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Effect of annealing on the hydrolysis of sago starch granules

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Sago starch annealed at varying temperatures, time intervals and pH was used to study granule hydrolysis by a glucoamylase (AMG) and α-amylase (Termamyl) mixture. Differential scanning calorimetry (DSC) indicated that there was a relationship between the extent of annealing and starch granule hydrolysis. Enthalpy of gelatinisation of annealed starch granules remained unchanged, suggesting that no gelatinisation had occurred. The degree of hydrolysis increased and the granule degradation pattern altered—from surface erosion to preferential digging of the internal regions of the granule. Sections of the hydrolysed granule residues revealed that enzymes attacked from one point on sufficiently annealed granules, and that after extensive hydrolysis, only an empty shell remained. © 1997 Elsevier Science Ltd

INTRODUCTION

Development of starch hydrolytic procedures without the need for gelatinisation would eliminate the need for energy input and circumvent the problems associated with the handling of highly viscous pastes. Sago starch has been used as a model for the action of enzymes on starch granules (Wang *et al.*, 1995). Interest in this material originated from the fact that these granules are hydrolysed in a manner that depends on the previous processing history of the starch. Susceptibility of the starch granules to enzyme attack can be modified by preheating the granules in aqueous solution at low pH (3.5) for 2 h. Following this treatment, granules are more sensitive to enzyme hydrolysis than in untreated material and the pattern of enzyme attack is also found to be highly dependent on the processing history (Wang *et al.*, 1995).

Thermal analysis of hydrolysed treated and untreated material suggested further differences in the properties of the treated starch granules (Wang *et al.*, 1995). Enthalpy of gelatinisation decreased in proportion with the extent of hydrolysis for the treated hydrolysed starch. In contrast, enthalpy of gelatinisation for untreated hydrolysed residues remained relatively unchanged despite an increase in hydrolysis.

Restricted heating of starch granules is known to result in a phenomenon known as annealing. In this process the arrangement of the macromolecules undergoes subtle changes resulting from rearrangement of hydrogen bonds which are broken and reformed as the granule components attain a state of more ordered crystal formation. The process of annealing has been well reported (Knutson, 1990; Larsson and Eliasson, 1991). However, information on the correlation between annealing and starch hydrolysis is scarce.

The present study investigated the relationship between starch thermal properties, resulting from different pretreatment or annealing conditions, and sago starch hydrolysis. Efforts were made to understand the detailed hydrolysis process of both raw and annealed granules.

MATERIALS AND METHODS

Starch

Sago starch (*Metroxylon* sp.) from Sarawak, Malaysia was supplied by a commercial producer, Wah Chang International Group of Companies (Singapore). It was processed specifically for our use.

Pretreatment or annealing was carried out by incubating a starch slurry (20%) with 0.1 M sodium acetate buffer in an oven at different temperatures, pH and time intervals.

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Enzyme

Enzymes were commercial preparations, kindly donated by Novo Nordisk, Regional Office, Kuala Lumpur. Glucoamylase (AMG, 300 units/ml) was derived from a strain of *Aspergillus nigor*. α-Amylase (Termamyl, 120 units/ml) was derived from a strain of *Bacillus licheniformmis*.

Hydrolysis of starch granules

A reaction mixture containing 2.0% starch granules, 0.1 M acetate buffer pH 5.0 and 40 ppm Ca^{2+} ($CaCl_2$) was incubated at 35°C with constant shaking. Hydrolysis was initiatated following addition of 1% (volume of enzyme per weight of starch) of each of the two enzymes (AMG and Termamyl) to the suspension. Aliquots were removed periodically, centrifuged and the supernatant mixed with an equal volume of 0.4 mM $HgCl_2$ and incubated in a water bath at 90°C for 20 min to inactivate the enzymes (Govindasamy *et al.*, 1992). This solution was then used for reducing sugar determination. The precipitate was washed, filtered through filter paper and dried at room temperature.

