showed no significant differences among incubation times; however, johnsongrass rhizomes were significantly lower in percent TNC at 0 h when compared to 24, 48, and 72 h (Table IV). A 24-h incubation was necessary to allow effective penetration by the enzyme to sites of starch localization within the rhizome tissue.

Abbreviations: TNC, total nonstructural carbohydrates; PAHBAH, *p*-hydroxybenzoic acid hydrazide; FCN, potassium ferricyanide; CUI, copper iodometric.

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Registry No. PAHBAH, 5351-23-5; amyloglucosidase, 9032-08-0; sucrose, 57-50-1; D-glucose, 50-99-7; D-fructose, 57-48-7; starch, 9005-25-8.

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Effect of Hydrolysis on Sapogenin Release in Soya

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The effect of various hydrolysis procedures on the sapogenin yield and profile of soya saponins was investigated. Hydrolysis for 3 h with 3% sulfuric acid in an anhydrous methanolic environment gave the highest yield of total sapogenins and also only liberated soyasapogenols A and B. The results show that soyasapogenols B_1, C, D and E are artifacts of the hydrolysis procedure employed.

Saponins are triterpene or spirostan glycosides that hydrolyze to liberate an aglycon (generally referred to as a sapogenin) and the composite sugar residues. Five sapogenins have been isolated from soya: soyasapogenols A, B, C, D, and E. Smith et al. (1958) proposed structures for soyasapogenols A, B, and C, Cainelli et al. (1958) proposed a structure for soyasapogenol D, and Willner et al. (1964) isolated and characterized a further sapogenin, soyasapogenol E. The originally proposed structures for soyasapogenols A, B, and E have recently been revised by Kitagawa et al. (1982). These sapogenins together with soyasapogenol C are shown in Figure 1.

We have recently reported a method for the analysis of soybean sapogenins by normal-phase, high-performance liquid chromatography (HPLC) and mass detection (Ireland and Dziedzic, 1985). Our work identified a previously unknown sapogenin, probably similar in structure to B, which we tentatively named soyasapogenol B_1. Of the five soya saponins that have been isolated and their structures elucidated, three contain soyasapogenol B and two soyasapogenol A as the aglycon (Kitagawa et al., 1976; Kitagawa et al., 1985 a,b). Saponins containing soyasapogenols C, D or E as the aglycon have not yet been isolated, and it has been suggested that some or all of these sapogenins may be artifacts (Dziedzic and Ireland, 1985; Heftmann et al., 1979).

Since sapogenin concentration is often used as a measure of the saponin concentration, it, therefore, seemed pertinent to examine how hydrolysis conditions affect sapogenin concentration and profile.

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EXPERIMENTAL SECTION

Extraction of Saponin Fraction. A single, defatted soya flour (Arkasoy 50; British Arkaday Co. Ltd., Manchester, U.K.) was used throughout this study. A sample of milled, defatted soybeans (USDA Grade II) was also used to monitor the effects of the hydrolysis procedures using aqueous sulfuric acid/1,4-dioxane and sulfuric acid in methanol. The samples were subjected to a 36-h methanol Soxhlet extraction to obtain an extract containing the saponin fraction. The saponin extracts were then hydrolyzed by the different hydrolysis methods outlined below.

Isolation of Standard Soyasapogenols A and B. Standard samples of soyasapogenols A and B were isolated and identified as previously described (Ireland and Dziedzic, 1985).

Effect of Time of Hydrolysis on Sapogenin Yield and Profile. Duplicate samples (15 g) of the flour were extracted, the methanol was evaporated under reduced pressure, and the extract obtained was dissolved in 133 mL of 3 N H_2SO_4 in 1,4-dioxane/water (1:3, v/v) and hydrolyzed for either 30 min or 1, 2, 4, 6, 9 or 12 h under reflux. After hydrolysis the samples were cooled, diluted with water (100 mL), and extracted with diethyl ether (200 mL). The hydrolysis solution was extracted with two further portions of diethyl ether (2 × 100 mL), and the combined ether extracts were washed with 2% aqueous KOH (m/v, 2 × 100 mL) and water (2 × 100 mL). The sapogenin extract was evaporated under reduced pressure and dis-

