substrate was free linoleic acid (Matoba et al., 1985). As elucidated in the present study, *n*-hexanal formation was not caused by nonenzymatic reaction, but enzymatically generated by hydroperoxide lyase. Galliard and Chan (1980) and Axelrod et al. (1981) imply that 9- and 13-hydroperoxides are produced from linoleic acid by the enzyme in soy milk. However, we have shown that only *n*-hexanal was most abundantly detected among carbonyl compounds having more than five carbons (*n*-pentanal) in the soybean homogenates (Matoba et al., 1985). This may be explained by the evidence that only 13-hydroperoxide lyase is present in soybean. There have been various types of hydroperoxide lyases in plants. The enzyme is specific for 13-hydroperoxide in tomato fruits (Galliard and Matthew, 1977) and tea chloroplast (Hatanaka et al., 1981), specific for 9-hydroperoxide in pear fruits (Kim and Grosch, 1982), and specific for both 9- and 13-hydroperoxides in cucumber fruits (Galliard et al., 1976). The soybean lyase did not act on 13-hydroperoxide of methyl linoleate (data not shown). This strongly suggests that *n*-hexanal is enzymatically generated through 13-hydroperoxide from free linoleic acid but not from bound linoleic acid such as triacylglycerols and phospholipids.

Under the usual conditions (pH 6–7), the level of *n*-hexanal formation in L-2 null seed was considerably lower than those in the other seeds, though the lyase activity occurred in the same level as those in all the seeds.

From several lines of the evidence as described above, it is postulated that L-2 isozyme predominantly reacts with linoleic acid to produce 13-L-*c*,*t*-HPO under the usual conditions (pH 6–7) and that 13-L-*c*,*t*-HPO is cleaved by the hydroperoxide lyase to produce *n*-hexanal.

Registry No. HPO, 71833-11-9; 13-L-*c*,*t*-HPO, 33964-75-9; 13-DL-*c*,*t*-HPO, 97672-38-3; lipoxygenase, 9029-60-1; *n*-hexanal, 66-25-1; linoleic acid, 60-33-3.

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Received for review March 15, 1985. Accepted June 5, 1985.

Formation of the Acid-Sensitive Fraction through the Interaction of Soybean Globulins and Lipids

Fumio Yamauchi, Yasuyuki Kurosawa, Katsumi Takahashi,* and Kazuyoshi Okubo

An acid-sensitive fraction (ASF) forming process was studied by using a model system with soybean proteins and lipids. ASF1 was formed as a result of adding glycolipids of phospholipids to β-conglycinin, precipitating the mixture at pH 4.5, and redissolving proteins in 1 M NaCl. ASF1 was gradually increased with an increase of lipids, whereas glycolipids gave more ASF1 than phospholipids. Most of the additional glycolipids migrate to the ASF1 precipitate. ASF2, which obtained as a 1 M NaCl insoluble fraction when adjusted to pH 4.5, was compared with ASF1 by using some model system consisting of soybean lecithin and soybean proteins. The yields of ASF1 were more than the yields of ASF2 in all cases and β-conglycinin gave more ASF1 and ASF2 than glycinin. ASF2 was formed from β-conglycinin but did not form from glycinin either with or without added lipids. Glycinin and mixtures of glycinin and β-conglycinin gave less ASF1 than β-conglycinin by itself; however, their yield of ASF1 increased when lipids were added. Both ASFs contain more α,α′ subunits and γ-conglycinin. The average hydrophobicity (AH) calculated from the amino acid composition of the α subunit was 940 and from γ-conglycinin was 929 cal/mol. The AH value was stronger than that of glycinin.

