Characterization of the Nonvolatile Minor Constituents Responsible for the Objectionable Taste of Defatted Soybean Flour

AN-SHUN HUANG, OLIVER A.-L. HSIEH, and STEPHEN S. CHANG

ABSTRACT

A scheme was developed for the separation of the nonvolatile minor constituents from soy flour which are responsible for the astringent an bitter tastes. The scheme involved solvent extraction, freeze-drying, and fractional crystallization. Two fractions, collected by semipreparative HPLC, had the characteristic bitter and astringent tastes. Further fractionation by reverse phase HPLC produced three pure compounds which were identified by UV, NMR spectrometry and elemental analysis, as daidzein, glycinecin 7-O-glucoside, and genistin. Preliminary sensory evaluation showed that these isofoamones might contribute additively to the undesirable bitter and astringent tastes of soy protein products.

INTRODUCTION

SOY PROTEIN PRODUCTS which can be produced in vast amounts with good functionality for uses in foods are a potential solution to world hunger. However, the incorporation of soy flour, concentrate, and isolate, and textured products into foods is hindered by their bitter and astringent tastes.

Arai et al. (1966) and his co-workers identified a series of nine phenolic acids from defatted soy flour. These phenolic acids may have some influence on soybean flavor since they possess sour, bitter and astringent tastes. Honig et al. (1971), isolated ethylo-D-galactopyranoside and L-cryptophan as bitter components in soy flakes produced by hexane-ethanol extraction. Evidence showed that galactoside was an artifact formed during extraction.

In 1976, Sessa et al., isolated three oxidized phosphatidylincholines which all possessed keto and hydroxy fatty acid esters. Based upon sensory evaluation and flavor threshold, they concluded that oxidized phosphatidylincholines were largely responsible for the bitter taste of soy protein products. Further experiments suggested that a bound oxidized fatty acid in the lecithin molecule, instead of the phosphocholine moiety is responsible for the bitter taste (Sessa, 1977).

This paper reports an attempt to characterize the minor constituents of soy flour which are responsible for its bitter and astringent tastes.

EXPERIMENTAL

Material used

Four commercially available defatted soy flours, two nontoasted and two toasted, were sensory evaluated by a trained eight-member panel. The samples were made into 2% dispersions in distilled water. Their odor strength, odor preference, flavor strength, and flavor preference were evaluated at 60°C. In order to maintain the sample temperature during the evaluation period, the samples were served to the panelist in the glass weighing bottles which were set in holes especially drilled into a heavy aluminum block preheated to 60°C.

A scale of 1 to 9 was used for the scoring of both strength and preference. The higher score indicated either the higher strength or the higher preference. The odor response included beany, bitter, green, chalky, musty, corn meal, and toasted. The flavor description included all the above, plus astringent. One of the nontoasted soy flours which had the strongest objectionable odor and flavor, as determined by sensory evaluation, was used for this study.

Isolation and fractionation of nonvolatile minor constituents with objectionable taste

The procedures used for the isolation and fractionation of the nonvolatile flavor constituents with objectionable tastes are outlined in Fig. 1.

Nontoasted, defatted soy flour (1.5 kg) was extracted with 7500 ml of 60% aqueous ethanol in a 5-gal stainless steel tank. The soy flour slurry was constantly stirred with a TALBOY 101 stirrer at room temperature for 2 hr. After standing at −20°C for 24 hr, the ethanol slurry was filtered through Whatman No. 1 filter paper and the residue was discarded. The filtrate was concentrated to approximately 1200 ml (I) with a Buchler rotary evaporator under reduced pressure (approx 27 mm Hg) at a temperature not exceeding 40°C.

Two hundred ml of the extract (I) were extracted in a 600 ml separatory funnel successively with two 100 ml portions of ethyl ether. This procedure was repeated six times in order to process all 1200 ml of the aqueous extract. The aqueous phase (II) containing the nonlipid fraction was collected. The ether phase (II) containing the lipid fraction was freed from solvent with a rotary evaporator under vacuum, and was dissolved in 70 ml of hexane (Baker Analyzed Reagent, J.T. Baker Chemical Co., Phillipsburg, NJ). The hexane solution was poured slowly into 350 ml of 0°C acetone (Baker Analyzed Reagent, J.T. Baker Chemical Co., Phillipsburg, NJ) with constant stirring. The precipitate, after filtration, was washed twice with two 55 ml portions of 0°C acetone.

