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Enzymatic modification of starches to improve their technological and functional properties

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Thesis presented by

Eugenia Yaiza Benavent Gil

Supervised by

Cristina Molina Rosell

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CRISTINA MOLINA ROSELL, Profesora de Investigación del Consejo Superior de Investigaciones Científicas (CSIC) en el Instituto de Agroquímica y Tecnología de Alimentos (IATA)

CERTIFICA:
Que la presente Tesis Doctoral, titulada **"Enzymatic modification of starches to improve their technological and functional properties"**, ha sido realizada bajo su dirección en el Departamento de Ciencia de Alimentos del Instituto de Agroquímica y Tecnología de los Alimentos, por EUGENIA YAIZA BENAVENT GIL, licenciada en Enología por la Universidad Politécnica de Valencia y en el Programa Oficial de Postgrado de Ciencias de la Alimentación; y que habiendo revisado el trabajo, considera que reúne las condiciones necesarias para optar al grado de Doctor Internacional en Ciencias de la Alimentación.

Y para que así conste a los efectos oportunos, se expide el presente escrito.

Valencia,

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Esta tesis ha sido desarrollada en el Instituto de Agroquíca y Tecnología de los Alimentos (IATA) que pertenece al Consejo Superior de Investigaciones Científicas (CSIC) de España.

C/ Catedrático Agustín Escardino, 7

46980 Paterna, Valencia, Spain

Tel: +34 963 900 022

Fax: +34 963 636 301

https://www.iata.csic.es/

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List of original publications

The present thesis is based on the following publications, which will be referred to in the text by their Roman numerals.

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(III) Performance of granular starch with controlled pore size during hydrolysis with digestive enzymes.
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A mi madre, mi hermano Javi y Emilio

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Sin duda la vida es un viaje de múltiples paradas. Cada parada te lleva a un sitio nuevo, o con suerte a un lugar ya visitado al que merece la pena volver. Eso es para mi el IATA, un lugar del que me iré con la esperanza de volver. Y es que al final, una parada no es el lugar en sí, sino su gente, sus experiencias, su día a día. Y como toda buena parada aquí empieza mi álbum de recuerdos.

Mi entrada en el IATA fue para entregar un currículo. Poco después para hacer una entrevista con Cristina M. Rosell, la que hoy por hoy es mi directora de tesis. Reconozco que aquel día me impresionó, hoy ese sentimiento se ha convertido en admiración y agradecimiento. Agradecimiento por todo el tiempo, paciencia y esfuerzo que me ha dedicado, pero sobre todo por permitirme formar parte de su equipo. Y así empezó mi aventura como pre-doctoral, animada por, o mejor dicho gracias a mi amiga Raquel (Pakel). Con ella he compartido mucho, fuera, dentro, antes, espero que después, porque al final un buen amigo es para siempre. Pero esta parada, laboratorio 109 del IATA, es una parada en hora punta. Allí conocí a Marivi, que ha conseguido sobrevivir al Cereal-Team (Pakel-Yai). La de paciencia que tiene, siempre intentando poner calma, pero es que, el trenecito es lo que tiene, que es muy goloso. También a Andrea, mi primer estudiante, hoy amiga, y de la que me siento tremendamente orgullosa. A Jehannara, mija, el terremoto cubano, capaz de sacarte de quicio en un segundo pero a la que no se puede dejar de querer. A María, la red social, esa persona que sin darse cuenta es

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Abstract

In the last decade, porous polymers have attracted the attention of the food and non-food industries owing their great absorption capacity, which is dependent on the number and sizes of the pores. In this type of materials have been incorporated diverse polymers like starches after being subjected to physical, chemical or enzymatical treatments. The latter are considered the most promising due to the resulting materials are clean label polymers. However, up to now, the reported studies do not allow controlling the starch porosity. The objective of this doctoral thesis was the integral study of the production of porous starches by enzymatic treatment in order to modulate their structural and technological properties. With that purpose, native starches from different origin (corn, wheat, rice, potato and cassava) and diverse amylases (amyloglucosidase, alpha amylase, cyclodextrin-glycosyltransferase, branching enzyme) were used for producing porous starches, which were then characterized according to their structural and technological properties, as well as their *in vitro* digestibility. In addition, their viability as probiotic carriers was evaluated. The surface analysis of the starch granules, carried out by scanning electronic microscopy (SEM) indicated that the porosity of the starches could be modulated by either using enzymes with different hydrolytic activity, changing of enzymatic levels, or employing starches from diverse botanical origin. The amyloglucosidase led to porous starches with bigger pore sizes, and those were deeper in the case of cereal starches. Moreover, the control of starch porosity allowed changing the functionality of the starches, significantly affecting the water and oil holding capacities, the pasting and thermal properties and even their behavior during *in vitro* digestibility. Porous starches obtained from corn or rice after treated with alpha amylase were tested as probiotic carriers, yielding an increase of the thermal stability of *Lactobacillus plantarum*, especially after being coated with gelatinized starch. Additionally, the study carried out with the porous starches after being subjected to gelatinization opens new opportunities to obtain hydrogels with diverse structural and functional properties.

Resumen

En la última década, los polímeros porosos han atraído la atención de las industrias alimentarias y no alimentarias principalmente por su gran capacidad absorbente, la cual está determinada por el número y tamaño de poros. En este tipo de materiales se han integrado biopolímeros como los almidones tras ser sometidos a tratamientos físicos, químicos o enzimáticos. Estos últimos, son los que se consideran más prometedores al originar biopolímeros etiqueta limpia. Sin embargo, los estudios realizados no permiten un control de la porosidad de los almidones. El objetivo de esta tesis doctoral fue el estudio integral de la producción de almidones porosos mediante tratamientos enzimáticos que permitieran modular las propiedades estructurales y tecnológicas de los almidones. Para conseguir dicho objetivo se utilizaron almidones nativos de distintos orígenes (maíz, trigo, arroz, patata, tapioca) y distintas enzimas hidrolíticas (amiloglucosidasa, alfa amilasa, ciclodextrin glicosil transferasa, enzima ramificante) para la obtención de almidones porosos, los cuales se caracterizaron atendiendo a sus propiedades estructurales, tecnológicas y digestibilidad *in vitro*. Asimismo, se determinó su viabilidad como vehículos de probióticos. El análisis de superficie de los gránulos de almidón realizado mediante microscopía electrónica de barrido (SEM) indicó que la porosidad de los almidones se puede modular utilizando diversas estrategias como el empleo de enzimas con diferente actividad hidrolítica, variando la concentración de estas enzimas o utilizando almidones de distinto origen botánico. La amiloglucosidasa fue la hidrolasa que originó poros de mayor tamaño, los cuales fueron más profundos en los almidones de cereales. Además, el control de la porosidad permitió modificar la funcionalidad de los almidones, afectándose significativamente su capacidad de retener agua y aceite, su capacidad de formación de pasta, propiedades térmicas, e incluso su digestibilidad *in vitro*. Los almidones de maíz y arroz porosos obtenidos con alfa amilasas se aplicaron como vehículos de probióticos, observándose un incremento de la estabilidad térmica de *Lactobacillus plantarum*, sobre todo tras ser recubiertos con almidón gelatinizado. Además, el estudio

realizado con los almidones porosos tras su gelatinización abre la posibilidad de obtener hidrogeles con propiedades estructurales y funcionales diversas.

Resum

En l'última dècada, els polímers porosos han atret l'atenció de les indústries alimentàries i no alimentàries principalment per la seua gran capacitat absorbent, la qual està determinada pel nombre i grandària de porus. En aquest tipus de materials s'han integrat biopolímers com els midons després de ser sotmesos a tractaments físics, químics o enzimàtics. Aquests últims són els que es consideren més prometedors en originar biopolímers etiqueta neta. No obstant això, els estudis realitzats no permeten un control de la porositat dels midons. L'objectiu d'aquesta tesi doctoral va ser l'estudi integral de la producció de midons porosos mitjançant tractaments enzimàtics que permetessen modular les propietats estructurals i tecnològiques dels midons. Per aconseguir dita objectiva es van utilitzar midons natius de diferents orígens (blat de moro, blat, arròs, patata, tapioca) i diferents enzims hidrolítics (amiloglucosidasa, alfa amilasa, ciclodextrin glicosil transferasa, enzim ramificant) per a l'obtenció de midons porosos, els quals es van caracteritzar atenent a les seues propietats estructurals, tecnològiques i digestibilitat *in vitro*. Així mateix, es va determinar la seua viabilitat com a vehicles de probiòtics. L'anàlisi de superfície dels grànuls de midó realitzat mitjançant microscòpia electrònica d'escombratge (SEM) va indicar que la porositat dels midons es pot modular utilitzant diverses estratègies com l'ocupació d'enzims amb diferent activitat hidrolítica, variant la concentració d'aquests enzims o utilitzant midons de diferent origen botànic. La amiloglucosidasa va ser la hidrolasa que va originar porus de major grandària, els quals van ser més profunds en els midons de cereals. A més, el control de la porositat va permetre modificar la funcionalitat dels midons, afectant-se significativament la seua capacitat de retenir aigua i oli, la seua capacitat de formació de pasta, propietats tèrmiques, i fins i tot la seua digestibilitat *in vitro*. Els midons de blat de moro i arròs porosos obtinguts amb alfa amilases es van aplicar com a vehicles de probiòtics, observant-se un increment de l'estabilitat tèrmica de *Lactobacillus plantarum*, sobretot després de ser recoberts amb midó gelatinitzat. A més, l'estudi realitzat amb els midons porosos després del

seu gelatinització obre la possibilitat d'obtenir hidrogels amb propietats estructurals i funcionals diverses.

Contents

List of Figures

List of Tables

Abbreviations

ΔH	Gelatinization Enthalpy
AACC	American Association of Cereal Chemists
AM	α-amylase
AMG	Amyloglucosidase
ANOVA	Analysis of Variance
AUC	Area Under the Curve
BE	Branching Enzyme
$\boldsymbol{C}$	Equilibrium Concentration of Hydrolyzed Starch
$\boldsymbol{C_\infty}$	Maximum Hydrolysis
CD	CycloDextrin
CGTase	Cyclodextrin-Glycosyltransferase
DS	Digestible Starch
DSC	Differential Scanning Calorimetry
EY	Encapsulation Yield
GG	Guar Gum
GI	Glycemic Index
GOPOD	Glucose Oxidase–Peroxidase
GX	Xanthan Gum
HI	Hydrolysis Index
HPAEC	High Performance Anion Exchange Chromatography
$\boldsymbol{k}$	Kinetic Constant
Pv	Peak Viscosity
Pv1	Additional Peak Viscosity
Fv	Final Viscosity
RS	Resistant Starch
RVA	Rapid Visco Analyzer
S	Gelatinized Starch
SEM	Scanning Electron Microscopy
Tc	Conclusion Temperature
To	Onset Temperature
Tp	Peak Temperature
TS	Total Starch Content

Chapter 1

Introduction

The present introduction chapter corresponds to a book chapter entitled "Porous starch" in "Starch-based materials: Principles and applications" edited by O.H. Campanella and M. Miao, published by Elsevier, that is currently under preparation.

Contents

1.1 Porous materials

In spite of the diversity of materials available in nature, there is remarkable repetition in the structures observed among them. Natural materials are often organized as porous structures, that is, materials whose structural geometry originates cavities/pores within a continuous solid phase (Liu and Chen, 2014). The morphology of these structures is broadly similar regardless of the type of solid matrix used. Therefore, the same structure can be employed for the same purpose by organisms with different base materials. In addition, these structures are related with large variety of functions. In this sense, porous structures can act as mechanical support, providing strong and low weight structures (Kennedy, 2012), capable of resisting buckling and bending and/or increasing toughness (Naleway et al., 2015). But they also play an important role in creating different functionalities such as permeability to fluids, maintenance of the heating and providing paths for mass transport (Wegst et al., 2015). These outstanding properties observed are often a product of the structural organization at different spatial scales. Structural design elements such as open and closed cell foams, tubules, scaffolds or other highly porous materials, are commonly found as the macrostructure of a bulk natural material, but also as nano- or microstructural elements within a natural composite material (Naleway et al., 2015). Examples of the structure–function relationships of these structural design elements can be located in a number of diverse organisms from varying biological classes, illustrating the extensive range of environments where these design elements are detected. For example, bones of birds and other flying organisms are more porous than those of animals crawled on the ground, which results in much lighter weight. However, porous structures to reduce weight in otherwise dense materials has also be found in many terrestrial and marine organisms.

With this knowledge in mind, scientists and engineers use natural systems to develop synthetic porous materials that not only mimic the functions of natural materials, but also have new and superior properties. In this way, different natural structures have been used as templates for the design of

artificial materials. Up to now, several kinds of natural structures, such as insect wings (Miyako et al., 2013), cotton (Boury et al., 2012), bamboo (Tao et al., 2011), wood (Dong et al., 2002), shells (Ogasawara et al., 2000), skeletons (Meldrum and Seshadri, 2000), living cells (Chia et al., 2000) and diatoms (Anderson et al., 2000) have been employed to synthesize man-made materials. All these templates make it possible to obtain synthetic materials with a large specific surface area and multiple pore size distribution. For example, Xia et al. (2012) used natural porous lotus pollen grains to synthesize hierarchically porous NiO/C microspheres. Interesting results were obtained by Huang and Kunitake (2003), who developed an artificial fossilization process to replicate from macroscopic to nanometer scales of natural cellulosic substances. Nevertheless, porous structures fabricated using natural biotemplates exhibit non-ordered hole structure with irregularly-shaped (Hoa, Lu, and Zhang, 2006).

From an industrial point of view, the primary concerns are functionality of porous materials. As pore size is often associated with the type and range of applications, scientists aspire to control the size, shape, uniformity and periodicity of the porous spaces, as well as the atoms or molecules that constitute them. For that reason, a series of technologies have been applied to produce such materials, along with nearly all classes of inorganic, organic and organic–inorganic hybrid materials (Yang et al., 2017). The control and adjustment of these properties allow different materials to be achieved for the performance of a desired function in a particular application.

1.1.1 Classification of porous materials

Many studies have attempted to sort out the porous materials into different categories. Nevertheless, it is not an easy task to give a consistent classification of porous structure in solid materials. This difficulty lies in the different ways of defining the pores whether as physical frames, pore origin, structure, size or accessibility to surroundings, which generates several classifications.

The porosity of solid materials is defined as the ratio of pore/void volume to the apparent volume of the porous sample (Dullien, 2012). Thus, porous materials can be classified as low porosity, middle porosity, or high porosity

(Liu and Chen, 2014). Porosity influences phenomena such as sorption, thermal conductivity, diffusivity of water as well as mechanical and texture properties (Juszczak, Fortuna, and Wodnicka, 2002; Włodarczyk-Stasiak and Jamroz, 2009). Nevertheless, porous materials with similar porosity may react in a different way under the same conditions due to a random or graded variation in size, shape and distribution of their pores.

A simple discrimination of porous materials is typically referring to their pore size, which is associated with the transport mechanisms, and thus plays an important role for most applications. Furthermore, pore size allows to selectively capture specific molecular species into their structure at the exclusion of others. The International Union of Pure and Applied Chemistry (IUPAC) (Dubinin, 1960) recommended a specific nomenclature based on pore size. Based on this recommendation, Figure 1.1 represents the classification of porous materials by pore size.

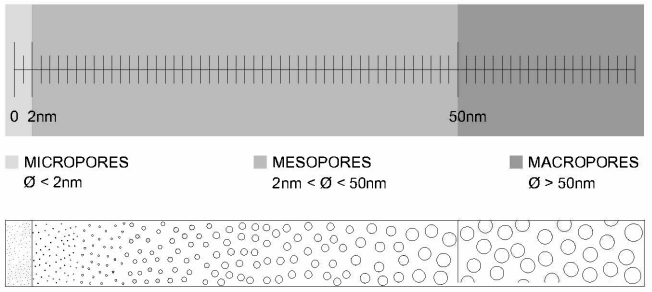

Figure 1.1: Classification of porous materials by pore size. According to The International Union of Pure and Applied Chemistry (IUPAC) pores can be classified as follows: pore sizes in the range of 2 nm and below are called micropores, those in the range of 2 nm to 50 nm are denoted mesopores, and those above 50 nm are macropores. Courtesy of Javier Benavent-Gil.

Regardless of porosity, pore size and pore shape, two kinds of pores are existent depending upon their accessibility to surroundings as depicted in Figure 1.2 (Zdravkov et al., 2007). According to this classification, the pores communicate with the surroundings in different forms with respect to the external surface. So-called open pores are those that communicate with the external surface (Figure 1.2 a-f), being accessible to molecules or ions in the

environment. In turn, these pores can be classified as blind pores, when the accessibility of pores is at only one side (Figure 1.2 a-c), or open pores at both ends (Figure 1.2 d-f). Conversely, so-called closed pores have no communication and association to the surroundings (Figure 1.2 g,h). The latter type is not associated with adsorption and permeability of molecules, but it has the potential to affect the mechanical properties of solid materials (Zdravkov et al., 2007).

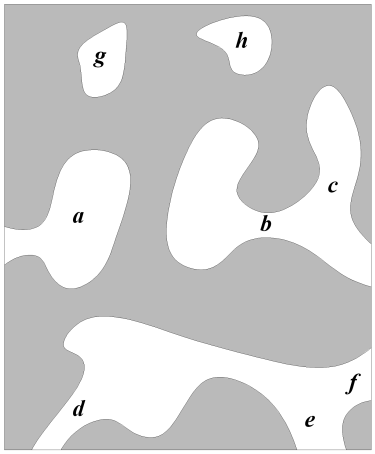

Figure 1.2: Schematic illustration of pores classification, according to their availability to surroundings. a-c: pores open only at one end; d-f: open pores at both ends; g,h: closed pores. Courtesy of Javier Benavent-Gil.

In addition to pore space, the type of building blocks (atoms, molecules, particles) and the way they create that space can be important. According to Liu and Chen (2014), two types of porous bodies can be defined. Natural porous bodies are those that can be found universally, while artificial porous bodies can be subclassified as porous metals, porous ceramics, and polymer foams.

1.1.2 Porous materials applications

Over the last decade, a wide range of materials along with some new designs have led to the production of diverse porous materials, which can find

potential applications beyond the traditional use as catalysts and adsorbents. As pore size of porous materials strongly determine their application, typical applications specific to the pore diameters are illustrated in Figure 1.3.

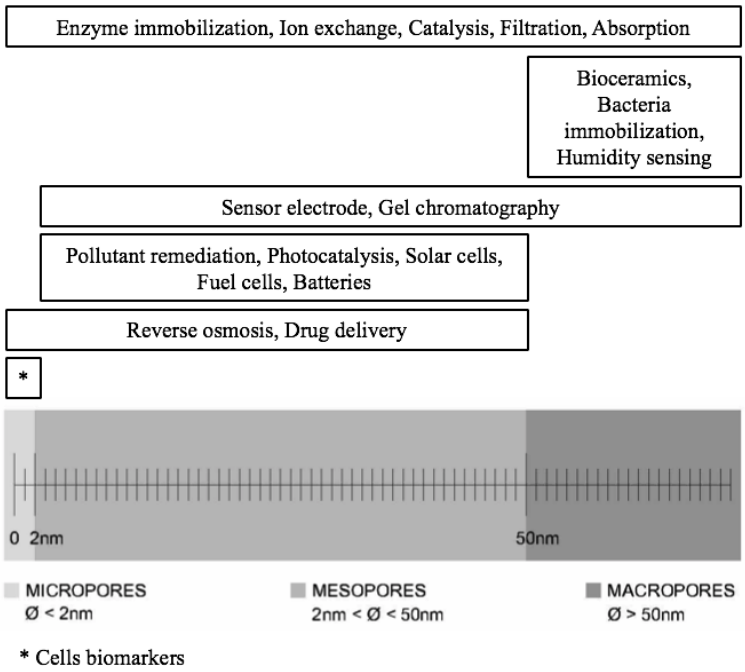

Figure 1.3: Classification of porous materials by pore size and corresponding typical applications (Davis, 2002; Ishizaki, Komarneni, and Nanko, 2013; Linares et al., 2014; Sharifi et al., 2014).

At the beginning, porous materials with porosities over lengths in the micro, meso to macro ranges have been mainly used as catalysts and catalyst supports. In many cases, mesoporous materials are employed as catalysts for the change of clean energy sources into electricity, fuels or valuable molecules. Some relevant examples include the transformation of biomass to high quality fuels, the efficient production of hydrogen or the valorization of CO_2 (Linares et al., 2014). Nevertheless, mesoporous materials are widely used beyond catalysis.

One of the most useful applications is in adsorption and separation processes

whereby pore wall can interact with different sized molecules, as well as the pore space can load or capture molecules in its different states (liquid, gas and solid). Porous materials possess unique properties such as high contact surface area, high storage volume, ready mass transport, shape selectivity and well controlled porosities over different length scales which lead to very high adsorption capacities and high separation efficiencies (Yang et al., 2017).

Owing to the high improvement of light absorption efficiency, porous materials are the prime choices for photosynthetic solar cells, photochemical bioreactors and photovoltaic solar cells (Yang et al., 2017). In life science issues, porous materials have become increasingly relevant with numerous potential applications. As an example, porous materials include applications in bio interface as cell culture substrates for cell adhesion, bone repair, biosensors with enhanced sensitivity, cell patterning, DNA/ protein microarrays or drug delivery (Hoa, Lu, and Zhang, 2006; Yang et al., 2017).

In spite of porous solids possess relatively high structural rigidity and low mass density, humans use porous materials more functionally than structurally, designing structural and functional applications to a complete use of them (Liu and Chen, 2014).

1.2 Starch as basis of porous starches

1.2.1 Form and surface structure

Granule size, shape and structure have been widely studied by a number of microscopy methods, as well as image analysis. Through these techniques, the botanical origin has been pointed out as a decisive factor in the size, shape, morphology, composition and supramolecular structure of the different starch granules. For instance, the main starches differ in their granule size, going from the big potato starch followed by wheat starch, tapioca starch, corn starch and finally the rice starch (Horstmann et al., 2016). Besides, these starches also differ in shape, including spheres, ellipsoids, polygon, platelets and irregular tubules. The micrographs have also revealed that

granules from wheat and potato are composed of two different populations, referred to large A-type granules and small B-type granules (Schirmer et al., 2013), that differ in both size and morphology (Lindeboom, Chang, and Tyler, 2004).

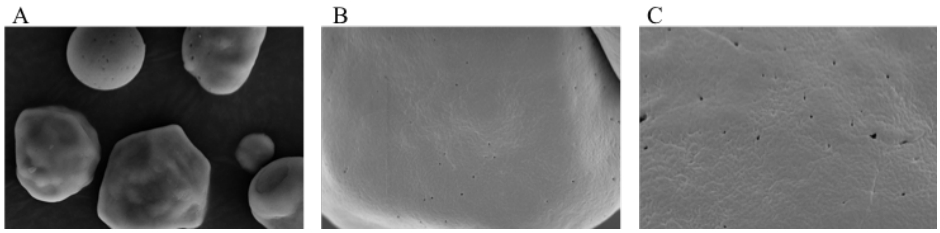

Figure 1.4: Scanning electron micrograph of porosity in native corn starch granules at different magnifications 3,500× (A), 10,000× (B) and 20,000× (C).

Apart from mentioned architectural features, the presence of pores at the granule surface, as well as internal cavities at the granule hilum and channels connecting the two have been also observed (Huber and BeMiller, 2000). Scanning electron microscopy (SEM) revealed that pores are normal, real, anatomical features of these starch granules and are not artifacts of processing or preparation of samples (Fannon, Hauber, and BeMiller, 1992; Fannon, Shull, and BeMiller, 1993). Nevertheless, not all starch granules display pores on the surface, thus, this is not a common feature of starches. For example, there was no pore evidence in tapioca, rice, oats, canna and arrowroot surface. While, pores were observed over the entire granule surface of corn, sorghum and millet starches or along the equatorial groove of wheat, rye, and barley starch granules (Baldwin et al., 1994; Fannon, Hauber, and BeMiller, 1992; Hall and Sayre, 1970). Overall, it seems that A-type cereal starches contain pores on the granules that lead to channels, which penetrate towards the center of granules drawn as hollow cavities (Dhital et al., 2013). Meanwhile, B-type polymorphic starches are linked to the absence of pores and channels.

In general, pores are randomly distributed and vary from starch to starch in terms of locations, dimensions and extent (Figure 1.4) (BeMiller, 1997; Fannon et al., 2004; Fannon, Hauber, and BeMiller, 1992). For instance,

maize and rice starches display porous diameters ranging from 100 to 200 nm, while wheat and potato range from 2 to 3 nm (Sujka and Jamroz, 2010). Nonetheless, pore ratios between 1-3 μm or less have also been reported in waxy and normal corn starch (Karathanos and Saravacos, 1993; Madene et al., 2006; Zhou et al., 2017).

1.2.2 Structure of starch granules

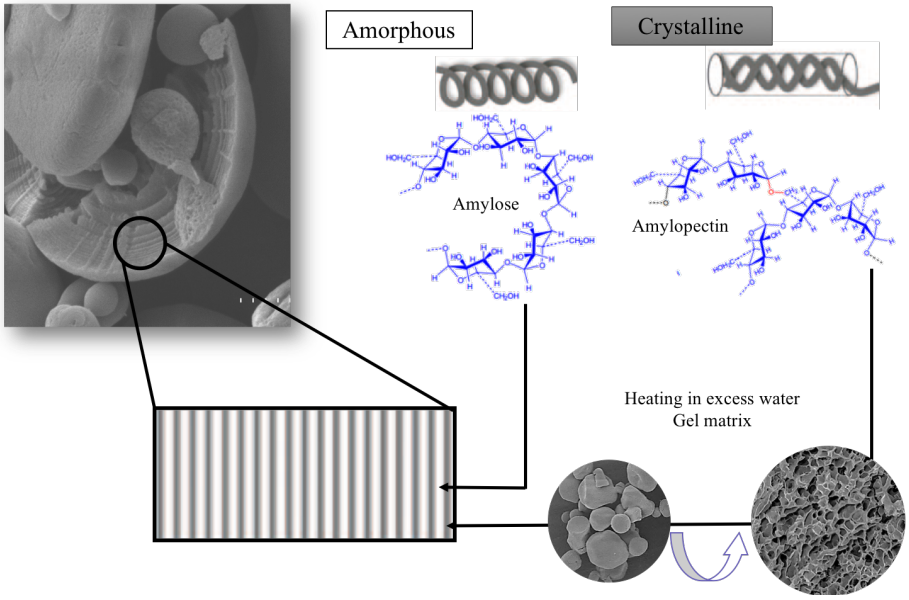

Figure 1.5: Composition and structure of starch granules.

Amylose and amylopectin are the two major biomacromolecules of starch. These polymers have the identical basic structure, having D glucose as a basic constituent unit, but changing in their length and degree of branching. Numerous studies have indicated that relationships between amylose and amylopectin content and molecular structure vary considerably amongst starches from the main botanical species, and even from the same plant cultivar grown under different conditions (Genkina et al., 2014; Iturriaga, Lopez, and Añon, 2004). Granular starch may be viewed in terms of a structure of alternating amorphous and semi-crystalline growth rings, with

a gradual transition between them (Figure 1.5). The amorphous rings are formed by amylose and amylopectin with a disordered conformation, whereas the semi-crystalline rings comprise a lamellar structure of alternating crystalline and amorphous regions with a repetition distance of 9-11 nm (Cameron and Donald, 1992). The crystalline lamellae are mainly double helices of amylopectin side chains packed laterally into a crystalline lattice (Blazek and Gilbert, 2011), while the amorphous regions contain amylose and the amylopectin branching points (Jane et al., 1992). According to Lopez-Rubio et al. (2008), the ratio of crystalline to amorphous material in native starch granules varies from 10% to 50% crystallinity, depending on starch source. Amylopectin clusters may contain amylose molecules, which join the crystalline and amorphous layers with a straightened conformation in crystalline regions and a disordered conformation in amorphous regions (Kozlov et al., 2007; Matveev et al., 1998). Molecular order within starch granules is often demonstrated by X-ray diffraction patterns corresponding to A- and B- polymorphs, or the intermediate C-form, depending on the packing of amylose and amylopectin. According to Hoover (2001) and Pérez and Bertoft (2010), the typical A-type X-ray pattern is presented in cereal starches, whereas the B-form and the mixed state pattern C are showed in tuber starches and legumes respectively.

The set of all these different characteristics provides starches of diverse properties (water retention capacity, peak viscosity, final viscosity and breakdown) (Schirmer et al., 2013; Singh et al., 2003; Witczak et al., 2016). Moreover, the starch isolation method induces changes in most of those properties (Correia, Nunes, and Costa, 2012).

1.3 Starch modifications to obtain porous starches

Initially, the aim of developing modified starches was to overcome the technological restriction that the use of native starches imposes. Nevertheless, some treatments employed not only improved the starch properties, but also caused structural modifications by forming pores in their surface that extended as cavities inside the granule. Following the discovery of the porous starches, some researches have been focused on their production. Up to now,

these starches have been conventionally accomplished by chemical, physical and enzymatic methods. It seems that starch treatment determines pore and cavity sizes (Fortuna, Juszczak, and Pałasiński, 1998), which can influence their further application. Nevertheless, reported literature is limited to the simple description of the presence of the pores on starch surface without applying quantitative analysis. Consequently, there is no information to make tailor-made porous starches regarding both the size and frequency of the pores. With the goal of simplifying the picture of published studies, in the present chapter, authors analyzed previously reported micrographs (Table 1.1 and Table 1.2), which will be discussed in the following paragraphs.

Porous starch preparation has been conducted by pure chemical synthesis. Hydrochloric acid promote the degradation of starch granules, resulting in porous structures due to the attack of the amorphous areas and the leaching caused by acid (Amaya-Llano et al., 2011). Similarly, it has been observed that oxidation treatment can create grooves on the surfaces of the granules (Zhou et al., 2016). Usually, oxidation is accomplished by the reaction of starch with sodium hypochlorite, but also with sodium periodate or hydrogen peroxide. As the oxidant concentration increases, the starch degrades and appears to crack when the oxidant concentration is high. Nevertheless, the effect is highly dependent on reaction time and starch source (Sangseethong, Termvejsayanon, and Sriroth, 2010), as can be observed in Table 1.1.

As for physical processing, porous structure of native starch materials has been conventionally produced through pre-gelatinization, annealing, ultra-high-pressure, ultrasonic, radiation, ball milling and freezing and thawing (Table 1.1). According to Table 1.1, starch source and treatment employed determine the resulting pore size. In addition, it seems that ratio amylose-amylopectin also play an important role at this concern (Rocha et al., 2012).

Therefore, chemical synthesis as well as by physical processing results in porous starch. Nevertheless, the main difficulty is still to create deep holes at the starch granules surface. Based on micrographs reported, external fissures or grooves can be clearly observed after chemical and physical treatments (Majzoobi, Hedayati, and Farahnaky, 2015; Sangseethong, Termvejsayanon,

and Sriroth, 2010). The role of depth is critical for developing specific applications in food and non-food industry, since the specific surface area is proportional to the specific pore volume (Sujka and Jamroz, 2007), influencing the absorption capacity (Zhang et al., 2012). To create deep holes, several studies carry out a preliminary gelatinization of the starch that serves as a matrix for the application of chemical or physical treatments. As can be seen in Table 1.2, the resulting pore sizes are increased by this technique. The difference with the previous ones is based on the preservation of granules integrity during treatment. When the starch is heated in excess of water, the gelatinization of the starch is produced, or what is the same, its loss of structure. Therefore, through these latest techniques, the shape of the porous starches will depend on the process used. For instance, porous starches can be obtained by replacing ice crystals in frozen starch gel with ethanol/water solvent using a solvent exchange technique. This technique allows obtaining porous starches with variable pore size using different proportions of ethanol/water volume (Chang, Yu, and Ma, 2011), but also changing the starch paste concentrations (Qian, Chang, and Ma, 2011). Both authors cut the gels in a cubic form, obtaining porous cubes.

Regarding enzymatic treatments, many enzymes can be used to change starch structure and to achieve preferred functionality (Rosell and Collar, 2008) (Table 1.1). Nevertheless, for obtaining porous starches the enzymes that might be used include α-amylase, β-amylase, glucoamylase, pullulanase and isoamylase (Dura, Błaszczak, and Rosell, 2014). In fact, SEM microscopy has revealed the formation of pin-holes, sponge-like erosion, numerous medium-sized holes, distinct loci leading to single holes in individual granules and surface erosion in starches of different origin treated by those enzymes (Dura, Błaszczak, and Rosell, 2014; Dura and Rosell, 2016; Tester, Qi, and Karkalas, 2006; Zhang et al., 2012). What is clear is that enzymatic treatments led to porous starches with random pore size, which seems that can be increased by using combined methodologies (Table 1.1). However, in the existing literature previously mentioned, the variability in the conditions of the enzymatic reactions, in terms of enzyme concentration, reaction time or pH do not allow to understand the role of those conditions in modulating the properties of porous starches and the resulting degree of porosity. Despite the

study of all these methodologies, unquestionably, obtaining porous starches through enzymatic modifications presents the greatest expansion due to environmental concerns.