INTRODUCTION

Approximately 90% of the protein precipitates when an aqueous extract of defatted soybean is adjusted to pH 4.6 (Smith and Circle, 1938). Although virtually all the water-extractable soybean proteins are soluble in a phosphate buffer (Wolf and Briggs, 1956), only about 80% of this acid-precipitated protein has been found to redissolve in the buffer (Wolf and Sly, 1964). The solubility of an extracted protein appears to be modified by exposure to an acidic pH. This acid-sensitive fraction (ASF) has been obtained by Wolf and Sly (1964). The decreased solubility is attributed to the acid-sensitive proteins of the 2S and

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7S ultracentrifuge components (Wolf and Sly, 1964; Nash and Kwolek, 1971). Anderson et al. (1973) demonstrated a similar acid-induced insolubility in the 2S and 7S components by examining the solubility of acid-precipitated proteins in 0.8 M NaCl at pH 4.5. The 7S (ASF) protein lacks the ability to form a dimer at low ionic strengths (Anderson, 1974). This acid-sensitive fraction binds non-protein material (Anderson, 1974) and off-flavors (Anderson and Warner, 1976). More recently, Lillford and Wright (1981) have reported that the factors controlling the solubility characteristics of acid-precipitated proteins and the insolubilization process were mainly of an electrostatic nature rather than involving a disulfide exchange reaction.

We (Yamauchi et al., 1980) have analyzed the composition of the acid-sensitive fraction and found appreciable amounts of lipid (8.2–8.8%) in which glycolipids were the major component. Some interaction between proteins and lipids was suggested (Kurosawa et al., 1981) as follows: further removal of lipids from defatted meals gave a lower yield of ASF and good solubility of ASF in sodium dodecyl sulfate indicated hydrophobic interactions were predominant in ASF.

This paper describes that ASF is formed not only by the insolubilization of soybean proteins themselves but also by the interaction of soybean globulins and lipids. This is demonstrated by using a model system.

MATERIALS AND METHODS

Defatted Soybean Meals. Soybeans (var. Raiden) harvested in 1974 were flaked and defatted with hexane in a batchwise manner several times until the filtrate showed nearly no color. Defatted soybean meals were ground further in a coffee mill to pass through 40-mesh sieves and defatted with hexane several more times. The final defatted meals contained 0.1–0.2% ether-extractable lipids determined by Soxhlet extraction.

Glycinin and β-Conglycinin. β-Conglycinin was prepared by a fractionation at pH 6.4 and then chromatographed on a DEAE-Sephadex A-50 column and a Sepharose 6B column (Thanh and Shibasaki, 1976). The β-conglycinin contained a small amount of γ-conglycinin on SDS–gel electrophoresis (Sato et al., 1984). Glycinin was prepared by rough fractionation at pH 6.4, fractionation at 66% saturation with ammonium sulfate, and chromatography on a Sepharose 6B column and a Con A Sepharose 4B column.

Lipids. A commercial lecithin from soybean was purchased from Nakarai Chemicals, Ltd. It was used for total lipids in this paper and mainly contained triglycerides, glycolipids, and phospholipids. Glycolipids and phospholipids were separated from the commercial lecithin by chromatography on a silicic acid column (Miura et al., 1982). Yields of neutral lipids, glycolipids, and phospholipids were 1.30, 1.26, 3.51 g, respectively, from 6.14 g of the commercial lecithin.

Lipid Dispersion. This dispersion was used for the interaction with protein solutions. The required amount of lipids was moved to a sonicator cell by dissolution with an appropriate amount of ether or $CHCl_3$–MeOH (1:1, v/v). The solvent was then removed by N_2 flow at 30 °C. The lipids were dispersed in a 0.01 M Tris-HCl buffer (pH 8.0) containing or not containing 1 M NaCl by sonication (Kubota Ltd., KMS-100) for 30 min. After sonication, the lipid dispersion was passed through sintered glass.

Preparation of ASF. For the preparation of ASF, two methods were used as shown in Figure 1 (Yamauch et al., 1980). ASF1 was prepared by precipitation at pH 4.5 followed by the removal of 1 M NaCl soluble materials from the precipitate. ASF2 was precipitated by adjusting the pH to 4.5 in the presence of 1 M NaCl. The latter method corresponds to that of Anderson and Warner (Anderson and Warner, 1976). Washings were followed by centrifugation. All centrifugations were preformed at 15000g for 30 min. Finally, each sample was dialyzed and lyophilized. ASF2 and its supernatant of globulins and whey proteins were prepared for amino acid analysis from the water extract of defatted soybean meals as described by the previous report (Yamauchi et al., 1980).

