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Obtention of amaranth resistant starch through succinylation and phosphorylation reaction

Obtención de almidón resistente de amaranto mediante reacción de succinatación y fosforilación

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Abstract
Starch is the human diet's primary energy source and is naturally found as a polysaccharide in grains. Starches from familiar sources such as corn, potato, and rice have been widely studied. However, starch extraction from pseudocereals such as the amaranth seed has also been studied due to their functional characteristics.

Starch is the principal component of the amaranth raw seed (50%-60%), and 0.5% of it corresponds to resistant starch (RS). RS is a fraction of starch not hydrolyzed in the small intestine within 120 min of consumption but can be fermented in the colon. This work studied the use of amaranth starch as a byproduct of protein and lipid extraction to produce RS. The starch was subjected to acid hydrolysis with HCl and modified with octenyl succinic anhydride (OSA) and sodium tripolyphosphate (STPP), obtaining a resistant starch yield of 47% and 56.7%, respectively. Nevertheless, the amaranth starch granules extract could be damaged either during their obtention or in the acid hydrolysis reaction, since water's low absorption and solubilization capacity was recorded. However, amaranth RS could display a low glycemic index and modulate metabolism, acting as dietary fiber.
Keywords: Amaranthus hypochondriacus, starch, resistant starch, succinylation and phosphorylation reaction.

Resumen
El almidón es la principal fuente de energía de la dieta humana y se encuentra de forma natural como polisacárido en los cereales. Los almidones de fuentes comunes como el maíz, la patata y el arroz han sido ampliamente estudiados. Sin embargo, la extracción de almidón de pseudocereales como la semilla de amaranto también se ha estudiado debido a sus características funcionales.

El almidón es el componente principal de la semilla cruda de amaranto (50%-60%), y un 0,5% corresponde a almidón resistente (AR). El AR es una fracción del almidón que no se hidroliza en el intestino delgado en los 120 minutos posteriores al consumo, pero que puede fermentar en el colon. Este trabajo estudió el uso del almidón de amaranto como subproducto de la extracción de proteínas y lípidos para producir AR. El almidón se sometió a hidrólisis ácida con HCl y se modificó con anhídrido octenil succínico (OSA) y tripolifosfato de sodio (STPP), obteniendo un rendimiento de almidón resistente del 47% y el 56,7%, respectivamente. Sin embargo, el extracto de gránulos de almidón de amaranto podría dañarse durante su obtención o en la reacción de hidrólisis ácida, debido a la baja capacidad de absorción y solubilización del agua. No obstante, el AR de amaranto podría presentar un bajo índice glucémico y modular el metabolismo, actuando como fibra dietética.
Palabras clave: *Amaranthus hypochondriacus*, almidón, almidón resistente, reacción de fosfatación, reacción de succinatación.

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1 Introduction

Amaranth belongs to the *Amaranthaceae* family and is widely spread in North America, Central America, and South America (Singh & Punia, 2020). Nevertheless, amaranth crops have been introduced to Africa, Asia, and Australia due to the ability to grow in extreme conditions (Assad *et al*., 2017). There is no stipulated number of *Amaranthus* genus; however, it is estimated to be between 60 and 75 (Ward *et al*., 2013). Being *A. hypochondriacus*, *A. cruentus,* and *A. caudatus*, those with the most significant food potential (Sarangi *et al*., 2021).

Amaranth seed contains a higher concentration of protein (13-19%) and is a good source of fat (5-13%). Nevertheless, the principal component of amaranth seed is the starch (50-60%) stored inside the perisperm (Montoya-Rodríguez *et al*., 2015). Amaranth starch granule diameter ranges from 0.8-2.5 μm, which are very small compared to other granules such as rice (3-8 μm) or potato starch (10-15 μm). Additionally, it is low in amylose concentration (1%), conferring low viscosity, high solubility, and low temperature gelatinization compared to other starch sources such as corn starch (Capriles *et al*., 2008; Arendt & Zannini, 2013). Recent studies report the use of amaranth starch as a thickener replacer, which can be used in the production of low-calorie food (Kierulf *et al*., 2024).

A fraction of starch is not hydrolyzed by amylases in the small intestine and passes to the colon, where the microbiota ferments it. This fraction is called resistant starch (RS; Birt *et al*., 2013). Amaranth has only 0.5% resistant starch in raw seed and increases to 1.36% when it is subjected to expansion with hot air (Gamel *et al*., 2005). RS has important advantages over conventional starches such as: improved solubility and adhesion, less tendency to retrogradation and stability at high temperatures (Bhosale & Singhal, 2006; Guerra-DellaValle *et al*., 2008) also compared to traditional fibers, RS has the advantage of not negatively affecting the acceptability of the product, in addition to providing improvements in appearance, texture and mouthfeel compared to fiber (Fuentes-Zaragoza *et al*., 2010).

RS's are divided into 4 categories depending on their nature: physically inaccessible such as some grains and seeds (RS1), resistant granules (RS2), retrograded starches (RS3) and chemically modified starches (RS4) such as starch esters, starch ethers and cross-linked starches (Gutierrez & Tovar, 2021).

