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Properties of protease-treated maize starches

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Abstract

Commercially produced maize starches were treated with protease (Promod 25P) and their composition and properties were compared with untreated controls. It was found that, although protease treatment reduced the starch protein contents by 41%, 21% and 37% for the waxy, normal and amylomaize starches, respectively, it also caused some pits on the granule surfaces, which were evident by scanning electron microscopy (SEM), but no obvious decrease in granule dimensions (Coulter Counter Multisizer). The protein extraction was associated with decreases in starch lipid content by 42%, 40% and 45% (waxy, normal and amylomaize starches, respectively) and a decrease in total amylose content (30.7–26.0%) for the normal maize starch. The gelatinisation parameters of the starches by differential scanning calorimetry (DSC) in water, 0.001 M HCl or NaOH were less obviously affected by protease treatment in common with the swelling factors at 80 °C. The amount of α-glucan leached by the swollen (80 °C) granules was, however, increased by the protease treatment by factors of 3.8, 1.4, and 1.1, for the waxy, normal and amylomaize starches, respectively. Although proteases provide a useful tool for the purification of native starches, commercial protease preparations need to be controlled in terms of amylase content to prevent modifications to starch structure and properties during industrial processing.

Keywords: Starch; Protease; Composition; Gelatinisation; Swelling

1. Introduction

In a previous paper (Tester, Yousuf, Kettlitz, & Röper, 2007), the use of the commercial protease, Promod 25P (P25P), to facilitate the production and purification of maize starch was described. This approach was developed as a potential replacement method for traditional wet steeping-type processing in dilute sulphur dioxide at ~50 °C for up to two days (Pomeranz, 1987; White & Johnston, 2003; Yousuf, 2004). The work was also undertaken to test the potential effectiveness and commercial viability of protease purification approaches with a view to developing a 'green' processing protocol. Highly pure starches, and typically the protein content of commercial starches is less than 0.4% (Appelqvist & Debet, 1997), have been prepared for compositional characterisation, involving protease steps (Morrison, Milligan, & Azudin, 1984), although the overall processing steps are not necessarily suitable for the production of edible materials or large scale production. Issues with respect to toxicity and extraction efficiency are highly relevant in this respect. Although protease costs could make starch purification more expensive (than existing methods) on a commercial scale (Lumdubwong & Seib, 2000) in the short term, hopefully the costs of the relevant proteases will decrease as and if more extensive use is made of these enzyme systems for starch production.

Some authors (Puchongkavarin, Varavinit, & Bergthaller, 2005; Radosavljevic, Jane, & Johnson, 1998) have reported that starch purification under neutral conditions (rice) is difficult with proteases alone and that sodium hydroxide or sodium dodecylsulphate (SDS) washing, subsequent to/ in conjunction with, protease treatment is necessary to purify the starch. Whilst the enzymatic processing

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undertaken by Puchongkavarin et al. (2005) produced low levels of starch damage, the addition of the alkali increased starch damage and both the sodium hydroxide and SDS affected (increased) granule swelling properties (at high temperatures). Protease treatment, in combination with sonication (Wang & Wang, 2004), similarly restricts starch damage, but the sonication does tend to increase peak viscosities (by the rapid visco-analyser, RVA). In a broad sense, however, the use of protease enzymes to aid starch purification is in its infancy. In part, this is because of the efficiency of existing methods, although it also relates to lack of knowledge regarding processing opportunities and availability of pure protease preparations for the purpose.

The following work was undertaken to identify how protease purification steps, developed by the authors for starch extraction from maize flour (Tester, Yousuf, Kettlitz, & Röper, 2007), when applied to commercially extracted (pre-extracted) starches (using conventional methodologies), affects the functionality of these starches. The objective was to use these data to help validate the extraction process in the commercial sector with a focus on the most effective protease preparation identified for this purpose.

