Protein-Lipid Interaction During Single-Screw Extrusion of Zein and Corn Oil

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ABSTRACT

Protein-lipid interaction in extruded model systems of zein and corn oil was investigated by analysis of lipids available to solvent extraction. Samples were extrusion cooked at barrel temperatures of 120 and 165°C with and without added corn oil. Two extraction methods were utilized: precipitation of solubilized extrudate into ethyl ether and slurrying of ground extrudates with hexane or ether. Extracts were then subjected to high-performance liquid chromatographic lipid class analysis. Both methods indicated a decrease in extractable lipid after extrusion cooking. Slurry extraction revealed triglyceride and diglyceride binding, whereas the more harsh extraction by precipitation was able to detect binding of fatty acid. Both methods showed protein-lipid interaction was not increased by elevating the temperature to 165°C, suggesting that heat favored reactions did not predominate. Noncovalent interaction appeared to cause the decrease in lipid extractability.

The extrusion cooking of foods can yield products with a variety of textures and flavors. The role of protein-lipid interaction in the development of these characteristics has essentially not been investigated and thus is not well understood. Protein-lipid interaction has been shown to occur during the breadmaking process, and gluten-lipid interaction is thought to be an important aspect in achieving baked products with the proper texture (Chung 1986). Similar interactions may play a role in attributes of extruded foods. Therefore, the purpose of the present study was to investigate whether interactions do occur in an extruded protein-lipid model system, to identify lipids involved in these interactions, and to determine the effect of temperature and added oil on degree of interaction, thus gaining insight into the mechanisms of interaction.

Protein-lipid interaction forces may involve covalent bonds, hydrophobic or van der Waals forces, electrostatic bonds, and hydrogen bonds. Electrostatic and hydrophobic bonds are thought to be especially important in protein-lipid interactions (Chapman 1969, Karel 1973). Van der Waals forces may occur between nonpolar lipid chains and nonpolar amino acid side chains in proteins. In the presence of water, such interaction will be strengthened by hydrophobic bonding, the existence of which is dependent upon the favorable energy resulting from the exclusion of water. Electrostatic binding may occur between positively charged protein residues and ionized fatty acids or negatively charged phosphate groups of phospholipids. Similarly, positively charged phosphatidycholine and negatively charged protein residues may also interact. Hydrogen bonding involving the hydroxyl groups of fatty acids, di- and monoglycerides, or the head groups of phosphatidylethanolamine and phosphatidylserine, may occur with carbonyl groups of protein.

Under the high temperature, pressure, and mixing encountered in extrusion processing, oxidation of unsaturated lipids may occur. Lipid hydroperoxides or their breakdown products may then attack proteins to form covalent bonds. Funes and Karel (1981) reported that linoleic acid incubated with lysozyme under oxidizing conditions produced small quantities of lysozyme-lipid and lysozyme-lysozyme-lipid. Gamage et al (1973) isolated hydroperoxides and secondary products of labeled linoleic acid. Upon incubation with protein, incorporation of radioactivity was detected. In addition to these reactions, malonaldehyde, a common oxidative breakdown product of polyunsaturated fatty acids with three double bonds or more, was shown by Chio and Tappel (1969) to cross-link amino acids by formation of a fluorescent 1-amino-3-iminopropene structure. Shin et al (1971) observed development of fluorescence in malonaldehyde-enzyme model systems, indicating formation of Schiff bases.

To investigate protein-lipid interaction during extrusion, a model system of the corn endosperm storage protein zein and corn oil was utilized. Zein was extruded with and without 5% added corn oil at 120 and 165°C. The temperatures were chosen over a range that would encourage protein denaturation and from a technical standpoint allow easily extrudable products with a favorable, snack stick type appearance. Two solvent extraction methods of differing degrees of severity were utilized to remove lipid from protein.

A high-performance liquid chromatography (HPLC) lipid class analysis designed to quantitate the amount of each lipid class available to extraction in the extrudates was developed. By comparing the classes and amounts present in various samples it was possible to see shifts in composition during extrusion due to binding or evolution of certain lipids.