Phosphorylated starch consists of introducing phosphate groups into the starch chains, which creates a repulsion between the phosphate groups on adjacent chains. Different reagents such as STMP (sodium trimetaphosphate), STPP (sodium tripolyphosphate), or a mixture of STMP-STTPP have been used to obtain phosphorylated starch, which has been classified as an RS4 (Malik *et al*., 2023). This chemical modification increases hydration and changes the physicochemical properties of the starch, such as gelling agent, thickener, colloidal stabilizer and water retention agent. Therefore, it has been used in the food industry such as additives in yogurt to decrease the syneresis, in bakery to increase the extensibility and viscoelasticity of the dough; stabilizer in frozen food; foam retention in foaming systems and improve roasting properties (Liu *et al*., 2022; Ramadan & Sitohy., 2020).

Chemically modified starches with octenyl succinic anhydride (OSA) are one of the chemically modified starches obtained by substitution modification by chemical groups (Bai *et al*., 2014). The Food and Drug Administration (FDA) restricts food grade succinate starches to a maximum incorporation of 3% OSA based on the weight of starch (Bhosale & Singhal, 2007). Succinate starches, unlike typical surfactants, form strong films at the oil-water interface, resulting in emulsions that are resistant to re-agglomeration, so they can be used to stabilize the flavor of emulsified beverages, oils in salad dressings, and to encapsulate flavors and fragrances (Altuna *et al*., 2018; Bhosale & Singhal, 2006). OSA starches display hydrophilic and hydrophobic character. Therefore, it has been used in the production of sauces, puddings, and baby foods (Bajaj *et al*., 2019). Nevertheless, OSA starches are used mostly in bakery, as they increase water absorption and resistance to deformation, enhancing the bread volume and texture (Korus *et al*., 2021).

This work aimed to obtain succinate and phosphate amaranth starch with a high resistant starch content and evaluate its physicochemical characteristics.

2 Materials and methods

2.1 Material

Acetic acid, boric acid, bromocresol green, chlorohydric acid, copper sulfate pentahydrate, hexanol, methyl red, phenol, potassium sulfate, selenium dioxide, sodium acetate anhydrous, sodium hydroxide, and sulfuric acid were purchased from J. T. Baker (Mexico State, Mexico).

2-Octen-1, succinyl anhydride (mixture of cis and trans 97%), sodium triphosphate pentabasic (purity ≥98%), Tris-hydroxymethyl aminomethane), were all purchased from Sigma Aldrich (St. Lois MO, USA)

Protease from *Bacillus licheninforomis,* (E.C. 3.4.21.62) at ≥2.4 U/g solid, α-amylase thermostable from *Bacillus licheninformis* (E.C. 3.2.1.1) at 1 U/mL, amyloglucosidase from *Aspergillus niger* (E.C. 3.2.1.3) at 30-60 U/mg of protein, pepsin from porcine gastric mucosa (E.C. 3.4.23.1) at ≥250 U/mg solid and

porcine pancreatic amylase type VI-B (E.C. 3.2.1.) at ≥ 10 U/mg were purchased from Sigma Aldrich (St. Lois MO, USA).

Glucose-SL (glucose-oxidase-peroxidase) was purchased from Sekisui Diagnostics (Burlington, Massachusetts, USA).

2.2 Physicochemical characterization of the amaranth starch

Bromatological analysis of the amaranth flour and amaranth starch was performed according to the Official Methods of Analysis (AOAC), which include lipids (920.30), proteins (920.10), crude fiber (978.10), soluble fiber (991.43) and starch concentration by the determination of total carbohydrates, which were quantified by the Dubois *et al*. (1956) technique.

2.3 Starch obtention

Amaranth popped seed flour (*A. hypochondriacus*) was obtained from a local producer of Santiago Tulyehualco (Mexico). An Amaranth:Water suspension (1:4) was made. To deactivate native enzymes in amaranth flour, the suspension was boiled for 10 min and then cooled to 50 °C. Later, 0.036 U/g of *Bacillus licheninformis* protease was added (E.C. 3.4.21.62. ≥2.4 U/g, Sigma Aldrich), and it was incubated in a thermoregulated bath at 50 °C with horizontal stirring at 100 rpm for 4 h (LabTech LSB-015S, Beijing, China). The suspension was brought to 90 °C for 10 min to deactivate the protease and centrifuged at 6000 rpm for 40 min (Avanti J-E, Beckman Coulter, Indianapolis, Indiana, USA). The supernatant containing lipids and proteins was separated, and the pellet consisting of a mix of starch-fiber was dried in a convection oven for 24 h at 45 °C (Felisa FE-291AD, Jalisco, México). Then it was milled and sieved, passed through a 250 μm mesh.

2.4 Starch quantification

Starch content was quantified as described by Soto-Azurduy (2010). This method allows the determination of the total starch content in a sample, ensuring complete availability of the starch for enzymatic degradation. In this procedure, the gelatinization and liquefaction steps were performed simultaneously.

A total of 100 mg of the sample was mixed with 6 mL of distilled water and 0.05 U of α-amylase. The suspension was incubated in a 97 °C water bath for 15 minutes, with gentle shaking every 5 minutes. After incubation, the tube was allowed to cool to room temperature while shaking was continued. Once cooled, the mixture was transferred to a 25 mL volumetric flask and diluted to volume with distilled water.

Next, 1 mL of this solution was transferred to a centrifuge tube, followed by the addition of 2 mL of sodium acetate buffer (0.1 M, pH 4.75). Then, 2.25 U of *Aspergillus niger* amyloglucosidase was added, and the sample was incubated at 60 °C for 30 min (Combi-SV12 FINE PCR), with gentle mixing every 5 minutes.

