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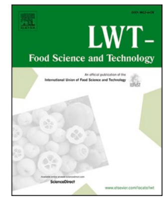

Chemical and microstructural characterization of easy- and hard-to-cook Jack bean (*Canavalia ensiformis* (L.) DC.) collections

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A R T I C L E I N F O

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A B S T R A C T

Differences in the microstructure and the chemical composition of pectic polymers were investigated from three Jack bean collections with different hard-to-cook (HTC) levels, as confirmed by the optimal cooking time analysis. These collections showed different cell and starch granule sizes, where the hardest-to-cook bean (Jombang collection) had the smallest maximum cell perimeter (285 μm), the smallest starch granule perimeter (98 μm), and the highest frequency of small intercellular space indicating more linkage in the middle lamella. No significant difference was observed in the frequency of small intercellular spaces between Malang and Cilacap despite having different optimal cooking times. Jack bean cotyledon cell walls were mainly composed of insoluble polysaccharide rich in arabinose and galactose. The beans contained high covalently bound pectin fractions (NEF) ranging from 78% to 83%. Higher bound phenolics (ferulic acid) were found with the increase in the optimal cooking times of the collections. Overall, the findings suggest the occurrence of cell wall linkage and highlight the importance of ferulic acid pectin crosslink in HTC Jack bean. This study could help develop strategies to reduce cooking time to promote the utilization of Jack bean.

1. Introduction

Due to sustainability and health issues, there is an increasing demand for alternatives of animal protein (Sá, Moreno, & Carciofi, 2020). Among plants, legumes represent an economic yet rich source of proteins (Friedman, 1996), and legumes are sustainable with lower technological challenges required for innovation (van der Weele, Feindt, Jan van der Goot, van Mierlo, & van Boekel, 2019). Legumes contribute substantially to the protein intake of a significant proportion of the world population, especially in developing countries, and are gaining more interest in developed countries. Whereas replacing animal proteins with plant proteins seems necessary for future food security, more alternatives to existing plant-based protein are needed, including promoting underutilized legumes such as Jack bean.

Jack bean (*Canavalia ensiformis* (L.) DC.) is one of the two utilized species from the genus *Canavalia* which counts approximately 60 other species (Schrire, 2005) that grow in the tropical and subtropical area (Ekanayake, Jansz, & Nair, 2000). They are grown in West Africa, Asia, South America, and the South Pacific (Sharasia, Garg, & Bhanderi, 2017). The plants are fast-growing and tolerant to drought, acidic, and nutrient-depleted soils (Sharasia et al., 2017; Udedibie & Carlini, 1998). Therefore, Jack bean has a good potential to tackle food security challenges in the future with its relatively high yield (2400–3400 kg/ha) compared to other legumes (Bressani, Brenes, García, & Elías, 1987). Jack bean is a cheap source of protein (content between 22 and 35 g/100g), carbohydrate (45–65 g/100g), and dietary fiber (4–17 g/100g) (Sridhar & Seena, 2006). Jack bean proteins contain high amounts of essential amino acids such as leucine, isoleucine, and threonine and are considered a good source of lysine (Bressani et al., 1987). It is rich in minerals and essential fatty acids such as linoleic and linolenic acid (Siddhuraju & Becker, 2001). Despite the nutritional benefits, the utilization of Jack bean is limited for human consumption and mostly destined for livestock feeding (Nwokolo, 1996). In addition, Jack beans are commonly hard-to-cook, which can be an obstacle for their utilization (Graham & Vance, 2003).

The hard-to-cook phenomenon (HTC) is a condition that does not

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allow beans to soften sufficiently after a reasonable soaking and cooking time. HTC phenomenon has been reported for several legumes, such as Pinto bean (*Phaseolus vulgaris* L.) (Njoroge et al., 2014), Jack bean (*Canavalia ensiformis* (L.) DC.) (Akpapunam & Sefa-Dedeh, 1997), Bambara groundnut (*Vigna subterranea* (L.) Verdc.) (Mubaiwa, Fogliano, Chidewe, & Linnemann, 2017), chickpea (*Cicer arietinum* L.) (Reyes--Moreno, Okamura-Esparza, Armienta-Rodelo, Gomez-Garza, & Milan-Carrillo, 2000), and kidney bean (*Phaseolus vulgaris* L.) (Parmar, Singh, Kaur, Virdi, & Shevkani, 2017). HTC is often the result of storage under both high temperature (≥25 °C) and high relative humidity (≥65%) (Chigwedere, Njoroge, Van Loey, & Hendrickx, 2019). Due to the HTC problem, cooking legumes become time-consuming, and less attractive due to its negative environmental impact since it requires more energy to process the legumes to reach acceptable palatability.

To explain the modifications of bean texture during cooking, several hypotheses have been put forward. The first suggests potential softening in the pectin-cation-phytate, implying linkage loss between demethylesterified pectin and cations (Reyes-Moreno, Paredes-Lapez, & Gonzalez, 1993). The second hypothesis involves phenolic compounds such as the ferulic acid linkage with arabinan and galactan residues of pectin (Fry, 1983) and the production of insoluble lignin as a result of oxidation and polymerization of polyphenols (Liu, 1995). The third hypothesis suggests the changes of starch and protein, which affect protein denaturation and starch gelatinization (Liu, 1995). Recent studies showed that changes in the cell wall, mainly pectin, may be the main contributor or the rate-limiting factor for tissue softening during cooking (Chigwedere, Nkonkola, et al., 2019; Shiga & Lajolo, 2006; Siqueira, Fernandes, Brito, & Santos, 2018). Tissue softening is a result of cell separation, which is facilitated by the solubilization of pectin in the middle lamella (Chigwedere et al., 2018). During relatively high temperature and high humidity storage, new interactions between cell wall polymers could occur and make cell separation more difficult.

To date, there is still limited information on the HTC problem of underutilized legumes, and none specifically on Jack bean. Moreover, understanding the molecular basis of HTC is essential to develop a processing strategy to overcome the HTC problem. Therefore, in this research, we investigated the cotyledon cellular morphology, the microstructure of the cell wall and the chemical composition in pectic polymers and related these observations to the severity of the HTC phenomenon of three collections of Jack bean.

