Nanostructure of starch high-pressure treated granules discovered by low temperature scanning electron microscopy

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Starch is an example of a naturally highly ordered structure formed by two polysaccharide polymers and used by plants for energy storage. Starch granules are so tightly packed that they are virtually inert and show little interactions. However, when granules are distorted, hydrogen bonds tying together amylose and amylopectin molecules are replaced with bonds with water and other molecules in the medium, and the packed structure gives rise to a much more open tangle of chains that are now prone to interactions. In this gelatinised form, starch is susceptible to the attack of digestive enzymes. A common way to destabilise starch structure is by thermal treatment: an irreversible process in presence of water. High pressure, a relatively novel approach to Food Processing, can also displace this reaction towards the gelatinised state. Some differences can be observed with the thermally generated product, among them the ease to obtain partially gelatinised starch when high pressure is employed. This procedure yields starch granules where only a fraction of the susceptible internal hydrogen bonds have been replaced by water bonds (as judged by differential scanning calorimetry). But the structural changes at micro and nano-scales can be dramatic, as revealed by scanning electron microscopy. Especially when water traces are present, a very rich network of polymer chains can be observed, remaining after the granule structure collapses. This is considered a suitable structural basis for different new products were interactions could be modelled, such as carriers for nutritional interesting substances.

Keywords starch; hydrostatic pressure; cryo-SEM; nanostructure; microstructure; gelatinization; tapioca

1. Introduction

Starch is not only one of the more abundant biopolymeric assemblies in nature but a major (may be the main) food component at world-wide scale and one of the main food ingredients, both in natural or manipulated states. Plants use starch granules as energy storage in a widely extended strategy to reduce the sugar concentration and control its availability. On one hand, soluble sugars can only be viably stored in vegetable cells up to a determined concentration, due to the increase in osmotic pressure that all soluble substances cause. A way to bypass this limitation is to remove sugar molecules from the solution equilibrium by binding them into this insoluble polymer. As the length of the glucose polymerised chains increase, their solubility decreases. For large chains, intramolecular interactions are favoured over those with water and molecules become non-soluble. A tighter and more ordered packaging adds the advantage of a closer control of the availability of energy. A complex enzymatic process packs starch granules alternating linear α1-4 bound chains (amylose) and branched (also with α1-6 bonds) polymers (amylopectin) in a way that can only be resolved by the right enzymatic systems, only present in plant cells. On this way, starch can be quickly mobilised and converted to sugar as required: for example, in the rapid processes associated to ripening, seed germination... Also the availability of the precious energy storage to predators is, so, limited, as they lack efficient enzymatic systems that could disentangle the starch granular packaging. This defence line failed when man tamed fire and cooking allowed the easy digestion of starch and so the plants with higher starch content were those favoured by agriculture, due to their rich apportions to diet. Man also profited from the inert quality of starch granules, as starch rich
food could be stored in dry state for long time without micro-organism founding an easy way to accede to it.

Thermal treatment is a procedure known from very old and extensively studied, in which a number of irreversible processes take place resulting in the starch granules losing their ordered structure and even their identity and starch becoming a gel-like substance in which a network of polymer chains inter-cross, moving with difficulty among the surrounding water. Starch gelatinization has been the subject of many good works and reviews [e.g. 1]. It can be studied in a number of ways, by monitoring the many physical characteristics than change, often dramatically, in this process. The ordered granule structure shows, as a curious consequence, a Maltese cross feature when observed by polarising optical microscopy, as a result of the birefringence of native starch granules (Fig. 1 shows an example of Maltese cross in potato starch granules). Gelatinization includes as a one of the initial effects the loss of this birefringence, so the process can be followed by optical microscopic methods, by simply counting the percentage of granules exhibiting a Maltese cross.

Fig. 1 Maltese cross on native potato starch granules as observed by optical microscopy under crossed polarisers. Scale bar corresponds to 20 µm.

