Effect of physical and thermal pretreatments on enzymatic activity in the production of microporous cassava starch

Efecto de pretratamientos físicos y térmicos sobre la actividad enzimática en la producción de almidón microporoso de yuca

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ABSTRACT

Cassava starch is modified to increase porosity and lacerations that are limited when only enzymatic treatments are used. This study proposes to improve enzymatic activity of α-amylase and amyloglucosidase on the polymer chains of cassava starch by implementing physical and thermal pretreatments below the gelatinization temperature and before the hydrolytic process. The pretreatments increased the biocatalytic action of the enzymes, causing significant changes in the morphology of the granules, and superficial lacerations were found in samples of starches pretreated with ultrasound (UTS) or annealing and ultra-rapid freezing (ANN-C). At the structural level, the modified starches revealed substantial changes as the infrared spectra reflected a displacement of the absorption bands in the region from 900 to 1100 cm^{-1}. This is associated with an alteration and reorganization of the amorphous and crystalline zones of the granules and is consistent with a decrease in amylose content (from 19.53% to 17.64%) and an increase in the crystallinity index. The thermal behavior of the starches was also modified by increasing the peak temperature (from 68.22°C to 75.38°C) and reducing the gelatinization enthalpy (from 19.34 to 15.79 J/g). UTS and ANN-C pretreatments significantly improved the mesoporous and hydrophilic properties of the modified cassava starches.

Key words: annealing, crystallinity, gelatinization, hydrolysis, hydro-thermal treatments.

RESUMEN

El almidón de yuca es modificado para aumentar su porosidad y laceraciones, las cuales son limitadas cuando sólo se utilizan tratamientos enzimáticos. Por lo tanto, este estudio propone mejorar la actividad enzimática de la α-amilasa y la amiloglucosidasa sobre las cadenas poliméricas del almidón de yuca utilizando pretratamientos físicos y térmicos por debajo de la temperatura de gelatinización antes del proceso hidrolítico. En este caso, los pretratamientos aumentaron la acción biocatalítica de las enzimas, provocando cambios significativos en la morfología de los gránulos, y se encontraron marcadas laceraciones superficiales en muestras de almidones pretratados con ultrasonido (UTS) o recocido y congelación ultrarrápida (ANN-C). A nivel estructural, los almidones modificados revelaron cambios sustanciales dado que los espectros infrarrojos reflejaron un desplazamiento de las bandas de absorción en la región de 900 a 1100 cm^{-1}. Esto está asociado con una alteración y reorganización de las zonas amorfa y cristalina de los gránulos y es consistente con la disminución del contenido de amilosa (desde 19.53% hasta 17.64%) y el aumento del índice de cristalinidad. El comportamiento térmico de los almidones también se modificó al aumentar la temperatura pico (desde 68.22 hasta 75.38°C) y reducir la entalpía de gelatinización (desde 19.34 hasta 15.79 J/g). Los pretratamientos UTS y ANN-C mejoraron significativamente las propiedades mesoporosas e hidrofílicas de los almidones de yuca procesados.

Palabras clave: recocido, cristalinidad, gelatinización, hidrólisis, tratamientos hidrotérmicos.

Introduction

Starch is a homopolymer composed of units of α-D-glucopyranose that can be linked by α-D-(1,4) and α-D-(1,6) glycosidic bonds to form two different polymeric structures: amylose and amylopectin. Amylose and amylopectin are densely packed in a semi-crystalline state that affects the susceptibility of starch to enzymatic attack (O'Brien & Wang, 2008). Compared to cereal starches, for example, tuberous root granules have a smoother surface free of porosities (Chen *et al*., 2011). Cassava starch has been widely incorporated in the formulation of food matrices as a stabilizer, texture modifier, fat emulator, or encapsulating material for bioactive compounds (Jayakody &

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Hoover, 2008). To improve the properties of thermo- or photosensitive substances as carriers of micronutrients or encapsulating materials, their adsorption properties should be enhanced through modification that promotes the development of structures with excellent micro- or mesoporous characteristics.

There are many methods to produce porous starches that involve physical, chemical, and enzymatic treatments, or a combination of them. Enzymatic modification is characterized by polymer degradation, total or partial loss of the native morphology, and molecular disorganization, altering the physicochemical properties of the granules (Rocha *et al*., 2010; Figueroa-Flórez *et al*., 2019). These biocatalytic processes offer several advantages: they are efficient, safe, and ecologically sustainable. In addition, the enzymatic modification involves the use of polypeptides in the production of porous starches, whose granules have lacerations, cracks, or pores that run from the surface to the interior (Xie *et al*., 2019) and are used in the industry for carrying or encapsulating pigments, antioxidants, fatty acids, etc. (Gao *et al*., 2013; Dura *et al*., 2014; Xie *et al*., 2019).

Several studies have reported the use of endo- or exo-amylases from different botanical sources in the production of porous starches (O'Brien & Wang, 2008; Wu *et al*., 2011; Zhang *et al*., 2012; Gao *et al*., 2013; Dura *et al*., 2014; Wang *et al*., 2016; Benavent-Gil & Rosell, 2017; Jung *et al*., 2017; Xie *et al*., 2019). Although few articles in the literature have examined the biocatalytic action of amylolytic enzymes in the production of porous cassava starches using α-amylase or amyloglucosidase, the articles have concluded that tuberous root starches are less susceptible to enzymatic attacks because they show small superficial lacerations and shallow holes (Rocha *et al*., 2010; Chen *et al*., 2011; Benavent-Gil & Rosell, 2017; Figueroa-Flórez *et al*., 2019).

