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Characterization of changes of sago starch components during hydrolysis by a thermostable alpha-amylase

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Apparent molecular weight profiles of native and hydrolysed sago starch were investigated. Components of sago starch were characterized by high-performance size-exclusion chromatography (HPSEC) and the relative peak areas of amylose and amylopectin calculated as 27·2 and 61·8%, respectively. Techniques to disperse molecularly the components of sago starch with minimal degradation were developed. Storage of samples at −20°C prior to analysis resulted in depolymerization. Polymer integrity was, however, maintained if samples were stored at 40°C in the presence of a suitable antimicrobial agent. The hydrolysis pattern of Termamyl 120L on sago starch was monitored using kinetic, HPSEC and scanning electron microscopy (SEM) studies. Kinetic studies showed that both K_m and V_{max} were temperature dependent; K_m at 90°C was lower than that at 40°C and the activity of 1 ml of Termamyl 120L solution on solubilized sago starch at 90°C, pH 6·0 and 30 ppm of Ca^{2+} was determined and found to hydrolyse an average of 7716 μequivalent glycosidic bonds of the starch per minute. Product spectra from HPSEC showed that the amylolysis was dependent on temperature, enzyme concentration, chain length and nature of substrate. SEM studies appeared to suggest that the enzyme tunnelled into the granular interior and then hydrolysed from within, along concentric rings.

INTRODUCTION

The sago palm is an agronomically important indigenous crop of the Southeast Asian and Oceanic regions, which has, until recently, remained unexploited (Flach, 1983). In recent years, however, the increasing rate of production of this starch has stimulated interest in its possible use for industrial processes. Products of hydrolysis, such as maltodextrins, corn syrup solids, glucose syrups and high-fructose syrups have wide applications in the food, textile, brewing and pharmaceutical industries (Griffin & Brooke, 1989). These products have mainly been derived from corn, barley or potato. It should be possible to obtain similar products from the processing of sago starch which could also include potential uses as ingredients for existing and/or new products.

The use of enzymes for the breakdown of starch has some distinct advantages over the less specific method of acid hydrolysis. Enzyme hydrolysis can be completed at relatively low temperatures and pressures whilst maintaining a high pH, thus minimizing the formation of undesirable components due to thermal effects and low pH (Griffin & Brooke, 1989). The comparative ease with which waste products of enzyme hydrolysis can be disposed of in contrast to those from acid hydrolysis would be an added advantage since the latter must first be neutralized prior to disposal (Yankov *et al.*, 1986).

The action of alpha-amylases has been the subject of numerous investigations and reports (Chang Rupp & Schwartz, 1988). It is therefore not surprising that the properties of these enzymes are well documented (Chang Rupp & Schwartz, 1988). In spite of this, detailed information relating to their mode of action is still incomplete, reflecting the wide variety of sources of alpha-amylase and the complex structure of starches (Chang Rupp & Schwartz, 1988). The morphology of starch granules, the molecular weights and the relative compositions of the component macromolecules amylose (Am) and amylopectin (Ap), are also known to

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differ according to the source. Such variations will ultimately affect the starch-amylase interaction (Bertoft & Henriksnas, 1982).

The distribution of products from starch hydrolysed by bacterial alpha-amylase is usually determined at temperatures lower than the optimum (Saito, 1973; Chiang *et al.*, 1979) or at temperatures similar to those employed industrially (Ramesh & Lonsane, 1989). The use of such limited temperatures is based on the belief that temperature does not affect product profiles (Ramesh & Lonsane, 1989). It is, however, to be expected that profiles obtained with conditions suitable for operation at the industrial scale will differ from those obtained with conditions employed during enzyme characterization studies (Ramesh & Lonsane, 1989).

Thus, the present investigation was undertaken to achieve a better understanding of sago starch hydrolysis by *Bacillus licheniformis* alpha-amylase at both conditions. The resulting dextran/oligosaccharide profiles were established using high-performance size-exclusion chromatography (HPSEC). Whilst HPSEC has many advantages in these characterization studies (Chang Rupp & Schwartz, 1988), problems associated with sample preparation have also been reported (Takagi & Hizukuri, 1984; Kobayashi *et al.*, 1985; Jackson *et al.*, 1988). Starches from different plant sources are known to behave differently reflecting the variable nature of their molecular conformations. The present study of the apparent molecular weight (MW) profiles of the native and hydrolysed sago starch therefore included the development of techniques for the molecular dispersion.

MATERIALS AND METHODS

Sago starch was obtained from a commercial producer, Wah Chang International Group of Companies. A commercial preparation of thermostable alpha-amylase (Termamyl 120L) was kindly donated by Novo Laboratories, Kuala Lumpur. The enzymatic preparation was reported to have an activity of 120 Kilo Novo alpha-amylase units (KNU), with one KNU being defined as the amount of enzyme necessary to break down 5·2 g of starch (Merck, Amylum soluble Erg. B.6, Batch 9947275) per hour to a non-iodine polymer under Novo's standard conditions (Anon., 1985). The malto-oligosaccharide and dextran standards were purchased from both Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan) and Sigma Chemical Co. (St Louis, MO). All other chemicals were of at least AR grade and obtained either from Merck, Darmstadt Co. or Sigma Chemical Co.

Analytical methods

Total carbohydrate was determined using a phenol-sulphuric acid procedure (Dubois *et al.*, 1956) and the reducing sugar assayed according to a modified Park-Johnson method (Hizukuri *et al.*, 1981) using D-glucose and D-maltose as the standards.

HPLC equipment

A Waters Associates (Milford, MA) series liquid chromatography system with a model 510 pump, WISP model 712 injector and a model 410 differential refractometer detector was used. The detector signal was electronically recorded and integrated by a Data Module Integrator Waters 746. The integrator was programmed to relate the response factors of the different oligosaccharides to their concentrations. The refractive index detector was set at a sensitivity of 128 and scale factor 30. The detector cell temperature was maintained at 40°C.

The WISP model 712 injector was preprogrammed to perform three injections of 50 μl of each sample. Three Ultrahydrogel HPSEC columns (dimensions of 7·8 mm i.d. × 30 cm) were connected in series. The columns included two Ultrahydrogel 120 and an Ultrahydrogel linear. The columns were maintained at 40°C. Samples were injected and eluted using a mobile phase of deionized water at a flow rate of 0·8 ml/min. The deionized water had been filtered with a Millipore filter of 0·22 μm pore size and degassed at room temperature with ULTRAsonik™ before use.