After incubation, the sample was centrifuged at 6000 rpm using an Eppendorf 5804R centrifuge. The supernatant was transferred to a 100 mL volumetric flask, the tube was rinsed with distilled water, and the rinsate was added to the flask, which was then filled to the mark with distilled water.

For glucose quantification, 20 μL of the resulting solution was mixed with 20 μL of distilled water and 2 mL of glucose quantifier reactive (Glucose-SL, Sekisui Diagnostics) in a centrifuge tube. Absorbance was measured at 500 nm using a spectrophotometer (Shimadzu UV-160A, Japan), interpolating the absorbance in a glucose anhydride calibration curve. Total starch quantification was performed according to *Equation 1*:

$$Total\ starch\ (\%) = \frac{Glucose\ (mg) x25 x100 x0.9}{Sample\ (mg)} x100(\%) \quad (1)$$

2.5 Modified starch obtention

2.5.1 Hydrolyzed starch

Starch suspension at 40% solids was prepared in a hydrochloric acid solution at 3.4% (concerning starch solids). Hydrolysis was performed at 50 °C in a thermoregulated bath with horizontal stirring at 100 rpm for 4 h. The suspension was centrifuged at 6000 rpm for 10 min. The supernatant was discarded, and the pellet was washed twice with distilled water. The sediment was dried at 45 °C for 24 h, ground, and sieved through a 250 μm mesh. The degree of hydrolysis (%) was calculated as the percentage ratio of the hydrolyzed starch solids based on the starch sample's previous hydrolysis, expressed as *Equation 2:*

$$Hydrolysis\ (\%) = \frac{Hydolyzed\ starch\ solids}{Initial\ starch\ solids} \quad (2)$$

2.5.2 Succinate starch

Succinate starch was prepared according to Bhosale & Singhal (2006) with slight modifications to optimize the reaction. A suspension of hydrolyzed starch: water (1:3.8) was prepared. An aliquot of 10 mL was taken and 3 mL of OSA was added and kept at 30 °C for 2 h under constant horizontal stirring at 100 rpm. Different reaction times (3, 4, 5, 6, and 7 h) and pH's

(6, 6.5, 7.5, and 8) were tested by adjusting the pH with NaOH (3%). At the end of the reaction, the pH was adjusted to 6.5 and centrifuged at 6000 rpm for 10 min. The sediment was washed twice with distilled water to remove reagent residues. The succinate starch was dried at 40 °C for 24 h, ground and sieved through a 250 μm mesh.

2.5.3 *Phosphate starch*

The phosphorylation of amaranth starch was performed according to Paschall (1964) with slight modifications. The hydrolyzed starch was dissolved in a 4% sodium tripolyphosphate solution (pent basic sodium triphosphate ≥98%; Sigma Aldrich) in a 1:5 ratio, and the pH was adjusted to 6 with 10% NaOH. Different temperatures (30, 40, 50, and 60 °C) and reaction times (1, 2, 3, 4, and 5 h) were tested. The suspension was heated at 25 °C in a thermoregulated bath with horizontal stirring at 100 rpm. At the end of the reaction, the suspension was centrifuged at 5000 rpm for 5 min. The supernatant was discarded, and the residue was washed with distilled water and centrifuged at 5,000 rpm for 5 minutes, repeating the procedure twice. The residue was dried at 40 °C for 48 h, ground, sieved through a 250 μm mesh, stored in cellophane bags, and subjected to dialysis in water at 4 °C for 4 days, changing the water daily. The phosphate starch was dried at 30 °C for 12 h.

2.6 *Degree of substitution quantification*

The degree of substitution (DS) of the OSA and phosphate modified starch were determined by a titration method. In brief, 1 g (dry basis) of the starch sample was combined with 10 mL of distilled water containing two drops of 1% (w/w) phenolphthalein in a flask. The suspension was then treated with 0.1 N NaOH until a pink coloration appeared. Subsequently, 25 mL of 0.5 N NaOH was added, and the mixture was stirred at 27 ± 2 °C for 40 minutes. The solution was then titrated with 0.5 N HCl to pH 7, indicated by the disappearance of the phenolphthalein color. A blank titration was performed using native starch for comparison.

The DS value of the succinylated starch was calculated according to *Equations 3* and *4*:

$$OSA\ sustitution\ (\%) = \frac{\left(V_{blank} - V_{sample}\right) x 0.1 N x 100\%}{Sample\ (g)} \tag{3}$$

$$DS = \frac{162\ x\ OSA\ sustituion\ (\%)}{21{,}000 - (209(OSA\ sustitution\ (\%))} \tag{4}$$

Where V is the volume of HCl used either in blank or sample titration.

The DS value of the phosphate starch was calculated according to *Equations 5* and 6.

$$A\ (\%) = \frac{\left(V_{blank} - V_{sample}\right) x 0.5 N x 158 x 100\%}{Sample\ (g)} \tag{5}$$

$$DS = \frac{162A}{15800 - 156A} \tag{6}$$

Where A is the content of esterified carboxyl groups (%), V is the volume of HCl used either in blank or sample titration.

