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Enzymatic hydrolysis of sago starch

S. Gorinstein[a]

[a] *Department of Pharmaceutical Chemistry, School of Pharmacy, The Hebrew University of Jerusalem, P.O.Box 12065, Jerusalem 91120, Israel*

C. G. Oates[b]

[b] *Department of Biochemistry, Faculty of Medicine, National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511*

Sh.-M. Chang[c] **& Ch.-Yi. Lii**[c]

[c] *Institute of Chemistry, Academia Sinica, Nankang, Taipei, Taiwan 11529, Republic of China*

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Various enzyme hydrolysis procedures were employed in this study. A kinetic model presents sago starch hydrolysis by *Bacillus licheniformis* α-amylase (Termamyl 120L), *Bacillus subtilis* β-glucanase (Cerefio 200L) and by *Bacillus* pullulanase (Promozyme 200L) at temperatures of 90°C and 60°C and substrate concentration of 15% solids. Quantitation of pH, temperature and enzyme dosage in different combinations was used in standard statistical techniques of experimental design and data analysis. Determination of the degree of hydrolysis was based on freezing point depression osmometry, high-performance size-exclusion chromatography (HPSEC), differential scanning calorimetry (DSC) and X-ray diffraction methods. Computer implementation of the model allows graphical evaluation and prediction in improvement of the conditions of enzyme hydrolysis of sago-resistant starch. Liquefaction and saccharification of sago starch were performed to establish the optimum conditions of enzymatic hydrolysis in comparison with other starches. Enzyme treatment resulted in a decrease in the degree of crystallinity of all hydrolysed starch samples. C-type crystals completely disappeared at 100°C.

INTRODUCTION

Enzyme hydrolysis of potato, sweet potato and cassava starches and other starches with thermostable endo-amylase has been described in many research investigations (Gallant *et al.*, 1982; Ostergaard, 1982; Kajiwara & Maeda, 1983; Franco *et al.*, 1987; Abraham *et al.*, 1988; Nebesny, 1989; Gorinstein & Lii, 1992; Gorinstein, 1993). Some of these studies used combinations of α-amylase with debranching enzymes, such as pullulanase and amyloglucosidase after achieving the liquefaction stage for saccharification (Lorenz & Kulp, 1982; Ostergaard, 1982; Novais *et al.*, 1984; Atkins & Kennedy, 1985; Slominska & Maczynski, 1985; Fumihide *et al.*, 1987; Przybyt & Sugier,1988). The fact that starch from different sources has proved acceptable for an enzyme hydrolysis process in the production of glucose suggests the possibility of applying a more efficient technology for new starch sources, such as sago starch (Alagarataam, 1977; Baker, 1980). Some preliminary kinetic studies on starch enzymatic hydrolysis were conducted in our previous investigations (Gorinstein, 1986; Govindasamy, *et al.*, 1992; Gorinstein, 1993). The conversion of cassava and potato starches and other starches into ethanol has attracted the attention of many researchers worldwide (Srikanta *et al.*, 1989), but few among them have been interested in the saccharide products. Consequently, an attempt was made to develop a process for sago starch hydrolysis for glucose production. The raw sago starch employed in our studies differs in chemical composition and physical properties from potato, cassava and amaranth starches with respect to fat, nitrogen and carbohydrate contents, gelatinization temperature, swelling power, solubility, water-binding capacity and amylograph consistency (Gorinstein & Lii, 1992; Govindasamy, *et al.*, 1992). Therefore our new approach included (a) investigating the suitability of sago starch for the production of glucose and (b) establishing the optimum conditions for enzymatic hydrolysis of enzyme-resistant sago starch.

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MATERIALS AND METHODS

Commercial sago starch was purchased from Wahchang Starch Products PTE Ltd, Singapore. The following enzymes were used in this study. Termamyl 120L, a thermostable bacterial α-amylase from *Bacillus licheniformis*, activity 120 KNU/g; Cereflo 200L, a bacterial β-glucanase from *Bacillus subtilis*, activity 200 BGU/g; and Promozyme 200L, a pullulanase from a novel species of *Bacillus*, activity 200 PUN/g, produced by Novo-Industria/s, Denmark. One kilo Novo α-amylase unit (1 KNU) is the amount of enzyme which breaks down 5.26 g starch per hour. One β-glucanase unit (BGU) is the amount of enzyme which, under standard conditions, degrades barley β-glucan to reducing carbohydrates with a reduction power corresponding to 1 μmol glucose per minute. One pullulanase unit Novo (PUN) is defined as the amount of enzyme which, under standard conditions, hydrolyses pullulan, liberating reducing carbohydrate with a reducing power equivalent to 1 μmol glucose per minute. Amyloglucosidase from *Aspergillus niger*, activity 5000–8000 units/ml was purchased from Sigma Chemical Co.

