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The moisture plasticizing effect on enzyme-catalyzed reactions in model and real systems in view of legume ageing and their hard to cook development

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Abstract

This study aims to understand the role of storage conditions (temperature, T and moisture content) in the reactions involved in the cation-pectin-phytate hypothesis, postulated to cause the Hard-to-cook (HTC) defect in beans as influenced by the plasticizing effect of moisture (glass transition (T_g) concept). The enzyme catalysed reactions, demethylesterification of pectin and hydrolysis of phytic acid, were monitored respectively in simplified pectin and phytic acid/protein model systems. In addition, both reactions were studied in a real system, red kidney bean cotyledon during storage at 35 °C and different relative humidity conditions (6%, 54%, 66%, 72% and 82 %) for 15 weeks. The T_g -moisture relation of the model systems and the bean cotyledon were established by Dynamic Mechanical Thermal Analysis (DMTA). The T_g -line, fitted using the Gordon-Taylor equation, was combined with the moisture sorption isotherm to create a stability diagram. The stability diagram obtained can be used to ascertain the biochemical stability during post-harvest ageing of beans. Results displayed that there was significant hydrolysis of phytic acid in the phytic acid/protein model system and the real system when stored above their glass transition values. On the other hand, in the model system the pectin degree of methylesterification (DM) did not significantly change during storage. In the real system, significant, although small, changes of the DM were noted when $T - T_g > 20$ °C. An examination of the glass transition lines revealed that the reactions were influenced by the plasticizing effect of moisture.

Keywords: Red kidney bean; Glass transition; cation-pectin-phytate hypothesis; DMTA; stability diagram

Abbreviations

PME-Pectin methylesterase, DMTA- Dynamic Mechanical Thermal analysis

1 Introduction

To feed the rising world population healthily and sustainably, great emphasis is placed on the increase in the consumption of plant-based foods. In this context, legumes and specifically beans have been receiving much attention due to their nutritional quality and sustainability aspects. However, one of the major factors which limit their consumer acceptability is their long cooking time which is attributed to the post-harvest storage at high temperatures and relative humidity (Temperature ≥ 25 °C and RH $\geq 65\%$).

The cation-pectin-phytate hypothesis is postulated to be the most probable cause of the 'Hard-to-cook' (HTC) development. It is hypothesized that pectin, a cell wall polymer may be demethylesterified by pectinmethylesterase (PME) to form pectin with a low degree of methoxylation (DM) and methanol. At the same time, increased phytase activity during storage leads to the hydrolysis of phytic acid resulting in a release of divalent cations like calcium and magnesium. It is postulated that these ions migrate to the cell wall and cross-link with low DM pectin forming insoluble or thermally stable pectates. The pectates hinder pectin solubilization and prevent cell separation and softening during cooking thereby leading to long cooking times (Galiotou-Panayotou et al., 2008; Njoroge et al., 2014; Reyes-Moreno et al., 2000). Since the individual reactions involved in the hypothesis occur in different matrices within the bean cotyledon (cell wall and protein respectively for PME and phytase), it is hypothesized that the influence of the individual matrix differs from that of the complex whole system in kinetically controlling these diffusion dependent reactions by mobility or viscosity.

Dry seeds exhibit an intracellular glassy state which helps them remain viable for a long period as the high viscosity of the glass restricts molecular mobility thereby inhibiting potential deteriorative reactions from occurring (Sun, 1997). This knowledge has provided a fresh perspective to explore the biochemical changes responsible for quality deteriorations (e.g. HTC development) that occur during post-harvest storage in the context of the glassy state. Studies have been carried out to understand the cation-pectin-phytate hypothesis by looking into the changes that occur in the pectin polysaccharide, mineral and phytic acid content but not from this point of view. So far, only the biochemical changes that occur in beans during germination and loss of cooking quality with storage have been linked to the glass transition concept (Chigwedere et al., 2019; Leprince and Walters-Vertucci, 1995; Murthy et al., 2003; Sun, 1997).

Differential Scanning Calorimetry is the most popular technique for the determination of T_g . However in the case of multicomponent foods like beans, the thermal responses are continuous and the transitions overlap (Mochizuki et al., 2019). Moreover, foods containing high molecular weight materials like polysaccharides (e.g. cell wall polymers) or proteins show only small to undetectable changes in the thermal properties during glass transition rendering DSC ineffective (Boonyai et al., 2007). Since the study focuses on biochemical stability, glass transition determination based on molecular mobility or mechanical changes (Dynamic Mechanical Thermal Analysis) is the practical choice. DMTA is able to detect short range motion prior to the glass-liquid transition and hence is more sensitive than DSC for the determination of T_g (Abiad et al., 2010; Hutchinson, 2009; O'Donoghue et al., 2020). A previous study (Chigwedere et al., 2019) reporting the cooking properties with the glass transition concept of stability has employed Thermal Mechanical Compression testing (TMCT) as the T_g determination method. It is of interest to evaluate whether the biochemical reactions responsible for the development of HTC defect can also be accounted for with T_g determined using TMCT.

