WHEAT BETA-AMYLASES

II. Characterization[1]

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ABSTRACT

Beta-amylase was isolated from wheat flour by a single $(NH_4)_2SO_4$ fractionation followed by ion-exchange chromatography to yield three components (A, B, and E) which differ only in their electrophoretic behavior and pH optima. The three components are homogeneous by sedimentation, Tiselius electrophoresis, and ion-exchange chromatography criteria. Optimum enzymatic activity occurs at pH of 5.4, 4.6, and 5.2 to 6.2 for A, B, and E components. The sedimentation coefficient at zero concentration is 4.56s, and the diffusion coefficient is 6.48×10^{-7} cm.^{2} sec.$^{-1}$. The partial specific volume, computed from the amino acid composition, is 0.733 ml./g. These data give a molecular weight of 64,200. Amino acid analysis indicated that the beta-amylase components have a similar amino acid composition, except that the E component has a higher glycine and methionine content. All three components are acidic molecules possessing four –SH groups and one S–S bond.

As amylases play an important role in the technology of bread production (1), it is essential that their properties and behavior be clearly understood. Before this is possible, the isolation and characterization of the various amylases present in wheat flour must be carried out. The previous paper (2) described the isolation, and the present paper will deal with the characterization (and some properties) of wheat-flour beta-amylase.

To avoid much repetition in describing the present study, the experimental procedures and the results obtained are described in one section.

Experimental Procedure and Results

1. Isolation

The isolation and purification of the beta-amylases used in this study was described previously (2). Three of the major beta-amylase components were isolated, each in amounts of approximately 200 to 300 mg. The samples were stored as concentrated solutions containing 1 to 2% protein at 2°–3°C., and were found to be stable for several months under these conditions.

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2. Crystallization Attempts

Numerous attempts were made to crystallize the beta-amylase fractions by the method of Meyer *et al.* (3). Particulate matter was obtained in most instances; however, the solid material obtained did not resemble the usual appearance of protein crystals but was in the form of oblong spheres (see Fig. 1). The solid nature of the spheres was

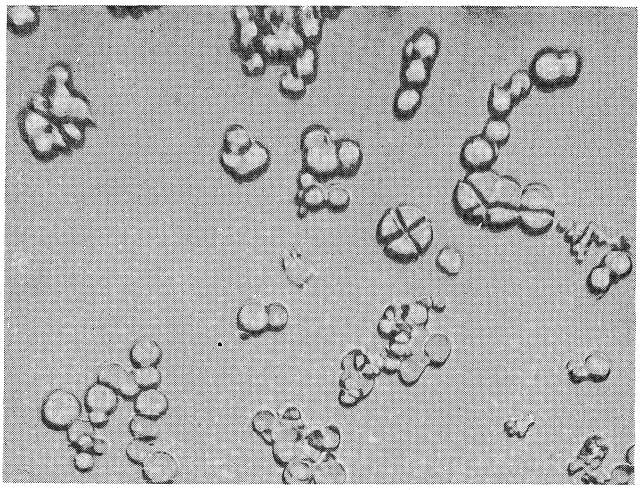

Fig. 1. Typical oblong spheres of wheat beta-amylase obtained on attempted crystallizations (× 560).

readily demonstrated by applying pressure on the microscope slide cover-glass, which crushed some of the spheres. Attempts to carry out crystallization without any movement of the crystallizing mixture so that fragile crystals would not become rounded, or by extraction with acetone prior to crystallization in order to remove any lipid materials, produced the same results.

3. Enzymatic Properties

Specific Activity. Modifications to the method of Noelting and Bernfeld (4) and definition of the unit of beta-amylase activity have been described previously (2). (See Table V for results.)

pH Optimum. Buffer solutions were made from 0.1*M* citric acid and 0.2*M* Na_2HPO_4. Final buffer concentrations of 0.04*M* were used in the determination of enzymatic activity. Optimum pH values of 5.4, 4.6, and 5.2 and 6.2 were obtained for components A, B, and E (Fig. 2).

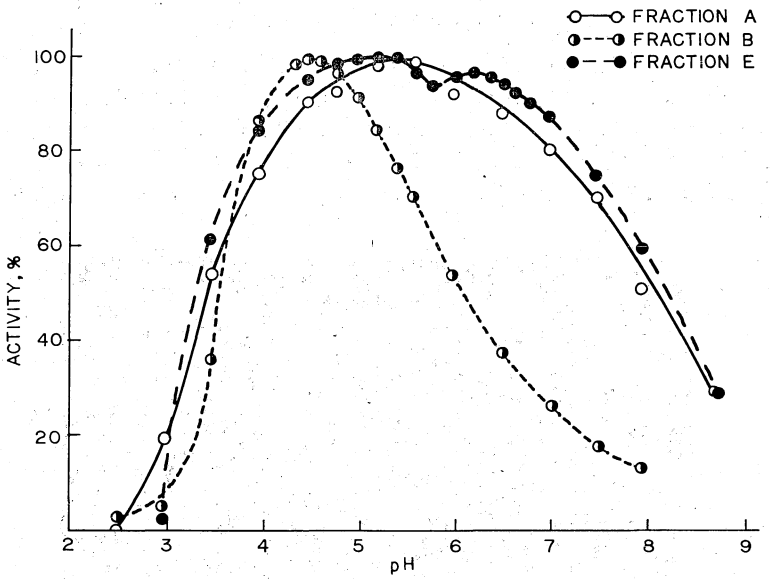

Fig. 2. Effect of pH on beta-amylase activity.

