Performance of granular starch with controlled pore size during hydrolysis with digestive enzymes

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Running head: Digestibility of porous starches

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Abstract

Studies on porous starch have been directed to explore different industrial applications as bio-adsorbents of a variety of compounds. However, the analysis of starch digestibility is essential for food application. The objective of this study was to determine the impact of porous structure on *in vitro* starch digestibility. Porous starches were obtained using a range of concentrations of amyloglucosidase (AMG), α-amylase (AM), cyclodextrin-glycosyltransferase (CGTase) or branching enzyme (BE). Porous starches exhibited major content of DS that increased with the intensity of the enzymatic treatment, and very low amount of RS. Porous starches behaved differently during *in vitro* hydrolysis depending on their enzymatic treatment. AMG was the unique treatment that increased the digestive amylolysis and estimated glycemic index, whereas AM, CGTase and BE reduced them. A significant relationship was found between the pore size and the severity of the amylolysis, suggesting that a specific pore size is required for the accessibility of the digestive amylase.

Therefore, pore size in the starch surface was a limiting factor for digestion of starch granules.

1 Introduction

Starch constitutes a biopolymer widely used in the food industry owing its unique thermal, structural and functional properties. Nevertheless, native starch is not always suitable for food production and diverse modifications have been reported to overcome those limitations [1]. Lately, enzymatic modification of starch is gaining attention as an environmentally friendly process that led to porous molecules with great adsorbent capacity [2], being them dependent on the enzymatic treatment and starch source [3,4]. However, scarce information is available about the impact of those modifications on nutrition and health.

Concerning nutritional implications, starch digestibility is an important property that can be altered by enzymatic modification. The surface organization, granular architecture, starch composition, type of crystal polymorph, granular size, and the presence of compound granules, affect the rate and extent of starch digestibility [5,6]. Among these factors, the presence of cracks on the surface layer has been related to the feasibility with which digestive enzymes can hydrolyze native starch granules [7]. Porous starches obtained enzymatically contain abundant surface pores that go to the inner center of the granules [8]. The number of pores is dependent on both the type and level of enzyme used for the production of porous starch [8]. Likewise, the number and size of the pores determine the morphological and physicochemical properties of the resulting porous starches and their subsequent applications in food industry as adsorbents.

In spite of the different applications reported for the porous starches [9-12], there is a dearth of information on their digestibility pattern. Dura et al. [13] and Dura and Rosell [14] studied the effects of AMG, AM and CGTase on digestibility behavior of corn starch at sub-gelatinization temperature (50 °C). High susceptibility to be digested was shown by porous

starches obtained after prolonged treatment with AM or AMG. Opposite trend was displayed on CGTase-modified starches that resulted less susceptible to be hydrolyzed by digestive enzymes. In addition, *in vivo* studies showed that porous starches obtained with CGTase had slower digestion, reducing the blood glucose levels, which was attributed to the presence of β- cyclodextrins that may impede the orientation of amylases [15]. Despite those initial studies, no information is available about the impact of granule morphology on the digestion pattern.

The aim of this study was to determine possible relationship among morphological structure of porous starches and digestibility performance. For that purpose, a range of enzymatically modified starches obtained in a previous study [9] were used to study their digestibility and glycemic index. In that previous study, porous starches were obtained with different enzymes (AMG, AM, CGTase and branching enzyme (BE)) using a range of enzyme concentrations, those treatments provided porous starches with varied size and frequency of surface pores. Those starches were subjected to digestive amylase and hydrolysis kinetics were compared.

2 Material and methods

Corn starch with a purity of 98.39% was purchased from Miwon (Seoul, Korea). Amyloglucosidase (EC 3.2.1.3), fungal α-amylase (EC 3.2.1.1), cyclodextrin-glycosyltransferase (EC 2.4.1.19) and branching enzyme (EC 2.4.1.18) activities were provided by commercial food grade preparations (Amyloglucosidase 1100 L declared activity 1100 AGU/g product, Fungamyl® 2500SG declared activity 2500 FAU/g product, Toruzyme® 3.0 L declared activity 3KNU/mL product and Branchzyme declares activity 50000 BEU/mL) supplied by Novozymes (Bagsværd, Denmark). All the other chemicals were analytical reagent grade. All solutions and standards were prepared by using deionized water.

