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Comparison of functional properties of porous starches produced with different enzyme combinations

Li Guo [a,1], Yuhan Yuan [b,2], Jiahao Li [a,1], Congping Tan [a,1], Srinivas Janaswamy [c,3], Lu Lu [a,1], Yishan Fang [a,1], Bo Cui [a,*,1]

[a] State Key Laboratory of Biobased Material and Green Papermaking, School of Food Sciences and Engineering, Qilu University of Technology, Shandong Academy of Sciences, Jinan, China
[b] Life Science and Technology College, Xinjiang University, Urumchi, China
[c] Dairy and Food Science Department, South Dakota State University, Brookings, SD 57007, USA

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ABSTRACT

To obtain porous starch granules with higher absorption capacities, three types of enzyme combinations were adopted to modify wheat and maize starches: (1) sequential α-amylase (AA) → glucoamylase (GA); (2) sequential branching enzyme (BE) → GA; and (3) sequential AA→BE→GA. The results indicated that AA→BE→GA treatment had a most optimal influence on porous starches. Compared to AA→GA and BE→GA, the mesopores in wheat starch granules treated with AA→BE→GA decreased by 52.82 and 48.70%, respectively. Conversely, the macropores increased by 216.68 and 138.18%, respectively. While for maize starch, the percentages of mesopores and macropores hardly changed after three enzyme combinations. Comparing the three enzyme treatments showed that pore volume (0.005 and 0.007 cm^3/g) and pore size (36.35 and 26.54 nm) were largest in the AA→BE→GA treated wheat and maize starches, respectively. Compared to the AA→GA and BE→GA, the adsorption capacities for oil, dye and heavy metal ions, wheat starch treated with AA→BE→GA increased by 46.61 and 242.33%, and 44.52 and 134.41%, and 28.83 and 271.72%, respectively. Correspondingly, that of maize starch increased by 29.71 and 133.29%, and 42.92 and 79.93%, and 28.16 and 161.43%, respectively. These results may provide a new and valuable enzyme combination for optimising porous starch granules with higher absorption capacities.

1. Introduction

Porous starch granules are biological adsorbents due to the abundant holes that extend from the exterior to interior of the granules, which increases the specific surface area [1]. Thus, porous starches are attractive alternatives as non-toxic absorbents [2,3]. However, the adsorption capacity of porous starch granules is smaller than inorganic adsorbents that have higher specific surface areas [4,5]. Therefore, increasing work has been directed toward improving the specific surface area of porous starches.

Enzymatic methods conducted under mild conditions are ideal for providing environmentally and consumer safe solutions. Currently, some enzymes, including α-amylase, glucoamylase, pullulanase, β-amylase, isoamylase, and cyclodextrin-glycosyltransferase have been used to form porous starches [6–9]. α-Amylase and glucoamylase are commonly used enzymes. Glucoamylase plays an important role in the enzymatic degradation of native starches. However, glucoamylase cannot completely hydrolyse α-1, 6-glycosidic bonds in amylopectin, resulting in incomplete starch hydrolysis [10]. α-Amylase can synergize glucoamylase catalysis, which improves starch hydrolysis [7]. However, the above enzyme methods cannot produce desirable results for higher rates of enzymatic hydrolysis, non-uniform pore distribution, large specific surface area, and pore size. Starch granules have different sizes, shapes, particle size distributions, amylose content, and crystal forms, all of which greatly influences the degree of enzymatic hydrolysis of starch granules [11,12]. Starch granules are found in many crops, notably cereals including maize, wheat, and other grains. Starches mainly consist of 20–30% α-1, 4-glucosidic-joined amylose and 70–80% of α-1,4- and α-1,6-glucosidic-joined amylopectin. Amylose and amylopectin are primarily located in amorphous and semi-crystalline regions of starch granules, respectively [13]. Maize and wheat are the first and

* Corresponding author.
E-mail addresses: guolizhuyuer@163.com (L. Guo), yyh_4868464@163.com (Y. Yuan), 772763647@qq.com (J. Li), tcp7411@163.com (C. Tan), Srinivas.Janaswamy@sdstate.edu (S. Janaswamy), lulu19861117@126.com (L. Lu), fangysh123@qlu.edu.cn (Y. Fang), cuiborr@163.com (B. Cui).
[1] No. 3501, Daxue Rd., Changqing District, Qilu University of Technology (Shandong Academy of Sciences), Jinan, Shandong Province 250353, China.
[2] No. 666, Shengli Rd., Xinjiang University, Urumchi, Xinjiang Province 830046, China.
[3] Dairy and Food Science Department, South Dakota State University, Brookings, SD 57007, USA.

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second most cultivated crops in the world [13]. Maize and wheat are 64–78% and 54–75% starch on a dry basis, respectively. The amylose level of common maize starch ranges from 25 to 29.4% [13,14]. The amylose level of common wheat starch varies from 18.2 to 28.8% [14,15]. Generally, starches with higher amylose content have lower degrees of enzyme hydrolysis. This may be due to amylose having only one non-reducing end for enzyme action. In contrast, amylopectin possesses more non-reducing ends, which facilitates higher rates of enzyme hydrolysis [16]. The number of non-reducing ends may depend on the degree of branching within starch molecules. Amylopectin starches with larger percentages of long branch chains have a higher resistance to enzymatic hydrolysis [17].

To improve the hydrolysis efficiency of starch, a novel strategy of a triple enzyme combination (α-amylase, branching enzyme, and glucoamylase) was adopted to modify starch to reduce amylose contents and to shorten the length of branch chains, and to increase the degree of branching. First, α-amylase is used to hydrolyse the (1 → 4) linkages of amylose and amylopectin, which shortens chain lengths and increases the quantity of short side chains [18,19]. Second, a branching enzyme is used to hydrolyse the α-1, 4-glucosidic bonds of starch and subsequently transfers linear chains to the C-6 hydroxyl positions of amylose and amylopectin via transglycosyl reaction, leading to the formation of new glucans with higher degree of branching (or more non-reducing ends) [20,21]. Through branching enzyme treatment, amylopectin contains more non-reducing ends, resulting in higher degree of hydrolysis of starch since amylopectin with more non-reducing ends has higher rates of enzyme hydrolysis than amylose with only one non-reducing end [16]. So, compared to starch without branching enzyme treatment (or starch with lower amounts of non-reducing ends), the amounts of non-reducing ends increased after branching enzyme treatment accelerate starch to be more easily hydrolysed by glucoamylase. Finally, to create more desirable porous starches, glucoamylase is used to effectively hydrolyse the new glucans with more non-reducing ends [22]. The intent of this method is to illuminate changes in the structure and absorption properties of wheat and maize starches after enzyme treatment. The enzyme treatments used in this study are (1) the conventional method, or sequential α-amylase from *porcine pancreatin* (AA) → glucoamylase from *Aspergillus niger* (GA); (2) sequential branching enzyme from *Rhodothermus obamensis* (BE) → GA; and (3) sequential AA→BE→GA.

2. Materials and methods

2.1. Materials

Wheat and maize starches, glucoamylase (GA, 24000 U/mL), isoamylase (142,000 U/mL), α-amylase (AA, 50 U/mg), and pullulanase (1100 U m/L), were ordered from Sigma-Aldrich. BE (4800 U/mL) came from a kind donation from Novozymes, Denmark. The standard samples (glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose) were ordered from Yuanye Biological Technology Co. (Shanghai, China).

