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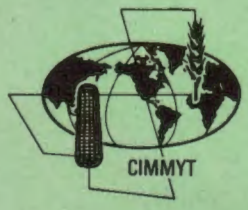

CENTRO INTERNACIONAL DE MEJORAMIENTO DE MAIZ Y TRIGO
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CHEMICAL SCREENING METHODS FOR MAIZE PROTEIN QUALITY AT CIMMYT

EVANGELINA VILLEGAS* AND EDWIN T. MERTZ**

INTRODUCTION

In 1964 Mertz, Bates and Nelson (1) found that the mutant gene opaque-2 of maize produces a modified amino acid balance of the grain endosperm protein with increased quantities of lysine and tryptophan. This discovery opened up exciting new vistas for plant scientists and human and animal nutritionists.

Feeding trials with diets of only opaque-2 or floury-2 maize, another high lysine mutant discovered subsequently, have confirmed that animals and humans, particularly the young, can gain weight very much faster than if fed normal maize diets. The implications of this discovery were immediately obvious to many scientists and the biochemical, physiological and genetic mechanism of the amino acid mutants in maize came under detailed study.

The discovery of the high lysine maize mutants has provided the possibility of combining high quality protein into superior maize lines which can be used within the traditional agricultural structure of extensive areas of the world.

The descriptive names "opaque" and "floury" indicate the dull, lusterless and chalk-like physical appearance of the kernels by which these mutants were originally isolated and identified. These characteristics represent an obstacle to acceptance by farmers accustomed to growing flint and dent types with their clean, shiny and lustrous appearance. In those areas where the floury grain types are traditionally grown such as the high Andean regions of South America, this would not be a problem, however, floury types are not grown in the majority of the maize areas of the world either for human or animal food.

The identification of high lysine lines within maize populations depends on careful chemical analysis. Until quite recently, most of the identification was made either by selecting the opaque kernels, which unfortunately precludes any opportunity to obtain better grain type, or by chemical analysis of small bulk samples which tended to obscure useful genetic variability containing the desirable combination.

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Several chemical methods have been recommended in the past but some of them are complicated and laborious, therefore not suitable for screening large populations, and others do not effectively indicate the levels of amino acid, i.e. in an opaque-2 segregating population in .which certain modifying genes are acting.

In an attempt to provide simple techniques suitable for screening large numbers of small samples, different analytical methods already available for the determination of tryptophan and lysine, have been studied and reevaluated at the Protein Quality Laboratory of the International Maize and Wheat Improvement Center (CIMMYT), and the Biochemistry Laboratory at Purdue University. Following this reevaluation, the following recommendations are made for routine evaluation of populations and segregating material produced in maize breeding programs.

METHODS *

1. Sample preparation for endosperm analysis

To readily identify the materials with improved protein quality, it is advisable to perform the analysis on the endosperm of the sample. The maize endosperm is deficient in lysine and trytophan, while the embryo on the other hand, has a relatively constant and well-balanced amino acid composition regardless of the genetic background. Also, when analyzing the whole kernel, the pericarp may contribute undesirable pigments which interfere with the colorimetric determinations. If whole kernel analysis are desirable therefore, these factors must be taken into consideration.

a) For the evaluation of genetic families

(i) Take a random sample of 10 seeds as representative of each ear.

(ii) Wash off any pesticide with distilled water if the seeds have been treated. If seeds are untreated, eliminate this step.

(iii) Soak seeds in distilled water for approximately 30 minutes. Peel off the pericarp and remove the germ with tweezers and scalpel. The remaining material of the kernel is considered as endosperm tissue. Air dry the endosperm sample overnight.

(iv) Grind the air dried sample in a burr mill at the finest setting.

(v) Defat ground samples in a Soxhlet continuous extractor with hexane for 6 hours.

(vi) Air dry the samples and grind to a fine powder with a Wig-L-Bug amalgamator.

b) For single kernel analysis

In certain cases, i.e. when one maize family with hard endosperm (normal endosperm type) is identified as possessing high levels of trypto-

* List of equipment needed for performance of these tests is available from the CIMMYT Protein Quality Laboratory on request.

phan in the protein, it is recommended that a single kernel analysis be performed on a further sub-sample to identify whether there is segregation within the family, and if so, which grains have the high levels of tryptophan. The following procedure does not destroy the embryo of the analyzed seed and thus allows the growing of those seeds selected as having the highest levels of tryptophan for further genetic analysis. This procedure was initiated by Bates in 1968 at CIMMYT.