The objective of this study is to gain insight into the cation-pectin-phytate hypothesis by understanding the individual enzyme catalysed reactions through controlled ageing at conditions that regulate the

development of the HTC defect. To distinguish the moisture plasticization of the individual biopolymers involved in the reactions, model systems depicting similar biochemical reactions as occurring in the local structures present in the real system were studied. It is emphasized that simplified model systems are used and thus only indicative of the biochemical reactions that occur in the real system. The model systems, namely a pectin and a phytic acid/protein model system, relate to the cation-pectin-phytate hypothesis related reactions. The action of PME on pectin and the hydrolysis of phytic acid by phytase in the model systems is analogous to the action of PME on pectin in the cell wall and hydrolysis of phytic acid in the protein bodies within the cotyledon. In parallel, these biochemical changes were evaluated in red kidney beans, a variety that is prone to be HTC development during storage. Thereafter the utilization of the T_g -moisture relationship of the matrices based on the DMTA measurement and the subsequent relationship between storage conditions, T_g and the biochemical reactions are examined in detail.

2 Materials and Methods

2.1 Raw materials

Citrus pectin with a DM of 75% and Puris™ Pea protein (protein content of 84 %) were obtained from Cargill, Belgium. PME from orange peel, phytase from wheat, Natamycin and Benzisothiazolinone were purchased from Sigma-Aldrich, Belgium. Phytic acid assay kits were from Megazyme, Ireland. Freshly harvested red kidney beans were sourced from Belgium and stored at -40 °C until further use. Unless mentioned otherwise, all chemicals used were of analytical grade. Ultrapure water used for the experiments was obtained by Simplicity™ water purification system (Millipore, USA).

2.2 Preparation of model systems

Model systems were selected with an emphasis on depicting enzymatic reactions following the same principles as in the real system. Thereby available components are utilized rather than extracting the specific enzymes, substrates and polymers involved in the phenomena locally occurring in the cotyledon. In this regard, model systems facilitating easier access of the enzyme to its substrate were created. The details of each model system are discussed in section 3. In the case of the pectin model system, a 1 % aqueous solution of citrus pectin was prepared. Its pH was adjusted to 6 using 0.1M NaOH to ensure complete ionization of the carboxylic groups to the pKa value of polygalacturonic acid (Manrique and Lajolo, 2002). This pectin solution was then dialyzed against demineralized water at 4 °C for 48 hours using a Spectra/Por® dialysis membrane (3.5 kDa, MWCO). Post dialysis, PME was added to the pectin solution (0.75U/mL) at 4 °C along with 5 ppm of Natamycin, an anti-mould agent, and the mixture was immediately frozen using liquid N₂ and subsequently lyophilized (Christ Alpha 2-4 LSC plus, Osterode, Germany) (Kyomugasho et al., 2015).

For the phytic acid/protein model system, a 10 % aqueous solution of pea protein was prepared with 100 ppm of Benzisothiazolinone as an antimicrobial agent. Phytase (8000U/kg of pea protein) was added to the solution at 4 °C and the mixture was immediately frozen and lyophilized (Luo et al., 2012).

The lyophilized model systems were then equilibrated over saturated salt solutions in hermetically sealed glass containers generating different relative humidity at 4 °C and on the attainment of equilibrium (confirmed by moisture contents) were aged at 35 °C. The salts used and the relative humidities conferred were: KOH-6%, NaBr- 54%, KI- 66%, NaCl- 74% and KCl- 82% (Greenspan, 1977). This generated samples with different moisture contents. Ageing was carried out for a total of 15 weeks with a sampling interval of 2 weeks. A storage temperature of 35 °C was chosen as the hard-to-cook defect is induced at such high

storage temperatures and the range of relative humidity might be capable of generating matrices below, around and greater than their T_g values.

2.3 Preparation of real system

Fresh red kidney beans were equilibrated over the saturated salt solutions (mentioned in section 2.2) in hermetically sealed chambers at 4 °C. Natamycin was added as an anti-mould agent. They attained equilibrium in 6 weeks which was confirmed by their moisture contents. Post equilibration, the set-up was shifted to incubators maintained at 35 °C for ageing. To ensure consistent conditions during the ageing period of 15 weeks, moisture content and a_w of the bean cotyledons were determined during sampling. Since the pectin-cation-phytate hypothesis occurs in the bean cotyledon (Chigwedere et al., 2019), the seed coat was removed before characterization.

2.4 Characterization of model systems and real system

2.4.1 Degree of methoxylation of pectin

The Degree of methoxylation (DM) of the pectin model system and the real system was determined by FT-IR spectroscopy (Shimadzu FTIR-8400S, Japan), according to the method described by Kyomugasho et al., (2015). Slices of bean cotyledon (40 µm) were washed in Phosphate buffer (pH 7.2-7.4) before the measurement to eliminate the starch granules and protein bodies and completely ionize the COOH group to COO⁻ to facilitate the DM measurement. Protein removal limits interference with the band due to COO⁻ stretching (Szymanska-Chargot and Zdunek, 2013). Five samples were analysed at each sampling point for the model system. In the case of the real system, 4 beans and 5 cotyledon slices per bean were analysed per sampling point.

2.4.2 Phytic acid content

The phytic acid content for the bean cotyledon and phytic acid/protein model system was determined using Megazyme Phytic acid assay kit, according to the method described and validated by McKie and McCleary (2016). Bean cotyledon powder, obtained by grinding and milling the cotyledons (Basic mill, IKA A10, Germany) was prepared before sample extraction. Sample extraction at each sampling moment was performed in duplicate with duplicate analysis for each extract.

