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Pattern of enzyme hydrolysis in raw sago starch: effects of processing history

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Hydrolysis patterns of five batches of sago starch were studied by using Novo Nordisk and Sigma α-amylases and glucoamylases. Native sago starch was a poor substrate to the enzymes and the hydrolysis patterns were surface erosion, pitting and crevassing. After incubation with pH 3·5 acetate buffer at 60°C for 2 h, the hydrolysis pattern was different: a single deep round hole developed regardless of the batch or enzyme(s) used. This step also significantly increased the degree of hydrolysis. Granule size distribution results indicated that at about 67% hydrolysis, treated granule residues were the same mean size as native granules while untreated granule residues had two major size populations. DSC results suggested that amorphous regions of the untreated granule were preferentially hydrolysed, however, upon pretreatment regions within the granule were more uniform towards enzymes' action.

INTRODUCTION

Starch is a plant storage reserve utilized during specific stages of plant morphogenesis, e.g. early post germination growth, fruit development. Although starch is mobilized from plant material at ambient temperatures, the susceptibility of starch granules from different botanical sources to hydrolytic enzymes *in vitro* varies widely (Dettori-Campus *et al.*, 1992).

In its native form, starch exists in relatively inert granular structures that are composed of macromolecules arranged in a polycrystalline state. Gelatinization, heating in the presence of water, enhances chemical reactivity toward hydrolytic enzymes. Alternative protocols eliminating the prior heat treatment could reduce the processing costs (Fogarty & Kelly, 1990). Recent research has focused on the use of microbial enzymes for the hydrolysis of starches at temperatures below the gelatinization temperature (Punpeng *et al.*, 1992; Haska & Ohta, 1993).

The initial interaction between amylases and starch granules can vary. Fungal amylases may first adsorb to the granule surface for efficient hydrolysis and the degree of adsorption is directly correlated to the extent of digestion (Fogarty & Kelly, 1990). Studies have shown that the affinity site is separate from the active site but is essential for hydrolysis (Nagashima *et al.*, 1992). A similar observation was found for a glucoamylase from *Aspergillus* (Chen *et al.*, 1988).

Bacterial amylases, on the other hand, are not always adsorbed onto the granule. An α-amylase from *Bacillus subtilis* 65 was able to digest raw potato starch as effectively as corn starch but did not adsorb to raw starches (Hayashida *et al.*, 1988). α-Amylases from *Clostridium butyricum* T-7, however, were adsorbed onto the granules and could not be liberated even when the granular structure was lost (Tanaka *et al.*, 1987).

The attack mode of amylases on starch granules has been widely described (MacGregor & Ballance, 1980; Bhat *et al.*, 1983). In general, the enzyme either erodes the entire/sections of the granule surface or digests channels from selected points on the surface toward the centre of the granule. On reaching the centre, enzyme attack proceeds outward over a broader front. It seems that in all the stages, from initial to later digestion, some regions of the granule are more readily digested than others. Five general patterns of attack have been identified by Evers (1979) as: pin-holes/pepper-potting; sponge-like erosion; many medium sized holes; distinct loci leading to single holes in individual granules and surface erosion.

Digestion of poorly hydrolysed starches and reactivity of amylases exhibiting low activity for raw starch can be improved by using 'mixed' enzyme systems (Fujii *et al.*, 1988; Monma *et al.*, 1989). Fujii *et al.* (1988) have attempted an explanation for this synergism phenomena

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between the enzymes: α-amylase attaches to the granule surface and peels away sections of the granule, which is then removed by the glucoamylase exposing new portions available for attack by the α-amylase.

This paper reports experiments on hydrolysis of sago starch which is resistant to enzyme or enzyme mixture action (Monma *et al.*, 1989; Haska & Ohta, 1991). Sago starch granules were chosen because they represent a good model for studying the effect of processing on hydrolysis.

MATERIALS AND METHODS

Starch and pretreatment

Five batches of sago starch from different botanical and geographic sources were used (Table 1). Batches 1 and 2 were supplied by a commercial producer, Wah Cheng International Group of Companies (Singapore). Another three batches were kindly donated by the Indonesia Institute of Sciences, Bogor, Indonesia. Batch 1 was specially processed for our laboratory use. Other batches were only used in SEM viewing of degraded starch granules. Corn starch was obtained from Sigma Chemical Co. Tapioca starch was also obtained from Wah Chang International Group of Companies.