2.2. Preparation of enzyme-treated starches

2.2.1. Sequential AA→GA treatment

Starch (8 g) was dispersed in 0.02 mol/L sodium acetate buffer to produce 15% (w/v) starch dispersion (pH 6.9). AA (100 U/g dry weight of starch) was added to the solution and agitated at 45 °C for 10 h. Next, 1 mol/L hydrochloric acid solution (2 mL) was added to cease the reactions and produce AA-treated starch. After the pH was adjusted to 5.5, the AA-treated starch solution was mixed with GA (1500 U/g dry weight of starch) and agitated at 48 °C for 12 h. The 1 mol/L hydrochloric acid solution (2 mL) was used to stop the enzyme reactions. The soluble sugars in the hydrolysate were determined (see Section 2.2.4). The hydrolysate was dialysed in deionised water to remove salt ions. Next, 98% ethanol was poured into the dialysate to form a precipitate. Finally, the precipitate was washed with distilled water and freeze-dried to obtain the AA→GA modified starch.

2.2.2. Sequential BE→GA treatment

Starch (8 g) was dispersed in 0.02 mol/L sodium acetate buffer to produce 15% (w/v) starch dispersion (pH 6.5). The starch dispersion was mixed with BE (100 U/g dry weight of starch) and agitated at 45 °C for 10 h to obtain BE-modified starches. The enzyme reactions were stopped through the addition of 1 mol/L hydrochloric acid solution (2 mL). After adjusting the pH to 5.5, the BE-modified starch liquid was mixed with GA (1500 U/g dry weight of starch) and agitated at 48 °C for 12 h. The 1 mol/L hydrochloric acid solution (2 mL) was used to terminate the enzyme reactions. The soluble sugars in the hydrolysate were determined (see Section 2.2.4). The hydrolysate was dialysed in distilled water to remove salt ions. Next, 98% ethanol was poured into the dialysate to form a precipitate. Finally, the precipitate was washed with distilled water and freeze-dried to obtain the BE→GA treated starch.

2.2.3. AA→BE→GA treatment

Starch (8 g) was dispersed in 0.02 mol/L sodium acetate buffer to produce 15% (w/v) starch dispersion (pH 6.9). The starch dispersion was agitated with AA (100 U/g dry weight of starch) at 45 °C for 10 h. The enzyme reactions were stopped through the addition of 1 mol/L hydrochloric acid solution (2 mL). After adjusting pH to 6.5, BE (100 U/g dry weight of starch) was added to the AA-treated hydrolysate and agitated at 45 °C for 10 h. The 2 mL hydrochloric acid solution of 1 mol/L was used to stop the enzyme reactions and obtain AA→BE treated starch. After adjusting the pH to 5.5, the AA→BE treated starch liquid was mixed with GA (1500 U/g dry weight of starch) and agitated at 48 °C for 12 h. The 1 mol/L hydrochloric acid solution (2 mL) was used to terminate enzyme activity. The soluble sugars in the hydrolysate were determined (see Section 2.2.4). The above hydrolysate was dialysed in distilled water to remove salt ions. Next, 98% ethanol was poured into the dialysate to form a precipitate. Finally, the precipitate was washed with distilled water and freeze-dried to obtain the AA→BE→GA treated starch.

2.2.4. Soluble sugars released after enzyme treatments

The hydrolysate obtained after each enzyme treatment was diluted to a concentration of 1.0 mg/mL with ultrapure water. The soluble sugars (mg/mL) in the diluted dialysate (20 μL) were measured using a Waters 1500 HPLC system with a refractive index detector (RID, Waters 2414) and a Sugar-pak™ column (6.5 mm × 300 mm, Bischoff). The ultrapure water was used as mobile phase at a flow rate of 0.5 mL/min. The soluble sugars were analysed from the calibration curve with the mixed standards (G1-G7).

2.3. The structure properties

The amylose levels of starch samples were measured according to the procedure of Biselli et al. [23]. The amylopectin levels from starch samples were prepared following the procedure of Guo [24]. A high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was used to measure the chain length distributions of amylopectin [25,26]. The elution time versus the degree of polymerisation of the sugars was calibrated with standard samples from glucose to maltotriose. A proton nuclear magnetic resonance (1HNMR) spectroscopy was utilised to determine the degree of branching (BD) according to a previous study [27].

2.4. Morphological characteristics

The morphological characteristics of the porous starches were measured using a scanning electron microscope (SEM). The samples were

placed on a double sticky tape, pasted on aluminium flakes, and overlain with 20 nm gold membranes in argon gas.

2.5. Structure features of porous starch

2.5.1. Particle size distributions

Particle size analyses of porous starches were measured using a laser light scattering particle size analyzer. The samples were added to distilled water and agitated. The refractive indices of particle and deionised water were 1.53 and 1.33, respectively. The absorption index was 0.01. The size of the starch granules determined to range from 0.1 to 100 μm. Particle size distributions included the surface weighted mean [$D(3, 2)$], volume weighted mean [$D(4, 3)$], 10th percentile[$d(0.1)$], 50th [$d(0.5)$], and 90th percentile [$d(0.9)$].

2.5.2. Pore size distribution and specific surface area

According to previous methods with proper modifications [5,28], The 4.84 g starch samples were heated to 80 °C for two days at 0.1 MPa to facilitate water evaporation prior to measurements of nitrogen adsorption/desorption. The pore size distributions and specific surface area were determined with nitrogen adsorption at 77 K using specific surface aperture analyzer (JW-BK100A, JWGB SCI &TECH Co., Beijing, China). The high purity of nitrogen (>99.99%) was utilised to establish adsorption and desorption isotherms. BET (Brunauer-Emmett-Teller) was adopted to calculate the specific surface area at $P/P_o = 0.2$. The pore diameter and pore volume were ranged from 2 to 100 nm and were calculated with BJH (Barrett-Joyner-Halenda).

2.6. Adsorption capacities

2.6.1. Adsorption capacities of oil (ACO)

Starch samples on a dry basis (1 g) were immersed in 10 mL oil for 25 min and constantly agitated at room temperature. Vacuum filtering was used to filter the mixtures and prevent oil loss. The starch adsorbing oil was weighed and Eq. (1) was used to calculate oil adsorption capacity [29]:

$$\mathrm{ACO}\ (\%) = (w - w_o)/w_o \tag{1}$$

where w is the mass of the starch adsorbing oil; w_o is the mass of starch sample.

2.6.2. Adsorption capacities of pigment (ACP)

Starch samples on a dry basis (1.5 g) were added to 15 mL neutral red (NR, 20 mg/L) or methylene blue (MB, 20 mg/L) solutions and constantly stirred for 90 min. NR and MB contents in the solutions were measured at 520 and 660 nm, respectively. Eq. (2) was utilised to calculate the pigment adsorption capacity [30]:

$$\mathrm{ACP}\ (\mathrm{mg/g}) = (c_1 - c_2) \times \mathrm{v} \times \mathrm{m/w} \tag{2}$$

where c_1 is pigment content in a solution before adsorption; c_2 is pigment content in a solution after adsorption; v is pigment solution volume; w is dry sample weight; m is pigment molecular mass.

