α-AMYLASES FROM TRITICALE 6A190: PURIFICATION AND CHARACTERIZATION[1]

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ABSTRACT

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α-Amylase was purified from malted triticale 6A190 by extraction, precipitation with glycogen, affinity chromatography on cyclohepta-amylose substituted epoxy-Sepharose 6B, and carboxymethyl cellulose ion-exchange chromatography. A yield of 25–30% was obtained with 150 to 200-fold purification. Two forms of α-amylase were separated by CM-cellulose ion-exchange chromatography at pH 5.5. Each form was composed of several enzyme species of approximately the same subunit molecular weight (4×10^4), but of different overall charge at pH 8.5. The two forms were further characterized in terms of pH optimum and stability, Km, V_{max}, activation energy, temperature stability, and isoelectric point.

Triticale (X *Triticosecale* Wittmack) is a synthetic species developed by the combination of *Triticum* (wheat) and *Secale* (rye) genomes (1,2). One of the most persistent problems in some lines of triticale has been poor kernel characteristics. The seed does not develop fully, but rather becomes shrivelled at maturity, resulting in low test weight. Hill *et al.* (3) found that α-amylase activity increased at maturity in some shrivelled lines (*e.g.*, cultivar 6A190) but not in nonshrivelled lines (*e.g.*, cultivar 6A250). This suggested that precocious germination may be occurring in some shrivelled varieties of triticale, and that the concomitant increase in α-amylase activity may be resulting in breakdown of starch in the kernel leading to shrinkage and partial collapse of the endosperm. Amylase attack on starch granules in developing triticale (cv. 6A190) has been observed (4).

This investigation was undertaken to isolate and characterize α-amylase from triticale (cv. 6A190) and to compare its properties to those of other cereal amylases.

MATERIALS AND METHODS

Materials

Triticale 6A190 (X *Triticosecale* Wittmack), rye (cv. Prolific) and wheat (cv. Stewart) were used. Mature samples came from field-grown material harvested on the head, frozen immediately in liquid nitrogen, and stored at −20° C. Malted triticale was prepared in a rotating drum laboratory malting unit by steeping for 36 hr at 10° C with several water changes, followed by 144 hr of germination at 17° C. The green malt was then frozen in liquid nitrogen and lyophilized.

Determination of α-Amylase Activity

Iodine-Dextrin Color (IDC) Method. α-Amylase activity was determined by the method of Briggs (5), using β-limit dextrin as the substrate. An IDC unit is

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defined as the amount of enzyme required to change the absorbance of a β-limit dextrin solution from 0.6 to 0.4 in 100 min at 540 nm. At all times the degree of hydrolysis was kept such that the normalized values never fell below 0.4 absorbance. The assay temperature was 35° C.

Dinitrosalicylic Acid Method. The method used was that of Fisher and Stein as described by Whelan (6) except that β-limit dextrin was used as substrate.

Protein Determination

The concentration of protein in solutions was determined by the method of Lowry *et al.* (7).

Disc Gel Electrophoresis

Polyacrylamide gels were prepared according to Davis (8). Upper gel was 3% polyacrylamide at pH 6.9; running gel was 7% polyacrylamide at pH 8.9 (Tris-Cl buffer). Reservoir buffer was Tris-glycine at pH 8.3. During electrophoresis, the current was maintained at 2 mA per gel.

Areas of α-amylase activity in the electrophoresis gels were detected using a method adapted from Doane (9). The disc gels were brought into contact with a 7% polyacrylamide film (on a glass plate) made with 0.4% β-limit dextrin at pH 5.5 (sodium acetate buffer with 0.001*M* $CaCl_2$) and incubated for 30–60 min at 35° C. The polyacrylamide film was then stained with iodine-potassium iodide solution (200 mg I_2, 2 g KI/500 ml H_2O). The areas of α-amylase activity remain clear while the background becomes colored.

Electrophoresis in gels containing sodium dodecyl sulphate (SDS) was performed using the method of Weber and Osborn (10). Samples were prepared by an adaptation of the method of Tzagaloff (11). A 7.5% gel was used and electrophoresis performed at 6–8 mA/gel for 3–4 hr. Gels were stained for 1 hr in 0.1% amido black in 7% acetic acid and destained with 7% acetic acid.

Subunit molecular weights were determined by comparing electrophoretic migration of the α-amylase subunit with the following molecular-weight standards: bovine serum albumin, γ-globulin, pyruvate kinase, lactic dehydrogenase, trypsin, and cytochrome C.

