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Enzymatic removal of zeins from the surface of maize starch granules

AM Belles[1,2], TJ Montville[1] and BP Wasserman[1,*]

[1]*Department of Food Science, Cook College, New Jersey Agricultural Experiment Station, Rutgers, The State University of New Jersey, 65 Dudley Road, New Brunswick, NJ 08901-8520, USA*

Alcohol-extractable, hydrophobic zein proteins contaminate starch granule surfaces and can be removed by enzymatic digestion with thermolysin. The goal of this research was to find practical alternatives to thermolysin that might be used during the corn wet-milling process. All of the commercial thermostable alkaline proteases studied (SP 709, Neutrase, and Spezyme FAN) removed the zein proteins from various types of cornstarch, as demonstrated by the lack of protein bands below 30 kDa under the reducing conditions of SDS-PAGE gel. Each enzyme removed the zein proteins as effectively as thermolysin removed them. However, the removal of the zein protein did not reduce the quantity of free fatty acids associated with the starch. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 71–74.

Keywords: zein; maize; wet milling; thermostable protease; thermolysin; fatty acid

Introduction

The wet milling of maize (corn) separates and refines the endosperm, the germ, and the bran components of the kernel. Cornstarch quality strongly correlates with its purity [5], so wet millers need to minimize the amount of protein and lipid in the starch. Furthermore, the proteins of the endosperm must be separated from the starch fraction. Proteins and lipids are found predominantly in the endosperm and germ tissues [9]. Although most of the proteins in the endosperm and the lipid-rich germ are removed through centrifugation and washing during wet-milling, proteins and lipids are still found within the starch granules and on the starch granule surfaces. The lipids can be on the granule surface or internal to the granule matrix [7], and we have recently been able to categorize cereal proteins as either intrinsic to the starch matrix or as surface contaminants [11,12]. The lipid components of the starch granule may influence starch functionality, milling properties, digestibility [2,7,8] and may be a source of thermally-generated off-flavors, off-colors, and off-odors [6]. A better understanding of surface components would facilitate the creation of economical, large-scale methodologies to reduce protein contamination of starches.

To date, the only known mechanism for removing these contaminants is solvent extraction, an impossibility for the industrial wet miller. However the zein proteins can be removed enzymatically using the heat-stable, neutral protease, thermolysin [12]. Thermolysin favors hydrophobic amino acid residues for digestion, making it particularly suited to hydrolyze zein proteins, but it is expensive and not available in process-magnitude quantity. The objective of this study was to determine if commercial enzymes could perform as well as thermolysin in removing surface proteins.

Correspondence: Dr TJ Montville, Department of Food Science, Cook College, New Jersey Agricultural Experiment Station, Rutgers, The State University of New Jersey, 65 Dudley Road, New Brunswick, NJ 08901-8520, USA. E-mail: Montville@aesop.rutgers.edu
[2]Present address: Danisco Cultor, 20 Lake Drive, East Windsor, NJ, USA
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Materials and methods

Starches

Cerestar, USA (Hammond, IN, USA), donated the commercial starches. Cerestar provided waxy maize (<2% amylose), two lines of normal cornstarch (25–30% amylose), and three lines of amylose extender (two 50–55% amylose lines and one 65–70% amylose line).

Enzymes

Thermolysin, Neutrase, SP709, and Spezyme FAN were compared in these studies. Thermolysin is a thermal stable endopeptidase produced by *Bacillus thermoproteolyticus rokko*. It has an absolute requirement for Ca^{2+} and has optimal conditions at pH 7 and 50–60°C. Thermolysin is also a metalo-protease [15] which hydrolyzes proteins at both protein-membrane and protein-carbohydrate interfaces. Thermolysin (protease X) was purchased from Sigma (St Louis, MO, USA). Neutrase, produced from *Bacillus amyloliquefaciens* is also a metalo-protease stabilized with Ca^{2+} and favors serine residues. Its activity is maximized at pH 5.5–7.5 at 45–55°C [13]. Neutrase is a food grade enzyme, and like SP 709, was donated by Novo Nordisk (Franklinton, NC, USA). SP 709, produced by *Aspergillus oryzae*, has both endoprotease and exopeptidase activity with optimal conditions at pH 5.0–7.0 at 45–50°C [14]. Spezyme FAN is produced by *Bacillus subtilis*. It is optimized at pH 5.5–6.5 while the temperature is 40–60°C. At temperatures of 30–40°C, its optimum pH is 4.0–4.5 [4]. It was donated by Genencor (Rochester, NY, USA). The commercial enzymes were shipped as liquids and stored at 4°C.

