Isolation and Characterization of α-Amylases from Endosperm of Germinating Maize

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ABSTRACT

Amylases from germinating maize (cv. B73) were fractionated by affinity chromatography, anion exchange chromatography, and chromatofocusing. Two groups of amylase enzymes were separated by affinity chromatography. About one half of the total amylase activity was bound on a cycloheptaamylose-Sepharose 6B column. Bound proteins were fractionated by anion exchange into four major α-amylase fractions, then further separated by chromatofocusing into eight fractions with apparent isoelectric point (pI) values ranging from 5.70 to 4.06. All affinity-bound fractions were confirmed as α-amylases by their action on β-limit dextrin. The affinity-bound α-amylases with lowest and highest pI values produced reaction products from soluble starch containing large amounts of dextrins with degrees of polymerization (DP) 2 and 6, with lesser amounts of intermediate oligosaccharides. Intermediate pI fractions produced primarily DP2, large amounts of DP3-5, and little DP6. Enzymes not bound by cycloheptaamylose affinity chromatography were purified by hydroxylapatite chromatography, then resolved by chromatofocusing into four subgroups of α-amylase, plus β-amylase. Among the affinity-unbound fractions, the lowest pI α-amylase had a unique action pattern, producing primarily DP7 and 8 oligosaccharides after exhaustive hydrolysis of soluble starch.

The study of cereal α-amylases is important from both fundamental and applied perspectives. The status of research on cereal α-amylases is the subject of a recent review (Hill and MacGregor 1988). Cereal grains synthesize multiple forms of α-amylase during germination to supply soluble carbohydrates for the developing seedling. Heterogeneity of starch-degrading enzymes in germinating seeds enhances the conversion of insoluble granules to soluble starch and dextrins (Beck and Ziegler 1989). The presence of multiple forms of α-amylase suggests that each form may have a particular metabolic function in situ, as the individual forms act cooperatively to degrade starch during germination.

The presence of multiple enzyme forms makes it difficult to assess reaction rates and biochemical parameters of individual forms. Each form must be isolated to accurately characterize its kinetic and regulatory properties, so that its role in the starch degradation process may be determined.

α-Amylases from different cereal grains have different biochemical properties, as do different forms that occur within a species (Frydenberg and Nielsen 1966, Kruger and Tkachuk 1969, Chao and Scandalios 1971, Goldstein and Jennings 1975, Okamoto and Akazawa 1979, Mundy 1982). Different forms within a species usually have similar molecular weights in the 42-45 kDa range (Tkachuk and Kruger 1974, Scandalios et al 1978, Jacobsen and Higgins 1982, Mundy 1982) but differ in electrophoretic mobility. Germinating barley and wheat each contain two groups of α-amylases, each of which contains several individual enzymes (Jacobsen and Higgins 1982, Callis and Ho 1983, Marchylo and MacGregor 1983, Kruger and Marchylo 1985). The two groups are products of two different gene families (Nishikawa and Nobuhara 1971, Gale et al 1983, Khursheed and Rogers 1988) and differ in biochemical and antigenic properties (Jacobsen and Chandler 1988). Two forms of α-amylase in germinating sorghum have partial immunological identity and different kinetic properties (Mundy 1982, Lecommandeur and Daussant 1989).

The study of amylases in germinating corn is less advanced than in other cereal grains. Polymorphism for α-amylase in germinating maize was first reported by Chao and Scandalios (1969, 1971, 1972), who identified eight amylase isoforms, two of which (α- and β-amylases) they classified as primary gene products. They recognized that maize amylases are different in many respects from those in wheat, barley, and rye; e.g., only 10% of the total maize amylase activity is β-amylase. Two allelic forms of α-amylase with isoelectric points (pIs) of 4.8 (Scandalios et al 1978) and 4.35 (Chao and Scandalios 1971) exhibit differential expression in developing kernels and germinating seedlings of maize (Chao and Scandalios 1969, 1971, 1972).

Goldstein and Jennings (1978) separated three forms of α-amylase from deembryonated sweet corn (cv. Seneca Chief). These forms vary in their sensitivities to Hg^{+2} and heating in the presence of Ca^{+2}.

Recently, MacGregor et al (1988) used a combination of isoelectric focusing (IEF) and chromatofocusing to identify α-amylases of several germinating cereal grains. Grains were classified into two groups: those that contain both a high (>5.8) and a low (<5.5) pI component (barley, wheat, rye, and triticale) and those with only low pI components (oats, maize, millet, sorghum, and rice). However, maize (cv. Sunnyvee) has intermediate (5.5-6.0) pI amylases as well as low pI components and does not strictly fit either classification.

More recently, extracts of germinating maize (cv. Dea) were found to contain six bands on IEF gels with three distinct amylase antigens (Lecommandeur and Daussant 1989).

Because relatively little fundamental study has been made of maize α-amylases, and no attempt has been made to use the endogenous amylases commercially, we have undertaken to isolate and characterize the enzymes found in germinating maize and to determine whether any of these might be commercially employed to hydrolyze granular starch at ambient temperatures, in a manner analogous to malting of barley.

Our goals in this project are to isolate the enzymes that hydrolyze granular starch during seed germination. We intend to determine the relative abundance and activity of these enzymes, to establish optimum conditions for their action, and to identify the reaction products formed by them. This report describes our initial efforts at isolation and characterization.

MATERIALS AND METHODS

Seed Germination and Enzyme Extraction

Samples of 100 kernels of inbred maize, cultivar B73, were surface sterilized with 1% NaOCl, steeped overnight in 10 m*M* $CaCl_2$, spread on filter paper saturated with steeping solution, sealed with plastic wrap in glass trays, and allowed to germinate in the dark for seven days at 25°C. The pericarp, scutellum, and embryonic axis were removed and discarded. Endosperm was ground in a mortar and pestle with 25 ml of 20 m*M* sodium acetate buffer, pH 4.5 or 5.0 (see note below), containing 1 m*M*

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$CaCl_2$. The mixture was centrifuged at 25,000 × *g* for 15 min, and the supernatant was decanted. The residue was washed with a second portion of buffer and recentrifuged. The combined supernatants were treated with two volumes of cold acetone on ice to precipitate proteins and centrifuged at 11,000 × *g* for 10 min. The pellet was redissolved in 10 ml of acetate buffer and centrifuged at 11,000 × *g* for 10 min to sediment insoluble material; the resulting solution thus contained enzyme from 10 kernels in each milliliter.