2. Materials and methods

2.1. Materials

Jack bean (*Canavalia ensiformis* (L.) DC.) was harvested from three different regions (Malang, Cilacap, and Jombang) in Indonesia and stored in the farmers'/collectors' warehouse at ambient temperature and moisture for 3, 2, and 14 months for Malang, Cilacap, and Jombang, respectively, before being shipped to the Netherlands. The storage in tropical conditions was known to trigger HTC development and represented the real condition we found in the Indonesian market. The cooking profile of each collection was determined upon their arrival at the laboratory, and the remaining beans were stored at −80 °C until further use. All chemicals used were of analytical grade unless otherwise specified.

2.2. Determination of Jack bean cooking profile

The cooking profile was determined using the finger-pressing method as described in our previous study (Purwandari, Westerbos, Lee, Fogliano, & Capuano, 2023). In brief, one hundred Jack bean whole seeds were soaked in demineralized water (3 mL/g) at 25 °C for 16 h. Subsequently, the soaked seeds were boiled in demineralized water (2 mL/g). During the cooking process, ten beans were taken out every 30 min and their texture was assessed by pressing between the thumb and forefinger. A bean was considered as cooked once it could be easily disintegrated. The cooking profile was shown as the percentage of cooked beans at different cooking times. The optimal cooking time was the time when 9 out of 10 beans could be crushed.

2.3. Preparation of Jack bean cotyledon powder

A preliminary study was conducted on another collection of Jack bean which showed that the presence of seed coat does not change the cooking time (data not shown). Therefore, only the cotyledon was used in the present study. To prepare the cotyledon powder, raw Jack beans were manually dehulled using a knife and the seed coat was removed. The cotyledon was freeze-dried to further lower the moisture content and ground using a Ball mill (MM 400, Retsh, Germany) at frequency 1/30 s for 60 s. Cotyledon powder that passed the 425 μm sieve was collected and stored in a desiccator until further use.

2.4. Proximate analysis

Nitrogen was measured using the Dumas combustion method using a Flash EA 1112 NC analyzer (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the crude protein content was calculated using 5.4 as conversion factor (Mariotti, Tomé, & Mirand, 2008). Crude fat content was determined gravimetrically by Soxhlet extraction using petroleum ether as a solvent, and moisture content was determined by drying the sample at 105 °C overnight until constant weight. Ash content was determined according to AOAC 923.03 in a muffle furnace at 550 °C overnight (AOAC, 2002). Carbohydrate content was calculated by difference. Phytic acid content was determined using the phytic acid assay kit (K-PHYT) from Megazyme Inc. (Bray, Ireland).

2.5. Microstructural analysis

Raw Jack beans were used for microstructural analysis. The samples for microstructural observation were prepared according to a previous report (Siqueira et al., 2018). A transverse midplane slice (thickness: 0.5–0.7 cm) was cut from Jack bean cotyledon before fixation in Karnovsky solution for 24 h (with a final concentration of 40 g/L paraformaldehyde, 20 mL/L glutaraldehyde, 0.1 mL/L Nonidet P-40 and 1 mmol/L 1,4-Dithiothreitol (DTT) prepared in 0.05 mol/L piperazine-N, N′-bis(2-ethanesulfonic acid) (PIPES) biological buffer (pH 6.9)). The combination of both aldehydes in the fixative preserves cellular morphology by crosslinking and minimizes shrinkage. The bean was rinsed with the buffer and distilled water, dehydrated in increasing ethanol and water mixtures (1:9, 3:7, 1:1, 7:3, 9:1, 96:4, 1:0 for 2 h, respectively) containing 1 mM DTT to minimize oxidation and continued with absolute ethanol overnight. The bean samples were infiltrated with mixtures of absolute ethanol and rising methacrylate solution (100 mL Technovit 7100, 1 g hardener I and 2 mL PEG 400) (3:1, 1:1, and 1:3 for 3 h, respectively), followed by pure methacrylate solution overnight. Samples were polymerized in a mixture of T7100 methacrylate with freshly added Hardener II (in ratio 15:1) and incubated for 1h at 37 °C. The polymerized block was glued with Technovit 3040 to the sample holder before cutting with a microtome to produce sections of 5 μm. Sections were stretched on water surface, lifted on an object slide, and dried for minimal 5 min at 80 °C hot plate to improve sticking prior to staining. Staining was performed with histological dyes that differentiate the composition of the cellular domain. Toluidine Blue (1 g/L) was applied for the overall staining of cell wall, cytoplasm, and nuclei and observed with bright field (Eclipse 80i, Nikon) with objective 20×/N.A. 0.50. For cell walls, Calcofluor White M2R (0.1 mL/L) was used as fluorescent dye and visualized with a confocal laser scanning microscope (LSM) (Stellaris 5 Confocal LSM, Leica, Germany) with argon laser excitation at 405 nm and objective 20×/N.A. 0.5. Images were acquired using LAS X software v.4.5.0 (Leica, Wetzlar, Germany) and were analyzed with ImageJ (Fiji) v.1.52 (NIH, Maryland, USA). To

ensure comparability between collections, all images were obtained on the mature cells located approximately 5000 μm from the epidermis.

The area of intercellular space, as well as the size of cells and starch granules, was measured on confocal LSM images. The area of intercellular space, i.e. the void space between cells, was used to assess the cell wall connections. The images were turned to 8 bit and inverted, segmented with the "Find maxima" and "Segmented particles" functions. The void areas were selected using the wand tool and measured. We calculated the total intercellular space per image plane by summing up the intercellular space of one image plane. In addition, we examined the frequency distribution of each calculated area of intercellular spaces. Two image planes were used for the quantification of intercellular space. The number of measurements ranged from 160 to 186 measurements/image plane. For measuring the cell size, the maximum perimeter of the cells, i.e. that referring to the outer surface of the cell walls, was selected on the segmented images. For measuring the starch granule size, the 8-bit images were adjusted for the threshold to identify the starch granules. The perimeter of the starch granules was selected and measured. Similar to the area of intercellular space, the cell and starch granule sizes were calculated on two different image planes for each collection. A total of 100 measurements were taken to analyze the cell and the starch granules size.

