Inhibition of Alpha-Amylase-Catalyzed Starch Granule Hydrolysis by Cycloheptaamylose

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ABSTRACT

Cycloheptaamylose (CHA) inhibited the adsorption of triticale and wheat α-amylases to wheat and waxy maize starch granules at 4°C. The adsorption of both amylases to wheat starch granules was inhibited 50% by 0.5 m*M* CHA. With waxy maize starch granules, however, 2m*M* CHA was required for 50% inhibition of triticale α-amylase and 30% inhibition of wheat α-amylase. The effect of CHA on adsorption of wheat α-I and wheat α-II amylase to wheat starch differed little. Hydrolysis of starch granules catalyzed by the cereal α-amylases was inhibited 20–60% by 0.88 m*M* CHA. At 15°C, the solubilization of wheat and of waxy maize starch granules by either triticale or wheat α-amylase was inhibited to the same extent by CHA. Solubilization of wheat starch by wheat α-amylase I was inhibited 40% by 0.88 m*M* CHA, whereas wheat α-amylase II solubilization was inhibited 35%. Inhibition of solubilization was similar for the cereal α-amylases and wheat starch granules at 35°C and comparable to that obtained at 15°C. The hydrolysis of waxy maize starch granules by either amylase was inhibited by CHA almost twice as effectively at 35 as at 15°C. CHA had similar effects on appearance of soluble carbohydrate and of reducing activity. The evidence suggests that a noncatalytic site on the cereal α-amylase might facilitate the hydrolysis of starch granules by adsorption of the enzyme to the granule at this site.

Evidence of the interaction of α-amylase with the starch granule through a noncatalytic site on the enzyme has existed for some time. Schwimmer and Balls (1949) studied the affinity of malt α-amylase for wheat starch granules at a low temperature. Malt α-amylase appeared to interact through a site different from the catalytic site. This was inferred because maltose noncompetitively inhibited the action of malt α-amylase on starch in solution and desorbed α-amylase from starch granules. In addition, Schwimmer and Balls demonstrated that the efficiency of adsorption was proportional to the surface area of the granules. Using α-amylase from various sources, Walker and Hope (1963) reached similar conclusions. In addition, they observed that *Aspergillus oryzae* α-amylase did not adsorb to starch granules and that the *Bacillus subtilis* enzyme only weakly adsorbed. There has, however, been little evidence to support suggestions on the functional role for a noncatalytic site on the enzyme.

Cycloheptaamylose (CHA) is a known inhibitor of α-amylase (Marshall 1973, Thoma and Koshland 1960) but does not always inhibit α-amylase digestion of soluble starch. Thus, pancreatic α-amylase is inhibited by CHA (Mora et al 1974), whereas cereal α-amylase is not (Weselake and Hill 1982). However, the ligand does interact with cereal α-amylase at a noncatalytic site as shown by the ability to purify the enzyme via affinity chromatography using CHA as the ligand (Silvanovich and Hill 1976, Weselake and Hill 1982). In addition, a dissociation constant of 19 μ*M* for the enzyme-ligand complex was determined by equilibrium dialysis (Weselake and Hill 1982).

Studies to determine the functionality of a noncatalytic site using starch or starch fractions are difficult to perform because these components can act as substrates in the catalytic process. CHA, on the other hand, is not cleaved to any degree by α-amylases (Thoma and Stewart 1965). In the case of wheat and triticale α-amylase, CHA was found not to interfere with the hydrolysis of soluble starch catalyzed by the enzyme (Weselake and Hill 1982). We used the ligand to demonstrate that it does interfere with binding of cereal α-amylase to starch granules and, furthermore, that it inhibits the release of soluble starch from starch granules catalyzed by the enzyme.

MATERIALS AND METHODS

Enzyme Purification

Total cereal α-amylase (α-I and α-II) from germinated and freeze-dried triticale (×*Triticosecale* Wittmack) line 6A190 and Neepawa wheat (*Triticum aestivum*) was prepared from crude kernel extracts according to a modification of the method of Silvanovich and Hill (1976). All operations were performed at 0–4°C. Crude extract was stirred in the presence of 10 mg/ml polyvinyl-polypyrrolidone (PVP) for 30 min, and the PVP was then removed by filtration through glass wool. The PVP-treated extract was then dialyzed overnight against 0.02*M* sodium acetate (pH 5.5, 0.001*M* $CaCl_2$). After dialysis, precipitated material was removed by centrifugation at 10,000 × *g* for 20 min. The supernatant was then fractionated on the CHA-epoxy-Sepharose 6B affinity column (1.6×7.6 cm). Total α-amylase, eluted from the affinity column with CHA in the moving phase, was rendered free of the cyclic dextrin by passing the enzyme from the affinity step through a column of Bio-Gel P-4 (200–400 mesh, 2.5× 28 cm) equilibrated with 0.05*M* sodium acetate (pH 5.5, 0.001*M* $CaCl_2$). α-Amylase was assayed by the Briggs method (1961) and protein by the method of Lowry et al (1951).

