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Resistant starch derived from processed legumes—purification and structural characterization

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Abstract

Purified resistant starch (RS), derived from processed (4 cycles) Bengal gram and red gram *dhals*, was of low molecular weight, 31.1 and 50.1 KDa, respectively, X-ray analysis revealed mixed polymorphic forms (C and V-types), which are characteristic of gelatinized starch suspensions. Permethylation analysis showed branched nature of RS having both 1,4-and 1,6-linkages, which was supported by ^{13}C NMR data.

Keywords: Legumes; Processing; Resistant starch; ^{13}CNMR; X-Ray diffraction

1. Introduction

Legumes occupy an important place in the diet of population in third world countries. They are rich sources of 'lente' carbohydrates, which are useful in some of their beneficial physiological effects (Tharanathan, 2002). Slow digestibility of legume starches has been explained as due to highly branched amylose and amylopectin, their molecular weight and chain length, and other intrinsic factors (Madhusudhan & Tharanathan, 1996a,b). Prior to consumption legumes are generally subjected to a wide variety of processing (Thermal processing and germination) treatments, and obviously they may bring in structural-functional property changes in some of their constituents. Resistant starch (RS) is the outcome of such a change induced by starch retrogradation, and it is attributed to extensive hydrogen bonding network leading to amylolytic resistance (Englyst, Kingman, & Cummings, 1992). Nevertheless, upon colonic fermentation RS is degraded to produce preferentially an enhanced butyric acid level, which is physiologically a very active and useful biomolecule of therapeutic significance (Phillips, Muir, Birkett, Lu, Jones, O'Dea, & Young, 1995; Scheppach, Sachs, & Kasper, 1988). Based on this attribute RS is considered as the new generation fibre, singled out as particularly valuable in maintaining intestinal balance. Very little information is available on the chemical nature of RS derived from processed legumes, and in this communication an attempt is made to bridge this gap.

2. Materials and methods

2.1. Materials

Standard varieties of Bengal gram (Chickpea, *Cicer arietinum*) and red gram (Pigeon pea, *Cajanus cajan*) dhals (dehusked split seeds) were purchased locally. The cleaned dhals were subjected to pressure-cooking with water (1:2, w/v) for 15 min (121 °C, 1 bar) and cooled to room temperature (1 h) followed by refrigeration cooling (4 °C) overnight (12 h). The entire processing treatment was repeated four times and finally the cooked dhals were oven dried and powdered (60 mesh) in Apex mill.

All chemicals/reagents were of analytical grade. Amyloglucosidase (E.C 3.2.1.3, 10 units mg^{-1} solid), D-glucose oxidase (E.C.1.11.1.7, 100 units mg^{-1} solid), protease (E.C.3.2.24) were from Sigma Chemical Co., USA. Thermostable α-amylase (Termamyl) was from Novo, Denmark.

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2.2. General methods

Total sugar, reducing sugar and D-Glc were determined by the modified phenol–H_2SO_4 (Rao & Pattabiraman, 1989), DNS (Miller, 1959) and D-Glc oxidase (Dahlqvist, 1964) methods, respectively using D-Glc or maltose as reference compounds. Powder X-ray diffraction patterns were obtained using a EG-7G solid state germanium liquid N_2 cooled detector Scintag XDS-200 instrument equipped with a θ–θ goniometer at 30 kV and 25 mA with a Cu kα, radiation (λ1.54104 Å). The relative intensity was recorded in the scattering range (2θ) of 4–40°. ^{13}C NMR spectra in d_6 DMSO were recorded at a probe temperature of 80 °C, 100 MHz in the Bruker AMX-400 spectrometer. A contact time of 1 ms and a pulse repetition time of 5 s were used and more than 8000 scans were accumulated in a pulsed FT mode with complete proton decoupling. Tetramethylsilane was the external standard used. IR spectra were recorded in KBr discs on a Perkin Elmer 2000 FTIR spectrometer under dry air at room temperature. GLC of alditol acetates was performed as before (Madhusudhan & Tharanathan, 1996b). DSC analysis was carried out with Rheometric Scientific, UK, instrument equipped with thermal software version 5.40. The samples (5 mg) were heated from 40–120 °C at 10 °C min^{-1} and a minimum of two measurements was done for each sample.