Other authors have proposed the use of physical treatments in the production of porous corn starches to increase the size and volume of the pores and improve the adsorption capacity of the starches. Zhao *et al.* (2018) explored a freezing-cooling combination and obtained significant changes in the crystallinity index, thermal, and morphological properties, as well as in the water adsorption capacity and solubility of corn granules. Thermal processes such as annealing have been implemented to optimize enzymatic hydrolysis; as a result, significant changes in structural properties and granules with a microporous morphology have been reported (Tukomane *et al*., 2007; O'Brien & Wang, 2008; Shariffa *et al*., 2017; Xie *et al*., 2019).

Previous studies have identified the presence of lacerations or small porosities in enzymatically modified cassava granules (Shariffa *et al*., 2009; Chen *et al*., 2011; Dura *et al*., 2014). However, few authors have evaluated the effect of physical pretreatments on the enzymatic hydrolysis to develop porous cassava starches. We hypothesize that physical-thermal pre-treatments alter the native semi-crystalline packing that favors the enzymatic absorption phenomenon, endo-corrosion processes, and the formation of micro-porous granules. The literature does not include reports on the characteristics of the pores in cassava-modified starches in terms of type, size, and pore volume. The aim of this study was to evaluate the effect of physical and thermal pretreatments on the degree of hydrolysis and the structural and morphological properties of native cassava starches during enzymatic modification.

Materials and methods

Materials

Native cassava starch (*Manihot esculenta* cv. M-Tai) was supplied by Almidones de Sucre S.A (Induyuca®, Sincelejo, Colombia). Amyloglucosidase from *Aspergillus niger* (Dextrozyme® GA, Novozymes, Denmark) and α-amylase from *Bacillus licheniformis* (Liquozyme® Supra 2.2X, Novozymes, Denmark) were used in the experiment. Analytical grade potato amylose (AO512, Sigma Aldrich, Germany) was also used.

Physical and thermal pretreatments

Native cassava starch (NCS) with a moisture content of ~50% (dry weight basis), was subjected to the following pretreatments:

- Annealing (ANN): Heating for 60 min at 60°C under constant agitation at 250 rpm in a thermostatic bath (MaxQ® 4450, Thermo Scientific, USA);
- Homogenization (HMG): Pretreatment using an ultra-turrax disperser (T25 Basic®, IKA, Germany) under stirring at 3000 rpm for 60 min at 60°C;
- Ultrasound (UTS): Process performed in an ultrasonic cleaner (Model CPX3800H, Branson, USA) at a frequency of 40 kHz and 60°C for 60 min;
- Annealing and ultra-rapid freezing (ANN-C): Heating at 60°C with shaking for 240 min with subsequent storage in an ultra-low temperature at -12°C for 8 h (Kaltis®, F390, USA). Pretreated starch was recovered and dried in a forced convection oven (UFB500, Memmert, Germany) at 35°C for 8 h.

Enzymatic hydrolysis of starch

Pretreated-starch suspensions at 20% (w/v) were subjected to enzymatic hydrolysis with the simultaneous application of α-amylase (15 U/g starch) and amyloglucosidase (10 U/g starch) in a sodium citrate/citric acid buffer solution at pH 5.0, 60°C and 250 rpm for 6 h (Figueroa-Flórez *et al*., 2019). Subsequently, hydrolysates were removed by centrifugation at 5000 rpm for 15 min. Samples were washed with ethanol and distilled water to remove the residual enzyme content. Finally, the starch was recovered and dried in a forced convection oven at 35°C for 8 h.

Native cassava starch (NCS) and non-physically pretreated starch granules (NPS) were referred to as control treatments. The NPS treatment corresponded to samples of enzymatically modified starch without physical and thermal pretreatment.

Hydrolysis degree (HD)

The degree of hydrolysis was evaluated from the reaction of reducing sugars released with 3,5-dinitrosalicylic acid (DNS) by UV-Vis spectrophotometry (UV-2550, Shimadzu, Japan) at a wavelength of 540 nm (Salcedo-Mendoza *et al*., 2018). We used 500 µl of sample to react with an excess amount of DNS for 8 min at 80°C with subsequent cooling to 10°C to stop the reaction. The degree of hydrolysis was expressed as the ratio between weight at the beginning and at the end of the period of enzymatic action (Figueroa-Flórez *et al*., 2019). The concentration of sugars was expressed in glucose equivalents (GE).

Starch morphology

Starch granules were fixed in a sample holder with electrically conductive carbon tape covered with a platinum/gold alloy (Chen *et al*., 2011). Samples were observed on a scanning electron microscope (JEOL, JSU LV-5600, Japan) under conditions set at 15 KV, 30 mA and an amplitude margin 3000X.

Determination of granular size

Granular size was determined by light scattering using a Mastersizer particle analyzer (Model 3000E, Malvern, UK) and expressed as a function of the average diameter D_X (50). Measurements were performed in triplicate at room temperature with a refractive index of 1.52 (Monroy *et al*., 2018). Pore characteristics were determined using an automatic specific surface area and porosity analyzer (Quantachrome Inc., NOVA 2000e, USA). Pure nitrogen (> 99.99%) was used to determine adsorption-desorption isotherms. The starch samples were dried for 20 h under vacuum at 100°C, degassed at 125°C for 24 h, and immersed in liquid nitrogen (-196°C) in the range of relative pressure P/Po ≈ 5-19% (Gao *et al*., 2013). The mean pore diameter and the specific surface area (A_S) were calculated with the BET method (Brunauer-Emmett-Teller). The total pore volume (v) was estimated with the BJH (Barrett-Joyner-Halenda) method (Guo *et al*., 2020).