The starch granules have shape and size (roughly) well defined that are characteristic of the biological species of origin. After the first intra-hydrogen bonds substitutions by water, the granules swell. Swelling can be followed by optical microscopy, directly or after specific dying with iodine, and also by light scattering or turbidity, which increases as the process progresses. Further in it, the molecules composing the granule migrate away form it. First the amylase linear, and so more movable molecule, exits wholly or partially the granule, and then amylopectin. This again can be observed by optical microscopy (both molecules being slightly different in colour after iodine dying). The exit of the polymer chains if the concentration of the paste is not too small results in interaction among the chains coming form different granules. This causes a strong increase in viscosity, and so, rheological methods can be employed to visualise the process. This viscosity increase as a reflection of the interactions inter- and intra-molecular is one of the main properties of starch when considered as a food ingredient.

Other associated phenomena can also be employed for the study of gelatinization. Such are volume, as it increases in the swelling process or x-ray diffraction properties, which are altered as a result of the changes undergone by the granular structure. As will be seen, scanning electron microscopy (SEM) can also be of utility but it is not possible to employ this powerful technique online, as the temperature driven gelatinization process takes place. The main tool to investigate it is differential scanning calorimetry (DSC): the heat required for the temperature scan to be constant is recorded, so that the “energetic history” of the process is truly considered. It is observed as a wide (because of the different processes associated and also as a reflection of the heterogeneous nature of natural starch granules) transition curve, with a maximum in the endothermic heat exchange at roughly the 50% of the area (energy). This is considered as corresponding loosely to the number of interactions that have been replaced by water. This gelatinization temperature is characteristic of the starch biological origin and modifications in its
structure, by chemical enzymatic or physical ways, are shown both in its value and in the associated enthalpy (area under the curve).

Gelatinised starch can be described as a paste where amylose and amylopectin molecules move slowly among a water matrix. Its properties are depending on the thermal history and also on the rheological history (degree of destruction of the original granular structure, chain breakage, chain orientation or intermingling...). A process a called retrogradation gives rise to a relatively ordered structure when temperature is again reduced, by partial crystallisation of the polymer chains. It is generally considered as deleterious for the quality of food and avoided when possible.

The utilities of starch as a food ingredient are related to this ability of forming a polymer network interacting with water. This gel structure is used to sustain in a paste-like state other food components and so as a stabilising or thickening agent, in many cases. These binding and gelling properties can be modified by acting on native starch granules or directly on the gelatinised resulting chains. Also binding to a wide range of molecules is of interest for the food technologist. Modern food formulation includes the introduction of non-natural components or unstable ones. This can be done by mixing with other components that keep them in suspension or bind to each other. Interesting substances from the food technologist point of view are colorant, flavours and aromas, on one side, but also nutritional additives: minerals, vitamins, antioxidants... Also pharmaceutically active compounds can be introduced in food in this way.

High pressure processing, though considered at laboratory scale from over a century now, is still a novel technology, as it is only now and slowly when it is becoming frequent in the food processing panorama. Most of its applications (virtually all of the industrial a working processes) are designed to reduce the microbial load of foodstuffs without resorting to more aggressive methods, such as heating (cooking, thermal sterilising...) or the addition of chemical preservatives. Though, other applications are also of interest. These include the modification of enzymatic properties of food (by increasing or reducing enzyme activity under pressure or by inactivating them irreversibly). High pressure-associated freezing and thawing are also of interest as, apart from being energetically favourable processes versus the classical approaches, they have been shown to result in reduced damage to food structure, limiting ice crystal growth destruction of the tisular microstructure. Action at different size and molecular complexity scales is achieved, for example, when parasites present in food are destroyed while preserving the delicate raw meat or fish muscle characteristics.

Macromolecular structural alterations base other processes. Both proteins and starch can have their structure altered by high pressure, roughly in a similar way, by displacing (Le Chatelier) the equilibria towards the smaller volume species. Proteins become pressure-denatured, a state not completely similar to the thermally or chemically denatured ones, and often reversible when pressure is released [2, 3]. Often in concentrated state such as it is common in food, proteins and other molecules aggregation is the resulting event. Starch suffers also a (this time irreversible) gelatinization process when water is in excess and the resulting state is very close to the thermally gelatinised systems [4]. Although still under study, the lower thermal agitation can be a cause for differences in the molecular separation and the resulting rheological behaviour of this product.

One of the differences of pressure-driven processes versus the thermal ones lies on the instantaneous transmission of pressure. While temperature slowly rises and falls transmitting itself from the reactor walls towards the sample centre, creating a gradient, pressure is virtually instantaneously propagated and homogeneously, so that gradients are absent. This would allow the elaboration of more homogeneously treated products and partially gelatinised starch (at a given and fixed time and pressure level) would be easier to obtain than its thermal equivalent.

