A Colorimetric Method of Screening Maize Seeds for Lysine Content[1]

C.-Y. TSAI, L. W. HANSEL, and O. E. NELSON[2], Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana 47907

ABSTRACT

A colorimetric method for screening lysine content in seeds of cereal grains is presented. The method utilizes 2-chloro-3,5-dinitropyridine as a reagent for the ε-amino group of lysine after enzymatic hydrolysis of the proteins and blocking the α-amino groups of the free amino acids with copper. After acidification, the excess 2-chloro-3,5-dinitropyridine is extracted with ethyl acetate. The absorbance at 400 nm. of the ε-dinitropyridyl-lysine formed is then measured. The method gives lysine determinations for *opaque-2* maize that agree well with lysine determinations for the same samples on the Beckman amino acid analyzer, but are somewhat higher.

The discovery (1) that the maize mutant *opaque-2* produces seeds with a higher content of lysine than does ordinary maize has stimulated interest both in the utilization of the *opaque-2* mutation to enhance the nutritional quality of maize and in the possibility that genetic differences improving protein quality exist in other cereal grains. In either endeavor, the necessity for large numbers of lysine-content estimates may be the limiting factor, particularly in developing nations. Accordingly, there is interest in methods that estimate the lysine content of total seed proteins by colorimetric techniques.

Several such techniques utilize the reagents used by protein chemists to identify the N-terminal amino acid of a polypeptide chain, since such reagents are also capable of reacting with the ε-amino group of lysine. Selim (2) employed Sanger's reagent, 1-fluoro-2,4-dinitrobenzene, to estimate the lysine content of protein hydrolysates after blocking the α-amino groups of the free amino acids with copper. Kakade and Liener (3) have utilized 2,4,6-trinitrobenzenesulfonic acid (TNBS) to determine the available lysine of foodstuffs. Subramanian et al. (4) combined the use of TNBS as a reagent with a simple protein-extraction procedure for the determination of lysine in maize seeds. In the Kakade and Liener technique and the Subramanian modification of it, the reaction with TNBS takes place before hydrolysis of the protein.

We report here another colorimetric technique for lysine determination in maize seeds that has been developed and employed successfully in our laboratory. It has also been used with success for lysine determination in maize and wheat at the CIMMYT laboratories in Mexico City[3], in rice at Academia Sinica, Taipei, Taiwan[4], and in maize at the Rural University of Minas Gerais, Viscosa, Minas Gerais, Brazil[5].

This method involves the use of 2-chloro-3,5-dinitropyridine as a reagent. This compound has been utilized to identify N-terminal amino acids in proteins by

[1]Supported by AID Contract 1A-449. Journal Paper No. 4535, Purdue University Agricultural Experiment Station.

[2]Present address: Department of Genetics, University of Wisconsin, Madison, Wis. 53706.

[3]E. Villegas, personal communication.

[4]H. W. Li, personal communication.

[5]R. Sant'Ana, personal communication.

Copyright © 1972 American Association of Cereal Chemists, Inc., 3340 Pilot Knob Road, St. Paul, Minnesota 55121. All rights reserved.

Signor et al. (5), who showed that ϵ-DNPyr-lysine would remain in the aqueous phase on extraction with ethyl acetate.

In this method, treatment of the defatted ground-corn sample with any of several proteolytic enzymes hydrolyzes the proteins present to free amino acids or low-molecular-weight peptides. The α-amino groups of the free amino acids are complexed with copper, but the ϵ-amino group of lysine remains reactive. The ϵ-dinitropyridyl derivative of lysine is then formed on reaction with the 2-chloro-3,5-dinitropyridine. Extraction of the reaction mixture with ethyl acetate removes the excess 2-chloro-3,5-dinitropyridine, leaving an aqueous solution of ϵ-DNPyr-lysine. The absorbance of this solution is then determined at 400 nm.

MATERIALS AND METHODS

Reagents

2-Chloro-3,5-dinitropyridine was purchased from Nutritional Biochemical Corporation. L-Alanyl-L-lysine•HCl, L-glycyl-L-lysine•HCl, and L-histidyl-L-lysine•HBr were obtained from Sigma Chemical Co., and pronase from Calbiochem. Alcalase (a proteolytic-enzyme preparation from *Bacillus subtilis*) and pancreatic trypsin were gifts from Novo Industri, Copenhagen.

