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Enzymic degradation of sorghum glucuronoarabinoxylans leading to tentative structures

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Abstract

Three glucuronoarabinoxylan (GAX) populations, obtained from water-unextractable cell wall material from sorghum by different alkali extractants, were digested by combinations of endo-xylanases (Xyl I, Xyl III and GXH), arabinofuranosidases (AXH and AraB) and an α-glucuronidase (GlcAase). All three GAX populations were shown to be rather poorly degradable, due to the very high degree of substitution, as well as the substitution pattern. The barium hydroxide-extracted GAX showed a maximum degree of degradation of almost 12%, using Xyl I combined with GXH and AXH. The GAX population extracted by 4 M KOH was hardly degraded by any of the tested combinations. In all cases, Xyl III showed lowest activity upon the three extracts. Synergistic effects were observed between Xyl I and AXH. Both neutral and acidic arabinoxylan oligomers were formed. The GlcAase acted only upon oligomeric material released by Xyl I. No synergistic effects were observed between the GXH and AXH. Combining the patterns of degradation with the modes of action of the enzymes, structures were proposed for the different populations of sorghum GAX. Evidence was obtained that the xylan backbone of especially the GAX extracted by 4 M KOH, is substituted by arabinose and glucuronic acid according to a strict pattern, which hinders the enzymes to act.

Keywords: Glucuronoarabinoxylan; Sorghum; Enzymic digestion; Model structure

1. Introduction

Arabinoxylan, being one of the major non-starch polysaccharides (NSP) present in cereal cell walls, plays an important role in the processing of cereals, such as baking [1] and brewing [2]. In brewing, they are associated with processing problems like poor wort and beer filtration rates [3,4] or the formation of beer hazes and precipitates [5]. A rather new development is the use of sorghum malt as a raw material for brewing lager type beers. EtokAkpan and Palmer [6] visualized, by scanning electron microscopy, the difference between germinating sorghum and barley grains. The cell wall structures of barley almost completely disappeared, whereas almost intact cell

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walls remained in the case of sorghum malt. Biochemical studies on barley malt have shown a partial degradation of arabinoxylans during malting [7]. This result seems to be in contradiction to the stable or increased amounts of arabinoxylans in barley malt described by EtokAkpan [8]. Research on sorghum also gave some contradicting results. EtokAkpan [8] found that the arabinoxylan content of sorghum decreased during malting, whereas unpublished results from our laboratory showed neither change in arabinoxylan content nor composition.

Degradation of polysaccharides during malting can only be explained by action of newly synthesized and activated endogenous enzymes during the malting process. Apart from α- and β-amylases, β-glucanases and proteases, also barley malt xylanases [9–11], xylosidases and arabinofuranosidases [12,13] have been identified. The malt from sorghum has been less well investigated. Despite reports about some varieties lacking β-amylase [3], this enzyme [14,15], as well as α-amylases [16,17], β-glucanases [18,19] and proteases [20] have been determined.

Although xylanase activity is present in sorghum malt [6], the arabinoxylans are hardly degraded. The NSP are composed of highly substituted glucuronoarabinoxylans (GAX), although minor amounts of other sugars occur [21–23]. Some of the side groups are composed of short arabinose-chains [24]. In the intact cell wall, GAX is esterified with acetic acid and ferulic acid; the latter may be involved in cross-linking polysaccharides and/or proteins [21]. The objective of this research was to obtain a better understanding of the chemical structure of GAX from sorghum kernels, by studying their enzymic degradability with purified enzymes. This knowledge may result in an explanation for the poor malting and brewing characteristics.

2. Experimental

Materials.—Three GAX populations were extracted from water-unextractable cell wall material (WUS) prepared from whole sorghum kernels [21] (*Sorghum vulgare* cv. Fara Fara) by saturated aqueous barium hydroxide, 1 M KOH and 4 M KOH solutions in sequence. The populations were named accordingly: BE1.1, 1K4 and 4K. The fractions together accounted for 67% of the total GAX present in the sorghum WUS [22]. They differ in molecular-mass distribution, in arabinose:xylose:glucuronic acid ratio (Ara:Xyl:GlcA), and degree of substitution at Xyl with Ara [22].

