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WHEAT BETA-AMYLASES

I. Isolation[1]

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ABSTRACT

Beta-amylase was isolated from Canadian hard red spring wheat flour by ion-exchange column chromatography of buffered saline extracts. Three major and two minor beta-amylase components were obtained. Maximum specific activity of the three main fractions was obtained by treatment with glutathione, which, however, did not cause chromatography patterns to be altered. "Free" beta-amylase, as extracted by water alone, contained only one main active component, whereas the "bound" beta-amylase, released by sodium chloride solution extraction of the residue from water extraction, contained several active components.

Wheat amylase, one of the first enzymes to be identified, was discovered by Kirchoff at the beginning of the last century (1,2). He produced a crystallizable sugar by incubating a wheat gluten preparation with potato starch. Since then the study of amylases has produced a vast literature. Much work has been carried out with impure concentrates, and only in the past decade or so have pure crystalline preparations of beta-amylase been obtained from sweet potatoes (3), barley malt (4), wheat (5), and soybeans (6). These methods essentially involved a protein fractionation by precipitation with salts and/or organic solvents and a final purification by repeated crystallization.

The method of Meyer *et al.* (5) for preparing wheat beta-amylase involves nine separate stages resulting in a yield, prior to crystallization attempts, of approximately 10% based on the original activity.

In the present study ion-exchange column chromatography was used to reduce losses during fractionation and to investigate the possible heterogeneity of wheat beta-amylase.

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Materials

A commercially milled, straight-grade, untreated flour from Canadian hard spring wheat was used. It contained 14.0% protein (N × 5.7) and 0.48% ash on a 14% moisture basis. The wheat had been steam-conditioned at 90°F. for 3 min. prior to milling. Quite severe heat-treatment of wheat has been shown (7,8) to have no deleterious effects on beta-amylase, and initial experiments confirmed that there was no loss of beta-amylase activity on heat-treatment.

Methods

Preparation of Beta-Amylase Extract. The extraction method of Meyer *et al.* (5) was followed except that a 1% sodium chloride solution was used as solvent since it has been shown to extract twice as much free beta-amylase as water alone (9). Violent agitation and mixing are unnecessary for maximum beta-amylase production (9), and after initial mixing the flour slurries were left without any subsequent stirring. To prevent copper-catalyzed oxidation of sulfhydryl groups, disodium ethylene diaminetetraacetate (EDTA) was used in the extractant.

The stages of initial purification were as follows:

Stage I. Flour (3 kg.) was extracted with 6 liters of 1% sodium chloride solution containing 0.0001*M* EDTA. The flour was gradually added to the salt solution with gentle stirring at room temperature, with care to avoid the formation of large lumps. When dispersed, the suspension was kept overnight at room temperature. The paste was centrifuged at 4°C. at 9,500 r.p.m. (12,000 × *g*) for 20 min. All centrifugations were carried out under these conditions unless otherwise stated. A cloudy, viscous, light-brown supernatant was obtained and the insoluble residue was discarded.

Stage II. The temperature of the extract was lowered to 4°C. and all subsequent operations were carried out at this temperature. The pH of the extract was adjusted to 3.7 with approximately 90 ml. of 5*N* acetic acid. The solution was held at this pH for 6 days to remove any traces of alpha-amylase. A grayish precipitate was discarded after centrifuging.

Stage III. The pH of the solution was adjusted to 5.3 with 4% ammonia, and protein material was precipitated by adding pH 5.3 saturated ammonium sulfate solution (SAS) dropwise to 0.66 saturation with stirring. On centrifuging, a grayish muddy precipitate was thrown down and the supernatant was discarded. The precipitate was stirred with 750 ml. of water and left overnight. After further stirring, the liquid was centrifuged and insoluble material was discarded.

Stage IV. The pH of the supernatant was adjusted to 5.5 with ammonia and the volume was made up to 1600 ml. with water. Over a 20-min. period, 425 ml. of pH 5.5 SAS was added slowly with stirring to give 0.21 saturation. After centrifugation, the precipitate was discarded and the supernatant was adjusted to 0.59 saturation by the addition of pH 5.5 SAS. After centrifugation again, the precipitate was dissolved in water and the solution made up to 200 ml. An aliquot of this solution (usually 60 ml.) was dialyzed against pH 6 buffer overnight prior to chromatography.

Ion-Exchange Column Chromatography. A schematic flow diagram of the chromatography system is shown in Fig 1. The buffer reservoirs were two 2-liter-capacity cylindrical containers made from acrylic sheet. Flow rates were regulated by means of a Milton-Roy mini-pump. A Vanguard model 1056 automatic ultraviolet analyzer and recorder were used for measuring protein concentrations which were recorded as optical density at 280 $m\mu$ in a 0.66-cm. cell on a logarithmic scale. A recorder chart speed of 0.5 in. per hr. was used. The effluent was collected in 25-ml. fractions in a fraction collector. A pH 6.0, 0.005*M* sodium phosphate buffer solution containing 0.001*M* EDTA was used for all chromatography work.

