Wheat Alpha-Amylases. I. Isolation[1]

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ABSTRACT

Wheat alpha-amylase was isolated from malted Canadian hard red spring wheat by heat-treating an extract at 70°C., followed by acetone fractionating, complexing with glycogen, and finally by ion-exchange chromatography. Four major alpha-amylase components were isolated, the total yield being 28% of the original alpha-amylase activity.

Wheat alpha-amylase is of considerable interest in cereal chemistry, as it is directly involved in the absorption properties and gassing power of dough and in the final properties of bread. Excess alpha-amylase such as that arising from sprout-damaged wheat can lead to bread having a sticky, excessively moist, and inadequate crumb. On the other hand, the addition of a small amount of alpha-amylase to a sound flour may lead to such breadmaking improvements as higher loaf volume, improved crumb color, increased moistness of the crumb and keeping power, and better flavor (1). It is understandable, then, that alpha-amylase has been the subject of considerable work; in fact, Jago, back in 1895, had formulated some of the concepts of alpha-amylase action (2). However, in spite of voluminous work on this subject, the role of alpha-amylase has not been clearly elucidated, perhaps because all the reported work on the subject has been done on crude extracts of the enzyme in which complications can arise because of contaminating enzymes.

The purpose of the present work was to isolate and characterize pure alpha-amylase from wheat so that future work with the pure enzyme would be possible. Alpha-amylase is present in sound wheat in trace amounts but increases greatly during germination. Consequently, malted wheat was chosen as the source of alpha-amylase. The importance of this form of the enzyme in malt supplementation is well know. The present paper describes the isolation of the enzyme.

MATERIALS AND METHODS

A commercial sample of malted Canadian HRS wheat used in the present study was purchased from Canada Malting Co., Ltd. After oats and other extraneous contaminants were carefully removed by hand, the malted wheat contained 13.1% protein (N × 5.7) and 1.5% ash on 13.5% moisture basis. The malted wheat was stored at 3°C. and was ground into a whole-wheat flour with a Wiley mill (equipped with a 1-mm.-mesh sieve) just prior to use.

Alpha-Amylase Activity

Activity was measured by the colorimetric method of Bernfeld using 3,5-dinitrosalicylic acid (3). The substrate used was soluble starch Merck (according to Lintner). Absorbance was measured at 525 millimicrons; a Beckman DU spectrophotometer was used. A unit of alpha-amylase activity was expressed in terms of the amount of enzyme necessary to liberate 1 mg. of maltose in 5 min. at 20°C.

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from 1% soluble starch at pH 4.6. In the case of alpha-amylase, the actual reaction products are dextrins rather than maltose, but the increase in reducing power increased linearly with increasing enzyme concentration when the hydrolysis of substrate was not allowed to proceed beyond 15%. Specific activity is expressed as amylase activity per mg. of protein.

Estimation of Protein

Interfering ammonium salts and other small nonprotein contaminants were first removed from samples by exhaustive dialysis or passage through a column of Biogel P-2. Protein (N × 5.7) in extracts was determined by the Kjeldahl method. Purified enzyme preparations were analyzed by Nessler's method as modified by Williams (4).

Polyacrylamide Disc Electrophoresis

Analytical polyacrylamide disc electrophoresis was carried out as described by Davis (5). Both upper and lower gels contained 0.1% soluble starch to detect alpha-amylase in electrophoretic bands. The analyses were carried out at 2°C. at 3 ma. per tube for approximately 2 hr. or until the bromphenol blue marker band had progressed to the bottom of the gel. The pH of the small-pore gel was 8.9 and of the large-pore gel, 6.7. After electrophoresis, protein bands were stained with Amido Schwarz. To determine if the protein bands were the ones containing amylase activity, some of the gels were incubated in 0.05M acetate buffer, pH 4.5, for approximately 1 hr. at room temperature. After incubation, the gels were placed in a solution containing 0.01% iodine and 1% potassium iodide. The background colors of the gels were deep blue, and a colorless band appeared where the starch had been degraded by alpha-amylase.