MATERIALS AND METHODS

Sample Preparation

Extrusion was carried out utilizing a type 2003 Brabender 3/4-in. single-screw extruder with a length-diameter ratio of 20:1, a die diameter of 0.6 cm, and a screw speed of 100 rpm (C. W. Brabender Co., Hackensack, NJ). Two temperature zones were utilized, with T1 held at 60°C and T2 either at 120 or 165°C. Zein (Sigma Chemical Co., St. Louis, MO) was extruded at both temperatures with and without added corn oil (Mazola). Product temperature was measured utilizing a thermocouple placed at the end of the barrel but before the die. Water was added to all samples in an amount equivalent to 30% of the dry material weight (oil + protein), and oil was added to appropriate samples in an amount equivalent to 5% of the zein weight. Conditions for extrusion of samples are shown in Table I.

Samples of zein (500 g) were prepared immediately prior to extrusion by mixing with corn oil, when appropriate, and then adding water dropwise while mixing with a Hobart blender. If water was added to samples without proper mixing, or samples were allowed to sit before extrusion, a solid mass of zein developed that could not be fed into the extruder by gravity. Extruded samples were mixed with dry ice and ground in a coffee grinding mill. These ground samples were stored in glass vessels at 4°C for further analysis. Zein, as obtained from Sigma in its granular form, was utilized as the standard to which extruded samples were compared.

TABLE I
Samples Utilized for Study of Protein-Lipid Interaction in Extrusion-Cooked Products

Feed % Moisture	Feed % Added Corn Oil	Barrel Temperature (°C)	Product Temperature (°C)	Feed Rate (g/min)
30	0	120	104-105	33.1
30	0	165	117-120	31.9
30	5	120	97-99	34.8
30	5	165	117	31.9

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Extraction by Precipitation

Figure 1 describes the extraction scheme adapted from Abe et al (1986), in which lipids are extracted from the protein by a rapid precipitation of zein in ethyl ether. One gram of sample was dissolved in 100 ml of chloroform/methanol (2:1). The resulting solution was added dropwise into 2 L of ethyl ether while being vigorously stirred with a large stir bar. The precipitated protein was separated by centrifugation at 2,500 rpm for 5 min (International Centrifuge Universal model UV, International Equipment, Boston, MA) and subsequent filtration through Whatman No. 40 filter paper. Residual solvent was removed from the precipitate under vacuum. The protein was dissolved in chloroform/methanol and extracted a second time. The ether fractions from both extractions were combined, and solvent was removed by rotary evaporation under reduced pressure. The samples were then gravimetrically quantitated. Moisture was determined on all samples (AOAC 1970) before extraction, and the percent extractable was calculated and reported on a dry basis. Extracts were brought to 15 mg/ml with chloroform and stored at −10° C.

Sequential Extraction—Slurry Method

Figure 2 describes the sequential extraction of lipids by the slurry method as adapted from Bekes et al (1983). Samples (2 g) were placed in Erlenmeyer flasks to which 25 ml of hexane was added (1:12.5 ratio of sample to solvent). The sample flask was then flushed with nitrogen and sealed. Flasks were incubated at 40° C for 13 hr while shaking at 150 rpm so as to obtain a slurrying of ground sample in the solvent. Samples were filtered through Whatman No. 40 filter paper, and the protein was again extracted with 25 ml of hexane, this time for 6 hr under the same conditions. The filtrate from the second extraction was combined with that from the first extraction and evaporated under reduced pressure in a rotary evaporator.

The evaporated residue was transferred with hexane to a 50-ml Erlenmeyer flask and the hexane was removed under nitrogen. This residue was then extracted with 10 ml of chloroform to remove any impurities (10 min, 30° C, 150 rpm). The sample was then filtered through Whatman No. 40 filter paper into a preweighed vial. Solvent was removed under nitrogen, and the extractable lipid was quantitated and again expressed on a percent basis. Samples were brought to 15 mg/ml with chloroform and stored at −10° C for further analysis.

