Wheat Proteases. I. Separation and Detection by Starch-Gel Electrophoresis[1]

E. KAMINSKI[2] and W. BUSHUK, Department of Plant Science, University of Manitoba, Winnipeg

ABSTRACT

A modified starch-gel electrophoresis technique with hemoglobin as substrate was used to study the proteolytic enzymes of the flour from one variety of Canadian HRS wheat. The fractions examined were water-, salt-, 70% ethanol-, and acetic acid-soluble, and six glutens washed out with different solvents. Proteolytically active components were detected in all flour fractions and were classified into four broad groups, A, B, C, and D, on the basis of mobility. The most active group is C which is localized mainly in the alcohol-soluble fraction. This study showed that wheat proteases either are highly heterogeneous or readily associate with other proteins. At low ionic strength, self-digestion occurred in the protein bands which represented the most active groups, C and D.

Many enzymes in natural systems occur in complex mixtures and are sometimes strongly bound to inhibitors or other protein molecules. Accordingly, their activity can be detected only by first removing the inhibitor or by dissociating the complex by fractionation or additions of dissociating agents. The proteolytic activity of flour from sound wheat as detected by chemical methods is considered to be very low and essentially insignificant insofar as the functional properties of the flour are concerned (1,2). Accordingly, very little is known about the distribution or the molecular properties of these enzymes in flour, mainly because of technical difficulties encountered in the detection of the very low activities. Recently the authors developed a highly sensitive technique for detecting proteolytic enzymes in starch gels after electrophoresis (3), which can be readily applied to studies of wheat proteases. The results of such a study are reported in this paper.

[1]Contribution No. 149 of the Department of Plant Science, University of Manitoba, with financial assistance from the National Research Council of Canada.

[2]National Research Council Postdoctorate Fellow. Permanent address: Department of Food Technology, Agricultural University, Poznan, Poland.

MATERIALS AND METHODS

The flour used for this study was milled from the Canadian HRS wheat variety Manitou of the 1966 crop, on the Buhler experimental mill, to an extraction of 72%. By visual examination and amylograph test, this wheat sample was judged perfectly sound. The ash and protein contents of the flour were 0.48 and 13.1% (N × 5.7) respectively (14% m.b.). To facilitate the extraction of the water-, salt-, and alcohol-solubles, the free lipids were removed from the flour by extracting 100 g. of flour with two 300-ml. portions of ethyl ether. Residual ether was removed from the defatted flour under vacuum.

Preparation of Water-, Salt-, and Alcohol-Soluble Fractions

Defatted flour (50 g.) was extracted with 200 ml. of distilled water in a Waring Blendor for 10 min. and the slurry was centrifuged for 30 min. at 1,700 × *g*. The supernatant was decanted and the residue was extracted similarly two more times. The supernatants from the first and second extractions were combined and freeze-dried. The freeze-dried material had a protein content (N × 5.7) of 29.1% (dry basis) and will be referred to as the water-soluble fraction.

Salt-soluble fraction was prepared by extracting the residue similarly with three portions of phosphate buffer of pH 7.6 containing 0.4*M* sodium chloride. The first two supernatants were combined and dialyzed against 0.01*N* acetic acid solution in a cold room (3°C.) for 24 hr. in two portions of fresh acetic acid solution. The resulting solution was freeze-dried.

Alcohol-soluble fraction was extracted from the residue remaining after extraction of water- and salt-solubles with three 200-ml. portions of 70% ethanol solution. The first two supernatants were combined, dialyzed against acetic acid solution as above, and freeze-dried. Protein contents of the salt- and alcohol-soluble fractions were 61.3 and 95.2% respectively.

Acetic Acid-Soluble Fraction

This fraction was prepared by extracting the flour with 0.05*N* acetic acid solution (50 g. flour to 200 ml. solution) in a Waring Blendor for 10 min., centrifuging the slurry, and freeze-drying the supernatant. The protein content of this extract was 67.5%.