Reaction Product. Soluble starch was incubated with the three beta-amylases until approximately 50% of the starch had been hydrolyzed. The reaction mixture was then chromatographed on Whatman No. 3 paper by elution for 18 hr. (5), with the nonaqueous layer a mixture of pyridine, ethyl acetate, water (40:100:100 v./v.). Maltose was the only sugar detected on development of the chromatogram with aniline phosphate reagent (6), modified by using water-saturated butanol in its preparation. It was estimated that the detection limit of glucose was approximately 1% of the maltose present.

Turnover Number. The beta-amylase possessing the highest specific activity was fraction A. One milliliter of solution containing 0.00441 mg. of this fraction when incubated with 1 ml. of 1% starch solution (Merck soluble starch according to Lintner) at pH 5.52 (0.04*M* acetate buffer) and 20°C. yields 1 mg. of maltose in 3 min. With 64,200 as the molecular weight, the turnover number is calculated to be 14,200, 11,800, and 11,800 for the A, B, and E components respectively. This is the number of glycosidic linkages hydrolyzed in 1 min. at 20°C. by one molecule of beta-amylase.

Effect of Sulfhydryl Inhibitors. When iodoacetate was added to the beta-amylase, enzymatic activity was not affected. The level of iodoacetate added was twice the equivalent amount of beta-amylase sulfhydryls. Similar results were obtained for N-ethylmaleimide. Mercuric

ion was a very effective inhibitor, since it decreased the enzymatic activity immediately on addition. From concentrated solutions of beta-amylase, the addition of mercuric ion caused immediate formation of a precipitate.

Contamination with Other Enzymes. The absence of alpha-amylase activity was confirmed by measuring for iodine color with soluble starch solution after incubation with samples of beta-amylase for 48 hr. at 25°C. as described previously (2). Traces of acid phosphatase activity were detected by measuring the hydrolysis of disodium *p*-nitrophenol-phosphate in 0.1*M* pH 5.5 acetate buffer.

4. Chemical Characterization

Amino Acid Composition. The beta-amylases were hydrolyzed by placing 5 to 6 mg. of the purified enzymes into a Pyrex test tube, adding 5 ml. of twice-redistilled 6*N* HCl, freezing the mixture, evacuating to less than 50 μ pressure, sealing the tube, and then heating for 24 hr. at 110° ± 1°C. in a forced-draft air oven. After hydrolysis the HCl was rapidly removed (7), usually within 12 hr., by placing the opened tube with the contents previously frozen into a desiccator containing fresh NaOH pellets and evacuating to approximately 0.1 mm. To the dry residue some 0.2*N* Na^+, pH 2.2 sodium citrate buffer, containing BRIJ-35 detergent and octanoic acid, was added. The humin was removed by filtration and the filtrate was accurately made up to volume with pH 2.2 buffer.

Acidic hydrolysis was also carried out on samples which had been previously oxidized with performic acid (8) in order to convert cystine plus cysteine to cysteic acid. The cysteic acid was analyzed by chromatography on the 150-cm. column of the amino acid analyzer.

Samples of beta-amylase were also hydrolyzed with 6*N* $Ba(OH)_2$ to obtain hydrolysates suitable for the analysis of tryptophan (9) by ion-exchange analysis on the 15-cm. column.

The amino acid analyses were carried out as described by Spackman *et al.* (10) with a Beckman-Spinco Model 120 amino acid analyzer. The results of analysis of the three beta-amylase components, which were treated with iodoacetate in 8*M* urea, are given in Table I.

Tryptophan content was determined both by a spectrophotometric (11) and a colorimetric (12) method. The results are listed in Table II, and it is seen that both methods were in good agreement. The results from chromatographic analysis of the $Ba(OH)_2$ hydrolysate are also shown in Table II, and it is seen that this method resulted in a poor recovery of tryptophan. Accordingly, the tryptophan content of the A component was assumed to be the average of the spectrophotometric

TABLE I

AMINO ACID COMPOSITION OF IODOACETATE-TREATED WHEAT BETA-AMYLASE COMPONENTS

	Component			Average [a]	Greatest Deviation from Average
	A	B	E		
	μm./g. protein	*μm./g. protein*	*μm./g. protein*	*μm./g. protein*	%
Lysine	298	288	262	283	7.4
Histidine	252	244	251	249	2.0
Ammonia	1,430	1,350	1,392	1,391	4.4
Arginine	387	375	355	372	4.6
S-Carboxymethyl-cysteine	49.0	52.0	50.2	50.4	3.2
Aspartic acid	824	831	828	828	0.5
Threonine	242	249	274	255	7.5
Serine	282	274	243	266	8.6
Glutamic acid	901	894	978	924	5.8
Proline	487	475	490	484	1.9
Glycine	741	745	888	791	12.3
Alanine	612	597	544	584	6.8
Cystine	...	..	...		
Valine	573	567	591	577	2.4
Methionine	155	177	202	178	13.5
Isoleucine	298	300	271	290	6.6
Leucine	671	660	624	652	4.3
Tyrosine	339	311	331	327	4.9
Phenylalanine	343	343	336	341	1.5

[a] The amino acid N recovery from the averaged results was 87%, an NH_3 content of 926 μm./g. protein being used in the computation.