Green type wheat α-amylase isozymes (α-I) were separated from the total α-amylase by carboxymethyl-cellulose (Whatman CM-32) chromatography on a column equilibrated with 0.02*M* sodium acetate (pH 5.5, 0.001*M* $CaCl_2$) (Silvanovich and Hill 1977). Germination type wheat α-amylase isozymes (α-II) were prepared by chromatography on a 0.5 × 10 cm column of diethylamino-ethylcellulose (Whatman DE-32) equilibrated with 0.05*M* imidazole-HCl (pH 7.4, 0.001*M* $CaCl_2$). Enzyme solution (½ ml) containing 72,000 IDC units as defined by Briggs (1961) in equilibrating buffer was applied to the ion-exchange column. The column was then eluted with a linear gradient consisting of 20 ml of equilibration buffer and 20 ml of equilibration buffer containing 0.2*M* NaCl. The fractions were analyzed by isoelectric focusing in an ampholyte gradient (pH 3.5–9.5) using commercially available polyacrylamide flatbed gels (Fisher Scientific). Isoelectric focusing was performed according to manufacturer specifications. The isozymes were visualized using starch zymograms based on a method adapted from Doane (1967). The fractions with α-II components were pooled and dialyzed against 0.05*M* sodium acetate (pH 5.5, 0.001*M* $CaCl_2$). All purified enzymes (total triticale α-amylase, total wheat α-amylase, wheat α-I, wheat α-II) were concentrated in an Amicon concentrator cell equipped with YM10 membrane. Bovine serum albumin (BSA, 0.1%) was incorporated into the concentrated enzyme preparations before freezing for future use.

Procedure for Studying Inhibition of Binding

Enzyme was mixed with 25 mg of starch granules in 5 ml of 0.05*M* sodium acetate (pH 5.5, 0.001*M* $CaCl_2$) in separate tubes containing concentrations of CHA ranging from 0 to 2 m*M*. All

operations were performed at 4° C to minimize hydrolysis. The test tubes were sealed and their contents tumbled slowly for 30 min at 4° C on a rotary shaker to allow for optimum enzyme adsorption (MacGregor 1979). The tubes were then centrifuged at 2,000 × *g* for 5 min and the supernatants assayed for α-amylase activity. Enzyme activity was determined by measuring the appearance of reducing activity from the hydrolysis of gelatinized starch at 30° C in the presence of 0.05*M* sodium acetate (pH 5.5, 0.001*M* $CaCl_2$). Reducing activity was determined using the Nelson (1944) adaptation of the Somogyi method for the determination of glucose using reagents recommended by Robyt and Whelan (1968). The amount of enzyme bound was determined from the difference between the amount of enzyme activity added and the amount present in the supernatant.

Five studies were conducted. Triticale α-amylase purified by affinity chromatography and containing 600 IDC units was introduced into each tube of a series with wheat starch granules (BDH) and into another series of tubes with waxy maize starch granules (American Maize Products). Systems with wheat α-I isozymes (510 IDC units) and wheat α-II isozymes (585 IDC units) were set up with wheat starch granules. Another system consisted of total wheat α-amylase (600 IDC units) from the affinity step and waxy maize starch granules. Wheat starch granules and waxy maize starch granules contained 10.8 and 9.3% moisture, respectively.

Procedure for Studying Inhibition of Granule Hydrolysis

The hydrolysis experiments were performed at 35 and 15° C. The starch granules were from the same batches used in the binding experiments. A time course study was conducted with triticale α-amylase and waxy maize starch granules at 35° C. Fifty milligrams of waxy maize starch granules were suspended in 10 ml of 0.05*M* sodium acetate (pH 5.5, 0.001*M* $CaCl_2$) containing 1 mg/ml of BSA. An identical system was prepared that contained 1 mg/ml of CHA by weight. The tubes were then warmed to 35° C in a water bath, and a small volume of triticale α-amylase mixture containing 3,300 IDC units was added. The tubes were sealed and gently rotated. At intervals, 1-ml aliquots were taken while the suspension was gently mixed. The aliquot was mixed with 0.02 ml of 5*N* HCl to stop the reaction. After enzyme deactivation the granules were sedimented using a Beckman Microfuge. The supernatants were retained at 4° C for analysis of soluble carbohydrate by the phenol-sulphuric acid method of Dubois et al (1956).