The starch samples were processed according to the methods of Franco *et al.* (1987), Nebesny (1989) and Sievert and Pomeranz (1990) with some changes and modifications of conditions of starch hydrolysis and enzymes. The following methods for starch hydrolysis were compared:

Method 1. 15% (w/w) of sago starch was mixed with 0.1 M acetate buffer at pH 6, containing 30 ppm Ca^{2+} ions and 0.1% (w/w) of Termamyl 120L. The reaction mixture was kept at 90°C for 5 min. Then the temperature was lowered to 60°C for 2 min.

Method 2. Samples were treated as described under method 1, but after 35 min (5 min at 90°C and 30 min at 60°C) 0.2% (w/w) of Cereflo 200L was added.

Method 3. Samples were treated as described under method 2, but after 35 min 0.4% (w/w) of Promozyme 200L was added.

Method 4. Samples were treated as described under method 2, but after 35 min 0.2% (w/w) Cereflo 200L and 0.4% (w/w) Promozyme 200L were added.

Method 5. Samples were treated as described under method 4, but after 45 min (15 min at 90°C and 30 min at 60°C) 0.2% (w/w) Cereflo 200L and 0.4% (w/w) Promozyme 200L were added.

In each of the five methods the reaction continued for 5 h after the addition of the enzymes. Substrates collected from samples undergoing each of the five methods were saccharified with amyloglucosidase at 60°C for 30 min at pH 4.5.

The equipment, previously described by Henderson and Teague (1988), and modified to fit our special needs for determining the optimal conditions of starch hydrolysis for the five methods shown here consist of a 1-litre reaction vessel (Exelo, United Scientific Co (Q–Q; F91/5)) with a flat flange lid, five necks F92/3 and a coil condenser 19/26, joint C18/1 and stirrer SS2 (Stuart Scientific, UK). Heat was supplied by a digital hot plate enclosed in a sand bath (Model 721-2; PMC Industries Inc., USA). The temperature was determined with a platinum probe, and pH was measured with a pH probe. Samples were taken from one of the necks using a pipette. Small-quantity substrate experiments were carried out in flasks, using the same conditions as in the sealed reaction vessel. During the incubation period, the dispersions were kept in motion in a rotatory shaker at 200 rev/min. Periodically, the incubated starch dispersions were removed after the first 15 min and then at intervals of 30 min during the 3.5 h of reaction. Later, the reaction hydrolysates were removed, freezed, freeze-dried and passed through a 60-mesh screen.

Table 1. Values assigned to process variables

Process variables	Coded level				
	−1·68	−1	0	1	1·68
X_1 pH	4·5	5·0	5·5	6·0	6·5
X_2 [Promozyme], w/w	0·05	0·25	0·45	0·65	0·85
X_3 [Cereflo], w/w	0·05	0·15	0·25	0·35	0·45

The treatments for determination of the parameters involved in the enzymatic degradation of sago starch by the five methods are given in Table 1. Concentration of Termamyl 0.1%(w/w), temperature 90°C for 15 min and 60°C for 3.5 h and 15% dry substance (starch) were kept constant during all 18 experiments. These represent a three-variable, five-level central composite (Table 2) response-surface methodology design, described by Cochran and Cox (1957).

Ranges for independent variables were determined from the initial experimental trial. Statistical calculation was achieved using a microcomputer (Toshiba T 1600) employing statistical package Statgraphics (Statistical Graphics Corporation, USA). Stepwise regression (forward and backward) using a least-squares fit procedure was performed on the data (Box *et al.*, 1978). Significant variables ($p < 0.005$) were graphically displayed by response-surface plotting using second-order polynomial functions. The extent of the degradation of the starch granules was determined by the percentage of hydrolysis through dextrose equivalent (DE) values using an osmometry method of Fitton (1979).