1.4 Porous starch applications

As native starches, porous starches exhibit numerous advantages such as nontoxicity, good biodegradability or biocompatibility. Nevertheless, the most important advantage of porous starches relies on their high absorption capacity because their large surface-to-volume ratio and accessible inner empty spaces (Li, Turner, and Dhital, 2016; Zhang et al., 2012; Zhao, Madson, and Whistler, 1996). In view of their absorption capacity, the use of porous starches as natural absorbers has undergone great expansion in various areas such as food, pharmaceuticals, tissue engineering and so on (Chang, Yu, and Ma, 2011; Glenn et al., 2010; Torres, Boccaccini, and Troncoso, 2007).

Theoretically, using materials with similar features to starch but with larger pores could open the door to absorb large molecules that are unable to enter into a native starch framework. With this in mind, porous starches as wall materials for encapsulation represents one of the most widespread applications of these solids. For illustration purposes, Table 1.3 describes the encapsulation methods used with porous starches for encapsulation a core material.

In the pharmaceuticals field, these materials can improve the oral delivery. This is a subject of great interest, mainly because despite being the preferred route for the treatment of many chronic diseases, many drugs are poorly soluble in water and exhibit a very low bioavailability after oral administration (Deveswaran et al., 2012). Successful use of commercially available porous starches, as well as porous starches from W/O emulsion – freeze thawing method has been demonstrated for the solubility improvement of different drugs (Table 1.3). Therefore, porous starches have a great potential for their use as a novel delivery system for poorly water soluble or lipophilic drugs.

Table 1.1: Pore size distribution after chemical, physical or enzymatic treatment from starch granules.

Starch treatment	Type of starch	Pore size (μm^2)	Process condition	Reference
Chemically-modified				
Acid-thinned	Corn	0.02 - 0.20	Aqueous HCl in a water bath at 40 °C	(Amaya-Llano et al., 2011)
Oxidized	Potato	3.65 - 24.75	Sodium hypochlorite oxidation at 35 °C for 0.5 h and pH 9.5	(Zhou et al., 2016)
	Cassava	0.05 - 0.49	Sodium hypochlorite oxidation at 30 °C for 1 h and pH 10	(Sangseethong, Termvejsayanon, and Sriroth, 2010)
		0.03 - 0.23	Hydrogen peroxide oxidation at 30 °C for 1 h and pH 10	(Sangseethong, Termvejsayanon, and Sriroth, 2010)
Physically-modified				
Annealed	Waxy barley	0.01 - 0.19	75% moisture content, heating at 50 °C for 72 h	(Waduge et al., 2006)
	Waxy corn	0.01 - 0.35	Heating at 63 °C for 24 h	(Rocha et al., 2012)
	Corn	0.01 - 0.37	Heating at 62 °C for 24 h	(Rocha et al., 2012)
High hydrostatic-pressure	Corn	0.01 - 0.81	400 MPa, at 40 °C for 35 h	(Deladino et al., 2015)
	Potato	0.16 - 3.47	600 MPa, for 2 min	(Błaszczak, Valverde, and Fornal, 2005)
Pulsed electric field	Waxy rice	0.08 - 0.29	Treatment at 30, 40 and 50 kV cm^1 with a flow rate of 60 mL/min	(Zeng et al., 2016)
Ultrasonic	Corn	<0.01 - 0.20	70% moisture content, stirring at 30 °C for 30 min	(Luo et al., 2008)
	Waxy corn	<0.01 - 0.55	70% moisture content, stirring at 30 °C for 30 min	(Luo et al., 2008)
	Amylomaize	<0.01 - 0.50	70% moisture content, stirring at 30 °C for 30 min	(Luo et al., 2008)
Enzymatically-modified				

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Table 1.1 – Continued

Starch treatment	Type of starch	Pore size (μm^2)	Process condition	Reference
Fungal amylase	Corn	0.05 - 0.50	Amount of enzyme: 400 U/g starch at 50 °C for 24 h and pH 4.6	(Qian, Chang, and Ma, 2011)
α-amylase	Corn	0.15	Amount of enzyme: 5 U/g starch at 50 °C for 24 h and pH 4.0	(Dura, Błaszczak, and Rosell, 2014)
	Wheat	0.09 - 2.94	Variable amount of enzyme at at 45 °C for 24 h and pH 6.2	(Majzoobi, Hedayati, and Farahnaky, 2015)
β-amylase	Corn	0.03 - 0.76	Amount of enzyme: 400 U/g starch at 55 °C for 24 h and pH 4.6	(Qian, Chang, and Ma, 2011)
Glucoamylase	Corn	1.72	Amount of enzyme: 4 U/g starch at 50 °C for 24 h and pH 6.0	(Dura, Błaszczak, and Rosell, 2014)
	Corn	0.32 - 5.42	At 25 °C for 6 h and pH 6.0	(Xu et al., 2017)
Dual-modifications				
Ultrasonic enzymatic hydrolysis	Corn	0.19 - 3.52	Pretreated: ultrasonic wave from 100 to 600 W Amount of enzyme: 400 U/g starch at 55 °C for 24 h and pH 4.6	(Qian et al., 2011)
	Wheat	0.09 - 2.77	Enzymatic treated starch was sonicated at 240 W and 35 kHz for 60 min	(Majzoobi, Hedayati, and Farahnaky, 2015)
Bioextrusion	Corn	0.09 - 62.71	Zn modified starch was mixed with an enzyme magnesium complex as biocatalyst prior to extrusion	(Xu et al., 2018)

Table 1.2: Pore size distribution after chemical, physical or enzymatic treatments of starch gels.

Starch treatment	Type of starch	Pore size (μm^2)	Process condition	Reference
Chemically-modified				
Acidized	Potato	0.23 - 67.58	Heating at 90 °C for 0.5 h with mercapto-succinic acid	(Bao et al., 2016)
Physically-modified				
Atomization + air classification	High-amylose corn	0.004 - 0.17	Atomizing starch gelatinous melt and air-classifying	(Glenn et al., 2010)
Frozen	Potato	122 - 6,735	Starch gel frozen at -10 °C was immersed in mixtures ethanol/water (10-1/0-9), dried at 50 °C for 6h and then heated at 105 °C for 2 h	(Chang, Yu, and Ma, 2011)
Solvent exchange	Unknown	0.06 - 4.50	Starch gel refrigerated at 5 °C was immersed in mixtures ethanol/water, dried at 30 °C	(Wu et al., 2011)
Pregelatinized	Rice	16 - 3,264	Double drum dryer at 110, 117 and 123 °C	(Nakorn, Tongdang, and Sirivongpaisal, 2009)

Following this approach, research in food science has used porous starches for the encapsulation of bioactive compounds (Table 1.3). Research efforts have been conducted to select the most appropriate starch modification, as well as to design the most suitable encapsulation method to maximize the yield and improve the encapsulation of various compounds. Commercially available porous starches and chemical, physical or enzymatical treated starches have been evaluated as wall materials (Table 1.3). Thus, the absorption capacity along with the porosity of these solids have allowed microorganisms, flavors, oils and so on can interact with the porous starches not only on the outer surface, but also inside the pores (Hoyos-Leyva et al., 2018). For instance, SEM and confocal laser scanning microscopy revealed that enzymatic hydrolysis extends natural cavities and channels present in corn starch, allowing probiotics to fill them (Li, Turner, and Dhital, 2016). Under this conditions, unstable compounds can be protected from adverse surrounding conditions such as oxidization, light-de-composition, temperature, pH or to confine an unpleasant odor or bitter taste (Ali et al., 2013; Belingheri, Ferrillo, and Vittadini, 2015; Belingheri et al., 2015; Li, Turner, and Dhital, 2016; Xing et al., 2015; Xing et al., 2014; Xu et al., 2007). Therefore, porous starches allow including sensitive compounds in foods or drugs, remaining stable for a long time, while maintaining their functionality.

Another application in which porous starches has shown a superior performance is in cleaning aquatic systems. Porous corn starches from enzymatic modification and loaded with Zr metal ions seem to be a good alternative for adsorption of fluoride from drinking water (Xu et al., 2017). Meanwhile, porous starch xanthate and porous starch citrate could be used as effective adsorbents for removal of heavy metals from contaminated liquid (Ma et al., 2015). In both studies, the simple contact of the porous materials with the target compounds allowed their absorption and consequently their removal.

Porous starches also possess excellent mechanical properties and stable structural integrity. Taking advantage of these features, Nakamatsu et al. (2006) proposed a starch-based scaffolds production for tissue engineering applications, obtaining promising results. The raw materials for starch-based

scaffolds and their blends with chitosan were treated by solvent exchange phase separation and thermally induced phase separation methods, leading to porous structures.

Table 1.3: Porous starches used for encapsulation.

Core material	Starch source	Modification	Encapsulation method	Reference
Drugs				
Insoluble probucol	Commercial	Unknown	Spraying	(Zhang et al., 2013)
Carbamazepine	Unknown	Solvent exchange	Immersion/solvent evaporation	(Ali et al., 2013)
Lovastatin	Unknown	Solvent exchange	Immersion/solvent evaporation	(Wu et al., 2011)
Lovastatin	Unknown	Solvent exchange	W/O emulsion – freeze thawing	(Jiang et al., 2014)
Microorganisms				
L. acidophilus	Commercial	Unknown	Ultrasonic	(Xing et al., 2014)
L. acidophilus	Commercial	Unknown	Ultrasonic + coating	(Xing et al., 2015)
L. plantarum	Corn	Enzymatic	Stirring	(Li, Turner, and Dhital, 2016)
Flavors				
Liquid flavors	Commercial	Unknown	Plating	(Belingheri et al., 2012)
Liquid tomato flavor	Commercial	Unknown	Plating	(Belingheri, Ferrillo, and Vittadini, 2015)
Oils				
Essential plant	High-amylose corn	Atomization + Air classification	Absorption	(Glenn et al., 2010)
High-oleic sunflower	Commercial	Unknown	Blending	(Belingheri et al., 2015)
Olive	Purple sweet potato	citric acid-disodium hydrogen phosphate	Stirring	(Lei et al., 2018)
Pigments				
Curcumin	Commercial	Unknown	Emulsion	(Wang et al., 2009)
Antioxidants				

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Table 1.3 – Continued

Core material	Starch source	Modification	Encapsulation method	Reference
Yerba mate	Corn	High hydrostatic pressure	High pressure	(Deladino et al., 2015)
Antibacterial				
Antibacterial coating	Corn	Heat + Solvent exchange + Alkali ketene dimer	Dispersion	(Dang et al., 2017)

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CHAPTER 2

Objectives

Starch in its native form has low surface area and pore volume and hence their industrial applications as an organic adsorbent are often limited. Modifications are aimed to extend the native starch applications by inducing several micropores on the surface of the granules, which can be extended to the inner part of the granules. These pores allow improving the retention of liquids throughout the starch granule, but also allow that certain molecules enter to the inner part of the granules through the pores obtained. In this context, the main objective of this work was the following:

Development of porous materials of interest for food industry applications that exhibit diverse structures containing functionalized pores, by using clean technologies like enzymatic modifications of granular starches testing the potential of different enzymes.

For this purpose, a sequential research was conducted. The first step (Chapter 3) consisted in comparing the effectivity of different hydrolases for producing corn porous starches and the impact of the enzymatic level on the intensity of the hydrolysis.

Although enzymatic modification of corn starch allows obtaining porous starches, starch source plays a fundamental role on defining their structure and properties. Therefore, the next step (Chapter 4) was focused on identifying the impact of the botanical origin of the starches on the production of porous starches by using different types of hydrolases.

Considering the extensive use of starch in food applications and the subsequent importance in human nutrition, the third objective (Chapter 5) consisted in determining the impact of porous structure on *in vitro* starch digestibility. In this case, corn porous starches obtained in the first stage were chosen.

As it has been described in the introduction, porous starches are already used for different purposes. In this research, applicability of the resulting porous starches was the most challenging objective and the one that could validate the effectivity of the proposed enzymatic strategies. Because of that, the next step (Chapter 6) was to identify the potential of controlled pore size starches from different botanical sources as carriers of probiotics. To this end, a novel system based on porous starches to stabilize probiotic bacteria was developed. Thus, prior to the incorporation of probiotics, an initial step was the optimization of the encapsulation process. The effect of different factors such as encapsulation time and previous hydrated samples on the encapsulation yield was also part of this specific objective. Based on the optimization performed for incorporation of probiotics using this encapsulation process, further stabilization was approached by studying the influence of different coating materials on the survival rate of probiotics cells. Finally, the influence of porous and coating materials on the survival rate of probiotics cells during heat treatment was tested.

In order to complete this thesis work (Chapter 7), a study of the effect of porosity on the texture, stability and properties of the starchy hydrogels obtained from previously developed porous starches was also carried out.

2 Objetivos

El almidón en su forma nativa tiene un área superficial y un volumen de poro bajos, lo que a menudo limita sus aplicaciones industriales como adsorbente orgánico. En este sentido, para aumentar las aplicaciones del almidón nativo se realizan modificaciones con el objetivo de generar microporos en la superficie de los gránulos, los cuales pueden extenderse hasta el interior de los gránulos. Estos poros permiten, no solo mejorar la retención de líquidos en los gránulos de almidón, sino también la incorporación de ciertas moléculas en el interior de los gránulos a través de los poros obtenidos. En este contexto, el principal objetivo de este trabajo fue el siguiente:

Desarrollar materiales porosos, de interés para aplicaciones en la industria alimentaria, que exhiban diferentes estructuras y que contengan poros funcionales mediante la utilización de tecnologías limpias como la modificación enzimática de almidones evaluando el potencial de diferentes enzimas.

Para ello se realizó una investigación secuencial. El primer paso (Chapter 3) consistió en comparar la efectividad de diferentes hidrolasas para producir almidones porosos de maíz y el impacto de la concentración enzimática sobre la intensidad de la hidrólisis.

Aunque la modificación enzimática del almidón de maíz permite obtener almidones porosos, la fuente de almidón juega un papel fundamental en la definición de su estructura y propiedades. Por lo tanto, el paso siguiente (Chapter 4) se centró en identificar el impacto del origen botánico de los almidones en la producción de almidones porosos mediante diferentes tipos de hidrolasas.

Considerando el amplio uso del almidón en aplicaciones alimentarias y la subsiguiente importancia en la nutrición humana, el tercer objetivo (Chapter

5) consistió en determinar el impacto de la estructura porosa en la digestibilidad *in vitro* de los almidones. Para este objetivo, se eligieron los almidones porosos de maíz obtenidos en la primera etapa.

Como se ha descrito en la introducción, los almidones porosos ya se utilizan para diferentes propósitos. En esta investigación, la aplicabilidad de los almidones porosos resultantes fue el objetivo más desafiante y el que pudo validar la efectividad de las estrategias enzimáticas propuestas. Por ello, el siguiente paso (Chapter 6) fue identificar el potencial de almidones con tamaño de poro controlado, procedentes de diferentes fuentes botánicas, como vehículos de probióticos. Con este fin, se desarrolló un nuevo sistema para estabilizar bacterias probióticas basado en almidones porosos. Así, antes de la incorporación del microorganismo probiótico, se optimizó el proceso de encapsulación como paso inicial. Como parte de este objetivo específico, se evaluó el efecto de diferentes factores, como el tiempo de encapsulación y la hidratación previa de las muestras sobre el rendimiento de encapsulación. En base a la optimización realizada para la incorporación de probióticos utilizando este proceso de encapsulación, se abordó una estabilización adicional mediante el estudio de la influencia de diferentes materiales de recubrimiento en la tasa de supervivencia de las células probióticas. Finalmente, se probó la influencia de los materiales porosos y de recubrimiento en la tasa de supervivencia de las células probióticas durante el tratamiento térmico.

A fin de completar este trabajo de tesis (Chapter 7), también se llevó a cabo un estudio sobre el efecto de la porosidad en la textura, estabilidad y propiedades de los hidrogeles de almidón obtenidos a partir de los almidones porosos previamente desarrollados con objeto de incrementar su aplicabilidad como absorbentes en su estado gelatinizado.

CHAPTER 3

Results I

Contents

Carbohydrate Polymers 157 (2017) 533–540

Comparison of porous starches obtained from different enzyme types and levels

Yaiza Benavent-Gil, Cristina M. Rosell*

Abstract

The objective was to compare the action of different hydrolases for producing porous corn starches. Amyloglucosidase (AMG),α-amylase (AM), cyclodextrin-glycosyltransferase (CGTase) and branching enzyme (BE) were tested using a range of concentrations. Microstructure, adsorptive capacity, pasting and thermal properties were assessed on the porous starches. SEM micrographs showed porous structures with diverse pore size distribution and pore area depending on the enzyme type and its level; AMG promoted the largest holes. Adsorptive capacity was significantly affected by enzymatic modification being greater influenced by AMG activity. Unexpectedly, amylose content increased in the starch treated with AMG and BE, and the opposite trend was observed in AM and CGTase treated samples, suggesting different mode of action. A heatmap illustrated the diverse pasting properties of the different porous starches, which also showed significant different thermal properties, with lower To and Tp. Porous starch properties could be modulated by using different enzymes and concentrations.

Keywords

Porous starch — Amyloglucosidase — α-amylase — CGTase — Branching enzyme — Microstructure

Institute of Agrochemistry and Food Technology (IATA-CSIC), C/Agustin Escardino, 7, Paterna 46980, Valencia, Spain

***Corresponding author**: crosell@iata.csic.es (C.M. Rosell).

3.1 Introduction

Porous starches are now attracting much attention due to their great adsorption ability (Zhang et al., 2012). Those starches contain abundant pores from the surface to the center of the granules, which increase the specific surface area, acting as excellent natural absorbents. In fact, there is a growing interest in exploiting their properties in different food and non-food areas. In food industry,porous starches are used as colorants, spices, flavorings, sweeteners carriers and also for protection of sensitive elements such as oils, minerals, vitamins, bioactive lipids, food pigments such as β-carotene and lycopene which are sensitive to light, oxidation or high temperature (Belingheri et al., 2015; Luo et al., 2013; Majzoobi, Hedayati, and Farahnaky, 2015).

Enzymatic treatments have been performed for obtaining porous starches, mainly applying glucoamylases and α-amylases (Sujka and Jamroz, 2007). Cassava starch granules were treated with α-amylase from *Bacillus amyloliquefaciens* without altering the size or morphology of the granules but significantly changing their properties (Ichihara et al., 2013). The combination of glucoamylase and α-amylase has been also proposed due to their synergistic action to hydrolyze raw starch completely very rapidly (Sun et al., 2010). In fact, porous starch was obtained using a combination of α-amylase and glucoamylases activity after optimizing the kinetic reaction to increase the reaction yield (Zhang et al., 2012). Later, Dura, Błaszczak, and Rosell (2014) compared the α-amylase and glucoamylase individual action to determine their effect on biochemical features, thermal and structural properties of corn starch. Researchers concluded that α-amylase or amyloglucosidase when acting on corn starch at subgelatinization temperatures for 24 or 48 h led to porous starch granules that differed in both, the microstructure surface and the internal morphology. Similarly, Chen and Zhang (2012) hydrolyzed native corn starch granules using glucoamylase at 50 °C for 1–8 h studying the impact of enzyme/granule ratio and hydrolysis time on the microstructure of porous starch. Research carried out on enzymatic treatments of starches has been accomplished using diverse enzymes and experimental conditions (Sorndech et al., 2016; Uthumporn, Zaidul, and Karim, 2010), which complicates results comparison and

a real understanding of the enzymes action on the structure and functionality of the starches.

In addition, other starch acting enzymes like α-glucanotransferases have received considerable attention to remodel parts of the amylose and amylopectin molecules by cleaving and reforming α-1,4- and α-1,6-glycosidic bond (Maarel and Leemhuis, 2013) or in the case of cycloamylose glucanotransferase for producing cyclodextrins (CDs) (Yamamoto, Zhang, and Kobayashi, 2000). Nevertheless, α-glucanotransferase such as branching enzyme or the cycloamylose glucanotransferase have been not tested for obtaining porous starches.

The aim of this study was to compare the effect of different enzymes on corn starch properties, taking also into account the impact of enzyme level. Amyloglucosidase (AMG), fungal α-amylase (AM), cyclodextrin-glycosyltransferase (CGTase) and branching enzyme (BE) were used to trigger particular starch functionalities.

3.2 Materials and methods

3.2.1 Materials

Corn starch was purchased from Miwon (Seoul, Korea). Amyloglucosidase (EC 3.2.1.3), fungal α-amylase (EC 3.2.1.1), cyclodextrin-glycosyltransferase (EC 2.4.1.19) and branching enzyme (EC 2.4.1.18) activities were provided by commercial food grade preparations (Amyloglucosidase 1100, Fungamyl 2500SG, Toruzyme® 3.0 L and Branchzyme) supplied by Novozymes (Bagsværd, Denmark). AMG activity was 1100 AGU/g (amyloglucosidase activity defined as the amount of enzyme that cleaves1 μmol of maltose per min at 37 °C); AM activity was 2500 FAU/g (fungal amylase activity); CGTase activity was 3 KNU/mL (kilo novo alpha amylase unit); BE activity was 50000 BEU/mL (branching enzyme units). All the other chemicals were analytical reagent grade and used without further purification. All solutions and standards were prepared by using deionized water.

3.2.2 Preparation of porous starch

The preparation of porous starch was based on the method of Dura, Błaszczak, and Rosell (2014) and

Dura and Rosell (2016) with minor modifications. Corn starch (20 g) was suspended in 100 mL of 20 mM sodium acetate buffer at pH 4.0 (AMG) or sodium phosphate buffer at pH 6.0 (AM, CGTase, BE). Then, different enzyme concentrations, expressed in units of enzyme stock solutions per grams of starch (U/g starch), were added to the starch suspensions, separately. The lowest enzyme concentration was the minimum recommended by the manufacturer (5.5 AMG U/g, 5.5 AMU/g, 0.1 CGTase U/g and 500 BE U/g), increasing concentrations (2, 3, 6 and 10 times the initial level) were also tested. Samples were kept in a shaking water bath (50 rpm) at 50 °C for 2 h. Then samples were centrifuged for 15 min at 7000 × g at 4 °C. Supernatants were boiled in a water bath for 10 min to inactivate the enzymes before any further analyses. Sediments were washed twice with 50 mL of water, homogenized with a Polytron Ultraturrax homogenizer IKA-T18 (IKA works,Wilmington, USA) for 1 min at speed 3, and then centrifuged at the same conditions as before. Washed sediments were freeze-dried and kept at 4 °C for subsequent analyses. Starch samples were subjected to the same procedure, without adding enzyme, at pH 4.0 (A-0) and pH 6.0 (P-0), and used as references. Two batches were prepared for each treatment.

3.2.3 Scanning electron microscopy (SEM)

The granule morphology of native, controls and treated starches was observed using a JSM 5200 scanning electron microscope (SEM) (JEOL, Tokyo, Japan). Samples were coated with gold in a vacuum evaporator (JEE 400, JEOL, Tokyo, Japan) prior to observation. The obtained samples were examined at an accelerating voltage of 10 kV and magnified 3500× times.

The microstructure analysis was carried out using the image analysis program (ImageJ, UTHSCSA Image Tool software). The SEM images were saved as 8-bit tiff format. Scale was initially set using the relationship between pixels and known distance. Threshold was assessed applying the default algorithm and then particle analysis was carried out. The following parameters were measured: granule size and the pore size. The area occupied by pores in a starch granule was calculated as the sum of the areas of all the pores of a starch granule divided by granule pore. Values were the average of 20 independent measurements.

3.2.4 High performance anion exchange chromatography (HPAEC)

The hydrolysis compounds (oligosaccharides and CDs) lixiviated during enzymatic treatment were quantified according to Dura and Rosell (2016). Samples were filtered through a 0.45 μm pore size membrane (Millex-HV) and then injected (10 μL) into HPAEC through a CarboPac PA-100 column (250 mm × 4 mm) at flow rate 1.0 mL/min, coupled to a pulsed amperometric detector (Dionex). Solutions included: A (water), B (1 mol/L NaOH) and C (1 mol/L $C_2H_3NaO_2$). Running profile applied was: time zero, 92.5% A, 5%B, 2.5% C; 25 min, 85% A, 5% B, 10% C; 1 min, 70% A, 15% B, 15% C;3 min, 66% A, 15% B, 19% C; 5 min, 57% A, 15% B, 28% C; 1.5 min, 37% A, 15% B, 48% C. Standards of known concentrations were previously analyzed.

3.2.5 Amylose content of enzymatically treated starches

The amount of amylose of the starches was analyzed in triplicate using a commercial assay kit (Megazyme International Ireland Ltd.,Bray, Co. Wicklow, Ireland) based on the concanavalin A method (Gibson, Solah, and McCleary, 1997).

3.2.6 Damaged starch

Damaged starch levels were estimated at least in duplicate following the Cereal Chemists (2000).

3.2.7 Adsorption of water and sunflower oil

Adsorptive capacity of starches for water and sunflower oil were determined according to the method described by Yousif, Gadallah, and Sorour (2012) with a slight modification. Starch (0.1 g) and solvent (1 mL, water or oil) were mixed and vortexed for 30 min at room temperature. Slurries were centrifuged 10 min at 3,000 × g and decanted. When no more water or sunflower oil was dropped off onto the filter paper, weight of the sediment was measured. The adsorption capacity was calculated as the weight of the wetted sediment divided by the dry weight of sample (g/g).

3.2.8 Viscosity measurement

The pasting properties of native and enzymatically modified starches were measured with the Rapid Visco Analyzer (RVA-4500, Perten Instruments, Hägersten, Sweden). Starch

(2 g based on 14% moisture content) was added to 20 mL of water placed into the aluminum RVA canister. Slurries underwent a controlled heating and cooling cycle, from 50 to 95 °C in 282 s, holding at 95 °C for 150 s and then cooling to 50 °C. The initial speed for mixing was 960 rpm for 10 s, followed by a 160 rpm paddle speed that was maintained for the rest of assay. Pasting parameters such as pasting temperature, peak viscosity, breakdown (peak viscosity-hot paste viscosity), final viscosity, setback (cold paste viscosity-peak viscosity) were recorded using Thermocline software for Windows (Perten Instruments, Hägersten, Sweden).

3.2.9 DSC thermal analysis

The gelatinization characteristics of modified starches were determined using a differential scanning calorimetry (DSC) from Perkin–Elmer (DSC 7, Perkin–Elmer Instruments, Norwalk,CT). The slurry of starch and water (3:1) was placed into stainless steel capsules. Capsules were hermetically sealed and equilibrated at room temperature for one hour before analysis. The samples were scanned from 30 to 120 °C at a heating rate of 10C/min under nitrogen atmosphere, using an empty stainless steel capsule as reference. The temperature values obtained were the onset temperature (To), peak temperature (Tp), and conclusion temperature(Tc). The enthalpy of gelatinization (ΔH) was estimated based on the area of the main endothermic peak, expressed as joule per gram sample (J/g).

3.2.10 Statistical analysis

All experiments were repeated at least in duplicate. Experimental data were statistically analyzed using an analysis of variance (ANOVA) and values were expressed as a mean $\pm$ standard deviation. Fisher's least significant differences test was used for assessment of significant differences among experimental mean values with 95% confidence. Statistical computations and analyses were conducted using Statgraphics Centurion XV software (Bitstream, Cambridge, N).

3.3 Results and Discussion

3.3.1 Microstructure analysis

The shape, size, structure and surface characteristics of corn starch granules tested (native, references and treated starches) were investigated using SEM (Figure 3.1). Native starch granules displayed an ir-

regular and mostly polygonal shape with relatively smooth surface (Figure 3.1 a). Reference starches (Figure 3.1 b,c) had similar appearance to native starch, showing no evidence of rupture, breakage or pores due to the incubation with buffer; results that were analogous to those reported previously (Dura, Błaszczak, and Rosell, 2014; Dura and Rosell, 2016).The effect of enzymatic treatment was readily visible in the modified starches microstructure, obtaining in all cases porous starch granules, without affecting the shape of the granule (Figure 3.1 d–w).

To give some objective results about the action of the enzymes, the pore size and the ratio pore area to starch granule area (related to the abundance of pore per granule) were quantified using image analysis (Figure 3.2). The pore size as well as pore area distribution was significantly affected by the type of enzyme and also their level. AMG action resulted in starch granules with larger pores and wider size distribution (Figure 3.2 A). In opposition, CGTase led to the lowest pore size. As the concentration of AMG increased, the size of the pores progressively augmented until 16.5 U of AMG were added; at higher enzyme level no further pore size increase was observed, although a significant increase in the ratio pore area to granule area was observed (Figure 3.2 B) indicating more pores per granule. Nevertheless, it was noted that at higher AMG concentrations appeared some depressions in the granules, which resulted from the eroding action of the enzyme onto the granule surface. Aggarwal and Dollimore (2000) also observed a visible increase in the size of the pores when augmented the AMG concentrations, till enzyme activity (800 U/g starch) was so pronounced that walls around pinholes were broken, leading to large irregular holes and broken structure. Similarly, pore size increased with the amount of AM or CGTase added, although both treatments resulted in smaller pore size than AMG treatment. The ratio pore to granule area of AM treated starches also maintained a similar pattern to the AMG samples, while it remained constant when CGTase enzyme was used. The BE enzyme produced very irregular pore sizes without any trend with the level of enzyme. It should be noted that the pore size was bigger when lower concentrations of enzymes were used, but in those cases pores resembled wide craters instead of deep holes. At higher enzyme concentration, smaller and deeper pinholes ap-

peared, leading a mixture of heterogeneous sizes.

When starch granules are incubated with amylolytic enzymes, the enzymes migrate through the channels and initiate hydrolysis leading to an inside out pattern of digestion (Chen and Zhang, 2012). Nevertheless, the present study reveals that different porous starches could be obtained depending on the type, thus it is possible to modulate the number and size of pores by using either different amylolytic enzyme or level of enzyme.

3.3.2 CDs and oligosaccharides released during enzymatic treatment

To understand the action of the enzymes on the starch granules, the released compounds after the incubation were analyzed.

Table 3.1 listed the oligosaccharides and cyclodextrins contents released per starch (mg 100 g^{-1}). As expected, neither oligosaccharides nor cyclodextrins (CDs) were released from the reference samples (data not shown), neither from AMG treatment. No oligosaccharides (from DP1 to DP5) were released when corn starches were subjected to BE hydrolysis. BE cleaves α-(1 $\rightarrow$ 4)-O-glycosidic bonds and transfers the cleaved-glucan to α-(1 $\rightarrow$ 6) position leading to branched glucan mixtures (Roussel et al., 2013).

Regarding the other amylolytic enzymes, starch-converting enzymes have been classified into exo-amylases and endo-amylases owing to their cleavage action, and results displayed that difference (Table 3.1). AMG treatment released exclusively glucose, and the amount remained constant independently on the enzyme concentration. Amyloglucosidase is a well-known exo-amylase, releasing only glucose residues from amylose or amylopectin chains (Bouchet-Spinelli et al., 2013). However, saturation of the non-reducing-ends of starch chains has been reported when enough glucoamylase is present (Chen and Zhang, 2012), which would explain the steady glucose level.

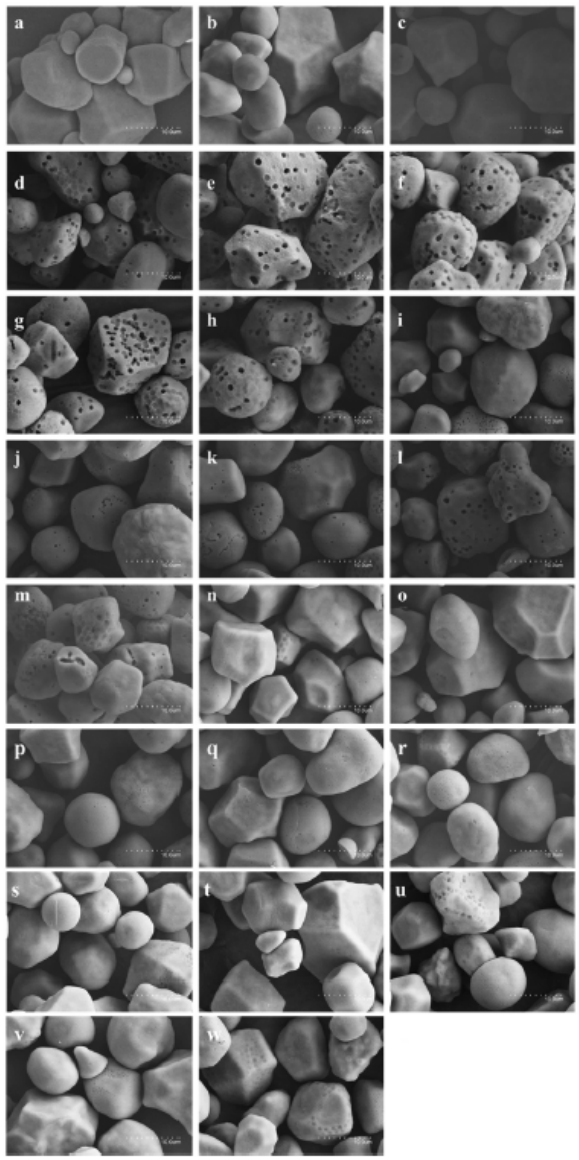

Figure 3.1: Scanning electron micrograph of and native corn starch (a), samples treated enzymatically (d–w) and their counterparts controls (b and c). Magnification 3500 ×.Reference A-0 (b); Reference P-0 (c); AMG 5.5, 11, 16.5, 33 and 55 (d-h); AM 5.5, 11,16.5, 33 and 55 (i-m); CGTase 0.1, 0.2, 0.3, 0.6 and 1 (n–r); BE 500, 1000, 1500,3000 and 5000 (s–w). Numbers following enzyme abbreviations are referred to the enzyme activity applied.

