Targeted hydrothermally induced cell biopolymer changes explain the *in vitro* digestion of starch and proteins in common bean (*Phaseolus vulgaris*) cotyledons

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Abstract

Digestion of macro-nutrients (protein and starch) in pulses is a consequence of the interplay of both extrinsic (process-related) and intrinsic (matrix-dependent) factors which influence their level of encapsulation and physical state, and therefore, their accessibility by the digestive enzymes. The current work aimed at understanding the consequences of hydrothermally induced changes in the physical state of cell biopolymers (cell wall, protein, and starch) in modulating the digestion kinetics of starch and proteins in common beans. The hydrothermal treatments were designed such that targeted microstructural/biopolymer changes occurred. Therefore, bean samples were processed at temperatures (between 60 – 95 °C) for 90 minutes. It was demonstrated that these treatments allowed to modulate starch gelatinization, protein denaturation and cell separation. The specific role of hydrothermally induced starch gelatinization, protein denaturation, alongside enhanced cell wall permeability on the digestion kinetics of common bean starch and proteins was illustrated. For instance, bean samples processed at T > 70 °C were marked by higher levels of starch digestibility (*Cf* values above 47%) compared to the partially (un-)gelatinized samples (processed at T ≤ 70 °C) (*Cf* values below 35%). Similarly, samples processed at T > 85 °C exhibited significantly higher levels of protein digestibility (*Cf* values above 47%) resulting from complete protein denaturation. Moreover, increased permeability of the cell wall to digestive enzymes in these samples (T > 85 °C) increased levels of digestibility of both gelatinized starch and denatured proteins. This study provides an understanding of the potential use of hydrothermal processing to obtain pulse-based ingredients with pre-determined microstructural and nutritional characteristics.

Keywords

Gelatinization, denaturation, kinetics, digestibility, microstructure, pulses.

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1 Introduction

Pulses such as common beans are regarded as vital components of the human diet. They are rich sources of dietary carbohydrates, proteins, vitamins, and minerals.[1] They also contain bioactive compounds including polyphenols, phytates and other compounds with various metabolic effects in the human body.[2] The link between consumption of pulses and reduced risk of diseases such as type 2 diabetes, cardiovascular diseases and obesity is well documented.[3–7] Of great interest recently is the relationship between consumption of pulses and glycemic control, which is an important factor determining the risk of type 2 diabetes.[8]

Nutrient digestibility, particularly the digestibility of starch and proteins, is paramount in evaluating the nutritional quality of common beans. Starch in common beans primarily occurs in a semi-crystalline granular structure and is built up of linear chains of amylose and highly branched amylopectin.[9] The presence of cell wall/protein barriers, the high amylose-amylopectin ratio, the starch crystallinity, the high content of viscous soluble dietary fibre, and the presence of antinutritional factors including phytic acid and polyphenols are among the factors limiting digestibility of starch in common beans.[10–14] On the other hand, digestibility of proteins in beans is hindered by the presence of a cell wall barrier, protein structure and the presence of antinutritional factors such as trypsin inhibitors, tannins and, lectins.[12,15]

Currently, various studies are focused on the use of processing techniques, including hydrothermal processing, to influence the release of cellular nutrients, particularly starch and proteins, from pulse-based foods thus modulating the rate and extent of their digestion.[16,17] Hydrothermal processing (cooking) is often used to achieve the desired palatability and nutritional quality in common beans. It promotes a sequence of cellular-level physicochemical events including starch gelatinization, protein denaturation and pectin solubilization. These changes cumulatively lead to softening of beans and improve nutrient digestibility in common

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beans.[13,18] Both temperature and time of cooking are important parameters influencing the sequence and occurrence of these changes.[18–20] Starch gelatinization, which is characterized by loss of its semi-crystalline structure (the swelling of starch granules and dissolution of starch polymers, although limited by the confinement in case of intact cells) occurs at an early phase of cooking (temperature of about 60 – 80 °C). This process enhances the susceptibility of starch to enzymatic hydrolysis by amylase, hence, increasing starch digestion.[16,21] This is succeeded by protein denaturation, which is characterized by unfolding of the protein molecules and changes in protein solubility, occurring above 80 °C.[22] These changes in the protein matrix lead to improved digestibility of proteins in beans.[23] Moreover, thermal inactivation of antinutrients such as trypsin inhibitors, lectins and tannins[15] during cooking enhances the hydrolytic activities of proteases thus enhancing digestibility of proteins. In contrast, it has been reported that, protein crosslinking and aggregation can occur upon increasing the thermal treatment intensity, rendering the protein less accessible and less susceptible to enzymatic hydrolysis.[23,24] At temperatures above 85 °C, gradual hydrothermal-mediated solubilization of pectin occurs causing the weakening of the middle lamella and subsequent cell separation upon application of a shear force such as chewing or mixing. Pectin solubilization is also linked to increased permeability of the cell wall to digestive enzymes.[17,25,26] Existing literature describes a well-established influence of these phenomena during the digestion of cellular proteins and starch. For instance, by reducing the cell wall and protein barriers, gelatinized starch becomes more exposed to amylases, thereby enhancing its digestibility.[19,27] Whereas many authors have used time-dependent hydrothermal processing techniques to modulate the starch and protein digestion kinetics for various pulse matrices including mechanically disintegrated whole cotyledon seeds,[13,19] individual cotyledon cells[19,27,28] and isolated macronutrients (starch and/or proteins),[11,29] the influence of temperature is often overlooked despite the known sensitivity of these events to temperature. Furthermore, the overlapping nature of the events of starch

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gelatinization, protein denaturation and pectin solubilization during a typical cooking process at around 100 °C, makes it difficult to accurately determine their specific role in modulating the digestion kinetics of a specific macronutrient (starch and/or proteins). Therefore, the goal of the current study was to use targeted hydrothermal processes to induce sequential changes in the physical state of these cellular components and to evaluate the role of these sequential changes in modulating the digestion kinetics of starch and proteins in common beans. It was hypothesized that hydrothermal processing carried out at distinct temperatures for a rather long time (samples reaching quasi equilibrium conditions) allowed generation of bean samples with varying physical state of the cell biopolymers. That is, samples characterized by different levels of starch gelatinization, protein denaturation and pectin solubilization were generated. Pectin solubilization was expected to be linked with weakening of the middle lamella and cell separation. It was hypothesized that this hydrothermal processing strategy and subsequent mechanical disintegration of the processed common bean samples (mimicking mastication) before the analysis of their digestion kinetics following the revised INFOGEST static *in vitro* digestion protocol[30] would enable us to better understand the role of the different polymer changes involved.

2 Materials and Methods

2.1 Raw materials

Freshly dried and packaged common beans (*Phaseolus vulgaris*) of Katumani B1 (KATB1) variety (locally referred to as yellow beans) were obtained from Kenya Agricultural and Livestock Research Organization (KALRO), Kenya. The freshly dried beans (moisture content of 11.68 ± 0.13%) were cleaned and sorted to remove spoiled and damaged seeds and foreign materials. To ensure stability of the beans against biochemical changes such as the development of hard-to-cook defect during storage, the beans were stored at -40°C until further use.

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2.2 Determination of the cooking profile and cooking time

The cooking profile was determined by measuring the hardness of the bean cotyledons as a function of cooking time.[26] Briefly, beans were soaked in deionized water (seed-water ratio of 1:5 w/v) at 25 °C for 16 h. The soaked beans were equally divided into 11 different containers (corresponding to the preselected cooking times) and hydrothermally processed (bean-water ratio of 1:5 w/v) at 96 °C for up to 120 minutes. The beans were cooled to room temperature and their respective texture (hardness) measured by compression test using a texture analyzer (TA-XTplus Stable Micro Systems, Godalming, UK) equipped with a 25 mm diameter aluminum probe and 25 Kg-f cell. The compression was done to reach a strain of 75% at the speed of 1 mm/s. The maximum compression force recorded for a half cotyledon was recorded as the hardness.[31] The cooking profile was obtained by plotting the average hardness of the bean cotyledons (n = 20) against cooking time. To determine the evolution of hardness during cooking, the fractional conversion model was used to fit the data as shown in **Equation 1**.

$H = H_f + (H_0 - H_f) * e^{-k*t}$	**Eq. 1**

Where, H is the relative hardness at time t, H_0 is the initial relative hardness, H_f is the maximum limit of the relative hardness at an infinite time, t is the cooking time (in minutes) and k is the softening rate constant during cooking.

2.3 Sample generation

From the cooking curve obtained in section 2.2 and presented in **Figure 1**, the cooking time of 90 minutes was identified as time when the bean texture reached a plateau value, the beans being sufficiently softened (95% of the beans were sufficiently cooked at 96 °C). To obtain bean samples with variable physicochemical characteristics (starch gelatinization, protein

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126 denaturation and cell separation), whole common bean seeds were subjected to varying levels
127 of heating intensities by heating the samples for 90 minutes at specific temperatures (isothermal
128 conditions). The 90 minutes treatment time was considered as a time for the samples to reach
129 a quasi-equilibrium of specific levels of starch gelatinization, protein denaturation and pectin
130 solubilization/cell separation at different temperatures. First, the beans were soaked in
131 deionized water (1:5 w/v) at 25 °C for 16 h. The soaked beans were separately processed (bean-
132 water ratio of 1:5 w/v) at selected temperatures (60, 65, 70, 75, 80, 85, 90 and 95 °C) for 90
133 minutes. This temperature range was selected as it corresponds to the range within which starch
134 gelatinization, protein denaturation and cell separation changes occur.[20,32] The thermally
135 treated common bean samples were drained of the cooking liquid and dehulled alongside the
136 untreated samples. The "untreated" bean samples in this context referred to raw bean samples
137 which did not undergo hydrothermal treatment after soaking. To mimic *in vivo* mastication, the
138 dehulled samples were mechanically disintegrated using mortar and pestle.[17,26] The samples
139 were then frozen in liquid nitrogen and stored at -40 °C until use.

140 2.4 Physical-chemical characterization

141 2.4.1 Chemical composition

142 The moisture content of the samples was determined in triplicate as the percentage water loss
143 upon oven drying at 100 °C for 24 h (Thermo Fisher Scientific Inc., Waltham, MA, USA). The
144 weight after drying was taken as the dry matter content of the sample.