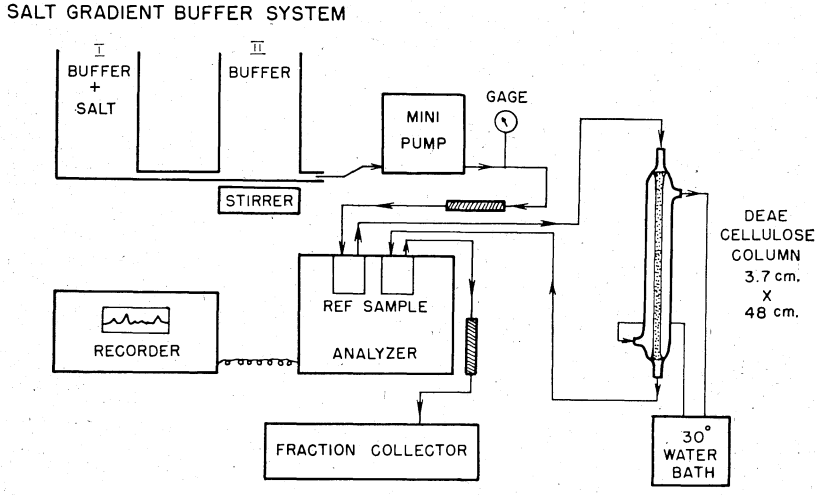

Fig. 1. Ion-exchange column chromatography system.

The jacketed Pyrex column had an internal diameter of 3.7 cm. and a length of 48 cm. Water at 30°C. was pumped through the outer jacket to maintain constant temperature. The column packing used

was Eastman N,N-diethylaminoethyl cellulose (DEAE cellulose) which was prepared for use by washing first with 0.1*N* sodium hydroxide solution, then with 0.1*N* hydrochloric acid, and finally with pH 6 buffer. "Fines" were removed by stirring in a large cylinder with buffer and decanting off material unsettled after 15 min. The cellulose was made into a slurry with pH 6 buffer and degassed by stirring under vacuum. The bottom of the column was plugged with glass wool and the cellulose slurry poured in until the settled material filled the column. A disk of filter paper was then placed on top to avoid disturbing the surface. Approximately 50 g. of dry DEAE cellulose was used for preparing a column.

The sample (containing up to 3.5 g. protein) was dialyzed overnight against pH 6 buffer before it was loaded on the column. Buffer of pH 6 was passed through the column at a flow rate of 140–190 ml. per hr. until an initial brownish-colored peak had been eluted. The salt gradient was then started by having 2 liters of pH 6 buffer in pot II, and 2 liters of 0.4*M* sodium chloride in pH 6 buffer in pot I (Fig. 1). A magnetic stirrer was used to mix the contents of pot II. When the gradient elution was complete, the eluant was changed to 0.1*N* sodium hydroxide solution to elute strongly bound material. All eluants were previously degassed by stirring under vacuum.

For rechromatography of particular fractions the salt gradient was begun immediately after the sample was loaded and no base elution was necessary.

Estimation of Beta-Amylase. Activity was measured by a colorimetric method with 3,5-dinitrosalicylic acid according to Noelting and Bernfield (10), except that soluble starch Merck (according to Lintner) was used and a Beckman DU spectrophotometer was used to measure absorbance at 520 mμ. The unit of beta-amylase activity was that amount of activity which liberated 1 mg. maltose in 3 min. at 20°C. from 1% soluble starch solution at pH 5.2, provided that starch degradation did not exceed 15%.

Alpha-Amylase Activity. To 5 ml. of the solution to be tested was added 2 ml. of 0.5% Merck soluble starch solution containing 0.01*N* calcium chloride solution. Toluene (3 drops) was added to prevent microbial growth. After the solutions had been incubated at 25°C. for 48 hr., 0.01*N* iodine solution was added drop by drop. A characteristic deep blue coloration occurred where little or no starch degradation had taken place, indicating the absence of alpha-amylase. Where alpha-amylase was present and had caused considerable starch breakdown, the solutions were colorless. Intermediate purples, reds, and browns denoted small traces of alpha-amylase. Included in each series

of tests were samples of two commercial preparations of barley beta-amylase supposedly free of alpha-amylase.

Nitrogen Estimation. Samples of enzyme preparations were dialyzed exhaustively against distilled water, freeze-dried, and analyzed for nitrogen with a Coleman nitrogen analyzer.

