PROTEOLYTIC ACTION OF WHEAT FLOUR ON NONFAT DRY MILK PROTEINS[1]

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ABSTRACT

In pH 3.1 aluminum lactate or 0.01*N* acetic acid, flour protease rapidly attacked the alpha$_s$-casein of nonfat dry milk. Starch-gel electrophoresis in aluminum lactate buffer and 7.5*M* urea readily separated three fragments that differed markedly in mobility. Starch-gel electrophoresis of the acidic digests in tris-citrate buffer, pH 8.6, confirmed the alteration of alpha$_s$-casein, although only two fragments were resolved. Upon prolonged digestion, the faster-moving components disappeared. Other milk proteins (beta- and kappa-caseins, beta-lactoglobulin, alpha-lactalbumin) were not observably attacked under conditions leading to alteration of all alpha$_s$-casein. The protease attacking alpha$_s$-casein was present in all flours examined (five HRS, five HRW). The pH 3.1 aluminum lactate buffer gave good resolution of milk-protein components, and of mixtures of milk- and flour-protein components.

The physical properties and baking performance of bread doughs can be significantly altered by adding nonfat dry milk (NFDM). Several properties of NFDM may be responsible; two that are recognized are its pH-buffering and water-absorbing capacities. NFDM for baking use must be heat-treated before drying to eliminate a depressing effect on loaf volume. The adequacy of this heat-treatment has been determined usually by the Harland-Ashworth test for undenatured whey proteins (1). Although the importance of these properties is recognized and variations in them are controlled, occasional unaccountable deviations in the effects of NFDM or in the tolerance of flours to addition of NFDM occur. The introduction of continuous-mix bread-baking processes appears to have increased interest in understanding the influences of NFDM on dough properties (2,3).

The nature and complexity of both flour and NFDM proteins suggest that protein-protein reactions or interactions may be involved.

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Previous studies show that the loss of sulfhydryl groups during dough mixing is affected by the presence of NFDM (4), although factors other than the properties of the proteins may be responsible. The present work was undertaken to determine whether changes in protein components could be demonstrated. The results show that a major casein component is fragmented rapidly by a protease present in flour.

Materials and Methods

Nonfat dry milk was a commercial product prepared for bakery use. It contained 36% acid-precipitable (pH 4.6) casein, and 6.2% N. Electrophoretic examination (see below) showed alpha$_s$-, beta-, and kappa-caseins and small amounts of alpha-lactalbumin and beta-lactoglobulin. No other bands were observable at the protein level applied to the gel. All NFDM proteins were electrophoretically identical with purified preparations of milk proteins prepared from fresh skimmilk.

Wheat flours were commercially milled, bleached, bakery flours; they included Hard Red Spring (HRS) and Hard Red Winter (HRW) wheat flours with protein contents ranging from 11.7 to 14.4% on a 14% moisture basis.

Aluminum lactate buffer (pH 3.1) was prepared according to Jones and Cluskey (5). A commercial preparation of aluminum lactate powder (Z. D. Gilman, Inc., Washington, D.C.) was used.

Electrophoresis was conducted in a vertical gel apparatus with water-cooled plates (E-C Apparatus Company, Philadelphia, Pa.). The gel dimensions were 120 mm. × 170 mm. × 3 mm. Starch-urea-aluminum lactate gels were prepared according to Woychik *et al.* (6) from 14% starch, 7.5*M* urea, and 0.017*M* aluminum lactate. The gels were stained with nigrosine dye. Tris-citrate gels were prepared according to Wake and Baldwin (7) from 14% starch, 7.5*M* urea, and 0.076*M* tris-citrate. Tris-citrate gels were stained with 0.1% amido black in 5% acetic acid. The electrode chambers contained the same buffer as that used in the gel. Electrophoresis was conducted for 2.5 hr. with 40 to 50 ma. current for aluminum lactate gels and 4 hr. with 40 to 50 ma. current for tris-citrate gels.

Extracts were prepared by adding aluminum lactate buffer to flour, NFDM, or flour plus NFDM (4 ml./g. flour or 4 ml./60 mg. NFDM) and mixing the suspension for 1 min. on a vortex mixer. The suspensions were allowed to stand in a water bath at a selected temperature, usually 30°C., for the specified times and then were centrifuged at 30,000 × *g* for 30 min. at 0°C. The supernatant was decanted and was either mixed with a NFDM extract or made 8*M* in urea and stored at 2°C. for electrophoresis. Acetic acid (0.01*M*) and tris-citrate (0.076*M*,

pH 8.6) buffer extractions were prepared using the same procedure.

