The Proteolytic Enzymes of Wheat and Flour and Their Effect on Bread Quality in the United Kingdom[1]

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ABSTRACT

Proteolytic activity was measured by following the rate at which a gluten ball softened when incubated in water at 30°C., and by variations on the Ayre-Anderson method, in which a number of ways of determining soluble nitrogen were investigated. The indanetrione hydrate method was finally chosen for use. Both techniques were used to determine the proteolytic activity of native wheat flours and proteinase preparations. There was no correlation between the softening of the gluten of different wheat flours and the increase in soluble nitrogen as measured by a modified Ayre-Anderson method. Total soluble nitrogen showed a correlation with the nitrogen supplementation requirements of flour doughs. The softening enzyme was affected by the flour grade and was inhibited when the gluten was washed with dilute sodium chloride solution. The Ayre-Anderson test was not a satisfactory method of measuring this enzyme. A concept of alpha- and beta-proteinases is proposed to explain these results, the gluten-softening enzyme being termed alpha-proteinase and the soluble nitrogen-producing enzyme, beta-proteinase.

Our knowledge of the proteinases present in wheat flour is meager compared to that of the starch-degrading enzymes. To some extent this lack of knowledge can be attributed to the findings of Hites, Sandstedt, and Schaumburg (1), that in normal sound wheat the proteinases had little effect on dough properties. This was based on experiments with papain, which showed that the amount of dough-softening detected by physical means was also measured by the Ayre-Anderson test.

Most wheats grown on the North American continent do not show gluten-softening to any marked degree. However, wheats grown in the USSR and in Eastern Europe show gluten-softening which is due to the attack of the wheat bug *(Eurygaster* and *Aelia* spp.), and in Great Britain the native wheats in a wet harvest also show significant softening of the gluten. This effect can cause trouble when flour made from such wheats is used for biscuit and bread production.

The investigation reported here was carried out to assess the value of tests for proteolytic activity as a measure of gluten-softening, and to examine the enzyme or enzymes responsible.

MATERIALS AND METHODS

Wheat

Wheat was obtained from average samples of commercial cargoes. The samples were blended on a Boerner blender and screenings (dockage) removed on a mechanical laboratory separator of our own design, and then picked by hand.

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[1]Presented at the 51st annual meeting, New York, April 1966. Present address of author: Ranks Hovis McDougall (Research) Ltd., Cressex Laboratories, Lincoln Road, High Wycombe, Bucks., England.

Flour

Flour was milled from wheat prepared as above on a pneumatic laboratory Buhler mill in an air-conditioned room at 70% r.h. and 21°C. The wheat was conditioned to give a final flour moisture of 14.5% and usually gave a yield of 69 to 73% of flour, at a color grade figure (Kent-Jones & Martin) of 2.0 – 3.0. It was used without any form of treatment.

Microbial Proteinases

The microbial proteinases used in these experiments were obtained from Norman Evans & Rais Ltd. (now Associated British Maltsters Ltd.), Stockport, Cheshire. Proteinase 18 and DS 2261 were of bacterial origin, and Panzyme 50 was of fungal origin.

Hemoglobin

The Bacto-hemoglobin was obtained from Difco Laboratories, Detroit, Michigan.

Determination of Gluten-Softening

Wheat (50 g.) was ground in a hand mill of the cone type and the resulting meal sieved through 8xx silk. A portion of the resulting flour (15 g.) was mixed with water (7.5 ml.) into a dough ball, which was placed in a gluten washer (Henry Simon Ltd.) for 15 min. The last traces of starch were removed by manipulating the ball in the hand under a running cold-water tap. Gluten (3.5 g.) thus formed was immersed in tap water (pH 7.0 – 8.0 at 30°C.) in a 100-ml. beaker, and the beaker was placed in a water bath at 30°C. The softness of gluten was measured on an arbitrary scale (shown in the table below[2]) by subjective assessment at hourly intervals after it was placed in the beaker. It is not possible to test more frequently because

Table for Assessment of Gluten-Softening

Marks Arbitrary Scale of 8	*Firmness/Softness*
0	Rotten, almost liquid
1	Very soft
2	Soft
3	Soft with some firmness
4	Firm with tendency towards softness
5	Fairly firm
6	Firm
7	Firm and solid
8	Very firm

the manipulations involved would affect the results. Proteolytic activity is expressed as the rate of softening of the gluten ball.