2.6. Isolation of water-soluble and water-insoluble polysaccharides

Jack bean cotyledon powders were used to generate water soluble polysaccharides (WSP) and water insoluble polysaccharides (WIP) (Njoroge et al., 2014; Rovalino-Córdova, Fogliano, & Capuano, 2018). An enzymatic hydrolysis was performed to remove starch and protein using heat stable α-amylase (3000 U/mL), protease (350 Tyrosine U/mL), and amyloglucosidase (3300 U/mL) from Total Dietary Fiber kit that was purchased from Megazyme Inc. (Bray, Ireland). According to the protocol, 1 g of raw Jack bean cotyledon powder was mixed with 40 mL 0.05 mol/L 2(N-morpholino) ethanesulfonic acid (MES)/tris (hydroxymethyl)aminomethane (TRIS) buffer pH 8.2, added with 50 μL heat-stable α-amylase and incubated in a shaking water bath (70 rpm) for 30 min at 98–100 °C to hydrolyze starch. After cooling to room temperature, 100 μL of protease was added and incubated in a shaking water bath for another 30 min at 60 °C. Subsequently, the pH of samples was adjusted to 4.8 by adding HCl and 200 μL amyloglucosidase was added, followed by incubation at 60 °C for 30 min. Suspension was centrifuged for 15 min at 4700g.

The supernatant was brought to ethanol and water mixture (80:20), followed by precipitation at −20 °C for 24 h. The precipitates were carefully collected and washed with ice-cold ethanol (ethanol and water mixture (80:20)) three times, suspended in deionized water and freeze-dried. This fraction is referred as WSP. The pellet from the centrifugation was collected and homogenized using an ultraturax homogenizer (3 × 6s) first in 64 mL, then 32 mL ethanol and water mixture (96:4), and finally in 32 mL ice-cooled acetone. After each homogenization step, samples were filtered under vacuum through 595½ filter paper (90 mm, Whatman). The residue, representing the WIP, was dried at 40 °C for 16 h and ground with mortar and pestle. WSP and WIP were stored in a desiccator until further use.

2.7. Fractionation of WIP

WIP from raw Jack bean cotyledon powder was fractionated into hot water extractable fractions (WEF), chelator extractable fractions (CEF), and Na_2CO_3 extractable fractions (NEF) (Chigwedere, Nkonkola, et al., 2019). Briefly, 0.5 g of WIP was suspended in 90 mL boiling demineralized water, followed by boiling the suspension for 5 min on a hot plate with stirring. The sample was cooled in cold water and vacuum filtered, as described in section 2.6. The obtained filtrate (Filtrate I) was adjusted to pH 5, topped up to 100 mL followed by dialysis against demineralized water at 4 °C for 72 h (with changes every 12 h). The residue was suspended in 90 mL 0.1 mol/L potassium acetate solution (pH 5) containing 0.05 mol/L cyclohexane-1,2-diaminetetraacetic acid (CDTA), incubated at 28 °C for 6 h on a plate stirrer and vacuum filtered. The filtrate obtained after CDTA addition (Filtrate II) was adjusted to pH 5, and top up to 100 mL, dialyzed against 0.1 mol/L sodium chloride at 4 °C, for 36 h (with changes every 12h) and then with demineralized water at 4 °C for 36 h (with changes every 12h). The residue was mixed with 90 mL of 0.05 mol/L sodium carbonate solution containing 0.02 mol/L sodium borohydride, incubated at 4 °C for 16 h and 28 °C for 6 h and vacuum filtered. The filtrate obtained after sodium carbonate addition (Filtrate III) was adjusted to pH 5, topped up to 100 mL, and dialyzed against demineralized water at 4 °C for 72 h (with changes every 12 h). After dialysis, Filtrate I, II, and III were freeze dried and labelled as WEF, CEF, and NEF, respectively. The flow diagram of cell wall isolation and fractionation is provided in Fig. 1.

2.8. Neutral sugar and galacturonic acid composition of WSP and WIP fractions

Neutral sugar and galacturonic acid composition of WSP, WIP, WEF, CEF, and NEF were determined by high-performance anion-exchange chromatography (HPAEC), using ICS-3000 ion chromatography high-performance liquid chromatography system equipped with a CarboPac PA-1 column (2 × 250 mm) in combination with a CarboPac PA guard column (2 × 25 mm) and a pulsed electrochemical detector in pulsed amperometric detection mode (Dionex, Sunnyvale, USA). One hundred milligrams of WSP, WIP, WEF, CEF, and NEF were weighed and hydrolyzed in sulphuric acid to generate monomeric sugars (Saeman, Moore, Mitchell, & Millet, 1954). The hydrolysis was carried out in 5 mL 12 mol/L H_2SO_4 at 35 °C for 1 h. After diluting with distilled water to a final concentration of 2 mol/L H_2SO_4, the mixture was incubated for 1 h at 100 °C, then cooled on ice. A portion of hydrolysate was diluted with Milli-Q water until 1 mL volume was reached, and 2.5 μL 1 g/L bromophenol blue in ethanol was added. The pH was adjusted with barium carbonate until a clear blue colour was obtained (pH > 4.6). The remaining solution was filtrated using a 0.45 μm PTFE filter. The amount of monomeric sugars was measured according to a previous report with modifications (Gilbert-López et al., 2015). A flow rate of 0.3 mL/min was used, and 0.1 mL/min for additional postcolumn (500 mol/L NaOH), column temperature was maintained at 17 °C. Elution was performed as follows: 0–63 min Milli-Q, 63–63.1 min 150 mmol/L NaOH, 63.1–83 min 500 mmol/L NaOH, 83–100 min Milli-Q. Monomeric sugars eluted from the CarboPac PA guard column were detected after adding 0.5 mol/L sodium hydroxide (0.15 mL/min). The monomeric sugars were quantified using a calibration curve generated with standards in the range of 0–0.1 mg/mL. Pectin content was estimated by the sum of polymeric Gal A and pectin-related neutral sugars (fucose, arabinose, rhamnose, galactose, xylose) from four fractions (WSP, WEF, CEF, and NEF). In this study, Jack bean contains 14.7–18.8 mg pectin/g dry cotyledon powder (Supplementary Table 1).