2.7 *Resistant starch quantification*

Conversion degree from amaranth native starch to resistant starch by succinate or phosphate was calculated according to Goñi *et al*. (1996). 100 mg of the sample was dissolved in 10 mL of KCl-HCl buffer (pH 1.5) and homogenized. Later, 60 UE of pepsin from porcine gastric mucosa (345 UE/mg solid) was added and incubated at 40 °C for 60 min with orbital agitation at 100 rpm (Combi-SV12, FinePCR, Gyeonggi-do, Korea) and allowed to cool at room temperature. Then, 9 mL of Tris-maleate buffer (0.1 M, pH 6.9) was added to the previous solution, mixed with 200 UE of α-amylase (10 UE/mg), and incubated at 37 °C for 16 h with orbital agitation. The samples were centrifuged for 15 min at 7000 rpm. Supernatants were discarded, and the pellet was resuspended in 3 mL of distilled water and 4 mL of KOH (4 M) and agitated for 30 min at room temperature. Then, 5.5 mL of HCl (2M) and 3 mL of acetate buffer (0.4 M, pH 4.75) were added to the previous solution to reach a pH of 6.9. Later 8 UE of amyloglucosidase from *Aspergillus niger* was added. The samples were centrifuged at 6,000 rpm for 15 min. Supernatants were collected. The pellets were washed with 10 mL of distilled water and centrifuged under the same conditions. The supernatants were mixed, and distilled water was added to reach a final volume of 25 mL. The glucose released was quantified using the glucose oxidase-peroxidase method. Briefly, 20 μL of the last sample was added to 2 mL of the glucose quantifier reactive (Glucose-SL, Sekisui Diagnostics), vortexed and incubated at 37 °C for 30 min. Glucose was spectrophotometrically quantified at a wavelength of 340 nm (Shimadzu UV 160-A, Japan), interpolating the absorbance in a glucose anhydride calibration curve. The resistant starch of the test sample was calculated according to *Equation 7*:

$$RS\ (\%) = \frac{Glucose\ concentration\ (mg)\ x\ 0.9}{Sample\ (mg)} x 100 \tag{7}$$

2.8 *Water absorption index (WAI) and water solubility index (WSI)*

Both indexes were calculated according to Anderson (1969). Briefly, 0.25 mg of the starch samples were resuspended in 3 mL of distilled water. The samples were incubated at 30 °C with orbital agitation at 200 rpm for 30 min and then centrifuged at 6,000 rpm for 10 min. The supernatant was dried until constant weight at 80 °C, and then, both the weight of the evaporated sample and the sedimented dried gel (pellet of the fermentation) were registered. WAI were calculated as indicated in *Equation 8* and WSI according to *Equation 9*:

$$WAI = \frac{Sedimented\ gel\ (g)}{Dry\ weigh\ of\ the\ sample\ (g) - Weight\ of\ the\ evaporated\ sample\ (g)} \tag{8}$$

$$WSI = \frac{Weigh\ of\ the\ evaporated\ sample\ (g)}{Dry\ weigh\ of\ the\ sample\ (g)} \tag{9}$$

2.9 *Scanning electron microscope (SEM)*

The starch samples were visualized in a scanning electron microscope and secondary emission with control of variable vacuum ranges of variable pressure (JEOL JSM-5900 LV), with a coupled EDS spectrometer (Oxford). The samples were mounted on aluminum SEM stubs with carbon adhesive tape and coated with gold (Dentum Vaccum Desk III). The conditions were: gold deposition for 60 seconds until 4 minutes of deposition were completed under vacuum conditions of 50 mTorr. The samples were kept in a sealed Petri dish and dried for 24 h. The samples were visualized under vacuum conditions using an acceleration voltage of 10 kV.

2.10 *Statistical analysis*

All experiments were performed in triplicate, and the results are reported as the mean value ± standard deviation. The statistical analysis was carried out with the IBM SPSS Statistic version 25.0 for Windows (IBM, New York, USA) software. A one-way analysis of variance (ANOVA) was performed to compare all pairs of groups, followed by Tukey's test. A p-value of 0.05 was considered statistically significant.

3 Results and discussion

3.1 *Physicochemical analysis*

Amaranth flour had 54±1.8% of starch, 15.41±1.6% of protein and 9.6±1% of lipids. Khan & Duta (2018) analyzed the popped amaranth flour from *A. hybridus*. The authors reported a carbohydrate concentration of 60.57%±0.06, protein of 11.59%±0.18, and lipids of 7.97±0.15, which differ from the concentrations in this work. Calva-Cruz *et al.* (2023) reported a proximal composition of popped amaranth (*A. hypochondriacus*) of protein: 15.8%±0.6, lipids: 6.7%±1.4 and carbohydrates: 63.4%±3.9. This can be attributed to the difference in the amaranth variety and the cultivation conditions (Venskutonis & Kraujalis, 2013; Shevkani *et al*., 2014).

The RS concentration was determined at 6.9±0.7% which is higher than the data reported by Capriles *et al*. (2008). Authors stated a concentration of RS of 0.50±0.02% for pooped amaranth seed (*A. caudatus*), differences can be related to the amaranth variety.

The method proposed in this work allows obtaining a starch extraction yield of 63.3% with high purity (95%±3.4), with a low protein (5%±0.4) and without lipids. Additionally, the fraction of soluble dietary fiber in the starch extract is 40%±0.4 and 3.0%±0.03 of crude fiber.

