Dialyzable Components Resulting from Proteolytic Activity in Extracts of Wheat Flour

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ABSTRACT

The dialyzable components produced at 30°C. by the action of indigenous enzymes upon the proteins in a flour extract were fractionated into ammonia, free amino acids, and peptides. Each was found in nearly equal proportions based upon the response to ninhydrin. The peptide fraction had an average of approximately seven amino acid residues per peptide. The free amino acid fraction was very low in glutamic acid and proline, although these are the two most abundant amino acids in the flour-extract proteins. Leucine and phenylalanine were the major components, and together comprised 50% or more of the free amino acids. When hemoglobin was added to the extract, six times as much dialyzable, ninhydrin-positive material was produced, 8% of which was ammonia, 62% free amino acids, and 30% peptides. Again, the average peptide contained approximately seven amino acid residues. The free amino acid fraction continued to be high in phenylalanine and leucine, but high proportions of lysine, histidine, and arginine were also present. These results imply the existence of both endo- and exoenzymatic activity as well as a considerable degree of specificity in regard to the peptide bonds that are hydrolyzed.

The existence of low levels of proteolytic activity in wheat flour has been recognized for many years (1). There are several lines of evidence that suggest that more than one species of protease may exist. By the electrophoresis of flour extracts in starch gels, Kaminski and Bushuk (2) have separated four different proteolytically active bands. Hanford (3) has speculated on the existence of both exo- and endoenzymes because of the poor correlation between the rate of dough softening and the rate of accumulation of trichloroacetic acid (TCA)-soluble nitrogen. Flour extracts apparently catalyzed a specific cleavage of α-casein (4).

Thermostability studies and chromatography of flour extracts on Sephadex gels (5) have produced results consistent with the existence of multiple protease species. However, it has also been proposed that there may be only a single enzyme, which is capable of forming complexes with other flour proteins. These complexes then appear to behave as different enzymes.

Something of the nature and specificity of an enzyme or group of enzymes may be determined by examining the products that accumulate as a result of their action. The dialyzable components that are produced in extracts of a hard red spring wheat flour have been isolated and subjected to various analyses. Some of the results are presented in this report.

MATERIALS AND METHODS

All chemicals were reagent grade unless otherwise specified. The flour used as a source of enzymes was milled from Manitou wheat on an Allis Chalmers experimental mill. The ash content and crude protein (N × 5.7) were 0.40 and 15.1%, respectively.

Proteolytic enzymes were extracted with 0.2M acetic acid from the residue remaining after two prior extractions of the flour with water, as described previously (5). The ratio of flour to extractant was 1:5. Flour protein concentrations were determined by the Folin-Ciocalteau method (6) as standardized by comparison with the Kjeldahl method. Proteolytic activity was measured by the modified Ayre-Anderson method (7), using bacto-hemoglobin (Difco Laboratories) as substrate. For certain experiments the method was further modified by varying the temperature or the digestion time, or by omitting the hemoglobin substrate.

Isolation of the Dialyzable Components Produced by Flour Proteases

To remove those low-molecular-weight components that were naturally occurring in the flour, the extracts were subjected to exhaustive dialysis vs. 10 volumes and 12 changes of 1.0M acetic acid solution at refrigerator temperature over a 72-hr. period. These conditions almost completely inhibit the enzyme activity. Following this, the dialyzed extract was adjusted to the optimum pH of 3.8 (7), which restored 90% of the original activity. From 45 to 60 ml. was then transferred into a 28-mm. diameter dialysis sac. A few drops of toluene were added to inhibit microorganisms. The sac was left open at the top to allow for continuous stirring, and was suspended in a similar volume of 0.2M acetate buffer at pH 3.8 in a graduated cylinder. A water bath controlled the temperature at 30°C. Enzymatic digestion with concomitant dialysis was allowed to progress for 24 hr. In some experiments the buffer outside the dialysis sac was replaced by fresh buffer at 8-hr. intervals. The dialysate was concentrated by evaporation at 40°C. aided by a stream of purified air, or by lyophilization.

A modification of the procedure was the inclusion of added substrate by mixing three parts of a 6% solution of bacto-hemoglobin (which had also been exhaustively dialyzed against the pH 3.8 acetate buffer) with five parts of flour extract, prior to the digestion period. To obtain a dialysate containing the naturally occurring low-molecular-weight nitrogenous compounds, the procedure was further modified by operation at 5° to 8°C., by dialysis against 1.0M acetic acid, and by starting with a fresh flour extract that had not been subjected to previous dialysis or pH adjustment.

