ELSEVIER

Food Chemistry 84 (2004) 53–64

Food Chemistry

www.elsevier.com/locate/foodchem

Changes in the cell wall polysaccharides during storage and hardening of beans

Tânia M. Shiga, Franco M. Lajolo, Tullia M.C.C. Filisetti*

Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo Av. Prof. Lineu Prestes 580, São Paulo, SP 05508-900, Brazil

Received 21 November 2002; received in revised form 18 March 2003; accepted 18 March 2003

Abstract

Experiments were carried out to study the changes induced by HTC development in the cell wall polymer sugar composition of beans stored under three different conditions. The cotyledon cell wall polysaccharide was composed, to a large extent, of Ara-rich polymers, released by hot-water, CDTA and NaOH. Cell wall polymers contained medium amounts of GalA, Xyl, Glc and minor amounts of rhamnose. The methylation analysis showed the presence of high amounts of terminal arabinose and 5-linked arabinose, and intermediate amounts of 2,5- and 3,5-linked arabinose probably derived from branched arabinans. Small amounts of terminal xylose, 2-linked xylose and 4,6-linked glucose indicated the presence of xyloglucan. There was an inverse relationship between loss of seed viability and increase in cooking time. Hard beans showed reduction of cell wall polysaccharide water-solubility. Beans stored under accelerated condition, mainly showed loss of uronic acid in the water-soluble polymer fractions. Mild storage condition, mainly induced increase of Ara-rich polysaccharide contents in the water-insoluble polymer fractions. Storage at 4 °C proved to be the best condition, since it prevents HTC development.

Keywords: Beans; *Phaseolus vulgaris*; Common bean; Hard-to-cook; Cell wall; Arabinans

1. Introduction

Common beans (*Phaseolus vulgaris* L.) are an important source of nutrients for populations of developing countries (Bressani, 1993). Most of these countries are located in tropical areas where the crops are subjected to inadequate storage conditions. Beans subjected to long periods of storage undergo gradual loss of quality, such as changes of the seed coat color, moisture content, soaking characteristics and cooking time (Hincks & Stanley, 1986; Liu, 1995). High temperature and humidity render beans susceptible to the hardening phenomenon known as a hard-to-cook (HTC) defect, which is characterized by extended cooking times for softening (Liu, 1995). Beans with HTC defect need more energy to prepare and have low nutritional and textural quality, which reduces consumer acceptability. Hence, a better understanding of hardening mechanisms could be useful for maintaining the seed quality during storage.

Bean hardening has been related to multiple mechanisms that comprise starch gelatinization, protein denaturation and changes of the cell wall components (Hincks & Stanley, 1986; Liu, 1995). The cell wall provides rigidity, strength and shape to the plant cell and is responsible for textural properties of plant-based foods (Brett & Waldron, 1996). The solubilization and depolymerization of polymers were suggested to be responsible for tissue softening during cooking (Brett & Waldron, 1996; Liu, 1995). Hence, changes in cell wall polysaccharide structure and organization may be considered to be the principal factors affecting HTC.

Plant cell walls, in general, are depicted as highly complex and dynamic, composed of polysaccharides,

Abbreviations: HTC, hard-to-cook; TFA, trifluoroacetic acid; RG, rhamnogalacturonan; PG, polygalacturonan; HG, homogalacturonan; UA, uronic acid; NS, neutral sugars; Linkages were deduced from partially methylated alditol acetates; *t*-ara*f*, terminal-arabinofuranose; *t*-xyl*p*, terminal-xylopyranose; 5-ara*f*, an arabinosyl residue containing a C-5 linkage; 2-xyl*p*, 2-xylopyranose; 3,5-ara*f*, an arabinosyl residue containing C-3 and C-5 linkages.

* Corresponding author. Tel.: +55-11-3091-3624; fax: +55-11-3815-4410.

E-mail address: tullia@usp.br (T.M.C.C. Filisetti).

0308-8146/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0308-8146(03)00166-3

phenolic compounds and proteins stabilized by ionic and covalent linkages (Carpita & Gibeaut, 1993). They mainly comprise two networks: a microfibrilar phase, composed of cellulose and a hemicellulose network embedded in an independent but coextensive pectin network (Carpita & Gibeaut, 1993). The pectic material comprises mainly acidic rhamnogalacturonan (RG) and homogalacturonan (HG), and associated neutral arabinan, galactan and arabinogalactan polymers.

During the cooking process it has been postulated that middle lamella pectin is depolymerized by β-elimination of methylesterified polygalacturonic acids, promoting an increase in cell separation and this softens the tissues. In legume seeds, tissue softening seems to be achieved mainly through the middle lamella polymer solubilization, which allows cell separation, producing a mealy texture (Ilker & Szczesniak, 1990). The starch swelling and gelatinization and protein denaturation seem to contribute to this process, forcing cells to round up (Brett & Waldron, 1996).

With aging, the formation of new interactions between wall polymers could make cell separation more difficult. For example, pectin demethoxylation and Ca^{2+} bridge formation may reduce cell separation of the plant tissue during cooking. Hence, the nature of the polymers and their linkages dictate the wall polysaccharide solubility and may be of relevance for development of HTC. Studies on ultra-structural and histochemical aspects of common bean revealed reduced cell separation in defective beans. Cell walls of HTC beans appear more compact, having smaller intercellular spaces (Garcia, Lajolo, & Swanson, 1993). Hard seed cells seems to be strongly bound, and they separate mainly by fracture of the cell wall, producing clusters with reduced cell separation (Bhatty, 1990; Jones & Bolter, 1983), which gives a ''sandy mouth'' feeling (Brett & Waldron, 1996).

A better knowledge of cell wall polysaccharide composition and solubilization patterns could be useful to understand how these polymers are interconnected and interact during storage. Changes in the cell wall polysaccharide structures could affect the solubility and the tissue disassembling mechanisms, making beans resistant to cooking. Moreover, the detailed composition of cell wall and middle lamella polysaccharides is helpful to have a better understanding of physical and physiological properties of dietary fibre for human health (McDougall, Morrison, Stewart, & Hillman, 1996). Hence, the aim of this work was to study possible changes in cell wall polysaccharide, of common beans stored under different conditions during the development of the hard-to-cook phenomenon.

This is the first report of isolated and analyzed changes in the cell wall polysaccharide composition, leading to HTC development.

2. Materials and methods

2.1. Materials

Common beans (*Phaseolus vulgaris* L. c.v. IAC, Carioca) seed, grown in Paranapanema valley, were harvested in July and kindly provided by Instituto Agronômico de Campinas (IAC)–Campinas, SP, Brazil. Carioca beans have a pale brown colour with tan stripes. The seeds were cleaned, separated into three lots and each lot was submitted to a different type of storage: (1) 4 °C storage condition: cold chamber at 4 °C for 24 months, in double layer of Kraft paper and polyethylene bags. (2) Room temperature (RT): 19 °C and 79% relative humidity (RH), for 24 months, in double layer Kraft paper bags. (3) 37 °C storage condition (accelerated condition): hot chamber at 37 °C in desiccators containing saturated NaCl solution (75% RH) under vacuum, for 6.5 months.

2.2. Moisture determination

Moisture content was taken as weight loss after heating whole bean flour ($n = 4$) at 105 °C for 12 h.

2.3. Germination test

Bean seeds ($n = 100$) were incubated in Petri dishes containing cotton fibre soaked with water. The plates containing beans seeds were allowed to stay at room temperature and after 2 weeks the numbers of germinated beans were counted.

