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Studies on Sorghum Proteins. 1. Solubilization of Proteins with Soaps

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It has been shown that up to 95% of sorghum flour proteins could be solubilized in distilled water in the presence of sodium salts of fatty acids. The most important parameters were the length of the hydrophobic chain of the soap, its concentration, and the extraction temperature. Soaps with longer hydrophobic chains (C18, C16) had lower dissolving ability than those with shorter chains (C10, C12, C14). Higher temperatures improved protein solubility, particularly at higher soap concentrations. The percentage of solubilized proteins increased with the flour protein content. Conversely, no significant difference could be observed in protein solubility between normal and high tannin sorghums. Results indicated that most of the sorghum proteins are tightly aggregated mainly through hydrophobic bonds. The main hindrance to sorghum protein solubilization would be the strong hydrophobic interactions between the proteins and the different flour components.

Most of the results reported on the solubilization of grain sorghum proteins were obtained either by the method of Osborne and Mendel (1914) or by that of Landry and Moureaux (1970). Both procedures were developed to solubilize maize proteins by using a sequence of solvents but modifications were usually introduced when the methods were applied to sorghum proteins.

According to Osborne and Mendel (1914), albumins and globulins are solubilized with a dilute salt solution, prolamins with an aqueous alcohol solution, and glutelins with a dilute alkali solution. About 50% of sorghum proteins remained insoluble by using this method (Naik, 1968; Skoch et al., 1970; Jones and Beckwith, 1970; Haikerwal and Mathieson, 1971). The procedure of Landry and Moureaux (1970) yields in addition to the albumins, globulins, and prolamins three glutelin fractions, all of them in the presence of a reducing agent: the alcohol, the alkali, and the detergent soluble reduced glutelins. About 5–10% of sorghum flour proteins were not extracted by this solvent system (Jambunathan and Mertz, 1973; Guiragossian et al., 1978; Chibber et al., 1978; Paulis and Wall, 1979; Neucere and Sumrell, 1979).

More recently it has been shown that wheat proteins, including glutenins, could be solubilized in distilled water in the presence of sodium salts of some fatty acids (Kobrehel, 1980). In the present work, the possibility of adapting this technique to solubilize sorghum flour proteins was investigated. The efficiency of different soaps was compared and optimal extraction conditions were determined. Sorghum varieties with different botanical and technological characteristics were analyzed. Results were compared to those obtained by others procedures.

MATERIALS AND METHODS

Sorghum Samples. Most of our analyses were carried out with two french sorghum varieties, *Sorghum bicolor*

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(Linn.) Moench: Inra 450 and Argence. For the comparative studies, senegalese grain sorghums with different characteristics were used.

Milling. Cleaned sorghum kernels were conditioned by adding water till the final moisture content of 15.5% was reached, and then the samples were ground in a Chopin-Dubois laboratory mill. The milling flow sheet included one break and three reductions. Depending on the samples, the yields of flours ranged from 30% to 65% of the grain weight.

Protein Solubilization. *Solubilization with Sodium Salts of Fatty Acids.* Various amounts of soap, from 0 to 900 mg, were added with 30 mL of distilled water to 2 g of sorghum flours. The samples were stirred overnight (for 15 h) at different temperatures and then centrifuged for 30 min at 4 °C, 38000 *g*. In some cases, the residues were washed, i.e., resuspended with 30 mL of distilled water and centrifuged after 2 h while stirring.

For subsequent extractions, different amounts of soap and 30 mL of distilled water were added to the residues and the experiments were carried out in the same conditions as previously.

In the different analyses, the following sodium salts of fatty acids were used: sodium decanoate, sodium dodecanoate, sodium tetradecanoate, sodium hexadecanoate, and sodium octodecanoate. The last two were commercial samples and the others were prepared in the laboratory from analytical grade chemicals.

Procedure of Osborne and Mendel (1914). Albumins and globulins were extracted from 5 g of flour with 30 mL of 0.5 M NaCl; the pH was adjusted to 6.8 with disodium phosphate. Samples were stirred for 1 h at 4 °C and then centrifuged at 38000 g for 30 min. Residues were washed twice with 20 mL of 0.5 M NaCl and all the supernatants were combined.

