A modified microbiological assay for lysine determination in Hordeum sp
by Robert Frank Waters

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF
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Abstract:
A modified microbiological assay technique is described for the identification and quantification of
high lysine mutants in Hordeum sp. Its potential application is the identification of high lysine
genotypes in cereal populations.

The method involves hydrolyzing ground grain samples hydrolyzed for a specific time in 6NHCL. An
aliquot from each hydrolyzate was microbiologically assayed using a lysine biosynthetic deficient
mutant in Leuconostoc mesenter-oides grown on barley hydrolyzates and known lysine substrates.
Using percent protein and the quantity of lysine in the hydrolyzate sample, the percent lysine in protein
and percent lysine in grain are computed.

The microbiological assay technique is rapid and inexpensive for genetic screening purposes.
Approximately 400-500 samples can be screened per week. The standard error (S.E.) for percent lysine
in protein for duplicate hydrolysis and duplicate bioassays of a sample was +.13 -±.17. The standard
errors of duplicate bioassays of one hydrolysis were ±.03 - ±.06. The coefficient of variation (C.V.) for
the bioassay was 4%.

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ABSTRACT

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ulations.

The method involves hydrolyzing ground grain samples
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INTRODUCTION

The improvement of the nutritional value of cereal crops through genetic manipulation is partially dependent upon an efficient method for identifying desired biochemical mutants.

Lysine is a limiting amino acid for feed in many cereal crops and is an essential amino acid to many animal systems (33). High lysine mutants in barley increase the nutritional value of the protein for animals (34).

Present methods such as UDY-Kjeldahl (32), D.B.C. technique (30), Stb gene system (14), Ninhydrin technique (25), and amino acid analyzer satisfy some of the requirements for screening of high lysine mutants. Major shortcomings, however, are high cost of operation, low numbers screened in a given period of time, inadequate quantification, and difficulties in estimation of respective technique variability. A technique having the least number of these deficiencies would facilitate the identification of high lysine mutants and the screenings of segregating populations.

The objectives of this study were 1) to develop a modified microbiological assay technique for rapid, efficient, and low cost quantification of high lysine mutants

in <u>Hordeum</u> <u>sp</u>., and 2) to describe the biochemical nature
of high lysine mutants themselves.

REVIEW OF LITERATURE

Microbiological Assay

Microbiological assay of amino acids has been used since the advent of this century. Early microbiologists developed specific amino acid media and isolated lysine requiring bacteria for this method. Some of the substances that can be analyzed are pantothenic acid (41), phenylalanine (8), glutamic acid (21), lysine (43), and tyrosine (4).

Versatility of the microbiological assay is evident in its application for amino acid analyses in plant and animal systems. Various procedures were developed for assay of amino acids in animal proteins, free amino acids, and determinations of micro-amounts of amino acids in plant and animal tissue samples (15,16,17).

Lysine is an amino acid that can be assayed microbiologically. Some of the specific lysine requiring bacteria are Leuconostoc mesenteroides P-60 (ATCC 8042), Lactobacillus plantarum (ATCC 8008), and Streptococcus faecalis R (ATCC 8043). These bacteria have a genetic block in the aspartic acid biosynthetic path in which lysine is a product.

L. mesenteroides is the lysine requiring bacterium generally used in microbiological assay of lysine. Certain

inherent physiological characteristics of this bacterium render it more advantageous for lysine assays in barley.

Lactic acid production of L. mesenteroides is greater than many other lysine requiring bacteria. It has been shown that L. mesenteroides produces about 50 percent more lactic acid in 74 hours of incubation than S. faecalis R (7). Since lactic acid is the growth parameter measured in the microbiological assay of lysine, greater acid production is advantageous.