Preparation of Sample

Maize endosperms or seeds were ground in a Waring Blendor for about 1 min. The meals were defatted for 48 hr. by refluxing with *n*-hexane in a Soxhlet apparatus. The dry, defatted samples were ground further in a Wig-L-Bug for about 3 min. Such preparations were then used for all further tests.

Preparation of Copper Phosphate Suspension

A copper phosphate suspension is prepared by a modification of the method originally suggested by Spies and Chambers (6) by dissolving 1.4 g. of $CuCl_2 \cdot 2H_2O$ and 6.8 g. of $Na_3PO_4 \cdot 12\ H_2O$ in 50 and 100 ml. of deionized water, respectively. These solutions are mixed together and centrifuged to collect the precipitate. The precipitate is washed four times with 0.05M sodium borate, followed by centrifugation. After the wash is completed, the pellet is then suspended in 40 ml. of 0.05M sodium borate. This suspension can be used for 2 weeks.

Procedure for the Colorimetric Determination of Lysine

After 200 mg. of sample is incubated overnight at room temperature with 5 ml. of 0.02M borate buffer, pH 8.4, containing 2.5 mg. of pronase or a mixture of alcalase and pancreatic trypsin (2 mg. each) and a few drops of chloroform, the hydrolysate is centrifuged for 15 min. at 18,800 × g. A control, containing proteolytic enzymes but no sample, is run simultaneously. From the supernatant fraction, 0.5 ml. is transferred into a centrifuge tube; to this, 1.0 ml. of 0.6M sodium carbonate-bicarbonate buffer, pH 9.0, and 0.5 ml. of copper phosphate suspension are added. The mixture is shaken occasionally for 5 min. and then centrifuged to precipitate the excess copper phosphate. From the supernatant fraction, 1 ml. is pipetted into a test tube, and 0.1 ml. of 2-chloro-3,5-dinitropyridine solution (30 mg. per 1 ml. of methanol or ethanol) added. The reaction is allowed to proceed at room temperature for 2 hr. before the reaction mixture is acidified by adding 4 ml. of 1N HCl. The ϵ-DNPyr-lysine formed

is freed from the excess of reagent and other DNPyr-amino acid derivatives by transferring the mixture to a 30-ml. separatory funnel and extracting with three 5-ml. portions of ethyl acetate. The aqueous phase containing ε-DNPyr-lysine is then measured in a Spectronic 20 at 400 nm. against a sample-free blank.

To obtain a standard curve representative of experimental conditions in the supernatant fraction, a constant volume (0.5 ml.) of any one sample supernatant fluid is added to various amounts (0 to 250 γ) of lysine•HCl (dissolved in 0.6M sodium carbonate-bicarbonate buffer, pH 9.0). In each of these tubes, the final volume is brought to 1.5 ml. with 0.6M sodium carbonate-bicarbonate buffer, pH 9.0, and 0.5 ml. of copper phosphate suspension is then added. The detailed procedure presented above is then applied and the standard curve is derived by subtracting the control absorbance (i.e., 0.5 ml. supernatant fraction plus 0 γ of lysine•HCl) from the absorbance of tubes to which various amounts of lysine•HCl have been added. For example, assuming that the contribution of 0.5 ml. of a particular sample supernatant fraction to the absorbance is 0.250, and that the absorbance of each tube in the standard series, 50, 100, and 250 γ of lysine•HCl, is 0.330, 0.415, and 0.650, respectively, the standard curve is derived from the values obtained by subtracting 0.250 from the absorbance of each tube in the standard series, i.e., 0.080, 0.165, and 0.400. Standard curves that are not based on enzymatic hydrolysates have a lower slope than those that do, and lysine content estimated from such curves is over-estimated. Reference to the standard curve than permits expression of the results as lysine (percent of total dry weight) or for samples for which the protein content is known as lysine (percent of protein).