Isolation of α-Amylases

Extraction. All steps of the procedure are carried out at 0°–4° C. α-Amylase was extracted using 0.2*M* sodium acetate buffer (pH 5.5, 0.001*M* $CaCl_2$). The sample was stirred for 1 hr on a magnetic stirrer and centrifuged at 10,000 × *g* for 10 min. Freshly germinated or growing kernels were extracted at a ratio of 25 ml/100 kernels using a Vir Tis 45 homogenizer to break up the tissue. Mature kernels were ground to flour in a microanalytical mill and extracted similarly. Lyophilized malt was extracted at a ratio of 30 g malt/100 ml of buffer. The supernatant recovered after centrifugation contains α-amylase and is called "crude extract."

Glycogen Precipitation. For further purification, a modification of the glycogen precipitation method of Schramm and Loyter (12) was used. Ethanol was added slowly, with stirring, to crude extract until a final concentration of 40% ethanol was reached and the precipitated protein was removed by centrifugation. Two per cent glycogen (Fisher Scientific Cat. No. G-47) was added dropwise to the stirred supernatant, at a ratio of 9.4 ml/100 ml of 40%

ethanol. After stirring for 1 hr, the precipitate was collected by centrifugation (20,000 × *g* for 15 min) and resuspended in a small volume of extraction buffer. This suspension was then left overnight and dialyzed against the appropriate buffer before chromatography. After dialysis, it was centrifuged at 20,000 × *g* for 15 min. The term "solubilized glycogen precipitate," when used in the text hereafter, refers to supernatant obtained by this final centrifugation.

Chromatography

Epoxy-activated Sepharose 6B (Pharmacia Fine Chemicals Ltd.) and cyclohepta-amylose (Schardinger-β-Dextrin, Sigma Chemical Co., St. Louis, MO, Cat. No. S-3503) were reacted using a method modified from that of Vretblad (13) and described elsewhere (14).

Solubilized glycogen precipitate dialyzed against 0.05*M* sodium acetate buffer (pH 5.5, 0.001*M* $CaCl_2$) and centrifuged was applied to a 2.2 × 3.8 cm column of cyclohepta-amylose eposy-Sepharose 6B at 4° C. The column was eluted with 120 ml of 0.05*M* sodium acetate buffer (pH 5.5, 0.001*M* $CaCl_2$) followed by the same buffer containing 8 mg/ml cyclohepta-amylose. Five milliliter fractions were collected at a flow rate of 35 ml/hr.

α-Amylase obtained by β-cyclodextrin elution of the affinity chromatography column (14) was dialyzed overnight against 0.02*M* sodium acetate buffer (pH 5.5, 0.001*M* $CaCl_2$) and applied to a 2.6 × 22 cm column of carboxymethyl cellulose (Whatman CM 32). The column was washed with the 0.02*M* sodium acetate buffer (pH 5.5, 0.001*M* $CaCl_2$), using an upward flow rate of 40 ml/hr. After the first peak was eluted, as indicated by uv absorption readings and qualitative determination of α-amylase activity, an NaCl gradient was started. It consisted of 100 ml of 0.02*M* NaCl *vs.* 100 ml 1.0*M* NaCl, both in column buffer followed by 100 ml of 1.0*M* NaCl in buffer. Five-milliliter fractions were collected.

Other Methods

pH Optimum. Barbital-sodium acetate buffers were prepared as described by Michaelis (15), but containing 0.001*M* $CaCl_2$. The pH of each buffer was measured before addition of the enzymes. Each enzyme was dialyzed against 4 m*M* $CaCl_2$ to remove acetate buffer (pH 5.5) and diluted to 1/4000 its original activity with the appropriate buffer before use.

pH Stability. The same buffers described in the pH optimum experiment were used. Dialyzed α-amylase was incubated for 2 hr at room temperature in buffers from pH 3 to 9. One set of buffers was prepared containing 0.375% β-limit dextrin. Protein concentration was less than 2 μg/ml. Prior to determination of activity the enzyme was diluted to the appropriate concentration using 0.2*M* sodium acetate (pH 5.5, 0.001*M* $CaCl_2$).

