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Starch granule porosity and its changes by means of amylolysis

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A b s t r a c t. The present work is a review on hydrolysis of native starch using α– and glucoamylases and its influence on starch granules porosity. Changes of specific surface area of starch granules were particularly taken into consideration as well as modifications of their surface observed by means of various microscope techniques. The most common methods for studying porosity of starch granules were briefly characterized. Scanning electron micrographs of hydrolysed granules were also presented as a part of authors' own research.

K e y w o r d s: starch, porosity, enzymatic hydrolysis

INTRODUCTION

Porosity and surface area are important characteristics of solid materials that determine their properties *eg* thermal conductivity, thermal diffusivity, mass diffusion coefficient. Mechanical and textural properties of food are also dependent on porosity (Marousis and Saravacos, 1990). Starch is the major energy reserve in higher plants and the main carbohydrate in human diet. It is laid down in the form of granules of different sizes (from 1 to over 100 μm in diameter) and shapes (Tester *et al*., 2004). Native starch granules are insoluble in cold water. Their density is about 1500 kg m^{-3}. Starch consists of two polysaccharides: amylose and amylopectin, and their ratio can vary in a very wide range, according to botanical origin. Starch granule is composed of three different regions: amorphous lamellae, crystalline lamellae, and amorphous growth ring. The crystalline lamellae consist of amylopectin double helix formation, whereas the amylopectin branch points form amorphous lamellae. At the lowest level of structure, the granule appears to be made up of alternating semi-crystalline and amorphous growth rings which are between 120 and 400 nm thick (Jenkins and Donald, 1995).

With the help of various modifications, *eg* enzymatic hydrolysis, the structure and properties of starch can be changed for diverse applications. In food industry starch is a very valuable functional ingredient which is added to sauces, confectionery, comminuted meat and fish products and a variety of low-fat products. It increases their viscosity and stability, and improves fat- and water-holding properties (Hermansson and Svegmark, 1996). Starch granules can also be utilized for non-food applications such as: plastic fillers, facial powder or carbonless copy paper (Röper, 1996; Burrell, 2003). Recently, interest has become focused on starch as an adsorbent for volatile compounds and as a fat substitute (Zeller *et al*., 1999; Boutboul *et al*., 2002; Jeon *et al*., 2003). For these applications microporous starches can be used. They are obtained from native starches hydrolysed by α-amylases at temperatures lower than gelatinization temperature (Röper, 1996; Zeller *et al*., 1999).

In this review we demonstrate how the process of amylolysis influences starch granule surface and its porosity, and show that this method of starch modification effectively alters this parameter in comparison to the other methods used.

STARCH POROSITY

Porosity is one of the characteristic features of starch. The porosity of solid materials is defined as the ratio of pore volume to the volume of solid phase. It is a network of both one- or two-side opened pores, combined with each other or not, and of closed pores. The classification of pores is based on the size of their diameter. According to IUPAC (Sing, 1985), pores can be classified as follows:
– macropores, with diameters larger than 50 nm,
– mesopores, with diameters between 2 and 50 nm,
– micropores, with diameters smaller than 2 nm.

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Starch researchers referred pores of starch granules of different origin to microscopic pores or holes. Such holes have been observed on the granule surface of the following starches: maize, sorghum, millet (over the entire granule surface), wheat, rye, and barley (along the equatorial groove of large granules). Granules of tapioca, rice, oat, canna, and arrowroot appear to have no pores, but there is disagreement about the presence of pores on the surface of potato starch granules (Fannon *et al*., 1992).

Apart from the surface pores, Fannon *et al.* (1993) observed specific channels and cavities in common maize starch granules and they suggested that surface pores are openings to serpentine channels penetrating into the granule interior. Huber and BeMiller (2000) observed granules of such starches as maize, millet, and sorghum by light, fluorescence and scanning electron microscopy. They found that most channels penetrate granule from external surface toward a cavity at the hilum but the depth of this penetration varies (Fig. 1). Channels appeared to be open whereas cavities were rather closed which, however, did not impede enzymatic hydrolysis. According to Huber and BeMiller (1997), central cavities were present in maize, waxy maize, and sorghum starch granules. They proved that the cavities were not formed by drying but could be enlarged by this process.