2.4.3 Moisture sorption isotherm

About 10 mg of sample (in duplicate) was placed in the Dynamic Vapour Sorption Advantage system climate chamber (Surface Measurement Systems, USA) at 35 °C while the relative humidity of the environment varied from 0% RH to 90% RH in a stepwise manner (van Donkelaar et al., 2015). The sample was defined to be in equilibrium when its mass did not change by 0.002mg/min for 10 min. The equilibrium moisture content determined at each step was used to construct the moisture sorption isotherm. Good agreement between the results obtained by DVSA as compared to the traditional methods have been illustrated in literature (Rudy et al., 2017; Schmidt and Lee, 2012).

2.5 T_g -moisture relation

2.5.1 Sample preparation

Samples containing un-freezable water: Bean cotyledon powder, pea protein and bean cell wall material were conditioned over saturated salt solutions in hermetically sealed containers for 4-5 weeks until they attained equilibrium and generated samples having different moisture contents. These samples were subsequently used for T_g determination. The bean cell wall material was obtained by wet sieving of milled

bean cotyledon powder over sieve sizes ranging from 40-80 μm separate the starch and protein fractions till cell wall material is the only component remaining (Kyomugasho et al., 2020).

Samples containing freezable water: 2-5 mg sample was weighed in a hermetic aluminium pan and a predetermined amount of water was added to achieve moisture contents of 0.7 kg water/kg sample and above. The pans were hermetically sealed and equilibrated for 24 hours at 25 °C. These samples were used to determine the critical moisture content.

2.5.2 Moisture content and water activity

The moisture content of the samples was measured gravimetrically by calculating the weight loss of the sample dried overnight in an oven at 103 °C (Bonner, 1981). The water activity was measured at 25 °C with LabMaster-aw neo (Novasina AG, Lachen, Switzerland). The analysis was performed in duplicate.

2.5.3 Combined TMCT and DMTA

The T_g was determined using the techniques as described by Chigwedere et al., (2019) and Monteyne et al., (2016) with some modifications. A modular compact rheometer MCR 302 (Anton Paar, Austria) with a convection temperature device (CTD 450) was used. The sample was placed in a sample container and was equilibrated at the starting temperature with an aluminium probe applying a constant normal force of 30 N and subsequently at compression-torsion mode with the same normal force, frequency of 1 Hz and strain of 0.5 %. Post equilibration, thermal scanning of the sample was carried out at 2 °C/min from -40 °C to 170 °C depending on the moisture content of the sample. Changes in the compressibility of the sample (mm) and storage (E') and loss modulus (E'') during the thermal scanning range was recorded using the software Rheocompass (Anton Paar, Austria). Loss factor ($\tan \delta$) is the ratio of loss to storage modulus.

The T_g obtained by a change in the compressibility of the sample (TMCT), due to displacement of the probe during thermal scanning was corrected for the thermal expansion of the sample holder by using microcrystalline cellulose (it has a high Glass transition temperature of 200 °C which is beyond the T_g of the samples used in the study). Additionally, the T_g was characterized by an increase in $\tan \delta$ of the sample during the thermal scanning range and the onset, endpoint (peak of $\tan \delta$) and mid-point of this change were noted (DMTA). All the measurements were carried out in duplicate. This improved methodology allowed the simultaneous measurement of T_g by DMTA and TMCT in the same experimental set-up.

2.5.4 Thermal transitions by DSC

Thermal transitions were measured using Q2000 heat flux differential scanning calorimeter with Advanced Tzero™ technology (TA Instruments, Delaware, USA). The DSC was calibrated for temperature and enthalpy of fusion by using Indium as a standard and an empty pan as a reference. Thermal analysis was performed according to the method described by (Pelgrom et al., 2013). Samples were cooled to -60 °C at 10 °C/min and held isothermally at that temperature for 1 minute. Subsequently, they were scanned to 90 °C at a heating rate of 10 °C/min. The heating rate was chosen based on literature (Gwala et al., 2019; Kyomugasho et al., 2020). A second heating and cooling cycle was performed on the samples (in duplicate) for glass transition measurements. The thermograms were analysed using the Universal Analysis 2000 software, version 4.5A (TA instruments-Waters LLC).

The latent heat of melting of ice (ΔH_m) was obtained by calculating the area of the melting endotherm in the first heating scan. The amount of unfrozen water was calculated by utilizing the plot of enthalpy of melting of ice versus moisture content and by extending the line of ΔH_m to zero. The T_g was the midpoint of the step change in the heat flow line during the second heating scan.

2.6 Data analysis

The moisture sorption properties of the materials were described by the three-parameter GAB equation (Equation 1) as it can be used to describe a range of a_w 0.0-0.9 and accounts for both monolayer and multilayer water in a substance:

$$M = \frac{CKM_0 a_w}{(1 - CKa_w)(1 - Ka_w + CKa_w)} \quad \text{Eq 1}$$

Where M is the moisture content (dry basis, db.) and a_w is the water activity. The parameters K and C are energy constants (dimensionless) referring to the heat of sorption of water in monolayer and multilayer respectively while M_0 is the moisture corresponding to the monolayer moisture content of the material.