Starch Gel Electrophoresis. This was carried out according to Smithies (11) using a 0.0175*M* aluminum lactate buffer.

Results and Discussion

Initial Purification of Extract. Typical nitrogen and activity data for the initial purification steps are given in Table I. Specific activity was increased approximately threefold in this initial purification, whereas the yield of beta-amylase activity was 55% of the original activity.

Chromatography of Purified Extracts. The first batch of extract to be prepared and chromatographed gave the separation shown in Fig. 2. The brownish unadsorbed material eluted by phosphate buffer alone was always slightly cloudy, presumably because of fatty substances it contained. This tended to give a falsely high optical density value. Almost half of the total nitrogen recovered by chromatography was eluted in this first peak which, on a dry weight basis, contained 15% nitrogen. The strongly adsorbed base-eluted peak was yellow-colored and accounted for only about 4% of the recovered nitrogen.

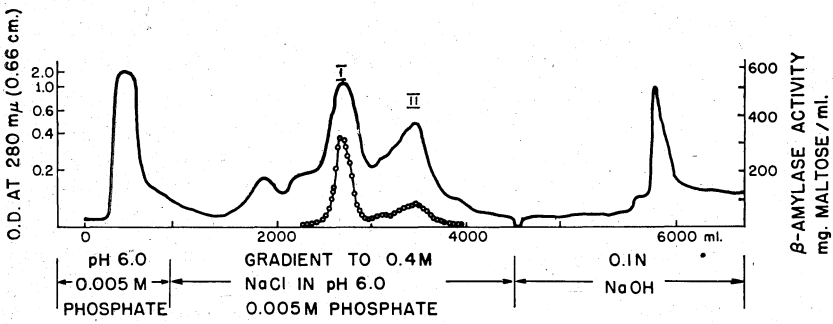

Fig. 2. Chromatography of first batch of wheat beta-amylase preparation. Load, 80 ml. of sample containing 1.7 g. protein. Flow rate, 146 ml./hr. Open circles, beta-amylase activity. Solid line, optical density.

From Fig. 2 it can be seen that beta-amylase activity was spread over two peaks. Rechromatography of peak I gave a single peak with a specific activity of approximately 1,200 units per mg. nitrogen. Peak II gave several active components with specific activities in the region of

800 units per mg. nitrogen. On fractionation of samples of peak I and peak II separately by starch gel electrophoresis, peak I was faster-running. However, when an original flour extract is run on starch gel, only one beta-amylase band is produced, probably because the starch itself is attacked by the beta-amylase which shows up as a dark black ridge. The leading component modifies the starch to such an extent that the starch pores are enlarged and made easier for succeeding components to pass through; hence, separation is prevented. Nimmo *et al.* (12) reported three beta-amylase bands from flour extracts using polyacrylamide gels which are not attacked by beta-amylase.

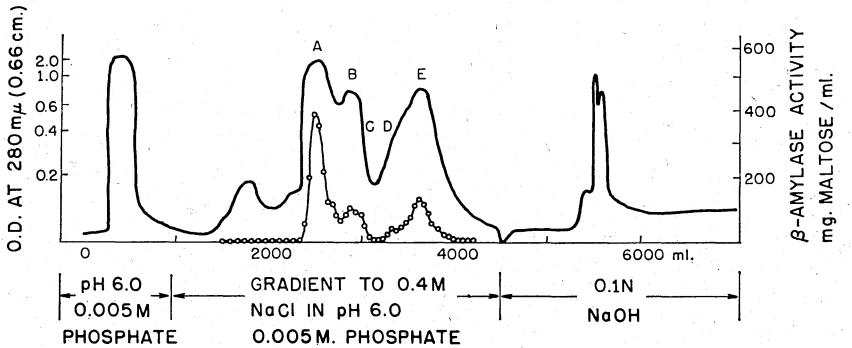

Fig. 3. Chromatography of second batch of wheat beta-amylase preparation. Load, 75 ml. of sample containing 3.7 g. protein with no glutathione added. Flow rate, 182 ml./hr. Open circles, beta-amylase activity. Solid line, optical density.

When a fresh batch of partially purified beta-amylase solution was chromatographed, a different separation pattern was produced (Fig. 3). At first glance, the logarithmic optical density scale of the recorder tends to create a false impression of the proportion of the various components, particularly with large loads, but it can be seen that three main beta-amylase peaks separated out together with two minor peaks, although in this case (Fig. 3) the minor components C and D are not as obvious as in other runs. Glutathione was added to a second aliquot of this batch of solution to give a threefold excess of sulfhydryl groups. The treated solution was then chromatographed (Fig. 4) and the over-all pattern was found to be the same except that the beta-amylase activities were considerably higher. The separation of components appeared to be somewhat sharper with glutathione treatment, and this was confirmed in subsequent experiments.

