THE TERMINAL AMINO ACIDS OF WHEAT GLIADIN[1]

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ABSTRACT

Wheat gliadin has been found by two different methods to contain three N-terminal histidine residues for each molecular weight of 27,000. Trace amounts of N-terminal aspartic acid, glutamic acid, alanine, valine, and serine were also detected in the preparation used. Hydrolysis in boiling hydrochloric acid partially destroyed the di-2,4-dinitrophenyl derivative of histidine. Losses of from 5% to 25% occurred depending upon the time and conditions of hydrolysis. Carboxypeptidase did not release free amino acids from wheat gliadin but qualitative evidence for the occurrence of C-terminal glutamic acid and C-terminal "leucine" was obtained.

It has been reported by Koroś (9) that wheat gliadin contains three free amino groups and that these belong to the three N-terminal histidine residues. Although doubt exists that gliadin is composed of a homogeneous and unique molecular species the observations of Koroś would point to the existence of at least one characteristic feature in gliadin. Because details of Koroś's work were unavailable independent experiments were done to determine the nature of the N-, and also of the C-, terminal amino acids of gliadin. Since the completion of the present work Koroś's results have been questioned (6) and it was reported that Sanger's method revealed one N-terminal tyrosine residue in gliadin from *Triticum durum*, and two N-terminal residues, one each of glutamic acid and tyrosine, in gliadin from *Triticum vulgare*; no N-terminal histidine was detected. Results recorded in the present communication support the earlier report (9) that histidine is the major N-terminal amino acid in gliadin. In addition, evidence presented indicates that glutamic acid and "leucine" occupy the C-terminal positions.

EXPERIMENTAL

Gliadin was prepared by the method of Blish and Sandstedt (4). The sample contained 17.64% nitrogen on a moisture and ash-free basis. The ninhydrin colorimetric procedure (8) and the Van Slyke nitrous acid method (11 min. reaction) indicated 3.18 and 3.32 free amino groups, respectively, for an assumed molecular weight of 27,000.

Identification of N-Terminal Groups Using 2,4-Dinitrofluorobenzene

DNP-gliadin was prepared using the reaction conditions described by Porter (14). Two grams of the protein and 2 gm. $NaHCO_3$ were suspended in 20 ml. water, 40 ml. of a 10% (w/v) solution of DNFB in ethanol were added, and the mixture was shaken for two hours at room temperature. The reaction mixture was exhaustively dialyzed against distilled water and the precipitated protein collected, washed, and dried (yield 2.0 gm.). It was calculated from

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the amide nitrogen content (14) that 119.9 mgm. of DNP-gliadin corresponded to 100 mgm. of the original gliadin. Samples of DNP-gliadin (100 mgm.) were hydrolyzed in 10 ml. of boiling 5.7 *N* hydrochloric acid for four hours or for 24 hr. Another sample was hydrolyzed in 12 *N* acid in a sealed tube at 105° C. for 24 hr. (14). The DNP-amino acids in the hydrolyzates were fractionated into ether-soluble and acid-soluble materials and the fractions were chromatographed by each of three previously used methods (2, 3, 13).

DNP-histidine was the only amino acid derivative found in the acid-soluble fraction. Trace amounts of DNP-aspartic acid, glutamic acid, alanine, valine, and serine were detected in the ether-soluble material and, although gliadin contains at least two residues of lysine per molecule, no ϵ-DNP-lysine was found. The identity of the DNP-histidine was established by hydrolyzing it with concentrated ammonia (12) in a sealed tube and chromatographing the resulting amino acid on paper.

The yields of various 2,4-dinitrophenyl derivatives obtained from the treated gliadin are recorded in Table I. The calculations for the histidine are

TABLE I

YIELD OF DNP-DERIVATIVES FROM ACID HYDROLYZATES OF DNP-GLIADIN

Derivative	Yield: moles/27,000 gm.	
	4 hr. hydrolysis	24 hr. hydrolysis
Di-DNP-histidine (N-terminal histidine)	3.1	2.9
Other DNP-amino acids*	0.17	0.11
Dinitrophenol, dinitroaniline, etc.†	1.3	1.0

**Calculated as DNP-aspartic acid.*
†Calculated as dinitrophenol.

based on optical density measurements at 360 mμ and reference to the calibration curve for di-DNP-histidine. The DNP-amino acids found in traces are calculated as DNP-aspartic acid. Values for histidine are corrected for hydrolytic losses by increasing them by 25% (see below). It was also found that only 71% of the expected histidine derivative could be estimated in a dinitrophenylation of histidine done under the same conditions as employed with gliadin. To compensate for this low recovery in dinitrophenylation the yields of di-DNP-histidine were also multiplied by the factor 1.41. Because of the large corrections the results in Table I are only approximate. Chromatography on a silicic acid column was used to separate trace amounts of DNP-amino acids from the relatively large amounts of dintrophenol and dinitroaniline found in the ether-soluble fraction (11). No corrections have been applied for hydrolytic losses of the latter materials.