RESULTS

The Effect of Buffers and pH on the Colorimetric Determination of Lysine

Selim (2) reported that the nature of the buffer employed seemed to affect greatly the condensation of the lysine-copper complex with 1-fluoro-2,4-dinitrobenzene, and that the presence of sodium bicarbonate in the mixture interfered significantly with the dinitrophenylation process as compared to the borate buffer. We observe no interference due to the presence of sodium bicarbonate for the ε-DNPyr-lysine formation when the pH values are lower than 9.0 (Table I). Table I also indicates that the pH optimum for the formation of

TABLE I. EFFECT OF BUFFERS AND pH ON FORMATION OF DNPyr-LYSINE[a]

pH	Absorbance	
	Carbonate buffer	Borate buffer
8.6	0.280	0.225
8.8	0.288	0.255
9.0	0.295	0.295
9.2	0.265	0.295
9.4	0.240	...
9.6	0.215	...
9.8	0.195	...

[a]Each tube contained either 1.0 ml. of 0.05M borate buffer or 1.0 ml. of 0.6M sodium carbonate-bicarbonate buffer at the pH indicated and 200 γ of lysine•HCl.

TABLE II. ABSORBANCE OF DNPyr-AMINO ACID DERIVATIVE AT 340 AND 400 NM.[a]

Amino Acid	Absorbance 340 nm. 0.6M buffer	Absorbance 400 nm. 0.6M buffer	Absorbance 400 nm. 0.8M buffer
Glycine	0.005	0.00	0.00
Alanine	0.005	0.00	0.00
Valine	0.010	0.00	0.00
Leucine	0.005	0.00	0.00
Isoleucine	0.00	0.00	0.00
Serine	0.030	0.00	0.00
Threonine	0.015	0.00	0.00
Cysteine	0.030	0.00	0.025
Methionine	0.00	0.00	0.005
Proline	0.00	0.00	0.00
Phenylalanine	0.005	0.00	0.00
Tyrosine	0.100	0.00	0.00
Tryptophan	0.00	0.008	0.015
Aspartic acid	0.005	0.00	0.00
Glutamic acid	0.010	0.00	0.00
Asparagine	...	0.00	0.00
Glutamine	...	0.00	0.00
Histidine	0.010	0.005	0.015
Arginine•HCl	0.035	0.020	0.020
Lysine•HCl	1.050	0.365	0.365

[a]Each assay tube contained 1.0 ml. of 0.6M or 0.8M sodium carbonate-bicarbonate buffer and 250 γ of the amino acid indicated.

ϵ-DNPyr-lysine is 9.0 when carbonate buffer is used, and that the formation of this derivative is affected significantly by the pH of the reaction mixture.

The Contribution of other Amino Acids to Absorbance

Although the maximum absorption of ϵ-DNPyr-lysine is at 340 nm., some DNPyr-amino acid derivatives, particularly DNPyr-tyrosine, do absorb at this wavelength (Table II). To minimize interference from these derivatives, absorbance is measured at 400 nm. The absorbance at 400 nm. attributable to each amino acid following standard procedures has been measured for reaction mixtures in which the buffer concentration was 0.6M and those in which the buffer concentration was 0.8M (Table II). At the 0.6M-buffer concentration, only lysine and arginine contribute materially to absorbance, with that due to arginine being approximately 6% of that due to lysine. At the 0.8M-buffer concentration, cysteine, tryptophan, histidine, and arginine contribute measurably to absorbance. The extent of possible contribution to absorbance by arginine depends on the buffer concentration in the reaction mixture (Fig. 1). As shown by the data plotted in Fig. 1, maximum formation of the ϵ-DNPyridyl derivative of lysine is found when the buffer concentration of lysine is 0.6M or higher. The contribution of arginine to absorbance decreases with an increasing concentration of the buffer in the reaction mixture. The choice of 0.6M sodium carbonate-bicarbonate buffer as a standard condition maximizes the contribution of lysine to absorbance and minimizes the possible contribution of other amino acids.

The Contribution of Amino Acyl-Lysine Peptides to Absorbance

Since the determination of lysine percentage for various maize samples is based on the treatment of the meal with proteolytic enzymes that releases free amino