2. Materials and methods

2.1. Maize starches

Waxy, normal and high amylose maize starches were obtained from Cerestar, Vilvoorde, Belgium (waxy and normal) and National Starch, Manchester, UK (amylomaize). The proteolytic enzyme, Promod 25P, a thermolysin from *Aspergillus* spp. [E.C.3.4.24.27], 0.4 U mg^{-1}, was sourced from Biocatalysts, Cardiff, UK. The commercial maize starches were treated with the protease according to the general 'optimised' method described by Tester et al. (2007). In addition, before protease treatment, sets of commercial maize starches were incubated with fungal (*Aspergillus oryzae*) α-amylase according to Karkalas, Tester, and Morrison (1992) at 30 or 45 °C for 15 or 60 min to observe whether the amylase facilitated protease activity on/in the granules. The amylase was inactivated by washing with 0.01 M HCl (~20 min) at room temperature, whereupon the starches were repeatedly washed with excess cold deionised water to remove the acid. These starches were then treated with protease, as above.

2.2. Analytical methods

The moisture content of the starches was determined gravimetrically; starch samples (100 mg ± 0.1 mg), in triplicate, were heated at 130 °C for 1 h in a fan-assisted oven and the moisture content was calculated by difference. The protein content was calculated from amino nitrogen determined using standard Kjeldahl methodology. The α-glucan (α-D-glucan) content of the starches was determined enzymatically according to Karkalas (1985). Swelling factors of starches were determined according to Tester and Morrison (1990a, 1990b). Using the same swelling system, the amount of solubilised α-glucan was determined (omitting the α-amylase digestion step) in the supernatant of granules heated at different temperatures according to Karkalas (1985).

2.3. Amylase assay of the protease preparation

The α-amylase assay procedure used to test side activity of the protease was adapted from the general procedure supplied by the Sigma Chemical Company, Poole, UK as follows: in triplicate, 1 ml aliquots of deionised water-solubilised protease (2.5 mg 10 ml^{-1}) were added by pipette to 1 ml aliquots of solubilised potato starch (1% soluble potato starch, Sigma S-2630) in 15 ml screw-capped tubes. The sealed and mixed tubes were incubated at 20 °C for 3 min in a mixing/cooled water bath. Next, 1 ml of a freshly made and carefully mixed solution of 5.3 M sodium potassium tartrate made in 2 M NaOH (8 ml) plus 96 mM 3.5-dinitrosalicylic acid (20 ml) plus deionised water (40 ml) was added by pipette to each tube. The sealed and mixed tubes were placed in a boiling water bath for 15 min, whereupon they were cooled on ice to room temperature. Next, 9 ml of deionised water were added to each tube by pipette and the sealed tubes were re mixed. Different controls were prepared with (i) the protease only and no starch, (ii) Sigma α-amylase (10065 from *A. oryzae*, 37 U mg^{-1}, 0.25 mg10 ml^{-1}) in place of the protease, with and without the starch and, a deionised water blank. A maltose standard series was also prepared (0.4–2 mg ml^{-1}) for calibration. The units of activity were calculated, where 1 U will liberate 1 mg maltose (reducing disaccharide) from starch after 3 min at 20 °C and pH 6.

2.4. Instrumental methods

Starch lipids, as fatty acid methyl esters (FAME), were extracted according to Morrison and Coventry (1985) using 75% propanol. As an internal standard, heptadecanoic acid (C17, Sigma H3500, corrected for impurities) was incorporated prior to methylation (of the starch lipids) using 14% boron trifluoride in methanol. The FAMEs, dissolved in a ~5 μl of diethyl ether, were fractionated using gas liquid chromatography (GLC) with a Perkin Elmer Autosystem (Perkin Elmer, High Wycombe) incorporating a flame ionisation detector (FID) and PE Nelson Model 1020 data capture module. Separation was achieved with a Supelco fused silica SP-2380 (30 m × 0.25 mm) column using helium as the carrier gas. The injector and detector ports of the system were set at 220 °C with an oven temperature of 185 °C. The system was pre-calibrated with FAMEs derived from C16:0, C16:1, C18:0, C18:1 and C18:2 (Supelco, 1891-1AMP). Factors reported by Morrison et al. (1984) were used to convert FAME to lipid.

