of plant-derived foods. The HPLC procedure reported here permits a direct assessment of the concentration of this compound and represents the first instrumental method that provides results that would be relavant to the bioavailability of vitamin $B_6$ in foods.

ACKNOWLEDGMENT

This research was supported in part by funds from the Florida Agricultural Experiment Stations and from Grant No. 83-CRCR-1-1240 from the Competitive Research Grants Office of the U.S. Department of Agriculture. The technical assistance of Doris Sartain is gratefully acknowledged. We thank Dr. John Gander and Sandi Bonetti of the University of Florida Department of Microbiology and Cell Science for their assistance in the NMR analysis.

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Received for review September 30, 1985. Revised manuscript received March 17, 1986. Accepted September 8, 1986.

# Application of in Vitro Methods To Assess the Nutritive Value of Leaf Protein Concentrates

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Sixteen leaf protein concentrates (LPC) were prepared from different crops by different processes and either freeze dried or oven dried in the 1983–1985 seasons. Total lysine and chemically available lysine of these samples were estimated. Biological assay parameters such as N retention and apparent digestibility were evaluated. Total lysine and chemically available lysine show good correlation with results from biological assay. Predicted biological nutritive values show good agreement with those from in vitro studies.

Processing conditions affect the nutritive value of food proteins. Rat assays are the best methods of nutritive value evaluation. Some in vitro methods such as digestibility with proteolytic enzymes (Buchnan, 1969; Saunders et al., 1973), growth of tetrahymena (Lexander et al., 1970; Smith and Pena, 1977), and microbial availability of essential amino acids (Henry and Ford, 1965) were used for nutritive evaluation of LPC. Shurpalekar et al. (1966) and Bickoff et al. (1975) using protein efficiency ratio assays with rats found considerable losses of nutritive value on thermal drying of LPC curd as compared with freeze drying. Henry (1964), who determined the biological value and true digestibility of LPC samples, found that hot-air drying in particular reduced the true digestibility of LPC. Byers (1971) found that damage to the LPC occurred during the heat coagulation stage, particularly to its lysine content. Proteins with lysine as a first limiting amino acid might, therefore, be subject to more severe reductions in nutritive value than would those deficient in methionine (Knipfel et al., 1975).

Biological nutritive evaluation methods are costly and time consuming. Hence, attempts are made to find quicker and simple chemical methods to predict the biological nutritive value parameters. In LPC preparations methionine is usually the first limiting amino acid. In some LPC preparations, due to low availability lysine could be the second deficient or limiting amino acid (Ohshima, 1985). Thus, digestibility and availability of methionine and lysine are the chief factors that govern the nutritive value. Processing conditions affect both sulfur amino acids and lysine in the same way. Since methods for determination of available lysine have worked out well for studies on factors affecting the nutritive value of LPC, a mathematical relation between available lysine and in vivo rat assay parameters such as apparent digestibility and N retention will be highly useful.

In order to evolve a suitable mathematical relationship between chemically available lysine and apparent diges-

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0021-8561/87/1435-0082$01.50/0 

