Corn Protein Subunits: Molecular Weights Determined by Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

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Reduced corn endosperm proteins or their subunits were resolved and molecular weights determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Heterogeneity, poor solubility, and molecular interactions make characterization of these proteins difficult by other methods. Albumins, globulins, and zein were extracted from endosperm meal by a modified Osborne-Mendel procedure. Then alcohol-soluble glutelin (ASG) was extracted with 70% ethanol–0.5% sodium acetate–0.1 *M* β-mercaptoethanol (β-ME), and alcohol-insoluble glutelin (AIG) was extracted with pH 8.9 Tris-borate buffer containing SDS and ME. Albumins and globulins were very heterogeneous and differed in polypeptide composition. Zein polypeptides, however, were homogeneous in mol wt (21,300). ASG contained primarily 24,700 mol wt subunits and differed uniquely from zein and from the heterogeneous AIG. ASG and AIG corresponded in polypeptide composition and mol wt to glutelin subunit fractions prepared from destarched residues and isolated on Sephadex G-200. Comparison of polypeptides of each protein class from normal corn endosperm to corresponding protein fractions from near-isogenic high-lysine *opaque-2* and *floury-2* endosperms revealed some quantitative differences. Thus, SDS–PAGE is useful for characterizing, comparing, and differentiating corn endosperm proteins in protein isolation studies and genetic investigations.

Corn proteins are a complex mixture whose heterogeneity and varying solubilities have impeded their characterization. Albumins, globulins, and zein fractions may be each in turn separated from corn meal by sequential extraction with water, saline, and ethanol solutions. The majority of the residual glutelin proteins are insoluble in even highly potent denaturing solvents, but upon reduction of glutelin disulfide bonds, the resulting polypeptides can be dissolved and dissociated to yield subunits in 8 *M* urea, 6 *M* guanidine hydrochloride, or sodium dodecyl sulfate (Nielsen *et al.*, 1970; Paulis and Wall, 1969; Landry and Moureaux, 1970). The alkylated-reduced subunits of glutelin have been partially fractionated by solvent extraction and gel filtration chromatography (Paulis and Wall, 1971). Amino acid analyses and starch gel electrophoresis suggest that the glutelin subunits may be different from those of other fractions of corn protein. Heterogeneity and tendency to associate have limited molecular weight determinations of these proteins (Nielsen and Beckwith, 1971).

To further establish the composition and molecular weights of reduced corn proteins or their subunits we have employed polyacrylamide gel electrophoresis (PAGE) in the presence of the anionic detergent sodium dodecyl sulfate (SDS) (Shapiro *et al.*, 1967). The method was used successfully to resolve wheat protein subunits and to compare their molecular weights (Bietz and Wall, 1972, 1973). The high resolving power of SDS–PAGE for proteins based on differences in molecular weight permitted distinction between some corn proteins of similar starch gel electrophoretic mobilities and gel filtration elution behavior. A rapid procedure for corn protein extraction was employed that is especially suitable for preparing samples for SDS–PAGE. SDS–PAGE patterns of subunits prepared by this extraction process were compared to those of glutelin subunits separated by other methods (Paulis and Wall, 1971).

Several investigators have studied possible composition differences between proteins of normal and high lysine corns and concluded that major variations are due to a change in proportions of the solubility classes (Mertz *et al.*, 1964; Mossé, 1966; Paulis *et al.*, 1969; Sodek and Wilson, 1971). Paulis *et al.* (1969) also observed that the starch gel electrophoresis patterns of reduced glutelins of normal corn exhibited more intense bands due to alcohol-soluble subunits than those from *opaque-2.* We have observed some additional quantitative differences in subunits of other endosperm proteins from near-isogenic lines of normal, *opaque-2,* and *floury-2* corns by means of SDS–PAGE.