Zein samples that were extracted with hexane were further extracted with ethyl ether by the same procedure, except that extractions were carried out at 25° C rather than 40° C due to the highly volatile nature of ether.

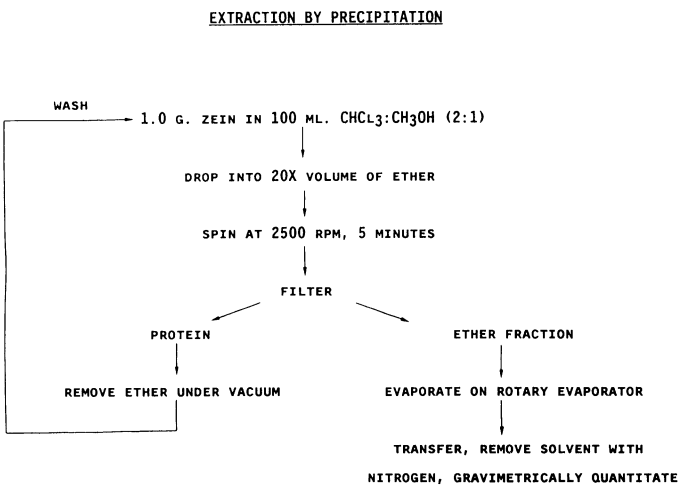

Fig. 1. Scheme for extraction by precipitation (adapted from Abe et al 1986).

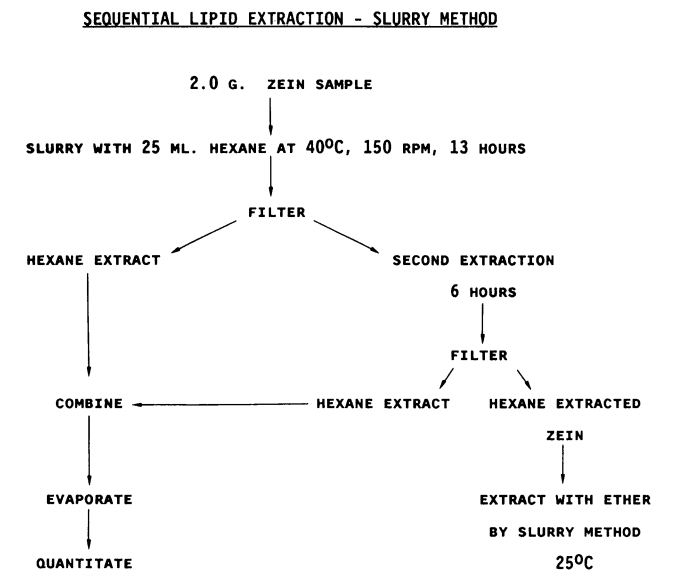

Fig. 2. Scheme for sequential lipid extraction by the slurry method (adapted from Bekes et al 1983).

HPLC Lipid Class Analysis

Lipid class analysis of extracts was performed utilizing the following conditions. The instrument used was a Varian model 5000 liquid chromatograph (Varian, Walnut Creek, CA) with an ACS model 750/14 mass detector (Peris Industries, State College, PA). The integrator was a Varian CDS 401 chromatography data system with chart speed set at 0.2 cm/min and attenuation at 16. A Partisil 5 microparticulate silica gel column, 25 cm long (Whatman, Clifton, NJ) was used. Mobile phase solvents were 1) 100% hexane; 2) 50% hexane, 50% ether with 0.5% acetic acid; 3) 35% hexane, 65% ether with 0.5% acetic acid; and 4) 100% ether with 0.5% acetic acid. The linear gradient program was solvent 1 to solvent 2, 15 min; sovent 2 to solvent 3, 10 min; and solvent 3 to solvent 4, 5 min. Sample concentration was 15 mg/ml of chloroform, and injection volume was 10 μl (loop). Flow rate was 1.2 ml/min. Peaks were tentatively identified by injection of standard solutions of triglycerides (tristearin, triolein, trilinolein, trilinolenin), diglycerides (dipalmitin, distearin, diolein), monoglycerides (monopalmitin, monostearin, monoolein), and fatty acids (palmitic acid, linolenic acid) obtained from Sigma. Standard curves of area counts versus amount of lipid injected were generated utilizing the standards above. From these data, the amount of each identified lipid class in a particular sample was determined.

