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MICROSTRUCTURAL CHANGES IN WHEAT STARCH DISPERSIONS DURING HEATING AND COOLING

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Abstract

Microstructural changes in 8-11% wheat starch dispersions during heating, cooling and cold storage have been evaluated by light microscopy and scanning electron microscopy.

Heat treatment of wheat starch dispersions gives rise to two stages of swelling and solubilization. During the first phase of swelling, solubilized amylose was observed in the centre of the granules and, to some extent, outside the granules. Further swelling deformed the granules and more amylose was released. When the temperature treatment took place under shear, the outer layer of the swollen granules fractured at 94°C and above, and amylopectin fragments were dispersed into the continuous amylose phase. Fragmentation of amylopectin was not observed when samples were heated with a minimum of mechanical action. There were also differences in the final gel structure between samples due to the mechanical treatment during preparation.

Aggregation of amylose took place on cooling and could be observed as irregularities in the gel structure. When the amount of released amylose was limited during the initial phase of swelling and below the critical concentration for gel formation, cooling resulted in deposition of amylose at the surface of the granules. This was expected to have an impact on the behaviour of the granules on further processing.

Introduction

Wheat starch is commonly used because of its thickening and gel forming properties in viscous foods and gels. Numerous studies have been made to characterize the rheological behaviour of heat-treated wheat starch dispersions in the concentration range 5-14% (Doublier, 1981; Wong and Lelievre, 1981; Bagley and Christianson, 1983; Eliasson, 1986 and Doublier *et al.*, 1987). Similar studies of the microstructure of wheat starch dispersions in this concentration range are surprisingly scarce. Heated starch systems can be described as colloidal systems in which the particles are dispersed in a macromolecular solution, and wheat starch dispersions in this concentration range have the ability to form gels on cooling.

Most studies of the microstructure have dealt with low concentrations (0.25-1.5% w/w) and have mainly concerned changes in the shape of lenticular wheat starch granules during heating (Ghiasi *et al.*, 1982; Varriano-Marston *et al.*, 1985; Bowler *et al.*, 1980, 1987 and Eliasson, 1985). In these studies, wheat starch granules at low concentrations were washed in distilled water before being examined under the microscope. The washing procedure removed exudated amylose as well as other components which may contribute both to the rheological properties and the microstructure of starch dispersions. The heating was usually done in tubes with a minimum amount of mechanical treatment. In all studies, the swelling and deformation of lenticular wheat starch granules followed the same pattern.

Bowler *et al.* (1980) used both light microscopy (LM) and scanning electron microscopy (SEM) in order to follow the heat-induced changes in the granules. On the basis of their own as well as previously published results; they proposed the following model for the swelling of lenticular wheat starch granules. The granule started to swell radially to about three times its original diameter at approximately 50°C. In the next stage the granules started to swell tangentially in the xy-plane. Little or no swelling took place in the z-direction, which meant that the disc started to pucker out of the xy-plane and the extent of puckering increased with temperature. The first type of change occurred relatively slowly and was well defined, but the second type took place rapidly and the resulting granules appeared flexible. They also noted that the equatorial groove prevails during the whole heating process up to 97°C. Later Bowler *et al.* (1987)

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investigated the effect of different preparation techniques for SEM, such as dehydration and critical point drying, freeze drying and cryo stage techniques. Even if differences were observed, the preparation techniques did not affect the above described swelling patterns of the granules. The effects of surfactants on the swelling of wheat starch granules have been investigated by Ghiasi et al. (1982) and by Eliasson (1985). They found that surfactants restricted the first but not the second stage of the swelling of wheat starch granules.

The amount of amylose released from wheat starch granules during heating has been investigated by means of iodine-binding at low concentrations in the range 0.25-2.5% (Doublier, 1981; Ghiasi et al., 1982; Eliasson, 1986). Here again two stages were observed. In the lower temperature range of about 55-80°C the release of amylose was slow, but it increased considerably at higher temperatures.

The aim of this study was to evaluate structural changes in wheat starch dispersions mainly by light microscopy and, to some extent, by SEM

- o during heating to 75-120°C, taking the effect of mechanical treatment during preparation into account
- o during cooling and cold storage

It is well known that, when evaluating complex food structures it is wise to use more than one technique for preparing the sample for electron microscopy (Hermansson and Buchheim, 1981; Bowler et al., 1987). The same holds true for light microscopy, and this study demonstrates the advantages of using three techniques in parallel.

### Experimental

#### Materials

In this study commercial wheat starch from CPC-Cerestar was used. Concentrations in the range of 2 to 11% w/v starch were investigated, but in the main study 8% w/v was used if not otherwise stated.

In a preliminary study different batches from three producers were tested to get some idea of the effects of the wheat quality, and of environmental and processing conditions. Samples were purchased from Raisio Tehtaat OY, Kröner and CPC. All wheat starch samples showed the same type of general behaviour even if there were some discrepancies in the temperatures at which different phenomena occurred.

#### Sample preparation

Samples were prepared by mixing 36 g wheat starch with 414 ml distilled water.

