α-AMYLASE FROM IMMATURE HARD RED SPRING WHEAT. I. PURIFICATION AND SOME CHEMICAL AND PHYSICAL PROPERTIES[1]

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ABSTRACT

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α-Amylase was purified from immature Canadian hard red spring wheat by extraction, heat treatment at 70°C, acetone fractionation, complexing with glycogen, and DEAE-cellulose ion-exchange chromatography. Purity at the glycogen-complex stage was indicated by SDS gel electrophoresis. A total yield of 18.4% was obtained with 1900-fold purification. Three multiple forms of α-amylase were separated by DEAE-cellulose ion-exchange chromatography at pH 8.2.

They were very similar in all of their properties, such as pH optimums, Km values, molecular weights, and activation energies, with the exception of their isoelectric points which were 4.65, 4.84, and 5.11 for the α-1, α-2, and α-3 components, respectively. The immature wheat α-amylases differed from malted wheat α-amylases in having broader pH optimums, greater heat labilities, lower isoelectric points, and a higher molecular weight.

Wheat α-amylase is now recognized as an important enzyme in affecting the quality of hard red spring (HRS) wheats. In breadmaking, the enzyme affects dough properties such as gassing power and consistency and, if in excess, will result in excessive liquefication and dextrinization, yielding a bread with a wet, sticky crumb (1). The amount of the enzyme increases enormously on germination. Because of this, germinated wheat has been the subject of intense investigations. Using agar-gel electrophoresis, Olered and Jonsson (2) found that germinated HRS wheat contained two electrophoretically distinct α-amylase forms. Further studies by Kruger (3) using polyacrylamide-slab electrophoresis at alkaline pH revealed that these two sets were composed of five and three distinct α-amylase components, with the latter set being the more electrophoretically mobile. The slower moving components were the major

components present in germinated wheat and were isolated and partially characterized (4,5).

On the other hand, very little is known concerning the nature of the α-amylase present in immature wheat and its significance in wheat quality. It has recently been established that in immature wheat there are three α-amylases present which are identical in electrophoretic behavior to the electrophoretically faster α-amylase components present in germinated wheat (6). In developing wheat kernels, the enzyme was found mainly in the pericarp (6,7,8) and its level decreased upon maturation. Olered (9) has found that the decrease was dependent on the moisture level present in the near mature kernels of wheat and this in turn affected the ultimate quality of a wheat. Thus, wheats harvested at a high level of moisture were found to have a lower falling number than similar samples harvested at a lower level of moisture. The final level of the enzyme may also be dependent on the variety. Thus, a Canadian wheat variety, Cypress, was found in this laboratory (6) to have a low amylograph viscosity and to contain only the α-amylase components present in immature wheat.

The manner in which immature wheat α-amylase is involved in the carbohydrate metabolism of the developing wheat kernel also remains to be elucidated. One suggested explanation is that it is involved in starch breakdown in the pericarp, possibly in combination with a β-amylase component which has been detected recently in this tissue (6).

To obtain further knowledge on this enzyme, therefore, it has been purified from a sample of immature Canadian HRS wheat and some of its chemical and physical properties determined.

MATERIALS AND METHODS

Heads of wheat were cut from a field of immature Canadian HRS wheat, variety Neepawa, at approximately 20 days after anthesis. The heads were immediately freeze-dried and stored in this state. Just prior to use, the freeze-dried wheat was ground into a whole wheat flour with a Wiley mill (equipped with a 1-mm mesh sieve).

α-Amylase Activity

Routine determinations of α-amylase activity were carried out using a semi-automated iodine-β-limit dextrin assay developed from the manual method of Briggs (10) as modified by MacGregor et al. (11). The basic Technicon AutoAnalyzer (Technicon Corp., Chauncy, N.Y.), was used for semi-automating the assay. The system consisted of a Sampler II; proportioning pump II; colorimeter with a 540-nm filter; and a recorder using absorbance paper. The substrate used was 0.08% β-limit dextrin in 0.2M sodium acetate buffer, pH 5.6, containing 0.001M calcium chloride. Two milliliters of enzyme or diluted enzyme was incubated with 2 ml of substrate for 10 to 20 min at 35°C, cooled rapidly to a low temperature, and sampled on the AutoAnalyzer. Sample pumping at 1.20 ml/min was joined with 0.005% iodine and 0.05% potassium iodide pumping at 1.6 ml/min and segmented by an air stream at 0.16 ml/min. The resulting stream was passed through a single mixing coil and then through the colorimeter. The pull-through on the 15-mm flow cell was 2.0 ml/min.

α-Amylase activity was calculated by the following equation which has been modified from that of Olered (9).

$$k\ (\alpha) = \frac{F}{t}\ (\log\ Ex_o - \log\ Ex)$$

Where k (α) is the activity per min at 35°C, t is the time of incubation in minutes, Ex_o is the extinction of the iodine-β-limit dextrin complex at t = 0, and Ex is the extinction after t minutes of enzyme incubation. F is the dilution factor necessary to bring the extinction within the linearity of the assay (0.2 − 0.8A).

For determining enzymatic properties, reduced starch was used as substrate and the liberated reducing sugars resulting from enzymatic action were measured by the method of Dygert et al. (12) with neocuproin. A Technicon AutoAnalyzer was used to automate the determination of reducing sugars. The reduced starch was prepared from soluble starch Merck (according to Lintner) by the procedure of Strumeyer and Romano (13) except that the starch-borohydride mixture was stirred overnight. This was followed by centrifugation at 10,000 × g for 10 min and freeze-drying of the supernatant.