2.4. Cooking time

The seed were soaked overnight in deionized water and a Mattson bean cooker (Mattson, 1946) was used to test 25 seeds at a time. Cooking time was the mean time over four replications, when 50% of the beans were cooked, as indicated by plunger dropping, penetrating each bean.

2.5. Cell wall isolation

2.5.1. Enzymatic–chemical treatment

The water-soluble and insoluble polymers were isolated according to the chemical–enzymatic method, adapted from the procedures of Carpita (1983), McLaughling and Gay (1990) and Prosky (1988) (Fig. 1). The seeds were manually dehulled and freed from germ. Seed coat and cotyledon were ground separately in a blade mill and the flours were passed through a 0.5-mm sieve. About 0.5 g of cotyledon and seed coat flour were incubated with 0.1 ml of α-amylase (Sigma Chemical Co., St. Louis, MO) in 25 ml of 0.08 M sodium phosphate buffer at pH 6.0 for 30 min in a

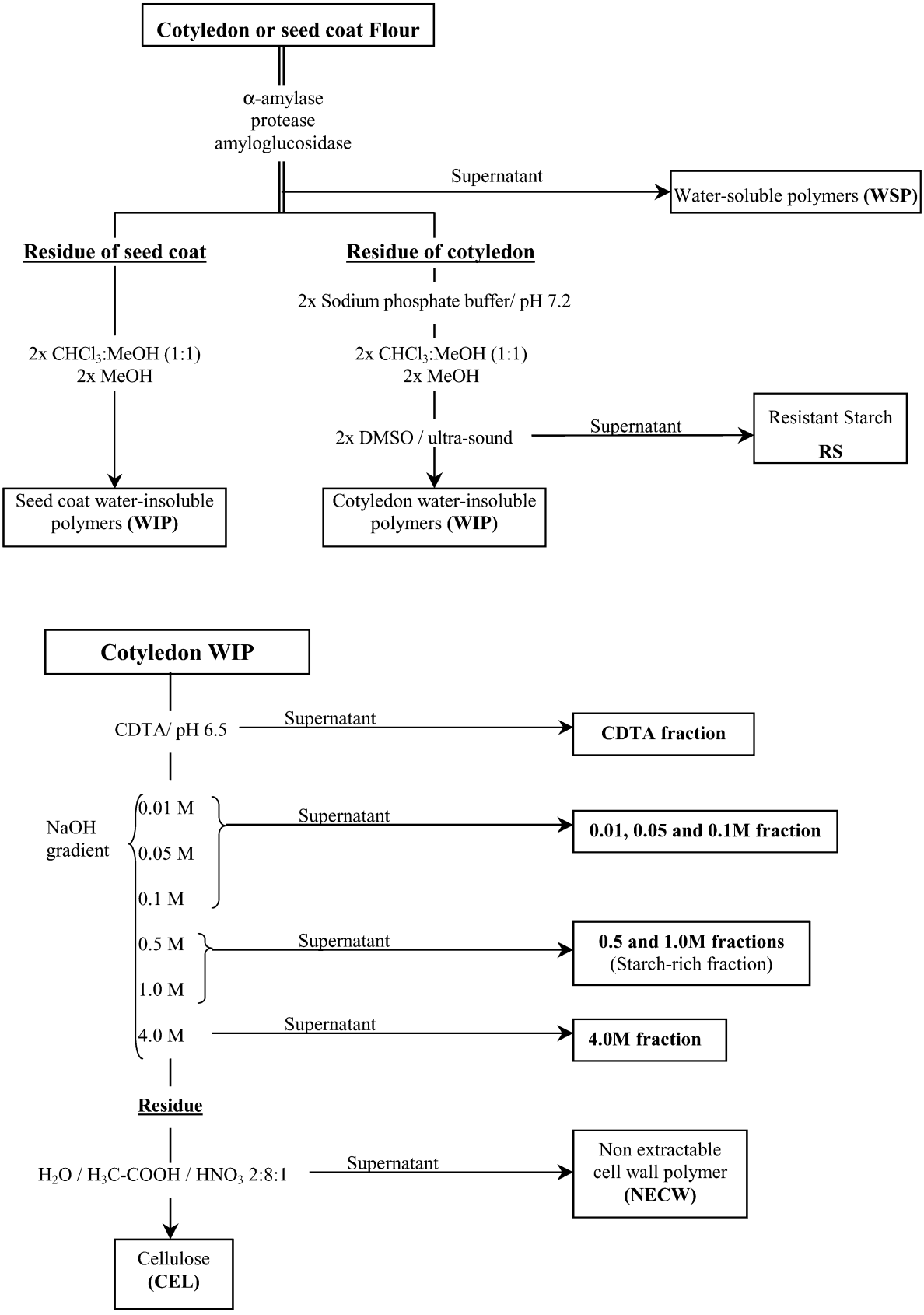

Fig. 1. General flow chart of cell wall polymer isolation procedure; extraction of the water-soluble polymers (WSP), water-insoluble polymers (WIP) and fractionation of WIP of cotyledon.

boiling water bath with manual stirring every 5 min. After cooling, the pH was adjusted to 7.5 and treated with 0.1 ml of protease (5 mg/ml) for 1 h at 60 °C. The pH was readjusted to 4.3 and 0.3 ml of amyloglucosidase (both from Sigma Chemical Co., St. Louis, MO) was added and incubated for 1 h at 60 °C (Fig. 1). The suspensions were cooled and pelleted by centrifugation at 8000 *g* for 10 min. The supernatants were passed through fibreglass filters (GF/F Whatmann) whenever necessary and brought to 80% (v/v) ethanol. The mixture was allowed to precipitate for 24 h at −20 °C and pelleted by centrifugation at 8000 *g* for 10 min. The precipitates were washed three times with 10 ml of ice-cold 80% ethanol, suspended in distilled water and freeze-dried (water-soluble polymers; WSP). The residues of the seed coat and cotyledon resulting from the enzymatic treatment, were submitted to a sequential extraction scheme (Fig. 1).

The residue of enzymatic hydrolysis of cotyledon was treated sequentially, twice, with each of the following: 10 ml of the 0.5 M sodium phosphate buffer (pH 7.2); chloroform : methanol (1:1; v/v) at 45 °C for 30 min, and methanol. The remaining residue of the cotyledon was added to 90% dimethyl sulfoxide (DMSO) and sonicated for 20 min. The residue obtained after centrifugation (8000 *g* for 10 min) was washed with 90% DMSO and rinsed exhaustively with distilled water and freeze-dried (water-insoluble polymers; WIP). The DMSO supernatants were pooled, dialyzed, using a Spectra/Por® Membrane, MWCO 12-14,000 (Spectrum®, Houston, TE), against distilled water and freeze-dried; this corresponded to the resistant-starch fraction (RS).

Seed coat residues from the enzymatic hydrolysis were treated twice with 10 ml of chloroform:methanol (1:1; v/v) at 45 °C for 30 min and with methanol. The remaining residue was rinsed with water and freeze-dried (WIP).