145 A duplicate analysis of starch, ash and nitrogen content was conducted on freeze-dried (Alpha
146 1– 4, LSCplus, Martin Christ, Germany) samples generated as explained in section 2.3 upon
147 ball milling (MM400, Retsch, Haan, Germany) of these samples. Total starch content was
148 determined using Megazyme Total Starch kit-DMSO format (AA/AMG, Megazyme Inc. Bray,
149 Ireland) following the procedure described by the manufacturer. To convert the glucose

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150 released from the sample to total starch (%), a conversion factor of 0.95 was used. Duma's
151 analysis method (CHNS-O Elemental Analyzer, CE instrument, Thermo Fischer Scientific,
152 Waltham, MA, USA) was used to determine the total nitrogen content of the sample. A
153 conversion factor of 5.4 (for legume seeds) was used to translate the determined total nitrogen
154 to crude protein content. [33] A modified Official AOAC Method 942.05 was used to determine
155 ash content.[16,34] Briefly, 200 mg of sample was incinerated in a muffle furnace (Nabertherm
156 Controller P330, Lilienthal, Germany) at 550 °C for 24 h. The measured weight of the residue
157 represented the ash content of the sample.

158 ### 2.4.2 Microstructural characterization

159 Microstructural properties including mode of tissue failure, starch gelatinization and particle
160 size distribution were assessed using differential interference contrast (DIC) light microscopy,
161 polarized light microscopy and laser diffraction.[28] Briefly, 2% suspension of each sample was
162 separately suspended in deionized water and mounted on a glass slide. Representative light
163 micrograph images were captured with an Olympus BX-51 light microscope (Olympus,
164 Optical Co. Ltd, Tokyo, Japan) fitted with cellSense Standard® photo-analyzing software.
165 Polarized light mode was used to evaluate the extent of starch gelatinization.

166 An LS 13 320 particle size analyzer (Beckman Coulter Inc., US) equipped with a Universal
167 Liquid Module was used to determine the volumetric particle size distribution of the samples.[28]
168 The analysis was conducted in triplicate.

169 ### 2.4.3 Thermal properties

170 Given the influence of starch gelatinization and protein denaturation on the digestibility of
171 starch and proteins, it was necessary to evaluate the extent of these phase transitions in the
172 different samples. Consequently, a Q2000 heat flux Differential Scanning Calorimeter (TA
173 Instruments, New Castle, DE, USA) with Advanced Tzero™ technology and RCS 90

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refrigeration system was used to determine gelatinization and denaturation parameters (residual enthalpies) as influenced by thermal processing.[35] High-volume DSC pans were filled with 10 mg of sample and deionized water (sample-water ratio of 1:3 w/v). The pans were then hermetically sealed and allowed to equilibrate overnight at 25 °C. A pan solely containing a known weight of indium standard, used to calibrate the temperature and enthalpy of the DSC equipment, was also prepared. An empty high-volume pan was used as the reference pan. Thereafter, each pan containing the sample/indium, alongside the reference pan, was loaded in the DSC oven and heated from 20 to 120 °C at the rate of 5 °C per minute while measuring the heat flux required to increase the temperature of the sample over time. The peak starch gelatinization and protein denaturation temperatures (T_p) of the duplicate samples were processed using TA Universal Analysis 2000 Software, (Version 4.5A, TA Instruments-Waters LLC, New Castle, DE, USA). The associated enthalpy change (ΔH) was determined upon deconvolution of the starch gelatinization and protein denaturation peaks using OriginPro software 2020 (Origin Lab Cooperation, USA).

2.5 Static *in vitro* digestion of hydrothermally processed beans

2.5.1 *In vitro* digestion procedure

Digestion simulation experiments were conducted based on the revised INFOGEST static *in vitro* digestion protocol.[30] The *in vitro* digestion was carried out in three phases: oral, gastric, and small intestinal. Prior to this, the enzymatic activities and bile salt concentration were assessed following the INFOGEST static *in vitro* protocol.[30] Similarly, all mass/volume ratios used during digestion were based on the targeted concentrations of solutes and enzyme activities in the digest at each of the phases of digestion as described in this protocol. In the oral phase, a meal-fluid ratio of 1:1 was used as this enhances the formation of a swallowable bolus. For gastric and intestinal phase, relatively dilute conditions were selected to ensure good

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198 mixing, so that all substrates are subject to the same pH and enzyme conditions. In this way
199 possible product inhibition within the digest can be prevented (since there is no absorption of
200 metabolites in the small intestinal phase of this model). As such, this makes the static digestion
201 method simple to use but does not truly mimic *in vivo* conditions which are dynamic. The
202 discussion and justification of the experimental parameters in relation to *in vivo* physiological
203 data has been described in detail.[30,36] This INFOGEST protocol has been widely applied to
204 study digestion of multiple nutrients in various food matrices.[10,12,16,37–43] To obtain the *in vitro*
205 digestion kinetics of starch and proteins in the samples generated in section 2.3, sampling at
206 predetermined time moments and post-digestion sample treatments followed a slightly
207 modified approach previously described.[28] A total of 11 digestion tubes (10 time points and a
208 blank) were used to assess the digestion kinetics with the sample in each tube digested
209 independently until achieving a predetermined time point in the small intestinal phase.

210 #### *2.5.1.1 Oral phase*

211 In the oral phase, each digestion tube (except for the blank) was filled with 1.25 g of
212 disintegrated sample (common bean puree, see section 2.3.). The particle size distribution was
213 analyzed. The results from this analysis (see section 3.2.2.) were in line with results obtained
214 for *in vivo* generated boluses of cooked common beans.[26]Thereafter, 1 ml of simulated salivary
215 fluid (SSF) stock solution (pH = 7), 0.0063 mL of 0.3 M $CaCl_2$ and 0.244 mL of deionized
216 water were added to the mixture and the digestion tube incubated for 2 minutes at 37 °C with
217 end over end rotation. This dilution resulted in the recommended food sample to fluid ratio of
218 1:1 (w/v) which is necessary to stimulate the wetting and lubrication of solid foods by saliva
219 into a swallowable bolus that has a paste-like consistency during the oral phase. Salivary
220 amylase was excluded from the oral phase due to the very short incubation time during the oral
221 phase (2 minutes) and rapid inactivation of the enzyme upon adjustment of the pH to 3.[31]
222 Moreover, this experiment primarily aimed to identify the influence of the processing

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conditions on the susceptibility of proteins and starch to enzymatic hydrolysis upon extended period of contact with the enzymes.

2.5.1.2 Gastric phase

The gastric phase was initiated by the addition of 2 mL of simulated gastric fluid (SGF, pH = 3) and 0.0013 mL of 0.3 M $CaCl_2$ to the oral bolus, after which the pH was adjusted to 3 using 2 M HCl. Thereafter, 0.125 mL of porcine pepsin enzyme was added to achieve an activity of 2000 U mL^{-1} in the final mixture. The mixture was topped up to a volume of 5 mL using deionized water and the digesta incubated (37 °C for 120 minutes) with end over end rotation. This results in a 1:4 (w/v) food sample to fluid ratio during the gastric phase. The static model uses relatively diluted conditions that allow for proper homogenization of the sample using an end over end rotation (this may not perfectly reflect the mixing of the gastric content *in vivo*).

2.5.1.3 Small intestinal phase

After the gastric phase, 2 mL of simulated intestinal fluid (SIF, pH = 7), 0.01 mL of 0.3 M $CaCl_2$ and 0.750 mL of 133.3 mM freshly prepared bile (to obtain bile concentration of 10mM in the final digest) were added to the gastric chyme. The pH was then adjusted to 7 using 1 M NaOH solution followed by the addition of 1.25 mL of SIF-prepared enzyme solution containing porcine pancreatin, trypsin and pure chymotrypsin enzymes to obtain 200 U mL^{-1} (for α-amylase), 100 U mL^{-1} and 25 U mL^{-1} respectively, in the final digest. Thereafter, the volume was topped up to 10 mL using predetermined volume of deionized water. Finally, the digesta was incubated at 37 °C for 180 minutes, with end over end rotation at 70 rpm. This results in a 1:8 (w/v) food sample to fluid ratio during the small intestinal phase. Here the static method used does not consider the fluxes of water and digestive products occurring *in vivo* and therefore a condition of low substrate concentration in a dilute system occurs (which may overcome product inhibition of the enzymes).

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To determine the static *in vitro* digestion kinetics of proteins and starch, digesta were withdrawn at 0, 5, 10, 20, 30, 45, 60, 90, 120, 150 and 180 minutes of digestion during the small intestinal phase. Separate digest tubes were used for each withdrawal time. Upon heat shock (98 °C for 5 minutes) to inactivate the enzymes, the samples were centrifuged at (2000 g for 5 minutes, 5 °C) (Sigma 4–16 KS centrifuge, Sigma, Osterode am Harz, Germany) to obtain a supernatant which was frozen and stored at -40 °C until analysis.

2.5.2 Quantification of *in vitro* digestion products

2.5.2.1 Starch digestion products

Digested starch (%) in the supernatant (section 2.5.1) was quantified by dinitrosalicylic (DNS) method,[44] as previously done in our lab.[16] Briefly, a mixture of 5% of the supernatant (0.1 mL supernatant in 1.9 mL deionized water) and 1 mL of DNS color reagent was incubated at 100 °C for 15 minutes. The solution was then diluted with 9 mL of deionized water, cooled to room temperature and the absorbance measured at 540 nm. The amount of reducing sugars was expressed in terms of maltose equivalent (employing a 0.5 to 2.0 mg maltose/ml calibration curve). Finally, a conversion factor of 0.95 was used to convert the amount of maltose obtained into digested starch (%) during the small intestinal phase of the *in vitro* digestion as shown in **Equation 2.**[19]

$$\text{Starch digestibility (\%)} = \frac{Maltose\ equivalent * 0.95}{Total\ starch\ content} * 100 \qquad \textbf{Eq. 2}$$

2.5.2.2 Protein digestion products

This study focused on understanding protein digestion kinetics mainly during the small intestinal phase of static *in vitro* digestion. This is because previous related investigations have reported low levels of proteolysis of pulse proteins during the gastric phase compared to the

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small intestinal phase of static *in vitro* digestion.[27,37–39] To this effect, small intestinal phase protein hydrolysis was analyzed and quantified as L-serine equivalent.