Estimation of Protein

Protein determinations were carried out by the colorimetric Folin method of Lowry et al. (14).

Polyacrylamide-Slab Electrophoresis

Electrophoresis was carried out on polyacrylamide slabs at pH 8.9 according to the method of Davis (15) and at pH 4.75 by the method of MacGregor and Meredith (16). An Ortec model 4200 slab electrophoresis system was employed with an Ortec Model 4200 pulsed power supply (Ortec Inc., Oak Ridge, Tenn.) as described previously (6). For electrophoresis of slabs containing sodium dodecyl sulfate (SDS), the procedure of Weber and Osborn (17) was employed. Protein samples for SDS electrophoresis were prepared under the strong reducing conditions of Robyt et al. (18) by incubation in 0.01 M sodium phosphate buffer, pH 8.5, containing 1.5 mM dithiothreitol. The gels were electrophoresed at a pulse rate of 225 pulses per sec at 325 V. The proteins used for calibration of the gel were ovalbumin, aldolase (Pharmacia, Uppsala, Sweden); bovine serum albumin, pepsin (Calbiochem, Los Angeles, Calif.); myoglobin, cytochrome C (Schwarz Mann, Orangeburg, N.Y.); and chymotrypsinogen A (Worthington Biochemicals, Freehold, N.J.). Following electrophoresis, the gels were stained for 16 hr in Coomassie Blue and destained with an Ortec Model 4216 destainer (Ortec Inc., Oak Ridge).

Molecular Sieve Chromatography

Chromatography on a 2.0 × 47-cm column of Biogel P-100 was also used to determine the molecular weight of immature wheat α-amylase. The column was equilibrated with 0.1 M tris-HCl buffer, pH 7.0, containing 0.001 M CaCl$_2$ at a flow rate of 15 ml/hr and calibrated with apoferritin, bovine serum albumin, myoglobin, cytochrome C, and ovalbumin (Pharmacia, Uppsala, Sweden). Protein was monitored with an LKB ultraviolet analyzer (LKB Producter, Uppsala, Sweden).

Isoelectric Focusing

Electrofocusing was carried out as described by Vesterberg and Svensson (19) with column, ampholytes, and gradient mixer purchased from LKB Products. A 110-ml column and temperature of 5°C were used in all experiments. Enzyme extracts were electrofocused at 300 V for 72 hr in a pH 4—6 gradient. Following electrofocusing, 2.1 ml fractions were collected from the column in a refrigerated fraction collector, and pH measurements were made at 5°—8°C with a Fisher Accumet Model 520 pH meter.

Detection of the Multiple Forms of α-Amylase

To determine the location of the multiple forms of α-amylase following polyacrylamide-slab electrophoresis, the method of Doane (20), as modified by MacGregor *et al.* (21), was employed. In preparation of the detection plates, starch (0.1 g) was used instead of β-limit dextrin. The polyacrylamide slab was sandwiched between two of the starch plates and incubated in a covered tray containing a small amount of $0.2M$ sodium acetate buffer, pH 5.5, containing $0.001M$ $CaCl_2$ at 35°C for 30 to 40 min, depending on the activity of the sample. The plates were then stained with 0.2% potassium iodide and 0.02% iodine solution. Colorless bands against a blue background indicated the location of α-amylase components.

EXPERIMENTAL PROCEDURES AND RESULTS

Purification of Immature Wheat α-Amylase

The isolation procedure was similar to that employed previously for malted wheat α-amylase (4) and involved extraction, heat treatment at 70°C, acetone fractionation, glycogen-complex formation, and DEAE-cellulose ion-exchange chromatography. A number of changes in experimental conditions were required, however, for isolating immature wheat α-amylase and the entire isolation procedure is described below.

Extraction of α-Amylase. Freeze-dried, ground, immature wheat (300 g) was slowly added to 2.4 l. of 0.2% $CaCl_2$ in a 128-oz jar. The jar was sealed and the suspension was shaken gently for 4 hr at 4°C with a mechanical shaker. Following this, the extract was centrifuged at 12,000 × g for 10 min and the resulting pale green supernatant retained. All further centrifugations were carried out under these conditions unless otherwise stated.

Heat Treatment. Immature wheat α-amylase in crude extracts was reasonably stable to heat and a heat-treatment step was advantageous, therefore, in removing a large amount of contaminating heat-labile proteins. Two hundred milliliters of extract was placed in a 95°C water bath and, with stirring, was rapidly brought to 70°C. The solution was transferred to a 70°C constant temperature bath and kept there until a total heating time of 15 min had elapsed. Following this, the extract was placed in an ice bath, rapidly cooled to 25°—35°C, and centrifuged.

Acetone Fractionation. Acetone at −10°C was slowly added to 1000 ml of stirring extract at 4°C to a concentration of 15% (v/v). The temperature of the extract was then lowered to −5°C and maintained there with an ice-salt bath. Acetone at −10°C was further added to a concentration of 25% (v/v). The mixture was allowed to stand for 20 min and then centrifuged. The precipitate

was discarded and acetone at $-10°C$ was added to the supernatant to a final concentration of 54% (v/v). After sitting for 20 min, the suspension was centrifuged at $-5°C$ and the precipitated protein containing the α-amylase was dissolved overnight in 75 ml of $0.07M$ tris-HCl, pH 8.2, containing $0.001M$ $CaCl_2$. Any undissolved protein was removed by centrifugation.