TABLE II

TRYPTOPHAN AND TYROSINE CONTENT

Amino Acid	Method of Analysis								
	Chromatographic: Component			Spectrophotometric: [a] Component			Colorimeter: [b] Component		
	A	B	E	A	B	E	A	B	E
	μm. [c]	*μm.*	*μm.*	*μm.*	*μm.*	*μm.*	*μm.*		
Tryptophan	66	...	109	208	157	182	220	..	..
Tyrosine	367	311	331	322	347	328	...	..	..

[a] See reference 11.
[b] See reference 12.
[c] μm./g. protein.

and colorimetric data. The spectrophotometric data were also used to calculate tyrosine content (11) of the beta-amylase components. This value is, in general (see Table II), in agreement with the chromatographic data.

The most reliable quantitative amino acid data were obtained for the A component, since it was possible to carry out two different analyses in duplicate with an averaged amino acid nitrogen recovery of 97% (see Table III).

The chromatographic data from Table III, in conjunction with

TABLE III

AMINO ACID RECOVERIES FROM WHEAT FLOUR BETA-AMYLASE (A COMPONENT)

	Recovered[a]			Nitrogen Recovered	
	Analysis 1	Analysis 2	Average		
	μm.	μm.	μm.	mg./g.	μm./g.[b]
Tryptophan	214[c]		214	6.00	214
Lysine	294	326	310	8.69	320
Histidine	278	317	298	12.52	307
Ammonia	864	988	843[d]	11.81	
Arginine	401	487	444	24.88	458
Cysteine					59
S-Carboxymethyl-cysteine	57			0.88	
Cysteic acid	123				
Aspartic acid	866	941	904	12.67	497
Asparagine					435[e]
Threonine	222	259	256[d]	3.59	264
Serine	268	332	324[d]	4.54	334
Glutamic acid	851	964	908	12.72	501
Glutamine					435[e]
Proline	486	517	502	7.03	518
Glycine	688	758	723	10.13	745
Alanine	647	667	657	9.21	677
Cystine			33	0.93	34
Valine	571	636	604	8.46	623
Methionine	180	200	190	2.66	196
Isoleucine	299	339	319	4.47	329
Leucine	684	754	719	10.07	741
Tyrosine	344	389	367	5.14	378
Phenylalanine	341	381	361	5.06	372
				161.38	

[a] μm. amino acid recovered/g. anhydrous enzyme hydrolyzed. The amino acid nitrogen recovery was 97.0%.
[b] Corrected to 100% N recovery by multiplying the third column by (16.6/16.1) or 1.031. The value of 16.6 was obtained by Dumas N analysis (see Table V), and the value of 16.1 refers to the amino acid nitrogen recovery in column 4.
[c] Average value from spectrophotometric and colorimetric analysis.
[d] Ammonia, threonine, and serine averages multiplied by 91,106, and 108% to correct for decomposition during hydrolysis.
[e] The amounts of glutamine and asparagine were each assumed to equal one-half of the ammonia content.

spectrophotometric and colorimetric data for tyrosine and tryptophan contents shown in Table II, were then used to calculate the representative amino acid composition for wheat beta-amylase, as shown in Table IV. The amino acid data may be used to calculate the nitrogen (N) content of wheat beta-amylase components. Thus, dividing the recovered weight of N (160.76 mg., Table III) by the weight of protein that this amino acid N corresponds to (952.3 mg., Table IV) and multiplying by 100, results in a value of 16.9% for the N content.

Sulfhydryl Content. The amount of S-carboxymethylcysteine present, obtained by reacting the sulfhydryl groups with iodoacetic acid, was used as an indication of the sulfhydryl content of the beta-amylase components (7,13). Excess iodoacetic acid in 8*M* urea at pH 8.7 was added to dry beta-amylase samples and allowed to stand at room

TABLE IV

AMINO ACID COMPOSITION OF WHEAT FLOUR BETA-AMYLASE

	Amino Acid Residue Weight w [a]	Residue Specific Volume [b] v	wv̄	Minimal Molecular Weight [c]	Amino Acid [d]	Nearest Integral No. of Amino Acid Residues
	mg./100 mg.				*μm.*	
Tryptophan	3.99	0.74	2.95	4,670	13.8	14
Lysine	4.10	.82	3.36	3,130	20.5	21
Histidine	4.21	.67	2.82	3,260	19.7	20
Arginine	7.15	.70	5.01	2,180	29.5	30
Cysteine	0.61	.86	0.52	16,900	3.8	4
Aspartic acid	5.72	.60	3.43	2,010	31.9	32
Asparagine	4.96	.62	3.08	2,300	27.9	28
Threonine	2.67	.70	1.87	3,790	16.9	17
Serine	2.91	.63	1.83	2,990	21.5	22
Glutamic acid	6.47	.66	4.27	2,000	32.1	32
Glutamine	5.57	.67	3.74	2,300	27.9	28
Proline	5.03	.76	3.82	1,930	33.3	33
Glycine	4.25	.64	2.72	1,340	47.9	48
Alanine	4.81	.74	3.56	1,480	43.4	43
Cystine	0.70	.61	0.42	29,400	2.2	2
Valine	6.18	.86	5.31	1,610	39.9	40
Methionine	2.57	.75	1.93	5,100	12.6	13
Isoleucine	3.72	.90	3.35	3,040	21.1	21
Leucine	8.38	.90	7.55	1,350	47.6	47
Tyrosine	6.17	.71	4.38	2,650	24.2	24
Phenylalanine	5.48	0.77	4.22	2,690	23.9	24
Totals	95.64		70.13			