Amylose content and infrared spectroscopy (FTIR)

Amylose content was estimated by the colorimetric iodine method using a UV-Vis spectrophotometer (UV-2550, Shimadzu, Japan) at a wavelength of 620 nm (Salcedo-Mendoza *et al*., 2018). Infrared spectra were obtained in the region of 500 to 4000 cm^{-1} making 32 readings at a resolution of 4 cm^{-1} using a spectrometer (Nicolet IS50 FT-IR, Thermo Scientific, USA). The order degree (OD) was estimated as the absorbance ratio in the 1047/1022 cm^{-1} bands, expressed as a percentage (Ma *et al*., 2018).

XRD diffraction patterns

Diffraction patterns were obtained with an XRD diffractometer (X'Pert Pro-MPD, Panalytical, Italy) in the range of 4-35°, operating at 1.8 kW with a current of 40 mA (Salcedo-Mendoza *et al*., 2018). The crystallinity index (CI) was calculated by the ratio of areas of the absorption peaks (crystalline zone) over the total area using Origin Lab v8.0 (OriginLab Corporation, USA).

Water solubility index (WSI), water absorption index (WAI), and swelling power (SP)

WSI, WAI, and SP were determined with slight modifications according to Rocha *et al.* (2010). One g of starch was dispersed in 25 ml of distilled water under agitation at 60°C for 30 min, then centrifugation was performed at 5000 rpm for 15 min. The pellet was removed, and weight was quantified. The SP was estimated as the ratio between the gel weight and the initial weight of dry starch, using the equation reported by Figueroa-Flórez *et al.* (2019). The resulting gel was weighed to estimate WAI. An aliquot of 10 ml was taken from the supernatant, poured into Petri dishes, and evaporated in an oven at 70°C for 16 h. WSI was calculated as the amount of dry solids recovered by evaporating the water absorption test supernatant.

Thermal properties

Gelatinization properties were determined using a differential scanning calorimeter (DSC-Q2000, TA Instruments, USA) and a suspension in an aluminum pan with a 2 mg of starch and 6 mg of water ratio (Xie *et al*., 2019). Capsules were sealed and stored at 25°C for 24 h to balance the system. Subsequently, they were heated from 20°C to 120°C at a speed of 10°C min^{-1} and cooled to a ramp of 25°C min^{-1}

under a nitrogen atmosphere. Onset temperature (T_O), peak temperature (T_P), and conclusion temperature (T_C) were determined from the DSC curves. The gelatinization enthalpy (ΔH) was estimated based on the endothermic peak area expressed in J/g.

Experimental design

A unifactorial categorical design was implemented where each level is related to a specific pretreatment. Results were analyzed using statistical tools such as analysis of variance (ANOVA) and Tukey test for comparison of means with a level of significance of 5% using the Statgraphics software (Centurion XVI, Statgraphics Technologies Inc., USA).

Results and discussion

Effect of pretreatments on the enzymatic hydrolysis of starch granules

The hydrolysis degree oscillated between 11.99 ± 0.65% and 24.92 ± 0.40% (Tab. 1). Similar results have been reported during hydrolysis with α-amylase and/or amyloglucosidase in starches from various amylaceous sources (Rocha *et al*., 2010; Shariffa *et al*., 2017). This behavior is probably due to the degradation of amylose and amylopectin polymer fractions by the synergistic action of amylolitic enzymes, affecting structural and morphological characteristics of the granules (Chen *et al*., 2011).

A decrease in the degree of hydrolysis was found in the UTS treatment (21.40 ± 0.82%) with respect to the NPS treatment (11.99 ± 0.65%). These results were lower than those reported in the hydrolysis of corn starch through the application of ultrasonic technology (Li *et al*., 2018). However, treatment of UTS can alter swelling and solubility properties, changing the interaction of polymer chains with water molecules and the semicrystalline order of the granules, and forming granules less susceptible to enzymatic attack (Wu *et al*., 2011). This same behavior associated with the redistribution or recrystallization of amylose and amylopectin chains during ANN-C treatment could explain the molecular resistance of the granules to enzymatic degradation and the decrease in the degree of hydrolysis (16.71 ± 0.60%).

The results of the degree of hydrolysis correlate with morphological changes of granules. In Figure 1, NCS granules with oval or spherical morphologies of relatively smooth surfaces without lacerations can be seen, with truncated ends possibly inherent to the extraction process (Salcedo-Mendoza *et al*., 2018). But, after the enzymatic treatment, granules showed erosion on their external surface and generalized cavities with apparent depth (Fig. 1NPS), as well as a rupture or fragmentation, probably associated with the physical and thermal pretreatments established before the biocatalytic process. Similar results have been reported previously, where the same pretreatments facilitated the release of starch components from the amorphous zone affecting the morphological characteristics of the granule on a superficial level (Chen *et al*., 2011; Wu *et al*., 2011; Shariffa *et al*., 2017; Zhao *et al*., 2018; Wang *et al*., 2022).

The microphotographs of the starches pretreated with UTS (Fig. 1UTS) suggest a granular surface and microstructural modifications. Likewise, the starches modified with the ANN treatment showed more generalized superficial granular lacerations with respect to NPS samples (Fig.1NPS), in line with the results reported for hydrolyzed cassava and sweet potato starches pretreated under gelatinization temperature for 72 h (Shariffa *et al*., 2017). With the HMG pretreatment there were deep surface erosions, presumably due to the enzymatic action (Fig. 1HMG) and a generalized loss of granule integrity due to exposure to shear stress. These data are consistent with those reported for mechanically modified cassava starches (He *et al*., 2014). After the ultrasonic pretreatment noticeable changes were observed in granular morphology that presented greater cracks and superficial lacerations at a lower degree of hydrolysis with respect to its NPS counterpart.