Glycogen Complex

The use of glycogen to purify α-amylases as originally reported by Loyter and Schramm in 1962 (22) was successful in purifying immature wheat α-amylase. Commercial rabbit liver glycogen (Sigma Chemical Co., St. Louis, Mo.) was first purified with Amberlite Monobed resin MV-3 (analytical grade) to remove contaminating protein. The purified glycogen was freeze-dried and stored at $8°C$.

Cold ethanol at $4°C$ was slowly added with stirring to 72 ml of extract for 1 hr to a 40% (v/v) concentration and the resulting suspension centrifuged. To the clear solution was added 6 mg of purified glycogen dissolved in 1 ml of water. After stirring for 10 min, the suspension was centrifuged at $1900 \times g$ at $4°C$ for 10 min. The supernatant was carefully drained from the precipitate and two more precipitations were carried out using 25 mg portions of glycogen. The glycogen-complex precipitates were combined in a total volume of $10-15$ ml of cold $0.07M$ tris-HCl, pH 8.2, containing $0.001M$ $CaCl_2$ and allowed to sit at room temperature for 2 hr and overnight at $8°C$ to allow the α-amylase to digest the glycogen. It was necessary to repeat the above purification in order to obtain pure α-amylase. The second glycogen complex was dissolved in $2-5$ ml of the above-mentioned tris-HCl buffer.

Typical protein and enzyme activities up to the second glycogen-complex stage are shown in Table I. An overall increase in specific activity of 1902 with an 18.4% recovery of enzyme was obtained. The specific activity at this stage was 760.8 k(α) per mg protein. The second glycogen complex effected substantial purification over the first one. Any further possible purification by the ion-exchange chromatography step to be described next could not be ascertained because of the minute amounts of protein which could not satisfactorily be determined. SDS polyacrylamide-slab electrophoresis (Fig. 1) indicated, however, that α-amylase free of contaminating proteins was present at the glycogen-complex stage.

TABLE I
Purification of α-Amylase from Immature Wheat

Stage	α-Amylase Concentration k(α)/ml	Total α-Amylase k(α)	Protein mg/ml	Specific Activity k(α)/mg protein	Yield %	Purification
	Volume ml					
Initial extract	1050 2.76	2898	6.87	0.40	100	1.00
Heat-treated extract	1000 2.44	2440	4.54	0.54	84.2	1.35
24–54% Acetone fraction	72 17.98	1294	3.53	5.09	44.7	12.72
Glycogen-complex I	10.4 77.22	803	0.40	193.05	27.7	482.63
Glycogen-complex II	5.0 106.51	533	0.14	760.8	18.4	1902.00

Ion-Exchange Chromatography. Ion-exchange chromatography was used as a final purification step to separate immature wheat α-amylase into three forms. A 0.9×59-cm column of microgranular N,N-diethylaminoethyl cellulose (DE-32) was equilibrated with degassed $0.07M$ tris-HCl buffer, pH 8.2, containing $0.03M$ NaCl and $0.001M$ CaCl$_2$. An enzyme extract was dialyzed against the above buffer and placed on the column. A linear gradient consisting of 1000 g of the above buffer and 1000 g of $0.07M$ tris-HCl, pH 8.2, containing $0.05M$ sodium chloride and $0.001M$ CaCl$_2$ was then used to elute two α-amylase forms from the column. Following this, $0.2M$ NaCl was passed through the column to remove the third form. The gradient was run through the column at 1.0 ml/min and 15-ml portions of the enzyme were collected in a fraction collector. Protein monitoring was carried out using an LKB Uvicord Ultraviolet Analyzer and α-amylase activity was determined on each fraction. The resulting elution profile is shown in Fig. 2. The peak first eluted was electrophoretically slowest on
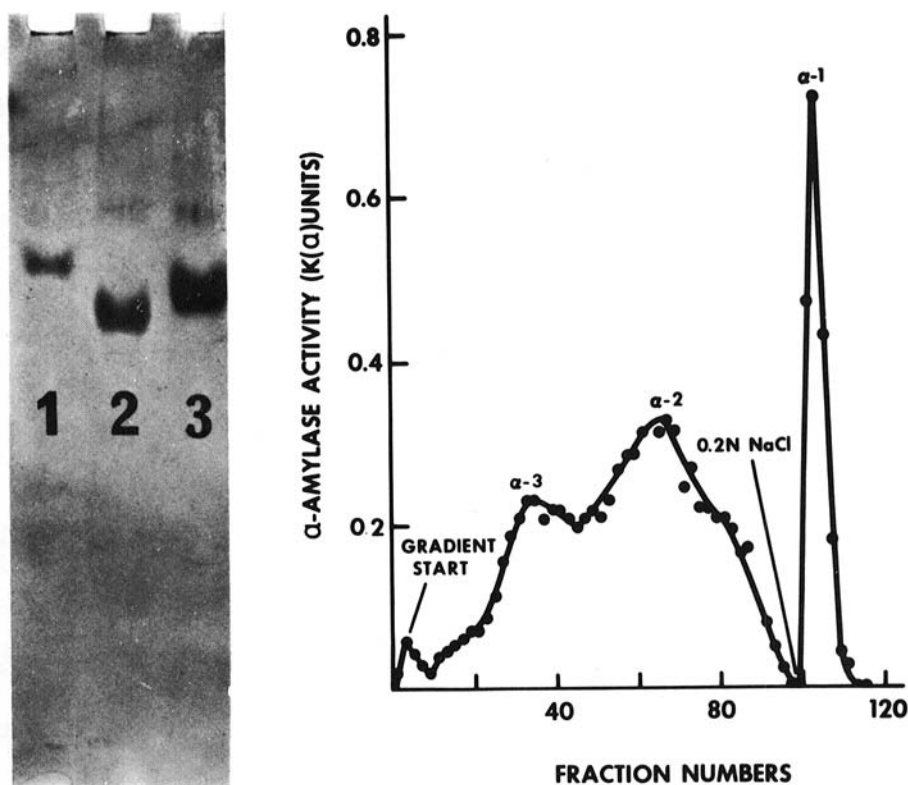