Proteolysis of starch granule surfaces

Unless otherwise indicated, 50 mg of starch granules were incubated with 10 μl of protease with 5 mM Ca^{2+} brought to 1 ml reaction volume with ddH_2O. Incubations were carried out for 4 h at 50°C [12]. The granules were washed four times with 50°C ddH_2O to remove protease. As thermolysin is sold lyophilized, it was used as a 10% (w/v) suspension. The other commercial proteases had similar protein contents and specific activities (0.24–0.34 Sigma Units mg^{-1}). These were used as supplied by the manufacturer (0.20 μl protease solution mg $starch^{-1}$) without further dilution.

Protein analysis

Proteins were extracted by gelatinizing granules with extraction buffer (3% SDS, 5% β-mercaptoethanol, 10% glycerol, and 62.5 mM Tris/HCl, pH 6.9, with the addition of 1% (v/v) of fresh 1 M dithiothreitol; 20 μl of buffer per mg starch) for 10 min at 72°C [11]. Unless otherwise indicated, total protein extracted from 5 mg of starch was loaded onto and analyzed by 9–18% SDS-PAGE gradient gels and visualized by double staining with Coomassie blue and silver stains (Integrated Separation Systems, Hyde Park, MA, USA).

Thermolysin stability

Thermolysin (10% w/v) was incubated at 55°C. Aliquots of 10 μl were removed every 6 h for 3 days. The ability of the heat-treated thermolysin to digest the surface proteins of 50 mg of normal starch granules was determined as described above.

Lipid extraction and quantification

Lipids were extracted from each sample of corn starch (500 mg ± 0.5 mg) with *n*-heptane (HPLC grade, Sigma) and the extracted fatty acids were converted to fatty acid methyl esters (FAMEs) [1,8,10] as detailed elsewhere [3]. FAMEs were identified by GC/MS by comparing their retention times to those of known standards at the Biotechnology Center for Agriculture and the Environment (Cook College, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA), with the assistance of Dr Max Häggblom using a Hewlett-Packard 5890 Series II GC (Hewlett Packard Co, Palo Alto, CA, USA) attached to a 5971A Mass Selective detector (Hewlett Packard Co) and a DB5MS capillary column (30 m × 0.25 mm ID × 0.25 m film thickness; J&W Scientific, Folsom, CA, USA) as detailed elsewhere [3]. Peak areas of the chromatograms were integrated and the weight in mg of each fatty acid methyl ester was calculated against an internal standard. Calculations were based on linear regression equations generated by spiking known amounts of fatty acid standards (C16:0, C18:0, C18:1, C18:2, and C18:3; Sigma) with the internal standard (C17:0).

Results and discussion

Surface proteolysis of starches from various genetic backgrounds

A group of commercially available starches was treated with thermolysin for 4 h at 55°C. The starch granule proteins were then extracted and fractionated by SDS-PAGE. Controls were identically treated, only thermolysin was omitted. Thermolysin effectiveness was defined as the ability to digest surface-localized, smaller molecular weight zein proteins of 27, 22, and 10 kDa (γ-, α-, and δ-zein, respectively) and was clearly demonstrated against all starch mutants (Figure 1). The lower molecular weight zein proteins were absent from each thermolysin-treated sample,

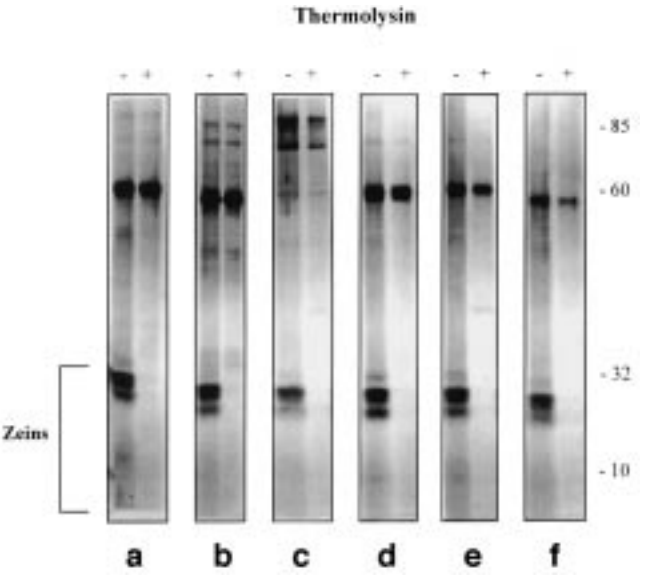

Figure 1 Effectiveness of thermolysin on commercial maize starch from various genetic sources. Lanes (a) and (b) are different preparations of normal (25% amylose) starch; (c) waxy maize; (d, e, and f) high amylose mutants, 50%, 55%, and 65–70% amylose, respectively. Proteins were extracted with extraction buffer, separated on 9–18% SDS-PAGE, and visualized by double staining with Coomassie and silver stains. Controls are indicated as (–) and thermolysin as (+).