2.9. Quantification of bound phenolics in WIP

Bound phenolics were extracted in duplicate using microscale alkaline treatment (Zavala-López & García-Lara, 2017). Firstly, 0.1 g of cotyledon powder was added with methanol and water mixture (80:20) to remove the soluble phenolics. The residue after centrifugation was added with NaOH and digested at 90 °C for 30 min under the nitrogen gas flush, followed by mixing for 5 min at 2500 rpm on Heidolph shaker. Samples were added with 2 mol/L HCl until the pH was 2, added with n-hexane to remove the fat layer. The bound phenolics were recovered by adding 1.5 mL ethyl acetate, mixed for 5 min at 2500 rpm, incubated at 25 °C for 10 min at 500 rpm and then centrifuged for 10 min at 2500 rpm. The ethyl acetate layer was removed carefully into a clean tube, and the extraction process was repeated twice. The extract was pooled and flushed with nitrogen until dry. The dry extract was resuspended

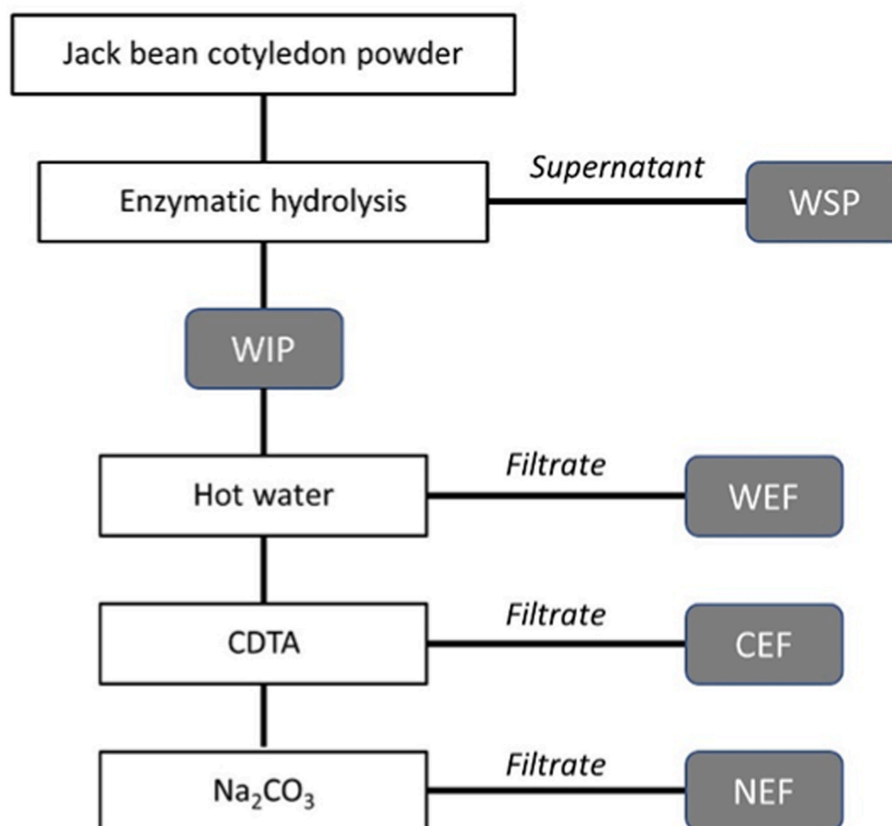

Fig. 1. Flow diagram of cell wall polysaccharide isolation and fractionation of Jack bean cotyledon powder. Water soluble polysaccharide (WSP), water insoluble polysaccharide (WIP), hot water extractable polysaccharide (WEF), chelator extractable polysaccharide (CEF), and Na_2CO_3 extractable polysaccharide (NEF) in WIP. CDTA: Cyclohexane-1,2-diaminetetraacetic acid.

with 1 mL methanol and water mixture (80:20) and stored at −20 °C until further analysis.

Total bound phenolics were analyzed using a colorimetric assay with Folin-Ciocalteau reagent, and gallic acid was used for generating a calibration curve (Urias-Lugo et al., 2015). The amount of bound phenolics was expressed as the relative amount of gallic acid equivalent to WIP by incorporating conversion factors from the yield of WIP/cotyledon powder in the calculation.

Ferulic acid was analyzed using a Dionex Ultimate 3000 apparatus (Thermo Fisher Scientific, Bleiswijk, The Netherlands). Prior to analysis, bound phenolic extracts were filtered through a 0.2 μm syringe filter. 10 μL extract was injected into the HPLC system with 1 mL/min flow rate. The separation was achieved on a RP-Polaris C18-A (46 × 150 mm) equipped with a Polar RP security guard cartridge (4 × 3 mm). The column temperature was set at 30 °C. A multistep gradient eluent was applied using water pH 2.5 (acidified with trifluoroacetic acid (TFA)) (A) and acetonitrile (B) for a total run time 35 min. The gradient program was 0 min 100:0 (A:B), 20–25 min 58:42 (A:B), 26–35 min 100:0 (A:B). Detection was performed at a wavelength of 290 nm. Ferulic acid standard was used to create calibration curve ranging from 0 to 0.1 mg/mL. Data processing was performed with Chromeleon v. 7.2 (Thermo Scientific, MA, USA).

2.10. Quantification of boron and calcium in WIP

Boron and calcium were measured on WIP of cotyledon powder. Sample (0.1 g) was digested using concentrated nitric acid, hydrochloric acid, and hydrogen-peroxide in a closed system microwave (MarsXpress, CEM corporation) (Novozamsky, Van Eck, Houba, & Van Der Lee, 1996). The supernatant was injected to inductively coupled plasma optical emission spectroscopy (ICP-OES) system (Thermo iCAP-6500 DV, Thermo Fisher Scientific), and the result was expressed as the relative amount of boron and calcium to WIP.

2.11. Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 software. A one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons was used to test the difference among the three seed collections on the quantitative data except for intercellular spaces. The frequency distributions of intercellular space were compared by applying Chi square test. Significant differences were defined at $p < 0.05$.

3. Results and discussion

3.1. Cooking profile of Jack bean from different collections

The cooking profile of the three collections of Jack beans selected in this study is presented in Fig. 2. The three collections of Jack bean showed different optimal cooking times, i.e. cooking times to achieve a 90% cooked stage. Malang collections showed the shortest optimal cooking time (120 min), followed by Cilacap (195 min) and Jombang (300 min). No bean was cooked after boiling for 30 min for any of the three Jack bean collections. Interestingly, Cilacap showed a higher % cooked beans compared to the Malang collections at 60 and 90 min of cooking. However, the situation was reversed after 90 min of cooking. In fact, after 120 min the percentage of cooked beans was almost 100% in Malang, but Cilacap took an additional 90 min to reach the same percentage of cooked beans as Malang. Jombang was the slowest to soften among the three collections, with 90% cooked beans after 300 min.