Flour was treated in exactly the same way as wheat except for the initial grinding and sieving.

Modified Ayre-Anderson Method

The modified Ayre-Anderson method was based on a paper by Hites *et al.* (2). Flour (10 g.) in a wide-necked, stoppered bottle was mixed with buffer solution (50 ml.) made by mixing 2.5 ml. of 4*M* acetate buffer,

[2]E. A. Farrand (unpublished).

pH 4.7, with 47.5 ml. water. When Bacto-hemoglobin was used, 1.25 g. of this was mixed with the flour before addition of the buffer solution. Two suspensions were made for each flour to be tested; one was treated immediately with 45% aqueous sodium tungstate solution (4 ml.) and 5*M* trichloroacetic acid solution (12 ml.); the other was incubated at 37°C. for 3 hr. before being treated. Fifteen minutes after the treatment, the suspensions were centrifuged at 11,000 r.p.m. (14,500×*g*) at 0°C. for 10 min. The supernatants were then decanted through No. 4 Whatman filter paper, and the solutions were tested for soluble nitrogen.

Biuret Method

The normal biuret method for testing protein solutions was insufficiently sensitive for the solutions obtained by the Sandstedt method, and the modification of Lowry *et al.* (3) was used.

A sample of the protein solution to be measured (0.2 ml.) was placed in a test tube and 1 ml. of reagent C was added. (Reagent C consists of a freshly prepared mixture of 2% sodium carbonate in 0.1*N* sodium hydroxide (50 ml.), and 0.5% copper sulfate pentahydrate in 1% sodium potassium tartrate (1 ml.).) The tubes were shaken well and allowed to stand for 10 min. at room temperature. Folin-Ciocalteu reagent (0.1 ml.), at concentration 0.1*N* to NaOH using phenolphthalein as indicator, was added rapidly, and after a further 30 min. at room temperature, the absorbances of the solutions were read on the spectrophotometer at 750 mμ.

Indanetrione Hydrate (Ninhydrin) Method

The method used required the preparation of a reagent (4) which consisted of indanetrione hydrate (4% solution in deionized 2-methoxyethanol, 50 ml.), deionized water (25 ml.), and 4*M* sodium acetate buffer, pH 5.5 (25 ml.). Stannous chloride (0.08 g.) was added and nitrogen was bubbled through the solution to remove residual oxygen. The protein solution to be tested (2 ml.) was mixed with the indanetrione hydrate solution (2 ml.) in a test tube, and the test tube was immediately placed in a boiling-water bath for 30 min. After cooling, the solution in the tubes was made up to 10 ml. with 50% aqueous ethanol solution, and the absorbance was determined at 570 mμ.

Determination of Absorbance

All readings of absorbance were carried out with a Uvispek spectrophotometer (Hilger & Watts Ltd.) with a glass prism for the visible wave length and a silica prism for the ultraviolet. If the absorbance exceeded a value of 1.0, dilutions were made to bring the reading below 1.0, and the reading was multiplied by the dilution factor.

RESULTS

Determination of Soluble Nitrogen in the Modified Ayre-Anderson Test

The published method for determination of proteinase in flour, AOAC (5) and AACC (6), uses Bacto-hemoglobin as a substrate for the proteolytic enzymes, and determines the amount of soluble nitrogen produced under standard conditions of temperature and time.

The soluble nitrogen is determined by micro-Kjeldahl. Because this is a time-consuming analysis which is not very sensitive, three other methods of determining soluble nitrogen were investigated. These were the ultraviolet absorption at 280 mμ (7), the Lowry modification of the biuret test (3), and the indanetrione hydrate (ninhydrin) method (4).

The ultraviolet absorption method relies on certain aromatic amino acids which absorb in the ultraviolet at 280 mμ. Unfortunately, nucleic acids, which have a peak absorption at 260 mμ, also absorb at 280 mμ to some extent. By measuring the ratio of the 260- and 280-mμ absorptions it is possible to make a correction for the presence of nucleic acids (7).