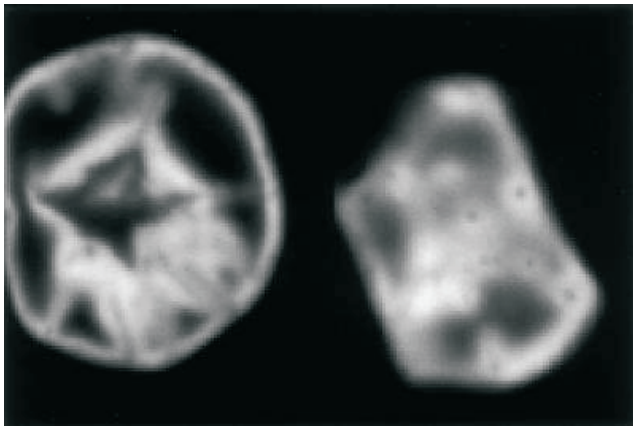

Fig. 1. Channels in the maize starch granules. On the left: granule sectioned along the length of the channels; on the right: granule sectioned across the channels (Huber and BeMiller, 2000, with permission from Elsevier).

SURFACE PORES ORIGIN AND ROLE

The origin of starch surface pores is various. They may appear as a result of natural processes that take place in plant tissue, as well as during mechanical treatment of grains (Niemann and Whistler, 1992). Starch researchers suggested that pores can occur as a result of drying processes of starch granules or that they are simply artefacts produced during the isolation and sample preparation for electron microscopy. Other considered possible origins of the pores were that they are produced by *in situ* amylases or by amylases produced during wet milling, or that they are a natural feature integral to the granule structure. Fannon *et al.* (1992) concluded after thorough observations that the presence of pores is a natural feature of starch granules and that they do not occur during mechanical treatment, although they do not deny that some factors can cause an increase in the number and/or size of pores on starch granules surface.

In general, native starch granules are practically inert toward chemical reactions unless they are pretreated to activate them, for example by enzymatic hydrolysis. It has been proven that the starch granules porosity significantly influences starch chemical reactivity. Presence of pores, channels and cavities increases the surface which is potentially available for chemical and enzymatic reactions. Fannon *et al.* (1992) suggested that pores may be the site of initial enzyme attack, openings that allow enzyme molecules direct access to the granule interior (hilum). Fortuna *et al.* (2000) reported that the larger specific surface area of the granules, the bigger their susceptibility to amylolysis.

Thomson *et al*. (1994) used atomic force microscopy to observe enzymatic attack on wheat starch granules in real time. They reported that the attack starts in the damaged, cracked regions or at surface pores. It proceeds toward the centre of the granule with little change in the lateral dimensions of the hole that has been formed.

METHODS OF EXAMINING STARCH POROSITY

Many methods are used to examine starch materials and their porosity, among others: scanning electron microscopy (SEM) (Fannon *et al*., 1992, 1993; Planchot *et al*., 1995; Helbert *et al*., 1996; Baldwin *et al*., 1997; Fortuna *et al*., 1998b, 2000; Huber and BeMiller, 2000; Sarikaya *et al*., 2000; Li *et al*., 2004), transmission electron microscopy (TEM) (Szymońska *et al*., 2003), atomic force microscopy (AFM) (Thomson *et al*., 1994; Baldwin *et al*., 1997), mercury porosimetry (Karathanos and Saravacos, 1993; Jamroz *et al.*, 1999), helium stereopicnometry (Marousis and Saravacos, 1990; Karathanos and Saravacos, 1993; Juszczak *et al*., 2002b), and methods based on physical adsorption from gaseous phase or liquid phase (Hellman and Melvin, 1950; Fortuna *et al.*, 1996, 1998a, 1998b; Juszczak *et al*., 2002a).