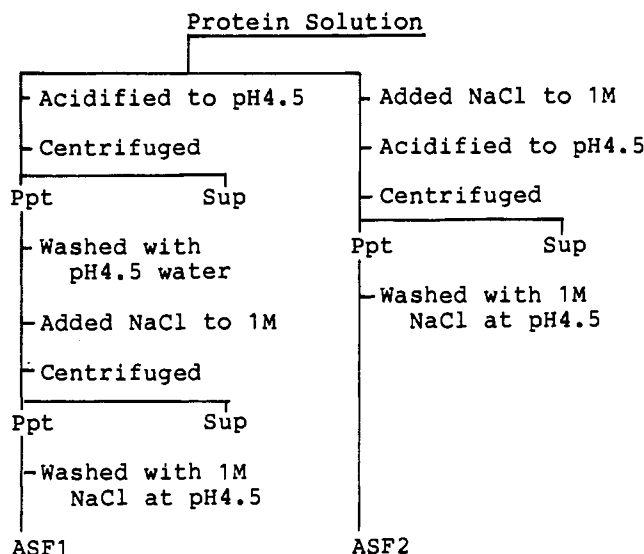

Figure 1. Schematic diagram for preparation of ASF1 and ASF2 from a protein solution (ASF, acid sensitive fraction).

Precipitation of Soybean Globulins under Acidic pH and at a High Ionic Strength. Proteins (1%) were dissolved in 0.01 M Tris-HCl (pH8.0) containing 1 M NaCl, 0.1 mM dithiothreitol, and 0.02% NaN_3. After stirring for 1 h, the insoluble materials were removed by centrifugation (15000g for 20 min at 10 °C). A portion of the protein solution was acidified with 1–2.5 M HCl to a pH range between 0.5–7.5 with stirring. After fillup, the protein solutions were kept for 1 h with occasional stirring and centrifuged (15000g for 20 min at 10 °C). The protein content of the supernatants was measured by the biuret method.

Assay of ASF by Interaction of Lipids and Proteins. For the formation of ASF1, proteins (about 2%) were dissolved in a 0.01 M Tris-HCl buffer (pH 8.0) and centrifuged to remove the insoluble materials.

Various concentrations of lipid dispersions (2 mL), which were prepared by the dillution with a 0.01 M Tris-HCl buffer (pH 8.0), were added to the protein solutions (2 mL) and kept for 1 h with occasional stirring. The mixtures were adjusted to pH 4.5 with 1 M HCl, and solid NaCl was added to the mixture to make it 1 M in NaCl. After dissolution, the mixtures were centrifuged. The protein content of the supernatants was measured by the biuret method. For the formation of ASF2, the proteins were dissolved in a 0.01 M Tris-HCl buffer (pH 8.0) containing 1 M NaCl. Lipids were dispersed in the buffer by the same procedure as ASF1. After setting 1 h, the mixtures were centrifuged, and the protein content of the supernatants was measured by the biuret method.

SDS–Gel Electrophoresis. Under the conditions for ASF1 formation, a portion of the supernatant, which was used for the protein measurement previously described, was dialyzed against water and lyophilized. The precipitates were twice washed with water, defatted with water-saturated *n*-butyl alcohol, and lyophilized. The

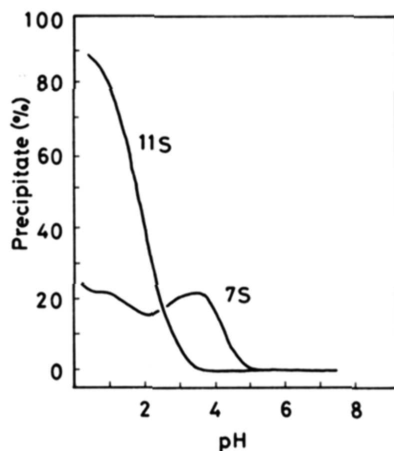

Figure 2. Precipitation profile of glycinin (11S) and β-conglycinin (7S) (0.6–0.9% proteins in 1 M NaCl).