The results in the table below were obtained after fivefold dilution of the hydrolysate from the modified Ayre-Anderson method; the flour was No. 2 Manitoba.

Absorbance of No. 2 Manitoba Flour Hydrolysate from the Ayre-Anderson Test

	Absorbance, 280 mμ	*Absorbance, 260 mμ*	*Ratio, 280/260 mμ*
Duplicate test	0.389	0.803	0.484
	0.331	0.732	0.452

The 280/260-mμ absorption ratio was significantly below 0.7, indicating that a considerable quantity of nucleic acid was present and that the method would not therefore be a reliable way of estimating soluble nitrogen (7). Further experiments with other wheats confirmed this conclusion.

The results obtained with the Lowry modification of the biuret test and the indanetrione hydrate method are shown in Table I.

TABLE I

COMPARISON OF THE INDANETRIONE HYDRATE AND LOWRY METHODS OF DETERMINING SOLUBLE NITROGEN IN THE AYRE-ANDERSON TEST

	INDANETRIONE HYDRATE (Absorbance 570 mμ)			LOWRY (Absorbance 570 mμ)		
SUBSTRATE	Zero Time	3 Hours	Difference	Zero Time	3 Hours	Difference
No. 2 Manitoba	0.477	0.641	0.164	0.180	0.220	0.040
No. 2 Manitoba + hemoglobin	1.257	2.840	1.583	.231	.331	.100
Russian	0.431	0.584	0.153	.177	.217	.040
Russian + hemoglobin	1.210	2.550	1.340	0.241	0.330	0.089

It can be seen that the indanetrione hydrate method was the most sensitive and that, as found by Hites *et al.* (2), the flour samples with added Bacto-hemoglobin gave the greatest increase in soluble nitrogen. The indanetrione hydrate method was consequently adopted for the determination of soluble protein in this investigation.

Changes in Soluble Nitrogen in Various Flours

The changes in the soluble nitrogen content of a number of Buhler-milled flours during autolysis of flour buffer mixtures, as determined by the modified Ayre-Anderson method, are shown in Table II. The proteolytic activity, as measured by the increase in soluble nitrogen, showed that the most proteolytically active wheats were Scotch, Weak English, and Koga, in

TABLE II

SOLUBLE NITROGEN DETERMINATIONS ON VARIOUS FLOURS
(Average figures; indanetrione hydrate method, absorbance at 570 mμ)

FLOUR AND NO. TESTED		ZERO TIME	3 HOURS	DIFFERENCE
5	Scotch	0.302	0.740	0.438
1	Plate	0.311	0.584	0.273
10	Manitoba No. 2	0.332	0.543	0.211
3	Russian	0.334	0.538	0.204
1	Koga	0.214	0.517	0.303
3	Hard winters	0.381	0.509	0.128
1	Weak English	0.200	0.506	0.306
1	French	0.243	0.500	0.257
2	Manitoba No. 3	0.320	0.470	0.150
3	U.S. soft white	0.200	0.448	0.248
1	Australian	0.194	0.382	0.188
2	Black Sea Russian	0.223	0.380	0.157
1	Canadian Eastern white	0.170	0.334	0.164

that order. It should be pointed out that the Scotch samples were probably not representative of the crop as a whole for that year (1961), as they were specially selected for their proteolytic activity. The Russian samples showed a relatively low level of proteolytic activity when measured by this test.

Significance of Soluble Nitrogen Determinations by the Ayre-Anderson Method

It has been known for some time (8) that yeast requires soluble nitrogenous compounds to grow and ferment at its optimum rate. Normally an ample amount of such compounds is present in doughs, but some flours are occasionally found to give poor gas production. In these cases the gassing rate can be increased markedly by the addition of a nitrogen-containing compound such as ammonium chloride.

The results shown in Table II were obtained under conditions of pH, temperature, and time which are similar to those of conventional dough-making. The zero-time reading gives the amount of soluble nitrogenous material originally present in the flour, and the 3-hr. figure represents the sum of the initial figure and the soluble nitrogen produced by the proteolytic enzymes. The total amount of soluble nitrogen available in the dough is therefore given by the 3-hr. figure.

