The Proteolytic Enzymes in Wheat Flour

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ABSTRACT

A single extraction of flour with acetate buffer will solubilize less than one-half the proteolytic activity found in a flour suspension. Unextracted activity can be accounted for in the residue. Repeated extractions will solubilize most if not all of the activity. The extracted enzyme was purified fourfold by precipitating inactive protein with 0.1M NaCl. Kinetic studies confirmed that activity did not increase linearly with enzyme concentration, but the deviation from linearity became smaller as the purity of the enzyme increased. Thermal denaturation studies revealed that a part of the extractable activity is unstable at 50°C., but another part is unaffected. Sephadex gel filtration of the extract showed two enzymatically active fractions, one associated with the glutenin. On the same column the most purified preparation showed no glutenin and only one broad activity peak. Results may be interpreted on the basis of more than one enzyme or, alternatively, one enzyme which forms one or more complexes with other flour proteins.

The existence of proteolytic enzymes in wheat flour has been recognized since early in the century (1). For several years there was considerable controversy about their effect on the bread-baking properties of flour during mixing and fermentation (2). More recently, efforts have been directed toward the characterization of these enzymes (3,4). In this paper results of further investigations in this direction are presented.

MATERIALS AND METHODS

All experiments were conducted with samples of the same flour: an unbleached, untreated, top patent grade produced from HRS wheat in a commercial mill. The flour protein and ash content were 13.1% and 0.38%, respectively[1]. Storage was at -20°C.

Hemoglobin was purchased from Difco Laboratories. Standard proteins, bovine serum albumin and pepsin were purchased from Calbiochem and Nutritional Biochemicals Corp. Sephadex was purchased from Pharmacia, Uppsala, Sweden. All other chemicals were reagent grade.

Extraction of Proteolytic Enzymes

Flour and extractant were stirred together for 30 min. with cooling in an ice bath. The extractants included water, 0.2M acetate buffer at pH 3.8, and 0.2M

[1] 14% moisture basis.

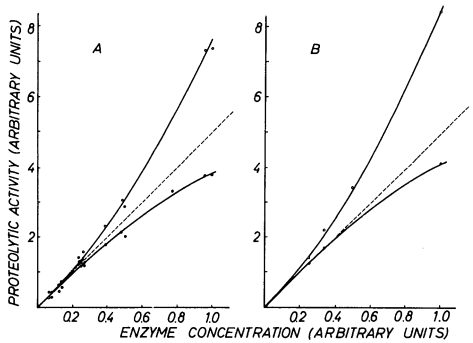


Fig. 1. The relation between proteolytic activity and enzyme concentration for a dilute acetic acid extract (A) and the most purified preparation (B). The uncorrected data fit the lower curved lines. The dashed lines are straight-line extrapolations through the uncorrected data at lower enzyme concentrations. The upper curved lines are fitted to the 3/2 power transformations of the uncorrected data.

acetic acid solution. The w./v. ratio of flour to extractant was 1:5 unless otherwise specified. The insoluble residue was removed by 10-min. centrifugation at 15,000 X g at 2°C., with the high-speed attachment of an International PR2 refrigerated centrifuge. In some cases the insoluble residue was subjected to further extraction. The residue was freeze-dried and ground to pass a No. 30 U.S. standard sieve before suspension in the extractant, or a Waring Blendor was used to form a uniform suspension with the wet residue.

Enzyme Assay

Proteinase activity was measured by the modified Ayre-Anderson method (3) with hemoglobin as the substrate. Absorbance was measured on a Beckman DU spectrophotometer. The activity was calculated with the use of a standard tyrosine curve and is expressed as μmoles of tyrosine (or equivalent) produced per min. per g. of flour (or 5 ml. of extract). A control analysis was performed with each sample analysis. These were given the same treatment as the samples, except that the 2-hr. digestion period was omitted. They are referred to as zero-time controls.