The WIP samples from cotyledon were fractionated (Fig. 1), following the methodology described by Carpita (1983). Thirty milligrammes of the WIP were extracted with 10 ml of 0.05 M cyclohexanediaminetetraacetic acid (CDTA) at pH 6.5 for 16 h. The suspension was pelleted by centrifugation at 1500 *g* for 10 min, and the supernatant filtered through a GF/F (Whatman) filter, dialyzed against distilled water and freeze-dried, resulting in the fraction called CDTA. The residue was treated with 10 ml of 0.01, 0.05, 0.1, 0.5 and 1.0 M NaOH containing $NaBH_4$ (3 mg/ml) for 1 h at 22 °C under an atmosphere of N_2, and with 4.0 M NaOH for 16 h under the same conditions. The NaOH extracts were neutralized with glacial acetic acid, dialyzed against distilled water and freeze-dried, resulting in the fractions called 0.01, 0.05, 0.1 and 4.0 M. The 0.5 and 1.0 M NaOH solution extracted mainly starch, since the Glc found in these fractions was almost completely hydrolysed by amyloglucosidase, and were not computed as cell wall polymer.

The cellulose-rich residue that remained after WIP fractionation was washed, suspended in distilled water, freeze-dried and weighed. Glucose derived from cellulose was not determined by GC, since it cannot be hydrolyzed by trifluoroacetic acid (TFA). Crystalline cellulose (CEL) was obtained after digestion of the non-cellulosic polymers with a mixture of H_2O:$HOAc^-$:HNO_3 (8:2:1; v/v/v). About 30 mg of residue was treated with 10 ml of nitric acid reagent (Updegraff, 1969) and incubated for 90 min in a boiling water bath (Fig. 1). The highly cross-linked polymers solubilised, corresponded to non-extractable cell wall polymers (NECW).

2.6. Neutral sugars, uronic acid and protein determination

The cotyledon and seed coat WSPs were analyzed for protein, neutral sugars and galacturonic acid (GalA) contents. The neutral sugars and uronic acids (UA) of the water-insoluble polymers were also determined.

Neutral sugars, rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), galactose (Gal) and glucose (Glc), excluding the glucose derived from the cellulose, were released by acid hydrolysis of the polysaccharides and quantitatively determined after reduction and acetylation (Blakeney, Harris, Henry, & Stone, 1983; Carpita & Whittern, 1986; Fox, Morgan, & Gilbart, 1989) using gas–liquid chromatography (GC). All sugar standards were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). The derivatives were dissolved in ethyl acetate; 1–3 μl samples were injected into a gas chromatograph equipped with a flame ionization detector (FID) and separated using a SP-2330 fused-silica capillary column, 30 m × 0.25 mm i.d., of 0.20 μm film thickness (Supelco, Inc., Bellefonte, PA) in a Hewlett-Packard 6890 gas-chromatograph. The column temperature was programmed from 170 to 240 °C at 10 °C/min with a 20 min hold at upper temperature. Injector and detector temperatures were set at 250 °C. Nitrogen was used as carrier gas at a flow rate of 30 ml/min.

The protein content was determined using the bicinchoninic acid (BCA) method (Smith et al., 1985; Wiechelman, Braun, & Fitzpatrick, 1988), using the BCA kit (Pearce, Rockford, IL) and the uronic acid was determined according to Filisetti-Cozzi and Carpita (1991).

2.7. Linkage analysis

2.7.1. Preparation of partially methylated alditol acetate (PMAA)

The PMAA were obtained according to Carpita and Whittern (1986). About 1 mg of each sample was placed in a 15 ml Corex® (Corning Glass from Fisher Scientific, Chicago, IL) tube and stored in a vaccum

desiccator over fresh P_2O_5 for at least 2 days. The tubes were sealed with serum sleeve stoppers and evacuated. About 1 ml of anhydrous silylation grade DMSO (Pearce, Rockford, IL) was added by syringe and the mixture was sonicated for 3 h/50 °C. Under N_2 flow, 0.5 ml of 2.5 M *n*-butyllithium in hexane (Aldrich Chemical Co., Milwaukee, WI, USA) was added drop by drop. The system was kept in a clean fume hood with no flammable material or water. Butyllithium is pyrophoric and may ignite on exposure to air; it is corrosive, and can cause skin burns and react violently with water.

The mixture was stirred for 2–3 h under N_2 flow and 0.5 ml of CH_3I (Aldrich Chemical Co., Milwaukee, WI, USA) was added. The N_2 flow was stopped and the solution was stirred for 1 h or until the solution colour turned to yellow. About 5 ml of water was added to the mixture and stirred vigorously. The methylated polysaccharide was extracted with 1.5 ml of chloroform and transferred to a screw cap vial. The aqueous solution was re-extracted with 1.5 ml of 2:1 (v/v) chloroform:methanol. The chloroform phases were pooled and washed five times with distilled water and evaporated under N_2 flow.

The per-*O*-methylated polymers were hydrolyzed, reduced with $NaBD_4$ and acetylated as described previously. The PMAA was cleaned-up according to Gibeaut and Carpita (1991) using a mixture of Spectrophotometric grade carbon tetrachloride (Carlo Erba Reagenti) [Spectrophotometric grade methanol 40% (v/v) in water, Aldrich Chemical Co.]. Separations were carried out in the same column as used for gas-chromatography using a Hewlett-Packard 6890 gas-chromatograph, coupled to a Hewlett-Packard 5973 quadrupole mass spectrometer. Temperature was programmed from 160 to 210 °C at 2 °C/min then to 240 °C at 5 °C/min with 10 min hold at the upper temperature. Helium was used as carrier gas.

2.7.2. Data treatment

The sugar and protein compositions were expressed as the percentage of total recovery. The WSP and the CDTA, 0.01 M and 4.0 M NaOH-soluble polymers extracted from WIP of cotyledon composed the bulk of wall polysaccharides. Changes in the sugar composition during beans storage were analyzed.

The use of enzyme resulted in contaminant addition in the WSP. In a recent study, Neves (1999) used the same enzyme kit to hydrolyze starch and protein of Carioca bean flour to extract cell wall polymers and verified that high amounts of protein and Man-rich polymers contaminated the WSP. The polymers derived from extraction enzymes were constituted of up to 62% of Man. This suggested that the Man content was derived from enzyme; hence this data was corrected in the calculation.

3. Results and discussion

3.1. Normal beans cell wall sugar composition

Hot water released about 9% of WSP from cotyledon (Table 1). About 12% of material remained after enzymatic–chemical treatment and was considered to be WIP. The WIP contained mainly neutral sugars and consisited of 66% non-starch polysaccharides, which was composed of about 13% CDTA soluble material, 23% weak alkali-soluble material (0.01, 0.05 and 0.1 M NaOH) and 45% 4.0 M NaOH-soluble polymers (Table 1). After alkaline extraction, the cellulose-rich residue that remained still contained 11% of pectic material (NECW) that was removed with the acetic-nitric reagent (Table 1). The residue obtained after acetic–nitric treatment corresponded to 9% of CEL. This was evidence of the existence of strong interactions between cellulose and pectic material. About 2% of material was extracted by DMSO and was considered as RS. This material contained 46% of Glc, mainly derived from starch, and considerable amounts of pectic material (54%), composed of Ara (23%), Gal (12%) and uronic acid (4%). The material extracted by 0.5 and 1.0 M alkali corresponded to 34% of the WIP. These fractions were composed of 92% of Glc, probably derived from starch, since they were almost totally hydrolyzed by amyloglucosidase. About 8–9% of these fractions corresponded to wall polysaccharide. For hard-to-cook studies, starch-rich fractions were not considered.

Bean cell wall was largely constituted of non-cellulosic polysaccharides extracted by hot water (WSP), CDTA, 0.01 and 4.0 M NaOH solution. A large proportion of these materials were water-insoluble and mostly composed by neutral sugars (NS), as can be seem by the high NS/UA ratio (Table 1). Much of the material, extracted by 4.0 M NaOH was neutral Ara-rich polysaccharide (Fig. 2), which showed the highest NS/UA ratio. Polymers released by 0.01 M NaOH and hot water showed low sugar recovery, attributed to the presence of high amounts of protein, as can be seem in Table 1 and also observed by Neves (1999).