The lowest figures for soluble nitrogen (Table II) after 3 hr. are for Canadian Eastern White, Black Sea Russian, Australian, and U.S. soft white wheats. Canadian Eastern and U.S. soft white wheats are not usually included in bread flour grists, but Australian and Black Sea Russian are so used, and our routine tests showed that these were the two wheats in this table which required nitrogen supplementation.

The commercial implications of this finding are not very great, since most bakers in the United Kingdom use bakery additives which contain nitrogen salts, and this would obviate any natural deficiency in the flour. However, if new varieties of wheats were being examined by a test-baking procedure which did not include soluble nitrogen salts, then it would be possible for a poor baking performance to be recorded because of lack of soluble nitrogen and consequently poor fermentation.

Gluten-Softening

The gluten-softening test as given in the Methods section is one which has been used in these laboratories for a number of years on routine samples of native and imported wheats. An untrained person can easily distinguish between two units on the scale (see "Table for Assessment" above), and some idea of the gluten-softening effects observed by this method can be seen in Fig. 1. In most experiments assessments were made every hour and overnight (about 20 hr.). The method has the advantage of requiring only a small sample of the material to be analyzed, and of being independent of artifacts caused by variations in water-absorption and starch damage. It was therefore chosen for the measurement of gluten-softening in preference to the dough farinograph method of Johnson and Miller (9).

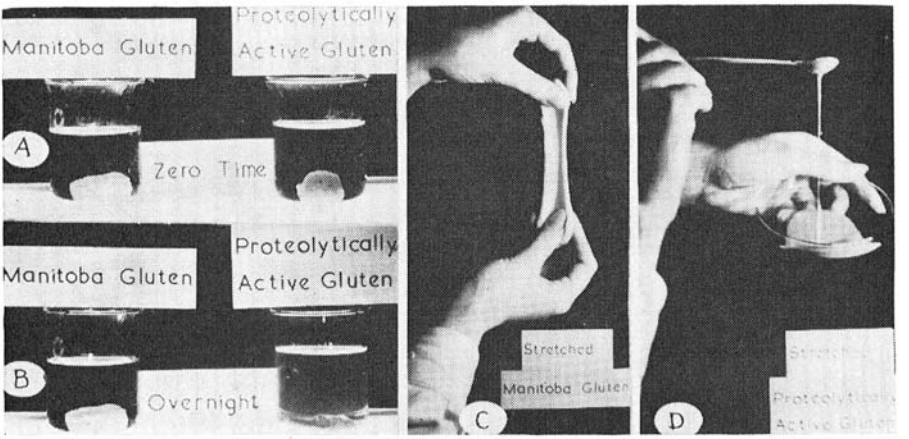

Fig. 1. A, Washed-out gluten, zero time; B, washed-out gluten 20 hours later; C, stretched Manitoba gluten; D, stretched proteolytically active gluten.

Comparison of Tests for Gluten-Softening and Soluble Nitrogen, Using Microbial Proteinases

The gluten-softening and modified Ayre-Anderson tests were compared in two experiments with microbial enzymes. In the first of these the effect of increasing concentrations of Proteinase 18 was examined (Table III and Fig. 2). It can be seen from these results that both methods can be used to measure the amount of enzyme added, although in neither case is there a directly proportional relationship.

In the second experiment the gluten-softening test was compared with

TABLE III

EFFECT OF QUANTITY OF PROTEINASE ON THE SOFTENING OF MANITOBA GLUTEN[a]

ADDITION	ZERO TIME	1 HOUR	2 HOURS	3 HOURS	4 HOURS	5 HOURS	20 HOURS
mg.							
No addition	5	5	5	5	5	5	5
11.2	5	5	4	4	4	3	3
56.0	5	5	4	3	2	2	0
112.0	5	4	3	2	2	1	0
168.0	5	3	2	2	1	1	0

[a] Softening expressed in units of the "Table for Assessment of Gluten-Softening."

