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SOLUBLE PROTEINS OF WINTER WHEAT CROWN TISSUES AND THEIR RELATIONSHIP TO COLD HARDINESS*

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Abstract—Soluble proteins from crown tissue of Minturki and Ponca winter wheats were studied as the plants cold hardened and dehardened. Cold hardiness measurements by a specific conductivity method indicated that Minturki was hardier than Ponca. The soluble proteins were fractionated by elution of DEAE-cellulose columns with NaCl solutions of varying molarity. There was no relationship between cold hardiness and the amount of protein eluted with 0·05 M, 0·1 M and 0·2 M NaCl. Correlation coefficients indicated that percentages of protein in the 0·3 M fraction increased as cold hardiness increased, that precentages of protein in the 1 M fraction decreased as cold hardiness increased, and that these two fractions may be related to cold hardiness. No association was found between cold hardiness and total soluble protein eluted from the columns. Seventeen amino acids were found in the protein from the 1 M fraction for both Minturki and Ponca. Ratios to glycine for given amino acids were similar between varieties and showed little variation from sampling date to sampling date, indicating that amino acid composition was the same for both varieties and did not change with cold hardening and dehardening.

INTRODUCTION

FOR MANY years attention has been focused on the phenomenon of cold hardening and dehardening of plant tissues. One of the principal areas of study has been the organic nitrogenous constituents of the plants. In 1949, Siminovitch and Briggs[1] reported an association between water-soluble protein nitrogen and cold hardiness of the cambial cells of the black-locust tree. Similar associations have been reported in winter wheat[2,3], alfalfa[4], red clover and sweet clover[4,5] and chrysanthemums.[6] Briggs and Siminovitch[7] studied the seasonal variation in the electrophoretic pattern of the soluble proteins from the black locust tree. Five major electrophoretic components were observed. No marked changes in these components occurred during the time of greatest changes in hardiness and in soluble protein increases. However, marked changes in the per cent concentration of 2 or 3 components did occur in midsummer.

Of importance in such studies is a method for measuring the hardiness of plants. Dexter *et al.*[8] developed a conductivity method for this purpose and showed it to be a reliable measure of cold hardiness by comparing it with plant survival in low temperature studies. With this method, specific conductance decreases as cold hardiness increases.

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[1] D. SIMINOVITCH and D. R. BRIGGS, *Arch. Biochem. Biophys.* **23**, 8 (1949).
[2] A. W. PAULI, B. J. KOLP and F. C. STICKLER, *Crop Sci.* **1**, 137 (1961).
[3] A. C. ZECH and A. W. PAULI, *Agron. J.* **52**, 334 (1960).
[4] R. J. BULA and DALE SMITH, *Agron. J.* **46**, 397 (1954).
[5] R. J. HODGSON and R. J. BULA, *Agron. J.* **48**, 157 (1956).
[6] D. B. MEADOR, Ph.D. dissertation, Kansas State University (1965).
[7] D. R. BRIGGS and D. SIMINOVITCH, *Arch. Biochem. Biophys.* **23**, 18 (1949).
[8] S. T. DEXTER, W. E. TOTTINGHAM and L. F. GRABER, *Plant Physiol.* **7**, 63 (1932).

The following study was concerned with fractionating the water-soluble proteins of winter wheat crown tissue, determining the amounts of protein in each fraction, and determining the amino acid content of certain protein fractions at different stages of cold hardiness in the plants. This was done with two varieties of winter wheat known to vary in cold hardiness.

RESULTS AND DISCUSSION

Cold Hardiness

Maximum hardiness in the first year was reached on January 6, 1965 for Minturki, and on January 6 and February 2, 1965 for Ponca (Fig. 1). Maximum hardiness the second year was recorded for both varieties on January 31, 1966 (Fig. 1). Per cent conductances for

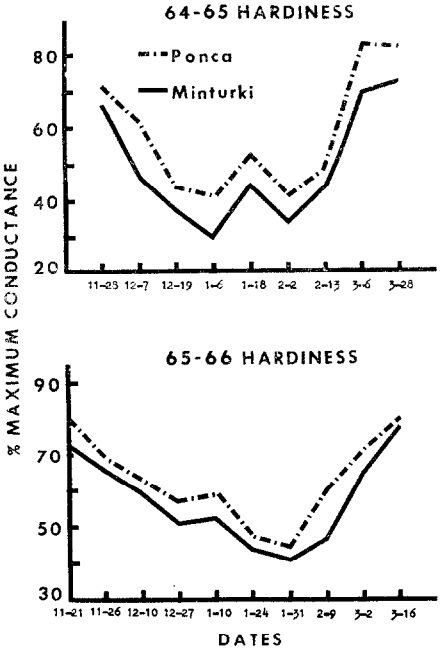

FIG. 1. PER CENT OF MAXIMUM CONDUCTANCE OF WHEAT CROWN TISSUE, 1964–65 AND 1965–66.