2.6.3. Adsorption capacities of heavy metal ions (ACHMI)

Starch samples on a dry basis (1 g) were added to 20 mL of a 10 mg/L metal ion solution containing Hg^{2+}, Cd^{2+}, Pb^{2+}, Cu^{2+}, and Zn^{2+} and constantly stirred for 14 h. An atomic adsorption spectrometer measured the heavy metal ion content in the solutions at 325 nm. The adsorption capacities of heavy metal ions were calculated according to Eq. (3) [31]:

$$\mathrm{ACHMI}\ (\mathrm{mg/g}) = (c_o - c_e) \times v/w \tag{3}$$

where c_o is the heavy metal ions content before adsorption; c_e is the heavy metal ions content after adsorption; v is solution volume; w is the mass of the sample.

2.7. Data statistics

Results are presented as average values ± standard errors from triplicate measures. Significant differences at 0.05 level of confidence between the average values were calculated using Tukey's test.

3. Results and discussion

3.1. Morphological characteristics of porous starch

The SEM of the starch samples is shown in Fig. 1. The different enzyme treatments change the surfaces of the starches at different levels due to the susceptibility of starches to enzyme action. Wheat starch granules display lenticular, disk, or spherical shapes, and the surfaces are smooth and nonporous (Fig. 1A1) [14]. Fig. 1A2 shows that wheat starch treated with the AA→GA treatment exhibits the development of large pores along the equatorial groove. This implies that enzyme hydrolysis mostly occurs along the equatorial groove and may result in the rupture of the granules [6,16]. A few small pores form on the surface of the starch particles. The BE→GA treatment results in many shallow pores developing on the surface of wheat starch particles (Fig. 1A3). These results indicate that AA can more effectively synergize the hydrolysis of GA as compared to BE [19,21,27]. Interestingly, the AA→BE→GA treatment results in more wheat starch granules with bigger and deeper holes on the surface and equatorial grooves (Fig. 1A4). Maize starch granules have spherical or polygonal shapes, with slightly more smaller pores on the surface of the starch granules (Fig. 1B1) [22]. The AA→GA treatment forms some deep holes in maize starch granules (Fig. 1B2), suggesting that the enzyme hydrolysis of maize starch may generate deep holes by starting at the surface of starch granules and subsequently penetrating into the granule interior. This could be due to the more numerous small pores on the surfaces of maize starch granules allowing enzymes access into the interior of the granules, whereas the large and non-porous surfaces of wheat starch create barriers to enzymes [19,22]. BE→GA forms fewer deep holes in starch granules than the AA→GA treated starch granules (Fig. 1B3). As is expected, the triple enzyme treatment (AA→BE→GA) produces more and deeper pores in maize starch granules than the other two treatments (Fig. 1B4). This implies that AA→BE treated starches will have a larger degree of branching and more short branch chains that may greatly accelerate subsequent GA hydrolysis. It may be because the combination of AA and BE treatment contributes to the formation of more new branch points in amylopectin, which makes amylopectin contain more non-reducing ends for enzyme action, resulting in higher hydrolysis through subsequent GA hydrolysis [16]. There is a remarkably synergistic action among AA, BE, and GA. Obviously, compared to wheat starch, maize starch is easier to be hydrolysed, resulting in the formation of a great number of bigger and deeper pores. It may be because larger wheat starch granules with smooth surface are much less susceptible to the enzymes than smaller maize starch granules with micropore surface [19,32]. While maize starch granules with micropore facilitate enzyme to enter into the granule interior, resulting in the generation of bigger and deeper holes [29].

3.2. Soluble sugars in enzymatic hydrolysates

The amounts of soluble sugars released after each enzyme treatment are shown in Table 1. Compared with native wheat starch (5.1 mg/mL), the total amounts of soluble sugars (G1-G7) released after AA→GA, BE→GA and AA→BE→GA treatment increase to 12.9, 8.9 and 30.5 mg/mL, respectively. Compared with native corn starch (5.3 mg/mL), the total amounts of soluble sugars (G1-G7) released after AA→GA, BE→GA and AA→BE→GA treatment increase to 27.7, 18.1 and 46.6 mg/mL, respectively. Generally, no oligosaccharides (from G3 to G7) were released when wheat or maize starch was subjected to AA→GA or BE→GA treatment. For AA→BE→GA treatment,

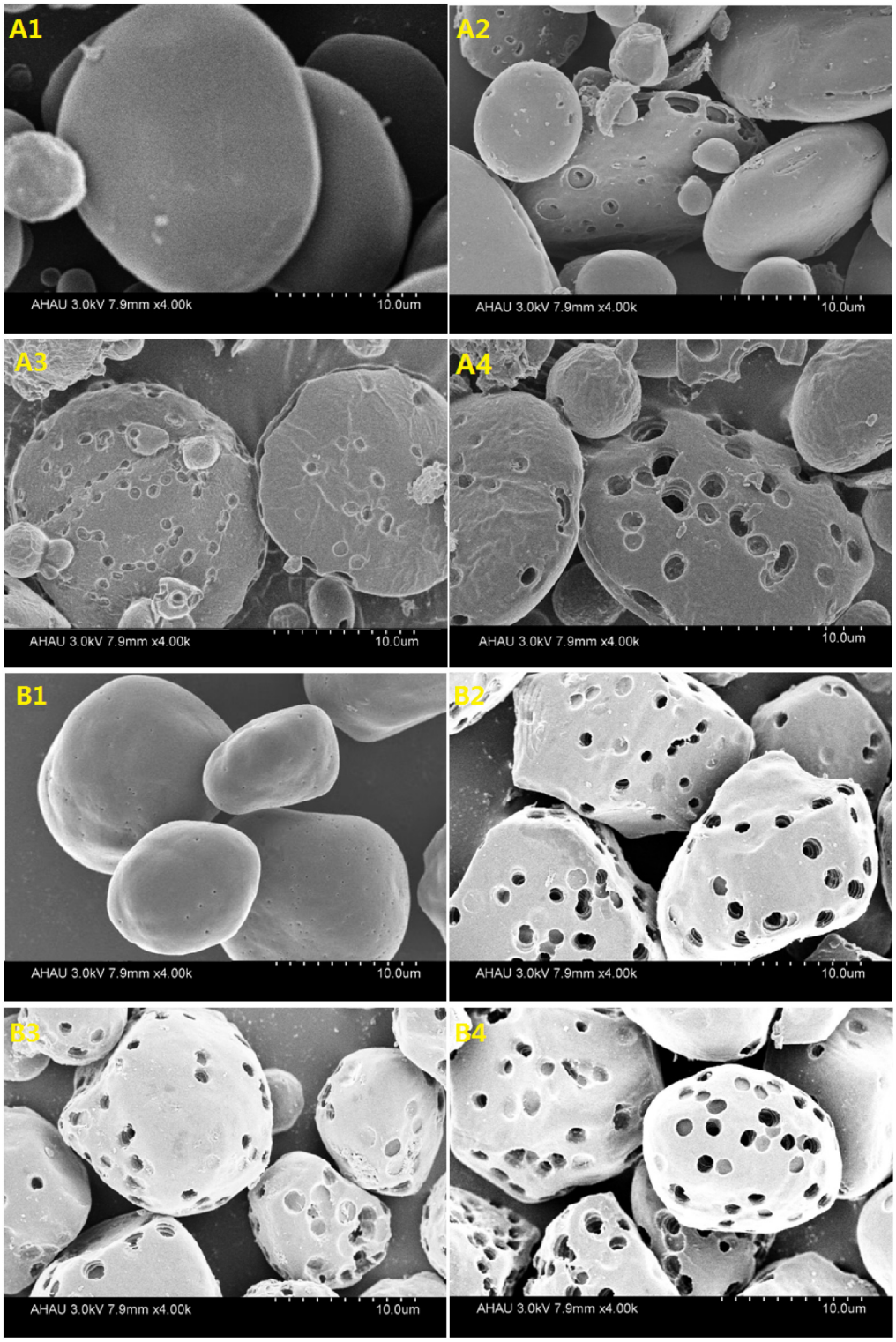

Fig. 1. SEM (A: wheat; B: maize) of (1) native starch; (2) AA→GA modified starch; (3) BE→GA modified starch; (4) AA→BE→GA modified starch.

