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Characterisation of legumes by enzymatic hydrolysis, microdialysis sampling, and micro-high-performance anion-exchange chromatography with electrospray ionisation mass spectrometry ☆

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Abstract

An assay based on enzymatic hydrolysis and microdialysis sampling, micro-high-performance anion-exchange chromatography (micro-HPAEC) with electrospray ionisation mass spectrometry (ESI-MS) for the characterisation of legumes is presented. Characterisation of two bean varieties; *Phaseolus mungo* and *P. acutifilous* was based upon enzymatic hydrolysis using an endo-β-mannanase from *Aspergillus niger* with subsequent analysis of the hydrolysates with HPAEC–MS. The hydrolysates were detected in the positive ionisation mode after desalting the chromatographic effluent, employing a cation-exchange membrane desalting device with water as the regenerating liquid. Mass chromatograms, acquired after hydrolysis of both bean samples for 12 h, showed two different profiles of hydrolysates. The *P. mungo* bean hydrolysate showed the presence of saccharides with a degree of polymerisation (DP) in the range of 2–6, whereas that of *P. acutifilous* showed only DPs of 2–5. Both bean samples had one type of DP 2, but showed different types of DPs 3, 4 and 5. Only the *P. mungo* sample showed the presence of DP 6. The most abundant fraction for *P. mungo* was DP 4, whereas that for *P. acutifilous* was DP 5. Tandem MS of the hydrolysates showed that the DP 2 hydrolysates observed for the samples were of the same type, having a 1,6 linkage. Also tandem MS data for DPs 3, 4, and 5 showed that similar hydrolysates were present within the same sample as well as among the two samples. The data also showed the existence of 1,6 linkages for DP 3, 4, and 5 hydrolysates. The single enzymatic hydrolysis in combination with microdialysis and HPAEC with ESI-MS proved to be sufficient and reproducible for profiling and showing the difference between the two bean samples.
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1. Introduction

In leguminous seeds, the carbohydrate polymer is mainly galactomannan, which is an energy reserve that is essential during germination of the seed. The main polymer backbone of galactomannan consists of β-1,4-linked mannan residues with side groups of α-1,6-galactospyranosyl units [1]. The main indus-

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trial application of galactomannans is as a gelling agent for foods and cosmetics [2,3]. Because of the industrial importance of this class of compounds, it is necessary to characterise legumes, as they are a main source of galactomannans.

To carry out effective characterisation of galactomannans, it is necessary to employ a methodology that will break down the polymer and allow subsequent analysis of the products in their native form. Microbial enzymes are the most preferred tool for breaking down the main polymer backbone because of their specificity and environmental friendliness compared to inorganic reagents. In the case of galactomannan, it is necessary to use a combination of β-mannosidase, α-1,6-galactosidase, and endo-β-1,4-mannanase to achieve total hydrolysis of the polymer. However, for diagnostic purposes, it might be worth exploring the use of a single enzyme in order to facilitate the characterisation of the polymer. A combination of an enzymatic hydrolysis and a technique that can allow in situ sampling and sample clean-up would be one component of a methodology that can be used to characterise these polymers.

One such technique that has been shown to be very versatile and can achieve in situ sampling and sample clean-up for enzymatic hydrolysates is microdialysis sampling [3–5]. Microdialysis sampling employs a semi-permeable membrane of a specific molecular mass cut-off, thereby introducing selectivity and achieving on-line sample clean-up. This eliminates laborious and time-consuming sample handling steps, such as centrifugation and filtration normally employed to eliminate enzymes and unhydrolysed substrates before injection into a separation system. Microdialysis can be directly coupled to a high-resolution separation methodology, such as high-performance anion-exchange chromatography (HPACE). HPAEC is ideally suited to the separation of carbohydrates in their enolate form with subsequent detection by mass spectrometry (MS).