Affinity Chromatography

All purification steps and liquid chromatography were performed on ice or at 4°C. Liquid column chromatography was performed using a Pharmacia fast protein liquid chromatography (FPLC) system. Affinity chromatography of the acetone-precipitated proteins was performed on cycloheptaamylose (CHA)-epoxy Sepharose 6B at either pH 4.5 or 5.0 according to the method of Silvanovich and Hill (1976). (Initial separations were done using pH 4.5. Investigation of the effect of pH on affinity binding [see Results] indicated that the binding was somewhat higher at pH 5.0–5.5; therefore, later separations were done at pH 5.0.) Ten milliliters of sample (i.e., enzyme extract from 100 kernels) in 20 m*M* acetate buffer containing 1 m*M* $CaCl_2$ was applied to a column with a bed volume of 7.5 ml. The sample was eluted at a flow rate of 1 ml/min. Fractions of 1 ml were collected. Elution was monitored by measuring absorbance at 280 nm. Elution continued until no protein was detected. Bound proteins were then eluted with a solution of CHA (10 mg/ml) in acetate buffer. The eluate and the retentate were tested for amylase activity using a soluble starch substrate.

Anion Exchange Chromatography: Affinity-Bound Enzymes

Amylases bound by the affinity column were further fractionated by ion exchange chromatography on Pharmacia Mono Q anion exchange resin at pH 8.5 in 50 m*M* TRIS buffer using a 0.0–0.1 *M* NaCl gradient at a flow rate of 1 ml/min. One-milliliter fractions were collected.

Hydroxylapatite Chromatography: Affinity-Unbound Enzymes

Eluate containing nonbinding activity was concentrated with an Amicon Centriprep 10 filtration concentrator, dialyzed in 10 m*M* potassium phosphate, pH 6.0, containing 0.1 m*M* $CaCl_2$, then purified by chromatography on a hydroxylapatite column with a bed volume of 7.5 ml, at a flow rate of 1 ml/min. Proteins were eluted in two steps with 45 and 300 m*M* potassium phosphate. α-Amylases from the second hydroxylapatite peak were concentrated and dialyzed against chromatofocusing start buffer for 24 hr.

Chromatofocusing

Fractions from anion exchange chromatography and from the second hydroxylapatite peak were further purified by chromatofocusing on a Pharmacia Mono P column. Samples were introduced in 25 m*M* Bis-Tris, pH 6.5, containing 0.1 m*M* $CaCl_2$. Then they were eluted with Polybuffer 74 that was diluted 1:10 and pH adjusted to 3.5, containing 0.1 m*M* $CaCl_2$. The flow rate was 1 ml/min. One-milliliter fractions were collected. Elution pH, approximately equivalent to the pI, was monitored with a flow-through pH electrode or by measuring the pH of individual fractions with a Beckman Expandomatic pH meter equipped with a combination electrode.

Enzyme Activity

Amylase activity was measured using the dinitrosalicylic acid (DNSA) assay (Bernfeld 1951). Depending on activity, 10–100 μl of enzyme was added to 0.5 ml of 2% (w/v) soluble potato starch (Sigma S-2004) in extraction buffer. The reaction mixture was incubated at 25°C. Aliquots (50 μl) of reaction mixture were taken after an appropriate reaction time and added to 1.0 ml of DNSA reagent. Assay solutions were placed in boiling water for 5 min. Water (4 ml) was added to the assay solution; the sample was mixed by vortexing; and the solutions were equilibrated to 25°C. Absorbance was measured at 540 nm and compared to values obtained from a maltose standard curve. One unit of activity was equivalent to 1 μmol of maltose released per minute. (Robyt and Whelan [1972] have shown that this method is not accurate for determining absolute activity because absorbance varies with the chain length of the oligosaccharides being analyzed. In spite of this limitation, we found the method to be satisfactory for estimating the relative activity values of different enzyme fractions or different preparations; the convenience of the method for our purposes outweighed the acknowledged lack of absolute accuracy.)

Protein Analysis

Protein was determined by the Bradford (1976) method using fatty acid-free bovine serum albumin as a standard. Samples for analysis (1 ml or less) were mixed with 2 ml of acetone, held overnight at 4°C, then centrifuged 2 min at 15,000 × *g* in an Eppendorf micro centrifuge (model 5415). The pellet was redissolved in 100 μl of 50% sucrose, and duplicate 25-μl aliquots were analyzed.

Effect of pH on Enzyme Activity

Effects of pH were determined by assaying amylase activity in 2% starch solutions prepared in 50 m*M* buffers with pH ranging from 3.0 to 8.0. Buffers used were glycine-HCl (pH 3.0), Na-acetate (pH 3.6–5.5), Bis-Tris-HCl (pH 5.5–7.0), and *N*-(2-hydroxyethyl)piperazine-*N*′-(α-ethanesulfonic acid)-NaOH (pH 7.0–8.0). All substrate solutions contained 1 m*M* $CaCl_2$. Reactions were run for 30 min at 25°C.

Effect of Calcium on Enzyme Activity

Major peaks from anion exchange (affinity-bound fractions) were evaluated for calcium dependence by dialyzing against 10 m*M* ethylendiamine tetraacetic acid (EDTA) at pH 5.2 for up to 138 hr. Aliquots of chromatofocusing fractions from the affinity-unbound peak were incubated directly with 1 m*M* or 10 m*M* ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetra-acetic acid (EGTA) in 50 m*M* Na-acetate, pH 4.5, for 85 min at 25°C. Reaction rates were then measured in 2% (w/v) starch in 50 m*M* Na-acetate, pH 4.5, containing no added $CaCl_2$.