Extraction of Crude Alpha-Amylase

A 0.2% calcium acetate solution was used as the extraction medium. The extraction was carried out as described by Kneen et al. (6). It has been shown by Walden that this procedure extracts 93% of the alpha-amylase (7, p.62).

Malted whole-wheat flour (3.2 kg.) was added slowly with stirring to 6 liters of 0.2% calcium acetate solution at room temperature. Stirring was continued for 2 hr. after addition of flour was completed. The suspension was then centrifuged at 4°C. at 9,500 r.p.m. (12,000 × g) for 10 min. All centrifugations were carried out under these conditions unless otherwise stated. The resulting clear dark-brown supernatant was stored at 2° to 3°C. prior to heat-treatment. To avoid subsequent fractionation difficulties, the next two fractionation stages should be carried out on the extract as soon as possible. Prolonged storage of the extract causes difficulties, possibly owing to oxidation and enzymatic reactions.

Heat-Treatment

Since beta-amylase interferes with the enzymatic determination of alpha-amylase used in this study, it was inactivated by heating the extract at 70°C. for 15 min. (6,8,9). Alpha-amylase between pH 6.5 and 8.0 is resistant to inactivation by this treatment (7, p. 67). In addition to beta-amylase, many other heat-labile protein components are denatured by this operation. The extract was first adjusted to pH 6.6 with cold 4% ammonium hydroxide. Heat-treatment was carried out in two 3-liter water baths, each heated by hot plates equipped with magnetic stirrers. One bath was maintained at 85° to 90°C. and the other at 72° to

74° C. Portions (500 ml.) of the extract in a 1-liter flask containing a magnetic stirrer were placed in the bath at 85° to 90°C. and kept in the bath with continuous stirring until the temperature rose to 69°C. The flask was then transferred to the bath at 72° to 74°C., with stirring maintained continuously. By means of manual manipulation of the flask in and out of the bath the temperature was maintained at 70° C. for 15 min. The extract was then rapidly cooled to 2° to 3°C.

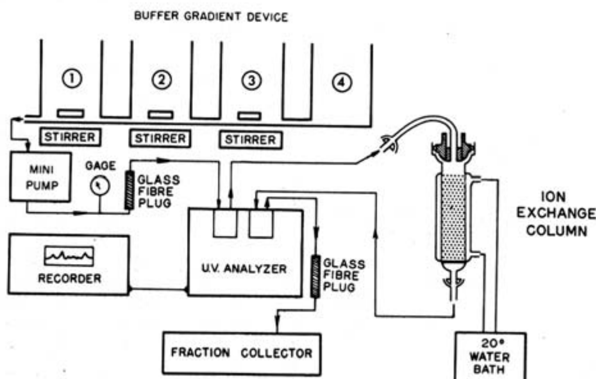

Fig. 1. Ion-exchange chromatography system.

Chromatography

Ion-exchange chromatography was carried out as described by Clayton and Bushuk (10), as illustrated in Fig. 1 (all chromatography experiments described in this paper were carried out with this apparatus). Protein monitoring was done on a Vanguard ultraviolet analyzer equipped with 0.66-cm. quartz cells. All buffers used for chromatography were degassed by stirring under vacuum for several minutes.

EXPERIMENTAL

Preliminary Investigations on Purification of Alpha-Amylase

Initially, an attempt was made to isolate alpha-amylase by heat-treating an extract of malted wheat, followed by ammonium sulfate fractionation and ion-exchange chromatography on carboxymethyl cellulose. Chromatography at pH 5.0, gradient 1 (Table I), resulted in partial separation of alpha-amylase. At pH values lower than 4.8, inactivation of alpha-amylase occurred, whereas at higher pH values there was poor retention of enzyme on the column. The chromatogram, as illustrated in Fig. 2 (chromatogram 1), indicated the presence of two alpha-amylase components; however, since the enzyme was not separated from other proteins occurring in relatively larger amounts, this separation scheme was abandoned.