RESULTS AND DISCUSSION

Extractions

Table II shows the results of lipid extraction by precipitation of protein from a chloroform-methanol solution into ethyl ether. During the extrusion process the availability of lipid to extraction was significantly decreased. It also can be seen that the zein used in our study was not pure protein, but contained substantial amounts of lipids. Therefore, even zein samples without added oil displayed a lipid-binding phenomenon during extrusion.

The temperatures studied had no significant effect on the amount of lipid extracted from extrusion-cooked zein with no oil

TABLE II
Results of Extraction By Precipitation

Sample	% Ether-Extractable Material[a]
Unextruded zein	17.3
Extruded zein, 120° C	10.9
Extruded zein, 165° C	11.0
Extruded zein + corn oil, 120° C	15.5
Extruded zein + corn oil, 165° C	14.8

[a]Weight of extract divided by total dry weight of sample.

added. Samples with oil added showed only a slight decrease in the amount extractable after extrusion cooking at the higher temperature. If heat-favored oxidative and degradative reactions of lipids leading to covalent incorporation of lipid into the protein are responsible for the decrease in extractability seen, one would expect decreased extractability at the higher temperature. This trend was not seen, suggesting that under the extrusion conditions studied, heat-induced reactions, such as oxidation and subsequent cross-linking, did not predominate. If this is the case, the decrease in extractable lipid was due to nondegradative, noncovalent interactions such as hydrophobic, hydrogen, or ionic bonding.

Table III shows the results of sequential lipid extraction by the slurry method, utilizing hexane followed by ether as previously described. Again, the same trend of decreased availability of lipids was seen after extrusion cooking. The method was less harsh and therefore much smaller amounts of lipids were extracted. Because the sample was not totally dissolved, the lipids were by nature less available. The hexane extractions revealed that oil added to the zein behaves differently than the lipids already present. Although no difference could be seen between samples without added oil at 120 and 165° C, when oil was added, significantly less lipid was available at the lower temperature. This again suggests that most of the protein-lipid interaction that occurred was not favored at higher temperatures, and thus was probably not a covalent interaction linked to oxidation.

Extraction of the residues of hexane-extracted samples with ether showed no significant differences between the standard unextruded sample and zein extrusion cooked at 120° C. In the sample extruded at 165° C, a slight decrease in ether-extractable material was seen that could not be detected simply by extraction with hexane. Ether would perturb hydrophobic lipid-protein interaction by mixing with water present in the zein and reducing the surface tension of the mixture. This would make it more energetically favorable for the ether-water system to solvate the nonpolar areas of the protein and lipids. More polar bonds, such as hydrogen or ionic interactions, would probably be unaffected by ether extractions. Therefore, it is suggested that the hexane extraction removed an equal amount of free lipids from zein extruded at 120° C and at 165° C. When extracted with ether, the sample extruded at 165° C displayed stronger interaction than that extruded at 120° C, possibly due to the presence of polar protein-lipid interactions, such as hydrogen or ionic bonding.

Examination of the percent of lipids extracted by ether from zein samples with oil added shows less lipids available at 120° C than at 165° C, in agreement with the results for the hexane extractions of the same samples. The lipid shows a greater interaction with the protein at 120° C than at 165° C. Again, the interaction seems to involve more polar bonds because ether cannot remove all the bound lipid. Thus, oil that is added and oil that is already associated with zein behave differently in relation to temperature and lipid binding.

Comparing information yielded by the two extraction methods, the slurry extraction shows interesting differences between samples extruded at different temperatures, whereas the precipitation extraction cannot distinguish such subtle differences. In addition, the slurry extraction yields itself to more rapid analysis of larger numbers of samples (for kinetic studies) than the precipitation method.

