Chemical Methods for Available Lysine[1]

J. W. FINLEY and MENDEL FRIEDMAN, Western Regional Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, Calif. 94710

ABSTRACT

Current procedures for available lysine are based on modification of the ϵ-amino groups with 1-fluoro-2,4-dinitrobenzene or 2,4,6-trinitrobenzenesulfonic acid. The proposed new method is based on modification of the lysine ϵ-amino group with activated vinyl compounds, such as methyl acrylate, followed by acid hydrolysis and ion-exchange chromatography on an amino acid analyzer. The methyl acrylate procedure for available lysine complements currently used methods and may have advantages in some applications.

The nutritive value of a food protein depends not only on its content of essential amino acids but also on physiological availability. Amino acids are unavailable if they are in regions of a protein protected (chemically or physically) from action of proteolytic enzymes or if they are linked to other chemical moieties through bonds not readily broken by digestion.

Cross-linking is probably the most important chemical mechanism restricting biological utilization. Because of its ϵ-amino group, lysine is particularly susceptible to side reaction and cross-linking making it unavailable. For more than 15 years the chemical and biological availabilities of lysine in processed foods and feedstuffs have been studied extensively. Lysine is especially important in wheat products because their lysine content is low to begin with and is liable to further degradation by moderate to severe heat treatments that some of these products receive.

Lysine becomes secondarily bound (nutritionally unavailable) as a result of chemical reaction of the ϵ-amino group of lysine including the following: First, lysine may be buried in a protein matrix in a particular sequence or conformation which is slow to hydrolyze or is not hydrolyzed at all by animal proteases. Such lysine may or may not appear as chemically available by hydrolysis methods and yet be totally unavailable nutritionally. Second, lysine can be cross-linked to an aspartyl or glutamyl residue on another protein or in the same protein molecule. Harding and Rogers (1) have isolated such a cross-link from hair and Asquith and Otteburn (2) and Holt and Milligan (3) demonstrated the presence of such cross-links in heated keratin proteins. Although the bond looks like a "normal" peptide bond it is not hydrolyzed by gut proteases. The aspartyl- or glutamyl-lysine remains after proteolysis and appears nutritionally unusable. The aspartyl- or glutamyl-lysine link is broken by acid hydrolysis which yields lysine and aspartic or glutamic acid. Another reaction of lysine is cross-linking with dehydroalanine residues. Bohak (4) and Patchornik and Sokolovsky (5) reported that dehydroalanine is formed by heating serine or cysteine under alkaline conditions.

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The dehydroalanine thus formed reacts with the ϵ-amino group to form lysinoalanine. According to Bohak, substantial losses of lysine resulted when proteins received moderate heat under alkaline conditions. A more common reason for losses of available lysine may be reaction with carbohydrates. Both reducing and nonreducing sugars react with lysine in proteins to render it unavailable. El-Nockrashy and Frampton (6) showed that sucrose, raffinose, and trehalose all react with lysine. Reaction with reducing sugars has been extensively studied. After formation of a Schiff's base, several further products can result from the Browning and Maillard reactions (7,8).

Paper-chromatographic, enzymatic, and microbiological methods for determining lysine (as well as other amino acids) have been studied by Pomeranz and Miller (9).

The principles of the currently used fluorodinitrobenzene (FDNB) and trinitrobenzenesulfonic acid (TNBS) chemical methods for available lysine are the same. First, the compounds are allowed to react with the lysyl side chain under mild conditions. Next, the protein is hydrolyzed, and the hydrolysis products are extracted with ether to remove excess reagent. Finally, the derivatives are measured spectrophotometrically.

The FDNB method is based on work by Carpenter and Bjarnason (10), but various modifications have been used to purify the resulting ϵ-dinitrophenyl-(DNP)-lysine, especially ion-exchange and other chromatographic procedures. In some cases, no further purification was considered necessary. Blom et al. (11) outline three detection procedures for DNP-lysine: a) direct photometry of the yellow color; b) photometry of the reaction product with ninhydrin; and c) polarographic measurement based on reducing the nitro group at the dropping mercury electrode. Blom and his associates also report significant differences in estimates of available lysine in certain proteins depending on sample size. Kakade and Liener (12) report that only 1 hr. is needed for analysis with TNBS as against 16 hr. with FDNB. Several workers have compared these methods with pure proteins and found that they agree quite well with biological determinations of available lysine.

In this paper we compare three methods for the determination of available lysine: The 1-fluoro-2,4-dinitrobenzene (FDNB) procedure, the trinitrobenzenesulfonic acid (TNBS) procedure, and a new method based on alkylation of ϵ-amino groups of lysine side chains by methyl acrylate. Our results indicate that the methyl acrylate method may find application in determining available and unavailable lysine in protein-containing materials.