Another isolation attempt was based on a report by Schwimmer and Balls (11) which utilized adsorption on starch for purifying barley alpha-amylase. To a heat-treated extract of malted wheat, saturated ammonium sulfate was added to 50% saturation at 2° to 3°C. The precipitate which formed was removed by centrifugation and dissolved in 0.2% calcium chloride. Ethanol at 2°C. was added to 40% concentration (v./v.) and the solution was passed through a starch column at 22°C. After washing with 100 ml. of cold 40% ethanol (v./v.), the starch column was eluted with 0.3% calcium acetate.. The calcium acetate effluent was chro-

matographed on carboxymethyl cellulose at pH 4.85; gradient 2 was used (described in Table I). As illustrated in Fig. 2 (chromatogram 2), two active alpha-amylase components were again detected, but once again the alpha-amylase components were not separated from other proteins. That the alpha-amylase components were contaminated with other proteins was confirmed by disc electrophoresis experiments. Further chromatographic experiments with microgranular carboxymethyl cellulose did not improve the resolution, and accordingly this separation scheme also was abandoned.

TABLE I. MOLARITIES OF BUFFERS USED FOR THE GRADIENT ELUTION OF ALPHA-AMYLASE ON N,N-DIETHYLAMINOETHYL CELLULOSE AND ON CARBOXYMETHYL CELLULOSE

Gradient	Buffer	Vessel 1	2	3	4
1	Disodium malonate, pH 5.0, being 0.006M in calcium acetate	0.005	0.050	0.005	0.195
2	Disodium malonate, pH 4.8, being 0.006M in calcium acetate	0.005	0.046	0.005	0.220
3 Tris-HCl,	pH 8.75, being 0.01M in calcium chloride	0.050	0.050	0.050	0.050
	sodium chloride	none	0.129	none	0.852
4 Tris-HCl,	pH 8.75, being 0.01M in calcium chloride	0.040	0.040	0.040	0.040
	sodium chloride	none	0.034	none	0.085

Isolation of Pure Alpha-Amylase

Further investigation on the purification of alpha-amylase led to a method volving acetone fractionation, complexing of alpha-amylase with glycogen, and ion-exchange chromatography. As this procedure was found successful, it is described in more detail.

Acetone Fractionation. A fractionation of the heat-treated alpha-amylase extract with acetone was found necessary for successful carrying out of the subsequent complexing of alpha-amylase with glycogen. The procedure adopted was as follows: To 1.5 liters of heat-treated extract at 2°C., with continuous stirring, acetone at −10°C. was added slowly from a dropping funnel. When the acetone concentration reached 10 to 15% (v./v.) the temperature of the mixture was further reduced to −5°C. and maintained there with an ice-salt bath. Acetone addition was continued until the concentration of the acetone was 39% (v./v.).

The mixture was allowed to stand for 20 min. and then centrifuged at −5°C. The precipitate was discarded and acetone at −5°C. was added to the supernatant, as described above, to 54% (v./v.). The fraction precipitating between acetone concentrations of 39 to 54% (v./v.) was found to contain most of the alpha-amylase activity. After standing for 20 min. the mixture was centrifuged. The supernatant was allowed to thoroughly drain away, and the precipitate was dissolved in 250 to 300 ml. of 0.005M Tris-HCl buffer, pH 8.0. The small amount of undissolved precipitate was removed by centrifugation.

Glycogen Complex. Schramm and Loyter reported in 1962 (12) that addition of glycogen to a crude extract of alpha-amylase from various sources causes the alpha-amylase to precipitate as a glycogen enzyme complex. Essentially pure

