Enzymatic Hydrolysis, Grease Permeation, and Water Barrier Properties of Zein Isolate Coated Paper

Nicholas Parris,*,† Leland C. Dickey,† Jack L. Wiles,‡ Robert A. Moreau,† and Peter H. Cooke†

Eastern Regional Research Center, U.S. Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038, and Clemson University, Clemson, South Carolina 29634

An inexpensive zein−lipid mixture was isolated from yellow dent, dry-milled corn. Grease permeation through zein isolate applied to brown Kraft paper was found to be independent of loading levels at zein isolate levels above 30 mg/16 in.^{2}. The data shows that water vapor transmission rates depended on the amount of coating applied. Triacylglycerols were the most abundant lipid in milled corn but were absent in the zein isolate (perhaps due to hydrolysis by lipases). Zein from the paper was hydrolyzed enzymatically and the hydrolysis monitored by SDS-capillary electrophoresis. At an E:S ratio of 1:100 no further increase in the hydrolysate peak occurred after 10 and 30 min for α-chymotrypsin and pancreatin 8×; however, zein and lipid were still present 1 h after hydrolysis by pancreatin 1×.

Keywords: *Zein; coating; paper; recycle; enzyme; hydrolysis; grease; water vapor*

INTRODUCTION

As part of an effort to develop a recyclable waxed paper product, we reported on the enzyme hydrolysis of zein−wax coated paper (Parris et al., 1998). We demonstrated that after treatment with α-chymotrypsin, the coating could be cleanly separated from the paper fibers by conventional pulping techniques. Adoption of this new technology, however, is severely limited, primarily because of the high cost of commercial zein. Dickey et al. (1999) have reported on the isolation of zein proteins from dry-milled corn. The proteins were less denatured and the projected cost was lower than commercial zein preparations from corn gluten.

In this study, an inexpensive zein−lipid mixture was isolated from dry-milled corn. Grease resistance and water barrier properties of brown Kraft paper coated with this zein isolate were examined as a function of coating level, method of application and time of exposure. Conditions required for various enzymes to cleanly separate the coating from the paper fibers were also investigated.

MATERIALS AND METHODS

Materials. Corn, yellow dent, was milled to a particle size of 20 mesh with a counter-rotating ribbed disk mill at Davis Feeds, Perkasie, PA. Corn zein isolates were prepared by batch extraction with 70% ethanol from dry-milled corn (Dickey et al., 1998). Typical composition (dry basis) of the isolates was 80−85% protein, 15−20% lipid, and <0.25% starch. Commercial corn zein F-4000 was obtained from Freeman Inc., Tuckahoe, NY. α-Chymotrypsin (type II, from bovine pancreas; activity, 40−60 units/mg of protein), pancreatin (from porcine pancreas, activity equivalent to U.S.P. specification), pancreatin (from porcine pancreas, activity equivalent to 8× U.S.P. specifications), and red oil dye were obtained from Sigma Chemical Co., St. Louis, MO. Oleic acid was from Fisher Scientific, Fair Lawn, NJ. A reciprocal shaking bath (Model 25), from Precision Scientific, Winchester, VA, an environmental chamber (model 317322) from Hotpack, Corp., Philadelphia, PA, a BioFocus Capillary Electrophoresis System, from Bio-Rad, Hercules, CA, and a Nalgene aerosol spray bottle (100 mL), from the Nalge Co., Rochester, NY, were also obtained. Natural hair brushes, 0.5 in. × 0.75 in. with 6 in. handles were obtained from a local supplier.

Chemical Analysis. Protein content (N × 6.5) for zein isolates was determined by the micro-Kjeldahl method (AACC, 1995; AOAC, 1995). The starch content was determined according to a previously published procedure (McCready et al., 1974) by measuring the amount of glucose present in trifluoroacetic acid (TFA)-hydrolyzed samples using HPLC and an Aminex HPX-87H column (Bio-Rad, Hercules, CA). The total lipid content was determined by packing a glass-wool plugged pipet with approximately 100−300 mg of sample, previously dried at 110 °C overnight. The microcolumn was eluted with 5 mL of hexane followed by 5 mL of chloroform. The eluates were collected in a tared vial and subsequently evaporated to a constant weight with a stream of nitrogen gas and the weights of hexane and chloroform extracts determined.