Oligosaccharide and dextran molecular weight standards (162–2 × 10^6) were dissolved in water and injected into the HPSEC system. A calibration plot was made using the relationship between retention time and log MW.

Sample preparation

The samples for HPLC injections were prepared using a modified method outlined by Jackson *et al.* (1988). Sago starch (0·2 g) was solubilized with 1 M NaOH (20 ml). Following purging with nitrogen, all tubes were subsequently stirred continuously at 25°C for 1 h. The pH was adjusted to 6·0 using 3 M acetic acid and the solution made to 50 ml (0·4%, w/v) with 100 mM acetate buffer (pH 6·0) containing 30 ppm Ca^{2+}. An aliquot of this starch suspension was dispersed by an ultrasonic processor (ULTRAsonik™ 300, Ney Co.) for 400 s. The solution was then subjected to centrifugation at 3400 *g* for 10 min. The supernatant was filtered through a Millipore filter (8·0 μm). The filtrate was diluted appropriately with deionized water and allowed to equilibrate at 40°C prior to HPSEC analysis.

Effect of ultrasonication

The effect of ultrasonication on the sago starch was determined by subjecting 1·0% solubilized starch

suspension to sonication for 0–800 s. At 80-s intervals, aliquots (25 μl) were removed and analysed by HPLC.

Effect of $HgCl_2$ on the storage of gelatinized sago starch

A mixture of equal volumes of 0·4 mM $HgCl_2$ and 0·4% gelatinized sago starch were incubated at 40°C for eight weeks. A control using 0·1 M acetate buffer (pH 6·0) instead of $HgCl_2$ was similarly treated. Aliquots (50 μl) of each were analysed by HPSEC analysis.

Effect of freezing on storage of gelatinized sago starch

Equal volumes of 0·4 mM $HgCl_2$ were added to two 5 ml aliquots of 1% gelatinized sago starch solution. One tube was stored at −20°C and the other at 40°C. After five days, the contents of both tubes were analysed using HPSEC.

Enzyme assays

Alpha-amylase activity (Termamyl 120L) was determined by assaying the reducing sugar released following hydrolysis of the sago starch. The standard assay involved incubating a reaction mixture, consisting of 4 ml (0·4%, w/v) substrate in 100 mM sodium acetate buffer, pH 6·0, 30 ppm Ca^{2+} and 20 μl of 100-fold diluted enzyme solution, for 15 min at either 40 or 90°C. The reaction was stopped by mixing an equal volume of 0·4 mM $HgCl_2$ and heating at 90°C for 20 min. The reducing sugars released were determined.

Determination of inhibitory potential of $HgCl_2$ at different temperatures

Enzyme assays were performed at a range of temperatures (0–90°C) in the presence of an equal volume of 0·1 mM $HgCl_2$. The reaction was stopped after 20 min following the addition of 165 μl of 5 M HCl and immersing the tubes in an ice bath. pH was adjusted to 6·0 with NaOH immediately before analysis.

Enzymatic hydrolysis on gelatinized sago starch

All solubilization of native sago starch was performed in 1 M NaOH in an oxygen-free environment as outlined previously. Aliquots (50 ml) of solubilized starch were inoculated with 450 μl of 10-fold diluted Termamyl 120L. The reaction mixtures were incubated at either 40 or 90°C. Aliquots (2·5 ml) were withdrawn at various intervals, mixed vigorously with equal volumes of 0·4 mM $HgCl_2$ and heated for 20 min at 90°C to stop the reaction. The tubes were cooled and the reducing and total sugars determined. The hydrolysis products were analysed by HPSEC.

Enzymatic hydrolysis on raw sago starch granules

Various amounts of raw sago starch granules (0·4–20%, w/v) were dispersed in 100 ml of 100 mM acetate buffer (pH 6·0) containing 30 ppm of Ca^{2+}. Solutions were incubated at either 40 or 90°C and stirred at 180 rpm. Enzymatic hydrolysis was initiated following addition of an appropriate amount of Termamyl 120L to the suspensions. Aliquots of the reaction mixtures were removed periodically, filtered immediately with a Millipore filter (8·0 μm) into an equal volume of 0·4 mM $HgCl_2$ and the reaction was stopped by heating at 90°C for 20 min. The reducing and total sugar contents were determined and the hydrolysis products analysed by HPSEC.

The solid residues were dispersed in distilled water, filtered, washed with water, dried under vacuum and subjected to scanning electron microscopy. The reducing sugar content of the filtrate provided an indication of the extent of starch granule degradation.

Scanning electron microscopy

The dried intact and degraded granules were sprinkled on to double-sided tape mounted on microscope stubs and coated with gold using Balzers SCD 004 sputter coater. Samples were examined on a scanning electron microscope (Phillips SEM 515) at an accelerating voltage of 10 kV. Photomicrographs were taken on AGFAPAN-APX 100 films.

RESULTS AND DISCUSSION

Dextran and maltooligosaccharide standards with known molecular weights (Hayashibara and Sigma Chemical Co.) were evaluated in the HPSEC. The elution times of these standards were used to determine the apparent MW of the components in the sample.

Figure 1 illustrates a typical HPSEC chromatogram of sago starch solubilized in 1 N NaOH. This profile depicts the intact native fractions and served as a reference throughout this investigation.

The profile shows two characteristic peaks, possibly representative of amylopectin and amylose. The first peak is probably that of amylopectin found to have a similar molecular weight range to that of those derived from potato and corn starches. It is likely that the second peak whose apparent MW was found to be about 8×10^5 was that of amylose. Similar MW values have been obtained for amylose of other starches (potato, maize, wheat, tapioca and waxy maize) determined by intrinsic viscosity and light scattering studies (Young, 1984).