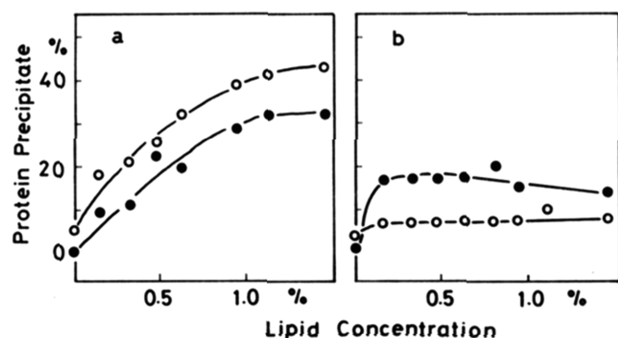

Figure 3. Effect of crude glycolipid or crude phospholipid on the precipitate formation under the ASF1 forming condition. Effect of glycolipid (a): β-conglycinin (—O), glycinin (—●). Effect of phospholipid (b): β-conglycinin (—O), glycinin (—●). Protein concentration: β-conglycinin 0.68%, glycinin 0.85%.

supernatants and precipitates, under the conditions for ASF2 formation, were treated by the same procedure as those for ASF1.

Slab gel electrophoresis was carried out in a SDS–urea system of 9% acrylamide by using a glass plate (14 × 14 cm) according to the previously described procedure. Gels were stained with 0.3% Coomassie blue G-250 and scanned at 570 nm with a Shimadzu CS-900 densitometer.

Quantitative Analysis. Glycinin and β-conglycinin were measured with ultraviolet at the $E_{280nm}^{1\%}$ 5.47 and $E_{280nm}^{1\%}$ 8.04, respectively. When precipitation occurred, the supernatant was measured for proteins by the biuret method.

Amino Acid Composition. Samples were defatted by water saturated butyl alcohol (Yamauchi et al., 1980) and hydrolyzed with 6 N HCl in an evacuated and sealed tube at 110 °C for 20 h. The hydrolyzate was subjected to chromatographic analysis with an amino acid analyzer (Nihondenshi, JLC-8AH).

The average hydrophobicities for the proteins were calculated from amino acid composition (Tanford, 1976).

RESULTS

Glycinin and β-conglycinin were precipitated at an acidic pH in 1 M NaCl solution. The results are shown in Figure 2. Glycinin was completely dissolved until pH 3.5; thereafter, it was extensively precipitated. The precipitated protein was composed of 86% glycinin at pH 0.5. On the other hand, precipitation of β-conglycinin started at pH 5.0 and gave a maximum amount at pH 3.5. The β-conglycinin was 5% precipitated at pH 4.5 and 24% at pH 0.5. When glycolipids or phospholipids were added to

Table I. Model System of ASF Formation[a]

model system	yield, %	
	ASF1	ASF2
β-7S	34	12
β-7S + lipid	36	10
11S	8	0.4
11S + lipid	25	0.0
β-7S + 11S	17	7
β-7S + 11S + lipid	29	11

[a] Commercial lecithin was used as lipid. Protein/lipid = 1.2–1.3.

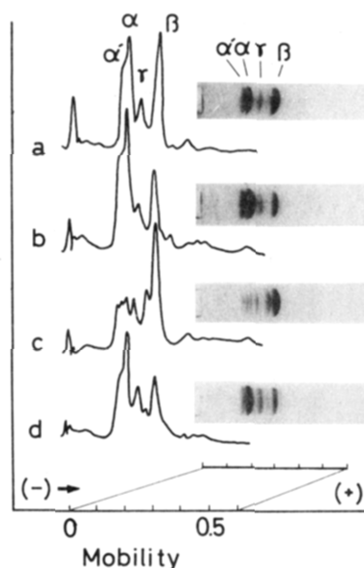

Figure 4. Densitometric scanning of SDS–gel electrophoretic patterns. Samples were prepared under the ASF1 forming condition: (a) β-conglycinin (sup), (b) β-conglycinin (ppt), (c) β-conglycinin + lipid (sup), (d) β-conglycinin + lipid (ppt).