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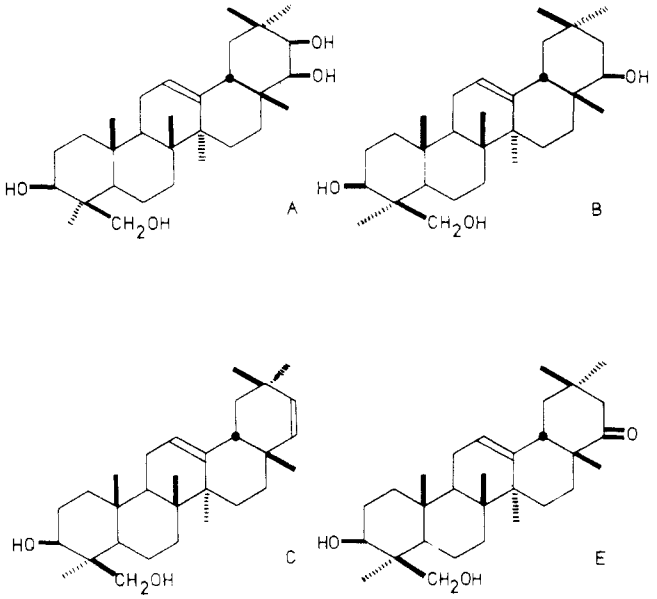

Figure 1. Structures of soyasapogenols A, B, C, and E.

solved in chloroform/methanol (1:1, v/v) to constant volume (5 mL).

Hydrolysis with Aqueous Sulfuric Acid/1,4-Dioxane. Triplicate samples (20 g) were hydrolyzed for 5 h as described above.

Hydrolysis with Aqueous Hydrochloric Acid/Ethanol. Triplicate samples (20 g) of the flour were extracted, and the methanol was removed. The samples were hydrolyzed for 5 h under reflux with concentrated HCl (60 mL) in water (30 mL) and ethanol (90 mL). After hydrolysis the samples were cooled and diluted with water (100 mL), and the sapogenins were extracted with diethyl ether as normal.

Hydrolysis in a Two-Phase Medium [Based on Tschesche et al. (1964)]. Triplicate samples (20 g) of the flour were extracted, and the methanol was removed. The samples were hydrolyzed for 5 h under reflux with a mixture of concentrated HCl (60 mL), water (60 mL), ethanol (60 mL), and toluene (60 mL). After cooling, the toluene layer was separated and the aqueous layer extracted with two portions of diethyl ether (2 × 150 mL). The toluene and ether extracts were pooled and washed with 2% aqueous KOH (2 × 100 mL) and water (2 × 100 mL), and the sapogenin fraction was obtained by evaporation of the solvent under reduced pressure.

Hydrolysis with Anhydrous Methanolic Hydrochloric Acid. Triplicate samples (20 g) of the flour were extracted, and the methanol was removed as above. A 5% solution (m/v) of HCl in dry methanol was obtained by bubbling hydrogen chloride gas into dry methanol. The samples were hydrolyzed under reflux for 3 h with 5% HCl in methanol (200 mL). After cooling, the sample was neutralized (pH 7) with ammonia solution (0.88 specific gravity), the methanol evaporated under reduced pressure, and the sample suspended in water (200 mL), and the sapogenins were extracted with diethyl ether as normal.

Hydrolysis with Sulfuric Acid in Methanol. Triplicate samples (20 g) were extracted. Hydrolysis was carried out under reflux for 3 h with concentrated H_2SO_4 in dry methanol (250 mL, 3% m/v). The sapogenins were isolated in the same manner as those obtained by hydrolysis with anhydrous methanolic HCl.