Gluten

Six types of gluten were prepared from untreated or extracted (as indicated below) flour by hand-kneading the dough in the following solutions or liquids until no more starch could be washed out: 1) 0.001*N* sodium chloride; 2) phosphate buffer-salt solution as used above to prepare the salt-soluble fraction (0.4*M* sodium chloride); 3) phosphate buffer of pH 7.6 as above containing 1*M* sodium chloride; 4) distilled water after extraction of the flour with phosphate buffer of pH 7.6 containing 0.4*M* sodium chloride, in Waring Blendor; 5) distilled water after an initial extraction of the flour with water in Waring Blendor; 6) tap water. After this washing, the glutens were freeze-dried. The protein contents of these glutens were: 1) 86.6%; 2) 82.6%; 3) 81.5%; 4) 73.5%; 5) 83.8%; and 6) 86.1%.

Solubilization of Protein Fractions

The freeze-dried extracts from the flour, except the glutens, were dissolved in 0.01*N* acetic acid solution to give 4% (or as otherwise stated) solutions. Because of the relative insolubility of the freeze-dried glutens, it was necessary to use a different procedure for solubilizing the glutens from that used with the other three fractions. The acetic acid solution (0.01*N*) used to dissolve the gluten contained 10 mg. per ml. of cysteine, and the dispersions were mixed at half-speed in a Lourdes Multi-mixer for 5 min. to facilitate solubilization. For each experiment, 0.8 g. gluten was dissolved in 10 ml. of solution. The solution was centrifuged to remove a small amount of insoluble material before the solution was applied to the gel.

Electrophoresis and Detection of Bands

The starch-gel electrophoresis used was, in general, similar to that originally developed by Smithies (4). The apparatus was constructed in the Laboratory from Plexiglas according to plans published by this author. Aluminum lactate buffer of pH 3.4 was used for the electrophoresis as applied to wheat proteins by Elton and Ewart (5). Both horizontal and vertical arrangements were used, with identical results. The protein solution (0.2 ml.) was applied to the proper slot in the gel and covered with a microscope slide cover. During electrophoresis the gel was covered with Saran film. Electrophoresis was at room temperature (25°C.) for 4 to 5 hr. at a potential gradient of 6 to 8 volts per cm. For detection of protein bands, the starch gel was stained with 0.02% nigrosin in 8*M* urea overnight. Unadsorbed dye was removed by washing in tap water.

The application of the starch-gel electrophoresis method to separate and detect proteolytic enzymes was described previously (3). According to the procedure developed, proteolytically active components are detected by their degradation of the hemoglobin substrate (0.3%) incorporated into the starch gel (14%) starch. To eliminate possible interference from amylase activity, this activity was inhibited by additions of 40 micromoles of ethylmercurithiosalicylic acid, sodium salt (Thimerosal), directly to the starch gel. This reagent in the concentration used had no noticeable effect on the activity of the proteolytic enzymes in the starch gels that were investigated. To eliminate boundary effects due to slight migration of hemoglobin, it is necessary to have the slots 7 to 8 cm. from the top of the gel.

For detection of proteolytically active bands the gel was incubated overnight in a phosphate (0.2*N*)-citrate (0.1*N*) buffer solution of pH 5.0 and stained for 4 to 6 hr. in 0.02% nigrosin in 0.2*N* phosphate buffer of pH 8.0. This step was not used with commercial proteolytic enzymes (3) but was introduced in the present study to facilitate detection of wheat proteases. After the gel is washed with tap water, the proteolytically active components can be detected as colorless areas in the dark violet-blue gel. To facilitate detection of weakly active components, fluorescent underlighting can be used.