**Table I. Lysine Content and Nutritive Value of Methionine-Supplemented LPC ($N$ = 16)**

| | matls and coagulation methods | year | total Lys, g/16 g N ($A$) | avail Lys, g/16 g N | app digestibility, % ($B$) | N retention, % | $A \times B$ | UN/DN |
|---|---|---|---|---|---|---|---|---|
| ethanol treated | Lucerne, heat | 1983 | 6.33 | 5.72 | 79.9 | 60.8 | 5.06 | 22.3 |
| | Lucerne, HCl heat | 1983 | 6.32 | 5.78 | 79.2 | 59.5 | 5.01 | 25.0 |
| | ladino, heat | 1983 | 6.79 | 6.20 | 78.2 | 60.3 | 5.31 | 20.0 |
| | ladino, HCl, heat | 1983 | 6.92 | 5.86 | 78.2 | 60.1 | 5.41 | 23.9 |
| | Italian, HCl, heat[a] | 1983 | 6.94 | 6.31 | 78.4 | 62.2 | 5.44 | 22.0 |
| | mean ± SE | | 6.66 ± 0.14 | 5.97 ± 0.12 | 78.8 ± 0.34 | 60.6 ± 0.46 | 5.25 ± 0.90 | 22.6 ± 0.86 |
| oven dried | Lucerne, heat | 1983 | 5.53 | 5.18 | 77.0 | 54.0 | 4.26 | 29.7 |
| | Lucerne, HCl, heat | 1983 | 5.57 | 5.10 | 76.6 | 50.4 | 4.27 | 31.4 |
| | ladino, heat | 1983 | 6.06 | 4.89 | 74.9 | 48.4 | 4.54 | 32.9 |
| | ladino, HCl, heat | 1983 | 6.27 | 4.73 | 75.8 | 52.9 | 4.73 | 29.1 |
| | Italian, HCl, heat | 1983 | 6.32 | 5.14 | 77.5 | 56.2 | 4.90 | 27.4 |
| | Italian, HCl, heat | 1984 | 6.21 | 6.00 | 80.6 | 56.0 | 5.01 | 32.3 |
| | ladino, HCl, heat[b] | 1984 | 6.71 | 4.98 | 79.2 | 53.0 | 5.31 | 33.0 |
| | mean ± SE | | 6.10 ± 0.16 | 5.15 ± 0.15 | 77.4 ± 0.74 | 53.0 ± 1.07 | 4.72 ± 0.15 | 30.8 ± 0.81 |
| freeze dried | ladino, HCl, heat | 1984 | 7.33 | 6.07 | 85.4 | 66.2 | 6.26 | 22.6 |
| | Italian, HCl, heat | 1984 | 6.83 | 6.84 | 83.7 | 61.7 | 5.72 | 25.5 |
| | Italian, HCl, heat | 1985 | 6.68 | 6.25 | 80.5 | 63.4 | 5.38 | 21.3 |
| | Italian, HCl[c] | 1985 | 6.81 | 6.78 | 81.4 | 64.0 | 5.54 | 21.7 |
| | mean ± SE | | 6.91 ± 0.14 | 6.49 ± 0.19 | 82.8 ± 1.11 | 63.8 ± 0.93 | 5.73 ± 0.19 | 22.8 ± 0.95 |

[a] $N$ = 5. [b] $N$ = 7. [c] $N$ = 4.

tibility, N retention, and urinary N/digested N (UN/DN), we prepared LPC samples from various crops under various conditions and treatments. The FDNB-reactive lysine and total lysine of these samples were estimated. The apparent digestibility, N retention, and UN/DN were estimated by rat assay methods.

## MATERIALS AND METHODS

**Preparation of Samples.** Lucerne (*Medicago sativa* L.), ladino clover (*Trifolium repens* L. var. giganteum), and Italian ryegrass (*Lolium multiflorum* Lam.) were used for preparation of leaf protein concentrates (LPC). At the time of harvest the crop had attained the height of 60 cm and was at early-bloom/early-heading or in an actively growing vegetative stage. Lucerne and ladino clover were harvested every month in the spring seasons of 1983 and 1984. Italian ryegrass was harvested in December 1983 and 1984 and April–May 1985. The vegetation was pulped in a pulp refiner (Kumagai Kogyo Ltd.), and juice was expressed by a hydraulic press (Kiya Seisakusho Ltd.).

Leaf protein concentrate was precipitated from juice by direct heating to 70 °C within 10 min, adjustment to pH 4.0 by using dilute HCl, or adjustment to pH 4.0 and subsequent heating. The coagulum was separated on a vertical decanter (Kokusan Enshinki H-600) at 16000 rpm. The wet cake contains 65% moisture.

A part of wet cake obtained from samples prepared in 1983 was soaked in 90% ethanol (1:4 w/v) for a few days with occasional stirring. The LPC was collected with a vertical decanter.

The wet cakes and ethanol-treated wet cakes were dried in a draft oven at 70 °C for 18 h. The dried cakes obtained in all the treatments except those freeze dried were given water and ethanol washings in a Buchner funnel, respectively, and then dried again. Some of the samples were freeze dried.