2.1.Preparation of porous starch

The preparation of porous starch was based on the method of Benavent-Gil, Rosell [8]. Enzyme stock solutions were added to the starch suspensions (U/g starch). The lowest enzyme level was the minimum recommended by the manufacturer (5.5 AMG U/g, 5.5 AM U/g, 0.1 CGTase U/g and 500 BE U/g) and increasing concentrations (2, 3, 6 and 10 times the initial level) were tested. Native starches were included for comparison, and starches subjected to treatment conditions in the absence of enzymes were used as controls.

2.2.Scanning electron microscopy (SEM)

The granule morphology of native and modified starches was observed using a JSM 5200 scanning electron microscope (SEM) (JEOL, Tokyo, Japan). Samples were examined at an accelerating voltage of 10 kV and magnified 2,000x times. The microstructure analysis was carried out using the methodology described by Benavent-Gil and Rosell [8]. The following parameters were measured: granule size and the pore area. The area occupied by pores in a starch granule (related to the abundance of pore per granule) was calculated as the sum of the areas of all the pores of a starch granule divided by granule area. Values were the average of 20 independent measurements.

2.3.*In vitro* starch digestibility and expected glycemic index

Digestibility of native, enzymatically treated and untreated starches was determined following the method described by Gularte, Rosell [16] with minor modifications. 100 mg sample were dissolved in 4 mL of 0.1 M sodium maleate buffer (pH 6.9) with porcine pancreatic α-amylase (0.2 U/mL) (Type VI-B, ≥10 units/mg solid, Sigma Chemical, St. Louis, USA) and incubated in a shaking water bath at 37 °C during three hours. Aliquots of 200 μL were taken at different incubation times and mixed with 200 μL ethanol (96%) in order to stop the enzymatic hydrolysis. Then, the sample was centrifuged for 5 min at

10,000 × *g* and 4 °C. The pellet was washed with 50% ethanol (200 µL) and the mixture of supernatants were kept together at 4 °C for further glucose determination.

Supernatant (100 µL) was diluted with 850 µL of 0.1 M sodium acetate buffer (pH 4.5) and incubated with 50 µL AMG (1.43 U/mL) at 50 °C for 30 min in a shaking water bath. After centrifuging at 2,000 × *g* for 10 min, supernatants were kept for glucose determination.

The remnant starch after 16 h hydrolysis was solubilized with 2 mL of 2 M KOH using a Polytron Ultraturrax homogenizer IKA-T18 (IKA works, Wilmington, USA) during 1 min at speed 3. The homogenate was diluted with 8 mL 1.2 M sodium acetate pH 3.8 and incubated with 100 µL AMG (143 U/mL) at 50 °C for 30 min in a shaking water bath. After centrifuging at 2,000 × *g* for 10 min, supernatant was kept for glucose determination.

The glucose content was measured using a glucose oxidase–peroxidase (GOPOD) kit (Megazyme, Dublin, Ireland). The absorbance was measured using an Epoch microplate reader (Biotek Instruments, Winooski, USA) at 510 nm. Starch was calculated as glucose (mg) × 0.9.

The *in vitro* digestion kinetics was calculated fitting experimental data to a first-order equation [17]: $C = C_{\infty}(1-e^{-kt})$ where C was the concentration at t time, C_{∞} was the equilibrium concentration or maximum hydrolysis extent, k was the kinetic constant and t was the time chosen. The hydrolysis index (HI) was obtained by dividing the area under the hydrolysis curve (0–180 min) of the sample by the area of a standard material (native starch) over the same period of time. The expected glycemic index (*eGI*) was calculated using the equation $eGI = 8.198 + 0.862HI$ [18].