Protein digested (%) was based on the quantification of the free α-amino groups present in the digest supernatant as determined using O-phthaldialdehyde (OPA) spectrophotometric assay.[45] Both OPA reagent preparation and spectrophotometric assay were done with minor modification.[46] Three main protein fractions were determined; the total α-amino groups in the undigested sample ($NH_{2(total)}$), readily bio-accessible protein fraction ($NH_{2(TCA)}$) and digested soluble protein fraction ($NH_{2(hydrolyzed)}$).

Briefly, $NH_{2(total)}$ refers to the total free α-amino groups present in the undigested sample, quantified upon complete acid hydrolysis of the undigested sample. To determine $NH_{2(total)}$, 5 mg of freeze-dried, ball milled (un-) treated common bean puree was hydrolyzed in 1 mL of 6 M HCl at 110 °C for 24 h. Thereafter, the acid was removed by rotary evaporation (Hei-VAP Advantage; Heidolph Instruments, Schwabach, Germany). The hydrolysate was then diluted in 5 mL deionized water and filtered (0.25 µm membrane) followed by OPA assay.[28]

On the other hand, $NH_{2(TCA)}$ refers to the fraction of small peptides (free α-amino groups, di- and tripeptides) capable of being directly absorbed without further hydrolysis by the brush border enzymes. To determine $NH_{2(TCA)}$, a mixture of 0.83 mL of 5% TCA and 0.5 mL of the digest supernatant was incubated for 15 minutes at room temperature and centrifuged (10000 g, 20 °C, 30 minutes) in a Microfuge 22R (Beckman Coulter Inc, Indianapolis, IN, USA). The supernatant was collected and subjected to OPA assay.

Protein digestion, however, also results in the generation of heterogenous group of compounds including oligo- and polypeptides which become bioavailable upon hydrolysis by the brush border proteases. These are not accounted for in the $NH_{2(TCA)}$ fraction. Consequently, $NH_{2(hydrolyzed)}$ obtained upon acid hydrolysis of the digest supernatant was analyzed as well.[27,28]

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To determine $NH_{2(hydrolyzed)}$, 1 mL of 6 M HCl was added to 0.5 mL of the digest supernatant and the mixture incubated at 110 °C for 16 h. After incubation, the acid was removed by rotary evaporation (Hei-VAP Advantage; Heidolph Instruments, Schwabach, Germany) and the hydrolysate diluted in 5 mL deionized water, filtered (0.25 µm membrane) and subjected to OPA assay.

During the OPA assay, 3 mL of OPA reagent was added to 25 µL deionized water (sample blank), sample or L-serine (standard) followed by incubation (2 minutes at room temperature) and the absorbance read at 340 nm. Free α-amino groups were expressed as L-serine equivalents (calibration with 12.5 – 100 mgL^{-1}). All analyses were conducted in duplicate. The degree of protein digestion (%) was expressed as shown **Equation 3** and **Equation 4** while taking into account the free α-amino group content of the sample at the start of the small intestinal phase ($NH_{2(t=0)}$) for the $NH_{2(TCA)}$ and $NH_{2(hydrolyzed)}$ fractions.

$$Readily\ bioaccessible\ proteins\ (\%) = \frac{NH_{2(TCA)} - NH_{2(TCA,\ t=0)}}{NH_{2\ (total)}} * 100 \quad \textbf{Eq. 3}$$

$$Digested\ soluble\ proteins\ (\%) = \frac{NH_{2(hydrolyzed)} - NH_{2(hydrolyzed,t=0)}}{NH_{2\ (total)}} * 100 \quad \textbf{Eq. 4}$$

2.6 Data analysis and modelling

All the data obtained was presented as means ± standard deviation. Where relevant, statistical differences between sample means were analyzed using one-way ANOVA and Tukey's HSD at 5% significance level using JMP software (JMP Pro 14, SAS Institute Inc., US).

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The fractional conversion model (**Equation 5**) was used to describe starch and protein digestion kinetics.

$C_t = C_f + (C_i - C_f) * e^{-k*t}$	**Eq. 5**

Where C_t represents digested starch/protein (%) at any digestion time, t represents time (minutes); C_i is the initial parameter value at $t = 0$, that is, the initial protein/starch concentration at the beginning of the gastric or the intestinal phase; k is the determined reaction rate constant (min^{-1}) and C_f is the estimated maximum theoretical limit at an infinite time for the concentration (%) of the digested starch/protein. When using this model to predict the digestion profiles, the changes of protein/starch during the small intestine digestion were modelled, thus subtracting the value at $t = 0$ with C_i becoming zero. The model parameters (C_f and k) were estimated using Proc NLIN in the SAS software version 9.4 (SAS Institute, Inc., Cary, NC, USA). The significant difference between the estimated kinetic parameters was assessed based on 95% confidence intervals. In addition, Joint Confidence Regions (JCR, 90% confidence) were calculated to evaluate whether the parameter sets obtained for the different treatments were significantly different. The fitness of the model was tested using adjusted R^2, sum of squares and visual analysis of residuals.

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3 Results and Discussion

3.1 Cooking profile

Softening is among the key macrostructural changes resulting from cooking beans.[46] Hydration and solubilization of the pectin-rich middle lamella alongside protein denaturation and starch gelatinization collectively contribute towards softening.[47] The cooking profile of KATB1 bean variety upon soaking and cooking is presented **Figure 1**.

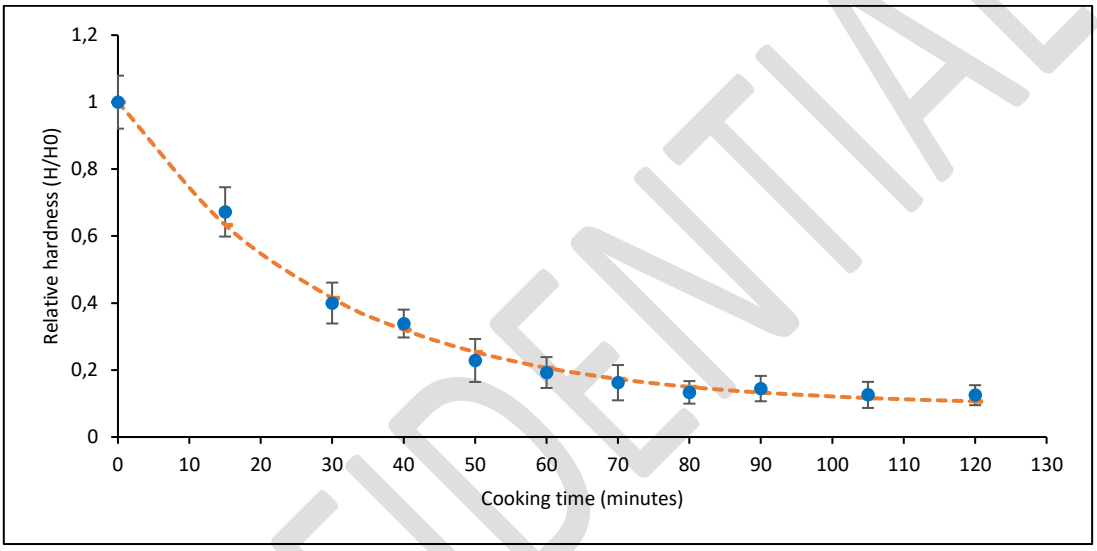


Figure 1. Cooking profile of fresh KATB1 variety of common bean soaked and then cooked in deionized water at 96 °C for 120 minutes. Markers denote experimentally obtained values (n =20) whereas the dotted line represents predicted values estimated from the fractional conversion model (**Equation 1**). The error bars represent ± standard deviations.

From this result, the relative hardness of beans decreased with cooking time. The cooking profile was characterized by rapid initial decrease in relative hardness (steeper slope) until 60 minutes of cooking before finally levelling off (plateauing). Similar cooking profiles have been reported for Canadian wonder beans cooked in demineralized water[48] and red kidney beans.[18] The plateauing of the curve is an indication that the beans achieved quasi equilibrium conditions upon cooking for 90 minutes at 96 °C. Thus, 90 minutes was selected as the treatment time for further experiments.

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3.2 Influence of hydrothermal processing temperature on chemical, microstructural and thermal properties of common beans

3.2.1 Chemical composition

The chemical compositions of common bean samples processed at different temperatures (for 90 minutes) are shown **Table 1**.

Table 1. Chemical composition of common bean samples hydrothermally processed at different temperatures for 90 minutes. The values represent means of triplicate analysis (g/100 g dry matter (dm)) ± standard deviation. Different superscripts within a column denote significantly different sample means ($p < 0.05$).

Sample processing condition	Moisture content (g/100g dm)	Protein content (g/100g dm)	Total starch (g/100g dm)	Ash content (g/100g dm)
Untreated	116.63 ± 3.81^{a}	20.45 ± 0.25^{a}	49.06 ± 1.24^{a}	3.25 ± 0.08^{a}
60 °C	122.23 ± 0.70^{a}	20.39 ± 0.32^{a}	49.19 ± 1.02^{a}	1.78 ± 0.06^{b}
65 °C	123.58 ± 5.40^{a}	21.29 ± 0.06^{a}	49.28 ± 0.81^{a}	1.95 ± 0.01^{b}
70 °C	122.46 ± 4.95^{a}	20.55 ± 0.70^{a}	49.26 ± 2.94^{a}	2.23 ± 0.50^{b}
75 °C	143.65 ± 9.01^{a}	21.24 ± 0.10^{a}	47.09 ± 0.71^{a}	2.29 ± 0.06^{b}
80 °C	133.45 ± 4.08^{a}	21.23 ± 0.05^{a}	48.17 ± 0.48^{a}	2.33 ± 0.16^{b}
85 °C	147.87 ± 9.90^{a}	20.49 ± 0.25^{a}	48.28 ± 0.78^{a}	2.02 ± 0.01^{b}
90 °C	206.25 ± 8.84^{b}	21.13 ± 0.05^{a}	47.81 ± 3.98^{a}	2.41 ± 0.06^{b}
95 °C	224.17 ± 10.64^{b}	20.99 ± 0.48^{a}	48.18 ± 1.00^{a}	2.06 ± 0.09^{b}

The chemical composition analysis revealed a temperature-dependent overall moisture uptake by bean seeds upon hydrothermal processing for 90 minutes. Generally, the net moisture content of the beans significantly increased with processing temperature ($p < 0.05$). Three distinct groups were observed. The first group of samples (processed at $T \leq 70$ °C) were characterized by low final water uptake. A second group of samples (processed between