Endo-(1 → 4)-β-D-xylanase I and III [25] (Xyl I, Xyl III), and (1 → 4)-β-D-arabinoxylan arabinofuranohydrolase [26] (AXH), were purified from *Aspergillus awamori* CMI 142717. α-L-Arabinofuranosidase B (AraB) was purified from *A. niger* by Rombouts et al. [27]. Glucuronoxylan xylanohydrolase [28] (GXH) was a kind gift from Dr. D.J. Nevins (University of California, Davis, USA), and purified from *Bacillus subtilis*. The α-glucuronidase (GlcAase) was purified from *Trichoderma viride* [29], and contained traces of β-xylosidase activity. This impurity did not affect the results.

GAX digest.—A solution of sorghum GAX (1 mg/mL) in 0.05 M NaOAc buffer (1 mL total incubation volume, pH 5.0) was digested with several combinations of Xyl I (0.22 μg protein/mL incubation volume), Xyl III (0.26 μg protein/mL), AXH (0.06 μg protein/mL), AraB (3.27 μg protein/mL), GlcAase (2.60 μg protein/mL), and GXH (0.14 μg protein/mL) for 24 h at 50 °C, mixed head-over-tail continuously, and inactivated (10 min, 100 °C). The degree of degradation was determined by estimating the release of reducing end-groups according to Somogyi [30]. The change in molecular-mass distribution was determined by high-performance size-exclusion chromatography (HPSEC) as described elsewhere [22]. The release of oligomeric end-products was studied by high-performance anion-exchange chromatography (HPAEC) as described elsewhere [24].

Methylation analysis was performed as described previously [22]. Samples were carboxyl reduced [31] using $NaBD_4$, prior to methylation by a modified Hakomori method [32]. On line liquid chromatography–mass spectrometry (LC–MS) was performed as described by Niessen et al. [33], using a HPAEC system run under the same conditions as described before [24] coupled to a Finnigan MAT (San Jose, CA, USA) thermospray interface fitted on to a Finnigan MAT TSQ-70 tandem mass spectrometer.

3. Results and discussion

Digestion by purified enzymes.—The GAX fractions isolated from sorghum appeared to be poorly xylanase-degradable (Table 1). A synergistic effect was observed by supplementing Xyl I with AXH showing an increase in the degree of degradation by 2 to 5 times. The maximum degree of degradation was observed with BE1.1 digested by a combination of Xyl I, GXH and AXH (11.7%), 4K was degraded

Table 1
Degradation (%; Nelson–Somogyi assay, standard Ara), and release of Ara (% of Ara present, HPAEC) of three populations of sorghum GAX by combinations of Xyl I[a], Xyl III[b], GXH[c], AXH[d], and GlcAase[e]

	Degradation (%)			Ara-release (%)		
	BE1.1[f]	1K4[g]	4K[h]	BE1.1	1K4	4K
Xyl I	1.6	3.0	0.4	0.0	0.0	0.1
Xyl III	0.3	0.4	0.6	0.2	0.0	0.1
GXH	1.4	0.0	0.0	–[i]	–	–
AXH	3.3	1.7	1.1	9.5	5.5	2.7
GlcAase	0.0	0.0	0.0	0.0	0.0	0.0
Xyl I + GXH	3.5	4.8	1.0	–	–	–
Xyl I + AXH	7.9	6.4	2.1	9.3	4.9	3.3
Xyl I + GlcAase	2.0	3.9	0.3	0.0	0.0	0.8
Xyl I + GXH + AXH	11.7	9.2	3.5	–	–	–
Xyl I + AXH + GlcAase	9.3	8.0	2.9	9.5	5.6	3.2
Xyl III + AXH	4.2	3.1	1.1	10.1	6.0	3.2
Xyl III + GlcAase	0.3	0.7	0.9	0.1	0.0	0.1
Xyl III + AXH + GlcAase	4.8	3.9	1.4	10.4	6.3	3.1
GXH + AXH	7.2	3.1	1.5	–	–	–
GXH + GlcAase	3.0	0.5	0.3	–	–	–
AXH + GlcAase	4.1	1.8	0.9	10.3	5.6	2.8