Periodate Oxidation [Based on Dugan and de Mayo (1965)]. A sample (20 g) was extracted and the methanol removed. The extract was suspended in water (100 mL)

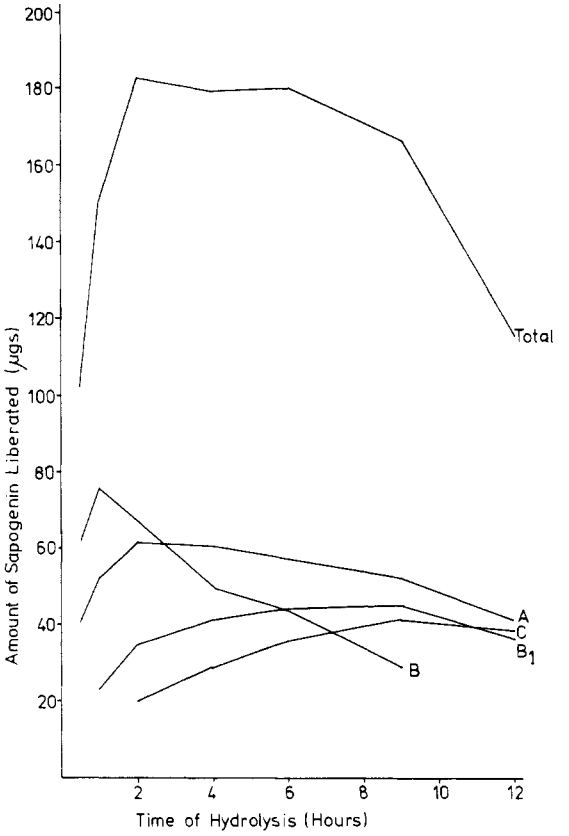

Figure 2. Effect of time of hydrolysis with H_2SO_4 in water/1,4-dioxane (3:1) on sapogenin profile and yield.

and cooled in an ice bath. Sodium periodate (4 g) was slowly added to the extract at 0 °C over 30 min with constant stirring. The reaction mixture was then kept in the dark for 24 h at room temperature (ca. 18 °C). Excess periodate was destroyed by addition of 1 ML of ethane-1,2-diol and stirring for 1 h. Potassium hydroxide (5 g) was added and the mixture heated at 90 °C for 30 min. After cooling to room temperature and careful acidification (pH 4 with HCl), the liberated aglycons were extracted with diethyl ether (2 × 100 mL). The ether extracts were washed with 2% KOH (2 × 50 mL), 2% sodium thiosulfate (2 × 50 mL), and water (2 × 50 mL). The aglycon fraction was obtained by removal of the ether and dissolving in chloroform/methanol (1:1, v/v).

Effect of Heating Standard Soyasapogenols A and B in Aqueous Sulfuric Acid/1,4-Dioxane. A sample (ca. 5 mg) of standard soyasapogenol B was dissolved in 1,4-dioxane (33 mL); aqueous sulfuric acid was added to produce 3 N sulfuric acid in 1,4-dioxane/water (1:3) and the resultant mixture heated under reflux for either 1, 2, 4, 6, or 18 h. After heating for the appropriate length of time, the sample was cooled and diluted with water (100 mL) and the sapogenin fraction isolated in the same manner as for the soya flour samples. Similarly, standard soyasapogenol A was heated under reflux in 3 N sulfuric acid in 1,4-dioxane/water (1:3) for either 1, 4, or 18 h and the sapogenin fraction isolated.

Analysis of Sapogenin Extracts by HPLC. The sapogenin extracts obtained were analyzed by HPLC with mass detection (Ireland and Dziedzic, 1985).

RESULTS AND DISCUSSION

The method recommended for soya saponin hydrolysis utilizes sulfuric acid in 1,4-dioxane/water (1:3) (Gestetner et al., 1966). Thus, this method was used to ascertain the optimal time for maximum yield of total and individual

Table I. Effect of Various Modes of Hydrolysis on Sapogenin Profile and Yield

hydrol type	sapogenin yield, %	compn of sapogenin fraction, % A	B	B_1	E	C
3 h dry HCl/MeOH[a]	0.330	28.0	72.0			
3 h dry H_2SO_4/MeOH[a]	0.342	30.4	69.6			
5 h aq H_2SO_4/1,4-dioxane[a]	0.304	27.6	30.0	16.5	10.1	15.8
5 h aq HCl/toluene[a]	0.299	21.7	14.9	13.2	22.3	27.9
5 h aq HCl/EtOH[a]	0.249	33.1		18.8		48.1
3 h dry H_2SO_4/MeOH[b]	0.328	25.4	74.6			
5 h aq H_2SO_4/1,4-dioxane[b]	0.319	25.6	23.8	19.8	12.5	18.3