β-conglycinin, ASF1 was formed as shown in Figure 3. ASF1 gradually increased with an increase of glycolipids. β-Conglycinin formed more ASF1 than glycinin in the presence of glycolipids. The formation of ASF1 was 40% from β-conglycinin and 30% from glycinin at 1% glycolipid. On the other hand, phospholipids formed less ASF1 than glycolipids. The formation of ASF1 was 17% from glycinin and only 5–7% from β-conglycinin at about 1% phospholipid.

ASF1 and ASF2 were measured in the model systems of various combinations by using the soybean lecithin and soybean proteins as shown in Table I. The yield of ASF1 was more than ASF2 in all cases. β-Conglycinin yield ASF1 and ASF2 more than glycinin. Addition of lipids was effective for both glycinin and β-conglycinin plus glycinin. ASF2 was formed from β-conglycinin but not from glycinin.

Proteins produced under ASF1 measurement conditions from β-conglycinin are shown in Figure 4. With the absence of lipids, a larger amount of α and α′ subunits of β-conglycinin was in the precipitate than the supernatant. When lipids were added, the precipitate had more α and α′ subunits than β subunit; therefore, α and α′ subunits decreased markedly in the supernatant.

Under ASF2 forming conditions, densitometric scannings are shown in Figure 5. When lipids were added to β-conglycinin, β-conglycinin also appeared in the precipitate (b) and entirely disappeared in the supernatant (a). When lipids were added to a mixture of β-conglycinin and glycinin, β-conglycinin moved to the precipitate (d); however, glycinin did not (c). Under ASF2 forming conditions, β subunits decreased slightly in the precipitate (b); how-

Table II. Amino Acid Composition and Average Hydrophobicity of Purified Soybean Proteins[a]

		amino acid composition, molecular %						
amino acid	$H\Phi_i$	WP + APP	ASF2	α^1	α'^1	β^1	γ-7S[2]	11S[3]
Lys	1500	5.5	6.7	6.2	7.3	5.3	6.9	4.1
His	500	1.8	2.2	1.2	3.7	2.0	2.8	1.8
Arg	750	6.0	5.0	8.7	7.2	7.1	6.3	5.9
Asp	0	11.0	9.6	11.8	11.0	13.0	10.1	11.8
Thr	400	3.3	4.0	2.2	2.3	2.7	4.2	4.2
Ser	-300	5.1	4.3	6.6	6.5	7.2	6.5	6.6
Glu	0	14.8	10.6	20.5	21.4	16.7	17.5	18.8
Pro	2600	5.8	5.6	7.1	6.5	5.1	5.9	6.3
Gly	0	6.0	6.8	4.4	5.0	4.4	6.1	7.8
Ala	500	4.9	6.6	4.6	4.3	5.3	4.8	6.7
Val	1500	4.7	3.3	4.4	4.8	5.4	6.4	5.6
Met	1300	1.0	1.3	0.4	0.5	0.1	1.4	1.0
Ile	2950	4.2	5.1	5.8	4.9	6.2	4.8	4.6
Leu	1800	6.5	7.7	8.7	7.5	10.4	7.7	7.2
Tyr	2300	2.0	2.4	2.2	2.2	2.6	2.1	2.5
Phe	2500	3.3	3.4	5.2	5.0	6.4	5.5	3.9
av	$H\Phi_i$	888	977	940	895	967	929	836

[a] $H\Phi_i$ = hydrophobicity of amino acid; WP = soybean whey protein. [1]Thanh and Shibasaki, 1977. [2]Sato et al., 1984. [3]Kitamura and Shibasaki, 1975.