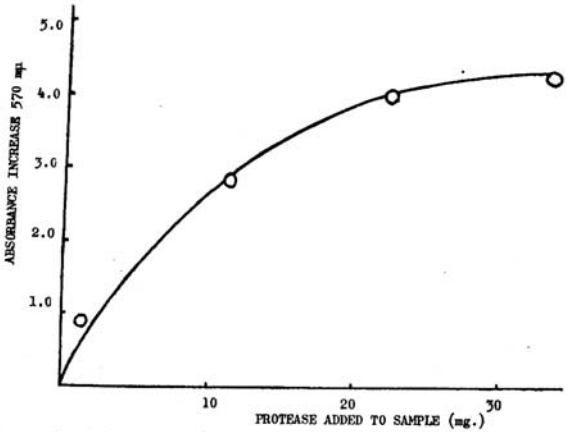

Fig. 2. Effect of addition of Proteinase 18 on production of soluble nitrogen in the Ayre-Anderson test, as measured by the increase in absorbance at 570 mμ.

the modified Ayre-Anderson method, with flour and flour with hemoglobin used as substrates, to determine the relative activity of three microbial proteinases. An aliquot (11.2 mg.) of each proteinase was added to the Ayre-Anderson reaction flasks and to the beakers containing the glutens. The results of the Ayre-Anderson determinations are shown in Table IV and those of the gluten-softening method in Table V.

The Ayre-Anderson results show that Proteinase 18 was the most active with the flour, and Panzyme 50 the most effective with hemoglobin. Under

TABLE IV

SOLUBLE NITROGEN DETERMINATIONS ON MICROBIAL PROTEINASES

EXPERIMENT	SOLUBLE NITROGEN INCREASE (ABSORBANCE 570 mμ)	CORRECTED [a]	EXPERIMENT	SOLUBLE NITROGEN INCREASE (ABSORBANCE 570 mμ)	CORRECTED [a]
Hemoglobin only	0.037	....	Russian	0.176	....
+ Enzyme DS 2261	0.185	0.148	+ Enzyme DS 2261	0.272	0.096
+ Proteinase 18	0.115	0.078	+ Proteinase 18	0.338	0.162
+ Panzyme 50	0.226	0.189	+ Panzyme 50	0.196	0.020
Manitoba	0.187	....	Enzyme DS 2261	—0.004	....
+ Enzyme DS 2261	0.223	0.036	Proteinase 18	0.007	....
+ Proteinase 18	0.374	0.187	Panzyme 50	—0.003	....
+ Panzyme 50	0.248	0.061			

[a] Corrected for soluble nitrogen produced by substrate alone.

TABLE V

SOFTENING ACTION OF MICROBIAL PROTEINASES ON NO. 2 MANITOBA GLUTEN [a]

	ZERO TIME	1 HOUR	2 HOURS	3 HOURS	4 HOURS	5 HOURS	20 HOURS
No addition	5	5	5	5	5	5	5
Enzyme DS 2261	5	5	3	3	2	2	0
Proteinase 18	5	5	3	2	2	1	0
Panzyme 50	5	5	4	4	4	4	1

[a] Softening expressed in units of the "Table for Assessment of Gluten-Softening."

the gluten softening method, Proteinase 18 showed the most proteolytic activity, whereas Panzyme 50 had little effect. The two methods therefore agreed fairly well when flour was used as a substrate, but not when hemoglobin was used. It was concluded that the use of hemoglobin as a substrate in the Ayre-Anderson test was likely to be seriously misleading as a measure of the action of the microbial proteinases on the flour. Since the two substrates (flour and hemoglobin) are very different proteins, this result is not surprising. As a corollary to this conclusion, it was assumed that the use of hemoglobin as a substrate would also be misleading as a measure of the activity of the natural proteolytic enzymes of flour on the flour proteins.

Comparison of Gluten-Softening and Ayre-Anderson Tests for Measuring Proteolytic Activity

An attempt was made to correlate the gluten-softening exhibited by some flours with the production of soluble nitrogen in the Ayre-Anderson test. As gluten-softening occurred only after about 5 hr. at 30°C. with certain Russian and English hand-ground flours, the same conditions were used for the soluble nitrogen test. The results are shown in Table VI.