[a]Endo-(1 → 4)-β-D-xylanase I; [b]endo-(1 → 4)-β-D-xylanase III; [c]glucuronoxylan xylanohydrolase; [d](1 → 4)-β-D-arabinoxylan arabinofuranohydrolase; [e]α-glucuronidase; [f]saturated barium hydroxide-extracted GAX; [g]GAX extracted with 1 M KOH; [h]GAX extracted with 4 M KOH; [i]not determined.

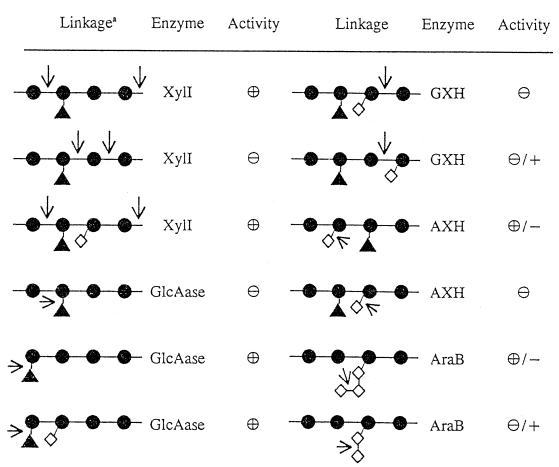
very poorly by any of the enzyme combinations. Degradation of 1K4 was intermediate with a degradation maximum of 9.2% (Table 1).

Another Ara-releasing enzyme, AraB, was also tested in sorghum GAX degradation. Interestingly, AraB could remove some Ara, although less than AXH. These Ara units, however, were different than the ones split off by AXH. This was observed in studies in which AraB and AXH were combined. The total amount of Ara was exactly the same as the sum of the Ara released by the two enzymes separately (results not shown).

The mode of action of Xyl I and Xyl III upon neutral wheat and barley arabinoxylans has been established [34]. In short, Ara-substitution restricts the action of both xylanases, however, Xyl I is affected less than Xyl III. These differences explain why Xyl I gives a higher degree of degradation of such a highly substituted arabinoxylan like sorghum GAX than Xyl III. Xyl III hardly acts upon the GAX populations. This means that these substrates essentially do not possess the three required adjacent unsubstituted Xyl residues [34]. The influence of a GlcA substituent on the mode of action of Xyl I and Xyl III is unknown.

GXH is only active on BE1.1. The GXH recog-

Table 2
Summary of the most important new insights, obtained in the present study, on the mode of action of Xyl I, GXH, AXH, AraB and GlcAase on some glucuronoarabinoxylan structures



[a]Linkage to be cleaved is indicated by the arrows; ⊕ activity; ⊖ no activity; ⊖/+ doubtful activity; ⊕/− expected activity; ●: Xyl *p*; ◇: α-Ara*f*; ▲: α-GlcA; ●-●: β-Xyl *p*-(1 → 4)-Xyl *p*; ◇●: α-Ara*f*-(1 → 3)-β-Xyl *p*; ▲●: α-GlcA-(1 → 2)-β-Xyl *p*; ◇◇: α-Ara *f*-(1 → 2)-α-Ara*f*; ◇◇: α-Ara*f*-(1 → 5)-α-Ara *f*.

