Inactivation of Cereal Alpha-Amylase by Brief Acidification: The Pasting Strength of Wheat Flour

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ABSTRACT

Acidification of a wheat flour slurry to pH 2.5 or below irreversibly inactivates flour alpha-amylase. Inactivation is complete in only a few minutes at room temperature. Flour beta-amylase is largely destroyed under the same conditions. A neutralized inactivated slurry is suitable for most thickening purposes but not for bread-baking. The acid-inactivation process may be incorporated in several analytical procedures. Inactivated flours vary in pasting strength measured by amylograph and in susceptibility to alpha-amylase attack. Flour alpha-amylase has survived storage for 60 years at low temperature and for 10 years at room temperature.

The destruction of alpha-amylase activity by acidification has been noted several times in the literature but does not so far seem to have been applied widely. It provides an alternative to the steaming processes (1,2) as a means of dramatically improving the thickening power of wheat flour (3) and has been successfully applied industrially.

Moss (4) has recently concluded that different varieties of wheat give different paste viscosities, even when alpha-amylase activity is very low.

This paper will show that alpha-amylase activity may be almost completely destroyed by a brief acidification; that beta-amylase is also considerably inactivated; that after inactivation the pasting properties of flours still vary; that this variation is not due to beta-amylase, nor is it a function of protein content or of damaged starch or of any factor represented by the ash content.

EXPERIMENTAL

Materials

To demonstrate the conditions for acid-inactivation, a blend of commercial bakers' flour with 20% of flour experimentally milled from sprouted wheat was used. The range over which inactivation would work is shown (Table I) by experimentally milled flours from pure lines of wheat of several different varieties and grades of baking performance of the 1967 and 1968 harvests. Additionally in Table I are data for some European, North American, and Australian experimental and commercial flours. The "aged" flour was a blend of New Zealand commercial flours stored 10 years at room temperature. The "Shackleton" flour had been taken to the Antarctic by an expedition of 1908. It was recovered from there, and assayed soon after its arrival in New Zealand in 1966.

Although the usual analytical data have been recorded for these flours, they are not pertinent to the discussion and will not be presented.

Methods

The methods for amylograph and for falling number (FN) have been described in the preceding paper (4a).

When acid-inactivation is to be carried out for the amylograph, 60 g. flour is shaken with a mixture of 400 ml. water and exactly 20 ml. of N HCl. After this has stood 20 min. at 80°F., exactly 20 ml. of N NaOH is pipetted in with vigorous swirling to avoid excessive local concentration. The suspension is then poured into the amylograph bowl and the procedure continued normally.

To apply acid-inactivation to the FN method, 5 g. flour is given 20 shakes with 12.5 ml. 0.1N HCl. After 20-min. standing at 80°F., 12.5 ml. 0.1N NaOH is added and 20 further shakes given. The remainder of the procedure is normal. The small amount of NaCl introduced by this technique has only a slight effect on FN, insignificant compared with the effect of inactivation of amylases.

The pH of an inactivated flour slurry is the same as that of a flour-water slurry of the same concentration.

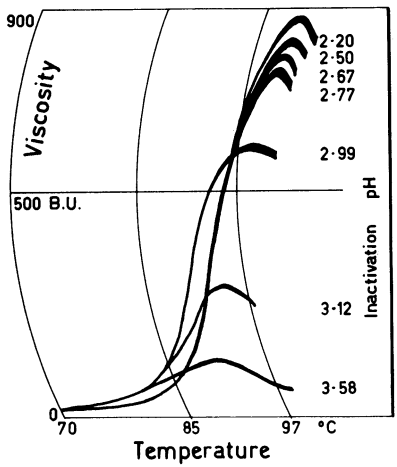
RESULTS AND DISCUSSION

Inactivation of Alpha-Amylase

Figures 1 to 3 show typical effects on the amylogram of holding the flour slurry for varying time and pH at 80°F. before neutralizing and determining the amylogram. Examples of amylogram maxima for a wide variety of flours, with and without acid-inactivation, are given in Table I.

It is necessary to distinguish clearly between reversible inhibition of alpha-amylase activity by pH less than optimum, and irreversible inactivation by lower pH. These two effects overlap in the region pH 4.5 to 3, the reversibility being dependent on time, temperature, and enzyme concentration. Most investigators of the effect of pH on alpha-amylase activity have not gone to sufficiently acid conditions to produce serious irreversible inactivation.

Figures 1 and 2 show that only below pH 3 does inactivation approach



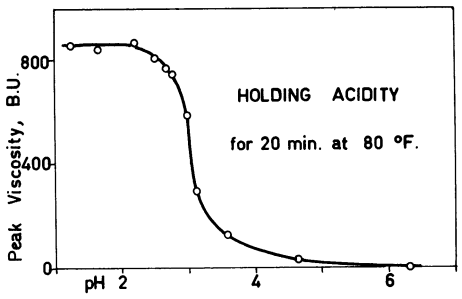


Fig. 1 (left). Family of amylograms, showing effects of holding flour slurry at stated pH for 20 min. at 80° F. before neutralizing and cooking.