The relative peak area for each component was 61·8% for amylopectin and 27·2% for amylose. The amylose content of sago starch agrees well with

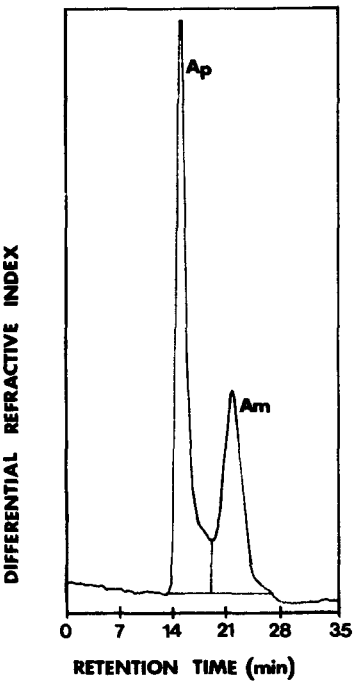

Fig. 1. Chromatogram of 0·4% (w/v) 1 N NaOH-solubilized sago starch, autoclaved for 15 min, sonicated for 400 s: amylopectin (Ap) and amylose (Am).

previously reported values of 24·4% (Takeda *et al.*, 1989), 21·7% (Kawabata *et al.*, 1984) and 26·1% (Takahashi, 1986) determined using iodine affinity studies. Occasionally, a smaller peak could be detected, which eluted immediately after amylopectin. Material of intermediate MW between Ap and Am has also been observed for corn starches characterized by HPSEC (Jackson *et al.*, 1988). The properties and structure of this intermediate fraction are being investigated.

Effect of ultrasonication

Ultrasonic vibrations are known to disrupt granule integrity, leading to a more complete dispersion of both Am and Ap. In addition, sonication will break down any amylose aggregates, thus slowing retrogradation (Jackson *et al.*, 1988).

Our results show that the amount of Ap liberated from sago starch increased with time reaching a maximum after 400 s (Fig. 2). Solubilization of Am, however, was not affected even after prolonged sonication of 800 s, indicating that the Am in native sago starch is readily solubilized in 1 N NaOH. Sonication for as long as 800 s did not cause either Am or Ap to depolymerize. On the other hand, for corn starch, Jackson *et al.* (1988) has demonstrated that a short sonication period of 20–80 s increased water solubility, whereas extended sonication of more than 80 s could result in depolymerization of Ap.

Effect of freezing on storage

Prior to HPLC injections, it is convenient to store samples. The temperature selected for storage was such that neither retrogradation nor bacterial degradation occurred.

HPSEC profiles of native starch stored at −20 and 40°C are shown in Fig. 3. The profile for samples stored at 40°C was similar to that of the reference except for the tailing of the Ap peak, indicating the presence of material of intermediate MW between Ap and Am (Jackson *et al.*, 1988). The Am peak was sharp. The presence of very small amounts of G6 was also detected. For samples stored at −20°C, retrogradation resulting from phase separations occurred. Retrogradation is evidenced by the loss of carbohydrate material upon filtration and the presence of separate Ap and Am peaks detected by HPSEC. The presence of increased amounts of G6 and the shifting of the retention times for both the Am and Ap peaks are suggestive of the breakdown of these components. Although the Ap peak had shifted only slightly in relation to the Am peak, small changes in retention

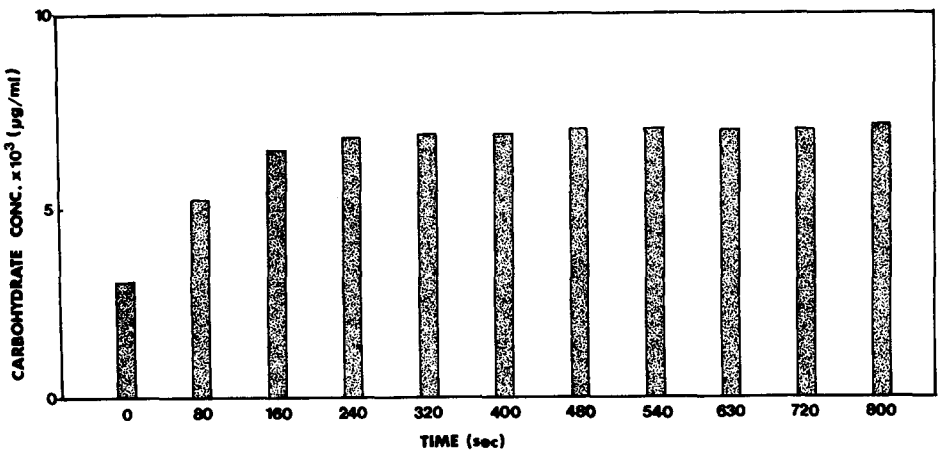

Fig. 2. Effect of ultrasonication on the solubilization of amylopectin (Ap) of NaOH-solubilized sago starch. The amounts had been determined from HPSEC chromatograms.

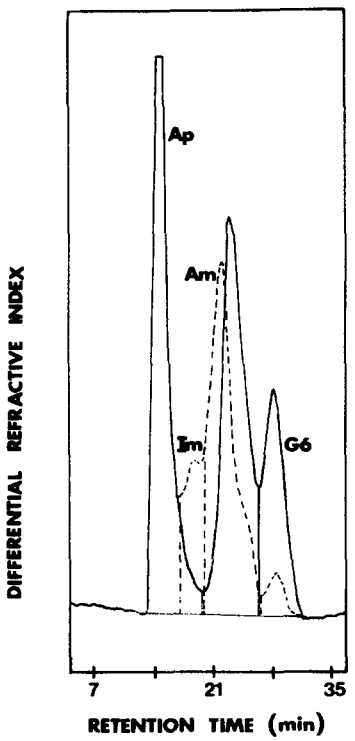

Fig. 3. Effect of temperature on the stability of amylopectin and amylose during storage: (———) −20°C, (– – – –) 40°C.

time at a higher MW range could well reflect a greater reduction in MW than a corresponding change at the lower MW range. Depolymerization of the starch components may have occurred as a consequence of breaking up of the retrograded material during filtration. Consequently, in view of polymer breakdown in samples stored at −20°C, the preferred temperature for storage of samples prior to HPSEC analysis was 40°C.