Densitometer tracings were obtained by scanning the dried gel (7) in a Beckman Analytrol, using the B 1 cam.

Experimental Results

Separation of NFDM and Flour Proteins. The complexity of wheat flour proteins is readily shown by electrophoresis in starch gels containing urea (6,8) and pH 3.1 aluminum lactate buffer (9). This buffer dissolves casein components of heat-treated NFDM and permits good separation of alpha$_s$- and beta-caseins by electrophoresis in starch gels. Furthermore, the mobilities of the principal milk-protein components allowed good resolution of milk from flour proteins. At the pH of the gel and with the electrophoresis apparatus used, kappa-casein did not migrate into the gel. Because the heat-treatment of NFDM for bakery use insolubilizes nearly all noncasein proteins, only traces of them appear in the starch-gel strips. These points are demonstrated in Figs. 1 and 2.

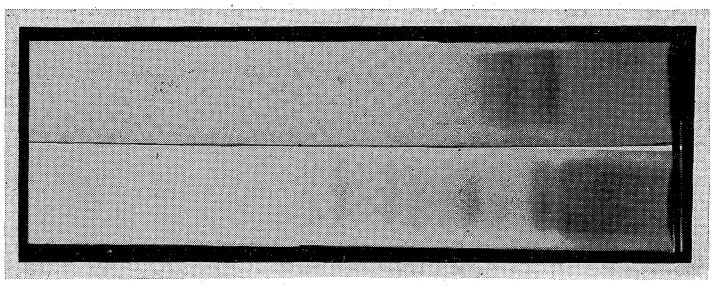

Fig. 1. Starch-gel electrophoresis in aluminum lactate buffer of 0.017*M* aluminum lactate extracts of commercial high-heat NFDM (top) and flour A (bottom). Migration is from right to left in all figures.

Starch-gel electrophoresis of milk proteins in tris-citrate buffer, pH 8.6 (7,10), was used for reference runs for identification of milk-protein components. This buffer has also been used for examination of gliadin proteins by Wright and co-workers (11), and in the present work was used to provide for examination of soluble flour proteins at a pH above their isoelectric point. Densitometer tracings of patterns given by material extracted with aluminum lactate buffer but run in tris-citrate are shown in Fig. 3.

Bands from the NFDM extracts were identified by comparisons with authentic samples of milk proteins prepared from unheated milk (obtained from C. A. Zittle, Eastern Regional Research Laboratory) as well as by reference to published information. Insofar as casein com-

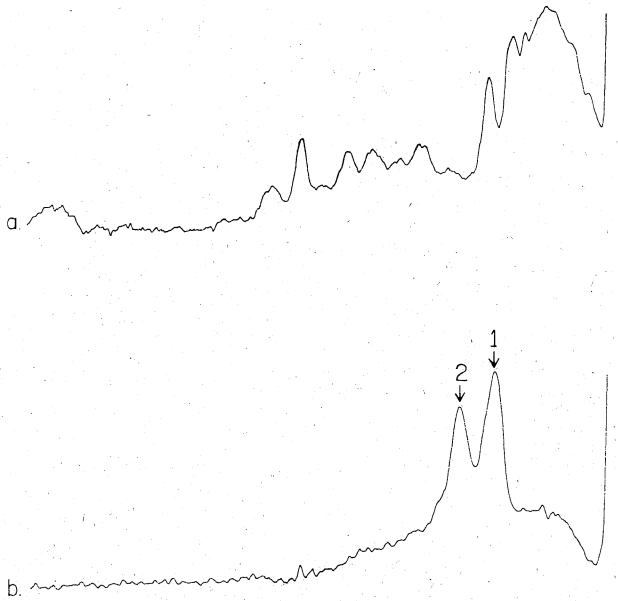

Fig. 2. Densitometer tracings of the electrophoresis patterns shown in Fig. 1: a, flour A; b, commercial high-heat NFDM; 1, beta-casein; 2, alpha$_s$-casein.