These results are similar to those reported by Resio *et al*. (2009), who obtained 67.7% by wet milling using sulfite solutions. Additionally, compared to Villarreal *et al*. (2013), they obtained a yield of 46.18% by wet grinding with prior protease and alkaline hydrolysis. The resistant starch content in amaranth seed (6.9%) was lower than in the starch extract (18%). Shoenlechner *et al*. (2008) reported a soluble dietary fiber in amaranth starch of 33.1 to 49.3 % for *A. hypochondriacus, whereas the crude fiber is reported in the range from 3-8%, depending on the extraction method and amaranth species* (Písaříková *et al*., 2009).

During the process of starch obtention, the high temperature and the prolonged reaction times could cause the gelatinization of the starch, resulting in the formation of RS3. This agrees with Hung *et al*. (2016), who reported an increase in slowly digestible starch and RS after subjecting rice starch to heat-humidity treatments.

3.2 *Acid hydrolysis*

Acid hydrolysis (also known as lintnerization) is one of the most used chemical methods to change the starch structure into a crystalline structure, which is resistant to enzymatic hydrolysis (Aparicio-Saguilán *et al*., 2014). The starch extract obtained was subjected to acid treatment, presenting a hydrolysis yield of 39.27%±1.35, which is lower than the data reported

by García *et al*. (2016). The authors stated a yield of 57.12% hydrolysis for gelatinized corn starch using 100 mL of HCl (1M) per gram of starch and a reaction condition of 50 °C for 2 h. The previous gelatinization of starch could influence the higher hydrolysis yield. Additionally, the small granule size of the amaranth starch contributes to a faster acid hydrolysis compared to bigger starch size granules (such as taro), as well as their amylose content, which is related to the hydrolysis rate; at a higher concentration of amylose, a lower rate of hydrolysis is registered. Furthermore, hydrolysis occurs faster in starch, with few amorphous zones (like amaranth starch). Nevertheless, crystalline regions are also hydrolyzed, indicating an alteration in all the zones of the starch granules. These zones are removed during acid hydrolysis (Bertoft, 2004; Chen *et al*., 2024).

3.3 Resistant starch and DS quantification by succinylation reaction

During succinylation, the hydroxyl groups of the starch molecules are substituted with carbonyl groups of the octenyl succinic anhydride (OSA). When modified OSA, naturally hydrophilic starch acquires hydrophobic properties due to the incorporation of octenyl groups. This modification results in amphiphilic starch molecules, which exhibit both hydrophilic and hydrophobic characteristics (Sweedman *et al*., 21013).

Fig. 1a shows an increment in the RS along with pH in the range 6-7.5, reaching the highest concentration at pH 7.5 (47.92 %) and the highest DS (0.02±0) in 6 h reaction time. Nevertheless, at higher pHs, the concentration decreased. Therefore, a pH of 7.5 was selected for further studies. Additionally, no change was detected in the RS content in the amaranth starch subjected to the same pH conditions but without the OSA reactive. The concentration of RS (47.92%) obtained in this work is higher than that reported by Bai *et al*. (2014) at a pH of 7.5. They recorded a yield of RS of 26.7% in waxy corn starch by using 3% of OSA and a reaction time of 30 min. Under slightly basic conditions, the reduction of hydrogen bonding between starch chains is promoted through the formation of alkoxide groups from starch hydroxyl (–OH) groups, while at pH higher than 8, the hydroxyl groups of starch are not sufficiently activated for the nucleophilic attack of the OSA residues to occur. This disruption of intermolecular bonds enhances the swelling of starch granules and facilitates the diffusion of OSA molecules into the expanded starch matrix (Bhosale & Singhal, 2006; Sweedman *et al*., 2013).

When working at a pH of 7.5, the RS starch in the reaction with OSA increased over time (Fig. 1b). The highest conversion to RS was recorded at 6 h (46.69%). Nevertheless, no significant changes were detected in the control starch (no OSA reagent). Furthermore, the maximum DS (0.02±0) was obtained at 6 h reaction time. Similar results have been reported by Bhosale and Singhal (2006). The authors evaluated the degree of OSA substitution in starch from *A. paniculatus* and reaction conditions of pH 8 and 30 °C and 6 to 30 h reaction times. The higher substitution degree was obtained at 6 h. When increasing the reaction time, the degree of OSA substitution decreased. Nevertheless, when working with waxy corn starch under the same reaction conditions, the higher degree of substitution was recorded when increasing the reaction time to 24 h.