Similar experiments were conducted at 15° C by studying wheat α-I and α-II isozyme groups independently in the hydrolysis of wheat starch granules. An enzyme activity of 2,300 IDC units was used in each study. A control system without enzyme was used to check for spontaneous release of soluble carbohydrate. Reducing activity released in the soluble fractions was also determined according to the described method.

In another series of experiments, triticale and wheat α-amylase, and wheat and waxy maize starch were studied in all combinations at 15 and 35° C. An enzyme activity of 2,160 IDC units was used in each case. After 5 hr the entire reaction mixture was treated with 0.2 ml of 5*N* HCl and centrifuged at 3,000 × *g* for 10 min at 0° C. Reducing activity was determined in the supernatants.

RESULTS

The enzymes prepared by the affinity chromatography procedure are purified about 275-fold from crude extracts with yields ranging from 40 to 120% depending upon the source of the enzyme. They are free of β-amylase contamination (Weselake and

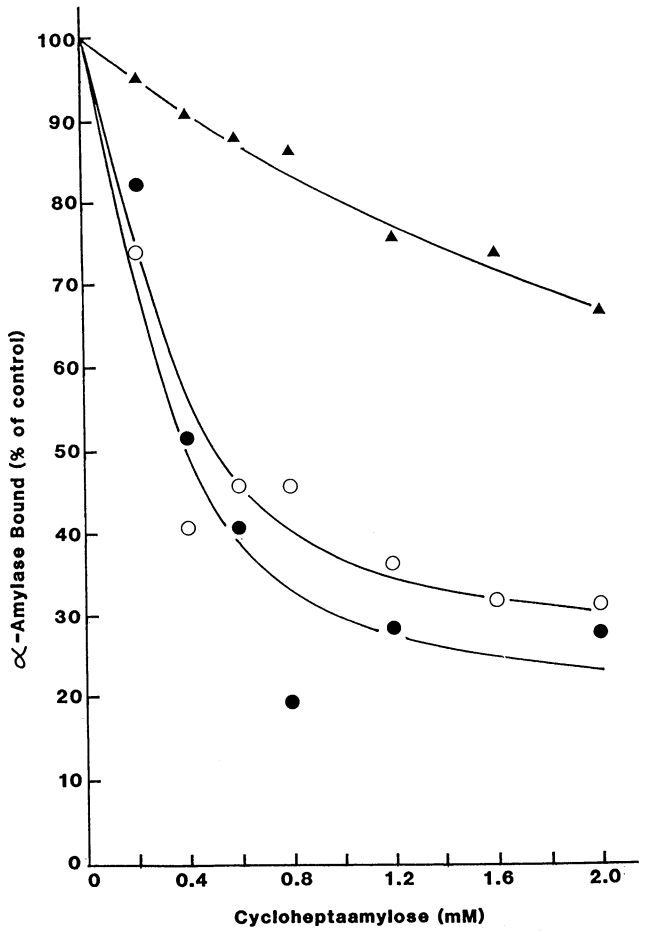

Fig. 1. Effect of cycloheptaamylose on the binding of wheat α-amylase to starch granules at 4° C. o = Wheat α-I and wheat starch, • = wheat α-II and wheat starch, ▲ = total wheat α-amylase and waxy maize starch.

