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Structural studies of linear and branched fractions of chickpea and finger millet starches [1]

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Abstract

Structural studies using beta-amylase and pullulanase were carried out to explain at a molecular level the differences in the digestibility of legume (chickpea, Bengal gram, BG) and cereal (finger millet, ragi, Rg) starches. In vitro, BG starch was less digestible (~ 45%) with glucoamylase than the Rg starch (> 70%). Of the various fractionation methods employed, concanavalin A precipitation gave pure amylopectin (Ap). The crude amylose on subfractionation with hot 1-butanol yielded pure amylose (Am) and an intermediate fraction (Ax). The content of "true" Am and Ax was higher in BG (33 and 6.5%) than in Rg starch (22 and 6%, respectively). The molecular weight of BG starch fractions was much higher than those of Rg starch fractions. Beta-amylolysis and debranching studies indicated BG-Am to contain sparsely distributed side-chain branches, whereas Rg-Am was more linear. The BG-Ap was comprised of very long B (dp 70), long B (dp 56), long A/short B (dp 25), and short A (dp 17) chains; whereas Rg-Ap had long B (dp 48), short B (dp 25), and short A (dp 17) chains. The structure of Ax lay between those of Ap and Am.

Keywords: Legume; Cereal; Starches; Digestibility; Structural studies

1. Introduction

Starchy foods have always been a dietary item of mankind, with both legumes and cereals constituting the major share of available (starch) and unavailable (dietary fibre) carbohydrates. The popular belief is that legume-based foods, in particular, are less

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digestible and their consumption leads to flatulence and other physiological discomforts [1,2]. It is well known that the digestibility of legume starches is lower than that of cereal starches, whether in the native or gelatinized form [3], and such differences could be attributed to subtle variations in the qualitative and quantitative make-up of starch per se. To a certain extent these variations are also manifested in the wide morphological characteristics of the starch granules; small spherical granules are shown to be more digestible than big hexagonal granules [4]. An attempt was therefore made to carry out enzyme debranching studies of chickpea (Bengal gram, *Cicer arietinum*) and finger millet (ragi, *Eleucine coracana*) starch fractions in order to understand at a molecular level the precise scientific basis for their differences in chemical nature and digestibility values.

2. Experimental

Materials.—Chickpea (Bengal gram, BG) and finger millet (ragi, Rg) were purchased in the local market. Starches were isolated from the 60-mesh flour by the water-steeping method and purified [5]. All chemicals used were of analytical reagent grade. Concanavalin A, Triton X-100, glucoamylase (E.C. 3.2.1.3, 10 units mg^{-1} solid), alpha-amylase (E.C. 3.2.1.1, 695 units mg^{-1} solid), beta-amylase (E.C. 3.2.1.2, 10 units mg^{-1} protein), pullulanase (E.C. 3.2.1.41, 35 units 0.58 mL^{-1}), D-glucose oxidase (E.C. 1.1.3.4, 19 units mg^{-1} solid), and peroxidase (E.C. 1.11.1.7, 100 units mg^{-1} solid) were purchased from Sigma Chemical Co., USA.

General methods.—Total sugar, reducing sugar, and D-glucose were determined by the modified phenol–H_2SO_4 [6], Nelson–Somogyi [7], and D-glucose oxidase [8] methods, respectively, using D-glucose or maltose as reference compound. Amylose was determined by the Chrastil method [9]. Limiting viscosity number $[\eta]$ was determined by extrapolation to zero concentration from a plot of reduced viscosity η_{sp}/C versus concentration (C) [10]. The approximate molecular weight (MW) of the starch fractions was determined by GPC on a precalibrated (with dextrans of known MW) Sepharose CL-2B column (1.7 × 92 cm) eluted (12–18 mL h^{-1}) with water containing 0.02% NaN_3. Size exclusion–high-performance liquid chromatography (SE–HPLC) was performed on a Shimadzu HIC-6A ion chromatograph equipped with a Shimadzu RID-6A refractive index (RI) detector, SCL-6A system controller, CR-2A Chromatopac integrator, and E-linear and E-1000 μ-Bondagel columns (ss, 30 cm × 3.9 mm, i.d., Waters Associates, Milford, USA) connected in series with a guard column [11]. Elution was done with water (0.2 mL min^{-1}) at 40 °C. The V_0 was measured using *Sesbanium* mosaic virus (MW, $\sim 6 \times 10^6$). The native and enzyme-treated starch fractions (20 mg) were separately solubilized in aq Me_2SO (85%, 1 mL) by heating at 95 °C for 5 min, each suspension was centrifuged (2000 rpm for 5 min), and 10 μL of the clear supernatant solution was injected into the SE-HPLC column.