Prolamins were then extracted by adding 25 mL of 78% ethanol (v/v) to the residues. The samples were left to stand overnight, stirred for 2 h, and then centrifuged under the same conditions as above. For a second extraction, the residues were stirred for 1 h with 25 mL of 70% ethanol (v/v) and then washed twice with 10 mL of 70% ethanol. All the supernatants were again combined.

To extract glutelins, the residues were thoroughly resuspended in 25 mL of 0.2% NaOH, allowed to stand overnight, then stirred for 2 h, and centrifuged. The samples were extracted once more under similar conditions, except the overnight contact, and finally washed twice with 20 mL of 0.2% NaOH.

Other Extraction Procedures. Sorghum proteins were extracted according to the method of Landry and Moureaux (1970) and also by the use of this procedure as modified by Feillet et al. (1977) and Paulis and Wall (1979).

Protein Determination. Protein contents ($N \times 6.25$) of sorghum flours and extracted fractions were determined in duplicate by a semiautomatic Kjeldahl method (Feillet, 1976).

RESULTS AND DISCUSSION

Solubility of Sorghum Flour Proteins with Different Soaps. Figure 1 shows the solubility curves obtained with sodium salts of different fatty acids.

The efficiency of the soaps varied according to the length of their hydrophobic chain. At their respective optimal concentration, soaps with shorter hydrophobic chain were more efficient to solubilize sorghum proteins. The optimal concentration of soap is defined as the lowest amount of soap necessary to solubilize the highest amount of proteins. With sodium octodecanoate only 25% of sorghum flour

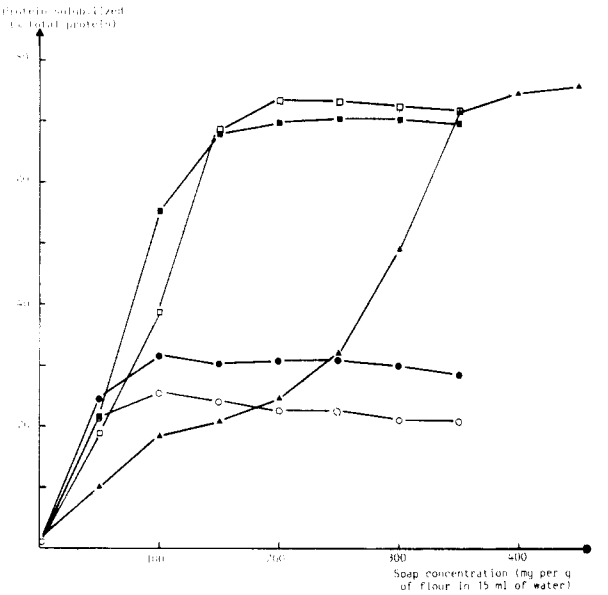

Figure 1. Solubility of sorghum flour proteins (variety Inra 450) in distilled water containing increasing amounts of soaps: (▲) sodium decanoate (C10); (□) sodium dodecanoate (C12); (■) sodium tetradecanoate (C14); (●) sodium hexadecanoate (C16); (○) sodium octodecanoate (C18).

proteins were solubilized while with hexa-, tetra-, and dodecanoate, 31%, 70%, and 73% of the total proteins were solubilized, respectively. The shape of the solubility curve obtained with sodium decanoate differed from the others. At the optimal concentration, 75% of the total proteins were solubilized but to reach that level a much higher amount of soap was needed than in the case of the other soaps.

According to earlier studies (Lundgren, 1945; Putman, 1948), the solubilization of proteins in the presence of soaps is due to the formation of water soluble complexes between proteins and detergents: the hydrophilic part of the soap molecules would combine through electrostatic interactions with charged groups along the polypeptide chains while hydrophobic interactions would take place between the hydrophobic chains of the soap molecules and the apolar groups of the proteins. Soaps do not cleave disulfide or other covalent bonds but cause protein solubilization by disrupting noncovalent bonds (Wasik et al., 1979; Hamauzu et al., 1979). It can be supposed therefore that, in the case of sorghum proteins, noncovalent bonds and especially hydrophobic interactions are involved in the formation of aggregates. Since relatively high soap concentrations were needed to solubilize higher amounts of proteins, it can be also postulated that these hydrophobic interactions are accessible with difficulty and/or are numerous between protein molecules and also between protein molecules and other grain components. Actually, Beckwith (1972) has suggested that the insolubility of sorghum glutelins can be at least partly due to the strong interactions between proteins and nonproteins.

