Influence of Storage on *in Vitro* Digestibility of Soybean Leaf Protein Concentrate

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Soybean leaf protein concentrates (LPC) prepared by acid (LPC(pI)) or heat (LPC(Δ)) precipitation were stored at ambient conditions for up to 24 weeks. The method of preparing LPC, its storage, and digestion time significantly influenced the *in vitro* digestibility of LPC by papain. The overall digestibility of soybean LPC(pI) and LPC(Δ) was 94.99 ± 0.70 and 91.47 ± 0.57, respectively. After digestion for 1 hr the LPC(pI) and LPC(Δ) stored 24 weeks were 35 and 42% less digestible than their respective controls. After a 24-hr digestion, however, all stored LPC samples were digested as readily as the controls.

The current protein shortage is expected to become accentuated in the next 20 years because of population increases (Abbott, 1973). Leaf protein concentrate (LPC) is one of several nutritious protein concentrates which may play an important role in combating global protein malnutrition. The favorable aspects of LPC as well as some of the problem areas have been summarized (Betschart and Kinsella, 1974; Protein Advisory Group Bulletin, 1970). Even on the domestic scene the contemporary shortage of food proteins will entail an objective reassessment of LPC as a potential source of food protein based on need and the economy of this source.

The extractability and solubility of soybean leaf protein under various conditions have been reported (Betschart and Kinsella, 1973). Recently the influence of storage upon the general composition, amino acid, and solubility profiles of soybean LPC was cited (Betschart and Kinsella, 1974). In addition to amino acid analyses, *in vitro* digestibility is often used to evaluate the nutritive value of LPC (Akeson and Stahmann, 1965; Byers, 1971; Buchanan, 1969b; Lexander *et al.*, 1970).

Enzymatic digestibility of LPC has been studied to ascertain the effects of plant species and variety, method of preparation, extraction, and fractionation, heat treatment, storage temperatures, and drying methods on this important criterion of protein quality. Digestion by pepsin–pancreatin has shown the nutritive value of LPC to be superior to beef, casein, soybean, and wheat protein, approximately equivalent to milk and lactalbumin, and inferior to egg and egg white (Akeson and Stahmann, 1965). Others have reported varying degrees of digestibility among LPC using a pepsin–pancreatin digest; *e.g.*, LPC from *Amaranthus caudatus* was nearly twice as digestible as other LPC preparations (Lexander *et al.*, 1970). Akeson and Stahmann (1965), however, found no major differences in the digestibility of LPC prepared from nine species, not including *Amaranthus caudatus*. Leaf maturity had little influence upon the digestibility of LPC by papain (Byers, 1971). Several workers have found the soluble "cytoplasmic" LPC to be more digestible than the less soluble "chloroplastic" LPC (Byers, 1971; Lexander *et al.*, 1970). Smith (1966) reported that "cytoplasmic" LPC was 95-100% digestible by pepsin–trypsin, whereas "chloroplastic" LPC was 60–70% digestible.

One of the problems associated with LPC which has been held under adverse conditions is the development of undesirable "grassy, hay-like" odors and flavors. The lipids of LPC and their oxidation products have been implicated as the source of these unpleasant properties (Buchanan, 1969b; Kohler and Bickoff, 1970; Lea and Parr, 1961; Shah, 1968). LPC lipids are quite susceptible to oxidation because 53–77% of the fatty acids are polyunsaturated (Betschart, 1971; Lima *et al.*, 1965). There is a marked reduction in digestibility of LPC following oxidation of the lipids when exposed to temperatures of 100–105° (Buchanan, 1969a; Shah *et al.*, 1967).

The objectives of the present study were to examine the *in vitro* digestibility of soybean LPC which had been stored at ambient temperatures, *i.e.*, 27° for up to 24 weeks.

Papain was the enzyme of choice in the present study since the digestibility of freeze-dried LPC by papain was reported to be more nearly correlated with true digestibility than values obtained by a pepsin–pancreatin digest (Buchanan, 1969a). Since the present study was completed, however, conflicting data regarding the correlation of *in vivo* data with papain and pepsin–pancreatin have been reported (Saunders *et al.*, 1973). The implications will be more thoroughly treated in the Discussion.

EXPERIMENTAL SECTION

Soybean LPC was prepared as previously described (Betschart and Kinsella, 1974). The leaf extracts were sequentially centrifuged at 1000, 10,000, and 20,000*g* prior to precipitation of the LPC by acid at pH 3.5 (LPC(pI)) or heat (80°) (LPC(Δ)). Two and three samples of LPC(pI) and LPC(Δ), respectively, were prepared for each 4-week storage period up to 24 weeks and stored at 27° in the presence of air. Freshly prepared samples served as controls.