RESULTS

Proteolytically Active Components in the Acetic Acid-Soluble Fraction

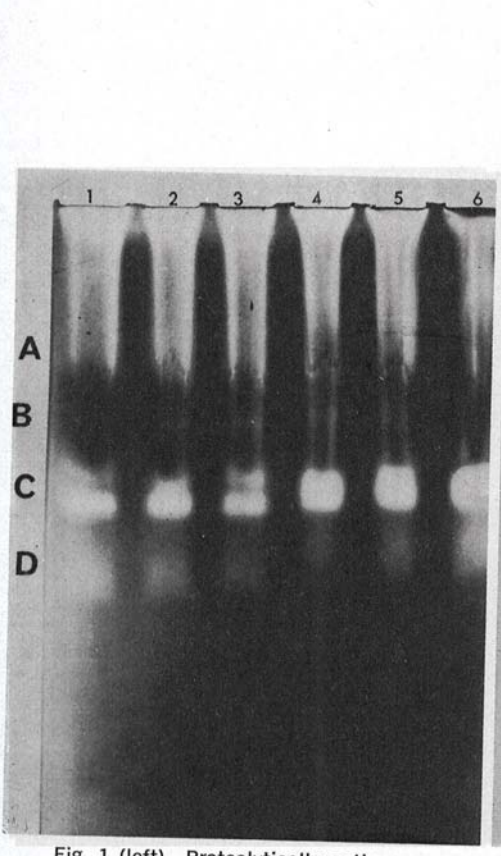
Since this fraction contains the major portion of the flour proteins (about

80%), it was examined first. Triplicate runs at two concentrations are shown in Fig. 1. It is apparent that the flour used contains a large number of proteolytically active components, some of which exist only in fractions of particular solubility (see below). To facilitate presentation of results, the active components were classified into four groups of similar electrophoretic mobility:

Group A had the lowest mobility and was detectable as a broad band starting from the point of application.

Group B had a slightly higher mobility than group A and was considerably lower in activity, but could be readily detected when low ionic strength of lactate buffer or high-concentration protein solution was used.

Group C had the next higher mobility and was the most active group, expressed in terms of activity per sq. cm.



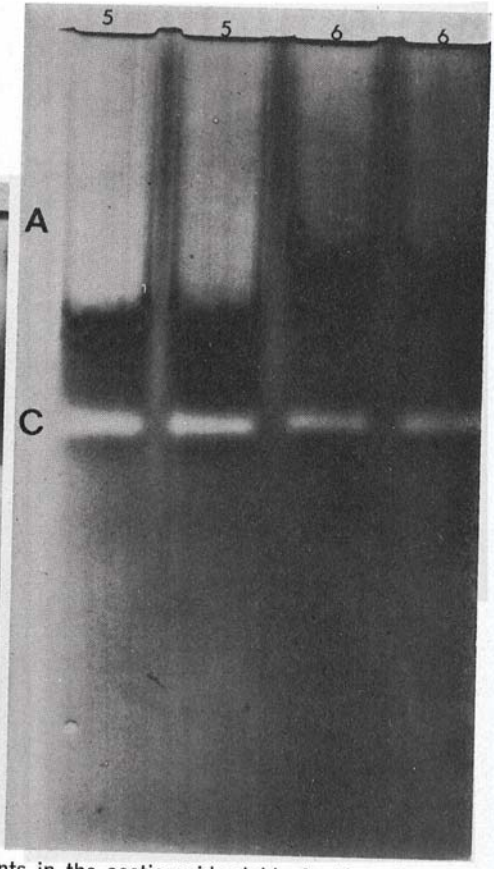


Fig. 1 (left). Proteolytically active components in the acetic acid-soluble fraction at 0.1 ionic strength and two concentrations: 1, 2, and 3, 4%; 4, 5, and 6, 8%.

Fig. 2 (right). Proteolytically active components in the water- and salt solution-soluble fractions at three ionic strengths: 1, 3, and 5, water-soluble at micron = 0.05, 0.1, and 0.2 respectively; 2, 4, and 6, salt-soluble at micron = 0.05, 0.1, and 0.2 respectively.

Group D had the highest mobility but was generally low in activity.