Effect of Heat on Enzyme Activity

Enzyme samples were heated at 70°C for 10 or 15 min in 50 m*M* acetate buffer (pH 4.5) containing 1 m*M* $CaCl_2$, and activity was measured to determine loss of activity due to heating.

Analysis of Reaction Products

Crude extracts, affinity chromatography fractions, and individual fractions from chromatofocusing were incubated with 4% (w/v) soluble starch in 20 m*M* acetate buffer at pH 4.5. For chromatofocusing fractions, 200 μl of enzyme from each 1-ml fraction was incubated with 10 ml of starch solution. At intervals between 0.5 and 144 hr, an aliquot was removed from the reaction mixture and mixed with two volumes of absolute ethanol to stop enzyme action and to precipitate unreacted starch and large oligosaccharides. The supernatant was filtered through a 0.2-μm filter. The composition of the reaction mixtures was determined by high-performance liquid chromatography (HPLC), using a Spectra-Physics SP8100 high-performance liquid chromatograph equipped with a Dionex PAD-2 pulsed amperometric detector and a Dionex Carbopak PA1 carbohydrate column. Oligosaccharide components were separated by gradient elution at 40°C; mobile phases were 100 m*M* NaOH (solution A) and 100 m*M* NaOH plus 500 m*M* sodium acetate (solution B); the flow rate was 1 ml/min. Maltooligosaccharides with degree of polymerization (DP) 1 to DP7 from Sigma Chemical Company were used as external standards for quantitation.

The acetone-precipitated proteins from the crude extract were tested with other substrates to detect activity of other carbohydrate-degrading enzymes. Maltase activity was detected by incubating 200 μl of extract with 225 mg of maltose in 10 ml of acetate buffer for 120 hr. Limit dextrinase activity was detected

by incubating 500 μl of extract with 400 mg of pullulan in 10 ml of acetate buffer for 48 hr. Aliquots were removed at regular intervals and processed in the same manner as for amylase activity. Reaction products were identified and quantitated by HPLC as described above.

Flatbed IEF and SDS-PAGE

IEF was performed according to the manufacturer's instructions, using Ampholine Pagplates, pH 4.0–6.5, and an LKB 2117 Multiphor II electrophoresis unit. Samples (5–80 μl) containing approximately 0.1 unit of amylase activity were applied on paper wicks 3 cm from the cathode. Focused gels were laid on 5.5% acrylamide gels 1 mm thick containing either 0.3% (w/v) soluble starch or 0.66% (w/v) amylopectin β-limit dextrin for 10 min at 25°C. Bands of amylase activity were visualized by flooding the gels containing starch and dextrin with a solution of 1 m*M* I_2 and 500 m*M* KI. The IEF gel was fixed immediately after the incubation period and silver-stained to visualize protein bands and markers for pI determination (Pharmacia LKB Biotechnology AB).

Enzyme purity and molecular weight were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) using Bio-Rad molecular weight markers and silver stain on a 1.5-mm, 10% acrylamide gel at pH 8, run at 60 mA, maximum voltage 600 V, using a Hoeffer electrophoresis system. Each well was filled with an 80-μl aliquot of the 1-ml chromatofocusing fraction, plus 20 μl of 50% sucrose with 0.1% bromphenol blue. Bio-Rad standard proteins were: rabbit muscle phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; hen egg white ovalbumin, 42.7 kDa; bovine carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; and hen egg white lysozyme, 14.4 kDa.

RESULTS

The redissolved portion of the acetone precipitate contained more than 50% of the amylase activity of the crude extract (Table I); activity of crude extracts ranged from six to eight units per kernel. A large portion of precipitate, presumably denatured protein plus complex carbohydrates, would not redissolve.

TABLE I
Purification of α-Amylase from Germinating Maize B73 Endosperm

Step or Fraction	pI[a]	Protein (μg)	Total Activity (units)	Specific Activity (units/mg)	Recovery (%)
Crude extract		11,000	803	73	100
Acetone precipitate		5,800	451	78	56
Affinity-bound		530	183	345	23
B-I[b]	5.70	34	4	145	<1
	5.50	99	20	207	2
B-II	5.37	232	36	157	4
	5.22	49	4	81	<1
B-III	4.82	10	17	1,707	2
	4.70	6	18	3,087	2
	4.57	4	4	1,025	<1
B-IV	4.14	13	11	865	1
	4.06	51	14	276	1
Affinity-unbound		3,500	202	58	25
Peak I[c]		530	21	39	3
Peak II		640	137	214	17
U-I[d]	5.65	7	4	571	<1
U-II	5.40	27	3	111	<1
U-III	5.18	13	3	231	<1
U-IV	5.13	13	13	1,000	2

[a] pI values for affinity-bound samples are from chromatofocusing elution on a Mono P column; those for unbound samples are from isoelectric focusing (IEF). Apparent pI values by chromatofocusing are about 0.3–0.4 pH units lower than by IEF.
[b] Fractions from anion exchange chromatography; Mono Q plus chromatofocusing on Mono P column.
[c] Hydroxylapatite fractions.
[d] Fractions from Mono P column.

Because the DNSA assay is not specific for α-amylase, and methods to inhibit or eliminate β-amylase activity were not employed, activities measured in crude extracts and in the affinity-unbound fraction overestimated the actual amount of α-amylase activity, due to the presence of β-amylase and other amylolytic enzymes (Maeda et al 1978). Maltase and limit dextrinase activities in the acetone precipitate were much lower than amylase activity. Maltase activity was 0.025 μmol of glucose per minute per kernel. Limit dextrinase activity was 0.04 μmol of maltotriose per minute per kernel. The reaction product mixture from pullulan substrate contained maltose, maltotriose, and maltotetraose in an approximate molar ratio of 1:3:1, as well as a trace of glucose.

Affinity Chromatography

Figure 1 shows a typical CHA column chromatogram. Effect of pH on amylase binding was tested at intervals of one-half pH unit from 4.5 to 6.5 (acetate buffer at pH 4.5–5.5, Bis-Tris buffer at pH 6.0 and 6.5). Multiple runs were made at pH 5.0 and 5.5 to determine variance in binding. Each run was made with a separate extract. Proportions of binding and nonbinding activities as percentages of loaded activity were, respectively: pH 4.5, 36/48; pH 5.0, 53/36; pH 5.5, 64/21; pH 6.0, 18/51; and pH 6.5, 26/61. The column was irreversibly inactivated at pH 4.0. Subsequent runs were made at pH 5.0 because the proportion of binding was less variable than at pH 5.5.