Minturki were lower than those for Ponca on all sampling dates, indicating that Minturki was the hardiest variety. This agrees with results reported by Pauli *et al.*[9, 3] Greater hardiness was obtained in 1964–65 than in 1965–66, probably due to the colder winter of 1964–65.

Protein Fractionation

Figures 2 and 3 represent typical elution curves obtained with DEAE-cellulose columns of soluble protein from Minturki. The curves represent two degrees of cold hardiness in the plants, non-hardy and hardy, respectively. The shapes of the elution curves were similar for both Minturki and Ponca varieties, and curves for Ponca therefore are not shown. Six fractions were obtained, which will be referred to as the buffer fraction, the 0·05 M fraction, the 0·1 M fraction, the 0·2 M fraction, the 0·3 M fraction and 1 M fraction. The front edge of the

[9] A. W. PAULI and A. C. ZECH, *Crop. Sci.* 6, 204 (1964).

0·05 M, the 0·1 M, the 0·2 M and 0·3 M fraction peaks increased sharply, but the back edge decreased slowly, producing a tailing effect. The broadness of these peaks indicated that a

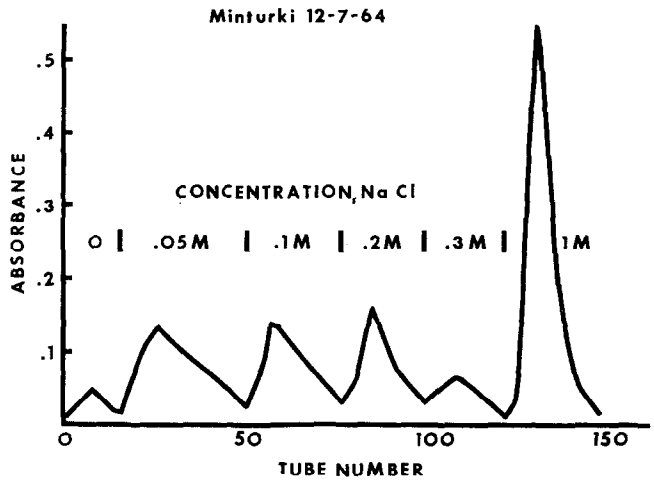

FIG. 2. ELUTION CURVE OF SOLUBLE PROTEIN OF MINTURKI WHEAT CROWN TISSUE SAMPLED DECEMBER 7, 1964. FRACTIONS WERE ELUTED FROM DEAE-CELLULOSE COLUMN WITH THE FOLLOWING ELUTING AGENTS, pH 7·3: (1) 0·005 M PHOSPHATE BUFFER (2) 0·005 M PHOSPHATE BUFFER AND 0·05 M NaCl (3) 0·005 M PHOSPHATE BUFFER AND 0·1 M NaCl (4) 0·005 M PHOSPHATE BUFFER AND 0·2 M NaCl (5) 0·005 M PHOSPHATE BUFFER AND 0·3 M NaCl (6) 0·005 M PHOSPHATE BUFFER AND 1 M NaCl.