Specific volume: 70.13/95.64 = 0.733

[a] Calculated from last column in Table III.
[b] See reference 20.
[c] Amino acid residue molecular weight × 100 / % amino acid residue in protein (column 1).
[d] Per 64,200 g. beta-amylase.

temperature for 1 hr. After the urea and excess iodoacetate was dialyzed away, the samples were hydrolyzed and analyzed on the amino acid analyzer. The sulfhydryl content was calculated from the amount of S-carboxymethylcysteine present. The S-carboxymethylcysteine content is shown in Table I, and is also reported as the number of sulfhydryl groups per molecule of beta-amylase in Table V.

Carbohydrate Content. The absence of carbohydrate material in the purified beta-amylase fractions was confirmed by tests with Molisch's Reagent and, after gentle acid hydrolysis, by the absence of sugars on paper chromatograms. In addition, levulinic acid was absent in all hydrolysates analyzed for their amino acid content. If any carbohydrate material was present in the beta-amylases, it would be less than 0.3%.

5. Physical Characterization

Electrophoretic Mobility. Zone electrophoresis was carried out with

a Beckman-Spinco Model H electrophoresis-diffusion instrument. A modified 11-ml. Tiselius cell as supplied by Beckman-Spinco was used. All experiments were made at 2°C. with a current of 13 ma. Acetate buffers of pH 4.0 and 5.0 and a veronal buffer of pH 8.0 and 8.5, all adjusted to ionic strength of 0.10 with NaCl, were used as described by Miller and Golder (14). Protein concentrations were in the region of 0.1 to 0.15%. Schlieren patterns were photographed at intervals, and electrophoretic mobilities were calculated by analysis of the photographic plates with a Gaertner microcomparator.

The electrophoretic patterns (Fig. 3) show that the components are

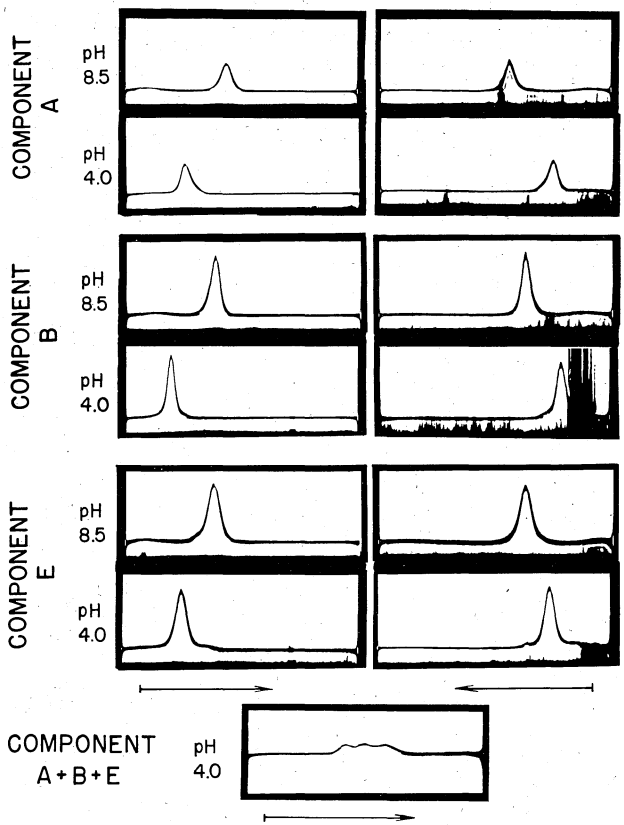

Fig. 3. Schlieren electrophoresis patterns. Descending boundaries on the left, ascending on the right. Descending photographs shown for component A at pH 8.5 and 4.0, component B at pH 8.5 and 4.0, component E at pH 8.5 and 4.0, and the mixture of components A+B+E at pH 4.0, were taken at 263.0 and 270.0 min., 270.0 and 270.0 min., 270.0 and 270.0 min., and 290 min. The ascending photographs were all taken 0.5 min. later. The ascending photograph for the A+B+E mixture is not shown.

homogeneous in all instances, except that the E component contains a faster-moving component at pH 4.0 which represents about 1–2% of the total protein. The computed electrophoretic mobilities are illustrated in Table V and are also shown in Fig. 4 in a plot of mobility *vs.* pH used to obtain the isoelectric points. Values of 4.9, 4.4, and 4.1 were obtained for the isoelectric points for the A, B, and E components.

An electrophoresis experiment was also carried out on a solution containing a prepared mixture of the A, B, and E components. The schlieren pattern obtained is illustrated in Fig. 3.

Free Diffusion. The free diffusion of the beta-amylases was studied in a Beckman-Spinco Model H electrophoresis-diffusion instrument. A modified 11-ml. Tiselius electrophoresis cell was used, the runs being conducted at 2.0°C. All runs were carried out in 0.1 ionic strength univalent buffers, the ionic strength being adjusted with NaCl (14).