wheat starch releases most soluble sugars except for G5 and G6, whereas maize starch releases all soluble sugars (from G1 to G7). It implies that AA→BE→GA treatment has a most significant influence on the hydrolysis of cereal starches followed by AA→GA and BE→GA treatments. Besides, for each enzyme treatment, the amounts of soluble sugars released from enzyme-treated maize starch are much more than that released from enzyme-treated wheat starch. It indicates that compared to wheat starch, maize starch is easier to be hydrolysed by enzymes, resulting in the production of more soluble sugars, especially for glucose (G1) and maltose (G2).

3.3. Structure features of enzyme-treated starches

3.3.1. Amylose content

Considering that the crystalline domains of starch are established by amylopectin branch chains and the amorphous domains are composed

Table 1
The amounts (mg/mL) of soluble sugars in enzyme-treated supernatant.

Sample		G1	G2	G3	G4	G5	G6	G7	Total
Wheat starch	Native starch	3.8 ± 0.1	1.3 ± 0.2	n.d	n.d	n.d	n.d	n.d	5.1 ± 0.2
	AA→GA	9.5 ± 0.3	2.7 ± 0.4	0.7 ± 0.1	n.d	n.d	n.d	n.d	12.9 ± 0.5
	BE→GA	6.4 ± 0.2	2.1 ± 0.2	n.d	n.d	n.d	n.d	n.d	8.5 ± 0.2
	AA→BE→GA	15.2 ± 0.4	10.5 ± 0.3	2.9 ± 0.3	1.6 ± 0.3	n.d	n.d	0.3 ± 0.2	30.5 ± 0.2
Maize starch	Native starch	4.1 ± 0.2	1.2 ± 0.2	n.d	n.d	n.d	n.d	n.d	5.3 ± 0.2
	AA→GA	18.8 ± 0.3	8.2 ± 0.4	0.7 ± 0.3	n.d	n.d	n.d	n.d	27.7 ± 0.3
	BE→GA	10.5 ± 0.2	7.6 ± 0.3	n.d	n.d	n.d	n.d	n.d	18.1 ± 0.2
	AA→BE→GA	23.6 ± 0.5	10.8 ± 0.4	5.4 ± 0.3	3.8 ± 0.2	2.1 ± 0.1	0.6 ± 0.4	0.3 ± 0.5	46.6 ± 0.4

n.d. means non detected.

of branching points and the majority of the amylose [13], it seems that amylose content and amylopectin branch chains greatly influence enzyme hydrolysis [11]. The amylose content of the enzyme-treated starch samples is listed in Table 2. The amylose content (28.12%) of native wheat starch is higher than that (20.72%) of maize starch. It indicates that wheat starch is more resistant to the enzymes than maize starch since higher amylose content result in lower enzyme hydrolysis due to the presence of only one non-reducing end for enzyme action [16]. After enzyme treatments, amylose content decreases. This may be due to AA preferentially hydrolysing amylose, resulting in a reduction in amylose levels [32]. Additionally, the α-1, 4, or α-1, 6 bonds of starch may be broken into more relatively short linear chains, with the external chain length of amylopectin becoming shorter. The AA→GA, BE→GA, or AA→BE→GA treatment decreases amylose levels of wheat starches by 12.66, 5.44 and 48.33%, respectively, and that of maize starches decreases by 18.23, 3.31 and 49.96% respectively. However, single enzyme treatment (AA, BE or GA) has different influence on amylose content in maize starch [11]. Benavent-Gil and Rosell have shown that GA at lower concentrations (≤5.5 U/g starch) slightly decreases amylose content, whereas GA at higher concentrations (≥11 U/g starch) increases amylose content. BE at lower concentrations (≤1 U/g starch) decreases amylose content, whereas BE at higher concentrations (500–5000 U/g starch) decreases or increases amylose content. While for AA, amylose content decreases by about 20% at lower or higher concentrations (5.5–55 U/g starch). Comparatively, the combinations of these enzymes result in the reduction in amylose content. It indicates that the enzyme combinations may attack more proportion of amylose, suggesting larger and deeper pores and the attack of amorphous and crystalline structure. Clearly, the AA→BE→GA treatment has a greater decrease in amylose content because starch is hydrolyzed for a longer time of period and more enzyme type.

3.3.2. Structure properties of amylopectins

Amylopectins consist of external and internal chains. External chains form double helices in crystalline lamellae, whereas internal chains between the branches and branch points build up the amorphous lamellae [33]. So, the chain length distribution of amylopectin is greatly correlated with enzyme hydrolysis and the formation of pores in starch granules. Table 2 also depicts the structure parameters of amylopectins from native and enzyme-modified starches. The chain length distributions of amylopectins are: A-chains (DP 6–12); B1-chains (13–24); B2-chains (25–36); and B3-chains (DP > 36) [26]. The proportion (70.27%) of longer chains (DP ≥ 13) of native wheat starch is larger than that (67.98%) of native maize starch, which implies that wheat starch has lower degree of hydrolysis. It can be explained by the fact that the longer chains (DP > 13) facilitate molecular entanglement to produce higher intermolecular associations and hence resulting in higher resistance to enzymes [20,34]. Enzyme modifications lead to an increase in the percentages of short chains (DP < 6) and A chains (DP 6–12), whereas the proportions of B chains decrease. Table 1 indicates that after AA→GA, BE→GA, or AA→BE→GA treatment, the relatively short chains (DP ≤ 12) in amylopectin from wheat starch increase by 25.69, 7.60, and 52.51%, respectively. The medium chains (DP 13–24) decrease by 7.17, 4.06 and 15.67%, respectively. However, the long chains (DP ≥ 25) reduce by 18.15, 1.56 and 35.08%, respectively. For amylopectin from maize starch, the AA→GA, BE→GA, or AA→BE→GA treatment increases the relatively short chains by 21.64, 9.12 and 59.68%, respectively, and the medium chains decrease by 9.34, 4.13 and 10.97%, respectively, whereas the long chains reduce by 12.43, 4.73, and 53.96%, respectively. These data indicate that compared to AA→GA and BE→GA treatments, the AA→BE→GA treatment produces a remarkable increase in short chains (DP ≤ 12) and a pronounced decrease in long chains (DP ≥ 25). It may be attributed to two reasons: (1) The increase in the ratio of short chains (DP < 13) to longer chains (DP > 13) of amylopectin after AA and BE treatment accelerate subsequent GA hydrolysis [20,34]; (2) More new branch points in amylopectin form after AA and BE treatment, which makes amylopectin contain more non-reducing ends for enzyme action, resulting in higher hydrolysis through subsequent GA hydrolysis [16]. The enzyme treatments decrease average chain length ($\overline{CL}$) by shortening the external chain lengths of amylopectins. The AA→GA, BE→GA, or AA→BE→GA treatment decreases the $\overline{CL}$ of wheat starch amylopectins by 16.68, 11.88, and 29.91%, respectively, and the $\overline{CL}$ of maize starch amylopectins decreases by 26.31, 20.26, and 34.73%, respectively. The AA→BE treatment appears to be the most efficient treatment for reducing average chain

Table 2
The amylose content, chain length distributions and branched degree of the enzyme-treated starches.

