Heterogeneity of the Beta-Amylase Enzymes of Barley[1,2]

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ABSTRACT

Eight major and several minor β-amylase components were separated reproducibly by chromatofocusing of thiol-containing extracts of barley. Each of these components had distinctive apparent pI values. During rechromatofocusing, each major component eluted reproducibly with the same apparent pI value but additionally produced one or several β-amylase components with apparent pI values similar to other major β-amylase components. Similar molecular rearrangements or dissociations were observed by isoelectric focusing. Heterogeneity of the β-amylase enzymes of extracts of barley containing low concentrations of thiolated reducing agents (10 m*M*) may result from the occurrence of several forms of "free" β-amylase, from the existence of several soluble forms of protein-bound β-amylase, or from partial dissociation of large molecular weight aggregates of β-amylase. Further characterization of each component isolated by chromatofocusing is required to determine the interrelationships among the various β-amylase components.

Key words: *Barley, β-amylase, Chromatofocusing, Isoelectric focusing*

INTRODUCTION

The polymorphic characteristics of β-amylase enzymes of barley have been observed by several techniques. Soluble β-amylase is heterogeneous relative to molecular size and surface charge properties, as demonstrated by gel filtration (17,18,19), ultracentrifugation (20), electrophoretic techniques (1,2,5,16), ion-exchange chromatography (11,12), and isoelectric focusing (21). Pure crystalline β-amylase from barley consisted of four isoenzymes with different isoelectric points (31).

Some of the heterogeneity results from aggregation of low molecular weight β-amylase molecules by disulfide bond formation to produce higher molecular weight components (17,18,19). The various molecular forms of barley β-amylases are immunochemically identical, as demonstrated by electroimmunodiffusion techniques (4). Relatively high concentrations (100 m*M*) of thiolated reducing agents such as mercaptoethanol (6,31), dithiothreitol (23,31) or monothioglycerol (11,12) are effective for extracting bound β-amylase and for maintaining the enzyme in reduced, monomeric forms. Lower concentrations of reducing agents produced only a partial reduction of polymeric forms of barley β-amylase (6,11).

There is now convincing evidence that the heterogeneity of barley β-amylase also results partially from the existence of several soluble forms of protein-bound β-amylase (15,24). This has been confirmed by two-dimensional immunoelectrophoretic techniques (6,7,8). A significant amount of β-amylase activity occurs as heterodimers composed of "free" enzymes and a nonactive protein referred to as protein Z (6,7,8). Protein Z was purified and characterized (9) and found to be the origin of a dominant protein found in beer (10).

The present study is an investigation of the use of a

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chromatofocusing technique (25,26,27,28) to isolate β-amylase enzymes from barley. Chromatofocusing utilizes amphoteric buffers to generate a pH gradient on an ion-exchanger without need for a gradient-making apparatus. Proteins elute from an ion exchanger in order of isoelectric pH, and focusing effects occur that concentrate proteins resulting in high resolution. Chromatofocusing should provide an efficient, cost-effective technique for isolating substantial quantities of β-amylase enzymes for further characterization.

EXPERIMENTAL

Extraction of Beta-Amylase Enzymes

Bonanza barley was ground in a Wiley mill equipped with a 1-mm sieve. Ground barley (75 g) was extracted with citrate buffer and dialyzed extensively with acetate buffer, as described previously (11,12,13). Buffers contained 10 m*M* monothioglycerol (Sigma Chemical Co., St. Louis, MO). Extractions and chromatofocusing experiments were performed at 4°C.

Chromatofocusing

Polybuffer™, Polybuffer exchangers PBE™, and Sephadex™ are exclusive trademarks of Pharmacia Fine Chemicals AB (Uppsala, Sweden). Polybuffer exchanger (PBE 94) was equilibrated with start buffer and packed in a Pharmacia K9/60 column to a bed height of 45 cm, as recommended by the manufacturer (22). The start buffer was 0.025*M* imidazole-HCl, pH 7.4, containing 10 m*M* monothioglycerol. A 2-cm layer of coarse Sephadex G-25 was poured on the top of the bed to ensure even sample application.