Effect of $HgCl_2$ on storage

Incubation of 0·4% gelatinized starch at 40°C over an eight-week period in the presence of $HgCl_2$ (0·4 mM) had no effect on its HPSEC profile (Table 1). In the absence of $HgCl_2$, both Ap and Am had been degraded into significant amounts of polymer of MW 10^4 and the oligosaccharide G7, and small amounts of G3. Low amounts of compounds of MW greater than Am were also detected and these were derived from the breakdown of Ap. It is interesting that the compound of 'intermediate' MW was comparatively stable with storage since there was no complete loss of this material.

Thus, whilst $HgCl_2$ probably inhibited the effects of microbial enzymes on the starch, it could also have had a dual function of deactivating alpha-amylases inherent in the starch granule.

Table 1. Effect of 0·1 mM $HgCl_2$ on the stability of amylopectin (Ap) and amylose (Am) fractions during extended storage at 40°C for 8 weeks

Component	With $HgCl_2$	Without $HgCl_2$
A	36·19	—
B	—	6·79
C	17·44	9·50
D	44·96	—
E	—	70·05
F	—	12·29
G	—	1·13

The percentage composition of each component was evaluated from HPSEC. (A) Ap; (B), (C) intermediate; (D) Am (MW = 10^5); (E) compound of MW = 10^4; (F) maltoheptaose (G7); (G) maltotriose (G3).

Enzyme assays

The activity of the enzyme Termamyl 120L from Novo Industri A/S was determined with potato amylose at 37°C and expressed in terms of commercial units KNU. Kinetic studies were carried out to relate the activity of this enzyme to that on sago starch at both 90 and 40°C as expressed in International units.

The Michaelis constants (K_m) and maximum velocities (V_{max}) for sago starch at both temperatures were determined from the Lineweaver-Burke plot (Fig. 4) and are shown in Table 2.

The K_m of the reaction at 90°C was relatively lower than that at 40°C, indicating a high enzyme-substrate affinity. Gelatinized starch in solution can exist in several physical forms other than as individual molecules. They could be trapped in granule remnants, entangled in gelled masses, or as recrystallized (retro-

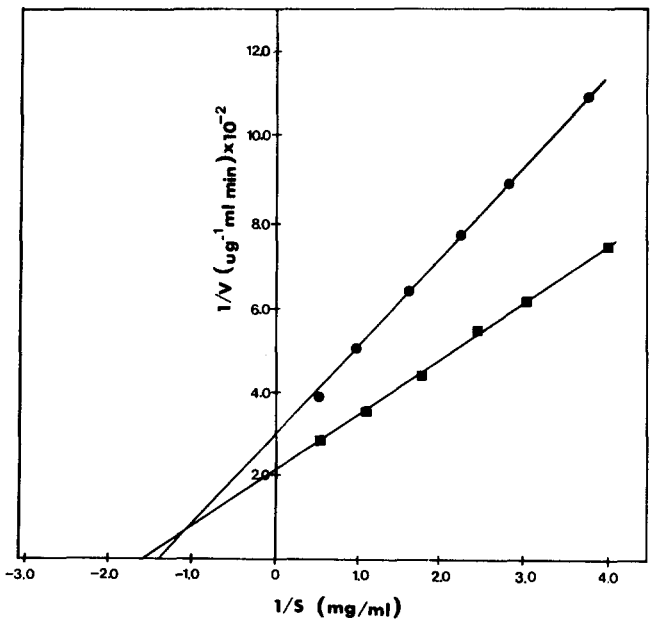

Fig. 4. Lineweaver-Burke plot for the hydrolysis of solubilized sago starch at 40°C (●———●) and 90°C (■———■) by 100-fold diluted Termamyl 120L, 0·1 M acetate buffer, pH 6·0, 30 ppm of Ca^{2+}.

Table 2. K_m and V_{max} values attained for the hydrolysis of solubilized sago starch at 40 and 90°C

Temperature (°C)	K_m (mg/ml)	V_{max} (μg/ml min)	Activity[a]
40	4·000	125	3858
90	3·077	250	7716

[a]Activity is expressed as μequivalents of glycosidic bonds of the starch per minute.

graded) polymers and perhaps as a combination of the above forms. Processing conditions are known to influence the distribution of such forms and consequently the 'water solubility' of the sample (Jackson *et al.*, 1990). Therefore, the higher affinity at 90°C could be due to an effect of the higher temperature on gelatinization as amylose and amylopectin chains still associated with the granules are relatively poor substrates.

At this temperature, pH 6 and 30 ppm Ca^{2+}, 1 ml of the enzyme Termamyl 120L solution was found to hydrolyse an average of 7716 μequivalent glycosidic bonds of the sago starch per minute.

Determination of inhibitory potential of $HgCl_2$ at different temperatures

Many alpha-amylases have been shown to be inhibited by the addition of 1 M acid (hydrochloric or acetic acids) (Manning *et al.*, 1961; Saito, 1973; Medda & Chandra, 1980; Morgan & Priest, 1981; Bajpai & Bajpai, 1989). Likewise, Termamyl 120L has also been shown to be inactivated by heating at 100°C at pH 3·7–3·9 for 6 min (Anon., 1985). However, inactivation of the enzymes under these conditions is undesirable as acid hydrolysis of the starch could occur during the inactivation step. As the purpose of the present investigation was to examine the effects of alpha-amylase hydrolysis on the structural changes of the sago starch granule in the absence of other contributing factors, the traditional procedure of acid hydrolysis at high temperatures was avoided.

Amylases in general are extremely sensitive to inactivation by heavy metals, especially copper (Myrback & Neumuller, 1950). Initial trials (not reported) showed that with $HgCl_2$, concentrations greater than 1·0 mM were necessary for inhibition of Termamyl 120L at 40°C. However an alpha-amylase isolated from *Thermus* sp. AMD33 has been reported to be 97% inhibited by only 0·1 mM Hg^{2+} at 40°C after 30 min incubation (Nakamura *et al.*, 1989).

In the authors' present study on Termamyl 120L, it was found that the enzyme was completely inhibited by a similar concentration of $HgCl_2$ but only at 90°C. The effect of 0·1 mM $HgCl_2$ on Termamyl 120L over the temperature range of 40–90°C is shown in Fig. 5.

Medda and Chandra (1980) have used $HgCl_2$ as an inhibitor at 110°C for reactions involving thermostable alpha-amylases from *B. licheniformis CUMC 305* and *B. coagulans CUMC 512*. It is possible that at high temperatures $HgCl_2$ was covalently bound to the enzyme or that the interaction itself was enhanced by the more frequent collisions between the enzyme and $HgCl_2$.