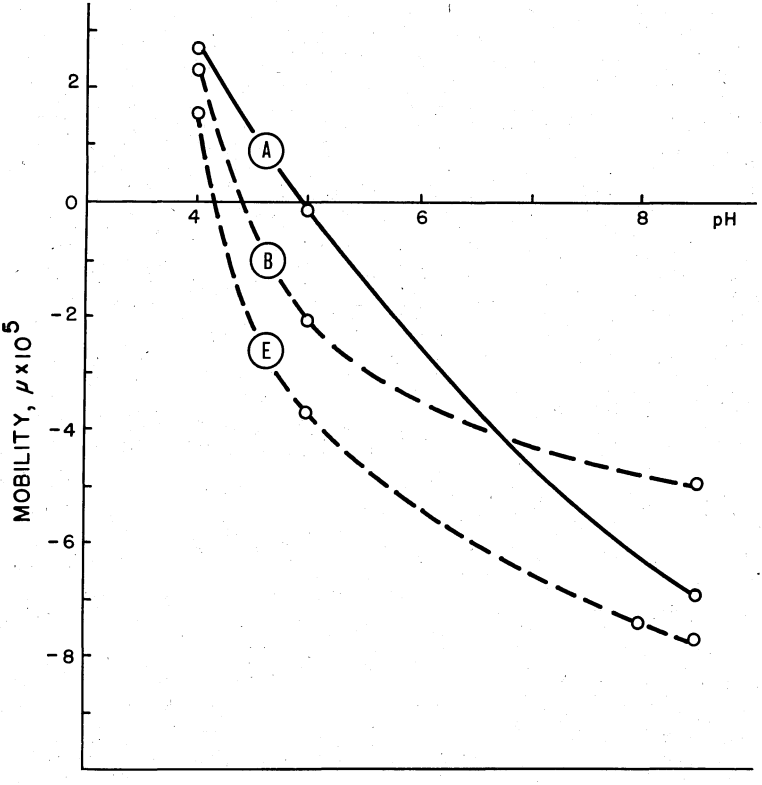

Fig. 4. Electrophoretic mobilities of wheat beta-amylases as a function of pH. All runs were carried out in univalent buffers of 0.1 ionic strength. Isoelectric points were found at pH 4.9, 4.4, and 4.1 for the A, B, and E components.

Automatic sequence photography was used to record the Rayleigh patterns. These were analyzed with a Gaertner microcomparator. The method described by Longsworth (15) and Schachman (16) was used to compute the diffusion coefficient with the aid of probability tables (17). The diffusion coefficients $D^{\circ}_{20,w}$ are listed in Table V.

The concentration of the beta-amylase solutions was high enough only in the instance of the A component to permit the use of the diffusion data as a criterion of homogeneity (18). The results showed that if a protein impurity with a different diffusion constant was present, then its concentration was less than 0.5%.

Sedimentation Velocity. Sedimentation velocities of the three major beta-amylases were determined using a Beckman-Spinco Model E ultracentrifuge. When the rotor speed reached 56,100 r.p.m., photographs were taken at 16-min. intervals. The temperature of the rotor was recorded during the run with a contact thermocouple, and the average value used was that of the temperature of the rotor once it had reached top speed and at the time when the final photograph was taken. The difference in the two temperatures was usually 0.5°C. Solutions containing 0.5 to 1.5% protein were analyzed using the same buffer solutions as in the diffusion experiments (univalent buffer solutions of 0.1 ionic strength, the adjusting salt being NaCl). The photographs (Fig. 5) were evaluated with a Gaertner microcomparator and the results calculated in the manner suggested by Kegeles and Gutter (19). Alternatively, the logarithm of the distance of the boundary to the axis of rotation was plotted against time, and the slope of the resulting line was used in calculating the sedimentation coefficient (16). The sedimentation coefficients were reduced to standard conditions ($s_{20,w}$), the unit 1×10^{-13} sec. being termed ls, where s is the Svedberg. The results are shown in Fig. 6 as a plot of s *vs.* concentration to obtain by extrapolation the zero concentration, $s^{\circ}_{20,w}$.

Because of the importance of –SH groups in flour protein chemistry, sedimentation experiments were also made on the A component dissolved in 0.001*N* KIO_3 and in 0.001*N* I_2. No changes in sedimentation velocities of the treated beta-amylases were observed when they were compared to untreated beta-amylase; thus, there was no intermolecular S–S cross-linking under these conditions. In the experiment with I_2, some beta-amylase precipitated out on addition of I_2.

Sedimentation experiments were also made in the presence of 8*M* urea. These showed only one component with a sedimentation coefficient of approximately 4.6s, indicating that the beta-amylase does not dissociate to components of lower molecular weight under these conditions.