From this experiment with glutathione it appeared that the various beta-amylases were not different oxidation forms of the same protein, since treatment with a reducing agent still gave the same components

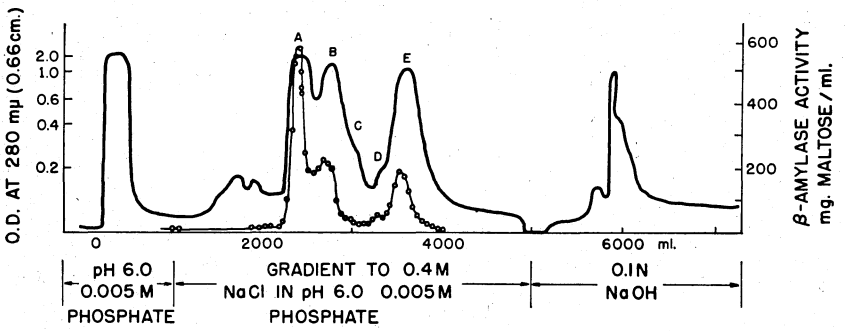

Fig. 4. Chromatography of second batch of wheat beta-amylase preparation. Load, 75 ml. of sample containing 3.7 g. protein and with 200 mg. glutathione added. Flow rate, 182 ml./hr. Open circles, beta-amylase activity. Solid line, optical density.

in approximately the same proportions. Treatment with glutathione increased the specific activities markedly, indicating that before chromatography, some of the sulfhydryl groups essential for activity had become oxidized, and restoration of these groups by glutathione reduction had caused the beta-amylase to regain full activity. That this higher activity was apparent after chromatography indicated that ion-exchange chromatography itself did not cause a loss of activity.

The main components, A, B, C, D, and E shown in Figs. 3 and 4 were purified by rechromatography on DEAE cellulose. Larger peaks were split up into smaller portions for rechromatography and only those fractions near the peak maxima were used. Figure 5 shows typical traces obtained. A single main peak was obtained in each rechromatography, although traces of other components, particularly on rechromatography of C and D, indicated the incomplete separation obtained in the first chromatography. This was due in part to the large loads used.

Fractions A, B, and E, obtained from initial runs of samples not treated with glutathione, upon treatment with glutathione yielded single peaks on rechromatography, thus further indicating that they were not oxidized forms of the same protein.

Possible Causes of Lack of Reproducibility. There are several points in the initial purification of the flour extract at which a variation in reproducibility of the proportion of components could be caused. These include the acid treatment, ammonium sulfate precipitation, storage of extracts, and the initial extraction using sodium chloride solution. These four possibilities will now be considered.

1. The 6-day pH 3.7 treatment to destroy alpha-amylase cannot be omitted because alpha-amylase came off the column with an activity