Pretreatment was carried by a modified Haska & Ohta method (1991). A suspension of 1 g of starch in 5 ml 0·1 M sodium acetate buffer (pH 3·5), was incubated in an oven at 60°C for 2 h.

Enzymes

Enzymes were commercial preparations, kindly donated by Novo Nordisk, Regional Office, Kuala Lumpur, Malaysia. Purified α-amylase and glucoamylase were from Sigma Chemical Co. (Table 2).

Hydrolysis of starch granules

A reaction mixture containing 2·0% starch granules, 0·1 M acetic acid–sodium acetate buffer solution pH 5·0 and 40 ppm Ca^{2+} ($CaCl_2$) was incubated at 35°C with constant shaking. Hydrolysis was initiated following addition of 1% (volume or weight of enzyme/weight of starch) of the enzyme(s) to be tested to the suspension. Aliquots were removed periodically and centrifuged at 2500 rpm for 10 min (Jouan BR.A 3.11). The supernatant was 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 enzyme(s) (Govindasamy *et al.*, 1991). This solution was then used for reducing sugar determination. The precipitate was washed with water, filtered through Whatman 1 filter paper and dried at room temperature.

Table 1. Sources of sago starch

Batch number	Palm	Geographic source	pH
1	*Metroxylon* sp	Sarawak, Malaysia	4·0
2	*Metroxylon* sp	Sarawak, Malaysia	5·6
3	*Arenga pinnata*	West Java, Indonesia	4·6
4	*Arenga pinnata*	Sumatra, Indonesia	4·6
5	*Metroxylon* sp	Malaku, Indonesia	4·5

Table 2. List of enzymes used in this work

Enzyme (commercial name)	Origin	Activity[a]
Glucoamylase (AMG)	*Aspergillus niger*	300 U/ml
Bacterial α-amylase (BAN)	*Bacillus subtilis*	480 U/g
Fungal α-amylase (Fungamyl)	*Aspergillus oryzae*	800 U/g
Thermostable α-amylase (Termamyl)	*Bacillus licheniformis*	120 U/g
Sigma glucoamylase	*Aspergillus niger*	6100 U/ml
Sigma α-amylase	*Bacillus subtilis*	1740 U/mg

[a]Enzyme activities were defined by Novo Nordisk or Sigma, respectively.

Analytical methods

The degree of hydrolysis was defined as follows:

$$\text{D.H.}(\%) = \frac{\text{Reducing sugar produced by enzyme hydrolysis}}{\text{Reducing sugar produced by acid hydrolysis}} \times 100\%.$$

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.

DSC

Gelatinization properties of the extracted starches were analysed using a Perkin Elmer differential scanning calorimeter, DSC7, (Norwalk, CT) equipped with an intercooler. Starch samples were placed in aluminium pans with a measured amount of water (water:starch ratio 60:40) and hermetically sealed. The sample was then heated from 20 to 110°C at a heating rate of 10°C/min. An empty pan was used as a reference for the run. Enthalpy changes, integrated using DSC7 software, were calibrated on the basis of the melting enthalpy of indium metal. All pans were cooled and reweighed after the run to ensure that no moisture was lost during the run.

Coulter Counter analysis

Starch granules or degraded granule residues (40–50 mg) were dispersed in 200 ml of electrolyte solution