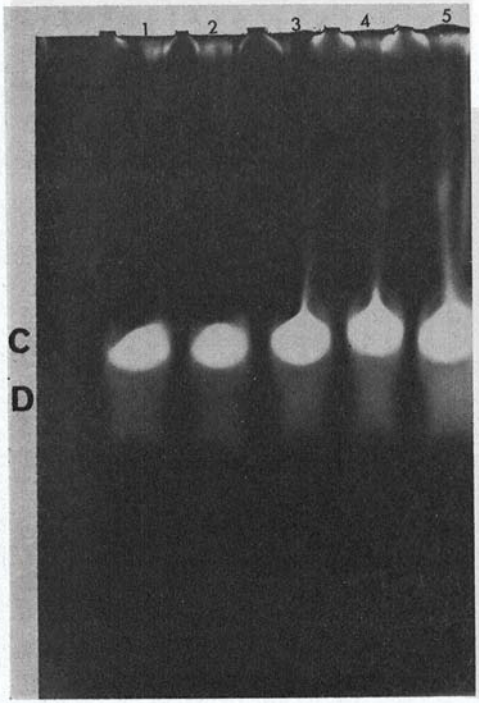
Each group of enzymes comprised two or three distinguishable bands of similar activity. In the acetic acid-soluble extract, groups C and A were the most active.

Proteolytically Active Components in the Water-Soluble and Salt Solution-Soluble Fractions

These fractions were similar and showed two groups of proteolytically active components (Fig. 2). In mobility, these were similar to groups A and C as defined above. In relative activity, these fractions were the lowest of the three soluble fractions examined.

Proteolytically Active Components in the Alcohol-Soluble Fractions

This fraction contained two groups of proteolytically active enzymes (Fig. 3). In mobility one group was similar to group C and the other to



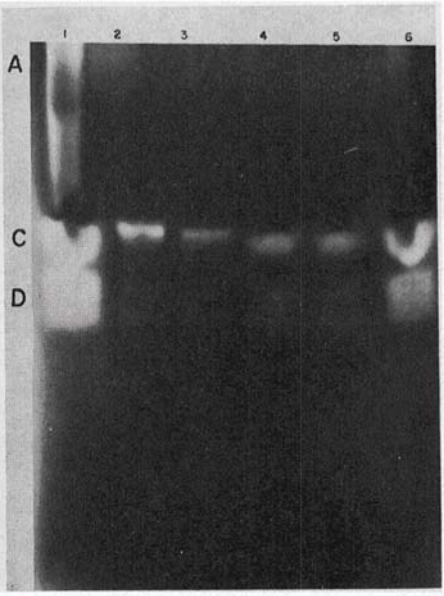


Fig. 3 (left). Proteolytically active components in alcohol-soluble fraction at 0.1 ionic strength at three concentrations (% solids): 1 and 2, 4%; 3 and 4, 6%; 5, 8%.

Fig. 4 (right). Proteolytically active components at 0.1 ionic strength in the six glutens shown in the same order as listed above.

group D as defined above. In this fraction group C was more active than group D.

Proteolytically Active Components in Gluten

Results obtained for the six gluten preparations described above are shown in Fig. 4. All six glutens showed three groups of proteolytically active com-

ponents: a slow-moving, low-activity group similar to A; a faster, highly active group similar to C; and the fastest group, similar to D. The activity of the two fastest groups depends on the method used for preparation of the gluten. Glutens washed with 0.001*N* sodium chloride and with tap water (1 and 6) showed the highest activities, whereas the two glutens washed with phosphate buffer-salt solution (2 and 3) had the lowest.

Self-Digestion of Proteolytically Active Components

With the use of starch-gel electrophoresis without hemoglobin at low ionic strength it is possible to observe self-digestion of some of the protein bands after development of the colored bands. Presumably this occurs after electrophoresis and during incubation. Self-digestion appears in the part of the protein where the most proteolytically active groups, C and D, are