Rechromatography of the unbound fraction resulted in no additional retention of activity, demonstrating the specificity of the affinity column for the bound enzymes and showing that the column was not overloaded. Inclusion of dithiothreitol (DTT) and phenylmethylsulfonyl fluoride in the extraction buffer and dialysis of the extract did not affect activity nor alter binding to the affinity column. After affinity chromatography, no maltase or limit dextrinase activity could be detected in either the unbound or the bound fraction; presumably the affinity chromatography procedure inactivated these enzymes or diluted them to a concentration below the limit of detection.

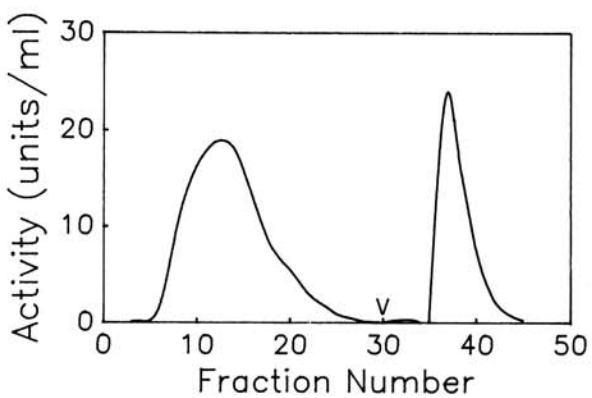

Fig. 1. Affinity chromatography of maize amylases on cycloheptaamylose. v = elution with cycloheptaamylose (10 mg/ml).

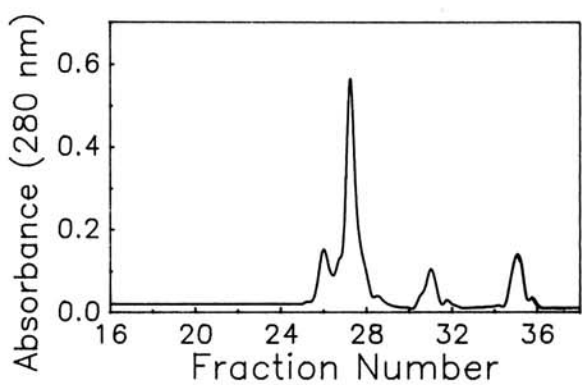

Fig. 2. Anion exchange chromatography of affinity-bound α-amylases.

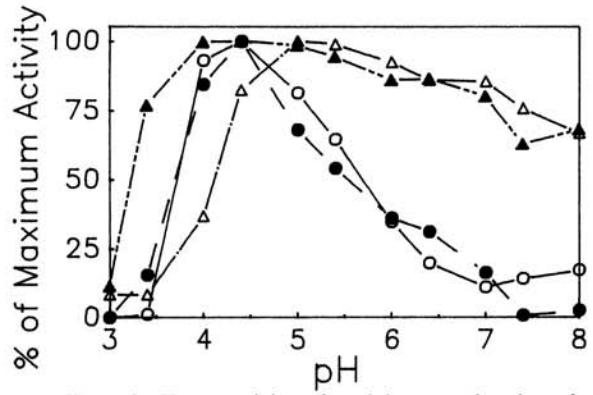

Fig. 3. Effect of pH on activity of cycloheptaamylose-bound anion exchange fractions: ○, fraction B-I; ●, fraction B-II; △, fraction B-III; ▲, fraction B-IV.

