an ion-trap mass spectrometer was used to analyse the products of polygalacturonase II, exopolygalacturonase, and pectin lyase digestions of isotopically labelled, partially methyl-esterified pectin [9].

In this paper, the mode of action of *A. niger* PME is studied using fully methyl-esterified oligogalacturonates and chemically synthesized monomethyl-esterified trigalacturonates [10]. The mode of action of PME was established by determining its activity on the different substrates, and by electrospray ionization tandem MS (ESI–MS/MS) analysis of the reaction products formed during the course of the reaction.

EXPERIMENTAL

Preparation of fully methyl-esterified and monomethyl-esterified oligogalacturonates

Oligogalacturonates of defined length [degree of polymerization (DP) 2–6] were prepared as described previously [11]. The

Abbreviations used: DP, degree of polymerization; ESI–MS/MS, electrospray ionization tandem MS; GalA, galacturonate; HPAEC, high-performance anion-exchange chromatography; MALDI–MS, matrix-assisted laser-desorption ionization–MS; PME, pectin methyl-esterase.

¹ These authors contributed equally to this work.

² To whom correspondence should be addressed (e-mail office@algemeen.mgim.wau.nl).

oligomers (50 mg) were dissolved in 5 ml of methanol/0.02 M HCl. After 14 days at 4 °C, the esterification reaction was stopped by the addition of 80 mg (0.3 mmol) of Ag₂CO₃. The silver-chloride precipitate was removed by centrifugation, and methanol was removed by evaporation under a stream of nitrogen. The dried material was dissolved in water (1 ml), loaded on to a 4-ml AG 501-X8 mixed-bed resin column (Bio-Rad Laboratories, Richmond, CA, U.S.A.), eluted with water, and freeze-dried to remove the partially methyl-esterified oligomers from the reaction mixture. The homogeneity of the oligogalacturonates with respect to the DP and the degree of esterification was analysed by HPAEC at pH 12 [12] and pH 5 [10] respectively. The dried oligomer preparations were dissolved in water, and the concentration was determined by measuring the uronate content [13]. Monomethyl-esterified trigalacturonates were synthesized chemically, as described previously [10].

Preparation of PME

PME was purified from the culture fluid of an *A. niger pkiA-pme* transformant grown on minimal medium with fructose as the carbon source (J. A. E. Benen, unpublished work). Before its use, the enzyme preparation was checked for contaminating endopolygalacturonase activity. This was accomplished by mixing 0.05 ml with 0.5 ml of 1% (w/v) polygalacturonic acid in 50 mM sodium acetate buffer, pH 4.2, and incubating at 30 °C for 24 h. Potential endopolygalacturonase activity in the PME preparation was monitored by analysing the oligomeric reaction products using HPLC, as described previously [12].

Measurement of PME activity on fully methyl-esterified oligogalacturonates with DP 2–6

Fully methyl-esterified oligogalacturonates with DP 2–6 (2.5 mM) were incubated with PME at 25 °C in 50 mM sodium acetate buffer, pH 5.0, in a total reaction volume of 0.2 ml. At timed intervals, 35 µl of the reaction mixture was analysed by HPAEC on a Carbowax PA-100 column (250 mm × 4 mm; Dionex, Sunnyvale, CA, U.S.A.) at pH 5, as described previously [10]. The activity of PME on these substrates was calculated from the initial decrease in concentration of the fully methyl-esterified substrates in the reaction mixture with respect to time.

Measurement of PME activity on fully methyl-esterified and monomethyl-esterified trigalacturonates

Fully methyl-esterified trigalacturonate and the three monomethyl-esterified trigalacturonates were incubated with PME at 25 °C in 50 mM sodium acetate buffer, pH 5.0. The substrate concentration was 0.5 mM in a total reaction volume of 0.2 ml. At timed intervals, 20 µl of the reaction mixture was used to analyse the reaction products by HPAEC, as described in the previous section. For fully methyl-esterified trigalacturonate, the PME activity was calculated as also described in the previous section. The activity on the monomethyl-esterified trigalacturonates was calculated from the initial increase of trigalacturonate, using trigalacturonate as an external standard.

Enzyme incubations and sample preparation for MS analysis

Fully methyl-esterified oligogalacturonates (DP 3–6) in 50 mM sodium acetate buffer, pH 5.0, were incubated with PME for 0.5 and 24 h. The substrate concentration was 5 mM in a 0.2-ml total reaction volume. The reaction was stopped by removal of the enzyme from the reaction mixture using a 0.2-ml Dowex 50W-X2 (H⁺) column, from which the oligomers were eluted with 0.6 ml of water, and subsequently freeze-dried. Use of the

cation-exchange column not only allowed removal of the enzyme, but also converted the products from the sodiated species into the protonated form, which is more amenable to MS.

