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Kinetic study of enzymatic α-galactoside hydrolysis in cowpea seeds

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ABSTRACT

The endogenous alpha-galactosidase activity of cowpea seeds was characterized and modelled assuming Michaelian behavior. The aim is to use the resulting knowledge to optimize alpha-galactoside degradation during the soaking-cooking process. In this study, the alpha-galactosidase enzyme from *Wankoun* cowpea was extracted and its enzymatic activity was analyzed as a function of temperature, pH and the presence of some inhibitors. Enzymatic activity was optimal around 35 °C and a pH of 5.8. Activation and inactivation energy was evaluated at 50 ± 3 and 103 ± 9 kJ.mol^{-1}, respectively. The strongest inhibitor was galactose with an inhibition constant K_I of 0.28 ± 0.03 mM. Incubation of the enzyme extract with alpha-galactosides revealed a 10-h lag phase in the early stages that could be due to low pH, the action of inhibitors including galactose and the biosynthesis of alpha-galactosides. After the lag phase, the degradation of each alpha-galactoside occurred without the appearance of any intermediary product. The degradation of alpha-galactosides was observed with a K_m of 1.7 ± 0.3 mM for raffinose; 3.6 ± 0.6 mM for stachyose and 15.9 ± 0.1 mM for verbascose. A long soaking step around 35 °C is suggested to maximize the alpha-galactosides enzymatic degradation.

1. Introduction

The legume cowpea is widely cropped and consumed in West Africa due to its drought tolerance and high nutritional value (Langyintuo et al., 2003). Cowpea seed has a high protein content (20–25% *w*/w), and could thus be a good alternative to meat (Boye, Zare, & Pletch, 2010). It is also rich in micronutrients such as vitamins and minerals (Campos-Vega, Loarca-Piña, & Oomah, 2010; Sreerama, Sashikala, Pratape, & Singh, 2012). However, cowpea also contains anti-nutritional factors such as alpha-galactosides. Three main alpha-galactosides are present in cowpea seeds depending on the number *n* of galactose units: raffinose (*n* = 1; 0.5–1 g/100 g db), stachyose (*n* = 2; 1.7–6 g/100 g db) and verbascose (*n* = 3; 0.6–1.3 g/100 g db) (Gonçalves et al., 2016). In terms of distribution within the seed, Sreerama, Neelam, Sashikala, and Pratape (2010) showed that in chickpea and horse gram, the alpha-galactosides were mainly present in the cotyledon (65% *w*/w) and in the embryonic axis (27% w/w) with a few in the seed coat (7% w/w). This distribution differs slightly depending on the alpha-galactoside concerned. Inside the cells, 85% (*w*/w) of alpha-galactosides are contained in cytosol and 15% w/w in storage vacuole (Obendorf & Gorecki, 2012). In the seeds, the role of these molecules is to provide energy through the action of the endogenous alpha-galactosidase enzyme that produces simple sugars for seed germination, but also to prevent hydric stress (Alani, Smith, & Markakis, 1989).

There are two types of complementary alpha-galactosidases in legumes. The first is an acidic form that accumulates during seed development and is mainly stored in cell vacuoles (Blöchl, Peterbauer, Hofmann, & Richter, 2008). During the early stage of germination, this enzyme mainly hydrolyzes the alpha-galactosides stored in the vacuoles (Obendorf & Gorecki, 2012) but also some of the alpha-galactosides present in cytosol, since this acidic form can be released from the vacuoles(Sekhar & DeMason, 1990). The other is an alkaline form that is synthesized after germination. However, its activity is observed only 40 h after the beginning of germination (Blöchl et al., 2008; Obendorf & Gorecki, 2012). This alkaline enzyme is present in cytosol and can hydrolyze the alpha-galactosides present in this medium.