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363 temperatures of 75 - 85 °C) showed a somewhat higher moisture uptake followed by a third
364 group of samples (processed at T = 90 °C and T = 95 °C) which showed a substantial increase
365 in water uptake. At lower process temperatures (with two distinct levels of moisture uptake in
366 the range of 60 - 70 °C and 75-85 °C) the absorption of water is mainly attributed to the heat-
367 mediated modifications occurring in starch and proteins, that is, starch gelatinization and
368 protein denaturation.[22,49] Thereafter, the hydration and solubilization of pectin at the middle
369 lamella and cell separation occurring at higher processing temperatures were described to
370 create more intercellular spaces with enhanced water holding capacity, alongside the capillary
371 channels.[49,50] Therefore, the thermal treatment at lower temperatures (< 85 °C) was expected to
372 only cause modifications in starch and protein. In contrast, treatments at higher temperatures
373 (> 85 °C) were expected to result in hydration and solubilization of pectin creating more
374 intercellular spaces that are linked with higher water uptake. This explains the significantly
375 higher water uptake observed for common beans processed at higher temperatures (90 and 95
376 °C) ($p < 0.05$). No significant differences in protein and total starch content were observed
377 among the different bean samples indicating an insignificant effect of hydrothermal processing
378 temperature on the amount of starch and protein in common beans. This observation could be
379 explained by presence of the seed coat which creates a protective barrier thus preventing
380 leakage of the dry matter content (proteins and starch) into the soaking and hydrothermal
381 processing liquid, thus maintaining their concentration in the seed during processing.[51] Similar
382 observations in starch and protein composition were made in cooked red kidney beans[52] and
383 lentils.[37] On the contrary, a decline in starch and protein content was reported in five pulses
384 (black grams, chickpeas, lentils, red and white kidney beans) upon soaking and cooking.[53] The
385 authors attributed the loss in protein to partial thermal elimination of certain amino acids with
386 other nitrogenous compounds while starch loss was linked to partial solubilization of starch
387 fractions in the cooking liquid. Additionally in this study, hydrothermal processing resulted in

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388 a significant decrease in the ash content of beans. This decrease can be attributed to leaching
389 out of minerals, in particular Mg^{++}, in the soaking and cooking liquids, in line with literature
390 findings.[37,53]

391 ### 3.2.2 Microstructural properties

392 Qualitative visualization of the microstructural changes occurring in bean samples (obtained
393 upon hydrothermal treatment and subsequent mechanical disintegration of the bean cotyledons
394 with mortar and pestle) were carried out microscopically and representative micrographs are
395 presented in **Figure 2A**. Three distinct groups could be observed in the samples treated at
396 different temperatures, demonstrating a transition from cell breakage to cell separation. The
397 first group consisted of samples processed at $T < 75$ °C (untreated, 60 °C, 65 °C and 70 °C)
398 and were mainly characterized by cell breakage as evidenced by the large number of free starch
399 granules and cell wall fragments in the matrix. The second group of samples (processed at $T =$
400 75 °C and $T = 80$ °C) were characterized by simultaneous cell breakage and cell separation as
401 evidenced by the occurrence of free starch granules and cell wall fragments alongside single
402 intact cells in the matrix. This group demonstrated the onset of pectin solubilization resulting
403 in cells with varying levels of cell wall strength.[54] The third group consisted of samples
404 processed at $T \geq 85$ °C (85, 90 and 95 °C) and was characterized by extensive cell separation
405 as evidenced by the large number of intact cells with a few free starch granules which disappear
406 completely in samples processed at 90 °C and higher.[49]

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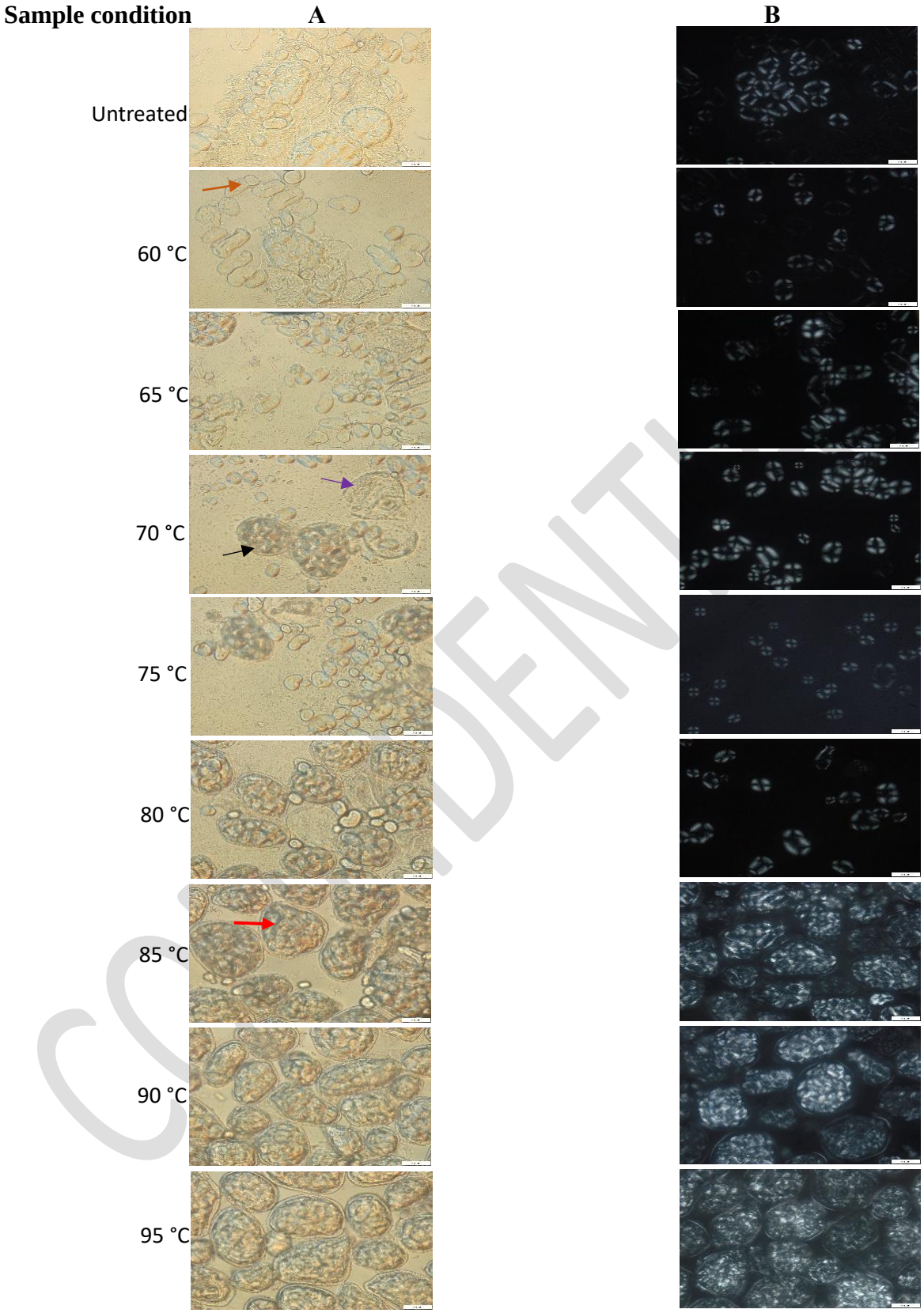


Figure 2. Micrographs of cotyledons from whole beans hydrothermally processed at different temperatures for 90 as observed under differential interference contrast (DIC) (A) and polarized (B) light microscopy. The untreated sample represents a soaked non-heat-treated sample. All micrographs were taken on a scale of 50 µm. The differently coloured arrows in the DIC micrographs represent cell clusters (black), starch granules (brown), broken cells (purple) and single intact cells (red).

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These observations can be explained by the variation in the mode of tissue failure upon mechanical disintegration of the hydrothermally processed bean tissues at varying temperatures. Like for other pulses, common bean cotyledons are composed of cells surrounded by the cell wall and glued together at the pectin-dense middle lamella.[47,54] Hydration and solubilization of pectin during wet heating compromises the integrity and strength of the middle lamella. The degree of pectin solubilization dictates the mechanism of tissue failure upon application of a mechanical force. For instance, a weakened middle lamella caused by pectin solubilization results in enhanced cell separation upon the application of a mechanical force, whereas exertion of a force against intact middle lamella results in cell breakage.[47,54] Therefore, the large number of free starch granules and cell wall fragments observed in bean samples processed at $T \leq 80$ °C was an indication of a stronger middle lamella (with little to no pectin solubilization at these conditions) which consequently resulted in cell breakage and release of cell contents upon exertion of force. On the other hand, samples processed at $T \geq 85$ °C exhibited a characteristic weak middle lamella (resulting from complete pectin solubilization at these conditions) which led to cell separation upon exertion of force as evidenced by the large number of intact/single cells.[54] Therefore, the above observations depict varying degrees of middle lamella and cell wall intactness/strength occurring in common bean seed processed at different temperatures.

Visualization of the bean samples under polarized light microscopy was intended to qualitatively explore the extent of starch gelatinization due to the heat-treatment process. The micrographs of this analysis are presented in **Figure 2B**. Ungelatinized starch was found in untreated samples as well as those treated at 60 and 65 °C as evidenced by the existence of Maltese crosses. A partial disappearance of the Maltese crosses was observed in samples processed at $T \geq 70$ °C indicating both onset of starch gelatinization and a variable sensitivity of starch granules to gelatinization during processing. Interestingly, some residual

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birefringence persisted in starch even upon processing at 95 °C for 90 minutes indicating a residual degree of ordering due to the confinement by the cell wall preventing the full completion of the gelatinization process.[55] Residual birefringence was also observed in Canadian wonder beans cooked at 95 °C for 270 minutes.[54] Whereas the influence of cooking time on gelatinization of starch in common beans has been reported,[48] the present study demonstrates the influence of process temperature on the gelatinization of common beans starch. This underpins the relevance of both temperature and time in processing of common beans. Given the qualitative nature of this analysis, a more quantitative approach using DSC was explored (section 3.2.3).

These microstructural characteristics were similarly reflected in the particle size distribution (PSD) data as shown in **Figure 3A** and **Figure 3B** below.

