Effects of Proteolytic Enzymes on Gluten as Measured by a Stretching Test[1]

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ABSTRACT

The stretching characteristics of gluten are found to be changed markedly upon incubation with proteolytic enzymes. This enables a determination of the relative softening effects owing to different proteolytic enzymes. A number of other enzymes have been tested and found to have no effect. Changes in physical properties of gluten upon incubation were also examined on the farinograph and alveograph. Increasing concentration of enzyme caused a progressive decrease in gluten consistency on the farinograph and a reduction in bubble size on the alveograph.

It is well known that gluten proteins are responsible for the peculiar and extremely important properties of doughs made from wheat flour. Any changes in the nature of these proteins can have an improving or deleterious effect on the quality of bread baked from such doughs. Proteolytic enzymes are often added as supplements in breadmaking and cause a "mellowing" effect on dough, undoubtedly through alterations of gluten proteins. Tests that are commonly used at present to measure proteolytic enzymes, based on the liberation of TCA-soluble nitrogen from large molecular-weight proteins such as hemoglobin or casein, do not always correlate with the observable softening effect of the proteolytic enzyme upon gluten. A reason for this is that such tests do not distinguish between exopeptidases, which cleave peptide bonds near the ends of the chain causing very little gluten softening, and endopeptidases which cleave proteins in the middle and are responsible for a softening effect upon gluten. Wheat is known to contain both kinds of proteolytic enzymes (1,2,3,4), and it has been observed that the modified Ayre-Anderson method with hemoglobin as substrate does not measure the enzyme responsible for gluten softening (4). Tests based on directly observable softening effects upon gluten are therefore required.

This paper describes a study on the reaction between proteolytic enzymes and gluten in order 1) to gain a better understanding of the changes in physical properties of gluten due to these enzymes, and 2) to see if such changes can be used as a test to assess the relative softening effect of proteolytic enzymes upon gluten. The stretching test, along with the farinograph and alveograph, is examined as a possible tool to observe these phenomena.

MATERIALS AND METHODS

The enzymes used in this study were: papain, bromelin, ficin, beta-amylase (barley), Mann Research Laboratories, New York, N.Y.; lipoxidase (soybean), peroxidase (horseradish), Sigma Chemical Co., St. Louis, Mo.; lipase (wheat germ), alpha-amylase (hog pancreas), Nutritional Biochemicals Co., Cleveland, Ohio;

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trypsin, Worthington Biochemical Corp., Freehold, N.J. Fungal protease sample was kindly supplied by Miles Chemical Co., Clifton, N.J. Gluten was prepared from commercially milled, straight-grade flour from Canadian hard red spring wheat. The flour contained 13.2% protein and 0.48% ash on a 14.0% moisture basis.

Preparation of Dried Gluten

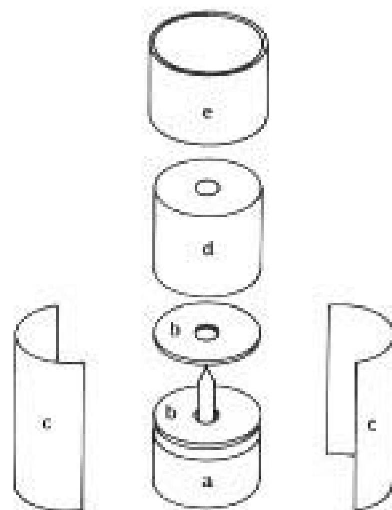
Dried gluten was prepared as described by Doguchi and Hlynka (5) with distilled water used in place of 0.001M sodium chloride to prevent possible enzyme inhibition by salt. Dried gluten used in the stretching tests contained 12.4% moisture and 84.4% protein on a dry basis; that used in farinograph and alveograph studies contained 11.3% moisture and 85.1% protein on a dry basis.

Proteolytic Enzyme Activity

Proteolytic enzymes were assayed by AACC Method 22-62 (6) with hemoglobin as substrate at pH 4.7 and temperature of 40°C. Under these conditions one HUT unit is the amount of enzyme that produces in 1 min. a hydrolysate whose absorbance at 275 nm. is the same as that of a solution containing 1.10 γ/ml. tyrosine in 0.006N HCl.