TABLE 1. Hydrolysis degree, amylose content, and physicochemical properties in native and modified cassava starches.

Treatment	HD (%)	Amylose (%)	WSI (g/g)	WAI (g/g)	SP (g/g)
NCS	-	19.53 ± 0.32^{a}	2.17 ± 0.08^{a}	3.84 ± 0.16^{a}	3.99 ± 0.10^{a}
NPS	11.99 ± 0.65^{a}	18.76 ± 0.19^{b}	3.50 ± 0.12^{b}	4.36 ± 0.11^{b}	3.20 ± 0.14^{b}
ANN	15.24 ± 0.47^{b}	18.71 ± 0.28^{b}	3.80 ± 0.17^{b}	4.29 ± 0.12^{b}	2.92 ± 0.06^{c}
HMG	24.92 ± 0.40^{c}	18.93 ± 0.14^{b}	4.22 ± 0.07^{c}	4.86 ± 0.05^{c}	2.65 ± 0.18^{d}
UTS	21.40 ± 0.82^{d}	17.97 ± 0.22^{c}	4.61 ± 0.11^{d}	4.85 ± 0.09^{c}	2.28 ± 0.10^{e}
ANN-C	16.71 ± 0.60^{be}	17.64 ± 0.16^{c}	4.84 ± 0.19^{d}	4.41 ± 0.07^{b}	2.55 ± 0.15^{df}

HD - hydrolysis degree; WSI - water solubility index; WAI - water absorption index; SP - swelling power. Native cassava starch (NCS); non-pretreated enzymatic modified starch (NPS); modified starch pretreated by annealing (ANN); modified starch pretreated by homogenization (HMG); modified starch pretreated by ultrasound (UTS), and modified starch pretreated by annealing and ultra-rapid freezing (ANN-C). Different superscripted letters in the same column indicate significant differences for Tukey test ($P<0.05$).

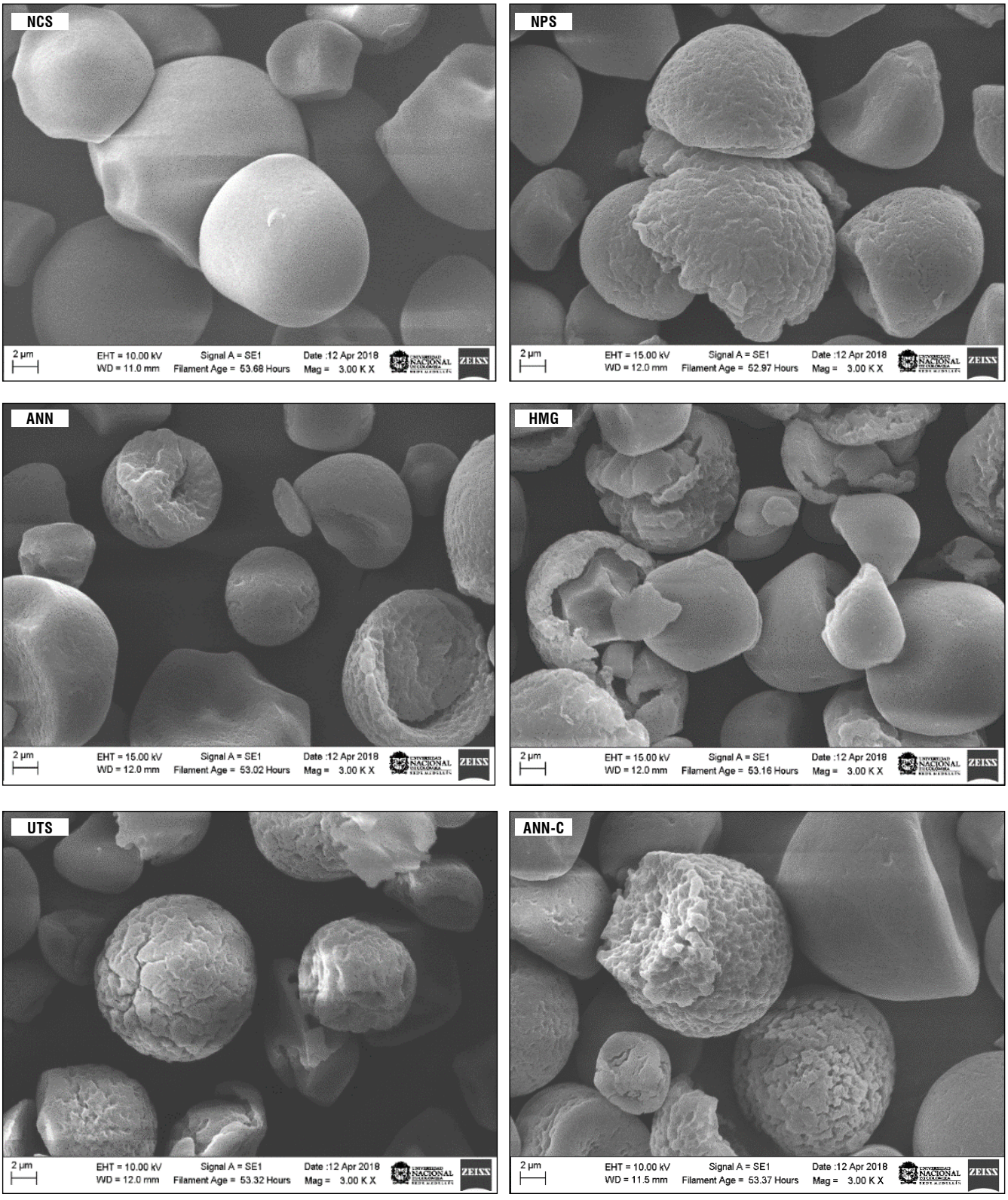

FIGURE 1. Microphotographs of native and modified cassava starches (3000x). Native cassava starch (NCS); non-pretreated enzymatic modified starch (NPS); modified starch pretreated by annealing (ANN); modified starch pretreated by homogenization (HMG); modified starch pretreated by ultrasound (UTS); modified starch pretreated by annealing and ultra-rapid freezing (ANN-C).