2.3. RS isolation

RS was isolated from processed legume flours as described earlier (Mangala, Malleshi, Mahadevamma, & Tharanathan, 1999; Sievert & Pomeranz, 1989). The crude RS was purified by $HClO_4$ solubilization followed by GPC on precalibrated (with T-series dextrans of known MW) Sepharose CL-2B. The separated fractions were analyzed for total sugar and starch-I_2 blue colour, and their MW determined. SE-HPLC on E-linear and E-1000 μ-Bondagel columns (Waters Associates, Millford, USA; ss, 3.9 mm × 30 cm) connected in series with a guard column was performed on a Shimadzu HIC-6A chromatograph as described before (Kobayashi et al., 1985).

2.4. Permethylation analysis

The purified RS was permethylated by the Hakomori method (Hakomori, 1964) and the products cleaned by passing through Sep Pak C 18 cartridges. After hydrolysis with formic acid-H_2SO_4, the partially methylated alditol acetates were prepared (using NaB^2H_4 in ^2H_2O) and analysed on a high performance quadrupole Shimadzu QP-5000 mass spectrometer combined with GC-17A and fitted with SP-2330 fused silica capillary column (30 m × 0.32 mm i.d., film coating thickness 0.02 μ; Supelco, USA). The operating conditions were temperature programme mode, 150–200 °C at 2 °C min^{-1}, ionizing voltage of 70 eV, detector voltage of 1.15 kV, mass range 40–400 amu, and 4 msec $scan^{-1}$.

3. Results and discussion

Among the different processing treatments (Mahadevamma, and Tharanathan, Unpublished data) attempted the five-cycle pressure-cooked legume dhal showed relatively a higher RS content (1.3–1.5%). It is known that $HClO_4$ is a very good solvent for the solubilization as well as separation of starch components, and the method has successfully been used earlier to extract as well as to separate starch components (Taki, 1962). The method causes no degradation and leaves behind the contaminating non-starch polysaccharides. The crude RS upon solubilization in cold $HClO_4$ gave an acidic supernatant which after dialysis and Sepharose CL-2B chromatography gave a single peak in both the legume RS. Nevertheless, their GLC analyses showed in addition to glucose (>85%) arabinose, xylose and mannose in varying proportions. The latter might have originated from strong polymer-polymer interactions between RS and other non-starch polymers, which could not be completely removed in spite of repeated purification steps. Profiles of SE-HPLC also showed a single peak, having MW of 50.1 and 31.1 kDa, respectively for red gram RS and Bengal gram RS, which corresponds to approximately 309 and 191 anhydroglucose residues. No such contaminating polysaccharides were observed in RS derived from processed cereals (Mangala & Tharanathan, 1999; Mangala, Malleshi, Mahadevamma, & Tharanathan, 1999; Sievert & Pomeranz, 1989). It is reported that a minimum chain length of 30–40 glucose residues is required for RS formation (Shukla, 1995). Nevertheless, longer glucan chains promote while their complexing with lipids (due to helical conformation) prevents RS formation.

X-ray diffraction patterns of RS were typical of mixed polymorphic forms i.e. C and V types. The latter is found especially in gelatinized starch suspensions (Buleon, Bizoi, Delags, & Pontoir, 1987; Zobel, 1964), and due to their poor crystallinity and poor water of hydration the diffraction peaks were not that sharp. Well-hydrated starches are known to give accentuated diffractogram with rather well defined peaks (Zobel, 1988). The crystallinity index normally increases when gelatinized starch suspensions are stored for long time at slightly elevated temperatures (Sosulski, Waczkowski, & Hoover, 1989). Nevertheless, the relative abundance of these diffractions and the scattering intensity (Table 1) are essentially dependent on the proportion and length of the branching residues (Nelson, 1961) as well as the nature of the processing regimen (temperature, duration and relative humidity), with the result there is a likelihood of obtaining a continuous transition from one form to the other form.

Bengal gram RS showed first endothermic peak at 63.2 °C and two sharp minor endothems at around 99.7° and

Table 1
Characteristics of RS of Bengal gram and red gram

RS	Yield (%)	MW kDa	X-Ray data			DSC data				
			d spacing, Å	2θ	Intensity[a]	T_0 (°C)	T_p (°C)	T_c (°C)	$\Delta T(T_c - T_0)$	ΔH (Cal g^{-1})
Bengal gram RS	1.31	31.1	7.92	11.16	S	61.1	63.2	73.5	12.4	1.53
			4.36	20.36	M					
			3.90	22.80	M					
			3.50	25.70	S					
			3.34	26.60	W					
			3.11	28.60	M					
			2.75	32.60	W					
			2.70	34.00	W					
Red gram RS	1.49	50.1	7.74	11.42	S	45.2	67.2	73.5	28.3	0.68
			5.03	17.60	M					
			4.31	20.00	W					
			3.42	20.60	M					
			3.35	26.00	S					
			3.11	28.60	W					
			2.84	31.40	W					
			2.73	32.10	W					

[a] S, strong; M, medium; W, weak.