The digestibility of stored soybean LPC by thioglycolic activated papain was studied using the method of Buchanan and Byers (1969) with minor modifications. The nonprotein nitrogen (NPN) or trichloroacetic acid (TCA) soluble nitrogen released during digestion was determined as follows. Duplicate samples (150 mg) of each stored lot were suspended in 50-ml glass-stoppered erlenmeyer flasks in a final volume of 20 ml of 0.2 *M* sodium phosphate–0.1 *M* citric acid buffer. To each buffered sample (pH 6.6) were added 20.4 mg of papain (twice crystallized, Nutritional Biochemicals Corporation), 60 μl of thioglycolic acid (80% in water), and 0.15 ml of toluene. The specific activity of the papain preparation was 16 units/mg. One unit hydrolyzes 1 μmol of benzoyl L-arginine ethyl ester/min at pH 6.2 and 25°. The LPC suspensions were incubated at 60° in a Dubnoff metabolic shaker. Two-milliliter aliquots were removed from each flask after 1, 2, 4, 8, 16, and 24 hr of incubation and immediately precipitated with 2 ml of cold (4°) 10% (w/v) TCA, thoroughly mixed, and stored at 4° for 1 hr. The samples were then centrifuged in a refrigerated centrifuge (4°) at 10,000*g* for 10 min. The NPN content of 2 ml of the supernatant was determined by micro-Kjeldahl (McKenzie and Wallace, 1954). Digestibility was calculated as follows

$$\%\,\text{N digested} = \frac{\text{mg of NPN in supernatant}}{\text{mg of N in LPC}} \times 100$$

This procedure will be referred to as method I.

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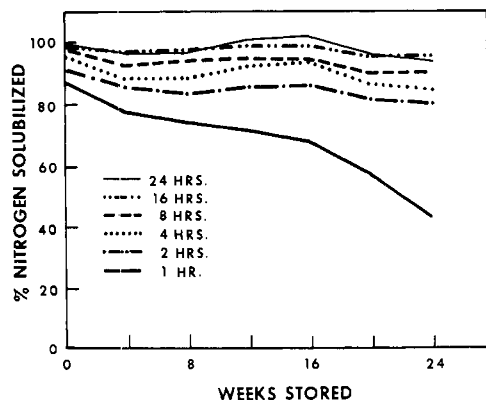

Figure 1. Digestibility, by papain, of heat-precipitated soybean leaf protein concentrate (LPC(Δ)) stored for progressive periods. The digestibility after 1, 2, 4, 8, 16, and 24 hr was determined by NPN release (method I).

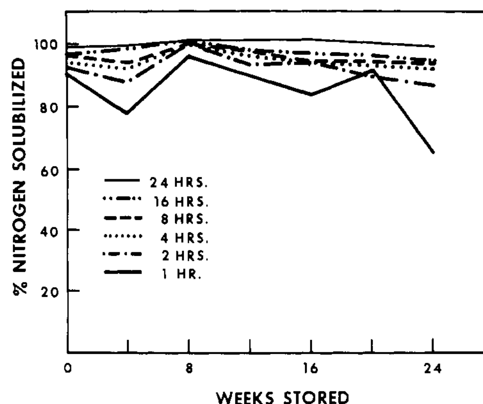

Figure 2. Digestibility, by papain, of acid-precipitated soybean leaf protein concentrate (LPC(pI)) stored for progressive periods. The digestibility after 1, 2, 4, 8, 16, and 24 hr was determined by NPN release (method I).

Protein nitrogen, or TCA-insoluble nitrogen, was also determined using a modification of Buchanan and Byers' method (1969). Duplicate samples (30 mg) of each stored LPC lot were weighed into 50-ml polypropylene centrifuge tubes; 1.63 mg of papain, 15 μl of thioglycolic acid, 0.05 ml of toluene, and the appropriate quantity of pH 6.6 buffer described in method I to bring the final volume to 5 ml were added in the order cited. After incubation at 60° for 24 hr, the protein nitrogen was immediately precipitated with 5 ml of 4° TCA, 10% (w/v), stored and centrifuged as in method I. The supernatant was carefully decanted and the TCA-insoluble precipitate was washed once with 5 ml of cold 5% TCA, stored, centrifuged, and decanted as above. The precipitate was solubilized in 2 ml of 1 *N* NaOH and transferred to a 30-ml micro-Kjeldahl flask together with two additional rinsings of the centrifuge tube. The enzymatic digestibility was expressed as

$$\% \text{ N digested} = \frac{\text{mg of N in LPC} - \text{mg of TCA-insoluble N}}{\text{mg of N in LPC}} \times 100$$

The latter procedure will be referred to as method II. These two complementary methods of determining digestibility were used on all stored samples. Method I provides detailed information on digestibility as a function of time, whereas method II may be used as a check for method I after digestion for 24 hr.