MS [6,7] in comparison to spectrophotometric detectors [8] and electrochemical detectors [9] is the most attractive detection technique because it yields information that leads to the unequivocal identification of carbohydrates. Because of the pioneering work by Simpson et al., carbohydrates can be detected directly with MS without any further sample work-up [10]. Simpson's group interfaced HPAEC with MS, using an anion micromembrane suppressor [10]. This is because separation of carbohydrates at high pH (14) is associated with high concentrations of sodium hydroxide and sodium acetate gradients up to 600 m*M*. If such a chromatographic effluent is introduced directly into the MS ion source, it results in blockage of capillaries that will not allow any further analysis unless the ion source is disassembled and cleaned thoroughly. In order to circumvent the limitation imposed by high salt content, several efforts have been reported [10,11] in an attempt to develop a routine method to effectively desalt chromatographic effluents before ionisation by ESI-MS.

In order to characterise two galactomannan polymers qualitatively, in *Phaseolus mungo* (mung) and *P. acutifilous* (tepary), a combination of enzymatic hydrolysis and micro-HPAEC with ESI-MS was used. The enzymatic hydrolysates of the two polymers were desalted after chromatographic separation, using a cation-exchange membrane desalting device with water as a regenerating liquid [4,5]. The mass chromatograms of the hydrolysates in their sodiated $[M+Na]^+$ form showed that the two polymers produced different hydrolysis products.

2. Experimental

2.1. Reagents

Galactomannan was obtained from Megazyme (Wicklow, Ireland). The sodium hydroxide (50%, w/w) and the sodium acetate were obtained from J.T. Baker (Deventer, The Netherlands). Propan-2-ol of HPLC grade was obtained from Ultrafine (London, UK). All solutions were prepared with ultrapure water from a Milli-Q system (Millipore, Bedford, MA, USA). The endomannanase enzyme was a gift from Dr. Evodia Setati (Department of Microbiology, University of Stellenbosch, South Africa).

2.2. Hydrolysis of bean samples

The substrate was dehulled manually, ground, and sieved through a 1-mm mesh. A 0.5-g amount of substrate was weighed into a 10-ml reaction vessel to which 9 ml of sodium citrate buffer was added to maintain a pH of 5.3. The reaction vessel (10 ml)

was housed in a Pierce react-Therm (heating/stirring module 18971: Pierce, Rockford, IL, USA). Enzyme loading was 270 nKat/g of substrate. The same treatment was applied to the control, except that no enzyme was added. Samples were hydrolysed for 12 h.

2.3. Microdialysis sampling

A polysulfone membrane, obtained as a gift from A/G Technology (Needham, MA, USA), with a molecular mass cut-off of 30 000, was mounted onto a laboratory-machined microdialysis probe [12]. The effective dialysis length of the hollow-fibre membrane was adjusted to 5 mm. The microdialysis probe was placed in the reaction vessel, housed in the Pierce react-Therm, and perfused at 2 μl/min with ultrapure water. The ultrapure water was delivered by a CMA/100 microinjection pump, CMA/Microdialysis (Stockholm, Sweden). Samples of 5-μl were either directly injected into the chromatographic system, using a CMA/160 on-line injector, or were collected with the CMA/142 microfraction collector (both from CMA/Microdialysis).

2.4. Chromatographic separation

The enzymatic hydrolysates were injected onto a Dionex 500 micro-chromatographic system (Dionex, Sunnyvale, CA, USA) described elsewhere [5]. Chromatographic separation was carried out at 0.15 ml/min, using a sodium hydroxide gradient. The mobile phase consisted of 500 m*M* sodium hydroxide, the composition of which was varied with the addition of water. The composition of sodium hydroxide from 0 to 5 min was 10%; 5–15 min it was reduced to 5%, and 20–90 min, the composition was maintained at 10%. Equilibration of the CarboPac PA1 pre- and analytical columns was achieved with 10% of the 500 m*M* sodium hydroxide solution.