96.2 °C (Table 1). The former may be attributed to the unmodified starch having a random coil amylose in retrograded crystallites. Red gram RS showed an endothermic peak temperature at 67.2 °C. In both RS, the endothermic peak was broad and variations in their enthalpy (ΔH) values were attributed to the overall gelatinization process, which is influenced by the associated polymeric constituents, which also decreases the net content of starch per se. Variation in the water content of the sample also alter the overall thermal characteristics, for instance, water exerts a plasticizing effect and results in a decrease in T_g and increase in ΔH values (Normand & Marshall, 1989).

The sharp appearance (due to –C=O absorption) of peaks at 1653/1644 cm^{-1} for Bengal gram RS and 1653/1555 cm^{-1} for red gram RS in the IR spectra may be due to the formation of more number of potential reducing end groups after thermal modification of starch into RS (Erlingen, Deccunick, & Delcour, 1993; Gidley, Cooke, Darks, Hoffmann, Russell, & Greenwell, 1995). It is plausible that during thermal processing a few amylose chains may leach out from the granules into the aqueous medium, which upon cooling begin to reassociate as double helices stabilized by innumerable hydrogen bonds.

The glycosidic linkage pattern in RS was ascertained by permethylation followed by GC-MS analysis. The results (Table 2) clearly showed the complexity of the molecular structures, especially of red gram RS. The presence of a major peak 2,3,6-tri-O-methyl-D-glucose indicated a 1,4-linked D-glucan backbone, which appears to be additionally involved in 1,6-branching (identification of 2,3-di-O-methyl-D-glucose). Possibly the legume RS might be derived from slightly branched amylose fraction (Madhusudhan & Tharanathan, 1996b) unlike in the case of cereals. Thus, a structure reminesent of amylopectin molecule (which has ~5% of branching) was observed for both RS fractions. Comparatively, Bengal gram RS appeared to be simpler in its structure, although it too has some contamination with non-starch polysaccharide(s). The latter (arabinoxylans-type) was indeed highly branched as evident from the identification of 2-mono-O-methyl-D-xylose in small amounts. Their mass fragmentation data were in good agreement with the assigned substitution pattern. The glucomannan type polysaccharide contaminating the red gram RS is having 1,2-and 1,6-linked D-mannose residues in the backbone, which are further involved in some branching (identification of 3,6-di-and 2-mono-O-methyl mannoses). In spite of repeated purifications by alkali and $HClO_4$ solubilization and GPC methods, these contaminating polysaccharides still persisted in the so-called 'purified' RS. Polymer-polymer association leading to strong macromolecular network is common in plant polysaccharides (Tharanathan, 1977). Close association of glucomannan, xylan and micro-fibrillar cellulose is well established in softwoods (Nelson, 1961; Timell, 1965). It is stated that the glucomannan is located deeper inside the framework of the cellulose (β-D-glucan) microfibrils than is the xylan, and is therefore difficult to purify. Being structurally more similar to the glucan molecule, their close association is very likely.

The RS isolated from both pea amylose and pea starch gels was almost entirely composed of linear amylose chains, and having a branched material comprising 0.7% enzyme resistant residue from the hydrolyzed amylose gel and 2.2% of the residue from the hydrolyzed starch gel (Cairns, Morris, Botham, & Ring, 1996). Methylation analysis of enzyme resistant residue from chickpea gel and in vivo RS recovered from the sample of ileostomy effluent showed somewhat a higher proportion of the branched materials,

Table 2
Permethylated alditol acetates derived from RS of Bengal gram and red gram