High-Performance Liquid Chromatography. Lipid in crude filtered hexane extracts from the milled corn and zein isolate 127-3 were separated and quantified by the method of Moreau et al. (1996). The separation was carried out on a LiChrosorb DIOL, column 5 μm (3 × 100 mm) using a ternary gradient system consisting of hexane/2-propanol/acetic acid and a constant flow rate of 0.5 mL/min. Eluting peaks were detected with two detectors connected in series. The first was a Hewlett-Packard Model 1050 fixed wavelength UV−visible detector set at 295 nm. The second an Alltech-Varex Mark III evaporative light scattering detector (ELSD) at 40 °C with nitrogen as the nebulizing gas at a flow rate of 1.6 L (STP)/min.

Sample Preparation. Zein isolate, 1 g, was dissolved with heating to 70 °C in 30 mL of 90% (v/v) ethanol. The solution was uniformly brushed or sprayed from an aerosol bottle onto brown Kraft paper (16 in.^{2}) and dried under ambient conditions. The dry paper was weighed after each application to determine the amount of zein isolate deposited.

Testing. Water vapor transmission rates (WVTR) of corn zein coated brown Kraft paper were determined according to

* To whom correspondence should be addressed. Tel: (215) 233-6453. Fax: (215) 233-6559. E-mail: nparris@arserrc.gov.
† U.S. Department of Agriculture.
‡ Clemson University.

10.1021/jf991079y CCC: $19.00
Published on Web 02/24/2000

ASTM E96-80 (ASTM, 1980). Test cups as described by Brandenburg et al. (1993) were filled with 18−20 mL of distilled water. This gave an air gap of 0.60 cm between the bottom of the film's surface and the water. Corn zein coated brown Kraft papers were mounted to the top of the test cups having a test area of 16.26 cm^2. The corn zein coated side of the paper was placed toward the distilled water for testing. Test cups were placed into an environmental chamber at 25 ± 2 °C and 50% relative humidity. This gave a water vapor gradient difference of 50% relative humidity (RH). WVTR measurements were taken by weighing the test cup with the coated paper every hour for 8 h and then at the 24th, 25th, and 26th hours. The measurements were taken to the nearest milligram. The slope of the linear portion of the amount of weight loss versus time curve represented the amount of water vapor diffusing through the corn zein coated paper per unit time. The linear fit yielded a regression coefficient of 0.99 or greater.

The grease permeation of corn zein coated Kraft paper was determined by using a modified TAPPI method T507 as described by Trezza and Vergano (1994). Oleic acid was used to simulate animal fat containing a red oil dye. The test was performed at 60 °C with sampling every hour for 4 h. The percentage area stained by the red dye on the blotter paper, which is used to absorb the grease as it permeates through the corn zein coated paper, was determined and recorded.

Enzyme Hydrolysis. Zein hydrolysis rate measurements, using α-chymotrypsin and pancreatin 8× and 1×, were begun by dissolving 10 mg of commercial zein F-4000 in 1 mL of 10 mM Tris buffer (pH 8.5), 0.1% SDS. The solution was diluted with 1 mL of water and incubated at 37 °C with shaking. The appropriate amount of enzyme, dissolved in water, was quickly added to the zein solution to yield enzyme-to-substrate ratios (E:S) of 1:10, 1:50, and 1:100. Samples (0.1 mL) were removed periodically and quenched in ice. Samples were analyzed within 1 h of being withdrawn from the reaction mixture. All measurements were repeated. Formation of zein hydrolysate was monitored by SDS-capillary electrophoresis (SDS-CE) using the method described by Parris et al. (1998). Electrophoretic separation was performed on an uncoated fused silica capillary (24 cm × 75 μm i.d.) at 15 kV and 20 °C for 15 min.

Removal of Zein−Lipid Coating. The zein coating was hydrolyzed as described previously by cutting the coated paper into 0.25 in. squares, placing them in a flask containing 25 mL of 10 mM Tris buffer (pH 8.5), 0.1% SDS, and enzyme. Flasks were shaken at 37 °C and samples, 0.1 mL, were taken periodically and mixed with an equal volume of water for hydrolysate analysis, as described above.

Completion of lipid removal from the coated paper was determined gravimetrically. Residual lipid on enzyme-treated paper squares was extracted for 0.5 h with 30 mL of hexane in a reciprocal shaking bath at 37 °C. The squares were rinsed two more times with 15 mL of hexane, combined with the original extract, dried under nitrogen, and weighed.