Michaelis Constants. The effect of substrate concentration on activity was determined using the dinitrosalicylic acid assay method (6). The substrate used was β-limit dextrin buffered with 0.2*M* sodium acetate (pH 5.5, 0.001*M* $CaCl_2$). The reaction was carried out at 35° C for 3 min (α-I) or 4 min (α-II) using a final volume of 1 ml. Blanks were run for each concentration of substrate. The apparent Michaelis constants were determined using the Lineweaver-Burk method (16).

Isoelectricfocusing. Flat-bed polyacrylamide-gel isoelectric focusing was

performed using equipment from LKB as described by Marchylo *et al.* (17). The pH range of the ampholine (LKB) extended from pH 3.5 to 11. An LKB multiphor power supply was used at a setting of 35 mA until the voltage reached 600 V and then left until the amperage reached a constant value of approximately 10 mA. α-Amylase activity was detected using a method adapted from Doane (9).

RESULTS AND DISCUSSION

Disc Gel Electrophoresis of the α-Amylases of Triticale and Its Parents

An electrophoretic survey of the α-amylase isozymes in Stewart wheat, Prolific rye, and triticale 6A190 was performed. Figure 1 is a diagram of the patterns obtained when β-limit dextrin zymograms were made of samples harvested at different developmental stages.

Two rapidly moving bands with R_f values between 0.5 and 0.6 were evident in all three species at all stages of development. They are the "green" α-amylase as described by Olered and Jönsson (18) and will be referred to as "α-I." Generally, four slower-moving bands were found in all germinated samples and are probably the "malt" or germination α-amylases (18). These will be called "α-II." The difference between triticale and its parents was the presence of α-II bands throughout the growth cycle of triticale 6A190. Their occurrence was not observed in immature or mature Prolific rye, and in Stewart wheat the α-II bands were less prominent than in triticale. Some samples of mature Stewart wheat

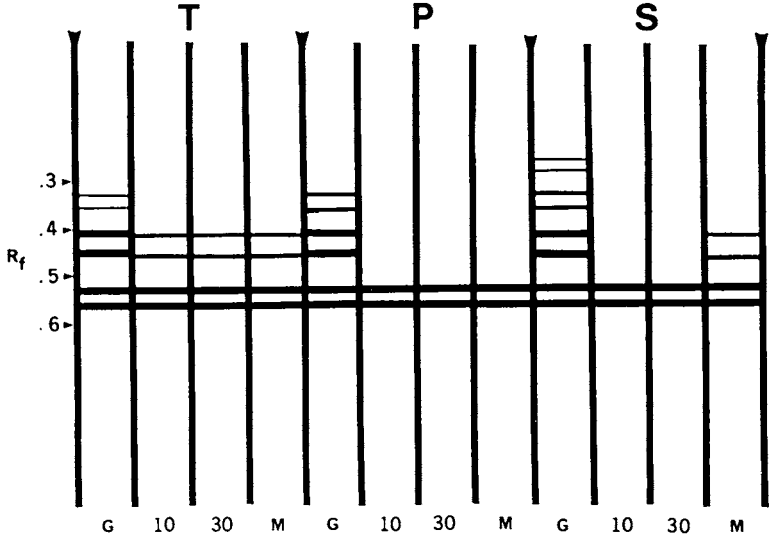

Fig. 1. Patterns of α-amylase activity as obtained by zymograms of polyacrylamide gel electrophoresis of extracts of triticale 6A190, Prolific rye, and Stewart wheat during different stages of growth. T = triticale 6A190; P = Prolific rye; S = Stewart wheat; G = germinated; 10 = 10 days post-anthesis; 30 = 30 days post-anthesis; M = mature.

TABLE I
Purification of α-Amylase from Triticale 6A190 by Glycogen Precipitation and Cyclohepta-Amylose Epoxy-Sepharose 6B Affinity Chromatography

Step	Fraction	Volume ml	Protein mg/ml	Activity IDC Units/ml	Activity IDC Units/ml	Total Units	Purification Fold	Recovery %
1	Crude extract	120	24.2	38,900	1,610	4,670,000	1	100
2	Glycogen precipitate	22	1.82	76,100	41,800	1,670,000	26	36
3	Affinity peak	50	0.132	30,400	230,000	1,520,000	143[a]	33

[a]Tube 34 (Fig. 1; 170 ml) was the most active fraction with 57,350 IDC units/ml and 296,000 IDC units/mg which represents a 184-fold increase in purity.