HPLC analysis was used to analyze samples obtained by both ether-precipitation extractions and hexane-ether slurry extractions. Figure 3 shows chromatograms for a standard mixture of glycerides and fatty acids, and for ether-precipitation extracts of unextruded zein and a representative extrusion-cooked zein sample.

All samples showed large triglyceride and free fatty acid peaks, with no monoglyceride detected. In addition, several unidentified peaks eluted before the triglyceride peaks and were reduced significantly during extrusion. In all zein samples, both extruded and unextruded, an unknown peak eluted immediately after the second diglyceride peak. The presence of a very small peak eluting at a similar position in the standard mixture, and the presence of a similar peak in samples of oxidized corn oil, suggest this peak may be either a molecular species of diglyceride that is low in our standard but high in corn, or some diglyceride-derived thermal product. This peak was designated peak B and quantitated utilizing a diglyceride standard curve.

Table IV shows the amount of various lipid classes extractable by ether precipitation from 2 g of sample on a dry basis. When no oil was added to zein samples, the extractable triglyceride level remained essentially the same in unextruded zein and zein extrusion cooked at 120 and 165° C. When oil was added, an obvious overall increase in triglycerides was seen, but this increase was not enough to account for the 5% added triglycerides. In addition, the level of extractable triglycerides was lower at 165 than at 120° C. A possible explanation for this is that during extrusion new sites for hydrophobic bonding of triglycerides became available, but only the added corn oil with its high degree of

TABLE III
Results of Hexane and Ether Extractions by the Slurry Method[a]

Sample	% Extracted By Hexane	% Extracted By Ether	Total
Unextruded zein	1.73 ± 0.01	0.26 ± 0.01	1.99
Extruded zein, 120° C	0.55 ± 0.03	0.25 ± 0.01	0.80
Extruded zein, 165° C	0.53 ± 0.04	0.16 ± 0.03	0.69
Extruded zein + corn oil, 120° C	0.73 ± 0.05	0.19 ± 0.04	0.92
Extruded zein + corn oil, 165° C	1.20 ± 0.05	0.34 ± 0.02	1.54

[a]Weight of extract divided by total dry weight of sample.

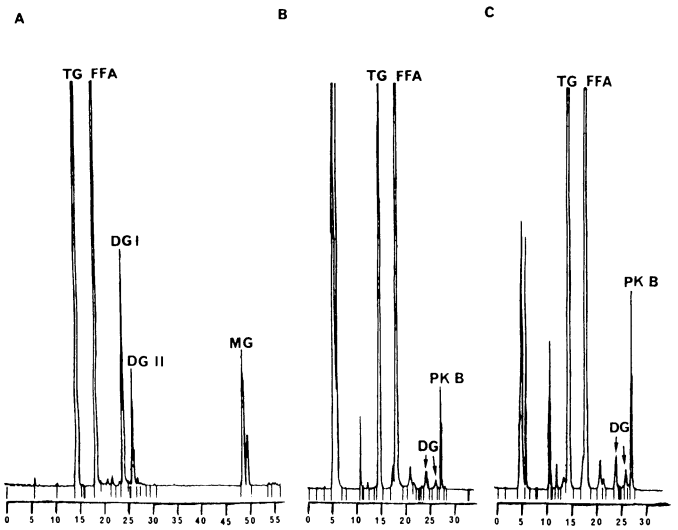

Fig. 3. High-performance liquid chromatography lipid class analysis of standard glyceride mixture (**A**), samples obtained by ether precipitation extraction of unextruded zein (**B**), and zein extruded at 165° C (**C**). **TG** = Triglyceride, **FFA** = free fatty acid, **DG** = diglyceride, and **MG** = monoglyceride; time is in minutes.