SEM. Square pieces of paper, ~1 cm on a side, were mounted on carbon stub, using carbon adhesive, and viewed in a JSM 840A scanning electron microscope as described previously (Parris et al., 1998).

RESULTS AND DISCUSSION

Enzyme hydrolysis of zein isolate coated paper was investigated as part of an effort to develop an inexpensive, recyclable, paper product exhibiting good water vapor and grease resistance. The composition (dry basis) of the isolate 127-3 selected for this study was: 83% protein; 16% lipid; 0.22% starch. The estimated cost of the isolate product is about $1/lb. The method used to apply zein isolate to the brown Kraft paper significantly affected its grease resistance. Both methods of coating application were exposed from 1 to 4 h and the stained areas measured (compare Table 1 and Table 2). The percent area stained for paper coated with approximately the same amount of zein isolate was lower when the coating was applied as a spray. On an hourly basis the area stained for eight coated samples was 13.8 ± 10.8% when the isolate was brushed on the paper and 3.0 ± 2.8% when applied as a spray. For the remainder of this study the isolate was sprayed on the paper. The grease permeation for zein isolate sprayed on brown Kraft paper was found to be independent of loading levels at concentrations above 30 mg/16 $in.^2$ (Figure 1). This critical loading level was lower than previously observed for zein−paraffin wax coated paper (Parris et al., 1998).

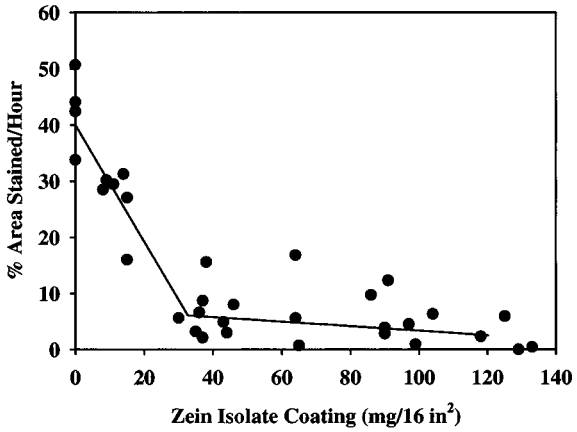

Figure 1. Effect of the amount of zein isolate coating on area stained.

Table 1. Effect of Brushing Zein Isolate Coating on Grease Permeation

isolate	coating (g/16 $in.^2$)	time (h)	area stained (%)	area per hour (%)
1	0.117	4	54.9	13.7
2	0.096	3	32.6	10.9
3	0.089	1	16.0	16.0
4	0.101	2	23.6	11.8
5	0.101	1	38.9	38.9
6	0.106	3	20.1	6.7
7	0.108	2	8.3	4.2
8	0.102	4	34.0	8.5
				13.8 ± 10.8 (av)

Table 2. Effect of Spraying Zein Isolate Coating on Grease Permeation

isolate	coating (g/16 $in.^2$)	time (h)	area stained (%)	area per hour (%)
1	0.127	1	7.6	7.6
2	0.127	3	12.5	4.2
3	0.114	4	20.8	5.2
4	0.106	2	6.9	3.5
5	0.115	2	<1	0
6	0.121	1	3.5	3.5
7	0.116	4	1.4	0
8	0.125	3	3.0	0
				3.0 ± 2.8 (av)

Kraft paper coated with zein isolates improved the water vapor barrier properties of the paper. For five different isolates, WVTR values ranged from 881.0 to 1521.9 (g/m^2 day) and were reduced approximately 40% when paper was coated with about 0.1 g/16 $in.^2$ of isolate (Table 3). Water vapor resistance is primarily attributed to the coating level and its lipid content. The lipid content and composition of the isolate were examined and compared to the lipids present in the milled corn. The lipid profiles for milled corn and zein isolate 127-3 are shown in Figures 2 and 3. The principal lipid components detected by evaporative light scattering