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EXPERIMENTAL SECTION

Unbroken mature kernels from a normal hybrid corn, P-A-G SX52, and its near-isogenic derived lines, P-A-G 50001 *opaque-2* (both inbred parent lines of modified stocks had undergone 6 and 4 backcrosses) and P-A-G 50101 *floury-2* (both parent inbred lines of modified stocks were backcrossed four times), were used in this study. The kernels were soaked in distilled water for 0.5 hr at room temperature and manually dehulled and degermed. The air-dried endosperms were rapidly ground in a Udy cyclone hammer mill through a 0.024-in. screen. The meals were partially defatted by intermittent shaking with petroleum ether at a 3:1 (v/w) solvent-to-meal ratio for 1 hr, the suspensions then were filtered, and the solids were washed on a Büchner funnel with cold solvent and air-dried.

A defatted normal corn endosperm meal served as a control for comparing extract nitrogens and SDS–PAGE patterns of the protein fractions obtained by different methods. Alkylated-reduced glutelin proteins from the corn and fractions of these proteins obtained by gel filtration chromatography were prepared as described earlier (Paulis and Wall, 1971).

Fractionation of Proteins. A modified Osborne-Mendel extraction method was used to separate the proteins from the corn endosperm samples into five fractions (albumins, globulins, zein, alcohol-soluble reduced glutelins, and alcohol-insoluble reduced glutelins). To avoid loss in transferring after centrifugation, all successive extractions were carried out in the same 250-ml polypropylene bottle.

Albumins and globulins were isolated from 10 g of defatted meal with 0.5 *M* NaCl according to the procedure described by Paulis and Wall (1969). The meal residues remaining after the 0.5 *M* NaCl extraction were washed free of salt with water.

Table I. Protein Distribution in Endosperms of Normal, *Floury-2*, and *Opaque-2* Corns

Fraction[a]	Proteins	% of total		
		Normal	*Floury-2*	*Opaque-2*
0.5 *M* NaCl	Albumins and globulins	6.1	12	17.8
70% ethanol–0.5% NaOAc	Zeins	53.2	43.2	32.6
70% ethanol–0.5% NaOAc–0.1 *M* β-mercaptoethanol	Alcohol-soluble reduced glutelins	12.5	12.4	13.8
Tris-borate–SDS–1% β-mercaptoethanol (pH 8.9)	Alcohol-insoluble reduced glutelins	26.8	29.8	37.2
Total nitrogen extracted		98.6	97.4	101.4

[a] Includes free amino acids and other nonprotein nitrogen which represent small amounts of the total nitrogen.

Zein was next extracted from the residue by vigorously shaking on an International bottle shaker at maximum speed with 100 ml of 70% ethanol (EtOH) containing 0.5% sodium acetate (NaOAc) at room temperature for 1 hr, three times. After each extraction, the suspension was centrifuged at room temperature at 1000*g* for 10 min. All zein extracts were combined and dialyzed at room temperature against several changes of 70% ethanol to possibly remove some pigments and/or phospholipids and NaOAc. After dialysis the ethanol was removed by evaporation and the water-insoluble zein was freeze-dried.

To obtain alcohol-soluble reduced glutelin (ASG), the residue was next extracted with 100 ml of 70% EtOH–0.5% NaOAc containing 0.1 *M* β-mercaptoethanol (β-ME) for 0.5 hr, two times, dialyzed, and freeze-dried using the conditions described for zein. The remaining residue, containing the alcohol-insoluble reduced glutelins (AIG), was washed free of salt with 70% ethanol and also freeze-dried. The residue material contained mainly starch in addition to AIG.

The amount of AIG extracted with the buffer used for preparation of protein for electrophoresis (0.125 *M* Tris-borate–0.1% SDS (pH 8.9) containing twice the protein weight of SDS and 1% β-mercaptoethanol) was determined by measuring the soluble nitrogen from the incubation of 2 g of AIG protein containing residue in 5 ml of buffer. The ratio of buffer to protein and conditions used are the same as those described for SDS–PAGE in the section on Analytical Methods.