Crude β-amylase extracts in acetate buffer were dialyzed extensively with elution buffer before application to chromatofocusing columns. The sample (20 ml) was applied with a pump at a flow rate of 0.5 ml/min. The elution buffer was Polybuffer 74, diluted 10 times with distilled water. The diluted Polybuffer contained a final concentration of 10 m*M* thioglycerol. This buffer was titrated to pH 4.3 with 5*N* HCl.

A total volume of 560 ml of elution buffer, equivalent to 14.5 column volumes, was applied to the column at a flow rate of 30 ml/hr. The column was equipped with a stream splitter so that a portion of column effluent was monitored continuously for β-amylase activity by means of a continuous flow analyzer. The remaining portion of column effluent was collected in a fraction collector at 8-min intervals.

Assay for Beta-Amylase

A manifold similar to that described by Bendelow (3) was used to assay for β-amylase. A portion of column effluent was taken up continuously by a Technicon AutoAnalyzer I™, mixed with 0.5% Lintner starch at pH 4.5 and incubated at 30° C in a bath containing a 20-ft delay coil. Reducing sugars were determined using neocuproine reagent (3). β-Amylase profiles of column effluents were monitored at 460 nm with a recorder chart speed of 3.92 in./hr.

Rechromatofocusing

Following initial chromatofocusing, appropriate fractions were pooled and concentrated in an Amicon ultrafiltration cell equipped with a PM-10 membrane. Concentrated, pooled fractions were not dialyzed with start buffer prior to rechromatofocusing.

Isoelectric Focusing

Precast thin-layer polyacrylamide gel plates (Ampholine PAG plate kits, LKB Productor AB, Bromma, Sweden) were used for isoelectric focusing, which was performed with a Desaga thin-layer electrophoresis apparatus (C. Desaga, Heidelberg, West Germany). Constant power (10 W) was maintained for 2½ hr with an ISCO model 494 power supply (Instrument Specialties Co., Lincoln, NE).

Isoelectric focusing was performed at 2°C with a pH gradient from 4.0 to 6.5. Crude extracts or solutions of purified β-amylase were deposited at the cathode end of the gel on pieces of Whatman No. 1 filter paper. β-Amylase bands were detected using a Lintner starch plate technique (14).

Action Patterns of Beta-Amylase Fractions

Each major β-amylase fraction was subjected to chromatofocusing three times. The final pooled, concentrated fractions were used to determine enzymic action patterns.

Fractions were incubated at 35°C for 20 hr with 1% amylose in 0.2*M* acetate buffer, pH 4.9. Hydrolyzates were frozen, freeze-dried, dissolved in 500 μl of distilled water and spotted on Whatman 3M chromatography paper. Glucose, maltose, and maltotriose were applied separately. Descending chromatography was performed, using equal proportions of water, butanol, and pyridine (30). Sugar spots were developed with a combination of treatments with silver nitrate, sodium hydroxide, and sodium thiosulfate (29).