It is interesting to note that conversly, the solubility of wheat glutenins increased in the presence of soaps with longer hydrophobic chains (Kobrehel and Bushuk, 1977). Structural differences between the proteins of these two cereals and the differences in the nature of their interactions with the other flour components are probably the reasons for their opposite behavior. More investigations are needed to give a full explanation for this phenomenon.

Effect of Soap Concentration and Successive Extractions with Soaps. Distilled water dissolved only about 2% of the flour proteins and, by increasing the

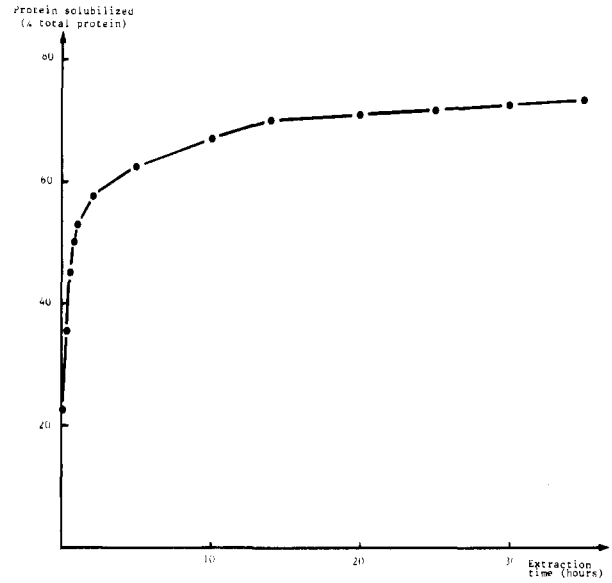

Figure 2. Effect of extraction time on solubility of sorghum flour proteins (variety Argence) in water in the presence of sodium dodecanoate (optimal soap concentration: 250 mg per 1 g of flour).

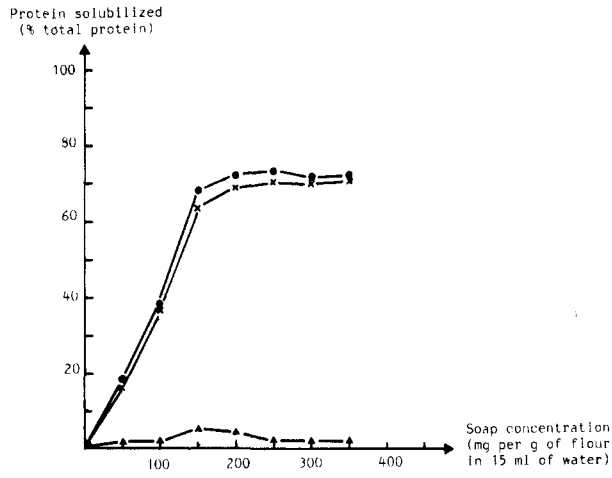

Figure 3. Solubility of sorghum flour proteins (variety Inra 450) in distilled water containing increasing amounts of sodium dodecanoate: (×) extraction with soap; (▲) washing with water; (●) extraction and washing. Extraction and washing temperature: 30 °C.

amount of soaps, the percentage of solubilized proteins increased (Figure 1). This increase was not linear, suggesting that proteins are more or less bond to each other or to the various flour components.

Beside soap concentration, protein solubility depended also on the stirring time. The highest solubility was reached after 15 h of stirring (Figure 2).

When, subsequent to the first extraction with soap, residues were washed with distilled water, some more proteins, from 1% to 5%, could be solubilized (Figure 3). Following that, however, practically no further quantities of proteins were extractible with distilled water alone. It seems that when residues were washed with water, only proteins which had already interacted with soaps entered into the solution.