The starch dispersion was heated at the rate of 1.5°C/min to a required temperature, held at that temperature for 30 minutes and cooled to 25°C at a rate of 1.5°C/min in a Brabender Amylograph. The temperature dependency was studied by performing experiments at the following max. temperatures 60, 70, 75, 80, 85, 90, 94, 95, 97 and 98°C. This temperature treatment was combined with mechanical treatment consisting of continuous stirring at 75 rpm. Mechanical treatment was kept to a minimum for two samples by preheating them in the Brabender, as described above. One sample was preheated to 85°C and then heated for 30 min at 95°C in a water bath without any stirring. The other sample was preheated to 85°C, whereafter it was canned and heated at 120°C for 30 min in a retort autoclave. After cooling, samples were stored at +4°C.

#### Light microscopy

Samples were taken immediately on reaching the required temperature, but also after being incubated for 30 minutes and after cooling to room temperature, as well as after storage in a refrigerator. Three techniques were used to prepare the samples for light microscopy: smearing, cryosectioning and embedding followed by thin sectioning. All three techniques were used on all samples, and the few micrographs presented here have been chosen out of several hundred.

All samples were stained with iodine in a diluted 1:1 Lugol's solution for one minute, whereafter the sample was covered and sealed. Preliminary studies were also made with other stains, such as safranin, Congo red, gentian violet and aniline blue with Orange G, in combination with different illumination techniques. The prepared samples were examined with a Nikon macrophot Fx microscope.

**Smears.** The starch dispersion was quickly and gently smeared out onto an object glass and stained directly. The advantage of this technique is that the hydrated sample can be observed without freezing or dehydration. It is also possible to observe intact swelled granules and obtain a three-dimensional impression by focusing through the depth of the sample. The disadvantage is that a sample with swelled granules gets rather thick, which decreases the resolution and results in a loss of detail.

**Cryosections.** Samples were frozen in liquid nitrogen and sectioned frozen. A Leitz cryostat was used and 7-8 µm thick sections were cut. The advantage of cryosections is that very quick changes in the microstructure are detectable as the structure is "frozen". A better resolution is obtained with this technique than with the smearing technique. The disadvantage is that the freezing can induce ice crystals that can damage the microstructure. This damage is more severe in the gel than in the fluid, viscous starch systems, where the dissolved macromolecules act as cryoprotectants.

**Embedded sections.** Samples were chemically fixed in 1% glutaraldehyde with 0.1% ruthenium red, dehydrated and embedded in historesin, LKB, as recommended by the manufacturer. A Reichert-Jung Ultracut E, with glass knives, was used to section the samples. Thin sections, 1 µm, were cut to obtain the best possible resolution, and thicker sections, 3-4 µm, were cut to achieve better contrast staining. This technique gives the best resolution for light microscopy, and no freeze damage or mechanical redistribution is induced in the sample. The samples were stained after sectioning, which means that microstructural changes due to the presence of iodine in the staining solution can be ruled out.

The disadvantage is that it may be difficult to interpret such thin sections with regard to dispersions with small and large irregular particles. The chemical fixation and dehydration used in this technique may also affect the macromolecular phase, but such effects cannot be seen from the resolution given by light microscopy.

#### Scanning electron microscopy

Samples of heat-treated starch dispersion were taken after they had reached room temperature as well as after storage. Two techniques were used: critical point drying and a cryo-stage technique.

The samples were examined in a Cambridge Stereoscan 200 equipped with a Hexland cold stage using accelerating voltage between 2 kV-10 kV.

Figures 1 and 2 are color micrographs at page 33.



Fig. 3. Scanning electron micrograph of a critical point dried 8% starch dispersion heated to 90°C and cooled to room temperature.

#### Critical point drying.

Small samples were chemically fixed in 1% glutaraldehyde with 0.1% ruthenium red, dehydrated, through graded alcohol, and critical point dried. The dried samples were fractured and mounted on SEM with "Leit-C", carbon glow. The samples were sputter coated, Au/Pd in a Polaron E5000 before examination in the SEM.

**Cryo-stage.** Very small samples about 1 mm<sup>3</sup> were placed in copper sandwich holders and extra rapidly fast frozen in a propane jet freezer, Balzer. Frozen samples were transferred to the cold stage in the microscope, fractured and etched at -90 - -100°C. Samples were examined at -150 - -176°C, both uncoated and gold coated at low accelerating voltage 2-3 kV.

#### Results and Discussion

##### Heat-induced changes in starch dispersions

Microstructural changes in wheat starch have been studied as a function of temperature combined with mechanical treatment. Samples were taken at various stages of heat treatment from 60 to 120°C for microstructural evaluation. Results from some heating temperatures were chosen to illustrate the most significant heat-induced changes. Wheat starch granules heated to 75°C are representative of the first stage of swelling, where the release of amylose is limited and the concentration of amylose is not high enough for gel formation. Heating temperatures of 90°C and above were chosen to illustrate phenomena occurring during the second stage of swelling.