Enzyme and substrate controls were carried through both methods. Duplicates of egg albumin were also digested with each experiment to evaluate the reproducibility of the method.

Statistical methods involved analyses of variance (Harvey, 1966) in which method of preparation of LPC, digestion time, storage time, and resultant interactions were sources of variation. An analysis of variance was also conducted on the two methods of determining digestibility after 24 hr.

RESULTS AND DISCUSSION

The data from this study provided information on the *in vitro* digestibility of freshly prepared and stored soybean LPC.

Digestibility of Freshly Prepared Soybean LPC. Unless otherwise stated, the papain digestibility discussed in this section was determined by method I, NPN release after 24 hr. Both soybean LPC preparations were highly digestible, *i.e.*, the LPC(Δ) and LPC(pI) were 98.6 ± 2.0 and 99.6 ± 1.1% digestible, respectively (Figures 1 and 2). The digestibility of unfractionated LPC by papain usually ranged from 70 to 78% (Buchanan, 1969a; Buchanan and Byers, 1969; Byers, 1971). Values of 43-63% have been recently reported for whole alfalfa LPC (Saunders *et al.*, 1973). The favorable digestibility of soybean LPC may be the result of centrifuging the supernatant sequentially at 1000, 10,000, and 20,000*g* during preparation of the protein extract (Betschart and Kinsella, 1974). Most of the less soluble, less digestible, highly colored "chloroplastic" fraction of leaf protein may be removed by this method of preparation. The report of Byers (1971) is in agreement with our data. The digestibilities of barley and lupin LPC increased from 78 to 97% and from 78 to 98%, respectively, when the "chloroplastic" fraction was removed by centrifugation at 50,000*g*. Byers (1971) also found that digestibility of LPC by papain was markedly enhanced when extracts were centrifuged at speeds as low as 70*g* prior to isolation of the protein. In contrast, Saunders *et al.* (1973) reported that cytoplasmic alfalfa LPC was only 80% digestible by papain. The lower values may be a result of species differences, the presence of inhibitors such as oxidizing agents, and/or the lack of definitive specific enzymatic activity of enzyme preparations described in the original method (Buchanan and Byers, 1969; Byers, 1967). Within the concentrations examined in the present study, the papain concentration had little influence on digestibility as assessed by method II (Figure 3). In general, the values obtained by method II were somewhat lower than those obtained using method I. In the present study total enzymatic activities of 2.18 and 0.87 units/mg of substrate LPC were used in methods I and II, respectively. The lower concentration was used in method II to avoid high blanks.

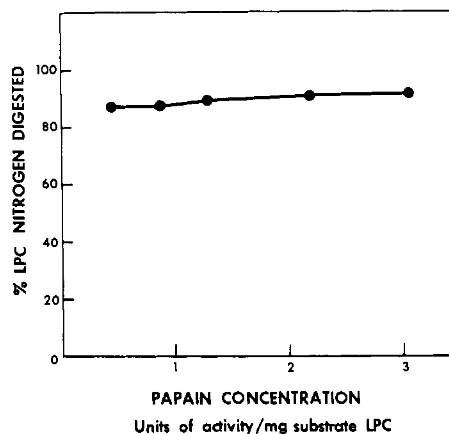

Figure 3. Influence of papain concentration on digestibility of LPC. Digestibility determined after 24 hr by TCA-insoluble nitrogen remaining (method II).

Digestibility of Stored Soybean LPC. Digestibility decreased with storage of LPC. This was especially noticeable after 1 hr of digestion (Figures 1 and 2). LPC(Δ)

Table I. Analysis of Variance; Papain Digestion for Model with Weeks Continuous

Source	df	Mean square	F
Preparation of LPC (acid or heat)	1	2005.92	87.63[a]
Digestion time (hr)	5	2203.91	96.28[a]
Storage time (weeks)			
Linear	1	1014.22	44.31[a]
Quadratic	1	391.79	17.12[a]
Preparation × digestion	5	267.80	11.70[a]
Preparation × storage			
Linear	1	205.74	8.99[a]
Quadratic	1	71.80	3.14
Digestion × storage			
Linear	5	314.60	13.74[a]
Quadratic	5	46.65	2.04
Preparation × digestion × storage			
Linear	5	76.30	3.33[a]
Residual	179	22.89	

[a] Significant at the 1% level.