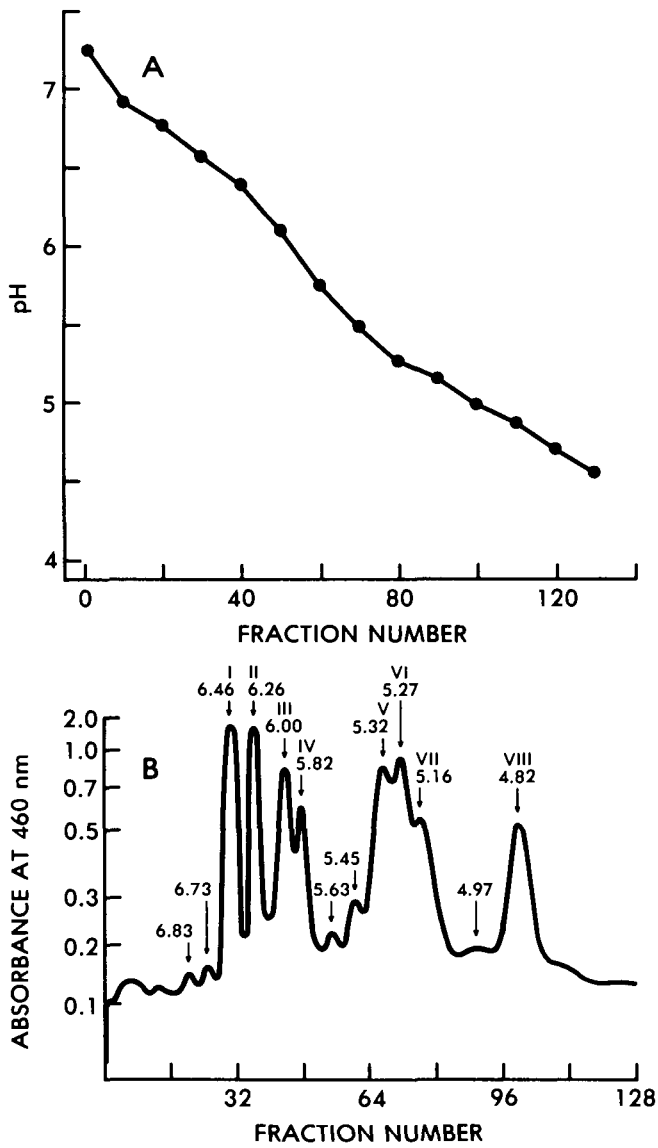

Fig. 1. A, A typical pH gradient generated by a chromatofocusing experiment. **B,** An elution profile of the β-amylase components of barley separated by chromatofocusing. Major and minor β-amylase components are identified by apparent pI values. Major β-amylase components are identified by Roman numerals I to VIII.

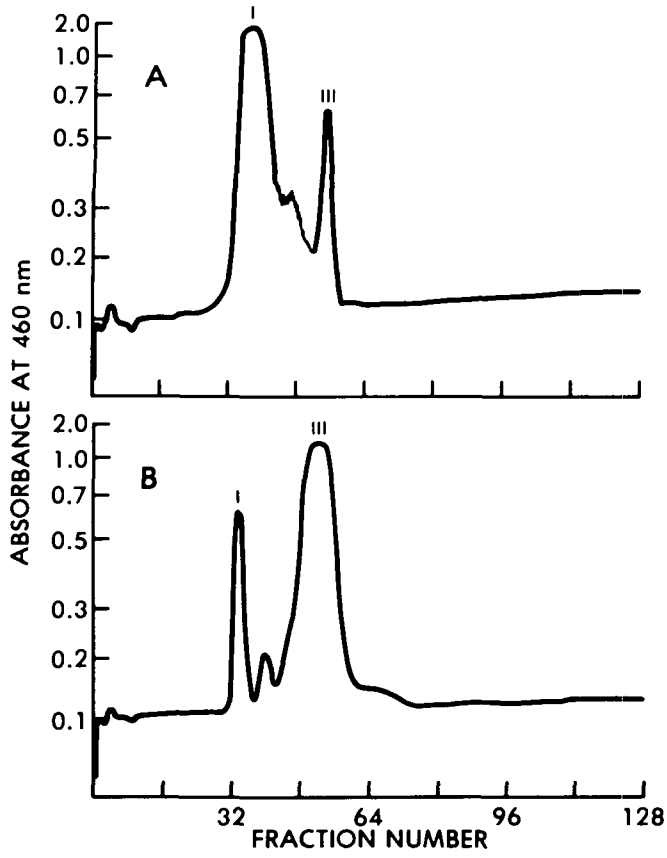

Fig. 2. Rechromatofocusing of β-amylase components I (A) and III (B).

RESULTS AND DISCUSSION

Chromatofocusing is a recently developed column chromatographic technique for eluting proteins from an ion-exchange column in order of isoelectric pH (22,25,26,27,28). An advantage of this technique is that focusing effects occur that result in band sharpening, sample concentration, and very high resolution, with peak widths ranging down to 0.04–0.05 pH units. Under certain conditions, proteins may elute from chromatofocusing columns at a pH slightly lower than the true pI value because of ionic effects, displacement effects, Donnan potentials, or varying solubilities of proteins at their isoelectric pH. For this reason, β-amylase components obtained by chromatofocusing will be identified according to apparent pI or elution pH values.

A typical pH gradient generated by the chromatofocusing procedure is shown in Fig. 1A. Minor variations of pH gradients were found among chromatofocusing experiments. Variations of pH gradients affect the elution volume or fraction number at which β-amylase components elute, but the apparent pI or elution pH of the various β-amylase components are not affected.

