Methionine-Rich Protein Fraction Prepared by Cryoprecipitation from Extracts of Corn Meal

By cryoprecipitation at −20 °C of a zein II extract obtained from corn meal by extraction with 70% ethanol, 0.6% sodium acetate, and 0.5% mercaptoethanol, a supernatant and precipitate fraction were obtained. The supernatant contained predominantly polypeptides of the same mobilities as those of peptides in a 70% ethanol–0.6% sodium acetate (zein I) extract of corn meal and some polypeptides unique to zein II. One zein II polypeptide that was not detected in the supernatant fraction and three zein II polypeptides whose abundance relative to zein II polypeptides was reduced were found as the major components of the precipitate fraction. The amino acid composition of the supernatant fraction resembled closely that of zein I while that of the precipitate was enriched for methionine, glycine, proline, and tyrosine relative to zein I.

Because in corn-based feeds supplemented with soybean protein methionine is the nutritionally limiting amino acid, there is some interest in improving the methionine content of corn (Paulis et al., 1978). In particular, interest has concentrated on a fraction of corn proteins variously designated zein II (Sodek and Wilson, 1971), G_1 (Landry and Moureaux, 1970), or alcohol-soluble glutelin (Paulis and Wall, 1971) and operationally defined as that protein which is soluble in aqueous alcohol containing a reducing agent after the alcohol-soluble zein I fraction has been removed from the meal. This fraction resembles the zein I fraction in the composition of most amino acids but has higher levels of some amino acids, notably methionine (Sodek and Wilson, 1971; Landry and Moureaux, 1970; Paulis and Wall, 1971; Misra et al., 1976). The high methionine content associated with the su_1 gene in some genetic backgrounds has been traced to the zein II fraction (Paulis et al., 1978). The suggestion (Sodek and Wilson, 1971; Landry and Moureaux, 1970) that this fraction may contain the same proteins as zein I and some additional proteins rich in methionine gained support from gel filtration studies (Paulis and Wall, 1971) in which a fraction of molecular weight of ~17 500 was found which was enriched for methionine. The zein II fraction could also be subfractionated by solubility in water (Paulis and Wall, 1977), again producing a methionine-enriched fraction. Two polypeptide fractions of 13.5×10^3 and 9.6×10^3 molecular weight prepared by electrophoresis were also shown to be rich in methionine (Gianazza et al., 1977).

In the course of the preparation of zein extracts for other studies, we have come across a procedure which allows the efficient separation of zein II into methionine-rich polypeptides and polypeptides resembling the proteins in the zein I extract. It is our hope that this simple method will facilitate screening for lines containing high levels of this methionine-rich zein fraction, as well as assist in the chemical characterization of these molecules.

EXPERIMENTAL SECTION

Kernels of W64A *Zea mays* L. were ground in a coffee mill, and the resulting meal was defatted by overnight stirring at room temperature with *n*-hexane. Preparation of extracts was by a modification of the procedure of Paulis et al. (1975). The air-dried meal was extracted 3 times for 30 min with 0.5 M NaCl (10 mL/g of meal) at room temperature. The residue, obtained by centrifugation for 10 min at 5000*g*, was then extracted 3 times with the same volume of 70% (v/v) ethanol–0.6% (w/v) sodium acetate at room temperature. This extract was termed zein I. The residue was then extracted 3 times with the same volume of 70% (v/v) ethanol–0.6% (w/v) sodium acetate–0.5% (v/v) 2-mercaptoethanol at room temperature. This extract was termed zein II. For cryoprecipitation, the zein II extract was placed in a −20 °C freezer overnight. The precipitate which formed was recovered by centrifugation for 10 min at 5000*g* at −20 °C. The supernatant was recovered and designated as the cryosupernatant of zein II. The precipitate redissolved readily in 70% (v/v) ethanol–0.6% (w/v) sodium acetate–0.5% (v/v) 2-mercaptoethanol. The resulting solution was then placed again at −20 °C. The precipitate which formed was again recovered by centrifugation at −20 °C and dissolved as described above. All extracts were then dialyzed extensively against water. The entire contents of the dialysis tubes were then frozen, lyophilized, and weighed.