The effect of heating an 8% wheat starch dispersion to 75°C is illustrated by the iodine-stained smear shown in Figure 1. There is a variation in the degree of swelling of the starch granules, and it is important



Fig. 4. Scanning electron micrograph of a 4% starch dispersion heated to 90°C and cooled to room temperature, frozen rapidly and examined frozen on a cryo stage.

to realize that starch consists of a population of granules which vary with regard to factors such as shape, deformation, release of amylose during heating, etc. From Figure 1 it can be seen that the majority of the granules heated to 75°C have retained their original shape and that they are stained dark blue which means that they are amylose-rich. These granules are in the first stage of swelling according to the swelling pattern described by Bowler et al. (1980). There is an exudated blue phase surrounding these granules, which means that the restricted leakage of amylose out of granules in the first stage of swelling can be detected by light microscopy. A number of granules in the second stage of swelling can also be seen from Figure 1. These are more brown and less blue in colour and have a more irregular shape. Iodine stains pure amylose blue and amylopectin beige-brown with varying intensity.

Figure 2 shows the structure of the 8% starch dispersion at 90°C. More amylose leaks out at this temperature, and the continuous phase surrounding the swelled granules is stained blue. There are also some blue-stained granules in the first stage of swelling at this temperature, but the majority of the granules are in the second stage of swelling. They are mainly beige in colour, and especially the outer layers are very weakly stained, indicating that amylose has leaked out. From Figures 1 and 2 it is also evident that the starch dispersion contains small granules of B-starch as well as lenticular shaped A-starch granules.

From the smears it is not possible to characterize the exact shapes of the lenticular A-starch granules at different stages of swelling. Corroborative SEM studies of heated dispersion show that the changes found in the shape of the granules in 4-8% dispersions were similar to those found in studies of

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Fig. 7. Schematic illustration of fragmentation of the outer layer of swollen granules.

dilute suspensions (Bowler et al., 1980; Ghiassi et al., 1982; Eliasson, 1985; Varriano-Marston et al., 1985). Figure 3 shows a SEM micrograph of a granule in an 8% dispersion after cooling from 90°C. The granule has a shape typical of the second stage of swelling and is surrounded by a network structure of amylose. This sample was prepared by chemical fixation, dehydration and critical point drying. Figure 4 shows a fractured granule in a 4% dispersion after cooling from 90°C, preparation by rapid freezing and examination on a cryostage. The use of low-temperature techniques for isolated granules has been discussed by Bowler et al. (1987). The advantage of this technique is that the sample can be investigated fully hydrated without additions of chemicals, provided that the freezing rate is high enough to prevent ice crystal formation, which can damage the structure. Some difficulties attached to working with highly aqueous samples of dispersions and gels are worth pointing out. Figure 4 shows a sample which should have been deeply etched under the etching conditions of 15 min at -90°C. However, the water is firmly held by the swollen granule and by the biopolymer-rich continuous phase. Therefore, it is very difficult to sublimate water from the surface, which makes the contrast low and makes it difficult to reveal fine details of the structure.

#### Fragmentation of amylopectin

When the starch dispersion was heated in the Brabender to 95°C, the outer layer of the swollen granules fractured and fragments were released into the blue stained amylose solution. These fragments are weakly stained and believed to be composed mainly of hydrated amylopectin and not any amylose with an affinity for iodine. Figures 5 and 6 show 8% wheat starch dispersion heated to 95°C. Amylopectin fragments of varying size, are dispersed in the exudated blue amylose phase as seen in Figure 5. The micrograph at a higher magnification shown in Figure 6 illustrates a granule where the outer layer has been partly sheared off. In three dimensions the outer layer that fractures would correspond to the outer part of the pucker granule as described by Bowler et al. (1980). The mechanism of fragmentation is schematically illustrated in Figure 7, showing the swelling of the granule, the formation of an outer layer and the fracture of the outer layer into fragments.

Fragmentation started at 94-95°C for samples prepared in the Brabender Amylograph at 75 rpm, and the amount of dispersed fragments increased with elevated temperatures and holding times at 94°C or above. This phenomenon has not previously been demonstrated by microscopy. The smear technique was chosen to introduce heat-induced changes and the presence of small fragments in the continuous amylose

Fig. 1. Smear of an 8% wheat starch dispersion heated to 75°C.

Fig. 2. Smear of an 8% wheat starch dispersion heated to 90°C.

Fig. 5. Smear of an 8% wheat starch dispersion heated to 95°C.

Fig. 6. Smear of an 8% wheat starch dispersion heated to 95°C.

Fig. 8. Cryosection of an 11% wheat starch dispersion heated at 94°C for 30 min and subjected to mechanical treatment.

Fig. 9. Cryosection of an 11% wheat starch dispersion heated at 94°C for 30 min and not subjected to mechanical treatment.

Fig. 10. Embedded section of an 8% gel, formed after heating at 95°C, cooling and storage at room temperature for 5 h.

Fig. 11. Embedded section of an 8% gel formed after heat treatment at 120°C for 30 min.

phase. With any of the sectioning techniques, a small particle as seen under the microscope may have been part of a bigger particle that has been sectioned close to its outer surface.