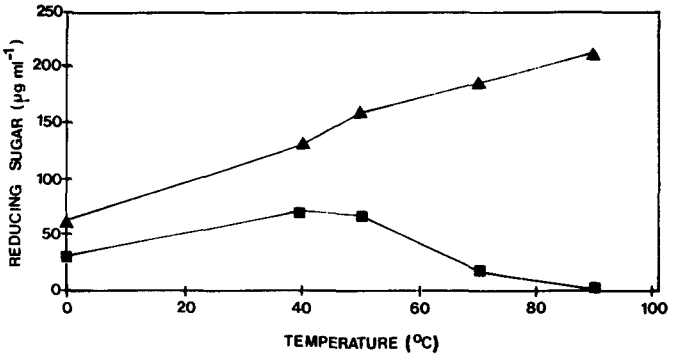

Fig. 5. Effect of temperature on the liberation of reducing end groups from sago starch (0·4%, w/v) hydrolyzed with Termamyl 120L (100-fold diluted) in the presence (■——■) or absence (▲——▲) of $HgCl_2$ (0·1 mM). Both systems were in acetate buffer (0·1 m, pH 6·0) and stabilized with 30 ppm Ca^{2+}.

Enzymatic hydrolysis of gelatinized sago starch

Time courses for hydrolysis of 0·4% gelatinized sago starch incubated at both 40 and 90°C are shown in Fig. 6. The rate of hydrolysis was temperature dependent and increased with time reaching a maximum after 30 min.

Product distributions

Product profiles of enzyme-hydrolysed starches are shown in Figs 7 and 8. The amount of each individual component was calculated from the HPLC chromatograms. For simplicity, these individual components have been categorized into three main groups based

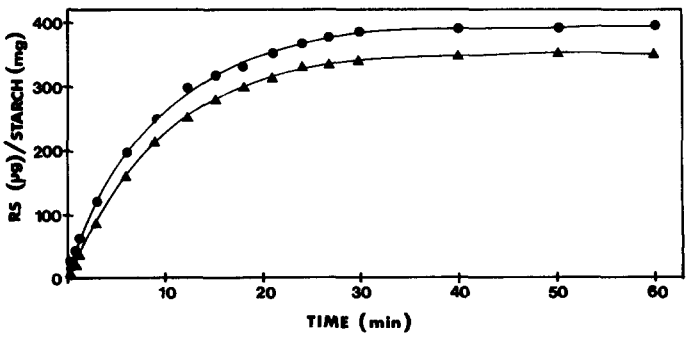

Fig. 6. Amount of reducing sugars (RS) produced by the hydrolysis of solubilized sago starch as a function of the incubation time by Termamyl 120L at 40°C (▲——▲) and 90°C (●——●).

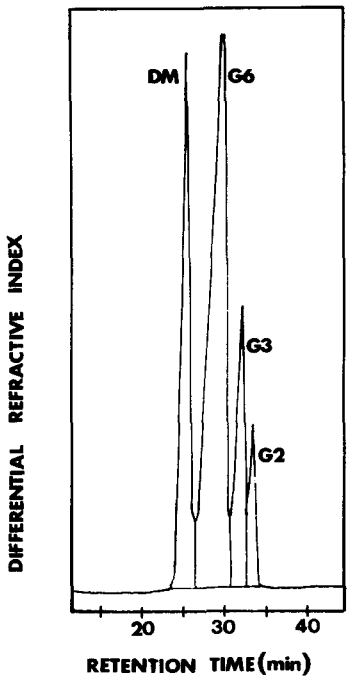

Fig. 7. A specimen chromatogram of sago starch (0·4%, w/v) hydrolysed with Termamyl 120L (10-fold diluted) for 6 min at 90°C: compound of MW = 10^4 (DM), maltohexaose (G6), maltotriose (G3) and maltose (G2).

upon their relative molecular weight derived from linear standards. Each of the main groups is further sub-divided as outlined:

(1) High molecular weight (HMW)
  (a) components with MW 10^7-10^{11}
  (b) components with MW 10^4-10^7
  (c) DP 16 oligosaccharides - material MW 10^4

(2) Intermediate MW (IMW)
  (a) oligosaccharide DP 12-15
  (b) oligosaccharide DP 8-11

(3) Low MW oligoosaccharides (LMW)
  (a) oligosaccharides DP 5-7
  (b) glucose-maltotetraose

The results are summarized as in points (a)-(h), as follows.

(a) At 0 min, the chromatogram was identical to that of the reference profile. After 30 s, hydrolysis of both amylose and amylopectin, although evident, was relatively mild with only a slight reduction in the molecular weight of both polysaccharides. Thus, during the early stages of hydrolysis, products of the HMW range of 10^7-10^{11} were largely present. These products were then utilized preferentially as substrates as depicted by the rapid decrease of such fractions. Detailed examination of the individual components in this HMW group showed that hydrolysis progressed relatively slower at 40°C than at 90°C from compounds of MW $10^{11} \rightarrow 10^{10} \rightarrow 10^9 \rightarrow 10^8 \rightarrow 10^4$.

At 90°C, hydrolysis was relatively faster and products were formed in a slightly different sequence from $10^{11} \rightarrow 10^{10} \rightarrow 10^6 \rightarrow 10^4$. Upon depletion of compounds of the first sub-group (10^{11}-10^7), the second group (10^7-10^4) was acted upon and rapidly hydrolysed to compounds of the third group G16-10^4.

(b) Increasing the temperature from 40 to 90°C resulted in a decrease in the level of HMW material by 41·4%, but at the end of 60 min the amounts of IMW and LMW components formed at the two temperatures were not significantly different.

(c) Resulting from the hydrolysis of HMW substrates, a compound of MW 10^4 was formed. This was further degraded only after prolonged incubation.

(d) One and a half minutes after the addition of alpha-amylase, amylopectin and amylose were totally hydrolysed. As degradation proceeded, well defined product profiles were formed, suggesting a non-random mode of action.