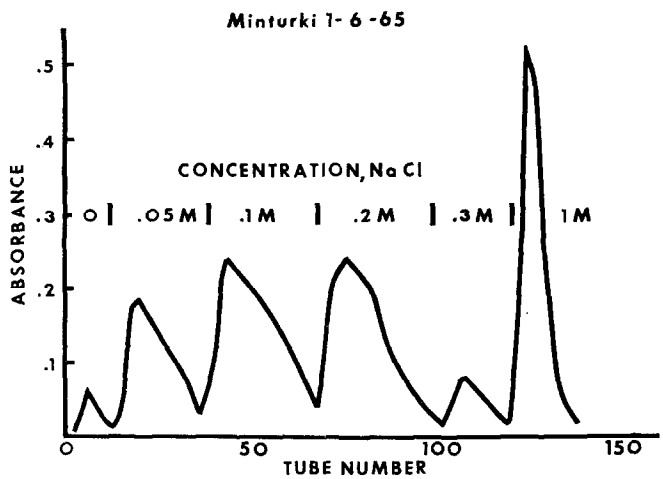

FIG. 3. ELUTION CURVE OF SOLUBLE PROTEIN OF MINTURKI WHEAT CROWN TISSUE SAMPLED JANUARY 6, 1965. FRACTIONS WERE ELUTED FROM A DEAE-CELLULOSE COLUMN WITH THE FOLLOWING ELUTING AGENTS, pH 7·3: (1) 0·005 M PHOSPHATE BUFFER (2) 0·005 M PHOSPHATE BUFFER AND 0·05 M NaCl (3) 0·005 M PHOSPHATE BUFFER AND 0·1 M NaCl (4) 0·005 M PHOSPHATE BUFFER AND 0·2 M NaCl (5) 0·005 M PHOSPHATE BUFFER AND 0·3 M NaCl (6) 0·005 M PHOSPHATE BUFFER AND 1 M NaCl.

mixture of proteins was involved in these fractions. This was further indicated by the fact that on a few sampling dates there was evidence of two peaks with some of these elution fractions. The 1 M fraction had a very sharp symmetrical peak on all sampling dates, indicating that it contained predominantly a single protein.

Buffer Fraction

In preliminary experiments, when protein extracts that had not been dialyzed were applied to DEAE-cellulose columns a large fraction was eluted with phosphate buffer. When extracts were dialyzed for 24 hr before being applied to columns, the buffer fractions were reduced considerably, but they were not eliminated completely. It was postulated that this fraction was composed of free amino acids, and paper chromatographic analyses of the dialysates and of the eluent from a dialyzed extract proved this to be true.

The total area (sq. in. × 10 per mg of nitrogen) under each elution curve varied from sampling date to sampling date. Therefore, for comparative purposes, it was more useful to express the results as per cent of the total area for each of the soluble protein fractions.

When comparable fractions from the two varieties were compared, percentages of total area for the 0·05 M, 0·1 M, and 0·2 M fractions were similar on given sampling dates during the 2 years, and it was concluded that there was no varietal difference in these fractions. Per cent conductance is inversely related to cold hardiness. Correlation coefficients between per cent maximum conductance and percentages of the total area for each of the above fractions were non-significant for both varieties both years. Thus, there was no relationship between cold hardiness and the proteins in these fractions.

The 0·3 M *Fraction*

Percentages for the 0·3 M fraction (Fig. 4) were low in November of 1964 for both varieties, but increased on December 7. Minturki continued to increase on December 19, while Ponca decreased on this date. Percentages for both varieties decreased on January 6, 1965, and increased on the next two sampling dates. Values for Minturki and Ponca decreased sharply

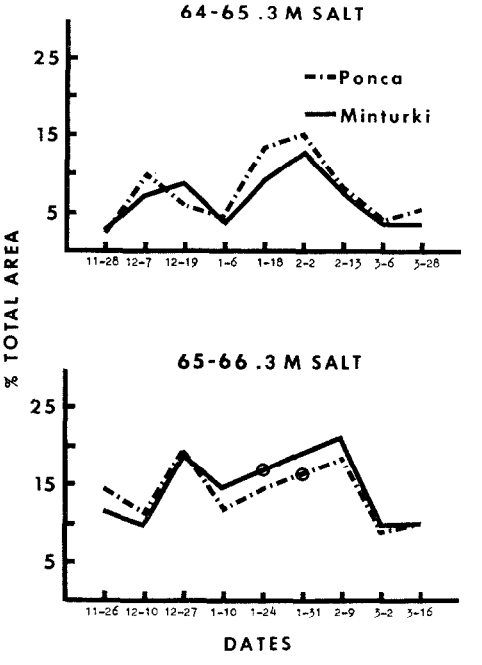

FIG. 4. PER CENT OF THE TOTAL AREA FOR THE 0·3 M FRACTION, 1964–65 AND 1965–66. CIRCLES ON THE GRAPH REPRESENT POINTS OF MISSING DATA.

on February 13 and March 6, 1965, and remained at a low level on the last sampling date. In 1965–66, percentages for both varieties were moderate in November. Although values decreased slightly for both varieties on December 10, they increased sharply on December 27 and decreased again on January 10, 1966. After this point, values for the two varieties increased through February 9 and then decreased on March 2 and remained at this level on the last sampling date. Little varietal difference was observed in this fraction.