Differential scanning calorimetry

Gelatinisation properties of washed and room temperature dried raw sago starch and sago starch retrieved from different annealing conditions were analysed using a Perkin Elmer differential scanning calorimeter, DSC 7, (Norwalk CT) equipped with an intercooler. Starch samples were placed in aluminium pans with a measured amount of water (water to starch ratio 60:40) and hermetically sealed. The sample was then heated from 30 to 110°C at a heating rate of 10°C/min. An empty pan was used as a reference for the run. Enthalpy changes and onset temperature, integrated using DSC 7 software, were calibrated on the basis of an indium standard. All pans were cooled and reweighed after the run to ensure that no moisture was lost during the run.

High performance size exclusion chromatography (HPSEC)

The analysis procedure was performed following the method of Govindasamy *et al.* (1992). A Waters Associates (Milford, MA) series liquid chromatography system and three Ultrahydrogel columns were used. The columns, maintained at 40°C, were connected in the order: Ultrahydrogel linear followed by two Ultrahydrogel 120. Deionised water was used as a mobile phase. Apparent molecular weights were determined using oligosaccharide and dextrin molecular weight standards.

Leached material from pretreated starch granules was analysed as follows: supernatant (20 ml) of the pretreatment suspension was concentrated and freeze dried and the dried powder was then dissolved in distilled water (3 ml). This solution was filtered through a 8.0 μm Millipore filter for HPSEC injections.

Raw starch HPSEC sample was prepared by a modified Jackson method (Jackson *et al.*, 1988). Distilled water (15 ml) was added to starch granules (30 mg) and the suspension was gelatinised by placing in a boiling water bath for 10 min. The suspension was cooled, dispersed further by sonication (30 s) and the final solution passed through an 8.0 μm Millipore filter prior to HPSEC analysis.

Scanning electron microscopy

Dried starch granules or hydrolysed granule residues were sprinkled onto double sided adhesive tape attached to a circular specimen stub and coated with gold using a Balzers SCD 004 sputter coater. The samples were viewed and photographed using a Phillips SEM 515 scanning electron microscope on AGFAPAN-APX 100 film.

Sectioning

Ethanol dehydrated starch granules or hydrolysed residues were embedded in Araldite resin and polymerised at 60°C for 24 h. Sections were cut to a thickness of 0.8 μm with a Sorvall Mt2-B Ultra-microtome using glass knives mounted in a rocking knife holder adapted for the microtome. The sections were dried onto glass slides and stained with a 6% KI and 0.3% iodine solution at 60°C.

Light microscopy

Starch granules were dispersed onto glass slides, viewed and photographed with an Olympus DAT-2 microscope. Photomicrographs of a calibration slide at the same magnification were taken and used as a reference for determining granule size. Stained sections were also viewed or photographed with this microscope.

Analytical methods

The degree of hydrolysis was defined as follows:

$$DH(\%) = \frac{\text{Reducing sugar producedby enzyme hydrolysis}}{\text{Reducing sugar produced by acid hydrolysis}} \times 100\%$$

Reducing sugar was determined by the method of Dygert *et al.* (1965) using glucose as standard. Acid hydrolysis was carried out by treating starch granules with 1.0 M HCl at 100°C for 2 h (Lee *et al.*, 1986). Complete hydrolysis was confirmed by HPSEC.

Total carbohydrate concentration of the pretreatment suspension was assayed by the method of

Dubois *et al.* (1956). Glucose was used to construct the standard curve.

RESULTS AND DISCUSSION

Effect of annealing on the degree of hydrolysis

Annealing starches, irrespective of source, alters the gelatinisation characteristics such that the gelatinisation range is narrowed and the peak temperature increased (Nakazawa *et al.*, 1984). Thermograms of preincubated sago starch are characterised by increased peak temperature (T_p) and onset temperature (T_o) (Table 1 and Fig. 1). In contrast, the gelatinisation conclusion temperature (T_c) remains relatively unchanged, a phenomenon previously reported by Nakazawa *et al.* (1984). Measurement of the gelatinisation range, $T_p - T_o$, was therefore adopted in this study as a comparative indicator for the extent of annealing, under a constant DSC scanning rate (10°C/min).

Enthalpy of gelatinisation (ΔH_G) did not change during the 5 h incubation at 60°C (Table 1), suggesting that no significant gelatinisation had occurred during annealing (Larsson and Eliasson, 1991; Zobel, 1992).