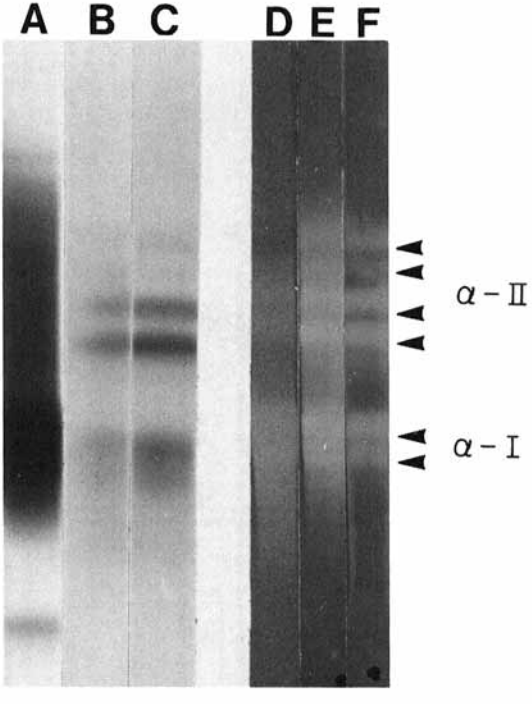

Fig. 2. Protein and α-amylase activity patterns of disc electrophoresis gels of crude extract, resuspended glycogen precipitate, and affinity chromatography purified α-amylase. The cathode is at the top of the gel. Legend: A, B, C: Protein stains of disc gels of A) Crude extract, B) Glycogen precipitate (350 μg), C) Affinity peak eluted with cyclodextrin (26 μg). D, E, F: Zymograms of α-amylase activity of crude extract, glycogen precipitate, and affinity peak, respectively.

failed to reveal α-II bands. No additional bands were observed when extracts of different species or of differing developmental stages were combined and analyzed electrophoretically.

In all species there were occasional variations in the number of α-II isozymes seen. Most preparations contained at least four isozymes, but occasionally a very faint third pair of α-II bands was observed that migrated more slowly.

Purification of Triticale α-Amylase

The majority of data in this work was obtained by using lyophilized malted triticale 6A190. The specific activity of α-amylase in crude extracts prepared from either germinated or malted triticale was in the order of 2000 IDC units/mg protein. Initial work with germinated tissue showed that heated crude extract (65° C for 10 min) lost anywhere from 20 to 40% of its α-amylase activity with increases in specific activity under 5% and occasional decreases in specific activity. Electrophoretic examination of the heated and unheated crude extracts revealed no visible change in the relative intensity of isozyme bands. Starch zymograms failed to reveal any change in the number or location of bands, indicating that β-amylase that may be present in the crude extract was in extremely low concentration. As a consequence, the normal heat purification step commonly used to free α-amylases of β-amylases was not frequently used.

The affinity chromatography method gave an α-amylase with a specific activity of 230,000 IDC units/mg, which represents an overall purification of 143-fold over crude extract, with a recovery of 33% of the activity in crude extract, or 92% of that in the solubilized glycogen precipitate (Table I). Figure 2 shows the protein stains and α-amylase activity patterns of disc electrophoresis gels of crude extract, glycogen precipitate, and the peak released by addition of cyclodextrin to the column buffer. The zymogram pattern of α-amylase activity

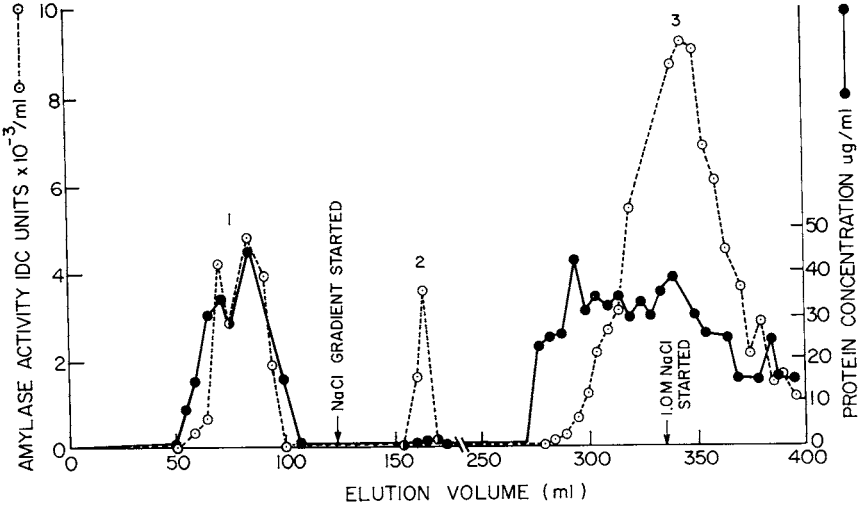

Fig. 3. Ion-exchange chromatography of triticale α-amylase on carboxymethyl cellulose.