2.4.Statistical analysis

All experiments were repeated at least in duplicate. Experimental data were statistically analyzed using an analysis of variance (ANOVA) and values were expressed as a mean ± standard deviation. Fisher's least significant differences test was used for assessment of

significant differences among experimental mean values with 95% confidence. Pearson correlation coefficient (r) and P-value were used to indicate correlations and their significance using Statgraphics Centurion XV software (Bitstream, Cambridge, N). Differences of $P < 0.05$ were considered significant.

3 Results and Discussion

Morphology of porous starches obtained after the action of different amylases and their technological properties were analyzed in detail by Benavent-Gil and Rosell [9] in a previous study. In this case, to show the variation of pore area, the quantification of pore area and total pore area (related to the abundance or frequency of pore per granule) was plotted (Fig. 1). The pore area as well as total pore area were significantly affected ($P < 0.05$) by the type of enzyme. AMG produced the largest pore and bigger total pore area, followed by AM, BE and CGTase. Overall, enzyme level (indicated by the size of the symbol in Fig. 1) had a significant impact ($P < 0.05$) on the pore area and total pore area, regardless enzyme type, with the exception of starch treated with CGTase. The area of the pores induced by AMG increased with the enzyme level until 16.5 U/g starch, likely due to saturation of the non-reducing-ends of starch chains [19], but the significant increase in the total area indicated more pores per granule. Similarly, pore area and total pore area increased with the amount of AM added, whereas CGTase level increased pore area but leading to similar total pore area independently on the amount of enzyme. In the case of BE, it was not possible to establish a trend among enzyme level and pore production, although it was detected that high amount of BE was required for obtaining deep pores.

Porous starches were subjected to hydrolysis with digestive amylase and TS, DS and RS were quantified (Fig. 2), observing a major content of DS and very low amount of RS. The DS fraction was significantly affected by the level of enzyme but hardly by the enzyme type. RS fraction did not significantly vary with the enzyme type or level. After enzymatic

1 treatment, TS was significantly reduced due to the release of hydrolysis products, which
2 agree with previous results [5,20].
3 Hydrolysis plots revealed different behavior of the porous starches depending on the type of
4 enzyme used for its production and the enzyme level (Fig. 3). AMG treated starches showed
5 greater susceptibility to be hydrolyzed by digestive amylase. Nevertheless, AM, CGTase and
6 BE treated starches offered great resistance to enzymatic hydrolysis. Similar effects were
7 reported when corn starch was treated with AMG and CGTase for 24 hours [13,14],
8 although opposite results have been observed with AM treated starch subjected to longer
9 production time that intensified changes in the crystalline areas [21].
10 The parameters derived from the *in vitro* digestion of porous starches including equilibrium
11 concentration of hydrolyzed starch (C_∞), kinetic constant (k), area under the hydrolysis curve
12 after 180 min (AUC 180), hydrolysis index (HI) and estimated glycemic index (*eGI*) are
13 summarized in Table 1. Those parameters were significantly ($P < 0.05$) affected by the
14 enzyme as well as the enzyme level used to produce the porous starches, with the exception
15 of k that was only influenced by enzyme level.
16 In general, the enzymatic modification for obtaining porous starches increased k, although
17 exceptions were the porous starches obtained with CGTase at levels of 0.1 and 0.6 U/g
18 starch and with the highest BE and AMG concentration (5000 U/g starch and 55 U/g starch,
19 respectively). The maximum hydrolysis, C_∞, were significantly decreased in the treated
20 starches with the exception of AMG and CGTase treatments when added 55 U/g starch
21 (AMG) and 0.6 U/g starch (CGTase). Porous starches obtained with AM, CGTase and BE
22 showed lower HI, whereas this parameter was significantly increased in the AMG treated
23 starches. Similar trend was observed for the total area under the hydrolysis curve (AUC)
24 over a hydrolysis period of 180 min. In consequence, the enzymatic modification lowered
25 the estimated glycemic index of the resulting starches, with the exception of AMG treated

samples. The strongest effect was observed with the BE treatment, likely due to the BE enzyme displayed a superficial attack causing the formation of wide craters instead of deep holes [8].