¹⁸O-Exchange of the oligogalacturonate anomeric oxygen

Sequencing carbohydrates by tandem MS requires the use of a label to differentiate between the reducing and non-reducing ends. Therefore, before MS analysis, the freeze-dried oligomeric reaction products were treated with H₂¹⁸O. This exchanges the anomeric oxygen with ¹⁸O, providing a label on the reducing end of the oligogalacturonate [9]. The exchange was accomplished by dissolving 50 µg of each sample in 50 µl of H₂¹⁸O (Isotec, Miamisburg, OH, U.S.A.). Formic acid [0.5% (v/v)], supplied by J. T. Baker, Phillipsburg, NJ, U.S.A., was added to catalyse the ring opening, and the reaction was allowed to proceed for 24 h at room temperature.

MS analysis

Mass spectra were obtained using a PE-Sciex (Norwalk, CT, U.S.A.) API-III triple quadrupole mass spectrometer equipped with an electrospray ion source. In a typical analysis, 0.5 µg of the ¹⁸O-labelled sample was introduced into the mass spectrometer by flow-injection through a 10-µl sample loop. The solvent, composed of methanol/water/acetic acid (49/49/2, by vol.), was delivered by an Applied Biosystems (Foster City, CA, U.S.A.) 140B solvent-delivery system at a flow rate of 5 µl/min. During MS experiments, the instrument scanned a range of 600 *m/z*, centred on the mass of the fully methyl-esterified trimer, tetramer, pentamer or hexamer at a rate of 12 scans/min for an average of 20 scans. The precursor ion was selected in tandem MS experiments by the first quadrupole (Q1); collision-induced dissociation (CID) was performed in the second quadrupole (Q2), and the fragments were analysed on the basis of their *m/z* ratios in the third quadrupole (Q3). Argon was used as the collision gas at a thickness of approx. 3.00 × 10¹⁴ molecules/cm². The third quadrupole scanned ranges of *m/z* of 100–600, 100–800, 100–1000 and 100–1300 for the trimer, tetramer, pentamer and hexamer respectively, at 12 scans/min.

RESULTS AND DISCUSSION

Activity of PME on fully methyl-esterified oligogalacturonates with DP 2–6

PME was active on the fully methyl-esterified oligogalacturonate trimer, tetramer, pentamer and hexamer, but was not active on the dimer. Additionally, the initial rate increased drastically with the DP of the substrate (Table 1), a phenomenon also observed for PME from *A. foetidus* [6]. Specifically, the activity with

Table 1 Initial reaction rates of *A. niger* PME on fully methyl-esterified oligogalacturonates with DP 2–6

The reaction conditions used were 2.5 mM oligomer in 50 mM sodium acetate buffer, pH 5.0, at 25 °C. Further details are given in the Experimental section.

DP	PME activity (units · mg ⁻¹)
2	0
3	0.1
4	1.5
5	5.4
6	10.7

Figure 1 Formation of products on incubation of fully methyl-esterified trigalacturonate with *A. niger* PME

Incubation conditions: 0.5 mM substrate in 50 mM sodium acetate buffer, pH 5.0, at 25 °C. Products were eluted in the order of trigalacturonate with three (peak 1), two (peak 2) and one (peak 3) methylester(s) respectively. (A) HPAEC analysis at pH 5.0. (B) Progression of product formation. For further experimental details, see the Experimental section.

the hexagalacturonate was 100-fold higher than that with the trigalacturonate (10.7 and 0.1 units·mg⁻¹ respectively). This indicates that the substrate-binding site of PME is composed of multiple subsites, which is a common feature of pectin-depolymerizing enzymes [12,14]. Oligomers with a higher DP interact with more subsites during substrate binding. This leads to a higher affinity and also, as a consequence, an increase in the reaction rate with increasing chain length.

Calculation of the initial rates was on the basis of a time-dependent decrease in concentration of the fully methyl-esterified substrates as analysed by HPAEC. Since HPAEC analysis is on the basis of charge differences and the products have the same DP, the separation is solely on the basis of the net charge of the oligomer, which is dependent on the degree of esterification. Under the conditions used for the separation, the fully methyl-esterified substrates were not retained by the column. In the same analysis, the product progression was monitored. HPAEC analysis revealed that a limited number of early-eluting, highly methyl-esterified products were formed. Prolonged incubation resulted in further de-esterification of the initially formed products. The products arising from fully methyl-esterified trigalacturonate

Table 2 Performance of *A. niger* PME on fully and monomethyl-esterified trigalacturonates

Incubation conditions used were 0.5 mM oligomer in 50 mM sodium acetate, pH 5.0, at 25 °C. The position of the methyl ester is numbered from the reducing end. G* signifies methyl-esterified oligogalacturonate. The reducing-end GalA is indicated in bold.