(i) Five or six randomly chosen seeds from each family are washed to eliminate any pesticide and then air dried.

(ii) A small portion of endosperm in different sites of the kernel is drilled out with an electric drill using a 1/16 inch bit.

(iii) Defat sample in Soxhlet with hexane.

(iv) Air dry the sample and grind to a fine powder with a Wig-L-Bug amalgamator.

2. Protein Determination

The nitrogen content may be estimated by the Micro-Kjeldahl procedure (2) and the percentage of protein calculated using the factor 6.25.

a) Reagents

(i) Sulphuric acid Sp. gr. 1.84, N-free.

(ii) Catalyst mixture 99.0 g of K_2SO_4, 4.1 g of HgO, and 0.8 g of Cu SO_4.

(iii) Sodium Hydroxide-sodium thiosulfate solution. Dissolve 50 g NaOH and 5 g $Na_2S_2O_3.5H_2O$ in distilled water and dilute to 100 ml.

(iv) Boric acid solution 4%.

(v) Methyl red-bromocresol green indicator solution. Mix one part 0.2% Methyl red in ethanol with 5 parts 0.2% bromo cresol green in ethanol.

(vi) Hydrochloric acid solution. 0.02 N.

b) Procedure

(i) Place 30-40 mg sample in digestion flask. Add 1.0 g of catalyst powder mixture and 2ml of concentrated sulfuric acid.

(ii) Digest 40 minutes, cool, add minimum quantity of water to dissolve solids, cool and place thin film of vaseline on rim of flask.

(iii) Transfer digest to distillation apparatus and rinse flask 5 or 6 times with 1-2 ml portions of distilled water.

(iv) Place 125 ml erlenmeyer flask with 6 ml of boric acid solution and 3 drops of Litmus indicator solution under condenser with tip extending below surface of solution.

(v) Add 8 ml sodium hydroxide-sodium thiosulfate solution to still and steam-distill until about 20 ml of distillate collects.

(vi) Titrate to gray end point or first appearance of violet.

(vii) Make blank determination using same quantity of reagents and same digestion and distillation period as for determination.

(viii) Calculate percentage of nitrogen.

$$\% \text{ Nitrogen} = \frac{(\text{ml HCl in detn.-ml blank}) \times \text{normality} \times 14.007 \times 100}{\text{mg sample}}$$

$$\% \text{ Protein} = \% \text{ N} \times 6.25$$

3. Tryptophan Determination

Because of the relationship observed by Hernandez and Bates (3) between tryptophan and lysine in the maize endosperm protein (approx. 1 to 4), the tryptophan may be used as a single parameter for maize quality evaluation.

For tryptophan estimation the Opienska-Blauth *et al.* colorimetric method modified by Hernandez and Bates (3) is recommended for its simplicity. With this method it is possible to analyze up to 75 samples with duplicates each day.

a) Reagents

(i) 270 mg of $FeCl_3.6H_2O$ is dissolved in 0.5 ml of distilled water and diluted to 1 liter with glacial acetic acid[1] (Reagent A).

(ii) 30 N sulfuric acid (Reagent B).

(iii) A volume to volume mixture of reagents A and B is prepared 1 to 2 hours prior to its use (Reagent C).

(iv) Papain solution. The enzyme[2] (4 mg/ml) is dissolved in 0.1 N sodium acetate buffer at pH 7.0. The enzyme solution is prepared daily.

b) Procedure

(i) 90 to 100 mg of finely ground defatted maize endosperm sample is weighed into a glass vial and 4 ml of papain solution added. The tubes are capped and carefully shaken, making sure that the sample is totally wetted. (Blanks must also be carried out with papain solution through this procedure.)

(ii) The samples are kept in an incubator oven at 65°C overnight.

(iii) The hydrolysates are removed from the incubator or oven and shaken, then allowed to adjust to room temperature by which time the supernatant should be clear (or if not clear, centrifuge the samples).

(iv) One ml of hydrolysate is pipetted into a test tube containing 4 ml of reagent C, the mixture is shaken vigorously and the color developed for 15 minutes at 65°C.

(v) After cooling, the solutions are transferred to calibrated tubes and read at 545 m μ on a Bausch and Lomb Spectronic 20.