Fig. 1 (*left*). SDS polyacrylamide-slab electrophoresis of immature wheat α-amylase and two reference proteins. 1) Immature wheat α-amylase, 2) aldolase, and 3) ovalbumin. Fig. 2 (*right*). DEAE-cellulose ion-exchange chromatography of immature wheat α-amylase purified to the glycogen-complex stage. α-Amylase activity is in terms of k(α)/ml.

polyacrylamide-slab electrophoresis at alkaline pH and, following previous terminology in this laboratory (6), was designated α-3. The second and third peaks corresponded to enzymes α-2 and α-1, respectively. Good resolution of the peaks can be seen although there is some overlapping present. The fractions containing each component were combined and concentrated to a small volume with Diaflo Ultrafiltration apparatus equipped with a UM-10 membrane (Amicon Corp., Lexington, Mass.). Polyacrylamide-slab electrophoresis at basic pH followed by α-amylase detection on a starch plate confirmed that immature wheat α-amylase purified to the glycogen-complex stage had been separated into individual components (Fig. 3). The electrophoretic positions of the three forms were as expected from their chromatographic behavior with the α-1 component being the most highly ionized at alkaline pH and α-3 the least. Detection of amylase with a β-limit dextrin plate was also carried out and confirmed that the components were α-amylase and not β-amylase. Polyacrylamide-slab electrophoresis at acid pH of the three components followed by α-amylase detection on a starch plate indicated only two bands of activity (Fig. 4) with α-2 and α-3 having the same mobility. Insufficient proteins were present in the
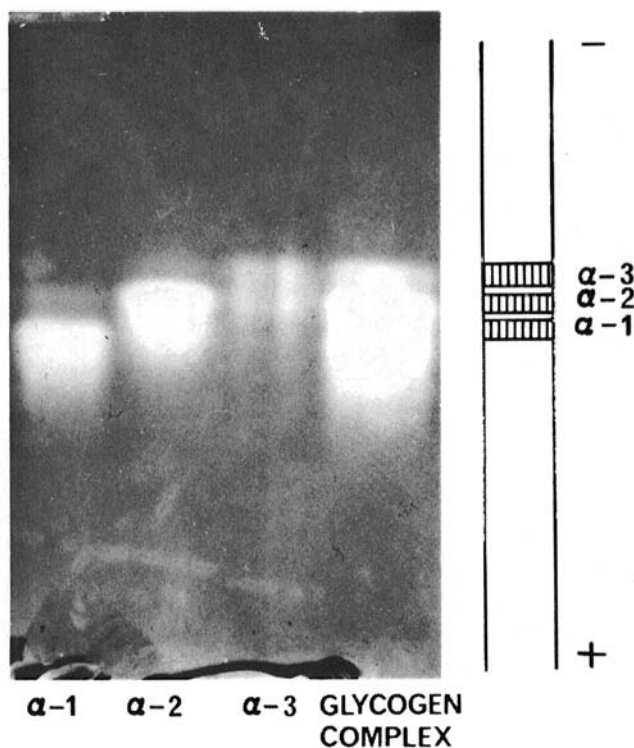

Fig. 3. Electrophoretic behavior on polyacrylamide slabs at pH 8.9 of α-amylase purified to the glycogen-complex stage and components α-1, α-2, and α-3 from DEAE-cellulose ion-exchange chromatography. Detection of isozymes was by incubation against a starch plate.

concentrated portions of each peak from DEAE chromatography to obtain suitable protein stains on polyacrylamide slabs. Protein staining of the glycogen complex, however, gave three bands on the basic gel and two on acid gel with mobilities in both cases corresponding to the location of the α-amylase components.

Enzymatic Properties

pH Optimum. The Michaelis (23) barbital-sodium acetate buffers (0.014M) containing 0.001M CaCl$_2$ were used for pH determinations. For components α-1 and α-3, 0.1 ml enzyme was added to 2 ml buffer plus 2 ml of 1.0% reduced starch and incubated for 4 min. The reaction was terminated by addition of 0.2 ml 4N HCl and the amount of reducing sugars determined. With component α-2, 0.25% starch was used as substrate and the reaction products measured continuously on the Technicon AutoAnalyzer. Substrate was in excess in all cases, as indicated by the linear formation of reducing sugars with time. As shown in Fig. 5, the pH optimums for all the multiple forms of the enzyme were almost identical and quite broad, ranging from pH 3.6 to 5.75.

pH Stability. The pH stability of the α-2 component was determined at pH values of 3.0, 4.5, 5.75, 7.0, and 8.0 in 0.014M barbital-sodium acetate buffers containing 0.001M CaCl$_2$. Enzyme (0.1 ml) was incubated in 3.8 ml of buffer for