Starch fractionation.—(*a*) *Butanol complexation method.* An aqueous slurry of starch (1 g in 6.5 mL water) was solubilized in dil aq NaOH (0.2 M, 82.5 mL containing 5% NaCl) or Me_2SO (85%, 50 mL) by stirring for ~ 2 h. Any insoluble (non-)starchy material was removed by a brief centrifugation. The alkaline extract was adjusted to pH

7 with dil HCl before use. To the clear supernatant solution was added 1-butanol (2–3 vol) and after 1 h at room temperature the Am–butanol complex [12] was centrifuged out and repeatedly washed with water-saturated butanol. The complex was dissociated by adding boiling water under N_2 atmosphere, cooled, and centrifuged. From the supernatant solution Ap was precipitated by adding EtOH (3 vol).

(*b*) *Concanavalin A precipitation method.* To the starch suspension (250 mg) prepared in NaOAc buffer (pH 6.4, 0.2 M) was added a solution of Con A (600 mg, in NaOAC buffer, pH 6.4, 0.2 M). The mixture was occasionally stirred for 2 h and then centrifuged (10,000 rpm for 15 min). The precipitated Ap was dissolved in NaOAc buffer, the solution heated in a boiling water bath for 10 min and then cooled, the protein removed by the $(NH_4)_2SO_4$ precipitation method, the solution dialyzed, and pure Ap lyophilized [13].

The supernatant solution was concentrated and precipitated with EtOH to recover Am.

(*c*) *Hot 1-butanol fractionation of Am.* Amylose (1 g) from (*b*) was suspended in hot 10% 1-butanol (100 mL, 40 °C for ~ 2 h) under N_2. The insoluble material was isolated by centrifugation and the process repeated twice. The final insoluble material was washed with ethanol and dried. The supernatant solutions were pooled, filtered through a G4 sintered funnel, and concentrated. The excess of butanol was removed by N_2 flushing, and the solution lyophilized [14].