Analytical Methods. Aliquots of extracts and amino acid hydrolysates or portions of weighed dried material were assayed for nitrogen by a semimicro-Kjeldahl method.

Lysine concentrations were determined on duplicate acid hydrolysates using a Beckman 121 amino acid analyzer as described earlier (Paulis *et al.*, 1974).

SDS–PAGE was performed as described by Bietz and Wall (1972) on 5% gels, using 0.125 *M* Tris-borate buffer (pH 8.9), containing 0.1% SDS. Isolated proteins (1–2 mg) were prepared for SDS–PAGE by incubation for 16 hr at 37°, or for 5 min at 95°, with 0.1 ml of buffer containing 1% β-ME and twice the protein's weight of SDS. AIG in 80 mg of the high-starch residues was incubated for 16 hr at 37° (to avoid starch gelatinization) in 0.2 ml of buffer (containing added β-ME and SDS). The mixture was then centrifuged and the protein in the supernatant was analyzed directly. Electrophoretic mobilities of all protein subunits (measured from photographic enlargements of the gels) were compared to that of standard proteins of known molecular weight (cytochrome *c*, chymotrypsinogen A, monomeric and polymeric ovalbumin, and bovine serum albumin) run simultaneously on each of the gels. Protein molecular weights were determined from a standard curve relating mobilities to molecular weights of the standard proteins as described by Bietz and Wall (1972). Each reported molecular weight value is an average of five or more separate SDS–PAGE runs on the same protein sample.

RESULTS AND DISCUSSION

Meal Composition. The defatted normal endosperm meal which served as a control (Paulis and Wall, 1971) and the near-isogenic normal, *opaque-2*, and *floury-2* endosperm meals contained 1.2, 1.6, 1.4, and 1.5% nitrogen, respectively. Based on a conversion factor of 6.25, these meals contained 7.5, 9.9, 8.6, and 9.4% protein, respectively. The *opaque-2* endosperm meal protein contained 3.0% lysine, whereas that of the normal and *floury-2* counterparts, only 1.6 and 2.4%.

Modified Fractionation Method. The new protein preparation was more rapid than that used previously for isolating glutelin (Paulis and Wall, 1971) but gave quantitative yields (Table I). Extracting the residues with 70% EtOH–0.5% NaOAc–0.1 *M* β-ME removed as much ASG as in the longer method requiring prior elimination of starch and alkylation of the reduced glutelin before 70% EtOH extraction. The reduced protein soluble in the ethanolic solvent is probably the same protein referred to by others as glutelin-1 or zein-2 (Landry and Moureaux, 1970; Sodek and Wilson, 1971; Misra *et al.*, 1972). Since the buffer used for preparing the proteins for electrophoresis contained β-ME and SDS which was found earlier by Moureaux and Landry (1968) to be capable of solubilizing residual glutelin without solubilizing starch, the protein extract obtained with this buffer was used directly for electrophoresis.

The relative amounts of protein fractions in the near-isogenic endosperms of normal, *floury-2*, and *opaque-2* are shown in Table I. The yields for saline and alcohol-soluble proteins are consistent with those reported by others (Jiménez, 1966; Mossé *et al.*, 1966; Misra *et al.*, 1972). Although the amount of ASG was slightly higher in *opaque-2* corn than in normal, it still constituted a smaller fraction of the total glutelin (alcohol-soluble and -insoluble reduced protein) than in normal corn.

The 0.125 *M* Tris-borate (pH 8.9) buffer containing 0.1% SDS plus twice the protein weight of SDS and 1% β-mercaptoethanol (Tris-borate–SDS–1% β-mercaptoethanol (pH 8.9)), which was used for preparing proteins for electrophoresis, solubilized approximately 100% of AIG protein. The total extracted nitrogen was 97–100%.