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[1] Each bottle of acetic acid must be tested for color development in the presence of tryptophan.

[2] Papain-Tech. powder from Nutritional Biochemicals Corp. (Cleveland, Ohio).

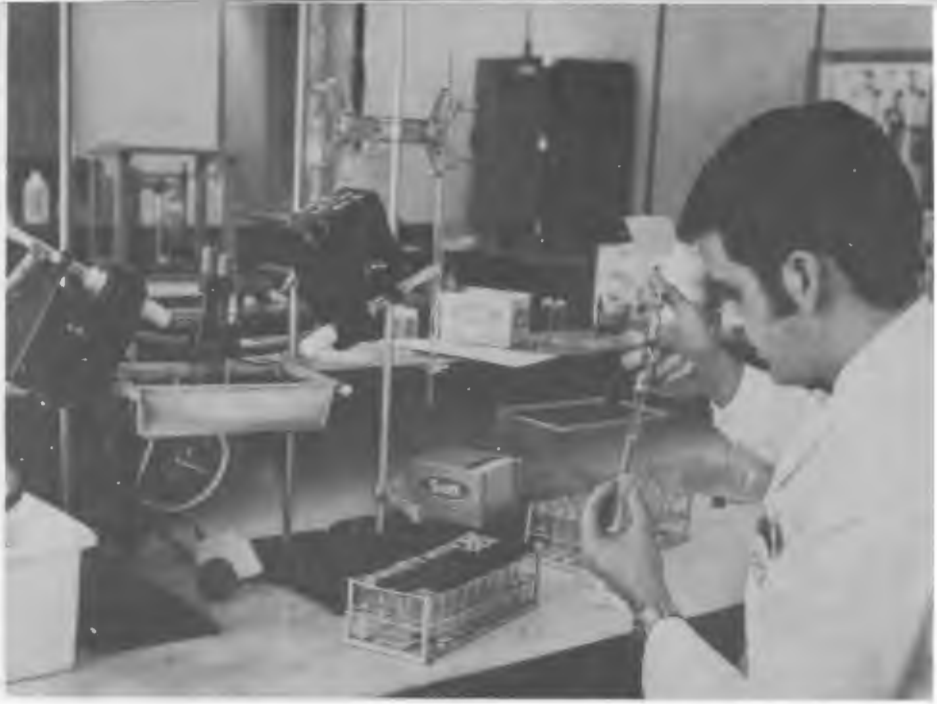
A random sample of ten seeds is taken from each ear for genetic family evaluation.

Above: *Peeling seeds and removing the embryo for endosperm sample.*

Below: *Small portions of the endosperm are drilled out for single kernel analysis.*

OPPOSITE PAGE:
Above: *Defatting samples with hexane in a soxhlet extractor.*

Below: *Regrinding the samples to a fine powder with a Wig-L-Bug amalgamator.*



Left: *Micro-Kjeldahl digestion for nitrogen determination.*

Center: *Distillation of nitrogen for protein estimation.*

Right: *Adding papain solution for protein hydrolysis.*

Below left: *Pipeting an aliquot of the hydrolysate for colorimetric estimation of tryptophan.*

OPPOSITE PAGE:

Above: *Shaking the copper complexes of the amino acids in the colorimetric estimation of lysine.*

Below: *Excess of reagent in the colorimetric estimation of lysine is extracted in the ethyl acetate phase with a syringe adapted to a polyethylene tube.*

Above right: *Reading absorbance in a Spectronic 20 in the tryptophan or lysine estimation.*

(vi) A standard curve is prepared in a range from 0 to 40 μg/ml usign DL-tryptophan.

(vii) The tryptophan content of the sample is calculated from the standard curve and reported on a protein basis.

4. Lysine Determination

The lysine determination is performed only on those materials selected as having high tryptophan values through the colorimetric procedure, or when the lysine value, in addition to the tryptophan is desired.

The colorimetric method designed by Tsai (Purdue University) and modified by Villegas (CIMMYT) is recommended. With this method up to 60 duplicate samples can be analyzed each day.

a) Reagents

(i) Papain solution, 4 mg of papain per ml of phosphate buffer 0.03 M, pH 7.4.

(ii) Carbonate buffer 0.05 M, pH 9.0.

(iii) Borate buffer 0.05 M, pH 9.0.