Fig. 1—Isolation and fractionation of defatted soy flour.

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The acetone filtrates and washings, both containing the neutral lipid fraction, were combined and freed from solvent to yield fraction IIIA. The precipitate which contained polar lipids, after being freed of acetone, was stored in an agar plate bottle under nitrogen at −20°C (Fraction IIIB).

The aqueous layer (III) was freeze-dried with a LABCONCO® FREEZE-DRY 12 freeze-drier (Labconco Corp., Kansas City, MO). The freeze-dried solids were extracted with ten times their weight of methanol at room temperature for 2 hr. The mixture, after being stored at −20°C for 24 hr, was filtered through Whatman No. 1 filter paper. The methanol insoluble residue (IIIB) was discarded after organoleptic evaluation. The filtrate (IIIA) was submitted to fractional crystallization by reducing the methanol/ sample ratio to 1:1 with a rotary evaporator. After sorting the solution at −20°C, for 24 hr, the white crystals formed were removed by filtration. The mother liquor (IIIA), after concentration, was diluted to 42 ml with methanol for further fractionation.

**Sensory evaluation**

All fractions obtained above, after being carefully freed from solvent with a mechanical vacuum pump, or after freeze-drying, were tasted for bitter and astringent flavor by a sensory panel consisting of three experienced persons. The water soluble fraction was made into a 2% solution in distilled water and sensory evaluated.

The water insoluble fraction was put on the tongue of the panel members, together with 2 ml of warm, distilled water. The mouth was thoroughly rinsed with warm water before and after tasting each sample.

**HPLC fractionation of fraction IIIAa**

Fraction IIIAa which was identified by the sensory panel as having a bitter and astringent taste was further fractionated by preparative HPLC with a Waters 6000A high performance liquid chromatograph (Waters Associates, Inc., Milford, MA) equipped with an M-660 solvent programmer and an M-440 UV absorbance detector. The conditions used in the preparative HPLC are given in Table 1. Thirty injections of 100 μl each were made for the preparative HPLC of Fraction IIIAa. The seven fractions obtained, P1 through P7, were individually and cumulatively collected. They were then freeze-dried and sensory evaluated.

**RESULTS & DISCUSSION**

**Yield and flavor characteristics of preliminary fractions**

The yield and flavor characteristics of the preliminary fractions of the soy flour are shown in Table 3. The total crude protein content (I) of the soy flour had a bitter, sweet, and biting taste. Both its lipid (II) and nonlipid fractions (III) showed bitter and astringent notes. However, the nonlipid fraction possessed a much stronger bitter and astringent taste than the lipid fraction.

By acetone precipitation, the lipid fraction was separated into a polar lipid fraction (IIIB) and a neutral lipid fraction (IIIA). The neutral lipids possessed a weak bitter and astringent taste with oiliness and waxy characteristics. The polar lipid fraction, which included phosphatides, had a plastic and creamy-like taste. These results, therefore, confirm the report that oxidized phosphatidylcholine is responsible for the bitter flavor of soy protein products (Sessa et al., 1974).

The methanol extract of the nonlipid fraction (IIIA) tasted bitter, astringent and sweet. After the removal of the sucrose crystals (IIIB), fraction IIIAa had an intense bitter and astringent taste. It was further fractionated by HPLC.

**HPLC fractionation of Fraction IIIAa**

Fraction IIIAa with a bitter and astringent taste was fractionated by preparative HPLC into seven fractions (Fig. 2). A reverse phase C18 BONDAPAK column was chosen due to the high solubility of the sample in polar...
solvents. Gradient elution was employed because Fraction IIIAa contained components with a wide range of polarity. The early separation was done isocratically at a high water concentration (70%). This relatively high polar mobile phase improved the separation between the early elution peaks (Region I). Decreasing the water concentration progressively (down to 55% v/v) reduced the fractionation time without sacrificing the resolution between the late elution peaks (Region III). In the final stage of fractionation, pure methanol was used to ensure that all the material was eluted out of the column (Region V).

The ultraviolet absorption detector used is a selective detector which does not respond to compounds without absorbance or with low absorbances at the monitoring wavelength used. Therefore, area collection, by which each succeeding fractional collection started right after the preceding fractional collection ended, was performed to cover the entire fractionation as shown in Fig. 2. Continuous area collection ensured that all components, which included compounds without absorbance at the monitoring wavelength, were collected for organoleptic evaluation.