The modified Ayre-Anderson method is suitable for the measurement of activity in suspensions as well as in solution. When activity measurements of different extracts and suspensions are compared, a correction must be applied to take into account the observed enzyme kinetics (discussed in a later section). For flour extracts the activity was adjusted as follows: A standard curve similar to that in Fig. 1, A, was employed. A straight line was fitted to the uncorrected data at lower enzyme concentrations and extrapolated to higher concentrations. Uncorrected data were known to obey a relationship depicted by the lower curved line and were adjusted by conversion to the values directly above on the extrapolated straight line.

To adjust the activity of suspensions of flour or extracted residues, 3/2 power transformations of the data were used. An extract, with an uncorrected activity of 0.050 units, was selected arbitrarily as a reference standard. This value was converted to the 3/2 power as were the uncorrected activities of all suspensions. The 3/2 power transformation now determined the ratios of activity between the standard extract and any suspension. The corrected activity of the standard extract

as determined by the standard curve method was 0.060 units. The quotient of 0.060, divided by the 3/2 power transformation of 0.050 was then used as a multiplier to convert the 3/2 power transformations of uncorrected suspension activities to corrected values. All tabulated proteolytic activities have been adjusted by one of these methods unless otherwise indicated.

Nitrogen analysis was performed by the Kjeldahl method or by the Folin-Ciocalteau method (5). The latter method was standardized against the former with a series of dilutions of a 0.2M acetic acid extract of the sample flour.

Sephadex Chromatography

Chromatography was performed on a 5 X 92-cm. column of Sephadex G-100. The eluant was pumped through the column with a Büchler Micro pump at room temperature at a rate of 1.0 ml. per min. The maximum volume of sample applied was 25 ml. Fractions of 22.5 ml. were collected. When necessary the sample solution was concentrated by a combination of dialysis and pervaporation (6) before chromatography.

RESULTS AND DISCUSSION

Extractability of the Proteinases in Flour

The proteolytic activities of various extracts, flour suspensions, and suspension of the insoluble residue remaining after extraction were determined. A comparison of some of these activities is shown in Table I.

A solution of 0.2M acetic acid was found to be the most efficient in solubilizing the proteolytic enzymes in flour. However, our observations confirm the finding of McDonald and Chen (3) that the total activity of a flour suspension is considerably higher than that of any single extract of the same amount of flour.

The observation that the residue remaining after extraction still exhibits a high level of activity is contrary to the report of McDonald and Chen (3). These authors state that such residues are inactive.

A single extraction of the flour leaves much protein in the residue. Repeated extractions of the residue can solubilize additional activity as well as additional protein. The results of one such extraction sequence are shown in Table II. Activity data are presented both before and after being corrected.

The amount of flour nitrogen solubilized by sequential extraction exceeds 80% of that present in the flour.

TABLE I. SOLUBILIZATION OF FLOUR PROTEOLYTIC ENZYMES IN A SINGLE EXTRACT

Sample	N Content[a]	Total N %	Proteolytic Activity	Total Activity %
Water Extract	3.1[b]	13	0.016[b]	8
Acetate buffer extract	12.6[b]	54	0.059[b]	29
Acetic acid extract[c]	7.0[b]	30	0.072[b]	35
Flour suspension	23.5	100	0.205	100
Flour residue[d]	13.4	57	0.140	69

[a]N, mg. per g. of flour or in the extract from 1 g. of flour.

[b]Average values for three separate extracts.

[c]Flour was extracted twice with water and the residue further extracted with 0.2M acetic acid.

[d]Residue from acetate buffer extraction.

TABLE II. SOLUBILIZATION OF FLOUR PROTEOLYTIC ENZYMES BY SEQUENTIAL EXTRACTION

Extraction Sequence	N Content[a]	Total N %	Proteolytic Activity[b]	Total Activity[b] %	Proteolytic Activity[c]	Total Activity[c] %
Water	3.1	13	0.016	14	0.016	8
Water	2.5	11	0.008	7	0.008	4
Acetic acid[d]	6.3	27	0.050	44	0.060	29
Acetic acid[d]	3.9	17	0.031	28	0.035	17
Acetic acid[d]	2.1	9	0.022	19	0.023	11
Acetic acid[d]	1.6	7	0.014	12	0.014	7
Residue			0.010	9	0.010	5
Total extracts	19.5	83	0.141	123	0.166	76
Flour suspension	23.5	100	0.114	100	0.205	100

[a]N, mg. per g. of flour or in the extract from 1 g. of flour.