The chromatofocusing profile of β-amylase components from Bonanza barley is shown in Fig. 1B. A number of major and minor β-amylase components with distinctive apparent pI values were separated reproducibly by this technique. For the purposes of the present study, only the major β-amylase fractions identified with Roman numerals from I to VIII were investigated further. Use of Roman numerals to identify β-amylase components is not intended as a system of nomenclature, but is used as a convenient method to discuss the properties of the various major β-amylase components.

Each major β-amylase component (I–VIII) from each of six chromatofocusing experiments was pooled together, concentrated by ultrafiltration, and subjected to rechromatofocusing. The elution profiles of each major β-amylase component obtained by rechromatofocusing are presented in Figs. 2, 3, 4, and 5. These

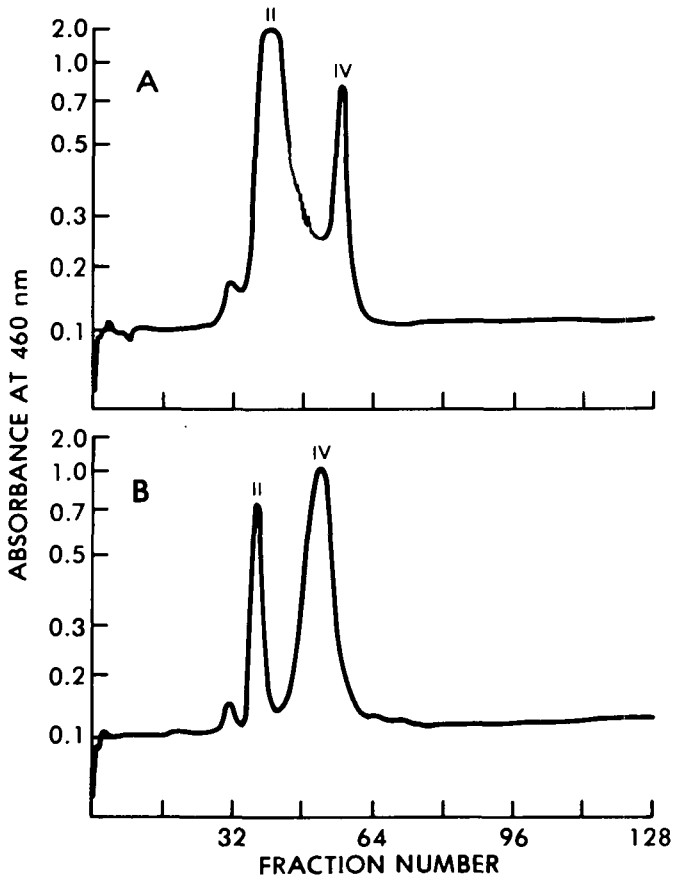

Fig. 3. Rechromatofocusing of β-amylase components II (A) and IV (B).

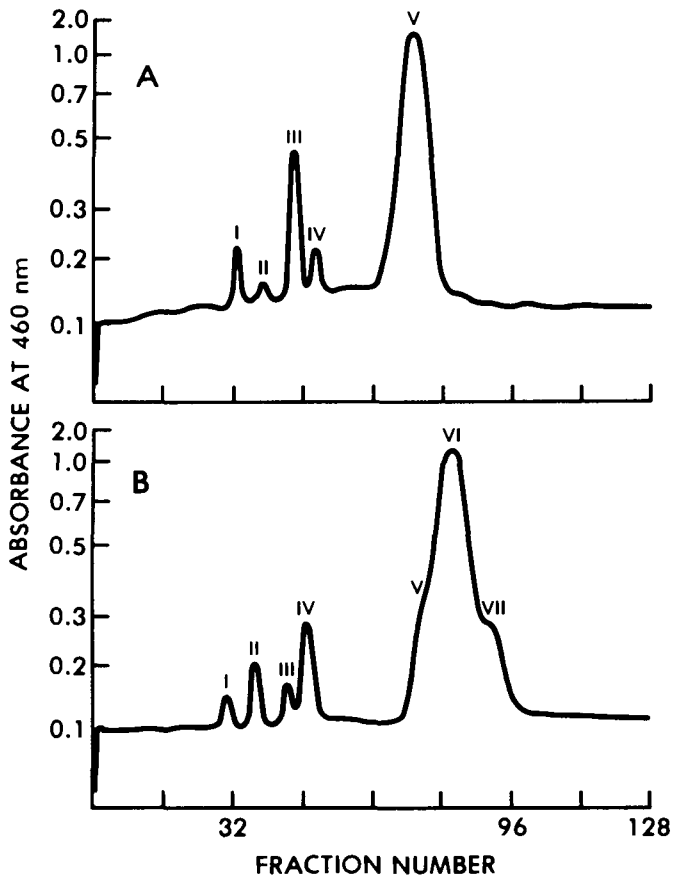

Fig. 4. Rechromatofocusing of β-amylase components V (A) and VI (B).

elution profiles demonstrate the extreme heterogeneity of β-amylase enzymes of barley.