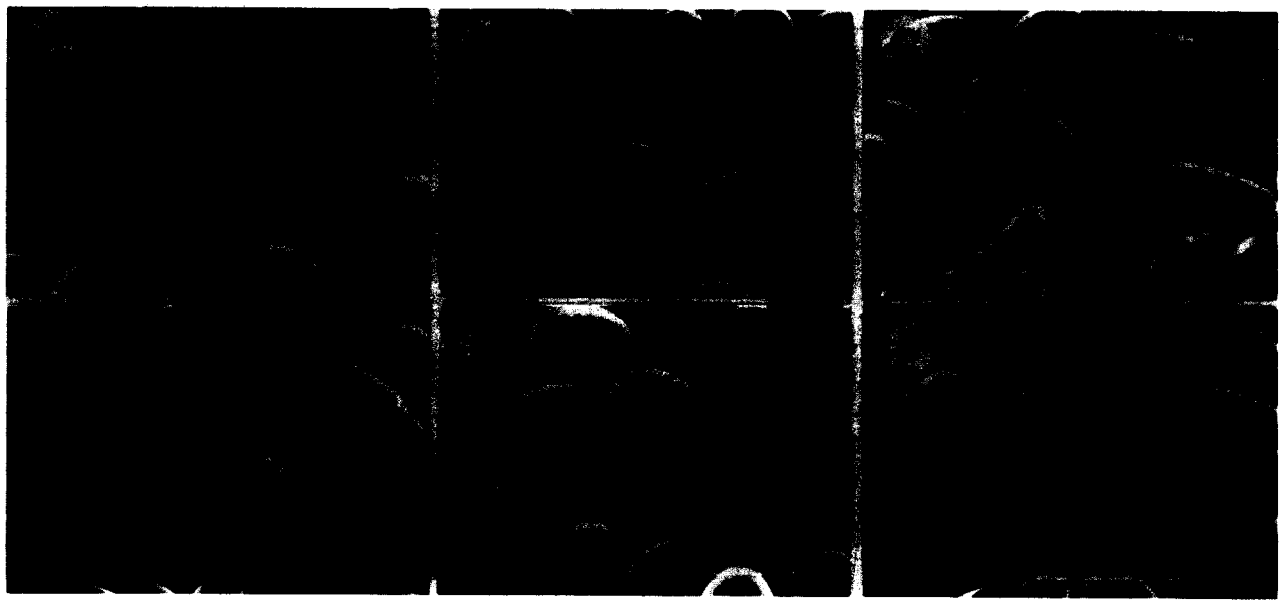

Fig. 1. SEM photomicrographs of five batches of native sago starch and treated batch 1 granules. A–E, batches 1–5; F, treated batch 1. Bar denotes 0·1 mm.

(ISOTON II diluant) and counted with a Coulter Counter (Industrial Model D, Coulter Electronics Ltd, UK). A 140 μm aperture was used. Size calibration was carried out by using a Coulter calibration standard particles (median diameter 14·7 μm).

Scanning electron microscopy

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

Light microscopy

Starch granules were dispersed onto a microscope slide and photographed. Photomicrographs of a calibration slide at the same magnification were taken and were used as a reference for determining granule size.

RESULTS AND DISCUSSION

Native sago starch: surface characteristics

Native granules extracted from *Metroxylon* spp grown in three different locations are characteristically smooth and ovoid (Fig. 1A, B and E). Size distribution of the batch 1 granules suggests a relatively narrow range of granular sizes (10–50 μm) with a mean size of 32 μm (Fig. 2). Small indentations are evident over the entire surface of many of the granules. Sections also appear to have broken off from some of the granules which may have occurred during processing (Fig. 1A, B and E).

Starch granules extracted from *Arenga pinnata* are more elongated (major:minor axis ratios 3:1 and 2:1 for batches 3 and 4, respectively) compared with the *Metroxylon* spp (Fig. 1C and D). Granules were either pear or cigar shaped and had a generally smooth outer surface with some shallow indentations. Many of the granules showed signs of damage, presumably resulting from processing.

In addition, a small, variable number of granules (< 1%) of batch 3, 4 and 5 samples had a honeycombed