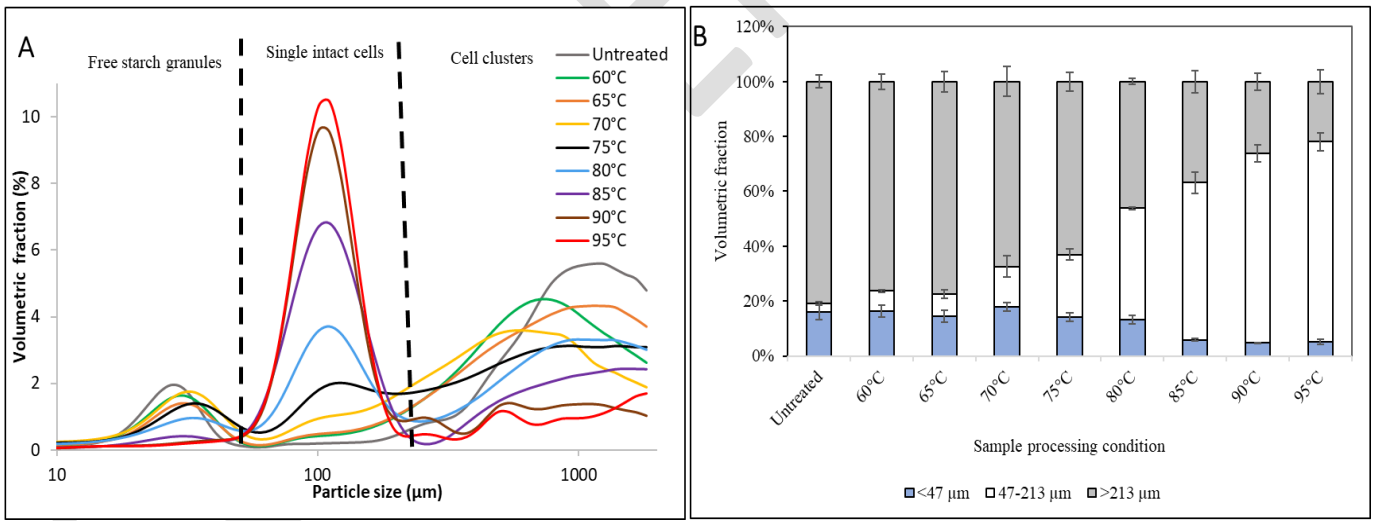


Figure 3. Mean particle size distribution (**A**) and associated relative proportions of the different particle size fractions (**B**) obtained from of whole common bean samples hydrothermally processed at different temperatures for 90 minutes. The error bars represent standard deviations.

Three regions, representing particle diameter of < 40 µm, 40-213 µm and > 213 µm, were discerned. The first region (< 40 um) was attributed to the occurrence of free starch granules and cell wall fragments, the second region (40 -213 µm) corresponded to single cells whereas the third region (> 213 µm) was mainly attributed to cell clusters.[12,56] Bean samples processed between 60 to 80 °C showed a clear peak representing starch granules (and most likely some

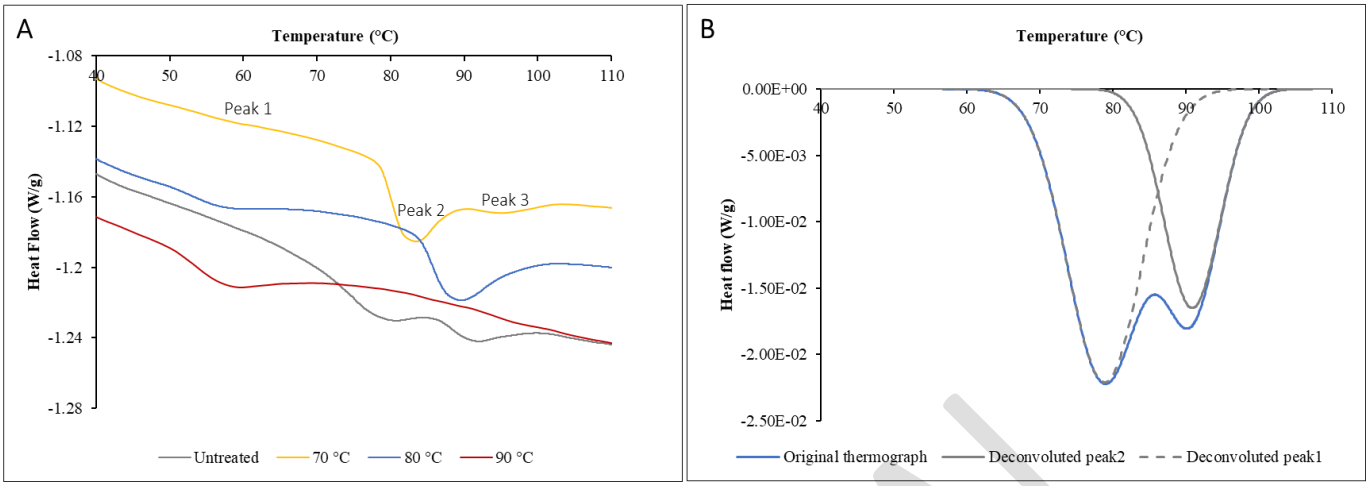
cell wall fragments) with a maximum shifting to the right (larger size) most likely representing starch granule swelling and a decreasing peak height and surface as the cell separation process starts to progress. Bean samples processed at $T \geq 80$ °C displayed a clear peak comprised of single intact cells indicating enhanced cell separation as a consequence of pectin solubilization in this temperature range. Moreover, the weakening of the middle lamella increased with an increase in processing temperature resulting in higher cell separation upon mechanical disintegration at temperatures above 80 °C. This observation is in line with the pattern observed in the micrographs. The current patterns of tissue failure are consistent with those reported for cooked lentils,[19] navy beans,[49] Canadian wonder common beans[54] and chickpea.[38]

3.2.3 Thermal properties

Representative DSC thermographs obtained for samples processed at different temperatures are presented in **Figure 4A** with their corresponding thermal properties in **Table 2.**

The DSC thermographs displayed three endothermic transitions corresponding to starch retrogradation (peak 1), starch gelatinization (peak 2) and protein denaturation (peak 3).[22,57] A clearer distinction in the endothermic transition between starch gelatinization and protein denaturation was obtained upon deconvolution of the DSC thermographs as illustrated in **Figure 4B**.

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488

489 **Figure 4.** Representative DSC thermographs (**A**) integrated to obtain the enthalpy associated with starch
490 retrogradation (peak 1), starch gelatinization (peak 2) and protein denaturation (peak 3) of common bean samples
491 processed at different temperatures for 90 minutes and representative deconvoluted DSC thermographs (**B**) of
492 untreated sample integrated to obtain the temperature and enthalpy associated with starch gelatinization (peak 1)
493 and protein denaturation (peak 2).

494 **Table 2.** DSC peak temperatures (Tp) and residual starch gelatinization and protein denaturation enthalpies of
495 common bean samples hydrothermally processed at different temperatures for 90 minutes. The enthalpy values
496 represent the mean of three replicates ±standard deviations. The abbreviation 'n.d' refers to not detected.

Sample processing condition	Starch gelatinization		Protein denaturation	
	Tp (°C)	Enthalpies (J/g)	Tp (°C)	Enthalpies (J/g)
Untreated	78.9	3.85 ± 0.37^{ab}	91.2	2.19 ± 0.20^{a}
60 °C	77.5	4.44 ± 0.29^{a}	92.6	3.34 ± 0.44^{b}
65 °C	80.0	4.09 ± 0.12^{ab}	91.8	3.01 ± 0.11^{ab}
70 °C	82.9	3.65 ± 0.10^{b}	93.5	2.66 ± 0.14^{ab}
75 °C	n.d	n.d	86.4	5.23 ± 0.49^{c}
80 °C	n.d	n.d	90.9	4.00 ± 0.34^{b}
85 °C	n.d	n.d	91.8	1.81 ± 0.05^{a}
90 °C	n.d	n.d	n.d	n.d
95 °C	n.d	n.d	n.d	n.d

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In this study, only peak temperatures and associated enthalpies attributed to starch gelatinization and protein denaturation were considered. Generally, the untreated sample was characterized by a narrower endothermic transition compared to treated samples which were characterized by wide and flat peaks. This is consistent with the observations made in Black 8025 and Pinto Durango beans.[13] There was complete starch gelatinization for all samples treated at $T > 70$ °C. A residual starch gelatinization peak was observed for samples treated at $T \leq 70$ °C with the peak starch gelatinization temperature ranging between 77.5 (untreated beans) - 82.9 °C (70 °C beans). Similar endothermic transition in flour obtained from beans have been reported. Peak starch gelatinization temperature of 77.5 °C for was reported for raw black beans,[57] while lower values of 70.1 to 75.4 °C were reported for raw Black 8025 and Pinto Durango beans.[13] In this study, the residual enthalpy of gelatinization ranged between 3.65 ± 0.096 J/g (for 70 °C sample) - 4.44 ± 0.291 J/g (for 60 °C sample). Whereas the observed shift in peak gelatinization temperatures to higher values with temperature could be a consequence of increasing stability of the non-gelatinized fractions of starch against heat, the decrease in enthalpy with processing intensity demonstrated an increasing fraction of gelatinized starch with treatment temperature.[58] One can also notice the lower enthalpy value for the non-treated sample (3.85 ± 0.37 J/g) compared to the samples treated at 60°C (4.44 ± 0.291 J/g) due to annealing resulting in some increase of the overall degree of ordering of the starch granules.[59] On the other hand, peak protein denaturation temperatures ranged between 86.4 – 93.5 °C with (nearly) complete protein denaturation observed at thermal treatment temperatures ≥ 90 °C as indicated by the absence of peaks associated with protein denaturation. Like starch gelatinization, a significant decrease in denaturation enthalpy can be explained by the decrease in the undenatured protein content in samples processed at $T \geq 85$ °C.[13] These observations are consistent with those obtained from the micrographs (section 3.2.2) in which the onset of starch gelatinization was detected in samples processed at $T \geq 70$ °C.

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3.3 Influence of hydrothermal processing temperature on *in vitro* starch and protein digestion in common beans

3.3.1 *In vitro* starch digestion kinetics

To understand the effect of varying processing temperatures and the resulting seed microstructural characteristics, on the extent and rate of static *in vitro* digestion of starch in common beans, starch digestion products were quantified as maltose equivalents during the small intestinal phase of digestion. Samples with different microstructural and thermal properties displayed variable patterns and levels of amylolysis (see **Figure 5)**.