The alpha-galactosidase enzyme does not exist in humans and alpha-galactosides ferment in the colon, causing intestinal disorders and flatulence (Obendorf & Gorecki, 2012). For the purpose of human consumption, it is possible to use the endogenous alpha-galactosidase activity of cowpea during soaking to reduce alpha-galactoside content. The intensity of endogenous alpha-galactosidase activity depends to a great extent on the soaking conditions (time, temperature, pH). A soaking period of 22 h at room temperature enabled Khattab and

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Arntfield (2009) to reduce total alpha-galactoside content by 41% in some Canadian cowpea cultivars. Coffigniez et al. (2018) showed that soaking cowpeas for 38 h at 30 °C reduced raffinose content by about 20% and stachyose content by 50%. This is explained by the fact that 30 °C is close to optimal for enzyme activity. Enzymatic degradation could be enhanced through a better understanding of enzyme activity as a function of soaking parameters (pH, temperature, etc.).

Only a few studies describing the alpha-galactosidase activity of legumes are available in the literature. Three alpha-galactosidase isoforms have been found in cowpea seeds: isoforms I (Mr = 111,000), II^1 (Mr = 29,000) and II^2 (Mr = 30,000) (Alani et al., 1989). Dey and Pridham (1969a) showed that alpha-galactosidase activity in *Vicia faba* was optimal between pH = 3 and pH = 6 with a PNP (*p*-nitrophenyl-α-D-galactopyranoside) substrate and between pH = 3 and pH = 5 with a raffinose substrate. Concerning the effect of temperature, Dey and Pridham (1969a) showed that the activity of the enzyme was stable up to 45 °C. Beyond this temperature, activity started to decrease, and stopped at 75 °C. Finally, Dey, Campillo, and Lezica (1983) showed that increasing the temperature in the range [20–50 °C] resulted in an increase in K_M and v_{max} with an activation energy of 62.1 kJ.mol^{-1}.

Alpha-galactosidase are Michaelian enzymes, and their affinity and activity both depend on the substrate. Alani et al. (1989) identified a K_M value of 4.6–5 mM for raffinose and 11–15 mM for stachyose in cowpea seeds, whereas Dey and Pridham (1969b) obtained a K_m value of 4–5 mM for raffinose and 5.26–7.5 mM for stachyose in *Vicia faba* seeds. The same authors found a v_{max} value of 4.18–28.4 μmol.min^{-1}.mg^{-1} for raffinose and 1.360–9.00 μmol.min^{-1}.mg^{-1} for stachyose at 30 °C. They also observed a strong impact of galactose, galactinol and myo-inositol as enzyme inhibitors. K_m and v_{max} are widely determined using the Linevewear-Burk method, however, this method of derivation leads to significant errors in the estimation of the K_m and v_{max} parameters since the y-axis takes the reciprocal of the rate of reaction – in turn increasing any small errors in measurement (Doeswijk & Keesman, 2009). A more robust method consists in integrating the Michaelis-Menten law. Another bias is due to the fact that enzyme characterization is mostly performed with purified enzyme, which, considering its chemical environment, is far from reflecting real behavior in actual applications.

Alpha-galactoside diffusion and chemical degradation in *Wankoun* cowpea seeds and during the soaking-cooking process has already been modelled (Coffigniez et al., 2018). However, enzymatic degradation was described assuming first-order kinetics whereas it is recommended to use Michaelis-Menten approach. The aim of the present study was to go further by characterizing and modelling the activity of alpha-galactosidase in cowpea as a function of substrate, pH, temperature and the presence of some inhibitors, while assuming Michaelian behavior.

2. Materials and methods

2.1. Material

Wankoun cowpea seeds was provided by IITA, Benin. Cowpea seeds were sown in November 2014 and harvested in February 2015. The seeds were stored in a vacuum pack at 4 °C in the dark until use. The cowpea seeds were freeze-dried and ground with a RETSCH Ultra Centrifugal Mill ZM 200. The grid size was 0.5 mm.

Raffinose, stachyose, galactose and galactinol standards (purity ≥98%), and myo-inositol (purity ≥99%) were purchased from Sigma (USA and China) and galactose from Fluka (Germany). The *P*-nitrophenyl-α-D-galactopyranoside (PNP, purity ≥98%) was purchased from Megazyme (Ireland). Ultrapure water was used in all the experiments (Simpak MilliQsystem, Millipore, USA). Sodium hydroxide solution (50–52% in water) used for HPAE was purchased from Sigma (Germany), and ethanol (purity ≥99.8%) from Honeywell Riedel-de-Häen (Germany). Potassium phosphate monobasic (purity ≥99%) was purchased from Sigma (Mexico) and potassium phosphate dibasic (purity ≥98%) from Honeywell (Spain). Borate (purity ≥99.5%) and sodium hydroxide (purity ≥98%) were purchased from Sigma (USA and Sweden).