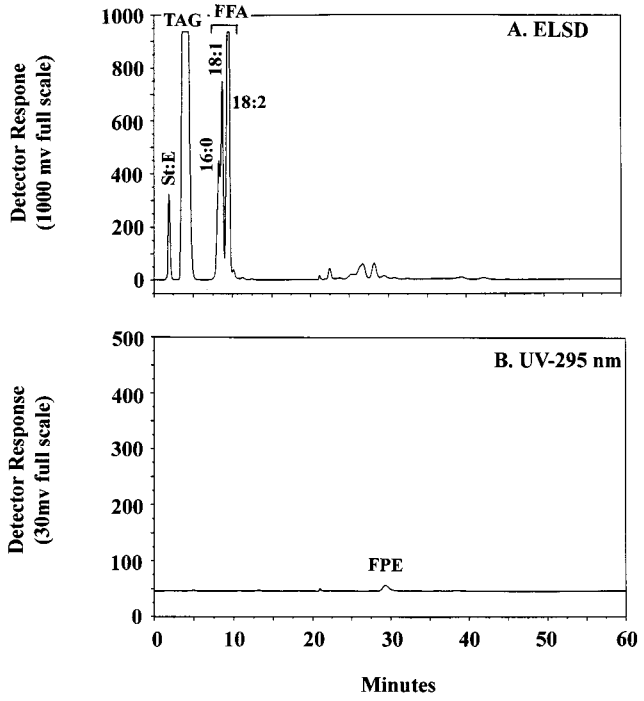

Figure 2. Normal phase HPLC chromatograms of the lipids in milled corn: (A) evaporative light-scattering detection (ELSD); (B) UV-absorbance, 295 nm.

Table 3. Water Vapor Transmission Rate of Zein Isolate

isolate	coating (g/16 in.^{2})	WVTR (g/m^2 day)	% reduction
control	0	1532.0	0
control	0	1515.7	0
123-B	0.092	1160.2	23.9
123-B	0.053	1397.2	8.3
124-2	0.032	1471.7	3.4
124-2	0.059	1226.7	19.5
125-2	0.022	1521.9	0.1
125-2	0.064	1284.3	15.7
125-4	0.012	1385.0	9.1
125-4	0.051	1384.3	9.2
127-3	0.116	881.0	42.2
127-3	0.050	1250.7	17.9
127-3	0.116	881.0	42.2
127-3	0.059	1447.7	5.0

(ELSD) in milled corn were triacylglycerols (TAG) and free fatty acids (FFA). Using this method, fatty acids are partially resolved and linoleic acid, as expected, is the principal FA (Figure 2A). No UV-absorbing compounds (295 nm) were detected in the hexane extract of milled corn (Figure 2B). The amount of TAG in the isolate appeared to be significantly less than that found in milled corn (compare Figures 2A and 3A). This can be attributed to lipase activity. No attempt was made to prevent hydrolysis of the oil before extracting the zein. There was, however, an unknown component in the isolate that had the same elution time as TAG and had an absorbance at 295 nm (Figure 3). This peak is not due to TAG since they do not absorb radiation at this wavelength. It was also determined by LC-MS that the molecular mass of this compound was 396.5 Da, significantly less than that of TAG. Two other major components in the lipid fraction of the isolate were phytosterols (St) and free phytosterol esters (FPE), which, apparently, were concentrated during the isolation procedure (Figure 3). A comparison of the lipid composition for the milled corn and isolate indicates that the TAG in milled corn was absent in the zein−lipid

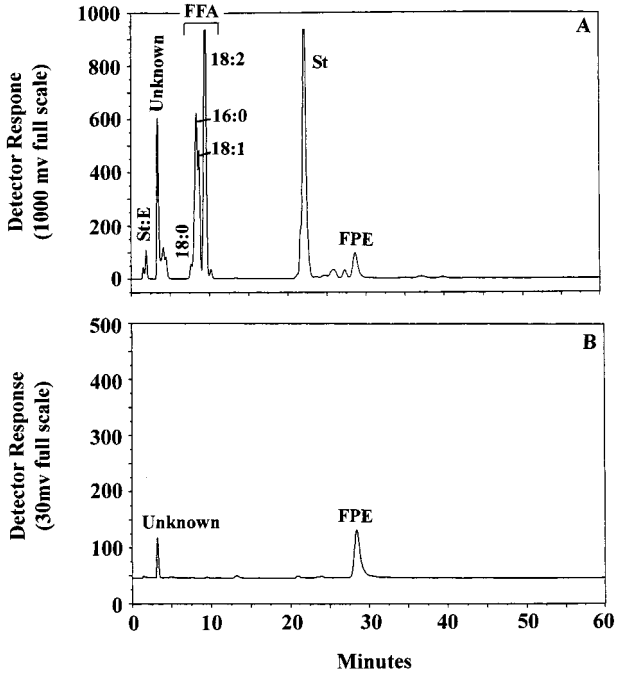

Figure 3. Normal phase HPLC chromatograms of the lipids in zein isolate 127-3: (A) ELSD; (B) UV absorbance.