Time of incubation had a significant effect on the apparent annealing process (Fig. 2). During the initial 2 h, the extent of annealing increased, measured as a reduction in peak width ($T_p - T_o$ value decreased by 50%) with a concomitant increase in the degree of hydrolysis (300% greater than for untreated starch). The first 2 h of incubation gave rise to the greatest effect on granular susceptibility with a less marked effect over an additional 3 h.

Temperature of incubation was found to be a critical factor for annealing (Fig. 3). A threshold temperature of 55°C was required before there was significant evidence of annealing. Above this threshold temperature, incubation of starch granules resulted in reduction of $T_p - T_o$ and increase in the degree of hydrolysis. At this annealing condition (55°C 2 h), the value of $T_p - T_o$ was equivalent to that for 60°C 0.5 h annealed starch. However, the degree of hydrolysis was only half of that for the latter.

Incubation of starch granules in hot water is known to result in the leaching of amylose from granules (John, 1992). HPSEC profiles for the supernatants of

Table 1. DSC thermogram values of 60°C annealed sago starch

Annealing time (h)	T_p (°C)	$T_p - T_o$ (°C)	ΔH (J/g)
0	70.09±0.25	7.03±0.23	15.15±0.29
0.5	70.22±0.23	5.25±0.08	15.24±0.24
1	70.94±0.33	4.06±0.07	15.22±0.33
2	71.73±0.29	3.54±0.07	15.19±0.21
3	72.00±0.18	3.37±0.08	15.09±0.33
4	72.50±0.19	3.28±0.05	15.08±0.38
5	72.77±0.31	3.17±0.01	15.16±0.14

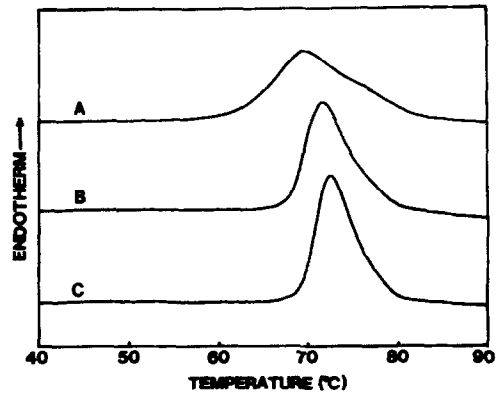

Fig. 1. DSC thermograms of (A) raw sago starch, (B) 60°C 2 h annealed sago starch, and (C) 5 h annealed sago starch.

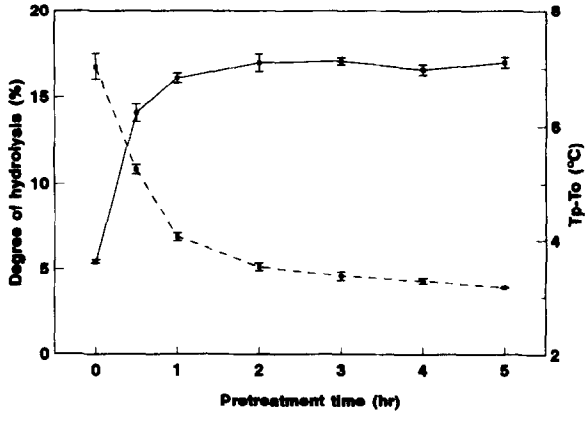

Fig. 2. Sago starch samples annealed at 60°C for different time intervals and the corresponding degrees of hydrolysis. (-) DH; (—) $T_p - T_o$; ±, standard error of mean.

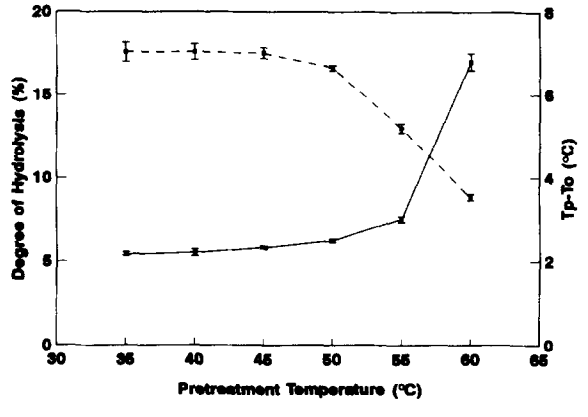

Fig. 3. Sago starch samples annealed at different temperatures for 2 h and the corresponding degrees of hydrolysis. (-) DH; (—) $T_p - T_o$; ±, standard error of mean.