Similar observations have been reported regarding corn starches modified using various ultrasound wave frequencies (Li *et al*., 2018). With the ANN-C treatment, an extended and uniform porosity was observed in the granules, accompanied by more pronounced lacerations or cracks in their surfaces (Fig. 1ANN-C). Experiments conducted with sweet potato and waxy rice starches have revealed an analogous behavior after applications of annealing or successive freezing (Tonon *et al*., 2009; Tao *et al*., 2015).

The annealing treatment produces cracks in the granules of sweet potato starch that exhibit a greater proportion of porous granules that preserve the integrity of their morphology (Tonon *et al*., 2009). Tao *et al*. (2015) consider that the presence of some fragmented granules is due to the powerful compression force exerted by the formation of ice crystals during freezing. Therefore, a greater generation of pores and lacerations is a consequence of thermal treatments that increase the surface area and favor the enzymatic action on polyglucan chains (Zhao *et al*., 2018).

The granule size had a bimodal distribution between 5 and 100 µm for NCS, and the mean diameter D_X (50) ranged from 17.05 to 21.65 µm for all samples (Fig. 2, Tab. 2). The diameters D_X (50) decreased after the enzymatic treatment in the following order: HMG<UTS<ANN-C<ANN<NPS. The samples of modified starch pretreated with HMG exhibited the smallest diameters (17.05 ± 0.35 µm), possibly caused by shear force action (Hossen *et al*., 2011). These results are consistent with the morphological changes of the granules analyzed by microscopy (Fig. 1HMG).

TABLE 2. Physical characteristics of starch granule populations in samples.

Treatment	D_X (50) (µm)	ν (cm^3/g) x 10^{-3}	∅ (nm)	As (m^2/g)
NCS	21.65 ± 0.35^a	0.14 ± 1E-5^a	0.90 ± 0.00^a	0.58 ± 0.02^a
NPS	20.45 ± 0.48^b	0.31 ± 1E-5^a	1.12 ± 0.01^b	0.88 ± 0.07^a
ANN	19.95 ± 0.26^b	0.67± 0E-5^b	0.97 ± 0.06^c	1.94 ± 0.01^a
HMG	17.05 ± 0.21^c	2.39 ± 3E-5^c	1.86 ± 0.03^b	1.03 ± 0.12^a
UTS	17.65 ± 0.37^d	2.05 ± 0E-5^d	2.01 ± 0.01^d	2.66 ± 0.06^a
ANN-C	17.55 ± 0.24^e	2.09 ± 1E-5^d	1.96 ± 0.05^e	3.19 ± 0.09^a

D_X (50) - average diameter of microporous granule; ν - total volume of microporous granule; ∅ - average diameter of microporous granule; A_S - specific surface area. Native cassava starch (NCS); non-pretreated enzymatic modified starch (NPS); modified starch pretreated by annealing (ANN); modified starch pretreated by homogenization (HMG); modified starch pretreated by ultrasound (UTS), and modified starch pretreated by annealing and ultra-rapid freezing (ANN-C). Different superscripted letters in the same column indicate significant differences for Tukey test (P<0.05).

UTS and ANN-C exhibited significantly reduced diameter D_X (50) in NCS. Ultrasonic cavitation and molecular reorganization (caused by ice crystals during freezing), probably, triggered structural changes (Wang *et al*., 2022)

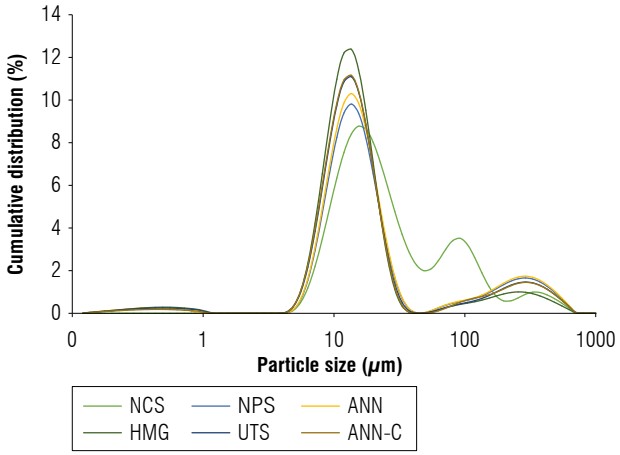

FIGURE 2. Particle size distribution of native and modified cassava starches. Native cassava starch (NCS); non-pretreated enzymatic modified starch (NPS); modified starch pretreated by annealing (ANN); modified starch pretreated by homogenization (HMG); modified starch pretreated by ultrasound (UTS), and modified starch pretreated by annealing and ultra-rapid freezing (ANN-C).

that favored hydrolysis, with morphological changes in granular size, pore size, and volume increase (Tab. 2). The ANN treatment only caused a slight reduction in the diameter D_X (50) with respect to its NPS counterpart. These results confirm that physical and thermal treatments reduce granular size and increase the porosity of the granules based on the depolymerization of the unstable amorphous materials present in the granular surface and the endogenous action of α-amylase hydrolyzing from the surface to the hilum (Tukomane *et al*., 2007; Monroy *et al*., 2018; Zhao *et al*., 2018).

