



# Multivariate model to characterise relations between maize mutant starches and hydrolysis kinetics



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## ABSTRACT

The many studies about amylolysis have collected considerable information regarding the contribution of the starch physico-chemical properties. But the inherent elaborate and variable structure of granular starch and, consequently, the multifactorial condition of the system hinders the interpretation of the experimental results. The immediate benefit of multivariate statistical analysis approaches with that regard is twofold: considering the factors, possibly interrelated, all together and not independently, providing a first estimation of the magnitude and confidence level of the relations between factors and amylolysis kinetic parameters. Based on data of amylolysis of 13 starch samples from wild type, single and double mutants of maize by porcine pancreatic  $\alpha$ -amylase (PPA), a multivariate analysis is proposed. Amylolysis progress-curves were fitted by a Weibull function, as proposed in a previous work, to extract three kinetic parameters: the reaction rate coefficient during the first time-unit,  $k$ , the reaction rate retardation over time,  $h$ , and the final hydrolysis extent,  $X_{\infty}$ . Multivariate models relate the macromolecular composition and the fractions of crystalline polymorphic types to the kinetic parameters.  $h$  and  $X_{\infty}$  are found to be highly related to the measured properties. Thus the amylose content appears to be significantly correlated to the hydrolysis rate retardation, which sheds some light on the probable contribution of the amylose molecules contained in the granules. The multivariate models give correct prediction performances except for  $k$  whose a part of variability remains unexplained. A further analysis points out the extent of the characterisation effort of the granule structure needed to extend the fraction of explained variability.

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## 1. Introduction

Many biological and industrial processes, such as mammal digestion, plant metabolisms, biofuel production, fermentation or malting rely on the hydrolysis of native starch by amyolytic enzymes. Amyolytic enzymes, mostly  $\alpha$ -amylases, break down the macromolecules into oligosaccharides later converted into glucose and then energy. The amyolytic process is a heterogeneous phase reaction highly dependent on the starch structure. The way the starch structure affects the amylolysis is still unclear nor the mechanisms fully understood, despite the accumulating findings.

Starch is a complex biological entity that displays hierarchical organisation levels represented by molecules, macromolecular arrangements, granule architecture or even granule population.

The starch granule form, size and structure also vary a lot with the botanical source. Insoluble starch granules of 1–100  $\mu\text{m}$  are a semi-crystalline arrangement of two  $\alpha$  D-glucose polymers built mainly on  $\alpha(1-4)$  linkages: amylose and amylopectin (Buléon, Colonna, Planchot, & Ball, 1998; Pérez & Bertoft, 2010). The major component, amylopectin, exhibits an arborescent branched structure with 5–6%  $\alpha(1-6)$  branching points; it is considered as the support of the crystalline domains in the starch granule (Pérez & Bertoft, 2010). Amylose is a mostly linear molecule of  $\alpha(1-4)$  D-glucopyranosyl units with less than 1% of  $\alpha(1-6)$  branching linkages. Native starches display mainly A-type and B-type crystalline structure. The former is mainly present in cereals while the latter is more common in tubers and high-amylose starches. In addition, amylose can form V-type complexes with monoacyl lipids in cereal starches. The fraction of crystalline materials varies from 15% to 45% depending on the plant source (Buléon et al., 1998). A native starch granule exhibits concentric “growth rings” consisting of alternate lamellae of crystalline and amorphous materials at a periodical distance of 9–10 nm.

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Several studies attempted to identify the factors affecting the susceptibility of native starch to  $\alpha$ -amylases in relation to the botanical origin or the  $\alpha$ -amylase source. Global properties of starch samples, such as relative surface area or granule sizes distribution, have been shown to have a significant influence (Tester, Qi, & Karkalas, 2006). Granule morphological features, such as granule surface condition with presence of pores or channels, are also suspected to play an important role in limiting or favouring the enzyme diffusion inside the granule and then the hydrolysis (Blazek & Gilbert, 2010; Dhital, Shrestha, & Gidley, 2010; Planchot, Colonna, Gallant, & Bouchet, 1995). The influence of factors at lower organisation levels, e.g. crystalline type, macromolecular composition, chain length distribution, have also been investigated in many papers (Blazek & Gilbert, 2010; Gérard, Colonna, Buléon, & Planchot, 2001; Huang et al., 2015; Lauro, Forssell, Suortti, Hulleman, & Poutanen, 1999; Tester et al., 2006). However the diversity of experimental conditions, the relation with the higher organisation levels limit and affect the interpretation of the experimental results. Thus, it is established that the porcine pancreatic  $\alpha$ -amylase (PPA) degrades A-type starches (wheat, rice, maize) much faster than B-type starches (potato, amylo maize). However starches from different botanical sources vary considerably on many structural aspects that can also explain the different susceptibilities to PPA (Blazek & Gilbert, 2010; Tester et al., 2006). For instance potato starch is characterised by high phosphate content and large granule size and then low accessibility for PPA compared to the smaller maize starch granules (Dhital et al., 2010; Warren, Royall, Gaisford, Butterworth, & Ellis, 2011). Another example is the unclear role of the amylose content. It is often seen as of minor importance under standard amylolysis conditions (Evans & Thompson, 2004; Gérard, Colonna, Buléon, et al., 2001; Shrestha et al., 2012), and yet recent results tend to show that for high substrate concentrations the amylose chains are actively involved in a resistance mechanism to hydrolysis (Lopez-Rubio, Flanagan, Shrestha, Gidley, & Gilbert, 2008; Tawil, Vikso-Nielsen, Rolland-Sabaté, Colonna, & Buléon, 2011). Starch granule is a complex biological object described by various properties, often inter-correlated, e.g. B-type starch is often high-amylose starch. Therefore focusing on the influence of a specific property can lead to wrong interpretations. Overall the problem is multifactorial and sorting out the different dependences at work during the hydrolytic process calls for appropriate data analysis technique.