TABLE VI

COMPARISON OF GLUTEN SOFTENING AND SOLUBLE NITROGEN DETERMINATIONS ON FLOUR

Flour	Soluble Nitrogen (Absorbance 570 mμ)			Gluten-Softening[a]		
	Zero Time	5 Hours	Diff.	Zero Time	5 Hours	Diff.
Hand-ground Russian	0.438	1.207	0.769	5	3	2
Hand-ground English	0.288	0.876	0.588	5	2	3
Hand-ground Manitoba	0.406	1.236	0.830	5	5	0

[a] Softening expressed in units of the "Table for Assessment of Gluten-Softening."

The most proteolytically active wheat on the gluten-softening test is the least active on the soluble nitrogen test; more significant still, the Manitoba wheat, which does not exhibit gluten-softening under these conditions, is the most proteolytically active wheat on the soluble nitrogen test.

In later experiments, two extremely active Scotch wheats were obtained which gave glutens showing considerable gluten-softening, even when made from Buhler-milled flours. These were also tested for soluble nitrogen production with the results shown in Table VII. In this case the Scotch wheat did show a greater production of soluble nitrogen than the Manitoba.

The proteolytic activity of the Russian sample was attributed to the wheat bug, since the grains exhibited the characteristic type of damage asso-

TABLE VII

COMPARISON OF GLUTEN-SOFTENING AND SOLUBLE NITROGEN DETERMINATIONS ON BUHLER-MILLED FLOUR

Flour	Soluble Nitrogen (Absorbance 570 mμ)			Gluten-Softening[a]		
	Zero Time	5 Hours	Diff.	Zero Time	5 Hours	Diff.
Manitoba No. 2	0.300	0.518	0.218	5	5	0
Scotch	0.300	0.750	0.450	5	3	2

[a] See "Table for Assessment of Gluten-Softening."

ciated with this pest. The activity of the Scotch wheat samples was due to native enzymes, since the wheat bug does not occur in Scotland.

It appeared from these experiments that gluten-softening and the production of soluble nitrogen were not directly related except where there was excessive proteolytic activity. The degree of gluten-softening in these cases was such that on the normal gluten test the firmness value fell to zero within an hour after the test.

Effect of Extraction Rate on Proteinase Activity

When flours from the same wheats produced by milling on the Buhler mill and hand-grinding and sieving were compared, it was found that the gluten-softening was much greater in the hand-milled flours. This is in agreement with previous work (10), and indicates that if proteolytic activity as evidenced by gluten-softening is a problem, a wheat which produced a satisfactory dough when made into a white flour might have a deleterious effect if used in a whole-meal flour.

Solubility of the Gluten-Softening Enzyme in Water

Since the gluten-washing process consists essentially of washing the dough with water, it seemed unlikely that the gluten-softening enzyme would be water-soluble: if this were the case it would be removed with the starch. To check this assumption, two proteolytically active glutens from a Scotch wheat were put into a beaker of water containing an inactive Manitoba gluten. The two glutens were arranged in the beaker so that they were not quite touching, and they were then tested for softening every hour. The results

TABLE VIII

SOFTENING OF PROTEOLYTICALLY ACTIVE AND NONPROTEOLYTICALLY ACTIVE GLUTENS IN WATER[a]

CONTENTS OF BEAKER	ZERO TIME	1 HOUR	2 HOURS	3 HOURS	4 HOURS	OVER-NIGHT
Manitoba alone	5	5	5	5	5	5
Scotch A alone	3	3	2	1	2	0
Scotch B alone	1	0	0	0	0	0
Manitoba	5	5	5	5	5	5
Scotch A	3	3	2	2	1	0
Manitoba	5	5	5	5	5	5
Scotch B	1	0	0	0	0	0

[a] Softening expressed in units of the "Table for Assessment of Gluten-Softening."

are shown in Table VIII. No transfer of proteolytic activity as measured by this test, occurred between the proteolytic Scotch and the Manitoba glutens, even after they had been left for 24 hr.