Most of the polymers of the WIP fraction were constituted of Ara (37–55%) and UA (19–30%), and Ara was mainly composed of high amounts of *t*-ara*f* and 5-ara*f* and intermediate amounts of 2,5-ara*f* and 3,5-ara*f* (Figs. 2 and 3). Only 10–11% of Xyl and 4–11% of Glc, derived from xyloglucan was found, deduced from intermediate amounts of *t*-xyl*p*, 2-xyl*p* and 4,6-glc*p* units. However, Fuc was not detected, maybe because the amount was too low to be detected (Figs. 2 and 3). The presence of high amounts of Ara was also reported in works carried out with kidney bean, lentil, chickpea and mung bean cell walls (Bhatty, 1990; Champ, Brillouet, & Rouau, 1986; Gooneratne, Needs, Ryden, & Selven-

Table 1
Polymer distribution of bean cotyledon and seed coat. The water-insoluble polymers composed by cell-wall polysaccharide rich fractions, extracted by CDTA solution and 0.01, 0.05, 0.1 and 4.0 M NaOH. Celullose (CEL) was determined after hidrolysis of non-cellulosic polysaccharides with nitric acid reagent (Updegraff, 1969). The starch-rich fractions was extracted by DMSO 90% (RS) and 0.5–1.0 M NaOH

Component	Fractions	(%)	NS	UA	Protein	Total	NS/UA
			μg/mg				
COTYLEDON							
	WIP	12.3±0.4[a]	639	91.2	145	875	
	WIP fractions						
Cell wall polymers	CDTA	13.2±0.28	649	225.2	nd	874	2.9
	0.01 M NaOH	16.1±0.38	243	103.2	nd	346	2.4
	0.05 M NaOH	3.8±0.54	nd	nd	nd		
	0.1 M NaOH	2.9±0.42	nd	nd	nd		
	4.0 M NaOH	44.5±0.37	736.3	172.0	nd	908	4.3
	CEL	8.6±0.41	nd	nd	nd		
	NECW	10.9±0.01	nd	nd	nd		
	WSP	9.0±0.4	384	117	214	715	3.3
Starch-rich fractions	0.5 M NaOH	16.8±0.9	675	26.0	nd	701	
	1.0 M NaOH	17.2±3.2	673	19.5	nd	692	
	RS	1.9±0.1	444	17.1	244	705	
SEED COAT							
	WIP	72.4±2.6	272	167	253	692	1.6
	WSP	5.5±1.6	164	144	184	492	1.1

NS, total neutral sugars; UA, uronic acids; WSP, water-soluble polymers; WIP, water-insoluble polymers; CEL, cellulose; NECW, non-extractable cell wall material, CDTA, polymers extracted by CDTA solution, 0.01–4.0 M NaOH extracted polymers; RS, resistant starch; nd = not determined.

[a] Means of three determinations.

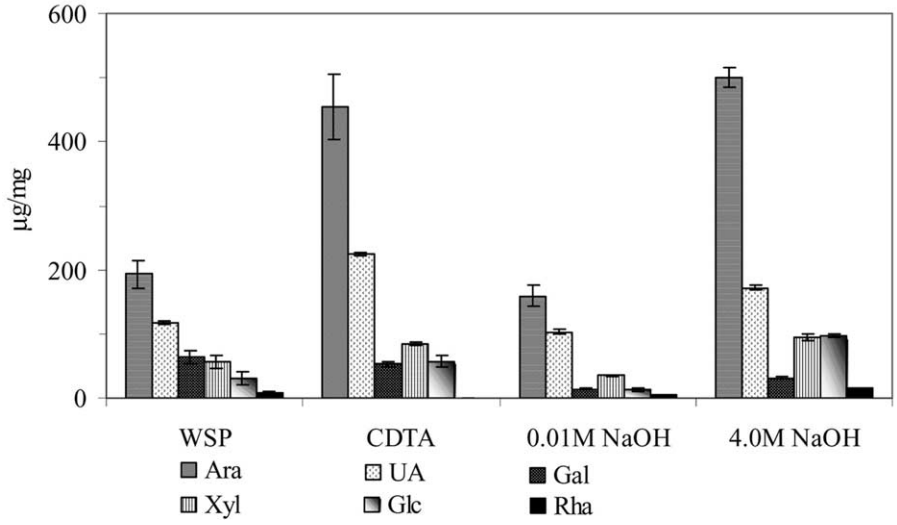

Fig. 2. Normal bean neutral sugar and uronic acid distribution in the cell wall polymer fractions. The cell wall polymers were extracted by hot-water (WSP), CDTA solution and 0.01–4 M NaOH. Sugars are expressed as Ara, arabinose; UA; uronic acid; Gal, galactose; Xyl, xylose; Glc, glucose; Rha, rhamnose.

dran, 1994). Bhatty (1990) showed that lentil cell wall was composed of arabinans and arabinogalactans because such pectins are commonly distributed in the cell wall of dicotyledonous plants. In bean cell wall, Ara-rich pectins were concentrated in the 4.0 M alkali fraction and seemed to be strongly bound and/or entangled. The linkages that predominated in these pectins are likely to be ether bonds, since these linkages