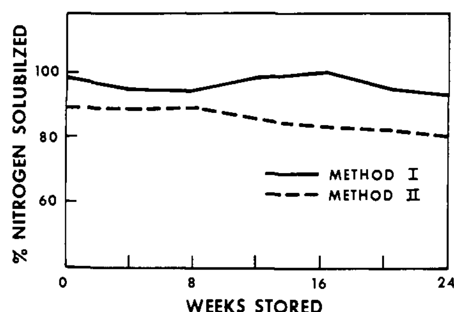

Figure 4. Digestibility, by papain, of heat-precipitated, stored soybean leaf protein concentrate (LPC(Δ)) after 24 hr of digestion as determined by NPN release (method I) and TCA-insoluble nitrogen remaining (method II).

samples stored for 24 weeks were 42% less digestible than the freshly prepared controls after digestion of 1 hr, 10–11% less digestible after 2 and 4 hr, and only 3% less digestible after a digestion period of 24 hr. Similarly, LPC(pI) stored for 24 weeks was 35% less digestible than the control after 1 hr of digestion by papain, but no differences were observed after digestion for 24 hr.

An analysis of variance revealed that the digestibility of soybean LPC was significantly (1% level of significance) influenced by the method of preparing LPC (acid or heat precipitation), digestion time, and storage time which contained linear as well as quandratic effects (Table I). The overall mean and standard error of digestibility for isoelectric soybean LPC (LPC(pI)) was 94.99 ± 0.70 as compared with 91.47 ± 0.57 for heat-precipitated soybean LPC (LPC(Δ)). Isoelectric precipitation apparently resulted in intramolecular aggregates which were more susceptible to enzymatic attack as opposed to the LPC precipitated at 80°.

A decrease in the *in vitro* digestibility of unfractionated LPC heated to 100 or 105° has been reported (Buchanan, 1969a; Shah *et al.*, 1967). However, the digestibility of LPC stored at lower temperatures such as 28 and 60° for 200 days was not markedly impaired (Buchanan, 1969b). The latter study observed papain digestibility after 24 hr and, thus, agrees with the present study in which storage effects were negligible after a 24-hr digestion period (Figures 1 and 2).

The impaired digestibility of LPC is hypothesized to be associated with the oxidation of the lipid fraction, *i.e.*, the lipid degradation products may form complexes with the

Table II. Analysis of Variance; Methods of Papain Digestion for 24 Hr

Source	df	Mean square	F
Preparation of LPC (acid or heat)	1	207.91	15.14[a]
Storage time (weeks)	6	21.78	1.59
Method of digestion (I, II)[b]	1	2072.15	150.93[a]
Preparation × storage	6	8.57	0.62
Preparation × method	1	0.01	0.00
Storage × method	6	16.93	1.23
Preparation × storage × method	6	17.53	1.28
Residual	42	13.73	

[a] Significant at the 1% level. [b] See text for equations.

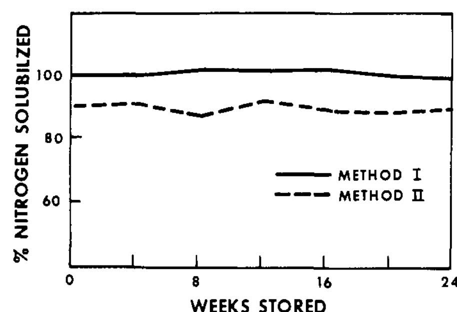

Figure 5. Digestibility, by papain, of acid-precipitated, stored soybean leaf protein concentrate (LPC(pI)) after 24 hr of digestion as determined by NPN release (method I) and TCA-insoluble nitrogen remaining (method II).

protein which interfere with protein digestibility (Buchanan, 1969a,b; Shah *et al.*, 1967). The effects of oxidizing lipids upon proteins and the formation of lipid–protein complexes have been studied in model systems, fish muscle, and fish protein concentrate (Crawford *et al.*, 1967; Pokorny, 1971; Roubal, 1971; Tannenbaum *et al.*, 1969). Although soybean LPC contained less lipid (8.0–9.5%) than unfractionated LPC studied by others, some oxidation may have occurred during storage giving rise to lipid–protein complexes and the resultant diminished digestibility after 1 hr of digestion. Buchanan (1969a) suggested an association between a decrease in lipid extractability and the oxidation of lipids in LPC. Such a decrease in extractability was not observed in any of the stored soybean LPC samples (Betschart and Kinsella, 1974). An analysis of the fatty acid composition of soybean LPC would aid in determining the stability of the lipid fraction. The data will appear in a subsequent paper.