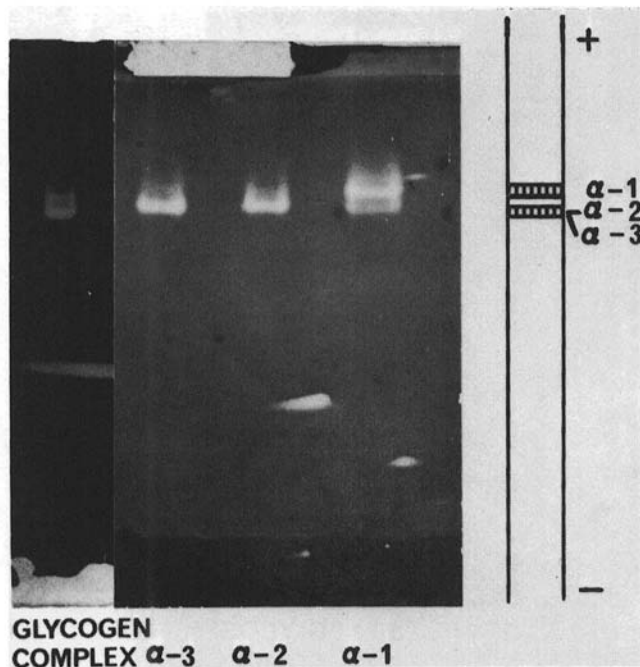Fig. 4. Electrophoretic behavior on polyacrylamide slabs at pH 4.75 of α-amylase purified to the glycogen-complex stage and components α-1, α-2, and α-3 from DEAE-cellulose ion-exchange chromatography. Detection of components was by incubation against a starch plate.

periods of 0, 2, 5, 10, and 30 min and then assayed for α-amylase activity using reduced starch substrate with continuous monitoring of hydrolysis products. No loss in activity was found after 30 min of incubation at any pH with the exception of pH 3.0. At this pH, the enzyme was completely inactivated in 2 min.

Effect of pH on Action Pattern. It has been observed with α-amylases from pork pancreas, human saliva, and *Aspergillus oryzae* that changes in action patterns may be observed at different pH values by plotting the change in iodine-amylose color *vs.* reducing power as per cent apparent maltose (24). In the present study, the action pattern of purified α-2 was examined in a similar manner except that reduced β-limit dextrin was used as substrate. The reduced β-limit dextrin substrate was prepared as described previously (25). Change in iodine-reduced β-limit dextrin was calculated from the absorbance at 540 nm at time t divided by the absorbance at time t = 0 multiplied by 100 and was determined with the semi-automated β-limit dextrin assay. Per cent apparent maltose was determined using the automated neocuproin reducing sugar assay. Immature wheat α-amylase components were examined at pH values of 4.0, 5.5, and 7.0 using 0.36% substrate in 0.014 M Michaelis barbital-sodium acetate buffers. The results (Fig. 6) indicated that no change in action pattern because of change in pH was observable under the conditions described. An extract of
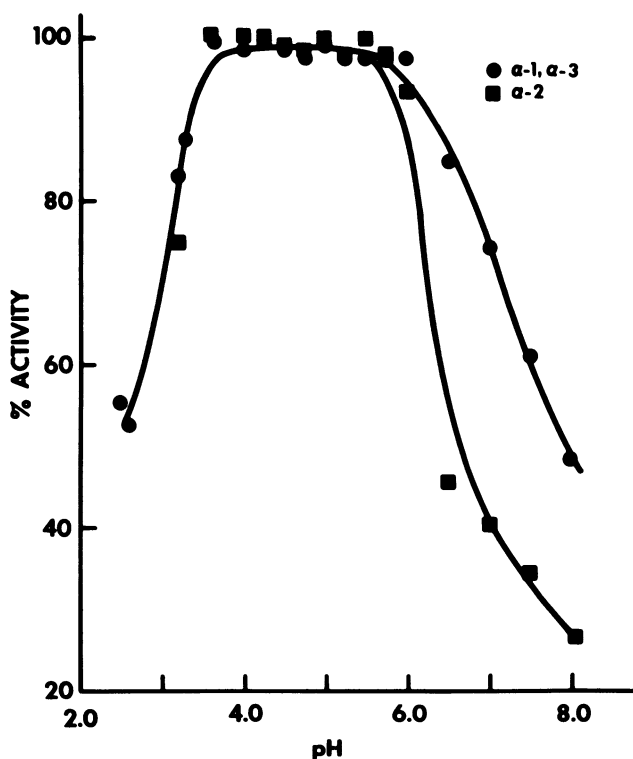

Fig. 5. Effect of pH on the activity of immature wheat α-amylases.

germinated Manitou wheat α-amylase, heat-treated at 70°C for 15 min to remove β-amylase, was also examined under the same conditions and, as shown in Fig. 6, behaved identically to the immature wheat α-amylase. The results presented here do not prove unequivocally that differences in action pattern are not present as β-limit dextrin is probably less sensitive than amylose in showing up such differences.