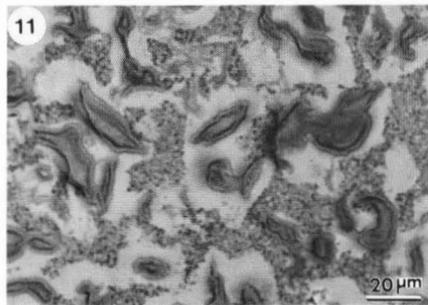
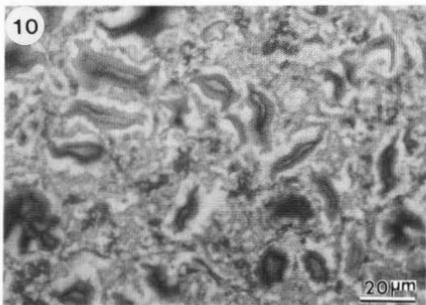
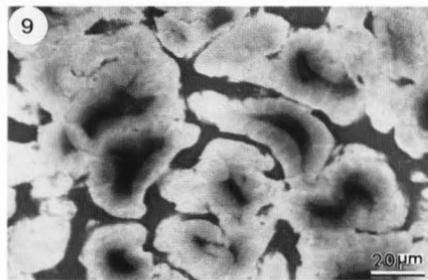
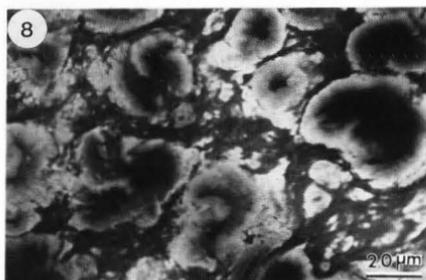
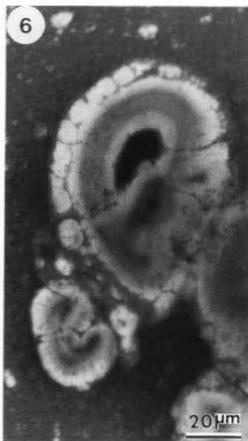
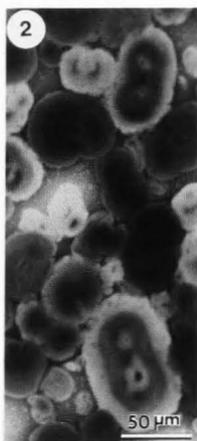
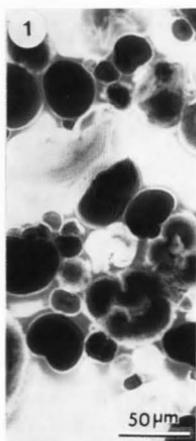
#### Fragmentation in dispersions and gels

The amylopectin fragments seemed to be fractured from the outer layer of the swollen granules, and it was considered of interest to study the effect of the mechanical treatment of the dispersion during preparation in the Brabender with regard to amylopectin fragmentation.

Figure 8 shows a cryosection of an 11% wheat starch dispersion heated to 94°C and held at 30 min in the Brabender. During continuous stirring a large number of small fragments can be seen as particles dispersed in the blue stained amylose phase. Figure 9 shows a cryosection of an 11% wheat starch dispersion where the sample has been heated to 85°C in the Brabender and then held at 94°C for 30 min in a water bath without any mechanical treatment. In this area hardly any fragments are seen. The difference with regard to the degree of fragmentation between the two samples is striking.

The higher concentration used in this experiment means a higher friction during preparation and a higher degree of fragmentation. Fragmentation of amylopectin will have a bearing on rheological properties (Svegmärk and Hermansson, to be published).

The mechanism of amylopectin fragmentation explains some observations from solubility studies made by Doublier (1981). By stirring at a rotation speed of 750 rpm and rapid heating, they found that all amylose was solubilized at 92-93°C and thereafter a large fraction of the amylopectin was "apparently solubilized" and recovered in the supernatant. When the rotation speed was lowered to 200 rpm, total solubilization was achieved at 94°C but no amylopectin was found in the supernatant. In a third experiment at 750 rpm and a heating rate of 1°C/min, which is closer to the heating rate used in this study, they found that the solubilization of amylose was not