The Gordon-Taylor equation (Gordon and Taylor, 1953) is used to describe the dependence of T_g on the fractions of the constituents (Equation 2).

$$T_g = \frac{w_s T_{gs} + k(1 - w_s)T_{gw}}{w_s + k(1 - w_s)} \quad \text{Eq 2}$$

In the above equation, T_g is the glass transition temperature of the mixture. T_{gw} and T_{gs} are the glass transition temperatures of water and the solid respectively with w_s being the weight fraction of solid. The T_{gw} of pure water is taken as 136 K (Capaccioli and Ngai, 2011). k is the fitting parameter describing the plasticizing effect of water.

Statistical analysis was performed using JMP Pro 15 statistical software (SAS Institute Inc, Cary, NC, USA) to obtain the rate constants of the reactions (sections 2.4.1 and 2.4.2) by linear regression. Statistical significance between the control and the aged samples of the model and real system was analysed using one-way ANOVA and the student's t -test for comparison of means at 95 % significance level ($p < 0.05$). Modelling of the glass line and sorption isotherm (Equations 1 and 2) was implemented using nonlinear regression in SAS version 9.3 (SAS Institute Inc., NC, USA). Significant differences in the estimated model parameters were analysed by comparing the 95 % confidence intervals. The goodness of fit between the experimentally obtained data and the mathematical models were evaluated by the adjusted coefficient of determination (R^2_{adj}) and root mean square error (RMSE). The estimated parameters and their standard error (a measure of the accuracy of predictions) are reported.

3 Results and Discussion

3.1 Biochemical changes

3.1.1 Pectin model system

In terms of structure, citrus pectin has a higher degree of linearity than bean pectin implying that the model system provided less interference of the pectin side chains to the pectin methylesterase enzyme. The easier access of the enzyme to the active sites on pectin as compared to the real system makes it a plausible model system. Besides, PME from citrus peel and bean both being plant PME have a similar mode of action, i.e. block-wise pectin de-esterification (resulting in blocks of free carboxyl groups). The initial DM of citrus pectin at 75 % is higher than that of bean pectin (approx. 50-60 %) but studies have shown that the action of PME depends to a larger extent on the carboxyl group distribution along the pectin backbone rather than the DM of pectin (Goldberg et al., 2001; Hotchkiss et al., 2002).

Based on the storage study, it was found that there is no statistically significant difference ($p > 0.05$) between the final DM of the pectin model system from its initial value after 15 weeks of storage at 35 °C (**Fig. 1A**). Additionally, the DM across the entire storage duration did not significantly differ among the model systems with the reaction showing non-negative slopes (**Table 1**). The reason for the limited demethylesterification could be attributed to the restricted accessibility of the enzyme to the pectin polymer. Although different reasons could have contributed to the low activity (e.g. possible change of pectin conformation from the native to a more coil-like structure during the extraction process) the main reason for these findings most likely is the polymer nature of both the enzyme and the substrate blocking the accessibility of the active sites of the enzyme or the substrate in these solid systems. The plasticizing effect of water and the molecular mobility seems too limited (limiting diffusivity) to allow enzyme catalysed substrate conversions.

3.1.2 *Phytic acid/protein model system*

The substrate phytic acid is associated with the protein bodies of the pea protein as in the case of the real system, although the protein bodies could have been partially disintegrated by extraction and milling during the production of pea protein thereby possibly increasing the accessibility of phytase to the phytic acid. Additionally, plant phytases exhibit a similar mode of action on phytic acid and its salt, though the activity is affected by the system moisture content and pH. These factors establish the model system as indicative of the real system.

The results of the storage experiment (**Fig. 1B**) indicate that there is a statistically significant difference ($p < 0.05$) between the initial and final phytic acid content in the model systems (stored for 13 weeks) having a moisture content of 9.6 % and above. This is higher than the estimated monolayer moisture content (section 3.2), signifying that the water over the monolayer water was available to participate in the reaction. The rate of hydrolysis also increased with increasing moisture content (**Table 1**). In this case, the plasticizing effect of water influenced the enzymatic conversion, the small size of the substrate (phytic acid) should have facilitated easier diffusion and the increasing moisture content played a role in facilitating this reaction.

3.1.3 *Real system*

The fresh red kidney bean cotyledons were confirmed by the cooking experiment (results not shown) as not being HTC before the storage experiment. A storage time of 13 weeks was considered due to the presence of mould growth beyond that. As indicated in **Fig. 2**, a significant difference ($p < 0.05$) between the initial and final DM was recorded only for the sample having the highest moisture content. The rate of the reactions was also higher with increasing moisture content (**Table 2**) but it is to be highlighted that the decrease in DM is quite limited. This limitation in the demethoxylation of pectin by PME during storage could be attributed to the large size of the pectin substrate preventing the enzyme from reaching the active site. The findings are in line with both the model system studied here as well as previous studies on beans (Njoroge et al., 2015), both reporting no significant changes in the DM during storage. Despite the hypothesis that suggests a decrease in the DM of pectin during storage under adverse conditions, the results in this study suggest that change in pectin DM is not involved in the cation-pectin-phytate hypothesis.

However, unlike the DM change the phytic acid content in the beans was significantly lower than their initial phytic acid levels for moisture contents of 12 % and above with the final values being 30-60 % lower than their initial values during the storage duration. The rates of phytic acid hydrolysis increased with

increasing moisture content (**Table 2**). These findings were similar to the observations in the phytic acid/protein model system and support the cation-pectin-phytate hypothesis; that is the release of divalent ions under the conditions of the HTC defect development as reported in previous studies (Coelho et al., 2007).