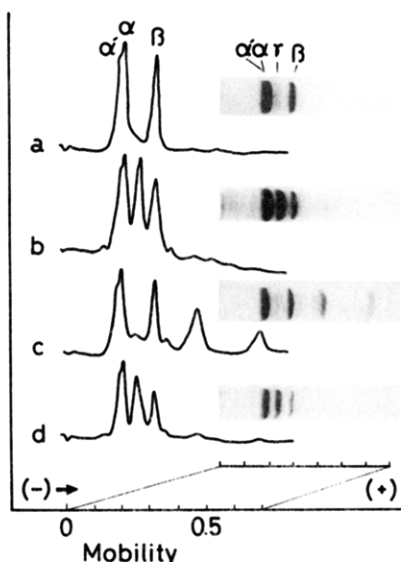

Figure 5. Densitometric scanning of SDS–gel electrophoretic patterns. Samples were prepared under the ASF2 forming condition: (a) β-conglycinin + lipids (sup), (b) β-conglycinin lipid (ppt), (c) β-conglycinin + glycinin + lipid (sup), (d) β-conglycinin + glycinin + lipid (ppt).

ever, α and α' subunits did not decrease (a) contrary to ASF1 forming conditions.

Amino acid composition and average hydrophobicity of ASF and the related proteins are shown in Table II. ASF2 gave a higher hydrophobicity than the mixture of whey proteins and globulins. ASF2 also showed the highest value among glycinin and subunits of β-conglycinin and γ-conglycinin.

DISCUSSION

In this study two types of ASF were prepared by different procedures. ASF1 has routinely been removed by most workers in the preparation of acid precipitated proteins. ASF2 was prepared according to the same procedure as that of Anderson and Warner (1976).

Proteins (2S and 7S) were obtained as the main components of ASF by ultracentrifugation (Anderson et al., 1973); however, as a small amount of glycinin was also contained in ASF, the solubility of globulins was examined in this report. Lillford and Wright (1981) described that glycinin was coagulated under these conditions without a reducing reagent. β-Conglycinin was precipitated at pH 4.5 but glycinin was soluble in the presence of 1 M NaCl, i.e., in ASF2 forming conditions (Figure 2). These results were consistent with those of Table I. On the contrary, glycinin was apparently coagulated when the pH was lowered. Koshiyama (1977) obtained similar results for the separation of β-conglycinin and glycinin.

In the resolubilization of precipitate with 1 M NaCl, i.e., in ASF1 forming condition, precipitates were increased in both β-conglycinin and glycinin samples with additional glycolipids. More β-conglycinin than glycinin was precipitated, and the precipitates amounted to more than 40%. Phospholipids were less effective for ASF precipitation than glycolipids (Figure 3). These results are consistent with our previous report that a remarkable amount of glycolipids were obtained in ASF (Yamauchi et al., 1980). It is presumed that an interaction exists between the sugars of glycoprotein (β-conglycinin) and glycolipids. The higher yield of ASF1 may be ascribed to the complex formation of the ASF2 corresponding materials which are soluble with 1 M NaCl. These materials, though, are located in the interior of the ASF1 structure and a 1 M NaCl solution may not permeate into the interior. In both ASF1 and ASF2 forming conditions, β-conglycinin was precipitated more than glycinin and their mixture showed an intermediate value between β-conglycinin and glycinin. It is considered that β-conglycinin has a higher average hydrophobicity than glycinin (Table II). When ASF2 was precipitated at pH 4.5, Cl^- may give a protein molecule a change which decreased hydrophobicity. Therefore, in the model system, the yield of ASF2 showed little difference with or without lipids but the yield of ASF1 increased with lipids (Table I). Although, glycinin and a mixture of glycinin and β-conglycinin gave lower ASF1 yields than β-conglycinin alone, the addition of lipids increased the ASF1 yield. It is considered that glycinin was precipitated effectively, and the resulting precipitates were difficult to redissolve with a NaCl solution.

Both α and α' subunits were the main constituents of ASF1 from β-conglycinin (Figure 4). β-Conglycinin, which is composed of three subunits, α, α', and β, has seven heterogenities from B_0 to B_6 (Thanh and Shibasaki, 1976, 1977). As the contents of α and α' subunits increase with increasing numbers (B_0 to B_6), mainly, B_5 and B_6 subunits may be precipitated. The addition of lipids enhanced this tendency. This enhancement was understood as follows. First step: the increase of relative hydrophobicity with the