MATERIALS AND METHODS

Lysozyme, egg albumin and ribonuclease were obtained from Sigma Chemical Co. (St. Louis, Mo.); casein, from Braun-Knecht-Heimann Co. (San Francisco, Calif.); and gluten were from Centennial Mills (Seattle, Wash.). Ardex 550 Soy was from Archer Daniels Midland Co., corn meal from Carnation Co. (Van Nuys, Calif.), wheat flour and bulgur were made from HRW wheat, and polished rice came from California. A 100-mg. sample of protein or flour was suspended in 10 ml. of 0.1 M sodium borate and sufficient methyl acrylate was added to yield a 10-fold molar excess over estimated total lysine in the sample. A larger excess of methyl acrylate accelerates the reaction. Reaction was usually carried out in tightly stoppered

25-ml. flasks. Before sealing, the flasks were flushed for 30 sec. with dry nitrogen. The samples were incubated at 22°C. with constant agitation for 24 hr. The samples were then dialyzed against five changes of distilled water for 24 hr. and lyophilized. The freeze-dried, alkylated protein was subjected to hydrolysis with 6N HCl for 22 hr. A nonalkylated control sample of each protein was dialyzed at the same time. All samples were subjected to amino acid analysis according to Spackman (13) and the results automatically computed (14). The FDNB procedure was carried out according to Carpenter and Bjarnason (10) and the TNBS method according to Kakade and Liener (12).

RESULTS AND DISCUSSION

The present method is based on the condensing of an α,β-unsaturated compound with the available amino groups as previously reported (15,16). The reaction of terminal amino groups in proteins with an excess methyl acrylate at pH 9.1 yields mainly ε,ε, N,N-dicarboxyethyllysine and a small amount of ε,N-monocarboxyethyllysine after hydrolysis of the derivatized protein (16). The reaction time can be cut to 4 hr. by employing 75% DMSO:25% (v./v.) buffer. This rate enhancement is achieved presumably because of the downward shift in the pK of the ε-amino groups (17,18).

$$(HOOCCH_2CH_2)_2NCH_2CH_2CH_2CH_2CH(NH_2)COOH$$

ε,ε,N,N-dicarboxyethyllysine

$$HOOCCH_2CH_2NHCH_2CH_2CH_2CH_2\ CH(NH_2)COOH$$

ε,N-monocarboxyethyllysine

Available lysine is measured by comparing lysine content before and after alkylation. The decrease in lysine content is inferred to measure the chemically available lysine or the lysine with a reactive ε-amino group. In Tables I and II results of this method are compared with results of the TNBS and FDNB procedures on a series of pure proteins. The data demonstrate reasonable agreement.

TABLE I. DETERMINATION OF AVAILABLE LYSINE IN VARIOUS PROTEINS BY REACTION OF FREE AMINO GROUPS WITH METHYL ACRYLATE[a]

Protein	Total Lysine g./100 g. protein	Lysine after Reaction with Methyl Acrylate g./100 g. protein	Available Lysine %
Bovine serum albumin	12.82	0.00	100
Lysozyme	5.75	0.11	98.1
Egg albumin	5.10	0.02	99.6
Ribonuclease	10.61	0.01	99.9
Gluten	1.25	0.011	99.1
Casein	8.31	0.233	97.2

[a]Average of triplicate determinations.

TABLE II. COMPARISON OF THREE METHODS FOR AVAILABLE LYSINE DETERMINATION

	Methyl Acrylate[a] g./100 g. protein	FDNB[a] g./100 g. protein	TNBS[a] g./100 g. protein
Bovine serum albumin	12.82	12.85	12.86
Lysozyme	5.64	5.60	5.78
Egg albumin	5.08	5.09	5.10
Ribonuclease	10.60	10.18	10.34
Gluten	1.24	1.26	1.24
Casein	8.08	8.17	8.06

[a]Average of triplicate determinations.

TABLE III. DETERMINATION OF AVAILABLE LYSINE IN HEAT-TREATED SAMPLES

Protein	Time at 205° C.[a] min.	Total Lysine g./100 g. material	Lysine after Alkylation[b] g./100 g. material	Available Lysine by Methyl Acrylate g./100 g. material
Casein	0	8.31	0.23	8.08
Casein + 20% dextrose	30		1.97	6.34
Casein + 20% dextrose	60		3.27	0.04
Gluten		1.25	0.06	1.19
+ 20% dextrose	30		0.27	0.98
Gluten + 20% dextrose	60		1.22	0.03

[a]Treated at 30% moisture.
[b]Average of triplicate determinations.

TABLE IV. APPARENT AVAILABLE LYSINE IN DRY-MILLED CEREAL PRODUCTS BY THE METHYL ACRYLATE PROCEDURE

Material	Lysine before Alkylation g./100 g. protein	After Alkylation g./16 g. N	Available Lysine %
Whole wheat[a]	2.63	0.36	86.3
Corn meal	2.20	0.30	86.5
Soy flour	6.15	0.73	88.0
Rice (polished)	2.25	0.41	81.5
Bulgur flour	2.12	0.29	86.0

[a]Whole wheat was ground to pass a 40-mesh screen before analysis.

Next, a study of heat-treated casein and gluten in the presence of large amounts of reducing carbohydrates was made (Table III). As expected, available lysine decreased rapidly as heat treatment increased. The total lysine dropped much more slowly. These samples show a characteristic limitation of the FDNB and TNBS

methods. The colored products formed during heating severely limit accurate spectrophotometry. Such samples require polarographic titration or chromatographic purification as described by Blom et al. (11). Another disadvantage of using FDNB is that it causes skin irritation in some individuals.

Finally, results shown in Table IV suggest that the described methyl acrylate procedure also appears useful for determining available and unavailable lysine in dry-milled cereal products.

In summary, reaction of α,β-unsaturated compounds appears attractive for estimating available lysine, for example, in high-carbohydrate foods, especially after heat processing. The potential exists for ascertaining total and available lysine in one determination.

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