nizes the presence of GlcA as single unit side chain and hydrolyses the β-(1 → 4)-Xyl backbone in such a way that the GlcA substituent is situated at the penultimate Xyl residue from the reducing end [28]. Preliminary results from our laboratory have shown that substitution by 4-O-methyl-glucuronic acid (4OMeGlcA) was not recognized by this enzyme (results not shown). The GXH preparation, active on sorghum GAX, was free from arabinofuranosidase, xylosidase and GlcAase, since neither Ara nor Xyl and GlcA were released. A typical substitution pattern in 1K4 and 4K might be the cause for their resistance to GXH attack. It can be hypothesized that, e.g., arabinosylated Xyl residues near the cleaving site for GXH inhibit degradation (Table 2). Another possibility is that O-3- and O-2-mono-arabinosylated Xyl at specific positions, probably near the GlcA-substituted Xyl residue towards the reducing terminus, may play a role. The activity of GXH is not influenced by AXH treatment. AXH in its turn might be hindered by the presence of GlcA-substitution or oligomeric Ara side chains in the vicinity of mono-arabinosylated Xyl residues, and will not split off these Ara's. This needs further clarification.

AXH acts specifically upon (glucurono-) arabinoxylans, not on arabinans or arabinogalactans [26], and is capable of releasing Ara from O-3-monosubstituted Xyl [35]. The Ara-releasing capacity of AXH in the case of sorghum GAX is presented in Table 1. Only small amounts of the total Ara units present were released by using the specified enzyme combinations. In incubations with BE1.1 ~10% of the Ara originally present was released. Relatively less Ara was released in incubations with 1K4 (~6% of total Ara) and 4K (~3% of total Ara) as substrates. Methylation analysis indicated that AXH alone released ~50% of all Ara-residues substituted at the O-3-monosubstituted Xyl units in the (1 → 4)-β-D-xylan backbone of BE1.1 (results not shown). For wheat arabinoxylan, this value was close to 100% [26], and this amount corresponds to 33% of the total amount of Ara present. Apparently, AXH is inhibited to remove the remaining Ara from the mono-arabinosylated Xyl residues in sorghum GAX, probably for reasons mentioned previously.

AraB is reported to split preferentially the α-(1 → 5)-linked Ara in arabinans [27]. Also action on wheat arabinoxylan has been observed [26]. Studies on oligomers showed that AraB cleaves the linkage between an Ara unit substituted at O-3 of a non-reducing terminal Xyl unit [35]. Sorghum GAX does contain small Ara chains as substituents [24]. Although

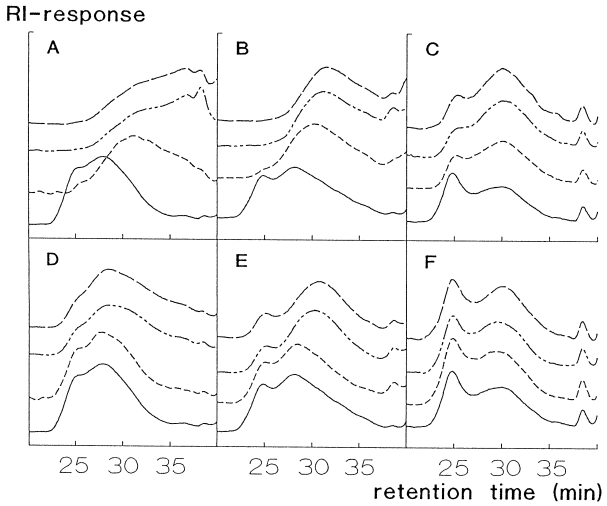


Fig. 1. HPSEC analysis of the incubation mixtures of GAX with combinations of Xyl I, Xyl III, AXH, and GlcAase. A and D substrate BE1.1; B and E substrate 1K4; C and F substrate 4K; A, B and C digested with Xyl I combined with accessory enzymes; D, E and F digested with Xyl III combined with accessory enzymes; ——— substrate blank; ----- + Xylanase; —·---·— + Xylanase + AXH; – + Xylanase + AXH + GlcAase.

only indicated by methylation analysis, these could be like α-Ara*f*-(1 → 5)-α-Ara*f*-(1 → 2)-α-Ara*f*-(1 → 3)-Xyl*p*, Xyl being part of the backbone. It is thought that AraB can cleave the α-(1 → 5)-linkage in the side chain, but not the other linkages present (see Table 2). This explains why AraB does not act synergistically with AXH, Xyl I or GXH.