Further amounts of soaps solubilized more and more proteins from the residue. Thus, four successive extractions with sodium dodecanoate, using each time 250 mg of soap per 1 g of flour, solubilized 83.5% of the total proteins (76.5%, 3.3%, 2.0% and 1.7%, respectively). It is interesting to note that sodium hexadecanoate was as efficient as sodium dodecanoate when used for the second

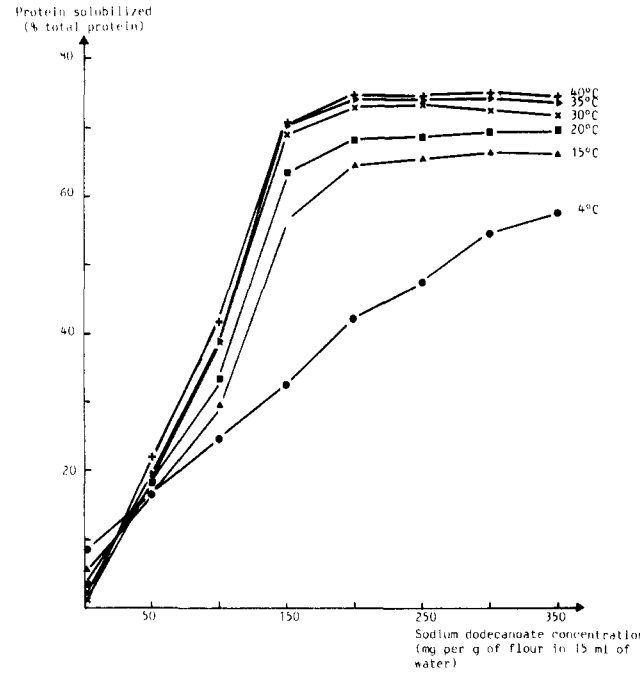

Figure 4. Effect of extraction temperature on solubility of sorghum flour proteins (variety Inra 450) in water containing increasing amounts of sodium dodecanoate.

and subsequent solubilizations, the first extraction being carried out with sodium dodecanoate. Moreover, the protein recovery was higher when less and less sodium hexadecanoate was used for the successive extractions. For example, a series of extractions involving 250 mg of sodium dodecanoate followed by 100, 50, and 10 mg of sodium hexadecanoate has solubilized 85.5% of the total proteins (76.5%, 4.1%, 2.8%, and 2.1%, respectively). Depending on the number of extractions and on the nature of soap used, up to 95% of the sorghum flour proteins could be solubilized.

These results suggest that at least 95% of the proteins, those solubilized with soaps, are bound noncovalently, mostly through numerous and more or less accessible hydrophobic interactions; hence the need is not only high soap concentrations but also successive additions of soaps for the solubilization.

Considering the insolubility of sorghum proteins in water and the action of soaps, it can be then postulated that sorghum proteins are surrounded by apolar components and have the tendency to turn their hydrophobic residues to the outside of the molecules. This hypothesis is supported by the findings of Jeanjean and Feillet (1977) showing that hydrated sorghum flours were able to form gel proteins after sheeting. The mechanical stresses would unfold polypeptide chains exposing hydrophilic groups to water.

Effect of pH. According to the soap used and to its concentration, the pH of the protein solutions obtained varied between 8 and 10.5. It should be stressed that the protein dissolving ability of a soap is not related to the pH value of the solution. Moreover, some solvents of high pH value are less efficient than soaps. For example, by using a sodium hydroxide solution at pH 11.8, only 43% of sorghum flour proteins could be solubilized (Wu, 1978). Solubility of sorghum proteins with soaps cannot be explained by an increase of the pH of the solution. The use of soaps to solubilize wheat proteins led to similar conclusions (Kobrehel and Bushuk, 1977).

Effect of Temperature. Figure 4 illustrates the effect of extraction temperature on the solubility of sorghum flour proteins.