Substrate	Specific activity (m-units·mg ⁻¹)	Relative activity (%)
Fully methyl-esterified trigalacturonate (G*-G*- G *)	70	100
1-Methyl trigalacturonate (G-G*- G *)	3.3	5
2-Methyl trigalacturonate (G-G*- G)	9.1	13
3-Methyl trigalacturonate (G*-G-G)	0	0

(Figure 1A, peaks 2 and 3) were assigned as di- and mono-methyl trigalacturonate respectively. These peak assignments were made by comparing the retention times of the products with those of a standard mixture of oligogalacturonates (DP 1–3) and previously synthesized monomethyl trigalacturonates (three isomers, methyl-esterified at positions 1, 2 and 3 relative to the reducing end). Using the HPLC method, it was not possible to determine whether peaks 2 and 3 were composed of more than one isomer with similar charge, since the three synthetic monomethyl trigalacturonate isomers were co-eluted at approx. 12 min, and pure dimethyl trigalacturonate isomers were not available.

Performance of PME on monomethyl-esterified and fully methyl-esterified trigalacturonates

As shown in the product-progression profile (Figure 1B), dimethyl trigalacturonate was the initial product formed from fully methyl-esterified trigalacturonate. Prolonged incubation with the enzyme resulted in the further production of dimethyl trigalacturonate, as well as the formation of monomethyl trigalacturonate as a second-generation product. This latter product is rather resistant to de-esterification, as evidenced by reaction rates of PME on monomethyl trigalacturonates (Table 2). Two of the monomethyl trigalacturonates showed reduced activity, whereas 3-methyl trigalacturonate was not a substrate. This latter result, in combination with those from the tandem MS analysis of the 24-h sample (Figure 2), demonstrates that the second-generation product is indeed 3-methyl trigalacturonate. These results demonstrate further that, although the methyl ester of 3-methyl trigalacturonate is resistant to hydrolysis by PME, its presence is not essential for hydrolysis of the other two methyl groups.

Analysis of reaction products by MS

ESI-MS/MS was used to characterize the products of the reaction of PME with fully methyl-esterified oligogalacturonates (DP 3–6) in order to understand the sequential de-esterification events. The incubation times were chosen, on the basis of pilot experiments, to cover all events: the preferential de-esterification after 0.5 h of incubation and the later reaction products after 24 h.

When subjected to tandem MS conditions, oligosaccharides dissociate in predictable patterns that are used to determine the structure of the intact sugar. Fragmentation can occur across the carbohydrate ring (cross-ring cleavage), but occurs typically along the glycosidic bond. For detection by MS, these fragments must be charged. Ions resulting from glycosidic bond cleavage are termed 'Y and Z fragment' ions if the charge is retained on

Figure 2 Product analysis by MS

(A) A tandem MS spectrum of dimethyl-esterified hexagalacturonate. 'Precursor ion' corresponds to the intact dimethyl-esterified hexagalacturonate. The diamonds, squares and circles represent the three isomers for this compound and Y_n and B_n correspond to fragment ions produced from the dissociation of each isomer. (B) A schematic diagram showing the sequence analysis on the basis of the fragment ions produced by tandem MS experiments. Further details are provided in the Results and Discussion section.

the reducing end and 'B and C fragment' ions if the charge lies on the non-reducing terminus [15]. The Y and B fragment ions lack one oxygen atom compared with the Z and C fragment ions respectively. In a triple quadrupole mass spectrometer, dissociation of the oligogalacturonate occurs along the glycosidic bond to form primarily series of B and Y fragment ions, with the

occasional loss of water (Figure 2A). This is in contrast with results obtained using an ion-trap mass spectrometer, in which compounds of similar structure have been reported to also produce C ions, Z ions and ions accounted for by cross-ring cleavages [9]. The lack of cross-ring fragmentation in the triple quadrupole instrument leads to simpler analysis of

Table 3 De-esterification of fully methyl-esterified oligogalacturonates with DP 3–6

Products analysed by tandem MS. For experimental details, see the Experimental section. The reducing end is shown in bold, Gn signifies the fully methyl-esterified substrate with DP=*n* and G* and G denote methyl galacturonate and GalA respectively. G? denotes that it is unclear whether or not the GalA is methyl-esterified. ms/ms, tandem MS.