A previous study classified beans that need 2 h or less to achieve 80%

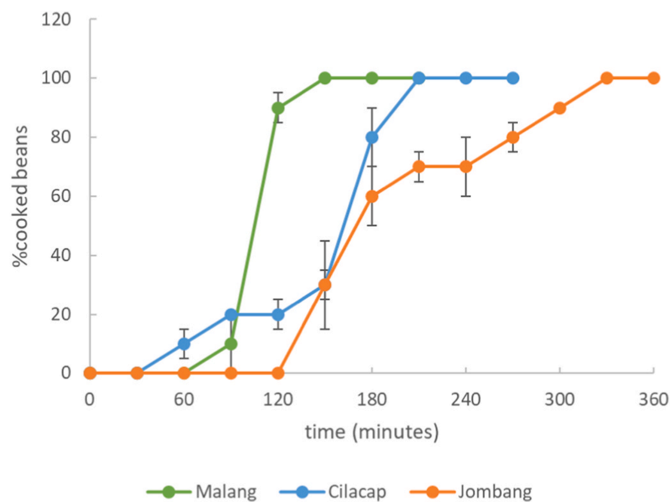

Fig. 2. Cooking profile of three Jack beans collections harvested and dried at different locations (N = 2, error bars indicate standard deviation of the mean).

cooked beans as easy-to-cook (ETC), and else was considered as hard-to-cook (HTC) (Kinyanjui et al., 2015). Based on this classification, both Cilacap and Jombang should be grouped as HTC. Elevated storage temperature and relative humidity as well as prolonged storage time were reported as the main factors for the development of HTC phenomenon (Njoroge et al., 2015; Siqueira & Pereira, 2014). In this study, the Jombang collection, which was stored with the longest time (14 months), showed the longest optimal cooking times. However, Cilacap showed a longer optimal cooking time compared to Malang despite being stored for a shorter time. Beside the storage factors, the growing region can contribute to HTC. Growing regions with longer warm climates were reported to impact cell wall properties and produce a harder-to-cook Navy bean (Chu, Ho, & Orfila, 2020). In this study, all the three collections were grown at different regions (Supplementary Table 2). Malang was grown in a mountainous area (elevation: 444 m) with lower temperature (<25 °C) compared to Cilacap and Jombang. This may partly explain the different longer cooking time of Cilacap (stored for 2 months) compared to Malang (stored for 3 months).

3.2. *Proximate composition and phytic acid content of the three Jack bean collections*

The proximate and phytic acid content of the three Jack bean collections are shown in Table 1. Proximate composition varied between collections except for ash content. Malang showed the highest carbohydrate content, followed by Jombang and Cilacap. Similar crude protein and crude fat content was found in Malang and Jombang collections, while Cilacap showed the highest crude protein and the lowest crude fat content among all collections.

Table 1
Proximate composition and phytic acid content of three different Jack bean collections (N = 2, Mean ± SD).

Collections	Carbohydrate (g/100 g db)	Protein (g/100 g db)	Fat (g/100 g db)	Ash (g/100 g db)	Phytic acid (g/100 g db)
Malang	67.2 ± 1.0[a]	26.2 ± 0.97[a]	3.4 ± 0.1[a]	3.2 ± 0.1[a]	1.6 ± 0.0[a]
Cilacap	61.8 ± 0.3[b]	32.0 ± 0.4[b]	2.9 ± 0.1[b]	3.3 ± 0.1[a]	2.2 ± 0.1[b]
Jombang	64.4 ± 0.1[c]	28.9 ± 0.3[a]	3.6 ± 0.1[a]	3.1 ± 0.0[a]	1.1 ± 0.0[c]

Different letters in the same column indicate significant differences ($p < 0.05$).
db: dry weight basis.

The highest phytic acid content was found in Cilacap, followed by Malang and Jombang. The pectin-phytic acid-pectate theory states that storage in elevated temperature and relative humidity is favorable for activating endogenous phytase, causing dephosphorylation of phytic acid and releasing divalent minerals. Divalent minerals would migrate from parenchyma cells and concentrate around the middle lamella of the cell wall, possibly binding to the free carboxyl groups of pectin to form insoluble pectate (Kruger, Minnis-Ndimba, Mtshali, & Minnaar, 2015). Here, we observed a lower phytic acid content in Jombang but a higher phytic acid content in Cilacap compared to Malang. It is important to note that the development of HTC is related to the decrease of phytic acid content according to pectin-phytic acid-pectate theory. Therefore, the phytic acid content alone is not predictive of the optimal cooking time in Jack beans.

3.3. *Microstructural characterization of the three Jack bean collections*

Fig. 3 gives an overview of Jack bean cotyledon cells cross planes. Cotyledon cells of Jack beans had a regular oval shape where Jombang had the lowest maximum perimeter (285 μm) compared to Malang (318 μm) and Cilacap (323 μm) (Supplementary Table 3). Spherical protein bodies could be observed in all three collections. Large starch granules embedded in a protein matrix could be observed in all the collections with the HTC ones (Cilacap and Jombang), showing smaller starch granule sizes (average: 100 and 98 μm) compared to Malang (106 μm) (Supplementary Table 3). To our knowledge, there are no reported data on the size of the starch granules in beans with different HTC levels. The degree of starch crystallinity was reported to increase during high temperature and high relative humidity storage, increasing the gelatinization temperature (Yousif, Kato, & Deeth, 2007). However, starch was reported to be completely gelatinized after 30 min of cooking of beans, irrespective of its HTC level, which suggests that starch properties play a minor role in HTC (Chigwedere et al., 2018).

An overview of the cell wall of the three Jack bean collections can be seen in Fig. 4. Cell integrity seems preserved in all Jack bean collections, i.e. we did not observe any cell wall fracture in any of the three collections. However, in Carioca beans the HTC bean showed evidence of cell wall rupture compared to the ETC bean (Siqueira et al., 2018). We observed cell contacts, i.e. the cell wall area where two adjacent cells were connected (as indicated by the yellow arrow) (Fig. 4) in all three collections. We examined the area of the intercellular space between cells as a measure of cell wall connections. It was reported that HTC bean showed less intercellular space compared to fresh bean due to the linkage in the middle lamella forming tricellular junctions (Perera, Devkota, Garnier, Panozzo, & Dhital, 2023). There was no significant difference in the total intercellular space areas of the three collections (Supplementary Table 3). However, the frequency distribution of intercellular spaces in the Jombang collection was significantly higher than Cilacap and Malang (Fig. 5) indicating more cell wall connections. However, the lack of a significant difference in the frequency distribution of the intercellular spaces between Malang and Cilacap demonstrates that the number of cell wall connections is not always correlated to the optimal cooking time.