The effect various barley grain components have on bacterial growth (i.e., lactic acid production) is important. Acid production by various lactic acid bacteria in the presence of carbohydrate and carbohydrate derivative substrates were studied (2). Results show that lactic acid production of L. mesenteroides is unaffected by the presence of certain monosacchrides (i.e., arabinose, maltose) and polysacchrides (i.e., amylose, dextrin). Relatively high levels of amylose are present in the grain of various barley lines (36). Therefore, the use of L. mesenteroides in the lysine assay of these barley lines is essential

The assay medium used for microbiological determination of lysine in barley has all components needed for bacterial growth except lysine. Lysine assay medium

(Difco) was formulated by determining growth requirements of L. mesenteroides such as vitamin and amino acid needs for the bacterium (7,39). A complete description of the lysine assay medium is shown in Appendix Table 1.

In the performance of the microbiological assay depletion of residual amino acid in the bacterial inoculum is essential. Removal of this amino acid ensures that the remaining amino acid being measured is in the sample. A depletion tube system has been proposed for the removal of residual amino acid in the inoculum (9).

Variability may be introduced into microbiological assay due to metabolite inhibition. Inhibitory effects are reported with the introduction of specific competitive analogs (13). Therefore, amino acid concentrations in the samples other than the amino acid assayed have to be monitored for inhibitory or stimulatory effects on growth of the bacterial inoculum in the samples

Procedural factors have been studied that alter results in microbiological assay. Some of the findings and recommendations from the study by Toennies and Gallant (44) were as follows.

a) Accuracy of microbiological assay is approximately 10%. They suggested running duplicate and triplicate bioassays.

b) Extended heating of medium adversely affects bacterial growth and lactic acid production.

c) Loss sustained of 10 ml of medium incubated for 24 hours at 42 C had a standard deviation (S.D.) of 2.13 ± .34 by weight.

d) The microbiological assay technique should be run in carefully cleaned and dried test tubes. A washing technique was developed and is described in Appendix Table 2.

e) An electric incubator was reported to be better for microbiological assay than a forced air or water-bath type incubator. The least temperature variation was noted with the electric incubator.

f) Reduction in early activity of the bacterial cultures was found if kept in refrigeration on agar for three months or more.

g) The variation of single drop inoculation in microbiological assay is negligible with respect to experimental error.

Early investigations by Mitchell and Block (27) indicate microbiological assay is useful in determination of numerous amino acids in various protein sources. They compared the percent of 13 amino acids in whole egg protein to sources of protein such as whole wheat, wheat germ, white flour and whole corn.

These results suggest it is possible that microbiological assay can be applied to amino acid analyses of barley protein.

High Lysine Mutants

Lysine is an essential amino acid to many animal systems including humans. The acquisition of this amino acid is through ingestion of food containing lysine. Although lysine is a limiting amino acid in barley protein for feed, a genetic biochemical mutant called "hiproly" or "high lysine" was found which increases the percent lysine in protein (31). The barley line (CI 3947) containing this mutant was added to the world collection of barley as a source for high lysine and inherent high protein.

High lysine mutants have also been found in maize and sorghum that express higher lysine levels than the "normal" lines (24,40). These sources of increased lysine

in protein have been added to their respective world collections.

In barley, additional high lysine mutants have been discovered that phenotypically express higher lysine levels in the protein than normal lines (5). These lines provide additional genetic variability in which plant breeders can work.

Lower grain yield and higher protein content in barley is expressed with the presence of a high lysine gene (32,35). The reason lower yield is associated with this gene has not been established. Differential protein deposition in grain with high lysine fractions in higher proportion has been proposed in maize (26). Other explanations range from specific histone fraction expression of high lysine to the actual genetic regulation of DNA transcription by the histones themselves in protein synthesis (19, 20,22,23).

For a number of years the amino acid analyzer has been used in determination of lysine in barley grain. Percent lysine in the protein for 'Hiproly Normal' (CI 4362) and 'Hiproly' (CI 3947) were shown to be 3.1 and 4.4, respectively (34).