TABLE IV
Quantitative Lipid Class Analysis of Extracts Obtained by the Precipitation Extraction Method

Extract Source	% Triglyceride	% Fatty Acid	% Diglyceride	% Peak B	% Unidentified
Unextruded zein	2.22	3.62	0.15	0.37	10.90
Extruded zein, 120° C	2.01	2.80	0.23	0.29	5.57
Extruded zein, 165° C	2.21	3.30	0.28	0.40	4.81
Extruded zein + corn oil, 120° C	6.18	2.75	0.21	0.36	6.00
Extruded zein + corn oil, 165° C	4.90	2.51	0.22	0.37	6.80

[a]Expressed as % extracted from 2.0 g. sample (db).

mobility was able to come in contact with these sites. The triglycerides that were present in the zein already may have been too highly associated or too thinly dispersed throughout the protein to come into contact with these newly formed sites.

Fatty acid levels were significantly decreased in all extruded samples, from 6 to 22 mg less than in the unextruded standard. Samples without oil added showed higher levels of fatty acids available at 165° C than at 120° C, denoting either less binding or more fatty acid evolution at the higher temperature. In samples with oil added, a decrease in extractable fatty acids was seen in comparison to the unextruded standard. The addition of oil into the extruder material should increase the amount of fatty acid present in the extrusion-cooked samples due to hydrolysis of the oil during the process. Because the opposite was observed, we attributed the decrease in available fatty acids to binding of the fatty acids during the extrusion process.

The level of extractable diglycerides rose during extrusion, possibly indicating an increased hydrolysis of triglycerides under extrusion conditions. The unidentified fraction, mostly composed of the three peaks eluting before the triglyceride peak, decreased significantly during extrusion and seemed to account for a large proportion of the loss of extractability observed between extruded and unextruded samples.

In summation, lipid class analysis of extracts obtained by precipitation of zein in ether revealed that 1) added triglycerides appeared to participate in binding to a greater extent than triglycerides already present; 2) free fatty acids and the unidentified lipid fraction were bound to zein in greatest proportions; and 3) diglycerides became available during extrusion, probably due to a hydrolysis of triglycerides.

HPLC chromatograms of samples obtained from slurry extraction with hexane are shown in Figure 4. Extracts of unextruded zein and zein extruded at 120° C, with and without corn oil, are shown. Extracts obtained from samples extruded at 165° C showed similar characteristics to those extruded at 120° C. Chromatograms show that composition of hexane extracts were similar to those obtained by precipitation of protein in ether, except for the presence of an unidentified peak (designated A) in extrusion-cooked samples that split off the front or the triglyceride peak.

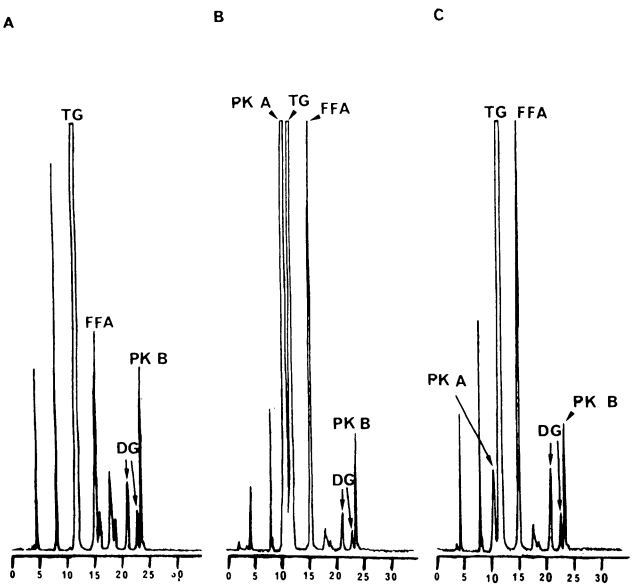

Fig. 4. High-performance liquid chromatography lipid class analysis of samples obtained by slurry extraction of zein samples with hexane from unextruded zein (**A**), from zein extruded at 120° C (**B**), and from zein + 5% corn oil extruded at 120° C (**C**).

Quantitation of the lipid classes is shown in Table V. A significant decrease in available triglycerides (18 mg) was seen during extrusion that could not be observed by precipitation extraction with ether. Since hexane removes free lipids, and ether will disrupt hydrophobic interactions, the triglyceride-binding effect shown here can be attributed to hydrophobic interaction. Even with addition of 5% corn oil, levels of triglycerides available in the extrudate were largely incorporated into the zein and rendered unavailable to extraction during extrusion.