The gelatinisation parameters, namely onset (T_o), peak (T_p) and conclusion (T_c) temperatures, plus enthalpy (ΔH) of starches (~3.5 mg, in triplicate) were obtained with a Mettler (Beaumont Leys, Leicester, UK) differential scanning calorimeter (DSC) model DSC 30 with a TA 3000 low temperature cell and TC 10A TA controller. The heat flow was calibrated with an indium standard. All samples were weighed into 40 μl standard aluminium pans to which 15 μl cold, deionised water (or 0.001 M HCl or 0.001 M NaOH) was added by syringe and, following mixing (mixing pin) of the contents, the pans were sealed. Samples were heated from 5 to 100 °C (130 °C for amylomaize) at 10 °C min^{-1} against a pierced, but empty sealed pan. Further details can be found elsewhere (Tester and Morrison (1990a, 1990b)). The use of the dilute acid or alkali was employed to gain an insight into how the starches would function during processing at a non-neutral pH.

Granule dimensions were recorded in 0.9% saline (filtered through 0.22 μm sterile filters) using a Coulter Counter ZM Microsizer (Coulter Electronics Limited, Luton, UK) operating with 256 channels and pre-calibrated with PDVP latex particles (Morrison & Scott, 1986; Tester, Morrison, Gidley, Kirkland, & Karkalas, 1994). For scanning electron microscopy examination (SEM), the general procedure described by Tester and Morrison (1990) was employed. Here, starch samples (~5 mg) were shaken onto a small section of filter paper attached by electrically conducting adhesive to a brass stub. The samples were sputter-coated with gold under an argon atmosphere and then examined with a JEOL JSM-T200 (Tokyo, Japan) microscope.

2.5. Analytical errors

Data included a coefficient of variation (CV) of typically 1% or less, except for the gelatinisation enthalpy which was 5% or less.

3. Results and discussion

3.1. Composition

The compositions of the native and protease-treated maize starches are presented in Table 1. It is apparent that the protease treatment reduces the protein content of the starches, corresponding to 41%, 21% and 37% for the waxy, normal and high amylose starches, respectively. The lipid was similarly reduced by 42%, 40% and 45%, respectively, for the same starches by the protease treatment. There was little effect of the treatment on the amylose content of the waxy starches although the apparent and total amylose content were reduced by 13% and 15%, respectively, for the normal starch and reduced by 8% for the apparent amylose content of the amylomaize starch but increased by 3% for the total amylose content for this starch. One assumes that the differences are caused by protein and lipid extraction, causing greater access of the protease to the starch α-glucans where any contaminating α-amylase would also have access to the α-glucans (discussed later). Possibly the extracted α-glucan was also associated with the protein and lipid (e.g. amylose–lipid complexes) which are reviewed in more detail elsewhere (Tester & Karkalas, 2002, 2003, 2005; Tester, Karkalas, & Qi, 2004).

Table 1
The composition[a] of commercial maize starches before and after treatment with the protease Promod 25P

	Protein (%)	Lipid (mg kg^{-1})	Amylose		
			Apparent (%)	Total (%)	Δ[b]
Waxy maize					
Native	0.41	4.5	0.3	0.3	0
Protease-treated	0.24	2.6	0.5	0.9	0.4
Normal maize					
Native	0.47	41.2	26.5	30.7	4.2
Protease-treated	0.37	37.8	23.1	26.0	2.9
Amylo maize					
Native	0.78	99.0	61.0	63.7	2.7
Protease-treated	0.49	54.2	60.5	65.9	5.4

[a] Calculated on an α-glucan basis.
[b] Difference between total and apparent amylose.

3.2. Physical properties

The gelatinisation properties of the starches are presented in Table 2. Compared to the native starches, protease treatment had little effect on the onset (T_o), peak (T_p) and conclusion (T_c) gelatinisation temperatures of the waxy (63.2, 70.5 and 78.1 °C versus 63.7, 70.5 and 78.1 °C, respectively), normal (63.2, 69.2 and 76.0 °C versus 63.7, 69.6 and 76.0 °C, respectively) or amylomaize (63.4, 93.4 and 104.9 °C versus 65.5, 87.5 and 103.5 °C) starches. The T_p for the amylomaize starches treated with the protease was lower than that for the native starch although these endotherms were broader than those of waxy and normal starches which always present difficulties with respect to defining the exact T_p. When compared to the native starches, the protease treatment for the waxy (14.7 versus 16.5 J g^{-1}), normal (19.3 versus 13.0 J g^{-1}) or amylomaize (12.6 versus 11.2 J g^{-1}) starches again did not show any major impact on the gelatinisation endotherm.