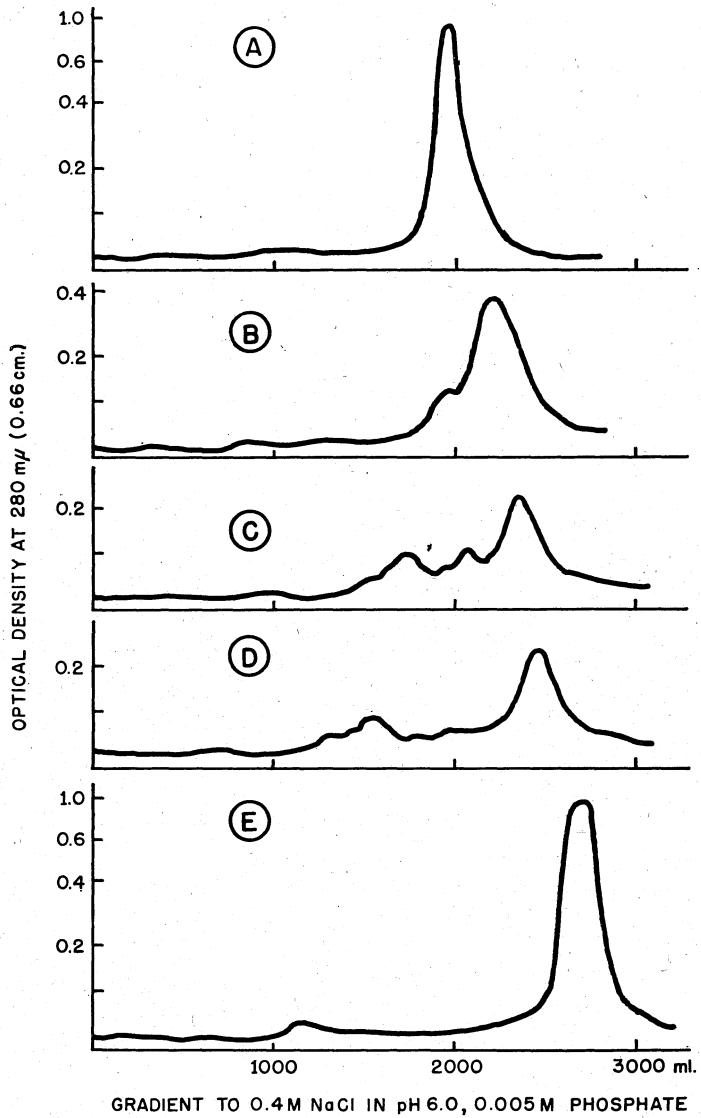

Fig. 5. Rechromatography of aliquots of beta-amylase peaks A–E on DEAE cellulose.

maximum occurring at a position approximately 100 ml. before peak A. This was too close for it to be separated chromatographically from beta-amylase under these conditions. During this acid treatment a variable amount of material is precipitated. Although a small part of the precipitation may be due to cooling of the initial extract from

room temperature to 4°C., this can be disregarded.

The variable proportions of components at first suggested the attractive hypothesis that acid treatment was causing amide groups to be cleaved from the beta-amylase, thus causing the apparent multiplicity of components. However, on chromatography of a solution of purified main peak A which had been kept at pH 3.7 for several hours previously, only one peak was obtained, showing that acid treatment did not appear to convert peak A to other soluble forms.

2. Ammonium sulfate fractionation was carried out after the acid treatment. SAS was added to the extract to precipitate all protein material. The resulting precipitate was extremely gummy and not readily soluble in water. Obviously material which is salt-soluble but not water-soluble (such as some globulins) will not dissolve in water, and it is very unlikely that stirring the precipitate with water effectively dissolved the same proportions of soluble and sparingly soluble components each time an extract was prepared. Again, when the aqueous solution of this first precipitation was further fractionated with SAS, the same proportions of components would be present each time in the precipitate and the supernatant only if pH, temperature, rate of stirring, and rate of SAS addition were strictly maintained.

Hence, it was considered that the difference in relative proportions of the beta-amylases produced in various preparations was probably due in part to a differential precipitation of these components by low pH and to solubility variations as mentioned above.

3. The effect of extraction solution was studied to further investigate the reasons for the difference in chromatography patterns of different batches of partially purified beta-amylase. An initial comparison was made between flour extracts made with water, salt solution, and glutathione solution. Samples of flour (65 g.) were extracted in a Waring Blendor at 4°C. with 3 × 130-ml. portions of each of (A) 1% sodium chloride solution, (B) water, and (C) 0.1% glutathione solution. The load on the column was much lower than in previous experiments, and the original extract was run without any initial purification. Figure 6 shows the results of this experiment.

Sodium chloride (A) or glutathione (C) solutions extracted approximately double the amount of activity that water alone (B) extracted, and the three extracts were quite different in over-all protein pattern as judged by optical density. The smaller beta-amylase components showed up more as a trailing off the main peak, and the water extract (C) appeared to contain one single peak rather than a mixture.

The water extract was repeated, with a much larger load, in order to magnify the smaller components, and this extract was made at 4°C.,