When β-amylase component I was rechromatofocused, a significant amount of activity was detected with an elution pH similar to that of β-amylase III (Fig. 2A). Conversely, β-amylase component III produced appreciable activity with an apparent pI of β-amylase component I (Fig. 2B). In a similar manner, β-amylase components II and IV appeared to be closely related (Fig. 3).

β-Amylase components V, VI, and VII behaved somewhat differently when rechromatofocused (Figs. 4 and 5A). In addition to a major peak for each of these components, varying amounts of activity were detected with apparent pI values similar to β-amylase components I, II, III, and IV. β-Amylase VI also contained β-amylase components V and VII (Fig. 4B), probably as a result of incomplete separation during the initial chromatofocusing. Nevertheless, β-amylase components V, VI, and VII undergo an apparent dissociation, giving rise to β-amylase components I, II, III, and IV.

β-Amylase component VIII was more complex. When subjected to rechromatofocusing, some of this component apparently dissociated, giving rise to β-amylase components V and VI, in addition to β-amylase components I, II, III, and IV.

To confirm the results of these rechromatofocusing experiments, each major β-amylase component (Figs. 2, 3, 4, and 5) was chromatofocused a third time. Each major β-amylase component produced an elution profile similar to those obtained during the second chromatofocusing experiments. For example, β-amylase VIII underwent a further partial dissociation, producing components with elution pH values similar to each of the other major β-amylase components I to VII (Fig. 6).

The elution pH values of each of the major β-amylase components determined from a number of chromatofocusing experiments are presented in Table I. Each of the components eluted with peak widths in the range of ±0.04 to ±0.06 pH units.

Studies of the action pattern of each of the major β-amylase components on amylose following three passes through chromatofocusing columns showed that maltose was the only sugar produced during hydrolysis (not shown). This confirms that elution profiles for each of the major components are a result of β-amylolysis.

The heterogeneity and complexity of β-amylase enzymes of Bonanza barley were confirmed by isoelectric focusing (Figs. 7 and 8). A multiplicity of major and minor β-amylase components were

TABLE I
Elution pH of Beta-Amylase Components from Barley

Component	No. of Determinations	Elution pH	Standard Deviation
I	17	6.46	0.04
II	17	6.26	0.04
III	15	6.00	0.05
IV	15	5.82	0.04
V	8	5.32	0.05
VI	7	5.27	0.05
VII	8	5.16	0.04
VIII	6	4.82	0.06