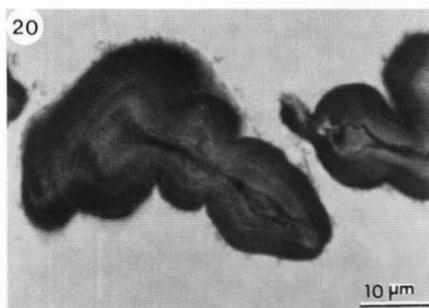
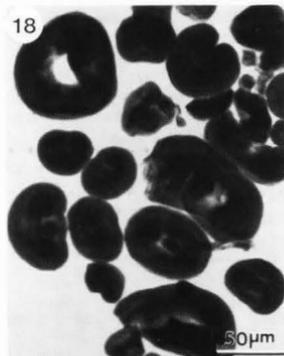
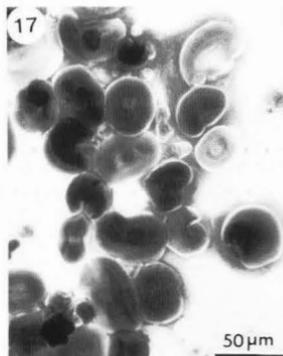
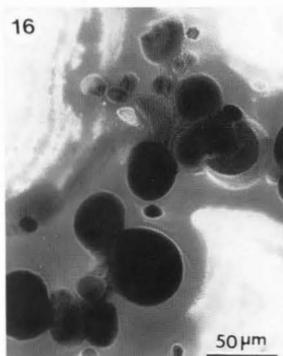
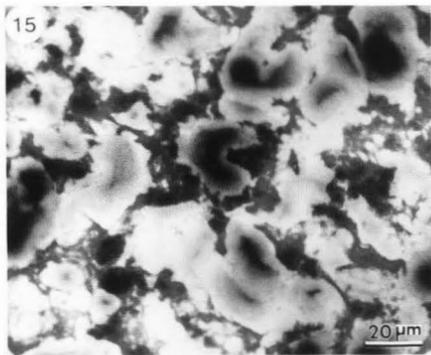
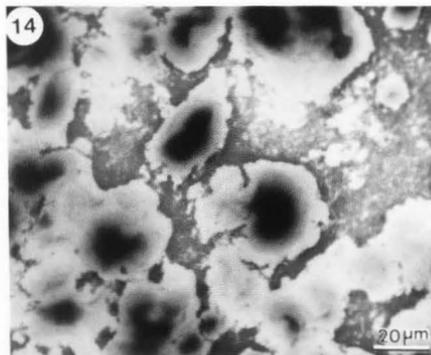


Fig. 14. Cryosection of an 8% wheat starch dispersion heated at 95°C for 30 min.

Fig. 15. Cryosection of an 8% wheat starch dispersion heated at 95°C for 30 min and cooled to room temperature.

Fig. 16. Smear of an 8% wheat starch dispersion heated at 75°C for 30 min.

Fig. 17. Smear of an 8% wheat starch dispersion heated at 75°C for 30 min and cooled to room temperature.

Fig. 18. Smear of an 8% wheat starch dispersion heated at 75°C for 30 min, cooled to room temperature and stored 15 days in a refrigerator.

Fig. 19. Embedded thin section of an 8% wheat starch dispersion heated at 75°C for 30 min cooled to room temperature and stored 4 days in a refrigerator.

Fig. 20. Embedded thin section of an 8% wheat starch dispersion heated at 75°C for 30 min, cooled to room temperature and stored for 24 h.

total and only a small part of the amylopectin was found in the soluble fraction. These are interesting observations, but direct comparisons are difficult to make, since the rotation speeds were considerably higher, the equipment different and the concentrations of wheat starch used in the solubility experiments lower than in this study.

Figures 8 and 9 show cryosections that are 7-8  $\mu\text{m}$  thick, which means that swollen lenticular granules are sectioned and only parts of the granules can be seen. Taking this into account, there is a good agreement between smears and cryosections. The cryosections are not thin enough to provide fine details of the granules. The appearance depends on how the granules have been sectioned and how structural components are super imposed. By embedding and sectioning 1  $\mu\text{m}$  sections, details of the interior of the swollen granules can be seen. The thin sections in Figures 10 and 11 show clearly that there is a blue stained amylose phase inside the swollen granules.

Figure 10 shows an embedded thin section of an 8% gel formed after cooling a dispersion heated to 95°C by continuously stirring in the Brabender. The amylopectin rich fragments in the thin sections stained very poorly and appear almost colourless in Figures 10 and 11. A large number of amylopectin fragments are dispersed in the amylose phase of the gel shown in Figure 10, and it is reasonable to assume that the degree of fragmentation increases due to the shearing during cooling. The large number of amylopectin fragments in the amylose phase will probably interfere with the gelation of amylose. Shearing will also disrupt the amylose network, and it can be noted that it took five h for this sample to form a gel after it had been cooled to room temperature. There is an unstained amylopectin-rich outer layer surrounding the granules in Figure 10.

Additional studies showed that there was a tendency towards phase separation of the small amylopectin fragments from the amylose phase when the gel was stored at +4°C.