Modification processes caused a significant increase in specific surface area, volume, and pore diameter (P<0.05) (Tab. 2). The N_2 adsorption isotherms of the modified starches were type IV with a hysteresis curve type H3 (results not shown) that is characteristic of mesoporous materials (Rouquerol *et al*., 1994). In this study, pore size was greater than 2 nm. Therefore, according to the classification presented by Xie *et al*. (2019), the material was mesoporous. The mesopores identified and estimated in NCS samples were probably caused by the presence of intrinsic porosities of the granule in its native state or the action of endogenous enzymes during the extraction process (Foresti *et al*., 2014). The results with the NCS treatment showed that enzymatic action increased the surface area, diameter, and volume of the pores compared to native starch (Tab. 2). However, no significant increase in mesoporous properties was detected with the HMG treatment, possibly because the mechanical action during the size reduction facilitated the amyloglucosidase

endo-corrosion phenomenon and led to a higher production of fragmented granules. Specifically, the granules pretreated with UTS and ANN-C exhibited more uniform porosity and larger pores that did not affect their native spherical or oval shape, as shown in the SEM microphotographs. Sharrifa *et al*. (2009) consider that temperatures close to gelatinization can swell the granule during pregelatinization, increasing the opening and size of the pores that could facilitate enzyme absorption and degrade the external part of the granule by exo-corrosion. As pore size grows due to exo-corrosion, it may also favor the phenomenon of endo-erosion toward the inside of the granule (Keeratiburana *et al*., 2020). All the enzymatic actions occurred at the surface or internal level, leading to the production of mesoporous granules with better hydrophilic properties.

The enzymatic process significantly altered the interaction of polymer chains with water molecules (Zhong *et al*., 2022). There was an increase in modified starches WSI and WAI with respect to the NCS samples (Tab. 1), possibly due to the breakdown of intermolecular bonds and the depolymerization of long-length chains, helping the release and availability of soluble polymer components during granules swelling process (Wang *et al*., 2016; Jung *et al*., 2017). The WSI values with respect to the control increased in the following order: HMG>UTS>ANN-C>ANN>NPS, while WAI values increased in the order: HMG>UTS>ANN-C>NPS>ANN. The increase of WSI and WAI in modified starches pretreated by HMG and UTS, possibly depends on granule size reduction by the HMG process or the increase of surface porosity, since those effects increase the surface area improving the interaction and penetration of water molecules, and other authors (Gao *et al*., 2013; He *et al*., 2014; Benavent-Gil & Rosell, 2017) report an increase in water solubility and absorption properties in corn starches subjected to a size reduction process. However, this contrasts with a decrease in WSI of modified starches that underwent annealing before the hydrolytic process (Gomes *et al*., 2005). The decrease in solubility may be due to molecular reorganization between amylose and/or amylopectin helices during annealing, producing a more stable structure that prevents the leaching of soluble components of the granules (Dias *et al*., 2010; Shariffa *et al*., 2017).

The SP decreased after enzymatic hydrolysis (Tab. 1), and similar results were reported in the production of porous corn starches with amyloglucosidase and glycosyltransferases (Dura *et al*., 2014; Benavent-Gil & Rosell, 2017). The decrease in SP has been attributed to the increase in crystal perfection, the intermolecular binding forces, and the decrease in WSI of polymer chains (Waduge *et al*., 2006; Jayakody & Hoover, 2008; Shariffa *et al*., 2017). The foregoing could explain the behavior in SP reduction in starches pretreated by UTS and ANN-C that showed higher relative crystallinity index and lower susceptibility to the enzymatic attack, as a consequence of strengthening between the amylose-amylose and amylose-amylopectin interactions. In relation to the pretreatment, SP values decreased in the following order: UTS<ANN-C<HMG<ANN<NPS. Several studies have reported the reduction of SP after annealing in starches from different starch sources associated with changes in amylose content and reordering of amylopectin double helices during cooling (Gomes *et al*., 2005; Waduge *et al*., 2006; Jayakody & Hoover, 2008; Dias *et al*., 2010; Shariffa *et al*., 2017).

Structural analysis of native and modified cassava starches

In FT-IR spectrum, characteristic peaks were observed in the 900 to 1200 cm^{-1} region (Fig. 3A), associated with crystalline and amorphous regions (Zhang *et al*., 2012; Salcedo-Mendoza *et al*., 2018; Figueroa-Flórez *et al*., 2019; Zhang *et al*., 2019). The absorption bands at 1130 and 1160 cm^{-1} attributed to the vibration of the C-O, C-C or O-H bonds present in amylose and amylopectin molecules, increased the intensity of the signal, possibly by the depolymerization and breakdown of glycosidic bonds with the enzymatic treatment (Salcedo-Mendoza *et al*., 2018). In addition, a characteristic peak of the starch is band length at 995 cm^{-1} associated with the presence of water bound in the structure, whose band possibly moved to a greater availability of OH groups after depolymerization, favoring the capacity of the starch granule to retain and absorb water (Zhang *et al*., 2012; Salcedo-Mendoza *et al*., 2018). Absorption peaks at 1047 and 1022 cm^{-1} are closely related to the crystalline and amorphous structure (Ma *et al*., 2018; Figueroa-Flórez *et al*., 2019). The FT-IR spectra depend on the changes in the starch structure in a short-range order, defined as the double helix order, and that reflects the number of double helices ratio (ordered domains) with simple helices (amorphous domains) (Xu *et al*., 2018). The determination of the order degree (OD) was made from the FT-IR spectra (Tab. 3), showing an increase after the enzymatic treatment (ANN-C>UTS>HMG>ANN>NPS) that are consistent results with those reported in previous studies on the enzymatic modification of cassava starch (Salcedo-Mendoza *et al*., 2018; Figueroa-Flórez *et al*., 2019). Modified starches pretreated by UTS and ANN-C showed the highest values of OD, possibly due to the release of free amylaceous materials and reorganization/recrystallization of amylose chains.