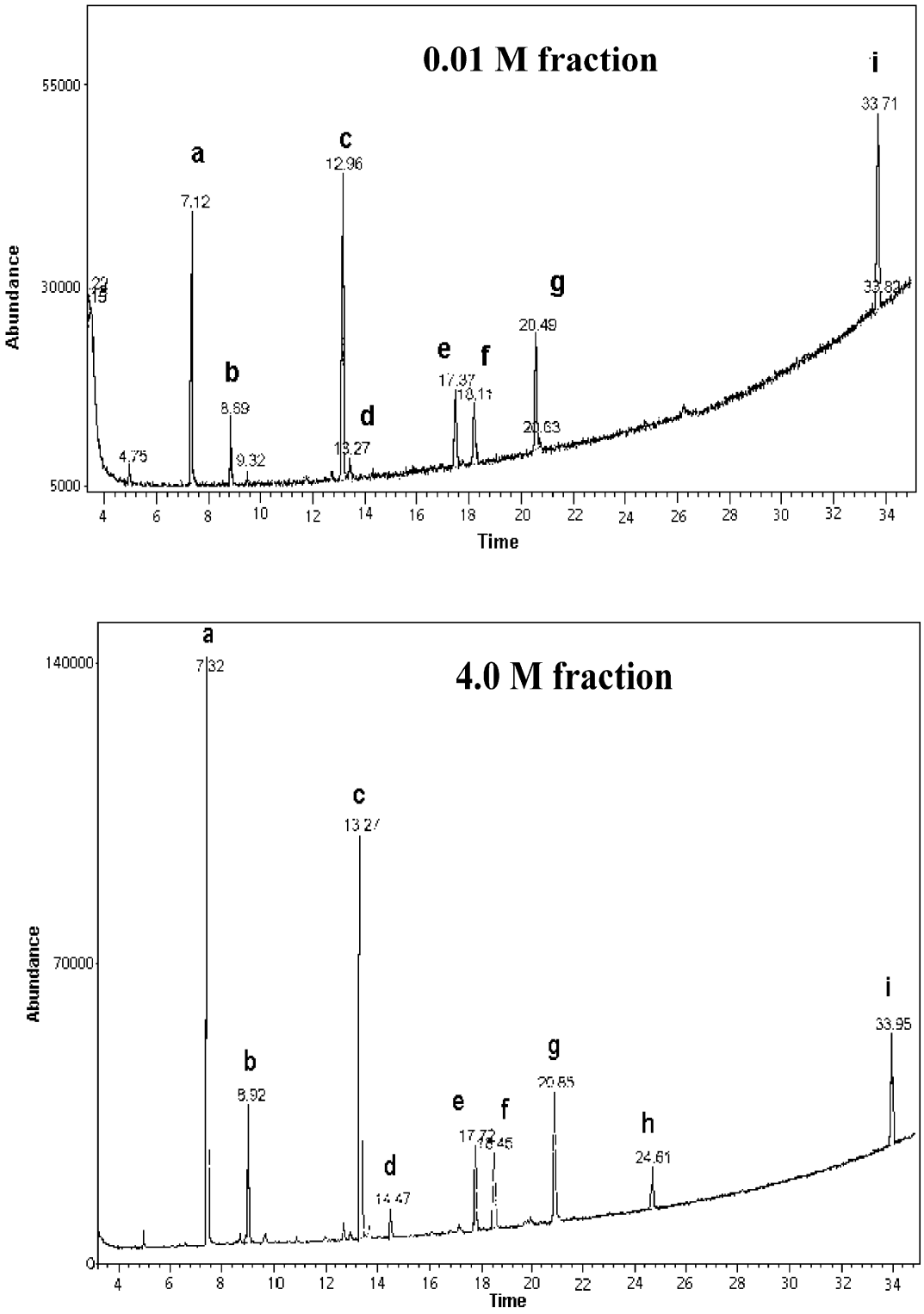

Fig. 3. Chromatogram showing the partial methylated alditol-acetate derivatives separated by gas–liquid chromatography and identified by electron-impact mass spectrometry. The main polymer fractions, obtained by 4.0 M and 0.01 M NaOH from WIP, were analyzed. Some of the derivatives identified are: (a) *t*-ara*f*, (b) *t*-xyl*p*, (c) 5-ara*f*, (d) 2-xyl*p*, (e) 3,5-ara*f*, (f) 2,5-ara*f*, (g) ara penta-OAc, (h) 4,6-glc*p*, (i) myo-inositol.

are known to be labile only in strong alkali solution (Fry, 1986; Lozovaya, Zabotina, & Widholm, 1999).

Bean cell wall is mainly composed of neutral non-cellulosic polysaccharides, most of them highly cross-linked and water-insoluble. These pectic polymers are constituted of branched arabinans, and acidic pectins containing intermediate amounts of Gal (3–12%) and minute amounts of Rha (0–2%) (Fig. 2). The presence

of minute amounts of Rha (0–2%) in Carioca bean suggests that the acidic pectins contain small "hairy" domains of branched RG_s interspaced by long stretches of polygalacturonan (PG). The high amounts of Ara-rich polysaccharides in the cell wall of Carioca beans are consistent with the sugar profile found in other legume seed cell walls.

The seed coat contained about 72% of WIP, composed of 69% of non-cellulosic polysaccharide. These polymers contained 21% of Xyl, 24% of UA residues and 37% of protein. Small amounts of Ara (12%), Glc (3%) and Gal (2%) were also found as minor components. The low recovery was probably due to the presence of high amounts of ash, lignin and cellulose that were not determined (Table 1). The WSP of seed coat constituted about 6% of the total and contained high amounts of acidic pectins (32%) and protein (40%). The high UA and protein recovery in the WSP and WIP of seed coat could be attributed to the interference of phenolic compounds in the colorimetric method adopted (Table 1).

3.2. Hard-to-cook phenomenon

3.2.1. Effects of storage on seed germination, moisture content and cooking time

There was an inverse relationship between cooking time and seed germination rate (Figs. 4 and 5). The higher the humidity and temperature of seed storage, the higher was the cooking time and lower the germination rate (Fig. 5). After 24 months at RT, germination had decreased to 17% and the cooking time increased by a factor of 2 (Fig. 4). When beans were stored under accelerated conditions, at the end of the sixth month there was total loss of seed viability and the cooking time had increased by a factor of 8 (Fig. 5). The

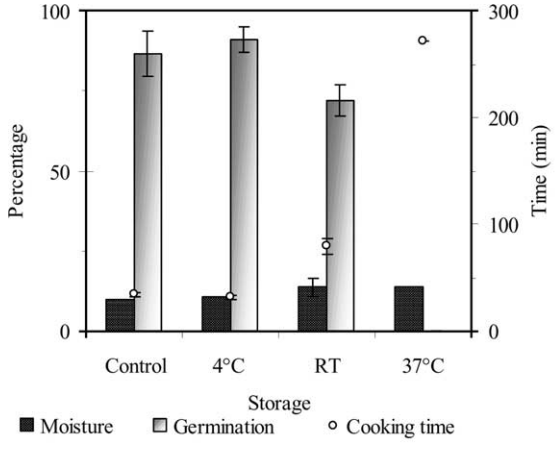

Fig. 4. Moisture content, seed germination and cooking time for seeds stored at 4 °C for 24 months, at room temperature (RT)/79% RH for 24 months, and at 37 °C/75% RH for 6.5 months. Values are means of four determinations.

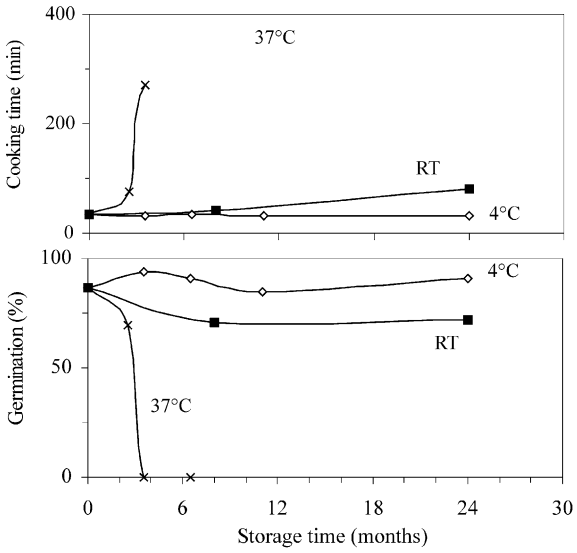

Fig. 5. Changes occurring in the cooking time and seed germination during bean storage at 4 °C for 24 months, 37 °C/75% RH for 6.5 months and RT/79% RH for 24 months.

best storage condition was 4 °C, at which germination rate, as well as cooking time, did not change, even after 24 months of storage (Fig. 5). Fig. 5 shows a clear inverse correlation between cooking time and seed germinability. Varriano-Marston and Jackson (1981) also observed that seeds with greatest loss of germinability took the longest to cook. Jones and Bolter (1983) observed that, in hard seeds, solute leakage during imbibition was 10 times higher than in soft beans, indicating loss of cell membrane integrity, required for normal cellular function. The reduction of seed viability is thought to be associated with extensive membrane damage and changes in the cotyledon intercellular components (Hincks & Stanley, 1986). Membrane damage and phytate degradation were associated with bivalent cation leakage and calcium pectate formation (Jones & Bolter, 1983).

3.3. Polymers recovery

The changes in the sugar composition of seed coat cell wall polymers during hard-to-cook development were not analyzed, because this work focussed on changes in the polymers of cotyledons.