[b]Uncorrected.

[c]Corrected.

[d]0.2M Acetic acid solution.

Because of the magnitude and also the rather arbitrary nature of the correction applied to flour suspension activity, some uncertainty in the calculation of total solubilized activity is inevitable. It is probable that from 75 to 100% of the activity of a suspension can be accounted for in the combined sequential extracts. This result is somewhat contradictory to the hypothesis of McDonald and Chen (3). They have proposed that wheat flour possesses an extractable and a nonextractable enzyme. However, it appears that much of what they considered to be nonextractable enzyme can actually be extracted. Certainly the residue from such a series of sequential extracts is very low in activity. Other experiments have indicated that the total amount of proteolytic enzyme solubilized in a single extract can be increased considerably by increasing the volume of extracting solvent.

We have confirmed that the proteolytic activity of a flour suspension has an optimum pH of 4.4, which is 0.6 units higher than that of flour extracts. A related observation concerns the pH effect upon the proteolytic activity remaining in the flour residue after a single acetate buffer extraction. It is somewhat surprising that such residues exhibit slightly higher activities at pH 3.8 than at 4.4. This too is inconsistent with McDonald and Chen's hypothesis (3).

Purification of the Enzyme

Classic fractionation procedures using ammonium sulfate, pH adjustment, and organic solvents were re-examined extensively. Most of the results were unsatisfactory.

As shown in Table I, water alone is not an efficient solvent for extracting proteolytic activity. By extracting flour twice with water and discarding the extracts, much inactive protein was removed with only small losses of total flour activity. Furthermore, when the residue was extracted with dilute acetic acid, protein solutions could be obtained with more total activity as well as higher specific activity than with direct acetate buffer extracts of the flour. Another advantage of the preliminary water-extraction is that the zero-time control readings of the eventual acetic acid extracts are much lower.

Much additional inactive protein could be precipitated from the dilute acetic acid extract by adding solid NaCl to 0.1M concentration at pH 3.5. Progressively

TABLE III. ISOLATION OF PROTEOLYTIC ENZYMES IN WHEAT FLOUR

Purification Stage	Proteolytic Activity	N Content[a]	Activity Recovered %	Specific[b] Activity
Flour	0.205	23.5	100	8.7
Acetic acid extract	0.068	7.2	33	9.6
NaCl supernatant	0.057	1.7	28	33.6
Concentrate 3.5X	0.152	5.0	21	30.5

[a]N, mg. per g. of flour or 5 ml. of solution.
[b]Proteolytic activity X 10^3/N content.

higher concentrations of NaCl resulted in a corresponding decrease in the amount of activity remaining in solution. The precipitated enzyme would redissolve with very little loss of total activity, but none of the redissolved fractions showed enhanced specific activity.

The enzyme solution remaining, after treatment with 0.1M NaCl, could readily be concentrated three- to fourfold by a combination of dialysis and pervaporation. The use of Sephadex G-25 as a method of concentration was not successful.

Typical data on enzyme purification are presented in Table III. The over-all purification is about fourfold on the basis of the activity of a flour suspension, or sixfold on the basis of the activity of a direct extract of the flour with pH 3.8 acetate buffer.

Enzyme Kinetics

When a series of dilutions are made on a flour extract and the proteolytic activities are measured, it is observed that activity does not vary directly with enzyme concentration. It tends to be lower than expected at higher concentrations. The same observation has been made by Miller and Johnson (7) and confirmed by McDonald and Chen (3). These authors report that a straight-line relationship can be achieved by transforming the activity data to the 3/2 power. In our studies the magnitude of the deviation from a direct relationship depended on the kind of enzyme preparation that was used. For enzyme solutions prepared by treating an acetate buffer extract of flour with two volumes of cold isopropanol, and re-dissolving the resulting precipitate, the 3/2 power transformation gave a reasonably straight-line relationship. This preparation was quite low in specific activity. As the purity of the enzyme increased, the deviation from linearity became smaller. Plots of relative activity vs. relative concentration for preparations at two different stages of purification are shown in Fig. 1. The uncorrected data fall on the lower curved line; the 3/2 power transformed data[2] fall on the upper curved line. Determination of the coefficients of correlation for the purest enzyme preparation (Fig. 1, B) reveals that the uncorrected data come closer to falling on a straight line than the transformed data.