pretreatment suspensions revealed that materials leached from both 60°C 0.5 h and 55°C 2 h annealed starch granules were mainly low molecular weight dextrins and oligosaccharides rather than amylose, compared to the HPSEC profile of raw sago starch. More significantly, the leached materials at these two different conditions had similar components and concentrations (Fig. 4). These results suggest that the hydrolysis difference between 60°C 0.5 h and 55°C 2 h annealed starch granules may not result from leaching of the starch materials. Reducing sugar and total carbohydrate analysis results supported this suggestion: the supernatant of 60°C 0.5 h incubated starch suspension had similar reducing sugar and total carbohydrate concentration to that of 55°C 2 h incubated suspension (data not presented).

The annealing process has been shown to be dependent on a number of factors including incubation temperature, starch to water ratio and time (Krueger *et al.*, 1987; Larsson and Eliasson, 1991). However, there are no reports on the effect of pH on the annealing process. For sago starch, the extent of annealing was found to be inversely proportional to the aqueous pH of the annealing system. Concomitant with the reduction in $T_p - T_o$, the hydrolysis potential of the starch granules increased (Fig. 5). Peak temperature (T_p) also decreased as pH was increased, from 71.7°C pH 3.5 to 70.8°C pH 6.0.

In all the above experiments, the degrees of hydrolysis and solubilisation of annealed sago starch were negligible, less than 0.2%, and there was no detectable change in gelatinisation enthalpy (data not presented). Minimal solubilisation and hydrolysis of starch on annealing indicated that the sago starch used in this study was not contaminated with an amylolytic enzyme.

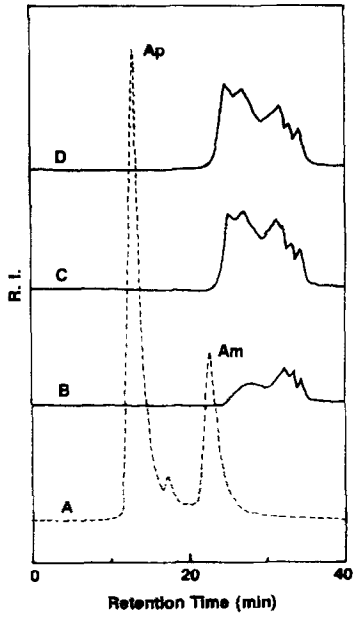

Fig. 4. HPSEC profiles of (A) raw sago starch; (B) supernatant of room temperature 2 h; (C) 55°C 2 h; and (D) 60°C 0.5 h, incubated starch suspensions (pH 3.5).

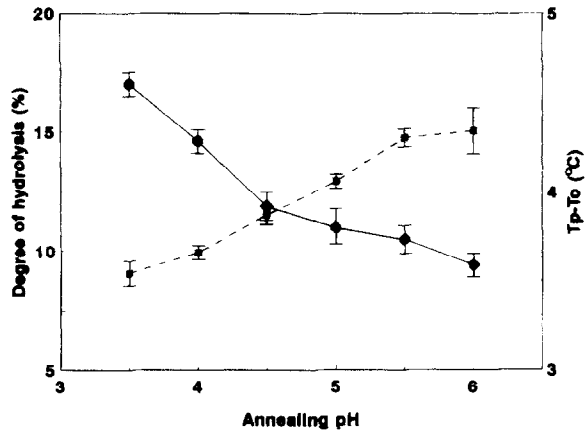

Fig. 5. Sago starch samples annealed at 60°C in different pH buffer solutions and the corresponding degree of hydrolysis. (-) DH; (—) $T_p - T_o$; ±, standard error of mean.