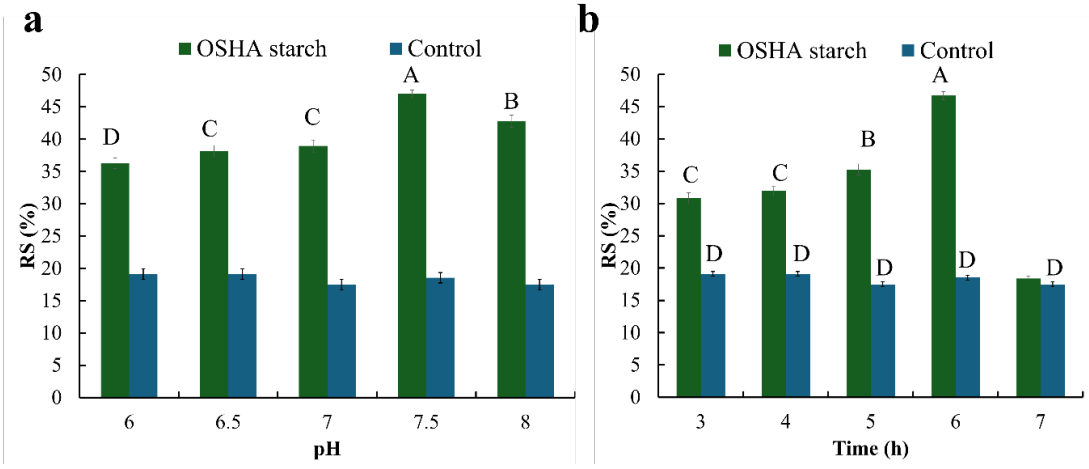

Fig. 1 Resistant starch concentration in the amaranth starch for the succinylation reaction a) Effect of pH, b) Effect of time. Error bars represent the standard deviation of the mean of triplicates.

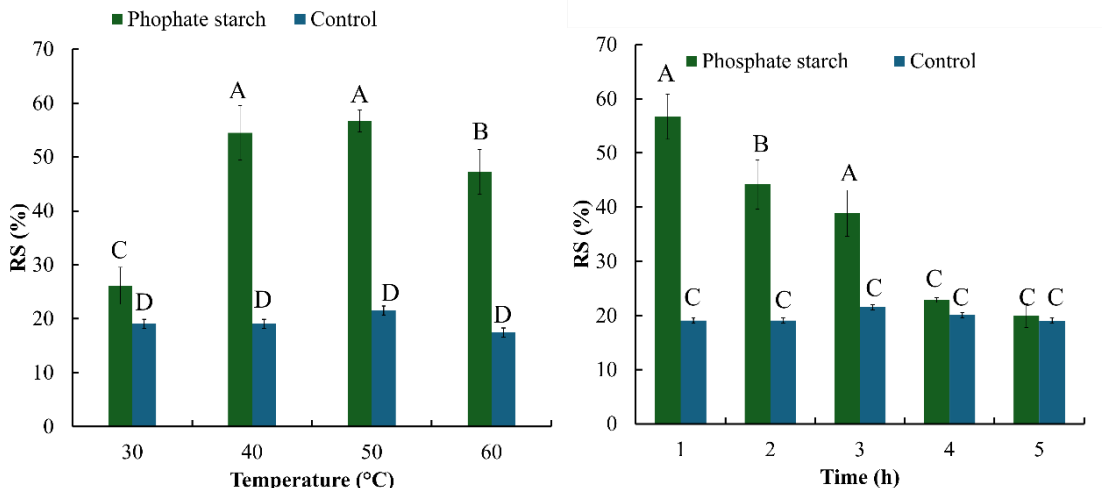

Fig. 2 Resistant starch concentration (RS) in the amaranth starch for the phosphorylation reaction a) Effect of temperature, b) Effect of time. Error bars represent the standard deviation of the mean of triplicates.

Additionally, by Fourier-transform infrared spectroscopy (FTIR), a strech in the 1245 cm^{-1} bands was observed in the fingerprint region between 1500-500 cm^{-1} of modified starches but absent in native starch (Suárez-Castillo *et al*., 2024). Furthermore, a marked band between 1450 cm^{-1} and 1300 cm^{-1} is observed, which is a torsion of the C-H bond of the aldehyde group as a result of the introduction of the OSA molecule into the starch structure (Sánchez de la Concha *et al*., 2020). Unfortunately, FTIR analysis is reliable only if the degree of substitution (DS) is ≥0.3, when OSA DS starches for food-grade products must be ~0.02 (Sweedman *et al*., 2013).

The improvement of the yield of RS during the succinylation reaction due to increasing the time is a direct consequence of the favorable effect of the diffusion and adsorption of the reagents and the starch molecule. A decrease follows the gradual increase in conversion to resistant starch because the OSA monomer is depleted as the succinate reaction proceeds. Furthermore, the reactive sites of starch decrease due to structural modification in the starch backbone (Murúa-Pagola *et al*., 2009). Additionally, the modification of the starch is related to the botanical and morphological origin of the starch granules.

3.4 Resistant starch and DS quantification by phosphorylation reaction

Phosphorylated starch, a chemically modified starch, is produced in large quantities and widely used. The incorporation of phosphate groups into starch chains creates repulsion between adjacent chains and enhances hydration. As a result, even at a low DS, increasing the phosphorylation degree can significantly alter the starch's physicochemical properties, such as rheological, thermal, and nutritional properties by increasing the ion-exchange, gel-forming, and intrinsic biological potential (such as prebiotic), respectively (Liu *et al*., 2022; Polnaya *et al*., 2013). The phosphorylation with STPP allows obtaining a monostich phosphate by an esterification reaction in which one phosphate is transferred into one hydroxyl group of a glucose unit (Ramadan & Sitohy, 2020).