Michaelis Constants. The effect of substrate concentration upon activity was determined for the three α-amylase components using reduced starch in 0.2M sodium acetate, pH 5.5, containing 0.001M CaCl$_2$. Substrate of varying concentration (4.0 ml) and 0.1 ml of enzyme were incubated for 5 min. The reaction was terminated with 0.2 ml of 5N HCl and the reaction mixture assayed for reducing sugars. The apparent Michaelis constant, Km, was determined by plotting 1/v *vs.* 1/S in the graphical representation of Lineweaver-Burke as
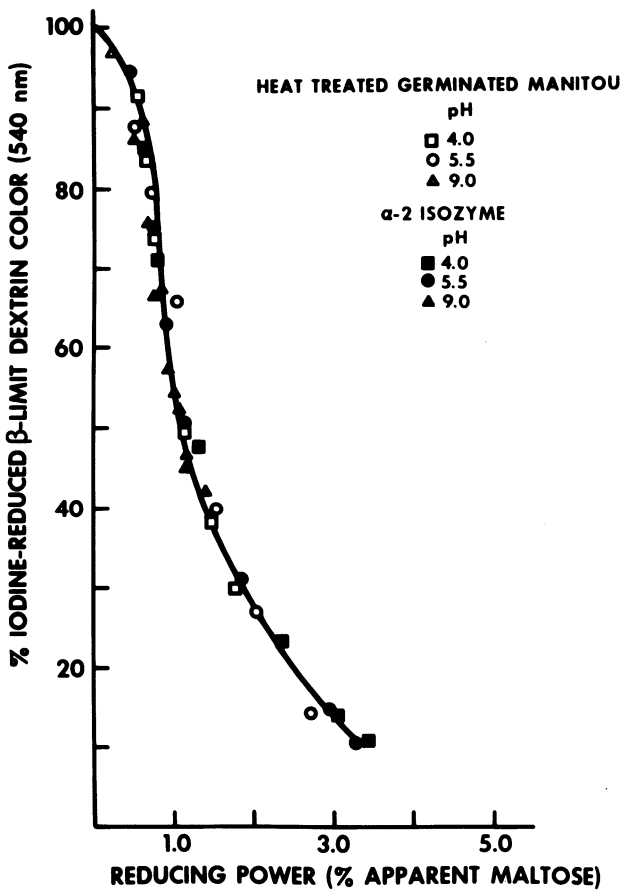

Fig. 6. Graph of decrease in iodine-reduced β-limit dextrin color *vs.* increase in reducing power for immature wheat, α-2, and heat-treated, germinated Manitou at pH values of 4.0, 5.5, and 9.0.

shown in Fig. 7. The reciprocal of the intercept of the plotted line with the abscissa indicated that the Km for the three multiple forms were α-1, 2.50×10^{-4} g/ml; α-2, 5.33×10^{-4} g/ml; α-3, 2.35×10^{-4} g/ml.

Thermal Stability. Thermal stabilities of the purified α-amylase components were determined in $0.2M$ acetate buffer, pH 5.5, containing $0.001M$ CaCl$_2$ for 30 min at temperatures between 25° and 80°C. As shown in Fig. 8, all behaved identically. Complete stability was found at 40°C but only 50% remained after a 30-min heat treatment at 55°C.

Effect of Sulfhydryl Reagents. Iodoacetic acid and N-ethylmaleimide at concentrations of $1 \times 10^{-4}M$ caused no inhibition of the immature wheat α-amylase after 15 min of incubation at 35°C, indicating that free sulfhydryl groups were not necessary to maintain the catalytic reactivity of the enzyme. On the other hand, $1 \times 10^{-4}M$ mercuric chloride effected a 90 to 96% inhibition with all three components but this was probably because of a heavy metal binding effect.

Physical Characterization

Determination of Molecular Weight by SDS Gel Electrophoresis. An extract of immature wheat α-amylase, purified to the second glycogen-complex stage, was subjected to SDS gel electrophoresis. One protein band was found, indicating that the three α-amylase components had identical molecular weights. The molecular weight, determined from a plot of the mobilities of the standard proteins against the logarithms of their molecular weights, was 52,000–54,000.

Determination of Molecular Weight by Molecular Sieve Chromatography. A calibrated column of Biogel P-100 was used also to determine the molecular weight of an extract of immature wheat α-amylase purified to the second
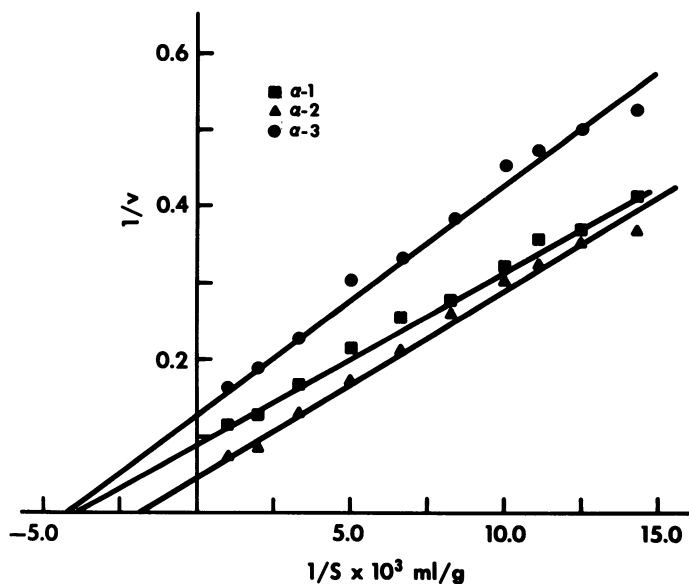

Fig. 7. Determination of Michaelis-Menten constants, Km, for immature wheat α-amylases.

glycogen-complex stage. A selectivity curve was determined by graphing the Km values for standard proteins *vs.* the logarithm of their molecular weight and from this the molecular weight of the enzyme complex was found to be 36,000—38,000.

Activation Energy. The effect of temperature on the hydrolysis rate of reduced starch by the three α-amylase components was determined over the temperature range 25°—80°C. Activation energies for α-1, α-2, and α-3, calculated from the Arrhenius plot shown in Fig. 9, were 9.66, 9.04, and 8.82 kcal/mol, respectively.