At the lowest pH investigated (pH 3.5), it is possible that lintnerisation may have taken place, giving rise to an apparent increase in hydrolysis. Effects of lintnerisation were investigated by steeping sago starch granules in 0.1 M acetic acid (10% w/v) at room temperature for one week. Granules treated in such a manner were subsequently hydrolysed as described previously but the degree of hydrolysis was 4% lower than that of the untreated starch granules. This suggests that lintnerisation contributed only marginally to the effects of low pH incubation on starch hydrolysis. The slight decrease in the degree of hydrolysis could result from peeling of the less compact surface layer, which is more easily hydrolysed by enzymes.

After pretreatment in pH 3.5, 0.1 M acetate buffer for 2 h at 60°C, the optimum pH of the enzyme mixture to hydrolyse starch granules was still the same as that for raw starch (pH 4.5), and the profiles of the two pH–hydrolysis curves were similar. The degree of hydrolysis of the annealed starch was greatly increased (Fig. 6), however.

Scanning electron microscope of the hydrolysis patterns

The raw starch granule surface was smooth with some indentations which may have resulted from endogenous enzyme action (palm) or contaminating microbial enzymes. Some granules appeared damaged, which may have occurred during extraction (Fig. 7(A)).

Granules were attacked slowly by AMG and Termamyl mixture, with 85% hydrolysis being achieved only after 8 days. After one day of hydrolysis (DH 25%) shallow indentations were visible on the

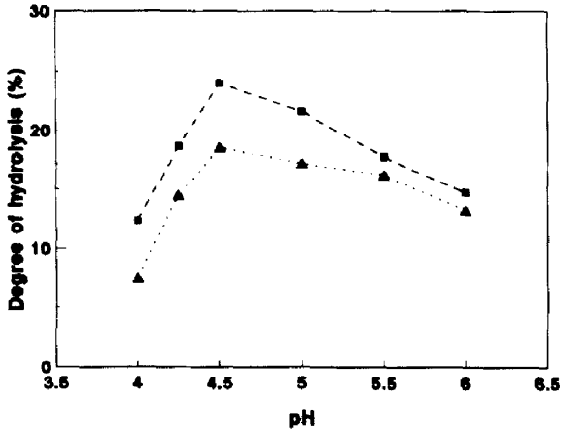

Fig. 6. The effect of hydrolysis pH on the degree of hydrolysis of raw starch (■) and 60°C 2 h pH 3.5 annealed sago starch (▲). Raw and annealed starch granules were hydrolysed for 20 h and 4 h respectively.

surface of the granules and some caving was evident in a small number of granules. Overall, the granule surface, including indentations, was smooth (Fig. 7(B)). Granules subjected to extended hydrolysis exhibited the same pattern of hydrolysis (surface erosion and caving). The result of this degradation was that most of the starch granules became smaller after 8 days of hydrolysis (Fig. 7(C)). At this stage, some of the granules were fragmented, probably due to the caving action of the enzymes. Light micrographs confirmed the hydrolysis pattern: starch granular size was significantly decreased after 8 days of hydrolysis as a result of surface erosion and breaking of the granules (results not presented). Hydrolysis for one week of lintnerised starch granules also exhibited the above hydrolysis pattern (results not presented).

Incubation of annealed starch (60°C, 2 h, pH 3.5) in the hydrolysis system for 32 h without enzyme did not alter the appearance of the granules (Fig. 8(A)). Hydrolysis by AMG and Termamyl mixture, however, proceeded at a greater rate in these granules, with degree of hydrolysis approximately 80% after 32 h. More significantly, this pretreatment altered the path of hydrolysis. For all samples annealed at 60°C, from 0.5 to 5 h and from pH 3.5 to 6.0, enzymes preferentially attacked the interior of the starch granules, leaving a deep round hole on the starch granule surface (Fig. 9). However, in the case of 2 h annealing at different temperatures, up to 55°C, only some of the granules observed this hydrolysis pattern. Others were degraded in a pattern similar to that for the untreated starch granules—mostly surface erosion and occasional caving (Fig. 8(B)).