Sample		Amylose content (%)	Chain length distributions of amylopectins (%)					Average chain length	Branched degree (%)
				A chain	B1chain	B2 chain	B3 chain		
			DP <6	(DP 6-12)	(DP 13-24)	(DP 25-36)	(DP ≥ 37)		
Wheat starch	Native starch	28.12±0.02^{a}	1.17±0.05^{d}	28.56±0.15^{d}	46.58±0.04^{a}	15.27±0.03^{a}	8.42±0.02^{a}	20.86±0.02^{a}	5.16±0.02^{c}
	AA→GA	24.56±0.03^{c}	3.79±0.03^{b}	33.58±0.08^{b}	43.24±0.02^{c}	13.05±0.03^{c}	6.34±0.05^{b}	17.38±0.07^{c}	5.04±0.03^{c}
	BE→GA	26.59±0.05^{b}	1.86±0.02^{c}	30.13±0.03^{c}	44.69±0.03^{b}	14.43±0.05^{b}	8.89±0.04^{a}	18.38±0.03^{b}	6.27±0.02^{b}
	AA→BE→GA	14.53±0.03^{d}	5.02±0.02^{a}	40.32±0.02^{a}	39.28±0.03^{d}	8.95±0.05^{d}	6.43±0.04^{b}	14.62±0.04^{d}	8.79±0.03^{a}
Maize starch	Native starch	25.34±0.04^{a}	1.48±0.04^{d}	30.54±0.04^{d}	49.15±0.04^{a}	12.36±0.12^{a}	6.47±0.05^{b}	19.84±0.06^{a}	5.37±0.04^{c}
	AA→GA	20.72±0.02^{c}	4.02±0.03^{b}	34.93±0.04^{b}	44.56±0.02^{c}	8.36±0.03^{c}	8.13±0.04^{a}	14.62±0.07^{c}	5.32±0.01^{c}
	BE→GA	24.85±0.04^{b}	2.86±0.02^{c}	32.08±0.02^{c}	47.12±0.03^{b}	9.84±0.05^{b}	8.10±0.03^{a}	15.82±0.03^{b}	7.86±0.05^{b}
	AA→BE→GA	12.68±0.05^{d}	6.48±0.02^{a}	44.65±0.02^{a}	43.76±0.03^{d}	6.89±0.05^{d}	1.78±0.04^{c}	12.95±0.03^{d}	10.86±0.02^{a}

length, which leads to the production of more relatively linear short chains beneficial to subsequent GA hydrolysis.

3.3.3. Degree of branching of amylopectins

The degree of branching (BD) of amylopectins in Table 2 demonstrates that BD enhances after BE→GA, or AA→BE→GA treatments. After BE→GA, or AA→BE→GA treatment, the BD of wheat starch increases by 21.51 and 70.35%, respectively, and the BD of maize starch increases by 46.37 and 102.23%, respectively. These results indicate that BE can significantly enhance starch BD. Additionally, AA has a synergistic effect on the transglycosylation of BE [11,35]. These results show that the AA→BE→GA treatment can remarkably catalyse starch hydrolysis. This may be related to the AA→BE treated starches having a larger degree of branching and more relatively short branch chains, which are beneficial for GA hydrolysis.

3.4. Structure characteristics of AA→GA, BE→GA, and AA→BE→GA modified starches

3.4.1. Particle size distributions

The particle size distributions are summarised in Table 3, which shows that $D[3, 2]$ and $D[4, 3]$ of wheat starch (8.32 and 16.58 μm) are higher than (6.34 and 14.89 μm) maize starch, respectively. Maize starch has smaller particle sizes compared to wheat starch, which makes maize starch easier to be hydrolysed. It has been reported that the susceptibility of starch to enzyme attack is influenced by factors such as the ratio of amylose to amylopectin, crystalline structure, granule size and relative surface area, granular integrity and porosity, and structural inhomogeneities. In terms of granule size, the smaller granule size is, the higher degree of hydrolysis is [36,37]. This implies that starch enzymolysis is greatly affected by particle size distribution. After the enzyme treatments, the $D[3, 2]$ and $D[4, 3]$ of wheat and maize starches are greater than native starches. The increase in $D[4, 3]$ may be due to the internal development of starch cavities during enzymic catalytic reactions, thereby leading to an increase in the surface area of the granules [11,33]. The [d(0.5)] and [d(0.9)]of native starches are: wheat starch (14.32 and 30.25 μm) and maize starch (12.53 and 20.75 μm), respectively, which are 50% or 90% lower than the particle diameter values listed above. Enzyme hydrolysis causes an increase in 50th [d(0.5)] and 90th [d(0.9)]. These findings may be explained by three reasons [38,39]: (1) Small fragments may be produced and accumulate on starch particle surfaces through enzyme modification; (2) Water permeates into the interior of starch granules causing them to swell; (3) Starch granules are combined.

3.4.2. Nitrogen adsorption-desorption isothermal curve

The nitrogen adsorption-desorption isothermal curves are depicted in Fig. 2. It can be seen from Fig. 2(A1 and B1) that the curves for native wheat and maize starches overlap and no hysteresis loop exists, displaying Type II features that is the unrestricted monolayer-multilayer adsorption up to high p/p_o [1,38,39]. Type II isotherms are exhibited by the physisorption of most gases on nonporous or macroporous adsorbents. It suggests that no hysteresis loop largely depends on the nonporous structure of native wheat and maize starches. It has been reported that the first layer adsorption is stronger than the multilayer adsorption when the acting force between adsorbent and adsorbate is larger than intermolecular reaction between adsorbates. At this point, the first layer adsorption is close to saturation and subsequently the second layer adsorption begins. Therefore, an obvious inflection point (Point B) occurs at lower relative pressure (P/P_o). Afterwards, the multimolecular layer adsorption begins to appear with increasing relative pressure. With increasing the number of the adsorption layer, the adsorbing capacity gradually increases until adsorption pressure reach gas saturation vapor pressure, resulting in liquification. At this time, the adsorbing capacity rises vertically under constant pressure (or Type II) [38,39]. Thus, Point B refers to the conversion of monolayer to multilayer adsorption. A gradual curvature (or a less distinctive Point B) is an indication of a significant amount of overlap of monolayer coverage and the onset of multilayer adsorption [38]. However, the adsorption-desorption isotherms of all enzyme-treated wheat and maize starches have hysteresis loops, suggesting Type IV curves and demonstrating that adsorption capacity obviously enhances due to capillary condensation. It can be seen from Fig. 2 that the hysteresis loops form after enzyme treatments. The hysteresis loops belong to Type H2 (b) that is associated with pore blocking, but the size distribution of pore neck widths is now much larger [38]. However, compared to inorganic porous materials [4,30,40], the hysteresis loops of enzyme-treated starches are very smaller. It may be attributed to the reason that starch as organic substance has much smaller specific surface area than inorganic materials [4,30,40], which results in a relatively smaller number of pores, displaying a smaller hysteresis loop [38,39]. Fig. 2 also indicates that compared to wheat and maize starches, the isotherms of enzyme-modified starches rapidly increase when the relative pressure approaches 1, indicating the formation of more pores on starch particle surfaces after enzyme treatment.