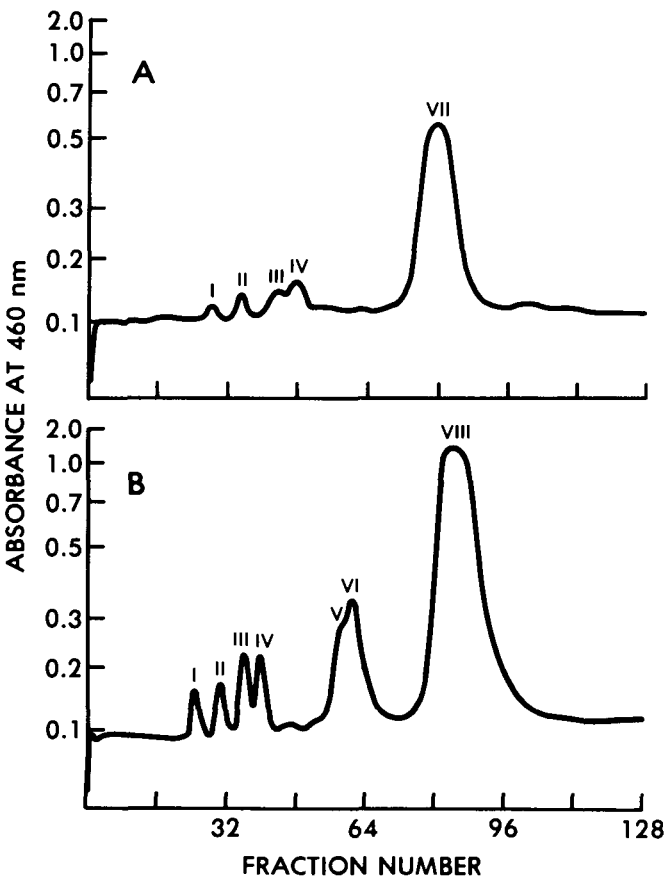

Fig. 5. Rechromatofocusing of β-amylase components VII (A) and VIII (B).

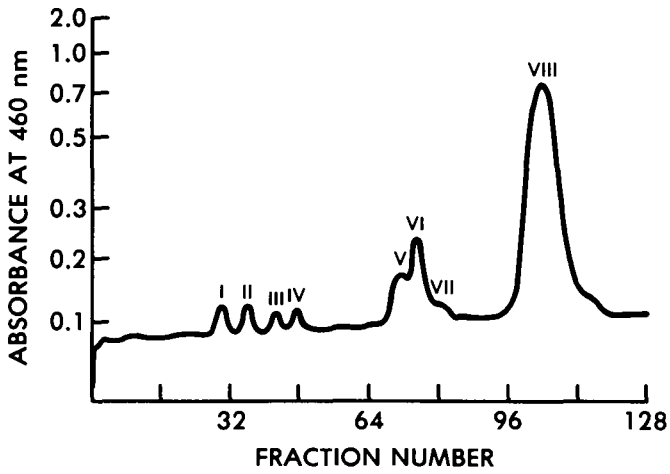

Fig. 6. Further apparent dissociation of β-amylase VIII (Fig. 5B) when subjected to another rechromatofocusing experiment.

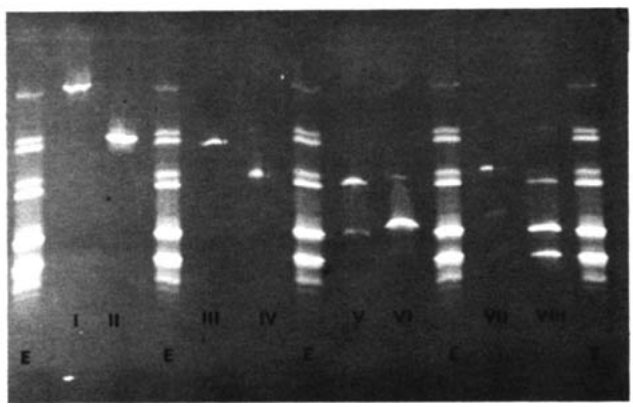

Fig. 7. Isoelectric focusing of barley extract (E) and β-amylase components I to VIII in a pH 6.5 (top) to pH 4.0 (bottom) gradient. Gel incubated on a Lintner starch plate for 8 min.

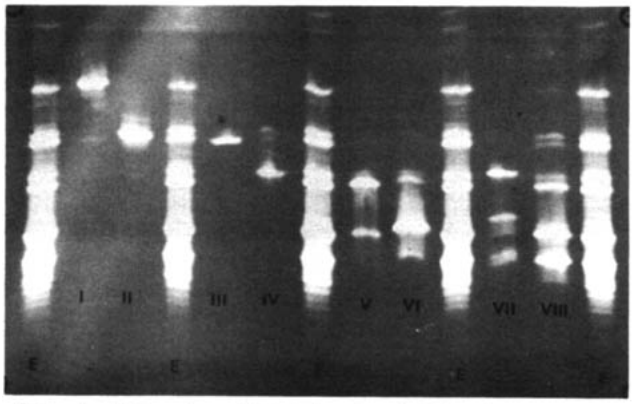

Fig. 8. Isoelectric focusing gel (Fig. 7) incubated with a Lintner starch plate for 40 min.

detected in crude extracts (E) of barley by this technique. Each of the major bands observed by isoelectric focusing corresponded to one of the major β-amylase components (I–VIII) isolated after three separations by chromatofocusing (Fig. 7). Long incubation of a starch gel plate by a sandwich technique with an isoelectric focusing gel (Fig. 8) reveals the heterogeneity of each of the β-amylase components isolated by chromatofocusing. Thus, results obtained by isoelectric focusing tend to confirm that purified β-amylase components undergo molecular rearrangements contributing to the heterogeneity of β-amylase components observed in crude preparations.