Several methods have been introduced to take the
place of the amino acid analyzer. For example, a ninhydrin
method has been developed for rapid screening of lysine in
barley (25). They found the normal barley line (Bomi) to
have 3.1 g of lysine per 100 g of protein. The high lysine
barley line (Risø 1508) had 5.3 g lysine per 100 g of pro-
tein.

The amino acid analyzer is a quantitative technique
for lysine determinations in barley protein. However, cost
per sample is much higher than the ninhydrin technique.
The latter method is semi-quantitative at best. Therefore,
if a method could be developed that is quantitative and
low in cost this system would be better than both amino
acid analyzer and ninhydrin techniques.

MATERIALS AND METHODS

Modified Microbiological Assay

Barley cultivars and sample origin. In this study three isogenic lines of barley were analyzed for lysine content. The 'normal' isogene refers to the barley line having lysine levels within the normal or usual range. The 'mutant' isogene has one or more genes that phenotypically exhibit higher than normal lysine content or higher amylose content (Table 1).

Table 1. Lysine isogenic barley pairs

Normal	Mutant
CI 5438 (Compana)	MT 294318 (Wapana)
CI 4362 (Hiproly Normal)	CI 3947 (Hiproly)
CI 6976 (Glacier)	CI 13993 (Hi-amylose Glacier)

The term 'sample' is referred to in this and subsequent sections in two manners. A 'barley sample' refers to the HPP (Hiproly Pair), WCP (Waxy Compana Pair), and AGP (Amylose Glacier Pair) from the geographical locations (Appendix Tables 3-5). A 'hydrolyzate sample' is a barley sample that has been hydrolyzed for modified microbiological assay.

The term 'bioassay' is the same as modified micro-
biological assay, and this procedure (unless specified
otherwise) is as described for barley samples in general.

Barley sample preparation and hydrolysis. The bar-
ley samples were prepared for hydrolysis by grinding
whole grain in a UDY cyclone mill. Approximately 2 g of
each ground grain sample were placed in a 250 ml Erlenmeyer
hydrolysis flask and 25 ml of analytical grade 6N HCL
(Baker) added. Each flask was fitted with a one meter
refluxing column. Using hot plates as a source of heat,
the temperature was raised to the constant boiling point
of the 6N HCL.

Determination of proper hydrolysis time for the ly-
sine assay involved utilization of the barley lysine iso-
genic pair, Hiproly (CI 3947) and Hiproly Normal (CI 4362)
grown at 15 locations (see Appendix Table 3 and exclude
last 5 locations). A barley sample of each isogene at the
15 locations was hydrolyzed for 4, 16, and 24 hours. Bio-
assays of all hydrolyzate samples within each hydrolysis
time were performed to determine which hydrolysis time
showed highest lysine yield.

Titration and volumization of hydrolyzate samples.

After hydrolysis each hydrolyzate sample was titrated to a pH of 6.3-6.5 utilizing analytical grade 6N NAOH and a Copenhagen Radiometer automatic titrator. All titrations were done in a cooling flask to stabilize temperature.

Each titrated hydrolyzate sample was brought to 100 ml volume with distilled water. An apparatus constructed in the laboratory automated this step. The sample aliquots were poured into 100 ml medicine type bottles, autoclaved, and stored at room temperature.

Thirty-two stored and 32 freshly prepared Compana hydrolyzate samples were bioassayed to determine the effect 6-month storage had on lysine recovery. Duplicate bioassays were run on both stored and freshly prepared samples. Variation in percent lysine was estimated using standard analysis of variance methods.

Assay medium preparation. Lysine assay medium (Difco) was used for the lysine determinations. The medium contained all components needed for bacterial growth except lysine and was made according to the manufacturer's recommended procedure. All medium was made on the same day the microbiological assay was performed.

Inoculum preparation. The bacterium used for micro-
biological assay of lysine was L. mesenteroides (ATCC 8042).
Stock cultures were obtained from Difco Laboratories and
stored at 0 - 3 C.

Inocula were prepared by the following procedure:

1) The stock culture was removed from refrigeration
and warmed to room temperature.