TABLE II
A Comparison of the Properties of Triticale 6A190 α-Amylases with those Reported for Other Cereals

Property	Values Obtained for Triticale α-I and α-II, Respectively	Values Reported in Literature[a]
pH Optimum	4.5 – 6.0	3.6 – 5.75 (19)
Km ($\times 10^{-4}$ g/ml)	2.5; 2.9	2.35, 2.50, 5.33 (19)
V_{max} (μmol maltose/min/mg enzyme at 25°C)	1250; 5000	1465 (20)
Activation energy (kcal)	15; 12	13 (22); 11, 11 (23)
Molecular weight	40,000; 41,000	41,500 – 42,500 (20)

[a]Number in parentheses indicates reference source.

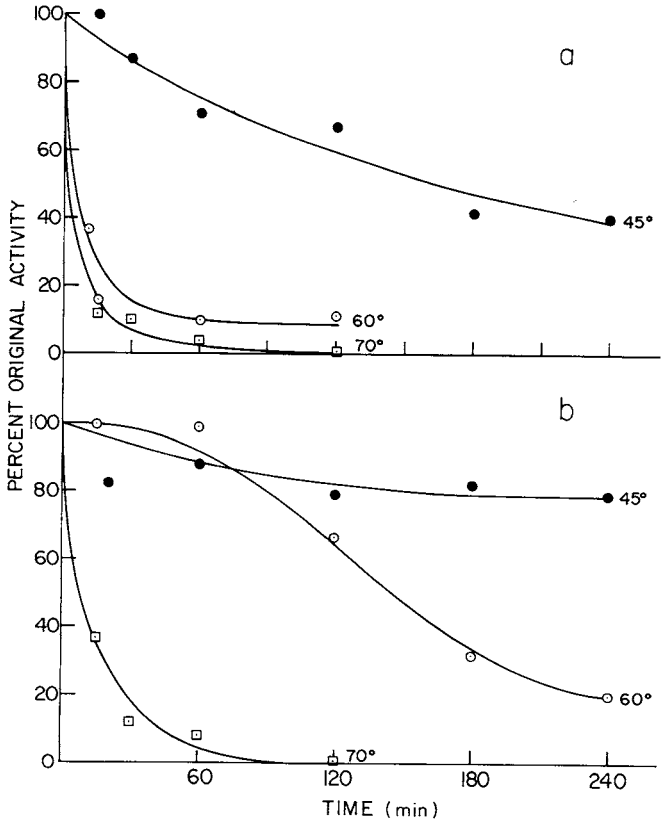

Fig. 4. Stability of triticale α-amylases at 45°, 60°, and 70°C; (a) α-I, (b) α-II.

was not affected by the purification procedure, whereas the number and intensity of protein bands decreased as purity was increased. Incubation of the disc gels on plates containing starch (not shown) rather than dextrin revealed no additional bands of activity, indicating that no detectable β-amylase was retained by the column.

Ion-Exchange Chromatography

Figure 3 shows the elution profile obtained when α-amylase procured by affinity chromatography was chromatographed on CM 32 cellulose. The protein levels were very low, making accurate determination difficult, but levels were monitored by uv light absorption. Three peaks of activity appeared. The second peak, eluting at approximately 170 ml (Fig. 3), is eluted after the NaCl entered the column. It lost activity very rapidly and represented only 0.3% of the protein loaded. Zymograms of the first peak showed it to have the bands characteristic of α-I while peak 3 had electrophoretic mobilities characteristic of the α-II amylase