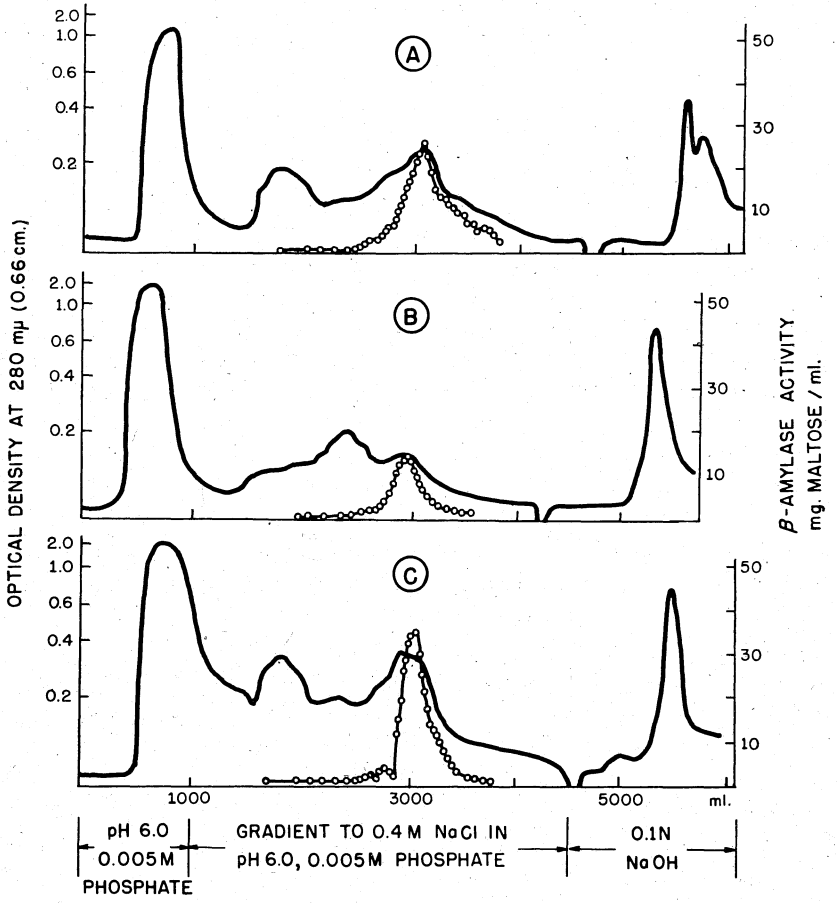

Fig. 6. Chromatography of extracts of 64-g. flour samples made with (A) 3 × 130 ml. 1% sodium chloride solution (B) 3 × 130 ml. water and (C) 3 × 130 ml. 0.1% glutathione solution. Flow rate, 205 ml./hr. Open circles, beta-amylase activity. Solid line, optical density.

the flour being stirred gradually into the water; this was allowed to stand for 3 hr. The residue was then extracted with 1% sodium chloride solution. Figure 7 shows the results of chromatography of these two extracts. The water extract (A) has one main beta-amylase component with only small traces of the others, whereas the salt extract of the residue (B), which can be presumed to contain released "bound" beta-amylase, showed quite a different proportion of active components. An initial peak, P, was also present in the salt extract but not in the water extract or in previous experiments.

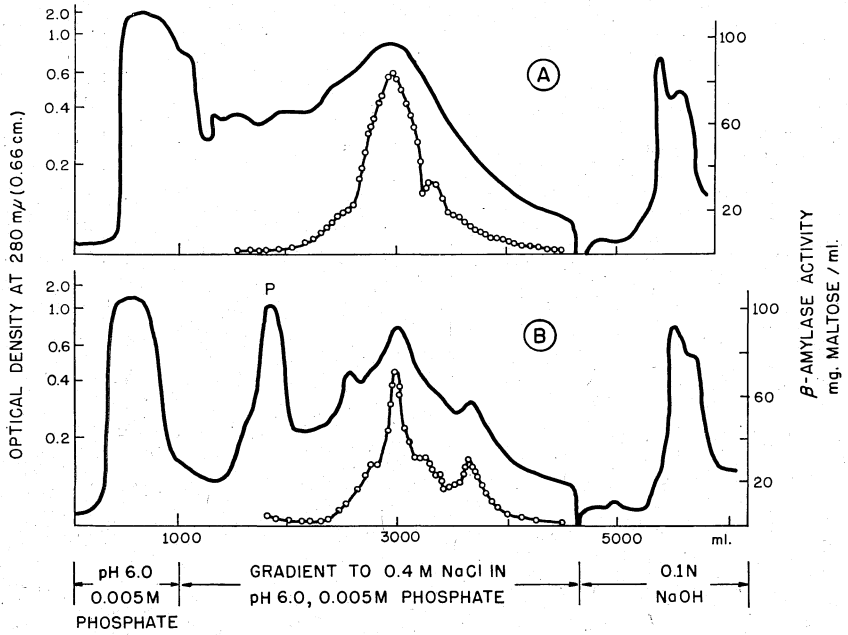

Fig. 7. Chromatography of (A) water extract of 800 g. flour and (B) 1% sodium chloride extract of residue from water extract of 540 g. flour. Flow rates, 205 ml./hr. Open circles, beta-amylase activity. Solid line, optical density.

4. An investigation was also made into whether standing of the original extract produced changes in the beta-amylases, possibly by proteolytic action. A slurry was made at 4°C. in the usual way, except that the flour suspension was allowed to stand for only 3 hr. at this temperature and was then centrifuged, dialyzed, and chromatographed immediately (Fig. 8A). This was compared with a similar extract made at room temperature and kept 24 hr. before it was centrifuged (Fig. 8B). An aliquot of this solution was stored for 10 days at 4°C. before it was run (Fig. 8C). The three beta-amylase traces shown in Fig. 8 were very similar except for small differences in load and sharpness of separation of secondary amylase components. One interesting difference was that in the "no-time" cold extract there was again an initial peak, P, which was not present after standing. Presumably this is destroyed or modified by standing, possibly by enzymatic reaction. The specific activities of the main peaks were around 760 units per mg. nitrogen.