The ability to extract polymers from the wall almost did not change with bean aging, especially with beans stored at 4 °C. The amount of material extracted by 4.0 M NaOH increased slightly in beans stored at RT, whereas in beans stored at 37 °C, the 0.01 M fraction rose considerably (Fig. 6). No observable change in the proportion of polymers extracted by CDTA or in the amounts of CEL and NECW was observed in all storage condition. The amount of WSP in beans stored at RT did not change, whereas in beans stored at 37C decreased slightly (Fig. 6).

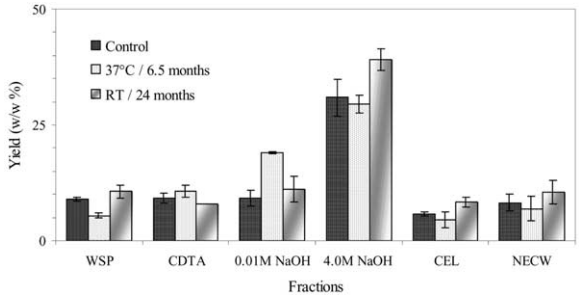

Fig. 6. Distribution of cell wall polymers of beans cotyledon after storage at room temperature (RT)/79% RH for 24 months and at 37 °C/75% RH for 6.5 months (accelerated condition). Polymers were extracted with hot-water (WSP), CDTA solution and gradient of NaOH. WSP, water-soluble polymers; CEL, cellulose; NECW, non-extractable cell wall material, CDTA, polymers extracted by CDTA solution; 0.01 and 4.0, 0.01–4.0 M NaOH-extracted polymers. Values are means of three determinations.

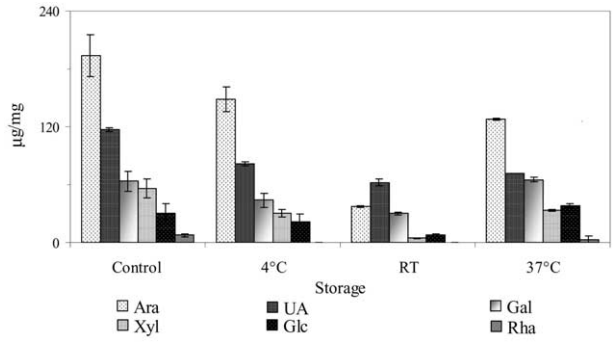

Fig. 8. Neutral sugars and uronic acid distribution of polymers extracted by hot water (WSP) from cotyledon of beans stored at 4 °C for 24 months, room temperature (RT)/79% RH for 24 months and 37 °C/75% RH for 6.5 months. The sugars are expressed as Ara, arabinose; Gal, galactose; Glc, glucose Rha, rhamnose; Xyl, xylose; UA, uronic acid. Cooking time at the end of storage.

3.4. Sugar distribution in the WSP

Although the proportion of polymers extracted by hot water were almost constant, the sugar composition changed considerably when compared with the controls. The sugar recovery in WSP decreased visibly in beans stored at RT and 37 °C, whereas protein content increased from 30 to 48 and 44%, respectively (Fig. 7). Storage at RT induced a significant reduction in the neutral sugars recovery. Total neutral sugar decreased from 54 to 32% in RT beans and to 46% in beans stored at 37 °C. Uronic acid especially decreased under accelerated storage conditions, from the initial 16% to 10% (Fig. 7).

Beans stored at RT showed considerable decrease in Ara content, whereas accelerated conditions showed only a slight decrease (Fig. 8). The WSP obtained from RT beans still contained high amounts of UA, while Ara content decreased considerably (Fig. 8). Hence, accelerated conditions mostly induced decrease in the UA while, in RT storage, the loss of Ara, Gal, Xyl and Glc contributed to a considerable decrease in the NS content (Fig. 8). Hence, under accelerated storage condition acidic pectins rather than neutral pectins become insoluble. Beans stored at 4 °C showed slight decrease in the sugars recovery, but no marked change in composition (Figs. 7 and 8).

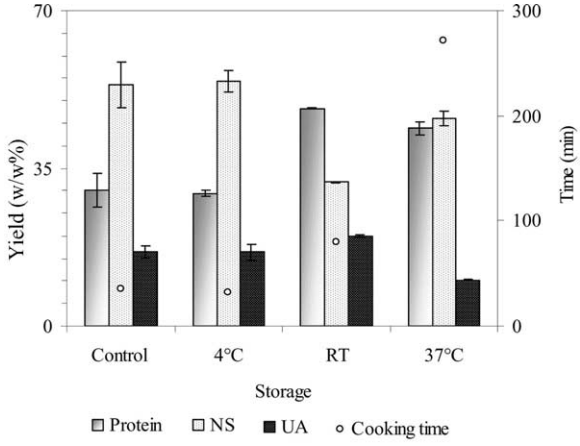

Fig. 7. Distribution of total neutral sugars (NS), uronic acid (UA) and protein in the water-soluble polymers (WSP) after storage at 4 °C for 24 months, room temperature (RT)/79% RH for 24 months and 37 °C/75% RH for 6.5 months.

Probably, during storage at RT, new interactions between Ara-rich polysaccharides were stated, producing aggregates with high molecular weight and low solubility. Contrarily, acidic pectins seem to retain solubility, remaining in the WSP. The starting of new interactions between Ara-rich cell wall polysaccharides is feasible, since ferulic acid is usually found esterified to Ara and Gal residues in pectin and two ferulic acids molecules can form diphenyl bonds (Fry, 1983, 1986). The ether bond formation between a phenolic –OH group of ferulate and a –OH group of polysaccharide or structural proteins (Brett & Waldron, 1996; Fry, 1983) could be involved in the wall polymer insolubilization.

Storage at low temperature (4 °C) seems to preserve the structure of the polymers, since the sugar and protein composition of WSP almost did not change (Figs. 7 and 8). The unaltered polysaccharide solubility and cooking time of beans stored at low temperature indicate that this would be the best condition for bean storage and also indicate the importance of the modifications in cell wall polymer solubility in the HTC situation.

3.5. Sugar distribution in the CDTA- and NaOH-soluble polymer fractions

The material extracted by CDTA showed almost no changes in the recovery of NS and UA for all storage conditions. However, it showed marked changes in the material extracted by 0.01 M NaOH from hard beans.

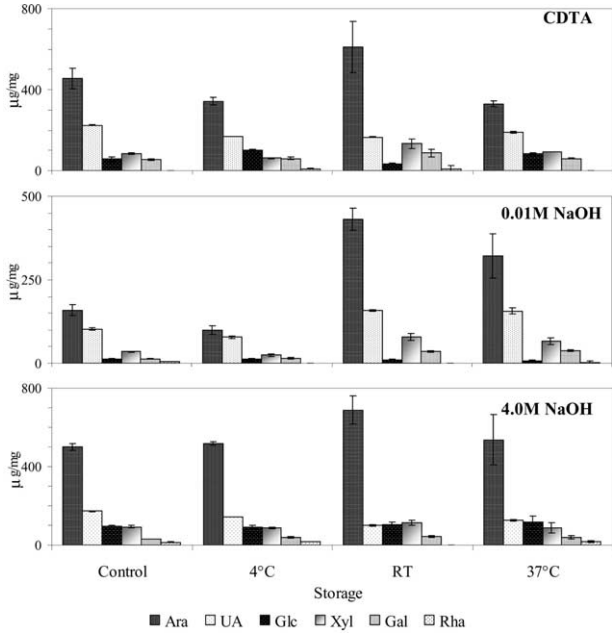

Fig. 9. Distribution of neutral sugars and uronic acid of the polymers extracted by (a) CDTA solution, (b) and (c) 0.01 and 4.0 M NaOH, from beans stored at 4 °C for 24 months, RT/79% RH for 24 months and 37 °C/ 75% RH for 6.5 months. The sugars are expressed as Ara, arabinose; Gal, galactose; Glc, glucose Rha, rhamnose; Xyl, xylose; UA, uronic acid.