2) Bacteria were transferred from the stock culture
to brain-heart infusion medium (Difco) prepared according
to manufacturer's recommended procedure.

3) After 72-96 hours of 37 C incubation, the brain-
heart infusion cultures were removed from the incubator.
A loopful of this culture was used to inoculate previously
prepared depletion tubes.

4) Depletion tubes were made by adding 5 ml of as-
say medium plus 5 ml deionized distilled water to as many
tubes as needed. All depletion tubes were autoclaved for
10 minutes at 121 C and 1.05 kg/cm^2 pressure, then cooled
to room temperature before inoculation. The inoculated
depletion tubes were incubated for 96 hours at 37 C.

5) The depletion tubes were checked for growth
after 96 hours of incubation. Another set of depletion

tubes were inoculated from this culture and incubated for 96 hours at 37 C. The original set of depletion tubes was refrigerated at 3 C.

6) The second set of depletion tubes was observed for bacterial growth after 96 hours incubation. If bacterial growth was noted, steps 4) and 5) were repeated. If no bacterial growth was observed, the original set of depletion tubes was removed from refrigeration, warmed to room temperature, and used as inoculum.

Hydrolyzate sample tube preparation. The sample tubes were prepared for microbiological assay by cleaning test tubes and caps. It was imperative that no residual soap or detergent remain in the test tubes. Each tube was thoroughly dried in drying ovens and placed into numbered test tube racks. Lysine assay medium (4.7 ml) and deionized distilled water (5 ml) were added to each tube. A 300λ aliquot from the hydrolyzate sample storage bottle was added to the corresponding sample assay tube using a 300λ Eppendorf pipette. Best results were obtained when a new tip was used for each transfer. However, washing the tip between transfers with deionized distilled water was satisfactory.

Thirty-six Glacier barley samples (Appendix Table 5) were analyzed for lysine in the 100λ, 200λ, and 300λ hydrolyzate sample aliquot size trial.

Analyses of variance of percent lysine in protein (untransformed) for bioassays and hydrolysis within each aliquot size were calculated.

Autoclaving. All filled sample tubes were stored in a refrigerator at 0-3 C until standard tubes were ready. Following standard tube completion, both sample and standard tubes were autoclaved simultaneously for 10 minutes and 1.05 kg/cm^2 pressure in an Amsco (General Purpose) autoclave.

Preparation of standard tubes. Standard tubes were a series of tubes with increasing concentrations of amino acid (lysine) added. Bacterial growth and lactic acid production by L. mesenteroides is proportional to the concentration of lysine in the buffered assay medium. Because of this, a standard curve can be developed by measuring pH with known concentration lysine.

Ten triplicate levels of lysine were used to generate the standard curve for each microbiological assay. The 10 levels were 0μg (control), 5μg, 10μg, 20μg, 30μg, 35μg,

40µg, 50µg, 70µg, and 80µg of L-lysine (General Biochemi-
cals). Five ml of assay medium were added to each tube.
Crystalline L-lysine (free base) was diluted with deionized
distilled water to 100µg/ml. Appropriate amounts of this
solution were added to standard assay tubes to yield the
desired levels. Deionized distilled water was used to
bring all tubes to 10 ml total volume.

pH determination. The pH of the standard and sample
tube media was determined by a combination electrode
coupled to a pH meter (Beckman Expandomatic SS-2). The
electrode was placed in an automatic sample changer system.
The sample changer, developed in this laboratory, included
a deionized distilled water jet for washing and a vacuum
nozzle to remove wash water and samples.

Standard tube readings were used to develop the
standard curve. The resulting pH values were plotted
against µg lysine to generate the standard curve (Figure 1).
After the sample tube pH values were read, the µg of lysine
in each sample was determined by interpolating from the
standard curve. The percent lysine in grain and percent
lysine in protein were then calculated.