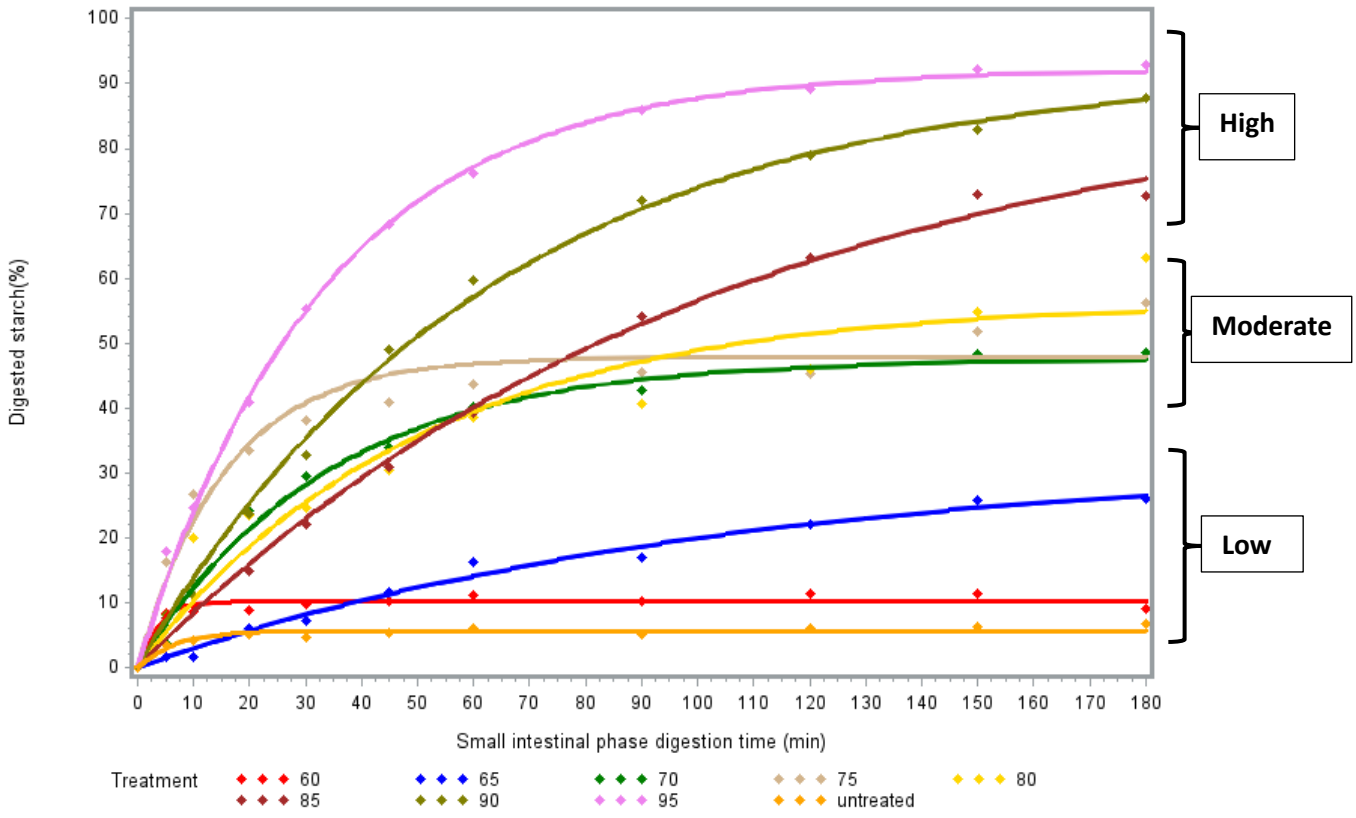


Figure 5. Static *in vitro* starch digestion kinetics (in the small intestinal phase) of cotyledons from common beans hydrothermally processed in deionized water at different temperatures for 90 minutes. The symbols represent the experimental values whereas the continuous lines represent the predicted values using fractional conversion model (**Equation 5**).

A qualitative analysis of this figure shows that the final extents of digestion increased with the treatment temperature. Three sets of curves can be distinguished, a first set with low digestibility for samples treated at $T \leq 65$ °C, a second set of curves with intermediate final extents of digestion representing treatments between 70 and 80 °C, and a third set of curves with a high final extent of digestion for samples treated at $T \geq 85$ °C.

In a first approach, the digestion curves were modelled using **Equation 5** obtaining a single rate constant (k) and a maximum theoretical limit for extent of digestion at an infinite digestion time (C_f) for each treatment temperature. The results (estimated kinetic parameters) are shown in **Table 3**. The 95% confidence intervals are added to evaluate the significant difference of the parameters. Additionally, the 90% joint confidence regions are depicted in **Supplementary Figure 1.** A characteristic very low digestibility was observed in samples treated at $T \leq 65$ °C. As discussed in section 3.2.2, these samples were characterized by broken cells with large amounts of exposed but ungelatinized starch. Untreated beans contain large amounts of tightly packed starch granules which are more resistant to digestion by amylase enzyme in their native (ungelatinized) forms. This means that, even though the free starch granules were easily accessible by pancreatic amylase, their digestibility was limited as the starch was mostly ungelatinized.[55,60] The increase of the C_f value for amylolysis in samples processed at 65 °C (C_f value of 32.25 ± 2.73%) compared to samples processed at 60 °C (10.13 ± 0.33%) could be attributed to the swelling of starch granules and/or the sensitivity of some starch granules to gelatinization even at this temperature as evidenced by the decrease in enthalpy of gelatinization (**Table 2**). On the other hand, the rate constant obtained at 65°C is lower than for the untreated or 60 °C treated sample, indicating presence of starch that can be hydrolyzed quite fast in the latter samples.

Samples processed between 70 – 80 °C experienced moderate C_f values of amylolysis with no significant differences ($p < 0.05$ in **Table 3**, and joint confidence region analysis in

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Supplementary Figure 1). However, the variation in the rate constant of digestion (**Table 3**) of these samples could be attributed to the differences in their thermal as well as microstructural properties. The sample processed at 75 °C seems to display a biphasic digestion behavior with a characteristic fast digestion of gelatinized free starch granules (the residual enthalpy of gelatinization for this sample being below detection limit) during the early phase followed by the slow digestion of starch contained in the intact cells in the second phase before reaching a plateau.

Table 3. Estimated kinetic parameters (C_f and k) of *in vitro* starch digestion of common beans hydrothermally processed at different temperatures. Fractional conversion model (**Equation 5**) was used to estimate starch digestion kinetic parameters. Values presented are parameter estimates ± approximate standard errors. The superscript letters represent significantly different parameter values within a column based on approximate 95% confidence limits.

Sample Processing Condition	**C_f (%)**	**k (min^{-1})**	**Adj. R^2**
Untreated	5.66 ± 0.24^{a}	0.146 ± 0.036^{a}	0.99
60°C	10.13 ± 0.33^{b}	0.290 ± 0.080^{a}	0.99
65°C	32.25 ± 2.73^{c}	0.009 ± 0.002^{b}	0.99
70°C	47.73 ± 1.09^{d}	0.030 ± 0.002^{c}	0.99
75°C	47.86 ± 1.88^{d}	0.064 ± 0.011^{d}	0.99
80°C	56.65 ± 5.82^{d}	$0.020 \pm 0.005^{b,c,e,f}$	0.97
85°C	91.78 ± 4.59^{e}	$0.010 \pm 0.001^{b,e}$	0.99
90°C	92.85 ± 1.94^{e}	0.016 ± 0.001^{e}	0.99
95°C	92.35 ± 1.08^{e}	$0.030 \pm 0.001^{c,f}$	0.99

**C_f is the maximum theoretical limit of starch digestion (%) at an infinite digestion time, k is digestion rate constant (min^{-1})*

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The characteristic biphasic behavior of the sample processed at 75 °C represented a transition in the digestion pattern between samples dominated with open versus closed cells (but in both cases the residual enthalpy of gelatinization was zero, non-detectable). The biphasic behavior makes the model less performant as reflected in the residue analysis (data not shown) and the broad range of uncertainty of the parameter estimates (which can be seen in the standard errors reported in **Table 3** and even better in the joint confidence regions reported in **Supplementary Figure 1**).

Although both samples (70 and 75 °C) contained relatively high amounts of free starch granules, the difference in the digestion rate constants could be due to the ratio of gelatinized versus ungelatinized starch in each sample. However, this was not the case for the sample processed at 80 °C which was overall slowly digested (k-value of 0.02 ± 0.005 min^{-1}) compared to the sample processed at 75 °C (0.06 ± 0.011 min^{-1}) but with both reaching significantly ($p < 0.05$) similar plateau values. This sample was dominated by single intact and, to a lesser extent, broken cells. Nevertheless, also for this sample a broad range of uncertainty was observed as reflected in the joint confidence region analysis (see **Supplementary Figure 1**), in particular on the C_f value. Similarly for this sample, a biphasic model could describe the data in a better way. The characteristic digestion phenomenon observed at this treatment temperature could possibly occur due to the gradual diffusion of amylase enzyme through the cell wall (and protein) barriers to the gelatinized starch trapped within the cell.[55] The highest C_f value on amylolysis (> 90%) was recorded among samples processed at $T \geq 85$ °C. Presence of completely gelatinized starch coupled with increased permeability of the cell wall and increased denaturation and enzymatic hydrolysis of proteins could be attributed to enhanced amylolysis.[12,37–39] In these cases, the rate constants are significantly lower compared to the sample processed at 75 °C because of the barriers involved. This barrier being less restrictive as the temperature increases from 85 °C to 95°C (significantly increasing rate constant). The

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joint confidence region analysis (see **Supplementary Figure 1**) shows less uncertainty in the parameter sets estimated, reflecting a single-phase behaviour of the digestion of completely gelatinized starch entrapped in closed cells with increasing permeability. Microstructurally, these samples were dominated by (nearly) completely separated cells, with the sample processed at 95 °C displaying a characteristically single cotyledon cells-like matrix under the microscope (**Figure 2A**). These outcomes are supported by previous findings reported on Navy beans,[12] chickpea,[28] lentils[37] and Bambara groundnuts,[27] although these data were obtained from cooking experiments (at high temperature) analyzing the events as a function of cooking time.