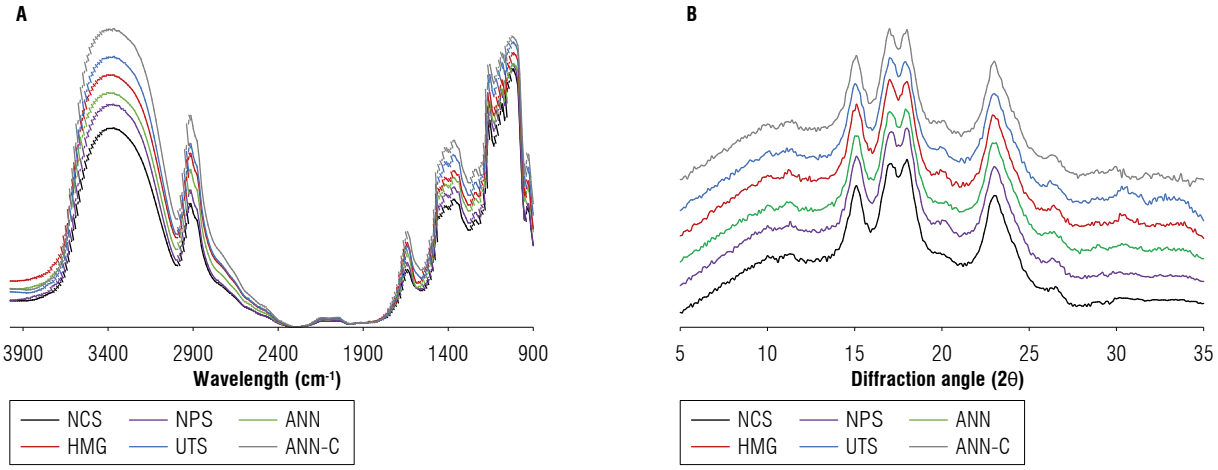

FIGURE 3. A) FTIR - infrared spectra and B) XRD - diffraction patterns in native and modified cassava starches. Native cassava starch (NCS); non-pretreated enzymatic modified starch (NPS); modified starch pretreated by annealing (ANN); modified starch pretreated by homogenization (HMG); modified starch pretreated by ultrasound (UTS), and modified starch pretreated by annealing and ultra-rapid freezing (ANN-C).

These results agree with the crystallinity index behavior estimated by XRD diffraction. In turn, Xu *et al*. (2018) linked the increase in the bands 1045/1024 ratio with the time and number of cycles of annealing treatment in bean starches and to the reorganization and a greater ordering of double helices of amylose and amylopectin in the crystalline domains of starch granules. By XRD diffraction, four crystallographic peaks were identified under angles of 15°, 17°, 17.8°, and 23° characteristic of tubular starches with diffraction pattern A-type (Fig. 3B) whose double amylopectin helices achieve a conformation in monoclinic form (Jayakody & Hoover, 2008). Starches showed higher intensity in peaks after physical and enzymatic treatments without evident changes in diffraction patterns. A similar behavior was found in previous studies possibly due to the decrease in amylose content in cassava starch granules (Salcedo-Mendoza *et al*., 2018; Figueroa-Flórez *et al*., 2019).

The crystallinity index increased in modified starches with respect to the control, in the following order: ANN-C>UTS>ANN>NPS>HMG (Tab. 3). The increase in CI of modified starches pretreated by UTS and ANN-C could be attributed initially to the removal of amorphous and unstable amylaceous materials by the action of the ultrasonic frequency, thermal energy, or enzymatic action (Li *et al*., 2018) affecting the structural and morphological properties as seen in FT-IR spectra and microscopy analysis.

Other research also has reported an increase in the crystallinity index of starch granules with the annealing process without affecting the characteristic diffraction pattern, due to the hydration and mobility of glucan chains by the action of temperature accelerate interaction and reorganization phenomena of amylose or amylopectin double helices (Jayakody & Hoover, 2008; Xu *et al*., 2018; Zhang *et al*., 2019), as well as the formation of additional intra-helical hydrogen bonds that result in an increase in crystalline sheet thickness during cooling (Jayakody & Hoover, 2008; Seetapan *et al*., 2016).

These results allow inferring that restructuring the short chains present in the amorphous and crystalline domains, together with the decrease of amylose content, altered the semicrystalline order of granules, and affected both the structural resistance of the granules to the enzymatic action and the behavior of thermal properties.

Thermal properties

In relation to gelatinization properties, significant changes in temperatures (T_O, T_P, and T_C) after the enzymatic process were estimated (Tab. 3), where the increase in T_O can be explained by the cleavage of glycosidic bonds that induce molecular and structural changes, affecting mainly the swelling process that regulates gelatinization (Chen *et al*., 2011; Dura *et al*., 2014). Likewise, recent investigations have explained that changes in transition temperatures are related to an increase of the crystallinity index, an indicator of structural stability and granular molecular resistance to gelatinization (Jung *et al*., 2017; Zhao *et al*., 2018; Xie *et al*., 2019). These results are consistent with those found from the values of OD and CI determined by FT-IR and XRD diffraction. No significant differences were detected in T_O values between samples of hydrolyzed starches with the NPS, ANN, HMG and UTS treatments, except for the exception of the ANN-C treatment. The difference in the behavior of T_O through the ANN-C cycle may be due to the

TABLE 3. Crystallinity index and gelatinization properties in native and modified cassava starches.