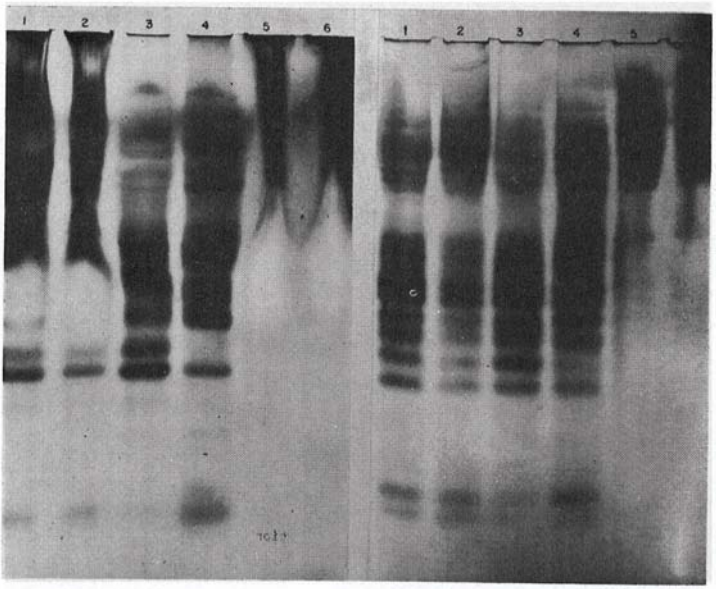


Fig. 5 (left). Starch-gel-electrophoresis patterns showing self-digestion of some protein components at 0.05 ionic strength: 1 and 2, acetic acid-soluble fraction; 3, water-soluble fraction; 4, salt-soluble fraction; 5 and 6, alcohol-soluble fraction.

Fig. 6 (right). Starch-gel-electrophoresis protein bands at 0.2 ionic strength: 1 and 2, acetic acid-soluble fraction; 3, water-soluble fraction; 4, salt-soluble fraction; 5 and 6, alcohol-soluble fraction.

located. It is possible to observe this effect by comparing results for fractions extracted from flour with alcohol or acetic acid solutions with those for water- and salt-soluble fractions which do not seem to show this effect (Fig. 5). The self-digestion shown in Fig. 5 can be eliminated by increasing the ionic strength from 0.05 to 0.2 (compare Fig. 6 with Fig. 5). Self-digestion of some protein components occurs at low ionic strength in the gluten also (Fig. 7). By increasing ionic strength of aluminum lactate buffer it is possible to eliminate this self-digestion also (Fig. 8). When the ionic strength of the aluminum lactate buffer was decreased from 0.2 to 0.05, the pH of

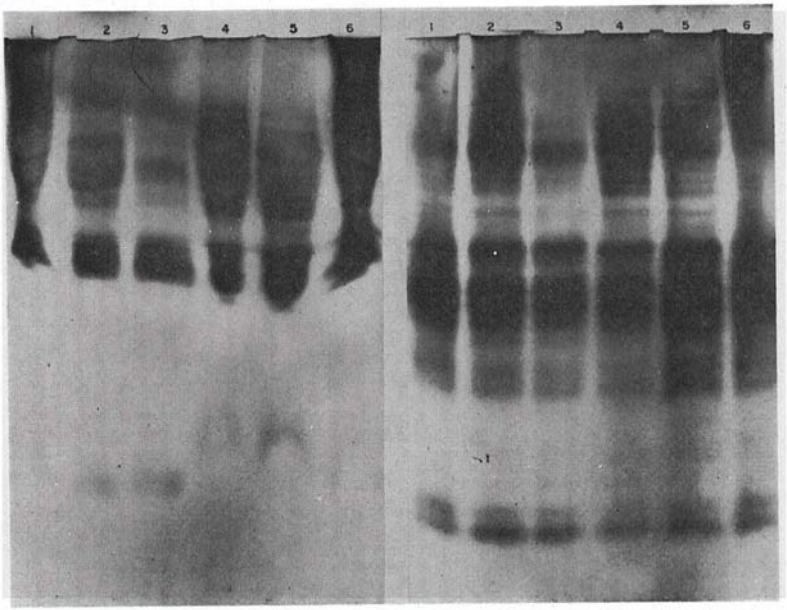


Fig. 7 (left). Starch-gel patterns showing self-digestion of some protein bands at 0.05 ionic strength for the six types of gluten shown in the same order as listed above.

Fig. 8 (right). Starch-gel-electrophoresis protein bands at 0.2 ionic strength for the six types of gluten shown in the order listed above.

the starch gel increased from 3.4 to 3.7, but this did not noticeably affect the mobility or separation of the protein bands.

DISCUSSION

The presence of several proteolytic enzymes in wheat has been reported by Wierzbowski and Skupin (6), Safonowa (7), and Hanford (1). These studies were with water- and salt-soluble extracts from sound or germinated grain.