Osmolalities (mOs/kg, where mOs is milli osmality) of hydrolysed samples were detected with a calibrated osmometer (Model 5004 from Precision System Inc., 16 Tech. Circle Natick MA, USA) using triplicate aliquots of 50μl. Instrument readings of molar concentration of glucose and glucose polymers at 15% dry substance (DS) were plotted against the DE previously calculated on a dry weight basis. The theoretical DE values were as follows: D-glucose (100); maltose (52.6); maltotriose (35.7); maltotetraose (27.0); maltopentaose (21.7); maltohexaose (19.2) and maltoheptaose (16.1). Then, sample DE was defined from the standard curve. This procedure gave corresponding dextrose

Table 2. Composite design employed in study

Independent variables			Dependent variables				
pH X_1	[Promozyme] X_2	[Cereflo] X_3	Solubility (%)	Oligosaccharide HPLC (%)			Enthalpy (J/g)
				G3	G5	G7	
−1	−1	−1	78·9	50·9	33·6	15·5	0·65
−1	1	1	80·0	65·8	34·2	—	0·50
1	−1	1	75·2	—	82·0	18·0	0·64
1	1	−1	77·8	47·5	37·5	15·0	0·67
0	0	0	77·9	44·8	38·3	16·9	2·58
0	0	0	80·5	—	100	—	2·82
−1	−1	1	76·6	—	100	—	1·62
−1	1	−1	71·5	47·8	35·1	17·1	0·77
1	−1	−1	71·8	—	100	—	1·59
1	1	1	77·5	46·4	36·8	16·8	0·84
0	0	0	76·9	—	80·0	20·0	2·38
0	0	0	76·9	—	79·6	20·4	2·81
1·68	0	0	78·9	—	79·2	20·8	3·55
−1·68	0	0	75·1	—	100	—	—
0	1·68	0	75·9	20·0	80·2	—	0·71
0	−1·68	0	76·8	—	79·0	21·0	2·65
0	0	1·68	81·5	—	79·5	20·5	1·43
0	0	−1·68	76·4	—	87·9	12·1	1·85

equivalent values of 15–25% dry substance (Fitton, 1979; Henderson & Teague, 1988). No correction was made for ash, but the readings for the standard curve were done using the acetate buffer with 30 ppm Ca^{2+} under the same conditions as the experiment. Dry substance of hydrolysed samples was determined by freeze-drying. For samples not at 15% DS, a correction of 0.09 DE was subtracted (added) for each DS percentage point over (below) 15% DS.

Carbohydrates were determined by the phenol-sulphuric acid method (Dubois *et al.*, 1956). The solubility of hydrolysed samples was calculated on dry weight of substance. The oligosaccharide profile was obtained by HPLC analysis (Waters HPLC System with Waters 712 WISP autosampler) on three Ultrahydrogel HPSEC columns, using H_2O as eluent at a flow rate of 0.5 ml/min with an RI detector. The columns were connected in series and included two Ultra-hydrogel 120 columns and an Ultra-hydrogel linear column. Column and detector temperatures were 40°C; injection volume was 50 μl. The calibration of oligosaccharides was made from G1 to G7. A differential scanning calorimeter (Perkin-Elmer DSC 7) was used to determine the gelatinization temperature of the starches. A 60% starch suspension was prepared. Samples of 4 mg were hermetically sealed in aluminium pans and heated from 20°C to 100°C at rate 10°C/min, according to the method of Chinachoti *et al.* (1990).

X-ray diffractograms of raw and hydrolysed starches were recorded by a Rigaku (MAX-IIIA, Rigaku Keisoku Co., Ltd, Japan) powder X-ray diffractometer according to Hizukuri's (1978) method. The X-ray, CuKα, irradiation was performed with a monochrometer. The operating conditions were: voltage, 35 kV; current, 25 mA; scanning speed, 6°C/min; chart speed, 2 mm/min; and time constant, 4 s. Samples were densely packed on a glass plate using an aluminium frame. Values of intensity were read from the curves over the angular range 4–30°, which includes most of the crystalline peaks. The *d*-spacings were computed by Bragg's law from $\lambda = 2d\sin\theta$, where λ is the wavelength of the X-ray beam (= 1.5405 Å), d is the spacing between the unit cell edges of the crystal being studied, and θ is the angle of diffraction.