3.2 Material properties

3.2.1 Moisture sorption isotherm

The moisture sorption isotherm for bean cotyledon, pea protein and citrus pectin determined at 35 °C in a water activity range from 0.0 to 0.9 is depicted in **Fig. 3**. The isotherms are of sigmoid type II shape according to the BET classification (Brunauer et al., 1938). At any given a_w , pea protein exhibited the lowest equilibrium moisture content (EMC) followed by bean cotyledon and citrus pectin. In zone II ($a_w > 0.2$) citrus pectin shows a sharp increase in EMC as compared to the others which only depicted a gradual increase. This could be due to the availability of stronger water binding sites on the citrus pectin which enables the added moisture to associate with the binding sites and neighbouring water molecules in the monolayer with hydrogen bonds. In zone III ($a_w > 0.7$) bean cotyledon absorbs higher moisture than citrus pectin and pea protein. The difference in the water adsorption capabilities of these materials can be attributed to the differences in the composition and the physical structure. Starch is the largest constituent of cotyledon followed by cell wall constituents and proteins but is a poor water binder. The moisture content of the biopolymers -protein and starch (as reflected by the model systems) are different despite similar a_w . This implication of this for a real system is that at a specific bean moisture content, bean pectin will possess a higher moisture content while protein will have a lower moisture content.

The experimental sorption data were modelled using the GAB equation. **Table 3** shows the values of the estimated GAB parameters and the goodness of fit of the model. Among the parameters, M_0 is considerably higher for citrus pectin ($\approx 10\%$) than pea protein and bean cotyledon which displayed almost similar values ($\approx 5\%$). Monolayer moisture represents the water that is strongly bound to the solutes and not available to participate in deterioration reactions and thus food matrices are stable at such moisture content values. The monolayer moisture content obtained in this study is within the range reported in literature being 1.3 to 7.4g/100 g db in legume-based protein (Alpizar-Reyes et al., 2018), 7-8g/100g db in bean cotyledon (Chigwedere et al., 2019) and about 8g/100g db in case of high DM pectin (Tsami et al., 1992).

3.2.2 Glass transition measurement

In this study, the T_g of bean cotyledon and pea protein was obtained by a change in the compressibility (T_g gap) as well as the change in $\tan \delta$ (T_g loss factor). In the case of loss factor, it is possible to visualize the range of the transition utilizing the onset, midpoint and end (**Fig. 4**). A change in the slope of E' and a decrease in E'' of the sample during the glass transition occurs as the system transitions from an amorphous to a crystalline state and it is the peak of the $\tan \delta$ that is usually reported as T_g (Rahman et al., 2007). Despite a lack of consensus on its definition, the onset is considered equally important as it marks the start of the glass transition. It is noteworthy that among the measurement methods, T_g estimated by loss factor differed greatly by the T_g values obtained by the change in compressibility (T_g gap) with the T_g gap being higher than T_g loss factor onset, almost equalling T_g loss factor mid and below T_g loss factor end. This is expected due to the fact the different material properties were being investigated by these methods— mechanical and rheological respectively.

In the case of citrus pectin, there wasn't a clear transition during thermal scanning by DMTA and DSC. This difficulty has been previously reported by other authors (Georget et al., 1998; Witczak et al., 2017) and may be caused by the fact that pectin may be partly occurring in a crystalline state complicating the detection of T_g (PRESTON, 1952). Considering that there were no biochemical changes taking place in the pectin model system in the chosen moisture range, the determination of T_g by other methods was not further explored and further discussion is limited to the phytic acid/protein model system and the real system.

3.2.3 T_g -moisture relation

To ensure that the glass transition is determined using samples having no frozen water, samples having a solid content fraction of 0.8 and above were chosen due to their moisture content being lower than their freezable water content.

Fig. 5 depicts the T_g (gap) moisture relationship for the matrices pea protein and bean cotyledon. At a given moisture content, pea protein depicted a higher T_g than bean cotyledon. T_g decreases with increasing moisture content due to the plasticizing effect of water which increases the interaction among the biomaterials and increases their free volume leading to a decrease in T_g . The Gordon-Taylor equation was fit to the data to obtain the parameters T_{gs} and k (**Table 4**) which allowed comparison among different materials. T_{gs} of pea protein was higher than that of bean cotyledon. The T_{gs} value of pea protein obtained was lower than that reported previously (Pelgrom et al., 2013) which could be due to the difference in the composition of the pea protein and the heating rate employed. The higher the k value, the higher is the decrease of T_g by water plasticization. k values were higher for bean cotyledon and its cell wall polymer than pea protein. The glass transition occurs in the amorphous hydrophilic part of the food system. Due to the presence of lipids and (partly) crystalline constituents (starch, pectin and cellulose) in the bean cotyledon and (pectin and cellulose) its cell wall, the T_g will be significantly decreased with a small increase in overall water content.