Endo-type degradation of GAX was followed by studying shifts in molecular-mass distribution by HPSEC which are summarized in Fig. 1. Graph A shows that Xyl I shifts the molecular-mass distribution of BE1.1 toward the lower molecular-mass ranges. Its average molecular-mass decreased from 210,000 [22] to ~45,000, thus, from dp ~1500 to ~300. These calculations were done using 140 as the average molecular-mass per sugar unit, since the average Xyl:GlcA ratio ≈4:1. The downward shift of the molecular-mass of BE1.1 is enhanced by supplementation with AXH. GlcAase had no effect on the molecular-mass distribution. Similar effects were seen for 1K4 (B), although the shift in retention time was not as extensive as for BE1.1. The average molecular-mass of the 1K4 population decreased from 917,000 [22] to ~160,000, a five- to six-fold reduction of the dp from 6500 to ~1100. Incubations of 4K with combinations of Xyl I and the accessory enzymes (C) showed no obvious shift in molecular-mass distribution, confirming the data in Table 1.

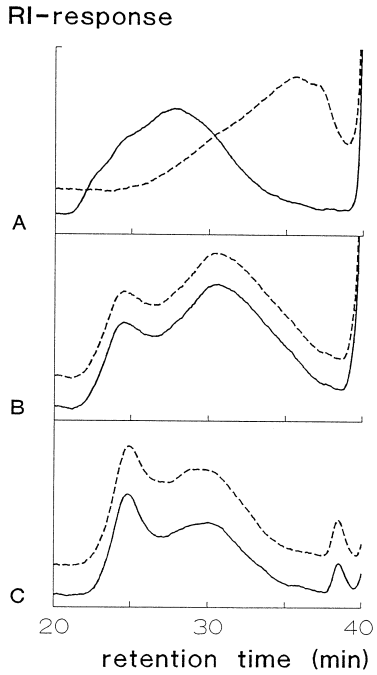


Fig. 2. HPSEC analysis of BE1.1 (A), 1K4 (B) and 4K (C) digested by GXH; ——— substrate blank; ---- + GXH.

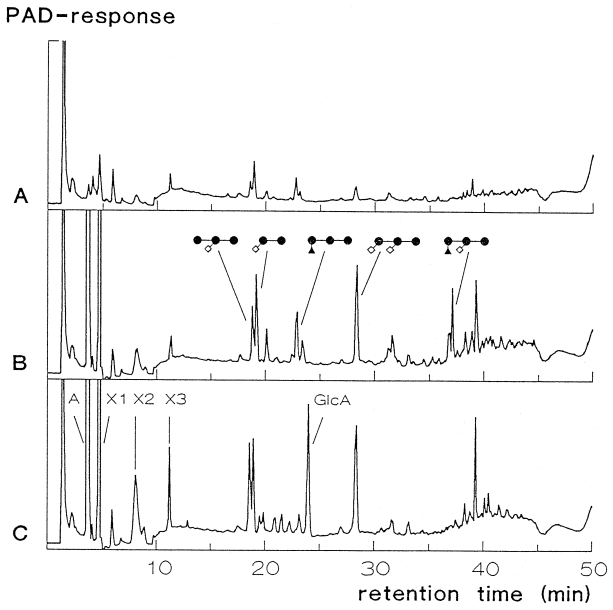


Fig. 3. HPAEC patterns of BE1.1 digested by A, Xyl I; B, Xyl I + AXH, and C, Xyl I + AXH + GlcAase. ●: Xyl *p*; ◇: α-Ara*f*; ▲: α-GlcA; ●-●: β-Xyl *p*-(1 → 4)-Xyl *p*; ◇-●: α-Ara*f*-(1 → 3)-β-Xyl *p*; I: α-GlcA-(1 → 2)-β-Xyl *p*.

Comparison of graphs D, E and F with the corresponding graphs A, B and C shows that the action of Xyl III was very limited on sorghum GAX. For BE1 and 1K4, only a slight downward shift in molecular-mass distribution could be observed, whereas for 4K no change was measured for any combination of enzymes. A shift in molecular-mass distribution by GXH was only seen with BE1.1 and neither with 1K4 nor 4K (Fig. 2). The effects of supplementing the incubation mixtures with the accessory enzymes was negligible for all the substrates.