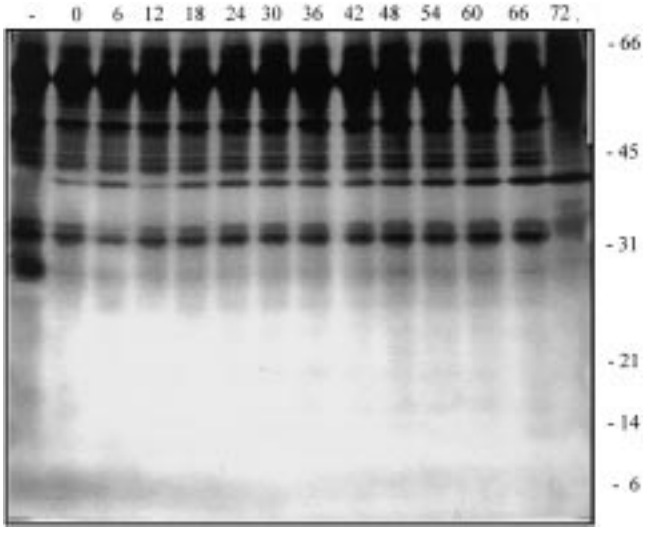

Figure 2 Thermolysin stability. Thermolysin (10 mg ml^{-1}) was incubated at 55°C for 3 days. At 6-h intervals, 10-ml aliquots were removed and used to digest the surface proteins of granules as described in the Materials and Methods section.

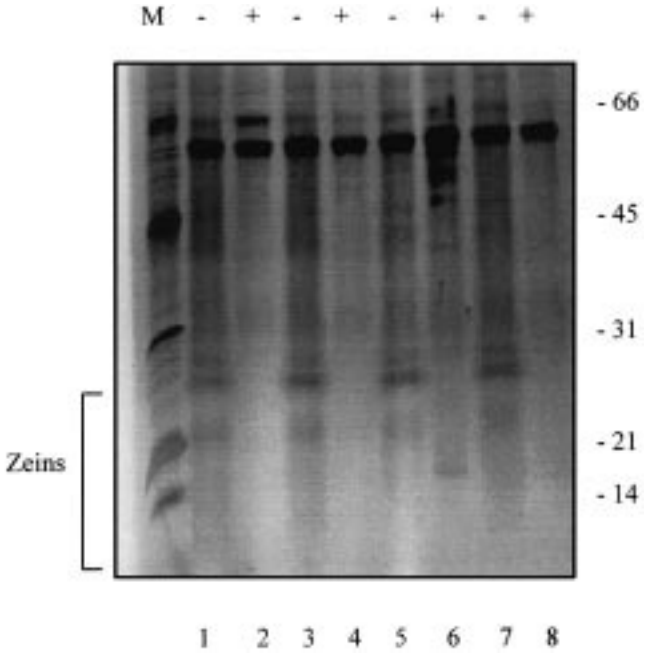

Figure 3 Deproteinization of high amylose starch. Control (–) and enzyme-treated (+) lanes are shown. (1 and 2) Thermolysin; (3 and 4) SP 709; (5 and 6) Neutrase; and (7 and 8) Spezyme. All enzymatic treatments hydrolyzed the surface zein proteins, as evidenced by the lack of low-molecular weight zein bands in (+) lanes.