pH close to the isoelectric point. Second step: absorption of the lipids and the formation of a complex precipitate by hydrophobicity. Third step: resolubilization of the weaker hydrophobic components by the addition of 1 M NaCl and neutralization. These three steps may cause the formation of the globulin precipitates with the hydrophobic interaction between the α and α' subunit-rich proteins, which mainly consist of β_5 and β_6 and lipids. The effect of heterogeneity of β-conglycinin on the ASF2 formation was less than ASF1 formation as judged by the subunit constitution (Figure 5). It is believed that the isoelectric point of glycinin was pH 5.8–6.4 and β-conglycinin was pH 4.8–5.8 (Thanh and Shibasaki, 1976), so that proteins with a slightly charged cation interacted with the Cl^- ion under the ASF2 conditions. These interactions perhaps weakened the hydrophobicity of the protein surface at pH 4.5. The reduced effect of hydrophobicity on the ASF2 formation may be due to these interactions. In the mixture of β-conglycinin and glycinin, glycinin which has weaker hydrophobicity (Table II), showed less precipitates. Moreover, the isoelectric point of glycinin is further from pH 4.5 than that of β-conglycinin; therefore glycinin gave nearly no precipitate with the ASF2 condition. γ-Conglycinin, present in β-conglycinin, was selectively precipitated. The average hydrophobicity or γ-conglycinin was stronger than β-conglycinin (Table II).

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Received for review February 22, 1985. Accepted June 13, 1985.

Production of Zearalenone, α- and β-Zearalenol, and α- and β-Zearalanol by *Fusarium* Spp. in Rice Culture

Kurt E. Richardson, Winston M. Hagler, Jr.,* and Chester J. Mirocha

Reverse-phase thin-layer chromatographic analysis of rice culture extracts of seven zearalenone producing isolates of *Fusarium* revealed several more polar compounds similar to zearalenone in fluorescence characteristics and/or reaction to 4-methoxybenzenediazonium fluoborate and Fast Violet B salt spray reagents. Capillary gas chromatography/mass spectrometric analysis of these extracts revealed six estrogenic compounds not previously reported as naturally produced, namely *cis*-zearalenone, *cis*-α-zearalenol, *cis*- and *trans*-β-zearalenol, and α- and β-zearalanol.

INTRODUCTION

Zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resorcylic acid μ-lactone] is an estrogenic secondary metabolite produced by various species of *Fusarium*, especially cultivars of *F. roseum* (Stipanovic and Schroeder, 1975; Hagler and Mirocha, 1980; Palyusik et al., 1980), colonizing corn, sorghum, oats, and other cereal grains. This fungal metabolite has been associated with hyperestrogensim and other reproductive disorders in cattle (Mirocha et al., 1968), swine (Miller et al., 1973), and poultry (Meronuck et al., 1970; Allen et al., 1981a,b).

Zearalenone was chemically characterized by Urry et al. (1966) and since then eight naturally produced derivatives of *trans*-zearalenone (Figure 1 part a) have been isolated from cultures of *F. roseum* "Gibbosum" and *F. roseum* "Graminearum": *trans*-α-zearalenol (Hagler et al., 1979; Watson et al., 1982), diastereomers of 8'-hydroxyzearalenone (Jackson et al., 1974; Stipanovic and Schroeder, 1975), diastereomers of 3'-hydroxyzearalenone (Pathre et al., 1980), 6',8'-dihydroxyzearalene (Steele et al., 1976), 5-formylzearalenone, and 7'-dehydrozearalenone (von Bolliger and Tamm, 1972). α-Zearalenol is estrogenic, having with three to four times more biological activity than zearalenone (Peters, 1972; Hagler et al., 1979).

Other derivatives of zearalenone have been chemically synthesized and some of these derivatives have been tested for use in animal nutrition as growth promoters (Hidy et al., 1977). Sodium borohydride reduction of zearalenone yields a diastereomeric mixture of α- and β-zearalenol. β-Zearalenol was somewhat less active estrogenically than zearalenone and the diastereomers of zearalenol were determined to have little anabolic activity when compared to their estrogenic effect (Hidy et al., 1977; Mirocha et al.,

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0021-8561/85/1433-0862$01.50/0 © 1985 American Chemical Society