LPC prepared in one or more alternative treatments for precipitation and dryness are shown in the flow diagram in Figure 1.

All the samples were ground in a ball mill (Yamato, Universal Ball Mill VB-51). The LPCs of various harvests were pooled for respective years and stored in deep freeze until use.

**Biological Assays.** Assays were carried out in different seasons on Wistar strain male growing rats weighing about 50 g. They were restrictively fed for 10 or 14 days. The diets contained 10% CP and were supplemented with methionine. In the later half of the experimental period, feces and urine were collected (Mitchell, 1924).

**Table II. Comparison of the Amount of Amino Acid in LPC with the FAO Pattern**

| amino acid | reference FAO value,[a] g/16 g N | mean value in LPC samples,[b] g/16 g N | arbitrary ratio index[c] |
|---|---|---|---|
| isoleucine | 4.2 | 5.59 | 100 |
| leucine | 4.8 | 10.09 | 159 |
| lysine | 4.2 | 5.59 (avail) | 100 |
| phenylalanine | 2.8 | 6.44 | 172 |
| tyrosine | 2.8 | 4.96 | 133 |
| sulfur amino acids | 4.2 | 3.07 | 55 |
| threonine | 2.8 | 5.19 | 139 |
| tyrptophane | 1.4 | | |
| valine | 4.2 | 7.24 | 133 |

[a] FAO-WHO Nutrition Meeting Report, Rome, 1965, FAO Series No. 37. [b] Byers, 1971. Quoted from: Ostrowski-Meissner, H. T. *J. Sci. Food Agric.* **1980**, *31*, 177. [c] Arbitrary ratio index = 100 × (content of AA in LPC)/(content of Ile in LPC)(ref FAO for Ile)/(ref FAO for AA).

**Chemical Assays.** The total lysine was estimated for the 6 N HCl hydrolyzed samples on a automatic amino acid analyzer (Hitachi KLA-5).

The chemical or FDNB- (1-fluorodinitrobenzene) reactive lysine was determined by modified method of Carpenter (Booth, 1971). The correction factor for LPC was determined by the addition of DNP-L [(dinitrophenyl)lysine] to the reaction mixture after the FDNB reaction had been stopped by acid.

## RESULTS AND DISCUSSION

Table I shows that oven-dried samples have significantly less total lysine than either ethanol-treated oven-dried or freeze-dried samples. It is also important to note that the ethanol-treated oven-dried samples show no significant difference in total lysine content when compared to freeze-dried samples.

The available lysine in all three groups of samples shows significant variation. The freeze-dried samples show a maximum amount of available lysine than the other two groups. The ethanol-treated samples show improvement over that of the untreated oven-dried samples. Thus, freeze drying appears to be protecting the free $\epsilon$-$NH_2$ of the lysine.

**Table III. Correlation Coefficients and Regression Equations between in Vitro and in Vivo Methods**

| total Lys | | | | available Lys | | | total Lys ($A$) × app digestibility ($B$) | |
|---|---|---|---|---|---|---|---|---|
| avail Lys | app digestibility | N retention | UN/DN | N retention | $A \times B$ | UN/DN | N retention | UN/DN |
| 0.627 | 0.658 | 0.771 | −0.639 | 0.847 | 0.714 | −0.717 | 0.839 | −0.624 |
| $y = 0.85x +$ 0.233 | $y = 3.696x +$ 55.217 | $y = 8.156x +$ 5.245 | $y = -5.297x +$ 64.646 | $y = 6.620x +$ 20.072 | $y = 5.652x +$ 1.889 | $y = -4.913x +$ 54.455 | $y = 8.279x +$ 15.558 | $y = -5.40x +$ 53.994 |