The binding of fatty acids that was observed in samples extracted by the ether precipitation method could not be detected by the hexane extractions. Fatty acids showed only a small extractability in all samples (0.9–2.3 mg), indicating the stronger nature of the fatty acid-protein bond, even in unextruded zein. Many of these bonds may involve hydrogen or ionic bonding regions and thus were not disrupted by hexane extraction.

Availability of diglycerides (and peak B) to hexane extraction was decreased during extrusion under all conditions studied. As noted previously, levels of diglycerides available to ether precipitation extraction were increased during extrusion, indicating hydrolysis of triglycerides to diglycerides during the process. Therefore, the decrease in diglycerides observed in the hexane extractions indicated a binding of the diglycerides that were generated during extrusion.

Analysis of samples obtained by further extraction of the above zein samples with ether revealed that fraction A (peak A) was the major constituent of all these samples from both extruded and unextruded zein. Extracts of extruded zein without added oil showed levels of fraction A twice as great as the unextruded standard, whereas extracts of extrusion-cooked zein plus corn oil showed about the same amounts of fraction A as the standard. Because fraction A was "diluted" by addition of corn oil it appears to be associated with the zein lipids. Levels of A increased during extrusion, denoting either a generation or release of this fraction during extrusion. It is strange that fraction A was not detected in extracts obtained by the ether precipitation method. One possibility is that fraction A was such a small proportion of total lipids extracted that its detection was masked.

CONCLUSION

Protein-lipid interaction was shown to occur during extrusion using both an extraction by precipitation and a slurry extraction method to determine binding. Slurry extraction of ground extrudates coupled with HPLC lipid class analysis enabled detection of triglyceride and diglyceride binding. Extraction by precipitation in ether was too harsh a method to detect such binding but did reveal a significant binding of fatty acids during extrusion. Analysis of data from both sets of extractions indicated that diglycerides were generated during extrusion and interacted with protein.

The less vigorous slurry extraction method had the capability to

TABLE V
Quantitative Lipid Class Analysis of Extracts Obtained by Slurry Extraction with Hexane[a]

Extract Source	% Peak A	% Triglyceride	% Fatty Acid	% Diglyceride	% Peak B	% Unidentified
Unextruded zein	NP[b]	1.05 ± 0.080	0.09 ± 0.002	0.07 ± 0.003	0.06 ± 0.006	0.59 ± 0.160
Extruded zein, 120° C	0.14 ± 0.040	0.13 ± 0.006	0.05 ± 0.002	0.01 ± 0.001	0.01 ± 0.001	0.21 ± 0.030
Extruded zein, 165° C	0.12 ± 0.008	0.09 ± 0.020	0.04 ± 0.004	0.006 ± 0.003	0.01 ± 0.001	0.30 ± 0.090
Extruded zein + corn oil, 120° C	0.02 ± 0.002	0.45 ± 0.035	0.06 ± 0.003	0.02 ± 0.002	0.02 ± 0.001	0.16 ± 0.070
Extruded zein + corn oil, 165° C	0.08 ± 0.005	0.60 ± 0.090	0.12 ± 0.020	0.04 ± 0.003	0.03 ± 0.007	0.16 ± 0.050

[a] Expressed as % extracted from 2.0-g sample (db).
[b] Not present.

distinguish differences in the amount of lipids bound at different barrel temperatures and might be useful in determining kinetics of protein-lipid interaction during extrusion. Results of our study indicated that protein-lipid interaction was not increased at a higher temperature. Therefore, we hypothesized the mechanism of these interactions did not involve degradation and covalent incorporation of lipids into protein. Lipids already present in the zein and added oil were found to behave differently, with the added oil becoming bound to a greater extent. The observation that different solvents removed different lipid fractions suggests that these fractions were associated with the protein by noncovalent interactions that may be encouraged by denaturation of the protein during extrusion cooking.

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