Figure 11 shows a thin section of an 8% gel formed after cooling from 120°C without mechanical

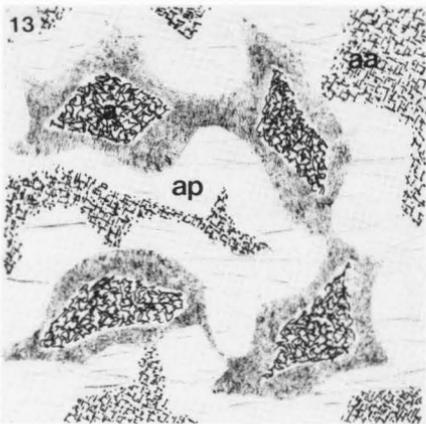
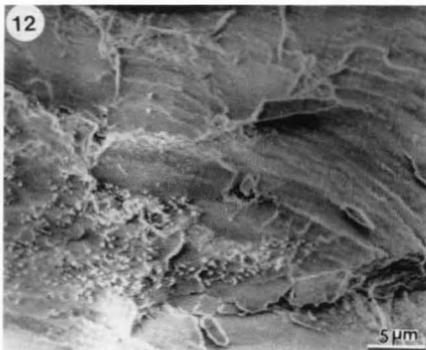


Fig. 12. Scanning electron micrograph of a gel formed after heat treatment at 120°C for 30 min and prepared by the rapid freezing technique.

Fig. 13. Illustration of the mixed gel structure formed after heat treatment at 120°C and not subjected to mechanical treatment showing amylose in the centre of the granule a, outer layer containing mainly amylopectin corresponding to the flaky structure in SEM, Figure 12 and the very weakly stained areas in LM, Figure 11 ap, and the aggregated released continuous amylose network corresponding to the aggregated structure in Figure 12 and to the blue continuous aggregated structure in Figure 11 aa.

treatment. Even after heat treatment at 120°C for 30 min, swollen granules still exist and their inner parts are larger than those shown in Figure 10. As in the dispersion shown in Figure 9, there are hardly any

visible amylopectin fragments in the gel formed without mechanical treatment. Instead the outer amylopectin-rich layer of the granules occupies a much larger volume than was the case in the gel shown in Figure 10, where these layers have been partly sheared off. The gel formed from 120°C without mechanical treatment seems to have two continuous structures separated from each other; one consisting of a network of released amylose and one consisting of granules with a highly swollen amylopectin-rich layer connecting them together. The network of released amylose formed from heat treatment at 120°C differed from that formed at 95°C, but evaluation of amylose network structures requires the resolution given by transmission electron microscopy (TEM) and will be the subject of a separate study.

The amylopectin-rich areas of the thin sections stained very poorly, and it can be questioned whether these areas really contain highly swollen amylopectin and are not just water-filled voids caused by phase separation. Corroborative studies of these regions were made by differential interference contrast of cryosections (LM) and cryo-SEM. The results showed that these regions had a structure which cannot have been caused by low molecular components in a water solution. Figure 12 is a SEM micrograph obtained by the low temperature technique, showing the smooth flaky structure of the amylopectin-rich outer layer and the small aggregates of released amylose formed by heat treatment at 120°C.

The structure of the gel formed by heat treatment at 120°C and not subjected to mechanical treatment is summarized by the schematic drawing in Figure 13. The swollen granules with amylose in the centre and the outer aqueous amylopectin-rich region form a continuum, and the network of aggregated released amylose forms another continuous phase. This gel was considered firmer than that formed by heat treatment at 95°C and mechanical treatment, and no storage at room temperature was necessary for a firm gel to form.

Apart from providing fine details of the structure, the embedding and thin-sectioning technique was more suitable for gels than the cryotechnique. The amylose network was sensitive to freezing, and freeze artifacts were often observed after cryosectioning of gels. The structure often fractured at the interface between the amylose and the amylopectin-rich regions. Before gel formation amylose acted as a cryoprotectant, and cryosectioning gave the best results for hot and cooled dispersions.

#### Aggregation of amylose during cooling from the second stage of swelling and solubilization

Figure 14 shows a cryosection of an 8% dispersion heated and treated mechanically at 95°C for 30 min. This dispersion was prepared in the same way as the 11% dispersion shown in Figure 8. The staining of amylopectin fragments was better at the higher concentration and the high water content may be one factor contributing to the lack of staining intensity. The degree of fragmentation was somewhat higher at the higher concentration due to the difference in shear force. Otherwise the two structures are similar in character.

When the dispersion was cooled to 25°C no gel was formed directly but amylose started to aggregate. Aggregates can be seen in Figure 15 as dark blue areas. The presence of amylose aggregation can also be seen from the thin section of the gel in Figure 10 even if the differences in colour intensity are not as

striking in the 1 µm section as in the 8 µm cryosection shown in Figure 15. The same type of amylose aggregation cannot be observed in Figure 11, and one possibility is that amylose is deposited on amylopectin fragments. From the cryosections it is not possible to say whether the amylose aggregates are solid or whether they cover an amylopectin fragment. The size of a fragment is about 10 µm in diameter which corresponds approximately to the thickness of the section. Despite the lack of resolution, cryosectioning is the best technique for dispersion due to the possibility of freezing the structure at any given state of aggregation.