2.5. Detection of hydrolysates

Desalting of the chromatographic effluents before detection was achieved using a cation-exchange membrane desalting device that employed electrolysis of water, as described elsewhere [4]. To ensure a constant water flow for the operation of the desalter, a nitrogen pressure of 15 p.s.i. was applied (1 p.s.i.=6894.76 Pa). The desalted sample proceeded to a three-way connection, where the sample combined with the sheath liquid, consisting of propan-2-ol–water (80:20). The sheath liquid, that also contained 1% 10^{-4} *M* sodium acetate, was introduced at a flow-rate of 50 μl/min. The sample was introduced into the electrospray interface of the Finnigan LCQ-Deca Ion trap MS system (Finnigan, San Jose, CA, USA) with sheath gas (nitrogen) at a flow-rate of 5 l/min. The capillary temperature and capillary voltage were optimised to 300 °C and 5 kV, respectively. Detection in the positive ionisation mode was carried out with a single-ion monitoring program.

For tandem mass spectrometry, the sheath gas flow-rate was optimised to 4 l/min, and spray voltage was maintained at 5 kV. The scan events were all MS–MS for degrees of polymerisation (DPs) 2–6. The collision energy used for all saccharides was optimised to 32%, except for DP 2, which was optimised to 34%.

3. Results and discussion

With the advent of ESI, the application of MS to the analysis of biolabile molecules, like carbohydrates, has increased [13]. ESI is regarded as a soft-ionisation technique that affords molecular mass information. In addition, due to the inherent loss of sensitivity of MS with increasing molecular mass, the ability of ESI to form multiply charged [14,15] pseudo-molecular ions enhances its sensitivity and extends the applicable mass range for the instrument. Therefore due to its mass selectivity, ESI-MS in combination with chromatography can be used to generate information facilitating the unequivocal identification of carbohydrates [5,16,17]. Combining the retention selectivity of chromatography and mass selectivity of MS enables differentiation of compounds with the same physico-chemical properties, such as carbohydrates.

Recent MS technology, particularly the introduction of ion traps [18–20], enables one to carry out collision-induced dissociation (CID) experiments at several levels up to 7 (MS^7). CID or tandem mass

spectrometry experiments for carbohydrates, coordinated to metal ions, is an effective method for acquiring stereochemical information [21]. Evaluation of CID data can reveal information about monosaccharide substitution [22], glycosidic linkage position [23–26], and anomericity [6]. From these patterns it may be possible to elucidate the structure of an unknown polymer. In this study, a single-ion monitoring (SIM) program, based on aldohexoses, was used to profile the hydrolysis products, due to its inherent sensitivity. Further experiments to distinguish hydrolysates having the same mass to charge values were carried out, using tandem MS, as discussed in the following sections.

3.1. Single-ion monitoring of the mung and tepary bean hydrolysates

For monitoring of the hydrolysis, positive ionization was used with a SIM procedure, based on singly $[M+Na]^+$ charged molecules up to a DP of 6, based on aldohexoses. Fig. 1 shows mass chromatograms of the mung bean hydrolysates that were acquired after 12 h hydrolysis, using micro-HPAEC with ESI-