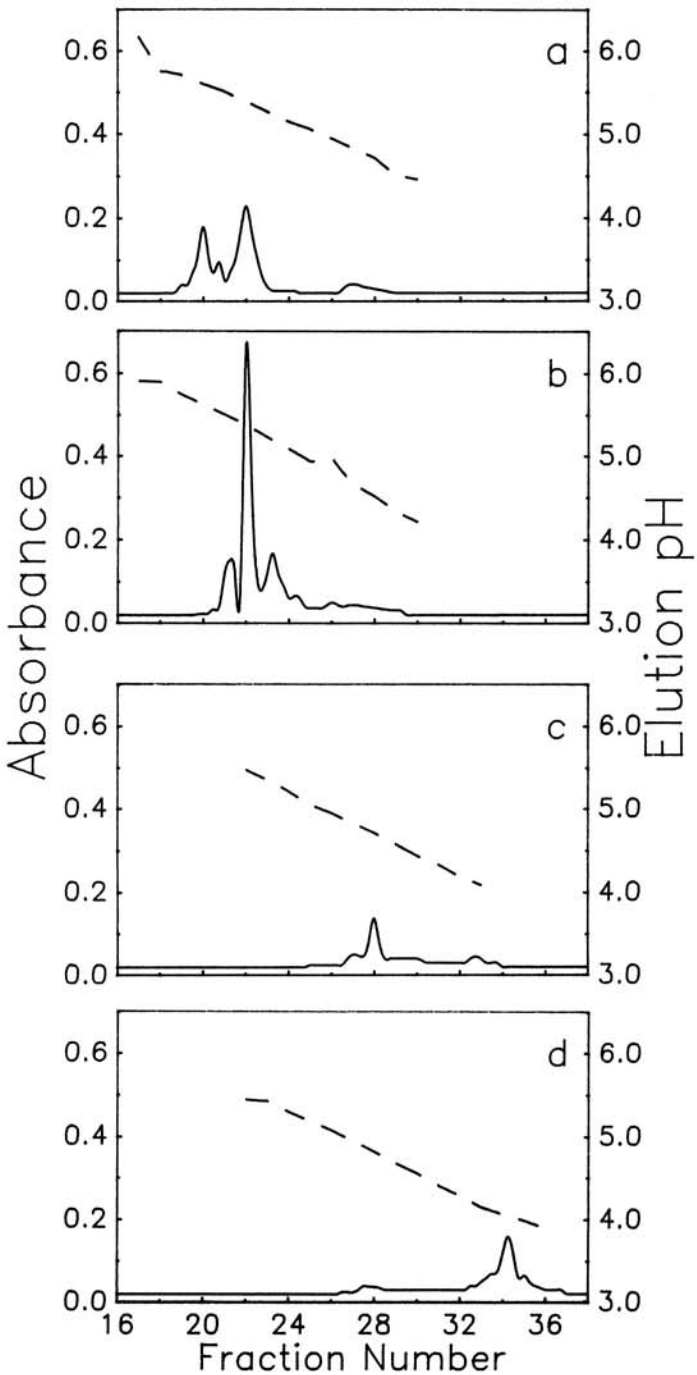

Fig. 4. Chromatofocusing of anion exchange fractions: **a,** fraction B-I; **b,** fraction B-II; **c,** fraction B-III; **d,** fraction B-IV. Dashed line is pH.

Characteristics of Affinity-Bound α-Amylases

Anion exchange chromatography. FPLC on a Mono Q column of the affinity-bound fraction yielded four subfractions (Fig. 2), which were designated B-I, B-II, B-III, and B-IV, corresponding to their order of elution.

After heating to 70°C for 15 min in the presence of 10 m*M* $CaCl_2$ at pH 4.5, fractions B-I through B-IV retained 88, 67, 76, and 68% of original activity, respectively. Dialysis against EDTA to remove calcium caused complete loss of activity in B-I and B-II in 24 hr; B-III and B-IV retained 82 and 52% activity, respectively, after 24 hr, 62 and 40% after 48 hr, and were completely inactive after 138 hr.

Optimum pH of all samples ranged from 4 to 5 (Fig. 3), but the activity at higher pH levels varied. Activity of fractions B-I and B-II declined sharply at pH values above 5 and was negligible at pH 7, whereas fractions B-III and B-IV retained 60% or more of their maximum activity up to pH 8.

Chromatofocusing. The four anion exchange fractions were each further fractionated by chromatofocusing (Fig. 4). The elution order is similar to that seen in anion exchange chromatography, but the resolution is improved by chromatofocusing. Theoretically, chromatofocusing fractions should elute at their pI. However, Marchylo and Kruger (1983) demonstrated that pI values determined by isoelectric focusing are somewhat higher than elution pH values measured on chromatofocusing fractions; elution pH values can therefore be considered only approximate pI values.

Fraction B-I (Fig. 4a) divided into two major peaks with pI values of 5.70 and 5.50 and a minor peak at pI 5.59.

Fraction B-II (Fig. 4b), the largest protein fraction on anion exchange, resolved into three fractions with pI values of 5.50, 5.37, and 5.22. Presumably, the presence of the pI 5.50 component in this sample was the result of incomplete resolution in the anion exchange step.

Chromatofocusing of fraction B-III (Fig. 4c) resulted in a major peak with a pI of 4.70; broad, flat shoulders on either side of it had pI values of 4.82 and 4.57.

Fraction B-IV (Fig. 4d) was separated into a main peak with pI of 4.06 and a preceding shoulder with pI of 4.14.

The four major groups all had comparable levels of total activity (Table I); however, protein recovery, and hence specific activity, varied substantially.

Isoelectric focusing. IEF of the chromatofocusing fractions resulted in numerous bands (Fig. 5). Amylases in subfractions of B-I and B-II were incompletely resolved by chromatofocusing; these displayed three major groups of "high pI" bands corresponding to the decreasing elution pH of the chromatofocusing fractions; the subfraction from fraction B-I eluting at pH 5.70, as well as that eluting at pH 5.59 (not shown), was enriched in a strong double band at the highest pI; subfractions from fraction B-I

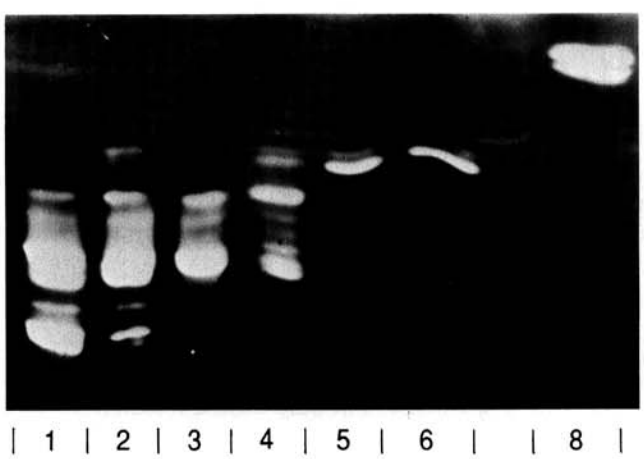

Fig. 5. Isoelectric focusing of chromatofocusing fractions. Elution pH values of fractions are as follows: lane 1, 5.70; lane 2, 5.50; lane 3, 5.37; lane 4, 5.22; lane 5, 4.82; lane 6, 4.70; and lane 8, 4.06.

eluting at pH 5.50, and those from fraction B-II eluting at pH 5.50 and 5.37, were enriched in the IEF band with the next highest pI; and the subfraction from B-II that eluted at pH 5.22 was enriched in the IEF fraction with the third highest pI. Amylases in chromatofocusing fractions from B-III and B-IV were well resolved. Subfractions from fraction B-III eluting at pH 4.57 (not shown), 4.70, and 4.82, all showed a single IEF band. The subfraction from fraction B-IV eluting at pH 4.06 consisted of a double band. The fraction that eluted at pH 4.14 (not shown) was identical to that of the pH 4.06 fraction. Silver-stained protein bands on IEF gels corresponded exactly to active amylase bands on starch-containing gels (data not shown).

SDS-PAGE. Chromatofocusing fractions all had an apparent molecular weight of about 42 kDa.