A parameter which is mainly used for determination of starch porosity is specific surface area (surface available for gas or liquid molecules; it includes the external as well as the internal surface of a solid body) which is defined as the actual surface of the adsorbent per unit mass and is usually expressed in $m^2 g^{-1}$. Specific surface area is proportional to specific pore volume, and inversely related to the pore diameter.

Among many methods of specific surface area examination the most common are those based on gas or liquid adsorption measurement. The measurements involve

determining the isotherms of nitrogen, argon, or krypton adsorption at the temperature of liquid nitrogen and calculating the mono-layer capacity basing on BET adsorption isotherm (Brunauer *et al*., 1938). Specific surface area can be calculated using the formula:

$$S_{BET} = V_m S_0 \, (m^2 \, g^{-1}),$$

where: S_0 – surface taken by the adsorbate molecule, V_m – mono-layer volume (Juszczak *et al*., 2002a).

The final stage of adsorption process also provides some information about pore shapes; they can be conical, cylindrical or open at both ends (Ościk, 1979).

Mercury porosimetry is useful in the case of meso- and macropored adsorbents. Its very serious defect is destruction of pores during the measurement which consists in pumping mercury into pores by continuous increase of pressure to 100 MPa or more. It is possible to determine the effective radius up to 15-20 nm and obtain pores volume distribution curve according to their diameter (Ościk, 1979). Specific surface areas of various starches determined by different methods are presented in Table 1. The values found by nitrogen adsorption and low-pressure mercury porosimetry are lower than those measured by water sorption and high-pressure mercury porosimetry because the latter techniques allow the estimation of very small pores (Karathanos and Saravacos, 1993). Scanning electron microscopy is widely used to observe details of granules' surface. This method requires, however, sample coating with gold-palladium alloy or carbon and silver. During sample preparation starch granules damage may occur. An alternative method for SEM is LVSEM (low-voltage scanning electron microscopy) that permits work on uncoated samples. Due to minimal sample preparation starch granules damage is greatly reduced and provided images are believed to represent the true native starch granule surfaces (Baldwin *et al*., 1997). Atomic force microscopy does not require any complicated sample preparation technique either and it allows direct observation of the starch granule surface details (Thomson *et al*., 1994; Krok *et al*., 2000). Krok *et al*. (2000) and Szymońska *et al*. (2003) used a non-contact mode of this technique to avoid any destructive effects on the samples surface.

ENZYMATIC HYDROLYSIS OF NATIVE STARCHES

Enzymatic hydrolysis is one of the most commonly used methods of starch modification. This very important industrial process enables to investigate starch ultrastructure (Gallant *et al*., 1997) and to change some of its properties *eg* reactivity.

Among the amylolytic enzymes, glucoamylases and α-amylases have the most practical importance. Glucoamylases are the exo-acting enzymes that produce D-glucose from the non-reducing ends of starch. α-Amylases are the endo-acting enzymes that randomly attack the internal α-1,4-D-glucosidic linkages of starch except those near the branch points that contain α-1,6-D-glucosidic linkages (Bryjak, 1999; Achremowicz and Wójcik, 2000).