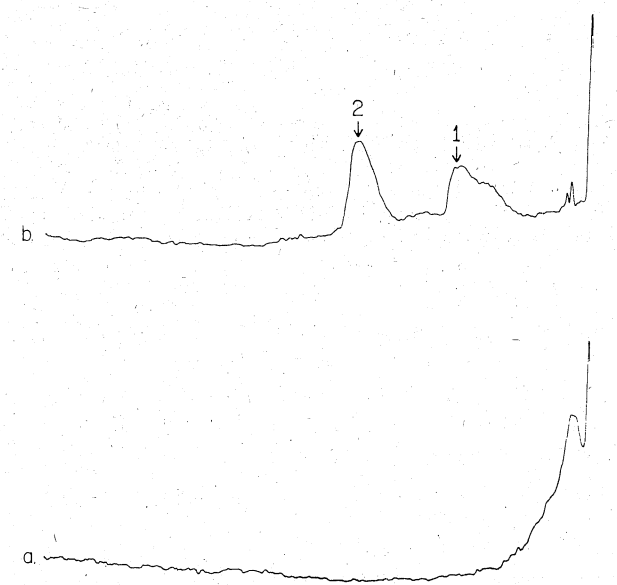

Fig. 3. Densitometer tracings of starch-gel electrophoresis patterns of aluminum lactate buffer extracts of flour and NFDM run in tris-citrate buffered gel: a, flour D; b, NFDM; 1, beta-casein; 2, alpha$_s$-casein.

ponents are concerned, the heat-treatments to which the NFDM has been subjected have no detectable effect on mobilities in 7.5*M* urea. Electrophoretic patterns are shown in Fig. 4.

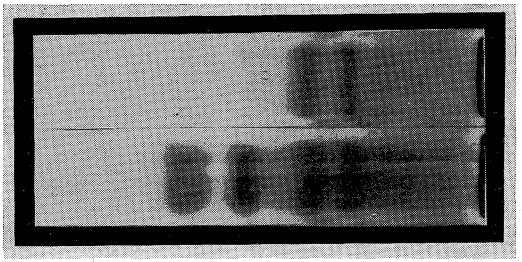

Fig. 4. Mobilities of milk protein components prepared from nonheated milk (bottom) compared with major components in aluminum lactate buffer extracts of commercial NFDM (top). Components from right to left; beta-casein; alpha$_s$-casein; alpha-lactalbumin; beta-lactoglobulin, genetic type A/B.

Examination of Flour-NFDM Combinations. When flour and NFDM were combined and extracted with the aluminum lactate buffer for a short time, the bands obtained by starch-gel electrophoresis did not show a simple superposition of the flour and NFDM patterns. Similar changes in patterns also were observed when extracts of flour and NFDM were prepared separately and the extracts combined. Depending upon extraction conditions and the ratio of NFDM to flour, the alpha$_s$-casein band disappeared or was markedly reduced in inten-

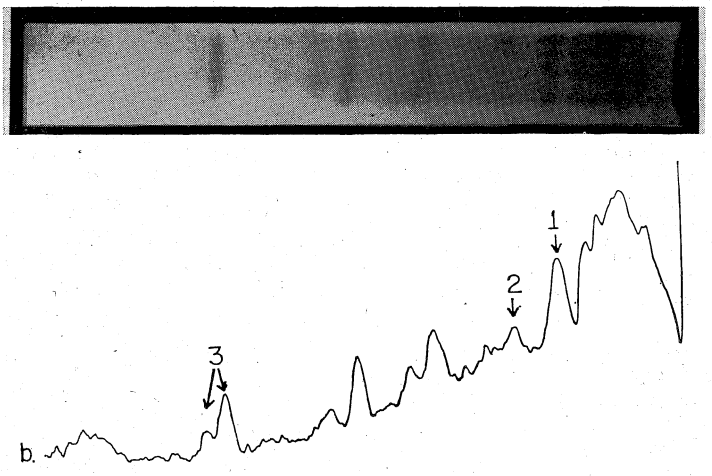

Fig. 5. Starch-gel electrophoresis patterns (top) and densitometer tracings (bottom) of an aluminum lactate extract of flour A plus 6% NFDM. Digestion for 30 min. at 30°C. 1, beta-casein; 2, marked decrease in alpha$_s$-casein band; 3, hydrolysis products of alpha$_s$-casein.

sity, and fast-moving bands appeared. That the bands of high mobility represented products from alpha$_s$-casein acted upon by a flour protease was shown by observations reported below. Corresponding changes were observed when the tris-citrate buffer system was used for electrophoresis, or when dilute acetic acid was used for extraction of proteins.