Table 4. Lipid Composition of Hexane Extract from Milled Corn and Zein Isolate

lipid class[a]	weight % lipid	
	milled corn	zein isolate
TAG	54.31 ± 0.71	14.87 ± 0.12[b]
FFA	24.58 ± 0.17	28.63 ± 0.79
St	0.51 ± 0.02	17.28 ± 0.19
FPE	0.31 ± 0.01	3.53 ± 0.04

[a] TAG, triacylglycerols; FFA, free fatty acids; St, phytosterols; FPE, free, phytosterol esters. [b] Unknown compound that coelutes with TAG.

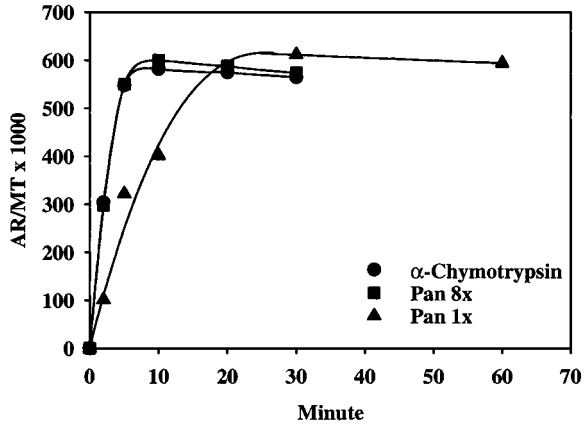

Figure 4. Effect of enzyme on the hydrolysis of commercial zein at E:S = 100: (●) α-chymotrypsin; (■) pancreatin 8×; (▲) pancreatin 1×.

isolate and a significant increase in the amount of St and FPE occurred (Table 4).

For coated paper to be recycled, it is necessary that the zein−lipid coating be removed enzymatically from paper. That this could be done was previously demonstrated for commercial zein−paraffin wax coated paper. Proteases such as papain, pepsin, and thermolysin have all been shown to be capable of digesting zein (Saito et al., 1988; Yamada et al., 1995; Yano et al., 1996). α-Chymotrypsin is preferable to the other enzymes

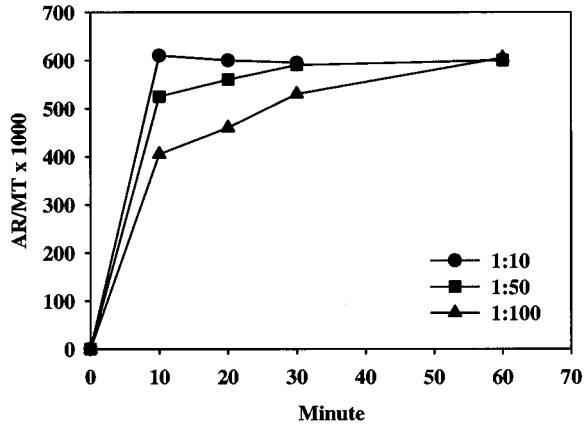

Figure 5. Effect of pancreatin 1× to zein ratio on commercial zein hydrolysis: (●) 1:10; (■) 1:50; (▲) 1:100.

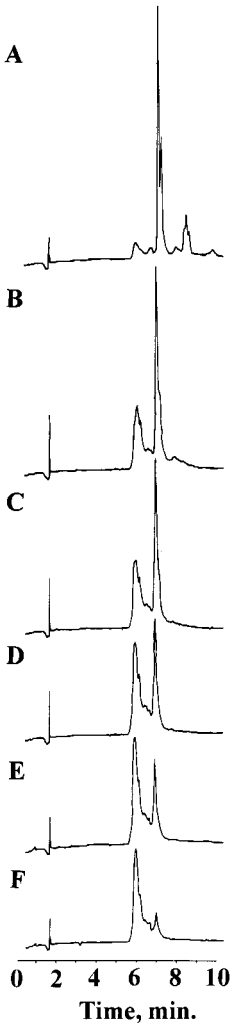

Figure 6. Electropherograms for the hydrolytic removal of zein isolate from coated kraft paper with pancreatin 1×: (A) 0 min; (B) 5 min; (C) 10 min; (D) 20 min; (E) 30 min; (F) 60 min.