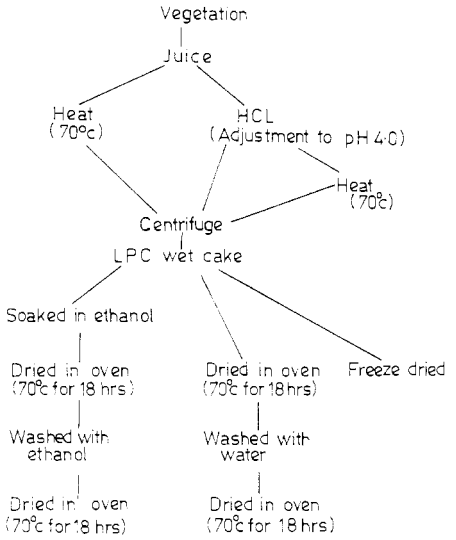


**Figure 1.** Flow diagram showing various methods used for the preparation of leaf protein concentrates.

When compared with FAO standards (FAO-WHO, 1965), as shown in Table II, available lysine is the second limiting amino acid in untreated oven-dried samples. But, when it is over 5.6 g/16 g of N, isoleucine is the second limiting amino acid and it is known that LPC contains enough isoleucine for rat growth (Henry and Ford, 1965). Available lysine of the ethanol-treated oven-dried sample is above the FAO level. Thus, for lysine being above FAO requirement, an ethanol-washing treatment will give the satisfactory result, where freeze driers are not feasible. However, if LPC is to be used to supplement the lysine content of other food ingredients, freeze-dried samples will give better results.

Variations in total lysine and available lysine content have reflected in correlated changes in apparent digestibilities, N retention, and urinary N/digested N. The correlation coefficients and regression equations between various combinations are given in Table III and are significant.

Maliwal (1983) obtained a correlation coefficient between the percent available lysine, true digestibility, and net protein ratio as 0.94 and 0.89, both significant at the 1% level of significance. The determination of percent available lysine is also tedious as it involves calculations of total lysine content, which makes nutritional evaluation more complicated. Walker (1979) obtained a regression equation for available lysine (FDNB reactive) as $y = -51 + 1.54x$ ($x$ = total lysine content). This equation differs from the equation in the present data. The lightness of the sample color (Walker, 1979) shows no correlation with available lysine. In the present exercise no systematic work was carried out on the colors of the samples, but it was observed that there appears to be no correlation between them.

Ohshima and Moriyama (1985) obtained regression equations of total lysine and N retention and UN/DN in LPC samples as $y = 14x - 34.8$ and $y = -13.3x + 112.6$ where $x$ = total lysine content, showing good agreement with our equations.

Bujard et al. (1967) obtained regression equations of total lysine and available lysine as available lysine: $y = 2.70x - 13.88$ ($r = 0.99$). Biologically available lysine: $W = 0.94V + 0.61$ ($r = 0.97$), $V$ = chemically (FDNB-) reactive lysine content.

Martinez et al. (1961) obtained a correlation coefficient between the nutritive index and FDNB-reactive lysine as $r = 0.86$ at 16 degrees of freedom.

Boyne et al. (1961) undertook a similar exercise with protein concentrates of animal and vegetable origin. They found a good correlation between available lysine estimated as per Carpenter et al. (1957) with gross protein value in meat and meat/bone meals, fish meals, whale meat meals, and dried whale solubles. No correlation of the available lysine with in vivo methods carried out on chicks was observed in groundnut and soy meals.

Carpenter and Woodham (1974) obtained a correlation coefficient $r = 0.97$ between FDNB-reactive lysine and the biological availability in chick assay in the protein concentrates of animal and vegetable origin.

Combs et al. (1968) reported the results of a large number of growth assays with chicks for the available lysine and methionine values of commercial samples of protein concentrates used in U.S. They found values that corresponded to 90% or more of chemical values for fish meals and soybean meals and 80% or more for meat meals.

When the regression equations from the present data were applied to the newly prepared LPC samples, the predicted values show good agreement with in vivo results obtained with rats. The available lysine of samples obtained was 6.93, the predicted N retention is 65.9, and the observed value is 60.

Thus, it should be possible to statistically predict with reasonable accuracy the in vivo nutritive value of LPC by determining available lysine content alone. However, since this method does not take into consideration the presence of unforeseen antinutritional factors, the method should be used for screening purposes only. The final decision on acceptability of LP as a good product should be confirmed only on the basis of an in vivo result.

ACKNOWLEDGMENT

C.S. thanks the Ministry of Education, Science & Culture, Japan, for financial assistance.