Solubility of the Gluten-Softening Enzyme in Salt Solutions

Since the enzyme did not appear to be water-soluble, an attempt was made to extract it with dilute salt solution, which is known to dissolve globular proteins. A proteolytically active Scotch wheat flour was washed in the gluten-washer with a 0.2*M* sodium chloride solution to remove the starch. After 15 min. the salt solution was replaced by tap water for a further 15

TABLE IX

EFFECT OF WASHING WITH SALT SOLUTION ON GLUTEN SOFTENING

FLOUR	WASHING		GLUTEN-SOFTENING[a]				
	Salt	Water	1 Hour	2 Hours	3 Hours	4 Hours	Overnight
	min.	*min.*					
Scotch	0	30	0	0	0	0	0
Scotch	15	15	4	3	3	3	0

[a] Softening expressed in units of the "Table for Assessment of Gluten-Softening."

min. of washing. The gluten ball was then placed in a beaker of water and tested every hour in the usual way. A control gluten was obtained from the same wheat washed for 30 min. with tap water. Tests showed that the residual salt in both glutens was below 0.3%. The results are shown in Table IX. The gluten washed with salt solution showed very much reduced proteolytic activity compared with the control. A further test on the same wheat (Table X) showed that a 0.01*M* solution had no detectable effect, and the inhibition rose to a maximum at a concentration of 0.1*M* sodium chloride.

TABLE X

INHIBITION OF GLUTEN-SOFTENING ENZYME BY SALT

SALT CONC., NaCl	GLUTEN-SOFTENING[a]				
	1 Hour	2 Hours	3 Hours	4 Hours	Overnight
M					
0.00	0	0	0	0	0
0.01	0	0	0	0	0
0.02	1	0	0	0	0
0.05	3	3	3	2	0
0.10	4	4	3	3	0
0.20	4	3	3	3	0

[a] Softening expressed in units of the "Table for Assessment of Gluten-Softening."

Since the washing of the gluten with dilute salt solution inhibited the proteolytic activity, it seemed reasonable to assume that the enzyme could have been leached out with the washings. Therefore, to examine these washings for proteolytic activity, they were added to a Manitoba gluten prepared in the normal way. No inhibition was observed. Attempts were also made to concentrate the enzyme by ammonium sulfate precipitation followed by dialysis to remove the residual ammonium sulfate. The results of this experiment were equivocal. No proteolytic activity could be detected by the softening of the gluten in the first 5 hr., but when it was left overnight some softening occurred. The degree of softening was difficult to assess, because the glutens usually floated to the surface and appeared to be fermenting. The long time necessary to produce the softening meant that the possibility of bacterial infection could not be ruled out, and no conclusive evidence of the presence of proteolytic enzymes in the washings was obtained.

Inhibition of Proteolytic Activity by Salt

It was possible that the effect of salt on gluten-softening was caused by inhibition of the enzymes (11) rather than their removal in the washings.

An experiment was therefore carried out in which a gluten washed in the normal way was immersed in salt solution. The effect of the immersion was to toughen the gluten considerably, and no decrease in the firmness figure could be obtained. In a further experiment, the Brabender resistance of a proteolytically active flour dough containing salt (after 5 min. of mixing on the farinograph) was determined at hourly intervals during incubation at 30°C. A control dough with no salt added was also used, but this became so sticky after 1 hr. that it could no longer be tested. The results (Table XI) showed that the dough with added salt still showed considerable proteolytic activity as measured by the decrease in farinograph resistance values, although its initial resistance was tougher than that of the dough with no salt.

TABLE XI

EFFECT OF SALT ON PROTEOLYTIC ACTIVITY OF DOUGH AS MEASURED BY THE DECREASE IN FARINOGRAPH RESISTANCE (B.U.) AFTER 5 MINUTES OF MIXING

	TIME AT 30°C.				
SAMPLE	Zero	1 Hour	2.5 Hours	3.5 Hours	4.5 Hours
Flour dough without salt (51% water)	580	360	.....	.....	.....
With 1.05% salt (51% salt solution)	630	450	390	340	310

The effect of salt on the proteolytic activity as measured in the test for soluble nitrogen was also investigated. Salt (to give 0.2*M* solution) was added to the buffer in which the samples were incubated, and the effect on the production of soluble nitrogen was noted. The results are given in Table XII. Whereas the salt increased the production of soluble nitrogen in the Manitoba flour, it decreased the production in the Scotch wheat flour.