T a b l e 1. Specific surface area of different starches

Type of starch	Specific surface area S_{BET} ($m^2 \, g^{-1}$)	Methods	References
Barley	0.599	Nitrogen adsorption	Juszczak *et al*., 2002a
Maize	0.687	Nitrogen adsorption	Juszczak *et al*., 2002a
	330	Water sorption	Hellman and Melvin, 1950
	0.70	Nitrogen adsorption	Hellman and Melvin, 1950
Amioca	0.39	Low-pressure mercury porosimetry	Karathanos and Saravacos, 1993
(waxy maize)	26.82	High-pressure mercury porosimetry	Karathanos and Saravacos, 1993
	0.675	Nitrogen adsorption	Juszczak *et al*., 2002a
Oat	1.224	Nitrogen adsorption	Juszczak *et al.*, 2002a
Wheat	0.534	Nitrogen adsorption	Juszczak *et al*., 2002a
Triticale	0.383	Nitrogen adsorption	Juszczak *et al*., 2002a
Rice	1.267	Nitrogen adsorption	Juszczak et al., 2002a
Potato	0.243	Nitrogen adsorption	Achremowicz *et al*., 1997
	0.110	Nitrogen adsorption	Hellman and Melvin, 1950
	422	Water sorption	Hellman and Melvin, 1950
Tapioca	0.280	Nitrogen adsorption	Hellman and Melvin, 1950
	348	Water sorption	Hellman and Melvin, 1950

Enzyme molecules influence the starch granule surface in different ways. Five patterns of enzymes attack have been identified: pin-holes, sponge-like erosion, numerous medium-sized holes, distinct loci leading to single holes in individual granules, and surface erosion (Evers, 1979). Generally, enzymes can erode the entire granule surface or sections of it (exo-corrosion) or digest channels from selected points on the surface towards the centre of the granule (endo-corrosion). Potato starch is slightly eroded by exo-corrosion, whereas such starches as wheat, barley, rye, and starches from some tropical tubers have specific susceptible zones which become pitted as a result of endo-corrosion. Pits become enlarged and form numerous channels inside the granule. Starch granules from normal maize and rice are hydrolysed in a similar way but pits are formed randomly and they are deeper (Oates, 1997). The mode of enzymatic degradation depends on the starch and enzyme used (Planchot *et al*., 1995).

According to Kimura and Robyt (1995), starches are characterized by a wide degree of variance in their susceptibility to hydrolysis by glucoamylase from *Rhizopus niveus*. They examined seven starches of different origin. Waxy maize starch was the most susceptible and showed typical structure called 'Swiss cheese', with numerous deep holes. Normal maize, barley and tapioca starches were only slightly eroded, but deep holes also occurred on their surfaces when the amount of enzyme was increased 100 x (from 20 to 200 U ml^{-1}). The most resistant starches: amylomaize, potato and shoti, showed only slight surface pitting at 100 x amount of glucoamylase. Quigley *et al.* (1998) used glucoamylase of *Cladosporium gossypiicola* to digest native potato, rice and maize starches. The enzyme attack resulted in many pin-holes on the surface of the maize and rice granules and digestion of their interior. Potato starch hydrolysis was characterized by the formation of a groove along the centre of the granule.

Matsubara *et al.* (2004) compared the α- and glucoamylases action on the granules of maize starch. α-Amylase digestion resulted in big holes formation, while glucoamylase formed small holes on the surface of starch granules. The researchers concluded that starch decomposition by glucoamylase occurred only on the surface of the granules.

Exo- and endo-corrosion patterns are presented in Fig. 2. Native potato, maize and wheat starch granules were treated by *B. subtilis* α-amylase at 50°C for 4 h. Before treatment the granules were smooth, except for maize starch that had some hollows on the surface. After hydrolysis, characteristic pits and cracks could be observed on the surface of wheat and maize granules, whereas potato starch granule surface was only eroded. Planchot *et al.* (1995) observed similar changes on the surface of maize starch granules of different amylose contents (high-amylose maize, normal maize, waxy maize) that were hydrolysed by α-amylase from *Aspergillus fumigatus*. A few stages of erosion could be observed in all studied starches. Initially, some scratches, cracks and pores were formed on the surface of starch granule. When these pores enlarged they produced internal corrosion channels and subsequently granules became degraded, revealing internal layered structure. It was possible to observe a blocklet structure defined by Gallant *et al.* (1997). The examinations showed that some regions of the granule are more susceptible to amylolysis than others. Hydrolysis occurred mainly in the more amorphous zones whereas crystalline lamellas were resistant to enzymatic action (Helbert *et al.*, 1996; Planchot *et al*., 1995).