Comparison of Glutelin Preparations. The SDS–PAGE patterns of alkylated-reduced whole glutelins isolated by the earlier procedure (Paulis and Wall, 1971) are compared in Figure 1 with that of reduced glutelin fractions isolated from the same normal endosperm meal by the present method. Both procedures gave similar patterns; differences in intensity may be due to concentration variations. SDS–PAGE patterns were similar for 70% EtOH extracts of destarched alkylated-reduced glutelins and the direct 70% EtOH–0.5% NaOAc–0.1 *M* ME extracts. The AIG prepared by the two methods also yielded

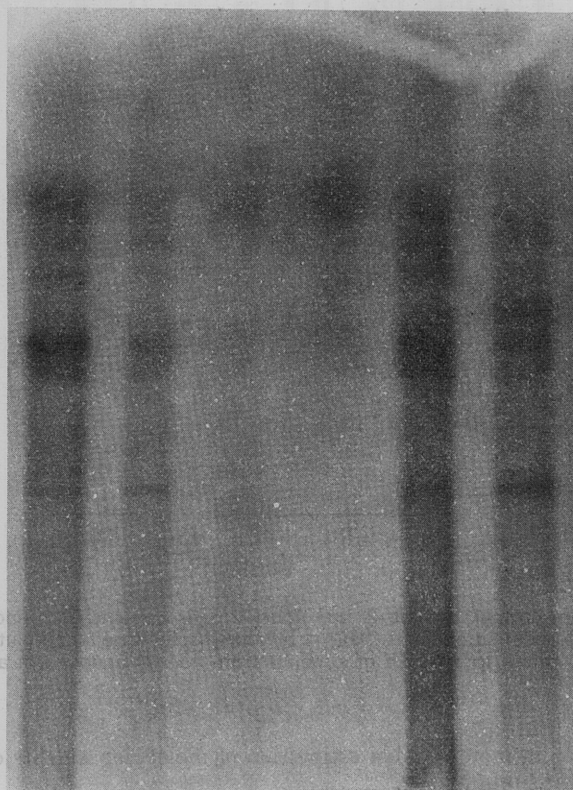

Figure 1. Comparison of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) patterns of glutelin preparations: A-R glutelin = destarched whole alkylated-reduced (A-R) glutelin; residue protein = whole glutelin–starch residue; alcohol-soluble fraction of A-R glutelin and 70% EtOH–0.5% NaOAc–0.1 *M* β-mercaptoethanol (β-ME) soluble glutelin (ASG); alcohol-insoluble fraction of A-R glutelin and 70% EtOH–0.5% NaOAc–0.1 *M* β-ME insoluble glutelin. All A-R glutelins are the same as described previously by Paulis and Wall (1971).

Figure 2. SDS–PAGE patterns of A-R glutelins separated by Sephadex G-200 chromatography. Fractions are described by Paulis and Wall (1971). Whole A-R glutelin is included for comparison.

similar patterns (Figure 1). Thus, for SDS–PAGE purposes there is no need to remove starch and to alkylate the reduced glutelins.

Comparison of SDS–PAGE patterns in Figure 2 of fractions from an earlier Sephadex G-200 separation of alkylated-reduced glutelins (Paulis and Wall, 1971) to those of the preparations (Figure 1) shows that the alcohol-insoluble and -soluble glutelin polypeptides are represented mainly by components in A to D and E and F, respectively. The lower mol wt subunits in fractions A to D appearing at similar mobilities to those in E and F indicate that some polypeptide association may have occurred in 6 *M* guanidine hydrochloride chromatographic solvent, causing early elution of these components, or that initial reduction of whole glutelin was incomplete. All fractions were heterogeneous by SDS–PAGE.