3.3 Stability diagram

3.3.1 Protein/Phytic acid model system

Temperature and moisture contents are mutual factors affecting the T - T_g . T_g decreases with increasing sample moisture content while storage temperature can vary below, at and above T_g . The experimental context for the model and real systems used in the study provided different T - T_g values using different moisture contents ($T = \text{constant}$, T_g varies with moisture and T - $T_g = \text{variable}$) which was used to interpret the biochemical reactions. The T_g line (T_g -moisture content relation) of pea protein (based on T_g gap and T_g loss factor onset) incorporating the moisture sorption isotherm and the positions of the model systems stored at 35 °C is presented in **Fig. 6**. The region below the T_g line is the glassy region while that above represents the rubbery state. The model systems are situated in the glassy state based on the T_g gap but the model system with the moisture content of around 16% is in the rubbery state while that with a moisture content of around 12 % was at the border of glass-rubber transition based on T_g loss factor (onset).

According to the glass transition theory, kinetically controlled processes are restricted in their glassy state, the implication being that the hydrolysis of phytic acid would be negligible as the storage temperature was below T_g (in all cases, except moisture content > 12 %). However, the hydrolysis of phytic acid was observed in all cases where the moisture content > 10 % irrespective of the state in which the model systems were present, where the rate of hydrolysis increases with T - T_g . The interesting occurrence of the

hydrolysis of phytic acid in the glassy state highlights that the plasticizing effect on the biochemical reactions is not identical to the plasticizing effect on the rheological properties of the materials. Moreover, the reactions could have been favoured due to the high diffusivity of the reactant molecule, phytic acid due to its small size. Previous studies in the field of biology (seed viability) have defined the concept of critical storage temperature (T_c) below which the seeds are stable with T_c/T_g being ~ 0.96 for pea (Sun, 1997) implying that there is a possibility of biochemical instability even below T_g in this matrix, which is in agreement with the present study.

Additionally, it must be pointed out that the T_g determination methods were not directly based on the molecular mobility of the matrix and reactants in this study. Therefore only a relationship exists between T_g and T_c and it is difficult to predict T_c accurately. Furthermore, the T_c can depend on whether the 'global' mobility of whole foods or the 'local' mobility of the matrix is taken into account. T_g obtained by DMTA (T_g loss factor) is different and can explain the hydrolysis of phytic acid better than T_g by TMCT (T_g gap). This is expected as the value of T_g depends on the method used with mechanical relaxation (response measured by DMTA) accounting for a better explanation of biochemical stability.

3.3.2 Real system

The stability diagram of bean cotyledon, highlighting the real system and the glassy and rubbery regions is shown in **Fig. 7**. At ambient temperature, fresh kidney beans (with a moisture content of approx.13%) exists in a glassy state or at the border of the glassy-rubbery region which is important in the context that the seeds need to remain viable for long term storage.

However, the experimental storage conditions have placed the real system below, at and beyond T_g . Based on the stability diagram, real systems with a moisture content of 12 % and above were in the rubbery state. The real system with a moisture content of about 10 % is in the rubbery state if T_g based on loss factor (onset) is considered. The hydrolysis of the phytic acid was observed in the real system when the storage temperature is beyond the glass transition temperature. The decrease in phytic acid content was dependent on $T-T_g$, with a higher decrease observed with increased $T-T_g$ highlighting that the hydrolysis of phytic acid was controlled by glass transition. Thus, the bean moisture content of 10 % appears to be the critical point, in agreement with T_g loss factor.

In the case of changes in DM, a slight change of DM was only observed in bean cotyledon having the highest moisture content of 18 % even though those with a moisture content of 11-15% were also in the rubbery state. The critical storage temperature or cross over temperature for the change in DM is 20 °C and 40° C higher than the T_g gap and T_g loss factor respectively. This coincides with the critical temperature of 40 °C, previously reported to describe the kinetics of seed ageing (Murthy et al., 2003). The difference in the extent to which T_c exceeds T_g for both reactions depends on the difference in the reactions' substrate, enzyme and matrix under consideration. This explains our hypothesis that different difference in water plasticization of the individual biopolymers can result in different extents of stability.

Beans being heterogeneous in composition, T_g values measured for the whole cotyledon would differ substantially from the T_g of the individual constituents' starch, protein and cell wall with mainly protein and cell wall polymers influencing the reactions involved in the cation-pectin-phytate hypothesis. Although the relationship between the biochemical changes in the real system was explained by the 'global' T_g of bean cotyledon, the separate constituents might not likely exist in the same state as indicated by the T_g values measured for the whole cotyledons. Among these constituents, protein and cell wall

constituents (pectin and hemicellulose) are expected to influence the T_g of bean cotyledon (Kyomugasho et al., 2020).

In this context, it is important to remember that in a heterogeneous matrix being stored at a particular temperature, under equilibrium conditions, the water activity will be the same for all constituents they might have different individual moisture contents (see sorption isotherms reported in **Fig. 2**). It is therefore useful to analyse the phytate conversion in model and real system by plotting the rate constant versus the water activity. As in the current study, the substrate concentration and enzyme units in both systems are not identical, a normalized representation is illustrated in **Fig. 8**. The rates for all storage conditions were calculated relative to RH 66 % to obtain the normalized rates. The prominent outcome shows that the plasticizing effect of water in both the model system and real system is identical.