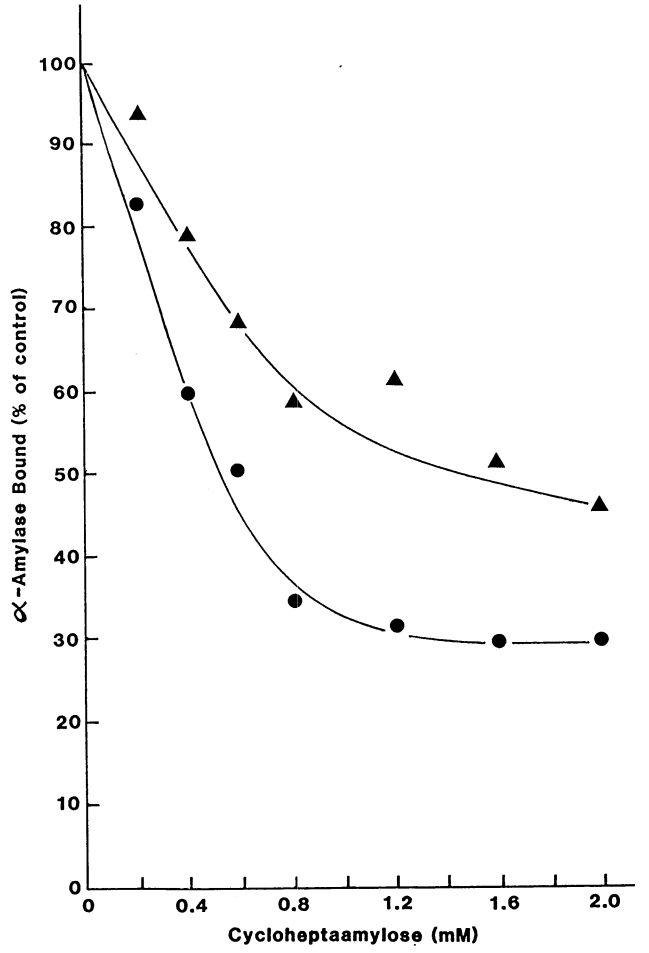

Fig. 2. Effect of cycloheptaamylose on the binding of triticale α-amylase to starch granules at 4° C. • = Wheat starch, ▲ = waxy maize starch.

Hill 1982).

CHA inhibited the binding of wheat α-amylase to starch granules at 4° C (Fig. 1). There appeared to be little difference in inhibition by the ligand of α-I and α-II wheat amylase binding to wheat starch granules. Fifty percent inhibition occurred at approximately 0.5 m*M* CHA, and the effect of increased CHA was minimal after about 0.8 m*M* CHA. In contrast, 2 m*M* CHA was required for 30% inhibition of adsorption of a mixture of wheat α-amylases to waxy maize starch granules (Fig. 1). Inhibition of triticale α-amylase binding to wheat starch granules at 4° C (Fig. 2) was similar to the system with wheat enzyme and wheat starch. The binding of the triticale enzyme to waxy maize starch granules was inhibited 50% at 2 m*M* CHA (Fig. 2).

The amounts of enzyme bound in the absence of CHA differed considerably. Waxy maize starch bound approximately 95% of wheat α-amylase or triticale α-amylase while wheat starch granules bound approximately 77% of either of the cereal enzymes. Wheat starch granules bound 73% of α-I wheat amylase and 34% of wheat α-II amylase.

The effect of CHA on the α-amylase-catalyzed release of soluble starch from waxy maize starch granules at 35° C is shown in Fig. 3. The release of soluble starch in the control (without CHA) was curvilinear over a 5-hr period. The presence of 0.88 m*M* CHA caused a 40–50% inhibition of starch solubilization after 1–5 hr of incubation. The effect of CHA with wheat α-amylase isozymes and wheat starch at 15° C is shown in Fig. 4. The curves were biphasic and consisted of a rapid phase solubilizing approximately 6–8% of the starch granules followed by a slow phase. Addition of 0.88 m*M* CHA caused a 40% inhibition of starch solubilization in the case of the α-I isozymes and a 35% inhibition of α-II isozymes after 5 hr of incubation. Release of soluble starch in the absence of enzyme was insignificant. The appearance of reducing activity in the same system was slightly lower when measuring reducing activity, and the shape of the curves changed.

The effect of CHA on the α-amylase-catalyzed release of reducing activity from starch granules is shown in Table I for eight systems where reducing activity determinations were made after 5 hr of hydrolysis. The inhibition by CHA at 15° C did not vary appreciably with the source of enzyme or starch ranging from 21–31%. The results at 35° C for wheat starch were similar to those obtained at 15° C. However, in the waxy maize starch systems the degree of inhibition at 35° C approximately doubled for both wheat and triticale α-amylases when compared to the degree of inhibition at 15° C.