Recovery and Activities of Purified Beta-Amylases. Details of a typical set of recoveries of beta-amylases at various purification stages are

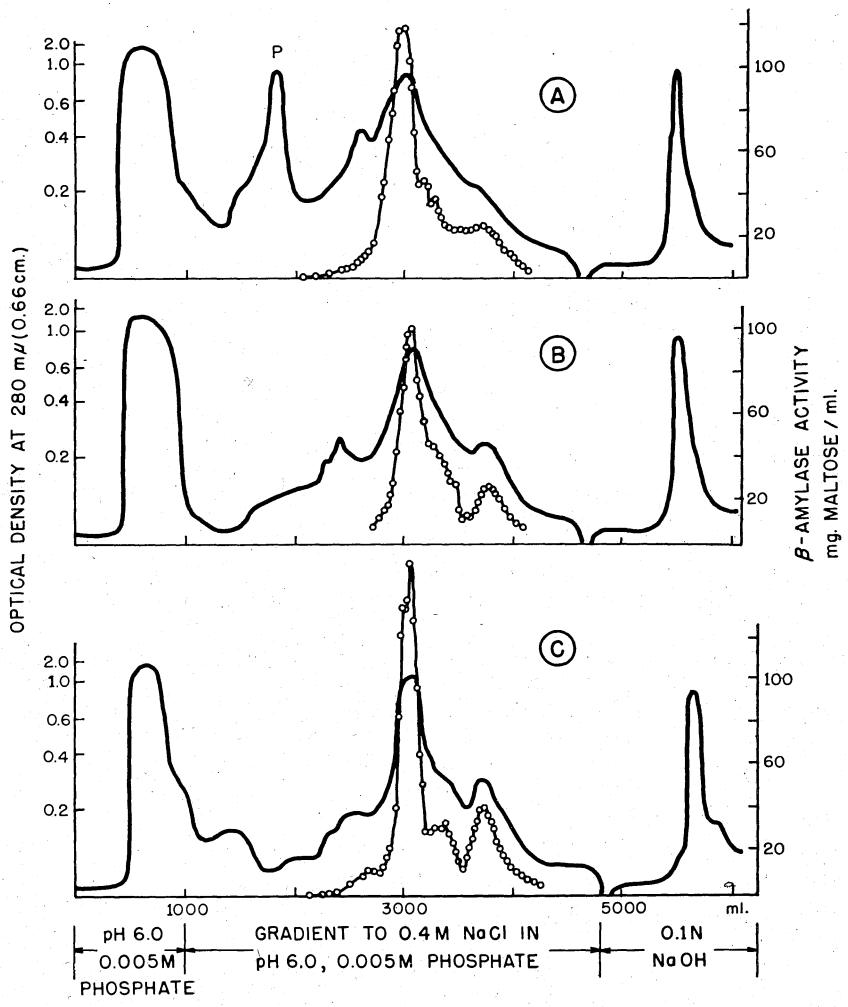

Fig. 8. Effect of time on chromatography of sodium chloride extracts of flour. A, "no-time" cold slurry; B, 24-hr. room-temperature slurry; C, aliquot of B stored 10 days at 4°C. Flow rates, 205 ml./hr. Open circles, beta-amylase activity. Solid line, optical density.

given in Table I. On the basis of activity of the original extract, the total recovery of beta-amylase activity contained in fractions A–E was 39%. This compares favorably with the yield of 8% of crystalline material obtained by Meyer *et al.* by their lengthy fractionation procedure.

Table II shows a comparison of nitrogen and specific activity data for purified fractions A, B, and E and crystalline wheat beta-amylase.

TABLE I

PURIFICATION OF WHEAT BETA-AMYLASE[a]

Stage	Operation	Volume	Total Beta-Amylase Activity		Yield Based on Starting Product	Total Nitrogen	Activity per mg. N	Degree of Enrichment
		ml.	*mg. maltose* $\times 10^{-3}$		%	*mg.*		
I	Salt extraction	4,560	1,182		100	8,580	138	..
II	Acid treatment	4,680	1,038		88	8,238	126	..
III	Ammonium sulfate 1	828	746		63	2,960	252	1.8
IV	Ammonium sulfate 2	200	657		55	1,827	360	2.6
V	Chromatography 1:							
	peak A		270					
	peak B		154					
	peak C		24	591	50			
	peak D		24					
	peak E		119					
VI	Chromatography 2:							
	peak A		210			153	1,370	9.9
	peak B		120			106	1,130	8.2
	peak C		19	460	39			
	peak D		18					
	peak E		93			81	1,150	8.3

[a] From 3 kg. flour.