Measured amounts of the various protein preparations were hydrolyzed with 6 N HCl–0.5% mercaptoacetic acid at 110 °C in vacuo for 18 h. Amino acid analysis was performed on a modified analyzer of the Spackman et al. (1958) type in a 7.8 mm diameter column (Liao et al., 1973). Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis was performed according to Laemmli (1970) on slab gels of 15% acrylamide monomer concentration. Visualization of polypeptide bands was achieved by staining with 0.5% (w/v) Coomassie brilliant blue R in 45% (v/v) methanol–10% (v/v) acetic acid. Molecular weights of polypeptides were estimated by comparison with the mobilities of bovine serum albumin, ovalbumin, chymotrypsinogen, cytochrome *c*, α-chymotrypsin, and cyanogen bromide fragments of cytochrome *c*.

RESULTS

When the alcoholic zein II extract was stored at −20 °C, a precipitate formed. The precipitate, after separation from the supernatant, could be readily dissolved in 70% ethanol containing mercaptoethanol. When this solution was recooled, the protein precipitated again, indicating that the precipitation was a reversible cryoprecipitation. Soluble and precipitated fractions of zein II were dialyzed against water and lyophilized. It was found gravimetrically that the cryoprecipitate accounted for 16.6% of the total zein II protein.

Amino acid analyses of the two zein II subfractions, as well as of zein II and zein I, were performed (Table I). As reported before (Sodek and Wilson, 1971; Landry and Moureaux, 1970), zein II differed from zein I in lower contents of aspartic acid (asparagine), leucine, isoleucine, and phenylalanine and higher contents of proline, methionine, glycine, and tyrosine. The zein II cryosupernatant proteins had an amino acid composition almost indistinguishable from that of the zein I proteins. By use

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Table I. Amino Acid Compositions of Zein Fractions

	composition, mol/100 mol			
	zein I	zein II cryosupernatant	zein II cryoprecipitate	zein II
Asp	5.0	4.2	2.4	3.8
Thr	3.1	3.1	3.0	3.0
Ser	6.4	6.3	5.0	6.1
Glu	21.1	20.6	20.1	19.7
Pro	10.8	12.3	14.1	13.1
Gly	2.6	3.5	8.1	4.3
Ala	13.7	12.4	12.1	12.4
Val	4.2	4.1	3.4	3.8
Met	1.1	2.2	7.1	4.4
Ile	3.6	2.9	1.1	2.6
Leu	17.0	16.6	11.1	15.4
Tyr	3.7	4.0	6.7	4.3
Phe	5.1	4.5	1.6	4.0
His	1.1	1.5	1.3	1.3
Lys	0.1	0.0	0.1	0.0
Arg	1.5	1.5	2.8	1.7

[a] All values are in moles of amino acid per 100 mol of recovered amino acids; the contribution of cysteine and tryptophan has been ignored.

of the method of Marchalonis and Weltman (1971) which gives $S\Delta Q$ values of less than 50 for closely related proteins, a comparison of the cryosupernatant composition with that of zein I resulted in $S\Delta Q = 11.0$. This value is close to that obtained by repeated analysis of the same protein (4). In contrast, when the compositions of zein I and zein II were compared by this method, $S\Delta Q$ was 173.0. Minor differences in aspartic acid (asparagine), proline, glycine, methionine, and isoleucine were still observed between cryosupernatant and zein I proteins, but these differences were much less than that between zein I and zein II compositions. The amino acid composition of the zein II cryoprecipitate proteins differed markedly from the composition of zein I in just those amino acids that differentiated zein I from zein II. Contents of aspartic acid (asparagine), leucine, isoleucine, and phenylalanine were 57, 65, 31, and 31% of the levels found in zein I. The levels of proline, tyrosine, glycine, and methionine in the zein II cryoprecipitate proteins were respectively 1.3-, 1.8-, 3.1-, and 6.4-fold higher than in zein I polypeptides. In addition, an increase in the content of arginine was noted.