(iv) Copper phosphate suspension. Solution A: 2.8 g of $CuC1_2.2H_2O$ are dissolved in 100 ml distilled water. Solution B: 13.6 g of $Na_3PO_4.12H_2O$ are dissolved in 200 ml distilled water Pour mixture A into B with swirling, centrifuge to bring out the precipitate and discard the supernatant. The pellet is then resuspended 3 times in 15 ml of borate buffer pH 9, centrifuging after each suspension. After the third washing resuspend the pellet in 80 ml of the borate buffer. The reagent can be used for one week.

(v) 2-Chloro-3,5-dinitropyridine solution. Prepare just prior to its use a solution containing 30 mg of 2-chloro-3,5-dinitropyridine per ml of methanol.

(vi) HC1 solution 1.2 N.

(vii) Mixture of amino acids:

Cystine	20 mg	Phenylalanine	40 mg
Methionine	20 mg	Valine	40 mg
Histidine	30 mg	Arginine	50 mg
Alanine	30 mg	Serine	50 mg
Isoleucine	30 mg	Aspartic acid	60 mg
Threonine	30 mg	Glutamic acid	300 mg
Tyrosine	30 mg	Leucine	80 mg
Glycine	40 mg	Proline	80 mg

Weigh 100 mg of the amino acid mixture and dissolve in 10 ml of carbonate buffer.

b) Procedure

(i) Weight 100 mg of finely defatted sample in a vial and add 5 ml of papain solution. Be sure to wet all the sample and shake it at least twice in the first hour of incubation. Carry on blanks with papain solution.

(ii) Incubate overnight at 65°C. Remove the hydrolysates from incubation oven and shake, then allow to settle and to adjust to room temperature by which time the supernatant should be clear, or centrifuge (One aliquot of 1 ml from this hydrolysate can be used for tryptophan determination).

(iii) Pipette 1 ml aliquot into a centrifuge tube and add 0.5 ml of carbonate buffer and 0.5 ml of the copper phosphate suspension.

(iv) Shake the mixture for 5 minutes and centrifuge.

(v) Pipette 1 ml aliquot of the supernatant into a large test tube, and add 0.1 ml of 2-chloro-3, 5 dinitropyridine-methanol solution, and shake well.

(vi) Allow the mixture to stand for 2 hours at room temperature (shake every 30 minutes).

(vii) Add 5 ml of HC1 1.2 N and shake well.

(viii) Add 5 ml of ethylacetate, stopper tubes, mix well inverting the tubes at least 10 times, then extract the upper phase using a syringe adapted with a polyethylene tube. This step must be performed 3 times.

(ix) Transfer the aqueous phase to calibrated tubes and read in the Spectronic 20 at 390 mμ against the blanks.

(x) Calculate lysine content of the samples by comparing with a standard curve and report on a protein basis.

c) Standard Curve

Prepare a standard curve in a range of 0 to 200 μg of lysine per ml. Stock solution of lysine: 62.5 mg of lysine-monohydrochloride in 20 ml of carbonate buffer (2,500 μg lysine/ml).

Dilute with carbonate buffer the stock solution of lysine to 0, 250, 500, 750, and 1,000 μg lysine/ml.

From each one of these solutions, take 1 ml and add 4 ml of papain solution (5 mg papain/ml of phosphate buffer).

Pipette 1 ml of each solution into centrifuge tube, add 0.5 ml of the amino acid mixture solution, and 0.5 ml of copper phosphate suspension. Continue with procedure on step (iv) in part b.

DISCUSSION

On the average, endosperm analyses give colorimetric values of:

a) Normal maize, about 0.45 g of tryptophan and 1.8 g of lysine per 100 g of protein (with approximately 9.0% protein in the samble), and

b) Opaque-2 maize, about 0.85 g of tryptophan and 3.6 g of lysine per 100 g of protein (with approximately 8.0% protein in the sample).

The values indicated above may vary due to the genetic background of the sample or to the content of protein in the sample. Intermediate val-

ues for tryptophan and lysine in the protein may be found in materials with the floury-2 gene or in some samples with hard endosperm.

Using the above analytical methods for screening large numbers of maize samples, it is possible to coordinate the work of the quality laboratory and the breeders, since the laboratory reports must be used by the geneticist and breeders to assist them in selecting the germ plasm to continue their breeding program, the results of the analyses must be reported on time.

These techniques are currently in use on a large scale within the CIMMYT maize program.

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