because hydrolysis can be carried out at relatively mild temperatures and at nearly neutral pH. Zein hydrolysis was monitored quantitatively by measuring the area/migration time (AR/MT) of the hydrolysate peak by SDS-CE. AR/MT was used because in CE peaks are moving through the detection window at their electrophoretic velocity plus the rate of electro-osmotic flow. Because the peak area depends on the UV response of the analyte and its residence time, it is necessary to compensate by normalizing peak area to migration time. The effect of α-chymotrypsin and pancreatin 8× and 1× on zein hydrolysis was examined at a constant enzyme-to-substrate ratio (Figure 4). Pancreatin was selected for comparison to α-chymotrypsin because it is much less expensive. It contains many enzymes, including amylase, trypsin, ribonuclease, lipase, and protease, and is useful in the hydrolysis of coating mixtures. At an E:S = 1:100 the hydrolysate peak reached its maximum within 10 min for α-chymotrypsin and pancreatin 8× and in approximately 30 min for pancreatin 1×. At a pancreatin 1× to commercial zein ratio of 1:10, 1:50, and 1:100 the hydrolysate peak reaches its maximum at approximately 10, 30, and 60 min, respectively (Figure 5). Characteristic peaks attributed to α-zeins were not observed after hydrolysis of the zein isolate coating and extraction after 10 and 30 min for α-chymotrypsin and pancreatin 8×. Electropherograms for the hydrolysis of the zein isolate coating with pancreatin 1×, however, indicated incomplete hydrolysis at E:S = 1:100 (Figure 6). Initially, two partially resolved α-zein proteins were extracted from the paper with the Tris−SDS buffer and had migration times of 6.8 and 6.9 min. After 5 min a hydrolysate peak formed with migration time of 6.1 min and an estimated molecular weight of 14 kDa. The hydrolysate peak became larger and, after 1 h, a small amount of zein remained about 14.7% of the original peak.

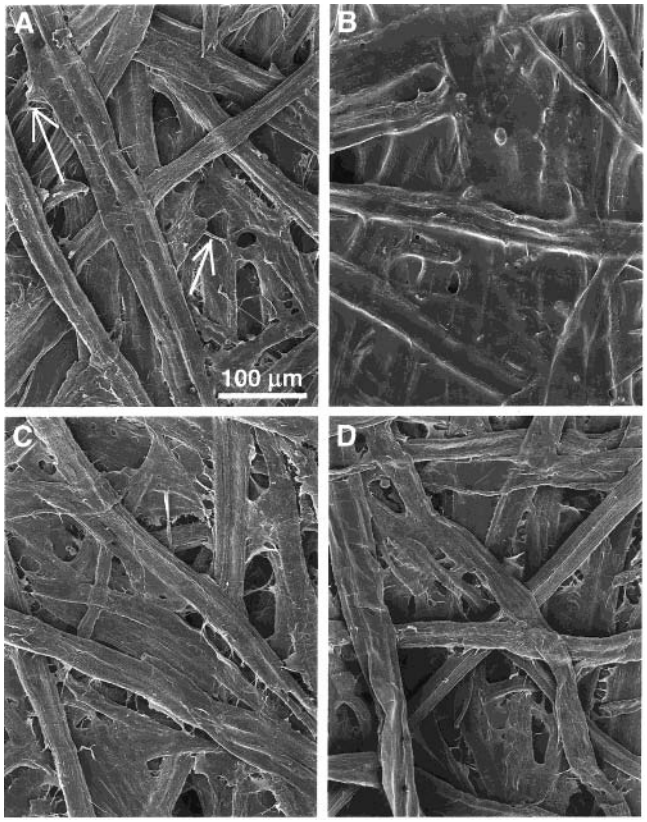

Figure 7. SEM images of (A) kraft paper, (B) zein isolate coated paper, (C) paper after extraction with pancreatin 1×, and (D) (C) after hexane extraction.