TABLE I
Effect of Cycloheptaamylose on the Release of Reducing Activity from Starch Granules Catalyzed by Cereal Alpha-Amylases at Two Temperatures

	Percent Inhibition			
	Wheat α-Amylase		Triticale α-Amylase	
	(15° C)	(35° C)	(15° C)	(35° C)
Wheat starch	25	32	21	29
Waxy maize starch	31	61	23	51

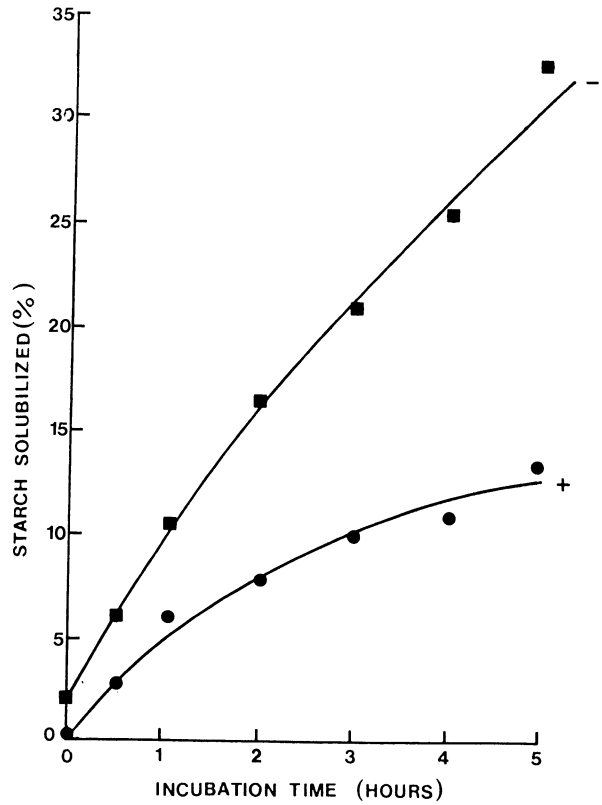

Fig. 3. Effect of cycloheptaamylose on the release of soluble carbohydrate from waxy maize starch granules by triticale α-amylase at 35° C. ■ = Control hydrolysis, • = hydrolysis in the presence of 0.88 m*M* cycloheptaamylose.

DISCUSSION

Schwimmer and Balls (1949) first suggested that α-amylase had a noncatalytic site for maltodextrin interaction based on evidence that maltose and limit dextrins interfered with ethanol precipitation of the starch-enzyme complex. The effect of CHA on binding of α-amylase to starch granules (Figs. 1 and 2) indicates that CHA interacted at this noncatalytic site. CHA had no effect on soluble starch hydrolysis (Weselake and Hill 1982) and, therefore, does not appear to bind at the active site. The enzyme has a strong

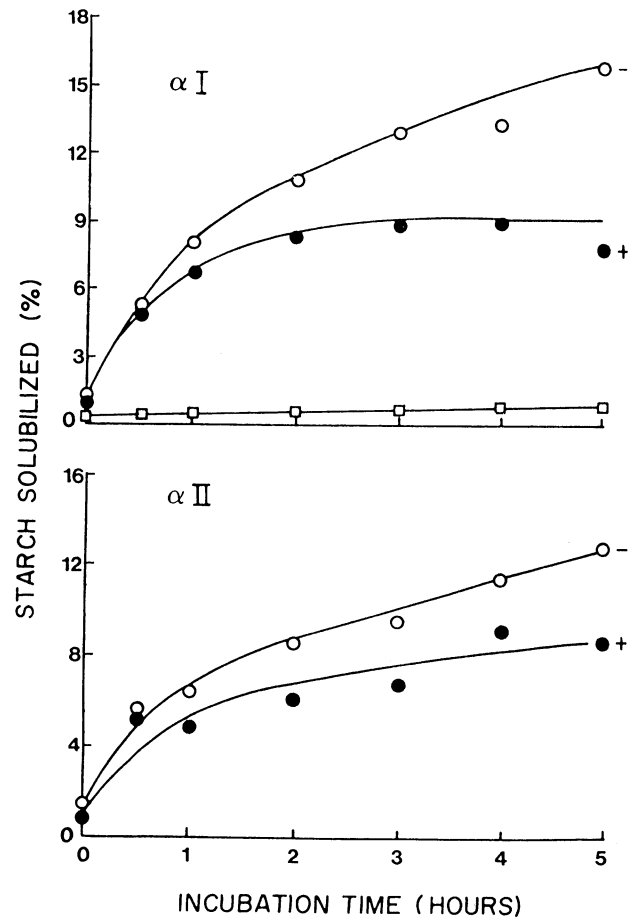

Fig. 4. Effect of cycloheptaamylose on the release of soluble carbohydrate from wheat starch granules by wheat α-amylase isozyme groups at 15° C. o = Control hydrolysis, • = hydrolysis in the presence of 0.88 m*M* cycloheptaamylose, □ = spontaneous release of soluble starch without enzyme present.

