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Use of commercial protease preparations to reduce protein and lipid content of maize starch

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Abstract

A method was developed to produce pure maize starch from maize flour using a protease processing step, and additional salt washing and sulphite steeping steps. A range of commercial protease enzymes were evaluated for this purpose. The laboratory scale procedure that was developed, using one protease in particular (Promod P25P, thermolysin), produced relatively pure starch (<0.45% protein). Using the same procedure, but applying to starches which had been produced in advance using traditional wet milling, starch protein contents could be reduced further by 25–50% with the lipid content reduced by up to 25%. The amount of starch damage was minimal using this approach (<1%). This procedure could be developed industrially for a 'greener approach' to starch extraction – although it may still be necessary to incorporate sulphite steeping stages to facilitate protein solubilisation and extraction.

Keywords: Starch; Purification; Protease; Lipid; Sulphite

1. Introduction

Although starch has been extracted commercially from starches for many decades, and in many cases even centuries, the same fundamental principles underlying extraction remain largely unchanged – maximisation of yield of pure starch at the lowest cost (Tester & Karkalas, 2002, 2005). The low cost production is achieved by using a very high capacity plant with a continuous throughput of plant material. Because of the relative ease of agricultural production, high yields, ease of extraction, genetic variability, low cost and universal acceptance of maize, it has become the main source of starch industrially – although other starches like wheat and potato are also significant commercial sources of starch.

Starch extraction from plant tissues requires that a number of interlinked processes are used to remove the non-starch fractions without causing appreciable damage to the starch granules themselves. In addition, the processes must be cost effective, applicable for vast rates of throughput and not cause any associated toxicity. Commercial starch (especially maize) extraction processes have been discussed and reviewed elsewhere (Pomeranz, 1987; White & Johnston, 2003; Yousuf, 2004). Typically, maize starch is softened by steeping in dilute sulphur dioxide at ~50 °C for up to two days. When the grain has been softened, the starch can be separated from the other materials by milling, screening and centrifugation (Pomeranz, 1987).

Starches are contaminated with materials derived from the plant cell matrices. These include lipids and proteins adhering to the surface of granules with non-starch fibres often associated between starch granules. Carbohydrates have the capacity to form different types of interactions with non-carbohydrates and readers are referred to other work on these issues (Appelqvist & Debet, 1997; Tester & Karkalas, 2003). Typically, the protein content of commercial starches is less than 0.4%, although this depends

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on the origin of the starch (Appelqvist & Debet, 1997). The non-α-glucan components associated with starch granules not only cause discolourisation and off-tastes, they are also associated with modification of the physical properties of the starches (Appelqvist & Debet, 1997; Debet and Gidley, 2006). These features include modification of: milling and baking properties; digestibility; swelling; solubilisation; retrogradation and retention of granular integrity. Hence, it is important that high starch purity is maintained.

Protease enzymes have been used in the laboratory and in pilot/commercial situations (in different formats and sometimes with other processing aids) to facilitate the extraction of pure starch from plant matrices (Belles, Montville, & Wasserman, 2000; Chiou, Martin, & Fitzgerald, 2002; Eckhoff & Tso, 1991; Lim, Liang, Seib, & Rao, 1992; Lumdubwong & Seib, 2000; Mezo-Villanueva & Serna-Saldivar, 2004; Puchongkavarin, Varavinit, & Bergthaller, 2005; Radosavljevic, Jane, & Johnson, 1998; Schulman & Kammiovirta, 1991; Singh & Johnston, 2002; Wang & Wang, 2001; Weegels, Marseille, & Hamer, 1992). These starches tend to be low protein (<1%) with low levels of starch damage although commercially focused processing protocols have not been optimised. Enzymatic (especially protease) processing has been used in conjunction with wet milling for maize starch purification and this appears to have many commercial advantages over other processes (Johnston & Singh, 2001, 2004; Singh & Johnston, 2002).

When utilising enzymatic processes for the purification of starch, a number of key issues must be addressed: (i) the purity of the final product; (ii) fermentation/degradation of the starch itself; (iii) yields; (iv) potential toxicity of the different processing steps and associated products; (v) availability of enzymes; (vi) enzyme, plant and processing costs and (vii) practicality. Because of the success of current processing methodologies, the enzymatic approaches need to be able to compete on the basis of cost and effectiveness. They do, however, have a 'green' image which can support adoption and utilisation – perhaps for specialist applications in the first instance.