RESULTS AND DISCUSSION

Figure 1 shows the effect of enzyme concentration in different combinations on dextrose equivalent during hydrolysis of sago starch (DE versus time). The lowest hydrolysis extent was observed when only Termamyl

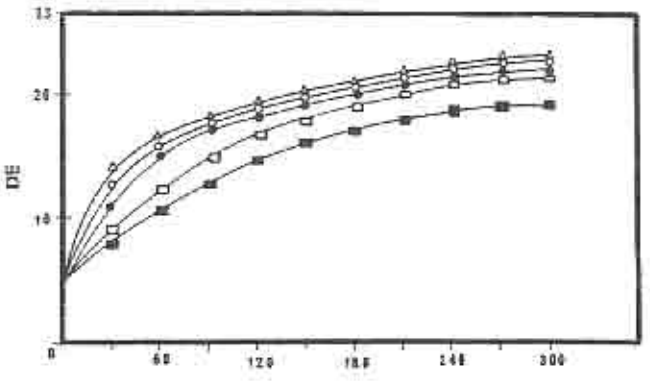

Fig. 1. Effect of different enzyme dosages on dextrose equivalent (DE) during sago starch hydrolysis. □ 0.1% Termamyl—method 1; ● 0.1% Termamyl and 0.2% Cereflo—method 2; ■ 0.1% Termamyl and 0.4% Promozyme—method 3; ○ 0.1% Termamyl, 0.2% Cereflo and 0.4% Promozyme method 4; Δ 0.1% Termamyl, 0.2% Cereflo and 0.4% Promozyme—method 5.

Table 3. Significant terms in regression analysis

[Cereflo] < 0·05
$[Cereflo]^2$ < 0·05
pH < 0·01
pH^2 < 0·01
[Cereflo] × pH < 0·05

For DE overall model, $p < 0{\cdot}01$, $R^2 = 77{\cdot}5$.

was used. The combination of Termamyl and Cereflo resulted in a higher degree of hydrolysis than that of Termamyl and Promozyme. The combination of Termamyl, Cereflo and Promozyme provided higher hydrolysis than that of Termamyl and Cereflo. The hydrolysis rate reached practically a plateau region after 180 min.

These results are in agreement with those of Robyt and Whelan (1968), Ishihara *et al.* (1982) and Griffin and Brooks (1989). Table 3 summarises the significant linear quadratic and interactive effects of the process variables on the dependent variables. DE and pH are the major contributors in the hydrolysed starch samples, and Cereflo concentration significantly affected DE. The following regression equation was derived.

$$DE = 21.24 - 0.50X_1 + 1.17X_2 - 0.40X_1^2 - 1.18X_2^2 + 0.59X_1X_2$$

Overall R^2 was comparatively low (77.5%) suggesting that the variables employed did not fully account for the dependent variable (DE). Examination of the response-surface (Figs 2 and 3) suggests that a high DE product could be obtained at a Cereflo concentration of 0.36% and pH 5.9.

A mixed enzyme system containing Promozyme and Cereflo produced hydrolysis mixtures with the highest DE. Optimal conditions for this mixture were determined using response–surface methodology. It was decided, on the basis of the narrow temperature range in which the enzymes could reasonably function and their overlapping optimal temperatures (Novo Enzymes, 1984), that the incubation temperature should be 60°C. DE is a convenient indicator of the extent of liquefaction, but little information on solu-