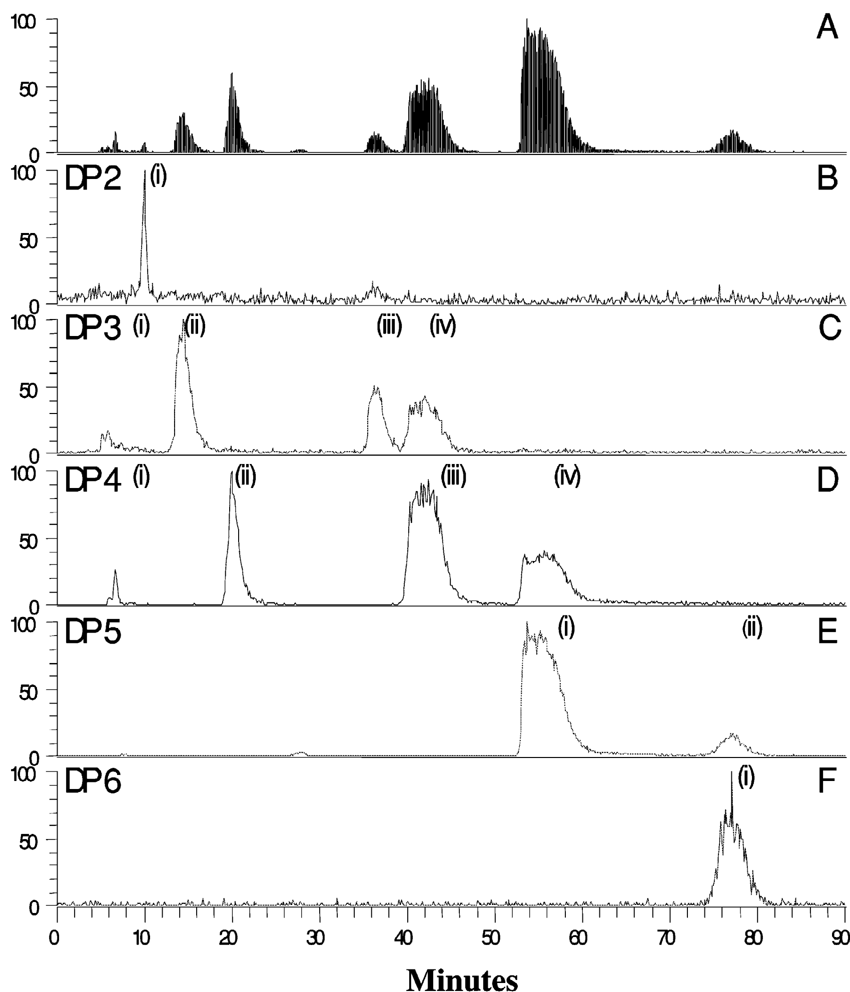

Fig. 1. Mass chromatogram of mung bean hydrolysates analysed after a 12-h hydrolysis period. The chromatograms represent: (A) the total hydrolysates detected after 12-h hydrolysis, (B) DP 2, (C) DP 3, (D) DP 4, (E) DP 5, and (F) DP 6, respectively. For DPs 3–5, the several peaks observed indicate either a different glycosidic linkage or a different degree of branching for the detected saccharides.

MS after clean-up by microdialysis. The chromatographic profile indicates the presence of DP 2 (m/z 365)–DP 6 (m/z 1013), as observed in the chromatograms (see Fig. 1B–F, respectively). The chromatographic profile shows that only DP 2 and DP 6 exist in one form as DP 3 (m/z 527); DP 4 (m/z 689) and DP 5 (m/z 851) exhibit multiple peaks. Because MS separates components by mass, the presence of several peaks for any particular DP indicates that the molecules might have a different type of glycosidic linkage or degree of branching (DP>2). The versatility of combining HPAEC with ESI-MS is also demonstrated by the chromatographic profile, as it is clear that some higher DPs are eluted before lower DPs, and also that some higher DPs are eluted together with lower DPs. Such a profile is not consistent with the elution order predicted for molecules of different sizes in anion-exchange chromatography. For example, the first two types (i and ii) of DP 4 (see Fig. 1D) are eluted before the two types (iii and iv) of DP 3 (see Fig. 1C). Also the fourth type (iv) of DP 3 (see Fig. 1C) is eluted together with the third type (iii) of DP 4 (see Fig. 1D). The fourth type (iv) of DP 4 is eluted together with the first type (i) of DP 5 (see Fig. 1E). DP 6 (see Fig. 1F) is also eluted with the second type (ii) of DP 5. The order of elution and the presence of several types of a particular saccharide further shows the complexity of carbohydrates.