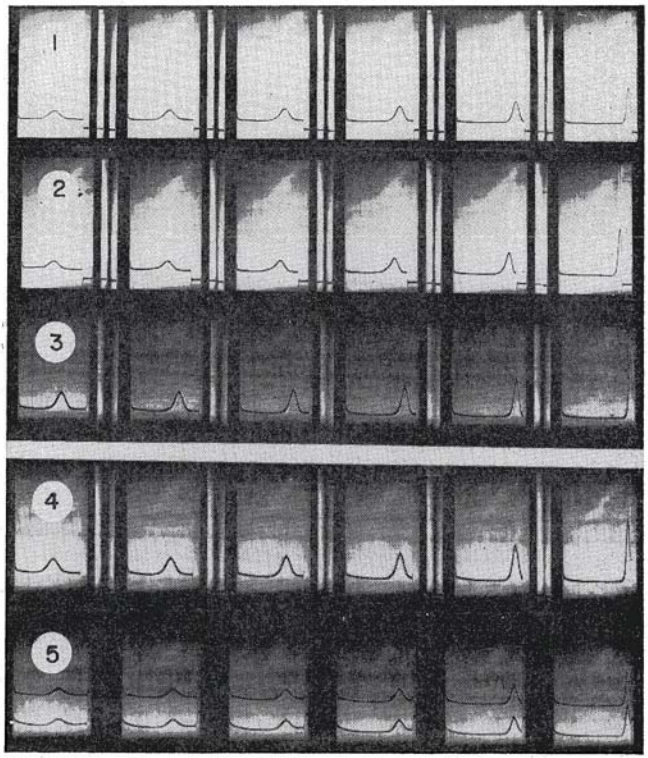

Fig. 5. Sedimentation velocity schlieren patterns: 1) A-beta-amylase component, 0.86%, pH 7.0; 2) B-beta-amylase component, 0.82%; 3) E-beta-amylase component, 0.69%, pH 9.0; 4) A-beta-amylase, 0.86%, in pH 5.0 buffer containing 0.015*M* KIO_3; 5) Top: A component, 0.43%, in pH 5.0, 0.01*M* glutathione; bottom: A component, 0.43%, in pH 5.0, 0.001*M* I_2. Every solution was adjusted to 0.1 ionic strength with NaCl.

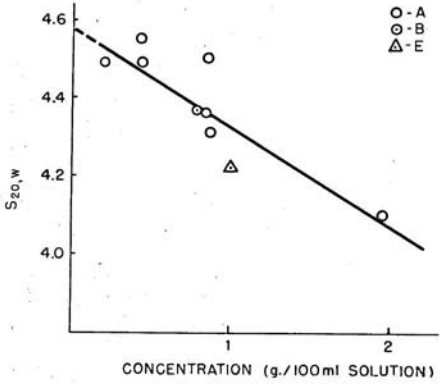

Fig. 6. Dependence of sedimentation coefficients on concentration. Before plotting, sedimentation coefficients were normalized to the viscosity and density of water at 20°C.

Specific Volume. The specific volume of beta-amylase was calculated from the specific volumes of the amino acid residues as described by Cohn and Edsall (20) and (see Table IV) was found to be 0.733 cc./g.

Molecular Weight. The molecular weights of the beta-amylases were calculated from the sedimentation and diffusion coefficients at infinite dilution by use of the Svedberg equation,

$$MW = \frac{RTs}{D(1-\bar{v}\rho)}$$

where R is the gas content, 8.314×10^7 ergs/mole/degree, T the absolute temperature, s the sedimentation coefficient, D the diffusion coefficient, $\bar{v}$ the apparent specific volume, and ρ the density of the medium. This calculation yielded the result:

$$MW = \frac{(8.314 \times 10^7)\ (293.2)\ (4.58 \times 10^{-13})}{6.48 \times 10^{-7}\ [1 - 0.733\ (0.9982)]} = 64{,}203 \text{ g./mole}$$

Ultraviolet Absorption Spectrum. Absorption spectra were obtained with a Beckman Model DU spectrophotometer. Typical spectra are illustrated in Fig. 7. Absorbancies, $A^{1\%}_{1\ cm.}$, at 290 mμ in 0.1*N* NaOH, of

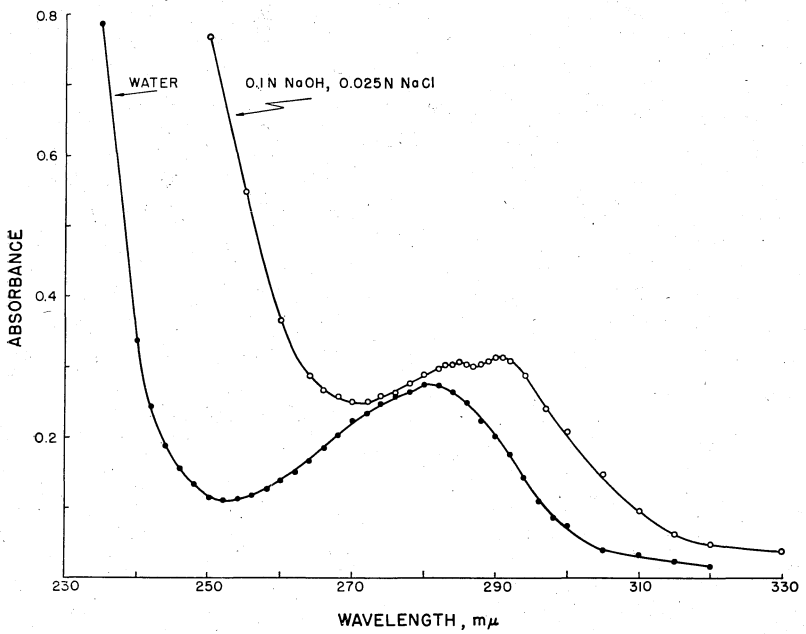

Fig. 7. Ultraviolet absorption spectra of the A component of wheat beta-amylase. Protein concentration was 0.192 mg./ml. in both instances.