During annealing, microregional gelatinisation of granule fractions may well occur. Available data, however, suggests that this is not the case: granules maintained their birefringence and the enthalpy of

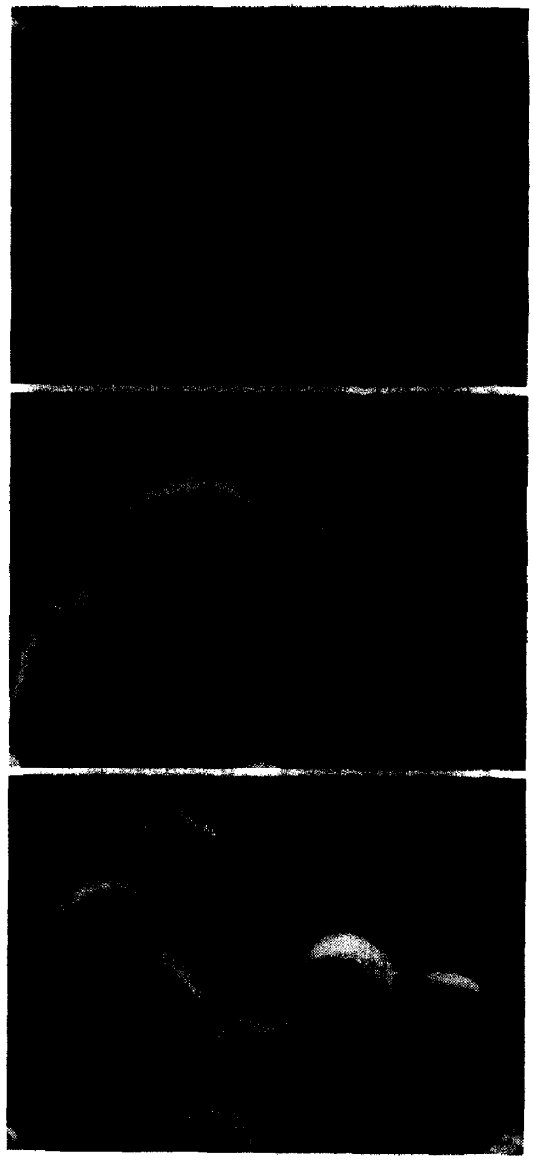

Fig. 7. SEM photographs of (A) raw starch granules; and granule residues hydrolysed by an AMG and Termamyl mixture for (B) 1 day, DH 25%, and (C) 8 days, DH 85%. Bar = 0.1 mm.

gelatinisation was not altered after annealing, suggesting little change in the degree of crystallinity.

As illustrated above, starch granules annealed at 60°C, in different pH buffer solutions and time intervals, showed a significant extent of annealing and greatly increased hydrolysis. For the different temperatures of annealing, 55°C was a critical point at which starch granules were annealed and the degree of hydrolysis increased significantly. A relationship might be expected between extent of annealing and granule hydrolysis. As observed in our previous work (Wang *et al.*, 1995), while the ΔH_G of untreated hydrolysed residues remains unchanged, the ΔH_G of pretreated hydrolysed residues decreases. This suggests that regions within the granule were apparently more

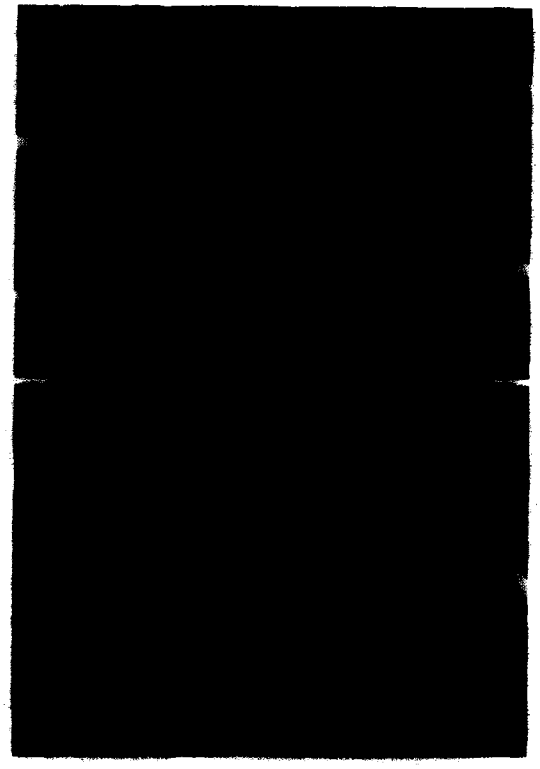

Fig. 8. SEM photographs of (A) treated (60°C, 2 h, pH 3.5) starch granules incubated in the hydrolysis system for 32 h without enzyme, and (B) 55°C 2 h annealed granules hydrolysed by enzymes for 24 h, DH 34%. Bar = 0.1 mm.

uniformly susceptible to enzyme digestion after pretreatment.