The amino acid analyses suggested that the cryoprecipitation had separated some zein I like polypeptides (in the supernatant) from other protein material. This suggestion was corroborated by $NaDodSO_4$–polyacrylamide gel electrophoretic comparison of the polypeptides in the two fractions resulting from cryoprecipitation with polypeptides in unfractionated zein II and zein I extracts (Figure 1). Because of difficulties in the detection of minor bands, samples were applied at two levels (20 and 100 μg/lane). Zein I consisted of three bands in the molecular weight range of 20 000. For purposes of discussion, they will be designated by their apparent molecular weights as 22K, 20K, and 19K. Even when the gel was overloaded with regard to these three bands, only trace amounts of other polypeptides could be detected. The same three bands (22K, 20K, and 19K) were present in the unfractionated zein II preparation. In addition, there were four additional bands in the 10 000–16 000 molecular weight range (12K, 13K, 15K, and 16K) and four bands greater than 23 000 in molecular weight (26K, 33K, 47K, and 55K). Of the three zein I bands only the 22K band was found in the zein II cryoprecipitate and this only in minor amounts. Also much diminished in this subfraction was the 55K band of the zein II sample. Four bands (12K, 15K, 33K, and 47K) accounted for most of the stained material

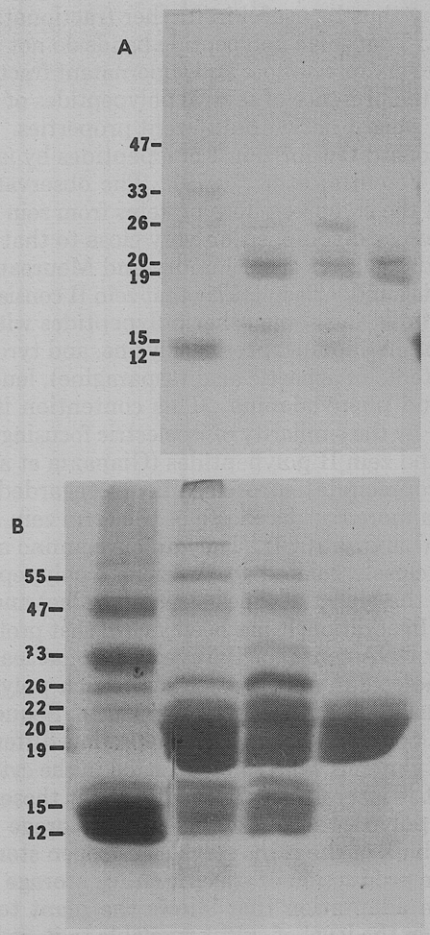

Figure 1. $NaDodSO_4$–polyacrylamide gel electrophoresis of zein fractions. (A) 20 μg of protein/lane; (B) 100 μg of protein/lane. In both cases, from left to right the lanes contained zein II cryoprecipitate, zein II cryosupernatant, zein II, and zein I. Numbers in left margin refer to approximate molecular weights ($\times 10^{-3}$) of the bands detected.

in the zein II cryoprecipitate preparation. The levels of those polypeptides that appeared as major polypeptides in the cryoprecipitate were correspondingly reduced in the supernatant of zein II cryoprecipitation. The 33K band was virtually undetectable and the relative amounts of the 12K, 15K, and 47K bands were reduced in the cryosupernatant relative to the total zein II fraction. The 16K and 26K bands were present in both the supernatant and the precipitate of cryoprecipitation. A high molecular weight band in the cryosupernatant fraction was not reproducibly observed and may thus be a contaminant.