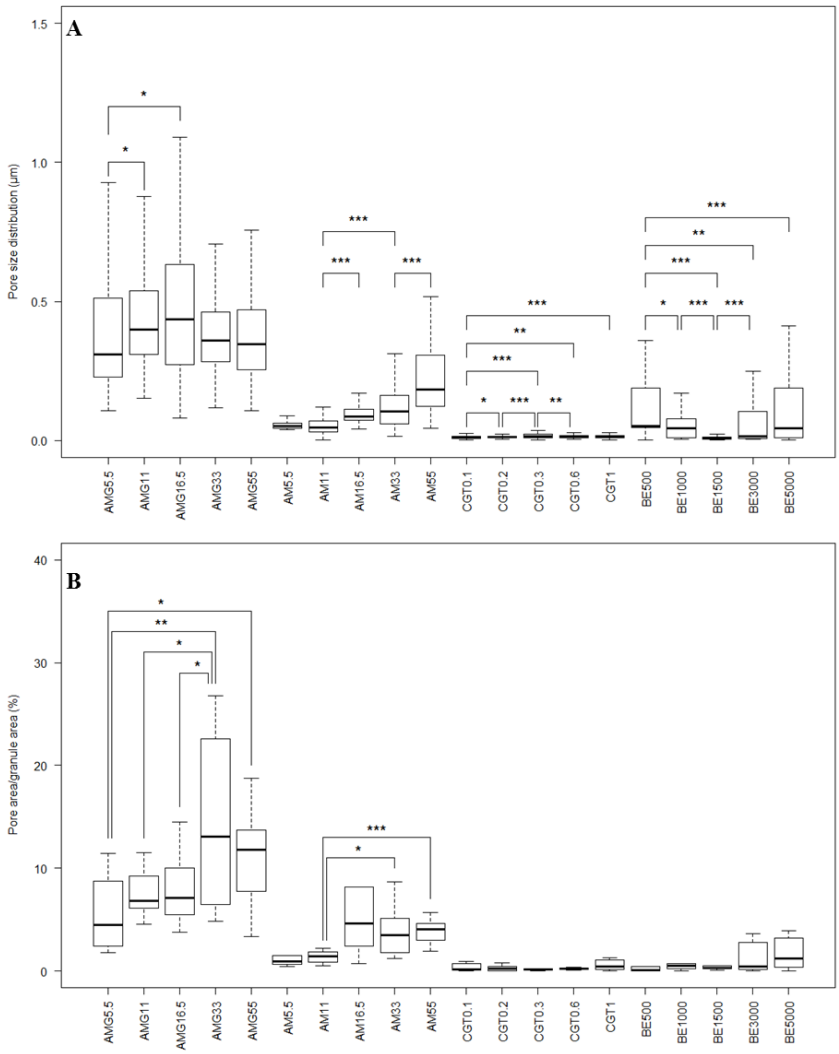

Figure 3.2: Image analysis from SEM photographs. A) Pore size and B) pore surface area distribution for each enzyme by boxplot. Numbers following enzyme abbreviations are referred to the enzyme activity applied.

Table 3.1: Oligosaccharides and cyclodextrins released after corn starch hydrolysis by AMG, AM and CGTase. Results are expressed in mg 100 g^{-1} of starch.

Enzyme type	Enzyme (U/g starch)	Glucose	Maltose	Maltotetriose	Maltotetraose	Maltopentose	α-CD	β-CD
AMG	5.5	16.19 ± 1.31	n.d	n.d	n.d	n.d	n.d	n.d
	11	15.64 ± 1.39	n.d	n.d	n.d	n.d	n.d	n.d
	16.5	16.16 ± 1.17	n.d	n.d	n.d	n.d	n.d	n.d
	33	15.57 ± 1.08	n.d	n.d	n.d	n.d	n.d	n.d
	55	15.49 ± 1.01	n.d	n.d	n.d	n.d	n.d	n.d
AM	5.5	9.76 ± 0.04	10.81 ± 0.20	7.68 ± 0.13	2.05 ± 0.02	n.d	n.d	n.d
	11	11.60 ± 0.27	8.82 ± 0.22	3.23 ± 0.40	1.90 ± 0.14	0.18 ± 0.00	n.d	n.d
	16.5	12.42 ± 0.06	9.48 ± 0.39	2.38 ± 0.17	1.57 ± 0.38	n.d	n.d	n.d
	33	13.94 ± 0.41	9.70 ± 0.13	0.55 ± 0.05	1.18 ± 0.01	n.d	n.d	n.d
	55	15.23 ± 0.16	10.49 ± 0.20	0.27 ± 0.09	0.42 ± 0.08	n.d	n.d	n.d
CGTase	0.1	1.23 ± 0.03	0.54 ± 0.05	0.51 ± 0.09	0.50 ± 0.13	0.01 ± 0.00	2.25 ± 0.09	n.d
	0.2	1.37 ± 0.03	1.07 ± 0.00	0.85 ± 0.04	0.96 ± 0.06	0.02 ± 0.00	2.33 ± 0.06	n.d
	0.3	0.83 ± 0.04	1.07 ± 0.05	0.93 ± 0.04	1.22 ± 0.09	0.02 ± 0.00	2.73 ± 0.24	n.d
	0.6	0.70 ± 0.08	1.19 ± 0.17	0.97 ± 0.13	1.00 ± 0.13	0.01 ± 0.00	1.73 ± 0.02	n.d
	1	1.27 ± 0.02	1.78 ± 0.00	1.37 ± 0.02	1.60 ± 0.05	0.03 ± 0.00	2.09 ± 0.14	n.d

n.d.: Not detected.

In addition, the endo-amylases, AM and CGTase, are able to cleave α-1→4 glycosidic bonds existing in the internal part (endo-) of a polysaccharide chain. As expected, AM majorly converted starch to glucose followed by maltose. Moreover, the amount of short chain oligosaccharides, ranging from DP1 to DP2 increased with the amount of AM added, whereas DP3, DP4 and α-CD chains decreased. Conversely, the amount of short chain oligosaccharides ranging from DP1 to DP5 decreased as increasing the level of CGTase added, with a simultaneous increase in α-CD. Overall, CGTases convert amylose or amylopectin into a mixture of α-, β- and γ-CD and some dextrins, and the proportion was dependent on the enzyme specificity (Terada et al., 1997), but also on the substrate, complexing agents and reaction conditions (Blackwood and Bucke, 2000).

3.3.3 Amylose, damaged starch content and adsorptive capacity

Amylose and damaged starch contents were determined in the treated starches (Table 3.2). The statistical analysis indicated that the enzymatic treatment significantly modified the amylose content, the amount of damage starch and the adsorption properties of the starches; but the enzyme level only prompted significant effect on the amount of damage starch and adsorptive water capacity. Amylose content showed a significant moderate correlation with the damaged starch content ($r = 0.6684$, $P < 0.0000$), mainly ascribed to the action of AMG and BE. Concerning the specific action of each enzyme, a significant reduction in amylose content, with the subsequent increase in amylopectin, was observed after AM and CGTase treatments, without observing any trend with the level of enzyme applied. These results are in agreement with the inverse relationship reported between the amylose content and the amount of hydrolyzed starch (Tester, Qi, and Karkalas, 2006), and also with the trend reported for CGTase modified starches (Dura and Rosell, 2016). Nevertheless, previous results with AM and AMG indicated that at lower concentrations than the one of the present study no change in the amylose content was observed even when increasing the enzymatic treatment to 24 or 48 h (Dura, Błaszczak, and Rosell, 2014).

Damaged starch was hardly affected by the action of AM and CGTase, al-

though a tendency to decrease it was observed in the case of CGTase. Considering that microstructure analysis confirmed the impairment of the granule, it seems that the experimental assay for quantifying damage starch was not sensible or reliable enough to distinguish the degree of damage. Conversely, AMG and BE treatment promoted the opposite trend, the amylose content appeared to increase but not always significantly, and the amount of damage starch significantly augmented, particularly in the case of BE. Regarding the level of BE applied, a clear decrease of damage starch content was observed when increasing the enzyme concentration. Starch granules have a unique semi-crystalline supramolecular structure with concentric layers of amorphous and crystalline regions radiating from the hilum (Ratnayake and Jackson, 2008). Taking into account that the amylopectin side chains form the framework of the crystalline lamellae, with branching points located in the amorphous domains, where the majority of the amylose is located (Copeland et al., 2009), it seems that depending on the enzymatic treatment amylose or amylopectin are preferentially hydrolyzed. Results on amylose content suggested that AM and CGTase attacked more proportion of amylose, leading an increase in the amount of amylopectin, suggesting deeper pinholes and the attack of amorphous and crystalline structure. In opposition, AMG and BE seem to hydrolyze preferentially the amylopectin chains, increasing the proportion of amylose in the surface of starch granule, thus bigger and less deep holes, which agrees with microstructure results.

The adsorptive capacity of modified starches for water and sun-flower oil are also summarized in Table 3.2. The hydrophilic nature was significantly dependent on both enzyme type and concentration, while hydrophobic nature depended only on the enzyme type. In general, all enzymatic treatments increased the water adsorption capacity of the starches; among them, AMG showed the greatest effect, followed by AM, CGTase and BE treatment. Likely, the size of the pores originated by AMG was responsible of this behavior due to the increase of the surface area. The adsorptive oil capacity of starch was only significantly modified when treated with AMG Chen and Zhang (2012) obtained an increase in both solvents retention ability respect to native starch, due to the increase in the surface area promoted

by the starch treatment with AMG (11 U/g starch), which agrees with results of the present study. Therefore, it seems that the pore size plays a fundamental role for oil adsorption, which was only sufficient in the case of AMG hydrolysis.

Table 3.2: Effect of enzymatic treatment on the water and oil adsorption capacity and chemical composition (amylose content and damaged starch) of the resulting porous starches.

Enzyme type	Enzyme (U/g starch)	Amylose content (%)	Damaged starch (%)	Adsorptive water (capacity g/g)	Adsorptive oil (capacity g/g)
Native	0	25.76 ± 0.82^{de}	15.41 ± 0.19^{cd}	0.74 ± 0.02^{a}	$1.14 \pm 0.05^{f,g}$
AMG	5.5	23.47 ± 0.35^{cd}	21.30 ± 0.05^{e}	1.12 ± 0.03^{hi}	$1.10 \pm 0.05^{e-h}$
	11	$27.36 \pm 1.31^{e-g}$	22.77 ± 0.17^{f}	1.25 ± 0.04^{j}	$1.27 \pm 0.00^{h-j}$
	16.5	$26.97 \pm 0.31^{e-g}$	23.64 ± 0.15^{f}	1.45 ± 0.08^{k}	1.41 ± 0.02^{j}
	33	$28.01 \pm 4.76^{e-g}$	21.51 ± 0.07^{e}	1.44 ± 0.08^{k}	1.35 ± 0.02^{j}
	55	26.91 ± 0.16^{g}	20.66 ± 0.05^{e}	1.46 ± 0.06^{k}	1.32 ± 0.03^{ij}
AM	5.5	19.53 ± 1.82^{ab}	$14.97 \pm 0.05^{a-d}$	1.16 ± 0.06^{ij}	$0.85 \pm 0.28^{a-d}$
	11	18.56 ± 0.46^{ab}	$15.01 \pm 0.63^{a-d}$	$1.07 \pm 0.01^{g-i}$	$0.96 \pm 0.08^{c-f}$
	16.5	18.95 ± 0.38^{ab}	15.40 ± 0.22^{cd}	$0.85 \pm 0.03^{b-e}$	$0.76 \pm 0.08^{a-c}$
	33	$21.24 \pm 0.41^{a-c}$	$15.13 \pm 0.37^{b-d}$	0.71 ± 0.06^{a}	$0.86 \pm 0.08^{a-d}$
	55	19.17 ± 0.82^{ab}	16.03 ± 0.73^{d}	$0.93 \pm 0.03^{d-f}$	0.71 ± 0.01^{ab}
CGTase	0.1	$21.26 \pm 0.19^{a-c}$	$14.38 \pm 0.05^{a-c}$	$0.90 \pm 0.07^{c-f}$	$0.86 \pm 0.05^{a-d}$
	0.2	19.45 ± 1.07^{ab}	$14.37 \pm 0.19^{a-c}$	$0.89 \pm 0.04^{f-h}$	$1.09 \pm 0.10^{e-h}$
	0.3	19.58 ± 2.39^{ab}	13.68 ± 0.07^{a}	$0.97 \pm 0.07^{c-f}$	$0.98 \pm 0.17^{d-g}$
	0.6	21.91 ± 0.14^{bc}	13.05 ± 0.91^{ab}	$0.80 \pm 0.03^{e-g}$	$1.13 \pm 0.33^{f-i}$
	1	21.66 ± 0.64^{bc}	$14.41 \pm 0.10^{a-c}$	$0.93 \pm 0.04^{a-c}$	$0.96 \pm 0.27^{c-g}$
BE	500	28.96 ± 0.15^{fg}	30.66 ± 0.11^{ij}	0.75 ± 0.07^{ab}	$0.85 \pm 0.01^{a-d}$
	1000	$18.90 \pm 0.84^{d-f}$	31.18 ± 0.63^{j}	$0.79 \pm 0.04^{a-c}$	0.66 ± 0.09^{a}

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Table 3.2 – Continued

Enzyme type	**Enzyme (U/g starch)**	**Amylose content** (%)	**Damaged starch** (%)	**Adsorptive water** (capacity g/g)	**Adsorptive oil** (capacity g/g)
	1500	$28.54 \pm 1.36^{\text{e-g}}$	$29.61 \pm 1.39^{\text{hi}}$	$0.88 \pm 0.02^{\text{c-f}}$	$0.90 \pm 0.00^{\text{b-e}}$
	3000	$27.11 \pm 1.65^{\text{d-f}}$	$29.06 \pm 1.22^{\text{h}}$	$0.82 \pm 0.05^{\text{a-d}}$	$0.85 \pm 0.11^{\text{a-d}}$
	5000	$27.25 \pm 0.65^{\text{e-g}}$	$27.76 \pm 2.06^{\text{g}}$	$0.87 \pm 0.12^{\text{b-f}}$	$0.84 \pm 0.12^{\text{a-d}}$
***P*-value**	Enzyme type	0.00	0.00	0.00	0.00
	Enzyme (U/g)	0.11	0.00	0.02	0.17

3.3.4 Enzymatic modification effects on pasting and thermal starch properties

To illustrate the pasting characteristics of the porous starches obtained from different type of enzymes a heatmap was constructed with the pasting properties (Figure 3.3). The heatmap of the hierarchical clustering of the RVA properties for the modified samples was analyzed on the basis of similarities and differences in starch pasting properties, including onset, peak viscosity, through, breakdown, final viscosity, setback, hydrolysis percentage at 95 °C and 50 °C (Figure 3.3). The dendrogram consisted of three major clusters. One cluster contained native, AM treated samples and the minor concentration of CGTase and BE treatments, up to 1500 U/g starch. Another cluster essentially included AMG treated starches and one AM treated sample. The last cluster comprised CGTase and BE treated starches using high enzyme levels.

It was evident from the heatmap that enzymes changed the pasting performance of starch suspensions and the effect was also dependent on their concentrations, particularly in the case of CGTase and BE. The onset temperature, indicative of the initial viscosity increase, was significantly decreased by all enzymes studied, independently of the concentration used. Therefore, lower cooking temperature was required for the gelatinization of porous starches, likely due to faster water absorption by the starch granules, since a negative correlation was observed between onset temperature and pore size (r = -0.4581, $P < 0.001$). AM treated samples showed similar pasting behavior to native starch, unless the maximum viscosity that decreased after treatment. AM acts on the starch molecules breaking α-(1 $\rightarrow$ 4) linkages and providing dextrins, which present lower swelling during gelatinization (Rocha, Carneiro, and Franco, 2010). Porous starches had significantly lower peak viscosity, through, final viscosity and setback compared to native, which agree with previous results (Dura, Błaszczak, and Rosell, 2014). In the case of AMG treated samples they were grouped due to their lower peak viscosity and breakdown and higher final viscosity and setback, besides the presence of an additional peak viscosity (Pv1) during heating, prior to the common peak viscosity at 95 °C. This additional peak was negatively correlated with peak viscosity, show-

ing a progressive increase in the first peak in parallel to the reduction of peak viscosity. The decrease of peak viscosity due to the joint action of α-amylase and glucoamylase has been explained by the disintegration of fragile granules owing to their porous structure, leading to less viscous slurries (Uthumporn, Zaidul, and Karim, 2010). In this regard, pore size, ratio of pore area to granule area and water adsorptive capacity was negatively correlated with peak viscosity, confirming this hypothesis.

Porous starches obtained with very high levels of CGTase or BE were mainly characterized by very low values of final viscosity and setback, and high breakdown values. Those effects have been reported when wheat starch was treated by CGTase Gujral and Rosell (2004).

The values for the thermal properties of native starch (Table 3.3) agrees with previous reported results for corn (Jane et al., 1999). In modified starches, To, Tp and ΔH significantly ($P < 0.05$) varied owing to the type of enzyme used and its level, but Tc was only significantly affected by the type of enzyme. Porous starches showed lower To and Tc than native starch. In the case of AMG treated starches those temperatures decreased when increasing the level of enzyme during treatment. Moreover, lower energy (ΔH) was required to promote starch gelatinization, likely due to less energy was needed to unravel and melt the unstable double helices during gelatinization (Chung, Liu, and Hoover, 2009).

On the other hand, BE enzyme produced starches with lower To and Tp, but similar Tc to native starch. Conclusion temperature (Tc) was only significantly reduced by AM. Correlation analysis indicated that all gelatinization parameters evaluated except enthalpy were positively correlated ($P < 0.05$) with amylose content, but not with damaged starch, pore size or pore area to starch granule, which are in agreement with previous observations (Stevenson et al., 2006). In addition, enthalpy was negatively correlated with water (r = -0.3555, $P < 0.05$) and oil adsorption capacity (r = -0.4078, $P < 0.01$).

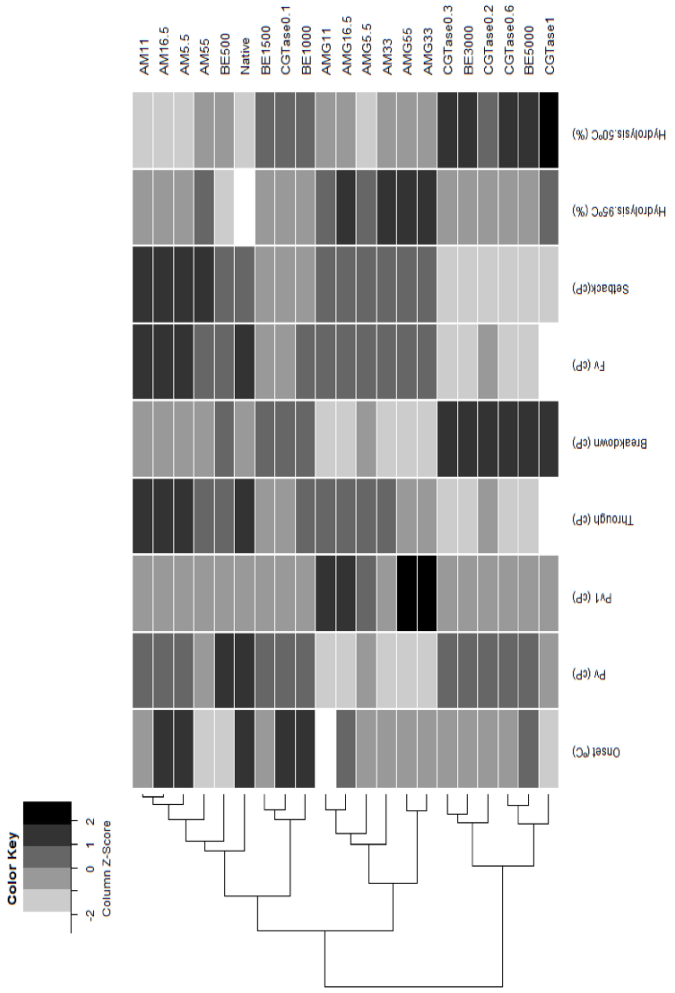

Figure 3.3: Hierarchical clustering of RVA profiles. A heatmap representing the hierarchical clustering of the Z scores of the enzyme activities related to viscoelastic properties, when compared AMG, AM, CGTase and BE enzyme treatment. The Z scores represent the dispersion around the overall mean of the viscoelastic properties and weighted by their standard errors. The scale of the intensity is shown in the top corner. Rows represent samples and column viscoelastic properties. Numbers following enzyme abbreviations are referred to the enzyme activity applied. Pv: peak viscosity; Pv1: additional peak viscosity; Fv: final viscosity.

Table 3.3: Thermal properties of enzymatically modified corn starches determined by DSC.

Enzyme type	Enzyme (U/g starch)	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)
Native	0	63.28 ± 0.14^{i}	68.20 ± 0.00^{h}	74.71 ± 0.17^{b}	$20.66 \pm 1.27^{c\text{-}e}$
AMG	5.5	$62.96 \pm 0.21^{g\text{-}i}$	$66.70 \pm 0.24^{a\text{-}e}$	74.32 ± 0.68^{b}	$20.26 \pm 1.08^{b\text{-}e}$
	11	63.26 ± 0.10^{hi}	67.53 ± 0.00^{g}	74.86 ± 0.08^{b}	19.18 ± 1.70^{bc}
	16.5	63.26 ± 0.15^{hi}	67.37 ± 0.47^{fg}	74.65 ± 0.11^{b}	16.64 ± 0.14^{a}
	33	$62.80 \pm 0.57^{f\text{-}h}$	$67.03 \pm 0.71^{c\text{-}g}$	74.45 ± 0.92^{b}	$19.64 \pm 1.75^{b\text{-}d}$
	55	$62.65 \pm 0.47^{d\text{-}g}$	$66.95 \pm 1.06^{b\text{-}g}$	73.88 ± 1.43^{b}	19.06 ± 0.38^{bc}
AM	5.5	$62.00 \pm 0.36^{a\text{-}c}$	$66.45 \pm 0.12^{a\text{-}c}$	73.93 ± 0.04^{a}	$20.77 \pm 0.18^{c\text{-}e}$
	11	61.86 ± 0.50^{a}	66.28 ± 0.35^{a}	73.81 ± 0.62^{a}	23.37 ± 1.13^{f}
	16.5	61.93 ± 0.20^{a}	66.37 ± 0.00^{ab}	73.86 ± 0.06^{a}	$19.43 \pm 0.49^{b\text{-}d}$
	33	$62.24 \pm 0.22^{a\text{-}e}$	$66.70 \pm 0.24^{a\text{-}e}$	73.12 ± 0.40^{a}	$19.82 \pm 2.70^{b\text{-}e}$
	55	61.98 ± 0.11^{ab}	66.37 ± 0.24^{ab}	73.62 ± 0.13^{a}	$21.67 \pm 0.94^{d\text{-}f}$
CGTase	0.1	$62.49 \pm 0.12^{c\text{-}g}$	$67.28 \pm 0.12^{e\text{-}g}$	73.98 ± 0.12^{ab}	19.35 ± 1.39^{bc}
	0.2	$61.99 \pm 0.01^{a\text{-}c}$	66.37 ± 0.24^{ab}	73.27 ± 0.46^{ab}	$20.99 \pm 0.87^{c\text{-}e}$
	0.3	$62.01 \pm 0.12^{a\text{-}c}$	66.37 ± 0.00^{ab}	73.34 ± 0.18^{ab}	18.15 ± 0.56^{ab}
	0.6	$62.20 \pm 0.08^{a\text{-}d}$	$66.62 \pm 0.12^{a\text{-}d}$	73.68 ± 0.09^{ab}	$19.47 \pm 1.02^{b\text{-}d}$
	1	$62.46 \pm 0.10^{b\text{-}g}$	$67.03 \pm 0.24^{c\text{-}g}$	73.67 ± 0.26^{ab}	19.11 ± 0.58^{bc}

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Table 3.3 – Continued

Enzyme type	Enzyme (U/g starch)	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)
BE	500	$62.81 \pm 0.28^{\text{f-h}}$	$67.28 \pm 0.12^{\text{e-g}}$	$74.25 \pm 0.46^{\text{ab}}$	$23.72 \pm 1.00^{\text{f}}$
	1000	$62.73 \pm 0.40^{\text{e-g}}$	$67.03 \pm 0.24^{\text{c-g}}$	$74.18 \pm 0.96^{\text{ab}}$	$21.95 \pm 1.43^{\text{ef}}$
	1500	$62.30 \pm 0.05^{\text{a-f}}$	$66.78 \pm 0.12^{\text{a-f}}$	$73.30 \pm 0.24^{\text{ab}}$	$20.31 \pm 0.84^{\text{b-e}}$
	3000	$62.48 \pm 0.28^{\text{b-g}}$	$67.12 \pm 0.35^{\text{d-g}}$	$74.04 \pm 0.77^{\text{ab}}$	$20.94 \pm 1.39^{\text{c-e}}$
	5000	$62.47 \pm 0.32^{\text{b-g}}$	$66.87 \pm 0.24^{\text{a-f}}$	$73.76 \pm 0.19^{\text{ab}}$	$20.02 \pm 0.70^{\text{b-e}}$
***P*-value**	Enzyme type	0.00	0.01	0.04	0.03
	Enzyme (U/g)	0.00	0.00	0.06	0.03

To = onset temperature, Tp = peak temperature, Tc = conclusion temperature, ΔH = enthalpy change. Values followed by different letters within a column denote significantly different levels ($P < 0.05$) (n = 3).

3.4 Conclusions

Porous starches could be obtained by enzymatic treatment of corn starch at sub-gelatinization temperature. The size distribution of the pores and their area were dependent on the type of enzyme used for the starch treatment, but also the level of enzyme. AMG led to porous starches with larger holes, whereas the smallest were obtained with CGTase. Porous starches differed in their pasting performance and thermal properties, besides adsorptive water or oil capacities. By selecting the type of enzyme and its level it could be modulated the degree of porosity.

Enzymatic treatment of native starch granules reveals as a powerful tool to modify the properties of starch. The added value and feasibility of this methodology on different sources of starch should be examined.

Acknowledgments

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CHAPTER 4

Results II

Contents

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Morphological and physicochemical characterization of porous starches obtained from different botanical sources and amylolytic enzymes

Yaiza Benavent-Gil, Cristina M.Rosell*

Abstract

Porous starches might offer an attractive alternative as bio-adsorbents of a variety of compounds. However, morphology and physicochemical properties of starches must be understood before exploring their applications. Objective was to study the action of different amylolytic enzymes for producing porous starches. Wheat, rice, potato and cassava starches were treated with Amyloglucosidase (AMG), α-amylase (AM) and cyclodextrin-glycosyltransferase (CGTase). Morphological characteristics, chemical composition, adsorptive capacity and pasting/thermal properties were assessed. Scanning Electron Microscopy (SEM) showed porous structures with diverse pore size distribution, which was dependent on the enzyme type and starch source, but no differences were observed in the total granule surface occupied by pores. The adsorptive capacity analysis revealed that modified starches had high water absorptive capacity and showed different oil adsorptive capacity depending on the enzyme type. Amylose content analysis revealed different hydrolysis pattern of the amylases, suggesting that AMG mainly affected crystalline region meanwhile AM and CGTase attacked amorphous area. A heatmap illustrated the diverse pasting properties of the different porous starches, which also showed significant different thermal properties,with different behavior between cereal and tuber starches. Therefore, it is possible to modulate the properties of starches through the use of different enzymes.

Keywords

Porous starch — Enzymes — Amyloglucosidase — α-amylase — CGTase — Microstructure

Institute of Agrochemistry and Food Technology (IATA-CSIC), C/Agustin Escardino, 7, Paterna 46980, Valencia, Spain

***Corresponding author**: crosell@iata.csic.es (C.M. Rosell).

4.1 Introduction

Porous starch granules are becoming of great interest such as non-toxic absorbents, owing to their great absorption capacity derived from the major specific surface area (Zhang et al., 2012). Pores can protect sensitive elements as oils, minerals, vitamins, bioactive lipids, food pigments such as β-carotene and lycopene that are sensitive to light, oxidation or high temperature (Luo et al., 2013; Majzoobi, Hedayati, and Farahnaky, 2015; Zhao, Madson, and Whistler, 1996). In fact, porous starches have been proposed as carriers or vehicles of colorants, spices, flavorings or sweeteners and pharmaceuticals (Belingheri et al., 2015). Nevertheless, very scarce information exists regarding the characteristics of the pores and how to modulate them to extend the application of the porous starches (Dona et al., 2010).

Up to now, several enzymes, such as α-amylase (AM), β-amylase, amyloglucosidase (AMG), pullulanase, isoamylase and cyclodextrin-glycosyltransferase (CGTase) have been used for producing porous starches (Dura, Błaszczak, and Rosell, 2014; Blazek and Gilbert, 2010; Chen and Zhang, 2012; Martínez, Pico, and Gómez, 2016). Pin-holes, sponge-like erosion, numerous medium-sized holes, distinct loci leading to single holes in individual granules and surface erosion are being observed after enzymatic action (Sujka and Jamroz, 2007), but there is no clear understanding about the role of either the botanical origin of starch or the enzyme used. Aggarwal and Dollimore (2000) observed an increase in the size of the pores on corn starch granules, when augmented the AMG concentrations, till the breakdown was so pronounced that walls around pinholes were broken, leading to large irregular holes and a disrupted structure. Recently, Benavent-Gil and Rosell (2017a) compared the effect of AMG, AM, CGTase and branching enzyme on corn starch properties, taking also into account the impact of enzyme level. Authors concluded that corn starches with varying number and size of pores could be obtained by controlling either the type of amylolytic enzyme or the level of enzyme.

In addition, it must be considered the intrinsic structural features of starches from different botanical origin, which might affect the amylolytic action. In fact, when corn, mung bean or sago starches were

treated with a mixture of AM and AMG at 35 °C for 24 h, porous granules were obtained, whereas only enzymatic erosion occurred on the surface of cassava starch granule (Uthumporn, Zaidul, and Karim, 2010). According to Rocha, Carneiro, and Franco (2010), AM degraded the external part of the granule surface of cassava, sweet potato, and potato starches after hydrolysis at 37 °C for 48 h; but Peruvian carrot starch showed only some granules with internal degradation.

In previous literature, substantial variation was found in terms of hydrolysis time and temperature, and enzyme type, which somewhat impedes the exploitation of porous starches; meanwhile there is no clear knowledge about the role of those factors on the pore development. Likewise, taking into account the variety of compounds to be adsorbed from foodstuffs, pharmaceutical, cosmetic and chemical products, the characterization of those starches would be needed from an industrial point of view.

Therefore, the main objective of this study was to identify the potential of starches from different botanical sources to obtain porous starches with different type of hydrolases. Particularly, to characterize and compare the effect of amyloglucosidase, fungal α-amylase and cyclodextrin-glycosyltransferase on the morphological and physicochemical properties of selected starches from cereals and tubers. In this study, morphological, chemical, thermal and pasting properties of different enzymatically modified starches were studied. Thereby, the granule characteristics as well as the enzyme attack on starch granules were visualized by scanning electron microscopy (SEM) and analyzed by a micrograph processing tool. In order to establish a possible correlation, these values were combined with chemical, pasting and thermal properties.

4.2 Materials and methods

4.2.1 Materials

Potato starch (Tereos Syral, Marckolsheim, France), wheat starch (NATILOR Chamtor company, Pomacle, France), intermediate amylose rice starch (Sigma-Aldrich, Spain) and cassava starch (local market) were used as substrates for enzymatic modification. Amyloglucosidase (EC 3.2.1.3), fungal α-amylase (EC 3.2.1.1) and cyclodextrin-glycosyltransferase (EC 2.4.1.19) activities were provided

by commercial food grade preparations (Amyloglucosidase 1100 L declared activity 1100 AGU/g product, Fungamyl® 2500SG declared activity 2500 FAU/g product and Toruzyme® 3.0 L declared activity 3KNU/mL product) supplied by Novozymes (Bagsværd, Denmark). All other reagents were of analytical grade. The water used was deionized.