The interrelationships of the various β-amylase enzymes of barley are not defined clearly in this study. However, chromatofocusing has proven to be a very useful technique for isolating substantial quantities of the various β-amylase components for further characterization.

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LITERATURE CITED

1. Allison, M. J., and Ellis, R. P. *Biochem. Genet.* 10:165, 1973.
2. Allison, M. J., and Swanston, J. S. *J. Inst. Brew.* 80:285, 1974.
3. Bendelow, V. M. *J. Am. Soc. Brew. Chem.* 35:81, 1977.
4. Daussant, J., and Grabar, P. *Eur. Brew. Conv., Proc. Congr. 10th, Stockholm, 1965*, p. 62.
5. Frydenberg, O., and Nielsen, G. *Hereditas* 54:123, 1965.
6. Hejgaard, J. *Physiol. Plant.* 38:293, 1976.
7. Hejgaard, J., and Carlsen, S. *J. Sci. Food Agric.* 28:900, 1977.
8. Hejgaard, J. *J. Inst. Brew.* 84:43, 1978.
9. Hejgaard, J. *Physiol. Plant.* 54:174, 1982.
10. Hejgaard, J. *J. Inst. Brew.* 83:94, 1977.
11. LaBerge, D. E., Clayton, J. W., and Meredith, W. O. S. *Am. Soc. Brew. Chem., Proc. 1967*, p. 18.
12. LaBerge, D. E., and Meredith, W. O. S. *J. Inst. Brew.* 75:19, 1969.
13. LaBerge, D. E., and Meredith, W. O. S. *J. Inst. Brew.* 77:436, 1971.
14. MacGregor, A. W., Thompson, R. G., and Meredith, W. O. S. *J. Inst. Brew.* 80:181, 1974.
15. Mugibayashi, N., and Shinke, R. *J. Agric. Chem. Soc. Jpn.* 41:295, 1967.
16. Niku-Paavola, M.-L., Skakoun, A., Nummi, M., and Daussant, J. *Biochim. Biophys. Acta* 322:181, 1973.
17. Nummi, M., Vilhunen, R., and Enari, T.-M. *Eur. Brew. Conv., Proc. Congr. 10th, Stockholm, 1965*, p. 52.
18. Nummi, M., Vilhunen, R., and Enari, T.-M. *Acta Chem. Scand.* 19:1793, 1965.
19. Nummi, M., Vilhunen, R., and Enari, T.-M. *Fin. Kemistsamf. Medd.* 74:49, 1965.
20. Nummi, M., Seppala, P., Vilhunen, R., and Enari, T.-M. *Acta Chem. Scand.* 19:2003, 1965.
21. Nummi, M., Niku-Paavola, M.-L., and Enari, T.-M. *Acta Chem. Scand.* 26:1731, 1972.
22. Pharmacia Fine Chemicals AB, Uppsala, Sweden. Chromatofocusing with Polybuffer™ and PBE™. 1980–81.
23. Sherman, R. A., and Lewis, M. J. *Proc. Soc. Brew. Chem.*, 33(1):26, 1975.
24. Shinke, R., and Mugibayashi, N. *Agric. Biol. Chem.* 35:1381, 1971.
25. Sluyterman, L. A. AE., and Elgersma, O. *J. Chromatogr.* 150:17, 1978
26. Sluyterman, L. A. AE., and Wijdenes, J. *J. Chromatogr.* 150:31, 1978.
27. Sluyterman, L. A. AE., and Wijdenes, J. *J. Chromatogr.* 206:429, 1981.
28. Sluyterman, L. A. AE., and Wijdenes, J. *J. Chromatogr.* 206:441, 1981.
29. Trevelyan, W. E., Procter, D. P., and Harrison, J. S. *Nature* 166:444, 1950.
30. Umeki, K., and Kainuma, K. *J. Chromatogr.* 150:242, 1978.
31. Visuri, K., and Nummi, M. *Eur. J. Biochem.* 28:555, 1972.

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