Correlation coefficients between percentage of the total area for the 0·3 M fraction and per cent maximum conductance were negative for both varieties both years. The coefficients for Minturki were significant at the 5 per cent level in 1964–65 and significant at the 1 per cent level in 1965–66. Coefficients for Ponca approached significance for both years, and when varieties were pooled the coefficients were significant at the 5 and 1 per cent levels, respectively, for 1964–65 and 1965–66. Thus, the data indicated that percentages of soluble protein in the 0·3 M fraction increased as cold hardiness increased and that this protein fraction may be related to cold hardiness.

The 1 M *Fraction*

In 1964–65, percentages for the 1 M fraction (Fig. 5) decreased in Minturki through January 6, then increased on the remaining sampling dates. Ponca values followed a similar

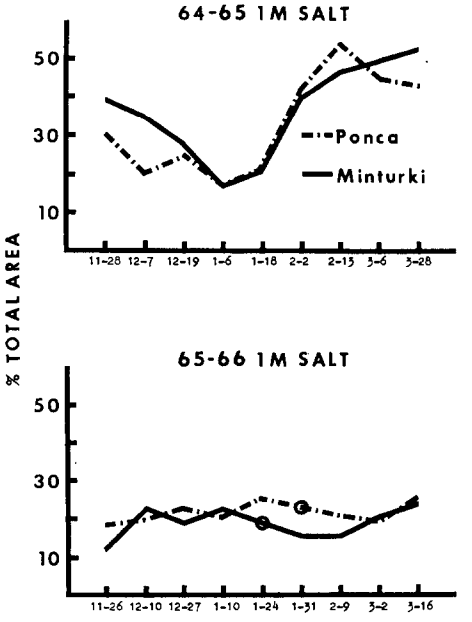

FIG. 5. PER CENT OF THE TOTAL AREA FOR THE 1 M FRACTION, 1964–65 AND 1965–66. CIRCLES ON THE GRAPH REPRESENT POINTS OF MISSING DATA.

pattern, with two exceptions. On December 19 Ponca increased while Minturki decreased, and on the last two sampling dates Ponca decreased while Minturki increased. The two varieties exhibited almost identical values from December 19 through February 2, but deviated somewhat on earlier and later dates. The second year, percentages were lower than the first year and differences from sampling date to sampling date were relatively small. Ponca values remained relatively level on most sampling dates, while Minturki values fluctuated

slightly. However, the value for Minturki was low in November and values decreased from mid-January to mid-February, after which percentages increased and reached the maximum on the last sampling date.

Correlation coefficients between percentages for the 1 M fraction and per cent conductances were positive for Minturki and Ponca both years. The coefficient for Minturki was significant at the 5 per cent level in 1964–65 and approached significance at the 5 per cent level in 1965–66. When Minturki was pooled across years, the coefficient was significant at the 1 per cent level. Coefficients for Ponca were non-significant for both years. However, when varieties were pooled in 1964–65 the coefficient approached significance at the 5 per cent level. The coefficients indicated that as cold hardiness increased the percentage of protein in the 1 M fraction decreased, and that the protein in this fraction may be related to cold hardiness. This relationship appeared to be stronger in Minturki than in Ponca.

The Total Area

The total areas under each elution curve (Fig. 6) were quite similar for Minturki and Ponca in November of 1964. Values for both varieties increased on December 7 and reached

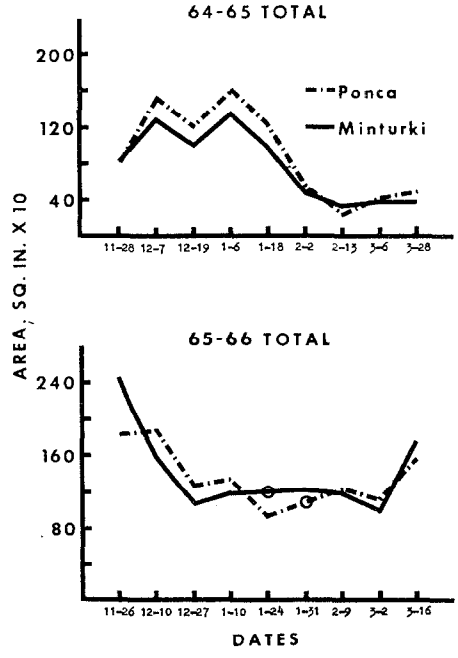

FIG. 6. TOTAL AREAS UNDER EACH ELUTION CURVE (SQ. IN. × 10 PER mg OF NITROGEN) 1964–65 AND 1965–66. CIRCLES ON THE GRAPH REPRESENT POINTS OF MISSING DATA.

maxima on January 6, 1965. The second year, areas were at maxima for both Minturki and Ponca in November and decreased the next few sampling dates, after which they leveled off. The patterns for the 2 years appeared to be quite different.