Methods of Separation and Analysis

The content of nitrogenous compounds in the dialysates and in fractions isolated from the dialysates was determined by reaction with ninhydrin in the modified Rosen method (8,9). Ammonia was separated and determined by chromatography according to Moore et al. (10).

Free amino acids, as a group, were separated from peptides by the chromatographic method of Tommel et al. (11). Only the pH 8.0 collidine-acetate buffer and the 0.17N acetic acid were used for elution of the column, as no attempt was made to use this method for the fractionation of peptides.

Acid hydrolysis was performed in evacuated sealed tubes with 5.7N glass distilled HCl at 110°C. for 6 hr. Amino acid analysis was performed on a Technicon sequential multi-sample amino acid analyzer Model No. TSM.

The "fingerprinting" technique developed for the analysis of tryptic digests of hemoglobin (12) was used in a preliminary investigation of the peptide fractions.

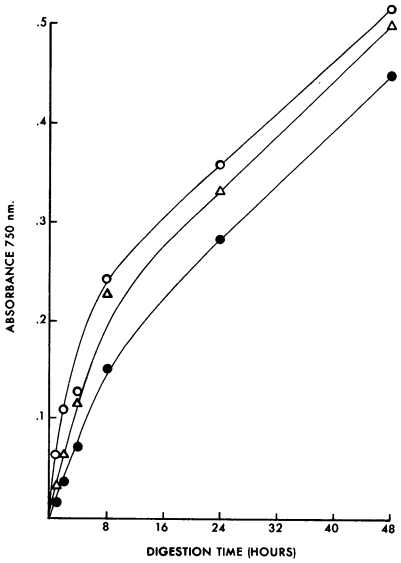
N-terminal amino acids of peptide mixtures were determined by the dansylation method (13) combined with high voltage electrophoresis (14).

RESULTS AND DISCUSSION

The method chosen for the extraction of flour has been shown to be quite efficient for solubilizing flour proteolytic enzymes (5). The resulting extracts contained an average of 1.9 mg. N per ml. This corresponds to 36% of the total N in the flour. Proteolytic activity vs. hemoglobin at 40°C. was 0.1 μeq. of tyrosine produced per g. of extracted flour per min., or 0.01 μeq. of tyrosine per mg. of N in the protein extract per min. This low level is very similar to values reported for extracts of similar flours (7). Exhaustive dialysis reduced the N content by 14% with only slight losses of proteolytic activity.

Temperature Selection for Enzymatic Digestion

Because of the low level of proteolytic activity, the time required to produce sufficient quantities of dialyzable material for further analysis was relatively long. The standard Ayre-Anderson assay is conducted at 40°C., but at this temperature flour extracts lose a substantial portion of their activity fairly rapidly (5). In an attempt to establish the most suitable temperature, the amounts of TCA-soluble



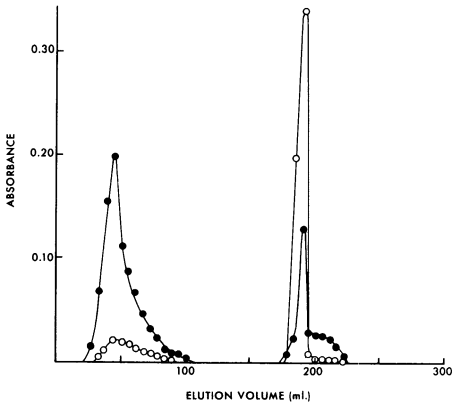


Fig. 1 (left). Effect of temperature on the accumulation of TCA-soluble, Folin-positive components in extracts of wheat flour, without any additional substrate at pH 3.8. Closed circles, triangles, and open circles are 30°, 40°, and 45°C., respectively.

Fig. 2 (right). Chromatographic separation of free amino acids and peptides as their Cu^{++} complexes, on a DEAE-cellulose column (11). The sample is dialysate I. The free amino acids are eluted first. The closed circles are colorimetric readings for aliquots of each fraction with ninhydrin. The Cu^{++} in each fraction was extracted with a chloroform solution of 8-hydroxyquinoline. The open circles are colorimetric readings of these extracts at 396 nm.

Folin-positive material that accumulate in flour extracts were determined at several temperatures over varying periods of time. The results are in Fig. 1.