The present study has shown that proteolytically active components are localized in water-, salt-, ethanol-, and acetic acid-solution extracts from wheat flour, and can be classified into four broad groups on the basis of mobility at pH 3.4 in starch gels containing 0.3% hemoglobin. The most active group is C, and it is extracted to the greatest extent by 70% ethanol. The water- and salt-soluble fractions contain groups A and C but of much lower activity. Under some conditions group C readily separates into two or three distinguishable forms. It appears from these results with one sample of flour that the most active wheat proteases are associated with gluten and therefore are not completely extracted with water or salt solution. This is in general agreement with the findings of Olcott and co-workers (8), who could not remove all the proteolytic enzymes from the gluten even by intensive washing with salt solutions using successive disintegrations in the Waring Blendor. McDonald and Chen (9) also found that they could not extract all the proteolytically active material from flour by water or by acetate buffer of pH 8.

However, the residual enzyme could not be extracted in active form by acetate buffer of pH 4.6, 0.2*M* sodium chloride solution of pH 7.5, borate-acetate buffer of pH 8.2, or 10% sodium chloride solution of pH 5.8. The possibility that the proteases in flour are normally adsorbed on inactive protein components is being investigated.

The phenomenon referred to above as self-digestion, that was observed for some bands, deserves further comment. This can be readily detected for bands of high specific activity, especially if the electrophoresis is carried out at low ionic strength. The existence of this effect is quite definite; however, its mechanism cannot be explained completely on the basis of available information. A number of questions remain to be answered. For example, is it digestion of some inactive protein that migrates with the enzyme? Experiments with purified proteolytic enzymes (3) showed that similar effects can be obtained with these enzymes, and therefore the possibility that self-digestion can occur cannot be ruled out. The effect of ionic strength, noted above, suggests that the enzyme must have a particular tertiary structure for self-digestion to occur. The small increase in pH that occurs with decrease in ionic strength would increase the activity of wheat proteases and this could increase self-digestion. Further work is necessary to clarify this point.

The present study of the proteolytic enzymes in one variety of HRS wheat by starch-gel electrophoresis has shown that the enzymes are highly heterogeneous but can be classified into four broad groups of similar electrophoretic mobility. Each group may comprise a number (two or three) of multiple forms of enzymes of very similar mobility. It is quite possible that some of these might be isoenzymes; however, further work is necessary to confirm this possibility.

Literature Cited

1. HANFORD, J. The proteolytic enzymes of wheat and flour and their effect on bread quality in the United Kingdom. Cereal Chem. 44: 499-511 (1967).
2. KOZMINA, N. P., and KRETOWICH, W. L. Biokhimiya zerna i produktov ego pererabotki. Moskva (1952).
3. KAMINSKI, E., and BUSHUK, W. Detection of multiple forms of proteolytic enzymes by starch gel electrophoresis. Can. J. Biochem. 46: 1317-1320 (1969).
4. SMITHIES, O. Zone electrophoresis in starch gel. Biochem. J. 61: 629-641 (1955).
5. ELTON, G. A. H., and EWART, J. A. D. Starch gel electrophoresis of cereal proteins. J. Sci. Food Agr. 13: 62 (1962).
6. WIERZBOWSKI, J., and SKUPIN, J. Determination of the relationship between sulfhydryl groups and amylolytic and proteolytic enzymes in wheat and wheat flour. Technical Report, Dept. of Food Technology, Agricultural University, Poznan, Poland (1964).
7. SAFONOWA, M. P. Proteolytic enzymes in wheat. Evolyutsionaya biokhimiya rastenii. Moskva, Izd-vo, Nauka, p. 70-80 (1964).
8. OLCOTT, H. S., SAPIRSTEIN, L. A., and BLISH, M. J. Stability of wheat gluten dispersions toward reducing agents in the presence and absence of a gluten proteinase. Cereal Chem. 20: 87-97 (1943).
9. McDONALD, C. E., and CHEN, LORA L. Properties of wheat flour proteinases. Cereal Chem. 41: 443-455 (1964).

[Received April 10, 1968. Accepted August 20, 1968.]