3.4.3. Pore diameter and volume distributions

Fig. 3 shows the pore diameter and volume distributions of starch samples, with corresponding structural characteristics listed in Table 4. In comparison with native starches, the specific surface areas of the enzyme-modified starches enlarge approximately 2–12 times. Pores are classified into micropores (<2 nm), mesopores (2–50 nm) and macropores (>50 nm) according to their size [39]. Table 4 indicates that native wheat and maize starches have no mesopores and macropores. While the pore diameter distributions of enzyme-treated starches have mesopores between 2 and 50 nm and macropores >50 nm. Following the AA→GA, BE→GA, and AA→BE→GA treatments, the percentages of wheat starch mesopores are 73.94, 80.40 and 37.93%, respectively, and the percentages of macropores are 26.06, 19.60, and 62.07%, respectively. For maize starch, the AA→GA, BE→GA, and AA→BE→GA treatments yield mesopore percentages of 61.56, 63.12, and 63.21%, respectively, and macropore percentages of 38.44, 36.88, and 36.79%, respectively. These data suggest that the AA→BE→GA treatment is conducive to forming more macropores. Table 4 also shows

Table 3
Particle size parameters of porous starch samples.

Sample		$d(0.1)^A$ (v/v, μm)	$d(0.5)^A$ (v/v, μm)	$d(0.9)^A$ (v/v, μm)	Surface weighted mean (μm, $D[3, 2]$)	Volume weighted mean (μm, $D[4, 3]$)
Wheat starch	Native starch	8.32 ± 0.01^a	14.32 ± 0.02^d	30.25 ± 0.05^d	8.32 ± 0.03^d	16.58 ± 0.03^d
	AA→GA	7.34 ± 0.05^b	16.86 ± 0.03^b	33.66 ± 0.05^b	11.49 ± 0.02^b	20.67 ± 0.02^b
	BE→GA	6.86 ± 0.02^c	15.37 ± 0.09^c	32.18 ± 0.04^c	9.62 ± 0.01^c	18.02 ± 0.01^c
	AA→BE→GA	4.16 ± 0.06^d	20.03 ± 0.02^a	39.04 ± 0.02^a	16.25 ± 0.02^a	25.86 ± 0.02^a
Maize starch	Native starch	8.49 ± 0.11^a	12.53 ± 0.12^c	20.75 ± 0.03^d	6.34 ± 0.05^d	14.89 ± 0.04^d
	AA→GA	5.49 ± 0.01^c	13.28 ± 0.02^b	23.96 ± 0.05^b	8.92 ± 0.03^c	19.36 ± 0.03^b
	BE→GA	6.28 ± 0.05^b	11.86 ± 0.03^d	22.68 ± 0.05^c	11.56 ± 0.02^b	16.72 ± 0.02^c
	AA→BE→GA	2.04 ± 0.02^d	14.84 ± 0.09^a	30.24 ± 0.04^a	18.93 ± 0.01^a	27.56 ± 0.03^a

A. These indicate 10th, 50th (median) and 90th percentiles.

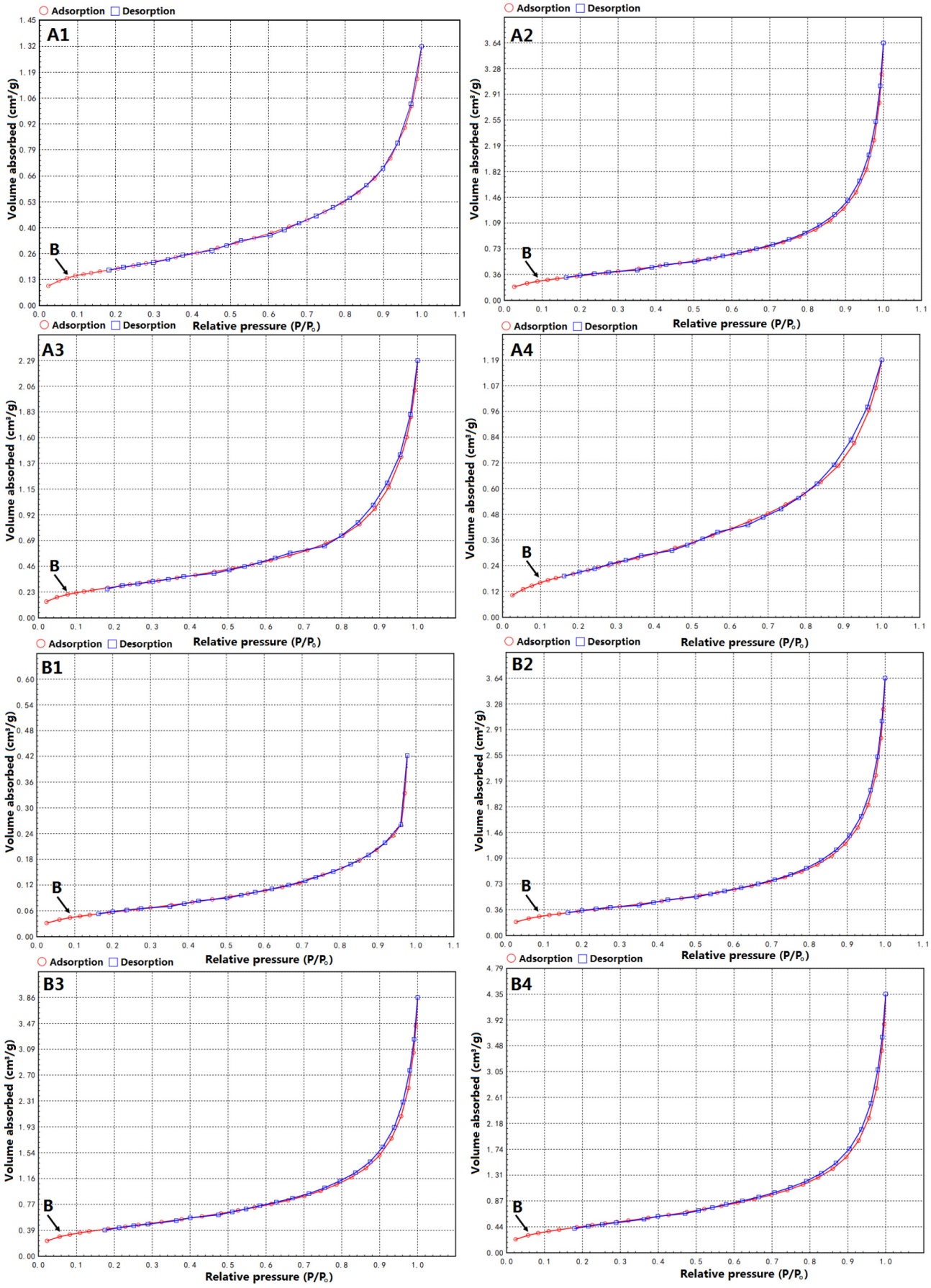

Fig. 2. Nitrogen adsorption-desorption isotherms (A: wheat; B: maize) of (1) native starch; (2) AA→GA modified starch; (3) BE→GA modified starch; (4) AA→BE→GA modified starch.