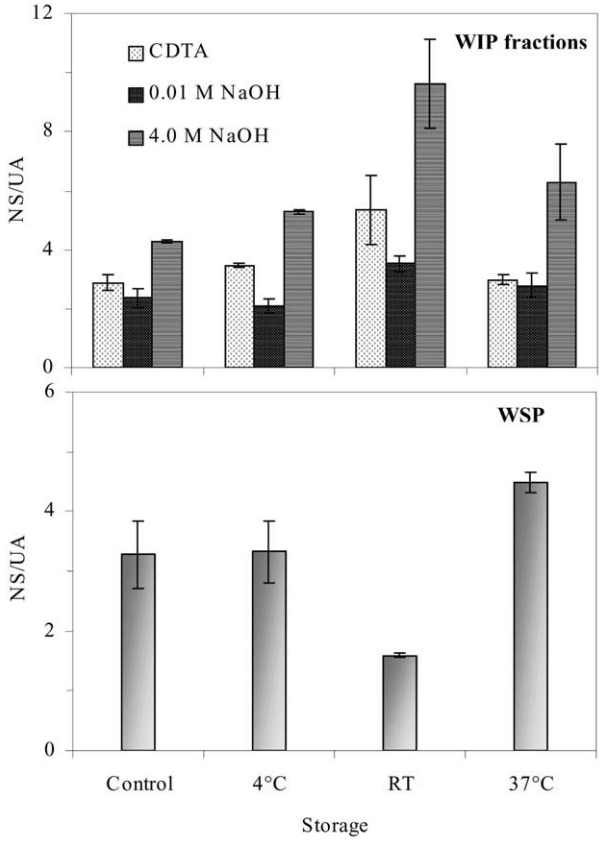

Fig. 10. Distribution of neutral sugars (NS) and uronic acid (UA) in the water-soluble polymers (WSP) and CDTA, 0.01 and 4.0 M NaOH-soluble polysaccharides in beans stored at 4 °C for 24 months, RT/ 79% RH for 24 months and 37 °C/75% RH for 6.5 months. The total neutral sugars are expressed as weight ratio to uronic acid (NS/UA).

The amount of Ara increased in beans stored at RT and 37 °C when compared to the control (Fig. 9). In beans stored at RT, Ara content increased 2.7 times, whereas at 37 °C, it doubled. Bhatty (1990) also reported that poor cooking lentils had higher amounts of Ara in the cell wall material than soft lentils. Also, Xyl content increased twice and UA 1.5 times in both storage conditions (Fig. 9). For material extracted by 4.0 M NaOH, Ara content rose 38% in beans stored at RT and only 7% at 37 °C. However, UA decreased 42% in RT beans and 27% in beans stored at 37 C (Fig. 9).

Fig. 10 shows the NS/UA ratio in the WSP and WIP and summarize the changes occurred during bean storage. Beans stored at 4 °C showed no changes, either in the cooking time or in the sugar composition of WSP and WIP fractions, indicating few cell wall polysaccharide alterations (Fig. 10). However, hard beans (RT and 37 °C) showed changes in the WSP and WIP sugar composition, probably resulting from neutral and acidic pectin insolubilization (Fig. 10). Acidic pectins took part in the cell wall insolubilization, mainly in beans stored under accelerated conditions, since amounts decreased in the WSP and increased in pectins extracted by 0.01 and 4.0 M NaOH (Fig. 10). However, in RT beans, cell wall polymer insolubilization seems to be mostly related to neutral pectin insolubilization, since the NS/UA ratio decreased in the WSP and increased in the WIP. The neutral pectin insolubilization, occurred mainly by insolubilization of Ara-rich polymers, since the amounts decreased in the WSP fraction and were distributed in the polymers extracted in the WIP fraction. The insolubilization of Ara-rich neutral polysaccharides, could be related to new interactions between cell wall components. Lozovaya et al. (1999) reported that a significant part of the primary cell wall phenolics in dicots is composed of hydroxycinnamic acid (0.01–0.19% of cell wall dry weight), the bulk of which (60–80%) is found to be ether linked to cell wall components. Ferulic acids is also found esterified to Ara and Gal in the pectin, and catalysed by peroxidase activity, can form diphenyl bonds or ether bonds between the phenolic –OH and the hydroxy group on a polysaccharide, increasing the molecular weight of arabinans (Fry, 1983; McNeil, Darvill, Fry, & Albersheim, 1984). Structural protein could also be involved in the interpolymeric linkages formation since ferulic acids esterified to Ara and Gal in the pectins may also form covalent linkages with extensin (Brett & Waldron, 1996; Fry, 1983). Garcia, Lajolo, Filisetti, and Udaeta (1998), verified that phenolics bound to pectin increased twice in the hard beans when compared with controls. Hence, ether and/or ester linkage formation is

a technically feasible event. We can conclude that cell wall components could undergo new interactions resulting in loss of polysaccharide solubility, maybe by increase in the number of linkages and/or entanglement of wall polymers.

According to the pectin–cation–phytate theory of bean hardening with aging, insoluble calcium-pectate is formed due to membrane damage and bivalent cation leakage from the cytosol (Jones & Bolter, 1983). The activation of pectin methylesterase (PME) also contributes de-esterifying the pectins and favouring cation binding. The pectin–cation–phytate model could explain the loss in UA recovery observed in the WSP of beans stored at 37 °C.

Aguilera and Rivera (1992), and Aguilera and Ballivian (1987) studied beans stored under accelerated conditions and verified that there is an initial hardening period of slower rate that may last for about 100 days that they called a pseudoinduction period. Aguilera and Rivera (1992) verified that the hardening achieved during the pseudoinduction period is reversible with cation chelating agents, indicating calcium pectate formation. Garcia et al. (1993) studied beans (cv. Carioca) stored under accelerated conditions, and observed that calcium was more abundant in the cell wall of HTC beans than in soft beans. The reduction of the uronic acid content in the WSP fraction of Carioca beans stored under accelerated conditions corroborates these studies. However, Liu (1995) concluded that only insoluble calcium pectate formation could not explain the middle lamella polysaccharide insolubilization observed in hard beans. Storage under RT conditions induced mainly neutral sugar insolubilization. The insolublization of neutral Ara-rich pectins also could be related to bean hardening. It is likely that different climates induce the development of the hard-to-cook defect by distinct mechanisms.

4. Conclusion

Common bean cell walls are composed of Ara-rich polysaccharides, extractable by hot water, CDTA, 0.01 and 4.0 M NaOH. The bulk of the pectic polysaccharides was solubilized by 4.0 M NaOH and showed the highest concentration of Ara-rich polymers. Intermediate amounts of Xyl, UA and Glc and minor amounts of Rha and Gal, were also found. Linkage analysis revealed an Ara-rich polysaccharide with branched structure, and the presence of xyloglucan. The data suggest a highly branched structure, containing high amounts of arabinans and low amounts of hemicellulose. The sugar composition of Carioca bean cell wall polysaccharide suggests that acidic pectins contain small hairy domains of branched RG_s, interspaced by long stretches of HG_s.