To date, and despite the generalisation, of the statistical tools, the application of multivariate statistical analysis method has rarely been attempted to relate native starch properties to amylolysis kinetics data. A challenging aspect is the small size of the datasets, in a domain where experimentations are time-consuming and can be costly. Another limitation is that many studies compare samples from different botanical origins. Using mutants from the same genetic background enables the analysis of samples with less structural changes. Several maize mutants have been produced with lack of elongation and/or branching activities, in particular to understand biosynthesis. In combining mutations, a wide range of granular and macromolecular structure can be obtained while global properties, such as granule size, remain comparable (Wang, White, Pollack, & Jane, 1993). Gérard, Colonna, Buléon, et al. (2001) analysed the amylolysis of 13 starches originated from single and double mutants of maize from the same genetic background, with varying macromolecular structures allowing the investigation of the impact of crystallinity type (A, B and V-type) and amylose/amylopectin content. Gérard, Colonna, Buléon, et al. (2001)'s data have two important features for building multivariate model linking starch properties to amylolysis: (1) starch materials required significant crop selection efforts to generate and control the genetic variability between the maize mutants, (2) valuable additional information can be extracted from such a

dataset by means of a multivariate analysis with regard to the initial paper.

In this work, a multivariate model is introduced to describe the relations between starch samples properties and amylolysis kinetic data and applied to Gérard, Colonna, Buléon, et al. (2001) data. The kinetic data are described by means of a simple empirical function (Weibull function), put forward in a previous work as a good compromise between the number of parameters to fit and the fitting performance (Kansou, Buléon, Gérard, & Rolland-Sabaté, 2015). A Partial Least Square Regression (PLSR) is used to analyse the relations between the parameters of the Weibull with the starches properties, ranking them by magnitudes. To deal with the small size of the dataset, a resampling technique is used to assess the outputs confidence intervals and the prediction capacity of the models.

## 2. Methods

### 2.1. Sample description

The data used in the present study were obtained by Gérard, Colonna, Buléon, et al. (2001) from the hydrolysis by porcine pancreas  $\alpha$ -amylase (PPA) of 13 starches produced from 9 different mutants of maize. Hydrolyses were carried out at 37 °C on 1 mg/mL starch suspensions in phosphate buffer with 5  $\mu$ g of enzyme per mg of starch (Gérard, Colonna, Buléon, et al., 2001). The kinetics data are 13 time-series of 11–13 measurements performed over a period of 45–50 h (progress-curves data in Fig. 4). Starch hydrolysis was duplicated, and measurements of the percentage of hydrolysis of starch data by orcinol–H<sub>2</sub>SO<sub>4</sub> method were triplicated. Crystallinity of the samples was determined by means of X-ray diffractometry, with 200 g kg<sup>-1</sup> H<sub>2</sub>O, adjusted with BaCl<sub>2</sub> solution (Gérard, Colonna, Buléon, et al., 2001). Relative crystallinity is determined after normalisation of all recorded diagrams at the same integrated scattering between 3° and 30° (2 $\theta$ ). A and B-type crystallites fractions are determined after scaled subtraction of an experimental amorphous curve.

Starches originated from single and double mutants of maize of the same genetic background, their nomenclature, process of extraction and general structural features are reported in Table 1. Generally, the wx mutation produces starches with 100% amylopectin and higher crystallinity proportion than normal maize (Gérard, Colonna, Buléon, et al., 2001). ae, du and su2 mutations induce modifications of the macromolecular structure with the presence of intermediate material, called IM2 (i.e. a macromolecule having longer chain length and lower molar mass than amylopectin) (Gérard, Barron, Colonna, & Planchot, 2001). ae mutant starch presents in addition a second type of intermediate material (IM1) essentially linear with lower molar mass than amylose. ae and su2 mutations induce a modification of the branching pattern of amylopectin; in particular, ae mutation leads to higher amylose content and B-type crystallinity (Gérard, Colonna, Buléon, et al., 2001; Inouchi, Glover, Sugimoto, & Fuwa, 1984) whereas su2 mutant starches have a lower crystallinity proportion. The size and morphology of the mutant starch granules remain rather close to the normal ones (Gérard, Barron, et al., 2001). In Table 1 one can notice the small proportion of V-type crystallites (6–13%) for some 1995 samples, likely due to the drying conditions at 50 °C.

### 2.2. Data modelling approach

The data modelling objectives are to quantify the relations between the starch properties of Table 1 and amylolysis kinetics, to establish the significance level by means of confidence intervals and to assess the model prediction capacity. The analytical process has three main steps:

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