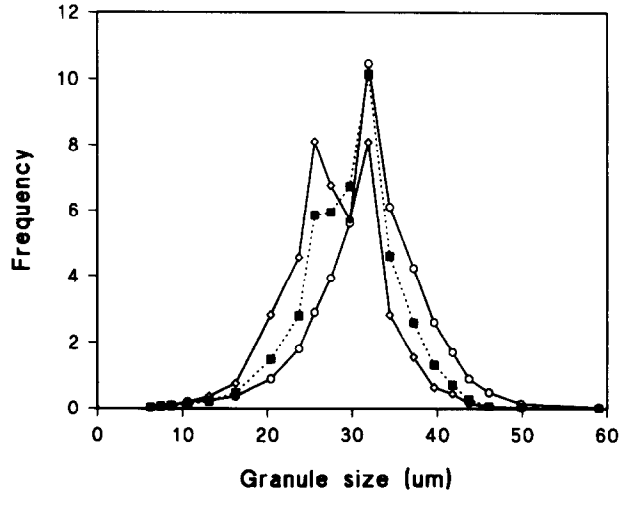

Fig. 2. Granule size distribution of batch 1 native (○) and hydrolysed residues: (◇), untreated and (■) treated. Hydrolysis was by an AMG and Termamyl mixture to about 67% digestion.

appearance (Fig. 1C). The precise cause of this is unclear but it could be the result of earlier endogenous enzyme activity or fungal/bacterial contamination.

The overall morphological appearance of the granules was not altered by pretreatment at pH 3·5 at 60°C (Fig. 1F) although there may have been more indentations on the surface of *Arenga* granules.

Hydrolysis of untreated granules

Granules of the five different batches were digested with glucoamylase, α-amylases and a mixture of these two types of enzymes. Batch 1 granules were poorly hydrolysed by single enzymes and despite a significant improvement, the mixed enzyme system was only able to attain a hydrolysis of about 85% after 8 days (Fig. 3). Similar observations were made for the other batches of starch from *Metroxylon* and *Arenga*.

Although the overall rate of hydrolysis differed with the enzyme systems used, the gross pattern of digestion of *Metroxylon* spp starch granules was similar irrespective of the enzyme(s) used. Isolated batch 1 starch granules incubated with amylases showed patterns of degradation such as surface erosion and crevassing (Fig. 4A). The size of a proportion of the granular population seemed to be much reduced after hydrolysis, those granules of reduced size looked to be abraded. After extended hydrolysis (67%) with a mixture of Termamyl and AMG, a greater variety of conformations was seen. The greater heterogeneity in granular shape may reflect breaking of the granule along fracture lines or possible shaving of the outer surfaces. Size distribution was broader with two major populations being evident, one centred at 25·5 μm the other at 32 μm (Fig. 2). The population of granules at 32 μm showed only slight surface erosion. It can be hypothesised that the two populations of granules exist with differing susceptibles to amylase action under these conditions. The progressive reduction in size as a consequence of shaving and breaking of the granules is also suggested by the light microscopy observations (data not presented).

Slight differences were evident between batches of sago starch following hydrolysis with a Termamyl and AMG mixture. Those batches obtained from *Metroxylon* showed surface erosion and occasional crevassing. This crevassing appeared to result in the breaking off of a portion of the granule. *Arenga* starch granules, on the other hand, showed some pitting with the exception of surface erosion and crevassing (Fig. 5A).

The enthalpy of gelatinization remained constant at about 15 J/g during the course of hydrolysis (Fig. 6). The onset temperature remained the same. The implication of this is that during hydrolysis under these conditions the amorphous regions are preferentially hydrolysed. Restricted access of the glycosidic bonds to α- amylase has been suggested previously (Govindasamy *et al.*, 1992).

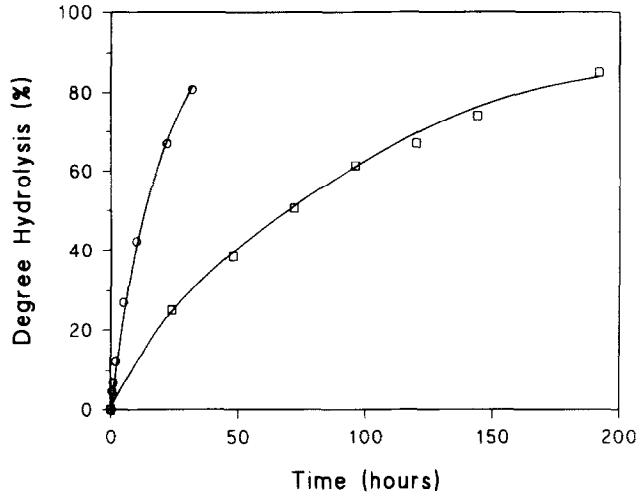

Fig. 3. Hydrolysis of untreated (□) and treated (○) batch 1 starch with a mixture of AMG and Termamyl at 35°C, pH 5·0.