(e) At 40°C, the lower maltooligosaccharides accumulate gradually, G5-G7 occurring at a greater rate than G1-G4 from 15 to 27 min, after which extensive degradation occurred. At 90°C the pattern is similar but the accumulated material was degraded relatively faster.

(f) Incubation at 90°C resulted in the appearance of glucose after only 6 min whilst at lower temperatures glucose appeared after 9 min.

(g) The levels of G9 remaining after 60 min at both temperatures was indicative of the amount of alpha-limit dextrin produced.

(h) G4 could not be detected in any of the samples analysed.

The wide range of initial HMW products formed during hydrolysis of sago starch, confirms the concept of a two-stage hydrolytic process (Robyt & French, 1964). First, the rapid initial hydrolysis of the sago starch components resulted in a wide spectrum of oligosaccharide fractions, some of which are known to be favoured products of enzymatic action and occur in large quantities (G7 and G6) (Atkins & Kennedy, 1985*a*). The second stage involves hydrolysis of the most susceptible oligosaccharides to produce G5, G3, G2 and G1. G5 is the predominant compound with G1, G2 and G3 produced in much lesser amounts. Even after an hour of incubation, G5 remained in greatest amounts followed by G9, G2, G3 and finally G1. This is similar to the action of *B. licheniformis 584* alpha-amylase which also predominantly produced G5 (Saito, 1973).

Initially when sago starch is hydrolysed, G7 is the most prominent oligosaccharide but as the reaction progresses, G6 becomes the largest single fraction. This

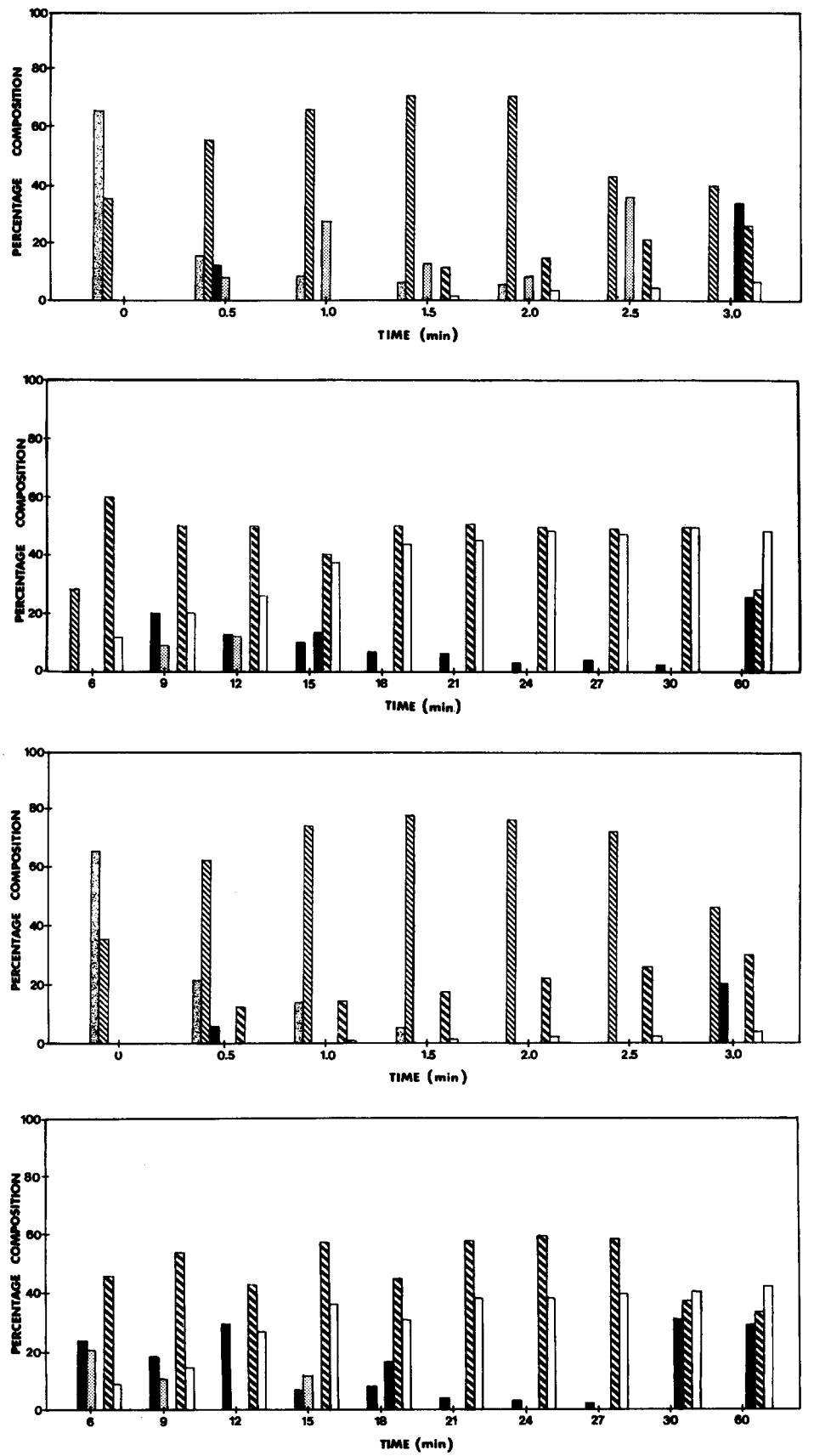

Fig. 8. Effect of temperature on the product profiles of enzyme hydrolysed sago starch (0·4%): (a, b) at 90°C, (c, d) at 40°C. Percentage composition of the following were calculated from HPSEC: 10^7–10^{11}, 10^4–10^7, DP 16–10^4, DP 12–15, DP 8–11, DP 5–7, DP 1–4.

shift from a molecular weight profile of material predominantly greater than G6 to that of material equal to or less than G6 is typical of the starch hydrolysis pattern of bacterial alpha-amylase (Atkins & Kennedy, 1985*a*).

Interestingly, G4 is virtually absent in the products formed by amylolysis of solubilized sago starch. This could be due either to the immediate hydrolysis of G4 to G2 + G2 and/or G1 + G3 (Saito, 1973) or to the complete absence of formation of this dextrin.