In view of the potential benefits of utilising proteases to extract pure maize starch, the following work was undertaken. The focus was to use commercially available protease systems – rather than highly purified and hence research focused enzymes – for obvious commercial reasons.

2. Materials and methods

2.1. Maize samples and proteolytic enzymes

Normal maize grain and maize starches were obtained from Cerestar, Vilvoorde, Belgium. Proteolytic enzymes were sourced from Biocatalysts as defined in Table 1.

2.2. Starch preparation – basic method

Maize grains were milled in a Tecator Cyclotec Sample Mill Model 1093. Samples (5 g) of the milled grain were weighed into 100 ml screw top flasks to which 50 ml of 1, 10 or 100 mg ml^{-1} protease (Table 1), dissolved in distilled water, were added. The sealed flasks were gently hand rotated to mix and then incubated in an orbital shaker (~30 or 90 cycles min^{-1}) at 45 or 50 °C for up to 24 h. The contents were transferred to 50 ml centrifuge tubes and centrifuged (1500*g*) for 5 min to recover the solids. This material was then washed five times with five volumes of cold distilled water (interspersed with centrifugation as above) to wash away the protein digest. The starch was then washed twice with five volumes of acetone (further interspersed with centrifugation) before spreading on glass plates to dry.

2.3. Salt washing to facilitate protein extraction

To the basic starch extraction process discussed above, a salt wash step was introduced. Here, the maize flour was incubated with 20 or 50 ml salt solution (sodium chloride and sodium metabisulphite, 1.5% w/v with respect to both salts) in an orbital shaker at 45 °C before or after protease treatment, then recovered and dried as above.

2.4. Salt incubation to facilitate protein extraction

The proteases (Table 1) were dissolved directly in the salt solution (Section 2.3) and they were used in this format to extract the protein from the starch as per the general method (Section 2.2).

2.5. Analytical methods

The moisture content of the starches (or dried maize flour, before or after protein extraction) was determined

Table 1
Proteolytic enzymes utilised to hydrolyse the protein component of milled maize

Protease	Supplier/code	Type	Source	Activity (U mg^{-1})
Promod 24P [E.C.3.4.24.28]	Biocatalysts[a]	Bacillolysin	*Bacillus subtilis*	0.1
Promod 25P [E.C.3.4.24.27]	Biocatalysts	Thermolysin	*Aspergillus* spp	0.4
Promod 144P [E.C.3.4.22.2]	Biocatalysts	Papain	*Carica papaya*	0.1–0.8
Promod 184P [E.C.3.4.22.32]	Biocatalysts	Bromelain	*Ananas cumusus*	0.1
Promod 194P [E.C.3.4.24.27]	Biocatalysts	Thermolysin	*Aspergillus* spp	0.2
Promod 278P [E.C.3.4.24.28]	Biocatalysts	Bacillolysin	*Aspergillus* and *Bacillus* spp	0.7

[a] Biocatalysts, Pontypridd, UK.

gravimetrically where starch/flour samples (1 g, accurately weighed), 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 the amino nitrogen, determined using standard Kjeldahl methodology. The α-glucan content of the starches was determined enzymatically according to Karkalas (1985) and the damaged starch content according to Karkalas, Tester, and Morrison (1992). 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 which were heated at different temperatures according to Karkalas (1985).

2.6. Instrumental methods

Starch lipids, as fatty acid methyl esters (FAME), were extracted according to the general method of 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 methanol. The FAMEs dissolved in ~5 μl diethyl ether and 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, Milligan, and Azudin (1984) were used to convert FAME to lipid.

2.7. Analytical errors

Data included a coefficient of variation (CV) of 1% or less.