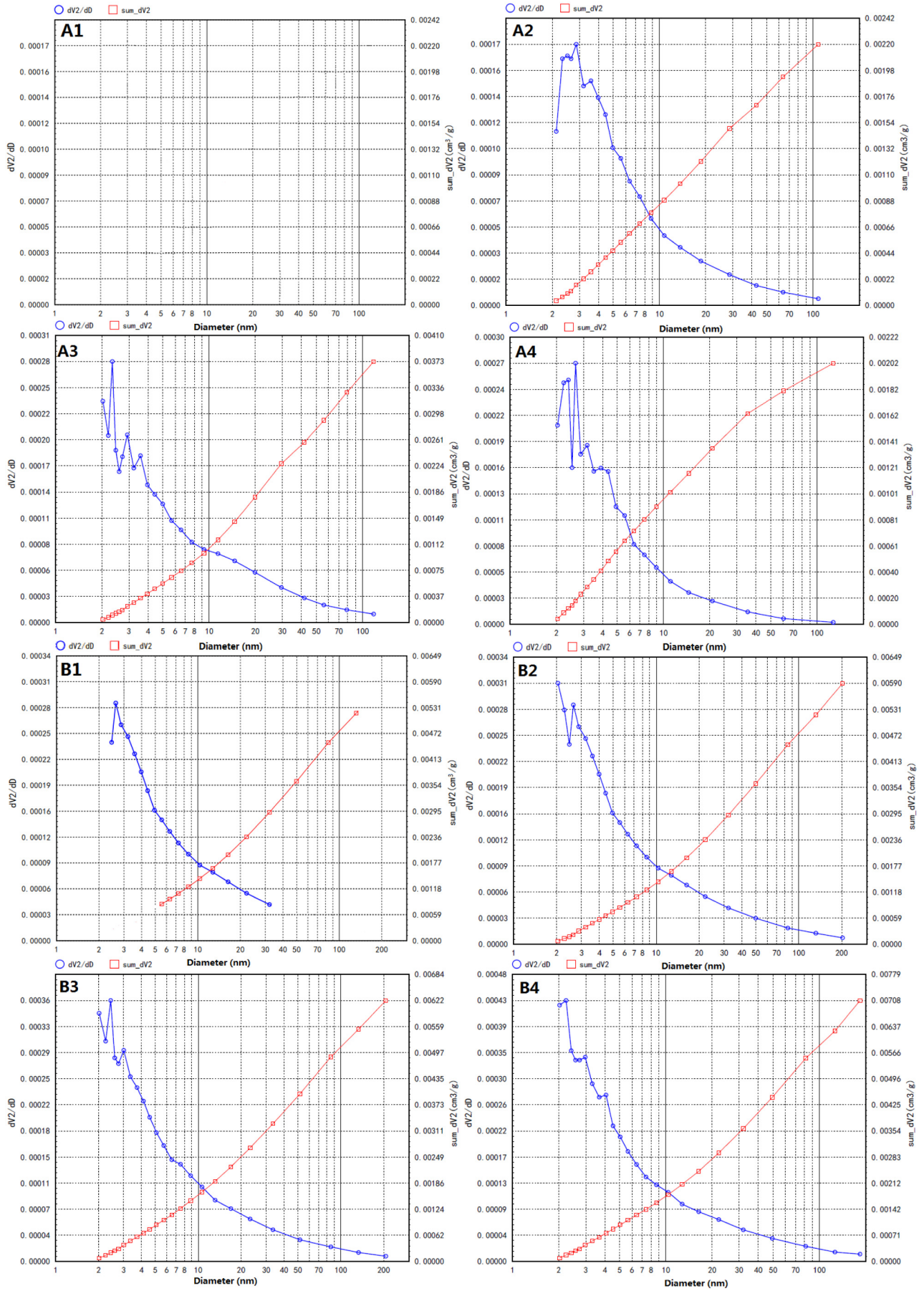

Fig. 3. Pore diameter and volume distributions with BJH (A: wheat; B: maize) of (1) native starch; (2) AA→GA modified starch; (3) BE→GA modified starch; (4) AA→BE→GA modified starch.

Table 4
Parameters derived from nitrogen sorption at 77 K.

Sample		Specific surface area (m^2/g)	Pore diameter distributions (%)			Pore size (nm)	Pore volume (cm^3/g)
			2–10 nm	10–50 nm	50–100 nm		
Wheat starch	Native starch	1.57 ± 0.03^{d}	–	–	–	–	–
	AA→GA	6.86 ± 0.09^{b}	28.36 ± 2.13^{b}	45.58 ± 1.50^{a}	26.06 ± 0.23^{b}	11.83 ± 0.04^{b}	0.003 ± 0.001^{b}
	BE→GA	3.59 ± 0.06^{c}	38.52 ± 1.02^{a}	41.88 ± 0.12^{b}	19.60 ± 0.07^{c}	8.69 ± 0.03^{c}	0.002 ± 0.001^{c}
	AA→BE→GA	11.33 ± 0.15^{a}	17.33 ± 1.18^{c}	20.60 ± 0.91^{c}	62.07 ± 0.06^{a}	36.35 ± 0.05^{a}	0.005 ± 0.001^{a}
Maize starch	Native starch	1.44 ± 0.18^{d}	–	–	–	1.82 ± 0.02^{d}	0.001 ± 0.000^{d}
	AA→GA	8.91 ± 0.43^{b}	23.36 ± 1.12^{b}	38.20 ± 1.05^{a}	38.44 ± 0.08^{a}	22.76 ± 0.02^{b}	0.006 ± 0.002^{b}
	BE→GA	5.56 ± 1.05^{c}	25.43 ± 0.06^{a}	37.69 ± 0.25^{b}	36.88 ± 1.12^{b}	17.45 ± 0.03^{c}	0.004 ± 0.001^{c}
	AA→BE→GA	16.32 ± 2.05^{a}	25.00 ± 0.06^{a}	38.21 ± 0.11^{a}	36.79 ± 1.54^{b}	26.54 ± 0.08^{a}	0.007 ± 0.001^{a}

that enzyme-treated starches have greater pore size and pore volume than native starches. Comparing the three enzyme treatments shows that pore volume (0.005 cm^3/g) and pore size (36.35 nm) are largest in the AA→BE→GA treated wheat starch, followed by pore volume (0.003 cm^3/g) and pore size (11.83 nm) of BE→GA treated wheat starch, and the smallest pore volume (0.002 cm^3/g) and pore size (8.69 nm) being found in the AA→GA treated wheat starch. Similarly, pore volume (0.007 cm^3/g) and pore size (26.54 nm) are largest in the AA→BE→GA treated maize starch, followed by pore volume (0.006 cm^3/g) and pore size (22.74 nm) of BE→GA treated maize starch, and the smallest pore volume (0.004 cm^3/g) and pore size (17.45 nm) being found in the AA→GA treated maize starch. The above data indicate all maize starch samples have much greater pore size and pore volume than all wheat starch samples. It may be attributed to the fact that maize starch granules are more accessible to enzymes and thus accelerate enzymes to permeate into the interior of granules, resulting in higher hydrolysis efficiency [41,42]. Moreover, BE and AA can facilitate efficiently GA catalysis of starch. To summarise, the increase in pore size, pore volume, and specific surface area of enzyme-modified starches may contribute to enhance adsorption properties of porous cereal starches (described later).