Effect of Acid Hydrolysis on Di-DNP-histidine

Di-DNP-histidine, with the reported physical constants (15), was prepared according to the method of Porter. Twenty-five milligrams was hydrolyzed with 20 ml. of 5.7 *N* HCl. The hydrolyzates were extracted with ether to remove artifacts like dinitroaniline and dinitrophenol, and the di-DNP-histidine

remaining in the acid phase was determined by spectrophotometry. For time intervals of 3.5, 6.5, and 24.0 hr. the destruction observed was 0.0, 5.0, and 12.0% respectively. When the same weight of the pure derivative was hydrolyzed in the presence of 40 mgm. gliadin the destruction observed was 25.0% for 24 hr. The latter figure has been used to correct for hydrolytic losses.

Application of the Edman Method (7) to N-Terminal Residue Identification

One gram gliadin was dissolved in 4 ml. 50% pyridine, the pH was adjusted to 8.6 with 0.1 *N* alkali, and 0.2 ml. of phenylisothiocyanate was added with vigorous stirring. The temperature was maintained at 40° C., and alkali was added as required to keep the pH at 8.6. When consumption of alkali ceased, the reaction mixture was extracted twice with benzene and the protein precipitated with acetone. The dry phenylthiocarbamyl protein was then suspended in 9 ml. of anyhydrous nitromethane containing 4% HCl by weight. After an hour of agitation, the material was filtered and washed with a few milliliters of the solvent. The combined filtrates were evaporated *in vacuo* to remove all traces of solvents and HCl. The residue was dissolved in EtOH. Absorption at 270 mμ indicated a content of 3.2 M. of phenylthiohydantoin per 27,000 gm. of the protein. Paper chromatography of the material (10) showed that PTH-histidine was the major component. Its identity was confirmed by barium hydroxide hydrolysis to the free amino acid. Stepwise degradation of the residual protein was carried out but during the second and third treatments an abnormally high yield of phenylthiohydantoin (of the order of five moles) was obtained. No detailed identification of the material was attempted, but paper chromatography of barium hydroxide hydrolyzates indicated the presence of several ninhydrin positive spots.

C-Terminal Residues of Gliadin

Action of carboxypeptidase on gliadin.—One-half gram of gliadin was suspended in 20 ml. water, the pH was adjusted to 7.7 with 0.2 *N* NaOH, and the mixture was incubated with 0.2 ml. of carboxypeptidase suspension (12.6 mgm./ml. Armour). In another experiment the same weight of protein was suspended in 20 ml. of 1% (w/v) sodium bicarbonate. No liberation of amino *N* could be detected by the Van Slyke method during a seven-hour period, and no free amino acids could be detected in the reaction mixture by paper chromatography. It was therefore concluded that the C-terminal amino acid residues in gliadin, if any, were not susceptible to hydrolysis by carboxypeptidase under the conditions used.

Application of the method of Schlack and Kumpf (1) to gliadin.—One gram of the protein was mixed with 0.3 gm. of pulverized anhydrous ammonium thiocyanate in 77 ml. of an AcOH: Ac_2O (9: 1) mixture, and the reaction mixture was stirred at 45° C. for four hours. At the end of this period 30 ml. of HCl was added dropwise with stirring and the material heated on a steam bath for one hour. The product was dried *in vacuo*, the residue dissolved in 60 ml. of 0.25 *M* phosphate (pH 6.5) and extracted five times with equal volumes of ethyl acetate. The combined extracts were washed once with an equal volume of water and dried, and the residue was hydrolyzed with 2.5 ml.

of 1.25 *N* $Ba(OH)_2$ at 140° C. in a sealed tube. The hydrolyzate was neutralized with carbon dioxide and heated for 10 min. on a steam bath to destroy carbamic acids. Aliquots of the suspension were examined by paper chromatography (BuOH–AcOH–H_2O, 4:1:5). The only amino acids detected were glutamic acid and "leucine". The amounts of these amino acids were estimated by ninhydrin colorimetry using a standard curve for the two amino acids obtained under the same conditions. One-half mole of glutamic acid and 0.45 mole of "leucine" were found for each 27,000 gm. gliadin. No corrections were applied.

Application of the lithium borohydride reduction method of Chibnall (5) indicated the presence of glutamic acid and "leucine" in C-terminal positions. Appreciable traces of other amino acids were also detected. Using a nine-fold excess of diazomethane for esterification of the protein and a 13-fold excess of $LiBH_4$ for the reduction in tetrahydrofuran, the total yield of α-amino alcohols was 0.654 mole per 27,000 gm. protein.

DISCUSSION

The present work supports the observations of Koroś (9) that gliadin of molecular weight 27,000 contains three free amino groups and that these belong to histidine. From this result, it was expected that three C-terminal amino acids could be detected. It was surprising, therefore, that carboxypeptidase did not liberate any free amino acids from the protein; particularly in view of the finding that leucine and glutamic acid were detected as C-terminal residues by the chemical method (1). Recoveries by the latter method are known to be very poor, and since only about 0.5 mole of C-terminal leucine and glutamic acid was found the data were not adequate for evaluation of the amounts of C-terminal leucine and C-terminal glutamic acid in gliadin. It is to be noted that Baptist and Bull (1) could not identify glutamic acid in glutathione or recover glutamic acid from glutamine by the Schlack and Kumpf method. The present identification of the C-terminal acids would therefore be tentative.

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