Characteristics of Affinity-Unbound α-Amylases

The affinity unbound fraction (Fig. 1) contained four zones of α-amylase activity, with pI values ranging from 5.1 to 5.6, and β-amylase, with pI 4.7 (Fig. 6, lane U). Unbound amylases were purified and resolved into two peaks of activity (eluting at 45 and 300 m*M* potassium phosphate) by hydroxylapatite (HAP) chromatography (Fig. 7a). The first peak contained β-amylase and minor amounts of α-amylase (Fig. 6, HAP I). The four subgroups in the second peak (Fig. 6, HAP II) were partially resolved by chromatofocusing into four fractions identified as U-I, U-II, U-III, and U-IV (Figs. 6 and 7b) in order of decreasing pI . Fraction U-I was enriched in the most basic subgroup, containing three individual bands at pI 5.6. Fraction U-II was enriched in a subgroup with two bands at pI 5.4. Fractions U-III and U-IV were each enriched in a single band, at pIs of 5.18 and 5.13, respectively. (A zone at pI 6.1 was sometimes present in the crude extract and the affinity bound fraction, but it did not appear consistently. It was never present in the affinity unbound fraction.) Banding patterns on β-limit dextrin gels were identical to those on starch-containing gels, except that the band at pI 4.7 (β-amylase) was absent.

SDS-PAGE showed that the chromatofocusing fractions were highly purified amylases with an apparent molecular weight of about 42 kDa. There was a minor contaminant of about 33 kDa in fractions U-II and U-III (data not shown).

Chromatofocusing fractions U-I, U-II, and U-III had optimum pH ranges from pH 3.6 to 5.0, with a peak at pH 4.0 for fraction U-II and at pH 4.5 for fractions U-I and U-III (Fig. 8). Fraction U-IV had highest activity at pH 5.0 and was more active at higher pH than the other bands. The mixture of affinity-unbound amylases had 50% or more of its maximum activity from pH 3.6 to 6.5, with a peak at pH 4.5 (data not shown). No buffer species effect was observed, as activity was the same at pH 5.5 using either acetate or Bis-Tris buffers and at pH 7.0 using Bis-Tris or HEPES buffers.

Activity of all forms was slightly inhibited by 1 m*M* EGTA

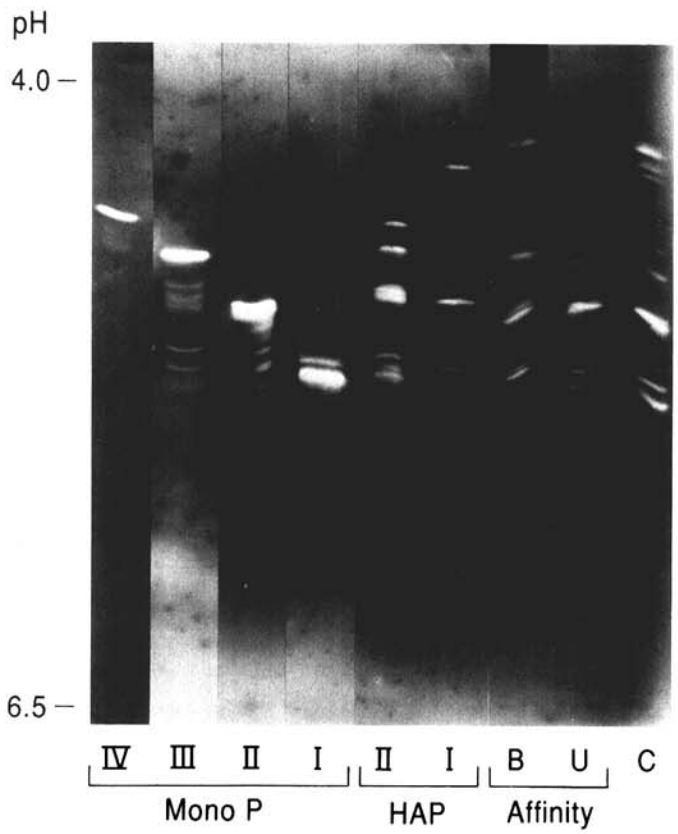

Fig. 6. Isoelectric focusing of crude amylase extract (C); affinity-unbound (U) and -bound (B) amylases; hydroxylapatite fractions (HAP I and II); and α-amylases from chromatofocusing of affinity-unbound HAP II fraction (Mono P I–IV).

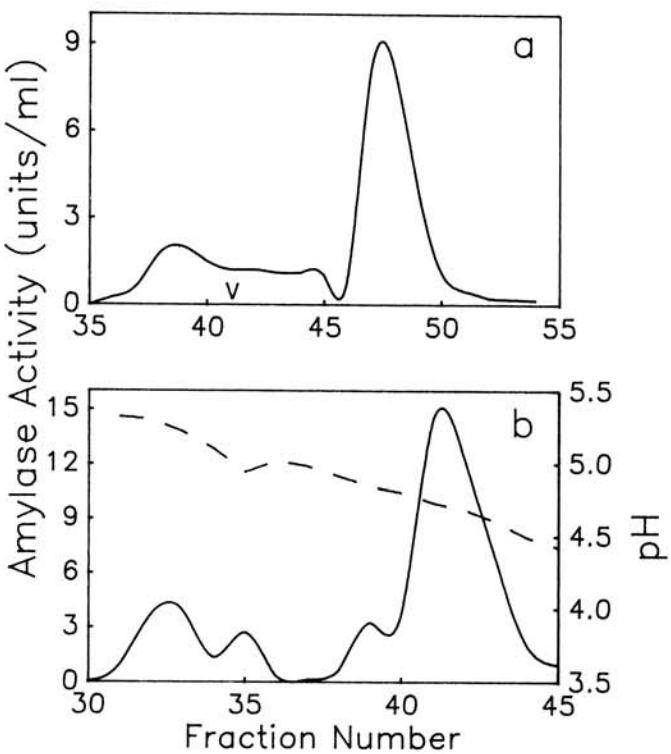

Fig. 7. Fractionation of cycloheptaamylose-unbound α-amylases. **a,** Hydroxylapatite chromatography of these amylases. Fractions 35–41 were eluted with 45 m*M* phosphate, fractions 42–55 with 300 m*M* phosphate buffer. v = start of elution with 300 m*M* buffer. **b,** Chromatofocusing of second peak from hydroxylapatite column, showing fractions U-I through U-IV. Dashed line is pH.