Fig. 2 shows mass chromatograms acquired by SIM for the tepary bean hydrolysates that were hydrolysed for 12 h and analysed by micro-HPAEC with ESI-MS after clean-up with microdialysis sampling. The chromatographic profile shows the presence of DP 2 (m/z 365)–DP 5 (m/z 851) in the hydrolysates. For the tepary bean, only one type of DP 2 (see Fig. 2B) and DP 5 (see Fig. 2E) are observed. As similarly observed for the mung bean, hydrolysates from the tepary bean show lack of separation as well as elution of higher DPs before the lower DPs. The first type (i) of DP 3 (m/z 527) is eluted before DP 2. The fifth type (v) of DP 3 is eluted with the first type (i) of DP 4 and the second type (ii) of DP 4 (m/z 689) is eluted with DP 5.

For two similar carbohydrate polymers expected to have the same backbone as well as substitution pattern and type of monosaccharides residues and substituents, hydrolysis of such a polymer with the same enzyme should give identical products. Qualitative characterisation can thus be achieved on the basis of the observed hydrolysis profile, particularly after analysis by micro-HPAEC with ESI-MS. Table 1 shows a summary of the profile of the mung and tepary bean samples. From the data in the table, it is clear that these two galactomannan polymers are distinguishable, because their hydrolysis products differ in composition and type (also see Fig. 1 and Fig. 2). The only similarity observed for both beans was the single type of DP 2 in the hydrolysates. For DP 3, the mung bean had four types compared with the five types observed for the tepary bean. The mung bean also had four types of DP 4, whereas the tepary bean had two types. The tepary bean had only one type of DP 5, and there was no DP 6 detected after hydrolysis with the mannanase. However, for the mung bean two types of DP 5 were observed as well as a DP 6.

MS data also showed that for the tepary bean the most intense signal was observed for DP 4. This suggests that the DP 4 hydrolysate is the most abundant and that DP 4 is the main product. This is consistent with the observation that hydrolysis of DP 4 by endo-β-mannanase is at a very slow rate [27,28]. However, for the mung bean the most abundant hydrolysate was DP 5. This suggests structural inhibition of the polymer cleavage by the endo-β-mannanase [29] and further confirms the difference between the tepary and mung beans.

If one was to screen several types of galactomannan polymers on the basis of a single enzymatic hydrolysis, using the assay presented, the results presented in Table 1 in combination with those in Figs. 1 and 2 could be used to establish criteria for characterisation. Further, information in Table 1 could also be expanded by distinguishing between similar DPs. Tandem MS experiments, even in the absence of authentic standards, have the ability to differentiate between saccharides based on the observed fragmentation pattern.

3.2. Tandem mass spectrometry of the mung and tepary bean hydrolysates

Glycosidic bonds that can be fragmented by low-energy collisions connect monosaccharide units. The observed fragmentation patterns are important, be-