During phosphorylation reaction at 40 and 50 °C, one hour and pH 6 (Fig. 2a), the highest conversion from native to resistant starch is obtained with no significant difference between temperatures (54.47±5.0 and 56.71±2.03, respectively). Additionally, the DS recorded was 0.03±0.001 and 0.03±0.001 for 40 and 50 °C, respectively. 1 Nevertheless, the concentration of RS is lower than the one reported by Sang *et al*. (2010) for wheat starch. The authors stated a 68% RS at reaction conditions of 40°C, pH 12, and a phosphate solution (sodium monophosphate, sodium sulfate, and sodium tripolyphosphate). The difference between the yields could be due to the starch's botanical origin, the reagent's type and concentration.

Fig. 2b shows that at 50 °C and one hour of reaction using STPP as a phosphating agent, the highest conversion to resistant starch was obtained compared to the succinylation reaction. As the reaction time increases, the conversion yield decreases. This could be because the amaranth starch begins to gelatinize at this temperature, and the long reaction time can cause the granules to swell, decreasing their reactivity to the chemical groups. Malik *et al*. (2023) stated the highest phosphorylation degree (51.71 %) at pH 9.5 and 70 °C in the phosphorylation reaction of mandua starch with a mix of STPP and Sodium STMP.

Additionally, FTIR studies showed a particular peak at 1417 $cm^{-1,}$ which is characteristic of the P=O stretching vibration (Liu *et al*., 2022). Esterified amaranth starch (such as phosphorylated) enhanced

their fat replacer characteristics. Therefore, they can be used to partially replace fat in fat-rich products, which can help to minimize health effects associated with the consumption of fatty foods (Fusuan *et al*., 2018).

3.5 Determination of WAI and WSI of the modified starch

The WAI of starch is an indicator of its application in the food industry and a parameter of the starch-polymer's stability against water, as well as an indicator of the starch degree gelatinization, since starch does not absorb water at room temperature (Seker *et al*., 2006; García-Cordero *et al*., 2024). The amaranth flour showed a WAI of 10.93±1.11 (Fig. 3). The WAI recorded in his work is higher than the data reported by Lux *et al*. (2022) for popped amaranth flour from *A. caudatus*. Authors stated a WAI of 6.77±0.21 at 80 °C and a soaking time of 1 h. The difference in WAI could be due to the amaranth variety. The highest WAI in the popped amaranth flour is a result of the gelatinization of the starch and denaturation of the proteins by the heat, resulting in a rapid and strong water absorption (Burgos & Armada, 2021; Gamel *et al*., 2005). Additionally, the WAI in the amaranth flour was higher compared to the starch; similar results have been reported by Zamudio-Flores *et al*. (2015). Authors recorded a higher WAI in oat flour compared to their starch extracts.

On the other hand, in the extract starch modified by acid hydrolysis, the WAI decreased (3.74 ±0.98). The treatment with HCl hydrolyzed the starch into small chains, decreasing its capacity to retain water molecules (Hung *et al*., 2016). After the succinylation and phosphorylation reaction, the WAI increased with no statistical difference between treatments ($p > 0.05$). Nevertheless, the WAI is still lower for all the modified starches than for the amaranth flour. Different results have been reported by Ashwar *et al*. (2017), the authors recorded a 2-2.8-fold increment in the WAI of the phosphorylated rice starch with STMP/STTP. Furthermore, the increase in the WAI of the starch from hybrid maize after the succinylation reaction with succinic anhydride was reported by Lawal (2004) compared to the native starch.

The WSI of starch refers to measuring how much of a starch sample can dissolve in water as a relation of the percentage of soluble polysaccharides released from the starch granules (Rojas-Molina *et al*., 2020). The starch extract has a lower WSI, this may be mainly due to two reasons: the first is that the amaranth seed used in this experiment was popped, a process that causes degradation in the starch polymers, reducing their solubility in water (Lara & Ruales, 1999). The second reason could be related to the starch obtention process that includes the action of protease,

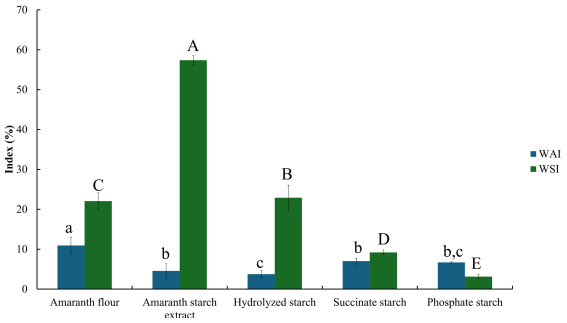

Fig. 3 Effect of the starch treatments in WAI and WSI. Error bars represent the standard deviation of the means of triplicate samples.

possibly attacking the protein matrix that protects the starch granules, causing damage to them. The nature of the granules is the factor that provides the hydration and solubilization capacity of starch in water (Contreras *et al*., 2013), regardless of the inclusion of chemical groups. Some authors have reported that as the RS content increases, there is a decrease in solubility values, which is attributed to the formation of crystalline zones; starches with a higher proportion of crystalline zones tend to have a lower capacity to solubilize in water (Neder-Suárez *et al*., 2016).