Fig. 2 (right). Plot of amylogram maximum against holding pH, for 20 min. at 80° F.

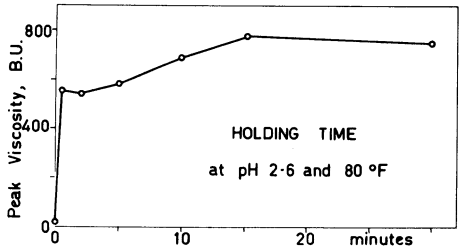


Fig. 3. Plot of amylogram maximum against holding time, for pH 2.6 and 80°F.

TABLE I. AMYLOGRAPH MAXIMUM VISCOSITIES OF FLOURS, WITHOUT AND WITH ACID-INACTIVATION

Variety or Origin	Active B.U.	Inactive B.U.	Variety or Origin	Active B.U.	Inactive B.U.
Sprouted blend, 1968	20	840	Raven (2)	330	1,100
Aotea, 1967	60	980	Arawa (2), 1967	410	1,070
Cracker flour (U.S.)	90	1,250	Capelle (Europe)	415	945
Shackleton (U.K. 1908)	120	1,010	Manitoba No. 3 (Canada)	420	1,280
Raven (1)	145	1,355	Manitoba No. 2 (Canada)	445	1,120
Line 946	170	1,025	Australian soft flour	460	1,140
Cookie flour (U.S.)	205	1,140	Arawa, 1968	475	1,020
Arawa (3), 1967	260	1,051	Lichtis Fruh (Europe)	680	990
Hilgendorf	290	1,040	Aged flour (N.Z. 1957)	985	1,490

maximum. Indeed, the plot of amylogram height against inactivation pH shows a very sharp rise at about pH 2.9. Acid-base titration of flour shows a marked inflection at about pH 2.9, shown even more clearly in the titration of flour proteins. It is the point at which ionization of the carboxyl groups of the proteins commences. This may be more than coincidence; the inactivation of alpha-amylase could be due to an irreversible change of configuration brought about by ionization. That the inactivation was mainly complete at room temperature in 0.5 min., the shortest time investigated, supports the hypothesis of ionization reconfiguration.

If this is so, we obviously cannot use the acid-inactivation method in a bread-baking process, because the enzyme properties and the desirable gluten properties are being destroyed by similar processes. Indeed, our attempts to apply acid-inactivation to bread-baking have given loaves with appearance similar to that caused by heat-damage of flour. Möttönen (5) has recently attempted the production of sour rye bread with an acid-inactivation process operating at pH 3.0. The acid-inactivation process is, however, entirely suitable for those food uses of flour that do not depend on gluten properties, such as soup thickening, gravies, and general thickening in the canning industry (3).

In comparing Fig. 2 with the data of other workers, one must consider that the pH of the inflection is a function of the sensitivity of the method by which the amylase activity is detected, and of the initial level of amylase activity.

Ford (6) in 1904 seems to have been the first to show an inhibitory effect of acid on malt diastatic power. Sherman et al. (7) in 1919 plotted the first curves of

pH against activity and showed that malt amylase lost its activity between pH 2.70 and 2.34, and that *Aspergillus* amylase lost its activity in the same region, 2.80 to 2.34. Pancreatic amylase was much more sensitive, losing activity between pH 4.75 and 4.02.

Venkata Giri and Subrahmanyan (8) held amylases at varying pH, then restored them to optimum before determining activity. Their results showed that barley amylase was not destroyed by brief treatment with pH 2.69 buffer, though pH 3.72 destroyed activity during 15 days of treatment. They showed Taka-diastase to be mainly inactivated at pH 4.2, a result apparently at variance with those of Sherman et al. (7) and Rau and Sreenivasan (9). However, it is known (10) that both acid-stable and acid-labile alpha-amylases are produced by Aspergilli.

The inactivation of cereal alpha-amylase by acid was first used by Ohlsson (11) and later used by Kneen et al. (12). Dadswell and Gardner (13) also used acidification to destroy alpha-amylase. Rau and Sreenivasan (9) described the irreversible inactivation by acid much more specifically, and incidentally showed that, by contrast, their alpha-amylase of fungal origin was reversibly inactivated under the same conditions. Stewart (14) has incorporated an acid-inactivation step in a method for damaged-starch determination.