No correlation was observed between structural attributes and the k and C_∞ parameters derived from the *in vitro* digestion. Nevertheless, analysis within each enzymatic treatment revealed that the pore size of AM treated starch presented a moderate significant positive correlation with k value ($r = 0.70$; $P < 0.05$) and negative with C_∞ ($r = -0.67$; $P < 0.05$). Similarly, the total pore area displayed a moderate significant positive correlation with k value ($r = 0.63$; $P < 0.05$) and a negative with C_∞ ($r = -0.64$; $P < 0.05$). It is generally accepted that the holes created in the starch surface after enzymatic treatment facilitate the access of digestive enzyme to the inner granule and in consequence the hydrolytic event [5,22,23]. The diffusion or access of the digestive amylase into the granule determines the way starch is disrupted [24]. Against previously reported assumptions, the relationships obtained in the present study indicate that a specific pore size is required for the accessibility of the digestive amylase. According to the structural analysis, AMG treatment induced larger pores ranged from 0.39 to 0.48 μm^2 [8], which are not limiting the accessibility of digestive enzymes. Conversely, AM led to smaller pores, whose size increases with the level of enzyme ranged from 0.05 to 0.24 μm^2 [8], and they are limiting the granule hydrolysis. Therefore, starch digestion could be modulated by obtaining certain pore size in the starch surface. On the other hand, the pore size and the total pore area presented a weak significant positive correlation with *eGI* parameter ($r = 0.54$; $P < 0.01$: $r = 0.49$; $P < 0.01$). No correlation between pore size and DS, RS and TS content was obtained, and only significant ($P < 0.01$) moderate correlation were found between the total pore area and RS ($r = 0.51$) DS ($r = -0.41$) and TS ($r = -0.40$).

4 Conclusions

In porous starches, the size and number of pores affected significantly their performance during *in vitro* digestion, showing significantly different amount of digestible starch, depending on the level of enzymatic treatment. AMG treated starches presented higher digestibility, whereas AM, CGTase and BE treatment reduced it, leading to lower estimated *GI*. Again, structural features of the pores also play a fundamental role controlling starch hydrolysis with digestive amylase. A specific pore size is required for the accessibility of the digestive amylase. Therefore, starch digestion could be modulated by obtaining certain pore size in the starch surface.

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Table 1: Kinetic constant (k), equilibrium concentration ($C\infty$), area under the hydrolysis curve after 180 min (AUC), hydrolysis index (HI) and estimated glycemic index (eGI) for native and modified corn starches.