4.2.2 Preparation of porous starch

The preparation of porous starch was based on the method of Benavent-Gil and Rosell (2017a). The selection of enzyme levels (16.5 AMG U/g, 11 AM U/g and 0.2 CGTase U/g) was based on preliminary experiments, which showed that under the experimental conditions used (50 °C, 2 h), maximum number of pores were obtained without distorting the granule. Native starches were included for comparison, and starches subjected to treatment conditions in the absence of enzymes were used as controls.

4.2.3 Scanning Electron Microscopy (SEM)

The granule morphology of native and modified starches was observed using a JSM 5200 scanning electron microscope (SEM) (JEOL, Tokyo, Japan). Samples were coated with gold in a vacuum evaporator (JEE 400, JEOL, Tokyo, Japan) prior to observation.The obtained samples were examined at an accelerating voltage of10 kV and magnified 3500 × times.

The microstructure analysis was carried out using the image analysis program (ImageJ, UTHSCSA Image Tool software). The SEM images were saved as 8-bit tiff format. Scale was initially set using the relationship between pixels and known distance. Threshold was assessed applying the default algorithm and then particle analysis was carried out. The following parameters were measured: granule and pore area. The area occupied by pores in a starch granule was calculated as the sum of the areas of all the pores of a starch granule divided by granule area. Values were the average of 20 independent measurements.

4.2.4 High performance anion exchange chromatography (HPAEC)

The hydrolysis compounds (oligosaccharides and cyclodextrins) lixiviated during enzymatic treatment were quantified using HPAEC (Dionex ICS3000, Thermo Fisher Scientific,

Waltham, MA,USA) according to the methodology described by Dura and Rosell (2016).

4.2.5 Analysis of chemical and physicochemical properties of modified starches

The amount of amylose/amylopectin in the starches was analyzed using a commercially available kit (Amylose/Amylopectin Assay Kit, Megazyme International Ireland Ltd., Bray, Co. Wicklow, Ireland) following supplier instructions. This enzymatic method is based on the concanavalin A method (Gibson, Solah, and McCleary, 1997). Water and sunflower oil adsorptive capacities of starches were determined following the method described by Yousif, Gadallah, and Sorour (2012), with slight modifications. Samples (0.100 g ± 0.005 g) were mixed with distilled water or oil (1 mL) and centrifuged at 3000× g for 10 min. Adsorptive capacities were expressed as percent weight of solvent retained by the sample. Each measurement was performed in duplicate.

4.2.6 Viscosity measurement

The pasting properties of native and enzymatically modified starches were measured using a Rapid Visco Analyzer (RVA-4500,Perten Instruments, Hägersten, Sweden). Starch (2.00 g ± 0.01 g based on 14% moisture content) was added to 20 mL of distilled water placed into the aluminum RVA canister. Slurries underwent a controlled heating and cooling cycle, from 50 to 95 °C in 282 s, holding at 95 °C for 150 s and then cooling to 50 °C. The initial speed for mixing was 960 rpm for 10 s, followed by a 160 rpm paddle speed that was maintained for the rest of assay. Peak viscosity, final viscosity, breakdown (peak viscosity-through), setback (final viscosity-through) and onset temperature for pasting formation were determined from the viscosity plot and recorded using Thermocline software for Windows (Perten Instruments, Hägersten, Sweden). The level of hydrolysis at 95 °C and 50 °C was defined as the %-change in paste viscosity recorded in the RVA at 50 °C and 95 °C.

4.2.7 DSC thermal analysis

Gelatinization properties of modified starches were measured using a differential scanning calorimeter (DSC) from Perkin-Elmer (DSC 7, Perkin-Elmer Instruments, Norwalk, CT). The slurry of starch and water (1:3) was placed into stainless steel capsules. The sealed capsules were equilibrated at room temperature for one

hour before analysis. The samples were then heated from 30 to 120 °C at a heating rate of 10 °C/min under nitrogen atmosphere, using an empty stainless steel capsule as reference. The onset (To), peak (Tp) and conclusion (Tc) temperatures were determined from the thermogram. The enthalpy of gelatinization (ΔH) was estimated based on the area of the main endothermic peak, expressed as joule per gram sample (J/g).

4.2.8 Statistical analysis

The data reported are the mean of replicates and expressed as a mean ± standard deviation. Statistical analyses were carried out with Fisher's least significant differences test with a significance level of 0.05. Pearson correlation coefficient (r) and P-value were used to indicate correlations and their significance using Statgraphics Centurion XV software (Bitstream, Cambridge, N). The correlation coefficient was classified in different levels of correlation: perfect ($|r|= 1.0$), strong ($0.80 \leq |r| \leq 1.0$), moderate ($0.50 \leq |r| \leq 0.80$), weak ($0.10 \leq |r| \leq 0.50$), and very weak (almost none) correlation ($|r| \leq 0.10$).

4.3 Results and Discussion

4.3.1 Microstructure of modified starches

Figure 4.1 shows SEM micrographs of native, references and modified starches. As expected, SEM micrographs revealed the broad variation in shape and area among native starches from different sources (Figure 4.1 A1, B1, C1 and D1). Granules from wheat were composed of two different populations. The large A-type granules exhibited lenticular or disk shapes, while the small B-type granules exhibited principally spherical or ellipsoidal shape. The granules average area was 242.90 μm^2 and 13.11 μm^2 for A-type and B-type granules,respectively (Figure 4.1 A1). Rice starch granules displayed polygonal shapes and 17.53 μm^2 in granules average area (Figure 4.1 B1). Potato starch was composed of large granules and similar to wheat, two different populations were observed (Figure 4.1C1). The largest granule fraction was ellipsoid in shape with a granules average area of 1098.04 μm^2, and the smallest fraction was basically spherical in shape and average area of 291.95 μm^2. Cassava starch granules showed many truncated granules and with several grooves on the surface and average

area of granules was 117.99 μm^2 (Figure 4.1D1).The observed microscopic appearances are in agreement with literature (Schirmer et al., 2013). The reference starch granules, subjected to treatment in the absence of enzymes at pH 4.5 (Figure 4.1A2, B2, C2 and D2) and pH 6.0 (Figure 4.1A3, B3, C3 and D3), kept their integrity, and there was not significant difference observed in rupture, breakage or pores due to the incubation with buffer (Figure 4.1A2,3, B2,3, C2,3 and D2,3) as has been previously reported (Dura, Błaszczak, and Rosell, 2014; Dura and Rosell, 2016).

Enzymatic treatments modified the surface of starches and the extent of the effect was highly dependent on the source of starch. Differences in the enzymatic action could be related to the starches susceptibility to be attacked (Figure 4.1A4–6, B4–6, C4–6 and D4–6). Generally, the enzymatic action on the starches provoked the formation of deep holes in cereal starches, while more superficial attacks were observed in the tuber starches (Gallant et al., 1992). In order to quantitatively establish possible differences associated to enzyme type and starch botanical origin, the pore size and the ratio pore area to starch granule area (related to the abundance of pores per granule) were assessed (Figure 4.2). Enzyme type and starch source significantly affected the pore size distribution (Figure 4.2A). Nevertheless, the ratio pore area to granule area (Figure 4.2B) was similar regardless starch source and enzyme type, with the exception of wheat starch treated with AMG that showed sponge-like erosion structures. Specifically,AMG treated wheat starch showed the formation of holes along the equatorial groove, suggesting main hydrolysis at these points, and in some cases leading to rupture of the granules. Rice, potato and cassava starches also showed pores on the surfaces of granules after AMG treatment, but in rice seems to be deeper than in cassava and potato starches. Aggarwal and Dollimore (2000) observed that potato starch offers greater resistance to AMG attack than wheat, rice, and corn starches. Image analysis did not reveal significant changes in the pore size distribution among starch types, but an increase in the abundance of pore per granule in treated wheat starch was observed.

The action of AM led to similar pore size distribution on the granules surface, independently on the

starch source, with the exception of potato starch that displayed bigger holes and wider distribution. In the case of treatment by CGTase, cereal starches exhibited wider distribution of pore sizes than tuber starches (Figure 4.2A). Overall, SEM suggested that enzymatic hydrolysis of cereal starches was initiated from granule surfaces and then spread toward the granule interior, producing deeper holes compared to tuber starches. Possibly the presence of pores and channels in cereal starches allowed enzymes to penetrate towards the granule interior, while the rigid and smooth surface of tuber starches acted as a barrier to enzymes (Gallant et al., 1992; Dhital et al., 2014). Moreover, tuber starches are more resistant to the enzymatic hydrolysis than cereal starches, due to a high number of branch points in non-crystalline regions, which lead to high density amorphous regions and stable crystallites (Dhital, Shrestha, and Gidley, 2010), yielding less deep holes.

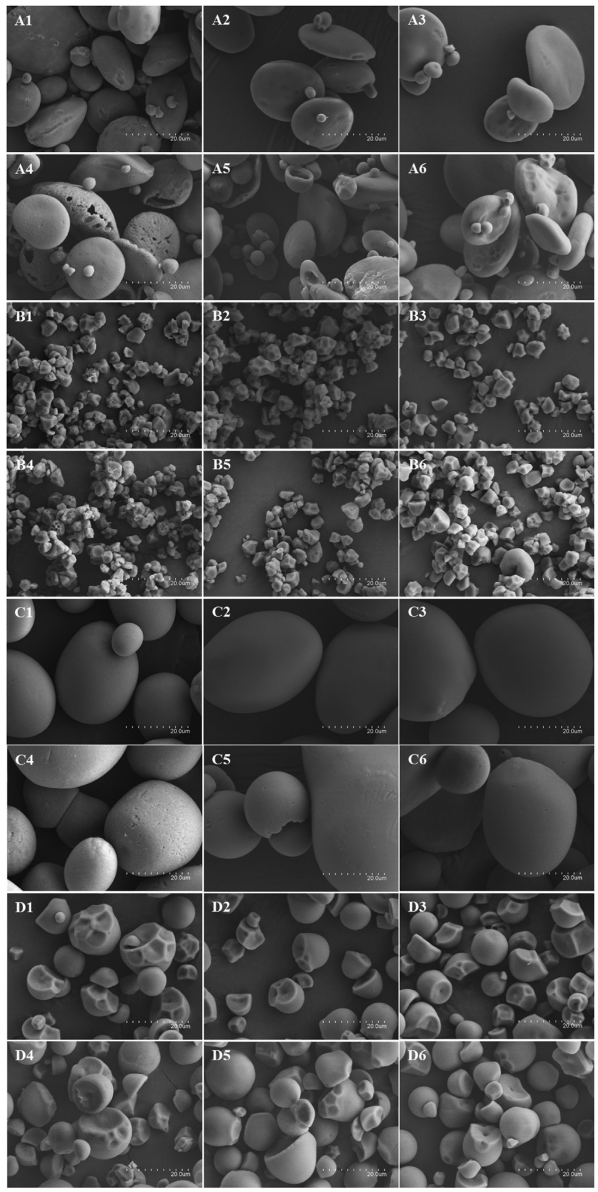

Figure 4.1: Scanning electron micrograph (wheat: A; rice: B; potato: C; cassava: D) of native starches (1), starches treated enzymatically (AMG: 4; AM: 5; CGTase: 6) and their counterparts subjected to treatment conditions without the presence of enzymes (2–3). Magnification 2000×.

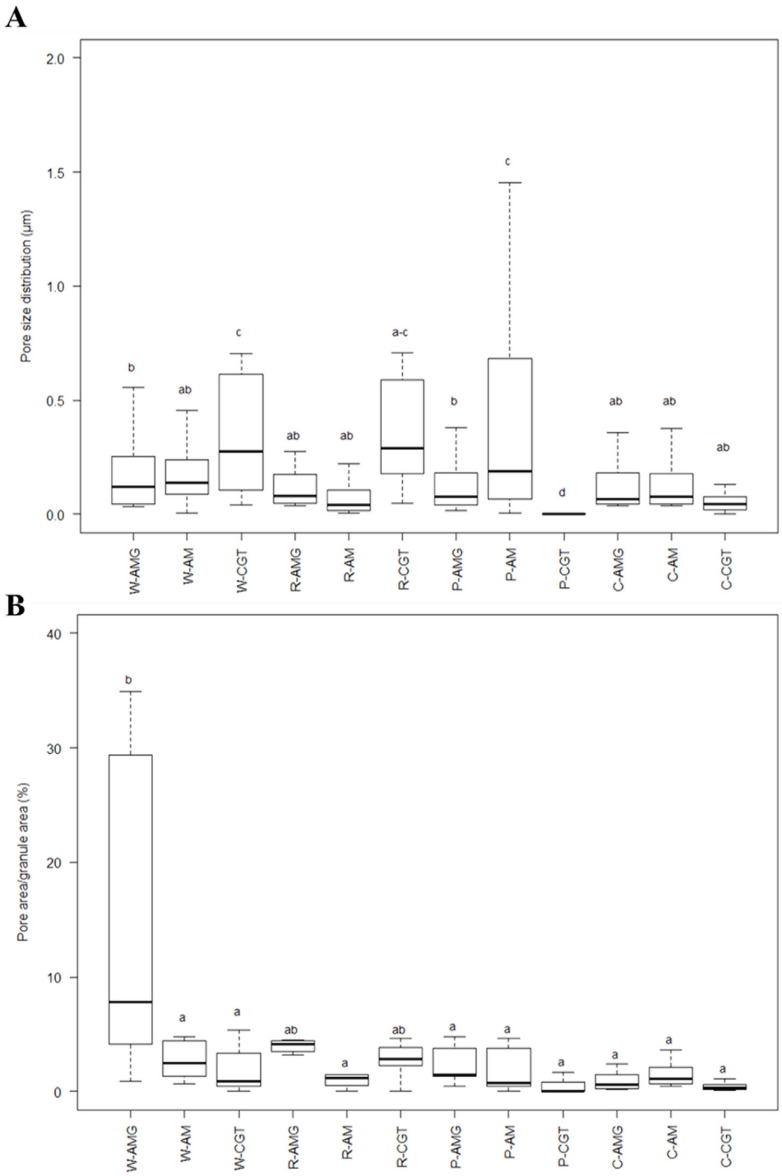

Figure 4.2: Pore size (A) and pore surface area distribution (B) obtained for each enzymatic treatment of starches from different origins. Notations are referred to the starch botanical source (Wheat, Rice, Potato, Cassava) followed by the abbreviations of the enzyme used (AM, AMG, CGT).

Table 4.1: Oligosaccharides and cyclodextrins released after corn starch hydrolysis by AMG, AM and CGTase. Results are expressed in mg 100 g^1 of starch.

Starch source	Enzyme type	Glucose	Maltose	Maltotetriose	Maltotetraose	Maltopentose	α-CD	β-CD	γ-CD
Wheat	AMG	23.94 ± 0.71^{e}	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	AM	8.46 ± 0.33^{c}	18.88 ± 0.30^{e}	2.07 ± 0.17^{e}	5.98 ± 0.38^{f}	n.d	n.d	n.d	n.d
	CGTase	0.86 ± 0.02^{a}	1.38 ± 0.00^{b}	1.09 ± 0.09^{c}	1.33 ± 0.05^{c}	0.02 ± 0.00^{b}	2.81 ± 0.04^{b}	0.56 ± 0.01^{a}	1.57 ± 0.00^{a}
Rice	AMG	25.42 ± 0.72^{f}	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	AM	7.04 ± 0.29^{b}	20.04 ± 0.34^{f}	1.59 ± 0.15^{d}	4.99 ± 0.38^{e}	0.04 ± 0.00^{c}	n.d	n.d	n.d
	CGTase	0.21 ± 0.02^{a}	0.38 ± 0.00^{a}	0.34 ± 0.04^{a}	0.28 ± 0.01^{a}	n.d	3.00 ± 0.05^{d}	1.11 ± 0.01^{c}	3.32 ± 0.00^{d}
Potato	AMG	24.03 ± 1.18^{e}	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	AM	10.12 ± 0.29^{d}	6.12 ± 0.41^{c}	5.83 ± 0.13^{g}	2.22 ± 0.20^{d}	0.01 ± 0.00^{a}	n.d	n.d	n.d
	CGTase	0.39 ± 0.03^{a}	0.64 ± 0.01^{a}	0.57 ± 0.04^{b}	0.67 ± 0.01^{b}	n.d	2.55 ± 0.04^{a}	1.84 ± 0.01^{d}	2.32 ± 0.00^{b}
Cassava	AMG	25.67 ± 0.72^{f}	n.d	n.d	n.d	n.d	n.d	n.d	n.d
	AM	8.48 ± 0.29^{c}	6.61 ± 0.34^{d}	3.15 ± 0.17^{f}	13.05 ± 0.38^{g}	n.d	n.d	n.d	n.d
	CGTase	0.92 ± 0.03^{a}	1.39 ± 0.10^{b}	1.01 ± 0.09^{c}	1.16 ± 0.06^{c}	n.d	2.88 ± 0.01^{c}	0.88 ± 0.01^{c}	2.94 ± 0.00^{c}
***P*-value**	Enzyme type	0.000	0.000	0.000	0.000	0.021	0.000	0.000	0.000
	Starch source	0.250	0.008	0.009	0.003	0.064	0.008	0.006	0.006

n.d.: Not detected.

Values followed by different letters within a column denote significant differences ($P < 0.05$) (n = 3).

4.3.2 Cyclodextrins and oligosaccharides released during enzymatic treatment of starches

Enzymes acted differently on the starch granules as indicated by the compounds released (glucose, oligosaccharides and cyclodextrins) during enzymatic treatment, and also depending on the botanical origin of starch (Table 4.1). In the absence of enzymes, no hydrolysis products were released (data not shown). In line with other reports, the only product of hydrolysis after AMG treatment was glucose. AMG is a well-known exo-amylase, releasing only glucose residues from amylose or amylopectin chains (Bouchet-Spinelli et al., 2013). Regardless of starch source, the amount of glucose seemed constant, likely due to glucoamylase level was enough to produce saturation of the non-reducing-ends of starch chains (Chen and Zhang, 2012). AM and CGTase are known to cleave α-1–4 glycosidic bonds existing in the internal part (endo-) of a polysaccharide chain. The main compounds produced during AM treatment differed between cereal and tuber starches.The products of hydrolysis were mainly maltose followed by glucose and oligosaccharides with a DP of 3–4. Conversely, the glucose was predominantly released from potato, whereas maltotetraose was the major hydrolysis product from cassava. CGTase treatment converted starch into a mixture of α-, β- and -CD and smaller amounts of oligosaccharides with a DP of 1–4, regardless starch source. Nevertheless, α-, β- and -CD contents varied between starches depending on the enzyme specificity (Terada et al., 1997) but also on the substrate (Blackwood and Bucke, 2000).

Table 4.2: Effect of enzymatic treatment on amylose content and the water and oil adsorption capacity of the resulting porous starches.

Starch source	Enzyme type	Amylose content (%)	Adsorptive water (capacity g/g)	Adsorptive oil (capacity g/g)
Wheat	Native	$21.20 \pm 0.16^{\text{d-f}}$	$0.77 \pm 0.03^{\text{b}}$	$0.65 \pm 0.01^{\text{c-e}}$
	AMG	$24.39 \pm 1.34^{\text{f-g}}$	$1.37 \pm 0.04^{\text{h}}$	$0.86 \pm 0.09^{\text{gh}}$
	AM	$19.98 \pm 2.60^{\text{de}}$	$1.13 \pm 0.02^{\text{ef}}$	$0.74 \pm 0.04^{\text{e-g}}$
	CGTase	$19.93 \pm 0.14^{\text{de}}$	$1.10 \pm 0.02^{\text{ef}}$	$0.81 \pm 0.06^{\text{f-h}}$
Rice	Native	$13.88 \pm 0.98^{\text{c}}$	$1.04 \pm 0.06^{\text{d}}$	$1.10 \pm 0.05^{\text{j}}$
	AMG	$23.67 \pm 3.94^{\text{e-g}}$	$1.15 \pm 0.02^{\text{e-g}}$	$0.98 \pm 0.01^{\text{ij}}$
	AM	$9.49 \pm 1.61^{\text{b}}$	$1.16 \pm 0.02^{\text{fg}}$	$0.92 \pm 0.01^{\text{hi}}$
	CGTase	$3.02 \pm 0.69^{\text{a}}$	$1.14 \pm 0.00^{\text{e-g}}$	$1.37 \pm 0.12^{\text{k}}$
Potato	Native	$26.53 \pm 1.19^{\text{g}}$	$0.62 \pm 0.02^{\text{a}}$	$0.50 \pm 0.01^{\text{ab}}$
	AMG	$22.81 \pm 3.22^{\text{e-g}}$	$1.09 \pm 0.05^{\text{de}}$	$0.55 \pm 0.08^{\text{a-c}}$
	AM	$21.81 \pm 1.69^{\text{d-f}}$	$1.20 \pm 0.01^{\text{g}}$	$0.48 \pm 0.03^{\text{a}}$
	CGTase	$18.89 \pm 1.20^{\text{d}}$	$1.20 \pm 0.01^{\text{g}}$	$0.61 \pm 0.04^{\text{b-d}}$
Cassava	Native	$24.66 \pm 1.18^{\text{fg}}$	$0.67 \pm 0.01^{\text{a}}$	$0.87 \pm 0.08^{\text{g-i}}$
	AMG	$24.63 \pm 0.05^{\text{fg}}$	$1.13 \pm 0.02^{\text{ef}}$	$0.72 \pm 0.03^{\text{d-f}}$
	AM	$22.70 \pm 2.07^{\text{d-g}}$	$0.91 \pm 0.02^{\text{c}}$	$0.67 \pm 0.08^{\text{de}}$
	CGTase	$20.09 \pm 2.22^{\text{de}}$	$0.91 \pm 0.04^{\text{c}}$	$0.63 \pm 0.04^{\text{c-e}}$
***P*-value**	Enzyme type	0.000	0.000	0.039

Continued on next page...

Table 4.2 – Continued

Starch source	**Enzyme type**	**Amylose content** (%)	**Adsorptive water** (capacity g/g)	**Adsorptive oil** (capacity g/g)
	Enzyme (U/g)	0.000	0.000	0.099

Values followed by different letters within a column denote significant differences ($P < 0.05$) (n = 3).

4.3.3 Amylose content and adsorptive capacity

In agreement with data reported in the literature (Singh et al., 2003), significant differences in amylose contents were detected among the starches from different sources (Table 4.2). The cereal starches (wheat and rice) contained lower average amylose content compared to the tuber starches.

Enzymatic treatment for obtaining porous starches affected amylose contents and the effect was significantly dependent on the starch origin and the enzyme type (Table 4.2). The ratio amylose/amylopectin remained unchanged in the case of wheat starch,whereas in the other starches the enzymatic treatment led to a decrease in the amylose content, with the exception of AMG modification. Specifically, AMG treatment increased the amylose content of rice starch, and no significant effect was detected in the other starches. Taking into account that a decrease in starch crystallinity has been correlated with an increase in amylose content (Hung, Maeda, and Morita, 2007), it seems that amylopectin of rice starch was preferentially hydrolyzed by AMG, since amylopectin has many more non-reducing ends. AM and CGTase preferentially hydrolyzed the amylose chains in rice and potato starches resulting in a decrease of this polymer, because the amylose is located in the amorphous regions. This observations agrees with the inverse relationship between the amylose content and the amount of hydrolyzed starch previously reported (Tester, Qi, and Karkalas, 2006). However, no significant effect was observed in the amylose content when cassava starch was treated with AM. It seems that AM and CGTase attacked amorphous domains, where the majority of the amylose is located (Copeland et al., 2009), leading an increase in the amount of amylopectin. Therefore, results on amylose content revealed that depending on enzymatic treatment amylose or amylopectin are primarily hydrolyzed.

The water adsorptive capacity (WAC) of starches was significantly dependent on the starch source, being higher for cereal starches; whereas no trend was observed for adsorptive oil capacity (OAC) (Table 4.2). Enzymatic treatment significantly affected the WAC and OAC (Table 4.2). All enzymatic treatments increased the ability of starch to bind water molecules, which suggested that hydrophilic tendency

of starch increased after enzymatic treatment. Among them, wheat starch treated with AMG showed the greatest absorption. Likely, the pore surface area originated by AMG was responsible of this behavior due to the increase of the surface area. It is generally assumed that the holes created in the starch surface after enzymatic treatment increase the surface area, having significant influence on starch water retention (Chen and Zhang, 2012).

Nevertheless, no clear tendency was observed for the OAC. AMG treatment did not significantly affect that property in rice and potato starch, while this enzyme enhanced and reduced the OAC of wheat and cassava starches, respectively. The addition of AM to rice and cassava starches resulted in lower values for their OAC, but no change was induced in wheat and potato starch. Conversely, the treatment of wheat and rice starches with CGTase led to porous starches with higher OAC, but this enzyme reduced this parameter in cassava starch and there is no change observed in potato starch.These observations suggested that rice after AM treatment and cassava porous starch, obtained with any of the tested enzymes, had more lipophilic surface. A significant negative and moderate correlation was identified between the OAC and the amylose content ($r = 0.684$, $P < 0.000$). Therefore, the ratio amylose/amylopectin must play an essential role in the OAC, being responsible of the different trend observed with each enzymatic treatment.

4.3.4 Pasting and thermal starch properties of porous starches

A heatmap was constructed (Figure 4.3) to visualize differences between pasting characteristics of the porous starches from different sources. The heatmap of the hierarchical clustering of the RVA properties was analyzed on the basis of similarities and differences in starch pasting properties, including onset, peak viscosity, through, breakdown, final viscosity, setback, hydrolysis percentage at 95 °C and 50 °C. In line with previous studies (Schirmer et al., 2013), the tuber starches displayed different paste viscosity patterns compared to their cereal counterparts. Starches from cereals showed lower peak viscosity, breakdown and final viscosity compared to tuber starches. The resulting dendrogram differentiated three different clusters. First cluster only contained cereal starches,

whereas second and third essentially comprised porous starches from cassava and potato, respectively. It was evident from the dendrogram that amylolysis changed the pasting performance of starch suspensions and the effect was highly dependent on the starch source. Nevertheless, there was more similarity among porous starches from same botanical source than among starches from different botanical origin treated with the same enzyme. This effect was more pronounced in the case of tuber starches than in cereal starches, which showed greater similarities between them. It has been reported that the enzymatic susceptibilities of starches varied depending on factors such as granule area, strength of association between starch components, ratio of amylose and amylopectin, crystallinity, polymorphic type (A, B, C), amylose-lipid complex, type of enzyme, and hydrolysis conditions (Dona et al., 2010; Dura, Błaszczak, and Rosell, 2014; Dura and Rosell, 2016). Significant correlations were found between pasting parameters and OAC of the starches. Particularly, OAC was significantly positive correlated with pasting temperature and setback ($r = 0.827$, $P < 0.000$; $r = 0.617$, $P < 0.000$),but showed a negative correlation with peak viscosity, through and breakdown ($r = 0.665$, $P < 0.000$; $r = 0.463$, $P < 0.008$; $r = 0.633$, $P < 0.000$). The onset temperature or temperature where viscosity started to increase was significantly ($P < 0.05$) augmented in cereal starches and decreased in tuber starches by AMG. Wheat and potato starches treated with AM had higher and lower onset temperature, respectively; while rice and cassava starches remained unchanged after hydrolysis. The CGTase action induced an enhancement of this parameter in all starches studied, except when added to cassava starch, which showed opposite behavior.

Porous starches obtained with AMG showed low peak viscosity compared to their native counterpart, particularly rice starch displayed the lowest peak viscosity, likely due to its large amylose contents after enzymatic hydrolysis as suggested Chung et al. (2011). Porous starches from cereals also showed a reduction of through and final viscosity values, but only low setback was observed on rice starch after AMG action. Besides, it was observed the presence of an additional peak viscosity (Pv1) during heating, prior to the common peak viscosity at 95 °C,

in the case of wheat starch treated with AMG. A similar result was recently reported by Benavent-Gil and Rosell (2017a) when studying the addition of different AMG levels to corn starches, observing a progressive increase of that peak with the level of AMG added, and a simultaneous decrease of the maximum peak viscosity. Pertaining to enzymatic treatment, diverse effect was promoted. Specifically, tuber starches showed a significant ($P < 0.05$) decrease of the peak viscosity, breakdown and setback after AM action. Moreover, only cassava starch decreased through and final viscosity, while potato starch enhanced these parameters after AMG treatment. It seems that AM preferentially disrupted the amorphous growth rings of cereal starches, but the amorphous and crystalline regions in tuber starches (Blazek and Gilbert, 2010). CGTase attack produced a significant ($P < 0.05$) decrease in pasting parameters of potato starch. In the case of wheat and cassava starches,CGTase treatment resulted in a low peak viscosity, through, final viscosity and setback, but a high breakdown. Similar effects were reported when wheat starch was treated with CGTase (Gujral and Rosell, 2004). Conversely, rice starch modified by CGTase showed an increase in breakdown and setback, but showed a decrease in through and final viscosity.

The gelatinization temperatures (To, Tp and Tc) as well as the enthalpy changes (ΔH) of native and modified starches are summarized in Table 4.3. A significant difference in gelatinization temperature was observed between cereal and tuber starches. The highest To, Tp and Tc values were found for rice starch followed by cassava, potato and wheat starches. Enzymatic treatment only promoted significant ($P < 0.05$) differences on the Tc (Table 4.3). Taking into account the interaction between botanical source and enzymatic treatment, it was observed that main differences were detected on To and Tc. Porous starches from cereals (wheat and rice) showed higher To, with the exception of rice starch treated with AM; whereas porous starches from tubers exhibited lower Tp and Tc. Similar results were obtained after partial hydrolysis using glucoamylases of wheat, corn and rice starches, which showed a high To (Aggarwal and Dollimore, 2000). The different behavior of the AM treated rice starch might be related to degradation of amorphous areas, as suggested the amylose content analysis. There-

fore, enzymatic treatment of cereal starches affected mainly the beginning of the gelatinization, in opposition to tuber starches where the last part of the gelatinization was more affected. Likely, factors such as granular pores and channels and length of amylopectin spacers and branches could be responsible of that behavior (Blazek and Gilbert, 2010).

Regarding the gelatinization enthalpy, no relationship was found neither with the botanical origin of the starches or the enzyme type. The highest ΔH values were noted in the potato starches followed by the cassava, wheat and rice starch. After the enzymatic treatment, porous starches showed lower ΔH compared to their native starches, except porous starches from rice that showed higher ΔH. Again, this result suggested that the state of the crystalline and amorphous regions of porous rice starches differed from the others. ΔH has been related to the amount of ordered carbohydrate structure in the granule that is disrupted during gelatinization (Warren et al., 2011). Therefore, the low ΔH values indicated that porous starches from wheat, potato and cassava required less energy to promote starch gelatinization, thus less energy was needed to uncoiling and melt the unstable double helices during gelatinization (Chung, Liu, and Hoover, 2009).

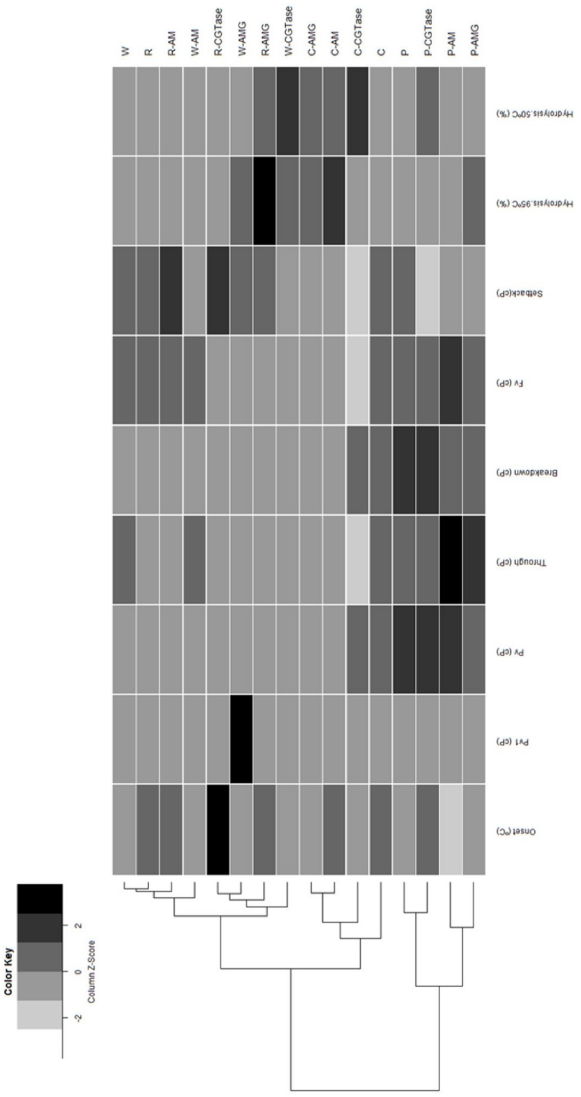

Figure 4.3: Hierarchical clustering of RVA profiles. A heatmap representing the hierarchical clustering of the Z-scores of the enzyme activities related to viscoelastic properties, when compared starches from different sources obtained from AMG, AM and CGTase enzymatic treatment. The Z-scores represent the dispersion around the overall mean of the viscoelastic properties and weighted by their standard errors. The scale of the intensity is shown in the top corner. Row represents samples and column represents viscoelastic properties. Notations are referred to the starch botanical source (Wheat, Rice, Potato, Cassava) followed by the abbreviations of the enzyme used.