A typical standard curve is shown in Figure 1. The
variation of triplicate determinations at 9 levels in three
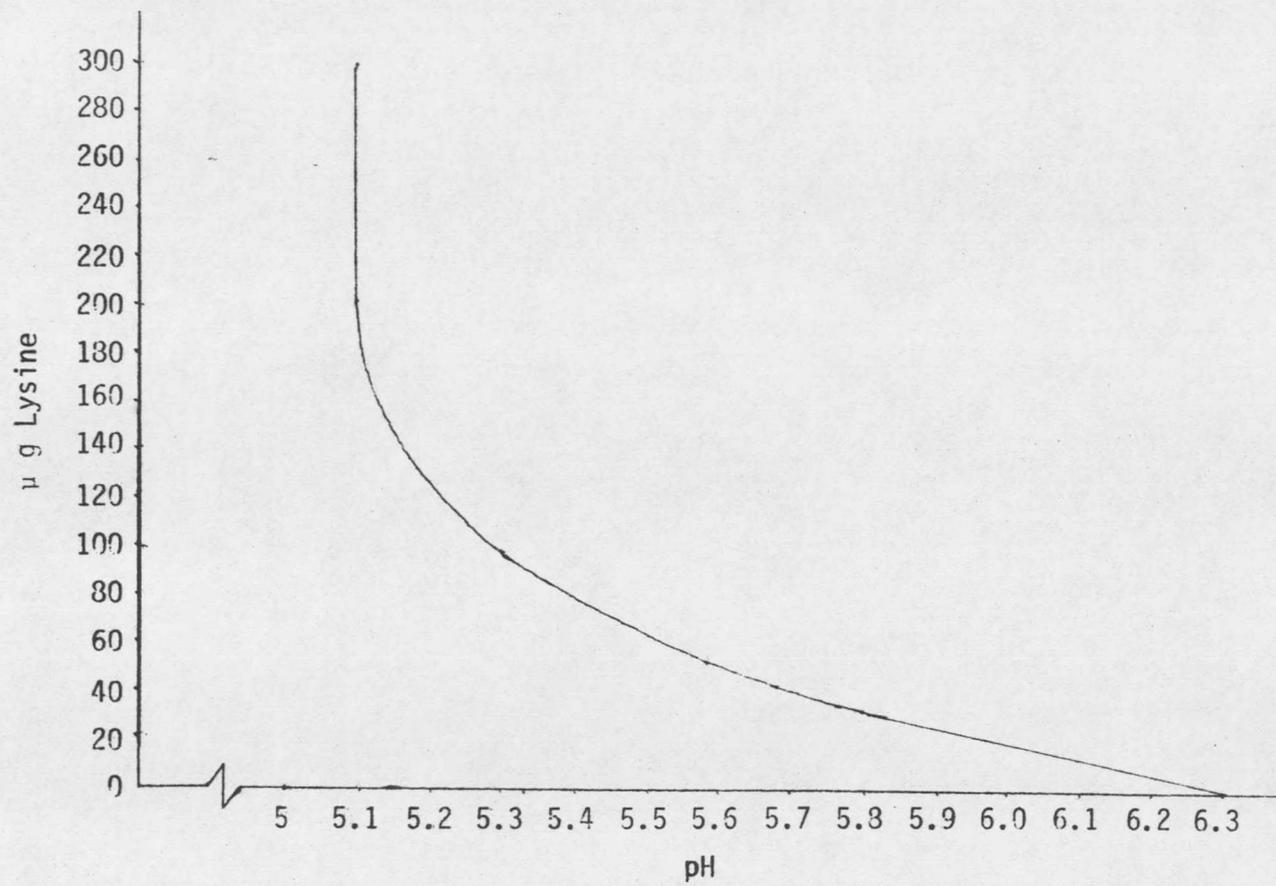
Figure 1. Typical Standard Curve

selected standard curves was determined and analyses of variance of pH for each level (triplicate) were performed.