Enzyme	Enzyme (U/g starch)	k	C_∞ [A]	AUC	HI	eGI [B]
Native	0	0.0046 ± 0.0003 [ab]	37.79 ± 0.12 [k]	2174 ± 121 [h]	100.00 ± 0.00 [i]	94.40 ± 0.00 [i]
AMG	5.5	0.0087 ± 0.0002 [fg]	26.44 ± 0.30 [gh]	2344 ± 2 [i]	107.82 ± 0.10 [j]	101.14 ± 0.08 [j]
	11	0.0099 ± 0.0010 [g]	24.74 ± 1.57 [g]	2359 ± 15 [i]	108.51 ± 0.68 [jk]	101.74 ± 0.59 [jk]
	16.5	0.0071 ± 0.0010 [d-f]	30.41 ± 2.16 [ij]	2366 ± 49 [i]	108.86 ± 2.28 [jk]	103.42 ± 1.96 [jk]
	33	0.0051 ± 0.0002 [a-c]	38.99 ± 0.30 [k]	2433 ± 45 [i]	111.94 ± 2.08 [k]	104.69 ± 1.79 [k]
	55	0.0032 ± 0.0000 [a]	63.65 ± 1.30 [m]	2763 ± 58 [j]	127.12 ± 2.67 [l]	117.78 ± 2.30 [l]
AM	5.5	0.0050 ± 0.0006 [a-c]	28.95 ± 2.46 [hi]	1760 ± 16 [ef]	80.98 ± 0.72 [e]	78.00 ± 0.62 [e]
	11	0.0065 ± 0.0008 [c-e]	23.48 ± 2.02 [fg]	1722 ± 1 [e]	79.23 ± 0.07 [e]	76.49 ± 0.06 [e]
	16.5	0.0118 ± 0.0011 [h]	16.15 ± 0.84 [ab]	1696 ± 8 [de]	78.03 ± 0.39 [e]	75.46 ± 0.34 [e]
	33	0.0080 ± 0.0005 [e-g]	20.58 ± 0.66 [ef]	1729 ± 18 [e]	79.55 ± 0.85 [e]	76.77 ± 0.73 [e]
	55	0.0133 ± 0.0006 [h]	16.67 ± 0.34 [a-c]	1857 ± 4 [f]	85.42 ± 0.16 [fg]	81.83 ± 0.14 [fg]
CGTase	0.1	0.0041 ± 0.0006 [ab]	33.83 ± 2.73 [j]	1777 ± 49 [ef]	81.73 ± 2.24 [ef]	78.65 ± 1.94 [ef]
	0.2	0.0083 ± 0.0007 [e-g]	19.94 ± 0.41 [c-e]	1725 ± 52 [e]	79.34 ± 2.37 [e]	76.59 ± 2.05 [e]
	0.3	0.0048 ± 0.0012 [a-c]	32.25 ± 4.77 [ij]	1870 ± 84 [fg]	86.01 ± 3.87 [g]	82.34 ± 3.34 [g]
	0.6	0.0034 ± 0.0003 [a]	43.83 ± 1.87 [l]	1990 ± 36 [g]	91.53 ± 1.66 [h]	87.10 ± 1.43 [h]
	1	0.0094 ± 0.0010 [g]	16.87 ± 0.47 [b-d]	1566 ± 52 [c]	72.03 ± 2.37 [d]	70.29 ± 2.05 [d]

BE	500	0.0091	±	0.0018	g	17.38	±	0.72	b-e	1576	±	122	cd	72.49	±	5.63	d	70.68	±	4.85	d
	1000	0.0058	±	0.0002	b-d	20.21	±	0.25	d-f	1376	±	53	ab	63.29	±	2.44	ab	62.75	±	2.10	ab
	1500	0.0133	±	0.0033	h	13.34	±	1.62	a	1463	±	8	bc	67.29	±	0.36	c	66.42	±	0.31	c
	3000	0.0065	±	0.0006	c-e	18.19	±	1.09	b-e	1333	±	6	a	61.33	±	0.26	a	61.06	±	0.22	a
	5000	0.0045	±	0.0010	ab	25.90	±	4.41	gh	1432	±	13	ab	65.88	±	0.59	bc	64.99	±	0.50	bc
P-value	**Enzyme**	0.361				0.004				0.000				0.000				0.000			
	Level (U/g)	0.009				0.005				0.000				0.000				0.000			

Values followed by a different superscript in each column are significantly different ($P < 0.05$).

[A] C_∞ and k were determined by the equation, $C = C_\infty(1-e^{-kt})$.

[B] *eGI* was calculated from equation proposed by Goñi et al. [17].

Fig 1. Individual pore area was plotted against the total pores area (related to the frequency and size of the pores in each starch granule) of starches obtained after each enzymatic treatment. The symbol size is related to the enzyme level applied for the starch modification.