2.2. Enzyme extraction

The enzyme was extracted from cowpea flour with 100 mM phosphate buffer (pH = 6.5) with a cowpea/buffer ratio of 1:4 (*w*/w) for 0.5 h at 4 °C under gentle stirring. The solution was then centrifuged at 1000*g* for 5 min at 4 °C and the supernatant, which constitutes the enzyme extract, was stored at 4 °C until use (up to 6 h).

2.3. Enzyme characterization

The initial activity of alpha-galactosidase extract (v) was measured as the amount of PNP hydrolyzed at 30 °C (no thermal degradation) in 0.1 M phosphate buffer (pH = 6.5, the same pH as in the seeds) after 5 min; 200 μL of PNP solution was hydrolyzed by 100 μL of enzyme extract with 700 μL of phosphate buffer. The concentration of PNP ranged from 0.02 mM to 3.96 mM (until ten times the K_M (Dey & Pridham, 1969b)). The enzyme extract was also pre-incubated for 600 min, 720 min, 840 min and 960 min at 30 °C before measuring the enzymatic activity on PNP at the concentration of 1.98 mM for 5 min. The reaction was stopped by adding 2 mL of 0.2 M borate solution (at pH = 9.8). The product of the reaction was quantified by measuring absorbance at 405 nm with a spectrophotometer (Schimadzu UV 2450, Japan). The initial enzyme activity v, or rate constant, expressed in μmol.min^{-1}.mL^{-1} of enzyme (or U.mL^{-1}) was calculated using the following equation (Sigma-Aldrich, 1997):

$$v = \frac{(Abs - Abs_0)V_{tot}}{\varepsilon V_{enz} t} \quad (1)$$

where Abs is the sample absorbance at 405 nm; Abs_0 is the blank absorbance; V_{tot} is the total volume of the assay (mL); V_{enz} is the volume of enzyme (mL); ε is the extinction coefficient of p-nitrophenol at 405 nm (18.5 mL μmol^{-1} cm^{-1}) and t is the time (min). The alpha-galactosidase activity was also evaluated by the direct consumption of verbascose, stachyose or raffinose in the extracted supernatant. The substrate consumption was calculated by difference (Eq. (2)).

$$HS = S_0 - S \quad (2)$$

where HS is the hydrolyzed substrate (mM), S_0 the initial substrate concentration (mM), S the substrate concentration at observed time (mM) and S could be raffinose, stachyose or verbascose. The initial concentrations of verbascose, stachyose, raffinose, and galactose in the enzymatic supernatant were 0.25 mM, 1.9 mM, 0.15 mM and 0.21 mM, respectively. Their concentrations were periodically assayed during hydrolysis at 30 °C for up to 2160 min. Changes in concentration were also quantified after spiking the enzymatic extract with 11 mM of stachyose, 5.0 mM or 37 mM of raffinose. The reaction was stopped by adding 2 mL of 0.2 M borate solution (pH = 9.8). The solution was filtered using a 0.45 μm membrane filter and alpha-galactosides were quantified after separation by high performance anion exchange (HPAE) chromatography using the procedure of Gangola, Jaiswal, Khedikar, and Chibbar (2014) modified by Coffigniez et al. (2018). Each experiment was performed in duplicate (two enzyme extractions and two enzyme activity measurements for each extraction).

The inhibition effect of galactose (spiked at 0.6 mM; 1.5 mM and 3.0 Mm) on alpha-galactosidase was determined by measuring enzymatic activity at different concentrations of PNP (between 0.02 and 3.96 mM) in phosphate buffer at 30 °C for 5 min. The inhibition induced by galactinol (spiked at 1.0 mM and 2.5 mM) and myo-inositol (spiked at 6.0 mM and 60 mM) was also tested on 1.98 mM of PNP in phosphate buffer for a time ranging from 5 to 300 min.

The effect of pH (in the range [2–8.5]) on alpha-galactosidase activity was determined by measuring enzymatic activity using 1.98 mM