Amylolytic Activity

This was determined by the dinitrosalicylic acid (DNS) method of Bernfield (7) as described previously (8).



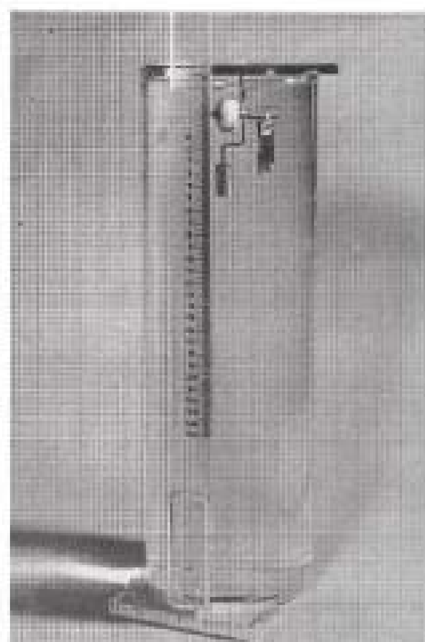


Fig. 1 (left). Apparatus for forming gluten doughnut.

Fig. 2 (right). Gluten-stretching apparatus with gluten doughnut in place.

Gluten-Stretching Test for Assay of Proteolytic Enzymes

The test consisted of incubating gluten with enzyme for 2 hr. followed by a determination of the stretching characteristics of the gluten in a manner similar to that described by Kaminski and Halton (9). The increase in rate of stretching of a gluten containing a certain amount of proteolytic enzyme compared to a gluten containing no enzyme can be used as a measure of the effectiveness of the enzyme to soften gluten.

Dried gluten (2.5 g.) was added with gentle stirring to 10 ml. of enzyme solution in a 20-ml. beaker. The resulting suspension was incubated for 2 hr. at 25°C. The gluten was removed from the beaker, formed into a ball, and excess water worked out of the gluten by hand until a weight of exactly 6.0 g. was obtained. The laboratory-designed device shown in Fig. 1 was next used to shape the gluten into a doughnut-like form. The device consisted of two aluminum cylinders, a and d, 2.5 cm. in diameter, with a pin 0.45 cm. in diameter and 2.6 cm. high in the lower cylinder which fitted into a corresponding hole in the upper cylinder. The gluten ball was impaled on the pin and sandwiched between the two cylinders. Teflon washers, b, were used to keep the gluten from sticking to the walls of the cylinder. Two polyethylene sleeves, c, held in place by a polyethylene ring, e, were used to encapsulate the cylinders. The entire assembly was placed in a 25°C. water bath and a 500-g. weight placed on top for 30 min. The formed gluten doughnut was then placed on the dough-stretching assembly under water as shown in Fig. 2. The apparatus consisted of a split pin assembly held together by a clip. A 9.8-g. weight (in air) was attached to the lower portion of the split pin. The gluten doughnut was placed on the split pin directly over the attached weight. The stretching experiment was started by removing the clip which in turn allowed the lower pin with attached weight to pull the gluten down. The extension of the gluten was read at various times using a centimeter scale. Experiments were carried out in duplicate. Control experiments were carried out with water or heat-inactivated enzymes.

RESULTS AND DISCUSSION

Table I indicates the enzymes that have been subjected to the described stretching test and denotes their proteolytic activity (AACC Method 22-62) and amylase activity (DNS method).

TABLE I. PROTEOLYTIC AND AMYLOLYTIC ACTIVITY OF ENZYMES USED IN GLUTEN STRETCHING TEST

Enzyme	Protease Activity HUT/mg.	Amylase Activity DNS units/mg.
Bromelin	192	0
Papain	13.1	0
Ficin	1,068	0
Trypsin	199	0
Fungal protease	34.1	15.2
Pronase	172	0
Lipase	0	6.5
Catalase	0	0
Lipoxidase	0	54
Beta-amylase	0	9.6
Peroxidase	0	0
Alpha-amylase	0	185

