Properties of Wheat Beta-Amylase Adsorbed on Glutenin[1]

JOHN A. ROTHFUS and STEPHEN J. KENNEL, Northern Regional Research Laboratory, Peoria, Illinois

ABSTRACT

Incubation of saline-soluble wheat beta-amylase with glutenin produces an insoluble enzymatically active complex. The pH profile of the bound enzyme indicates that binding may be selective for only certain wheat amylases. Association with glutenin reduces enzyme activity, but similarity between the kinetic properties of bound and unbound beta-amylase suggests that binding involves a portion of the enzyme that is remote from its catalytic site. Elevated temperatures increase the activity of bound beta-amylase, but the relation between activity and temperature changes at 20°C. Calculated activation energies are 11.7 kcal./mol. between 4° and 20°C. and 8.8 kcal./mol. between 20° and 55°C. The apparent Michaelis constant for the bound enzyme is 0.15% (w./v.). Little or no active enzyme is released from the complex by disulfide-reducing agents, 0.1M NaCl, or temperatures up to 55°C.

Beta-amylase is, perhaps, the best known enzyme in wheat endosperm. Considerable information has accumulated on its properties in solution (1,2). Comparatively little is known, however, about the molecular disposition and behavior of the enzyme adsorbed on nonactive materials in dough, pasta, or other semisolid forms in which wheat is usually processed. In general, cereal-grain enzymology dealing with activities in aggregates or insoluble complexes remains relatively unexplored, even though important roles have long been assigned to biologically active proteins in such endeavors as baking and brewing.

This communication describes properties of the active insoluble complex that is formed when wheat beta-amylase is mixed with glutenin.

MATERIALS AND METHODS

Flour was from laboratory stock that was milled from sound HRW wheat (Ponca variety) and stored for 3 years at −15°C. Glutenin was isolated by the method of Jones et al. (3). Portions of this material were granulated as follows:

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Freeze-dried glutenin (100 mg.) was dispersed, with stirring, in 25 ml. of 1M acetic acid at 75°C. on a heated magnetic stirrer for 1 hr. The hot solution was brought to pH 5.5 with 2N NaOH, and the precipitation of granular glutenin was completed with stirring at 4°C. for 16 hr. Either the resulting suspension was used directly or the granulated glutenin was dialyzed against 4 liters of water for 18 hr., lyophilized, and stored. Before use, this stored glutenin (100 mg.) was suspended in 1M acetic acid (25 ml.) and neutralized to pH 5.5 with 2N NaOH. All preparations of granulated glutenin were free of reducing sugar as measured by reaction with 3,5-dinitrosalicylic acid. Treatment of the glutenin with either papain (0.1 mg./ml. in 0.025M cysteine, pH 5.1) or 10^{-4}M dithiothreitol (Calbiochem, Los Angeles, Calif.) failed to produce any amylase activity.

Crude beta-amylase was prepared by extraction of 10 g. of wheat flour with 100 ml. of 0.1M NaCl for 4 hr. at 4°C. The extraction mixture was centrifuged for 20 min. at 4°C. in an International centrifuge with head No. 824a at 3,200 r.p.m. (1,800 X g), and the supernatant solution was separated and brought to 35% saturation with solid $(NH_4)_2SO_4$. The protein, which precipitated overnight at 4°C., was separated by centrifugation at 1,800 X g, dissolved in 5 ml. of cold water, and then lyophilized to dryness. This preparation was free of reducing sugar as measured by reaction with 3,5-dinitrosalicylic acid. The activity of the crude beta-amylase was 3.0 units per mg. One unit of activity was the amount of enzyme that liberated 1 μmol. of maltose per min. at 25°C.

Stirring 40 mg. of wheat beta-amylase at 4°C. overnight with granulated glutenin (100 mg.) in sodium acetate solution at pH 5.5 produced a solid enzyme-glutenin complex. The solid was separated by centrifugation at 1,800 X g for 5 min. at 4°C., resuspended in 25 ml. of ice-cold 0.1M NaCl, and recovered again by centrifugation. Washing with cold salt solution was repeated until the supernatant showed no beta-amylase activity.

Soluble beta-amylase was assayed by determination of liberated maltose with 3,5-dinitrosalicylic acid according to the method of Bernfeld (4). Substrate was generally 1% soluble starch (Difco Laboratories, Detroit, Mich.) in 0.016M sodium acetate buffer. Starch suspensions were heated to 70°C. to effect solution and filtered through Celite on Whatman No. 1 filter paper after cooling to room temperature. Unless indicated differently, activity of the glutenin-bound enzyme was measured by determination of maltose in the effluent of starch solutions passed through a column of the bound enzyme. Flow rates were controlled by a Beckman Accu-Flo pump attached to the column outlet.