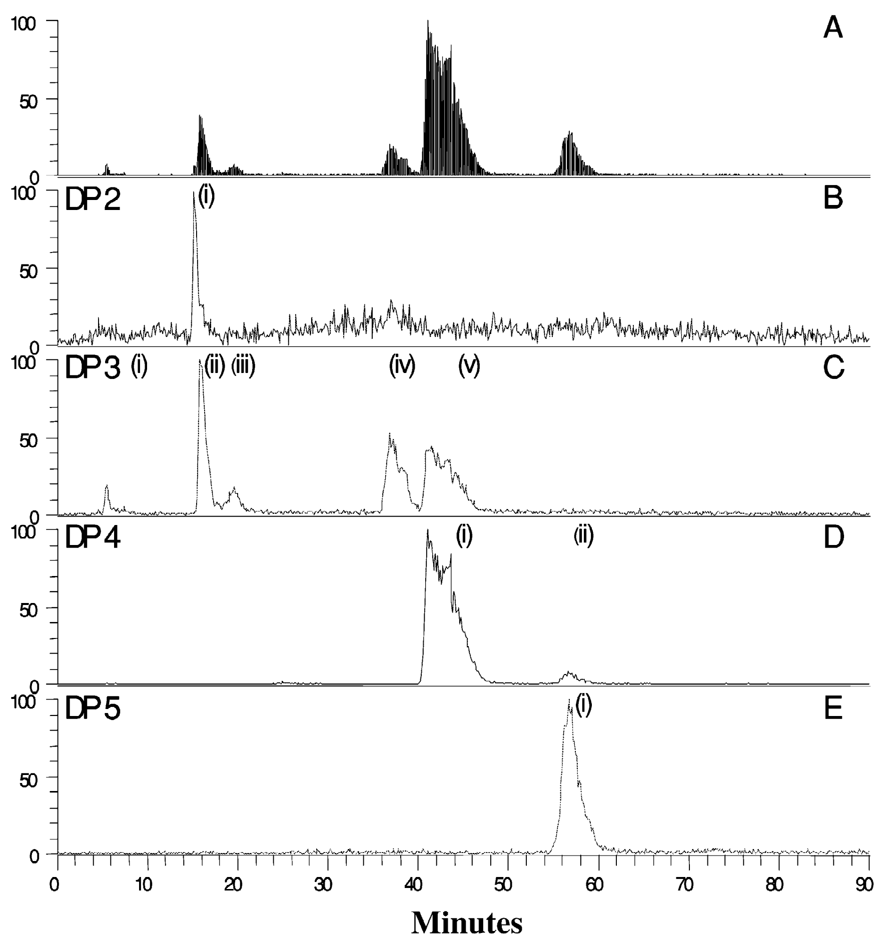

Fig. 2. Mass chromatogram of tepary bean hydrolysates analysed after a 12-h hydrolysis period. The chromatograms represent: (A) the total hydrolysates detected after 12-h hydrolysis, (B) DP 2, (C) DP 3, (D) DP 4, and (E) DP 5, respectively. For DPs 3–4, the several peaks observed indicate either a different glycosidic linkage or a different degree of branching for the detected saccharides.

cause they can generate information leading to monomer sequencing and, hence, characterisation. Tandem experiments were carried out on the DP 2 hydrolysates for both the mung and tepary bean in the MS–MS mode. Only the fragments with a relative intensity of 10% or more were used for data analysis. For both beans, the major fragments observed were for losses of the following: water (*m/z* 347), $C_2H_4O_2$ (*m/z* 305), $C_3H_6O_3$ (*m/z* 275) and a glucose unit (*m/z* 203). The most abundant fragment was that of $C_2H_4O_2$. According to work carried out by Asam and Glish [25], losses of $C_2H_4O_2$, $C_3H_6O_3$, and $C_4H_8O_4$ indicate the presence of a 1,6 linkage. The observed fragmentation of DP 2 for both the mung and tepary bean was associated with a 1,6 glycosidic linkage, confirming that the single DP 2 observed for both beans was of a similar type.

Table 1
Summary of the hydrolysates obtained from the two bean samples

DP	Observed saccharides from mung bean hydrolysis	Observed saccharides from tepary bean hydrolysis
2	One type	One type
3	Four types	Five types
4	Four types	Two types
5	Two types	One type
6	One type	–

Tandem MS experiments were also carried out in

order to distinguish amongst the different DP 3 for both samples. Evaluation of the DP 3 data is complicated by the fact that DP 3 is the smallest oligomer in which branching can occur. From Table 2 (see also Figs. 1 and 2), hydrolysates (i) and (iii) of the mung bean have a fragment resulting from the loss of a glycosidic unit as the only fragment, *m/z* 365. Hydrolysates (ii) and (iv) for the mung bean show a similar fragmentation pattern. The similar mass-to-charge ratios shown by DP 3 units with different retention times can be associated with a difference in structure, i.e., branched versus linear DP 3 unit. The hydrolysates for the mung bean seem to be different from each other, although the fragmentation loss of hydrolysate (ii) is comparable to that of (iv).