Gn	Time (h)	No. of methyl groups	Isomer 1					Isomer 2					Isomer 3					Isomer 4											
G3	blank	3	G*	G*	G*																								
	0.5 h	1	G*	G	G																								
		2	G*	G*	G																								
		3	G*	G*	G*					G*	G	G*																	
	24 h	1	G*	G	G																								
		2	G*	G	G*																								
		3	G*	G*	G*																								
	<hr/>																												
	G4	blank	4	G*	G*	G*	G*																						
0.5 h		2	G*	G*	G	G																							
		3	G*	G*	G?	G?																							
		4	G*	G*	G*	G*					G*	G	G*	G*															
24 h		1	G*	G	G	G																							
		2	G*	G*	G	G																							
		3	G*	G	G*	G*					G*	G	G?	G?															
		4	G*	G*	G*	G*																							
<hr/>																													
G5	blank	5	G*	G*	G*	G*	G*																						
	0.5 h	2	G*	G*	G	G	G																						
		3	G*	G*	G	G?	G?																						
		4	G*	G*	G	G*	G*					G*	G*	G	G?	G?			G*	G	G	G*	G*						
		5	G*	G*	G*	G*	G*					G*	G*	G*	G	G*													
	24 h	1	G*	G	G	G	G																						
		2	G*	G*	G	G	G																						
		3	G*	G	G	G*	G*					G*	G	G	G?	G?													
		4	Signal too weak; unable to get good ms/ms fragmentation																										
<hr/>																													
G6	blank	6	G*	G*	G*	G*	G*	G*																					
	0.5 h	2	Signal too weak; unable to get good ms/ms fragmentation																										
		3	G*	G*	G	G	G?	G?					G*	G	G?	G	G?	G?			G*	G	G	G	G*	G*			
		4	G*	G*	G*	G	G?	G?					G*	G*	G	G?	G?	G?			G*	G	G	G*	G*				
		5	G*	G*	G*	G*	G?	G?					G*	G*	G	G*	G*	G*			G*	G	G	G*	G*				
		6	G*	G*	G*	G*	G*	G*																					
	24 h	2	G*	G*	G	G	G	G					G*	G	G*	G	G	G			G*	G	G	G	G				
		3	G*	G*	G	G	G?	G?					G*	G	G	G	G*	G*											
		4	G*	G*	G	G	G*	G*					G*	G	G*	G	G*	G*			G*	G	G	G*	G*				

the fragmentation patterns. This is extremely useful when analysing samples containing multiple isomers, as was the case in these samples.

Tandem MS experiments on most of the precursor ions produced multiple series of Y and B fragment ions, with each series being produced by a single isomer of the precursor ion. For example, analysis of the tandem MS spectrum of a dimethyl-esterified hexagalacturonate revealed three series of Y fragment ions and some B fragment ions. Each series of fragment ions were produced by a different positional isomer of dimethyl-esterified hexagalacturonate. The sites of methyl esterification were located in a straightforward manner by observing the mass differences between fragment ions, and between the precursor ion and the Y_n ion of a given series, as illustrated in Figure 2(B). Mass differences of m/z 190 and 176 between the fragment ions correspond to methyl-esterified GalA residues or GalA residues respectively. This is illustrated for one isomer (symbolized by a circle) of the dimethyl-esterified hexagalacturonate (Figures 2A and 2B), which produced a series of Y fragment ions (Y_2 to Y_5) and a series of B fragment ions (B_1 , B_2 and B_3). A mass difference of 190 m/z between the precursor ion and the Y_5 fragment ion identified a methyl-esterified GalA as the non-reducing residue. The remaining residues were identified on the basis of mass differences between Y_n and $Y_{(n-1)}$ fragment ions. Mass differences of 176 between the fragment ions Y_5-Y_4 and Y_4-Y_3 demonstrate that both the fourth and fifth residues, relative to the reducing end, are GalA. Furthermore, a mass difference of 190 between Y_3 and Y_2 fragment ions identifies a methyl-esterified GalA as the third residue. The calculations from the B-ion series confirm the validity of results obtained from the Y-ion series. The peak at m/z 191 corresponds to the B_1 fragment ion of a methyl-esterified GalA on the non-reducing residue. Similarly, mass differences of 176 between the fragment ion pairs B_1-B_2 and B_2-B_3 confirms that the fourth and fifth residues are GalA.