Table 4.3: Thermal properties of enzymatically modified starches from different botanical sources.

Starch source	Enzyme type	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)
Wheat	Native	53.16 ± 1.74^{b}	58.45 ± 1.06^{bc}	64.66 ± 0.93^{b}	20.88 ± 1.05^{ef}
	AMG	58.58 ± 0.10^{ef}	60.78 ± 0.12^{de}	64.93 ± 0.32^{b}	19.33 ± 0.83^{cd}
	AM	57.51 ± 0.11^{c-e}	60.37 ± 0.24^{d}	64.48 ± 0.37^{b}	18.99 ± 0.45^{c}
	CGTase	56.68 ± 0.12^{cd}	59.62 ± 0.12^{cd}	63.49 ± 0.16^{b}	18.18 ± 0.59^{bc}
Rice	Native	58.84 ± 1.05^{ef}	66.62 ± 0.83^{h}	75.22 ± 0.28^{ef}	14.84 ± 0.17^{a}
	AMG	60.83 ± 0.38^{g}	66.78 ± 0.12^{h}	75.53 ± 0.49^{f}	20.62 ± 0.42^{de}
	AM	59.37 ± 0.52^{f}	67.20 ± 1.18^{h}	74.68 ± 1.19^{ef}	19.50 ± 0.53^{c-e}
	CGTase	61.74 ± 1.59^{g}	64.45 ± 0.35^{g}	73.78 ± 0.47^{de}	19.34 ± 0.84^{cd}
Potato	Native	56.35 ± 0.19^{c}	61.79 ± 0.12^{ef}	69.12 ± 0.56^{c}	27.59 ± 0.54^{i}
	AMG	52.60 ± 1.70^{ab}	56.20 ± 0.47^{a}	61.91 ± 0.51^{a}	22.55 ± 0.63^{g}
	AM	50.88 ± 0.18^{a}	55.37 ± 1.41^{a}	61.15 ± 1.71^{a}	15.64 ± 0.36^{a}
	CGTase	51.03 ± 0.11^{a}	57.78 ± 1.06^{a}	64.29 ± 0.73^{b}	22.22 ± 0.00^{fg}
Cassava	Native	57.40 ± 0.02^{c-e}	65.85 ± 0.21^{gh}	75.26 ± 0.18^{ef}	24.04 ± 1.04^{h}
	AMG	58.38 ± 0.32^{d-f}	62.53 ± 0.47^{f}	72.32 ± 0.68^{d}	17.18 ± 0.57^{b}
	AM	57.49 ± 0.14^{c-e}	62.70 ± 0.00^{f}	72.53 ± 0.32^{d}	18.98 ± 1.11^{c}
	CGTase	57.82 ± 0.19^{c-f}	63.03 ± 0.00^{f}	72.86 ± 0.70^{d}	18.85 ± 0.59^{c}
***P*-value**	Enzyme type	0.4431	0.1021	0.0051	0.1191
	Enzyme (U/g)	0.0000	0.0000	0.0000	0.1167

To = onset temperature, Tp = peak temperature, Tc = conclusion temperature, ΔH = enthalpy change. Values followed by different letters within a column denote significant differences ($P < 0.05$) (n = 3).

4.4 Conclusions

Starches from cereal and tuber sources could be used to obtain porous starches with different structural and functional features,which also depended on the enzyme used to produce the surface pores or cavities. Cereal starches were more susceptible to enzymatic hydrolysis than tuber starches, presenting deep holes with some degradation of its internal part. The size distribution of the pores was dependent on the type of enzyme and botanical source of starch, but the number of pores per granule was independent of the above. The right combination of type of starch and enzyme could provide porous starches with different degree of porosity, as well as varied pasting performance, thermal properties, WAC and OAC.

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Chapter 5

Results III

Contents

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

Yaiza Benavent-Gil, Cristina M. Rosell*

Abstract

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

Keywords

Digestibility — Enzymes — Glycemic index — Porous starch

Institute of Agrochemistry and Food Technology (IATA-CSIC), C/Agustin Escardino, 7, Paterna 46980, Valencia, Spain

***Corresponding author**: crosell@iata.csic.es (C.M. Rosell).

5.1 Introduction

Starch constitutes a biopolymer widely used in the food industry owing its unique thermal, structural and functional properties. Nevertheless, native starch is not always suitable for food production and diverse modifications have been reported to overcome those limitations (Singh, Kaur, and McCarthy, 2007). Lately, enzymatic modification of starch is gaining attention as an environmentally friendly process that led to porous molecules with great adsorbent capacity (Zhang et al., 2012), being dependent on the enzymatic treatment and starch source (Uthumporn, Zaidul, and Karim, 2010; Sujka and Jamroz, 2007). However, scarce information is available about the impact of those modifications on nutrition and health.

Concerning nutritional implications, starch digestibility is an important property that can be altered by enzymatic modification. The surface organization, granular architecture, starch composition, type of crystal polymorph, granular size, and the presence of compound granules, affect the rate and extent of starch digestibility (Dona et al., 2010; Tahir, Ellis, and Butterworth, 2010). Among these factors, the presence of cracks on the surface layer has been related to the feasibility of digestive enzymes to hydrolyze native starch granules (Singh, Dartois, and Kaur, 2010). Porous starches obtained enzymatically contain abundant surface pores that go to the inner center of the granules (Benavent-Gil and Rosell, 2017a). The number of pores is dependent on both the type and level of enzyme used for the production of porous starch (Benavent-Gil and Rosell, 2017a). Likewise, the number and size of the pores determine the morphological and physicochemical properties of the resulting porous starches and their subsequent applications in food industry as adsorbents.

In spite of the different applications reported for the porous starches (Belingheri, Ferrillo, and Vittadini, 2015; Kaur et al., 2012; Luo et al., 2013; Majzoobi, Hedayati, and Farahnaky, 2015), there is a dearth of information on their digestibility pattern. Dura, Błaszczak, and Rosell (2014) and Dura and Rosell (2016) studied the effects of AMG, AM and CGTase on digestibility behavior of corn starch at sub-gelatinization temperature (50 °C). High susceptibility to be digested was shown by porous starches obtained after pro-

longed treatment with AM or AMG. An opposite trend was displayed on CGTase-modified starches that resulted less susceptible to be hydrolyzed by digestive enzymes. In addition, *in vivo* studies showed that porous starches obtained with CGTase had slower digestion, reducing the blood glucose levels, which was attributed to the presence of β-cyclodextrins that may impede the orientation of amylases Dura, Yokoyama, and Rosell (2016). Despite those initial studies, no information is available about the impact of granule morphology on the digestion pattern.

The aim of this study was to determine possible relationship among morphological structure of porous starches and digestibility performance. For that purpose, a range of enzymatically modified starches obtained in a previous study (Belingheri, Ferrillo, and Vittadini, 2015) were used to study their digestibility and glycemic index. In that previous study, porous starches were obtained with different enzymes (AMG, AM, CGTase and BE) using a range of enzyme concentrations; those treatments provided porous starches with varied size and frequency of surface pores. Those starches were subjected to digestive amylase and hydrolysis kinetics were compared.

5.2 Materials and methods

Corn starch with a purity of 98.39% was purchased from Miwon (Seoul, Korea). Amyloglucosidase (EC 3.2.1.3), fungal α-amylase (EC 3.2.1.1), cyclodextrin-glycosyltransferase (EC 2.4.1.19) and branching enzyme (EC 2.4.1.18) activities were provided by commercial food grade preparations (Amyloglucosidase 1100 L declared activity 1100 AGU/g product, Fungamyl® 2500SG declared activity 2500 FAU/g product, Toruzyme® 3.0 L declared activity 3KNU/mL product and Branchzyme declares activity 50,000 BEU/mL) supplied by Novozymes (Bagsværd, Denmark). All the other chemicals were analytical reagent grade. All solutions and standards were prepared by using deionized water.

5.2.1 Preparation of porous starch

The preparation of porous starch was based on the method of Benavent-Gil and Rosell (2017a). Enzyme stock solutions were added to the starch suspensions (U/g starch). The lowest enzyme level was the minimum recommended by the manufacturer (5.5

AMG U/g, 5.5 AM U/g, 0.1 CGTase U/g and 500 BE U/g) and increasing concentrations (2, 3, 6 and 10 times the initial level) were tested. Native starches were included for comparison, and starches subjected to treatment conditions in the absence of enzymes were used as controls.

5.2.2 Scanning electron microscopy (SEM)

The granule morphology of native and modified starches was observed using a JSM 5200 scanning electron microscope (SEM) (JEOL, Tokyo, Japan). Samples were examined at an accelerating voltage of 10 kV and magnified 2000× times. The microstructure analysis was carried out using the methodology described by Benavent-Gil and Rosell (2017a). The following parameters were measured: granule size and the pore area. The area occupied by pores in a starch granule (related to the abundance of pore per granule) was calculated as the sum of the areas of all the pores of a starch granule divided by granule area. Values were the average of 20 independent measurements.

5.2.3 *In vitro* starch digestibility and expected glycemic index

Digestibility of native, enzymatically treated and untreated starches was determined following the method described by Gularte and Rosell (2011) with minor modifications. 100 mg sample were dissolved in 4 mL of 0.1 M sodium maleate buffer (pH 6.9) with porcine pancreatic α-amylase (0.2 U/mL) (Type VI-B, $\geq$ 10 units/mg solid, Sigma Chemical, St. Louis, USA) and incubated in a shaking water bath at 37 °C during 3 h. Aliquots of 200 μL were taken at different incubation times and mixed with 200 μL ethanol (96%) in order to stop the enzymatic hydrolysis. Then, the sample was centrifuged for 5 min at 10,000× g and 4 °C. The pellet was washed with 50% ethanol (200 μL) and the mixture of supernatants were kept together at 4 °C for further glucose determination.

The remnant starch after 16 h hydrolysis was solubilized with 2 mL of 2 M KOH using a Polytron Ultraturrax homogenizer IKA-T18 (IKA-works,Wilmington, USA) during 1 min at 14,000 rpm. The homogenate was diluted with 8 mL 1.2 M sodium acetate pH 3.8 and incubated with 100 μL AMG (143 U/mL) at 50 °C

for 30 min in a shaking water bath. After centrifuging at 2000× *g* for 10 min, supernatant was kept for glucose determination.

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

The *in vitro* digestion kinetics was calculated fitting experimental data to a first-order eq. (Goñi, García-Alonso, and Saura-Calixto, 1997): $C = C_{\infty}(1 - e^{-kt})$ where C was the concentration at t time, C_{∞} was the equilibrium concentration or maximum hydrolysis extent, k was the kinetic constant and t was the time chosen. The hydrolysis index (HI) was obtained by dividing the area under the hydrolysis curve (0-180 min) of the sample by the area of a standard material (native starch) over the same period of time. The expected glycemic index (eGI) was calculated using the equation $eGI = 8.198 + 0.862\text{HI}$ (Granfeldt et al., 1994).

5.2.4 Statistical analysis

All experiments were repeated at least in duplicate. Experimental data were statistically analyzed using an analysis of variance (ANOVA) and values were expressed as a mean ± standard deviation. Fisher's least significant differences test was used for assessment of significant differences among experimental mean values with 95% confidence. Pearson correlation coefficient (r) and P-value were used to indicate correlations and their significance using Statgraphics Centurion XV software (Bitstream, Cambridge, N). Differences of $P < 0.05$ were considered significant.

5.3 Results and Discussion

Morphology of porous starches obtained after the action of different amylases and their technological properties were analyzed in detail by Belingheri et al. (2015) in a previous study. In this case, to show the variation of pore area, the quantification of pore area and total pore area (related to the abundance or frequency of pore per granule) was plotted (Figure 5.1). The pore area as well as total pore area were significantly affected ($P < 0.05$) by the type of enzyme. AMG produced the largest pore and bigger total pore

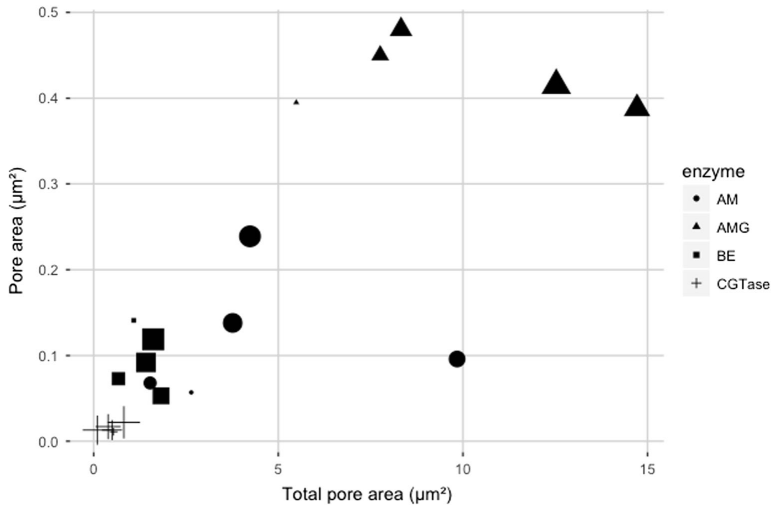

Figure 5.1: Individual pore area was plotted against the total pores area (related to the frequency and size of the pores in each starch granule) of starches obtained after each enzymatic treatment. The symbol size is related to the enzyme level applied for the starch modification.

area, followed by AM, BE and CGTase. Overall, enzyme level (indicated by the size of the symbol in Figure 5.1) had a significant impact ($P < 0.05$) on the pore area and total pore area, regardless enzyme type, with the exception of starch treated with CGTase. The area of the pores induced by AMG increased with the enzyme level until 16.5 U/g starch, likely due to saturation of the non-reducing-ends of starch chains (Chen and Zhang, 2012), but the significant increase in the total area indicated more pores per granule. Similarly, pore area and total pore area increased with the amount of AM added, whereas CGTase level increased pore area but leading to similar total pore area independently on the amount of enzyme. In the case of BE, it was not possible to establish a trend among enzyme level and pore production, although it was detected that high amount of BE was required for obtaining deep pores.

Porous starches were subjected to hydrolysis with digestive amylase and TS, DS and RS were quantified (Figure 5.2), observing a major content of DS and very low amount of RS. The DS fraction was significantly affected

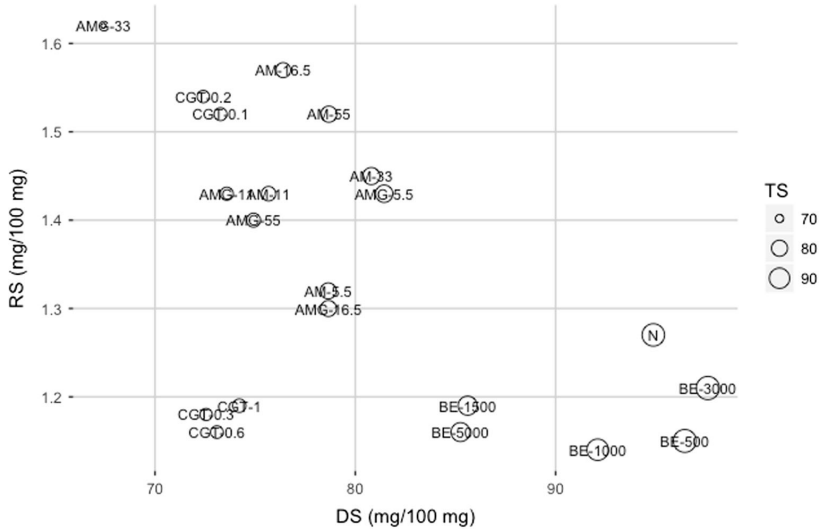

Figure 5.2: Bubble charts for digestible starch (DS) and resistant starch (RS) for each enzyme. The bubble size represents the total starch content (TS). Numbers following enzyme abbreviations are referred to the enzyme activity applied.

by the level of enzyme but hardly by the enzyme type. RS fraction did not significantly vary with the enzyme type or level. After enzymatic treatment, TS was significantly reduced due to the release of hydrolysis products, which agree with previous results (Dona et al., 2010; Kasprzak et al., 2012).

Hydrolysis plots revealed different behavior of the porous starches depending on the type of enzyme used for its production and the enzyme level (Figure 5.3). AMG treated starches showed greater susceptibility to be hydrolyzed by digestive amylase. Nevertheless, AM, CGTase and BE treated starches offered great resistance to enzymatic hydrolysis. Similar effects were reported when corn starch was treated with AMG and CGTase for 24 h (Dura, Błaszczak, and Rosell, 2014; Dura and Rosell, 2016), although opposite results have been observed with AM treated starch subjected to longer production time that intensified changes in the crystalline areas (Wang, Powell, and Oates, 1995).

The parameters derived from the *in*

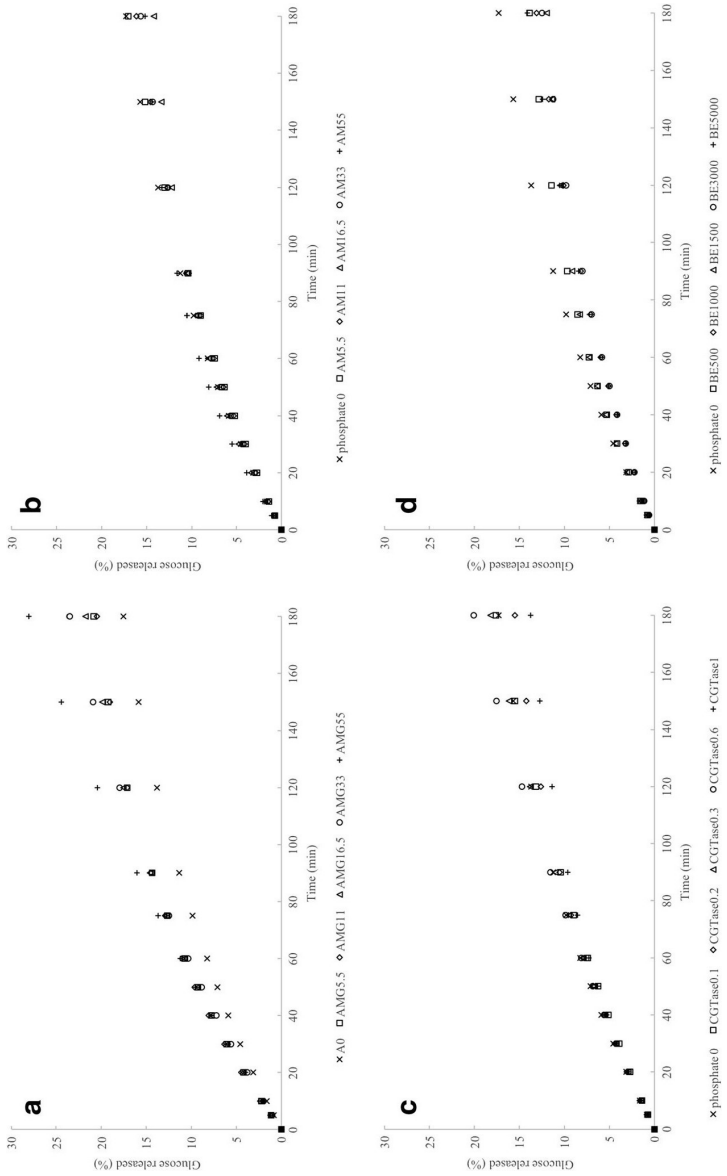

Figure 5.3: Hydrolysis of modified corn starch treated with (a) AMG (b) AM (c) CGTase and (d) BE treatment. Numbers following enzyme abbreviations are referred to the enzyme activity applied expressed in enzyme unit/g starch.

vitro digestion of porous starches in- cluding equilibrium concentration of

hydrolyzed starch (C_∞), kinetic constant (k), area under the hydrolysis curve after 180 min (AUC 180), hydrolysis index (HI) and estimated glycemic index (eGI) are summarized in Table 5.1. Those parameters were significantly ($P < 0.05$) affected by the enzyme as well as the enzyme level used to produce the porous starches, with the exception of k that was only influenced by enzyme level.

In general, the enzymatic modification for obtaining porous starches increased k, although exceptions were the porous starches obtained with CGTase at levels of 0.1 and 0.6 U/g starch and with the highest BE and AMG concentration (5000 U/g starch and 55 U/g starch, respectively). The maximum hydrolysis, C_∞, were significantly decreased in the treated starches with the exception of AMG and CGTase treatments when added 55 U/g starch (AMG) and 0.6 U/g starch (CGTase). Porous starches obtained with AM, CGTase and BE showed lower HI, whereas this parameter was significantly increased in the AMG treated starches. Similar trend was observed for the total area under the hydrolysis curve (AUC) over a hydrolysis period of 180 min. In consequence, the enzymatic modification lowered the estimated glycemic index of the resulting starches, with the exception of AMG treated samples. The strongest effect was observed with the BE treatment, likely due to the BE enzyme displayed a superficial attack causing the formation of wide craters instead of deep holes (Benavent-Gil and Rosell, 2017a).

Table 5.1: Kinetic constant (k), equilibrium concentration (C_∞), area under the hydrolysis curve after 180 min (AUC), hydrolysis index (HI) and estimated glycemic index (eGI) for native and modified corn starches.

Enzyme	Enzyme (U/g starch)	k	C_∞ [A]	AUC	HI	eGI [B]
Native	0	0.0046 ± 0.0003^{ab}	37.79 ± 0.12^{k}	2174 ± 121^{h}	100.00 ± 0.00^{i}	94.40 ± 0.00^{i}
AMG	5.5	0.0087 ± 0.0002 fg	26.44 ± 0.30 gh	2344 ± 2 i	107.82 ± 0.10 j	101.14 ± 0.08 j
	11	0.0099 ± 0.0010 g	24.74 ± 1.57 g	2359 ± 15 i	108.51 ± 0.68 jk	101.74 ± 0.59 jk
	16.5	0.0071 ± 0.0010 d-f	30.41 ± 2.16 ij	2366 ± 49 i	108.86 ± 2.28 jk	103.42 ± 1.96 jk
	33	0.0051 ± 0.0002 a-c	38.99 ± 0.30 k	2433 ± 45 i	111.94 ± 2.08 k	104.69 ± 1.79 k
	55	0.0032 ± 0.0000 a	63.65 ± 1.30 m	2763 ± 58 j	127.12 ± 2.67 l	117.78 ± 2.30 l
AM	5.5	0.0050 ± 0.0006 a-c	28.95 ± 2.46 hi	1760 ± 16 ef	80.98 ± 0.72 e	78.00 ± 0.62 e
	11	0.0065 ± 0.0008 c-e	23.48 ± 2.02 fg	1722 ± 1 e	79.23 ± 0.07 e	76.49 ± 0.06 e
	16.5	0.0118 ± 0.0011 h	16.15 ± 0.84 ab	1696 ± 8 de	78.03 ± 0.39 e	75.46 ± 0.34 e
	33	0.0080 ± 0.0005 e-g	20.58 ± 0.66 ef	1729 ± 18 e	79.55 ± 0.85 e	76.77 ± 0.73 e
	55	0.0133 ± 0.0006 h	16.67 ± 0.34 a-c	1857 ± 4 f	85.42 ± 0.16 fg	81.83 ± 0.14 fg
CGTase	0.1	0.0041 ± 0.0006 ab	33.83 ± 2.73 j	1777 ± 49 ef	81.73 ± 2.24 ef	78.65 ± 1.94 ef
	0.2	0.0083 ± 0.0007 e-g	19.94 ± 0.41 c-e	1725 ± 52 e	79.34 ± 2.37 e	76.59 ± 2.05 e
	0.3	0.0048 ± 0.0012 a-c	32.25 ± 4.77 ij	1870 ± 84 fg	86.01 ± 3.87 g	82.34 ± 3.34 g
	0.6	0.0034 ± 0.0003 a	43.83 ± 1.87 l	1990 ± 36 g	91.53 ± 1.66 h	87.10 ± 1.43 h
	1	0.0094 ± 0.0010 g	16.87 ± 0.47 b-d	1566 ± 52 c	72.03 ± 2.37 d	70.29 ± 2.05 d
BE	500	0.0091 ± 0.0018 g	17.38 ± 0.72 b-e	1576 ± 122 cd	72.49 ± 5.63 d	70.68 ± 4.85 d
	1000	0.0058 ± 0.0002 b-d	20.21 ± 0.25 d-f	1376 ± 53 ab	63.29 ± 2.44 ab	62.75 ± 2.10 ab
	1500	0.0133 ± 0.0033 h	13.34 ± 1.62 a	1463 ± 8 bc	67.29 ± 0.36 c	66.42 ± 0.31 c
	3000	0.0065 ± 0.0006 c-e	18.19 ± 1.09 b-e	1333 ± 6 a	61.33 ± 0.26 a	61.06 ± 0.22 a
	5000	0.0045 ± 0.0010 ab	25.90 ± 4.41 gh	1432 ± 13 ab	65.88 ± 0.59 bc	64.99 ± 0.50 bc

Continued on next page...

Table 5.1 – Continued

Enzyme	Enzyme (U/g starch)	k	C_∞ [A]	AUC	HI	eGI [B]
P-value	Enzyme	0.361	0.004	0.000	0.000	0.000
	Level (U/g)	0.009	0.005	0.000	0.000	0.000

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

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

[B] eGI was calculated from equation proposed by Goñi, García-Alonso, and Saura-Calixto (1997)

No correlation was observed between structural attributes and the k and C_∞ parameters derived from the *in vitro* digestion (Figure 5.4). Nevertheless, analysis within each enzymatic treatment revealed that the pore size of AM treated starch presented a moderate significant positive correlation with k value (r = 0.70; $P < 0.05$) and negative with C_∞ (r = -0.67; $P < 0.05$). Similarly, the total pore area displayed a moderate significant positive correlation with k value (r = 0.63; $P < 0.05$) and a negative with C_∞ (r = -0.64; $P <$ 0.05). It is generally accepted that the holes created in the starch surface after enzymatic treatment facilitate the access of digestive enzyme to the inner granule and in consequence the hydrolytic event (Dona et al., 2010; Fannon, Hauber, and BeMiller, 1992; Utrilla-Coello et al., 2009). The diffusion or access of the digestive amylase into the granule determines the way starch is disrupted (Blazek and Gilbert, 2010). Against previously reported assumptions, the relationships obtained in the present study indicate that a specific pore size is required for the accessibility of the digestive amylase. According to the structural analysis, AMG treatment induced larger pores ranging from 0.39 to 0.48 μm^2 (Benavent-Gil and Rosell, 2017a), which are not limiting the accessibility of digestive enzymes. Conversely, AM led to smaller pores, whose size increases with the level of enzyme ranging from 0.05 to 0.24 μm^2 (Benavent-Gil and Rosell, 2017a), and they are limiting the granule hydrolysis. Therefore, starch digestion could be modulated by obtaining certain pore size in the starch surface. On the other hand, the pore size and the total pore area presented a weak significant positive correlation with *eGI* parameter (r = 0.54; $P < 0.01$: r = 0.49; $P < 0.01$). No correlation between pore size and DS, RS and TS content was obtained, and only significant ($P < 0.01$) moderate correlation were found between the total pore area and RS (r = 0.51) DS (r = -0.41) and TS (r = -0.40).

5.4 Conclusions

In porous starches, the size and number of pores affected significantly their performance during *in vitro* digestion, showing significantly different amount of digestible starch, depending on the level of enzymatic treatment. AMG treated starches presented higher digestibility, whereas AM, CGTase and BE treatment reduced it, leading to lower estimated *GI*. Again, structural features of the pores also play a fun-

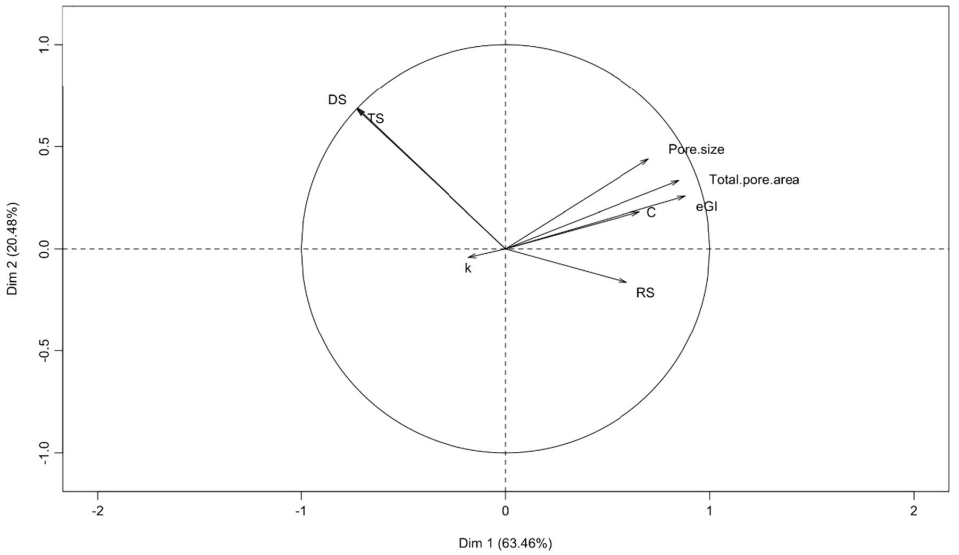

Figure 5.4: Multi factor analysis plot relating pasting properties and structural attributes with digestive parameters of enzymatically modified corn starches.

damental role controlling starch hydrolysis with digestive amylase. A specific pore size is required for the accessibility of the digestive amylase. Therefore, starch digestion could be modulated by obtaining certain pore size in the starch surface.

Acknowledgments

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CHAPTER 6

Results IV

Contents

Carbohydrate Polymers 197 (2018) 558–564

Thermal stabilization of probiotics by adsorption onto porous starches

Yaiza Benavent-Gil, Dolores Rodrigo, Cristina M. Rosell*

Abstract

Industrial processing factors, such as temperature, compromise the viability of probiotic cells. Objective was to develop a system to thermally stabilize probiotic bacteria based on porous starches and using biopolymers as coating materials (gelatinized starch, guar gum and xanthan gum). Porous starches from corn and rice starches, having controlled number and size of porous were used as supporting material. Scanning electron microscopy confirmed the adsorption of the microorganism, leading microcapsules with corn starch but aggregates with rice starch. Surface pores of rice starch increased the encapsulation yield of rice starch around 10%, but that effect was not observed in porous corn starch. The highest encapsulation yield was obtained with porous starches coated with gelatinized starch, which ranged from 92 to 100%. Microencapsulates made with porous starches with small pores, like the ones obtained with α-amylase, and coated with gelatinized starch resulted in the highest thermal resistance at 55 °C.

Keywords

Porous starch — Enzymes — Probiotics — Amylase — Amyloglucosidase — *L. plantarum* — Thermal stability

Institute of Agrochemistry and Food Technology (IATA-CSIC), C/Agustin Escardino, 7, Paterna 46980, Valencia, Spain

***Corresponding author**: crosell@iata.csic.es (C.M. Rosell).

6.1 Introduction

The emphasis on the use of food to promote well-being and a healthy state have driven to the development of the so-called "functional foods" (Roberfroid, 2000). Nearly all segments of the food industry offer functional products, opening the door to dairy products, soft drinks, juices, pastries and infant food (Miñana and Serra, 2009), with functional ingredients such as probiotics, prebiotics, vitamins and minerals (Stanton et al., 2001). Nevertheless, a constant major challenge is to ensure that these products preserve the viability of probiotic microorganisms under the harsh conditions associated to product processing. In this regard, microencapsulation techniques are used to protect microorganisms in food.

In general, starches, particularly modified starches, have been used as coating materials for encapsulation. A non-chemical way to modify starch granules is by applying enzymatic treatment using amylolytic enzymes. Those modified starches led to porous molecules with great adsorbent capacity, due to their large surface area (Zhang et al., 2012), but also those pores provide an expandable space that could be used as a protective microenvironment for probiotic encapsulation. It might be expected that the probiotic bacteria would be physically adsorbed in the pores and cavities without any covalent binding, allowing their complete released in a sustained manner (Li, Turner, and Dhital, 2016) whenever having the right size and shape of pores. However, size and shape of the microporous structure largely depends on the amylase type and amylolysis level. Dura, Błaszczak, and Rosell (2014) studied the enzymatic modification of corn starch by using α-amylase (AM) and amyloglucosidase (AMG) enzymes revealing the formation of superficial micropores with diverse pore size depending on the amylolytic enzyme. Authors concluded that AMG yielded starch granules with more abundant and larger pores than those obtained after AM treatment. Recently, Benavent-Gil and Rosell (2017a) compared the effect of a range of amylases on the properties of corn starch, also taking into account the impact of the enzymatic level, revealing that the number and size of the pores could be modulated by controlling the amylase type and level. Additionally, the intrinsic structural characteristics of starches from different botanical origin can offer extended possi-

bilities for obtaining porous starches. In fact, studies carried out with different amylolytic enzymes on cereal and tuber starches indicated that starches of cereal origin have deeper and larger pores compared to the superficial cavities observed in tuber starches after enzymatic modification (Benavent-Gil and Rosell, 2017b). Therefore, the resulting porous starches might have different technological performance and, consequently, different industrial applications.