Enzymatic reaction consists of a few stages: diffusion towards the surface of solid phase, adsorption, and finally catalytic reaction. Helbert *et al*. (1996) investigated the diffusion of α-amylase into maize starch granules. They divided this process into several phases. After random adsorption of the enzymes at the granule surface, the process of hydrolysis starts, but only at these points. The hydrolysis

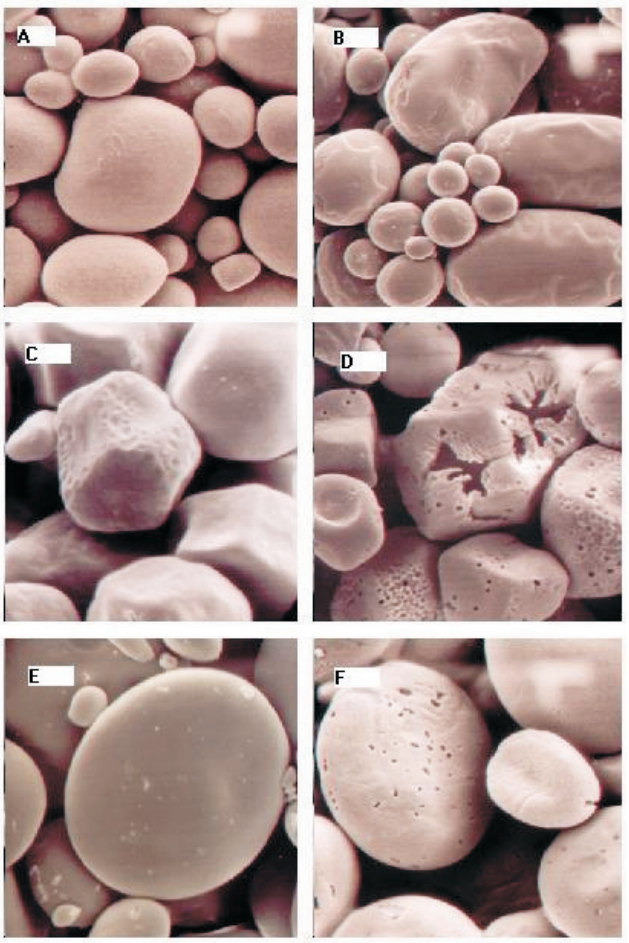

Fig. 2. Scanning electron micrographs of native: A – potato, C – maize, E – wheat starch granules and granules hydrolyzed by *B. subtilis* α-amylase at 50°C for 4 h: B – potato, D – maize and F – wheat. Magnification 5000 x (potato starch 1500 x).

proceeds radially, forming pores which prevent free diffusion of the enzymes into the granule. This leads to the formation of channels which finally reach the centre of the granule. The enzyme molecules are entrapped inside the granule and can only hydrolyse the substrate which is available in their limited diffusion range.

The adsorption of enzyme onto the granule surface does not necessarily cause the occurrence of subsequent catalytic activity. According to Hamilton *et al.* (1998), there is also α-amylase of *Bacillus* sp. IMD 434 that can hydrolyse native maize, potato, wheat, and rice starches, although it does not adsorb onto the granule surfaces, even after foregoing acid treatment. In spite of that fact, some interaction must occur because affinity sites on this amylase are present.