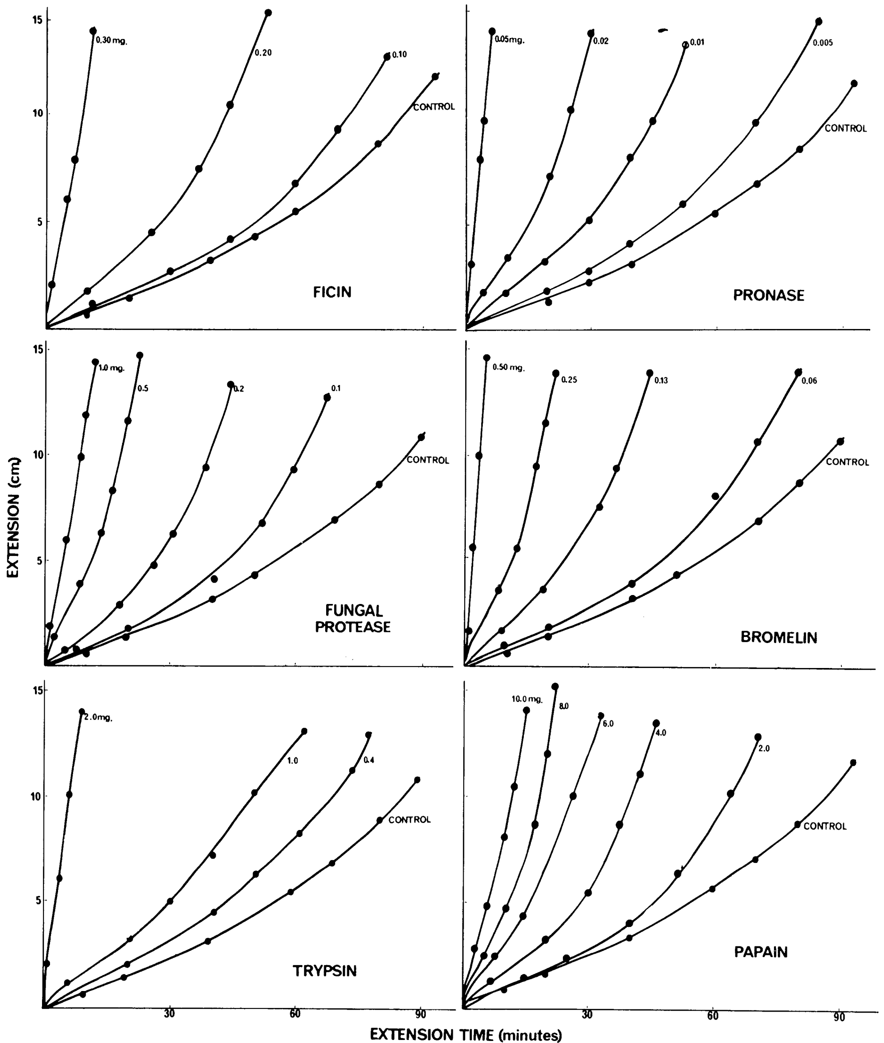


Fig. 3. Effect of ficin, pronase, fungal protease, bromelin, trypsin, and papain on the stretching characteristics of gluten.

The effects that papain, bromelin, ficin, pronase, trypsin, and fungal protease at various concentrations (mg. per 10 ml. solution) have on the stretching characteristics of gluten are shown in Fig. 3. It can be seen that the rate of gluten extension increases with enzyme concentration. The various enzymes vary enormously in their ability to bring about such changes, i.e., 0.05 mg. pronase having a larger effect than 10.0 mg. papain. Heat-treated enzyme samples (100°C. for 10 min. in a water bath) at the highest concentration shown in the figure were

tested and found to have no effect, indicating that nonenzymatic components were not responsible for these differences.

The mean rate of stretching of these enzymes based on the first 5 to 20 min. of stretching vs. their respective proteolytic activities toward hemoglobin is shown in Fig. 4. It is seen that the effect of different proteolytic enzymes as judged by the modified Ayre-Anderson method does not reflect the same ability to degrade gluten. This is now generally recognized and should not be expected in view of the difference in structure between hemoglobin and gluten proteins. Small amounts of pronase, bromelin, and papain in terms of HUT units are particularly effective in degrading gluten, perhaps reflecting their nonspecific nature in hydrolyzing peptide bonds.