Further stability of the probiotic cells can be obtained by applying coating materials. This technology allows enclosing the probiotics cells inside the microcapsules that are subsequently coated by an additional layer. Overall, hydrolyzed starches (Brinques and Ayub, 2011; Lahtinen et al., 2007; Li, Turner, and Dhital, 2016; Xing et al., 2014), porous starches and starches/alginates (Brinques and Ayub, 2011; Lahtinen et al., 2007; Li, Turner, and Dhital, 2016; Xing et al., 2014) have been the elected encapsulating agents for many authors. Meanwhile, hydrocolloids are frequently used for coating microcapsules. Hydrocolloids produce a gel network structure that can easily adhere to the surface of the microcapsules to control external and internal mass transfer (Morreale, Garzón, and Rosell, 2018). This additional shell to the encapsulated cells can provide better barrier and protection against harsh environmental conditions (Li, Turner, and Dhital, 2016; Xing et al., 2015). Nevertheless, a careful selection of the coating materials must be made to obtain capsules with different physical properties. For instance, the type and level of the hydrocolloid applied for coating can alter the starch granule properties (Gularte and Rosell, 2011).

In spite of the great possibilities that porous starches could offer for probiotics production, as far as authors knowledge, up to now there are no previous studies about the effect of different coating materials on the stability of microcapsules based on enzymatically modified starches with different structure. Therefore, the objective of this study was to identify the potential of controlled pore size starches from different botanical sources, obtained in a previous study (Benavent-Gil and Rosell, 2017b), as carriers of probiotics. Particularly, to investigate the role of different enzymatic treatments (AMG and AM) on two different starches (corn and rice) on the *Lactobacillus plantarum*

viability, as probiotic microorganism, during the encapsulation process and to establish the possible correlation between the morphological properties and the thermal stability of the bacteria within the microcapsules. The influence of different coating materials on the survival rate of *L. plantarum* was also evaluated during exposure to heating treatment at different times.

6.2 Materials and methods

6.2.1 Starch samples

The starch samples were selected from previous studies (Benavent-Gil and Rosell, 2017a; Benavent-Gil and Rosell, 2017b). The starch sources were corn starch (C) (Miwon, Seoul, Korea) and intermediate amylose rice starch (R) (Sigma-Aldrich, Spain). Their respective enzymatic modifications were carried out with amyloglucosidase (AMG) (EC 3.2.1.3) and fungal α-amylase (AM) (EC 3.2.1.1) treatment (Novozymes, Bagsværd, Denmark), using 16.5 U AMG / g starch and 11 U AM / g starch. The selected starches evinced the microstructure characteristics summarized in Table 1, which were obtained from the image analysis of the scanning electron micrographs using ImageJ software (ImageJ, UTH-SCSA Image Tool software). Surface starch characteristics were previously reported by Benavent-Gil and Rosell (2017a) and Benavent-Gil and Rosell (2017b). Granule and pore size, as well as pore frequency (ratio of the sum of the areas of all the pores in a granule and the granule area).

6.2.2 Strains, media and growth conditions

The bacterial strain used in this study was *Lactobacillus plantarum* CECT 230. The strain was grown in de Man, Rogosa and Sharpe (MRS) broth (Scharlab, Barcelona, Spain) at 30 °C for 24 h. Cells were harvested and washed by centrifugation at 4000 × g for 10 min and resuspended with sterile peptone water, resulting in a cell suspension containing approximately 2×10^{10} CFU mL^{-1}.

6.2.3 Encapsulation of *Lactobacillus plantarum* cells

L. plantarum cells were encapsulated in the native and modified starches. Starch (2 g) was transferred into sterile tubes containing 6 mL of bacterial culture. The encapsulation process was carried out in four different stages: microorganism adsorption (S1), vacuum filtering (S2), freezing (S3) and freeze drying (S4). Process

Table 6.1: Structural characteristics of native and modified starches used as supporting materials. C: Corn, R: rice, AMG: porous starch from corn or rice obtained with amyloglucosidase, AM: porous starch from corn or rice obtained with α-amylase.

	Granule Size (μm^2)	Pore Size (μm^2)	Pore frequency (%)
C	87.68	n.d	n.d
C-AMG	87.68	0.59	4.47
C-AM	87.68	0.13	1.57
R	17.55	n.d	n.d
R-AMG	17.55	0.19	3.69
R-AM	17.55	0.03	0.43

n.d.: Not detected.

was as follows: the mixture was kept in a shaking water bath (600× g) at 30 °C for 90 min (S1). Then, samples were vacuum filtered through Whatman n° 2 filter paper mounted in a Buchner filter (S2). After that, microcapsules were frozen and kept at -20 °C for 1 h (S3). Microcapsules were freeze-dried for 24 h and kept at 4 °C for subsequent analysis (S4). The encapsulation process was conducted in duplicate, separately using two batches of prepared starches. When coating was applied onto the surface of the microcapsules the same procedure described above was carried out, but coating material was added to the microcapsules in the stage S3, before freezing. Specifically, microcapsules and coating material were gently homogenized (3 mL g^{-1} coating material) with a Polytron Ultraturrax homogenizer IKA-T18 (IKA works, Wilmington, USA) for 0.5 min at speed 3 and then frozen.

6.2.4 Edible coating material preparation

Three different coating material were prepared. Gelatinized starch (GS) was prepared by heating native starch (6% w/v) in water for 15 min at 90 °C. Guar gum (GG) (Guar gum - 3500 from EPSA, Spain) and xanthan gum food grade (GX) (Jungbunzlauer, Austria) suspensions (2%, w/v) were used as coating material, separately. Preliminary tests were carried out to optimize the level of coating material suspension in order

to cover the largest proportion of granule.

6.2.5 Encapsulation yield

To determine the encapsulation yield (EY) at the different process stages (S1-S4) microorganism viability in starch samples was studied by plate counting on MRS agar. The microcapsules (0.10 g) were first added into 0.9 mL peptone water (0.1% w/v) containing pancreatin (0.9 mg/100 mg starch). The pancreatin was added to hydrolyze the starch releasing the encapsulated bacteria (Li, Turner, and Dhital, 2016). The volume of 0.1 mL of decimal serial dilutions in peptone water were plated in duplicate on MRS agar and incubated at 30 °C for 48 h. The microbial count data was expressed as decimal-log of colony-forming units per gram (CFU g^{-1}). Encapsulation yield (EY) (%) was calculated by using the equation of Ashwar et al. (2018)

$$EY = N/N_0 \times 100$$

Where N is the log cell count (CFU/g) of viable entrapped cells released from the microcapsules, and N_0 is the log cell count (CFU/g) of free cells added to the production of microcapsules.

6.2.6 Scanning electron microscopy (SEM)

A JSM 5200 scanning electron microscope (SEM) (JEOL, Tokyo, Japan) was used to visualize the distribution of probiotic bacteria in native starches and enzymatically modified starches. Samples were coated with gold in a vacuum evaporator (JEE 400, JEOL, Tokyo, Japan) prior to observation. The obtained samples were examined at an accelerating voltage of 10 kV and magnified 3,500× times.

6.2.7 Thermal stability studies

The heat resistance of the encapsulated *L. plantarum* was evaluated by a thermal treatment at 55 °C for 20 and 35 min, which were set up in preliminary studies (data not showed). Before treatment, 100 mg microcapsules were inoculated into 20 mL peptone water (0.2%, w/v) and introduced in Thermal-Death-Time (TDT) stainless steel tubes. A thermocouple connected to a data logger was introduced through the sealed screwed top to follow the process temperature. After 0, 20 and 35 min incubation, the tubes were rapidly cooled in an ice-water bath to room temperature, centrifuged and

supernatant removed. The pellet obtained was used to determine the total number of viable cells as described in Section 2.5.

6.2.8 Statistical analysis

The data reported are the mean of replicates and expressed as a mean ± standard deviation. Statistical analyses were carried out with Fisher's least significant differences test with a significance level of 0.05. Pearson correlation coefficient (r) and P-value were used to indicate correlations and their significance using Statgraphics Centurion XV software (Bitstream, Cambridge, N). The correlation coefficient was classified in different levels of correlation: perfect ($|r|$=1.0), strong ($0.80 \leq |r| \leq 1.0$), moderate ($0.50 \leq |r| \leq 0.80$), weak ($0.10 \leq |r| \leq 0.50$), and very weak (almost none) correlation ($|r| \leq 0.10$).

6.3 Results and Discussion

6.3.1 Microstructure of the microcapsulates

The microstructure of native and porous starches was widely described in previous studies (Benavent-Gil and Rosell, 2017a; Benavent-Gil and Rosell, 2017b), but since the morphology of microcapsules from raw and modified materials could affect the encapsulation process, some details about those starches are included in Table 6.1. Figure 6.1 exhibits the microcapsules obtained after S4 stage. SEM was used to investigate the distribution of *L. plantarum* in modified and unmodified starch materials (Figure 6.1). As expected, the microorganism adhesion onto the granular surface was clearly visualized in all samples. Nevertheless, the microcapsules obtained from corn or rice sources revealed a markedly different pattern. In the corn microcapsules (Figure 6.1 A-C), the microorganism adhesion was more pronounced than onto the rice granular surface (Figure 6.1 D-F). After freeze drying (S4), microcapsules from treated and untreated rice starches gave aggregates, whereas in the case of corn they appeared as individual entities. Those morphological characteristics have been previously reported for native starches and rice based encapsulates obtained by spray drying process (Avila-Reyes et al., 2014; Horstmann et al., 2016), and those starchy aggregates have been related to the residual protein nearby rice starch granules (Costa et al., 2011).

The native starches from corn and rice showed lower adhesion onto the

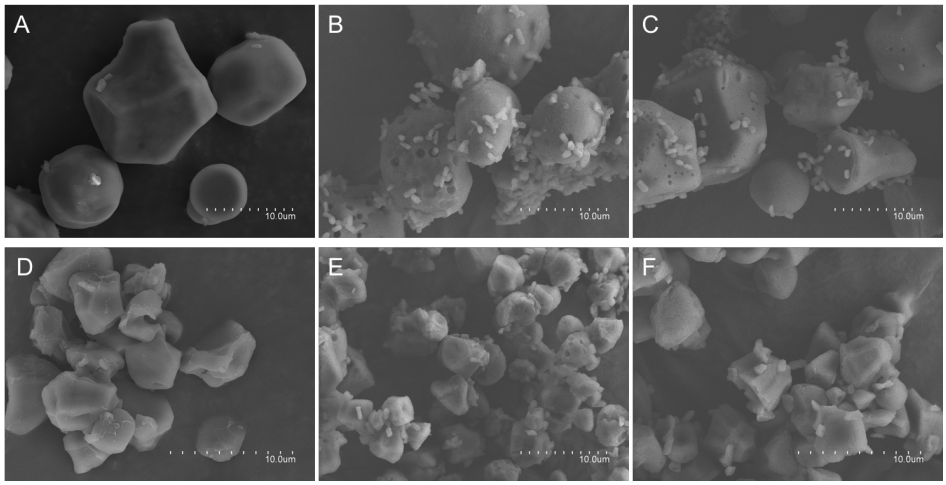

Figure 6.1: SEM micrographs of native corn (A) and rice (D) starches and the resulting microencapsulated *L. plantarum* with different supporting materials: porous corn starch obtained with amyloglucosidase (B); porous corn starch obtained with amylase (C); porous rice starch obtained with amyloglucosidase (E); porous rice starch obtained with amylase (F).

granule surface (Figure 6.1 A, D). In contrast, higher load of bacteria was observed when using AMG or AM treated starches (Figure 6.1 B, C, E, F), which exhibited a porous surface with internal cavities due to the 'inside out' hydrolysis (Dhital, Shrestha, and Gidley, 2010). This is an indication that the enzymatic modification and the consequent formation of deep pores affected the adhesion capacity of the bacteria onto the surface of the granules. It has been described that unmodified starches only were able to adsorb bacteria onto the granular surface (Conrad et al., 2000). Meanwhile, superficial holes might facilitate the bacterial entrapment, due to the expanded space, which can be filled with bacteria (Li, Turner, and Dhital, 2016). In this sense, Wu et al. (2011) demonstrated an increase in the oil adsorption capacity related to the degree of hydrolysis, i.e., greater degree of hydrolysis produced during the formation of porous starch led to larger surface for the adsorption of different components. In addition, Zhang et al. (2012) stated that when producing porous starches, enzyme concentration should be optimized

to increase their adsorption capacity, because excessive hydrolysis would be detrimental.

6.3.2 Effect of enzymatic treatment on encapsulation yield

Encapsulation yields (EY) (%) onto the different starches through the different stages are given in Figure 6.2. Porous starches after AMG or AM treatment of corn or rice were used as the supporting material. The statistical analysis indicated that the starch source had a significant ($P < 0.05$) effect on EY, but no the enzymatic treatment applied to obtain the porous starches. Nevertheless, when analyzing each microencapsulation stage, significant differences in the EY were observed due to the starch source at stage S1, meanwhile the enzyme type prompted significant different effect at S1 and S4 stages.

Regarding S1 stage, the encapsulation efficiency of *L. plantarum* was similar for the native starches regardless of their origin, with an EY of 93.02% for native corn starch and 92.92% for native rice starch. Porous rice starch did not improve the encapsulation efficiency compared to its native counterpart, which suggested that the increase in porosity did not improve the adsorption, supporting the importance of the granules aggregates to entrap the microorganism. Conversely, in the case of porous corn starches, the EY of *L. plantarum* reached 100%, indicating better adsorption due to the superficial pores. Nevertheless, no significant difference ($P > 0.05$) in the EY was observed due to the procedure for obtaining porous with AM or AMG, thus the pore size and pore frequency hardly affected the microorganism adsorption onto the surface. It can be also taken into account that Crittenden et al. (2001) described the adherence of different strains of lactic acid bacteria onto starch, which was promoted by the starchy hydrolysis products released from the action of the strains on the starch. The results obtained in the present study indicate that the cell adhesion capacity for *L. plantarum* depends on the supporting material used for the microencapsulation. Similar results have been previously described for the oil absorption capacity of porous starches from different sources, concluding that the pore size plays a fundamental role for the adsorption of oil and water molecules (Benavent-Gil and Rosell, 2017a; Benavent-Gil and Rosell, 2017b).

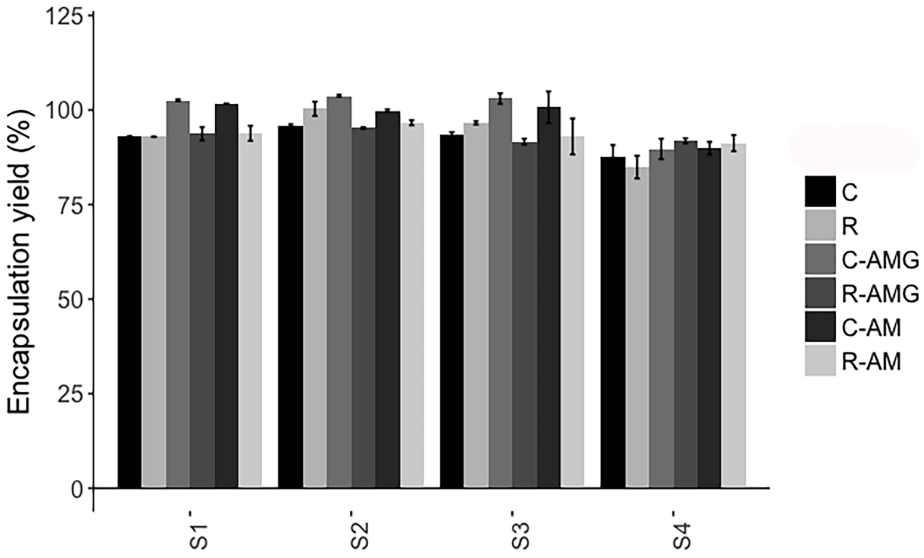

Figure 6.2: Effect of supporting materials (C: Corn, R: rice, AMG: porous starch obtained with amyloglucosidase, AM: porous starch obtained with α-amylase) on *L. plantarum* encapsulation yield at different process stages (S1. immediately after starch inoculation; S2. after vacuum filtering; S3. after freezing; S4. after freeze drying). Mean bars with different letters within the same supporting material differed significantly ($P < 0.05$).

During S2 and S3 stages, which comprised the vacuum filtering and freezing, samples showed similar trends. The EY remained constant during the S2 and S3 stages, except for native rice sample that showed an increase in the viability of the microorganisms after filtration, which remained constant during freezing. Similar results were found by Heidebach, Först, and Kulozik (2010).

Regarding S4, freeze drying is frequently used as an effective way to produce probiotic products with high stability rates during preservation and convenient handling (Li, Turner, and Dhital, 2016). Nevertheless, S4 stage induced a decrease of the encapsulation efficiency, except in the case of porous rice starches. The lowest EY was obtained with native starches, presenting an encapsulation efficiency of 87.67% for corn and 84.91% for rice starch. A pos-

sible explanation might be that the absence of pores allowed the inclusion of cells only at the very superficial level of the microcapsule. Bacteria adhered onto the granular surface presumably had greater exposure to low temperature and the subsequent formation of ice crystals (Li, Turner, and Dhital, 2016). The removal of water during the sublimation process after the formation of intracellular ice crystals during the freezing process can damage the cellular membrane (Conrad et al., 2000). Thus, the absence of pores resulted in the reduction microorganism viability during the freeze drying process. The enzymatic modification of corn starch did not result in an increase of the EY, having similar yields compared to the native starch (87 ± 3%). Comparable results were obtained for native and porous corn starches obtained with pancreatic α-amylase, pancreatin, and fungal α-amylase, without significant differences on the relative survival (Li, Turner, and Dhital, 2016). Nevertheless, the enzymatic treatment of rice starch improved the EY (91.86 ± 0.67 and 91.24 ± 2.14% for AMG and AM treatments, respectively), observing an increase in the viable cell count compared with the native starch (84.91 ± 3.00%). As it was observed in the micrographs, porous and native rice starches formed aggregates that might protect the microorganisms located within the interstitial spaces (Avila-Reyes et al., 2014). Nevertheless, in the present study, the formation of aggregates in the native rice starch was not enough to improve the protection of the microorganisms after freeze drying process. Porous rice starches showed higher EY after freeze drying process than its native counterpart. Thus, it can be assumed that in rice, porous structure correlates with an increase in EY (Li, Turner, and Dhital, 2016). The results also suggested that porous structure in rice starch together with its ability to agglomerate, contributed to the microorganism protection.

6.3.3 Effect of coating material on encapsulation yield

In order to provide better protection to the microencapsulated cells, different polymers like gelatinized starch, guar gum and xanthan gum were used as coating materials. The protective effect against the damage induced during the freeze drying process is presented in Figure 6.3 (SEM micrographs can be displayed in supplementary mate-

rial). After freeze drying stage, the *L. plantarum* EY when coated with gelatinized starch, GG or GX were 92–100%, 75.76–98.79% and 84.09–99.18%, respectively, versus 84.91–91.86% found in the absence of coating materials. Generally, drying processes have a typical survivability rate around 70–85% (Lahtinen et al., 2011). The higher survival rate of the cells confirmed that coating materials maintained the integrity of cells and enhanced the microencapsulated cell number.

Coating polymers had a significant effect on *L. plantarum* EY ($P < 0.01$). Gelatinized starch was the unique coating material that increased the EY, regardless starch source and enzymatic treatment compared to non-coated encapsulated samples (Figure 6.3). This result is in agreement with findings of Xing et al. (2015), who indicated that pores in the modified starch and the presence of mannitol, glycerol and sodium alginate as complex coating materials offer better protection for *L. acidophilus* cells. Nevertheless, results indicated that protective effect was greatly dependent on the coating material. In fact, GX was only effective with the microcapsules from porous starches, with the exception of those from rice porous starch obtained with AMG treatment. In contrast, GG tended to decrease the EY in all samples, except in the case of porous corn starches.

This variability in the number of encapsulated cells encountered among starches might be attributed to the different interaction of the coating material with the starch surface (Xing et al., 2015). The porous structure of modified starches could improve the adhesiveness and absorbability of the coating materials (Nagashima, Hirose, and Matsuyama, 2011; Wang et al., 2012), but that effect was not related to the pore size or frequency because very weak correlation was observed between EY and pore size ($r = 0.26$; $P < 0.05$) and no correlation between EY and pore frequency. Only in the microcapsules coated with GG, a moderate significant positive correlation was found between EY and the pore size ($r = 0.63$; $P < 0.05$), and pore frequency presented ($r = 0.55$; $P < 0.05$), suggesting that GG accessibility to large pores favors the release of microorganism. Therefore, the effect of coating materials on EY was rather dependent on the starch–coated material interaction, promoting reinforcement of microcapsules or competing

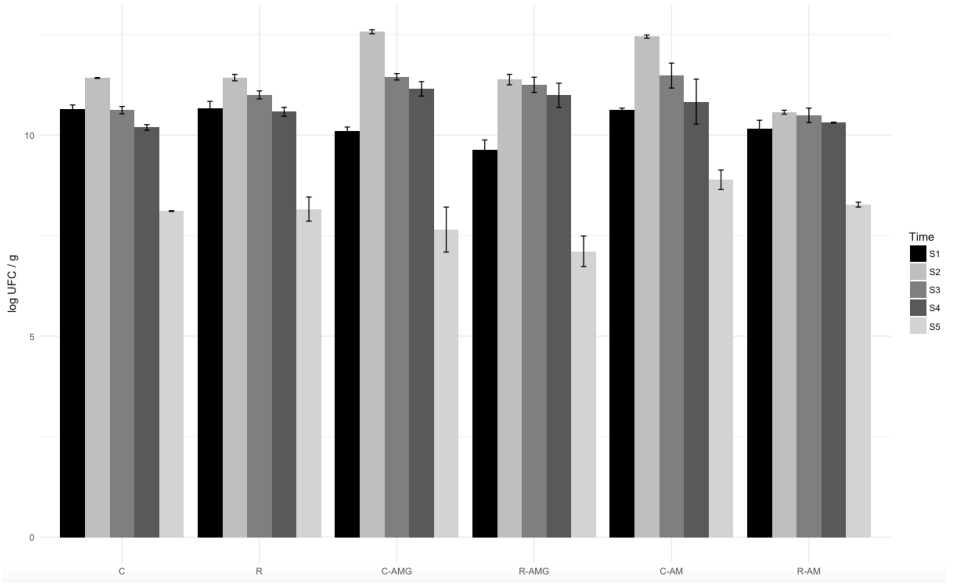

Figure 6.3: Effect of coating material on L. plantarum encapsulation yield (EY) at S4 (freeze drying stage). Different letters inside the symbols differ significantly ($P < 0.05$). C: Corn, R: rice, AMG: porous starch obtained with amyloglucosidase, AM: porous starch obtained with α-amylase.

with the microorganism for filling the pores.

6.3.4 Survival of microencapsulated cells under heat treatments

The thermal stability of *L. plantarum* microcapsules was tested at 55 °C (Table tab:4.2). Statistical analysis revealed that the type of starch, the enzyme applied to obtain porous starches and the coating material had significant ($P < 0.01$) effects on the cell stability, even after prolonged tests, with the exception of coating materials when 35 min were applied. Previous studies indicate that the survival rate of microencapsulated cells depends on the contact time and temperature gradient (Avila-Reyes et al., 2014), decreasing the survival of *L. plantarum* up to 63% at 55 °C after 10 min (Ashwar et al., 2018).

Table 6.2: Cells viability (%) of *L. plantarum* at 55 °C after 20 and 35 min using native and porous starches as supporting material and either gelatinized starch (GS), guar gum (GG) or xanthan gum (GX) as coating material.

Starch	Enzyme	Coating	t=20	t=35
Corn	Native		0 ± 0^{a}	0 ± 0^{a}
		S	45.96 ± 2.3^{cd}	0 ± 0^{a}
		GG	44.94 ± 4.07^{c}	0 ± 0^{a}
		GX	55.28 ± 1.03^{fg}	0 ± 0^{a}
		AM	$47.53 \pm 1.88^{c-e}$	43.28 ± 1.73^{gh}
		S	61.39 ± 4.69^{hi}	61.57 ± 4.43^{k}
		GG	82.4 ± 3.89^{l}	52.1 ± 0.98^{j}
		GX	$51.4 \pm 0.54^{d-f}$	$39.84 \pm 1.65^{d-g}$
		AMG	63.95 ± 0.98^{ij}	40.4 ± 5.73^{fg}
		S	$52.67 \pm 10.79^{e-g}$	0 ± 0^{a}
		GG	57.82 ± 5.81^{gh}	46.78 ± 4.54^{hi}
		GX	33.24 ± 1.57^{b}	26.64 ± 3.9^{b}
Rice	Native		57.17 ± 0.08^{gh}	34.06 ± 3.08^{cd}
		S	63.89 ± 2.97^{ij}	53.16 ± 5.32^{j}
		GG	69.64 ± 4.55^{j}	53.69 ± 1.8^{j}
		GX	56.18 ± 1.36^{gh}	49.87 ± 2.92^{ij}
		AM	$51.24 \pm 1.01^{d-f}$	$37.41 \pm 0.95^{d-f}$
		S	80.32 ± 2.54^{kl}	67.36 ± 2.32^{l}
		GG	76.56 ± 3.82^{k}	37.72 ± 0.53^{ef}
		GX	78.69 ± 0.9^{kl}	68.62 ± 0.76^{l}
		AMG	55.04 ± 1.37^{fg}	38.84 ± 1.96^{ef}
		S	0 ± 0^{a}	0 ± 0^{a}

		GG	54.14 ± 2.48^{fg}	36.02 ± 2.36^{de}
		GX	57.63 ± 6.38 ^{gh}	31.92 ± 4.14 ^{c}
***P*-value**	Starch		0.0052	0.001
	Enzyme		0.0002	0.0000
	Coating		0.0027	0.3291

Numbers followed by different letters within a column indicated significant differences.

In this study, non viable cells were detected after heating microencapsulates in native corn starch, while the survival of entrapped cells in native rice starch was approximately 60% after incubation at 55 °C for 20 min, which further decreased till 34% after 35 min at that temperature. The stability conferred by rice starch granules could be understood considering the inclusion of the cells in the interstitial spaces of the aggregates that offers protection against the external environment (Avila-Reyes et al., 2014). In fact, Zhao and Whistler (1994) described the stabilization of food ingredients when those were entrapped within the spherical aggregate of small starch granules, like amaranth starch, rice starch or small wheat starch.

With regard to porous starches, also a significant effect was observed depending on the supporting material (rice or corn) (Table 6.2). Encapsulation with corn porous starches conferred stability to the cells, which survived even after 35 min at 55 °C. Conversely, encapsulates with rice porous starches showed lower stability after 20 min than the one observed when adhering to native starch, but opposite behavior was observed after 35 min heat treatment. According to the investigation of Xing et al. (2014), the collapse of the microcapsules can promote the release of the cells and their subsequent inactivation.

Considering coating, it has been previously reported that multilayers coating enhances the tolerance of cells to thermal treatment (Wang et al., 2012; Xing et al., 2015), thus it would be expected higher survival on the coated encapsulated materials. Nevertheless, significant differences were observed depending on the coating material (Table 2). All coating materials tested allowed maintaining greater number of *L. plantarum* viable cells after 20 min heat treatment when encapsulated in native starches, and even after 35 min in the case of rice supporting material. Consequently, coating materials provided additional protection to the aggregated structure of the rice microcapsules. The protection provided by the coating material varied depending on the characteristics of the porous starch, that is on the enzymatic treatment carried out to obtain the porous starches. Coating did not provide additional protection to the microorganism encapsulated into the starch pores obtained with AMG. Likely, the higher pore size and frequency obtained when starches were

treated with AMG could increase the adhesion surface for the cells and the coating material, but the hydrophilic nature of the coating materials could favored the diffusion of hot water molecules through the capsule (Mandal et al., 2014), resulting in large reduction of the probiotic viability. Conversely, immobilized cells in the pores of starches modified with AM exhibited extended thermal resistance, probably the smaller superficial pores covered by coating material provides better fitting for protecting the cells. For this reason, the knowledge of the structures formed after the enzymatic modification of the starch could be taken as a basis for the choice of the coating material. It has been already reported that the survival of microencapsulated bacteria coated with porous starches was higher than that of non-coated microcapsules (Li, Turner, and Dhital, 2016; Wang et al., 2012; Xing et al., 2015), and that protection is highly dependent on the porous starch concentration (Xing et al., 2014). However, it must be stressed that even when using the same starch concentration, like in the present study, the size and number of pores on the starch surface (Table 6.1) can significantly affect the stability of the cells. Therefore, the degree of hydrolysis of the starches resulting from the enzymatic modifications may be used for modulating the starch morphology, with subsequent effect on the cells encapsulation and stabilization.

6.4 Conclusions

The potential of rice and corn starches in their native state or as porous granules to be used as supporting material for obtaining probiotic foods was evaluated using *L. plantarum*. The yield during the encapsulation process indicated the different entrapment undergone by the microorganism depending on the type of starch, being adsorbed on the surface of corn starch but entrapped within the rice granules aggregates. The porosity of the porous starches contributed to increase the encapsulation yield, particularly in the case of rice starch. Coating of the encapsulates provided additional protection, but the effect was dependent on the supporting and coating materials. In general, best encapsulation yield was obtained with encapsulates in porous starches coated with gelatinized starch. Thermal stability of the encapsulates revealed that microencapsulation using porous starches with small pores, like the ones obtained with α-amylase, and coated with gelatinized starch

resulted in the highest heat resistance. Microcapsules produced with the mixture of porous starches, obtained with α-amylase, and coating materials can be incorporated in probiotic food formulation to maintain the integrity of the cells.

Acknowledgments

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CHAPTER 7

Results V

Contents

Starch-Stärke
graphicx

Physicochemical properties of gels obtained from corn porous starches with different levels of porosity

Yaiza Benavent-Gil[a], Laura Román[b], Manuel Gómez[b], Cristina M. Rosell[a*]

Abstract

Porous starches are attracting much attention owing to their adsorption ability of different compounds. However, only their granular structure has been exploited. The objective of the present research was to analyze the structure and properties of the gels obtained from porous starches having diverse degree of porosity (0-15%). Gels were obtained from corn starches with different degree of porosity and their microstructure, gel rheology, hardness and syneresis during storage was determined. SEM micrographs revealed honeycomb structures with diverse size and number of holes depending on the porosity of the initial porous starches. In addition, when increasing porosity, gels showed lower viscoelasticity, decreasing G' and G" and leading to low elastic gels, with also soft texture. Syneresis of starches was accelerated during the storage of the samples during the first week. However, no significant differences were observed during the second week. Therefore, porous starches with diverse porosity offer an attractive alternative to obtain hydrogels with diverse network matrix.

Keywords

Gel matrix — Microstructure — Porous starch — Viscoeslastic behavior

[a] *Institute of Agrochemistry and Food Technology (IATA-CSIC), C/Agustin Escardino, 7, Paterna 46980, Valencia, Spain*

[b] *Food Technology Area. College of Agricultural Engineering. University of Valladolid, 34004 Palencia, Spain.*

*__Corresponding author__: crosell@iata.csic.es (C.M. Rosell).

7.1 Introduction

Starch is an important ingredient widely used in the food industry due to its biodegradable nature, competitive cost, accessibility, and feasibility to modify its properties. Prior to its use, starch can be structurally modified to increase its applicability as an ingredient by decreasing retrogradation and improving paste properties and gel texture during processing (Majzoobi, Hedayati, and Farahnaky, 2015). Among the various starch modification methods, enzymatic modification is applied to alter starch granular and molecular structure. Research carried out on enzymatic treatments of starches has been accomplished using diverse enzymes and experimental conditions (Uthumporn, Zaidul, and Karim, 2010; Sorndech et al., 2016), most of them focused on applying glucoamylase (AMG) (Sujka and Jamroz, 2007). Lately, many studies have been focused on assessing the effects of AMG treatment on starch granules, including functional properties, biochemical features, thermal and structural analyses (Aggarwal and Dollimore, 2000; Southall et al., 1999; Polakovič and Bryjak, 2004; Zhang et al., 2012; Benavent-Gil and Rosell, 2017a), as well as digestibility behavior (Dura, Błaszczak, and Rosell, 2014; Benavent-Gil and Rosell, 2017b).