Sample	CI	OD	To (°C)	Tp (°C)	Tc (°C)	(Tc-To) (°C)	ΔH (J/g)
NCS	0.487±0.006^{a}	0.475±0.000^{a}	64.49 ± 0.13^{a}	68.22 ± 0.22^{a}	86.72 ± 0.44^{a}	22.23 ± 0.20^{a}	19.34 ± 0.33^{a}
NPS	0.496±0.007^{b}	0.483±0.009^{b}	65.30 ± 0.11^{b}	70.14 ± 0.19^{b}	84.77 ± 0.27^{b}	19.45 ± 0.11^{b}	15.19 ± 0.41^{b}
ANN	0.491±0.010^{b}	0.486±0.004^{b}	70.27 ± 0.15^{bc}	73.17 ± 0.25^{c}	85.79 ± 0.76^{b}	15.52 ± 0.03^{c}	14.51 ± 0.71^{ab}
HMG	0.495±0.000^{b}	0.472±0.005^{a}	70.36 ± 0.09^{bc}	73.01 ± 0.17^{bc}	87.41 ± 0.52^{c}	17.05 ± 0.17^{d}	14.98 ± 0.50^{abc}
UTS	0.502±0.004^{c}	0.497±0.002^{c}	70.43 ± 0.16^{c}	73.28 ± 0.23^{c}	87.77 ± 0.53^{c}	17.34 ± 0.06^{e}	15.69 ± 0.54^{ac}
ANN-C	0.511±0.008^{d}	0.506±0.009^{c}	72.96 ± 0.10^{d}	75.38 ± 0.17^{d}	86.13 ± 0.72^{b}	13.16 ± 0.15^{f}	15.79 ± 0.63^{acd}

OD - order degree; CI - crystallinity index; To - onset temperature; Tp - peak temperature; Tc - conclusion temperature; ΔH - gelatinization enthalpy. Native cassava starch (NCS); non-pretreated enzymatic modified starch (NPS); modified starch pretreated by annealing (ANN); modified starch pretreated by homogenization (HMG); modified starch pretreated by ultrasound (UTS), and modified starch pretreated by annealing and ultra-rapid freezing (ANN-C). Different superscripted letters in the same column indicate significant differences for Tukey test ($P<0.05$).

initial endothermic transition caused in granules during the annealing process, where water diffuses freely in the amorphous and crystalline regions altering the semicrystalline order, together with the reorganization of short chains during ultra-rapid-freezing process (Waduge *et al*., 2006; Jayakody & Hoover, 2008; Seetapan *et al*., 2016; Zhao *et al*., 2018). Authors argue that a greater reorganization of double helices during annealing resulting from an accentuation in the mobility of the glucan chain leads to the formation of intra-helical hydrogen bonds changing the thickness of the crystalline sheet and ultimately the melting temperature of double helices, delaying gelatinization (Kiseleva *et al*., 2004). Analogous results have been found in the SP behavior (Tab. 1).

The temperature range (T_O-T_C) showed a marked difference with the implementation of pretreatments followed by the enzymatic process with respect to the NCS samples. This behavior is supported by the premise that enzymatic hydrolysis causes both morphological and structural changes that limit the thermal behavior of starch granules (Gao *et al*., 2013; Li *et al*., 2018). Likewise, the increase in gelatinization temperature is more pronounced for T_O (fusion of the weakest crystallites) and less for T_C (fusion of stable and more perfect crystals) during the annealing process (Kiseleva *et al*., 2004; Jayakody & Hoover, 2008). For this reason, the decrease of T_O -T_C range with the ANN-C treatment, possibly due to a higher homogeneity, cooperative fusion, and more perfect crystals. This allows us to understand the most notorious changes on properties such as swelling, crystallinity, and susceptibility to enzymatic action after the hydrothermal pretreatment ANN-C. These same premises could explain the increase of ΔH in modified starches that is influenced by the hydration of the macromolecule and the interaction between polymer chains that alter the assembly of amorphous and crystalline lamellar structures (Jayakody & Hoover, 2008; Shariffa *et al*., 2017; Xie *et al*., 2019). However, the increase of ΔH in starches with different amylose contents after annealing can be correlated with an interaction of the short chains that can form new double helices or the granular conformation of the smectic type, whose reorganization will require higher values of energy to melt and start the gelatinization process (Kiseleva *et al*., 2004; Waduge *et al*., 2006; O'Brien & Wang, 2008; Dias *et al*., 2010).

Conclusions

The integrated processes of enzymatic biocatalysis and physical-thermal pre-treatment introduced here allowed the development of modified cassava starches with microporous surfaces. The enzymatic process caused significant changes in the structural characteristics of such starches, altering their semicrystalline order and gelatinization. The HMG decreased the size of the granules, thus, affecting their swelling capacity and gelatinization properties that are defined by a flexible and unstable granular configuration of small and fragmented granules. In turn, UTS pretreatments and the ANN-C cycle caused changes in morphological properties and homogeneously increased the size and volume of the pores in the granular surface, thus decreasing the range and enthalpy of the gelatinization. The ANN-C cycle can be an affordable and effective pretreatment in the production of thermally stable microporous cassava starches. As a result, we recommend optimizing the hydrothermal annealing process in the production of porous cassava starches considering variables such as starch/water ratio, heating time, and temperature.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author's contributions

JAFF, ECC, and ERS designed the experiments; JAF and EDAD carried out the experiments and data collection in laboratory experiments. JAFF, EDAD, and ECC contributed to the data analysis. JAFF, EDD, ECC, HJC, JGSM, and ERS wrote the article. All authors reviewed the final version of the manuscript.

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