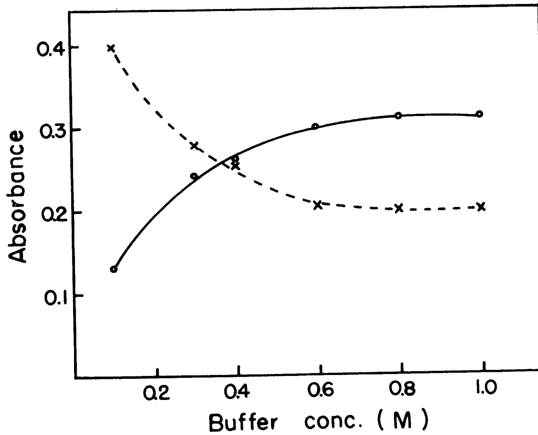

Fig. 1. The effect of buffer concentration on the formation of ϵ-DNPyr-lysine and DNPyr-arginine. Each assay tube contained 1.0 ml. of sodium carbonate-bicarbonate buffer at various concentrations and 0.2 mg. for lysine•HCl or 2 mg. for arginine•HCl. Broken line = arginine•HCl; solid line = lysine•HCl.

acids or low-molecular-weight peptides, it is essential to measure the contribution of various amino acyl-lysine peptides to absorbance. Table III indicates that on an equimolar basis, the amino acyl-lysine peptides tested, L-alanyl-L-lysine, L-glycyl-L-lysine, and L-histidyl-L-lysine, make the same contribution to absorbance as does free lysine.

Absorbance due to Added Lysine

The method was evaluated by determining the absorbance of known amounts of lysine incubated together with a sample during hydrolysis with pronase. Lysine•HCl (2.5, 5, and 10 μmoles) is incubated, respectively, with 200 mg. of the standard *opaque-2* maize endosperm sample during treatment with pronase. After incubation, lysine was determined as described in "Procedure". The added lysine raised the absorbance in a manner linearly proportional to the amount of lysine added.

TABLE III. THE CONTRIBUTION OF AMINO ACYL-LYSINE PEPTIDES TO ABSORBANCE[a]

Additives	Absorbance
L-Lysine	0.520
L-Alanyl-L-lysine	0.530
L-Glycyl-L-lysine	0.535
L-Histidyl-L-lysine	0.515

[a]Each assay tube contained 1.0 ml. of 0.6M sodium carbonate-bicarbonate buffer and 0.1 ml. of either 0.02M L-lysine, L-alanyl-L-lysine, L-glycyl-L-lysine, or L-histidyl-L-lysine.

Accuracy of Determining the Lysine Content in Maize Samples

Table IV shows that the estimates of lysine percentage for various samples determined by the colorimetric method agree well in duplicate samples in the same run and in the samples run at different times. Also, the results obtained are in reasonable agreement with those derived from the amino acid analyzer; however, somewhat higher estimates of lysine content are obtained. This is particularly marked for those samples containing low quantities of lysine (548, W64A+, and 549). This could be due to the contribution of arginine present in the sample, since arginine has an absorbance about 6% of that of lysine (Table II). In all samples examined, arginine stated as a percentage of protein ranges from 3.5 to 5.0%. The absorbance due to the arginine present would contribute a more marked bias in the samples with a low quantity of lysine.

The method has also been evaluated by assaying varying amounts of the same *opaque-2* maize sample. As shown in Fig. 2, absorbance is linearly proportional to the amount of meal taken for the assay.

TABLE IV. LYSINE CONCENTRATION IN MAIZE SEED OF VARIOUS GENOTYPES AS ESTIMATED BY THE COLORIMETRIC METHOD AND BY COLUMN CHROMATOGRAPHY

Sample	Lysine (% protein)				
	Colorimetric			Selim method	Amino acid analyzer[a]
548	2.28[b]	2.28[c]	2.22[d]		1.89
W64A+	2.80[b]	2.83[c]	2.85[d]		2.54
W64Ao_2	4.60[b]	4.69[c]	4.52[d]		4.51
103	5.43[b]	5.54[c]	5.36[d]		5.17
116	5.12[b]	5.09[c]	5.12[d]	8.62	4.90
549			2.22[d]		1.91
369			3.30[d]		3.00
571			3.30[d]		3.07
81			4.88[d]		4.60
425			4.77[d]		4.59
473			4.80[d]		4.60
346			5.19[d]		4.89
368			5.45[d]		4.85
114			4.68[d]	7.08	4.25
242			4.40[d]	7.33	4.25

[a]Amino acid analyzer assays were made in the laboratory of E. T. Mertz.
[b,c]Duplicate samples in the same run.
[d]Sample run at different times.