**Registry No.** L-Lysine, 56-87-1; EtOH, 64-17-5.

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Received for review February 19, 1986. Accepted August 11, 1986.

# Interaction of Proteins with Allyl Isothiocyanate

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Interaction of allyl isothiocyanate (AITC) with proteins and the digestibility of a protein–AITC adduct were studied in detail as a model reaction of isothiocyanate and protein in crushed *Brassica* seeds. Insulin, bovine serum albumin (BSA), ovalbumin and lysozyme as model proteins reacted slowly with AITC to cleave the disulfide bond in their cystine moieties followed by polymer formation. Besides this action, AITC also attacked free amino groups of lysine and arginine residues in protein to form their thiourea-like derivatives, detected by amino acid analyses and determination of the free amino group. Proteolytic digestibility of the protein–AITC adduct was investigated on a BSA–AITC adduct using trypsin, chymotrypsin, and pepsin. Digestibilities with trypsin and chymotrypsin were decreased markedly, but not so significant with pepsin. The reasons were considered as follows: The former attacked the peptide moieties containing basic and aromatic amino acids, which were easily modified with AITC, but the latter have a wide action program compared with the former.

The seeds of the Cruciferae family contain many kinds of glucosinolates that are degraded to alkyl isothiocyanate (mustard oil), glucose, and sulfate by the action of β-thioglucosidase (myrosinase) localized in same plant cell (Ettlinger et al., 1961). This enzyme reaction usually forms thiocyanates and cyanides as minor products besides isothiocyanate.

The isothiocyanates have a strong pungent taste and easily react with some nucleophiles to give thiocarbamoyl derivatives and several kinds of degradation products (Kawakishi and Muramatsu, 1966; Kawakishi et al., 1967; Kawakishi and Namiki, 1969). Especially, isothiocyanates formed in crushed *Brassica* seeds during the processing of rapeseed oil may participate in some chemical reaction with amino acids and proteins in the seed under mild condition. Rapeseed (*Brassica nupus* or *Brassica campetris*) has been utilized as an oil seed in worldwide oil production, but its defatted meal is underestimated as an animal feed even though it contains a high-quality protein (Matsumoto, 1977). Among many reasons, there are two problems related to glucosinolates and their degradation products: one is the contents of goitrin (5-vinyloxazolidine-2-thione), which is well-known for its goitrogenic action (Kjaer et al., 1956; Greer, 1962); the other one seems to be related to the interaction between isothiocyanates and meal proteins. If such interaction arises in the processing of rapeseed oil, a part of seed protein will be transformed to the modified one with isothiocyanate, and it may change to an indigestible and/or some cytotoxic one.

From these situations, we have studied in detail the interaction of protein with isothiocyanate, and in this study allyl isothiocyanate (AITC), well-known for its formation from sinigrin and most widely distributed in the Cruciferae family, was used. The interaction of AITC with cystine (Kawakishi and Namiki, 1982; Kawakishi et al., 1983) and oxidized glutathione (Kawakishi and Kaneko, 1985) under physiological conditions has been reported, and the oxidative cleavage in their disulfide bonds with the electrophilic action of AITC was clearly demonstrated.

This paper is concerned with the interaction between some model proteins and AITC and digestibilities of protein–AITC adducts with some proteolytic enzymes.

## EXPERIMENTAL SECTION

**Materials.** BSA (crystallized) and lysozyme (crystallized from egg white) were purchased from Seikagaku Kogyo Co., and insulin (from bovine pancreas) and ovalbumin (grade V) were purchased from Sigma Chemical Co. Trypsin (type IX), α-chymotrypsin (type I-S), and pepsin were obtained from Sigma Chemical Co. AITC was used a commercial guaranteed grade and redistilled immediately before using. DTNB, TNBS, and other reagents were obtained commercially.

**Reaction Mixtures.** Reaction mixtures were prepared in 25 mL of a $^1/_{30}$ M phosphate buffer, and the compositions of reaction mixtures were as follows: insulin (5 μmol)–AITC (0.5 mmol) at pH 7.5; BSA (1.25 μmol)–AITC

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0021-8561/87/1435-0085$01.50/0 