The time course for hydrolysis of sago starch shows that a plateau is reached after an initial linear rate. This plateau may be the region at which G7 is utilized as the substrate. Also at this stage, glucose is also produced which is known to inhibit Termamyl (Yankov *et al.*, 1986). The profile at 40°C is similar to that obtained at 90°C, except that upon reaching a maximum at 30 min, there is a noticeable accumulation of G6 and G5 compounds. These dextrins may not be as susceptible as the HMW material or G7 towards hydrolysis.

Hydrolysis of native granules

Figure 9 indicates the extent of hydrolysis of native starch granule suspensions (0·4–20%) at 40°C. It is apparent that the extent of hydrolysis was consistently low especially in relation to that reported for wheat, corn and maize, which had been hydrolysed with an alpha-amylase of *Chalara paradoxa*, a black mould isolated from sago starch granules (Kainuma, 1986).

The extent of starch hydrolysis increased slightly with substrate concentration. A 10-fold increase in the Termamyl 120L concentration resulted in the doubling of hydrolysis.

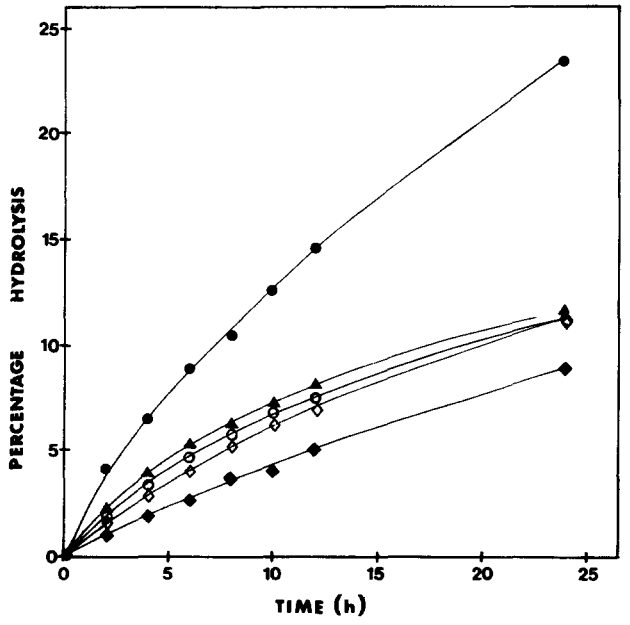

Fig. 9. Extent of hydrolysis of different concentrations of raw starch granules by Termamyl 120L. (♦——♦) 0·4% with 10-fold diluted enzyme, (●——●) 0·4% with undiluted enzyme, (◇——◇) 2·0%, (○——○) 10·0%, (▲——▲) 20·0%.

Raw starch hydrolysis conducted at 90°C further emphasized the fact that native sago starch granules were poor substrates for enzyme hydrolysis compared with gelatinized substrate. Again the rate of hydrolysis was observed to be dependent on the enzyme concentration. Hydrolysis of 60% was achieved with 10-fold dilution of the enzyme solution (771·6 μequivalent glycosidic bonds per minute) as compared with 90% with the undiluted enzyme solution after five hours' incubation with 0·4% substrate.

Product distribution

Product distribution in the incubation medium was found to be dependent on temperature and enzyme concentration. Reactivity of dispersions of native starch granules (0·4%) with low concentration of enzyme (10-fold diluted) at 40°C resulted in the following products: G9 (8·68%), G5 (9·33%), G3 (17·43%) and G2 (64·56%), whilst hydrolysis with a high enzyme concentration (undiluted) brought about a predominance of G3 (46·74%), followed by G2 (32·69%), G14 (10·76%) and finally G5 (9·81%) (Table 3). G9 was virtually absent. The high levels of G3 produced suggested a more extensive breakdown of initial products of granule hydrolysis.

Surprisingly, at the low substrate concentration (0·4%) free glucose could not be detected throughout the 24 h incubation but was found to be present if a higher substrate concentration (2–20%) was used. This phenomenon probably reflects that the K_m for the production of glucose is high. Thus, at high substrate concentrations productive complexes between the enzyme and substrate could be formed resulting in glucose production. The different substrate concentrations exhibited a general trend, as listed in points (a) and (b).

Table 3. Percentage composition of each individual oligosaccharide evaluated from HPSEC following hydrolysis of raw starch granule by Termamyl 120L at 40°C (24 h sample) and 90°C (5 h sample)

Oligosaccharide	40°C		90°C	
	E1	E2	E1	E2
G14	—	10·76	—	—
G9	8·68	—	—	—
G7	—	—	—	18·74
G6	—	—	42·50	—
G5	9·33	9·81	—	—
G4	—	—	9·18	—
G3	17·43	46·74	—	—
G2	64·56	32·69	48·30	81·26
G1	—	—	—	—

(a) The increasing levels of G1, G2, G3 and G5 with the progression of the hydrolytic reaction denoted that these products were not susceptible to further enzymatic attack. The enzyme was preferentially using the high MW polymers associated with starch granule as the substrate.
(b) Higher levels of the smaller oligosaccharides G1, G2 and incompletely degraded granules were detected in relation to the low levels of G3, G5, G7 and G9. Atkins and Kennedy (1985*b*) suggested that such an oligosaccharide profile would reflect the fact that the starch was highly branched. A highly branched starch structure would then tend to restrict the accessibility of the substrate to alpha-amylase resulting in an increased rate of hydrolysis of the otherwise less favoured substrates G7 and G8 (Atkins & Kennedy, 1985*b*). On the other hand, hydrolysis of gelatinized starch resulted in higher levels of G5, G9 and G3 than G1, G2 and undegraded polysaccharides. Current studies are under way to elucidate this phenomenon.

At 90°C following five hours of reaction, G6 (42·5%), G4 (9·18%) and G2 (48·3%) were detected whilst G3 and G5 were virtually absent. An increase in enzyme concentration brought about a shift in the product profile with the major oligosaccharides detected being G7 (18·74%) and G2 (81·26%) (Table 3). On examination of the products at hourly intervals it could be seen that Ap was rapidly degraded within the first hour resulting in the formation of intermediate products larger than amylose and the oligosaccharide G7. On the other hand, Am was hydrolysed at a much slower rate and was completely degraded only after 2 h of incubation.