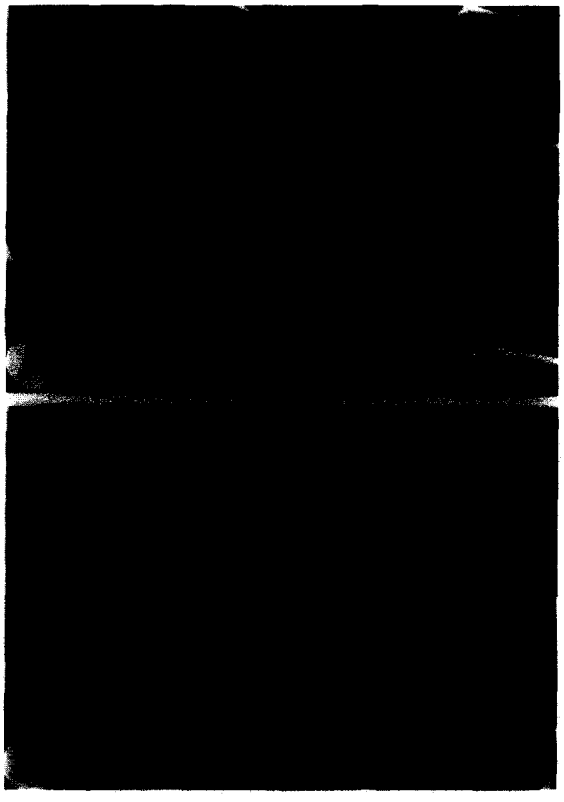

Fig. 4. SEM photomicrographs of untreated (A) and treated (B) batch 1 granule residues attacked by an AMG and Termamyl mixture at about 67% hydrolysis. Bar denotes 0·1 mm.

Hydrolysis of treated starch

Treatment of all starch batches prior to addition of the enzyme(s) improved hydrolysis dramatically (Fig. 3). The enthalpy of gelatinization was the same as that of native starch (Fig. 6), however, peak width was significantly decreased and peak temperature elevated, 70·1

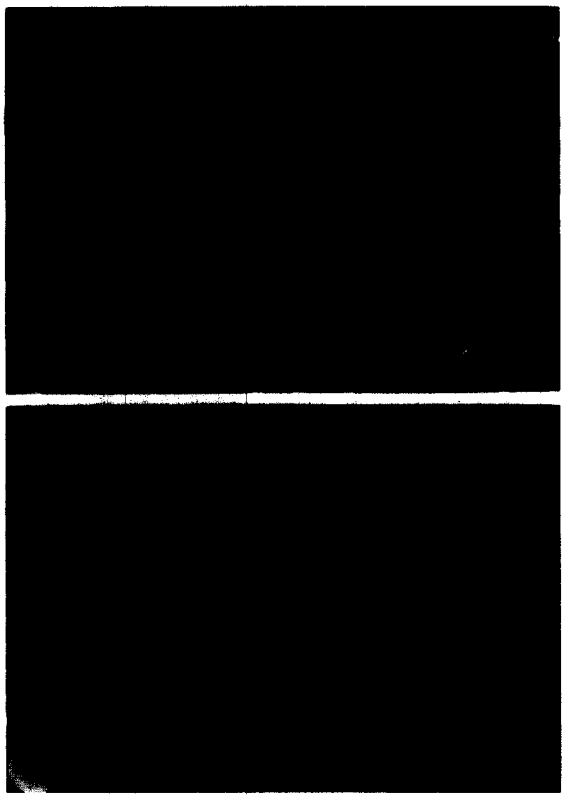

Fig. 5. SEM photomicrographs of batch 3 granule residues hydrolysed by a mixture of AMG and Termamyl. A, untreated after 24 h hydrolysis; B, treated after 5 h hydrolysis. Bar denotes 0·1 mm.