Enzymatic hydrolysates of starch were analyzed by descending chromatography on Whatman No. 1 filter paper in butanol:pyridine:water (6:4:3). Sugars were visualized by spraying the paper with ammoniacal silver nitrate (0.3M $AgNO_3$ in 0.9M NH_4OH) and drying at 100°C. for 5 min.

EXPERIMENTAL AND RESULTS

Glutenin-Bound Beta-Amylase

Incubation of beta-amylase with glutenin produced an insoluble active complex, but only 0.5% of the activity originally in the incubation mixture was recovered in the solid regardless of whether incubation was carried out for 16 or 96 hr. (Table I). Comparison of activities recovered in supernatants from glutenin-enzyme

TABLE I. EFFECTS OF GLUTENIN AND A CROSS-LINKING AGENT ON BETA-AMYLASE[a]

Component Mixed with Beta-amylase	Temperature and Time °C.	hr.	Concentration %	Enzyme Activity (Percent of Total Added) Supernatant %	Precipitate %
Glutenin	4	16		59	0.5
Glutenin	4	96		47	0.5
Glutenin plus glutaric dialdehyde	4	16	0.0015	50	0.08
Glutaric dialdehyde	4	17	0.05	98	
Glutaric dialdehyde	4	1	5.0	0	

[a]Values are averages of at least duplicate experiments. Enzyme activity was measured at pH 5.5 by the method of Bernfield (4). After development of color, assay solutions containing glutenin-amylase were clarified by centrifugation.

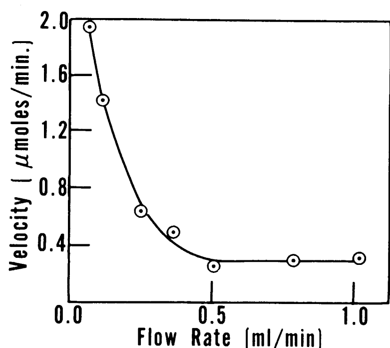
incubation mixtures with the activities of enzyme solutions exposed to the same conditions indicated that 40 to 50% of the enzyme could have been bound. Presumably, only those amylase molecules that are sterically unhindered at the surface of the glutenin aggregate remain active. This contention is supported by the fact that a short-chain cross-linking agent readily inactivated bound beta-amylase (Table I). Whereas the free enzyme was fully active after 17 hr. in 0.05% glutaric dialdehyde solution, activity of the bound enzyme was reduced some sixfold by incubation in only 0.0015% glutaric dialdehyde. In more concentrated glutaric dialdehyde solution (5%), beta-amylase was completely inactivated after only 1 hr. at 4°C.

Other properties of glutenin-bound beta-amylase were measured by monitoring changes in starch solutions as they passed through small columns containing the enzyme. A suitable column was prepared by placing 100 mg. of glutenin-bound beta-amylase into a standard jacketed glass column (0.9 X 15 cm.) that contained 2.5 g. of 4-mm. glass beads. Glass beads prevented the bound enzyme from packing into an impervious mass and made it possible to sustain high flow rates through the column. Column packings maintained at 4°C. and stored in 0.1M NaCl when not in use remained active for more than 8 weeks.

The hydrolysis of starch solutions passed through columns of bound enzyme depended in part upon the flow rate. An example of the relation between flow rate and apparent activity at pH 5.2 is illustrated in Fig. 1. At slower flow rates, the longer contact times produced corresponding increases in the apparent velocity of hydrolysis. At rates above 0.5 ml. per min., in this particular example, the amount of starch hydrolyzed was essentially independent of flow rate. Generally, a rate of 1 ml. per min. provided the best combination of convenience and conditions ideal for measuring initial enzyme velocities. Chromatography of hydrolysates showed that maltose was the principal product.

pH Optimum

The effects of pH on bound beta-amylase were measured with a column of the enzyme washed with 0.1M NaCl (20 ml.) and then equilibrated at 4°C. with starch solution at the specified pH. After eight 1-ml. fractions were collected at a flow rate of 1 ml. per min., the column was washed again with 0.1M NaCl and then



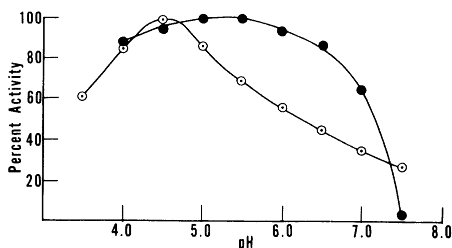


Fig. 1 (left). Effect of flow rate on the apparent velocity of starch hydrolysis by glutenin-bound beta-amylase in a column at 4°C. in 0.1M acetate buffer, pH 5.2.