Effect of Other Enzymes on the Gluten-Stretching Test

A number of other enzymes were tested to see if they would also influence this test. Beta-amylase, lipase, lipoxidase, and catalase were tested at a concentration of 20.0 mg. and found to have no effect. Peroxidase at a concentration of 20.0 mg. was found to have a substantial effect on the stretching characteristics of gluten but a heat-inactivated preparation had the same effect (Fig. 5), indicating that nonenzymatic components were responsible for this change. Alpha-amylase at a concentration of 4.1 mg. (759 units) was also without effect.

Effect of Proteolytic Enzyme from Malt Syrup and Malted Wheat

Malted wheat and malt syrup are sometimes used as supplements in breadmaking processes and are known to contain proteolytic enzymes. The test was therefore applied to these sources to ascertain the relative effects of these enzymes upon gluten. Malt syrup had no effect with quantities up to 2.0 g. As it was found that the sugars and dextrins present in the syrup influenced the stretching test itself by greatly increasing the mean rate of stretching of the controls, an additional experiment was carried out in which the malt syrup was first dialyzed against 0.001M $CaCl_2$ to remove these components. An amount of the resulting extract equivalent to 1 g. of malt syrup was tested and had no effect. This suggests that the dough softening observed with additions of malt syrup in long fermentation breadmaking processes is due largely to amylases acting on starch rather than proteases acting on gluten.

Malted wheat proteases were extracted from finely ground malted wheat with 0.05M acetate buffer, pH 4.5 (2 ml. liquid per g. solid), and tested for their influence on the rate of gluten extension. Although 2 ml. of this extract caused an increase in the mean rate of stretching as compared to a water control, it was found that a heat-treated extract behaved similarly. There are in malted wheat, therefore, other heat-stable components that are able to influence the rheological properties of gluten.

Another experiment was carried out in which the malted wheat extract was concentrated and purified using an Amicon ultrafiltration assembly (Amicon Corp., Lexington, Mass.). A large amount of small molecular-weight material was removed and the proteolytic activity was largely retained. The extract had a specific activity of 948 HUT per ml. The extract caused substantial changes in the mean rate of gluten stretching at concentrations of 0.25 and 0.5 ml. as shown in Fig. 6. The control contained 0.5 ml. of heat-treated extract.

Increase in Mean Rate of Gluten Stretching of Control Samples with Time

Control samples containing water in place of enzyme solution were found to have an increase in rate of gluten extension with longer incubation times. Control glutens after 2, 30, and 48 hr. are shown in Fig. 7. Present research is being done to ascertain whether these changes are due to the presence of a gluten-softening enzyme bound in the gluten, to bacterial contamination, or even to slow relaxation.

Measurement by Other Methods of Changes in Gluten Caused by Proteolytic Activity

The farinograph and the alveograph have also been examined as possible alternates to the gluten-stretching test for measuring changes in gluten owing to proteolytic enzymes.

Farinograph. The technique of Bushuk (10) was used to make farinograms of gluten. The small bowl was used in the farinograph with the sensitivity set in the