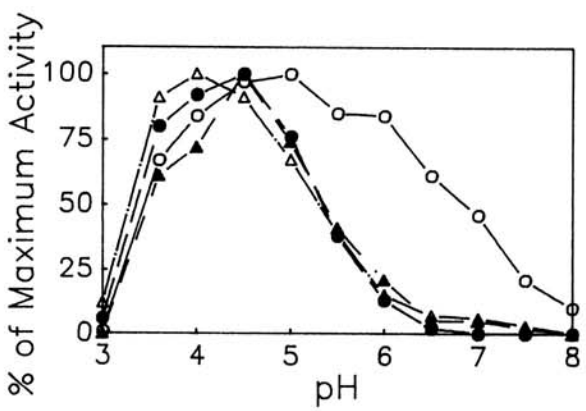

Fig. 8. Effect of pH on activity of cycloheptaamylose-unbound α-amylases: ▲, fraction U-I; △, fraction U-II; ●, fraction U-III; ○, fraction U-IV.

(Table II). Inhibition increased with 10 m*M* EGTA except for fraction U-II. Heating to 70°C for 10 min in the presence of 10 m*M* $CaCl_2$ at pH 4.5 completely eliminated activity of the individual fractions (data not shown). Incubation of individual chromatofocusing fractions with 1 m*M* DTT for 20 min at 25°C did not alter amylase activity (data not shown). Neither the affinity-bound nor unbound amylases were inhibited by up to 10 m*M* CHA (data not shown).

The HAP II and chromatofocusing fractions were free of β-amylase (Fig. 6).

Reaction Products of CHA-Bound and -Unbound Enzymes

Bound fractions. Equal volumes (200-μl aliquots from 1-ml fractions) of individual chromatofocusing fractions of the affinity-bound enzymes were incubated with soluble starch; thus, for each enzyme form, the amount of activity used was proportional to its abundance in the acetone precipitate from maize endosperm (as given in Table I). Because equal activities were not used, these action patterns should be regarded as tentative. However, our experience with these enzymes has shown that, for any given fraction, although substantial variation in reaction products may be found during the early stages of reaction, final reaction product mixtures do not vary significantly within the range of activity levels found in these samples. After 96 hr of reaction, all fractions had hydrolyzed a substantial portion of the substrate; all reactions had slowed to a very low rate; and relative proportions of reaction products were changing slowly or not at all, so the reactions could be considered to be complete. At this reaction time (Table III), the major fractions gave distinct product profiles. Note that values in Table III represent that proportion of substrate not precipitated by addition of ethanol, nominally consisting of oligosaccharides of DP less than 20. Therefore, values designated ">DP 8" actually are DP 9 to about DP 20.

Subfractions with elution pH between 5.37 and 5.70 all produced similar proportions of reaction products, with DP2 and DP6 being the predominant products; these fractions were shown by IEF (Fig. 5) to be incompletely resolved, so any differences in action patterns may be indiscernible until fractions of higher purity are available. Fractions with elution pH 4.70 and 4.82 exhibited a different pattern, producing primarily DP2 plus substantial DP4, with low amounts of DP6. Reaction products at times greater than 96 hr (data not shown) showed that the quantity of DP6 was still decreasing in those samples that still contained substantial DP6 at 96 hr. Fractions with elution pH of 4.14 and 4.06 produced predominantly DP6; the quantity of DP2 was also large and still increasing, whereas DP7 was still relatively large but decreasing.

Unbound fractions. Unbound fractions other than U-IV had lower activity than bound fractions, so larger volumes of these enzymes were necessary for hydrolysis studies. For each fraction, a volume sufficient to provide activity equivalent to 200 μl of fraction U-IV was used. The action patterns of the CHA-unbound α-amylases were quite different from those of the bound forms. In particular, the end-products after 96 hr of hydrolysis of soluble starch by affinity-unbound fractions U-III and U-IV contained high proportions of DP7 and DP8. Substantial amounts of DP7 also remained in U-I and U-II hydrolysates. The action of fraction U-IV also differed from affinity-bound fractions in that the reaction proceeded very rapidly (<24 hr) to >70% hydrolysis of the starch substrate, with no subsequent action on the remaining higher oligosaccharides.

For both bound and unbound amylases, granular starch was hydrolyzed much more slowly than was soluble starch treated with an equal amount of activity; as a result, any large soluble oligosaccharides that were produced were consumed relatively rapidly and thus were not detected in substantial amounts. The major component detected in each case was DP2, with substantial amounts of DP1 and 3 (data not shown). After extensive hydrolysis (one to two weeks), granular starch was hydrolyzed to 25–30% by affinity-bound enzymes, and hydrolysis was still proceeding; in contrast, equal activity of affinity-unbound amylases had accomplished hydrolysis of 3% or less.

TABLE II
Effect (% Inhibition) of Ethylene Glycol-Bis(β-Aminoethyl Ether)*N,N,N′,N′*-Tetraacetic Acid (EGTA) of Affinity-Unbound Forms of α-Amylase

	EGTA	
Fraction	1 mM	10 mM
U-I	14	4
U-II	19	19
U-III	13	49
U-IV	21	39

DISCUSSION

Affinity chromatography on CHA is highly specific for α-amylase (Silvanovich and Hill 1976), and recovery of activity in the bound fraction from wheat and barley extracts is usually >80%. However, only 37% of the activity extracted from sorghum bound to a CHA affinity column (Mundy 1982), and, in this study, about 40–50% of the amylase activity bound to the affinity column.