[a] Defatted soya flour. [b] Milled, defatted soya bean.

sapogenins, shown in Figure 2. The release of total sapogenins appears to reach a maximum after 2 h, remaining at that maximal yield for a further 4 h and then subsequent refluxing under the hydrolysis conditions results in loss of sapogenins. Thus, it appears that an excessively long (greater than 6 h hydrolysis under the aqueous acid conditions used can result in the degradation of sapogenins. However, a hydrolysis time of 4 h with this procedure as previously recommended (Gestetner et al., 1966) appears to be consistent with the maximum release of sapogenins.

The release of soyasapogenol A appears to display a similar pattern to that obtained for total sapogenins: an initial release of sapogenin that reaches a maximum after 2 h and remains at that level before gradually declining. The release of soyasapogenols B, B_1, and C, however, follows different patterns. The maximum yield of soyasapogenol B is obtained after 1 h after which soyasapogenol B appears to be degraded, showing a much faster rate of degradation than soyasapogenol A. Soyasapogenols C and B_1, however, are released at a much slower rate and reach their maximum yields after 9 h of hydrolysis before beginning to slowly degrade. It should be noted here that soyasapogenol E was observed in this study but at levels too low for accurate quantification and consequently is not shown in Figure 2, but a slower rate of release similar to soyasapognols B_1 and C was observed.

A possible explanation for the slow release of soyasapogenols B_1 and C is that the sugar residue and/or mode of attachment to the sapogenin results in a saponin more resistant to acid hydrolysis. An alternative, more attractive explanation takes into account the rapid degradation of soyasapogenol B, which is readily released from its parent saponin, as can be seen by its initial position as the major sapogenin, but appears to be unstable under the hydrolysis conditions used. As soyasapogenol B degrades, it must be converted into a different chemical species whose rate of formation would be slower than that of soyasapogenols B or A. Thus, the slower rate of release of soyasapogenols B_1 and C may be due to the fact that they are artifacts formed by the degradation of soyasapogenol B. A third possibility is that soyasapogenols B_1 and C are naturally present in low amounts and are also found under the hydrolysis conditions used by the degradation of soyasapogenol B.

If soyasapogenols B_1 and C are true sapogenins and not artifacts, then they would be expected to appear in the sapogenin fraction obtained under a variety of hydrolysis conditions. Consequently the effect of a number of methods of hydrolysis on the total sapogenin yield and sapogenin profile were studied; the results are presented in Table I.

A significant observation from the results in Table I is that the method of hydrolysis influences the sapogenin

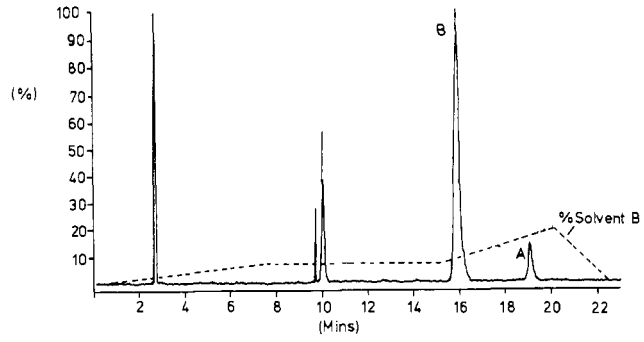

Figure 3. HPLC separation of sapogenin extract obtained by hydrolysis with H_2SO_4 in methanol. Conditions: normal-phase silica column; flow rate 1.5 mL/min; solvent A = light petroleum ether (bp 60–80 °C); solvent B = ethanol; mass detection. For full details see Ireland and Dziedzic (1985).