A more ordered crystalline region resulting from annealing implies a more compact crystalline region. Therefore, there may be a slight shrinkage of this region and an increase in enthalpy. As a result, the amorphous region may expand slightly and some hydrogen bonds between the amorphous and crystalline regions could be broken. This may reduce the enthalpy. The combined result is that apparent enthalpy is unchanged by the annealing. However, the slight swelling of the amorphous region may render this region more susceptible to enzyme action, and hydrolysis of this region will expose the crystalline region to the enzyme. This mechanism may explain why the hydrolysis of the unprotected crystalline region is also improved.

Initial enzyme attack developed from a depression at one end of the granule, which had a pumiced appearance immediately prior to penetration (Fig. 9(A)). Later stages of hydrolysis resulted in the development of a hole from this point. Hydrolysis continued with the apparent excavation of the internal regions with little removal of the outer surface of the granule (Fig. 9(B)). After extended incubation, a sharp edge was developed as the hole size increased. Development of this sharp edge may reflect erosion by the enzymes on the very pumiced and possibly weak

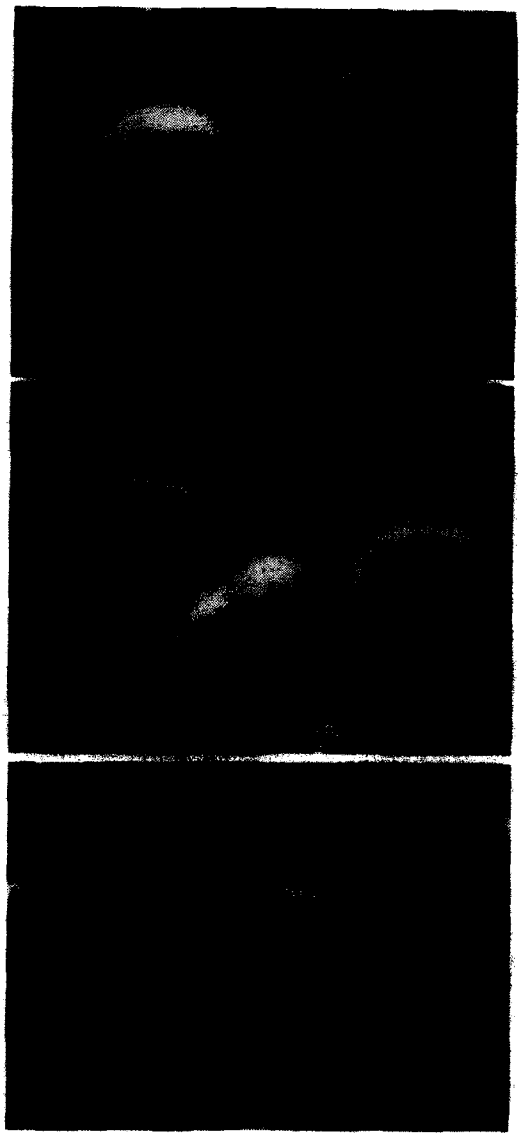

Fig. 9. SEM photographs of 60°C 2 h annealed starch granules hydrolysed by enzymes for (A) 0.5 h, DH 4.5% (bar = 10 μm), (B) 1 h, DH 7.0% (bar = 10 μm), and (C) 22 h, DH 67% (bar = 0.1 mm).

points around the original point of entry into the granule. The whole outer surface after extensive hydrolysis (DH 67%) was pumiced and had shallow indentations similar to those seen in the enzyme hydrolysed raw starch granule residues. This may reflect hydrolysis of the granule surface, although it proceeded at a slower rate. At this stage the granule was simply an empty shell (Fig. 9(C)). Further hydrolysis led to the granule breaking down into curved fragments.