Molecular Weight of Subunits. SDS–PAGE patterns of protein subunits of the five fractions from normal, *opaque-2,* and *floury-2* endosperms are shown in Figure 3. Most polypeptides in each fraction differ from those in preceding or succeeding fractions from the same endosperm with the possible exception of some albumin and globulin subunits. Thus, each fraction primarily represents different proteins and not residual protein from a previous extraction or disulfide interchanged polypeptides arising previously during mixing. The SDS–PAGE patterns for normal endosperm are also illustrated diagramatically in Figure 3; each polypeptide is numbered for identification and their molecular weights are listed in Table II. The accuracy of mol wt determined by SDS–PAGE is normally ±5% (Dunker and Rueckert, 1969).

The albumins, globulins, and AIG subunits represent a broad heterogeneous range of mol wt, whereas the alcohol-soluble polypeptides, zein and ASG, constitute a narrow range of smaller mol wt. Zein consists mainly of one band at 21,300 mol wt. This mol wt compares closely with the value found for reduced zein by ultracentrifugation analysis by Turner *et al.* (1965). ASG has a slightly different mol wt, 24,700. Earlier, these proteins were thought to be of the same mol wt since they eluted in the Sephadex G-200 fractionation of alkylated-reduced glutelins in the same volume. This difference in mol wt and differences in amino acid compositions observed earlier (Paulis and Wall, 1971) further support the nonidentity of these two protein groups. Also, the different mobility of the zein band from other bands in the ASG fraction indicates that practically all zein was removed by 70% EtOH–0.5% NaOAc extraction before isolating the reduced protein by addition of 0.1 *M* ME to this solvent. The zein band and bands 3 and 4 of ASG (fractions E or F in Figure 2) were shown to be heterogeneous by starch gel electrophoresis (Paulis and Wall, 1971).

The mean molecular weights obtained for fractions of reduced glutelin polypeptides separated on Sephadex G-200 columns as determined by calibration of the column with known proteins are fairly consistent with the molecular weights obtained by SDS–PAGE for the constituent polypeptides. Comparison of electrophoretic mobilities of glutelin polypeptides in Figure 2 to those in Figure 3 was used to identify the Sephadex G-200 fractions and determine molecular weights of components from Table II. Fraction A, which is greater than 70,000 mol wt by Sephadex G-200 analysis, consists mostly of AIG bands 1 to 8 and minor bands 15 to 17. Fraction C,