3.4. *Chemical characterization of Jack bean cell wall polysaccharides*

The WSP and WIP yields obtained from the three collections of Jack bean are shown in Table 2. Representative chromatograms of sugar of the collections can be seen in Supplementary Fig. 1. There was a difference ($p < 0.05$) between the amount of WSP extracted from the three collections where Jombang showed the least amount of WSP followed by Cilacap and Malang. This is in line with previous studies that reported a negative correlation between WSP and cooking time in common beans and Carioca beans (Chigwedere, Nkonkola, et al., 2019; Shiga, Lajolo, & Filisetti, 2004). Unlike WSP, WIP yield was not significantly different among the three Jack bean collections and ranged from 135 to 151 mg/g

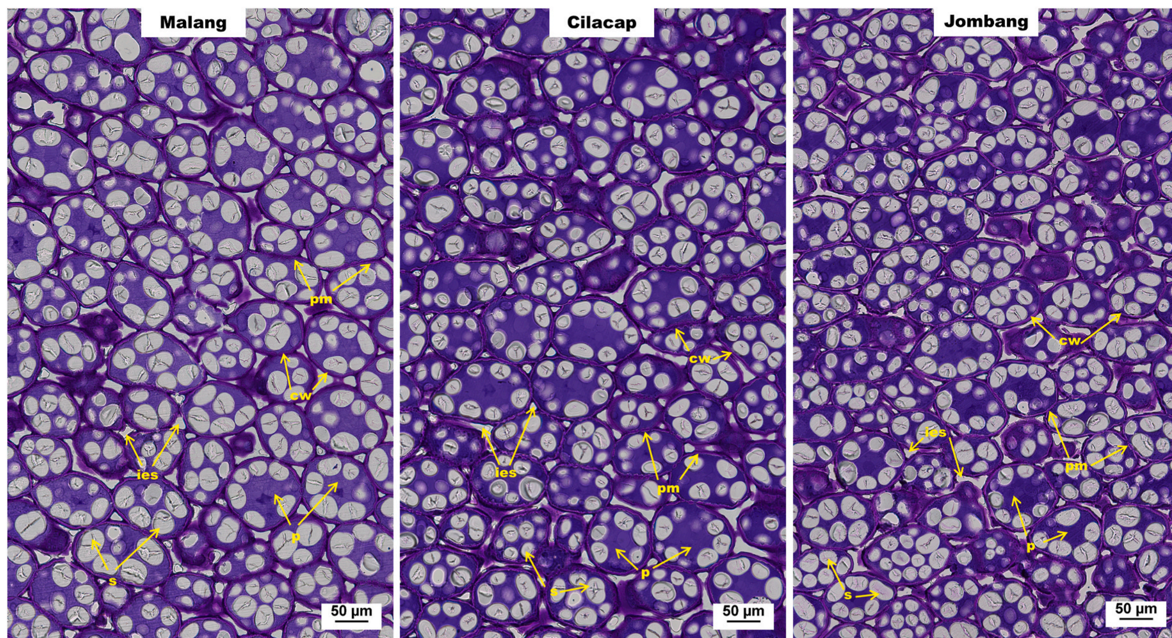

Fig. 3. Overview of light microscope images of cotyledons of Malang (panel Malang), Cilacap (panel Cilacap), and Jombang (panel Jombang) stained with toluidine blue. s: starch granule, p: protein bodies, cw: cell wall, pm: plasmamembrane, ies: intercellular space. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

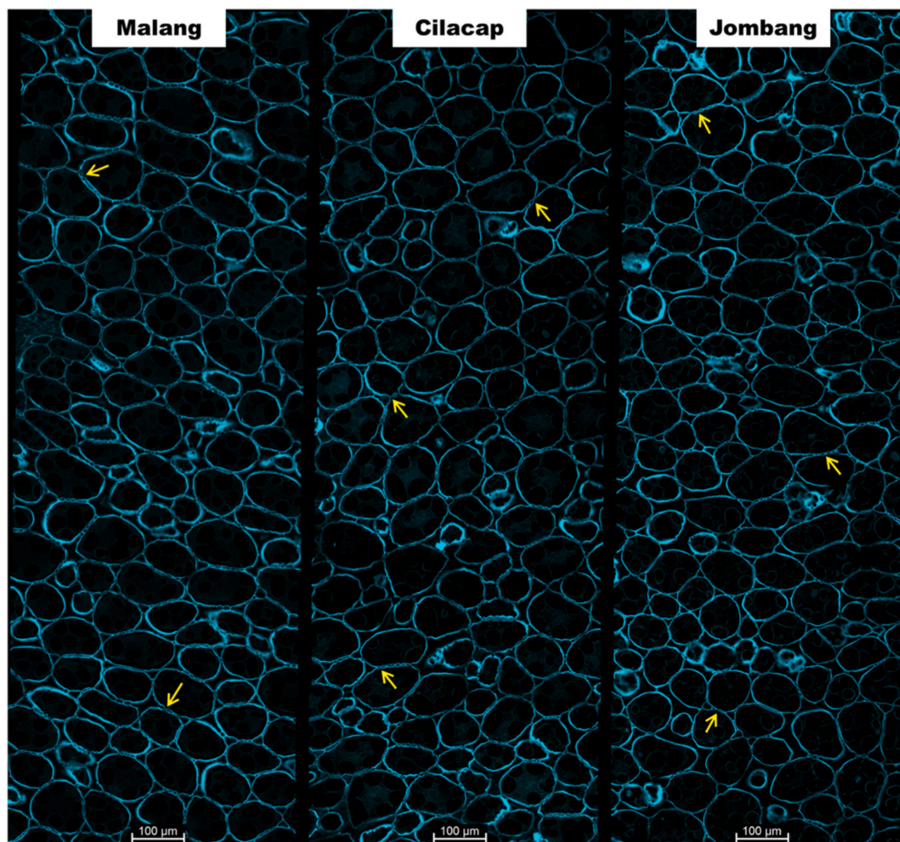

Fig. 4. Overview of confocal microscopy images of cotyledon of Malang, Cilacap, and Jombang collections. Cell wall (green) are stained with calcofluor white. Yellow arrow indicates the cell wall at the cell contacts. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

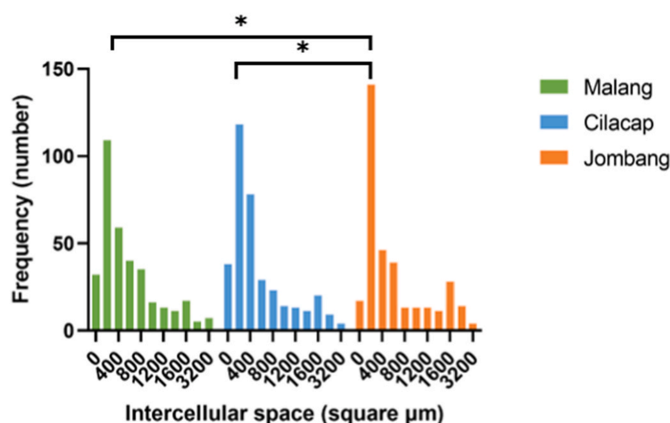

Fig. 5. Frequency distribution of the intercellular spaces of three Jack bean collections. Significant differences are indicated by an asterisk symbol ($p < 0.05$).

dry cotyledon.