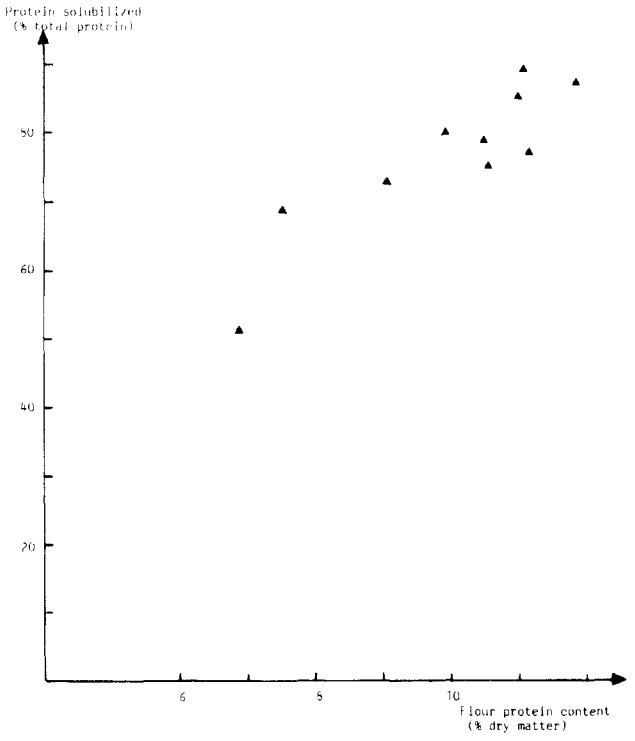

Figure 5. Effect of protein content of different sorghum cultivars on protein solubility in water in the presence of sodium dodecanoate (respective optimal soap concentration: 200–300 mg per 1 g of flour).

In distilled water, protein solubility was somewhat higher at lower temperatures and, at the lowest soap concentrations, the extraction temperature had no effect on protein solubility. However, extractibility increased with the increase of the temperature when higher amounts of soap were used. At low temperatures, the maximum protein solubility was reached with higher soap concentrations; this was particularly clear at 4 °C. These results can be explained by the endothermic character of hydrophobic bonds; thus at higher temperatures, the bonds between soaps and hydrophobic protein residues should be stronger. Consequently, these results confirm the important role played by hydrophobic interactions on sorghum protein solubilization.

Effect of Sorghum Protein Content. The percentage of proteins solubilized with soaps varied according to the protein content of the sample.

Figure 5 shows that in the case of sorghum varieties with protein content ranging from 5.8% to 10.8% dry matter, the amount of proteins solubilized in a single step with an optimal sodium dodecanoate concentration varied from 50% to 90%. In general, protein solubility increased with an increase of the flour protein content.

It should be mentioned that, conversly, Skoch et al. (1970) found a decrease in sorghum protein solubility when the protein content of the samples increased. Their results cannot be compared with ours since these authors used the method of Osborne and Mendel (1914). However, sorghum with higher protein levels generally have more kafirins and glutelins which are more easily soluble in detergents than in the solvents of Osborne and Mendel (1914). The fact that the amount of each protein group changes as the total protein content changes may explain these different results.

Protein–protein interactions are probably more numerous in flours when protein content is higher. Consequently, our results would indicate that interactions between proteins are weaker, and therefore more easily