Sections of hydrolysed granule residues

Sections of treated starch and enzymatic hydrolysed annealed granule residues revealed the internal regions of the granule digested by the enzymes (Fig. 10).

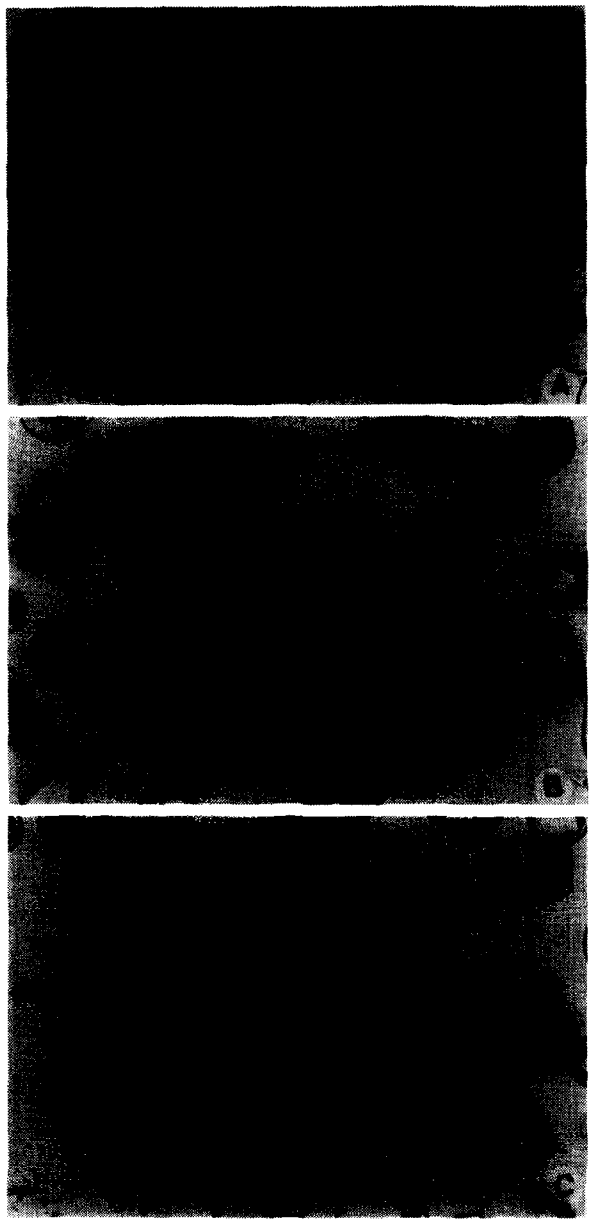

Fig. 10. Micrographs of sections of (A) treated starch granules, and granule residues hydrolysed by an AMG and Termamyl mixture for (B) 2 h, DH 12%, and (C) 22 h, DH 67%. Starch granules were annealed at 60°C pH 3.5 for 2 h.

Initially, the enzyme appeared to erode granular material from one point. The front edge of the enzymes digestion was rough. When the starch granule was extensively hydrolysed (DH 67%), only a smooth shell—the outer layer of the starch—remained. The same annealed granules were also hydrolysed with a Sigma purified (four times crystallised) α-amylase and the residues were cut into sections. The pattern of hydrolysis was identical to that observed with the commercial enzyme mixture attack (results not presented), suggesting that the hydrolysis pattern observed was not mediated by contaminating enzymes present in the commercial enzymes.

CONCLUSION

The degradation pattern of starch granules pretreated for extended periods of time appeared to be a combination of rapid digestion of internal material with slow surface erosion. At 60°C, prolonged annealing times (>2 h) did not significantly affect the gelatinisation range. It appears that at a high temperature, annealing rapidly approached its maximum degree. This agrees with the results of other studies (Krueger *et al.*, 1987; Larsson and Eliasson, 1991).

Incubation temperature had to be greater than 55°C before annealing was induced and consequently the degree of hydrolysis increased and the degradation pattern altered. Although 55°C 2 h annealed starch had a similar extent of annealing to 60°C 0.5 h annealed starch, the degree of hydrolysis was only half that of the latter, with many of the granules degraded in the same manner as raw starch granules. Further studies are needed to understand the mechanism of sago starch hydrolysis.

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