#### Aggregation of amylose during the first stage of swelling and solubilization

Interesting observations were made from dispersions heated to 75°C. At this temperature the amount of amylose released from the granules is limited, gels do not form, and there is no amylopectin fragmentation. Figure 16 shows a smear of a dispersion kept at 75°C for 30 min. More amylose has leaked out due to the holding time than observed in Figure 1 when the dispersion had just reached 75°C and the majority of the granules are in the first stage of swelling. When this dispersion was cooled to room temperature, shown in Figure 17, the interesting observation was made that there was less amylose outside the granules than at 75°C. Thus, the released amylose seemed to have been reabsorbed by the swollen granules. After 15 days of storage at +4°C this effect was striking and no amylose was visible outside the granules, which can be seen from Figure 18. Some of the granules in this sample had a hollow appearance.

Preparation by embedding and thin-sectioning gave information about the structural states of granules after heat treatment at 75°C, cooling and storage at +4°C. Figure 19 shows granules stored for 24 h and Figure 20 granules stored for 4 days. The granules already have an amylose-rich zone in the centre after heating to 75°C. There seems to be a passage through the equatorial groove facilitating transport of amylose from the interior zone out of the granules and vice versa. It may then be possible that released amylose can be reabsorbed to the central zone on cooling from 75°C.

It can also be seen from Figures 19 and 20 that a thin layer of amylose has been deposited on the surface of the granules. It is possible that such a layer of amylose can form a film around the granules, especially if the dispersion is dried. This amylose film may have a similar effect as hardening of the granules and results in a delay of the swelling of the granules on rehydration and reheating.

Iodine has been added at different stages of preparation, so possible effects of iodine on the state of aggregation of amylose as seen by the light microscope can be excluded. In the thin sections iodine was added after the structure was completely fixed by chemical fixation, dehydration and embedding and no rearrangements were possible.

#### New aspects of the release of amylose

As described above, amylose is already released from the structure and concentrated in the centre of the granules at 75°C when the solubilization of amylose determined by analysis of the supernatant is still limited (Doublie, 1981; Ghiasi et al., 1982; Eliasson, 1986). The reason for this can be the adsorption of water and the concentration of solubilized amylose in the amorphous central part of the

granules. It is postulated that there are openings in the equatorial groove allowing for transport of amylose out of and into the central zone without diffusion through the granular structure; as depicted by the schematic drawing in Figure 21. Amylose is probably squeezed out of the central zone during mechanical shear or centrifugation during preparation of samples. A comparison of Figures 10 and 11 shows that the sheared granules are more deformed and elongated and have a smaller amylose zone in the centre than the unshaded sample. The effect of processing conditions on the amount of amylose in the centre may be a reason for the differences in the amount of amylose in the sediment as described by Doublier (1981) even if it is very difficult to compare experimental conditions.

Amylose also diffuses through the granular structure and very little amylose is left in the outer layer of the granules in the second stage of swelling and solubilization as shown by Figures 6, 10, 11, 14 and 15.

There seems to be a difference in the state of aggregation between amylose in the centre and amylose outside the granules, as well as differences in the network due to processing conditions, but transmission electron microscopy is needed to reveal differences at this dimensional level. It is impossible to say whether a difference between the amylose structure inside the granules and that outside the granules is due to concentration, fractionation of amylose molecules of different molecular weights and degree of branching or if such a difference is due to environmental factors during processing.

#### Conclusions

By a combination of microscopy techniques the following observations have been made on heating, cooling and cold storage of 8-11% starch dispersions.

- \* The presence of an amylose-rich phase in the centre of the granules already in the first stage of swelling when the amount of amylose released from the granules is limited.
- \* The fragmentation of the outer amylopectin layer induced by shear during the heating process.
- \* The deformation of swollen granules and release of amylose from the central zone on mechanical treatment.
- \* Aggregation of amylose on cooling. Amylose was deposited on the surface of the granules and was expected to affect their behaviour when they were reheated.

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Fig. 21. Schematic drawing of a swollen granule with an enriched amylose centre with a passage through the granule at the equatorial groove.

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#### Discussion with Reviewers

E.A. Davis: When smears were made on slides of hydrated material did this result in dehydration by air drying? What care was taken to ensure that the sample was hydrated when viewed?

Authors: Samples were applied on the object glass and stained immediately. Excess of stain solution was gently removed when the cover glass was adapted. The

cover glass was sealed with entellan to prevent evaporation and the sample was examined in the hydrated state. This is a very important point since only fully hydrated smears provide evidence for the fragmentation of granules and the distribution of fragments in the continuous amylose phase. With any of the sectioning techniques, a small particle as seen under the microscope may have been part of a bigger particle as stated in the text. Techniques were combined made and compared in order to make sure that the preparation technique did not induce any structural changes that could be misleading. The drawback of smears is the lack of resolution due to the sample thickness.

E.A. Davis: When cryosections were made, were they viewed on a cryostage for a light microscope or were they freeze-dried?

J. Grider: To fully understand the findings from the light microscopy cryosections, it is necessary to know whether the cryosections were examined at low temperatures or air dried and viewed at room temperature. When was the staining procedure done in the cryosection method?

Authors: Cryosectioning was used as a routine method for screening a large number of wheat starch samples. The cryosections were picked up on slides at room temperature and kept in a desiccator before staining. Thereafter the staining solution was added and the sections were rehydrated before examination under the light microscope. In preliminary experiments staining was done directly, before drying. No structural differences could be observed in the wheat starch samples between sections stained before and after drying.