Correlation coefficients between total area and per cent conductance were negative and non-significant for the two varieties in 1965–65 and were positive and non-significant for the two varieties in 1965–66, indicating no relationship between cold hardiness and total soluble protein eluted from the column. This is not in agreement with results from other investiga-

tions on wheat in which data were expressed on a fresh weight basis.[2,3] However, the results reported here were expressed as protein per mg. of nitrogen and do agree with results reported by Pauli and Mitchell whose data were expressed as percentages of total nitrogen.[10]

Amino Acid Analysis

The 1 M protein fractions from Minturki and Ponca wheats were analyzed on 4 and 5 sampling dates, respectively, in 1965–66. The 1 M protein fraction was chosen because it appeared to be the purest. Results of these analyses were expressed as ratios of a given amino acid to glycine and are presented in Table 1. Seventeen amino acids were found in the protein from this fraction for both Minturki and Ponca. Other than glycine, 15 are listed in Table 1. Cystine also was found in all samples, but only in trace amounts. Tryptophan was not deter-

TABLE 1. RATIOS OF VARIOUS AMINO ACIDS TO GLYCINE FROM HYDROLYSATES OF THE 1 M FRACTION OF WHEAT CROWN TISSUE FROM CERTAIN SAMPLING DATES IN 1965–66

	Ponca Date					Minturki Date			
Amino Acid	11–26	12–27	1–24	2–9	3–16	12–27	1–24	2–9	3–16
	Ratio (per cent)								
Lysine	51	55	41	55	51	55	58	56	50
Histidine	10	12	10	11	10	10	11	10	10
Arginine	10	8	11	9	9	7	11	9	8
Aspartic Acid	40	39	34	48	47	41	43	49	54
Threonine	25	24	24	25	23	25	29	26	26
Serine	98	111	69	97	109	121	102	109	99
Glutamic Acid	82	87	82	97	85	91	68	100	87
Proline	18	22	14	20	18	18	17	19	21
Alanine	51	52	49	56	47	52	54	56	50
Valine	23	21	23	24	22	20	27	26	26
Methionine	5	6	6	7	6	6	6	4	8
Isoleucine	12	12	11	13	13	12	14	15	14
Leucine	18	17	18	20	18	18	23	22	21
Tyrosine	5	4	2	4	3	5	8	5	4
Phenylalanine	11	9	8	11	10	9	12	12	11

mined. These results partially agree with Pauli and Zech who reported 16 amino acids in the total soluble protein from wheat crown tissue.[9]

Comparison of the ratios for given amino acids between wheat varieties indicated no varietal difference in amino acid composition of the protein in the 1 M fractions. Serine, glycine and glutamic acid concentrations, in that order, were the highest, while concentrations of alanine, lysine and aspartic acid were also rather high. Cystine, tyrosine and methionine concentrations were the lowest, but histidine, arginine, isoleucine and phenylalanine were also low, Amino acid ratios varied little from sampling date to sampling date. Serine was somewhat of an exception, but it is one of the more labile amino acids and more variation might be expected. These results indicate that the amino acid composition of this protein fraction did not change with cold hardening and dehardening. This differs from results reported by Pauli and Zech.[9] They found that aspartic acid, glutamic acid, alanine, arginine and histidine increased with cold hardening and decreased again with dehardening. However, they used

[10] A. W. PAULI and H. L. MITCHELL, *Plant Physiol.* **35**, 539 (1960).

the total soluble protein from wheat crown tissue and their data were expressed as micrograms per gram fresh weight.