Isoelectric Focusing. Preliminary isoelectric focusing between pH 3 and 10 indicated that the pI values of immature wheat α-amylases were between four and six. A pH 4—6 gradient was used, therefore, in subsequent experiments. The isoelectric focusing profiles of each of the purified components as well as at the glycogen-complex stage are shown in Fig. 10. pI values for α-1, α-2, and α-3 were 4.65, 4.84, and 5.11, respectively. Three peaks were visible in the isoelectric

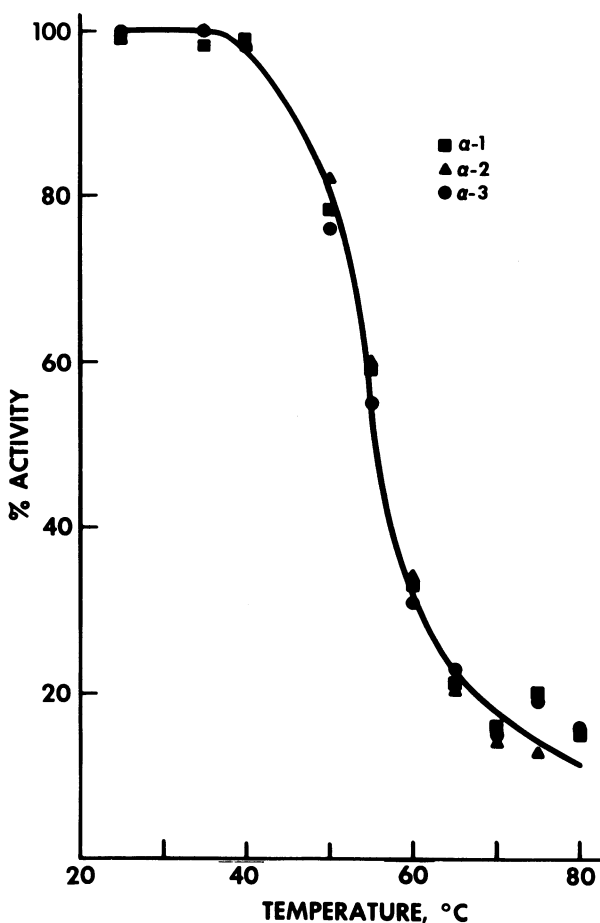
Fig. 8. Effect of temperature on immature wheat α-amylases.

focusing profile of the glycogen-complex preparation with isoelectric points of 4.64, 4.90, and 5.08. It should be possible, therefore, to use this technique as a purification step to obtain individual α-amylase components by using a narrower pH gradient in the isoelectric focusing.

DISCUSSION

α-Amylase was purified from the immature HRS wheat variety, Neepawa, by a process involving extraction, heat treatment, and complexing with glycogen. The amount of enzyme present in immature wheat was very small compared to that in malted wheat. Because of this, strict attention to optimum isolation conditions was very important to obtain enough enzyme for characterization studies.

Three multiple forms of the enzyme were separable by DEAE-cellulose ion-exchange chromatography and some of their chemical and physical properties determined. A summary of some of these properties is shown in Table II, with the properties of malted wheat and immature barley included for comparisons.

The three immature wheat α-amylase forms behaved similarly with respect to most of their properties such as pH optimums, molecular weights, thermal stabilities, and lack of inhibition by sulfhydryl reagents. Small differences were found in their Km value and activation energies. Perhaps the most significant differences were found in their isoelectric points with pI values of 4.65, 4.84, and 5.11 for the α-1, α-2, and α-3 components, respectively. These differences account for the observed differences in electrophoretic mobility of the three
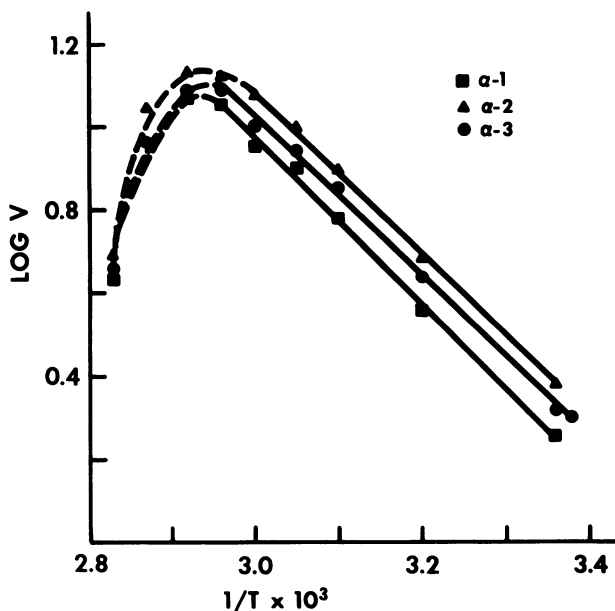

Fig. 9. Arrhenius plot for immature wheat α-amylases.

components on polyacrylamide slabs at pH 8.9 and order of elution from DEAE-cellulose ion-exchange columns at pH 8.2.

Comparing immature and malted wheat α-amylases (Table II), it is seen that the immature wheat α-amylases were similar to the malted wheat α-amylase components in activation energies. In addition, no observable change in action pattern could be distinguished between them by graphing the reduction in iodine-β-limit dextrin color due to enzyme action vs. the concomitant increase in reducing power. On the other hand, there were a number of notable differences between the immature and malted α-amylases. For immature wheat α-amylases, a broad pH optimum was found between pH 3.6 and 5.75. By comparison, the malted wheat α-amylases had sharp maxima around pH 5.5. This suggested that immature wheat α-amylases are more stable under acidic conditions. pH stability experiments confirmed the acid stabilities of immature wheat α-amylases with complete activity remaining after 30 min of incubation at pH 4.5.