Computation formulae. The formulae for calculating percent lysine in grain and percent lysine in protein are:

$$\frac{\mu g \ (\text{sample}) \cdot 10^{-6} \cdot 10^{2} \cdot \frac{100 \ (\text{Total volume})}{\text{Aliquot size}}}{\text{Barley grain sample weight (g)}} = \% \ \text{lysine in grain}$$

$$\frac{\% \ \text{lysine in grain}}{\% \ \text{protein}} \times 100 = \% \ \text{lysine in protein}$$

Standard curve fitting and coding. In order to expedite computation on a large scale, computerization was needed. The equation used that approximated the standard curve for curve fitting is the following multiple linear regression.

$$\mu g \ \text{amino acid} = d + \beta_1 \ (\text{pH}) + \beta_2 \ \log \ (\text{pH})$$

The following coding factor was used to aid in stability of computation and uniformity of standard curve in sequential bioassay analyses.

The formula for the coding factor (CF) was:

$$CF = \text{pH @ 35 } \mu g - \frac{(\text{pH@10 } \mu g - \text{pH@70 } \mu g)}{2}$$

(See Appendix Table 6 for modification)

The coding factor was subtracted from the pH values of the standard curve and a new standard curve was developed where µg of amino acid was plotted versus the corresponding pH values minus CF. The CR was subtracted from each sample pH value so that sample (hydrolyzate sample) pH values correspond to the standard coded pH value. The µg of amino acid was used in the computation of percent lysine in grain and percent lysine in protein. Samples with lower pH than the CF indicate too much lysine in the assay and should be rerun using less sample.

Comparison of UDY, amino acid analyzer, and modified microbiological assay methods. Thirty-two Compana samples (Appendix Table 4) were analyzed for lysine by UDY, amino acid analyzer, and modified microbiological assay. Correlation coefficients, standard errors of a single determination and coefficients of variation were estimated. Due to the nature of the amino acid analyzer data, a standard error of a single determination could not be calculated.

Amino acyl t-RNA synthetase experiment. The barley synthetase enzyme was isolated by mixing 6 g of barley sample in a Vor-Tex grinder. Fifty ml of 0.02M Tris-HCL (pH 7.5)-0.01M Mg Cl_2-0.05 MKCL-0.01M 2-mercaptoethanol

buffer were added. This homogenate was then blended for approximately 5 minutes. Differential centrifugation was done according to the method of Ghosh (10).

Fractionation of protein with ammonium sulphate was performed and the 75% fraction collected was dialyzed against the buffer 0.01M Tris-HCL (pH 7.5)-0.001M $MgCL_2$-0.05M KCL-0.01M 2-mercaptoethanol for 12 hours. This purified fraction was used as the enzyme component in the cold T.C.A. precipitation reaction system.

Isolation and purification of lysine t-RNA synthetase enzyme involved the lysine isogenic barley pair, CI 4362, the normal lysine, and the high lysine cultivar, CI 3947. The source of unacylated t-RNA was Escherichia coli strain B.

A reaction preparation was made in bulk with 25λ each of ATP, GTP, PEP, PEPkinase, E. coli B t-RNA, and 75λ of the same buffer used in dialysis described earlier. Two-tenths ml of this reaction mixture was added to each of 16 reaction vessels. Eight vessels were for the mutant and eight for the normal line (Table 2).

The final concentrations in the reaction preparation were 0.25 μg ATP, 0.0075 μmole GTP, 0.125 μmoles PEP, .1 mg t-RNA, and 0.75 enzyme units Pepkinase in the reaction

mixture. The controls were made by the addition of 0.5 ml
of .2M KOH to the control tubes to block acylation of
t-RNA's. The reaction was initiated by addition of 25λ of
radioactive lysine which gave a final concentration of
0.25 μCi. These aliquots were added in sequential time
block to each of the reaction vessels incubated at 37 C in
the water bath.

Table 2. Volume (λ) of components in reaction mixture.