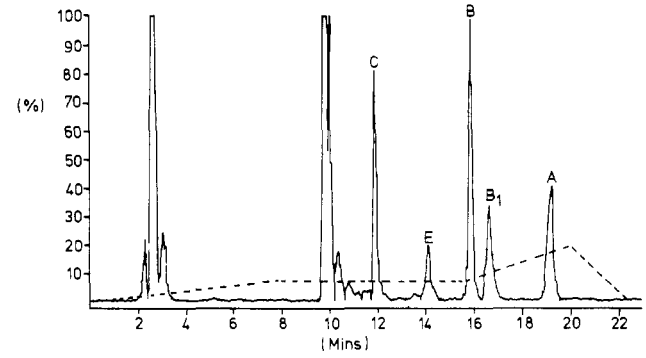

Figure 4. HPLC separation of sapogenin extract obtained by hydrolysis with H_2SO_4 in water/1,4-dioxane (3:1). Conditions as in Figure 3.

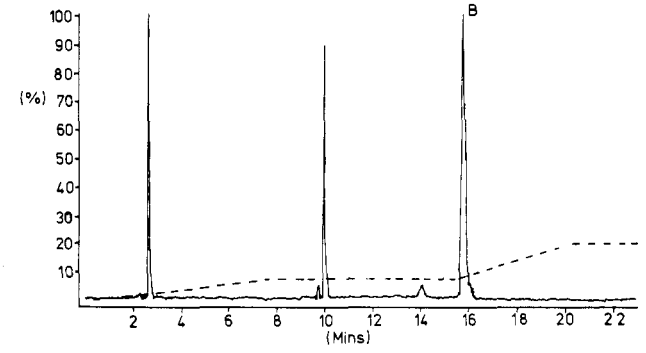

Figure 5. HPLC separation of sapogenin extract obtained by periodate oxidative hydrolysis. Conditions as in Figure 3.

profile. Only two methods give consistent results, and these involve the use of acids in a methanol rather than an aqueous environment. The use of anhydrous methanolic conditions with either hydrochloric or sulfuric acids (see Figure 3) yields only soyasapogenols A and B. When hydrolysis occurs in an aqueous or partially aqueous environment, soyasapogenols B_1, C, and E are also present in the sapogenin profile (e.g., Figure 4). The conditions of hydrolysis affect the sapogenin profile when hydrolysis is carried out in the presence of water as is seen by the effect of time of hydrolysis (Figure 2) and type and strength of acid (Table I).

Periodate oxidation of a soybean saponin extract yielded only soyasapogenol B (see Figure 5). Periodate oxidation cleaves α-glycols, thus degrading the carbohydrate moiety to liberate the aglycon. In this respect, the technique would not be expected to liberate soyasapogenol A as the two saponins containing this aglycon (soyasaponins A_1 and

Table II. Effect of Mode of Hydrolysis on Sapogenin Profile from Previous Studies

hydrol type	soyasapogenols obsd[a,b]
30 h aq HCl/EtOH[c]	A, B, C, D
10 h aq H_2SO_4[d]	A, B, C, E
20 h aq H_2SO_4/MeOH[e]	A, B, C, D, E
4 h dry HCl/MeOH[f]	A, B, D, E
4 h aq H_2SO_4/1,4-dioxane[g]	A, B, C, D, E

[a] Relative proportions of each sapogenin not reported. [b] Analyzed by comparative TLC with authentic samples. [c] Smith et al. (1958). [d] Peri et al. (1979). [e] Kitagawa et al. (1976). [f] Nash et al. (1967). [g] Gestetner et al. (1966).

Table III. Effect of Heating Soyasapogenol A in Aqueous Sulfuric Acid/1,4-Dioxane

time, h	soyasapogenol,[a] %	
	A	E
0	100	
1	100	
4	97	3
18	77	23

[a] Only soyasapogenols A and E observed.

A_2) also contain an arabinose moiety (Kitagawa et al., 1985a,b) that inhibits periodate degradation of the disaccharide attached to the C-22 hydroxyl group. Thus, the absence of soyasapogenols B_1, C, and E after periodate oxidative hydrolysis may be due to the fact that they are artifacts or a result of saponins containing these aglycons having a sugar attached directly to the aglycon that is similarly resistant to periodate oxidation.