Fig. 2 (right). Comparison of the activities of unbound wheat beta-amylase (solid circles) and glutenin-bound beta-amylase (dotted circles) at different pH values in 0.05M acetate (pH 3.5 to 5.5) or 0.05M phosphate buffers (pH 6.0 to 7.5) at 4°C. Values for the bound beta-amylase are averages of eight fractions taken from column effluent at a flow rate of 1 ml. per min. Before samples were collected, the column was equilibrated at each pH value by washing with 20 ml. of 0.1M NaCl and 10 ml. of buffered 1% starch solution.

re-equilibrated at another pH value. In Fig. 2, the pH activity profiles of glutenin-bound wheat beta-amylase and the unbound enzyme are compared. The free enzyme was active over a broad pH range with an optimum near pH 5.4. In general, the curve was similar to the profile recorded for water-soluble wheat beta-amylase by Meyer et al. (5). The activity of bound beta-amylase, in contrast, reached an optimum near pH 4.6, and then decreased rapidly at higher pH's, but remained more active than the soluble enzyme at pH 7.5.

Effect of Temperature

Experiments illustrating how temperature affects activity were conducted with a jacketed column of glutenin-bound beta-amylase in much the same way that changes with pH were studied. Temperatures were read from a thermometer suspended just above the packing inside the column. The column, operating at a flow rate of 1 ml. per min., was brought to the desired temperature and maintained there for at least 15 min., after which four 1-ml. fractions of effluent were collected and analyzed.

An inflection in the plot of activity vs. temperature near 20°C. (Fig. 3, A) corresponds to a change in the activation energy of the reaction, which, in similar cases, has been attributed by Dixon and Webb (6) and Massey et al. (7) to a transition of the enzyme between active conformations. Between 4° and 20°C., the activation energy was 11.7 kcal. per mol., whereas between 20° and 55°C. it was 8.8 kcal. per mol. The relation between activity and temperature exhibited by glutenin-bound beta-amylase was similar to that observed with water-soluble beta-amylase by Meyer et al. (5), even though their values at pH 4.8 were slightly higher.

After the enzyme had been returned to 4°C., the level of activity was about 25% lower. Presumably, this change was the result of irreversible denaturation of some of the enzyme at higher temperatures. It is unlikely that the lower activity was due

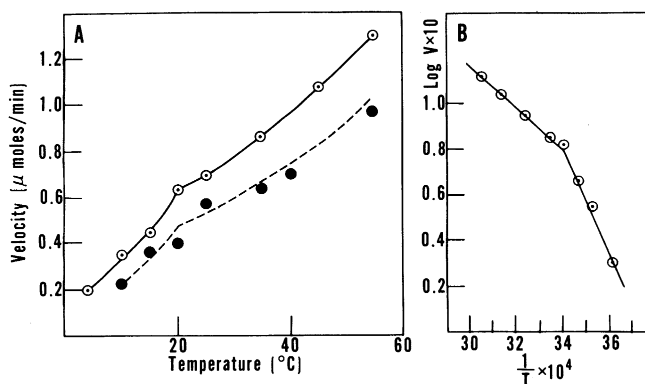


Fig. 3. A, Effect of temperature on the velocity of starch hydrolysis by glutenin-bound beta-amylase. Values are averages from four 1-ml. fractions taken at a constant flow rate of 1 ml. per min. Substrate was 0.5% starch in 0.01M acetate buffer, pH 5.2. The initial experiment (dotted circles) was conducted from 4° to 55°C. and then repeated (solid circles) with the same enzyme. B, Arrhenius plot of effect of temperature on velocity.

to a release of active enzyme from the glutenin, since analytical values obtained with duplicate samples of column effluent that had been kept at 4°C. for an additional 48 hr. varied from the original values by an average of only ±4.2%.

Lineweaver-Burk Analysis of Activity

Figure 4 compares Lineweaver-Burk plots of the activities of free and glutenin-bound beta-amylase. At pH 5.1, the Michaelis constants (K_m) of the bound and unbound enzyme preparations are nearly the same with numerical values