16.3, 15.2, and 15.6 were obtained for the A, B, and E beta-amylase components. Absorbancies, $A_{1\,cm.}^{1\%}$, at 281 mμ in water at pH 6.0, were in the vicinity of 14.0 ± 0.2 for all the beta-amylases. Tyrosine and tryptophan contents of the beta-amylases were also calculated from the absorption data (8) (Table IV).

Discussion

A comparison of the various properties of the three main beta-amylase components and purified beta-amylases from barley and barley-malt is summarized in Table V. The nitrogen content is appreciably higher than the published value for crystalline wheat beta-amylase. Consequently, the specific activity expressed per mg. N is somewhat lower than that of the crystalline beta-amylase, whereas when expressed per mg. enzyme, the specific activity results are in good agreement. Component A has a higher specific activity than component B or E.

It was not possible to obtain well-formed crystals of any of the

TABLE V

SUMMARY OF PROPERTIES OF PURIFIED CEREAL BETA-AMYLASES

	Wheat Flour				Barley Malt	
	Present Work Component			Meyer, Spahr, Fischer (3)	Danielsson and Sandegren (23)	Danielsson (29)
	A	B	E			
Nitrogen, %						
(Dumas N analysis)	16.6	16.7	16.5	14.3		
(Amino acid analysis)	16.9	. .	. .			
Activity per mg. N	1,370	1,130	1,150	1,450		
Activity per mg. enzyme	227	189	189	198		
Sedimentation constant, $s^0_{20,w}$	4.58	4.58	4.58		4.52	4.6
Diffusion constant, ($\times 10^7$)cm.^{2}sec.$^{-1}$	6.49	6.51	6.44			6.53
Partial specific volume, ml./g.	0.733	0.733	0.733			0.69
Molecular weight, g./mole	64,200	64,200	64,200			54,000
Mobility						
pH 4.0	2.67	2.26	1.50		2.3	
pH 8.0	−6.97	−4.95	−7.71		−4.4	
Isoelectric point, pH	4.9	4.4	4.1	6.0	5.75	
pH optimum	5.4	4.6	5.2–6.2	5.3		
$E_{1\,cm.}^{1\%}$, at 290 mμ, 0.1*N* NaOH	16.3	15.2	15.6	11.8 (at 278 mμ)		
Turnover number	14,200	11,800	11,800			
Sulfhydryl content, –SH/molecule	3.6	3.8	3.7			

beta-amylase components, other than in the form of spherical particles, illustrated in Fig. 1. Beta-amylase is possibly a difficult enzyme to crystallize, since in the three instances where it has been crystallized from wheat flour (3), barley (21), and barley-malt (22), only ill-defined crystals were obtained.

The three beta-amylases gave quite different pH optimum curves (Fig. 1). Component A produced a broad curve with a maximum at approximately pH 5.4. The enzyme exhibited 80% or more of its potential activity between pH 4 and 7. Component B showed a narrower pH optimum range with a maximum at pH 4.6. Component E gave a broad curve similar to that of A, except that it had a double maximum at pH 5.2 and 6.2. These pH optimum values compare with the published values of 5.3, 5.2, and 4.5 for wheat, barley-malt, and sweet-potato beta-amylase. The curve for A is very similar to that published by Meyer *et al.* (3) for wheat beta-amylase. This is to be expected, as peak A is the predominant component of "free" beta-amylase extractable with water (2), and Meyer's beta-amylase was prepared from a water extract of flour.

Alpha-amylase activity was found to be absent from all the three main components. The absence of alpha-amylase was further substantiated by paper chromatography of the products formed by the three components on soluble starch solution. In all cases maltose was the only product, whereas the presence of alpha-amylase would have caused the production of glucose and maltotriose. Two commercial samples of barley beta-amylase, allegedly free of alpha-amylase, were actually found to be contaminated with alpha-amylase, since no iodine color was given by a soluble-starch solution which was incubated with the commercial enzymes. Some contamination by acid phosphatase was detected. While the extent of contamination was difficult to evaluate quantitatively because the specific activity of wheat acid phosphatase is not known and it is markedly unstable, it is considered that the contamination was not significant.

Electrophoresis experiments at pH 4.0, 5.0, and 8.5 all indicated that the three beta-amylase components behaved as single, relatively homogeneous components. Component E had a trace of an impurity present, probably component A, which moved faster at pH 4 and slower at 8.5. Isoelectric pH values of 4.9, 4.4, and 4.1 were obtained for the A, B, and E components. These values are appreciably lower than the published values of 6.0 and 5.75 for wheat (3) and barley-malt (23) beta-amylases.

To confirm that the three components were in fact electrophoretically different, mixtures of components A, B, and E were examined

electrophoretically at pH 4.0 and 8.5. Both experiments showed that three separate peaks were clearly discernible. These findings are consistent with previous reports of three beta-amylase-active electrophoretic components in wheat flour (24) and multiple beta-amylase components in barley and barley-malt (25–28). Evidence for multiple forms of one enzyme differing only in electrophoretic behavior is becoming a common finding, and the term *isozymes* is used to describe them.