Based on the experimental observations, in a second approach a global model was constructed. This global model was formulated based on the following considerations:

- The monophasic behavior of the digestion of starch released by cell breakage for the samples treated at temperatures below 75 °C is described by a fractional conversion model with a k and C_f value of digestion being treatment temperature dependent. The relation of these two parameters with treatment temperature was not specified.
- A biphasic behavior of starch digestion at 75 and 80 °C due to the presence of completely gelatinized starch partly released due to cell breakage and partially encapsulated in closed cells. The rate constant of digestion of the released starch after both treatments was considered equal and the C_f value of digestion of the released starch was considered dependent on the treatment temperature. The rate constant of the digestion of the starch encapsulated in the cells was considered temperature dependent and the C_f value was considered equal to the extent reached at the higher temperatures (so an equal, temperature independent C_f value of digestion for treatments ranging between 75 and 95 °C, all samples showing complete starch gelatinization).

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- A monophasic behavior of digestion of samples treated at 85 °C and higher, due to completely gelatinized starch being encapsulated in cells (due to cell separation). The rate constant of digestion was considered dependent on the treatment temperature. Based on an initial parameter estimate, the dependence of the rate constant with temperature was described by an exponential relation with treatment temperature. This equation describes the evolution of the digestive barrier (of cell wall and possibly protein) with treatment temperature. The C_f value of digestion was considered treatment temperature independent.

The model formulation and the results obtained are summarized in **Supplementary Table 1** (estimated set of parameters based on all data modelled in a single step non-linear regression) and **Supplementary Figure 2** (experimental data and predicted digestion curves). This model adequately described all data and supports the approach based on the biological background information presented here.

3.3.2 *In vitro* protein digestion kinetics

In vitro protein digestion, expressed as soluble proteins based on OPA assay and readily bioaccessible protein, was analyzed to evaluate the role of varying processing temperatures in modulating digestion kinetics of proteins in common beans during the small intestinal phase of digestion. In **Supplementary table 2**, the total soluble protein and readily bioaccessible protein at the start of the intestinal digestion (at the end of the gastric digestion) are reported. The data show that the total soluble protein decreased with the treatment temperature, (due to protein denaturation). The readily bioaccessible protein was very low and independent of the treatment temperature indicating protein digestion during the gastric phase being negligible (independent on the degree of denaturation).

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3.3.2.1 *Digested soluble protein fraction*

The kinetics of proteolysis quantified as the release of soluble proteins (in the supernatant of the digest) and their accompanying kinetic parameters are presented in **Figure 6** and **Table 4,** respectively.

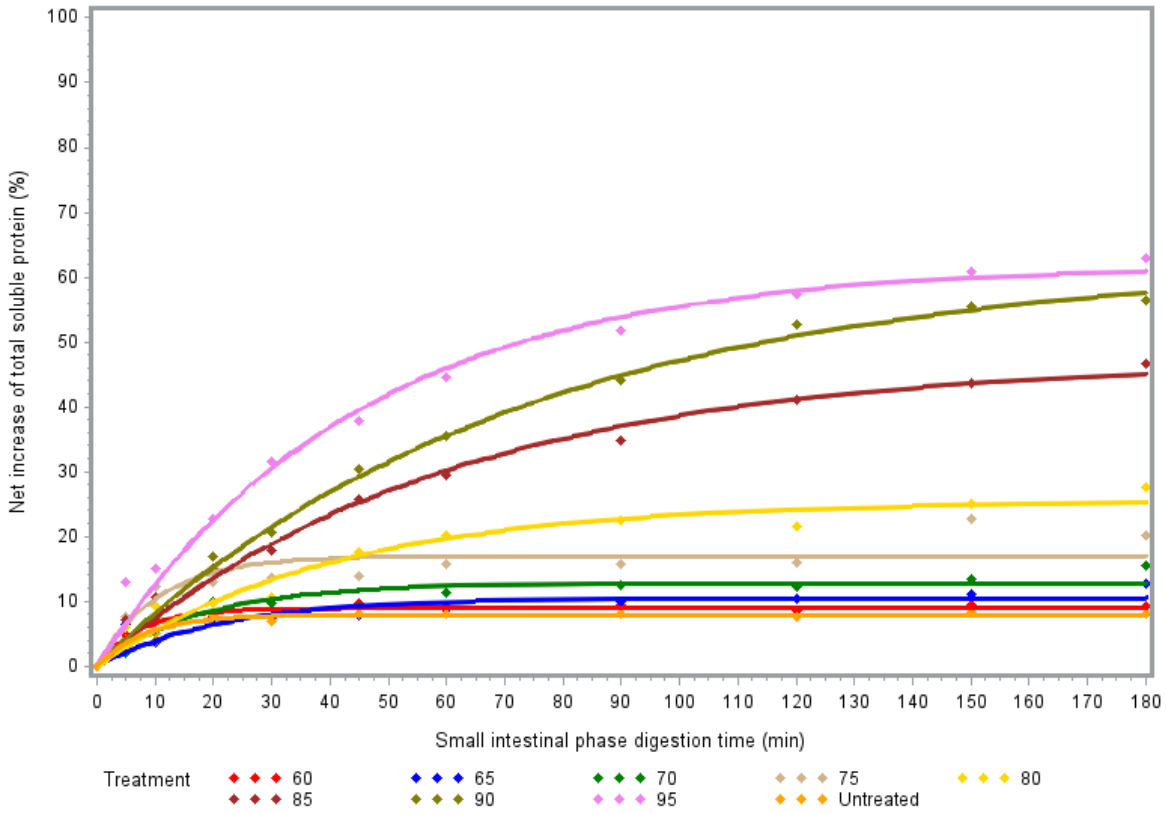


Figure 6. Kinetics of the net increase of the digested soluble protein fraction during the small intestinal phase of static *in vitro* digestion of cotyledons from common beans hydrothermally processed at different temperatures for 90 minutes. The symbols represent the experimental values whereas the continuous lines represent the predicted values using fractional conversion model (**Equation 5**).

The extent of proteolysis followed a pattern very similar to starch digestion (section 3.3.1). Processing temperature exhibited a significant effect on the rate constant of proteolysis similar to the digestion pattern depicted by starch. Significant differences can be assessed based on the

95% upper and lower limits **(Table 4)** and the joint confidence region analysis **(Supplementary Figure 3)** for the parameters estimated.

Table 4. Kinetic parameters (C_f and k) for the net increase of soluble proteins during the small intestinal phase of static *in vitro* digestion of cotyledons from common beans hydrothermally processed at different temperatures. Fractional conversion model (**Equation 5**) was used to estimate the digestion kinetic parameters. Values presented are parameter estimates ± approximate standard errors. The superscript letters represent significantly different parameter values within a column based on approximate 95% confidence limits.

Sample Processing Condition	Cf (%)	k (min^{-1})	Adj. R^2
Untreated	7.96 ± 0.17^{a}	0.12 ± 0.01^{a}	0.98
60 °C	9.00 ± 0.26^{b}	0.14 ± 0.02^{a}	0.99
65 °C	$10.61 \pm 0.88^{b,c}$	0.047 ± 0.014^{b}	0.96
70 °C	12.78 ± 0.74^{c}	0.056 ± 0.013^{b}	0.98
75 °C	17.07 ± 1.11^{d}	$0.094 \pm 0.030^{a,b}$	0.97
80 °C	25.54 ± 1.49^{e}	0.025 ± 0.001^{b}	0.99
85 °C	47.37 ± 1.94^{f}	0.017 ± 0.002^{c}	0.99
90 °C	62.68 ± 2.27^{g}	0.014 ± 0.011^{c}	0.99
95 °C	62.12 ± 1.97^{g}	0.026 ± 0.002^{c}	0.99

**C_f is the maximum theoretical limit of soluble protein increase at an infinite digestion time in the small intestine (%), k is digestion rate constant (min^{-1})*

Overall, the C_f value for protein hydrolysis at extended digestion time (C_f values) increased with processing temperature which could be attributed to the increased protein denaturation,[23,37] the improved cell wall permeability to proteases at higher processing temperatures[39] as well as possibly a decrease in antinutrients such as trypsin inhibitors and lectins.[40,53]

At temperatures below 80 °C a rather low level of C_f value of protein hydrolysis together with a rather high rate constant observed are explained by the microstructural properties of broken cells and an onset of protein denaturation (conformational changes). Generally, native pulse

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676 proteins are primarily composed of albumins and globulins. The compact globular structure of
677 albumins which is further stabilized by the presence of disulfide bonds provides an inherent
678 structural barrier to proteolytic enzyme access.[23] Additionally, the presence of antinutrients
679 such as lectins and protease inhibitors could be a potential reason for the low digestibility of
680 proteins in untreated and low temperature treated samples.[61] Nevertheless, the observed
681 marginal increase in proteolysis with processing temperature (below 80 °C) could possibly be
682 due to changes in protein conformation during heating which favored binding of proteolytic
683 enzymes and/or inactivation of the heat sensitive antinutritional factors present in beans.[39,62]

684 At temperatures of 80 – 95 °C a high (and increasing with treatment temperature) C_f value of
685 protein hydrolysis together with a rather low rate constant, but increasing with treatment
686 temperature, are observed. This can be explained by the microstructural properties of closed
687 cells (the cell wall creating a barrier for the proteases), and proteins being denatured. A close
688 look at the data of samples processed at 75 °C shows some biphasic behavior (although less
689 pronounced than in case of starch digestion, see section 3.3.1) due to the presence of both
690 microstructural organizations at the same time. The lower performance of the fractional
691 conversion model (considering a single phase) at this treatment temperature is reflected by the
692 residue plot (data not shown) and the uncertainty on the parameter estimates (see **Table 4** and
693 **Supplementary Figure 3**). This can most likely be explained by starch digestion being
694 complete at 75 °C, while this is not the case for protein denaturation.

695 Increase in proteolysis with processing intensity has also been reported for other pulses.[15,27,40,56]
696 Contrary to observations made in cooked lentils[37], no decrease in protein digestibility (C_f
697 values) was observed even upon processing at the highest temperature (95 °C) indicating low
698 effects of thermally mediated cross-links on enzymatic access to proteins during digestion in
699 KATB1 variety of beans.