However due to the lack of DM reduction of pectin in the pectin model system, an attempt to relate the observation of DM in the real system was carried out using the T_g -moisture relation of bean cell wall polymers. The whole-cell wall matrix, not just limiting to pectin was considered as the demethoxylation of pectin occurred in the cell wall with PME being a cell wall-bound enzyme. Based on the state diagram of the bean cell wall polymer fraction as shown in **Fig. 9**, it is clear that the cell wall of beans were all in a rubbery state during storage, despite the lack of a significant DM reduction observed in the real system (except M.C. of 18 %). Therefore, the demethoxylation of pectin needs a higher moisture content to provoke sufficient molecular mobility for these enzymatic conversion reactions to take place. The findings in this study combined with researches that have linked the cooking ability to the state of bean cotyledon (Chigwedere et al., 2019; Kyomugasho et al., 2020), can lead to the conclusion that there is a relation between the degradation of phytic acid and reduction of cooking ability in the rubbery state.

While the critical temperature or moisture content can change depending on whether the whole cotyledon or the individual constituents are considered, the results point towards the acceptability of T_g (loss factor) of the bean cotyledon in suitably explaining the biochemical stability. Chigwedere et al., (2019) has demonstrated that T_g obtained by TMCT is a good indicator in linking the loss of cooking ability to the state of bean cotyledon. However, TMTC values do not seem to explain the biochemical changes causing the HTC defect. The cooking defect is manifested as textural changes (that require the displacement of divalent cations next to the biochemical changes) while the biochemical changes depend on the local molecular mobility. In addition, TMTC based T_g data have commonly been used to explain changes in mechanical properties like milling, fracture behaviour, hardness etc (Ebara et al., 2019; Mochizuki et al., 2019; Pelgrom et al., 2013). Therefore, it is suggested to use T_g determination methods based on molecular mobility (NMR) and changes in the physical state like viscosity (DMTA) to account for biochemical stability.

Conclusion

This study aimed to understand the individual reactions involved in the cation-pectin-phytate hypothesis from a glass transition point of view. This study clearly shows that the demethoxylation of pectin is limited in beans during storage under conditions leading to HTC development despite the bean cell wall polymers being in a rubbery state. The pectin model system displayed similar results and thus it can be established that reduction in DM of pectin during storage is not a major cause of the HTC defect. The reaction requires temperatures far above the system's glass transition temperature. However, under the given storage conditions, phytic acid was significantly hydrolysed in both the model system and real system indicating

that the conditions studied suffice the activation of phytase and lead to the liberation of divalent mineral ions. Based on the stability diagram of bean cotyledon, it is evident that the hydrolysis of phytic acid occurred in the rubbery state and the reaction increases with increasing $T-T_g$ and is therefore controlled by the glass state. Further research is necessary to understand the migration and cross-linking of the liberated ions with pectin in the middle lamella.

The results highlight that the storage of beans in their glassy state prevents/slows down biochemical changes associated with hardening. Therefore, T_g -moisture relation and stability maps are key in identifying suitable storage conditions for post-harvest storage of beans. Beans can either be stored at lower temperatures or can be dried to lower moisture content such that they are below their T_g values. This would enable longer storage times with delayed ageing and consequently delayed hardening.

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Declarations of interest

None.

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Figure Captions:

Fig. 1. DM (A) and phytic acid content (B) in control and aged samples of the pectin and phytic acid/protein model system respectively stored at 35 °C for 13 and 15 weeks. Samples not sharing similar letters are significantly different ($p < 0.05$).

Fig. 2. DM (A) and phytic acid content (B) in control and aged samples of the real system stored at 35 °C for 13 weeks. Samples not sharing similar letters are significantly different ($p < 0.05$).

Fig. 3. Moisture sorption isotherm of Citrus pectin (■), Bean cotyledon (◆) and Pea protein (●) at 35 °C fit to GAB model.

Fig. 4. Corrected displacement (mm) of the probe (A) and Loss factor (B) during the thermal scan and demonstration of T_g determination in bean cotyledon powder having a moisture content of 10 %.

Fig. 5. Variation of glass transition temperature with the moisture content of bean cotyledon (■) and pea protein (●) fit to the Gordon-Taylor model.

Fig. 6. Stability diagram of phytic acid/protein model system depicting the T_g gap (●) and T_g loss factor (▲) of pea protein (fit to Gordon-Taylor model), moisture sorption isotherm (◆) fit to GAB model and position of the model systems (*) at 35 °C.

Fig. 7. Stability diagram of bean cotyledon depicting the T_g gap (●) and T_g loss factor (▲) of bean cotyledon (fit to Gordon-Taylor model), moisture sorption isotherm (◆) fit to GAB model and position of the real systems (*) at 35 °C.

Fig. 8. Normalized rate of phytic acid hydrolysis in the model (●) and real system (●).

Fig. 9. Variation of glass transition temperature with the moisture content of bean cotyledon cell wall polymers (▲) obtained by DSC fit to the Gordon-Taylor model.

Table 1. The rate of change (rate constant $\pm$ standard error) in DM of pectin and hydrolysis of phytic acid in the pectin and protein/phytic acid model systems respectively stored at 35 °C for 15 and 13 weeks. The DM (mean $\pm$ standard deviation) of the model system for the entire storage duration is also reported. The moisture content is expressed on wet basis. Values within a column followed by the same letter are not significantly different ($p < 0.05$).