The Desirability of Defatting before Analysis

The preferred method involves defatting with *n*-hexane prior to enzymatic hydrolysis. It is not an essential step, although higher values are obtained if this is carried out as shown in Table V. Since protein percentage was determined both before and after defatting, and lysine content was expressed as a percentage of protein, the increased lysine content is not due to loss of weight from defatting. It is possible that defatting allows the enzymatic hydrolysis of a relatively lysine-rich protein (or proteins) otherwise protected by association with the lipid fraction.

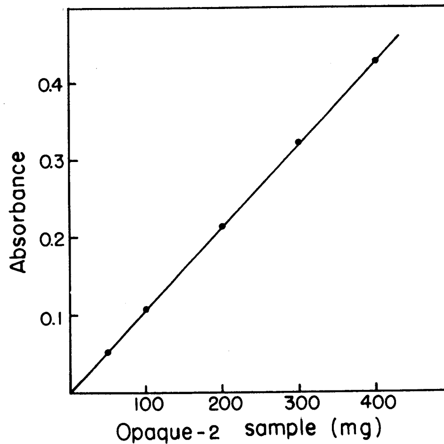

Fig. 2. Absorbance (400 nm.) following the standard procedure for different amounts of meal from the same *opaque-2* maize sample.

TABLE V. EFFECT OF DEFATTING ON LYSINE DETERMINATION

Sample	Lysine (% protein)[a]	
	Defatted	Nondefatted
114	4.98	4.23
103	5.27	4.71
81	4.96	4.56
116	5.13	4.51

[a]Percent protein was determined both before and after defatting.

DISCUSSION

Several colorimetric techniques are now available for the estimation of available lysine in cereal grains. This allows the individual laboratories concerned with the estimation of lysine content a choice of inexpensive methods suitable for the determination of large numbers of samples. This is particularly important in conjunction with plant-breeding efforts designed to incorporate a gene (or genes) affecting protein quality into various genetic backgrounds or with the screening of large numbers of accessions of a particular cereal grain with the hope of detecting a variant conditioning a substantial increase in lysine content.

The colorimetric method presented here has been evaluated by comparing the results with those derived from the amino acid analyzer. Lysine content as estimated by this colorimetric method is usually higher than the estimate of lysine content from the amino acid analyzer, but is in good general agreement (Table IV). The higher lysine values found in the colorimetric assays may be a consequence of the relatively minor contribution made to absorbance by the arginine present.

This method has several advantages for analytical laboratories that are supporting plant-breeding efforts: a) only a small amount of sample is required; b) inexpensive enzyme preparations such as the alcalase plus pancreatic trypsin of Novo Industri yield satisfactory results (papain has also given good results in the laboratory of E. Villegas at CIMMYT in Mexico City[6]); c) the enzymatic hydrolysis allows an estimation of tryptophan content from the same hydrolysate; d) as with the Kakade and Liener method (3), one obtains an estimate of "available" lysine (those lysine moieties in which the ε-amino group is free); e) it requires relatively simple and inexpensive equipment; and f) a number of samples can be processed simultaneously, sparing both time and expense.

---

[6]Personal communication.

Acknowledgment

We wish to thank E. T. Mertz, in whose laboratory the column chromatographic (Beckman automatic amino acid analyzer) assays of the amino acids were made.

Literature Cited

1. MERTZ, E. T., BATES, L. S., and NELSON, O. E. Mutant gene that changes protein composition and increases lysine content of maize endosperm. Science 145: 279 (1964).
2. SELIM, A. S. M. Microdetermination of lysine in protein hydrolyzates. J. Agr. Food Chem. 13: 435 (1965).
3. KAKADE, M. L., and LIENER, I. E. Determination of available lysine in proteins. Anal. Biochem. 27: 273 (1969).
4. SUBRAMANIAN, S. S., JAMBUNATHAN, R., CONCON, J. M., and MERTZ, E. T. Simple methods for determining lysine and tryptophan in high lysine and normal maize. Fed. Proc. 29: 761 (1970).
5. SIGNOR, A., PREVIERO, A., and TERBOJEVICH, M. Determination of the N-terminal amino-acid in polypeptides and proteins. Nature 205: 596 (1965).
6. SPIES, J. R., and CHAMBERS, D. C. Spectrophotometric analysis of amino acids and peptides with their copper salts. J. Biol. Chem. 191: 787 (1951).

[Received September 2, 1971. Accepted March 6, 1972]