Numerous researchers have conducted their studies selecting corn starch as substrate to produce porous starch by AMG treatment, since it is easier to be hydrolyzed and has more inerratic matrices as compared to other starches (Wang, Yuan, and Yue, 2015). It is well known that glucoamylase is an exo-acting enzyme that catalyzes the hydrolysis of both α-D-(1 $\rightarrow$ 4) and α-D-(1 $\rightarrow$ 6)-linkages from the non-reducing ends of the starch chain. As a result of AMG action, the molecular chains were shortened by hydrolysis (Wang, Yuan, and Yue, 2015). Studies carried out on granular starch after AMG treatment suggested that AMG action affected amorphous and crystalline regions following a cooperative process (Dura, Błaszczak, and Rosell, 2014), hydrolyzing preferentially the amylopectin chains (Benavent-Gil and Rosell, 2017b). The general behavior of corn starch under AMG treatment is characterized by pores developed from the surface to the center of the starch granule (Zhang et al., 2013), resulting in greatly perforated granules. These structural character-

Table 7.1: Structural characteristics of native and modified starches used as supporting materials (data from (Benavent-Gil and Rosell, 2017a))

Name	Enzyme (U/g starch)	Porosity (%)
C-0	0	0.00
C-5.5	5.5	5.49
C-7.8	11	7.76
C-8	16.5	8.32
C-13	33	12.53
C-15	55	14.67

istics can be modulated by varying the enzyme dosages, so that the resulting starches have different functional properties (Benavent-Gil and Rosell, 2017b). The expandable space within the granule significantly increases the specific surface area (Zhang et al., 2012), because of that those porous structures are used as carriers or vehicles of diverse compounds (Wang, Yuan, and Yue, 2015). Because of that all studies carried out on porous starches have been focused on their granular structure, despite in industrial settings, gelatinization is a crucial stage. For many processing applications involving starch, starch granules are heated in excess of water. This process (gelatinization) results in the loss of granular integrity and gives rise to the formation of a network, which turns the solution into a gel. Therefore, the properties of starch gel determine the exploitation of starch in an increasing number of applications. Nevertheless, little information has been provided about how granule porosity present on porous starches affects gelatinization behavior and gel properties, as well as the resulting microstructure.

In the present work, a selection of porous corn starches with varied structure changes (Benavent-Gil and Rosell, 2017b), have been used to determine the impact of pores intensity on the gel properties. Porous starches were obtained from a previous study using increasing levels of AMG to modulate the severity of the surface perforation. To achieve this goal, resulting starch gels were characterized based on their microstructure and physical behavior. This work will provide a greater understanding of the use of porous starches

as a main component or additive in food applications, where the texture and stability of the gels, as well as the properties of the paste are fundamental.

7.2 Materials and methods

7.2.1 Materials

Native corn starch (Miwon, Seoul, Korea) without being subjected to the action of the enzyme were used as reference. The development and preparation of enzymatically modified porous starches were described in detail in a previous publication by Benavent-Gil and Rosell (2017b). The AMG modified starches were used for the study since they produced the largest difference in microstructure characteristics, which are summarized in Table 7.1. The notation used for the samples was C to denote corn starch and a number that specified the porosity of the porous starches.

All the other chemicals were analytical reagent grade and used without further purification. All solutions and standards were prepared by using deionized water.

7.2.2 Flow behavior of corn starch slurries (granular state)

Corn starch slurries were prepared by dispersing 20 mg of starch in 40 mL of distilled water in aluminum canisters and vigorously mixing the content with a wire rod until complete starch suspension. Shear stress versus shear rate data was recorded using a rheometer (Haake RheoStress 1, Thermo Fischer Scientific, Scheverte, Germany) with a Z34 DIN Ti concentric cylinder system (1 mm gap) at a temperature of 30 °C. Shear rate was logarithmically increased from 1 to 500 s^{-1} in step mode (30 seconds per point) and data were fitted to the Ostwald-de Waele model (R equal to or higher than 0.99):

$$\sigma = K.\gamma^{\,n}$$

where σ is the shear stress (Pa), γ is the shear rate (s^{-1}), K is the consistency coefficient ($Pa.s^n$), and n is the flow behavior index (dimensionless). In this model, the apparent viscosity (η) can be calculated as (η=$K.\gamma^{(n-1)}$). All measurements were made in duplicate.

7.2.3 Porous starch gel preparation

Corn starch was gelatinized by running the standard method 61-02.01 for pasting properties (AACC, 2015) with modifications according to Martínez, Pico, and Gómez (2015) using a Rapid Visco Analyser (RVA-4) (Perten Instruments, Macquarie Park, Australia). Briefly, the starch-water mixture, prepared by adding 3.5 g of starch to 25 mL of distilled water, was held at 50 °C for 1 min, then heated from 50 to 95 °C, and held at this temperature for 2 min 30 s. Subsequently, the sample was cooled down to 30 °C, and held at 30 °C for 4 min. Gel/paste preparation was made in duplicate.

7.2.4 Scanning Electron Microscopy (SEM)

Morphology of the gelatinized starch samples was observed with a scanning electron microscope (SEM) (S-4800, Hitachi, Ibaraki, Japan). The freeze-dried gelatinized samples were split, and then the sections were mounted on specimen holders followed by coating with gold in a vacuum evaporator (JEE 400, JEOL, Tokyo, Japan). All micrographs were recorded at an accelerating voltage of 10 kV and at 300× magnification.

The microstructure analysis was carried out using the image analysis program (ImageJ, UTHSCSA Image Tool software). The SEM images were saved as 8-bit tiff format. Scale was initially set using the relationship between pixels and known distance. Threshold was assessed applying the Otsu's algorithm according to Gonzales-Barron and Butler (2006) and then particle analysis was carried out. The following parameters were measured: void size and void frequency.

7.2.5 Viscoelastic behavior of porous gels/pastes

To assess the linear viscoelastic behavior of corn starch gels, the gels were loaded in a Haake RheoStress1 rheometer (Thermo Fischer Scientific, Scheverte, Germany) immediately after RVA cycle. Rheological tests were conducted with a titanium parallel plate geometry sensor PP60 Ti (60 mm diameter, and 1 mm gap). After gap adjustment, sample surface between plates was covered with Panreac vaseline oil (Panreac Quimica S.A., Castellar del Valles, Spain) to avoid drying. Before conducting any rheological test, the pastes were allowed to rest for 500 s in the measurement position. Dynamic linear viscoelastic range at 30 °C was esti-

mated by performing a stress sweep from 0.1 to 100 Pa at 1 Hz frequency. Frequency dependence experiments were conducted from 10 to 0.01 Hz at 30 °C. The applied stress in the frequency sweep was always selected to guarantee the existence of linear viscoelastic response. The storage (G') and loss (G") modulus as a function of frequency (ω) were obtained. All measurements were performed in duplicated.

7.2.6 Gel hardness

Gel hardness was evaluated using a TA.XT-Plus Texture Analyses (Stable Micro Systems Ltd., Godalming, UK) equipped with a 5 kg load cell and a 2-mm aluminum cylindrical probe. Briefly, the paste obtained from RVA was transferred in 13 g portions into disposable sample cups with a height of 15 mm. These were allowed to cool to room temperature and then stored at 4 °C for 24 h. Gel penetration measurement was performed by placing perpendicularly each gel in the equipment and compressed at a speed of 1 mm/s.

7.2.7 Syneresis

Syneresis was measured by a centrifugation test (Ribotta et al., 2007) using a Eppendorf 5415 R centrifuge, (Eppendorf, Germany). Starch gels were stored 10 days at 4 °C. After storage, the gels were tempered at 25 °C for 2 h and centrifuged at 3000× g for 10 min at 25 °C. After centrifugation the free water was separated, weighed, and expressed as percentage of amount of water released from gels. Measurements were the mean of three repetitions for each duplicated gel.

7.2.8 Statistical analysis

All experiments were repeated at least in duplicate. Experimental data were statistically analyzed using an analysis of variance (ANOVA) and values were expressed as a mean ± standard deviation. Fisher's least significant differences test was used for assessment of significant differences among experimental mean values with 95% confidence. Pearson correlation coefficient (r) and P-value were used to indicate correlations and their significance using Statgraphics Centurion XV software (Bitstream, Cambridge, N). Differences of $P < 0.05$ were considered significant.

Table 7.2: Flow behavior of corn starch slurries

	Flow behavior	
	K (Pa·s^n)	n
C-0	0.0003^{a}	1.52^{e}
C-5.5	0.0008^{a}	1.36^{d}
C-7.8	0.0010^{a}	1.32^{cd}
C-8	0.0013^{a}	1.28^{c}
C-13	0.0043^{b}	1.09^{b}
C-15	0.0081^{c}	0.98^{a}
St. error	0.0006	0.02

7.3 Results and Discussion

7.3.1 Flow behavior index of non-gelatinized starch dispersions

The flow behavior of starch slurries prepared in the absence of heating are reported in Table 7.2. The consistency index (K) of the non-gelatinized pastes augmented with increasing starch porosity, although no significant differences were visible up to a porosity of 13% (starch C-13). Likewise, the values for the flow behavior index (n) decreased progressively as the porosity increased, but in this case, differences were significant even with the smallest porosity. These results are in agreement with those found in α-amylase hydrolyzed corn starches in which the overall resistance of the sample to flow decreases (Khatoon et al., 2009). Regarding the flow behavior of the starch dispersions, it was visible that the native corn starch dispersion corresponded to the model for shear-thickening fluids (also incorrectly known as dilatant), i.e. an increase in apparent viscosity is observed at higher shear rates (n>1), while this behavior was steadily lost with increasing the severity of the porosity. A decrease in the severity of shear-thickening can be attributed to a number of factors including polydispersity in granule size, granule volume fraction, and deformability of the dispersed granules (Rao et al., 1997). It is noteworthy that with the maximum porosity the flow behavior became shear-thinning (n <1), close to Newtonian, meaning that the apparent viscosity decreases at higher

shear rates. This behavior may be due to better alignment properties of more deformable starch granules with porous on its surface or to a reduction in the granule volume fraction as Rao et al. (1997) suggested. Likewise, Kwon et al. (1999) reported an almost Newtonian behavior of liquefied corn starches treated by maltogenic amylases, probably due to the increase of the molecular mobility. Shear-thickening is a typical behavior of suspensions of smooth spheres in Newtonian suspending fluids (Denn, Morris, and Bonn, 2018). Thus, it is known that severe starch treatments can destroy the starch ability to form shear-thickened fluids (Dintzis et al., 1996; Tattiyakul and Rao, 2000). It has been shown that shear-thickening behavior of starch pastes shifts to a more shear thinning one when starch granules are disrupted by gelatinization process (Rao et al., 1997). Similarly, the change to shear-thinning behavior could steadily occur as the starch surface is modified by the action of the AMG hydrolytic enzyme. The presence of more surface porous may account for a greater water uptake (as the higher consistency index indicates), turning the insoluble starch granule into a more soluble one, facilitating interaction among granules, and, making water more difficult to be squeezed out from the granules at higher shear rates. In fact, a more hydrophilic nature (higher water absorption capacity) of these enzymatically treated corn starches was found in a previous study (Benavent-Gil and Rosell, 2017b). The more swollen starch granules would be better aligned along the flow direction; reducing in this form the drag experimented by the microstructure (Carrillo-Navas et al., 2014).

7.3.2 Gel matrix structure

Native and AMG treated corn starch granules had an irregular and mostly polygonal shape. Nevertheless, enzymatic treatments modify the surface of starch granules by producing pores in a random way (Benavent-Gil and Rosell, 2017b). In addition, no significant change in relative crystallinity has been reported among native corn starch and AMG treated starch (Martinez-Alejo et al., 2018), neither in the amylose content (Table 7.1) (Benavent-Gil and Rosell, 2017a). SEM characterization of the sample gel matrices was carried out to investigate any possible structural changes induced by the sample porosity (Figure 7.1). In line with previous reports (Htoon et al., 2009), SEM micrographs revealed a dense structure,

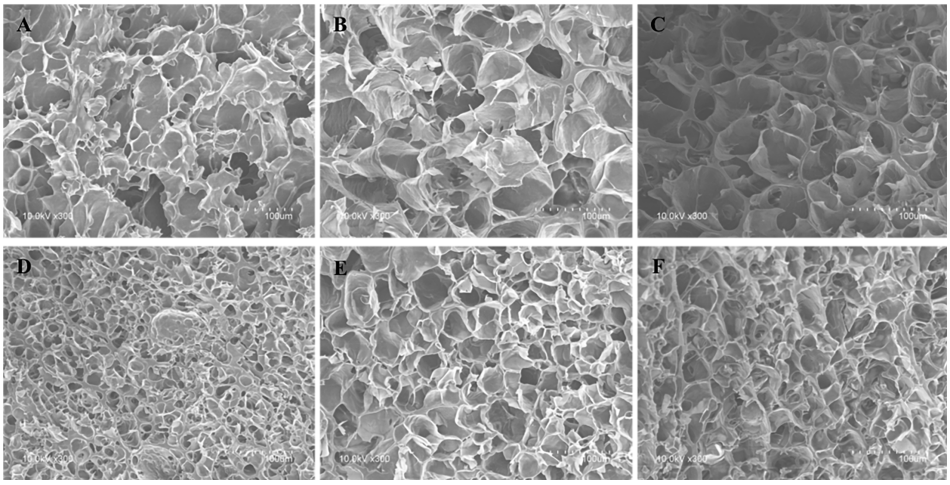

Figure 7.1: Scanning electron micrograph of starch gels obtained from native corn (A) and porous starches with increasing level of porosity (5.5, 7.8, 8.0, 13, 15) (B–F). Magnification 300×.

similar to a honeycomb construction, due to the loss of granular structure. It has been described that heating starch in the presence of sufficient water leads to the mobilized amylose molecules being dispersed from the interior of the granule to the surrounding aqueous medium (Schirmer et al., 2013). As a consequence of this process, an amorphous structure is generated, which is composed of spherical or oval holes and surrounded by a gel matrix (Wang et al., 2008).

From SEM micrographs, it was clear the effect of porosity on starch gel microstructure. The network obtained from the different porous starches was significantly different regarding the hole size distribution within the matrix and the number of voids (Figure 7.2). Overall, the starch porosity caused a reduction in the hole size distribution compared with non-porous sample, except in the case of the lowest porosity (carried out with 5.5% porosity). The smallest hole size was observed in the sample with a porosity of 8% (Figure 7.2 A). Meanwhile the larger hole size was displayed by the sample with the porosity of 5.5%. The other samples studied in this study did not show significant differences in terms of hole size among them. As a conse-

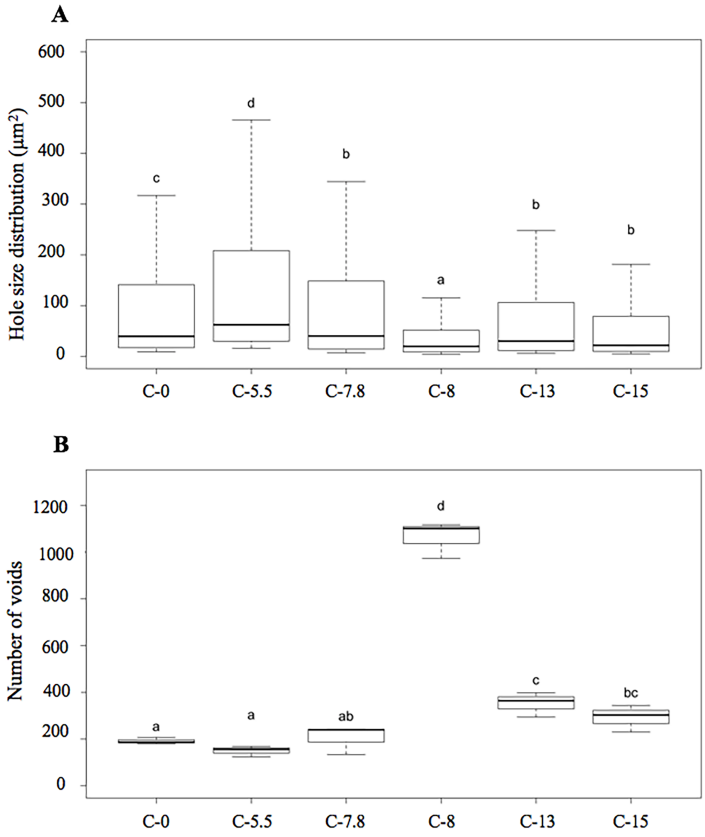

Figure 7.2: Image analysis from SEM photographs. A) Hole size and B) number of holes distribution by boxplot. Numbers following the abbreviation are referred to the porosity of the starches (5.5, 7.8, 8.0, 13, 15).

quence, the number of voids significantly varied among samples (Figure 7.2 B). Nevertheless, no correlation was observed between both parameters. The lowest porosity did not differ in the number of holes respect to non-porous gel matrix. The largest number of holes was obtained with the porous starch with 8% of porosity, which led to the smallest pores, followed by the gels with 13% and 15% of porosity. Therefore, although enzymatic treatment is initially carried out to obtain porous starches, results revealed that enzymatic treatment allowed obtaining gels with significant

different microstructures.

7.3.3 Viscoelastic behavior of gelatinized starch pastes

The mechanical spectra of gelatinized corn starch pastes (starch in non-granular form) in Figure 7.3 showed the existence of solid-like starch gels, with values of G' greater that G" for all the studied starches and with increasing values for increasing frequencies. This behavior has already been described for other starch gels (Carrillo-Navas et al., 2014). It is known that any damage of the starch prior to gelatinization decreases the values of the moduli as indicated by Kochkina and Khokhlova (2016) when modifying the starches by means of solid state milling. In this sense, it was observed that both moduli, elastic and viscous modulus, decreased as the porosity increased. The lower value of the viscous and elastic moduli is expected for porous starches according to their higher starch damage produced due to the AMG hydrolytic action on the granule surface (Southall et al., 1999), which may lead to a granular weakening that increases the cooking loss and reduces the water uptake during and after gelatinization (Roman et al., 2017).

However, no significant changes on the moduli values with respect to the non-porous material (C-0) were observed until the porosity of 8%, values (C-8), which seems to reach a plateau from 13% porosity (Table 7.3). Therefore, a minimum enzymatic treatment seems to be necessary to modify the rheology of the cooked starch pastes, this is, to affect the changes of the starch after the gelatinization and retrogradation processes, reducing the values of G' and G", but these changes did not continue from a certain treatment.

Regarding the value of tan δ, the smaller porosities (mildest treatments) reduced the values of this parameter, but as soon as the porosity is increased, the tan δ value increased progressively, exceeding the value of the non-porous starch in the highest porosities, which indicates a decrease in the elastic character. These results agree again with those found by Kochkina and Khokhlova (2016), with changes only being observed when they damaged the starch for a certain time of treatment. They associated these results to the more destructed starch molecules, which would form a weaker network with a low entanglement density.

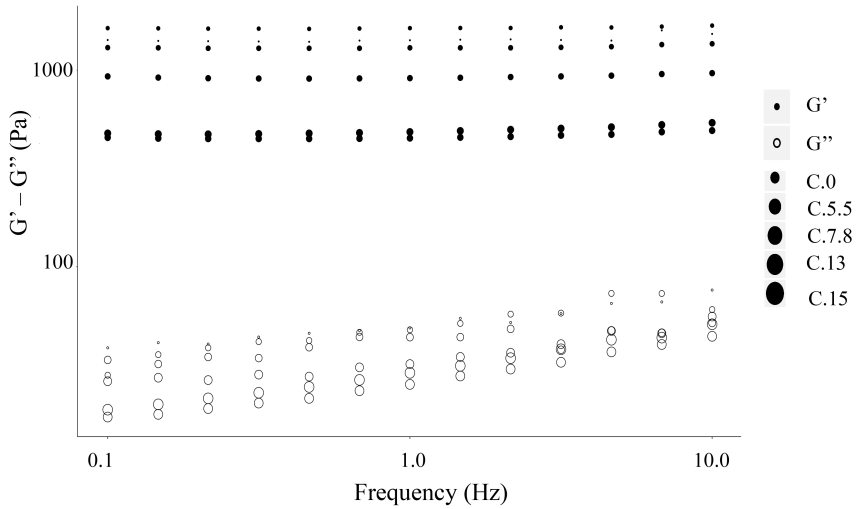

Figure 7.3: Variation of storage (G') and loss (G") moduli with frequency of corn starch gels.

7.3.4 Texture profile analysis (TPA)

The hardness of non-porous and porous gel matrices after storage at 4 °C for 24 h is shown in Figure 7.4. The statistical analysis indicated that gels from diverse porous starches had significant different hardness.

Table 7.3: Viscoelastic behavior of gelatinized starch pastes

	Viscoelastic behavior		
	G' (Pa)	G" (Pa)	tan δ
C-0	1300^{c}	48.75^{b}	0.041^{c}
C-5.5	1637^{d}	44.83^{b}	0.027^{a}
C-7.8	1300^{c}	43.73^{b}	0.034^{b}
C-8	911^{b}	31.90^{a}	0.035^{bc}
C-13	451^{a}	25.15^{a}	0.056^{d}
C-15	485^{a}	28.80^{a}	0.059^{d}
St. error	92	2.65	0.002

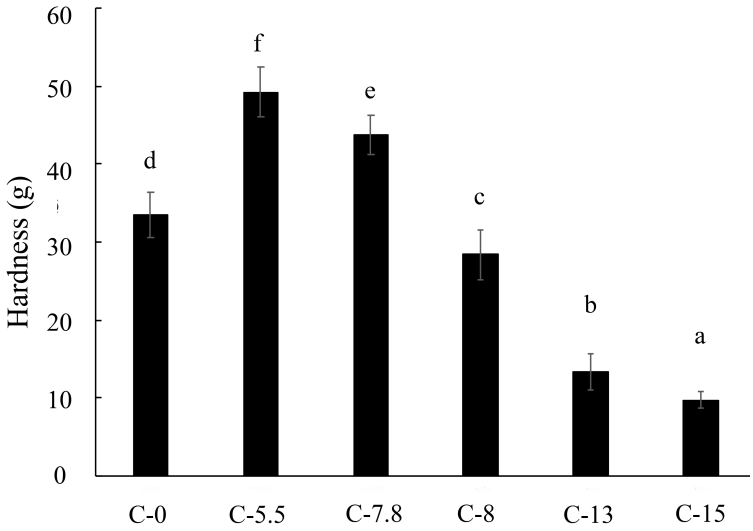

Figure 7.4: Hardness (g) of the gels produced from different porous starches. Numbers following the abbreviation are referred to the porosity of the starches (5.5, 7.8, 8.0, 13, 15).

The trend observed was the same as the one described for the microstructure and the viscoelastic behavior, observing different tendencies between the lowest and the highest porosities compared with non-porous starch. Specifically, it was observed that porous starches with lower porosity resulted in gels with a significant increase in hardness (from 33.43 ± 2.89 g in C-0 to 49.28 ± 3.28 and 43.68 ± 2.52 g for C-5.5 and C-7.8, respectively). But after certain porosity (8 %), a steady decrease of gels hardness was obtained; consequently, more intensive porosity of the granular starches led to softer gels. These results suggested that enzyme dosage in the enzymatic treatment with AMG could potentially be used to increase or reduce the hardness of corn starch gels.

7.3.5 Effect of enzymatic treatment on syneresis

After cooling, the release of the bulk phase water from the polymer network occurs, which is the so-called syneresis (Zhang et al., 2018), and has been directly related to the starch ability to hold water and its tendency to retrograde (Amani et al.,

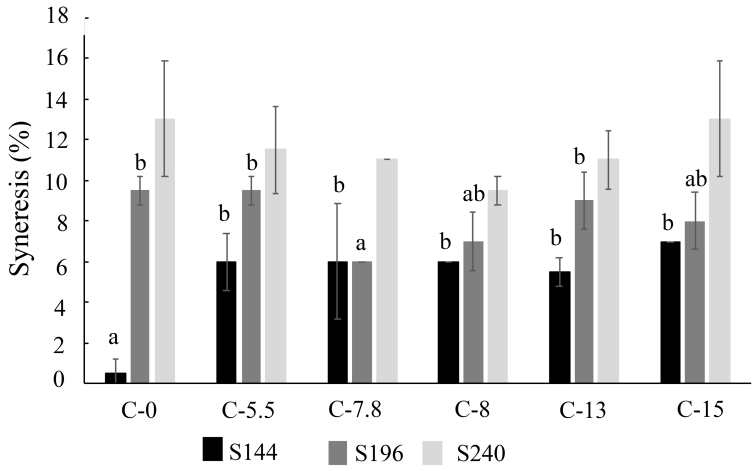

Figure 7.5: Syneresis (%) of the starch gels obtained from porous starches after 144, 196 and 240 hours of storage. Numbers following the abbreviation are referred to the porosity of the starches (5.5, 7.8, 8.0, 13, 15).

2002; Majzoobi, Kaveh, and Farahnaky, 2016). The syneresis of the gels was measured over a period of up to 240 h at 4 °C (Figure 7.5). The syneresis was virtually zero for all samples at the onset. In line with previous reports (Zhang et al., 2018; Amani et al., 2002; Majzoobi, Kaveh, and Farahnaky, 2016; Liu et al., 2015; Zortéa-Guidolin et al., 2017), the syneresis of all samples increased significantly with storage time. Previous reports observed an increase in syneresis during the first two weeks of storage, but then did not change for up to 8 weeks (Dreher et al., 1983). The gels obtained from porous starches had higher syneresis values than the non-porous corn starch after 144 h, with no significant differences regarding the porosity observed in porous starches. In spite of the lower initial ability of the gels obtained from porous starches to retain the water molecules, they exhibited better performance after longer storage periods. Especially, those gels that had closed microstructure with small voids (C-8), which might help to retain the water molecules.

7.4 Conclusions

Gels obtained from porous starches exhibited a network microstructure

similar to the one displayed by native starch. However, the size and number of holes within the gel structure was significantly dependent on the initial porosity of the porous starches. Starch with 8% porosity produced gels with closed microstructure, which were softer than the gel obtained from native starch but were able to retain better the water molecules at longer storage. In addition, higher surface porosity resulted in less viscoelastic starch gels. Therefore, porous starches not only have different granular structure, surface pores also induced the production of diverse gels matrixes, and their structure will be dependent on the porosity of the original porous starch.

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Chapter 8

General Discussion

Porous starches represent alternative modified starches with abundant pores and accessible inner empty spaces (Li, Turner, and Dhital, 2016; Zhang et al., 2012; Zhao, Madson, and Whistler, 1996). Among the different starch treatments employed to obtain porous starches, enzymatic treatments are those that lead to larger pore and cavity sizes (Fortuna, Juszczak, and Pałasiński, 1998). The enzymatic modification of starch involves the reaction of one or more enzymes that act on a solid substrate represented by the starch granules. Porous are formed during enzymatic treatment, because of that the initial understanding of enzymes action on starch granule is necessary prior to propose any further application. In this sense, the current investigation describes diverse studies for understanding porous starch properties regarding its characterization and application. Specifically, studies have been conducted to evaluate the effect of enzymatic treatment on starch granules, to establish the main technological properties that allow differentiation among resulting porous starches, besides to evaluate their potential nutritional benefits assessed by simulated enzymatic digestion, and finally to confirm their applicability for protecting bioactive compounds or for being used as additives in the food industries.

Many enzymes can degrade starch (Dona et al., 2010), being the most commonly used the hydrolases (Dura, Błaszczak, and Rosell, 2014). Therefore, structural changes are greatly dependent on enzyme used to obtain porous starch. In fact, studies carried out applying different techniques like light microscopy, confocal laser scanning microscopy and scanning electron microscopy (SEM) stated several structural changes, comprising pin-holes, sponge-like erosion, numerous medium-sized holes, distinct loci leading to single holes in individual granules and surface erosion (Dura, Błaszczak, and Rosell, 2014; Dura and Rosell, 2016; Apinan et al., 2007; Zhang et al., 2012; Zhang, Ao, and Hamaker, 2006; Tester, Qi, and Karkalas, 2006).

Nevertheless, the diversity of the experimental conditions reported in the literature makes very difficult to really understand the enzymes action on the structure of the starches or how to modulate the starch structure by using starch-hydrolases.

A proposed strategy for understanding the influence of enzymatic treatments on the structure of porous starches was the application of different concentrations (increasing 2, 3, 6 and 10 times the minimum concentrations recommended by the manufacturer) of AMG, AM, CGTase and BE enzymes, individually. In this research, corn starch was selected as a substrate to be modified enzymatically due to its availability, and hydrolysis susceptibility compared to other starches (Wang, Yuan, and Yue, 2015). Morphological evaluation of the treated starches was conducted by SEM to observe their granular structure and to confirm the enzymatic action on starch granules. All samples exhibited pores on the granular surface, showing different porosity degree depending on the enzymatic treatment applied. The effect of AMG and AM enzymes on creating pores was clearly visible on the granules surface, which agree with results of Dura, Błaszczak, and Rosell (2014), who compared the individual effect of AMG and AM on corn starch granules when the enzymatic reaction was carried out at different pHs. Results from image analysis supported that both enzyme and its concentration, affected the pore size and number of pores per granule. In general, pore size increased progressively due to the enzyme concentration, except in the case of BE enzyme. This increase might be consequence of larger available surface area due to more pronounced enzymatic action, which lead to better accessibility (Aggarwal and Dollimore, 2000). Regarding the number of pores, AMG and AM enzymes presented the same trend previously described for the pore size, whereas CGTase and BE enzymes did not show an increase with the increase of the concentration applied.

Besides enzymes, substrate used, that is the starch origin, is greatly responsible for the different ways in which the starch is disrupted. A great variability among starch properties has been observed, mainly ascribed to botanical species and even to diverse growing conditions of the same plant cultivar. Among them, granule size and shape, starch crystallinity, amylose-amylopectin ratio, swelling power and solubility, gelatinization and

rheological properties have been pointed out as major factors affecting starch functionality (Witczak et al., 2016). In order to obtain porous starches with different size, shape, uniformity and periodicity of the porous spaces, starches from wheat, rice, potato and cassava sources were subjected to enzymatic treatment with AMG, AM and CGTase, individually. Starch structure was captured using SEM and then analyzed to obtain the porous size distribution by image analysis. Several porous structures with diverse pore size distribution depending on the enzyme type and starch source were observed, presenting similar pore area per granule. Generally, the amylolytic action on granular starch provokes the formation of deep holes in cereal starches, while more superficial attacks occur in the tuber starches.

All of these changes suffered during the enzymatic modification affected to a greater or lesser extent the functional properties of the treated starches. The amylopectin/amylose relationship that is closely related to the functional properties of starch (Schirmer et al., 2013; Witczak et al., 2016) was markedly modified, depending on the enzymatic treatment and starch source. AM and CGTase mainly led to a decrease in the amount of amylose, while AMG and BE increased the amylose proportion but only in cereal starches, likely due to existing defects of the crystalline regions (Koroteeva et al., 2007a; Koroteeva et al., 2007b; Kozlov et al., 2007). With amylose changes in mind, differential scanning calorimetry (DSC) thermal transition temperatures were analyzed because they are valuable indicators of the stability of starch crystallites (Liu et al., 2015). A decrease of the melting temperature of the amylopectin crystallites in AMG and BE-treated cereal starches was expected (Koroteeva et al., 2007a; Koroteeva et al., 2007b; Kozlov et al., 2007). Nevertheless, when DSC data of the group of treated starches used in this study were compared with their respective native starches, it was observed that To was dependent on the hydrolase type and the concentration applied but also on the starch source. Meanwhile, Tc only was significantly influenced by enzymatic treatment and starch source. Both parameters (To and Tc) have been related with the melting of the weakest crystallites (To) and the melting of crystallites of high stability (Tc), respectively (Jacobs et al., 1998). Therefore, the changes observed after enzymatic treatments indicate a more or less widespread attack on the crystalline regions independently

on the enzyme used. Nevertheless, in spite of the enzyme attacks the crystalline portion, the concentration of the enzyme did not affect the more stable crystallites. This result suggested that the attack of the crystalline region depends on the mechanism of action of the hydrolase used. DSC also measures the gelatinization enthalpy (ΔH) change, which is directly related to the amount of ordered carbohydrate structure in the granule. A decrease in the molecular order was favored by the hydrolytic action of the enzymes, since lower ΔH was observed compared to their native starches, except porous starches obtained from rice that showed higher ΔH. Having the amylose content and DSC data in mind, it can be concluded that AM and CGTase mainly attacked amorphous and crystalline structure. In opposition, AMG and BE acted predominantly on crystalline areas.

All mentioned structural changes resulted in increasing the ability of porous starches to bind water molecules, that effect was principally observed in AMG-treated starches, which also increased their oil absorption capacity. Therefore, it seems that the pore size plays a fundamental role in determining the adsorption capacity. In fact, in a porous domain, the smaller the pore sizes in the matrix the slower the molecules migration (Labuza and Hyman, 1998).