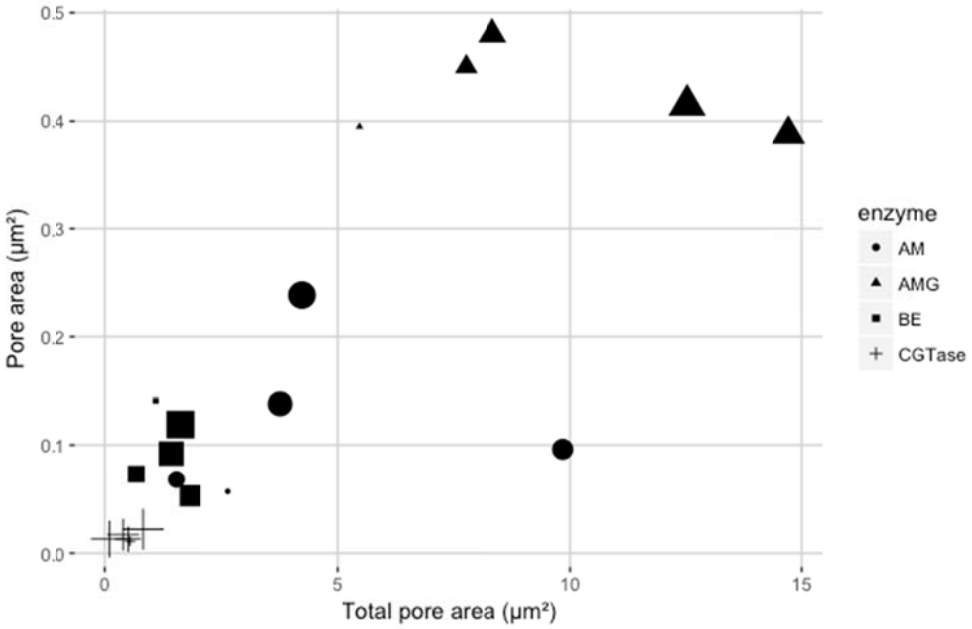

Fig 2: Bubble charts for digestible starch (DS) and resistant starch (RS) for each enzyme. The bubble size represents the total starch content (TS). Numbers following enzyme abbreviations are referred to the enzyme activity applied.

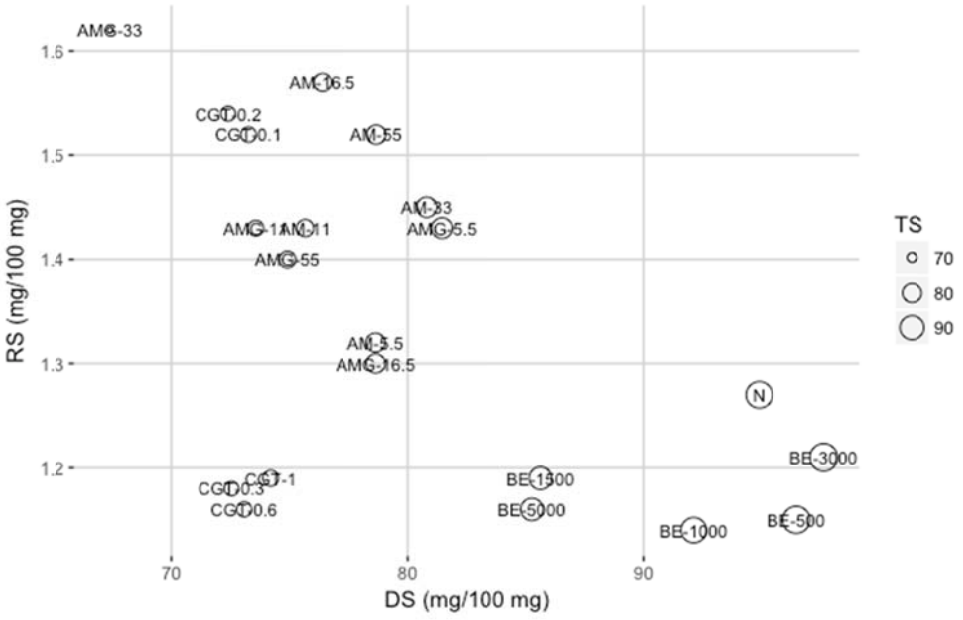

Fig 3: Hydrolysis of modified corn starch treated with (A) AMG (B) AM (C) CGTase and (D) BE treatment. Numbers following enzyme abbreviations are referred to the enzyme activity applied expressed in enzyme unit/g starch.