The presence of two fast-moving components is shown in Fig 5. Comparison with Fig. 1 shows they were not present in either flour or NFDM extracts. By comparison of samples in which the flour and NFDM extracts were combined and allowed to stand for various time intervals before addition of urea, the change was shown to be a progressive one. Densitometer tracings illustrating this are presented in Fig. 6. With the extracts held at 30°C., one fast-moving band was present after 30 min., two were present and at about maximum inten-

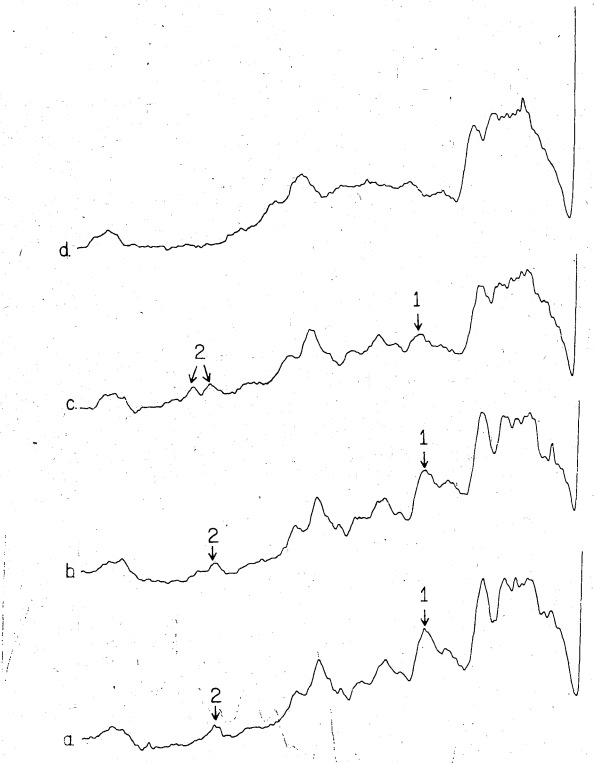

Fig. 6. Densitometer tracings of starch-gel electrophoresis patterns showing changes in the distribution of protein components with time of digestion; aluminum lactate extract of flour B plus 6% NFDM. a, 30 min.; b, 1 hr.; c, 2 hr.; d, 3 hr. 1, alpha$_s$-casein hydrolysis product decreasing from 30 min. to 3 hr.; 2, high-mobility hydrolysis product of alpha$_s$-casein showing the appearance of one band at 30 min.; increasing to two bands at 2 hr., and with no evidence of peptides remaining at 3 hr.

sity after 2 hr., and after longer periods the intensities of the bands decreased. During the 2- to 3-hr. incubation, the alpha$_s$-casein band had disappeared (or, at higher levels of NFDM, been reduced in intensity), and two new bands had appeared.

The changes with time indicate that proteolytic activity was responsible. This was confirmed by extracting flour and NFDM separately, heating portions of the extracts at 100°C. for 2 min., and examining the various combinations of heated and unheated extracts. The changes in patterns occurred only when an unheated extract of flour was combined with a NFDM extract. Heating of the NFDM extract had no effect, as would be expected in view of the heat-treatments used in NFDM preparation.

It was found also that addition of urea to about 7.5*M* concentration to flour extracts prevented or stopped the changes. Extracts made with the pH 8.5 tris-citrate buffer also showed no evidence of proteolytic activity.

The alteration of the alpha$_s$-casein component indicates that the changes observed were the result of action of a flour protease on the casein component, rather than, e.g., activation of a flour protease acting on a flour protein. More evidence that alpha$_s$-casein served as a substrate was obtained by use of a flour-protease concentrate (obtained from C. E. McDonald). An aluminum lactate extract of NFDM plus flour protease, after 30 min. of digestion at 30°C., was adjusted to pH 4.6 to precipitate casein proteins. Examination of the precipitate showed that it contained the fast-moving components. Also, because of the absence of flour proteins, it was possible to observe that another component of slower mobility had formed from the alpha$_s$-casein. When an alpha$_s$-casein preparation and the flour-protease concentrate were combined, the electrophoretic pattern showed three hydrolysis products of alpha$_s$-casein. The latter observations are illustrated in Fig. 7.

Although changes did not occur at pH 8.5, samples digested in the aluminum lactate buffer (or in dilute acetic acid) and then examined at pH 8.5 showed characteristic changes, confirming that alpha$_s$-casein was the component attacked. In the tris-citrate buffer, two components were detected, both of higher mobility than alpha$_s$-casein, with complete disappearance of the alpha$_s$-casein. Some results are shown in Fig. 8. Purified preparations of alpha$_s$-, beta-, and kappa-casein, beta-lactoglobulin, and alpha-lactalbumin also were treated with the protease concentrate. Examination of the reaction mixtures by starch-gel electrophoresis with either aluminum lactate or tris-citrate buffer showed alpha$_s$-casein to be the only component that had been modified.