When the starches were heated in DSC pans containing 0.001 M HCl (Table 2) were again compared to the native starches, protease treatment had little effect on the onset (T_o), peak (T_p) and conclusion (T_c) gelatinisation temperatures of the waxy (62.4, 71.0 and 78.0 °C versus 63.7, 71.0 and 78.8 °C, respectively), normal (63.2, 70.2 and 76.2 °C versus 63.7, 70.2 and 76.2 °C, respectively) or amylomaize (66.3, 94.4 and 104.0 °C versus 66.2, 81.4 and 102.2 °C)

Table 2
The gelatinisation parameters[a] of commercial maize starches before and after treatment with the protease Promod 25P in water, 0.001 M HCl or 0.001 M NaOH

Starch	Gelatinisation temperatures (°C)			
	Onset (T_o)	Peak (T_p)	Conclusion (T_c)	Enthalpy (ΔH, J g^{-1})
Water				
Waxy maize				
Native	63.2	70.5	78.1	14.7
Protease-treated	63.7	70.5	78.1	16.5
Normal maize				
Native	63.2	69.2	76.0	13.9
Protease-treated	63.7	69.6	76.0	13.0
Amylo maize				
Native	63.4	93.4	104.9	12.6
Protease-treated	65.5	87.5	103.5	11.2
0.001 M HCl				
Waxy				
Native	62.4	71.0	78.0	14.1
Protease-treated	63.7	71.0	78.8	15.6
Normal				
Native	63.2	70.2	76.2	13.8
Protease-treated	63.7	70.2	76.2	14.8
Amylo				
Native	66.3	94.4	104.0	9.7
Protease-treated	66.2	81.4	102.2	8.1
0.001 M NaOH				
Waxy				
Native	62.6	71.3	79.1	14.9
Protease-treated	63.0	71.7	79.1	15.7
Normal				
Native	63.0	70.4	76.1	12.0
Protease-treated	63.0	70.4	76.1	11.5
Amylo				
Native	68.7	91.3	105.6	11.5
Protease-treated	65.6	93.0	104.3	16.1

[a] Calculated on an α-glucan basis.

starches. Again, the T_p for the amylomaize starches treated with the protease was lower than that for the corresponding native starch. When compared to the native starches, the protease treatment for the waxy (14.1 versus 15.6 J g^{-1}), normal (13.8 versus 14.8 J g^{-1}) or amylomaize (9.7 versus 8.1 J g^{-1}) starches again did not show any major impact on the gelatinisation endotherm. The enthalpy of gelatinisation for the native and protease-treated amylomaize starches were, hence, both lower than those for the corresponding starches heated in water.

When the starches were heated in DSC pans containing 0.001 M NaOH (Table 2) and once again compared to the native starches, protease treatment had little effect on the onset (T_o), peak (T_p) and conclusion (T_c) gelatinisation temperatures of the waxy (62.6, 71.3 and 79.1 °C versus 63.0, 71.7 and 79.1 °C, respectively), normal (63.0, 70.4 and 76.1 °C versus 63.0, 70.4 and 76.1 °C, respectively) or amylomaize (68.7, 91.3 and 105.6 °C versus 65.6, 93.0 and 104.3 °C) starches. Here, the T_p for the amylomaize starches treated with the protease was slightly higher than that for the corresponding native starch. When compared to the native starches, the protease treatment for the waxy (14.9 versus 15.7 J g^{-1}), normal (12.0 versus 11.5 J g^{-1}) or amylomaize (11.5 versus 16.1 J g^{-1}) starches again did not show any major impact on the gelatinisation endotherm. The enthalpy of gelatinisation enthalpy for the protease-treated amylomaize starch was, however, high compared to the control in NaOH and for the starches heated in water or acid.