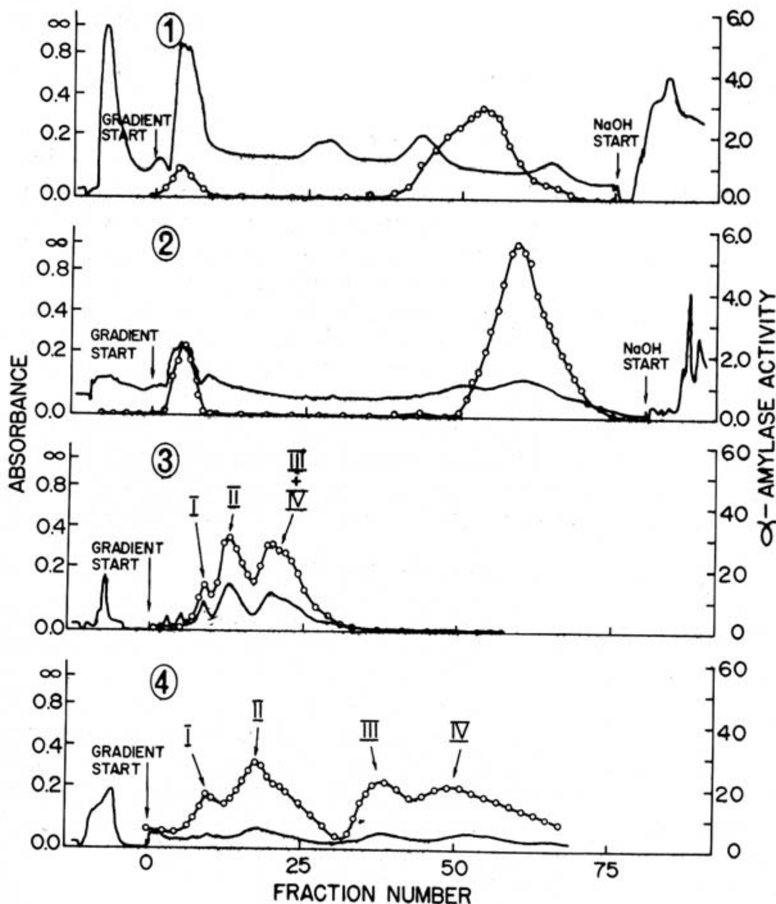

Fig. 2. Ion-exchange chromatography of wheat alpha-amylase extracts. Sections 1 and 2 show chromatography on carboxymethyl cellulose. Sections 3 and 4 show chromatography on N,N-diethylaminoethyl cellulose. Flow rates, 60 ml. per hr., 22-ml. fractions. Open circles, alpha-amylase activity; solid line, absorbance at 280 millimicrons.

alpha-amylase was obtainable from the complex by removal of the glycogen. This method, with some modification, was found suitable for purifying malt wheat alpha-amylase from crude extracts and is described below.

To 300 ml. of the above extract at 2° to 3°C., cold ethanol was added slowly with stirring to 40% concentration (v./v.). A small amount of precipitate which formed during addition of ethanol was removed by centrifugation. To the ethanol solution at 2° to 3°C. next was added dropwise 130 to 140 mg. of oyster glycogen (Nutritional Biochemicals Co.) dissolved in 2 to 3 ml. of 0.005M Tris-HCl buffer, pH 8.0 (a precipitate forms immediately). The mixture was stirred for 10 min. and then centrifuged at 2°C. for 10 min. at 4,000 r.p.m. (1,900 × g). The supernatant was allowed to drain away thoroughly, and the precipitate was dissolved in approximately 30 ml. of cold 0.005M Tris-HCl pH 6.7 buffer, containing 0.005M calcium chloride. As a rule, it was found necessary to repeat the above precipitation

procedure two more times before most of the alpha-amylase activity was removed from the ethanol solution. The alpha-amylase solutions were combined and then allowed to stand overnight at 15° to 20°C. to permit the glycogen to be digested by the alpha-amylase.

Ion-Exchange Chromatography. The stability of alpha-amylase was much better on N,N-diethylaminoethyl cellulose than on carboxymethyl cellulose, and this was the ion-exchange resin chosen for the final purification step. A column 0.9 × 64 cm. was used, packed with microgranular N,N-diethylaminoethyl cellulose (Whatman DE-32). Before the column was prepared, the cellulose was conditioned by successive washes with 0.1N NaOH, then with 0.1N HCl, followed by several washes with 0.05M Tris-HCl buffer pH 8.75 containing 0.01M calcium chloride. After each wash the fines were removed by decantation after the cellulose had settled. The pH 8.75 buffer was then pumped through the column until the column effluent had a pH value of 8.75.

The buffers used in the gradient device (Fig. 1) are described in Table I (gradients 3 + 4), 400 g. of each buffer being used. The alpha-amylase solution was adjusted to pH 8.75 with 2% ammonium hydroxide and then dialyzed overnight against several liters of the starting buffer prior to a chromatographic run.