Quantitative removal of lipid from the paper was determined gravimetrically, and the paper fibers were examined by SEM. Residual wax on the pancreatin 1× treated paper was extracted with hexane, as described earlier. Residual lipid remaining on the paper after

extraction was 16 ± 5% (n = 6) of the original amount. SEM digital images of the paper surfaces were prepared and recorded at 250× (Figure 7, figured reduced 50% for publication). At this magnification, unraveled fibrils on the paper fibers (arrows) are no longer visible when the paper is coated with the zein isolate (compare Figure 7A,B). After treatment for 1 h with pancreatin 1×, the fibrils on the paper fibers are again visible (Figure 7C). The SEM image after extraction with hexane appeared similar to the image after enzyme treatment (compare Figure 7D,C).

In conclusion, treatment of zein isolate coated paper with α-chymotrypsin and pancreatin 8× effectively removed the zein−lipid coating from the paper fibers. After treatment of the coated paper for 1 h with pancreatin 1×, approximately 15% α-zeins were present by SDS-CE and 16% lipid, by gravimetric analysis. Qualitative examination of SEM images indicate that the paper fibers for coated paper treated with pancreatin 1× were similar in appearance to the uncoated paper.

Results of this study indicate that paper coated with the zein−lipid mixture could be a viable replacement for nonrecyclable wax paper and other synthetic packaging materials. The mixture is inexpensive and the coated paper exhibited good grease proofing and water vapor barrier properties. The coating can readily be removed with an inexpensive enzyme mixture, thus allowing the paper to be pulped and recycled.

ACKNOWLEDGMENT

We express our appreciation to M. F. Dallmer, M. J. Powell and A. J. McAloon for their assistance in this research.

LITERATURE CITED

American Association of Cereal Chemists. *Approved Methods of the AACC*, 9th ed.; The Association: St. Paul, MN, 1995; Method 46-13.

AOAC. *Official Methods of Analysis of the Association of Official Analytical Chemists*, 16th ed.; The Association: Arlington, VA 1995; Method 32.203.

ASTM. Standard test method for water vapor transmission of materials. In *ASTM Book of Standards*; American Society from Testing and Materials: Philadelphia, PA, 1980; pp E96-80.

Brandenburg, A. H.; Weller, C. L.; Testin, R. F. Edible films and coating from soy protein. *J. Food Sci.* **1993**, *58* (5), 1086−1089.

Dickey, L. C.; Dallmer, M. F.; Radewonuk, E. R.; Parris, N.; Kurantz, M.; Craig, J. C. Zein batch extraction from dry-milled corn cereal disintegration by dissolving fluid shear. *Cereal Chem.* **1998**, *75*, 443−448.

Dickey, L. C.; McAloon, A.; Craig, J. C.; Parris, N. Estimating the cost of extracting cereal with ethanol. *Ind. Crops Prod.* **1999**, *10*, 137−143.

McCready, R. M.; Ducay, E. D.; Gauger, M. A. Automated analysis of sugar, starch and amylose in potatoes by measuring sugar-dinitrosalicylate and amylose-iodine color reactions. *J. AOAC* **1974**, *57*, 336−342.

Moreau, R. A.; Powell, M. J.; Hicks, K. B. Extraction and quantitative analysis of oil from commercial corn fiber. *J. Agric. Food Chem.* **1996**, *44*, 2149−2154.

Parris, N.; Vergano, P. J.; Dickey, L. C.; Cooke, P. H.; Craig, J. C. Enzymatic hydrolysis of zein-wax-coated paper. *J. Agric. Food Chem.* **1998**, *46*, 4056−4059.

Saito, H.; Shinimi, O.; Watanabe, Y.; Nishimura, K.; Aso, K. Papain-catalyzed hydrolysis of zein in an aqueous organic system. *Agric Biol. Chem.* **1988**, *53*, 855−856.

Trezza, T. A.; Vergano, P. J. Grease resistance of corn zein coated paper. *J. Food Sci.* **1994**, *59* (4), 912−915.

Yamada, K.; Takahahi, H.; Noguchi, A. Improved water resistance in edible zein films and composites for biodegradable food packaging. *Int. J. Food Sci. Technol.* **1995**, *30*, 599−608.

Yano, S.; Suzuki, K.; Funatsu, G. Isolation from α-zein of thermolysin peptides with angiotensin I-converting enzyme inhibitory activity. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 661−663.

Received for review September 30, 1999. Revised manuscript received January 4, 2000. Accepted January 6, 2000. Mention of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture above others of a similar nature not mentioned.

JF991079Y