Sugar composition of WSP and WIP are presented in Table 2. The cell wall of Jack bean cotyledon was composed of mainly insoluble fractions. Among the three collections, Malang had a significantly higher total sugar content in the WSP fraction, while no significant difference was observed between Cilacap and Jombang. Arabinose and galactose were the main pectin-related neutral sugar in both polysaccharide extracts, probably derived from arabinans or arabinogalactan. High arabinose content is also reported in other legume cotyledon such as lentil, kidney bean, and Carioca bean (Bhatty, 1990; Rovalino-Córdova et al., 2018; Shiga & Lajolo, 2006). In this study, a lower uronic acid content was observed compared to other varieties of Jack beans, as previously reported (Benítez et al., 2013). The difference is possibly due to the use of different part of the seeds. It is not clear whether only the cotyledon or the whole seed was used in the reported study, and GalA was mainly presented in the seed coat rather than in the cotyledon (Yi et al., 2016).

3.5. Chemical characterization of the pectin fraction

WIP was fractionated into WEF, CEF, and NEF fractions in which relative content and composition in neutral sugar, galacturonic acid, and glucuronic acid are displayed in Fig. 6. We found low GalA in all three fractions of Jack bean cotyledon, suggesting that Jack beans contain limited homogalacturonan, as can be seen in soybean (Voragen, Coenen, Verhoef, & Schols, 2009).

All in all, there were small differences in the distribution of WEF, CEF and NEF among the three collections. Jombang showed the least relative amount of WEF and the highest relative amount of NEF (Fig. 6A). WEF fraction contains the loosely bound pectin that can easily be solubilized with hot water extraction. No differences were observed in the sugar composition of the WEF fractions from the three collections (Fig. 6B). CEF represents the calcium crosslinked pectin fraction. We observed a higher content in arabinose in Malang compared to the other two collections (Fig. 6C). Cell wall connection is widely attributed to the properties of pectin, typically through the calcium crosslinked homogalacturonan (Daher & Braybrook, 2015; Jarvis, Briggs, & Knox, 2003). NEF represents the ester-linked pectin fractions. The link occurs between arabinan and galactan residues in Rhamnogalacturonan I with phenolic acids (such as ferulic acid); leading to the formation of ferulate dimers catalyzed by peroxidase and consequently enhancing the cell wall strength (Fry, 1983; Shiga et al., 2004). In addition, apiofuranosyl residue of xylose-containing side chains in Rhamnogalacturonan II may ester link with borate (Ishii et al., 1999). Arabinose and galactose in NEF fraction were higher in Cilacap and Jombang compared to Malang, showing that these two sugars occur more in the ester-linked form in the first two collections than in the Malang collection (Fig. 6D). Previously, it was postulated that some loosely bound pectin is converted into covalently bound pectin during development of HTC (Chigwedere, Nkonkola, et al., 2019). We must mention, however, that the differences in NEF content are rather small ranging from 78% to 83%.

In WIP fractions, we found a similar amount of calcium and boron content but higher bound phenolic/ferulic acid ($p < 0.05$) with increasing optimal cooking times (Table 3). This corresponds to the covalently bond pectin crosslink, possibly the ferulic acid pectin crosslink. Ferulic acid has been linked to the crosslink of pectic arabinans and galactans in several dicotyledons (Fry, 1983; Wefers, Tyl, & Bunzel, 2014). A recent study with immunolabelling microscopy of red Haricot bean showed evidence of higher feruloylated pectin crosslinking at intercellular junction zones of aged beans compared to non-aged beans (Chen et al., 2021). It is important to note that the amount of ferulic acid that has been measured may give an approximate indication of the actual level of crosslinks in the Jack bean cell wall. The crosslink of ferulic acid and polysaccharides occurs via ferulic acid dimers and trimers (Waterstraat, Bunzel, & Bunzel, 2016), and those are not quantified by our method. In sugarbeet and beetroot, the feruloylated moieties have an impact on the textural degradation during heat treatment (Waldron, Ng, Parker, & Parr, 1997). Sugarbeet, where 20% of the feruloyl moieties are integrated into dimers, does not completely soften after several hours of heating at 100 °C, whereas beetroot, where 10% of the feruloyl moieties are integrated into dimers, softens in 20–30 min.

4. Conclusion

This paper provides, for the first time, a characterization of microstructure and composition in Jack bean collections with different levels of HTC induced by different storage times and growing regions. We found modest differences in the microstructure of these three Jack bean collections. Our finding, however, reveal that the hardest-to-cook Jack

Table 2
Yield, sugars composition and total sugars of water-soluble (WSP) and water-insoluble polysaccharides (WIP) from the three Jack bean collections (mg/g dry cotyledon) (N = 2, Mean ± SD).

Collections	Yield	Sugars									Total sugars
		Fuc	Ara	Rha	Gal	Glc	Xyl	Man	GalA	Glu	
Malang											
WSP	141.7^a	0.3 ± 0.1^a	8.2 ± 0.5^a	0.3 ± 0.1^a	4.5 ± 0.1^a	3.5 ± 0.2^a	0.9 ± 0.1^a	5.4 ± 0.0^a	1.3 ± 0.2^a	0.6 ± 0.1^a	24.9 ± 1.1^a
WIP	144.3^A	0.4 ± 0.0^A	38.7 ± 1.1^B	0.0 ± 0.0^A	7.4 ± 0.3^A	25.0 ± 0.2^A	4.0 ± 0.1^A	0.0 ± 0.0^A	1.5 ± 0.0^A	0.7 ± 0.1^A	77.6 ± 1.3^A
Cilacap											
WSP	129.0^a	0.2 ± 0.0^a	4.9 ± 0.2^b	0.2 ± 0.0^a	3.4 ± 0.1^b	3.0 ± 0.1^{ab}	0.5 ± 0.0^a	4.4 ± 0.0^b	0.6 ± 0.0^a	0.3 ± 0.0^a	17.5 ± 0.5^b
WIP	135.1^A	0.3 ± 0.0^A	37.0 ± 1.9^B	0.0 ± 0.0^A	7.4 ± 0.4^A	17.1 ± 4.6^B	3.9 ± 0.1^A	0.0 ± 0.0^A	1.3 ± 0.1^A	0.7 ± 0.0^A	67.7 ± 7.1^B
Jombang											
WSP	103.4^b	0.1 ± 0.0^a	5.3 ± 0.3^b	0.2 ± 0.0^a	3.5 ± 0.3^b	2.3 ± 0.1^b	0.5 ± 0.0^a	4.1 ± 0.1^b	0.7 ± 0.1^a	0.4 ± 0.0^a	17.1 ± 0.5^b
WIP	151.1^A	0.4 ± 0.0^A	42.1 ± 1.3^A	0.0 ± 0.0^A	8.1 ± 0.4^A	24.1 ± 1.7^A	4.1 ± 0.2^A	0.0 ± 0.0^A	1.6 ± 0.1^A	0.7 ± 0.0^A	80.9 ± 3.5^A