3.5. Adsorption capacity

The adsorption capacities of porous starches are summarised in Table 5 and shows that compared to wheat starches, the oil adsorption capacities of the AA→GA, BE→GA, and AA→BE→GA modified starches increase by 972.81, 359.45 and 1472.81%, respectively. The oil adsorption capacities of the AA→GA, BE→GA, and AA→BE→GA treated maize starches increase by 916.08, 464.93 and 1217.92%, respectively. Compared to all enzyme-treated starches, the most significant increases in oil adsorption capacity occur in the AA→BE→GA treated porous starches that have higher specific surface areas and more pores with larger sizes and volumes. In terms of single enzyme treatment (AA, BE or GA) [11], compared to native maize starch, the oil adsorption capacity of AA-treated starch increases by about 25%, whereas that of BE or GA-treated starch decreased. Comparatively, the above data indicate that the combinations of these enzymes in this study result in a significant increase in oil adsorption capacity that is far higher than single enzyme [11]. It further confirms that the enzyme combination is an efficient method for preparing porous starches with higher absorption capacities. What are synergistic effects between AA, BE and GA will be studied in detail later. Compared to native starches, the MB adsorption capacities of AA→GA, BE→GA, and AA→BE→GA treated wheat starches increase by 292.26, 138.83 and 458.54%, respectively and the NR adsorption capacities increase by 303.51, 152.08 and 492.33%, respectively. For maize starch, the MB of the AA→GA treated, BE→GA treated, and AA→BE→GA treated starches increase by 337.30, 247.34 and 523.53%, respectively, and the NR adsorption capacities increase by 374.80, 277.17 and 580.22%, respectively. The larger pore size and specific surface area of the AA→BE→GA modified starches are significantly greater than the AA→GA treated and BE→GA treated starches. The metal adsorption capacities of enzyme-modified starches notably increase, especially for AA→BE→GA treated porous starches that display the highest adsorption capacities for the tested metal ions. Compared to native wheat starch, the adsorption capacities of Cu^{2+}, Pb^{2+}, Cd^{2+}, Hg^{2+}, and Zn^{2+} are enhanced by 723.40, 598.29, 375.50, 524.36 and 454.38%, respectively, after AA→BE→GA treatment. Compared to native maize starch, the adsorption capacities of Cu^{2+}, Pb^{2+}, Cd^{2+}, Hg^{2+}, and Zn^{2+} are enhanced by 753.29, 500.12, 489.69, 334.09 and 383.29%, respectively, after AA→BE→GA treatment. In the mixture of metal ions, Cu^{2+} exhibits a stronger competitive adsorption behavior with AA→BE→GA modified porous starches. It may be attributed to the reason that the structural properties of copper such as molecular weight, atomic radius and size, atomic shape, etc. may be easier to enter the interior of pore in porous starches, hence resulting in a higher affinity for Cu^{2+} adsorption. We are planning to study the mechanism about the adsorption of enzyme-modified starches on heavy metals in detail later. Overall, the combined effects of AA and BE may create larger amorphous fields that contribute to GA hydrolysis. The synergistic actions of AA, BE, and GA increases with increasing pore number, the appropriate pore size and volume, and specific surface area, thereby leading to remarkable increase in the adsorption capacities of porous starches. Besides, Table 5 obviously shows that the adsorption capacities of porous maize starches are far larger than that of porous wheat starches, which verifies the results from SEM and nitrogen adsorption/desorption.

Table 5
Adsorptive capacity of enzyme-treated starches.

Sample		Oil (%)	MB (mg/g)	NR(mg/g)	Cu^{2+}(mg/g)	Pb^{2+}(mg/g)	Cd^{2+}(mg/g)	Hg^{2+}(mg/g)	Zn^{2+}(mg/g)
Wheat starch	Native starch	4.34 ± 1.15^{d}	6.85 ± 2.54^{d}	6.26 ± 1.13^{d}	8.42 ± 2.31^{d}	14.06 ± 2.25^{d}	18.37 ± 5.21^{d}	14.78 ± 2.34^{d}	17.71 ± 5.22^{d}
	AA→GA	46.56 ± 3.06^{b}	26.87 ± 2.54^{b}	25.26 ± 3.12^{b}	49.28 ± 2.56^{b}	75.25 ± 3.15^{b}	76.88 ± 3.23^{b}	72.89 ± 4.23^{b}	79.12 ± 4.52^{b}
	BE→GA	19.94 ± 4.12^{c}	16.36 ± 4.52^{c}	15.78 ± 2.12^{c}	16.29 ± 6.35^{c}	23.12 ± 5.03^{c}	26.49 ± 7.12^{c}	29.31 ± 1.13^{c}	27.28 ± 1.93^{c}
	AA→BE→GA	68.26 ± 8.15^{a}	38.26 ± 4.15^{a}	37.08 ± 3.23^{a}	69.33 ± 4.16^{a}	98.18 ± 8.22^{a}	87.35 ± 2.25^{a}	92.28 ± 6.31^{a}	98.18 ± 5.56^{a}
Maize starch	Native starch	6.53 ± 2.22^{d}	11.26 ± 2.03^{d}	10.16 ± 2.54^{d}	8.65 ± 2.56^{d}	16.79 ± 5.23^{d}	17.17 ± 4.16^{d}	22.56 ± 3.09^{d}	20.83 ± 3.12^{d}
	AA→GA	66.35 ± 5.12^{b}	49.24 ± 4.09^{b}	48.24 ± 3.35^{b}	50.24 ± 4.15^{b}	80.12 ± 8.12^{b}	82.43 ± 9.14^{b}	78.22 ± 5.15^{b}	79.16 ± 2.32^{b}
	BE→GA	36.89 ± 3.08^{c}	39.11 ± 3.25^{c}	38.32 ± 4.56^{c}	29.27 ± 4.14^{c}	38.12 ± 2.05^{c}	39.27 ± 2.02^{c}	36.44 ± 7.21^{c}	38.37 ± 1.23^{c}
	AA→BE→GA	86.06 ± 3.54^{a}	70.21 ± 5.29^{a}	69.11 ± 3.55^{a}	73.81 ± 3.12^{a}	100.76 ± 8.30^{a}	101.25 ± 6.23^{a}	97.93 ± 6.82^{a}	100.67 ± 5.22^{a}

MB refers to methylene blue and NR refers to neutral red.

4. Conclusions

The three enzyme types, AA→GA, BE→GA, and AA→BE→GA, were used to modify the external and internal structures of wheat and maize starch granules and prepare porous starches with different structure and adsorption characteristics. The results indicate that all enzyme treatments increase pore number, pore volume, and specific surface area of porous starches, which leads to a notable increase in adsorption capacities of the porous starches. The AA→BE→GA treatment has the maximum adsorption capacity, followed by AA→GA and BE→GA. Following AA→BE treatment, there is a decrease in amylose levels, amylopectin branch chain lengths are shortened, and the degree of branching increases, all of which facilitates subsequent GA hydrolysis. Thus, a novel AA→BE→GA treatment may be an efficient method for preparing porous starches as organic adsorbents with greatly improved absorption capacities.

CRediT authorship contribution statement

All persons who have made substantial contributions to the work reported in the manuscript. Li Guo: Conceptualization, Methodology, Funding acquisition, Writing Yuhan Yuan: Investigation, Formal analysis. Jiahao Li: Investigation, Formal analysis. Congping Tan: Investigation. Srinivas Janaswamy: Investigation. Lu Lu: Investigation. Yishan Fang: Investigation. Bo Cui: Conceptualization, Funding acquisition.

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