Based on the fragmentation patterns, similarities between the bean samples can be identified. For both bean samples, either fragment associated with a $C_2H_4O_2$ (*m/z* 467) or glycosidic unit (*m/z* 365) loss were the most abundant for any hydrolysate. Between the two bean samples, hydrolysates (i) and (iii) of the mung bean sample is similar to that for hydrolysates (i) and (iv) of the tepary sample. Hydrolysates (ii) and (iv) of the mung bean is similar to that of hydrolysate (v) of the tepary bean. Because both beans are built upon the galactomannan polymer, it is not surprising that there were some saccharide units that showed similar fragmentation patterns. However, tandem MS data also revealed that not all the observed saccharides types for the sample were similar. Both mung (ii, iv) and tepary (v) hydrolysates showed fragmentations that were consistent with a 1,6 linkage at the reducing end. This confirms the expected high degree of substitution on the polymer backbone, by the galactosyl substituent.

Separate tandem MS experiments were also carried out for DP 4 and DP 5 (results not shown). A similar trend was observed for these oligasaccharides, where there were some fragments that were common for both bean samples as well as within each bean sample. This confirmed that the complexity of saccharides, which increases with the DP. The observed fragmentations could be associated with 1,6-type of linkages for both bean samples. The results from these studies clearly show that a profile based on hydrolysis and subsequent analysis with micro-HPAEC with ESI-MS can give enough qualitative information to distinguish between complicated polymers from each other without acquiring expensive authentic standards.

4. Conclusion

The essential role of MS in the analysis of carbohydrate hydrolysates and legume characterisation is recognised. An on-line, reliable and reproducible system, employing microdialysis sampling and micro-HPAEC with ESI-MS was used to characterise two bean samples. The mass chromatograms of the hydrolysates showed the elution profile of the hydrolysates. For both samples, there was lack of

Table 2
The fragmentation pathways of all the DP 3 hydrolysates for both bean varieties

DP 3		Loss associated with					
		Water (*m/z* 509)	$C_2H_4O_2$ (*m/z* 467)	$C_3H_6O_3$ (*m/z* 437)	$C_4H_8O_4$ (*m/z* 407)	Glycosidic unit (*m/z* 365)	Water (*m/z* 347)
Mung	(i)					100	20
	(ii)	10	100	25	10	70	
	(iii)					100	
	(iv)	12	100	28	10	65	
Tepary	(i)					100	17
	(ii)		100	25	10	60	
	(iii)		100	15		43	
	(iv)					100	
	(v)	16	100	25	10	60	

separation of some of the saccharides, reversed elution order that was not consistent with the DP, as well as the presence of a variety of sugars for specific DPs. For any particular hydrolysate, the number of different types of saccharides observed was not the same. The information generated from the hydrolysates in the SIM mode confirmed that the two polymers were significantly different from each other. The most abundant saccharide for the mung bean was DP 4, but DP 5 for the tepary bean. The only similarity observed was the presence of a single DP 2 for both polymers. Tandem mass spectrometry further illustrated the presence of structural differences within hydrolysates of the same DP. From the dissociation pathways observed the interglycosidic linkages at the reducing ends of the hydrolysates could be predicted. Also the tandem MS data showed that some of the different types of hydrolysates observed within the same polymer had a similar glycosidic linkage that was also observed for the other polymer. Further work is in progress to complete characterisation of the beans by determining the glycosidic linkages throughout the oligomers by carrying out multiple MS (MS^n) experiments.

The data presented show that despite the need for the cooperative effect of β-mannosidase, α-1,6-galactosidase, and endo-β-1,4-mannanase for the complete breakdown of the galactomannan polymer, a simple hydrolysis, combined with a versatile combination of techniques, enabled the characterisation of the carbohydrates in the legume seeds. The information obtained in this study can be used to screen similar polymers from legumes.

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