TABLE II

SPECIFIC ACTIVITIES OF BETA-AMYLASES

Source	Beta-Amylase Activity per mg. N	Activity per mg. Enzyme	Nitrogen
			%
Peak A	1,370	227	16.6
Peak B	1,130	189	16.7
Peak E	1,150	189	16.5
Wheat (crystalline (5))	1,450	198	14.3

Nitrogen figures obtained for A, B, and E were appreciably higher than the figure published by Meyer *et al.* Consequently, specific activities expressed per mg. nitrogen are lower than that of the crystalline enzyme, whereas, expressed per mg. enzyme, results are in good agreement. Peak A is seen to be slightly more active than peaks B and E.

Conclusions

The method of ion-exchange column chromatography with DEAE cellulose has been shown to be suitable for preparing pure wheat beta-amylase forms without the lengthy fractional precipitation steps of classic methods, and to give a much greater yield of enzyme. However, initial purification of the extract is necessary.

There is a difference between the latent or "bound" beta-amylase,

which contains multiple forms of the enzyme, and "free" beta-amylase, which contains predominantly one enzyme.

Several unanswered questions arise from this work. Why does sodium chloride release bound beta-amylase, and is the bound beta-amylase that is released by sodium chloride the same as that released by glutathione or other thiols? Rowsell and Goad (13,14) concluded that the latent beta-amylase of wheat was chemically bound to glutenin by disulfide linkages. This "bound" enzyme could be released by splitting the disulfide attachment with such reagents as sodium sulfite and thiols, or by splitting peptide bonds in the glutenin. It is difficult to imagine how sodium chloride solution can bring about either of these two means of release. Increase in ionic strength can cause an unfolding of protein molecules; perhaps this unfolding makes the molecule more susceptible to proteolytic enzyme attack. Walden (9) produced evidence that sodium chloride activates an enzyme system which then releases latent beta-amylase. However, more work is necessary to elucidate the full picture of beta-amylase binding and release.

Literature Cited

1. KIRCHHOFF, G. S. C. Observations, expériences, et notices à interéssantes, faites et communiquées l'Académie. Académie Imperiale des Sciences de St. Pétersbourg. Mémoires **4:** 27 (1811).
2. KIRCHHOFF, G. S. C. Über die Zuckerbildung beim Malzen des Getreides, und beim Bebrühen seines Mehls mit Kochendem Wasser. J. Chem. Phys., Schweigger's, Nürnberg **14:** 389–398 (1815).
3. BALLS, A. K., WALDEN, M. K., and THOMPSON, R. R. A crystalline beta-amylase from sweet potatoes. J. Biol. Chem. **173:** 9–19 (1948).
4. FISCHER, E. H., MEYER, K. H., NOELTING, G., and PIGUET, A. The purification and crystallisation of malt beta-amylase. Arch. Biochem. **27:** 235–237 (1950).
5. MEYER, K. H., SPAHR, P. F., and FISCHER, E. H. Purification, cristallisation et propriétés de la beta-amylase de blé. Helv. Chim. Acta **36:** 1924–1936 (1953).
6. FUKUMOTO, J., and TSUJISAKA, Y. Soybean amylase. I. Purification and crystallisation of beta-amylase from soybeans. Kagaku To Kogyo (Osaka) **28:** 282–287 (1954).
7. KRETOVICH, V. L., and FLORENSKAYA, T. G. Effect of heating on proteins and enzymes of wheat grain. Biokhim. Zerna, SG No. 4: 56–85 (1958).
8. KYDREV, T. G., and KOLEV, V. M. Thermal treatment of dry wheat seeds and its effect on the plants. Fiziol. Rast. **8:** 576–581 (1961).
9. WALDEN, C. C. Purification of wheat alpha- and beta-amylases. Ph.D. Thesis, Univ. of Minnesota (1954).
10. NOELTING, G., and BERNFELD, P. Diastatic enzymes. III. Beta-amylase: Determination of activity and control of absence of α-amylase. Helv. Chim. Acta **31:** 286–290 (1948).
11. SMITHIES, O. An improved procedure for starch-gel electrophoresis. Further variations in the serum proteins of normal individuals. Biochem. J. **71:** 585–587 (1959).
12. NIMMO, C. C., O'SULLIVAN, MARY T., MOHAMMAD, A., and PENCE, J. W. Fractionation and zone electrophoresis of proteins of water-soluble materials of flour. Cereal Chem. **40:** 390–398 (1963).
13. ROWSELL, E. V., and GOAD, L. J. The constituent of wheat binding latent beta-amylase. Biochem. J. **84:** 73P (1962).
14. ROWSELL, E. V., and GOAD, L. J. Latent beta-amylase of wheat: Its mode of attachment to glutenin and its release. Biochem. J. **84:** 73P–74P (1962).