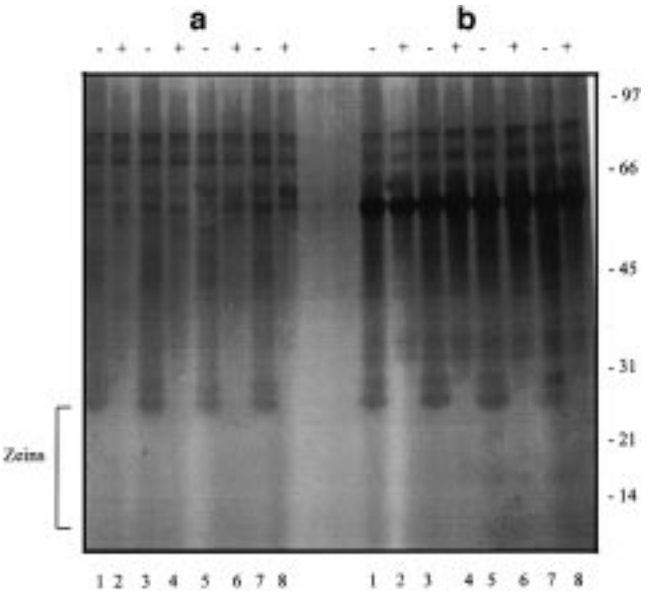

Figure 4 Deproteinization of waxy and wild-type starch. Control (–) and enzyme-treated (+) lanes are shown. (a) Waxy starch; (b) normal starch. (1 and 2) Thermolysin; (3 and 4) SP 709; (5 and 6) Neutrase; and (7 and 8) Spezyme. All enzymatic treatments hydrolyzed the surface zein proteins, as evidenced by the lack of low-molecular weight zein bands in (+) lanes.

independent of starch type, but were visible in the control lanes. These results extend the recent findings [12] of the effectiveness of thermolysin on normal (25–30% amylose) laboratory-prepared starch to a variety of commercially available starches.

Thermolysin stability

Aliquots of thermolysin held at 55°C were taken every 6 hours and used to digest the surface proteins of starch granules as described above. Even after 3 days at 55°C, thermolysin was able to digest the zein bands in the treatment lanes (Figure 2). If thermolysin had been inactivated, the low molecular weight bands seen in the control lanes would be visible also in the treatment lanes. This stability at elevated temperatures indicates that thermolysin might retain its activity throughout normal corn wet milling conditions and be difficult to inactivate in treated starch. This, combined with thermolysin's lack of commercial availability, lack of regulatory approval for this use, and cost argue against its large-scale use.

Commercial protease preparations

Commercially available alternatives to thermolysin were also investigated. The surface proteins of the *ae* (65–70%), *wt*, and *wx* starches were effectively digested by Spezyme FAN, Neutrase, and SP 709 (Figures 3 and 4). Samples were prepared and visualized as described above. The low molecular weight zein proteins are visible in the control lanes but not in the enzymatically treated lanes, similar to the results with thermolysin.

In time course experiments, starch granules were incubated with enzymes for up to 4 h. At 1-h intervals the enzymes were removed and the proteins extracted and fractionated on SDS-PAGE gels. All the commercial enzymes effectively removed zein proteins in 1 h (results not

Table 1 Enzymatic removal of surface protein and subsequent fatty acid analysis of starch. Controls are indicated as (–ENZ) and treatments as (+ENZ)

Starch	Enzyme	μg fatty acid mg starch^{-1}		Percent change
		–ENZ	+ENZ	
65–70% Amylose	Thermolysin[a]	49.5 ± 13.2	45.3 ± 10.9	–8.4%
	SP 709[a]	51.7 ± 16.3	49.3 ± 15.3	–4.7%
25–30% Amylose	Thermolysin[a]	24.2 ± 10.0	26.5 ± 12.7	+9.3%
	SP 709[b]	26.0 ± 3.0	19.2 ± 4.0	–26.0%
Waxy	Thermolysin[b]	25.6 ± 3.8	25.6 ± 0.65	+0.3%
	SP 709[b]	24.6 ± 2.8	24.6 ± 1.6	+0.5%

[a]Average of 12 trials.
[b]Average of triplicates.

shown). Experiments demonstrated that the amount of enzyme used was in excess. All enzymes completely removed the low molecular weight protein when diluted up to 8-fold (results not shown). These experiments demonstrate that this system can be further optimized during scale-up.

Effect of surface deproteinization on fatty acid levels

Because zeins are hydrophobic, other hydrophobic moieties, such as fatty acids, might associate with these proteins. If this hypothesis were true, removing those proteins should decrease the amount of fatty acid. However, there was no decrease in fatty acid content when zein proteins were removed by enzymatic digestion (Table 1). In high amylose starch (65–70% amylose), surface fatty acid content was approximately 45–52 μg g^{-1} starch in both control and treatment starches (Table 1). Although thermolysin and SP 709 decreased fatty acid content in this starch, it was not statistically significant ($\alpha = 0.05$). The changes in fatty acid content of normal and waxy starches were also not statistically significant. Normal and waxy starches contained on average only 25–30 μg fatty acid g^{-1} starch compared to 45–52 μg fatty acid g^{-1} high amylose starch. This observation was consistant with the relatively low amounts of lipids in both normal and waxy starches compared to the high amylose varieties [16].

Conclusion

All of the commercially available enzymes examined removed surface zein proteins under conditions that can be improved by optimization during scale-up. Thus, this research may provide novel methodologies for producing higher quality cornstarch.

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