DISCUSSION

The separation of the zein II fraction obtained by cryoprecipitation differs from that obtained by previously reported methods. The fractionation of zein II by gel filtration (Paulis and Wall, 1971) resulted in a 17 500 molecular weight fraction that contained methionine-enriched polypeptides, while cryoprecipitation selectively enriched for two of the low molecular weight components and two of the higher molecular weight components. Fractionation of zein II into water-soluble and insoluble subfractions (Paulis and Wall, 1977) did result in a fraction which was enriched in methionine and had reduced amounts of polypeptides of molecular weight ~20 000. However, the amino acid compositions resulting from this fractionation differed substantially from those reported here, particularly in proline, leucine, arginine, and aspartic acid (asparagine) contents. A combination of these tech-

niques may thus be useful in further fractionating these molecules. That some polypeptide bands do not segregate cleanly into cryoprecipitate and supernatant fractions may be due to the presence of several polypeptides of the same molecular weight but with different properties. This has been demonstrated for zein I polypeptides by isoelectric focusing (Valentini et al., 1979). The observation that removal of the cryoprecipitate proteins from zein II results in an amino acid composition very close to that of zein I supports the hypothesis of Landry and Moureaux (1970) and of Sodek and Wilson (1971) that zein II consists of zein I polypeptides and some other polypeptides with higher contents of methionine, proline, glycine, and tyrosine and lower contents of aspartic acid (asparagine), leucine, isoleucine, and phenylalanine. This contention is further supported by the similarity of isoelectric focusing patterns of zein I and zein II polypeptides (Gianazza et al., 1976).

The cryoprecipitate proteins may be regarded as zeins although a more restricted use of the term zein has been proposed (Landry, 1979). They do have amino acid compositions closely related to the zein I polypeptides, in particular the high glutamic acid (glutamine) and proline contents. In addition, it has been shown that protein body messenger RNA codes for the synthesis of at least one of the low molecular weight methionine-rich polypeptides when translated in vitro (Melcher, 1979). Sequestration in protein bodies is one characteristic that differentiates zeins from glutelins which are deposited in the cytoplasmic matrix. It is interesting to speculate that these methionine-rich polypeptides serve as sulfur storage proteins while the bulk of the zeins serve as nitrogen storage proteins. The production of specific sulfur storage proteins may be an adaptation that allows the plant to survive variations in the level of sulfur available to it, as has been suggested for pea storage proteins (Millerd et al., 1979).

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2-Hexyl-3-methylmaleic Anhydride: An Unusual Volatile Component of Raisins and Almond Hulls

2-Hexyl-3-methylmaleic anhydride has been identified in the volatile oils of California raisins and almond hulls by using capillary GLC–MS, IR, and ^{1}H NMR spectrometries. An authentic sample for comparison was synthesized by the Stobbe condensation of hexanal with diethyl methylsuccinate, followed by hydrolysis, anhydride formation, and acid-catalyzed double-bond rearrangement. This seems to be the first detection of a natural volatile acid anhydride in a food.

The authors are involved in a study to determine the identities of the volatile components of raisins (and other products) and to test whether these components attract certain insect pests. Some of the authors had already reported on a study of the volatile components of almond hulls (Buttery et al., 1980). An unusual component previously detected in almond hulls was also found in raisins. The present communication reports the identification of this compound.

EXPERIMENTAL SECTION

Materials. High-quality raisins (dried Thompson's seedless grapes) were obtained from a local raisin processor in the Fresno area of California. Raisins were also obtained from local markets. Starting materials for the synthesis of the anhydride were obtained from Aldrich Chemical Co.

Isolation of Volatile Oil from Raisins. Raisins (1500 g) were placed in a 12-L round-bottomed flask together with 6 L of odor-free water. A Likens-Nickerson steam distillation continuous extraction head was attached to the flask. Purified hexane (100 mL) was placed in a 250-mL flask attached to the solvent arm of the head. The extraction head condenser was cooled with a water–ethanol mixture at 0 °C. A dry ice cooled reflux condenser was

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