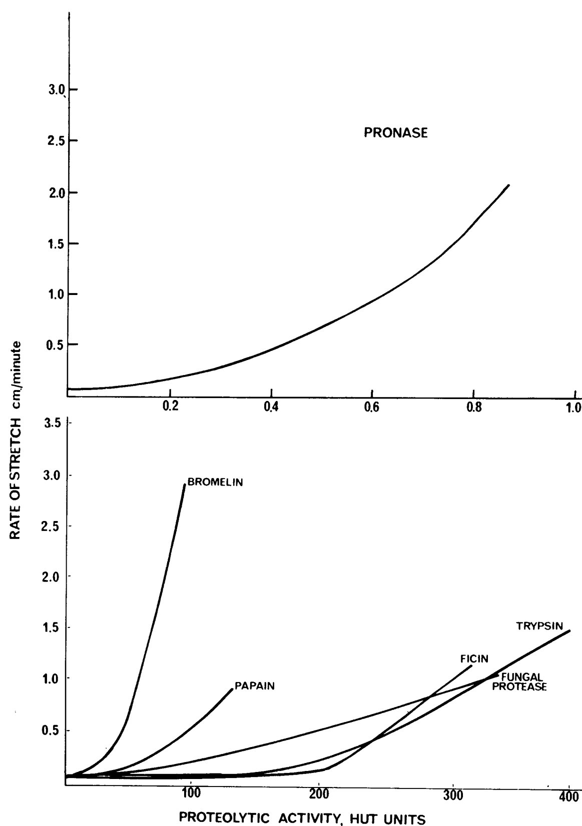


Fig. 4. Effect of pronase, bromelin, papain, ficin, trypsin, and fungal protease, expressed in HUT units, on the rate of stretch (cm./min.) of gluten.

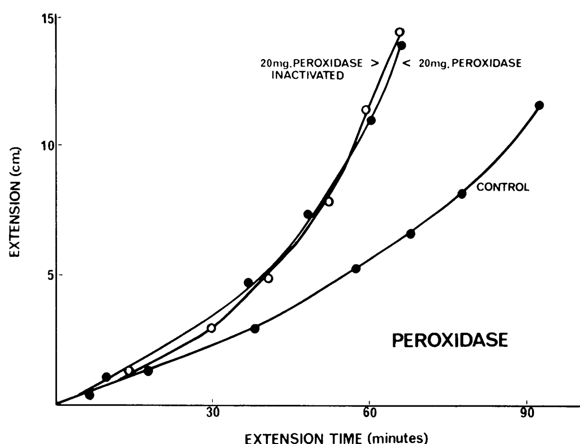


Fig. 5. Effect of peroxidase and heat-inactivated peroxidase on the stretching characteristics of gluten. A control containing no enzyme is shown for comparison.

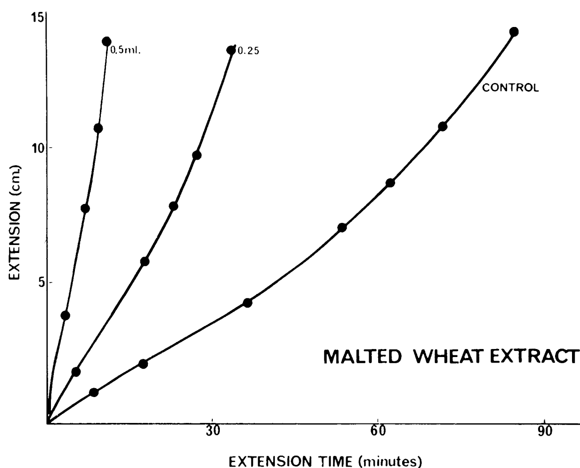


Fig. 6. Effect of a concentrated malted wheat extract at a concentration of 0.25 and 0.5 ml. on the stretching characteristics of gluten.

position normally used with the large bowl (1:5 ratio). At zero time, 35.0 ml. of enzyme solution was added with mixing to 45.0 g. of dried gluten to give a constant wet weight of 80.0 g. After 3 min., the mixing was stopped and the gluten allowed to rest for 2 hr. at 25°C. in the mixing bowl. Mixing was then continued and the consistency recorded after 15 min. of total mixing.

Pronase, papain, and fungal protease were examined by this technique. Typical farinographs illustrating the effect of increasing concentration of proteolytic enzyme for pronase are shown in Fig. 8. A progressive drop in consistency is found

with increasing concentration of enzyme. The relation between enzyme concentration and consistency drop for pronase, papain, and fungal protease is shown in Fig. 9. Heat-treated solutions of fungal protease were found to have a small effect on the consistency of the gluten and a curve corrected for this nonenzymatic effect is also shown. Pronase was found to have a linear decrease in consistency with increasing concentration of enzyme over a very large consistency range. The effect with papain is similar although the relation is more curvilinear. Increasing concentration of fungal protease caused a linear decrease in consistency