Fig. 3 shows HPAEC elution patterns for BE1.1 digested with Xyl I, Xyl I + AXH and Xyl I + AXH + GlcAase. Incubation with only Xyl I (A) resulted in small amounts of oligomeric reaction products. The concentration of most of these products could be increased by addition of AXH (B) to the reaction mixture. Taking the PAD response factors into account, both Ara (RT = 3.3 min) and Xyl (RT = 4.3 min) were released in relatively high amounts by this enzyme combination, whereas xylobiose (RT = 7.8 min) and xylotriose (RT = 11.7 min) occurred in only low amounts. The neutral compounds were identified by using purified, ^{1}H NMR identified arabinoxylan oligomers [36] as standards. They were eluted in one peak with the corresponding indicated peaks. Further confirmation was performed by LC–MS (data not shown).

Digestion of the GAX populations by Xyl I + AXH resulted in some oligomers which served as substrates for GlcAase (Fig. 3C). GlcA was clearly detected as degradation product by HPAEC, and no

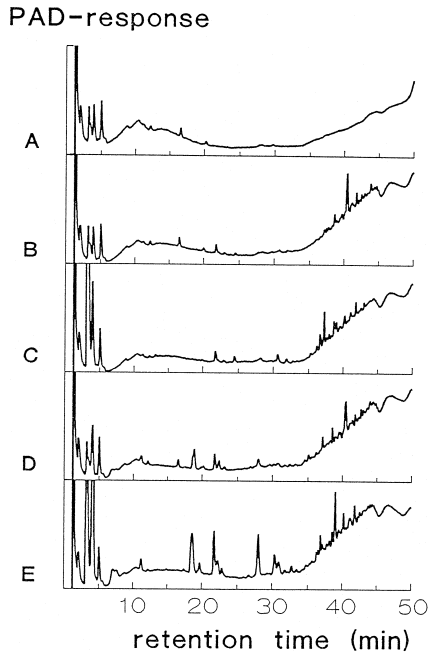


Fig. 4. HPAEC pattern of BE1.1 (A, blank) digested by GXH (B); GXH + AXH (C); GXH + Xyl I (D), and GXH + Xyl I + AXH (E).