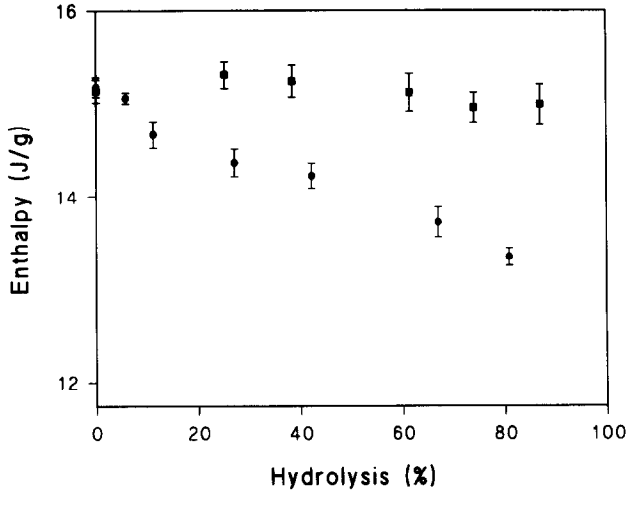

Fig. 6. Enthalpy of gelatinization of differing extents of hydrolysis for batch 1 untreated (■) and treated (●) granules. ±, standard error of mean.

and 71·7°C for native and treated granules, respectively (Fig. 7). Changes in the thermal properties of the granular material suggest modification to the internal architecture of the granule, the specifics of which are under review. One possibility is that the short time at 60°C allows an incomplete annealing process to occur whereby realignment of the starch chains in the amorphous regions occurs. The changes in thermal properties suggest that materials adhering to the outer surface of the granule may not be responsible for the poor hydrolysis of raw, non-treated granules.

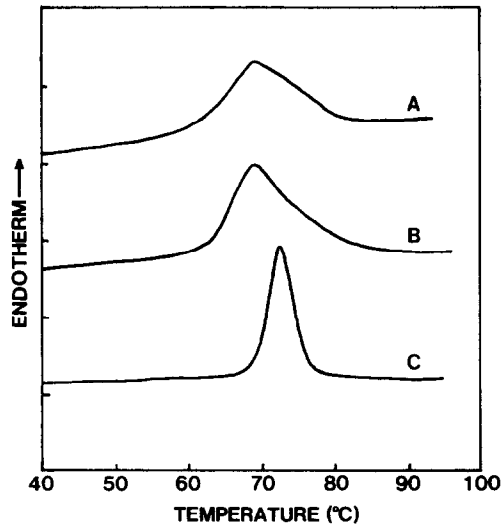

Fig. 7. Thermograms of batch 1 starch. Native granules (A) and digested residues of untreated (B) and treated (C) granules. Hydrolysis was by an AMG and Termamyl mixture to about 67% digestion.

Hydrolysis of all the batches after pretreatment occurred at single specific loci regardless of the enzyme or enzymes used (Figs 4B, 5B and 8; Table 3). The consequence of this hydrolysis was the development of a single deep round hole. Areas on the granule of obvious damage prior to hydrolysis did not correspond to the sites of enzyme attack. There was a noticeable pumice appearance on the surface of the granule which was more pronounced near the hole. The hole size at about 67% hydrolysis was similar for all the *Metroxylon* batches considered but the time to reach this extent of hydrolysis depended on the enzyme(s) used. The holes in *Arenga* granules tended to be smaller, more ragged and were located randomly on the granules. There was a greater extent of pumicing in both the treated batches of *Arenga* and some pitting was noted.

Irrespective of the enzyme used, single enzyme hydrolysis with commercial or purified amylase and glucoamylase resulted in holes of the same shape—rounded with smooth edges (Fig. 8). Double enzyme hydrolysis resulted in holes with ragged or serrated edges (Figs 4B and 5B). The production of smooth edges after single enzyme action may result from restricted access to the glycosidic bonds away from the immediate site of hydrolysis. In contrast the double enzyme systems, acting in a synergistic manner, attack portions of the surface which can be removed from the granule and hydrolysed *ex situ* (Fujii *et al.*, 1988).