3. Results and discussion

3.1. Method development

When milled maize samples (5 g) were incubated in 50 ml water, salt or different protease solutions for up to 4 h (deliberately limited at this stage to 4 h to make potential commercialisation of the process efficient with respect to time) at 45 °C, the protein contents were reduced to a greater or lesser extent as shown in Fig. 1. Three of the proteases (P25P, P184P and P278P) were particularly effective with respect to extracting the protein from the milled maize. These proteases are thermolysin, bromelain and bacillolysin types, respectively (Table 1) with little difference in proteolytic activity, in commercial terms. Undoubtedly these enzymes are not particularly pure with respect to the activity of other enzymes, but could be the types of products which might be used industrially for this purpose. In some cases, certain proteases tested were no more effective at removing the maize protein than washing with water or salt solution (Fig. 1).

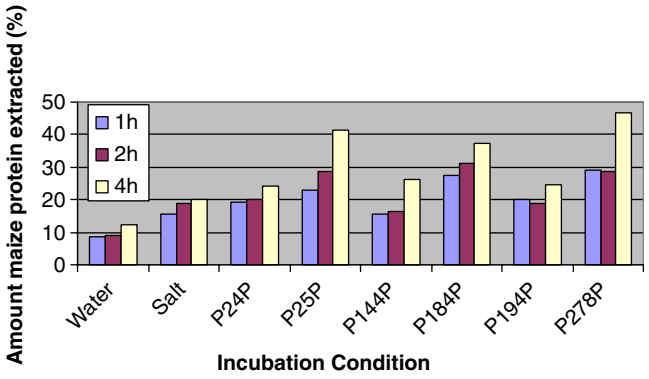

Fig. 1. Extraction of protein from milled maize (5 g) in an orbital shaker (30 rpm) using 50 ml deionised water, salt solution (1.5% w/v NaCl and $Na_2S_2O_5$) or protease (1 mg ml^{-1}, pH 7) at 45 °C for 1–4 h.

The effect of incubation time, increased enzyme concentration and temperature (Fig. 2) was tested with respect to maize protein extraction, using the most effective proteolytic enzymes identified for this purpose (P25P, P184P and P278P) as discussed above (Fig. 1). The incubation time was increased from 4 h up to 24 h and enzyme concentration was increased to 10 mg ml^{-1}, with a small increase in temperature to 50 °C plus a faster rate of shaking/mixing. From these data (Fig. 2) there was a clear difference between the enzymes with respect to their capacity to extract the maize protein where P25P > P278P > P184P. In fact, the increased protease concentration and rate of mixing were more effective with respect to protein removal than time, where increasing incubation beyond 4 h made little overall difference with respect to extraction. Clearly,

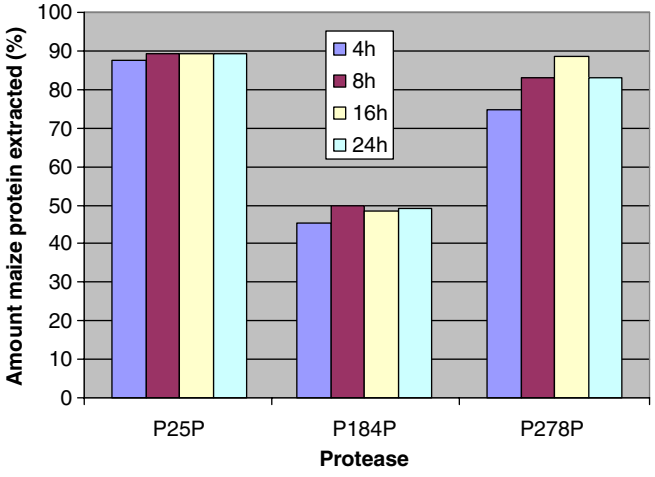

Fig. 2. Extraction of protein from milled maize (5 g) in an orbital shaker (90 rpm) using 50 ml protease (10 mg ml^{-1}, pH 7) at 50 °C for up to 24 h.

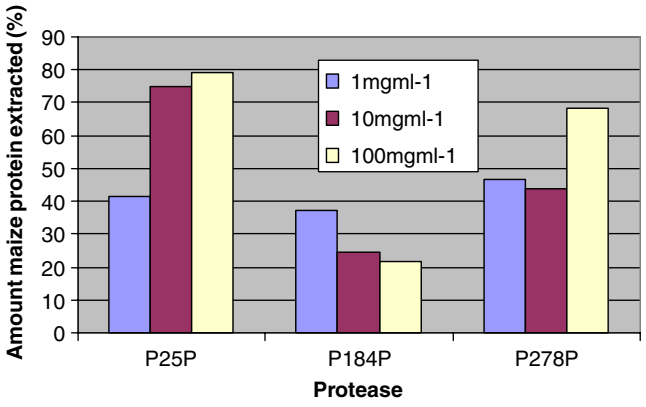

Fig. 3. Extraction of protein from milled maize (5 g) in an orbital shaker (90 rpm) using 50 ml protease (1, 10 or 100 mg ml^{-1}, pH 7) at 45 °C for 4 h.