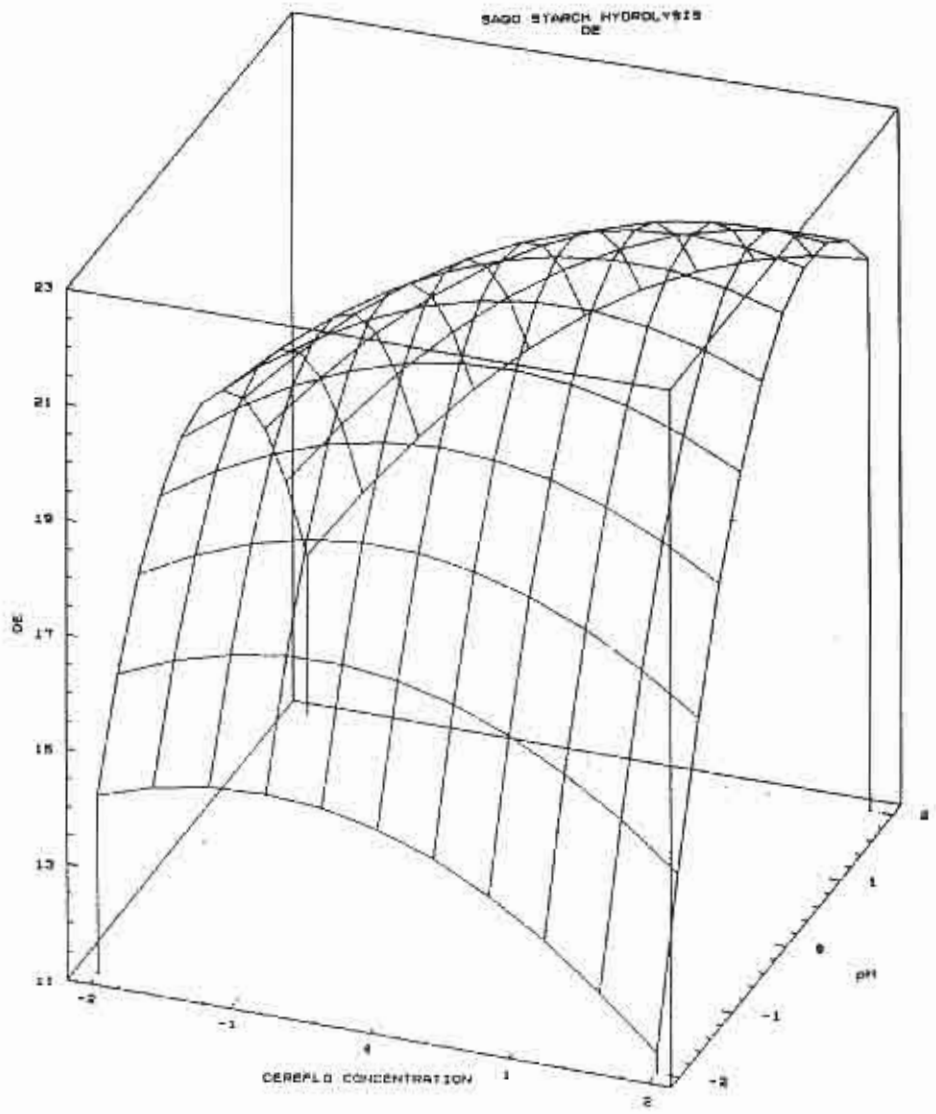

Fig. 2. Three-dimensional presentation of the model, showing pH and enzyme concentration.