Method II which employed TCA-insoluble nitrogen remaining after digestion for 24 hr served as a check for the 24-hr digestion period of method I which was based upon NPN release. Although Buchanan and Byers (1969) found the methods agreed within 4% of each other, differences of from 5 to 17% were observed in the present study (Figures 4 and 5). The values obtained using method I in the present study were significantly higher (1% level of significance) than those obtained by method II (Table II). The general means and standard errors for digestibility were 98.78 ± 0.63 and 87.68 ± 0.58 for methods I and II, respectively. Initially, the use of 2.18 *vs.* 0.87 units of total enzymatic activity/mg of LPC in methods I and II may appear to be a major factor. However, Figure 3 clearly shows that the use of quantities in excess of 3 units/mg of LPC in method II increased digestibility to a maximum of 4% above that achieved with 0.87 unit. Thus, the quantity of enzyme used does not appear to be a critical factor. Since the authors offer no plausible explanation for the

discrepancy between the two methods, it is recommended that method II be used in conjunction with method I, and not as the sole determinant of digestibility.

The *in vitro* digestibility data in the present study provided a criterion for evaluating the relative digestibility of stored soybean LPC. Although Buchanan (1969a) showed favorable agreement between papain digestibility and true digestibility, the recent report of Saunders *et al.* (1973) advises that values obtained by papain digestibility are poorly correlated with *in vivo* data. It is of interest that the values obtained by Saunders *et al.* (1973) were considerably lower (in general, 40-60% digestible) than values reported by the authors and others (Buchanan, 1969; Byers, 1971). Since there are conflicting reports regarding the correlation of papain digestibility with *in vivo* digestibility, *in vitro* digestibility by papain should be viewed as providing comparative data within experiments, but should not be viewed as having direct implications for *in vivo* digestibility.

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Parameters Affecting the Binding of Volatile Flavor Compounds in Model Food Systems. I. Proteins

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The binding of a homologous series of aldehydes and methyl ketones by various food proteins was studied in model systems by headspace analysis using gas chromatography. The amount of flavor bound depended on the type, amount, and composition of the protein, and the presence of solvents such as water and lipids. The addition of water to proteins, *i.e.*, α-lactalbumin, bovine serum albumin, leaf protein concentrate, single-cell protein, and various soy protein preparations, decreased the volatilities *via* increased adsorption or solubilization of flavors by the protein–water mixture. The concentration of headspace volatiles in model systems containing flavor and leaf protein concentrate increased upon removal of lipids. Flavor binding by the concentrate, isolate, and textured forms of soy protein was influenced by their compositions. The effect of proteins on volatility was similar in systems containing either dilute or concentrated flavors.

The problem of flavoring foods, excessive flavor binding by specific food components, and loss of flavor is assuming increasing importance because of the growing use of fabricated foods. Desirable organoleptic qualities are required for the eventual large scale utilization of new food proteins including leaf proteins, single-cell protein, and fish protein concentrate. In developing artificial flavoring systems and modifying or enhancing natural ones, a knowledge of the parameters influencing flavor volatility, binding, and the interaction of flavoring compounds with different food constituents is necessary.

Foods are complex mixtures of proteins, carbohydrates, lipids, water, and other organic compounds which can interact with and bind flavors. Nawar (1966) listed the factors affecting the headspace concentration of a volatile flavor compound, *i.e.*, vapor pressure and temperature, type of medium, degree of solubility, concentration, miscibility with other organic compounds, and the presence of salts or sugars. Sodium chloride increased the volatilities of dilute ester solutions (Jennings, 1965), and saturated aqueous sodium sulfate solutions increased the vapor pressures of aldehydes, ketones, esters, and alcohols (Nelson and Hoff, 1968). Unlike salts, carbohydrates and proteins either increase or decrease the volatility of flavor compounds. Wientjes (1968) found that the addition of glucose, sucrose, fructose, or invert sugar to dilute aqueous flavor solutions increased the volatility of a number of compounds while it decreased the volatility of other compounds. Sucrose increased the headspace volatility of aqueous acetone solutions; however, it decreased the volatility of 2-heptanone and heptanal solutions (Nawar, 1971). Maier (1970) investigated the influences of casein, gelatin, ovalbumin, and various carbohydrates on the headspace concentrations of acetone, ethanol, acetalde-

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