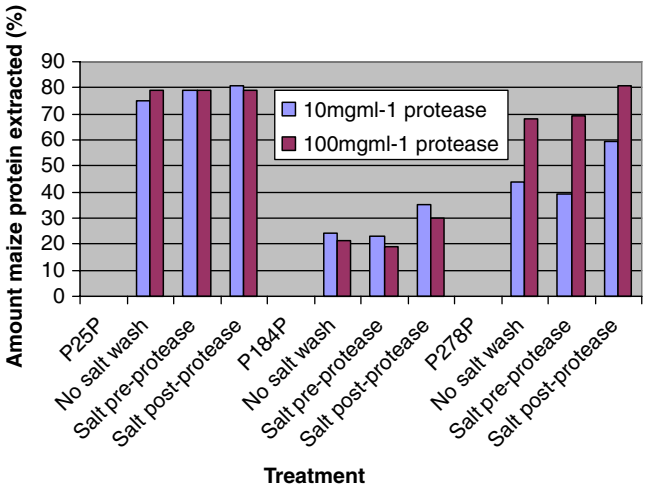

Fig. 4. Extraction of protein from milled maize (5 g) in an orbital shaker (90 rpm) using 50 ml protease (4 h, 10 or 100 mg ml^{-1}) with or without salt washing (20 ml for 1 h, 1.5% with respect to NaCl and $Na_2S_2O_5$) at 45 °C.

there was also an opportunity to reduce the extraction time below 4 h for extraction optimisation.

Clearly, for the proteases to work effectively, the concentration, time and temperature utilised during processing were very important. It was a deliberate choice not to add salts to buffer pH because of the necessity to remove these materials from the starch post-extraction from the grain. However, when the enzyme concentration was varied to investigate the affect on extraction (45 °C), as shown in Fig. 3, P184P showed little sensitivity. This suggests that the enzyme solution was simply washing the protein from the ground maize rather than solubilising as a consequence of hydrolysis. It is possible that the neutral pH did not suit the activity of this protease preparation. However, P278P and especially P25P showed a broad enzyme concentration response with respect to the extraction of the protein under these conditions. This data (and the data discussed above) also indicates that 50 °C is significantly better than 45 °C with respect to extraction of the protein.

When salt solution (NaCl plus $Na_2S_2O_5$) washing of the milled grain was introduced, before or after protease (P25P, P184P or P278P) treatment (Fig. 4), there was a small improvement for extraction using 10 mg ml^{-1} P25P, but no difference for extraction using the higher concentration 100 mg ml^{-1}. For the P184P protease, salt washing