Initially the rate of proteolysis showed a marked temperature dependence, but after 8 hr. the accumulation of TCA-soluble nitrogenous material had slowed considerably, particularly at the higher temperatures. Subsequently it appeared to be nearly independent of temperature over the range from 30° to 45°C. The choice of 30°C. as the temperature for further study was based on these results.

Analysis of Dialysates

Three types of dialysate were examined. The first contained components which were produced by proteolytic enzymes in the extract acting on indigenous flour proteins. The second contained the components resulting from the action of these enzymes upon an extract which contained added hemoglobin as substrate. The third contained those dialyzable components that were naturally present in the flour. These three types have been coded as dialysates I, II, and III, respectively. Dialysate II would almost certainly contain a portion of the same components as dialysate I, but since the amount of ninhydrin-positive material that was produced was found to be at least six times greater with added hemoglobin, the components that result from hemoglobin digestion will be in considerably higher concentration than those resulting from the digestion of flour proteins.

Analysis indicated that the average amount of ninhydrin-positive material in dialysate I was slightly less than the equivalent of 0.05 μmoles of leucine per mg. of flour protein in the extract. The volume of dialysate was approximately equal to the volume of extract, but the dialysis conditions were such that equilibrium was not attained. Therefore the total amount of dialyzable ninhydrin-positive material produced in the extract at the end of the digestion period would probably exceed the equivalent of 0.1 μmoles of leucine per mg. of flour protein. As 1.0 mg. of flour protein on complete hydrolysis would yield approximately 10 μmoles of amino acid, it is estimated that the extent of flour proteolysis that occurred during the 24-hr. digestion period was less than 2%.

Ammonia was found in all dialysates. For example, it accounted for 30% of the ninhydrin-positive material in dialysate I. The possibility cannot be excluded that the ammonia was a contaminant, or it may have resulted from chemical hydrolysis of amide groups under the experimental conditions that were employed. However, it appears equally possible that it results from amidase activity in the extracts. Duplicate experiments resulted in essentially identical amounts of ammonia.

The separation of the residue obtained upon evaporation of dialysate I into free amino acid and peptide fractions is shown in Fig. 2. The average proportions of ammonia, free amino acids, and peptides in all three types of dialysates are shown in Table I.

To obtain an estimate of the average size of the peptides, the ratios between the amounts of ninhydrin-positive material before and after acid hydrolysis were determined. Results from several experiments indicated that the average number of amino acid residues per peptide for the peptide fractions isolated from both dialysates I and II ranged from 6 to 8. Similar analysis for all three types of unfractionated dialysates indicated an average value of approximately three residues per peptide. These values are consistent with the proportions of free amino acid and peptide reported in Table I.

TABLE I. COMPOSITION[a] OF DIALYZABLE MATERIAL

Type of Dialysate	Ammonia	Free Amino Acids	Peptides
I (from proteolysis of soluble flour proteins)	30	34	36
II (proteolysis with added hemoglobin substrate)	8	62	30
III (indigenous to the flour extract)	20	30	50

[a]As a percentage of the total ninhydrin-positive components and based on the response to ninhydrin by the modified Rosen method (8,9).

Amino acid analysis of the free amino acid fractions found in the three types of dialysate are shown in Table II. The flour extract and the peptide fractions were subjected to amino acid analysis after acid hydrolysis, and these results are included in Table II.

A comparison of the results for duplicate runs 1 and 2 reveals that there was considerable variation from run to run. For example, no detectable arginine was found in the peptide fraction of run 2, compared to over 8% in run 1. This discrepancy may be partly explained by the further observation that the free amino acid fraction was considerably higher in arginine in run 2 than in run 1.

In spite of this variation between runs, a few observations are noteworthy. The amounts of glutamine, glutamic acid, and proline in the free amino acid fraction of dialysate I are very low, especially in view of the high levels of these amino acids in the proteins of the flour extract. The method used for separating peptides from free amino acids is not considered to be particularly good for the acidic amino acids. Because of this a paper-chromatographic examination of the peptide fraction from dialysate I for free glutamic acid was performed. This did not reveal any spot corresponding to glutamic acid. Even the acid hydrolysate of this peptide fraction contains less than one half the level of glutamic acid found in the flour extract, and here again proline occurs in only trace amounts. The failure to detect glutamine may be a result of the conversion of this amino acid to pyrrolidone carboxylic acid during the isolation procedures (15). This possibility has not yet been investigated.