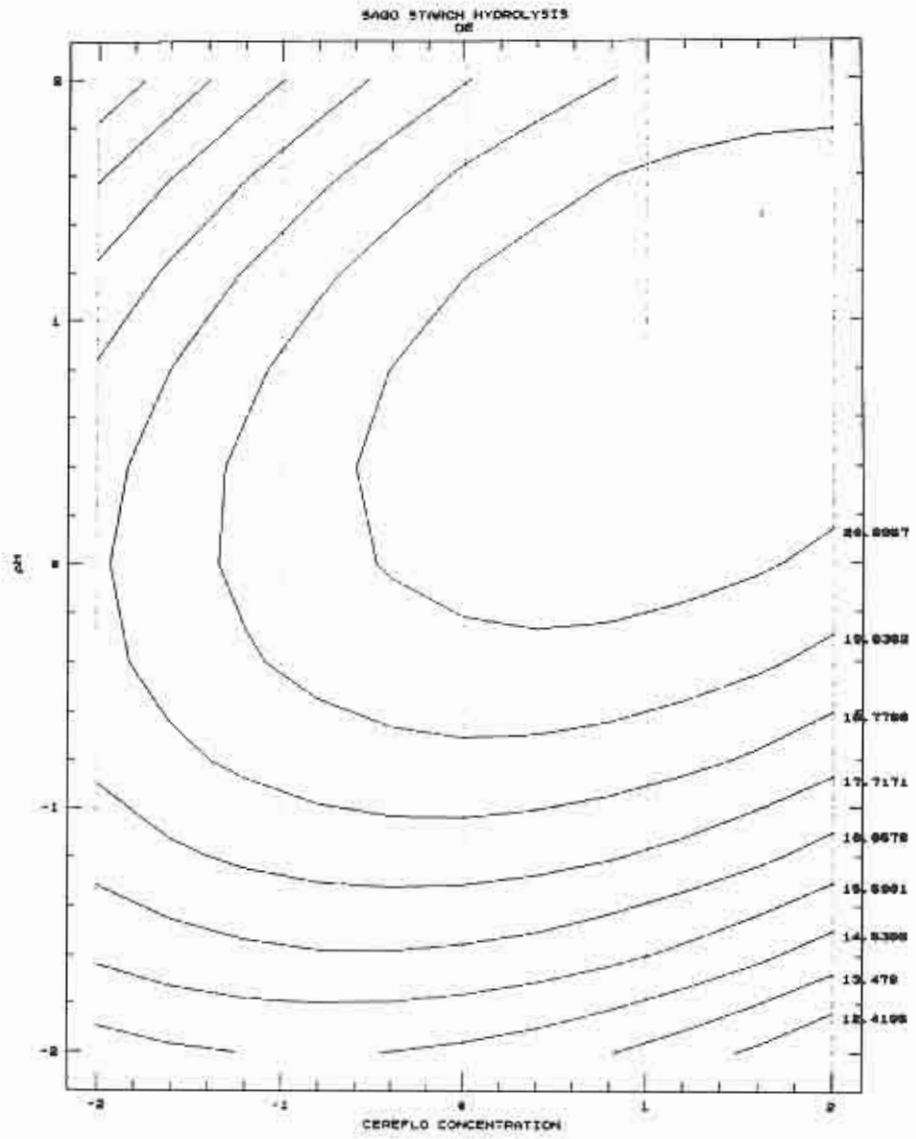

Fig. 3. Presentation of the model, showing pH and enzyme concentration.

bilization and oligosaccharide profile of the soluble material can be found in the literature or by experiment. Hydrolysed samples were subsequently analysed to understand the nature of the soluble and insoluble fractions. Percentage solubility, oligosaccharide profiles of the soluble material and differential scanning calorimetry (DSC) analysis of the hydrolysis mixtures are presented in Table 2.

It is apparent that, in all cases, the material was well solubilised (71.5–82.5%) which compares with 69% solubility of sago starch liquefied only with Termamyl. The soluble material for most samples contained only G5 or G5 and G7 oligosaccharides. Sago starch hydrolysed with Termamyl alone gave a final product composed mainly of G1, G2, medium and high molecular weight material but low in G3, G5, G7 and G9 (Govindasamy *et al.*, 1992).

The oligosaccharide profile of Termamyl-hydrolysed material is in keeping with that of a highly branched structure (Sandstedt & Ueda, 1969; Atkins & Kennedy, 1985). The suggestion by high-performance size-exclusion chromatography (HPSEC) and supported by DSC that the higher molecular weight material is absent and the presence of the preferred products G5 and G7 emphasize the usefulness of Promozyme in debranching the structure. Although the concentration of this enzyme would seem not to be significant, it is apparent that it should be present. Two experiments were based on the assumption that Promozyme concentration is held at its centre point (0.45%) with an optimum concentration of Cereflo of 0.35%. Therefore, the first experiment was carried out with Promozyme and Cereflo and the second without Promozyme. The experiment with Promozyme and Cereflo showed a higher DE (23.2) than that without Promozyme (20.6), which is consistent with the results of Lorenz and Kulp (1982), Atkins and Kennedy (1985), Słominska and Maczynski (1985) and Fumihide *et al.* (1987). It is interesting to note that some samples which contained significant amounts of G3, had been hydrolysed by high levels of Cereflo. Furthermore, the enthalpies of dissociation of non-hydrolysed fractions in these mixtures were signifi-

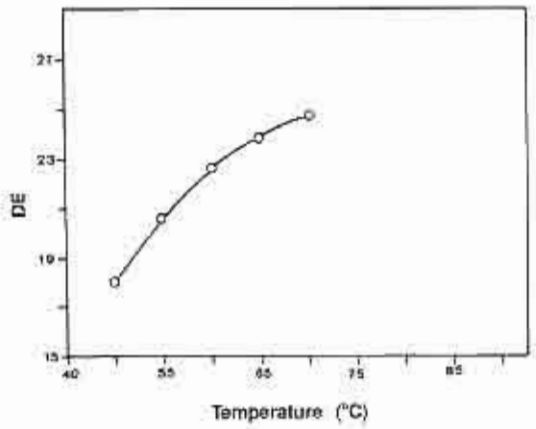

Fig. 4. Dependence of the dextrose equivalent of the starch solution during hydrolysis at different temperatures.