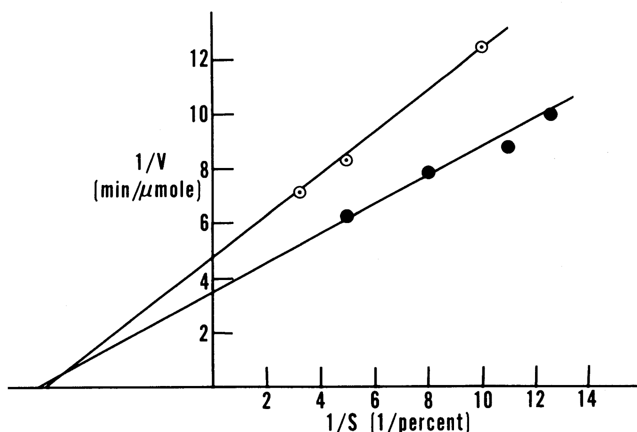


Fig. 4. Lineweaver-Burk plots of initial velocities of unbound wheat beta-amylase (solid circles) and glutenin-bound beta-amylase (dotted circles) at different starch concentrations in 0.01M acetate buffer, pH 5.1 at 4°C. Unbound enzyme concentration was 7.5 γ/ml. Activity represents maltose liberated in 3 min. Bound enzyme activities are averages from eight 1-ml. fractions of effluent from a column of enzyme operated at a flow rate of 1 ml. per min.

TABLE II. EFFECT OF MERCAPTANS ON GLUTENIN-BOUND BETA-AMYLASE

Mixture	Enzyme Activity[a]	
	Supernatant	Precipitate
Glutenin-amylase	0.00	0.50
Plus 0.5 µmol. cysteine	0.00	0.50
Plus 10 µmol. cysteine	0.06	0.50
Plus 20 µmol. cysteine	0.06	0.50
Plus 20 µmol. cysteine plus 120 µmol. mercaptoethanol	0.09	0.50

[a]Expressed as the percentage of total used in preparation of glutenin-amylase.

near 0.15%. This percentage is of the same order of magnitude as the K_m determined at 35°C. for water-soluble beta-amylase from whole-wheat flour by Meyer and Press (8).

Influence of Sulfhydryl Groups on Bound Beta-Amylase

Glutenin-bound beta-amylase was treated with cysteine and mercaptoethanol (Table II) to test the possibility that the glutenin-enzyme complex was stabilized by disulfide bonding, as suggested for latent beta-amylase by Rowsell and Goad (9). Ionic bonding appeared to be of minor importance, since the bound enzyme remained insoluble in 0.1M NaCl.

Addition of 0.5 µmol. of cysteine hydrochloride to 100 mg. of glutenin-bound enzyme in 5 ml. of water at pH 5.5 failed to release any activity into the supernatant during 30 min. at 4°C. An additional 10 µmol. of cysteine liberated activity equivalent to 0.06% of the total activity that was incubated with glutenin or about 0.1% of the activity that could have been bound to the glutenin (Table I). Another 10 µmol. of cysteine did not increase activity further. At this point, the amount of cysteine added (20.5 µmol.) was 46% in excess of that theoretically required to rupture all disulfide bonds in the glutenin (12 µmol.) and beta-amylase (2 µmol.). To keep the accumulated ionic strength low, the mixture was treated thereafter with mercaptoethanol to a tenfold excess of reducing agent. At this level, the amount of soluble beta-amylase in the supernatant solution increased to only 0.09%, and the residual solid was as active as it had been before treatment with disulfide bond-cleaving reagents. Thus, forces other than disulfide bonding, perhaps hydrophobic interactions or a combination of bonds of different types, must cause the enzyme to adhere to glutenin.

DISCUSSION

The stability of the beta-amylase-glutenin complex is surprising, in consideration of the ease with which the enzyme can be extracted from flour. This raises a question whether beta-amylase is altered upon adsorption or whether other endosperm components separated in preparing the glutenin and enzyme are important in solubilizing and activating beta-amylase. In this connection, there is little doubt that the enzyme molecules that remain active after adsorption retain much of their native configuration, since the bound enzyme has nearly the same apparent Michaelis constant as unbound beta-amylase and behaves remarkably like soluble beta-amylase in response to temperature.

The unusual pH-activity profile exhibited by the bound enzyme (Fig. 2) is

especially interesting because it nearly duplicates the pH-activity profile of one of the wheat beta-amylases isolated by Tkachuk and Tipples (2). Equally noteworthy is the fact that the amount of enzyme apparently bound to glutenin in our experiments and the amount of "bound amylase" enzyme soluble in salt solution, found in wheat by Tipples and Tkachuk (10), both represent approximately 45% of the extractable amylase in flour.

Obviously, our experimental conditions do not duplicate the native relation between glutenin and the wheat amylases, but the characteristics just described, when considered together, suggest that the binding of beta-amylase by glutenin may be a selective process, specific for certain wheat enzymes. We will investigate the possibility of specific interaction and control of enzyme activity as quantities of the individual enzymes and glutenin proteins become available.

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