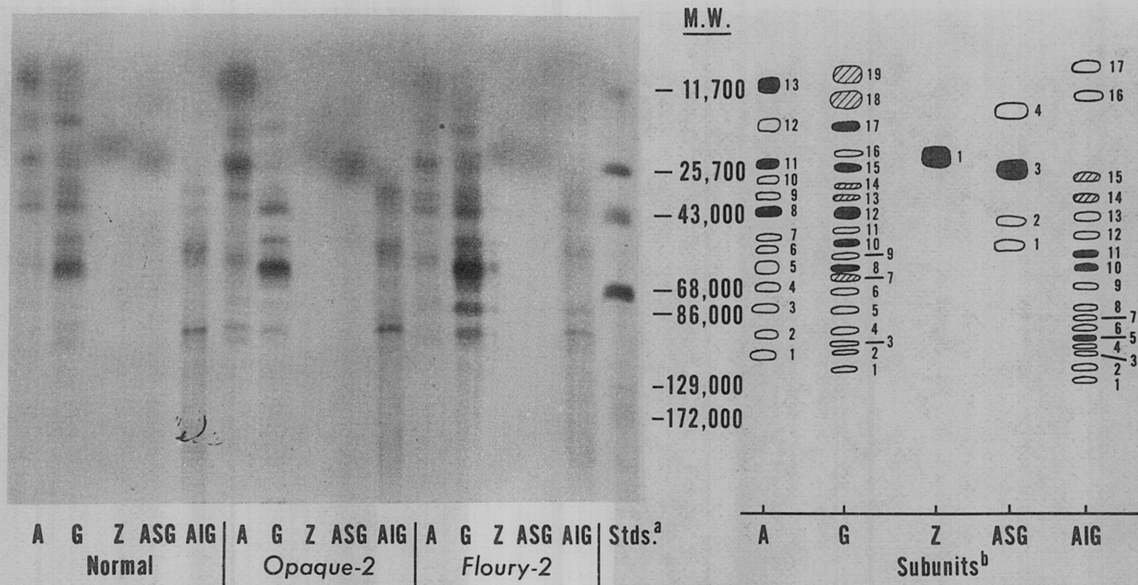

Figure 3. Comparison of SDS–PAGE patterns of protein polypeptides from normal, *opaque-2*, and *floury-2* near-isogenic corn endosperms (A = albumins, G = globulins, Z = zein, ASG = alcohol-soluble reduced glutelins, AIG = alcohol-insoluble reduced glutelins; (a) std., refers to standard protein calibration mixture; (b) subunits, refers to diagram of subunits from normal endosperm and number system used in text and Table II for protein polypeptides.

Table II. Molecular Weights of Polypeptides from Reduced Endosperm Protein Fractions[a] from Normal Corn

	Mol wt $\times 10^{-3}$				
Band no.[b]	Albumins (A)	Globulins (G)	Zeins (Z)	Alcohol-soluble reduced glutelins (ASG)	Alcohol-insoluble reduced glutelins (AIG)
1	105	119	21.3	52.6	127
2	93.3	106		43.9	120
3	78.4	101		24.7	109
4	68.5	92.3		13.6	102
5	59.3	81.4			97.2
6	53.9	71.7			91.1
7	49.2	65.7			85.3
8	41.0	61.9			78.9
9	35.4	56.6			67.3
10	30.1	51.6			59.9
11	24.5	47.8			54.8
12	16.5	40.7			47.8
13	10.8	35.7			41.4
14		32.2			34.2
15		25.8			27.7
16		22.0			14.2
17		16.3			11.6
18		12.8			
19		10.5			

[a] Molecular weights were obtained with a calibration curve of standard proteins from the averaged mobilities of polypeptides in the protein fractions in five gels. [b] Band numbers refer to diagram in Figure 3.

53,000 mol wt, contains mainly polypeptides 9 to 11. Fraction D, 35,000 mol wt, is composed mainly of polypeptides 12 to 14; E, 25,000 mol wt, contains mainly band 3 of ASG. F is 17,500 mol wt and consists mostly of band 4 of the ASG. The better resolution obtained by SDS–PAGE results in more reliable estimation of molecular weights of specific subunits.

Polypeptides of Mutant Endosperm Proteins. Comparison of polypeptides in the protein fractions from normal, *opaque-2,* and *floury-2* endosperms in Figure 3 reveals that a few minor differences in band intensities occur between some of the same fractions of the various genotypes. Band 8 of the albumin fraction is present in low concentration in *opaque-2.* Globulin bands 4, 5, and 6 are barely visible in normal; band 5 is most intense in *floury-2.* Since the albumins and globulins represent most of the enzymes of the endosperm, the difference in concentration of polypeptides might relate to a difference in activities of certain enzymes of the grains (Dalby and Davies, 1967; Wilson and Alexander, 1967). These bands of reduced intensity in either the albumins or globulins of one genotype must be due to a protein found in only albumins or globulins since, if it occurred in both fractions, a decrease of bands with similar mobility would be anticipated in both fractions. The only difference between normal and mutants in the ASG was a lighter band 3, and in the AIG a darker band 7, in fractions from *floury-2.* The increased methionine in *floury-2* corn endosperm glutelins noted by Hansel *et al.* (1973) may be related to differences found here in the glutelin polypeptides of *floury-2* compared to that of normal grain.

The finding of only minor differences in the SDS–PAGE patterns of the same genotype fractions is anticipated for proteins of near-isogenic endosperms in which only one gene has been changed. These studies support earlier findings that main differences in high lysine from normal corns are due to changes in proportions of protein which contain high levels of lysine (Mertz *et al.*, 1964; Mossé, 1966; Mossé *et al.*, 1966; Paulis *et al.*, 1969; Sodek and Wilson, 1971). The possibility is not excluded that new proteins but of similar molecular weight are produced in high lysine corns.