cantly lower than with reaction mixtures containing low concentrations of Cereflo. Unexpectedly, the amount of soluble material in these mixtures was the same as the other samples; therefore the low enthalpies are not the result of low amounts of non-soluble material. This suggests that this material is structurally different. Further work is being carried out to understand the structure of the insoluble material of the different systems. The enthalpies of all hydrolyzed samples were lower than that of raw sago starch (23.2 J/g). The enthalpies of sago-hydrolysed samples were similar to that of cassava-hydrolysed samples (Gorinstein & Lii, 1992). Some of the DSC results are similar to those of Wootton and Bamunuarachchi (1980), Donovan *et al.* (1983) and Sievert and Pomeranz (1990)

Regression analysis suggested that variable factors which had not been included in the investigation were necessary for the determination of optimal conditions. The effects of different incubation temperatures were therefore investigated. The highest degree of hydrolysis was at 70°C (Fig. 4).

Figure 5 shows the X-ray patterns for raw starches and three enzymatically treated starches. The raw sago starch samples gave type-C patterns similar to cassava. The strongest peaks in sample A were located between 15 and 24° with the *d* spacing corresponding to 5.84, 5.21, 4.94 and 3.78 Å. Samples a,b, c, d, e and f had only one broad peak, between 4.00 and 4.67 Å. The treatment with thermostable α-amylase at 100°C clearly destroyed the crystallinity of cassava starch. Samples of cassava starch treated with α-amylase and amyloglucosidase had only one broad peak between 3.90 and 4.87 Å (Gorinstein & Lii, 1992). Cassava and sago starches displayed very similar X-ray diffraction patterns, characterized as type C, as was shown in our previous results (Gorinstein & Lii, 1992). The highest percentage of hydrolysis was obtained using the bacterial thermostable α-amylase and amyloglucosidase which is in agreement with other investigators (Gallant *et al.*, 1982; Franco *et al.*, 1987). Enzymatic attack probably occurred throughout the amorphous granular areas; therefore sample c was completely amorphous.

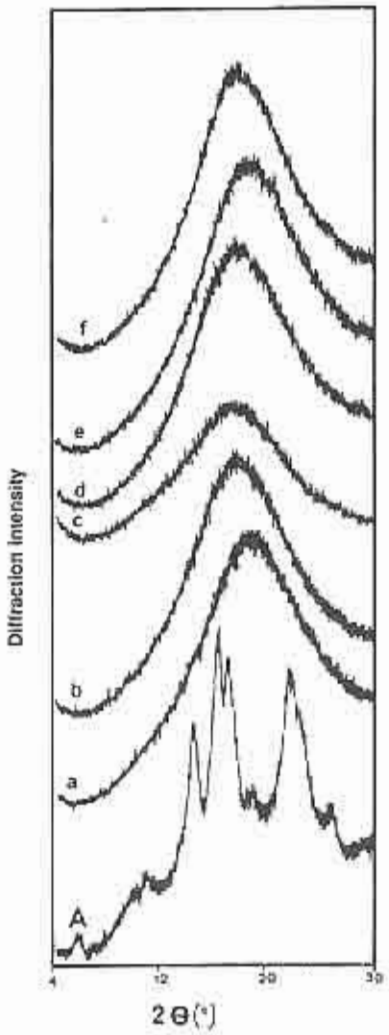

Fig. 5. X-ray diffraction patterns of sago starch with different hydrolysis treatments. A, raw. Hydrolysed starch samples with (a) 0.1% Termamyl, 0.25% Promozyme, 0.15% Cereflo, pH 5.0; (b) 0.1% Termamyl, 0.25% Promozyme, 0.35% Cereflo, pH 6.0; (c) 0.1% Termamyl, 0.65% Promozyme, 0.35% Cereflo, pH 6.0; (d) 0.1% Termamyl, 0.45% Promozyme, 0.25% Cereflo, pH 5.5; (e) 0.1% Termamyl, pH 6.0; (f) 0.35% Cereflo, pH 6.0.

Even samples treated only with thermostable α-amylase appeared largely amorphous. The effectiveness of enzymes in reducing the crystallinity followed the order: Termamyl > Cereflo > Promozyme.

Liquefaction of sago starch with Termamyl alone gave products which contained high molecular weight material and which had low overall solubility. Such products are not good substrates for amyloglucosidase; therefore low yields of glucose can be expected (70%) while the liquefied products using the mixed enzyme system proposed in this study would be suitable substrates for amyloglucosidase.

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