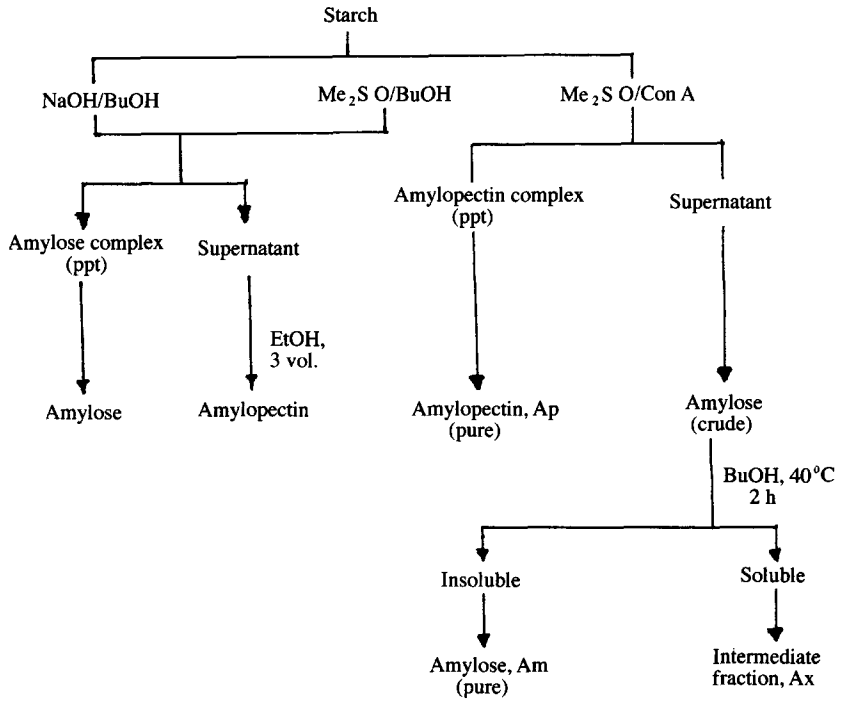
In vitro digestibility of native and gelatinized starches.—Starch (100 mg) suspended in NaOAc buffer (pH 4.8, 0.05 M, 4 mL) was gelatinized by heating in a boiling water bath, cooled to 60 °C, and incubated with glucoamylase (11 units mg^{-1} starch) for 30 min. The excess of enzyme was heat inactivated (~ 100 °C for 5 min), the suspension centrifuged, and the supernatant solution assayed for glucose [8].

Beta-amylolysis.—The starch fraction (50 mg) was solubilized in aq Me_2SO (85%, 2 mL) by heating for 10 min, and the resulting suspension was cooled and centrifuged. The clear supernatant solution was made up to 10 mL with NaOAc buffer (0.1 M, pH 4.8) and incubated at 37 °C for 24 h with beta-amylase (1500 units). The enzyme was heat inactivated and the percentage beta-amylolysis was calculated [15]. The beta-limit dextrin (beta-LD) obtained was subjected to a second beta-amylolysis. The final beta-LD was subjected to GPC on a precalibrated Biogel P-10 column (1.7 × 80 cm).

Debranching with pullulanase.—The starch fractions as well as beta-LDs (20 mg) were debranched with pullulanase (3.2 units in 2 mL of 0.1 M NaOAc buffer, pH 5.5) for 24 h at 37 °C. Each digest was heated and centrifuged [16], and the supernatant solution was analyzed by GPC.

3. Results and discussion

Three independent methods of fractionation (Scheme 1) were employed to separate the individual fractions in BG and Rg starches. The amylose–butanol complexing method, though very simple, did not give pure fractions, unlike the recent Con A precipitation method [13], which gave homogeneous Ap. Hot 1-butanol extraction of crude amylose gave pure Am (butanol insoluble) and an intermediate fraction (Ax,



Scheme 1. Fractionation scheme employed to separate the constituent molecules of BG and Rg starches.

butanol soluble). The amylose content of Rg starch (22%) was considerably less than that of BG starch (39%), whereas the Ax fraction was present in comparable amounts in both starches. However, the "true" Am content, as obtained by the hot butanol extraction method [14], was comparatively much less than that deduced by the "blue value" method [17] (42 and 28%, respectively, for BG and Rg starches). This was indicative of a possible contribution of extended long and unbranched A–B chains of the Ap molecule to the total I_2–KI blue colour yield, as also reported by others [18,19]. As possible corroboration of this, the λ_{max} of BG starch (625 nm) was higher than that of Rg starch (616 nm) (see Fig. 1). In SE-HPLC, the Ax fraction was eluted at 16.26 min, Am at 22.21 min, and Ap at 14.32 min (Fig. 2).

From Table 1, it is clear that the λ_{max} and MW of BG starch fractions were much higher than their corresponding Rg counterparts. However, the limiting viscosity value of BG-Ap, though slightly more than that of Rg-Ap, remained about the same for both the Am fractions.