Once confirmed the enzymatic modification of the granular starch, its technological properties were evaluated, since they determine the exploitation of starch in an increasing number of applications. Viscosity profiles along a heating-cooling cycle, which was represented in a heatmap, were used to characterize the gelatinization and gelling behavior of the different modified starches. All samples showed a typical viscosity pattern, i.e., an increase of viscosity up to peak (during heating) followed by a drop of viscosity (during cooling) under agitation and heating. Nevertheless, enzymatically modified samples exhibited changes in their viscosity values revealing diverse pasting behavior, related to enzyme action and concentration but also to starch source. AM-treated starches from cereal source exhibited similar viscoelastic behavior than native starch, followed by AMG-treated samples. Interestingly, the most differentiated behavior was mainly shown by CGTase- and BE-treated samples, although the enzyme concentration of the latter played an important role in the behavior of the samples. Meanwhile, the treated sam-

ples from tuber source displayed a completely opposite scenario, where the more similar character to the native starches was found in CGTase-treated samples.

Structural changes promoted by the starch modification induced variations on the technofunctional properties of the starches, but likely those structural changes could lead to diverse physiological response. In fact, enzymatic modification of starches has been recently proposed as a way to reduce the glycemic index (GI) (Dura, Yokoyama, and Rosell, 2016). However, the precise impact of structural modifications on the digestive behavior of modified starches is not always very well defined. Because of that, corn modified starches were selected for assessing their potential nutritional benefits by *in vitro* hydrolysis test. The starch digestion curves were analyzed to determine the rate and extent of hydrolysis during enzymatic digestion. Taking into account the morphological and structural changes promoted by enzymatic treatment, it was expected an increase of the starch digestibility. However, diverse behavior was observed depending on the enzymatic treatment. AMG-treated starches displayed the highest susceptibility to be hydrolyzed. Conversely, AM- CGTase- and BE-modified samples offered great resistance to be hydrolyzed by digestion enzymes. Therefore, in general, the application of enzymatic treatments for producing porous starches, prompted lower GI, with the exception of AMG treated samples.

To get a general picture of the importance of the pores size on the morphological and functional properties of the resulting starches, a multi factor analysis was conducted with all the parameters. It is clear that the pores created in the starch surface play a critical role in the hydrolytic event, allowing the access of the enzymes, involved in the digestion process, to the inner granule (Dona et al., 2010; Fannon, Hauber, and BeMiller, 1992; Utrilla-Coello et al., 2009). The different hydrolysis patterns in the simulated digestibility were significantly related to the pore size of the samples, thus the diffusion or access of the digestive amylase was strongly influenced by the pore size of the samples, inducing different starch disruption (Blazek and Gilbert, 2010), and thus leading to faster or slower hydrolysis. This opens promising ways for the integration of enzymatically modified starches into minimally processed cereal products contributing to healthier and improved products.

Nowadays, the development of functional foods is attracting much attention of the food industry due to the consumers' growing demand for healthful foods (Biström and Nordström, 2002; Siro et al., 2008). In this context, the market for functional foods is dominated by those that contain probiotics in their formulation (Stanton et al., 2001). Probiotics are live microorganisms, which when administered in adequate amounts exert a health benefit on the host (WHO/FAO, 2002). Therefore, probiotic foods should contain a critical concentration of viable bacteria, recommending a daily dosage of 10^6-10^9 viable organisms reaching the intestinal tract in humans (Shah, 2000). However, the development of probiotic foods continues to be a challenge for the food industry, since probiotics incorporated in processed foods must overcome certain technological aspects such as temperature, which compromises the viability of probiotic cells. In this sense, the encapsulation of probiotics allows cells to be protected from the adverse environment, remaining in a viable and metabolically active state (Burgain et al., 2011; Garti, 2008).

The high absorption capacity of the enzymatically modified starches could provide a natural absorptive matrix to protect probiotic microorganisms in their porous surface. Moreover, further stability of the probiotic cells could be obtained by the combination of porous starches and coating materials. So far, several enzymatic treatments conducted in the present research have been discussed to obtain porous starches using diverse starch sources, including cereal and tuber origin. From the obtained data, it was observed that AMG and AM treatments in cereal starches resulted in porous starches with different pore size, but also deep cavities. Therefore, corn and rice starches after AMG (16.5 U AMG / g starch) and AM (11 U AMG / g starch) treatments were selected for encapsulating *Lactobacillus plantarum*. The encapsulation process, based on preliminary assays, was carried out in four different stages: microorganism adsorption (S1), vacuum filtering (S2), freezing (S3) and freeze drying (S4). Respect to encapsulation yield (EY) of *L. plantarum* in the microcapsules, the S4 stage was the one that had the greatest influence on the final EY depending on the starch source. The choice of wall material has a significant impact on their stability and application (Favaro-Trindade and Grosso, 2002; Oliveira et al., 2007; Tonon et al., 2009).

Therefore, porous microcapsules from rice starch kept higher EY after S4 stage, likely due to the porous structure in the granule surface along with the agglomerate character of the rice starch, protected the microorganisms. Namely, this was corroborated by microscopy analysis from microcapsules after S4 stage, where different entrapment by the microorganism onto the surface of the granules was observed. The microorganism adhesion occurred onto the corn granular surface but they were also entrapped within the rice granules aggregates, since that effect was also observed when native rice starch granules were used.

Coatings study also revealed significant differences in the EY depending on the gel used, comprising GS (gelatinized starch), GG (guar gum) and GX (xanthan gum). In general, microcapsules coated with GS displayed an increase in this parameter. Meanwhile, GG and GS coatings differed in their protecting effect, which seems to be related to the different interaction of the coating material with the starch surface (Xing et al., 2015). Therefore, coating induces a reinforcement of the microcapsules or a competition with the microorganism to fill the pores, increasing or decreasing the EY.

During heat treatment, all the samples were affected. The microcapsules from corn porous starch conferred more thermal stability to the *L. plantarum* cells, not achieving this effect with microcapsules from rice porous starches. This might be due to the release of the cells and their subsequent inactivation due to the collapse of the microcapsules (Xing et al., 2014). Considering coatings, all of them provided additional protection and allowed maintaining greater number of *L. plantarum* viable cells after short (20 min) thermal treatment. However, after long (35 min) thermal constraints, only rice supporting material microcapsules exhibited that stabilization effect. In addition, starch porosity degree obtained in the case of AMG- and AM-treated starches significantly affected the protection provided by coating. Thus, porous starches with small pores, that is AM-treated starches, and GS coating brought about higher thermal resistance.

In spite of the great possibilities that porous starch displays in their granular state, in industrial settings, the properties of starch gel are crucial for their exploitation in a number of applications. Processed products involving

starch require heating of the starch granules in excess water, transforming the granule into a gel. Nevertheless, there is no information about how granule porosity present on porous starches affects gelatinization behavior and gel properties, as well as the resulting microstructure. According to previous studies (Schirmer et al., 2013; Singh et al., 2003; Witczak et al., 2016), both the granules size and the amylose content, significantly affect the functional properties of starch (water retention capacity, peak viscosity, final viscosity and breakdown). Then, it is possible to hypothesize that due to the structural and functional changes of porous starches after enzymatic treatments, their gel properties will be significative influenced. For that reason, AMG-treated corn starches, previously characterized, were gelatinized and analyzed based on the texture and stability of the gels, as well as the properties of the resulting pastes. These samples were chosen owing to their diverse degree of porosity ranging between 0 and 15%. Gels obtained displayed honeycomb structures; however, the size and number of holes contained in these structures differed depending on the porosity of the initial porous starches. Among all sample, treated starch with an initial porosity of 8% produced gels with closed microstructure. This porosity was of particular relevance in all the parameters analyzed, since in general was presented as a turning point between changes. In fact, the viscoelastic behavior did not show significant changes on the moduli values until the porosity of 8% was reached. Regarding the hardness of gel matrices, an increasing trend was observed till 8%-porosity, but higher porosity led to softer gels. Therefore, previous assumptions were corroborated since results revealed that enzymatic treatment allowed obtaining gels with significant different microstructures and textures.

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8 Discusión General

El término almidón poroso se refiere a almidones que han sido modificados para incrementar la presencia de poros y espacios vacíos en el interior del gránulo accesibles a distintas moléculas (Li, Turner, and Dhital, 2016; Zhang et al., 2012; Zhao, Madson, and Whistler, 1996). Entre los diferentes tratamientos empleados para modificar los almidones y obtener almidones porosos, los tratamientos enzimáticos son los que proporcionan tamaños de poros y cavidades más grandes (Fortuna, Juszczak, and Pałasiński, 1998). La modificación enzimática del almidón implica la reacción de una o más enzimas que actúan sobre un sustrato sólido representado por los gránulos de almidón. Los poros se forman durante el tratamiento enzimático, por lo que la comprensión de la acción de las enzimas sobre el gránulo de almidón es necesaria antes de proponer cualquier aplicación. En este sentido, la presente investigación describe diversos estudios para comprender las propiedades de los almidones porosos con respecto a su caracterización y aplicación. Concretamente, se han realizado estudios para evaluar el efecto del tratamiento enzimático en los gránulos de almidón, y de esta manera establecer las principales propiedades tecnológicas que permiten la diferenciación entre los almidones porosos resultantes, además de evaluar los beneficios nutricionales potenciales evaluados mediante la digestión enzimática simulada y, finalmente, confirmar su aplicabilidad para proteger compuestos bioactivos o para ser utilizados como aditivos en la industria alimentaria.

Muchas enzimas son capaces de degradar el almidón (Dona et al., 2010), siendo las amilasas las más comúnmente utilizadas (Dura, Błaszczak, and Rosell, 2014). Por lo tanto, los cambios estructurales dependen en gran medida de la enzima utilizada para obtener el almidón poroso. De hecho, estudios realizados aplicando diferentes técnicas como la microscopía óptica, la microscopía confocal de barrido y la microscopía electrónica de barrido (SEM) indicaron varios cambios estructurales, que incluyen orificios, erosión

similar a una esponja, numerosos orificios de tamaño intermedio o distintos loci que conducen a agujeros aislados en gránulos individuales y erosión superficial (Dura, Błaszczak, and Rosell, 2014; Dura and Rosell, 2016; Apinan et al., 2007; Zhang et al., 2012; Zhang, Ao, and Hamaker, 2006; Tester, Qi, and Karkalas, 2006). Sin embargo, las diferentes condiciones experimentales descritas en la literatura hacen muy difícil entender realmente la acción de las enzimas sobre la estructura de los almidones o como modular la estructura del almidón utilizando almidón-hidrolasas.

Una de las estrategias propuestas para comprender la influencia de los tratamientos enzimáticos en la estructura de los almidones porosos fue la aplicación de diferentes concentraciones (aumentando 2, 3, 6 y 10 veces las concentraciones mínimas recomendadas por el fabricante) de las enzimas AMG, AM, CGTase y BE, individualmente. En esta investigación, el almidón de maíz fue seleccionado como sustrato para ser modificado enzimáticamente debido a su disponibilidad y susceptibilidad a la hidrólisis en comparación con otros almidones (Wang, Yuan, and Yue, 2015). La evaluación morfológica de los almidones tratados se realizó mediante SEM para observar su estructura granular y para confirmar la acción enzimática sobre los gránulos de almidón. Todas las muestras mostraron poros en la superficie granular, mostrando diferente grado de porosidad dependiendo del tratamiento enzimático empleado. El efecto de las enzimas AMG y AM en la creación de poros fue claramente visible en la superficie de los gránulos, lo que concuerda con los resultados de Dura, Błaszczak, and Rosell (2014), quienes compararon el efecto individual de las enzimas AMG y AM en los gránulos de almidón de maíz cuando la reacción enzimática se llevó a cabo bajo diferentes pHs. Los resultados obtenidos por análisis de imagen confirmaron que, tanto la enzima como su concentración afectaron el tamaño de los poros así como el número de poros por gránulo. En general, el tamaño de los poros aumentó progresivamente debido a la concentración de la enzima, excepto en el caso de la enzima BE. Este aumento podría ser consecuencia de un mayor área de superficie disponible debido a una acción enzimática más pronunciada, lo que conduce a una mejor accesibilidad (Aggarwal and Dollimore, 2000). Con respecto al número de poros, las enzimas AMG y AM presentaron la misma tendencia descrita anteriormente para el tamaño de poro, mientras

que las enzimas CGTase y BE no mostraron un aumento al aumentar la concentración aplicada.

Además de las enzimas, el sustrato utilizado, o lo que es lo mismo, el origen del almidón, es en gran medida responsable de las diferentes formas en que se degrada el almidón. Existe una gran variabilidad entre las propiedades de los almidones, principalmente atribuidas a las especies botánicas e incluso a las diversas condiciones de crecimiento de una misma planta. Entre ellos, el tamaño y la forma de los gránulos, la cristalinidad del almidón, la relación amilosa-amilopectina, el poder de hinchamiento y la solubilidad, la gelatinización y las propiedades reológicas han sido señaladas como los principales factores que afectan la funcionalidad del almidón (Witczak et al., 2016). Con el fin de obtener almidones porosos con diferente tamaño, forma, uniformidad y periodicidad de los poros, los almidones de trigo, arroz, patata y yuca se sometieron a tratamiento enzimático con AMG, AM y CGTasa, individualmente. La estructura del almidón se capturó mediante SEM y luego se analizó para obtener la distribución del tamaño de poro mediante análisis de imagen. Esta metodología mostró varias estructuras porosas con una distribución diversa de tamaños de poro según el tipo de enzima y la fuente de almidón, presentando un área de poro similar por gránulo. En general, la acción amilolítica sobre el almidón granular provocó la formación de agujeros profundos en los almidones de cereales, mientras que se produjeron ataques más superficiales en los almidones de tubérculo.

Todos estos cambios sufridos durante la modificación enzimática afectaron en mayor o menor medida las propiedades funcionales de los almidones tratados. La relación amilosa-amilopectina, que está estrechamente relacionada con las propiedades funcionales del almidón (Schirmer et al., 2013; Witczak et al., 2016), se modificó notablemente dependiendo del tratamiento enzimático y la fuente de almidón. Las enzimas AM y CGTase condujeron principalmente a una disminución en la cantidad de amilosa, mientras que las enzimas AMG y BE aumentaron la proporción de amilosa, pero solo en almidones de cereales, probablemente debido a defectos existentes de las regiones cristalinas (Koroteeva et al., 2007a; Koroteeva et al., 2007b; Kozlov et al., 2007). Teniendo en cuenta los cambios en la cantidad de amilosa, se analizaron las temperaturas de transición térmica mediante calorimetría

diferencial de barrido (DSC) ya que son indicadores valiosos de la estabilidad de los cristalitos de almidón (Liu et al., 2015). Se esperaba una disminución de la temperatura de fusión de los cristalitos de amilopectina en los almidones de cereales tratados con las enzimas AMG y BE (Koroteeva et al., 2007a; Koroteeva et al., 2007b; Kozlov et al., 2007). Sin embargo, cuando se compararon los datos de DSC del grupo de almidones tratados utilizados en este estudio con sus respectivos almidones nativos, se observó que To dependía del tipo de hidrolasa y la concentración de enzima aplicada, pero también de la fuente de almidón. Mientras que, Tc solo fue significativamente influenciada por el tratamiento enzimático y la fuente de almidón. Ambos parámetros (To y Tc) se han relacionado con la fusión de los cristalitos más débiles (To) y la fusión de los cristales con alta estabilidad (Tc), respectivamente (Jacobs et al., 1998). Por lo tanto, los cambios observados después de los tratamientos enzimáticos indican un ataque más o menos generalizado en las regiones cristalinas independientemente de la enzima utilizada. Sin embargo, a pesar de que la enzima ataca la porción cristalina, la concentración de la enzima no afectó a los cristalitos más estables. Este resultado sugiere que el ataque de la región cristalina depende del mecanismo de acción de la hidrolasa utilizada. Mediante DSC también es posible medir el cambio de entalpía de gelatinización (ΔH), que está directamente relacionado con la estructura ordenada en el gránulo. Una disminución en el orden molecular se vio favorecida por la acción hidrolítica de las enzimas, ya que se observó una ΔH inferior en comparación con sus almidones nativos, excepto los almidones porosos obtenidos del arroz que mostraron una ΔH superior. Teniendo en cuenta el contenido de amilosa y los datos del DSC, se puede concluir que las enzimas AM y CGTase atacaron principalmente la estructura amorfa y cristalina. Por el contrario, las enzimas AMG y BE actuaron predominantemente sobre las áreas cristalinas.

Todos los cambios estructurales mencionados resultaron en un aumento de la capacidad de los almidones porosos para unirse a las moléculas de agua, ese efecto se observó principalmente en los almidones tratados con la enzima AMG, que también aumentó la capacidad de estos almidones para absorber aceite. Por lo tanto, parece que el tamaño de los poros juega un papel fundamental en la capacidad de adsorción. De hecho, en un espacio poroso,

cuanto más pequeños son los tamaños de poro en la matriz, más lenta es la migración de las moléculas (Labuza and Hyman, 1998).

Una vez confirmada la modificación enzimática en los gránulos de almidón, se evaluaron sus propiedades tecnológicas, puesto que son determinantes para la explotación del almidón en un gran número de aplicaciones. Los perfiles de viscosidad a lo largo de un ciclo de calentamiento-enfriamiento, que se representó en un mapa de calor, se utilizaron para caracterizar el comportamiento de gelatinización y gelificación de los diferentes almidones modificados. Todas las muestras mostraron un patrón de viscosidad típico, es decir, un aumento de la viscosidad (durante el calentamiento) seguido de una caída de la viscosidad (durante el enfriamiento) bajo agitación y calentamiento. Sin embargo, las muestras modificadas enzimáticamente mostraron cambios en sus valores de viscosidad, revelando diversos comportamientos de pegado, relacionados con la acción y concentración de las enzimas, pero también con la fuente de almidón. Los almidones de cereales tratados con la enzima AM mostraron un comportamiento viscoelástico similar al del almidón nativo, seguido de las muestras tratadas con AMG. Curiosamente, el comportamiento más diferenciado lo mostraron principalmente las muestras tratadas con CGTasa y BE, aunque la concentración de esta última enzima desempeñó un papel importante en el comportamiento de las muestras. Mientras que, las muestras de tubérculo tratadas mostraron un escenario completamente opuesto, donde se observó un comportamiento más similar a los almidones nativos en las muestras tratadas con CGTasa.

Los cambios estructurales promovidos por la modificación del almidón indujeron variaciones en las propiedades tecnofuncionales de los almidones, pero es probable que esos cambios estructurales también pudieran conducir a una respuesta fisiológica diversa. De hecho, la modificación enzimática de los almidones se ha propuesto recientemente como una forma de reducir el índice glucémico (GI) (Dura, Yokoyama, and Rosell, 2016). Sin embargo, el impacto de las modificaciones estructurales en el comportamiento digestivo de los almidones modificados no siempre está bien definido. Debido a eso, los almidones de maíz modificados fueron seleccionados para evaluar sus beneficios nutricionales potenciales mediante hidrólisis *in vitro*. Las curvas

de digestión del almidón se analizaron para determinar la velocidad y el grado de hidrólisis durante la digestión enzimática.

Teniendo en cuenta los cambios morfológicos y estructurales promovidos por el tratamiento enzimático, se anticipó un aumento de la digestibilidad del almidón. Sin embargo, se observó un comportamiento diferenciado dependiendo del tratamiento enzimático. Los almidones tratados con AMG mostraron la mayor susceptibilidad a ser hidrolizados. Por el contrario, las muestras modificadas con AM CGTasa y BE ofrecieron una gran resistencia a ser hidrolizadas por las enzimas digestivas. En general, la aplicación de tratamientos enzimáticos para producir almidones porosos provocó un GI más bajo, con la excepción de las muestras tratadas con AMG.

Para determinar de forma global la importancia del tamaño de los poros en las propiedades morfológicas y funcionales de los almidones resultantes, se realizó un análisis multifactorial con todos los parámetros. Está claro que los poros creados en la superficie del almidón juegan un papel crítico en el evento hidrolítico, permitiendo el acceso de las enzimas involucradas en el proceso de digestión al interior del gránulo (Dona et al., 2010; Fannon, Hauber, and BeMiller, 1992; Utrilla-Coello et al., 2009). Los diferentes comportamientos de hidrólisis en la digestibilidad simulada se relacionaron significativamente con el tamaño de los poros de las muestras, por lo que la difusión o el acceso de la amilasa digestiva se vio fuertemente influenciada por el tamaño de los poros de las muestras, lo que indujo una degradación diferente del almidón (Blazek and Gilbert, 2010), provocando una hidrólisis más rápida o más lenta. Esto abre la posibilidad de la integración de almidones modificados enzimáticamente en productos de cereales mínimamente procesados contribuyendo al desarrollo de productos más saludables y mejorados.

Hoy en día, el desarrollo de alimentos funcionales está atrayendo la atención de la industria alimentaria debido a la creciente demanda de alimentos saludables por los consumidores (Biström and Nordström, 2002; Siro et al., 2008). En este contexto, el mercado de alimentos funcionales está dominado por los productos que contienen probióticos en su formulación (Stanton et al., 2001). Los probióticos son microorganismos vivos, que cuando se administran en cantidades adecuadas ejercen un beneficio para la salud en el

huésped (WHO/FAO, 2002). Por lo tanto, los alimentos probióticos deben contener una concentración mínima de bacterias viables, recomendando una dosis diaria de 10^6-10^9 organismos viables capaces de llegar al tracto intestinal en humanos (Shah, 2000). Sin embargo, el desarrollo de alimentos probióticos continúa siendo un desafío para la industria alimentaria, ya que los microorganismos probióticos incorporados en alimentos procesados deben superar ciertos aspectos tecnológicos, como la temperatura, que comprometen la viabilidad de las células probióticas. En este sentido, la encapsulación de probióticos permite que las células se protejan frente a un ambiente adverso, permaneciendo en un estado viable y metabólicamente activo (Burgain et al., 2011; Garti, 2008).

La alta capacidad de absorción de los almidones modificados enzimáticamente podría proporcionar una matriz de absorción natural para proteger los microorganismos probióticos en su estructura porosa. Además, se podría obtener una estabilidad adicional de las células probióticas mediante la combinación de almidones porosos y materiales de recubrimiento. Hasta ahora, varios tratamientos enzimáticos realizados han sido discutidos en la presente investigación para obtener almidones porosos utilizando diversas fuentes de almidón, incluyendo cereales y tubérculos. A partir de los datos obtenidos, se observó que los tratamientos con las enzimas AMG y AM en almidones de cereales dieron como resultado almidones porosos con diferentes tamaños de poros, pero también cavidades profundas. Por lo tanto, los almidones de maíz y arroz después de los tratamientos con AMG (16.5 U AMG / g de almidón) y AM (11 U AMG / g de almidón) se seleccionaron para encapsular células de *Lactobacillus plantarum*. El proceso de encapsulación, basado en ensayos preliminares, se llevó a cabo en cuatro etapas diferentes: adsorción de microorganismos (S1), filtrado a vacío (S2), congelación (S3) y liofilización (S4). Respecto al rendimiento de encapsulación (EY) de *L. plantarum* en las microcápsulas, la etapa S4 fue la que tuvo una mayor influencia en el EY final dependiendo de la fuente de almidón. La elección del material de pared tiene un impacto significativo en su estabilidad y aplicación (Favaro-Trindade and Grosso, 2002; Oliveira et al., 2007; Tonon et al., 2009). Así, las microcápsulas porosas del almidón de arroz mantuvieron un EY más alto después de la etapa S4, probablemente debido a que la estructura porosa en la superficie

del gránulo, junto con la formación de aglomerados que presentaron los gránulos de arroz protegieron a los microorganismos. Este hecho fue corroborado por el análisis microscópico de las microcápsulas después de la etapa S4, donde se observaron diferentes atrapamientos del microorganismo en la superficie de los gránulos. La adhesión del microorganismo se produjo de forma superficial en los gránulos de maíz, mientras que los gránulos de arroz permitieron que el microorganismo quedara atrapado en el interior de los aglomerados, observándose este efecto también en los gránulos de almidón de arroz nativo.

El estudio de los recubrimientos también reveló diferencias significativas en el EY dependiendo del gel utilizado GS (almidón gelatinizado), GG (goma guar) y GX (goma xantana). En general, las microcápsulas recubiertas con GS mostraron un aumento de este parámetro. Mientras que, los recubrimientos GG y GX difirieron en su efecto protector, el cual parece estar relacionado con el modo de interacción del material de recubrimiento y la superficie de almidón (Xing et al., 2015). Por lo tanto, el recubrimiento puede actuar como refuerzo de las microcápsulas o como una competencia del microorganismo para llenar los poros, aumentando o disminuyendo el EY.

Durante el tratamiento térmico, todas las muestras se vieron afectadas. Las microcápsulas de almidón de maíz poroso confirieron más estabilidad térmica a las células de *L. plantarum*, no lograndose este efecto con las microcápsulas de los almidones porosos de arroz. Esto podría deberse una liberación de las células debido al colapso de las microcápsulas que propició su posterior inactivación (Xing et al., 2014). Teniendo en cuenta los recubrimientos, todos ellos brindaron una protección adicional y permitieron mantener un mayor número de células viables de *L. plantarum* después de un tratamiento térmico corto (20 min). Sin embargo, bajo tratamientos más largos (35 min) solo las microcápsulas a base de arroz mostraron ese efecto de estabilizador. Además, el grado de porosidad del almidón obtenido en el caso de los almidones tratados con AMG y AM afectó significativamente la protección proporcionada por el recubrimiento. Por lo tanto, los almidones porosos con poros pequeños, es decir, los almidones tratados con AM y recubiertos con GS proporcionaron una mayor resistencia térmica.

A pesar de las grandes posibilidades que presenta el almidón poroso en su estado granular, en entornos industriales, las propiedades del gel de almidón son cruciales para su explotación en múltiples aplicaciones. Los productos procesados que involucran almidón requieren del calentamiento de los gránulos de almidón en exceso de agua, transformando el gránulo en un gel. Sin embargo, no hay información acerca de como la porosidad del gránulo presente en los almidones porosos afecta al comportamiento de la gelatinización y las propiedades de los geles, así como la microestructura resultante. Según estudios previos (Schirmer et al., 2013; Singh et al., 2003; Witczak et al., 2016), tanto el tamaño de los gránulos como el contenido en amilosa afectan significativamente las propiedades funcionales del almidón (capacidad de retención de agua, viscosidad máxima, viscosidad final y descomposición). Por lo tanto, es posible suponer que los cambios estructurales y funcionales de los almidones porosos después de los tratamientos enzimáticos pueden influenciar significativamente las propiedades de sus geles. Por esa razón, los almidones de maíz tratados con AMG, y previamente caracterizados, se gelatinizaron y analizaron en función de la textura y estabilidad de sus geles, así como de las propiedades de las pastas resultantes. Estas muestras fueron elegidas debido a su diverso grado de porosidad que osciló entre un 0 y un 15%. Los geles obtenidos exhibieron estructuras similares a las de un panal de aveja; sin embargo, el tamaño y el número de orificios contenidos en estas estructuras difirió dependiendo de la porosidad de los almidones porosos iniciales. Entre todas las muestras, el almidón tratado con una porosidad inicial del 8% produjo geles con una microestructura más cerrada. Esta porosidad fue de particular relevancia en todos los parámetros analizados, ya que en general se presentó como un punto de inflexión entre los cambios observados. De hecho, el comportamiento viscoelástico no mostró cambios significativos en los valores de módulo hasta que se alcanzó la porosidad del 8%. Con respecto a la dureza de las matrices de gel, se observó una tendencia al alza hasta el 8% de porosidad, pero un aumento de esta porosidad llevó a geles más blandos. Por lo tanto, las suposiciones anteriores se corroboraron, ya que los resultados revelaron que el tratamiento enzimático permitió obtener geles con microestructuras y texturas diferentes.

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CHAPTER 9

Conclusions

Research conducted through the different chapters is conclusive about the production of tailor-made porous starches with selected amylolytic enzymes, for modulating either the size of the pores on granular starches or network structure of starchy hydrogels, opening new and multiple possibilities of using starch as porous materials.

Particularly, the following concluding remarks can be drawn:

1. Enzymatic modification of corn starch by glucoamylase, α-amylase, cyclodextrin-glycosyltransferase or branching enzyme at sub-gelatinization temperatures results in porous starch granules with specific functional properties. The degree of porosity could be modulated by using different enzymes or changing enzyme concentration, which offers a suitable possible application in starch-based food matrices. Glucoamylase prompted larger pores, whereas cyclodextrin-glycosyltransferase was less active obtaining smaller porous sizes.

2. Porous starches from cereal and tuber sources modified by enzymatic treatment showed to be enzyme and starch source dependent, being cereal starches more susceptible to enzymatic hydrolysis. The deeper pores obtained in the cereal starches improved the main benefits of the starch granules and, therefore, their application as natural absorbent. A good combination between type of starch and enzyme could provide porous starches with diverse structural characteristics and own functional properties, increasing the versatility of the porous starches and thus their subsequent application.

3. The structural features of the pores obtained by enzymatic modification of corn starch with amylolytic enzymes at different concentrations, provoked different *in vitro* digestion behavior. The presence of a specific pore size hindered the digestive amylase action, leading to lower digestibility. Overall, food products with healthy properties can be obtained by including enzymatic modified starches with certain pore size in the starch surface.

4. Modified corn and rice starches with different porosity degree provide a powerful tool for being used as a protective microenvironment for probiotic encapsulation. The porosity of the modified starches contributes to increase the encapsulation yield by different ways, ie, increasing the surface absorption of the corn starch or entrapping the probiotics within the aggregates of rice granules. Coating of the encapsulates provide additional protection but a careful selection of the coating and the enzymatic treatment are needed. Under thermal treatment, microcapsules produced with α-amylase treated-starches along with gelatinized starch as coating material can be incorporated in probiotic food formulation to maintain the integrity of the cells.

5. The initial porosity of the enzymatically treated starches induces the obtaining of different gel matrixes, modifying the porous structure of the hydrogels and consequently the viscoelastic character and texture of them.

9 Conclusiones

La investigación realizada a través de los diferentes capítulos es concluyente sobre la producción de almidones porosos hechos a medida con las enzimas amilolíticas seleccionadas, modulando tanto el tamaño de los poros en los gránulos de almidón como la estructura de red en los hidrogeles de almidón, lo que abre nuevas y múltiples posibilidades de usar el almidón como un material poroso.

Concretamente, se pueden extraer las siguientes conclusiones finales:

1. La modificación enzimática del almidón de maíz con glucoamilasa, α-amilasa, ciclodextrina-glicosiltransferasa o una enzima ramificadora a temperaturas de sub-gelatinización da como resultado gránulos de almidón poroso con propiedades funcionales específicas. La utilización de enzimas diferentes o el cambio de la concentración enzimática permite modular el grado de porosidad, lo que ofrece la posibilidad de su aplicación en matrices de alimentos a base de almidón. La acción de la glucoamilasa sobre el almidón provocó los poros más grandes, mientras que la ciclodextrina-glicosiltransferasa fue menos activa y obtuvo tamaños de poro más pequeños.

2. La porosidad de los almidones de cereales y tubérculos tras el tratamiento enzimático mostró ser dependiente de las enzimas y de la fuente de los almidones, siendo los almidones de cereales más susceptibles a la hidrólisis enzimática. Los poros más profundos obtenidos en los almidones de cereales mejoraron las principales ventajas de los gránulos de almidón y, por tanto, su aplicación como absorbente

natural. Una buena combinación entre el tipo de almidón y la enzima podría proporcionar almidones porosos con diversas características estructurales y propiedades funcionales propias, aumentando la versatilidad de los almidones porosos y, por lo tanto, su posterior aplicación.

3. Las características estructurales de los poros obtenidos por modificación enzimática del almidón de maíz con enzimas amilolíticas utilizando diferentes concentraciones, provocaron comportamientos diferentes durante la digestión *in vitro* de los almidones porosos. La presencia de un determinado tamaño de poro obstaculiza la acción de la amilasa digestiva, llevando a una menor digestibilidad. En general, los productos alimenticios con propiedades saludables pueden obtenerse mediante la inclusión de los almidones modificados enzimáticamente con cierto tamaño de poro en la superficie de almidón.

4. Los almidones modificados de maíz y arroz con diferente grado de porosidad proporcionan una herramienta poderosa para ser utilizados como un microambiente protector para la encapsulación de probióticos. La porosidad de los almidones modificados contribuye a aumentar el rendimiento de encapsulación de diferentes maneras, es decir, aumentando la absorción en la superficie del almidón de maíz o atrapando a los probióticos dentro de los agregados de los gránulos de arroz. El recubrimiento de los encapsulados proporciona una protección adicional, pero es necesaria una selección cuidadosa del recubrimiento y el tratamiento enzimático. Bajo tratamiento térmico, las microcápsulas producidas con almidones tratados con α-amilasa junto con almidón gelatinizado como material de recubrimiento pueden incorporarse en la formulación de alimentos probióticos para mantener la integridad de las células.

5. La porosidad inicial de los almidones tratados enzimáticamente induce la obtención de diferentes matrices de gel, modificando la estructura porosa de los hidrogeles y, por consiguiente, el carácter viscoelástico y la textura de los mismos.

Chapter 10

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