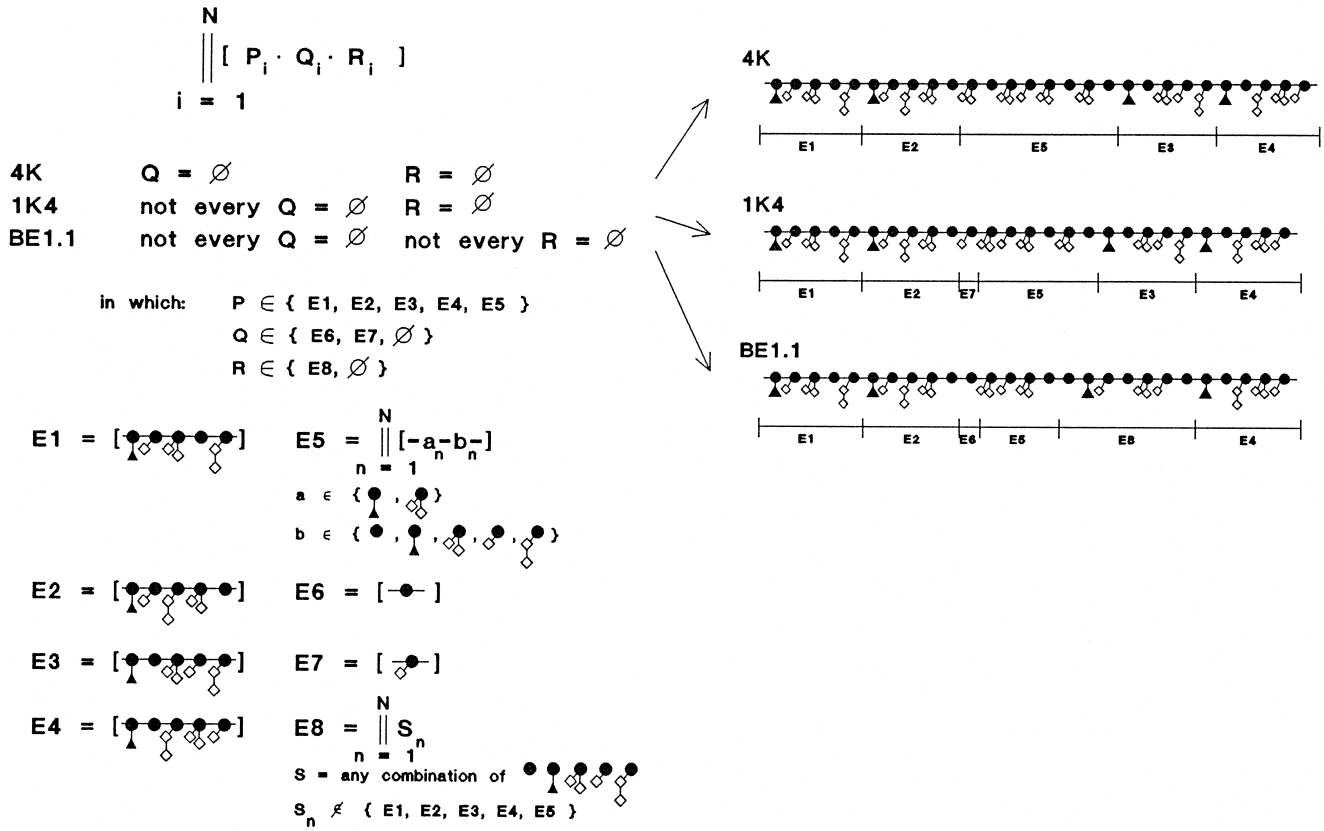


Fig. 5. Tentative model for sorghum glucuronoarabinoxylans built of 8 elements (E1 to E8). E1 to E5 represent undegradable combinations of substituted and unsubstituted Xyl units. E6 and E7 represent interrupting elements. E8 represents any degradable combination of Xyl units, either substituted or not. These elements are collected in P (responsible for an undegradable structure), Q (responsible for a degradable structure, however, hardly resulting in small oligomers) and R (responsible for a degradable structure, resulting in small oligomers). (See *Structural features of sorghum GAX* for further explanation). ∅: Empty set; N: Number of repetitions starting from 1 (*n*, *i*); ●: Xyl *p*; ◇: α-Ara*f*; ▲: α-GlcA; ●-●: β-Xyl *p*-(1 → 4)-Xyl *p*; : α-Ara*f*-(1 → 2)-β-Xyl *p*; α-Ara*f*-(1 → 3)-β-Xyl *p*; : α-GlcA-(1 → 2)-β-Xyl *p*; : α-Ara*f*-(1 → 2)-α-Ara*f*.

other uronic acids could be identified. The identity of the oligomer which was eluted at RT = 37 min was α-D-Glc*p*A-(1 → 2)-β-D-Xyl *p*-(1 → 4)-[α-L-Ara*f*-(1 → 3)]-β-D-Xyl *p*-(1 → 4)-β-D-Xyl *p* [30]. The addition of GlcAase to the reaction mixture resulted in the formation of β-D-Xyl *p*-(1 → 4)-[α-L-Ara*f*-(1 → 3)]-β-D-Xyl *p*-(1 → 4)-β-D-Xyl *p*. The peak area of this compound increased relatively to that of α-L-Ara*f*-(1 → 3)-β-D-Xyl *p*-(1 → 4)-β-D-Xyl *p*, and no other peaks were formed. Also α-D-Glc*p*A-(1 → 2)-β-D-Xyl *p*-(1 → 4)-β-D-Xyl *p*-(1 → 4)-β-D-Xyl *p* disappeared upon GlcAase treatment, leading to the formation of xylotriose. The GlcAase can split off a GlcA unit from a Xyl unit at the non-reducing side (Table 2).