The data from the tandem MS experiments are presented in Table 3. Examination of the products produced from the oligomers with DP 4–6 during the initial stage of the reactions (0.5-h incubations) demonstrates that the first event is the hydrolysis of a methyl group located on an internal residue (Table 3). Hydrolysis of one methyl group from the fully methyl-esterified tetramer results in 1,2,4-trimethyltetragalacturonate, and probably 1,3,4-trimethyltetragalacturonate, whereas the fully methyl-esterified pentamer yields 1,2,4,5-tetramethyl- and 1,3,4,5-tetramethyl-pentagalacturonate. The position of the methyl ester is numbered from the reducing end of the oligogalacturonate. The methyl groups at positions 4 and 5, and probably at position 2, are the first to be hydrolysed in the fully methyl-esterified hexamer. Under the tandem MS conditions used, we were occasionally unable to observe fragment ions, which would allow location of all the methyl esters. This was especially true when the site of methyl esterification was located at or near to the reducing GalA residue, as exemplified in isomer 2 of the dimethyl-esterified tetragalacturonate (24 h) with the given structure G*-G-G?-G? (Table 3). The location of one methyl ester, symbolized by G*, was determined to be on the non-reducing end sugar. However the location of the second methyl

ester (G?) has been limited to either the reducing residue or the residue adjacent to the reducing GalA residue.

The trimer appears to be unique in that initial hydrolysis of the methyl ester may occur at the internal residue or the reducing-end residue, leading to 1,3-dimethyl trigalacturonate and 2,3-dimethyl trigalacturonate respectively (Table 3). HPAEC analysis or tandem MS without appropriate standards cannot establish the relative abundance of the two isomers. Hence, it is not clear which of the two methyl esters is preferentially hydrolysed by PME.

Further de-esterification of the trimer leads to a residual methyl ester at the non-reducing end GalA. The residual methyl ester is also located at the non-reducing end GalA for the monomethyl trimer, the tetramer and the pentamer. This observation leads to the conclusion that PME does not hydrolyse the methyl ester at this position. This is in agreement with data obtained previously for the trimer, in which 3-methyl trigalacturonate was not hydrolysed by PME (Table 2), and supports the earlier finding that PME is not able to hydrolyse all methyl esters in the homogalacturonan part of pectin [5].

In the present study it is shown that incubation of the fully methyl-esterified oligogalacturonates with PME results in a mixture of partially methyl-esterified isomers. It is demonstrated further that ESI-MS/MS is a very powerful technique by which the positions of the methyl esters can be determined without prior fractionation of the reaction mixture. On the basis of this sequence information for the specific reaction products, the mode of action of *A. niger* PME has been established.

This work was financially supported by grants from the European Economic Community (AIR2-CT9413450) and the North Atlantic Treaty Organization (CRG 973086).

REFERENCES

- Sakai, T., Sakamoto, T., Hallaert, J. and Vandamme, E. J. (1993) Adv. Appl. Microbiol. **39**, 213–250
- Versteeg, C. (1979) Ph.D. Thesis, Agricultural University Wageningen, Wageningen, The Netherlands
- Markovic, O., Machová, E. and Slezárik, A. (1983) Carbohydr. Res. **116**, 105–111
- Dongowski, G. and Bock, W. (1984) Die Nahrung **28**, 507–516
- Kohn, R., Dongowski, G. and Bock, W. (1985) Die Nahrung **29**, 75–85
- Kester, H. C. M., Benen, J. A. E. and Visser, J. (1999) Biotechnol. Appl. Biochem. **30**, 53–57
- Daas, P. J. H., Arisz, P. W., Schols, H. A., De Ruiter, G. A. and Voragen, A. G. J. (1998) Anal. Biochem. **257**, 195–202
- Körner, R., Limberg, G., Mikkelsen, J. D. and Roepstorff, J. (1998) Mass Spectrom. **33**, 836–842
- Körner, R., Limberg, G., Christensen, T. M. I. E., Mikkelsen, J. D. and Roepstorff, P. (1999) Anal. Chem. **71**, 1421–1427
- Kester, H. C. M., Magaud, D., Roy, C., Anker, D., Doutheau, A., Shevchik, V., Hugouvieux-Cotte-Pattat, N., Benen, J. A. E. and Visser, J. (1999) J. Biol. Chem. **274**, 37053–37059
- Kester, H. C. M. and Visser, J. (1990) Appl. Biochem. **12**, 150–160
- Pařenicová, L., Benen, J. A. E., Kester, H. C. M. and Visser, J. (1998) Eur. J. Biochem. **251**, 72–80
- El Rayah Ahmed, A. and Labavitch, J. M. (1977) J. Food Biochem. **1**, 361–365
- Benen, J. A. E., Kester, H. C. M. and Visser, J. (1999) Eur. J. Biochem. **259**, 577–585
- Domon, B. and Costello, C. E. (1988) Glycoconj. J. **5**, 397–409