The deviation from linearity is typical of systems where some factor other than enzyme concentration is involved in limiting the reaction rate. The effect of the concentration of hemoglobin has been investigated and, under the standard conditions of the assay, the substrate concentration is not rate-limiting. The reaction mixture routinely included 10^{-3}M cysteine as an enzyme activator. The effect of cysteine concentration on proteolytic activity was also investigated. Dilute acetic acid extracts of flour showed a positive response to cysteine, the maximum

activity occurring at 4×10^{-3}M. Increasing the concentration of cysteine above 4×10^{-3} resulted in a gradual reduction in activity. The presence of less than the optimum amount of cysteine in the standard assay medium probably had a limiting effect on the rate of proteolysis at higher enzyme concentrations. However, the activity in the presence of 10^{-3}M cysteine varied from 92 to 95% of the activity with optimum cysteine. This relatively small difference is not sufficient to explain all of the observed deviation from linearity. Furthermore, the purest enzyme preparation showed no response to cysteine at all.

With increasing purity of the enzyme preparations, the data approach a linear relationship between enzyme activity and concentration. Hence, the following simple hypothesis is proposed. The enzyme is capable of forming one or more complexes with other proteins in the solution.

$$E + P \leftrightharpoons EP$$

$$K = \frac{[EP]}{[E]\ [P]}$$

The fraction of the total enzyme in the free state must decrease with increasing protein concentration in order to maintain a constant value for K. If it is assumed that the free enzyme is more active than any enzyme-protein complex, the kinetic results are readily explainable.

Thermal Denaturation of Proteolytic Activity

The proteolytic enzymes in air-dried flour are quite resistant to heat-denaturation. Heating flour in an oven at 100°C. for 12 hr. reduced the activity by only 30%. In contrast, the proteolytic activity of a dilute acetic acid extract of the flour is much less stable. At 70°C. all activity is lost in 5 min. The decrease in activity with time of heating at 50°C. is illustrated in Fig. 2. Activity decreases rapidly initially, but after approximately 30 min. and for up to 2 hr. it remains constant at a level that is more than one-half that of the unheated extract. At 40°C. a similar situation prevails on a somewhat longer time scale.

These results suggest the presence of two enzymes, one stable and the other unstable at 50°C. However, it is also consistent with the hypothesis presented earlier. The free enzyme is unstable at 50°C., but when complexed with other flour proteins it is protected against heat-denaturation.

Preheating the extracts before determining their activity results in an increase in

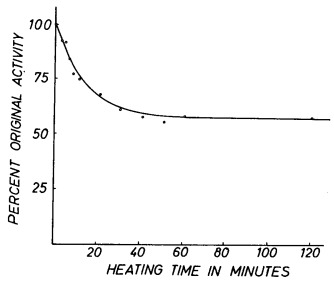


Fig. 2. Effect of heating at 50°C. on the proteolytic activity of a 0.2M acetic acid extract of wheat flour.

the zero-time control readings, no doubt because of autolysis of the flour proteins. When preheating was conducted at 40°C., it was noted that autolysis proceeded only until all the heat-labile portion of the activity had disappeared.

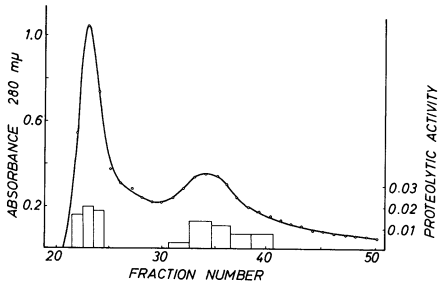
Sephadex Chromatography

The result of column chromatography on Sephadex G-100, for dilute acetic acid extracts and the most purified enzyme preparation are shown in Figs. 3 and 4. An acetic acid extract produces two peaks for both protein and proteolytic activity. The most purified preparation shows only one broad peak for both. We have applied the methods of Andrews (8) and of Leach and O'Shea (9) to estimate the molecular weights of the peak fractions. Pepsin and bovine serum albumin were used to standardize the column. The first peak for the acetic acid extract will contain the glutenin fraction and has a molecular weight too high to be estimated by this method. The second peak corresponds to a molecular weight of approximately 80,000. The protein peak and the activity peak for the most purified preparation correspond to molecular weights of 50,000 and 65,000 respectively.