Binding of amylase enzymes to cycloamyloses is thought to be either through the formation of inclusion complexes between functional groups on the enzyme and the hydrophobic cavity of the cycloamylose (Gibson and Svensson 1986) or through an interaction of a catalytic or noncatalytic starch-binding site on the enzyme with a portion of the cycloamylose ring itself (Weselake and Hill 1983). Thus, changes in primary amino acid sequence may prevent binding to cycloamylose by altering the exposure

TABLE III
Reaction Products from 96-hr Hydrolysis of Soluble Starch

Anion Exchange Fraction	Mono P Elution pH	Percent of Original Substrate Converted to Each Oligosaccharide									
		DP1[a]	DP2	DP3	DP4	DP5	DP6	DP7	DP8	>DP8	Total
Affinity-bound											
B-I	5.7	5	18	7	3	11	19	9	1	0	72
	5.59	8	19	5	4	12	20	3	1	3	75
B-II	5.5	11	22	7	9	12	17	1	2	8	88
	5.37	6	18	6	4	10	17	6	1	1	67
B-III	4.82	2	16	7	12	11	12	11	4	4	78
	4.7	3	22	14	17	11	6	3	2	6	85
B-IV	4.14	9	15	7	9	17	25	10	1	0	93
	4.06	5	9	4	5	11	18	14	2	7	76
Affinity-unbound											
U-I	5.65	1	7	2	2	3	8	8	1	3	35
U-II	5.40	1	7	2	2	3	7	9	1	5	36
U-III	5.18	1	11	4	2	6	13	16	1	2	58
U-IV	5.13	0	5	3	5	4	8	20	15	10	71

[a]DP = Degree of polymerization.

of aromatic side chains on the surface of the enzyme or by changing the structure of a starch-binding site to prevent the binding to carbohydrate moieties of the cycloamylose. The last possibility is supported by the low activity on granular starch and the novel action patterns of the unbound amylases. On the other hand, barley α-amylase 2 has a tryptophan residue in a noncatalytic surface site that binds to CHA (Gibson and Svensson 1986). Nonbinding amylases from maize (and sorghum) may lack a corresponding tryptophan residue.

The difference in binding could also be due to posttranslational modifications such as methylation or glycosylation that interfere with affinity binding. Recent evidence indicates that a form of maize α-amylase is glycosylated (Lecommandeur et al 1990). A tight-binding carbohydrate occupying the starch-binding site could also affect binding to the affinity column. Sorghum α-2 amylase contains 5.7% (w/w) carbohydrate, while α-1 amylase contains only 0.7% (Mundy 1982). Alternatively, complexation of enzyme with protein factors in the crude extract, comparable to the enzyme-inhibitor complex found in extracts of barley (Weselake et al 1983), could affect affinity binding. An endogenous protein α-amylase inhibitor from maize has been isolated (Blanco-Labra and Iturbe-Chinas, 1981).

Our results demonstrate that the multiple forms of amylase from germinating maize varied significantly in their action patterns on soluble starch. At least three forms of α-amylase enzymes, comprised of eight or nine identifiable IEF bands, were identified in the CHA affinity-bound fraction. The three groups were: the B-I and B-II forms, with apparent pIs in the range of 5.2–5.7, which produce a mixture of reaction products containing large amounts of DP2 and 6; B-III forms, with a pI range of 4.6–4.8, which produce a mixture of reaction products containing primarily DP2, with substantial amounts of DP3–5 but very small amounts of DP6; and B-IV forms, with a pI range of 4.0–4.2, which produce a product mixture that is primarily DP6. The product mixtures of the highest and lowest pI groups were consistent with those of other cereal α-amylases (MacGregor and MacGregor 1985). There was, however, a significant and reproducible difference between the products of the two enzyme groups, in that the high pI group produced a mixture in which DP2 and DP6 were nearly equal, whereas the low pI group produced about 50% more DP6 than DP 2. The intermediate pI group displayed an action pattern significantly different from that of typical cereal α-amylases, in that very little DP6 was found in the final reaction product mixture. This fraction could be contaminated with minute amounts of β-amylase. This possibility has not been unequivocally ruled out but is unlikely in view of the facts that this enzyme group was shown to be pure on IEF (Fig. 5) and that β-amylase isolated from the affinity unbound fraction displayed a lower pI (Fig. 7).

Two forms of CHA-unbound α-amylase purified in this study produced DP7 as a major stable product after extensive hydrolysis of soluble starch. These enzymes may possess a unique catalytic site for cereal amylases, since maltohexaose is the largest major oligosaccharide produced by other cereal α-amylases (MacGregor 1983). Many amylases produce oligosaccharides with chain lengths from six to eight as early products, but DP7 and DP8 are not present after extended hydrolysis. The possibility remains that other cereal α-amylases exist that do produce DP7 as a final product but that this has not been observed because of contamination with other enzyme forms that degrade DP7. In this study, chromatofocusing fraction U-IV contained only a single band of activity, whereas most previous studies have analyzed forms of amylase that contain several bands of activity.

The CHA-unbound amylases in this study appeared identical on IEF gels to the B-III amylase group from the bound fraction, and the pH curves (Figs. 2 and 7) were similar for unbound and bound amylase fractions that appeared the same on IEF. However, reaction products of amylases isolated from the bound fraction contained little, if any, oligosaccharides larger than DP6 after 96 hr of digestion (Table III). In addition, unbound forms were much less thermostable than bound forms, and the extent of hydrolysis of granular starch by unbound amylases was less than that by bound forms, providing further evidence of the unique character of the unbound amylases.

Lack of binding to CHA and low activity on granular starch indicates that amylases in the affinity-unbound fraction are not involved in the initial attack on starch granules in vivo. The rapid degradation of soluble starch and the inability to hydrolyze DP7 suggested that the form of amylase in fraction U-IV is involved in the breakdown of large soluble dextrins released from starch granules early in germination. Further characterization of the kinetics and substrate specificities of individual forms of α-amylase will elucidate the role of each form in the pathway of starch degradation.

The chromatofocusing fractions in the range of pI 5.7–5.5 are similar to those described by MacGregor et al (1988). However, in our sample, these constituted the major portion of total protein as well as a substantial portion of total activity, whereas in the sample studied by MacGregor, these fractions were a relatively minor component. This difference may be due to differences in isolation procedures or in the source of activity; our samples were isolated from endosperm, whereas MacGregor's samples were obtained from the whole kernel. Alternatively, it appears likely that proportions of the different amylases may vary significantly from one maize variety to another.

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