RESULTS AND DISCUSSIONS

Initial attempts to purify alpha-amylase by ammonium sulfate fractionation, starch adsorption, and chromatography on carboxymethyl cellulose were not successful.

Successful isolation of alpha-amylase from malted wheat was achieved by acetone fractionation of a heat-treated malted wheat extract, complexing with glycogen, and ion-exchange chromatography on microgranular N,N-diethylaminoethyl cellulose. Typical protein and enzymatic activity recoveries are given in Table II. At the glycogen complex stage, approximately 28% of the alpha-enzyme was recovered with a 77-fold increase in specific activity.

The last stage of alpha-amylase purification involved ion-exchange chromatography of the material complexed by glycogen. Typical chromatography results obtained by using gradients 3 and 4 (Table I) are illustrated in chromatograms 3 and 4 in Fig. 2. The first peak in both chromatograms is due to elution of a yellow-colored material, containing little protein as indicated by Kjeldahl nitrogen analysis. Comparison of chromatograms 3 and 4 showed that the shallower gradient (gradient 4) resulted in the best resolution of the alpha-amylase components, and accordingly gradient 4 was used to isolate the four alpha-amylase components. Rechromatography of each of the four alpha-amylase components showed that most of the component eluted out again as a single peak at its original elution position in chromatogram 4, Fig. 2. The specific activity of each of the four components in chromatogram 4 was approximately 618 units, an increase of 174 units over the specific activity of the glycogen complex.

The center portions of the four peaks in chromatogram 4 containing alpha-amylase activity (see Fig. 2) were examined for their purity by disc electrophoresis. The electrophoresis patterns revealed the presence of only one stained major component, in peaks I, II, and III; peak IV contained a trace of impurity which was presumably peak III. Gel electrophoresis of peaks I, II, III, and IV also

TABLE II. PURIFICATION OF ALPHA-AMYLASE FROM MALTED WHEAT

Procedure	Volume	Concentration	Total Units	Specific Activity	Protein	Yield	Purification
	ml.	units/ml.	X10−3	units/mg. protein	mg./ml.	%	
Initial extract	5,200	097.0[a]	504[a]	005.74[a]	16.90	100.0	001.0
Heat-treated extract	4,752	097.0	461.0	015.80	06.14	091.5	002.8
39−54% acetone ppt.	0,831	352.0	292.0	103.50	03.40	057.9	018.0
Glycogen complex	0,268	523.6	140.0	443.70	01.18	027.8	077.3
Ion-exchange chromatography							
Peak I			12.9	640.00			111.5
Peak II			46.5	611.00			106.4
Peak III			29.2	608.00		027.5	105.9
Peak IV			50.0	614.00			107.0

[a]The solution used to determine the alpha-amylase activity of the original extract contained phenylmercuric chloride to inactivate beta-amylase.

was carried out in the presence of starch. Incubation of the gel strips in iodine revealed clear areas which indicate no iodine absorption in areas where the starch was hydrolyzed by alpha-amylase. The clear areas corresponded to the positions of the stained major protein zones.

The distribution of alpha-amylase activity among the four peaks, I, II, III, and IV, was 9, 34, 21, and 36% of the total alpha-amylase activity eluted from the ion-exchange column. Virtually all of the alpha-amylase activity placed on the column can be recovered after the chromatographic stage. The sum of enzymatic activities of the four alpha-amylase components indicates that over-all recovery of alpha-amylase activity is 27%. This does not mean that the four individual pure alpha-amylase components can be isolated with an efficiency of 27%, since many of the chromatographic fractions contain two of the alpha-amylase components, as is apparent in Fig. 2.

The results described in this work indicated that there are three major and one minor alpha-amylase component in malted wheat. It is interesting that Frydenberg and Nielsen report that germinated barley contains five electrophoretically different alpha-amylases (13). It remains for future research to answer the interesting question whether these alpha-amylase components form a family of isozymes with identical enzymatic activities or whether they are enzymes which may catalyze different carbohydrate hydrolase reactions.

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