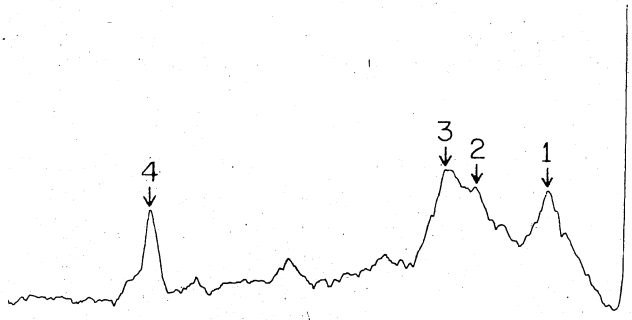

Fig. 7. Densitometer tracings of starch-gel electrophoresis in aluminum lactate buffer of flour protease-alpha$_s$-casein digest to demonstrate products of alpha$_s$-casein digestion. 1, 3, and 4, hydrolysis products of alpha$_s$-casein; 2, remaining alpha$_s$-casein. The small peak between components 1 and 2 is a small amount of beta-casein in the alpha$_s$-casein preparation.

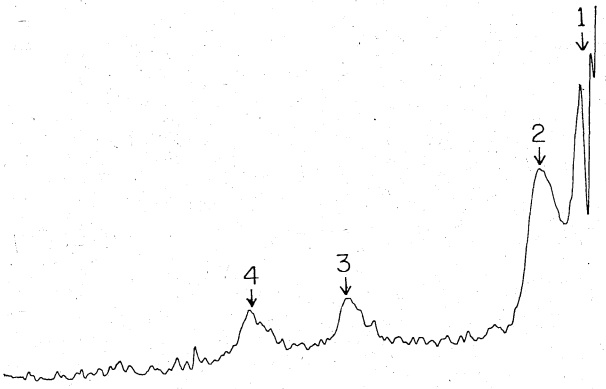

Fig. 8. Densitometer tracings of starch-gel electrophoresis patterns of aluminum lactate extracts of flour plus 6% NFDM examined in tris-citrate-buffered gel. 1, flour proteins (the large dip in the peak resulted from break in the dried gel); 2, beta-casein; 3 and 4, hydrolysis products of alpha$_s$-casein.

Comparisons of Flours and NFDM Preparations. Ten flours in all were examined. In every case, an aluminum lactate buffer extract modified the starch-gel electrophoresis pattern when combined with NFDM extracts in the ways described above. Also, a low-heat NFDM (a commercial product for home use) and two high-heat NFDM preparations (commercial products for bakery use) gave similar responses to flour extracts.

It should be pointed out that while this report has been concerned only with flour protease action on alpha$_s$-casein, other observations suggest additional reactions or interactions of milk and flour proteins. For example, combined extracts give precipitates on partial neutraliza-

tion or the addition of salt, and these precipitates in some cases appear to contain only specific milk or flour proteins. These have not been investigated beyond initial observations.

Discussion

The changes in alpha$_s$-casein reported above would be difficult to detect without starch-gel (or similar) electrophoretic techniques, and the clarity with which changes were demonstrated illustrates the value of applying such techniques to follow modifications of dough components. In particular, changes in a specific component have been shown to occur rather quickly. The presence of proteases in wheat flour has been recognized for years (12), but the methods of measurement used, such as various modifications of the Ayre-Anderson procedure (13), indicate rather low levels of activity even with nonflour substrates. The present work indicates that some specific types of change may occur rapidly. In fact, the precipitability at pH 4.6 of the various components derived from alpha$_s$-casein suggests that the rapid and specific changes involved would not be reflected in measurements of the Ayre-Anderson type. Also, the disappearance of the fast-moving components with long digestions raises the question of whether a different protease degrades them, particularly since evidence for the occurrence of at least two proteases in flour has been presented by McDonald *et al.* (14).

On the basis of information so far obtained, no technological importance of the proteolytic changes in NFDM can be claimed. Before such significance can be established, or its possibility eliminated, procedures for measurement of the extent of change under conditions existing in dough or for preparation of the modified alpha$_s$-casein components for use in baking need to be developed. However, the presence in flour of a protease with this apparently unsuspected activity seems noteworthy in itself.

Acknowledgments

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