The chromatographic results suggest the possibility of at least two proteolytic enzymes but can also be explained on the basis of one free enzyme plus enzyme complexed to other proteins. The fact that treatment of an acetic acid extract with 0.1M NaCl causes complete disappearance of the glutenin peak but very small losses of total proteolytic activity would appear to indicate that the salt is promoting dissociation of the enzyme from some fraction of the glutenin. This evidence cannot be regarded as conclusive, because the recovery of total activity from the G-100 columns never exceeded 50%, although protein recoveries were essentially quantitative.

GENERAL DISCUSSION

Very recently Kaminski and Bushuk (4) reported the presence of four soluble proteolytic enzymes in wheat flour. This conclusion was reached on the basis of the separation of four zones of activity by starch-gel electrophoresis. Our results are not inconsistent with the existence of more than one enzyme, but they do suggest an alternate explanation. Only one enzyme is present, but it is capable of forming



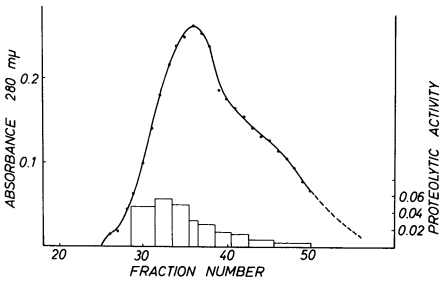


Fig. 3(left). G-100 Sephadex chromatography of a 0.2M acetic acid extract of wheat flour. The bar graph indicates the relative proteolytic activity of individual or pooled fractions.

Fig. 4(right). G-100 Sephadex chromatography of the most purified enzyme preparation. The bar graph indicates the relative proteolytic activity of individual or pooled fractions.

complexes with other proteins. This could account for several active zones on starch-gel electrophoresis.

Complex formation could also account for McDonald and Chen's observation of an extractable and a nonextractable enzyme. In a single extraction, a portion of the enzyme would remain in combination with insoluble flour proteins and thus would appear to be nonextractable.

Acknowledgment

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Literature Cited

1. FORD, J. S., and GUTHRIE, J. M. The amylolytic and proteolytic ferments of wheat flours and their relation to baking strength. J. Soc. Chem. Ind. 27: 389-393 (1908).
2. ELION, E. The action of glutathione and wheat germ on dough in relation to proteolytic enzymes in wheat flour. Cereal Chem. 20: 234-250 (1943).
3. McDONALD, C. E., and CHEN, LORA L. Properties of wheat flour proteinases. Cereal Chem. 41: 443-455 (1964).
4. KAMINSKI, E., and BUSHUK, W. Wheat proteases. I. Separation and detection by starch-gel electrophoresis. Cereal Chem. 46: 317-324 (1969).
5. COWGILL, R. W., and PARDEE, A. B. Experiments in biochemical research techniques, p. 176. Wiley: New York (1957).
6. HOFSTEN, B. V., and FALKBRINY, S. U. A simple arrangement for the concentration of protein solutions. Anal. Biochem. 1: 436-439 (1960).
7. MILLER, B. S., and JOHNSON, J. A. A single linear relationship and definition of a unit for proteinase activity. Arch. Biochem. 32: 200-206 (1951).
8. ANDREWS, P. Estimation of the molecular weights of proteins by sephadex gel-filtration. Biochem. J. 91: 222-233 (1964).
9. LEACH, A. A., and O'SHEA, P. C. The determination of molecular weights of up to 225,000 by gel filtration on a single column of sephadex G-200 at 25° and 40°. J. Chromatog. 17: 245-251 (1965).

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