O-Methyl ether	R_t[a]	Mode of linkage	Approximate percentage		Diagnostic mass fragments, *m/Z*
			Bengal gram RS	Red gram RS	
2,3,4-Me_3-Xyl	0.64	Xyl(1 →	2.62	2.40	43, 87, 101, 102, 118, 129, 145, 162, 205,
2,3,4,6-Me_4-Glc[b]	1.00	Glc(1 →	6.56	7.10	43, 45, 87, 101, 102, 118, 161, 162, 205
2,3-Me_2-Ara′	1.17	→ 5)Ara(1 →	4.80	3.84	43, 87, 99, 102, 118, 129, 162, 201, 233
2,3-Me_2-Xyl′	1.28	→ 4)Xyl(1 →	2.46	5.47	43, 87, 102, 118, 129, 162, 201, 233
3,4,6-Me_3-Man	1.45	→ 2)Man(1 →	–	2.50	43, 45, 87, 101, 129, 130, 161, 190, 205, 234
2,3,4-Me_3-Man	1.55	→ 6)Man(1 →	–	11.34	43, 87, 99, 102, 118, 129, 131, 162, 201, 203, 233
2,3,6-Me_3-Glc	1.80	→ 4)Glc(1 →	74.62	49.26	43, 45, 87, 99, 102, 118, 129, 162, 233
2-Me-Xyl′	1.89	→ 3,4)Xyl(1 →	3.35	2.49	43, 87, 118, 129, 143, 201, 261
2,3-Me_2-Glc	2.21	→ 4,6)Glc(1 →	5.59	5.15	43, 87, 99, 102, 118, 162, 201, 261, 305
3,6-Me_2-Man	2.39	→ 2,4)Man(1 →	–	3.35	43, 45, 87, 99, 113, 130, 190, 201, 233
2-Me-Man	2.81	→ 3,4,6)Man(1 →	–	7.04	43, 87, 99, 102, 118, 173, 303

[a] Relative to 2,3,4,6-tetra-O-methyl-1,5-di-O-acetyl-D-glucitol.
[b] 2,3,4,6-Me_4-Man also has the same retention time.

4.2 and 5.5%, respectively. On the other hand the RS isolated from processed cereals rice and ragi (Mangala, Malleshi, Mahadevamma, & Tharanathan, 1999) and wheat (Mahadevamma & Tharanathan, 2001) was shown to be essentially a linear 1,4-D-glucan with a molecular weight of ~40 kDa.

Additional information on the linkage and the anomeric ring carbons in the RS was deduced by ^{13}C NMR spectral data (Table 3). In agreement with literature data (Colquhoun, Parker, Ring, Sun, & Tang, 1995; Gidley, 1985; McIntyre, Ho, & Vogel, 1990), the spectral signals in the region 100.38, 71–79 and 60–62 ppm were assigned to carbon nuclei C1–C6 of α 1,4-D-glucan. The presence of doublet in the region 100–102 ppm was attributed to C1 of the B-type crystalline polymorph of RS (Gidley & Bociek, 1988). Less crystalline starches are however devoid of this doublet. The anomeric signal at ~100 ppm (well resolved from signals of other nuclei) was assigned to α-configuration. In their study of genetically modified potato starch (waxy types), new chemical shift values of 102.1 and 102.4 ppm have been assigned for 1,4-and 1,6-linked C1, respectively (Dais & Perlin, 1982). The signals in the region 71–76 ppm are attributed to C4, C2, C3 and C5, whereas those in the region 60–62 ppm are assigned to C6. Bengal gram RS showed two separate peaks for C3 and C5 at 71.9 and 71.6 ppm, respectively. Unlike the multitude of signals observed in the ^{13}CNMR spectrum of cyclodextrins (Gidley & Bociek, 1988), the spectrum of RS like that of V-amylose contained no more than one signal for each carbon nucleus. Compared to solid-state ^{13}CNMR data, all the signals in the solution state in general showed slight chemical shift variations. The two carbon nuclei C1 and C4, both involved in glycosidic linkages, usually are most sensitive for changes in polymer conformations. The solution of red gram RS (in d_6-DMSO) was viscous, probably because of the contaminating polymers and its spectrum was of relatively poor resolution.

Table 3
^{13}C NMR data of RS derived from Bengal gram and red gram

	C1	C4	C2	C3/C5	C6
Bengal gram RS	100.1	78.6	73.4	71.9, 71.6	60.8
Red gram RS	100.4	79.0	73.5	71.8	60.7

Chemical shift values in ppm

4. Conclusions

Thus, it can be concluded that the RS derived from processed Bengal gram and red gram dhals is a branched α D-glucan, possibly derived from the branched amylose fraction of starch. It is likely that the ratio of Am to Ap, their degree of branching and the length of the extended outer chains of Ap may all contribute to the formation of RS from processed legumes.

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