Component	Tube Number							
	1	2	3	4	5	6	7	8
Reaction Prep.	200	200	200	200	200	200	200	200
Enzyme Prep Hiproly Normal	25	25	50	50	75	75	75	75
buffer	250	250	225	200	200	200	200	200
							control	
	9	10	11	12	13	14	15	16
Reaction Prep.	200	200	200	200	200	200	200	200
Enzyme Prep. Hiproly	50	50	100	100	150	150	150	150
buffer	225	225	175	175	125	125	125	125
							control	

Loading was stopped by the addition of three ml of
cold T.C.A. (20%) in 15-second intervals to each of the re-
action vessels. The precipitation reaction was allowed to
continue for 30 minutes at 37 C in each of the reaction ves-
sels. The reaction mixtures were then filtered through

H.A.W.P. millipore filters (0.45 μm nitrocellulose) and washed three times with cold T.C.A. (5%). Planchettes were made from the filters and counts per minute (CPM) were recorded on a low background gas flow counter (Nuclear Chicago Gas Flow Counter).

RESULTS AND DISCUSSION

Modified Microbiological Assay

Evaluation of the modified microbiological assay was feasible only if adequate differentiation of lysine levels among the isogenic barley cultivars could be achieved. Many variations for the proper modifications were investigated. Data on three initial experiments are included 1) proper hydrolysis time, 2) storage effect on hydrolyzate samples, and 3) proper hydrolyzate aliquot size. Reliability of standard curves associated with the modified microbiological assay were examined.

Determination of hydrolysis time. Hydrolysis times of 4, 16, and 24 hours were imposed on 30 Hiproly samples grown at 15 locations (Appendix Table 3). The hydrolysis time can affect the evaluation of lysine mutants by enhancing or reducing the apparent lysine difference between the high and low isogenic cultivars.

A composite analysis of variance of percent lysine in the protein was generated for 4-, 16-, and 24-hour hydrolysis. Variation among the three hydrolysis times was not significant, cultivar differences were highly significant (Table 3).

Table 3. Analysis of variance of percent lysine in protein
for 4-, 16-, and 24-hour hydrolysis of 15 Hiproly
and Hiproly Normal isogenic pairs: single
hydrolysis and single bioassay.

Source	Sum of squares	Degrees of freedom	Mean squares	F Calculation
Total	34.0953	89		
Hydrolysis (hours)	0.7080	2	0.3540	2.01
Cultivars	23.1946	29	0.7998	4.55**
Error	10.1927	58	0.1757	

**Greater than .01 significance level.

Cultivars differed significantly for percent lysine
in protein at each hydrolysis time. Significance among cultivars at the .05 level was noted for 4- and 24-hour hydrolysis compared to .01 significance for 16-hour hydrolysis
(Tables 4-6).

Additionally, standard errors of a single determination were calculated for each hydrolysis time. The variation within 16-hour hydrolysis for percent lysine in protein was less than for either 4- or 24-hour hydrolysis
times.

The 16-hour hydrolysis was found to be the most reliable of the three for differentiating levels of lysine

Table 4. Analysis of variance of percent lysine in protein
for 4-hour hydrolysis of 15 Hiproly and Hiproly
Normal isogenic pairs: single hydrolysis and
single bioassay.

Source	Sums of squares	Degrees of freedom	Mean squares	Calculated F value
Total	12.6509	29		
Cultivars	2.3632	1	2.3632	6.4322*
Samples within cultivars	10.2877	28	0.3674	

*Greater than .05 significance level.

Table 5. Analysis of variance of percent lysine in protein
for 16-hour hydrolysis of 15 Hiproly and Hiproly
Normal isogenic pairs: single hydrolysis and
single bioassay.

Source	Sums of squares	Degrees of freedom	Mean squares	Calculated F value
Total	9.8756	29		
Cultivars	2.1440	1	2.1440	7.7653**
Samples within cultivars	7.7316	28	0.2761	

**Greater than .01 significance level.

and was adopted as the standard hydrolysis time for all

further experimentation.