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3.3.2.2 *Readily Bioaccessible Protein fraction*

The kinetics for the generation of readily bioaccessible proteins (in the digest supernatant) during static *in vitro* digestion and their corresponding parameters are displayed in **Figure 7** and **Table 5**, respectively.

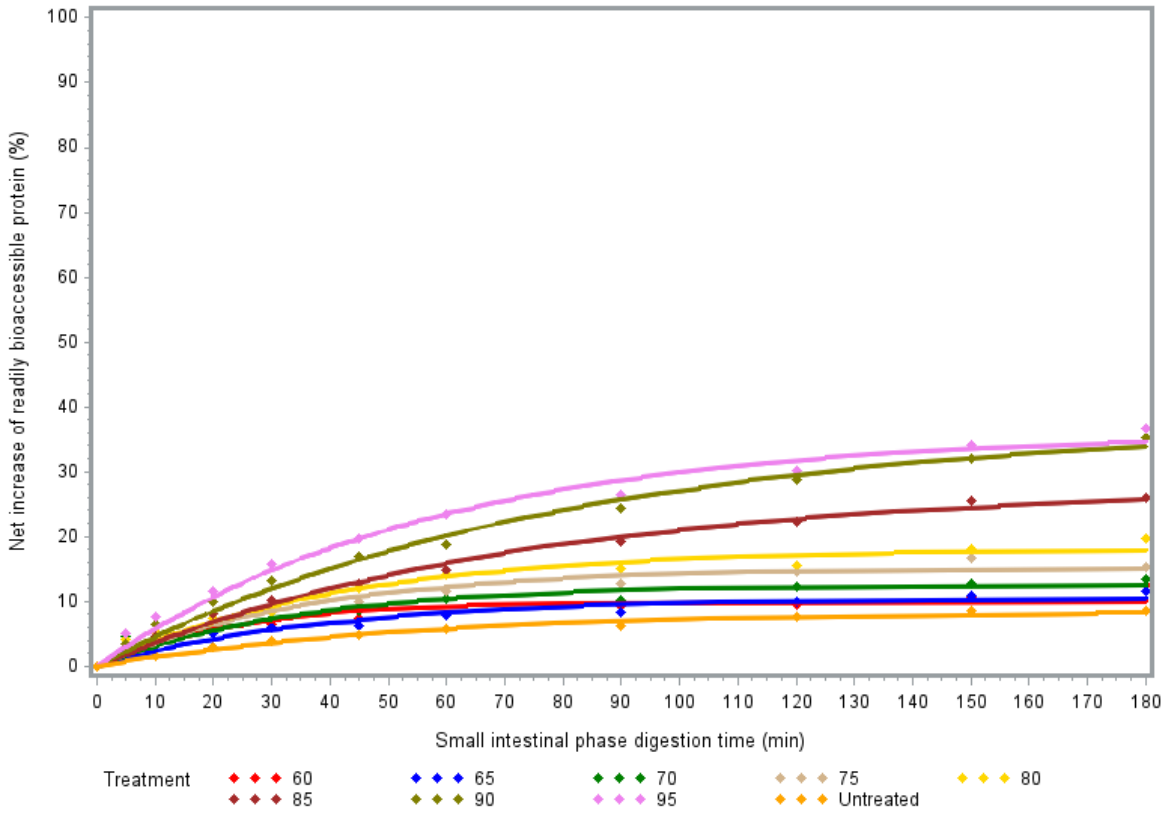


Figure 7. Kinetics of the net increase of readily bioaccessible protein fraction during the small intestinal phase of static *in vitro* digestion of common beans hydrothermally processed at different temperatures for 90 minutes. The symbols represent the experimental values whereas the continuous lines represent the predicted values using fractional conversion model (**Equation 5**).

These kinetics during the small intestinal phase were here characterized by similar rate constants (confirmed by the 95% upper and lower limits **(Table 5)** and the joint confidence region analysis **(Supplementary Figure 4)** for the parameters estimated).

713 **Table 5.** Kinetic parameters (C_f and k) for the net increase of readily bioaccessible proteins during the small
714 intestinal phase of static *in vitro* digestion of cotyledons from common beans hydrothermally processed at
715 different temperatures. Fractional conversion model (**Equation 5**) was used to estimate the digestion kinetic
716 parameters. Values presented are parameter estimates ± approximate standard errors. The superscript letters
717 represent significantly different parameter values within a column based on approximate 95% confidence limits.

Sample Processing Condition	Cf (%)	k (min^{-1})	Adj. R^2
Untreated	8.73 ± 0.38[a]	0.018 ± 0.002[a]	0.99
60 °C	10.12 ± 0.76[a]	0.041 ± 0.011[a]	0.97
65 °C	10.67 ± 0.65[a]	0.025 ± 0.004[b]	0.98
70 °C	12.56 ± 0.73[a,b]	0.029 ± 0.005[b]	0.98
75 °C	15.32 ± 0.75[b]	0.027 ± 0.004[a]	0.99
80 °C	18.20 ± 0.90[b]	0.024 ± 0.003[a]	0.99
85 °C	27.98 ± 1.21[c]	0.014 ± 0.001[a]	0.99
90 °C	37.69 ± 1.93[d]	0.013 ± 0.001[a]	0.99
95 °C	36.26 ± 1.48[d]	0.018 ± 0.002[a]	0.99

718 **C_f is the maximum theoretical limit of readily bioaccessible protein increase at an infinite digestion time in the small intestine*
719 *(%), k is digestion rate constant (min^{-1})*

720

721 However, increasing processing temperature significantly increased C_f-value for proteolysis
722 levels (C_f values) ($p < 0.05$). Rather low estimated C_f-values ($C_f < 20\%$) for digestion were
723 observed in samples processed at $T \leq 80$ °C, even though these samples were characterized by
724 an open cell matrix, but proteins not being denatured and antinutritional factors not yet
725 inactivated. Higher C_f-value were observed at temperatures from 80 °C on reaching a C_f-value
726 of 36.26 ± 1.48% at 95 °C. The low molecular weight hydrolysis products considered here most
727 likely result from the larger peptides (due to initial protein hydrolysis by pepsin enzyme), with
728 the low molecular weight fraction also containing free amino acids due to the action of trypsin
729 and chymotrypsin. Therefore, the influence of the different microstructural properties discussed

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730 for the soluble proteins are less applicable as the protein fragments analyzed in the readily
731 bioaccessible part are not directly formed from the proteins in the sample. Consequently, the
732 rate constants for the release of readily bioaccessible protein of the samples treated at different
733 temperatures are very similar because the process of hydrolysis to obtain the smaller fragments
734 uses the same intermediate hydrolysis products. The C_f-value for the readily bioaccessible
735 protein fraction depends on the thermal treatment of the sample, as this level depends on the
736 level of hydrolysis that is reached for the total protein hydrolysis (thus depending on the amount
737 of initial products formed due to enzymatic hydrolysis). The C_f-value for readily bioaccessible
738 protein can indirectly be explained by the same factors as the soluble protein i.e. heat
739 denaturation of the protein, the complete inactivation of endogenous protease inhibitors,
740 reduced proteins-antinutrient interactions resulting from thermal-related loss of antinutrients
741 such as phytic acid, tannins, and polyphenols which crosslink with proteins, change in albumin-
742 globulin ratio with heating intensity and improved cell wall permeability to digestive enzymes
743 resulting from solubilization of pectin.[16,19,23,39,61] Some additional insights can be obtained by
744 analyzing the ratio of the net increase in readily bioaccessible fraction over the net increase of
745 the total soluble protein fraction during the small intestinal digestion. This ratio, both for the
746 experimental data points and the predicted values resulting from modelling the respective
747 digestion curves are illustrated in **Supplementary Figure 5.**

748 Nevertheless, the readily bioaccessible fraction remained low with end values being lower than
749 40 % for all samples. This is consistent with values reported for cooked lentils[37] but much
750 lower compared to those obtained in chickpea flour[38] and Bambara groundnut.[27]

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4 Conclusion

The aim of this study was to investigate the influence of temperature induced physicochemical changes in modulating the *in vitro* digestion kinetics of starch and proteins in common beans. Specific thermal treatments at different temperature levels (with a long heating time of 90 minutes) were designed to create targeted microstructural changes (different levels of starch gelatinization, protein denaturation and pectin solubilisation resulting in cell separation upon mechanical stress). The study confirmed temperature as a key processing parameter affecting both the microstructural and thermal properties of common beans biomolecules (starch, proteins, and cell wall) with consequent effects on the digestibility of starch and proteins in common beans. It was demonstrated that both the physical state and the limitations of enzymatic access provoked by the cell wall affect the digestion of proteins and starch in common beans. For instance, starch and proteins which were directly and readily exposed to digestive enzymes as a result of cell breakage remained largely undigested due to their native physical states (ungelatinized and undenatured). Under these conditions the rate constants were rather high and final levels of digestion were low. However, upon starch gelatinization and protein denaturation during a treatment step, these biomolecules became more susceptible to enzymatic attack with characteristic increases in the extent of hydrolysis as evidenced in samples processed ≥ 80 °C. At these temperatures, the rate constant of starch digestion and protein digestion were lower than those observed at lower temperatures due to the cell wall barrier. Additionally, the role of the cell wall as a physical barrier (and denatured protein in case of starch digestion) to digestion became evident as the digestion of gelatinized starch and protein was limited until the onset of cell separation as evidenced by the sample processed at 80 °C.

The results of this study provide an improved understanding of the role of the physical state of starch and proteins, and the barriers created by the cell wall in modulating digestion kinetics

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of starch and proteins. It also provides a further basis for using targeted hydrothermal processing of pulses to obtain ingredients and derived products with pre-determined microstructural and associated nutritional characteristics in the production of healthy and nutritious foods for consumers. Furthermore, the use of *in vivo* models in a follow up study would be a significant step towards the application of these ingredients in real food systems to meet specific therapeutic and nutritional goals such as generation of food products with specific glycemic characteristics.

Credit authorship contribution statement.

Erick Omodho: Investigation, formal analysis, writing – original draft, Writing -review and editing. Irene Wainaina: Methodology, writing: review and editing. Dorine Duijsens: Methodology, formal analysis, writing: review and editing. Arnold Onyango: Writing – review and editing, Daniel Sila: Project Administration, supervision, writing – review and editing, Tara Grauwet: Methodology, writing: review and editing. Marc Hendrickx: Project administration, supervision, conceptualization, methodology, formal analysis, writing – review and editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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