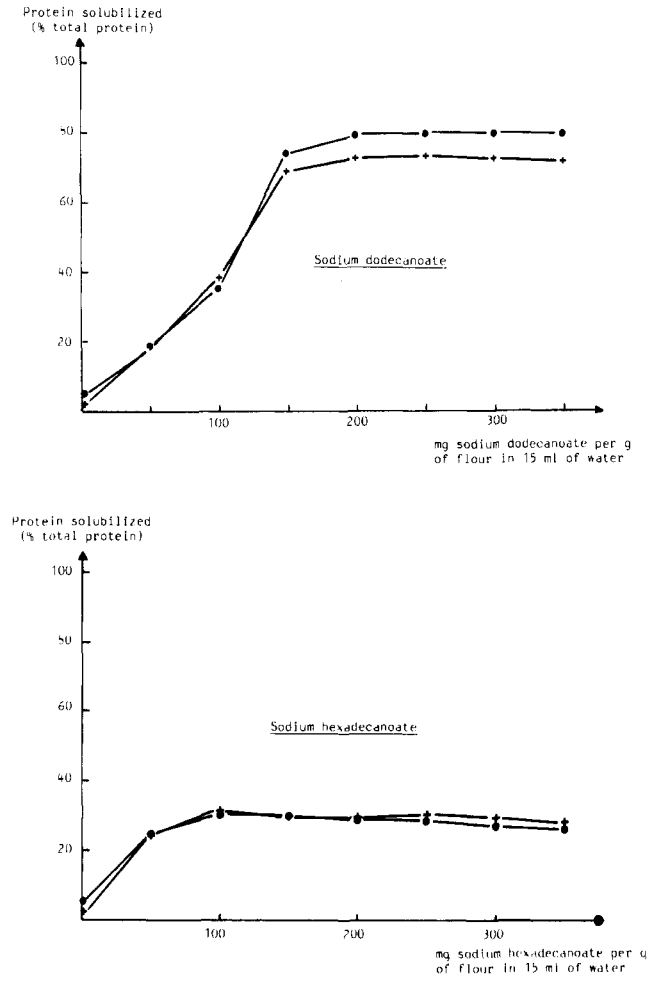

Figure 6. Influence of sorghum tannin content on solubility of proteins in water containing increasing amounts of sodium dodecanoate or sodium hexadecanoate: (●) variety CE 151 248 A2 (white pericarp, no testa); (+) variety Inra 450 (red pericarp, colored testa).

disrupted with soaps, than interactions between proteins and other flour components. It can be also postulated that hydrophobic interactions between protein chains on one hand, and between proteins and other components on the other, are not equally accessible. According to the protein content of the samples, the percentage of these proteins, linked either through stronger or through less accessible bonds, varies.

Effect of Anthocyanins and Tannins. Two sorghum varieties Inra 450 and Ce 151-248 A2 with similar protein content, 8.0% and 8.9%, respectively, were compared in this study. The former one is a high anthocyanin and high tannin variety, while the latter is devoid of anthocyanin and testa. The protein solubility curves obtained with sodium dodecanoate and sodium hexadecanoate are presented on Figure 6. No significant difference could be noticed between the protein solubility of the two varieties independently of the quantity or the nature of soap used.

These results are consistent with those of Jambunathan et al. (1972) where the total amounts of proteins solubilized by the procedure of Landry and Moureaux (1970) were similar for a normal and a pigmented sorghum variety. However, these workers found differences between the two varieties in the respective proportions of the five protein fractions obtained by this procedure. Many authors postulated that such differences in protein distribution could be explained by the existence of protein–phenolic compounds interactions altering protein solubility. Thus,

Table I. Sequential Extraction of Sorghum Flour Proteins (Variety Argence) According to Different Procedures

sequences of solvents	protein solubilized by various extraction procedures[c] as % of total protein				
	A	B	C	D	E
0.5 M NaCl	15	15	15	15	
60% ethanol			7		
70% ethanol	9				
70% isopropyl alcohol		11			
60% *tert*-butyl alcohol				25	
0.2 M NaOH	13				
70% isopropyl alcohol–0.6% ME[a]		20			
60% *tert*-butyl alcohol–0.1 M ME[a]				24	
borate pH 10–0.5% SDS[b]			34		
borate pH 10–0.6% ME[a]		5			
borate pH 10–0.5% SDS[b]–0.6% ME[a]		42	40	30	
total protein solubilized, %	37	93	96	94	75–95

[a] ME = 2-mercaptoethanol. [b] SDS = Sodium dodecyl sulfate. [c] Extraction procedures (ref): A (Osborne and Mendel, 1914); B (Landry and Moureaux, 1970); C (Feillet et al., 1977); D (Paulis and Wall, 1979); E (the sodium dodecanoate method).

the salino or alcohol soluble fractions can behave as glutelins (Jambunathan and Mertz, 1973; Chibber et al., 1978; Fishman and Neucere, 1980; Daiber and Taylor, 1982).

The similar protein extractibility between the two varieties we have studied can be attributed to the hydrophobic nature of the tannin–protein interactions which would be disrupted in the presence of soaps. This hypothesis is supported by the studies of Oh et al. (1980) and Hagerman and Butler (1980). They proved that mainly hydrophobic bounds were involved in the formation and stabilization of tannin–protein complexes.

Comparison of Protein Extraction Procedures. Our method to solubilize sorghum proteins was compared with extraction procedures used by different authors. Results obtained on the same sorghum flour are presented in Table I.