E.M. Varriano-Marston: Liquid nitrogen is not necessarily quick-freezing because the boiling vapor causes insulation and reduced cooling. It would have been better to freeze rapidly in isopentane cooled in liquid nitrogen.

Authors: We are aware of this and small samples were rapidly frozen in a propane jet freezer for cryo-SEM. However, in our experience, rapid freezing often results in cracking of the larger samples (8 x 8 mm) prepared for light microscopy. Furthermore, rapid freezing is only possible over a very small distance and not relevant for larger samples. In order to prevent the sample from cracking, it was repeatedly dipped in liquid nitrogen.

As stated in the text, freeze artifacts were observed for wheat starch gels and no cryosections of gels are presented in the paper. Freeze artifacts were not observed in hot or cooled dispersions. This indicates that solubilized starch has a cryoprotective effect and that this effect is lost when the gel network is formed.

E.M. Varriano-Marston: Figure 4 is more like the real structure than Figure 3, where the polymers have been obviously disorganized by the fixation and critical point drying techniques. For Figure 3, you cannot tell if the particles surrounding the starch are amylose when using the SEM technique. Chemical fixation and CPD techniques have been shown to alter the polymer structure. Carbohydrate chemists have long known that polymer morphology and functionality are dramatically affected by drying techniques. These problems should be mentioned with the CPD technique.

Authors: There is a lot of documentation about drawbacks of chemical fixation, dehydration and critical

point drying of biological specimens (see e.g. Boyle and Wood, 1969 or Cohen, 1977). Bearing this in mind, the cryotechnique for SEM is a very attractive approach. However, we have encountered practical problems with highly aqueous samples and we thought it would be of general interest to mention some of the difficulties. The crucial question is whether it is possible to control the sublimation of amorphous ice during preparation of highly aqueous biopolymer network structures. Despite the documented disadvantages of the CPD technique, we have obtained good results with this technique for biopolymer network structures and we have also compared this technique with other EM techniques (see e.g. Hermansson and Buchheim, 1981; Hermansson and Langton, 1988; Hermansson, 1988). In this paper we mainly wanted to illustrate the shape of the swollen granules by SEM. For detailed studies of amylose aggregates and network structures, it would be better to prepare amylose samples for TEM, for example, by the sandwich mica technique (Hermansson, 1989).

D.D. Christianson: Is the retrograded amylose in beads? It looks like this from the structures at the surface of the granules. Is there a temperature dependence with regard to the structure of amylose aggregates?

Authors: A separate study is planned to evaluate the fine structure of the amylose phase. As indicated above, we will use TEM rather than SEM for this study.

D.D. Christianson: You make a very intriguing point about phase separation of small amylopectin fragments from amylose. The kinetics should be studied of separation of both small and large fragments.

J. Grider: You make reference to additional studies showing the tendency towards phase separation of small amylopectin fragments from the amylose phase when the gel was stored at +40°C. What evidence from these studies support this conclusion?

Authors: The number of colour micrographs presented had to be limited and phase separation of amylose on cooling is illustrated by a cryosection of a cooled dispersion. The effect of cooling and cold storage of gels was also studied by embedding and thin sectioning. From these micrographs we observed a tendency towards phase separation with storage. Amylopectin fragments clustered together and the amylose phase became more even. The kinetics of this process needs to be studied further.

E.A. Davis: Do you think that as amylopectin is solubilized it no longer looks "brownish" in the presence of iodine or is it mainly a concentration effect?

Authors: In this case we think that the concentration is an important factor. However, other factors may also contribute to the ability of amylopectin to stain. We have observed differences in staining intensity between wheat starch from different types of wheat and differences between potato starch subjected to different degrees of chemical modification and native potato starch.

D.D. Christianson: Is readsorption of amylose evidence for the annealing process in starch? What correlations can be expected with DSC measurements?

Authors: The annealing process of starch is not fully understood. Generally, annealing refers to rearrangements of molecules inside the starch granule to a more

ordered structure. An alternative is reabsorption of amylose and the formation of an amylose film on the surface of the granule. This may have a similar effect on physical properties in heating as changes inside the granules, and it is possible that both phenomena are involved in the, so called annealing process.

K. Ghiasi: The authors suggest the possibility of amylose coming out of the granules and going back in. This is a very interesting hypothesis. Do the authors have any other data on this?

Authors: This is a hypothesis based on observations of differences between the size and shape of the amylose zone in the centre of the granules. The thin sections obtained by the plastic embedding technique have made it possible to observe the inner zone of the granules, which is not the case for smears or cryosections. The size and shape of the central amylose zone of mechanically treated granules are smaller and narrower than for granules heated under static conditions, indicating that amylose has been squeezed out of the granules. The thin sections show that amylose is adsorbed on the granule surface on cooling. The possibility of amylose going back into the granule is a speculation and further studies are needed in order to elucidate the transport mechanisms of amylose.