affinity for CHA (Weselake and Hill 1982) that is borne out by its ability to interfere at low concentrations with binding of the enzyme to starch granules (Figs. 1 and 2). CHA not only inhibited binding of the enzyme, but also inhibited enzyme-catalyzed starch solubilization from starch granules (Figs. 3 and 4, Table I), suggesting that the noncatalytic site functions to bind the enzyme to the starch granule during hydrolysis. Similar suggestions were made for the noncatalytic site of glycogen phosphorylase (Fletterick et al 1976).

The effects of CHA on binding were greater in cereal α-amylases and wheat starch when compared to the waxy maize starch system. Researchers noted (Schwimmer and Balls 1949, Walker and Hope 1963) that waxy-maize starch adsorbed α-amylase more efficiently than other starches. Walker and Hope observed that the degree of adsorption was a function of the surface area of the granule. In addition, several workers (Goering and Eslick 1976, Leach and Schoch 1961, MacGregor and Ballance 1980) observed that waxy maize starches were more susceptible to α-amylase hydrolysis. Wheat starch, therefore, probably has fewer binding sites and does not compete with CHA as effectively as waxy starch for the noncatalytic site.

There is some discrepancy between the results presented here and those of Sargeant and Walker (1978) on the adsorption of α-I and α-II isozymes to starch granules. Sargeant and Walker reported greater adsorption of "germination" (α-II) α-amylase, whereas our results demonstrated that "green" (α-I) α-amylase adsorbed more to starch granules. We used affinity chromatography to purify the enzyme, whereas Sargeant and Walker used glycogen precipitation and preparative isoelectric focusing. The two methods could impart different binding characteristics to the isozymes. Affinity chromatography would displace dextrin from the noncatalytic site that interacts with the starch granule. Thus, with isozymes relatively free of β-limit dextrin, the binding to starch granules may be more effective. Stabilization of the "green" isozyme of triticale is enhanced by the presence of β-limit dextrin (Silvanovich and Hill 1977).

Wheat α-I was more efficient at solubilizing starch granules than α-II (Fig. 4). Similar results were reported for barley α-amylase (MacGregor and Ballance 1980). CHA inhibited α-I slightly more than α-II. Because only a single CHA concentration was used, the extent of this differential effect might be altered as concentration is varied. Measurement of reducing activity instead of appearance of starch in solution had only a slight effect on the inhibition by CHA, indicating little inhibitor effect on the further hydrolysis of solubilized starch by the enzyme. This agrees with previous findings (Weselake and Hill 1982).

Reducing activity measurements after 5 hr of hydrolysis (Table I) with various enzyme-starch-temperature combinations indicated that the type of starch influenced the degree of inhibition by CHA at 35°C. This effect was common to both wheat and triticale α-amylase systems, thus ruling out the possibility that major differences are due to the source of the enzymes. The increased inhibition at the higher temperature was also shown in the time course release of soluble carbohydrate from waxy maize starch hydrolysis catalyzed by triticale α-amylase (Fig. 3). Efficient binding of cereal α-amylase to waxy maize starch granules may be more critically affected at higher temperatures than for starches with high levels of amylose.

An interesting observation from our results concerns the effect of CHA on α-amylase binding to and hydrolysis of starch granules. CHA inhibited the binding of α-amylase to wheat starch granules to a greater extent than waxy maize starch. However, at 35°C the hydrolysis of waxy maize starch granules by α-amylase was inhibited to a greater extent by CHA than wheat starch. Possibly, the sites on the waxy maize starch granules compete more effectively with CHA for the binding site on α-amylase than wheat starch. This agrees with the observation that waxy maize starch granules adsorbed α-amylase more efficiently (Schwimmer and Balls 1949, Walker and Hope 1963). The results at 35°C may indicate that the binding site on the enzyme is of greater importance in hydrolyzing waxy starch granules. This may be a function of the amylopectin in the granule. The noncatalytic site of α-amylase has little or no effect on the catalytic properties of the enzyme as it degrades soluble dextrins. Apparently, however, the enzyme acts largely on insoluble starch granules both during germination of the seed and during handling processes associated with end-product use of the flour. The presence of hydrolysis products, the nature of the starch granules, and the temperature of the system all have pronounced effects on the action of the enzyme whenever insoluble starch is present.

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