Inactivation of Beta-Amylase

Looking more closely at the family of amylograms of Fig. 1, it is evident that as amylase is inactivated by acid, the pasting temperature suddenly rises about 2°C. This has not been explained, but it does have the important consequence that beta-amylase becomes considerably more liable to heat-inactivation before it can cause damage to the pasting properties, as described by Yasunaga et al. (15). These authors also showed the significant effect that beta-amylase can have on the amylogram of a flour that is low in alpha-amylase. Hanes and Cattle (16) in 1938 confirmed the suggestion of Ohlsson (11) that beta-amylase possess some starch-degrading ability, just as alpha-amylase has some saccharifying power. Collison and Elton (17), in discussing the raising of pasting temperature by surfactants, distinguished two mechanisms that could bring about the effect: inhibition of starch swelling and reduction of intergranular adhesion.

Ohlsson (11) demonstrated that at pH 2.5 both amylases are almost entirely destroyed in a short time, but at pH 2.8 the beta-amylase was noticeably more resistant to degradation. He also used the thermal instability of beta-amylase for its differential destruction, showing its activity to be little at 60° and nil at 70°C., regardless of pH.

Beta-amylase activity was determined by the method of Schwimmer (18) for two flours of contrasted pasting strength. The activities were reduced by acid treatment from 111 to 10 and from 115 to 11 units, demonstrating that acid treatment used to inactivate alpha-amylase had also removed more than 90% of the total beta-amylase activity.

To completely remove beta-amylase activity from the amylogram, thiomersal was added to active and to acid-inactivated slurries of three flours before heating commenced (Table II). Organomercurials are highly specific and effective inhibitors of beta- but not alpha-amylase (19,20,21). No effect of beta-amylase was evident in acid-inactivated flours. The suggestion of Yasunaga et al. (15) has also been tested,

TABLE II. INHIBITION BY ACIDIFICATION OR BY THIOMERSAL OF BETA-AMYLASE EFFECT ON AMYLOGRAMS

	Aged Flour B.U.	Commercial Flour B.U.	Sprouted Wheat Flour B.U.
Active flour	627	110	24
Active flour + thiomersal	684	118	30
Acid-inactivated flour	1,282	868	973
Acid-inactivated flour + thiomersal	1,280	870	941

that addition of salt to the amylogram mixture raises the pasting temperature sufficiently to ensure thermal inactivation of beta-activity. Additions of salt up to 3% flour basis had no effect on acid-inactivated amylogram height, and even 10% salt had scarcely a discernible effect.

Pasting Strength of Starch

The variation of pasting properties of flours, independent of amylase activities, has been demonstrated by statistical means. A series of flours were analyzed for alpha-amylase activity by the Farrand method (22) and for amylogram height and FN. Additional analyses were made for protein, damaged starch, and ash. There was good correlation between the amylograph and FN results ($r = 0.876^{**}$). The FN results were significantly correlated with the Farrand assay of activity but at a lower degree of correlation ($r = -0.532^{**}$). The amylograph results were not significantly correlated with Farrand units ($r = -0.295^{NS}$). We can conclude that, although the amylograph and FN results are in reasonable agreement with one another, both are dependent on factors additional to alpha-amylase as assayed. Taking the additional factors protein, damaged starch, and ash into consideration did not appreciably increase any of the correlations, suggesting that these three factors were not of importance here.

Moss (4) almost certainly obtained amylase effects in his amylograms of flours; one of the present series of flours, acid-inactivated but otherwise determined by Moss's technique, gave a maximum of 920 B.U. in Moss's units, well above the values quoted by him.

Using the acid-inactivation process on a variety of flours, it is immediately obvious (Table I) that pasting properties vary independently of amylase. This is true of the amylograph technique. The FN method, being less sensitive than the amylograph to low levels of alpha-amylase, tends to attain a normal level of about 240 sec., above which it does not rise except in the presence of gel-strengthing factors such as aging.

Amylograms carried out on starches washed from a series of flours demonstrated that the starches also varied in pasting properties. One cannot assume that washed starch is free of alpha-amylase, since Dodds and Knight (23) showed that damaged-starch granules adsorb considerable amounts of the enzyme. Fractionation of the washed starches into "prime" starch and "gum" starch suggested that the variation was mainly in the "gum" starch fraction. Analyses were made for total pentosan; this was not correlated with pasting variation.

The pasting properties of cereal flours are known to be affected by pentosans (24), surfactants (17,25,26), fats (26), fatty acids (27), and residual protein of the starch granules (28,29).

Horiuchi (30) showed that, in rice, alpha-amylase did not have a close relation with peak amylograph viscosity of the flour. Moss (4), Günzel (31), and Medcalf and Gilles (32) also showed that maximum viscosity of wheat flour pastes varied apart from alpha-amylase activity. Horiuchi's papers have suggested that many of the pasting characteristics of rice starches can be correlated with the amylose (i.e., linear polymer) content. Shukhnov and Maslova (33) also concluded that there is a definite relation between the amylose content and the viscosity of cooked suspensions of rice flour from different varieties.