The sapogenins obtained and type of hydrolysis used in previous studies are presented in Table II. The only sapogenins found in all studies are soyasapogenols A and B. With the exception of the study of Nash et al. (1967), hydrolysis was carried out in an aqueous or partially aqueous environment. Nash et al. (1967) used an anhydrous methanolic hydrochloric acid procedure and obtained soyasapogenols D and E as well as soyasapogenols A and B. The results presented in Table I show that when an anhydrous methanolic acid hydrolysis was used in this study, only soyasapogenols A and B were found. In this respect, our results contradict those of Nash et al. (1967).

The effect of using an aqueous sulfuric acid or anhydrous methanolic sulfuric acid hydrolysis procedure on the sapogenin profile and yield of the soybean sample can be seen in Table I. As with the soya flour sample (Table I), aqueous hydrolysis conditions yield soyasapogenols A, B, B_1, C, and E while anhydrous conditions yield soyasapogenols A and B only, and this result has consistently been obtained in our laboratory with a number of different soya flours. This indicates that the observed effect on sapogenin profile is due to the hydrolysis conditions rather than being an artifact of the particular sample of soya flour analysed.

If soyasapogenols B_1, C, and E are artifacts formed by the action of aqueous acids on soyasapogenols A and B, then heating pure standards of soyasapogenols A and B in aqueous acid should indicate which sapogenins are artifacts. Consequently, standard soyasapogenols A and B were subjected to the aqueous sulfuric acid/1,4-dioxane hydrolysis conditions used for saponin hydrolysis, and the sapogenin fraction was recovered after various times of heating and analyzed by HPLC (Tables III and IV). Clearly, it can be seen that the aqueous acid conditions previously recommended (Gestetner et al., 1966) for the hydrolysis of soya saponins results in transformation of soyasapogenols A and B. Soyasapogenol B is transformed

Table IV. Effect of Heating Soyasapogenol B in Aqueous Sulfuric Acid/1,4-Dioxane

time, h	soyasapogenol,[a] %		
	B	B_1	C
0	100		
1	49.9	16.6	33.5
2	15.4	45.5	39.1
4	6.5	42.4	51.1
6		32.4	67.6
18		7.5	92.5

[a] Only soyasapogenols B, B_1, and C observed.

to soyasapogenols B_1 and C; indeed after refluxing for a period of 6 h, soyasapogenol B is completely transformed (Table IV). Continuation of heating of the mixture of soyasapogenols B_1 and C results in the transformation of soyasapogenol B_1 to C.

Although soyasapogenol A appears to be more stable under these conditions, it is partially transformed to soyasapogenol E if the hydrolysis time is sufficiently long (Table III).

CONCLUSIONS

It can clearly be seen that the mode of hydrolysis of soybean saponin influences not only the sapogenins obtained but also their relative proportions. The use of an aqueous hydrolysis environment results in the formation of soyasapogenols A, B, B_1, C, and E as found in this study and soyasapogenol D as found previously. An anhydrous methanolic acid hydrolysis environment liberates only soyasapogenols A and B and also results in the highest yield of total sapogenins. The results represented suggest that soyasapogenols A and B are true aglycons and that soyasapogenols B_1, C, D, and E are artifacts formed during the hydrolysis step. This conclusion is supported by the fact that the soya saponins that have been isolated and characterized (Kitagawa et al., 1976, 1985a, 1985b) contain either soyasapogenol A or B as the aglycon while saponins containing soyasapogenols B_1, C, D, or E have not been isolated from soya.

Thus, it is recommended that the hydrolysis of saponins should be carried out in an anhydrous methanolic environment to prevent artifact formation and obtain the maximum yield of sapogenins.

Registry No. Soyasapogenol A, 508-01-0; soyasapogenol B, 595-15-3; soyasapogenol B_1, 104033-83-2; soyasapogenol C, 595-14-2; soyasapogenol D, 65892-76-4; soyasapogenol E, 6750-59-0.