To understand further their molecular architecture, Am, Ax, and Ap fractions were subjected to beta-amylolysis and pullulanase debranching. In all reactions, the beta-amylase action was complete at the first beta-amylolysis, since repeated enzyme action on the first beta-LD, freed from maltose, did not occur. The absence of glucose in the digests revealed the purity of the enzyme, since α-D-glucosidase is the usual contaminating enzyme found in some beta-amylase preparations [20]. From the beta-amylolysis

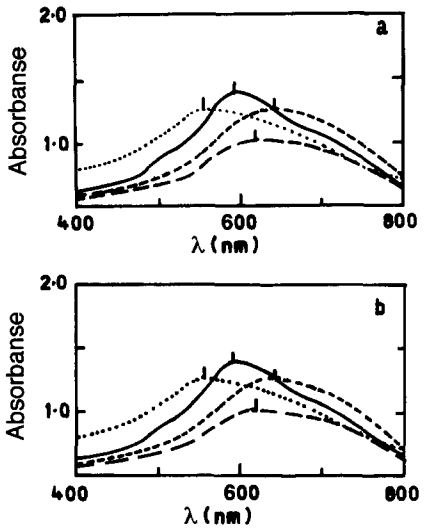


Fig. 1. λ_{max} of starch–I_2 blue colour of (a) BG and (b) Rg starches and their fractions: (---) starch; (· · ·) Ap; (— — —) Am; (————) Ax.

limit values (Table 1), it is implied that none of the fractions are essentially linear, instead they are branched to varied extents and the branching appeared to be much less in Rg-Am. The beta-amylolysis limit value of Rg-Ax was greater than that of BG-Ax, which was reflected in the nature of side-chain branches (see later). On the other hand, the value for Ap was very much within the range (~ 52–56%), similar to those of Ap from several other sources [20].

Pullulanase debranching of native Ap as well as the derived beta-LD showed a chain profile, as depicted in Fig. 3. A considerable difference was discernible in the relative proportions of various peaks. The absence of a V_0 peak indicated complete debranching. The results indicated BG-Ap to have chains with dp values 19 (short A), 27 (long

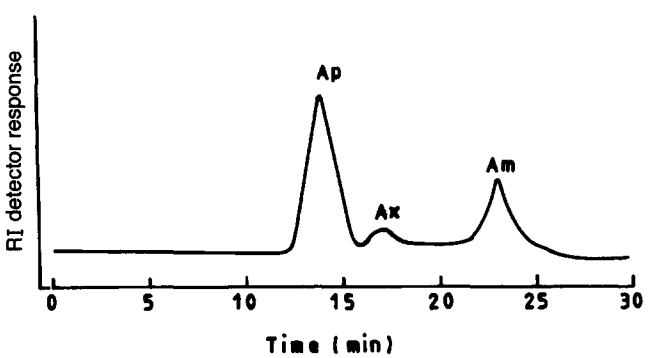


Fig. 2. SE-HPLC profile of isolated Ap, Am, and Ax.

Table 1
Characteristics of amylose, amylopectin, and the intermediate fraction derived from BG and Rg starches

	Amylose		Amylopectin		Intermediate fraction	
	BG	Rg	BG	Rg	BG	Rg
I_2–KI colour	Deep blue	Deep blue	Purple	Purple	Blue	Light purple
λ_{max} (nm)	646	632	566	551	602	591
$[\eta]$	10	15	105	75	– [a]	–
MW	6.3×10^6	1.6×10^6	1.5×10^7	1.3×10^7	2.2×10^7	4.5×10^6
Beta-amylosis limit (%)	79.1	93.7	56.3	52.1	65.8	75.0
$\overline{CL}$	–	–	26.0	20.7	–	–
ECL [b]	–	–	16.6	12.1	–	–
ICL [c]	–	–	8.4	6.3	–	–
General structure	Sparsely branched	Unbranched	Highly branched	Highly branched	Branched	Branched

[a] –, Not determined.
[b] ECL, External chain length.
[c] ICL, Internal chain length.