Susceptibility of Starch to Amylase Attack

A high-pasting flour and a normal flour were compared by the FN method for the effect of added alpha-amylase from sprouted flour. A linear plot of reciprocal of FN against added enzyme showed that the normal flour was more susceptible than the high-pasting flour to the action of amylase. We can conclude that the high-pasting flour contains either additional structure in the starch or additional components favorably affecting starch structure, to make it more resistant.

Those flours having a FN in excess of the "normal" value of about 240 sec. we have called "supersound." This effect is not a function of lack of amylase but of an additional strength of pasting of the starch. Additional to this increased strength may be increased resistance to alpha-amylase attack, but at low enzyme levels this affects only the amylogram, not the FN.

Conversely, low FN is not necessarily to be interpreted as due to alpha-amylase activity. Olered and Augustin (34) interpreted low values in one year's crop in Sweden as a lower degree of starch polymerization.

It is generally assumed that variation of starch properties, other than granule mechanical damage, plays little part in the variation of bread-baking qualities of different wheat flours. However, Williams (35) recently showed that the starch of hard wheat flours is apparently more susceptible to attack by diastatic enzymes than is starch from soft wheat flours.

The variation of pasting properties reported here suggests that the status of starch in bread quality should be re-examined with respect to inherent gel strength of the minimally hydrated, cooked, and cooled starch of bread crumb and with respect to the susceptibility of different wheat starches to alpha-amylase attack. The work of Mitchell (36) and further unpublished work on New Zealand commercial flours has already shown that the correlation between Hagberg penetrometer assessment of alpha-amylase damage and the bread-baker's assessment of crumb damage is good only for the average of many samples; individual samples show wide deviations from the average correlation. This suggests that either gel structure or paste susceptibility is a significant factor in the effect of sprout damage on bread quality.

Stability of Alpha-Amylase

In aged flour free fatty acids are known to increase (37). In the present work it has been repeatedly observed that aged wheats and flours give high amylograms and

high FN's. The high amylogram of an aged flour does not necessarily reflect absence of alpha-amylase, but is due to increase of pasting strength of the starch, with perhaps also increased resistance to enzyme attack. One example will demonstrate this. A sample of flour stored 10 years at room temperature, smelling rather rancid and with ruined baking quality, had an amylogram maximum of 985 B.U. When the acid-inactivation process was applied, the amylogram maximum rose to 1,490 B.U., suggesting that considerable amylase activity was still present in the flour.

Alpha-amylase in flour does have remarkable stability, particularly at lower temperatures. A sample of flour recovered from the Antarctic in 1966, part of the provisions taken there by Shackleton's expedition of 1908, had an amylogram height of only 120 B.U. whereas the inactivated amylogram height was 1,010 B.U. Alpha-amylase determined by Farrand's method (22) was 18.0 units, suggesting that the flour was made either from slightly sprouted wheat or that it had been malted. For comparison, a modern Canadian flour we have analyzed had an amylogram height of 80 B.U. and amylase activity of 28 Farrand units.

Uses of Acid-Inactivation

We can conclude that the acid-inactivation process has effectively removed both alpha- and beta-amylase actions from the amylogram. Then, by addition of standard amylase preparations, we can assess the various susceptibilities of different flours to attack. In the absence of added enzyme we can assess the inherent pasting properties of different flours. These may not reflect variation of pasting of the starches themselves, however, but variation of other constituents that affect starch pasting. We have already noted above that fungal alpha-amylase may not be destroyed by this process, so that we have a possible method for determining fungal amylase additions to flour. Enzyme-digestibility methods for determining damaged-starch content of flour require, first, that the naturally occurring amylases be destroyed. This has previously been done by refluxing with ethanol (38), but acid-inactivation is easily applicable with consequent simplification of technique, as shown by Stewart (14).

The observations of aged flours suggest another approach to the combatting of sprout damage; namely, to increase the strength of the starch gel and its resistance to amylase attack. The use of fatty and surfactant compounds seems a reasonable approach and this will be explored in a subsequent paper.

Hutchinson (1) suggests that for satisfactory soup-making properties a flour should give an amylogram exceeding 450 B.U., for a 10% solids slurry. That figure corresponds to about 520 B.U. in the units of this paper. It is evident (Table I) that acid-inactivation enables this criterion to be easily satisfied. The lower possible paste concentration enables consequent saving in material costs. Kent-Jones and Amos (personal communication) suggest for the same purpose that 750 B.U. maximum should result from 60 g. flour plus 450 ml. water. That is 820 B.U. in the present terms. Again acid-inactivation easily satisfies this. We have applied the acid-inactivation process to commercial paste manufacture with outstanding success. A similar acid-inactivation of degradative enzymes in tomato pulp manufacture has been patented (39).

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