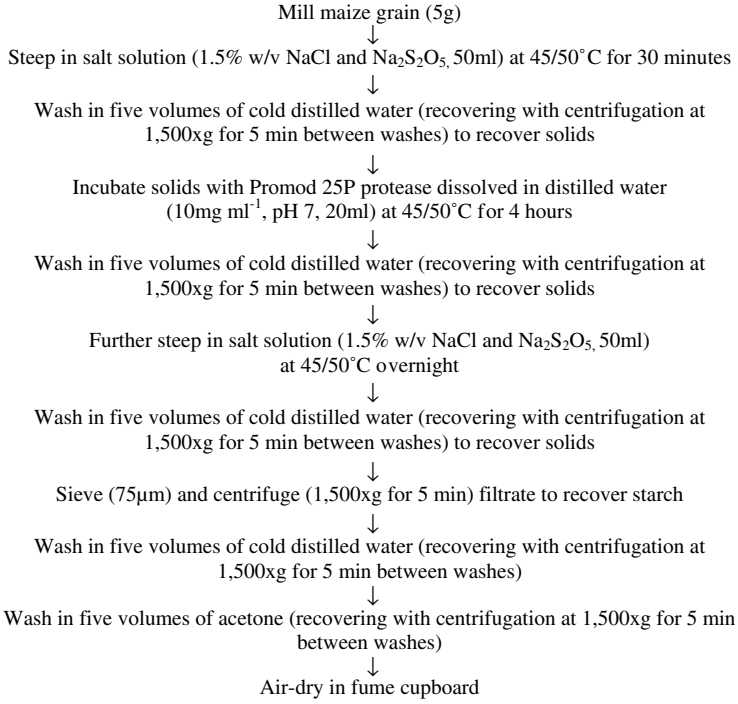

Fig. 5. Protease procedure used to produce pure maize starch.

Table 2
Purification of commercial maize starches (5 g) using Promod 25P[a,b,c]

Commercial starch	Protein content, %	Lipid content, mg 100 g^{-1}
Normal maize		
Native	0.47 ± 0.02	412 ± 12
Protease treated	0.37 ± 0.01	378 ± 10
Waxy		
Native	0.41 ± 0.03	45 ± 2
Protease treated	0.24 ± 0.01	26 ± 2
High amylose		
Native	0.78 ± 0.03	990 ± 21
Protease treated	0.49 ± 0.01	542 ± 10

[a] According to Fig. 1.
[b] Incubation at 90 rpm in Orbital Shaker.
[c] Salt solution was prior or subsequent to protease treatment.

post-protease treatment facilitated protein extraction, but again overall less than for P25P. Finally, for the P278P protease, salt washing post-protease treatment also facilitated protein extraction at both enzyme concentrations. From this data it was clear that P25P was the most effective protease and that there was some advantage with respect to salt washing, in the process to extract protein.

Utilising the approaches mentioned for maize starch production, an enzymatic purification procedure was constructed to produce pure maize starch (from milled grain) as presented in Fig. 5. The final starch product contained <0.45% protein which is fairly typical of commercial starches (Tester, Karkalas, & Qi, 2004) and a damaged starch content (Karkalas et al., 1992) of <1%. Probably the true (integral) protein content of highly purified maize starch granules is <0.35% (Baldwin, 2001), where other proteins are surface contaminants. The approach was also used to purify commercial maize starches further, where the milled grain (as shown in Fig. 1) was replaced with maize starch (Table 2). It is apparent that further purification of starch in terms of protein removal is possible using this approach – and hence could be used as an additional step after wet milling if required. The lipid content of protease treated starches also decreased (Table 2). Presumably the protein and lipid must be associated where protein extraction facilitates lipid extraction.

4. General discussion

Although the wet milling process for extracting starch from maize is well established industrially, there are potential 'green' advantages associated with utilising protease extraction processes to extract and purify the starch. These tend to focus on the use of specific chemical processes although energy usage is becoming an ever increasing issue. There may also be technical and physiological advantages associated with using this approach to produce pure starch (e.g. reducing off colours and tastes) – although these would need to be investigated. The availability of appropriate commercial enzyme preparations is facilitating this development.

The data discussed in this publication indicates that protease purification of starches is a possible option, although developments are necessary if other processing aids such as limited metabisulphite treatments are to be avoided. Enzyme development dedicated to this application is necessary together with appropriate processing methodology development.

Apart from the desirability to produce pure starch using enzyme based procedures, the enzymatic procedures lend themselves to reducing the amount of chemicals carried over from the grain processing steps – which is another commercial advantage. On the other hand, the value of the non-starch components extracted from the starch using enzymes may be reduced. The obvious focus in this context is the plant protein which, post-hydrolysis, could have less desirable functionality and hence down grading of commercial value.

5. Conclusions

Pure maize starch can be extracted and purified from grain using processing steps incorporating protease hydrolysis. There are advantages with respect to utilising this approach commercially in terms of reduction of chemical exposure, with an associated green image. However, the protein extraction protocols employed required additional chemical processing steps to facilitate protease hydrolysis (salt washing, including sodium metabisulphite) and hence may not resolve all of the issues associated with traditional wet milling processing of maize. In addition to the production of starch from maize flour using this approach, it is apparent that commercial starch can be further purified using this approach and there may be commercial advantages using this approach in certain applications – for example maize starch for syrup production where α-glucan components can cause discolouring and off flavours.

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