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Binding of Diacetyl by Pea Proteins

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Binding of diacetyl to pea protein was studied, keeping the ligand concentration within the range practically found in foods. The bound ligand concentration was found to depend upon both the free ligand and the protein concentrations in a quasi-linear manner. Decrease in the pH value of the protein solution and particularly isoelectric precipitation of the protein result in dramatic reductions of its retention properties and led to a partial release of the previously bound ligand. The overall binding capacity of the isolate appeared to amount to nearly the sum of the weighted abilities of the individual protein fractions, ruling out the chance of any significant contribution by the residual fat. Taking into account findings previously reported about the affinity between diacetyl and some amino acids, it is postulated that the binding of the diacetyl to the pea protein may result from the interaction of diacetyl with arginyl residues.

INTRODUCTION

Several studies have demonstrated marked binding effects of certain nonvolatile food components, in particular proteins, on the vapor pressure of some flavor volatiles in model systems. Most previous studies in which proteins have been shown to bind volatile substances have been conducted at high concentration of the volatile substance (Reineccius and Coulter, 1969; Arai et al., 1970; Gremli, 1974; Beyeler and Solms, 1974; Franzen and Kinsella, 1974) or in dry systems (Maier, 1975; Aspelund and Wilson, 1983). However, most important volatile flavor substances are very potent odorants present at levels below ppm, and because some deviations from Henry's law can occur at low but not at higher concentrations (Land, 1979), studies of the effects on flavor should be made at sensorily relevant concentrations. Furthermore, as flavor by mouth is always perceived from aqueous stimuli, dry systems are not relevant to flavor perception although they are relevant to flavor retention in food processing. Although more recent studies (Damodaran and Kinsella, 1980; Kinsella and Damodaran, 1980; King and Solms, 1979, 1980, 1981) have used lower concentrations of volatile substances, only one (Land and Reynolds, 1981) was conducted at levels directly related to the concentration range of sensory significance in aqueous solution.

Legumes are now recognized as an increasingly important source of protein for both human and animal nutrition (Fauconneau, 1983). Species that grow successfully in Western Europe and are being actively investigated include the pea (*Pisum spp.*), protein concentrates and isolates from which show promise as functional food additives.

The present study of the effects of various forms of pea protein on the volatility of the important flavor compound diacetyl (butane-2,3-dione) was undertaken with the criteria of Land and Reynolds (1981) to provide information relevant to flavoring of products containing pea proteins.

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EXPERIMENTAL SECTION

Protein Isolates. Samples of protein were prepared at the INRA pilot plant facility at Nantes from seeding grade peas (*Pisum sativum* var. amino) grown under commercial conditions in the Ile de France district. Pea flour was extracted at room temperature by dilute NaOH at either pH 7 or pH 9 according to the procedure described by Gueguen (1983). The protein content of the isolates was always higher than 93%.

Purified protein fractions consisted of the two oligomeric globulins vicilin (M_r = 180000) and legumin (M_r = 320000) and were provided by J. Gueguen (Laboratorie de Biochimie et Technologie des Protéines, Nantes).

Diacetyl. Diacetyl (Fluka, puriss. >99.5% by GC) freshly purchased was redistilled under vacuum, and samples of the main fraction were sealed under nitrogen in glass ampules and stored at −20 °C until required. A fresh ampule was opened every few weeks, when additional peaks had increased to a level that interfered with the analysis. Solutions were made in freshly distilled water daily as required.

Headspace Vials. Glass screw-top bottles of 30-mL volume, with Bakelite tops that were drilled and fitted with aluminum foil wrapped 3-mm-thick silicone rubber septa, were used. Samples were taken with dedicated gas-tight syringes (Hamilton 100 or 500 μL) kept in a clean oven at 50 °C at all times except during filling and injection.

Gas Chromatography. A Pye Unican Model 104 gas chromatograph with standard electron capture detector and GCV pulsed supply (optimized detector current, ×32) was used with a stainless-steel SCOT column (150 m × 0.64 mm i.d.) coated with Carbowax 20M. Conditions: carrier gas argon (0.8 kg/cm^2; 35 cm/s) with 10% methane in argon as quench (5 mL/min); oven 85 °C isothermal; in-

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