A preliminary experiment had indicated that phenylalanine and leucine were the only free amino acids in a type I dialysate that occurred in greater than trace amounts. The results in Table II confirm that the levels of these two are higher than all other free amino acids, and in run 1 they together accounted for over 70% of the total free amino acids. The levels of these two free amino acids are also quite high when hemoglobin is provided as an added substrate as well as in the indigenous dialyzable material in the flour extract.

Another observation is the comparatively high levels of free lysine, histidine, and arginine produced with hemoglobin as an added substrate. It may be speculated that the relatively high activity of flour proteases toward hemoglobin in comparison to the activity toward flour proteins is related to the higher levels of basic amino acids in hemoglobin.

Preliminary indications of the numbers of peptides in the peptide fractions from all three types of dialysate have been obtained by a combination of electrophoresis and chromatography on filter-paper sheets (12). The resulting peptide maps show

TABLE II. AMINO ACID COMPOSITION[a] OF VARIOUS FRACTIONS

Amino Acid	Flour Extract[b]	Free Amino Acid Fractions				Dialyzable Peptide Fractions[b]				
		From proteolysis of soluble flour proteins[c]		With added hemoglobin	Indigenous to flour extract	From proteolysis of soluble flour proteins[c]			With added hemoglobin	Indigenous to flour extract
		run 1	run 2			run 1A	run 1B	run 2		
Asp	1.9	0.0	0.0	0.0	12.9	2.5	1.2	11.5	0.4	10.1
Thr	1.7	2.3	2.7	0.2	2.2	4.7	4.6	6.8	3.1	5.2
Ser	4.4	0.0	2.2	0.5	7.2	5.5	6.6	9.2	6.8	7.4
Glu	47.6	3.1	0.0	0.0	8.9	15.7	21.9	16.9	3.3	19.6
Pro	15.8	0.0	0.0	0.0	0.0	trace	0.0	0.0	0.0	5.2
Gly	3.5	2.0	2.0	0.0	14.1	11.9	13.4	12.1	1.5	12.9
Ala	2.5	2.3	3.6	0.0	6.4	4.2	5.5	13.6	8.4	6.6
1/2 Cys	trace	trace	0.0	0.0	0.0	0.0	0.0	6.7	0.0	1.5
Val	3.5	1.6	6.0	1.2	4.1	9.3	9.0	9.4	9.1	7.4
Met	0.8	3.1	3.1	3.1	trace	0.0	0.0	0.0	2.0	0.0
Ile	3.0	2.2	7.0	0.0	0.7	4.7	6.9	3.4	0.0	6.4
Leu	5.5	25.8	25.9	14.7	22.7	6.8	5.5	3.4	20.7	6.6
Tyr	1.7	1.3	8.3	9.2	3.0	8.1	6.7	2.4	2.2	1.9
Phe	4.5	48.6	22.3	19.5	11.7	18.2	9.9	3.6	19.9	3.0
Lys	0.7	3.8	7.4	17.3	trace	trace	trace	1.0	4.6	1.2
His	1.2	0.0	0.0	11.2	trace	0.0	0.0	0.0	11.3	0.0
Arg	1.5	4.1	9.6	23.2	6.0	8.5	8.8	0.0	6.8	5.0

[a] Mole percent.

[b] Analysis after acid hydrolysis.

[c] Data from two separate experiments are presented for the dialyzable products resulting from the proteolysis of soluble flour proteins. 1A and 1B represent the leading and trailing halves, respectively, of the peptide peak from run 1.

that dialysates I and II each have only five major spots, although these maps are otherwise considerably different. Dialysate III is somewhat more complex. Five N-terminal amino acids were detected in the total peptide fractions from each of the three types of dialysate. The end groups were the same in each case, and consisted of phenylalanine, leucine, valine, glutamic acid, and lysine.

GENERAL DISCUSSION

The production of peptides averaging six to eight amino acid units in length confirms that endoprotease activity exists in the flour extracts. The occurrence of a substantial proportion of free amino acids as proteolysis products infers that exoenzymatic activity also exists. Whether the same enzyme was responsible for both types of activity remains an open question.

The general indication is that there is a considerable degree of specificity with regard to the nature of the peptide bonds that are hydrolyzed. Peptide bonds adjacent to leucine, phenylalanine, lysine, histidine, and arginine apparently show the highest degree of susceptibility to proteolysis by the flour enzymes.

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