MATERIALS AND METHODS

Minturki and Ponca winter wheats were planted on October 2, 1964, and September 29, 1965. These genotypes were chosen because Minturki is a cold hardy variety and Ponca is moderate in hardiness.[3]

Collection of crown tissue the first year started on November 28, 1964, and continued at approximate two-week intervals until March 28, 1965. The first sample the second year was taken November 21, 1965, and the last sample on March 16, 1966. The plants were brought from the field to the laboratory where they were washed free of soil with cold tap water. The crowns, defined as that portion of tissue above the roots and below the soil surface, were removed, washed with cold tap water and rinsed with distilled water. Excess surface water was removed by blotting the crowns between towels.

Crowns were tested for cold hardiness by using the specific conductivity method outlined by Dexter, *et al.*[8] 5 g of fresh crown tissue were placed in an uncovered beaker and frozen 4 hr at $-12°$ in a controlled temperature chamber. The tissue was removed, placed in 50 ml of double distilled water and allowed to stand for at least 12 hr in a chamber maintained at approximately 5°. Using a Wheatstone bridge, resistance readings were made on the solution containing the exosmosed solutes from the cells damaged by freezing. The crown tissue then was blended to macerate the tissues, extracting all the cell solutes. Resistance readings were taken on these solutions containing the total cell electrolytes. All resistance readings were made at 20°. Results were expressed as percentages of maximum conductance, reflecting the per cent of total electrolyte exosmosed following freezing.

Soluble proteins were extracted by a modification of the method described by Pauli and Zech.[9] 5 g of fresh crown tissue were blended in 50 ml of 0·005 M phosphate buffer, pH 7·3. The blending was accomplished with a Servall Omni-mixer with the blender cup immersed in an ice bath. The extract was centrifuged in a refrigerated centrifuge for 30 min at approximately $30{,}000 \times g$. The supernatant was dialyzed against 0·005 M phosphate buffer for 24 hr at 1–2°. After dialysis, the protein solution was centrifuged as before. The supernatant was used as the protein extract.

Protein fractionation was accomplished in diethylaminoethyl (DEAE) cellulose columns by a modification of the method of Dechary, *et al.*[11] Whatman DE 11 cellulose ion-exchange powder was used to prepare columns that were approximately 2·2 by 30 cm. 20 ml of protein extract, containing 2–2·5 mg of nitrogen, were applied to each column. Proteins were removed from the column by echelon elution with the following solutions at pH 7·3: (1) 0·005 M phosphate buffer, (2) 0·005 M phosphate buffer and 0·05 M NaCl, (3) 0·005 M phosphate buffer and 0·10 M NaCl, (4) 0·005 M phosphate buffer and 0·20 M NaCl, (5) 0·005 M phosphate buffer and 0·30 M NaCl and (6) 0·005 M phosphate buffer and 1·00 M NaCl. The eluent was collected in 10 ml volumes by an ISCO automatic volumeter and fraction collector. The protein fractions coming off were monitored by an ISCO u.v. absorption scanner at 254 nm.

The relative protein content of each fraction was obtained by measuring the absorbance of each 10 ml volume of eluent at 254 nm. The absorption values were plotted against tube number, and the area under each fraction peak was determined with a planimeter. This area was used as a measure of protein in each fraction. Results were expressed as area in square inches × 10 per mg of nitrogen applied to the column.

[11] J. M. DECHARY, K. F. TALLUTO, W. J. EVANS, W. B. CARNEY and A. M. ALTSCHUL, *Nature* **190**, 1125 (1961).

In preliminary experiments with this procedure, 75–80% of the nitrogen applied to the columns was recovered, and good agreement was obtained between duplicate samples of crown tissue for the various fractions.

The total nitrogen content of the protein extracts was determined by a modification of the nesslerization method of Thompson and Morrison.[12]

Protein hydrolysates were prepared by a modification of the method of Pauli and Zech.[9] Prior to hydrolysis each fraction was dialyzed against double distilled water until the salt was removed.

Amino acid analyses were made by the procedures of Spackman, *et al.*[13] and Moore, *et al.*[14] A model 120 B Beckman amino acid analyzer was used.

Correlation coefficients were calculated according to the procedure of Snedecor.[15]

[12] J. F. Thompson and G. R. Morrison, *Anal. Chem.* **23**, 1153 (1951).
[13] D. H. Spackman, W. H. Stein and S. Moore, *Anal. Chem.* **30**, 1190 (1958).
[14] S. Moore, D. H. Spackman and W. H. Stein, *Anal. Chem.* **30**, 1185 (1958).
[15] G. W. Snedecor, *Statistical Methods*, fifth edition. Iowa State University Press, Ames, Iowa (1956).