The swelling factors for the starches heated at 80 °C are shown in Table 3. The native and protease-treated waxy starches typically swelled more (45.0 and 45.8, respectively) than did the normal starches (8.8 and 9.2) which themselves swelled more than did the amylomaize starches (2.3 and 2.3). The protease treatment, thus, was causing the granules to swell a little more than the native starches. The equivalent starches leached progressively less α-glucan as the amylose content increased from waxy (3.4% and 13.0%), to normal (5.1% and 7.0%) to amylomaize (2.9% and 3.1%). Normally, waxy starches would be expected to leach more α-glucan than would normal starches, which themselves leach more than high amylose starches (Tester and Morrison (1990a, 1990b)). The 'cross over' between the native waxy and normal starches may reflect different amounts of starch damage (Karkalas et al., 1992) although the protease treatment itself, according to the same method, has apparently little effect on starch damage (Tester et al., 2007).

The dimensions of the native and protease-treated granules are presented in Table 4 where the mean diameters of

Table 3
The swelling factors and amounts of α-glucan leached from commercial maize starches[a] swollen at 80 °C before and after treatment with the protease Promod 25P

Starch	Swelling factor	Amount of α-glucan leached (%)
Waxy maize		
Native	45.0	3.4
Protease-treated	45.8	13.0
Normal maize		
Native	8.8	5.1
Protease-treated	9.2	7.0
Amylo maize		
Native	2.3	2.9
Protease-treated	2.3	3.1

[a] Calculated on an α-glucan basis.

Table 4
The dimensions of commercial maize starches determined with a Coulter Counter Multisizer before and after treatment with the protease Promod 25P

Starch	Dimension (mean)		
	Diameter (μm)	Surface area (μm^2)	Volume (μm^3)
Waxy maize			
Native	12.04	456.5	913.9
Protease-treated	11.48	414.0	792.2
Normal maize			
Native	12.04	455.4	913.9
Protease-treated	11.76	434.5	851.6
Amylo maize			
Native	7.84	193.1	252.3
Protease-treated	10.08	319.2	536.3

the granules before and after protease treatment were 12.04 and 11.48 μm (waxy), 12.04 and 11.76 (normal) and 7.84 and 10.08 (amylomaize), respectively. The granule dimensions are generally comparable to data reported elsewhere for commercial waxy, normal and amylomaize starches (Tester, Debon, & Sommerville, 2000) although the native amylomaize starch granules are smaller. After protease treatment of the granules, they appeared pitted when viewed by scanning electron microscopy (SEM, data not shown) which is typical for α-amylase treatment although the nature of the erosion and extent are both starch- and amylase-dependent (Planchot, Colonna, Gallant, & Bouchet, 1995; Sarikaya, Higasa, Adachi, & Mikami; 2000; Tester, Qi, & Karkalas, 2006). Singh and Johnston (2002) reported that certain protease enzymes which they used to purify starches, also caused granule pitting – again due to the presence of amylolytic side activity (contamination).

4. General discussion

The protease used for this work was capable of extracting proteins from commercial maize starches which had been produced using traditional processing methodologies. The protein extraction was associated with lipid extraction and this suggests that the proteins and lipids are somehow associated on/in the maize starches. This is contrary to the work of Belles, Montville and Wassermann (2000) who treated maize (starch) with thermolysin and found that removal of zein proteins was not associated with a reduction in free fatty acids associated with the starch.

When the α-amylase content of the Promod 25P protease was assayed, it was found to contain 0.8 U mg^{-1} α-amylase, compared to the Sigma α-amylase (10065 from *A. oryzae*) used as an assay reference which, according the manufacturers, contained 37 U mg^{-1} (actually 46.8 U mg^{-1} according to the assay used here). It was not, therefore, surprising, that the protease-associated removal of granule protein caused a variation in the amylose to amylopectin ratio and probably also the molecular integrity of the α-glucans themselves (discussed in more detail in a subsequent paper) as a consequence of amylolytic activity. However, when the starches were pre-treated with this *A. oryzae* α-amylase (Karkalas et al., 1992), there was actually no increase in the amount of protein extracted from the granules. This indicates that the protease and any α-amylase activity on native starch granules are acting independently on different regions of starch granules or that, for the amylase to promote protease access to granules, the two enzymes must be used together (at the same sites).