In agreement with earlier reports by Sargeant and Walker (1978) and MacGregor and Morgan (1986), maltose was found to be a major end-product of hydrolysis of the starch granule. As the overall hydrolysis of granules is relatively low such high levels of maltose could only have arisen from other sources.

The possible explanations for the significant effects of temperature and enzyme concentrations on the product profiles might be due to various mechanisms such as a shift in the route of substrate hydrolysis and the alterations in the enzyme–substrate complexes (Ramesh & Lonsane, 1989).

A number of factors may well be responsible for the low levels of enzyme hydrolysis of sago starch granules, namely:

(a) The alpha-amylase is unable to attain access to the granular components. The enzyme may be restricted by the specific molecular orientation of the component starch chains at the granular surface, extensive degree of crystallinity, tight packing or the presence of minor components which may be 'coating' the surface of the granule (Bowler *et al.*, 1985).
(b) There is a limited surface available for adsorption. This may reflect the comparatively small size of the granule or the fact that only a few suitable chain segments are available as this enzyme hydrolyses only alpha-1,4 linkages which are remote from both the chain end and the branch points.

Scanning electron microscopy

Morphological changes in sago starch granules resulting from the action of Termamyl 120L were studied using SEM. There were pits, pin-holes and craters observed on the surface of the granules after 2 h of incubation with the enzyme but the pitting was more extensive only after 4 h (Fig. 10(b)). Though the pits were not all equal in depth and width, these characteristic dish-like depressions were evenly distributed on the surface. The exterior surface was rough as a result of mild surface erosion over the whole surface, although penetration seemed confined to certain areas as reported for wheat (Evers *et al.*, 1971). On closer examination of the action of the enzyme on the granules after prolonged period of degradation (24 h), it was observed that the enzyme appeared to tunnel into the granular interior along the equatorial regions before hydrolysing the granule from within along concentric rings (Fig. 10(c)).

CONCLUSION

Native sago starch granules were found to be a poor substrate for the thermostable alpha-amylase investigated at a temperature lower than its gelatinization temperature. There is therefore a need to identify factors responsible for the lowered hydrolysis rate especially with a view to utilize the abundant starch source for industrial applications. Further structural studies have to be carried out to relate the structure of the starch components to the functional properties of the enzyme. The role of the minor components on the hydrolysis rate could perhaps be verified by incorporating mixed enzyme systems. The product spectra data, obtained for the raw starch granule hydrolysis, seem to indicate that the starch could be highly branched. Hence, the concomitant use of a debranching enzyme together with Termamyl 120L would probably improve the rate of hydrolysis.

Although the sago starch is a poor substrate for Termamyl 120L, judicious selection of the appropriate enzyme parameters, together with the blending of hydrolysates obtained under various conditions, could provide desirable products with specific characteristics.

HPSEC has proved to be a valuable technique in the characterization of the hydrolysis products of sago starch following incubation with alpha-amylase. This

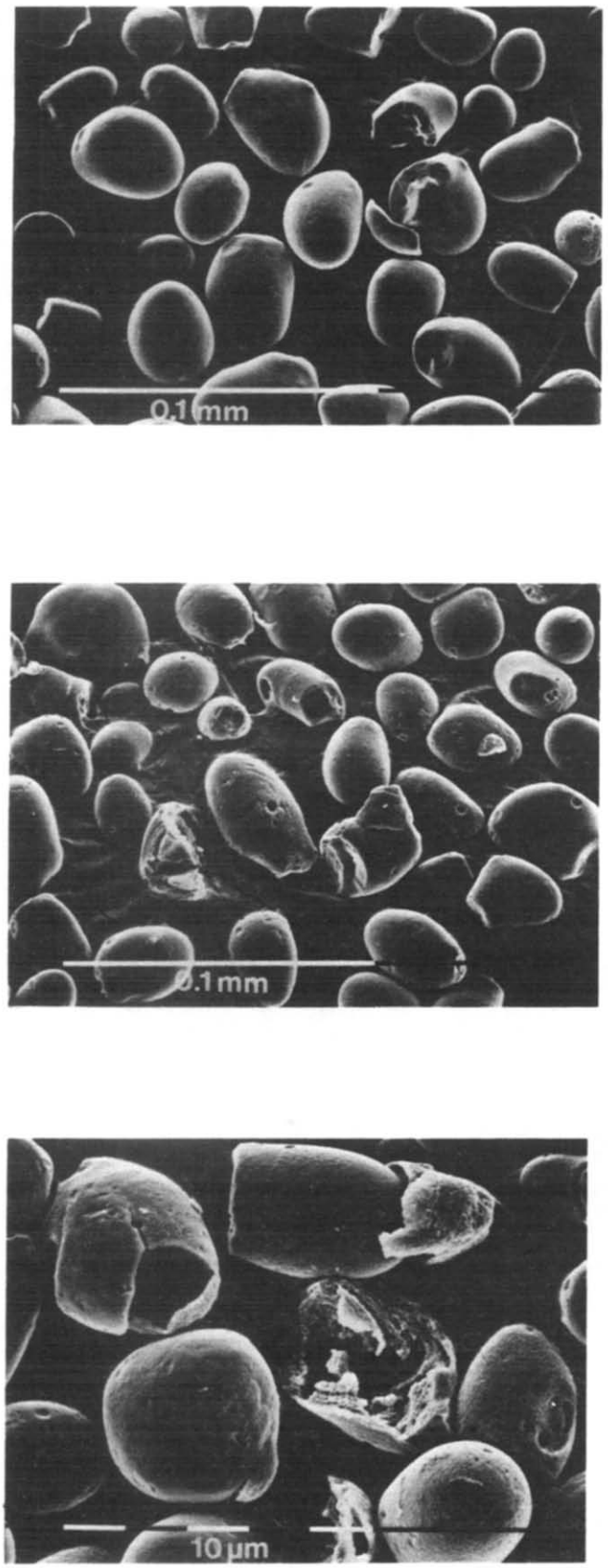

Fig. 10. Scanning electron photomicrograph of the intact and degraded sago starch granules. (a) Intact granules (0 h); (b) degraded granules after 4 h incubation with Termamyl 120L; (c) after 24 h hydrolysis.

technique could have potentials for the monitoring of products of a specified oligosaccharide composition.

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