EFFECTS OF DIFFERENT METHODS OF MODIFICATION ON STARCH GRANULE SURFACE

Starch porosity can be modified by physical, chemical and enzymatic treatment. Fortuna *et al.* (1998b) used convection heating (130 and 200°C for 2 h), irradiation with microwaves, chemical modification using sodium trimetaphosphate, and enzymatic hydrolysis with bacterial α-amylase. They modified potato, wheat, maize, and oat starches. After each process specific surface area, volume of mesopores and their medium diameter were determined using low-temperature adsorption of nitrogen. The highest surface area was found after α-amylase treatment for all examined starches. The highest increase of specific surface area occurred in oat starch and the lowest in potato starch (Table 2). Volumes of mesopores and their medium diameter also increased. The authors of the present review used the same method to determine S_{BET} changes of maize and potato starches after α-amylolysis (*B. subtilis* α-amylase, 500 U g^{-1} starch, at 50ºC for 1 h). Their results, presented in Table 3, are similar to those obtained by Fortuna *et al.* (1998b). Sarikaya *et al.* (2000) treated native rice, potato, maize, wheat, and sweet potato starches with α-amylase (from *B. amyloliquefaciens*) and β-amylases (from *B. cereus*). The changes they observed were similar to the above-mentioned. The surfaces of untreated granules were smooth, except maize starch granules whose surface had small pores. α -Amylase attacked native starches more efficiently than β-amylases. In maize, wheat, and rice granules degraded by α-amylase, big and deep holes were formed on the surface and granule interiors could be seen. Maize granules presented the so-called 'Swiss cheese' structure. Only small pits occurred on the surface of starch granules treated by β-amylase. Potato starch granules were considered to be resistant to amylase hydrolysis. However, investigations showed that it is hydrolysed by *Bacillus firmus/lentus* (Wijbenga *et al*., 1991), *A. fumigatus* (Planchot *et al*., 1995) and *Bacillus subtilis* (Heitmann *et al*., 1997) α-amylases.

Changes of starch porosity occur also during air-drying. Marousis and Saravacos (1990) obtained a linear increase in porosity of maize starch (native and gelatinized) during air-drying at 60°C as a function of the moisture content. Microscopic observations of granular starches revealed the presence of radial channels and cracks on the surface of a sample. Drying at low temperatures gives similar effects. Krok *et al.* (2000) investigated the influence of the freezing

T a b l e 2. Specific surface area of initial and modified starch granules (Fortuna *et al*., 1998b)

Starch source	Specific surface area S_{BET} (m^2 g^{-1})
Potato	
Initial	0.24
Convectional heating at 130°C	0.34
Convectional heating at 200°C	0.35
Microwave heating	0.51
Chemical modification	0.40
Enzymatic modification*	0.62
Wheat	
Initial	0.53
Chemical modification	0.70
Enzymatic modification*	3.74
Maize	
Initial	0.69
Convectional heating at 130°C	0.71
Convectional heating at 200°C	0.77
Microwave heating	0.68
Chemical modification	0.65
Enzymatic modification*	3.12
Oat	
Initial	1.22
Chemical modification	1.26
Enzymatic modification*	8.41

*α-amylase, Maxamyl, 20 μl g^{-1} d.s. starch, 40°C.

T a b l e 3. Specific surface area of potato and maize starches before and after α-amylolysis at 50°C for 1 h

Type of starch	Specific surface area S_{BET} (m^2 g^{-1})
Potato	
Initial	0.164
Enzymatic modification	0.400
Maize	
Initial	0.580
Enzymatic modification	1.093

process on the surface structure of potato starch granules. Freezing starches of different water content caused granule surface crushing and destroyed the granule inner structure acting in a way similar to high pressure. The multiple freezing and thawing process was used by Szymońska *et al.* (2003) to modify the surface of potato starch granules. The granule surface before freezing was smooth, but some scratches and roughness could be observed after the modification. Another way of modification is the use of ultrasounds which cause corrosion of starch granules, similar to that observed after amylolysis. Surface changes become more distinct as the intensity of ultrasounds increases, and long-lasting sonification may cause starch granule disintegration (Nowotny, 1969). Similar effects are observed after the use of high pressure (Tomasik, 2002). It is also possible to apply α-rays for modifying starch granule surface (Tomasik, 2000). Initially their action causes cracks on the surface, but then the granules are fragmented.

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