3.6 Granule morphology of the treated amaranth starches

Figure 4a shows the morphology of the popped amaranth flour; irregular patterns can be observed in the flour particles. Alonso-Miravalles *et al*. (2020) recorded irregular asymmetrical particles in pseudocereals such as amaranth. Figure 4b shows that the amaranth starch aggregates are more rounded and spaced apart from each other, which differs from that reported by other authors, where its morphology is described as polygonal grouped granules without fissures on the surface (Paredes, *et al*., 1989; Shindu & Singh, 2016). There could be two reasons for this. The first is that the amaranth used in this work was subjected to an expansion process that causes a change in the morphology of the starch granule. And the second is by the process of obtaining starch since conventional processes are carried out with a sodium hydroxide solution at low temperatures, which allows obtaining starch granules with minor changes in the physical, chemical, and rheological properties (Perez *et al*., 1993). In this research, the removal of protein and lipids from the starch granules influenced the final morphology of the granule, since these two constituents are considered to be responsible for the agglomeration of starch molecules (Qian & Kuhn, 1999).

When the amaranth starch extract is subjected to acid hydrolysis, a notable change in its morphology is observed.

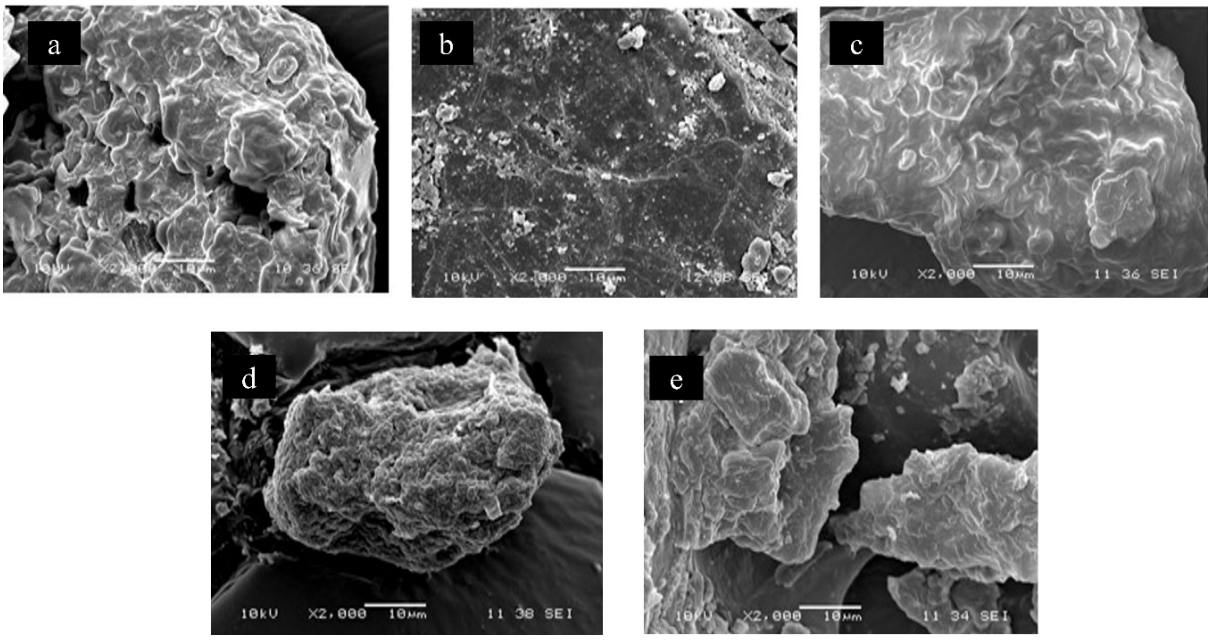

Fig. 4 SEM images of the a) amaranth flour, b) amaranth starch extract, c) hydrolyzed amaranth, d) phosphorylated amaranth starch, e) succinylated amaranth starch.

The rounded shape is lost, and a rougher, aggregate shape is now seen (Figure 4c). Martín & López (2009) stated changes in the external area of the cassava starch, modifying its smooth surface for slightly rougher regions. Additionally, the smaller granules hydrolyze quickly because of the more excellent available surface (Sanguanpong *et al*., 2003), likewise this starch presents a wrinkled surface, probably due to the contraction of the starch agglomerate caused by the high temperatures of the process of both obtaining the starch and its acid hydrolysis (Altay & Gunasekaran, 2006). In Fig. 4d and 4e, when chemical groups were introduced into the molecule, the size of the starch agglomerates increased. In general, these increases are due to the inclusion of phosphate and succinate groups within the starch agglomerates, which create specific repulsive forces that could increase the inter- and intra-molecular spaces, allowing the inclusion of a more significant number of water molecules (Liu *et al*., 2022). In Fig. 4e, it is observed that the succinate starch presents a rough surface, where some small particles are attached to the surface of the starch. This happens as the degree of substitution increases, its characteristic structure is replaced by a fiber-like structure (Xu, Miladino & Hanna, 2004). Liu *et al*. (2022) reported a rough surface and aggregation in the chestnut starch granules. The authors stated this morphological change to the gelatinization of the starch during the acetic anhydride addition. Additionally, Bajaj *et al*. (2019) reported that OSA modified starches from different sources, such as corn, potato, and rice, displayed rough surfaces, loss of the starch edges, development of superficial pores, and pronounced depression on the surface.

Conclusions

The methodology proposed in this work allowed the obtaining of 63.6% of starch from popped amaranth seed. Through the succinylation and phosphorylation reaction, a concentration of resistant starch of 47.92% and 56.7%, respectively, was obtained. Therefore, it could be added to food formulas granting health benefits such as low glycemic index, modulating metabolism, and increasing post-meal satiety.

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