The flavor characteristics of the seven fractions collected by HPLC are shown in Table 4. Both Fractions P3 and P4 had bitter and astringent tastes. Whether this was due to overlapping between the two adjacent fractions or to different bitter and astringent substances, was difficult to ascertain. Therefore, Fractions P3 and P4 were combined as Fraction P34 for further fractionation.

**Analytical HPLC of Fraction P34**

Methanol extraction of Fraction P34 yielded 57% of insoluble materials (P34I) and 43% of soluble materials (P34S). The methanol-soluble fraction tasted bitter and astringent, while the insoluble fraction did not (Table 5). Since Fraction P34 originated from Fraction IIIAa, which was methanol-soluble, these results indicated that fractionation of trace amounts of flavor constituents of soy flour depends not only upon the solvent employed, but also upon other factors, including concentration, temperature, and mutual solubility of other constituents.

Both Fraction P34 and Subfraction P34S were analyzed by an analytical reverse phase BONDAPAK C18 column under identical conditions. The chromatograms of P34 and P34S are shown in Fig. 3 and 4, respectively. By injecting several different mixtures of various ratios of P34 and P34S, the three major peaks, C, D, and E, in P34S were confirmed as the same components as peaks C, D, and E in Fraction P34. Using the same technique in comparing the chromatograms of P34I and P34S, small peaks A and B were confirmed as residual peaks from P34I. Since only P34S tasted bitter and astringent, Subfractions C, D, and E of P34S were collected and identified.

**Table 3—Yield and flavor characteristics of the preliminary fractions of the extract of soy flour**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield (g)</th>
<th>Flavor characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>6.4(^b)</td>
<td>bitter, sweet, biting</td>
</tr>
<tr>
<td>II</td>
<td>2.3</td>
<td>oily, waxy, slight bitter and astringent</td>
</tr>
<tr>
<td>IIIA</td>
<td>1.6</td>
<td>rye bread-like, oily, weak bitter and astringent</td>
</tr>
<tr>
<td>IIIB</td>
<td>0.7</td>
<td>plastic, corn meal</td>
</tr>
<tr>
<td>III</td>
<td>4.1</td>
<td>bitter, sweet, mealy, weak astringent</td>
</tr>
<tr>
<td>IIIAa</td>
<td>2.5</td>
<td>bitter, astringent, sweet</td>
</tr>
<tr>
<td>IIIAb</td>
<td>0.9</td>
<td>intense bitter, astringent lingering after taste</td>
</tr>
<tr>
<td>IIIB</td>
<td>1.6</td>
<td>meaty, hay-like</td>
</tr>
</tbody>
</table>

\(^{a}\)Weight percentage based on starting material (1500g)

\(^{b}\)Sum of Fraction II and Fraction III (by calculation)

**Chemical characterization of the subfractions of P34S**

The chemical structures of the three subfractions of P34S, as identified in this study, are shown in Fig. 5. They all imparted a bitter and astringent flavor when tasted. Due to the very limited amount of these samples obtained, they could not be sensory evaluated. All three isoflavones identified in this study have been previously identified in defatted soybean products or raw soybeans. However, they were studied because of their physiological and nutritional effects, particularly their estrogenic activity. No report could be found on the relationship of isoflavones with objectionable flavor of soybean products. Based upon the organoleptic evaluations in this study, these isoflavones, particularly, glycine 7-β-D-glucoside which possess a herb-like astringency and bitterness, might contribute additively to the nonvolatile objectionable flavor of soy protein products. These isoflavones were considered to be naturally occurring components, originally existing in soybeans, which have not been removed during processing.