The absorption spectra of the amylases in water showed a maximum at 281 mμ; in alkaline solution a double peak was observed with maxima at approximately 290 and 285 mμ. Extinction coefficients, $E_{1\text{ cm.}}^{1\%}$, at 290 mμ in 0.1*N* NaOH, were calculated to be 16.3, 15.2, and 15.6 for the A, B, and E components. These values are higher than the value of 11.8 at 287 mμ obtained by Meyer *et al.* (3).

Analytical ultracentrifuge experiments revealed that the beta-amylases possessed sedimentation coefficients which fell on a straight line when plotted against concentration, and which increased with decreasing concentration (see Fig. 6). This relationship was obtained at three different pH levels for each component and also when the A component was treated with I_2, KIO_3, or glutathione. Extrapolation to zero concentration yielded a value for $s^\circ_{20,w}$ of 4.58. It is interesting to note that this value is virtually identical with the values of 4.52 and 4.6 obtained by Danielsson and Sandegren (23) and Danielsson (29) for barley-malt beta-amylase. Sedimentation experiments with A component in 8*M* urea solution showed that it did not dissociate into subunits.

Sedimentation experiments on the A component which was oxidized with I_2 showed no change in s. This indicates that oxidation of the sulfhydryl (28,29) either does not result in disulfide formation, or if disulfide does form it must occur intramolecularly. Experiments with the A component treated with 0.015*M* KIO_3, either in the absence or presence of 7*M* urea, indicated no change in the sedimentation coefficient, which indicates either absence of oxidation or intramolecular oxidation. Evidence that KIO_3 does not oxidize the beta-amylase sulfhydryl was also indicated by the observation that the presence of KIO_3 did not alter the enzymatic specific activity. It has been previously reported that wheat-flour beta-amylase (3) requires free sulfhydryl groups for enzymatic activity. Barley and barley-malt beta-amylase also require free sulfhydryl groups for enzymatic activity, and it has been reported that iodine inactivates the enzyme, probably by reaction with sulfhydryl groups (30,31).

One experiment was also carried out with the A component which

had been treated with glutathione at pH 5.0. The sedimentation behavior was examined simultaneously with the iodate experiment in a double-sector rotor and, as illustrated in Fig. 5, it showed that the glutathione-treated enzyme sedimented at a rate equivalent to that of the untreated enzyme. This indicates either that the one disulfide bond present in beta-amylase is unreactive under these conditions, or that it is an intramolecular bond.

The amino acid compositions of the three beta-amylase components analyzed are generally similar within the limits of an experimental error of ± 4% except in the case of the E component, which has a significantly higher amount of glycine and methionine. The E component may also contain more threonine and less serine than the other two components, although the amounts of these amino acids are known with less certainty owing to destruction during hydrolysis. The differences in methionine content of the beta-amylases cannot be due to varying amounts of reaction with iodoacetate (32), since similar amounts of S-carboxymethylhomocysteine and homoserine lactone were present in the hydrolysates of all the beta-amylases. Moreover, the extent of reaction of methionine with iodoacetate was only about 5%. Analysis of an unmodified sample substantiated the finding that the E component contained more glycine and methionine.

As seen from the data in Tables I and II, it is apparent that the iodoacetate-treated beta-amylases have been contaminated with ammonia. The recovery of amino acids from the iodoacetate-treated beta-amylases is only 87%, presumably owing to losses of the enzyme when it was extensively dialyzed prior to hydrolysis to remove the urea and excess iodoacetate.

With the value of 64,200 for the molecular weight, the amino acid composition for a typical wheat beta-amylase molecule was calculated to the nearest integral number of amino acid residues (Table IV).

Examination of Table IV suggests that there are 32 each of aspartic and glutamic acid residues as compared to 21, 20, and 30 lysine, histidine, and arginine residues, giving an excess of seven cationic groups. This result is in conflict with the ion-exchange behavior of the beta-amylase components (2), which reveals that they are acidic in nature; they are, in fact, the most acidic of the water-soluble flour proteins. In addition, the beta-amylase components possess isoelectric points of pH 4.1 to 4.9 – which is additional evidence that there must be an excess of anionic groups. The reason is unknown, but this discrepancy is most probably due to analytical results which are too high for the ammonia content. This possibility would result in a too-high amide content and therefore a too-low free carboxyl content.

The specific volume of wheat beta-amylase of 0.733 cc./g. does not agree with the value of 0.69 used by Danielsson for barley-malt beta-amylase (29). The latter value is probably too low, since inspection of the amino acid composition for purified wheat and barley albumins which possess beta-amylase activity reveals that the glycine, serine, NH_3, aspartic acid, and glutamic acid contents are not significantly different (26). This observation, together with the fact that, to our knowledge, no other value as low as 0.69 has been reported for a naturally occurring protein or enzyme (33), would suggest that the value of 0.733 is more accurate.

The molecular weight of 64,200 is somewhat higher than that obtained by Danielsson for barley-malt beta-amylase (29). If the value of 0.733 for the partial specific volume of barley-malt beta-amylase is used in conjunction with the Danielsson values for the sedimentation and diffusion constants, then a molecular weight of 62,600 is obtained which is in excellent agreement with the molecular weight obtained in the present study.

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