Albumins and globulins represented about 15% of sorghum flour proteins. The amount of alcohol fraction depended on the nature of alcohol used. Higher amounts of kafirins were extracted with 60% *tert*-butyl alcohol than with 70% isopropyl alcohol or with 70% ethanol, 25% compared to 11.5% and 9%, respectively. The greater efficiency of *tert*-butyl alcohol may be attributed to the hydrophobic character of kafirins (Sastry and Virupaksha, 1969; Jones and Beckwith, 1970; Wu et al., 1971; Beckwith and Jones, 1972). In any case, without using a reducing agent or a detergent, such as mercaptoethanol or sodium dodecyl sulfate, no more than about 40% of the total proteins could be solubilized (procedure of Osborne and Mendel, 1914). Similar results were obtained by Naik (1968), Skoch et al. (1970), Haikerwal and Mathieson (1971), Jones and Beckwith (1970).

When protein extraction was carried out according to the procedure of Landry and Moureaux (1970), about 5% of the total proteins remained insoluble independently of the procedure used (procedures of Landry and Moureaux (1970), Feillet et al. (1977), or Paulis and Wall (1979)). It should be noted that authors that used these different procedures to extract sorghum proteins obtained comparable results (Jambunathan and Mertz, 1973; Guiragossian et al., 1978; Chibber et al., 1978).

Similary, when proteins were first solubilized with different amounts of soap followed by a buffered solution containing a reducing agent (see Table II) or by using successive extractions with soaps as mentioned above, up to 95% of the total proteins could also be solubilized.

Table II. Successive Extractions of Sorghum Flour Proteins (Variety Inra 450) with a Soap and a Buffer Containing a Reducing Agent

extraction conditions		protein solubilized as % of total protein				
sodium dodecanoate (mg/g of flour)	50	19				
	100		39			
	150			69		
	200				73	
	350					72
borate pH 10–0.5% SDS–0.6% ME[a]		74	51	28	27	26
total protein solubilized, %		93	90	97	100	98

[a] SDS = sodium dodecyl sulfate, ME = 2-mercaptoethanol.

The role of reducing agents in solubilizing sorghum proteins is rather uncertain. Considering the efficiency of soaps, the hypothesis of the cleavage of interchain disulfide bonds through the action of reducing agents should be discarded. They probably disrupt intrachain disulfide bonds, unfolding polypeptide chains and thus rendering them more accessible to solvents. However, results do not give any explanation concerning the insolubility of about 5% of sorghum proteins.

When comparing methods of solubilization, our method involving the use of soaps has the advantage that it does not disrupt covalent bonds. Solubilized proteins are therefore more suitable for further characterization. Such studies are being carried out in our laboratory.

CONCLUSION

The difficulty to solubilize sorghum proteins, independently of the method used, cannot be due to the presence of interchain disulfide bonds. Results obtained by using soaps underline the importance of hydrophobic interactions between kernel components. The electrophoretic characterization of these proteins solubilized in water in the presence of soaps bring further informations concerning their structure. The results will be reported in the next communication.

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Registry No. Sodium decanoate, 1002-62-6; sodium dodecanoate, 629-25-4; sodium tetradecanoate, 822-12-8; sodium hexadecanoate, 408-35-5; sodium octadecanoate, 822-16-2.

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Synthesis and Synergistic Activity of Oxime Ethers Containing a Benzo-1,3-dioxole Group

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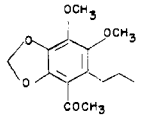
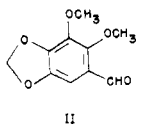
A large number of *O*-alkyl, *O*-alkenyl, and *O*-propargyl oxime ethers having a benzo-1,3-dioxole group as a common feature have been synthesized from piperonal, dillaldehyde, and acetyldihydrodillapiole as potential pyrethrum synergists. Their factors of synergism, R_m values, and structure-activity relationships are being reported. Piperonal oxime *n*-pentyl ether (XXXI) shows remarkably high activity.