In general, the pattern of hydrolysis appears to progress along a single pathway and the internal regions hydrolysed until at advanced states of hydrolysis only

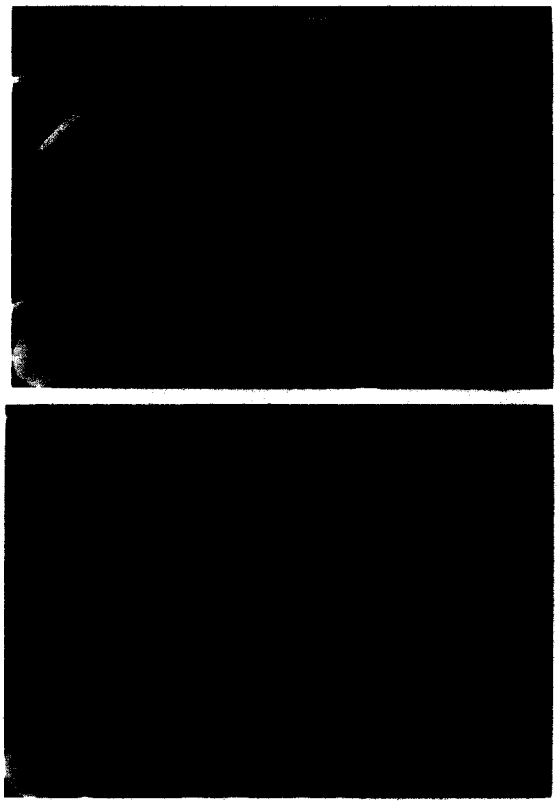

Fig. 8. SEM photomicrographs of treated batch 1 starch hydrolysed by AMG (A) and Sigma glucoamylase (B) after 32 h. Bar denotes 0·1 mm.

Table 3. Detection of the 'one deep round hole' hydrolysis pattern for different enzyme(s)/treated starch combinations

	Novo Nordisk enzyme							Sigma	Enzyme
Starch	B	F	T	A	A+B	A+F	A+T	SA	SG
Batch 1	D	D	D	D	D	D	D	D	D
Batch 2			D	D			D		
Batch 3			D	D			D		
Batch 4							D		
Batch 5							D		
Corn				N			N		
Tapioca				N			N		

Enzymes: A, AMG; B, Ban; F, Fungamyl; T, Termamyl; SA and SB, Sigma α-amylase and glucoamylase. D, detected, N, not detected.

an empty shell remains. This type of pattern for hydrolysis is similar to that seen for non-waxy rice granules hydrolysed with hog pancreatic α-amylases (Evers, 1979).

Non-treated and treated corn and tapioca starch granules were also hydrolysed with AMG and an AMG and Termamyl mixture. After pretreatment there is no obvious change of the hydrolysis pattern — random pitting and crevassing (Table 3).

After extensive hydrolysis, treated batch 1 granules were the same mean size (32 μm) as native starch granules (Fig. 2). There was, however, a small shoulder appearing at approximately 25·5 μm. This may result from fragmentation along original lines of weakness in partially digested granules.

The enzyme mixture attacks mostly the amorphous regions of the untreated starch granule. Regions within the granule were apparently more uniformly susceptible to enzyme digestion after pretreatment, suggested by the marked decrease of the enthalpy of gelatinization (ΔH) upon hydrolysis (Fig. 6). ΔH decreased by approximately 11% but weak width remained constant. We have used ΔH as an indicator of degree of molecular order for comparative purposes (Cooke & Gidley, 1992). We assumed that in both treated and untreated starches the extent of swelling of the granule was the same. The amounts of carbohydrate at any given point are the same. However, the times to achieve a similar extent of hydrolysis were markedly different. Prolonging incubation for the non-treated granules may have resulted in the use of greater quantities of amylose as substrate. Narrowing of the endotherm occurred in the treated sample but not for the untreated granules (Fig. 7). This may reflect the removal of both crystalline and amorphous regions in the treated starch granules.

In conclusion, the processing history of sago starch has a profound effect on the pattern and extent of hydrolysis. In contrast to the non-random path of hydrolysis in non-treated granules, pretreatment resulted in a less restricted hydrolysis.

We propose that the disparity in these observations and multiplicity of patterns reported for enzymatic hydrolysis may be a consequence of processing in addition to differences in granular architecture and enzyme preparations.