A/short B), 33 (long B), 56 and 62 (very long B); whereas its beta-LD was composed of large amounts of maltose and chains of dp 32 and 47. The chain profile (Fig. 4) for Rg-derived fractions appeared to be simpler, in that they had chains with lower dp values. Accordingly, from the average chain-length values (assuming an equal number of A and B chains), the ECL and ICL values (see Table 1) deduced were larger for BG-Ap than for Rg-Ap.

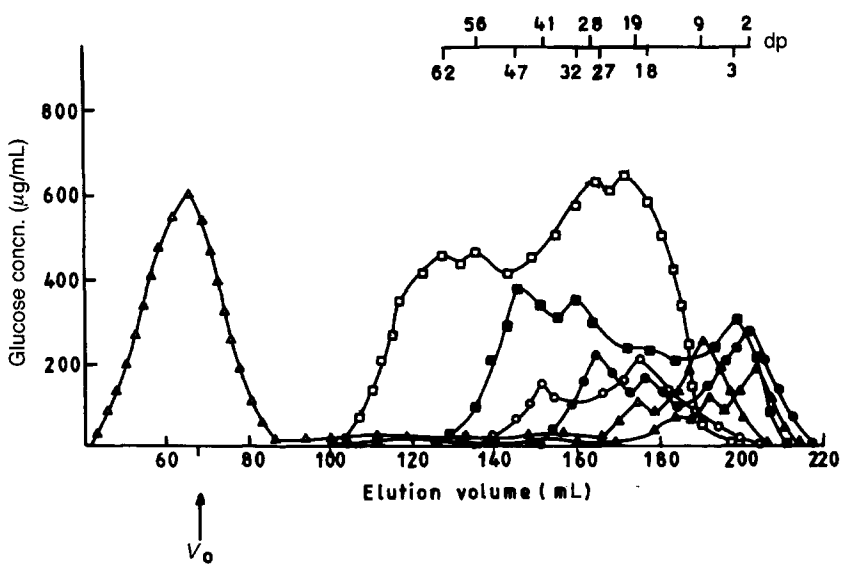


Fig. 3. GPC on Biogel P-10 of debranched BG starch fractions: (□) Ap; (■) its beta-LD; (△) Am; (▲) its beta-LD; (○) Ax; (●) its beta-LD.

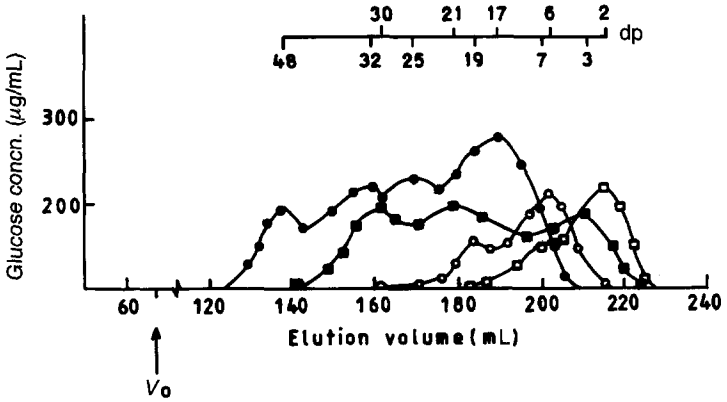


Fig. 4. GPC on Biogel P-10 of debranched Rg starch fractions: (●) Ap; (■) its beta-LD; (○) Ax; (□) its beta-LD.

In contrast to the above, debranching of BG-Am (see Fig. 3) gave a prominent V_0 peak together with small amounts of peaks of dp 9, 11, and 19. The former indicates a high MW linear molecule arising after the removal of short-chain stubs. Its beta-LD contained essentially maltose and a small peak of dp 9. Pullulanase treatment of Rg-Am did not reveal any major changes, as the molecule was more or less linear.