**Compound P34S-C identified as Daidzein, 4',7-dihydroxy isoflavone**

Elemental analysis found C, 72.90; H, 4.18; and O, 22.92, which suggested an empirical formula, C\(_{15}\)H\(_{10}\)O\(_{4}\). (Continued on next page)

![Fig. 2—Chromatography of the HPLC analysis of Fraction IIIAa.](image)

**Table 4—Flavor characteristics of the HPLC fractions of IIIAa**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield (g)</th>
<th>Flavor</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>15.7%</td>
<td>sweet, molasses</td>
<td>light yellow powder</td>
</tr>
<tr>
<td>P2</td>
<td>5.3%</td>
<td>woody, phenol-like</td>
<td>bright yellow powder</td>
</tr>
<tr>
<td>P3</td>
<td>17.5%</td>
<td>bitter, salty</td>
<td>light yellow powder</td>
</tr>
<tr>
<td>P4</td>
<td>24.0%</td>
<td>bitter, astringent</td>
<td>yellow green powder</td>
</tr>
<tr>
<td>P5</td>
<td>19.9%</td>
<td>woody, biting</td>
<td>light brown powder</td>
</tr>
<tr>
<td>P6</td>
<td>17.1%</td>
<td>tasteless</td>
<td>grey powder</td>
</tr>
<tr>
<td>P7</td>
<td>15.6%</td>
<td>tasteless</td>
<td>light brown powder</td>
</tr>
</tbody>
</table>

\(^{a}\)Weight percentage based on Fraction IIIAa

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These values are close to the theoretical value of Daidzein whose C%, H% and O% are 73.17, 4.06, and 22.76, respectively. Color reactions showed colorless under daylight, light yellow green under UV irradiation at 366 nm, colorless under daylight after ammonia fume treatment, yellowish blue under UV after ammonia fume treatment, violet under daylight after FeCl₃ treatment. These color reactions were compared with the data published by Seikel (1962) and Mabry et al. (1970).

Spectral analyses: UV λmax (MeOH) 237sh, 249, 259sh, 303sh; λmax (MeOH + MeONa) 259, 289sh, 325; λmax (MeOH + AlCl₃) 249sh, 249, 260sh, 300sh; λmax (MeOH + AlCl₃ - HCl) 240sh, 249, 262sh, 302sh. NMR (trimethylsilyl ether derivative) showed: δ7.9 singlet (H-2); δ7.40 doublet (H-2, H-5); δ7.1 doublet (H-6); δ6.9 singlet (H-8); δ6.7 doublet (H-3, H-5). These spectral data were compared to the standard spectra published by Mabry et al. (1970).

Table 5—Yield and flavor characteristics of the subfractions of P34

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yieldb</th>
<th>Appearance</th>
<th>Flavor</th>
</tr>
</thead>
<tbody>
<tr>
<td>P34l</td>
<td>57%</td>
<td>pale yellow powder</td>
<td>woody, flour-like</td>
</tr>
<tr>
<td>P34S</td>
<td>43%</td>
<td>bright green yellow solid</td>
<td>bitter, astringent, weak phenol-like</td>
</tr>
</tbody>
</table>

b Weight percentage based on subfraction P34

Compound P34S-D identified as Glycitein 7-O-β-glucoside, 4'-hydroxy-6-methoxy-7-O-β-glucopyranosyl isoflavone

Elemental analysis found C, 58.42; H, 5.10; and O, 36.57, which suggested an empirical formula, C₁₁₂H₁₇₁O₁₀. These values are close to the theoretical values of glycitein 7-O-β-glucoside, whose C%, H%, and O% are 59.19, 4.92, and 35.87, respectively. Color reactions showed colorless under daylight, yellow green under UV irradiation at 366 nm, colorless under daylight after ammonia fume treatment, yellowish blue under UV after ammonia fume treatment, reddish violet under daylight after FeCl₃ treatment. These color reactions were compared with the data published by Seikel (1962) and Mabry et al. (1970).

Spectral analyses: UV λmax (MeOH), 228sh, 259, 319; λmax (MeOH + MeONa), 223, 271, 322sh; λmax (MeOH + AlCl₃) 228sh, 264, 312; λmax (MeOH + AlCl₃ - HCl) 240sh
Spectral analyses: UV λmax (MeOH), 260, 320sh; λmax (MeOH + MeONa), 270, 355sh, λmax (MeOH + AlCl₃), 272, 305sh, 375; λmax (MeOH + AlCl₃ - HCl), 272, 307sh, 374; NMR (trimethylsilyl ether derivative) showed 87.64 singlet (H-2); 67.44 doublet (H-2', H-6'); 86.84 doublet (H-3', H-5'); 86.4 singlet (H-8); 86.4 singlet (H-6); 53.4-3.8 broad (glucosyl protons). These spectra were compared with and confirmed by the standard spectra published by Mabry et al. (1970).

REFERENCES


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