Insecticide synergists play an important role in efficient and economic formulations of pesticides. Several new pyrethrum synergists have been earlier synthesized in this laboratory by chemical modification of dillapiole (Tomar et al., 1979a; b) and dihydrodillapiole and furapiole (Mukerjee et al., 1982). Synergistic activity of all these compounds has been mainly attributed to the presence of a benzo-1,3-dioxole group. Since oxime ethers have also been reported to possess synergistic insecticidal activity with pyrethroids (Hennessy, 1969), we now report the synthesis, synergistic properties, and structure-activity relationships of a large number of *O*-alkyl oxime ethers having a benzo-1,3-dioxole group as a common feature from three structurally similar carbonyl compounds, namely, piperonal (I), dillaldehyde (II), and acetyldihydrodillapiole (III).



I



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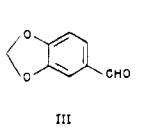

III

MATERIALS AND METHODS

Acetyldihydrodillapiole (I; Mukerjee et al., 1982) and Dillaldehyde (II; Tomar et al., 1979b) needed for this work were synthesized by literature procedures. All melting points are uncorrected. All liquid compounds were purified by column chromatography over activated silica gel followed by short-path distillation whereever possible under reduced pressure (bath temperature 150 °C). The position of all the oxime ethers on TLC plates was visualized by spraying with 2,4-dinitrophenylhydrazine reagent or H_2SO_4 spray followed by heating. NMR spectra were recorded in CCl_4 or $CDCl_3$ on a Varian EM-360 60-MHz spectrometer by using Me_4Si as the internal reference. Chemical shifts are given in δ values.

Synthesis of Test Chemicals. *Acetyldihydrodillapiole Oxime (IV).* Acetyldihydrodillapiole (I) (22.4 g), hydroxylamine hydrochloride (10.5 g), and sodium carbonate (20 g) were refluxed in ethanol for 5 h. After completion of reaction (TLC), the bulk of solvent was removed by distillation, were (500 mL) added, and mixture cooled in an ice bath. The product so separated was crystallized from ethanol as white crystals: mp 99 °C; NMR ($CDCl_3$) δ 0.9 (3 H, t, $-CH_2CH_3$), 1.5 (2 H, m, $-CH_2CH_2CH_3$), 2.2 (3 H, s, $-N{=}CCH_3$), 2.55 (2 H, t, $ArCH_2-$), 3.8 (3 H, s, $-OCH_3$), 4.0 (3 H, s, $-OCH_3$), 5.85 (2 H, s, $-OCH_2O-$). Anal. Calcd for $C_{14}H_{19}O_5N$: C, 59.9; H, 6.8. Found: C, 59.5; H, 7.1.

Dillaldehyde Oxime (XV). Dillaldehyde (II) (21 g) and hydroxylamine hydrochloride (10.5 g) were refluxed in ethanol (500 mL) containing sodium carbonate for 5 h. After working up as above, the product was crystallized from ethanol as white crystals: mp 95 °C; NMR ($CDCl_3$) δ 3.9 (3 H, s, $-OCH_3$), 4.1 (3 H, s, $-OCH_3$), 5.95 (2 H, s, $-OCH_2O-$), 6.9 (1 H, s, aromatic), 8.1 (1 H, s, $-CH{=}N-$). Anal. Calcd for $C_{10}H_{11}O_5N$: C, 53.3; H, 4.9. Found: C, 53.6; H, 5.1.

Piperonal Oxime (XXVI). Piperonal (III) (12.4 g) and hydroxylamine hydrochloride (5.25 g) were refluxed in ethanol (500 mL) in the presence of sodium carbonate for 5 h. After working up as usual, the product was crystallized from ethanol as white crystals: mp 105 °C; NMR ($CDCl_3$) δ 5.95 (2 H, s, $-OCH_2O-$), 6.75 (3 H, m, aromatic), 7.85 (1 H, s, $-CH{=}N-$). Anal. Calcd for $C_8H_7O_3N$: C, 58.2; H, 4.2. Found: C, 58.4; H, 4.5.

General Procedure for the Synthesis of Oxime Ethers. A solution of the appropriate oxime (IV, XV, or XXVI) (0.1 mol) in dry acetone containing potassium carbonate was refluxed with alkyl halides, alkenyl halides, and propargyl bromide for 3-5 h. After completion of the reaction (TLC), solvent was distilled off and water (500

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