The loss of solubilities of acidic and neutral Ara-rich pectins were associated with the storage conditions and also with the development of the HTC phenomenon. Long-time storage under mild conditions seems to be involved with neutral, Ara-rich polysaccharide insolubilization, while accelerated storage was more associated with loss in the solubility of acidic polymers, which is in accord with the pectin–cation–phytate model discussed by Liu (1995). Changes in cell wall polymer solubility are reflected mainly in the hot water, 0.01 and 4.0 M NaOH-extracted material. The results of this research suggest that the increase of seed hardness was correlated, even if not proportionally, with Ara-rich neutral polymer insolubilization. Accelerated conditions, induced neutral Ara-rich polymer insolubilization, as well as extensive cell damage, resulting in higher hardening degree.

Acknowledgements

The authors would like to acknowledge Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), SP-Brazil and Conselho Nacional de Pesquisa (CNPq) for the financial support and scholarship. We thank Dr. Nicholas C. Carpita, Department of Botany and Plant Pathology, Purdue University for his valuable advice on cell wall characterization.

References

Aguilera, J. M., & Ballivian, A. (1987). A kinetic interpretation of textural changes in black beans during prolongued storage. *Journal of Food Science*, *52*, 691–695.

Aguilera, J. M., & Rivera, R. (1992). Hard-to-cook defect in black beans: hardening rates, water imbibition and multiple mechanism hypothesis. *Food Research Internation*, *25*, 101–108.

Bhatty, R. S. (1990). Cooking quality of lentils: the role of structure and composition of cell walls. *Journal of Agricultural & Food Chemistry*, *38*, 376–383.

Blakeney, A. B., Harris, P. J., Henry, R. J., & Stone, B. A. (1983). A simple and rapid preparation of alditol acetates for monosaccharides analysis. *Carbohydrate Research*, *113*, 291–299.

Bressani, R. (1993). Grain quality of common beans. *Food Reveiw International*, *9*, 237–297.

Brett, C., & Waldron, K. (1996). *Physiology and biochemistry of plant cell walls* (2nd ed.). London: Chapman and Hall.

Carpita, N. C. (1983). Hemicellulosic polymers of cell walls of Zea coleoptiles. *Plant Physiology*, *72*, 515–521.

Carpita, N. C., & Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant Journal*, *3*, 1–30.

Carpita, N. C., & Whittern, D. (1986). A highly substituted glucuronoarabinoxylan from developing maize coleoptile. *Carbohydrate Research*, *146*, 129–140.

Champ, M., Brillouet, J. M., & Rouau, X. (1986). Non-starchy polysaccharides of *Phaseolus vulgaris*, *Lens esculenta*, and *Ciccer arietinum* seeds. *Journal of Agricultural Food Chemistry*, *34*, 326–329.

Filisetti-Cozzi, T. M. C. C., & Carpita, N. C. (1991). Measurement of uronic acid without interference from neutral sugars. *Analytical Biochemistry*, *197*, 57–162.

Fox, A., Morgan, S. L., & Gilbart, J. (1989). Preparation of alditol acetate and their analysis by gas chromatography (CG) and mass spectrometry (MS). In C. J. Biermann, & G. D. McGinnis (Eds.), *Analysis of carbohydrates by GLC and MS* (Vol. 1, 1st ed., pp. 87–11). Boca Raton: CRC Press.

Fry, S. C. (1983). Feruloylated pectins from the primary cell wall: their structure and possible functions. *Planta*, *157*, 111–123.

Fry, S. C. (1986). Cross-linking of matrix polymers in the growing cell walls of angiosperms. *Annual Review of Plant Physiology and Plant Molecular Biology*, *37*, 165–186.

Garcia, E., Lajolo, F. M., Filisetti, T. M. C. C., & Udaeta, J. E. M. (1998). Hard-to-cook beans (*Phaseolus vulgaris*): involvement of phenolic compounds and pectates. *Journal of Agricultural Food Chemistry*, *46*, 2110–2116.

Garcia, E., Lajolo, F., & Swanson, B. G. (1993). A comparative study of normal and hard-to-cook brazilian common bean (*Phaseolus vulgaris*): ultrastructural and histochemical aspects. *Food Structure*, *12*, 147–154.

Gibeaut, D. M., & Carpita, N. C. (1991). Clean-up procedure for partially methylated alditol acetate derivatives of polysaccharides. *Journal of Chromatography*, *587*, 284–287.

Gooneratne, J., Needs, P. W., Ryden, P., & Selvendran, R. R. (1994b). Structural features of cell wall polysaccharides from the cotyledons of mung bean Vigna radiata. *Carbohydrate Research*, *265*, 61–77.

Hincks, M. J., & Stanley, D. W. (1986). Multiple mechanisms of bean hardening. *Journal of Food Technology*, *21*, 731–750.

Ilker, R., & Szczesniak, A. (1990). Structural and chemical base for texture of plant foodstuffs. *Journal of Texture Studies*, *21*, 1–36.

Jones, P. M. B., & Boulter, D. (1983). The cause of reduced cooking rate in *Phaseolus vulgaris* following adverse storage conditions. *Journal of Food Science*, *48*, 623–649.

Liu, K. (1995). Cellular, biological, and physicochemical basis for the hard-to-cook defect in legume seeds. *Critical Reviews of Food Science and Nutrition*, *35*, 263–298.

Lozovaya, V. V., Zabotina, O. A., & Widholm, J. M. (1996). Synthesis and turnover of cell wall polysaccharides and starch in photosynthetic soybean suspension cultures. *Plant Physiology*, *111*, 921–929.

Mattson, S. (1946). The cookability of yellow peas: a colloid-chemical and biochemical study. *Acta Agricula Suecana II*, *2*, 185–231.

McDougall, G. J., Morrison, I. M., Stewart, D., & Hillman, J. R. (1996). Plant cell wall as dietary fibre: range, structure, processing and function. *Journal of the Science of Food and Agriculture*, *70*, 133–150.

McLaughlin, M. A., & Gay, M. L. (1990). Differentiation of dietary fibre sources by chemical characterization. In I. Furda, & C. J. Brine (Eds.), *New developments in dietary fibre* (Vol. 270, pp. 295–310). New York: Plenum.

McNeil, M., Darvill, A. G., Fry, S. C., & Albersheim, P. (1984). Structure and function of the primary cell walls of plants. *Annual Reviews of Biochemistry*, *53*, 625–663.

Neves, T. R. M. (2000). *Endurecimento de feijão (*Phaseolus vulgaris *L.): proteínas da parede celular ricas em hidroxiprolina*. Tese de doutorado (Doutora em Ciência dos Alimentos), Faculdade de Ciências Farmacêuticas, Universidade de São Paulo (USP).

Prosky, L., Asp, N. G., Schweizer, T. F., DeVries, J. W., & Furda, I. (1988). Determination of insoluble, soluble, and total dietary fibre in foods and food products: interlaboratory study. *Journal of the Association of Official Analytical Chemists*, *71*, 1017–1023.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, *150*, 76–85.

Updegraff, D. M. (1969). Semimicro determination of cellulose in biological materials. *Analytical Biochemistry*, *32*, 420–424.

Varriano-Martson, E., & Jackson, G. M. (1981). Hard-to-cook phenomenon in beans. Structural changes during storage and imbibition. *Journal of Food Science*, *46*, 1379–1385.

Wiechelman, K. J., Braun, R. D., & Fitzpatrick, J. D. (1988). Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. *Analytical Biochemistry*, *175*, 231–237.