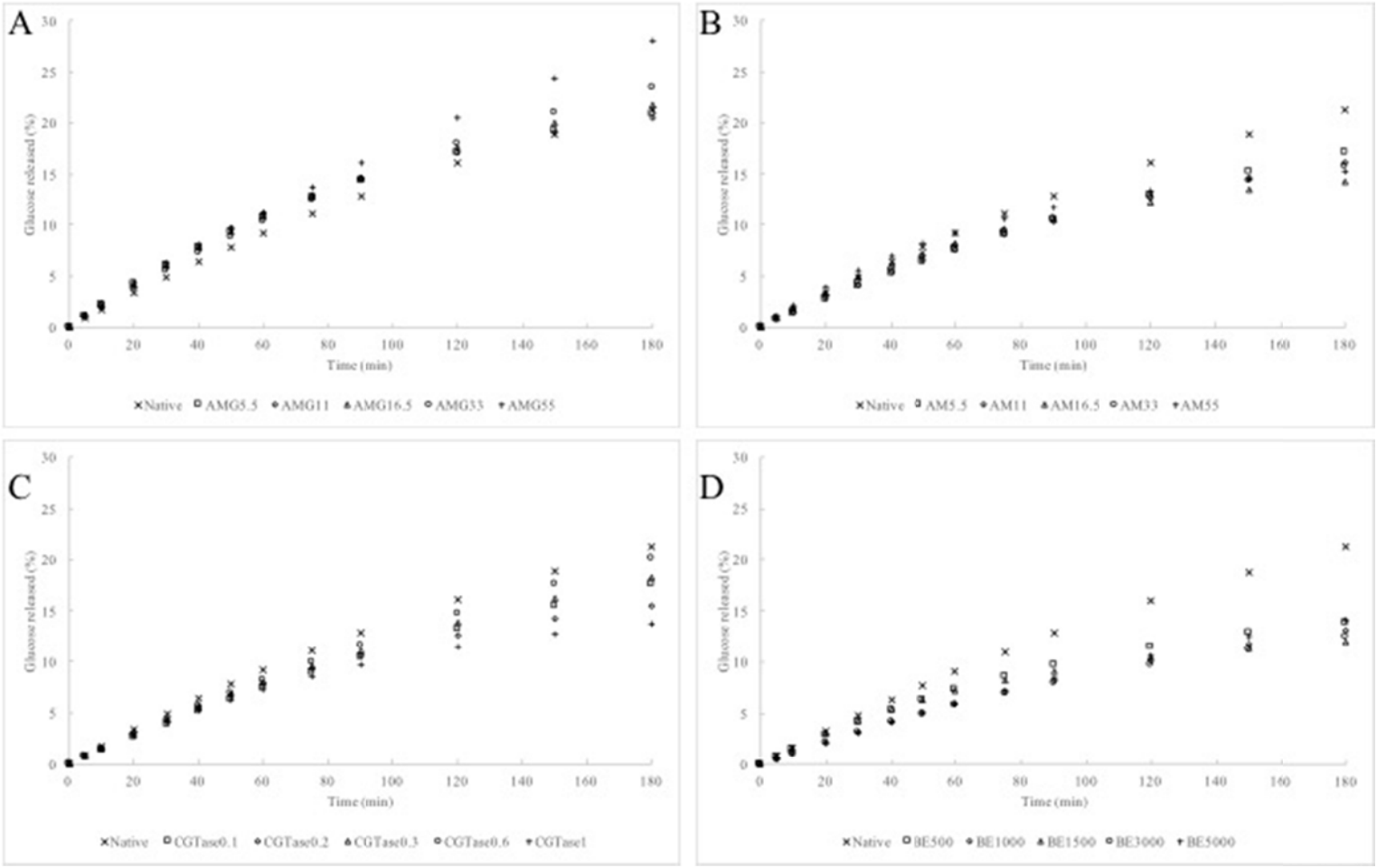

Fig 4: Multi factor analysis plot relating pasting properties and structural attributes with digestive parameters of enzymatically modified corn starches.