TABLE XII

EFFECT OF SALT ON PRODUCTION OF SOLUBLE NITROGEN[a]

	ABSORBANCE AT 570 mμ		
FLOUR AND TREATMENT	Zero Time	3 Hours	Difference
Manitoba	0.311	0.541	0.230
Manitoba + salt	.295	.559	.264
Scotch	.303	.734	.431
Scotch + salt	0.319	0.712	0.393

[a] All results the mean of three separate determinations.

DISCUSSION

It is now clear that the proteolytic activity exhibited by wheat and flour is due to the action of more than one enzyme. McDonald and Chen (12) found evidence for extractable and unextractable enzymes with differing pH optima by a variation on the Ayre-Anderson technique for determination of soluble nitrogen. Johnson and Miller (9), using the method of decrease in farinograph consistency, also found two proteinases, one of which was in-

hibited by oxidizing agents; and a number of enzymes in malted wheat flour with differing activities on different substrates were found by Johnson *et al.* (13).

The results presented in this paper indicate the presence of at least two enzymes with differing action, one of which results in gluten-softening and the other in production of soluble nitrogen as measured by the modified Ayre-Anderson test. It is proposed to refer to these enzymes as alpha- and beta-proteinases respectively, a terminology similar to that employed for the amylases.

The alpha-proteinase is postulated as an endopeptidase, breaking protein chains in the middle and giving rise to a high degree of gluten-softening, but little or no production of soluble nitrogen fragments as measured by the Ayre-Anderson test. There is no direct evidence that this enzyme is a proteinase, and other types of enzymes (such as reductases acting on sulfhydryl groups) could be envisaged. The beta-proteinase is postulated as an exopeptidase, removing small fragments of protein or amino acids which would be soluble under the conditions of the Ayre-Anderson test, but (unless present in excessive amounts) unable to produce any measurable degree of gluten-softening.

The hypothesis of alpha- and beta-proteinases as described above would be in accordance with the results found in this investigation. Manitoba wheats can be envisaged as having a large amount of the beta-enzyme, but no detectable alpha-enzyme. The English and Scotch wheats, on the other hand, contain sufficient alpha-enzyme to soften the gluten, but insufficient beta-enzyme to make their figures for soluble nitrogen greater than that of the Manitoba wheat. When excessive enzyme activity is present (see table VII) there is an increase in production of soluble nitrogen; this could be due to the excess of alpha-proteinase, resulting in the availability of more end chains for the beta-enzyme or to concurrent production of more beta-proteinase under the conditions leading to the excess of the alpha-enzyme. The Russian samples which exhibited gluten-softening all showed evidence of wheat-bug damage to the grain. The enzyme secreted by the wheat bug could therefore be classified as an alpha-proteinase, although it probably differs considerably from the native alpha-proteinase of wheat. It is possible that variation in the activity of the alpha-proteinase could be responsible for the differences attributable to "gluten quality" in the baking and rheological properties of flours with otherwise similar analyses.

The gluten-softening technique can be used to determine the amount of natural proteinase, proteinase due to the wheat bug, or added proteinase. With added proteinase it is possible to use more accurate and less subjective methods, but the effect of the proteinases on the rheological properties of the dough will vary and therefore must first be established by some similar technique, measuring gluten or dough-softening.

The modified Ayre-Anderson test does not measure the alpha-enzyme, although by definition it is used to measure the production of soluble nitrogen by the beta-proteinase. The use of hemoglobin in the official method could be misleading, since the results giving relative activities of microbial pro-

teinases indicated that their action on hemoglobin was different from their action on the native flour proteins. This applied to both the bacterial and fungal enzymes, in contrast to the findings of Bowlby *et al.* (14) that fungal and wheat malt enzymes gave similar results with both the Ayre-Anderson hemoglobin method and dough-softening measured by the farinograph technique. In view of the knowledge now available on the structure of hemoglobin (15) and flour proteins (16), which indicates that they are very dissimilar proteins indeed, any correlation of the action of proteinases on the two substrates must be purely coincidental, and it must not be assumed that results obtained with hemoglobin as a substrate are necessarily applicable to the action of enzymes on the native flour proteins.

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