Starch partially gelatinised is considered as an interesting substance, as it may preserve some of the intact granule properties. By maintaining some granular structural elements, the digestibility would be reduced or slowed, which could contribute to the now considered valuable property of starch resistance (lack of digestibility and so of energetic contribution) and also can drive bound molecules further into the digestive track. Also, as it will be revealed by SEM, this procedure gives rise to nanoscale structures of a very promising character when the number and type of molecular interactions that can be formed is considered.
2. Material and Methods

2.1 Starch samples
Starch of different biological origin was used for comparison purposes. Tapioca and potato starch were obtained in dry state (containing 10-15% of humidity, though). Starch is also known to contain, unless a defatting treatment is applied (not the case here) a small amount of tightly bound phospholipids. It was used without further purification and suspended in deionized water at a concentration of 8% w/w. Though no stirring is available during pressure treatments, and starch granules quickly sediment, the overlying water is considered to be in equilibrium when the gelatinization process requires it, as water diffusion is a much quicker process than gelatinization.

2.2 High pressure treatment
A pilot-scale high pressure equipment was employed (ACB GEC Alsthon, Nantes, France). Temperature was controlled through a fluid mixture travelling through a jacket around the pressure vessel and monitored by means of internal thermocouples. In spite of this, the thermal rises and falls associated to pressure changes make that temperatures quoted are only temporal averages. Pressure was monitored also at a point in the pressure circuit close to the pressure vessel. This experimental set-up is described with more detail elsewhere [5].

Samples in plastic containers were pressure-treated immersed in pressure-transmitting medium, in this case water. An experimental temperature of 25ºC was used, and a maximum pressure level of 430 MPa was selected, known to cause partial denaturation of most starch types.

After treatment, starch was decanted and freeze dried before further studies were carried out.

2.3 Differential scanning calorimetry studies
Calorimetric determinations were carried out with a TA-1000 differential scanning calorimeter, at a scanning rate of 10 ºC/min. Starch samples where thoroughly dispersed in water (10% starch in water, w/w) to avoid non-sufficiently hydrated regions, at least 24 hours before DSC experimentation. Then, about 15 µl of slurry were introduced in weighted aluminium pans which were sealed and re-weighted. After DSC, pans were punctured and dried at 110 ºC to constant weight. This procedure yielded accurate water and starch weights while ensuring a good mixing of both substances. The average starch mass in each experiment was 1.5 µg. Thermograms were analysed by standard procedures.

2.4 Scanning electron microscopy
Scanning electron microscopy (SEM) observations were performed with a Zeiss DSN-960 scanning microscope equipped with a Cryotrans CT-1500 cold plate (Oxford, UK). Cryo-SEM permits sample observation without the need of prior chemical fixing or drying techniques. The procedure consists in physical fixing, breaking and etching the sample. Sample holders were fitted into a special bracket on the microscope and placed in the pre-chamber of the Cryotrans cold plate (-180 ºC), where samples were broken to obtain a suitable observable surface. Then, samples were inserted in the microscope and etching was performed for three minutes at -90ºC. After etching, samples were coated with gold and observed at -150/-160ºC under secondary and backscattering electron modes.

Native or pressure treated starch samples were allowed to dry under reduced pressure (a vacuum tap pump) at room temperature for two hours before observation. This procedure was not adopted for a number of samples, which consequently contained increased amounts of water.
3. Results and Discussion

Starch samples treated at different pressures (200-430 MPa), temperatures (20-40 °C) and times (15-60 min) were studied by DCS and observed by cryo-SEM, in order to evaluate the structural changes taking place in their granules. Pressures and temperatures were in all cases lower than those reported by other authors as causing complete starch gelatinization, considering the biological origin of each starch sample [2, 4, 6]. In good agreement with this, all DSC curves obtained showed a DSC thermal gelatinization curve, as a proof that not all the gelatinization process had already taken place. Figure 2 show a typical DSC curve for a 430 MPa treated potato starch sample, as compared with the thermogram of the native sample. It can be observed a small but consistent displacement of the process towards lower temperatures and a reduction in the area under the transition curve, proportional to the enthalpy of the process. All pressure-treated samples showed some degree of temperature displacement and enthalpy reduction - between 10 and 30% - (data not shown). The enthalpy reduction can be related to the fraction of intramolecular hydrogen bonds that have been already replaced by water bonds. Then, even those samples studied at the harsher conditions (430 MPa, 25 °C, 60 min) maintained still about a 70% of their original structural intramolecular bonds.