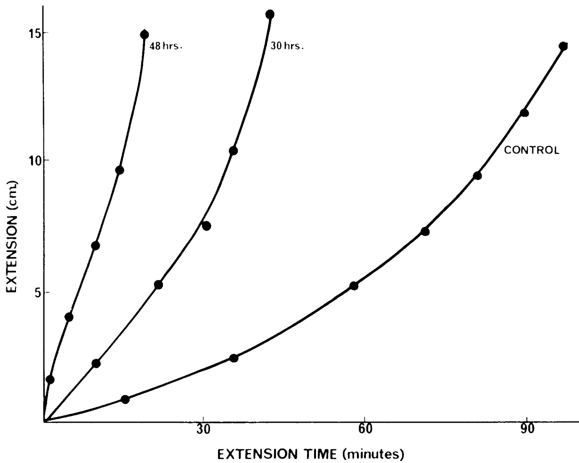


Fig. 7. Effect of 30 and 48 hr. of incubation on the stretching characteristics of the control gluten.

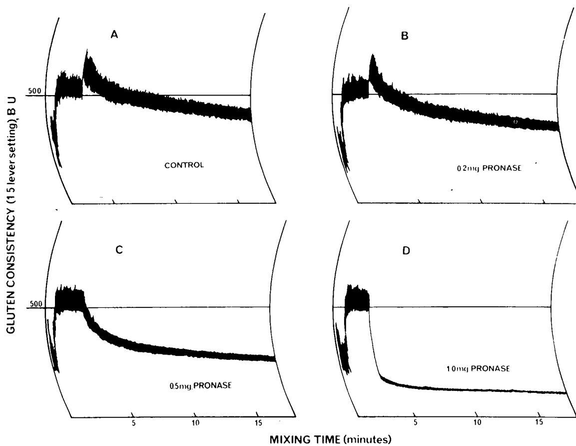


Fig. 8. Farinograph mixing curves for gluten upon incubation for 2 hr. with pronase at levels of: A, 0 mg.; B, 0.2 mg.; C, 0.5 mg.; D, 1.0 mg. Incubation was started after 3 min. of mixing.

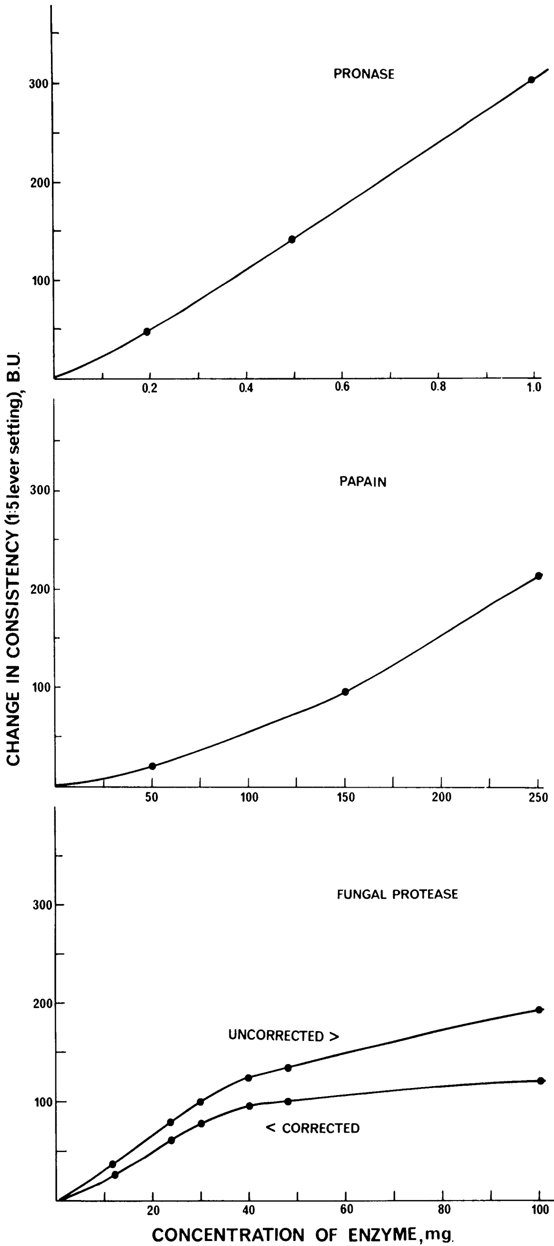


Fig. 9. Plot of change in consistency in Brabender units (1:5 lever setting) vs. concentration of enzyme for pronase, papain, and fungal protease. A plot corrected for consistency changes due to nonenzymatic components is shown for fungal protease.