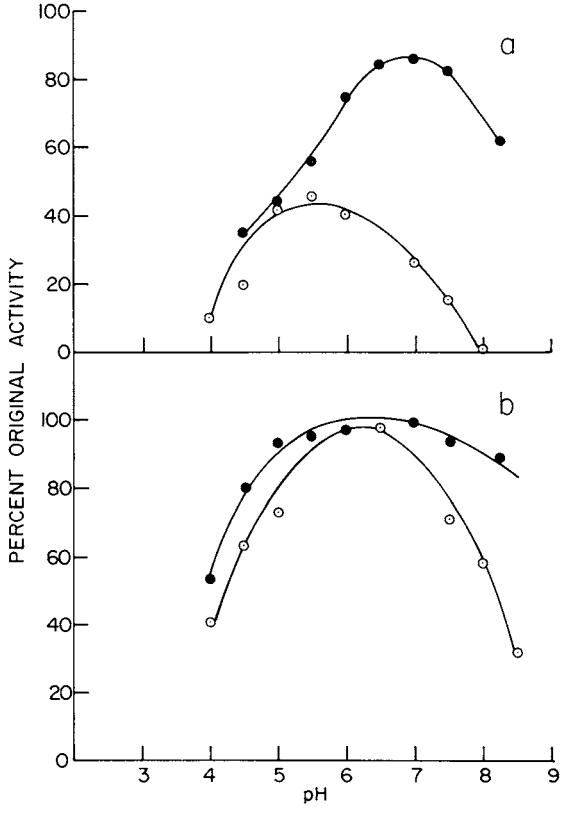

Fig. 5. pH stability of triticale α-amylases with and without β-limit dextrin; (a) α-I; (b) α-II. Closed circles represent stability in presence of β-limit dextrin.

group. Approximately 20% of the activity recovered was in the first peak, 4% in the second peak, and 76% in the third peak. The total activity recovered, in terms of units applied to the column, was 88%. The first peak appeared to be two peaks shouldered together, but electrophoretic examination of the two peaks at their centers and extreme shoulders showed no difference in the zymograms. The specific activity of α-I was 130,000 IDC units/mg, that of α-II 299,000 IDC units/mg.

Reaction Properties of Triticale α-Amylase

Both α-I and α-II showed optimal activity between pH 4.5 and 6.0 (Table II). Marchylo *et al.* (17), using immature wheat α-amylases, has observed an optimum from pH 3.6 to 5.75. Tkachuk and Kruger (19) separated the four isozymes of malted wheat and found no difference in the pH optima or profiles.

The effect of substrate concentration on activity was determined for both α-I and α-II. The Km for α-I was 2.5×10^{-4} g/ml and for α-II it was 2.9×10^{-4} g/ml (Table II). This agrees favorably with the values reported by Marchylo *et al.* (17) for immature wheat α-amylases (2.35, 2.50, and 5.33×10^{-4} g/ml), and values for pig pancreas (1.8×10^{-4} g/ml), malted barley ($>6.3 \times 10^{-4}$ g/ml), and *Bacillus subtilis* (6.3×10^{-4} g/ml) α-amylases as reported by Bernfeld (20 and references therein).

The V_{max} for α-I and α-II were determined at 35° C, but for comparative purposes have been corrected to 25° C using data from the Arrhenius plot. α-I has a V_{max} of 1250 μmol maltose/min/mg enzyme at 25° C, pH 5.5. The value for α-II is 5000 μmol/min/mg under the same conditions. One report (19) on malted wheat α-amylases gave an average value of 1465 μmol/mg.

The effect of temperature on the hydrolysis rate of β-limit dextrin substrate was determined for both α-I and α-II isozymes. The temperature range 25° to 50° C was examined. The activation energies determined from the Arrhenius plot were 15 and 12 Kcal/mol for α-I and α-II, respectively (Table II). The values are similar to those obtained for malted barley (21), 13 Kcal; rye; 11 Kcal; and wheat, 11 Kcal (as described in reference 22).

Temperature and pH Stability

Purified α-amylases (α-I and α-II) were incubated at pH 5.5 in 0.2*M* sodium acetate containing 0.001*M* $CaCl_2$ for various lengths of time at 45°, 60°, and 70° C, in the absence of substrate and a protein concentration of less than 2 μg/ml. The activity of the enzyme was then determined. Figure 4 shows the stability of α-I and α-II to various temperatures. α-I was less stable than α-II. Especially noticeable is the fact that after 15 min at 60° C, the activity of α-I decreased much more than that of α-II.