![DSC thermograms obtained with native (a) and pressure-treated (430 MPa, 25 °C, 15 min) (b) potato starch. Scanning speed was 10 °C/min and samples contained 1.5 µg of starch in 4.5 µl of water. The pressure–treated sample shows a transition whose area is approx. 30% smaller and displaced towards lower temperatures.](image)

Low temperature scanning electron microscopy allowed the observation of starch samples with little additional manipulation. Fig. 3 shows cryo-SEM micrographs obtained after sample thoroughly vacuum drying. Increased pressure levels and treatment duration do not alter significantly the granular integrity for tapioca starch (similar results were obtained for potato starch, not shown here). Samples treated at the higher pressure levels present an appreciable dissolution of granule borders, with apparition of a continuous substrate, binding granules.
If the same cryo-SEM procedure is applied to samples where a small amount of water is allowed, the observed structures change completely. In this state, the starch molecular chains separated from the granule in the incipient pressure-gelatinization process get resolved and confer the granules a much more detailed structure in which they can be seen as dissolving structures surrounded by a network of carbohydrate branches. Pressure level and processing time have a role in the advance of this granular dissolution, as can be seen in Fig. 4. This richly branched star-like structure which can be traced to nanometric scale, is considered as a very promising substrate for all kind of molecular interactions. The numerous water hydrogen bonds could be replaced by appropriate polar molecules while the large carbohydrate chains could offer non-polar substances a harbouring place. Additionally, a number of internal hydrogen bonds are still ready to be interchanged with water, an interaction that can take place with more ease than departing form native structures (as indicated by the DSC thermograms displacement towards lower temperatures). These bonds could also be employed for constructing additional interactions.

The persistence of the granular structure (though weakened) could allow the formation of chelate-type bonds. So, large molecules or atoms could be attached by different freely moving starch branches. Also the products formulated from partially gelatinised starch could be treated partially also as native starch: i.e., they would not get readily dissolved in water, forming a solid, precipitating phase; they would combine and react in a similar way to native starch and they would keep their integrity (together with the molecules bound to them) until their progressive digestion by the appropriate enzymatic system. This incipient gelatinization degree would be enough, though, for allowing digestive enzymes to hydrolyse its structure, releasing, so whichever substances had been bound to them.

Consequently, it is considered that the partially gelatinised starch granules could be employed for the transport and delivery of substances of interest for the Food and Pharmaceutical Industry, such as nutrients, oligoelements, vitamins, antioxidants, colorants, flavours and aromas, as well as medical active principles. Although thermal processes can also yield similar partially degraded starch granules, the pressure gelatinization can be an appropriate production procedure as the pressure process characteristics are more suitable for yielding starch granules homogeneously partially gelatinised. Pressure being a...
property transmitted in a quasi-instantaneous way, it can be homogeneously risen in a few minutes and maintained exactly constant at given levels for the desired time periods, to be released in a few instants, all irrespective of the sample size and dimensions. This is not easy in thermal processing, due to the very slow heat transmission, which gives rise to a slowly equilibrating thermal gradient, which would result in a very wide distribution of gelatinization degrees.

![Fig. 4](image)

**Fig. 4** Pressure-treated (430 MPa, 25 °C) tapioca starch observed by cryo-SEM while containing small amounts of water. a and c were treated for 15 minutes while b was treated 60 minutes. It can be seen that, while granular identity is maintained, their structure is expanded and partially dismantled (this effect is more evident in the 60 min treatment sample (b)). As can be especially observed in c, the granule interior is the region whose structure is firstly altered, so that it could be employed as a container for bound molecules. Scale bars correspond to 10 µm.

4. Conclusions

Homogeneously partially gelatinised starch can be obtained in an easy and controlled way by the use of high hydrostatic pressure processing. This physically modified starch product maintains its granular integrity while a large number of carbohydrate branches appear ready to interact with other molecules. This is suggestive of many possibilities of use as food additive and drug carriers for its controlled release.

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