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Degradation of Methionine in Heated Soybean Protein and the Formation of β-Methylmercaptopropionaldehyde

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Methionine, added to soy protein isolate, when heated in boiling water is destroyed under aerobic conditions. A portion of the added methionine was degraded to the corresponding β-methylmercaptopropionate. This was determined by gas chromatographic separation of the butyl ester of heated soybean protein hydrolysate. A characteristic peak was eluted after 15.30 min from the heated protein hydrolysate. The mass spectrum of this peak indicated that it was *n*-butyl β-methylmercaptopropionate.

The production of flavors in foods upon processing as a result of reactions between sulfur-containing amino acids and sugars has been suggested in several reports. Many studies reporting losses or binding of methionine during thermal processing have been done on meat proteins. Miller *et al.* (1965) reported loss of methionine in cod muscle processed for 27 hr at 85°. Loss of methionine was also reported in defatted herring cake processed for 27 hr at 130°. Similar observations of loss of methionine were observed by Horn (*Agr. Res.*, 1969) who suggested that this was the result of a condensation between the sulfur in the methionine and the carbonyl of the carbohydrate present. Arroyo and Lillard (1970) attributed the overcooked egg odor of a heated cystine–glucose mixture to hydrogen sulfide and mercaptans. Gruenwedel and Patnaik (1971) stated that the black deposits in the headspace area of canned protein rich foods resulted from heat-induced decomposition of the sulfur containing amino acids which produced hydrogen sulfide and, in turn, iron sulfide during the thermal processing.

Wainwright *et al.* (1972) proposed a variety of intermediate compounds of methionine resulting from degradation. The authors suggested that one of these compounds was methional. A general review concerning the Strecker degradation of α-amino acids has been published by Schonberg and Moubacher (1952). According to these authors, the formation of the intermediate Schiff compound between the carbonyl and the amino group is an essential step in the reactions followed by decarboxylation and deamination.

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In the present study, the thermal degradation of methionine, when supplemented to soybean protein and the subsequent formation of methional (β-methylmercaptopropional), was investigated.

EXPERIMENTAL SECTION

Materials. The protein source that was used in the experiments reported herein was Promine D, sodium soy proteinate, a commercially available product, courtesy of the Central Soya Co., Chemurgy Division, Chicago, Ill.

Heat Treatment Procedures. Four methods of heating the protein samples were investigated: (1) dry air heating with a chamber air dryer at 100° for 60 min; (2) steam heating with a steam blancher at atmospheric pressure for 60 min; (3) heating of the protein dispersion (10%) in water at 100° for 60 min; (4) heating under vacuum at 100° for 60 min.

Amino Acid Analysis. Acid hydrolysates were obtained by dissolving 20 mg of protein in 10% 6 *N* HCl in a Pyrex tube. The tube was flushed with nitrogen and closed securely with a Teflon lined cap. Hydrolysis was carried out at 100° for 22 hr. The hydrolysate was filtered through glass wool and the 6 *N* HCl was evaporated by heating in an oil bath at 100° under a stream of nitrogen. The hydrolyzed amino acid residue was dissolved in 10 ml of 0.1 *N* HCl. Ornithine (5 mg) was added as an internal standard and the 0.1 *N* HCl was evaporated again. The sample was redissolved in 10 ml of 3 *N* HCl in *n*-butyl alcohol with the aid of an ultrasonic mixer. Esterification of the amino acids was carried out in open vials, heated at 100° for 15 min. The excess butanol was removed by evaporation and the residue dissolved in 4 ml of methylene chloride–trifluoroacetic anhydride (3:1) solution and acylated for 5 min at 150° in a sealed acylation tube.

Gas Chromatography Conditions. In general, the