To test for pH stability, the enzyme was incubated at various pH levels from pH 3 to 9 with and without substrate, and tested for activity at a pH of 5.5. Figure 5 gives a profile of activities remaining after treatment at varying pH. The presence of β-limit dextrin particularly at pH values greater than 6 stabilized both α-I and α-II with the effect most striking for α-I. For example, incubation for 2 hr at room temperature at a pH of 7.5 without substrate resulted in about 15% of the initial α-I activity remaining whereas approximately 80% of the α-II activity remained under the same conditions. Incubation of α-I in the presence of substrate at the same pH resulted in retention of about 80% of the activity.

Determination of Molecular Weight by SDS Gel Electrophoresis

α-Amylases α-I and α-II were subjected to SDS gel electrophoresis. One protein band was found, indicating that despite the appearance of multiple bands with regular disc gel electrophoresis with either α-I or α-II, the subunit molecular weight observed was 40,000 for α-I and 41,000 for α-II (Table II). These values are not significantly different from each other, and agree favorably with those obtained in wheat by Tkachuk and Kruger (19) which ranged from 41,500 to 42,500.

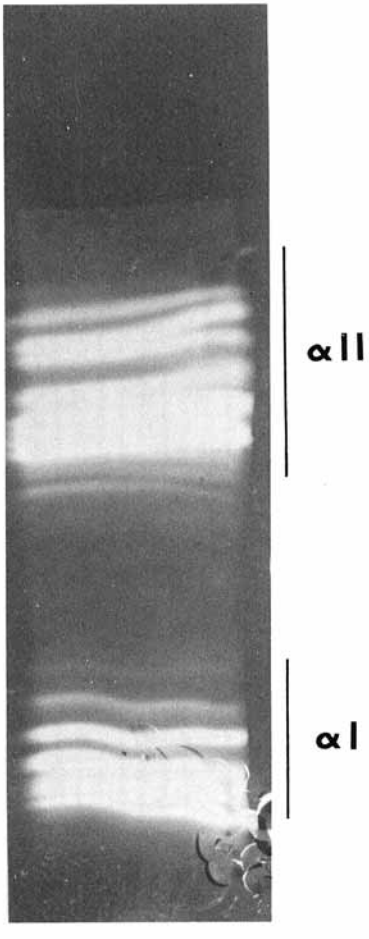

Fig. 6. Isoelectric focusing of triticale α-amylase using a pH gradient of 3.5 to 11. α-I isozymes have an isoelectric point in the range pH 4.6 to 5. α-II isozymes have an isoelectric point near 6.2. The slab is stained for amylase activity using β-limit dextrin as substrate.

Isoelectric Focusing

Polyacrylamide flat-bed isoelectric focusing (Fig. 6) of α-I and α-II on a gradient of pH from 3.5 to 11 indicated that α-I isozymes have an isoelectric point in the range of 4.6 to 5 whereas α-II isozymes are centered near pH 6.2. Zymograms were used to reveal the isozymes. There were at least five bands in α-I and at least nine in α-II. Such a phenomenon with the α-amylases of cereals is not uncommon. Nishikawa and Nobuhara (23) in a survey of over 30 lines of germinated wheat, found up to 15 bands of α-amylase activity using a pH gradient of pH 5 to 8. Such a large number of true isozymes seems unlikely. Tkachuk and Kruger (19) found very little difference in the amino acid composition of the four isozymes they separated from malted wheat, and the similarities of the four isozymes in terms of pH optimum, molecular weight, etc., suggests the differences are small. Similar observations were made by Marchylo *et al.* (17).

DISCUSSION

The cyclohepta-amylose affinity chromatography column offers a consistently high recovery rate (>90%), with a high purification factor. Its use eliminates the need for purification steps such as ammonium sulfate precipitation, or acetone fractionation. The column has proved stable for several months at 4° C and has been used successfully with crude extracts.

Some general conclusions may be drawn from the chromatographic and electrophoretic data. The α-I isozymes are definitely more acidic than the α-II. This is borne out by their isoelectric points, the fact that α-I is retarded on diethylaminoethyl chromatography compared to α-II (unpublished observations), and that α-I is not held back on the CMC column. These facts and the fact that it migrates most rapidly in a basic pH disc gel are consistent with α-I, green type α-amylase being more acidic than the α-II germination type.

From the disc-electrophoresis patterns obtained at various stages of growth, it appears that triticale 6A190 is different from its parental species in that germination type α-amylase is present during all stages of growth.

The presence of a germination type of α-amylase supports the proposal that a lack of dormancy in the seed of 6A190 triticale may be responsible for elevated levels of α-amylase activity observed with this cultivar (4).

Acknowledgments

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