According to the work of Chiou, Martin, and Fitzgerald (2002), who used protease enzymes to purify rice starches, protease purification of starches could be achieved without modification of the fine structure of the α-glucans. This is to be expected if the proteases used are free of contamination with amylases. Clearly this degree of purity is much easier to achieve when using pure enzymes under laboratory conditions than when using commercial preparations focussed towards industrial processes. Work on chromatographic separation of native starch α-glucans from protease-purified starches has also been reported to have no effect on the molecular weight profiles of the amylose and amylopectin molecules (Radosavljevic et al., 1998).

In terms of gelatinisation parameters, the protease treatment with Promod 25P had little overall effect on the gelatinisation temperatures or enthalpy of gelatinisation when heated in water, 0.001 M HCl or NaOH, which indicates that the treatment has had little effect (hydrolysis) on the double helical arrays in the starches forming the crystalline regions. This is probably to be expected, assuming that the protease enzyme, whether contaminated or not with α-amylase, is less likely to disrupt the crystalline regions of the starches than amorphous regions, as a consequence of granule hydrolysis and extraction. These data also indicate that the apparent loss of amylose (Table 1), as a consequence of protease (presumably α-amylase contamination) hydrolysis, is focussed on the amorphous and probably surface-orientated (amylose) regions rather than the crystallites.

Protease treatment did not tend to affect starch swelling properties at 80 °C (slight increase) although it did tend to increase the amount of α-glucan leached from the treated granules. Again, presumably α-amylase contamination caused hydrolysis and disruption to amorphous regions within the granules, facilitating extraction when the granules were heated.

Maize starch granules tend to be spherical/polyhedral (waxy and normal) with some irregularity (amylomaize) where there is a unimodal distribution of the 2–30 μm diameter granules (Tester & Karkalas, 2002; Tester et al., 2006). The granules studied here were generally within this range and were not too dissimilar before or after treatment with the protease (although some small granule loss may have occurred after protease treatment for the amylomaize starch). It is important that, for commercial purification of starches, no major selective losses of granules occur during sedimentation type recovery (where small granules tend to be lost most easily) or due to fragmentation (which is

associated with mechanical processing). Enzyme (α-amylase in particular) hydrolysis of native granule α-glucans is to be avoided during production – especially where granules may already be slightly mechanically damaged (Karkalas et al., 1992), which allows more extensive hydrolysis. The actual hydrolytic pattern will be more or less evident, depending on the origin and composition of the native starch granules (Tester et al., 2006), where small granules tend to be more subject to amylase hydrolysis than do large granules, due to their relatively large surface area to volume ratio.

The proteins associated with starch granules are essentially surface contaminants or integral to the starch granules (Baldwin, 2001). Ideally, any form of protease purification/extraction of starches should optimise protein (and lipid) extraction without causing modification to the α-glucans. This approach may be useful with respect to reducing the protein content of starches where the consumer has an adverse reaction to the protein. An obvious example is the removal of wheat proteins associated with starch granules that may be associated with celiac disease. This is also discussed in more detail in a subsequent paper.

5. Conclusions

The data presented in this work indicate that proteases, such as Promod 25P, do have a role to play with respect to starch purification but such proteases – probably through α-amylase contamination – can cause modification of starch composition and properties, especially solubilisation of α-glucan from swollen granules. All processing methodologies are compromises and inevitably modify granule structure compared to the native forms within plant tissues. If the objective is to extract the granules with commercial constraints, with as 'natural' a structure as possible, care must be taken with respect to the choice of the protease.

This publication represents the second in a series concerning the effects of commercial proteases for the extraction of maize starch and the effect of such proteases on the structure and properties of the starch. The next paper in this series focusses on the types (size, functionality and location) of proteins extracted from native maize starch granules using different types of proteases with a final paper concerning the effects of maize protein addition (when added back) on the structure and properties of commercially purified starches.

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