Purified immature wheat α-amylases were more heat-labile than their malted counterparts. As shown in Fig. 8, the activities of the immature wheat α-amylases decreased rapidly upon heat treatment for 30 min past 37° C. By comparison, germinated wheat α-amylases lost less than 10% activity after heating for the same period at 60° C (5).

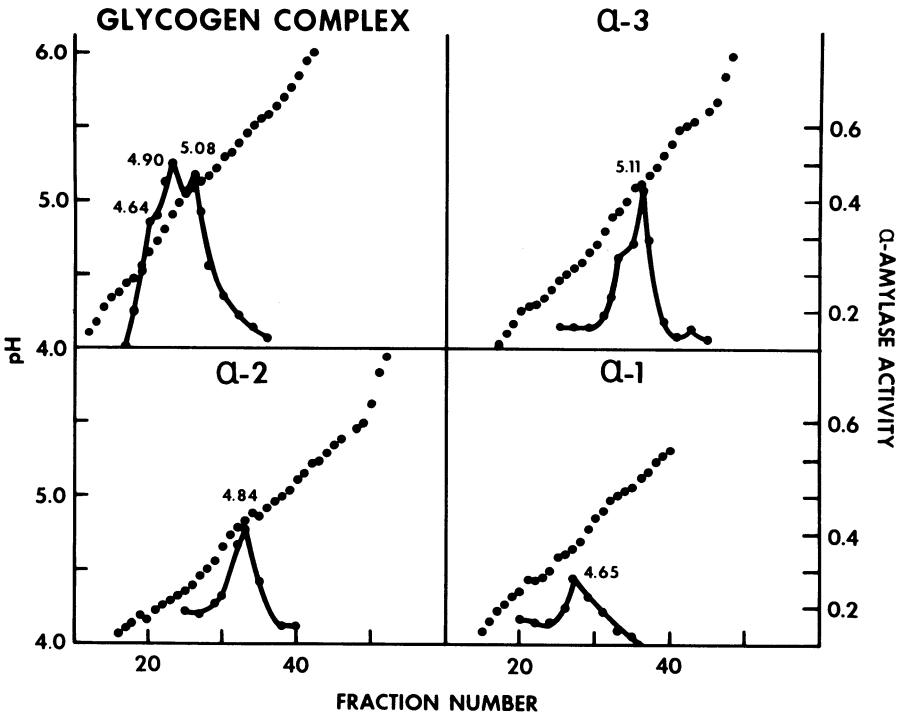Fig. 10. Isoelectric focusing of immature wheat α-amylase purified to the second glycogen complex and components α-1, α-2, and α-3. Solid line = α-amylase activity; unjoined circles = pH.

TABLE II
Summary of α-Amylase Properties

	Immature Wheat α-Amylase			Malted Wheat α-Amylase Components (5)				Immature Barley α-Amylase (21)
	α-1	α-2	α-3	I	II	III	IV	
pH optimum	3.6−5.75	3.6−5.75	3.6−5.75	5.5	5.7	5.5	5.5	5.5
Km, g/ml	2.50×10^{-4}	5.33×10^{-4}	2.35×10^{-4}	n.d.[a]	n.d.	n.d.	n.d.	n.d.
Molecular weight								
SDS gel electrophoresis		52,000−54,000			41,500−42,500			46,000
Biogel P-100		36,000−38,000			23,000			
Activation energy kcal/mol	9.66	9.04	8.82	8.47	7.01	9.28	7.01	9.5
Isoelectric point, pI	4.65	4.84	5.11	6.16	6.20	6.05	6.17	4.83

[a]n.d. = Not determined.

The molecular weight of immature wheat α-amylases (52,000–54,000) was higher than malted wheat α-amylase (42,000) as determined by SDS gel electrophoresis. Molecular sieve chromatography on Biogel P-100, as shown in Table II, gave anomalously low values for both the immature and malted wheat α-amylase; the reason for this phenomenon is not known. As suggested by Fischer and Stein (26), it may be that α-amylase has a very tight compact molecular structure. By molecular sieve chromatography, therefore, the molecule would give the appearance of having a lower molecular weight. SDS would be expected to open up the molecule and give a true value for the molecular weight.

As indicated in Table II, the isoelectric points of the immature wheat α-amylases, which ranged from 4.65 to 5.11, were lower than those of the malted wheat α-amylases (pI values from 6.05 to 6.16). As a consequence, the immature wheat α-amylases would be more highly ionized at alkaline pH and this would explain their greater mobility on polyacrylamide slabs at alkaline pH relative to malted wheat α-amylase components (3).

It is not known whether the three multiple forms of immature wheat α-amylase are true isoenzymes, arising from genetically determined differences in primary structure, or simply artifacts of the isolation procedure. The finding that all three have similar molecular weights and catalytic properties suggests that the three forms may be artifacts. Even in crude extracts, however, all are present as shown by polyacrylamide gel electrophoresis or DEAE chromatography. Furthermore, the three forms are always present in the same proportions. Artifactal changes, such as removal of amide side-groups from glutamic or aspartic acid, would be expected to affect the proportions of the three forms from one isolation to the next. All hexaploid wheats that have been examined contain the three multiple forms, whereas tetraploid wheats contain only two forms.

An α-amylase component isolated recently from immature barley (21) had a molecular weight, activation energy, and isoelectric point very similar to that of the immature wheat α-amylases (Table II). Although the pH optimum was sharper for the barley enzyme, it was similar to immature wheat α-amylase in having a greater pH stability at lower values of pH than other α-amylases.

Further research is presently being carried out to determine if the α-amylases from immature and malted wheat differ in their effects on flour rheological and breadmaking properties.

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