Digestion of BE1.1 with GXH resulted in larger oligomeric reaction products (Fig. 4, RT > 37 min). The main component eluted at RT = 40.2 min, and contained Ara since this peak disappeared when AXH was added to the reaction mixture. In this case, a newly formed peak at RT = 37 min, and an Ara peak were noticed. Interestingly, AXH and GXH showed no synergism, and GXH degradation products were not degraded by Xyl I, and vice versa. The oligomers originating from GXH digestion were not degraded by GlcAase (results not shown). It can be concluded that the GlcAase is not capable of splitting off the GlcA, α-(1 → 2)-linked to a Xyl residue adjacent to the reducing Xyl residue.

This research has been carried out on alkali-extracted GAX populations. In the intact cell walls, these polysaccharides are much more complex, because they contain acetyl groups, probably linked to Xyl residues and feruloyl groups linked to the Ara side groups [21]. Moreover, the accessibility of the polymers for enzyme action will be further reduced

by their 'fixation' in the cell wall matrix by hydrogen bonds and probably covalent (diferulic bridges) and/or ionic bonds. The above, together with the poor degradability of the polymers may explain why cell walls remain intact during malting.

Structural features of sorghum GAX.—Taking in consideration the chemical and structural features of the sorghum GAX populations (sugar and glycosidic linkage composition [22]) and the modes of action of Xyl I [34] and AXH [35], and also the new insights summarized in Table 2, we propose an 'overall' sorghum GAX structure composed of structural units presented in Fig. 5. We have used 4K as a starting point, because this population is very enzyme-resistant. This means that the distribution of side chains is such, that incubation with Xyl I and AXH gives no degradation. Only a few ways of substitution can meet this condition, i.e., elements E1 to E5. It is thought that the structure of 4K GAX is strictly built up of these five structural units, resulting in the presented example (Fig. 5). The backbone of this structure will not be degraded by Xyl I and AXH. In addition, these kind of structures can theoretically not be degraded by GXH either [28], which is supported by the results. The Ara present as single substituent at O-3 of Xyl can theoretically be released by AXH. Most probably, however, the Ara at an O-3-monosubstituted Xyl adjacent to the reducing side of the GlcA-substituted Xyl unit, will not be removed by AXH (Table 2). In the presented model for 4K, AXH activity will not create cleavage sites for Xyl I.

Although indications were obtained, that Ara units and short side chains linked at O-2 of Xyl in the backbone were present [24], these structures were not integrated in the overall model for sorghum GAX. From the methylation analyses of the GAX populations [22], it can be expected that these structures are present in rather low amounts.

The structures of BE1.1 and 1K4 are probably (small) modifications of the 4K model. It is believed that 1K4 is built by the same structural elements as 4K. This '4K like polymer' however, is probably interrupted by an introduction of an un- or O-3-substituted Xyl unit (E6 and E7) between the elements E1 to E5, resulting in structures like presented in Fig. 5B. This may explain that 1K4 is degraded to a certain extent (shift in molecular-mass distribution) with little release of small oligomers. Degradation of the BE1.1 fraction resulted in the release of small oligomers. Therefore, it is believed that, additionally to the 1K4 structure, interruptions also occur within the elements E1 to E5, and thereby introducing degradable parts in the polymer. These smaller degradable parts are represented by E8 in Fig. 5: any combination of Xyl units, substituted or not, other than E1 to E5, will result in degradable parts.

With this knowledge about the structure of sorghum GAX, it can be understood that sorghum cell walls are hard to degrade when the right enzymes are not present. The enzymes used in the present study failed in degrading extracted GAX. Other enzymes are necessary, such as a glucuronidase active on polymeric substrates or arabinofuranosidases which can cleave Ara units from O-2-substituted, or disubstituted Xyl residues. For the degradation of crude cell wall material also acetyl- or feruloyl-esterases might be important. These enzymes are probably not synthesized or activated sufficiently in sorghum during malting, since the cell walls are poorly broken down.

Acknowledgements

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