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REFERENCES

Bhat, U.R., Parmahans, S.V. & Tharanathan, R.N. (1983). Scanning electron microscopy of enzyme digested starch granules. *Starch/Starke*, **35**, 261–265.

Chen, L., Ford, C., Niklolov, Z., Reilly, P. & Svensson, B. (1988). Genetic analysis of the starch-binding region of *Aspergillus glucoamylase*. *Genome*, **30**, Suppl 1, 486.

Cooke, D. & Gidley, M.J. (1992). Loss of crystalline and molecular order during starch gelatinization: origin of the enthalpic transition. *Carbohydr. Res.*, **227**, 103–112.

Dettori-Campus, B.G., Priest, F.G. & Stark, J.R. (1992). Hydrolysis of starch granules by the amylase from *Bacillus stearothermophilus* NCA26. *Process Biochem.*, **27**, 17–21.

Dygert, S., Li, L.H. & Thoma, J.A. (1965). Determination of reducing sugars with improved precision. *Analyt. Biochem.*, **13,** 367–374.

Evers, A.D. (1979). Cereal starches and proteins. In *Food Microscopy*, ed. J.G. Vaughan. Academic Press, London, pp. 139–191.

Fogarty, W.M. & Kelly, C.T. (1990). Recent advances in microbial amylases. In *Microbial Enzymes and Biotechnology*, eds W.M. Fogarty & C.T. Kelly. Elsevier, London, pp. 71–132.

Fujii, M., Homma, T. & Tanigushi, M. (1988). Synergism of α-amylase and glucoamylase on hydrolysis of native starch granules. *Biotechnol. Bioengny*, **32,** 910–915.

Govindasamy, S., Oates, C.G. & Wong, H.A. (1992). Characterization of changes of sago starch components during hydrolysis by a thermostable alpha-amylase. *Carbohydr. Polym.*, **18,** 89–100.

Haska, N. & Ohta, Y. (1991). Glucose production from treated sago starch granules by raw starch digesting amylase from *Penicillium brunneum*. *Starch/Starke*, **43,** 102–107.

Haska, N. & Ohta, Y. (1993). Effect of cellulase addition on hydrolysis of sago starch granules by raw starch digesting amylase from *Penicillium brunnenum* No. 24. *Starch/Starke*, **45,** 237–241.

Hayashida, S., Teramoto, Y. & Inoue, T. (1988). Production and characteristics of raw potato starch digesting α-amylase from *Bacillus subtitis* 65. *Appl. Environ. Microbiol.*, **54,** 1516–1522.

Lee, G.M., Kim, C.H., Lee, K.J., Yusof, Z.A.M., Han, M.H. & Rhee, S.K. (1986). Simultaneous saccharification and ethanol fermentation of sago starch using immobilized *Zymomonas mobilis*. *J. Ferment. Technol.*, **64,** 293–297.

MacGregor, A.W. & Ballance, D.L. (1980). Hydrolysis of large and small starch granules from normal and waxy barley cultivars by alpha-amylases from barley malt. *Cereal Chem.*, **57,** 397–402.

Monma, M., Yamamoto, Y., Kagei, N. & Kainuma, K. (1989). Raw starch digestion by α-amylase and glucoamylase from *Chalara paradoxa*. *Starch/Starke*, **41,** 382–385.

Nagashima, T., Tada, S., Kitamoto, K., Gomi, K., Kumagai, C. & Toda, H. (1992). Site directed mutagenesis of catalytic active site residues of Taka amylase A. *Biosci. Biotechnol. Biochem.*, **56,** 207–210.

Punpeng, B., Nakata, Y., Goto, M., Teramoto, Y. & Hayashida, S. (1992). A novel raw starch digesting yeast alpha-amylase from *Lipomyces starkeyi* HN-606. *J. Ferment. Bioengng*, **73,** 108–111.

Tanaka, T., Ishimoto, E., Shimomura, Y., Taniguchi, M. & Oi, S. (1987). Purification and some properties of raw starch binding amylase of *Clostridium butyricum* T-7 isolated from mesophyllic methane sludge. *Agric. Biol. Chem.*, **51,** 399–405.