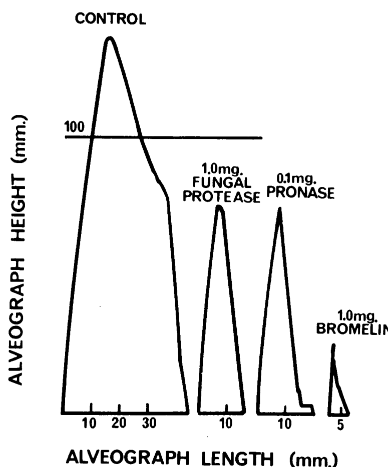


Fig. 10. Alveograms of gluten incubated with no enzyme, 1.0 mg. fungal protease, 0.1 mg. pronase, and 1.0 mg. bromelin.

with additions up to 35 mg. beyond which the consistency drop became much less sensitive to enzyme concentration.

Alpha-amylase at a concentration of 8.1 mg. (1,518 units) did not cause any decrease in consistency of the gluten after a 2-hr. rest period. Johnson and Miller (11) have also devised a farinograph technique for measuring proteolytic activity in which the drop in consistency of a 4-hr. sponge dough, measured after 15 min. of mixing, was found to be proportional to enzyme concentration. Alpha-amylase, acting upon starch, is partially responsible for the drop and is usually added in excess to keep its effect constant. The farinograph technique described here, using gluten in place of dough, obviates such difficulty in that no correction for the presence of alpha-amylase need be applied.

Alveograph. The procedure devised by Matsuo and Irvine (12) for making alveograms of gluten was used except that the gluten was rested for 30 min. instead of 1 hr. on the alveograph. Gluten and proteolytic enzymes were incubated as described in the stretching experiments. Fungal protease, pronase, and bromelin were tested and were found to cause a substantial reduction in the volume of the gluten bubble (Fig. 10). This reduction increased with increasing concentration of enzyme. Reproducibility, however, was found to be exceedingly poor and this technique was discarded as a possible method to assess the effect of proteolytic enzymes on gluten.

Comparison of Methods

Use of the alveograph to determine the effects of proteolytic enzyme preparations on gluten has been found unsatisfactory. Small amounts of proteolytic enzymes cause large decreases in the gas retention of gluten, however, and thus indicate that this technique could serve as the basis for a possible test if refinements can be made in adapting the alveograph to gluten studies. The farinograph

technique with gluten is certainly a satisfactory method as has been illustrated with pronase, papain, and fungal protease. It is necessary, however, to examine the effect of various concentrations of each different proteolytic enzyme on the drop in consistency so as to determine the range of concentrations which can be satisfactorily used to obtain quantitative results. The gluten stretching test as described here is somewhat harder to quantify than the farinograph technique but appears to be superior as a qualitative tool. It is much more sensitive and consequently much smaller amounts of proteolytic enzymes can be detected. The test could be made even more sensitive by extending the incubation time. Smaller amounts of substrate are required (2.5 vs. 45 g.) and hence much time is saved in preparation of gluten. The equipment required to carry out the test is also less costly and can easily be made.

CONCLUSIONS

The results presented in this paper illustrate the large effects that small amounts of proteolytic enzymes can have on changing the physical properties of gluten. Such changes are manifested by an increase in the mean rate of stretching as measured by the stretching test, a decrease in consistency as measured by the farinograph, and a reduction in volume of a gluten bubble as measured by the alveograph. The changes found would undoubtedly also occur in dough upon incubation with proteolytic enzymes, although the possible presence of inhibitors or activators could alter the magnitude of the change.

The stretching test and the farinograph both appear suitable to measure the activities of proteolytic enzymes with the former being more sensitive. Longer incubation times would achieve even greater sensitivity.

It has been found that part of the proteolytic enzymes of wheat are poorly soluble in common buffers and can only be extracted with some difficulty (4,13,14). It may be that methods such as the stretching test described here, and carried out directly on the gluten from the test flour, may be the only satisfactory way to measure the activities of wheat proteolytic enzymes.

Acknowledgment

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