<i>Relative humidity</i>	Pectin model system			Protein/phytic acid model system	
	<i>Moisture content (%)</i>	<i>Rate constant (% DM/week)</i>	<i>DM (%)</i>	<i>Moisture content (%)</i>	<i>Rate constant (% phytic acid/week)</i>
Rh 82 %	21.29	0.132 ± 0.03^a	74.6 ± 1.28^a	13.69	-0.165 ± 0.018^a
Rh 74 %	18.34	0.130 ± 0.03^{ab}	74.9 ± 1.22^a	11.38	-0.137 ± 0.012^a
Rh 66 %	15.98	0.127 ± 0.03^{ab}	75 ± 1.24^a	9.67	-0.079 ± 0.004^b
Rh 54 %	13.14	0.043 ± 0.02^{ab}	75.1 ± 0.84^a	7.7	-0.032 ± 0.005^c
Rh 7 %	2.89	0.034 ± 0.02^b	75.1 ± 0.78^a	1.86	-0.014 ± 0.004^d

Table 2. The rate of change (rate constant $\pm$ standard error) in DM of pectin and hydrolysis of phytic acid in the real system stored at 35 °C for 13 weeks. The moisture content is expressed on wet basis. Values within a column followed by the same letter are not significantly different ($p < 0.05$).

<i>Relative humidity</i>	<i>Moisture content (%)</i>	DM of pectin <i>Rate (% DM/week)</i>	Hydrolysis of phytic acid <i>Rate (% phytic acid/week)</i>
Rh 82 %	18.54	-0.277 ± 0.04^a	-0.047 ± 0.003^a
Rh 74 %	14.82	-0.190 ± 0.05^{ab}	-0.046 ± 0.003^a
Rh 66 %	12.29	-0.111 ± 0.06^{ab}	-0.026 ± 0.002^b
Rh 54 %	9.66	-0.125 ± 0.04^{ab}	-0.008 ± 0.002^c
Rh 7 %	2.72	-0.050 ± 0.06^b	-0.002 ± 0.002^d

Table 3. The values of the parameters ($\pm$ standard error) estimated by the GAB model for the sorption isotherm of bean cotyledon, citrus pectin and pea protein at 35 °C. C and K are dimensionless constants and M_o is the monolayer moisture content (g water/100g). The adjusted coefficient of determination (R^2_{adj}) and root mean square error (RMSE) are the criteria for the adequacy of the model. Values within the column not sharing a similar letter are significantly different ($p < 0.05$).

Material	M_o	C	K	R^2_{adj}	RMSE
Bean cotyledon	5.7 ± 0.1^a	14.4 ± 1.7^a	0.91 ± 0.00^a	1.00	0.19
Citrus pectin	10.2 ± 0.5^b	7.8 ± 1.4^b	0.79 ± 0.01^b	0.99	0.50
Pea protein	5.2 ± 0.6^a	10.0 ± 5.2^{ab}	0.84 ± 0.03^b	0.99	0.17

Table 4. The estimated Gordon-Taylor parameters ($\pm$ standard error) of Bean cotyledon, its cell wall polymer and Pea protein. Goodness of fit is presented as R^2_{adj} . Values within a column not sharing a same letter are significantly different ($p < 0.05$).

Sample	T_g measurement	T_{gs}	k	R^2_{adj}
Bean cotyledon	Change in the compressibility	98.5 ± 6.9^{ab}	3.2 ± 0.4^{ab}	0.98
	Loss factor onset	98.2 ± 7.9^{ab}	4.8 ± 0.5^b	0.98
Pea protein	Change in the compressibility	118.4 ± 3.9^b	2.2 ± 0.2^a	0.99
	Loss factor onset	121.3 ± 9.1^b	3.7 ± 0.5^{ab}	0.99
Bean cotyledon cell wall polymer	Change in heat flow	70.4 ± 7.4	4.7 ± 0.6^b	0.98

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Fig. 4. Corrected displacement (mm) of the probe (A) and Loss factor (B) during the thermal scan and demonstration of T_g determination in bean cotyledon powder having a moisture content of 10 %.

Fig. 5. Variation of glass transition temperature with the moisture content of bean cotyledon (■) and pea protein (●) fit to the Gordon-Taylor model.

Fig. 6. Stability diagram of phytic acid/protein model system depicting the T_g gap (●) and T_g loss factor (▲) of pea protein (fit to Gordon-Taylor model), moisture sorption isotherm (◆) fit to GAB model and position of the model system (*) at 35 °C.

Fig. 7. Stability diagram of bean cotyledon depicting the T_g gap (●) and T_g loss factor (▲) of bean cotyledon (fit to Gordon-Taylor model), moisture sorption isotherm (◆, fit to GAB model) and position of the real systems (*) at 35 °C.

Fig. 8. Normalized rate of phytic acid hydrolysis in the model (●) and real system (●).

Fig. 9. Variation of glass transition temperature with the moisture content of bean cotyledon cell wall polymers (▲) obtained by DSC fit to the Gordon-Taylor model.

Highlights

- The reactions in the cation-pectin-phytate hypothesis were studied in model and real systems.
- Hydrolysis of phytic acid is controlled by glass transition.
- Glass transition lines of red kidney bean cotyledon and its' cell wall polymers were created.
- DMTA based T_g lines are better suited for explaining (bio)chemical stability rather than TMCT.
- Stability map supports in establishing suitable post-harvest storage conditions.

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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