Different lowercase and capital superscript letters within a column indicate significant differences ($p < 0.05$). Fuc: fucose, Ara: arabinose, Rha: rhamnose, Gal: galactose, Glc: glucose, Xyl: xylose, Man: mannose, GalA: galacturonic acid, Glu: glucuronic acid.

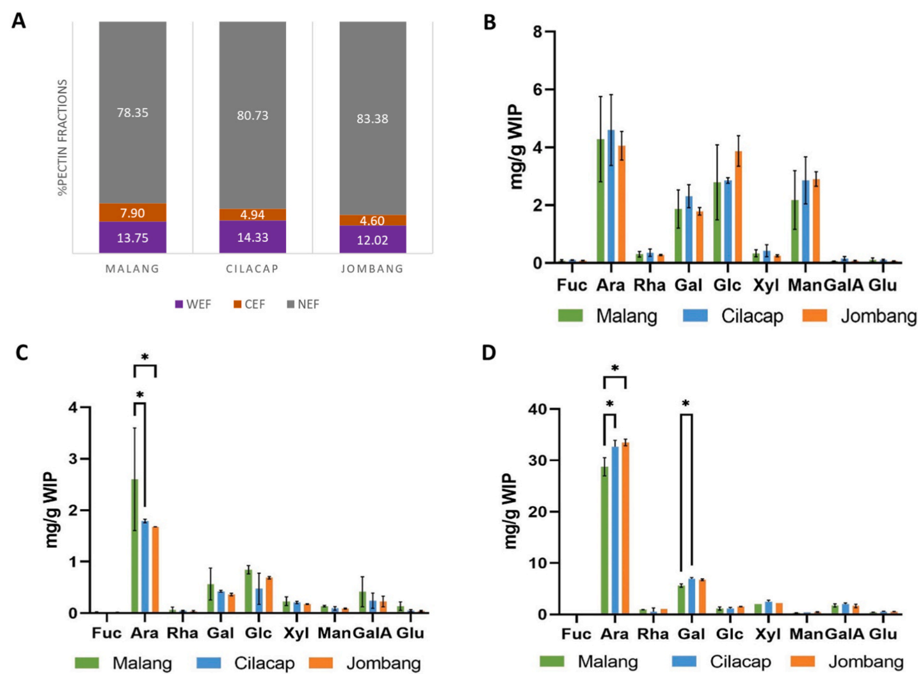

Fig. 6. Relative amount of hot water extractable polysaccharide (WEF), chelator extractable polysaccharide (CEF), and Na_2CO_3 extractable polysaccharide (NEF) in WIP fraction from the three Jack bean collections (panel A). Neutral sugar, GalA and Glu composition of WEF (panel B), CEF (panel C) and NEF (panel D) from three different Jack bean collections. Fuc: fucose, Ara: arabinose, Rha: rhamnose, Gal: galactose, Glc: glucose, Xyl: xylose, Man: mannose, GalA: galacturonic acid, Glu: glucuronic acid. Significant differences are indicated by asterisk symbol ($p < 0.05$). (N = 2, error bars indicate standard deviation of the mean).

Table 3
Mean and standard deviation of calcium, boron, bound phenolic, and ferulic acid of three Jack bean collections (N = 2).

Collections	Calcium (g/kg WIP)	Boron (mg/kg WIP)	Bound phenolic (μg GAE/g WIP)	Ferulic acid (μg/g WIP)
Malang	1.2 ± 0.0[b]	26.0 ± 0.0[a]	341.0 ± 26.8[c]	12.0 ± 1.3[c]
Cilacap	1.5 ± 0.1[a]	27.5 ± 2.5[a]	502.9 ± 25.1[b]	20.5 ± 0.2[b]
Jombang	1.4 ± 0.0[ab]	22.5 ± 1.5[a]	636.8 ± 17.1[a]	29.5 ± 1.9[a]

Different letters indicate significant differences ($p < 0.05$).

bean collection (Jombang) has more cell wall connections, as showed by a higher frequency number of the fractions of small intercellular space, compared to the other two collections. The proximate and phytic acid content differs among collections, with no apparent correlation to the optimal cooking time. The water-soluble polysaccharides (WSP) and the covalently bond pectin fractions (NEF) are associated to the increasing optimal cooking times in Jack bean. Whether this difference in the cooking behaviour of Jack bean can be linked to molecular modifications remains to be studied. Another possible tool, such as FTIR, can be utilized to evaluate the chemical bond caused by HTC on the cell wall fractions. To date, there is still limited study on the ferulic dimers or oligomers on legume cell wall related to the development of HTC. Furthermore, it is necessary to investigate the role of ferulic-pectin crosslinking on the development of textural softening upon cooking to gain a mechanistic understanding of the HTC Jack bean. This can be done, for instance, by quantifying the ferulic dimer or oligomer in the pectin fractions during different cooking times and eventually finding collections or providing solutions to reduce optimal cooking times.

CRediT authorship contribution statement

Fiametta Ayu Purwandari: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Vincenzo Fogliano:** Conceptualization, Writing – review & editing, Visualization, Supervision, Project administration. **Norbert C.A. de Ruijter:** Methodology, Investigation, Data curation, Writing – review & editing. **Edoardo Capuano:** Conceptualization, Methodology, Visualization, Supervision, Writing – review & editing, Project administration, Resources.

Declaration of competing interest

The authors declare no conflict of interests that may influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.115451.

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