The results obtained with Ax were of different nature (see Fig. 3), in that the BG-Ax gave peaks of dp 8, 32, and 46 along with a V_0 peak. The last peak could represent the amylose impurity (from an incomplete fractionation, most unlikely) or it may constitute a truly linear high MW material as a result of the enzyme debranching. The beta-LD profile showed large amounts of maltose and maltotriose together with chains of dp 18 and 28. The chain profile of Rg-Ax and its beta-LD (see Fig. 4) indicated only a very limited degree of branching with short and long A chains, which resulted in high amounts of maltose after debranching.

From these results it is obvious that the BG and Rg starches are very dissimilar in their structures. Unlike the amyloses of wheat, triticale, and rye (beta-amylolysis values 77 to 82%) [21], the Rg-Am was more linear. The Ap of triticale with an A:B chain ratio of ~ 2.1 has a greater extent of branching than in other cereal starch amylopectins known so far [21].

Structural details in relation to in vitro digestibility.—Comparatively, BG starch was less digestible (45%) than Rg starch (> 70%). Rice starch used as a reference standard had an in vitro digestibility value of $\sim$ 88%. Legume starches, in general, are known to be poorly digested and this has been related to their high amylose content [3], as in the case of BG starch. The low solubility and swelling power in water and Me_2SO, low viscosity, and high content of non-carbohydrate constituents (viz., protein, lipid, enzyme inhibitors, etc.) would all contribute to the low digestibility of legume starches. In part, it could also be explained on the basis of damaged starch and also resistant starch formed during plate milling and starch preparation steps. Resistant starch is shown to be essentially retrograded amylose, and therefore it appears reasonable that the higher the

content of amylose the higher is the content of resistant starch formed and consequently the lower the starch digestibility [22]. Indeed, there exists a positive correlation between the amylose content and the formation of resistant starch in vitro [23]. High amylose varieties of maize are poorly digested in both mice and humans, and their starches are far more refractory for swelling and gelatinization than normal starches [24].

The implications of the molecular details could be correlated to a considerable extent with the differences in the in vitro digestibility of BG and Rg starches. The polymodal distribution of A and B chains in BG starch Ap and Ax fractions, all of relatively high chain length, and its relatively branched amylose could be a cause for the low digestibility. In comparison, the Rg starch fractions were relatively less densely branched and also of low MW, and were therefore easily digestible.

Several studies have shown that the structure of cell walls in legumes restricts the access of enzymes to the starch. Further, dietary fibre present in the form of an intact structural network acts as a steric hindrance to enzymic attack in vivo [25]. The nutritional significance of heat-stable amylase inhibitors and antinutrients is probably of greater magnitude in legumes than in cereals [26]. In a study aimed at understanding the role of gastric factors in enhancing the rate of in vivo digestibility of four legume samples, it was observed that green gram was more digestible and Bengal gram the least digestible [27]. It was shown that the amylose fraction contributed significantly to the digestibility differences, green gram having ~ 35% amylose of dp 667 and Bengal gram having ~ 45% amylose with a much higher dp of 1667, in agreement with our findings. The poor digestibility of Bengal gram is the primary cause for flatulence. Not only the non-reducing saccharides, such as raffinose, stachyose, and verbascose, are involved in the flatus property of Bengal gram, but even the low digestibility of its starch could contribute to flatulence, as reported by El Faki et al. [28].

4. Conclusions

It may be argued that amylose plays a crucial role as far as starch digestibility per se is concerned. Amylose is involved in a variety of in situ modifications, such as complexing with lipid, retrogradation, and resistant starch formation, in addition to the differences in its content, molecular size, and architecture, which all together decrease the starch digestibility as a whole. The subtle architectural variation due to side-chain branches in the otherwise "linear" molecule is yet another contributing factor to the low digestibility of legume starch. Such a phenomenon is not observed in waxy-starch varieties, which are naturally low in, or virtually devoid of, amylose.

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