Separation of Barley Malt Alpha-Amylase by Chromatofocusing[1]

B. A. MARCHYLO and A. W. MacGREGOR, Canadian Grain Commission, Grain Research Laboratory, Winnipeg, Manitoba, Canada R3C 3G8

Cereal Chem. 60(4):311-314

ABSTRACT

Chromatofocusing was used to resolve green malted barley α-amylase into individual components. Five peaks were eluted at 4°C, whereas four peaks were eluted at room temperature (22°C). Analysis of eluent fractions by polyacrylamide gel isoelectric focusing showed that the α-amylase III fraction was converted to a specific α-amylase II component under chromatofocusing conditions. Evidence for interconversion of α-amylase II components also was obtained by chromatofocusing and polyacrylamide gel isoelectric focusing analysis.

Germinated or malted barley α-amylase multiple forms have been separated by agar gel electrophoresis (Frydenberg and Nielsen 1965), polyacrylamide gel electrophoresis (Van Onckelen and Verbeek 1969), ion-exchange column chromatography (MacGregor et al 1971), and polyacrylamide gel isoelectric focusing (PAG-IEF) (MacGregor 1977). By PAG-IEF, malted barley α-amylase has been resolved into three main groups, designated α-amylases I, II, and III (MacGregor 1977). Each group has been shown to be heterogeneous (MacGregor and Daussant 1981). Quantitative PAG-IEF (MacGregor and Ballance 1980) and immunochemical studies (MacGregor and Daussant 1981) have shown that α-amylase III components can be converted to α-amylase II components by appropriate heat treatment or kilning.

Chromatofocusing is a new column chromatographic technique (Sluyterman and Elgersma 1978; Sluyterman and Wijdenes 1978, 1981a, 1981b; Pharmacia Fine Chemicals, 1980) that separates proteins on the basis of isoelectric pH. This technique has been used to resolve germinated wheat α-amylase isoenzymes (Marchylo and Kruger, 1983). The purpose of this study was to determine whether chromatofocusing could be used to resolve individual malt barley α-amylase components.

MATERIALS AND METHODS

Five-day green malt was prepared from six-rowed barley cultivar Bonanza, using steeping and germination units previously described (Bettner et al 1962). The green malt was stored frozen.

Extraction

Ten kernels of green malt were homogenized with a pestle in a mortar containing 1 g of sand and 10 ml of cold buffer (0.025*M* imidazole-HCl, pH 7.4). The extract was centrifuged twice (23,600 × *g*) at 4°C for 15 min.

Column Preparation

Polybuffer exchanger™ 94[2] (PBE) was equilibrated and packed as recommended by the manufacturer (Pharmacia Fine Chemicals 1980). The start buffer used was 0.025*M* imidazole-HCl, pH 7.4. Pharmacia K9/60 columns with bed heights of 45 cm were used. Columns were packed at flow rates of 2.5 ml/min. Two to four centimeters of Sephadex™ G-25 (coarse) was layered on top of the bed to ensure even sample application.

Sample Application and Elution

Samples were applied by gravity. Following sample application, eluent buffer (Polybuffer™ 74 diluted 10 times with distilled water, pH 5.0, containing $10^{-4}M$ $CaCl_2$) was pumped through the column at 0.5 ml/min. Fractions (2 ml) were collected with an LKB UltroRac fraction collector (LKB Producter, Bromma, Sweden) equipped with a drop counter. Columns were regenerated as described by the manufacturer (Pharmacia Fine Chemicals 1980) and were repacked after every three to four runs or two weeks of use. All analyses were duplicated.

Rechromatofocusing

Appropriate fractions were pooled and frozen following five chromatofocusing analyses at 4°C. Before rechromatofocusing, pooled fractions were thawed, and a 10-ml aliquot was applied to the column. Pooled fractions were not dialyzed against start buffer before rechromatofocusing.

α-Amylase Activity Analysis

Total α-amylase activity per fraction was determined, as described by Kruger and Tipples (1981), with a Perkin-Elmer model 191 Grain Amylase Analyzer. When required, fractions were diluted with 0.2*M* sodium acetate buffer, pH 5.5, containing $10^{-3}M$ $CaCl_2$, before activity analysis.

Isoelectric Focusing

Prepared thin-layer polyacrylamide gel plates (Ampholine PAG plate kits, pH 3.5–9.5, LKB Producter AB, Bromma, Sweden) were used for isoelectric focusing, which was done with a Desaga TLE-Double Chamber 12 20 00 (C. Desaga, Heidelberg, Germany). A 450-g weight was placed across the electrodes to facilitate contact between gel, wick, and electrode. Constant power (10 W) was maintained throughout the 2-hr run by an Isco model 494 electrophoresis power supply (Instrument Specialties Company, Lincoln, NE). The pH gradient across the PAG plate was determined with a Multiphor pH surface electrode (LKB Producter AB, Bromma, Sweden).

Detection of α-Amylases

α-Amylases were detected by use of the β-limit dextrin-plate technique described previously (Marchylo et al 1980, MacGregor et al 1974). Incubation time for the β-limit dextrin-polyacrylamide gel sandwich was 15–20 min for eluent fractions and 5–7 min for extracts.

RESULTS AND DISCUSSION

Chromatofocusing at room temperature (22°C) and at 4°C resolved green malt α-amylase into four and five peaks of activity (Fig. 1A, B), respectively. As determined in 12 chromatofocusing analyses, peaks 1–5 eluted reproducibly at average elution pHs of 6.33 ± 0.05, 6.03 ± 0.04, 5.84 ± 0.04, 5.68 ± 0.08, and 5.25 ± 0.07. Of the total α-amylase activity applied to the column, 45.6% was recovered in room temperature analyses, whereas 77.1% was recovered at 4°C. Low enzyme recovery at room temperature also has been reported in chromatofocusing analyses of germinated wheat α-amylases (Marchylo and Kruger 1983). Peaks 1 and 2 displayed substantial decreases in relative peak heights at room temperature (Fig. 1A) as compared to 4°C (Fig. 1B). This decrease may be attributed to differential heat stability of these peaks at room temperature as compared to 4°C. An alternate explanation

[1] Paper no. 510 of the Canadian Grain Commission, Grain Research Laboratory, 1404-303 Main Street, Winnipeg, Manitoba R3C 3G8.

[2] Polybuffer exchanger 94, Polybuffer 74, and Sephadex are the exclusive trademarks of Pharmacia Fine Chemicals AB, Uppsala, Sweden.

will be presented later in this report.

Resolution of peaks was better at room temperature than at 4°C. In particular, peaks 3 and 4 showed less overlap at room temperature. Peak 5 was well separated from peaks 1 to 4 at both temperatures.

Malt extract was analyzed by PAG-IEF to determine the pattern of α-amylase components (Figs. 2A, 3A). The three main groups of α-amylase detected were designated α-amylase I, II, and III, as described previously (MacGregor and Daussant 1981). To facilitate discussion, these groups were subdivided further, as shown in Figs. 2A and 3A. Eluent fractions from the chromatofocusing runs then were analyzed by PAG-IEF to determine which α-amylase components were eluted in each peak (Figs. 2B,C and 3B,C). All major α-amylase components and a majority of minor α-amylase components were recovered following chromatofocusing. Minor components that were not recovered were present only in trace levels in the original extract (Figs. 2A and 3A), and these may have been lost due to dilution during chromatofocusing.

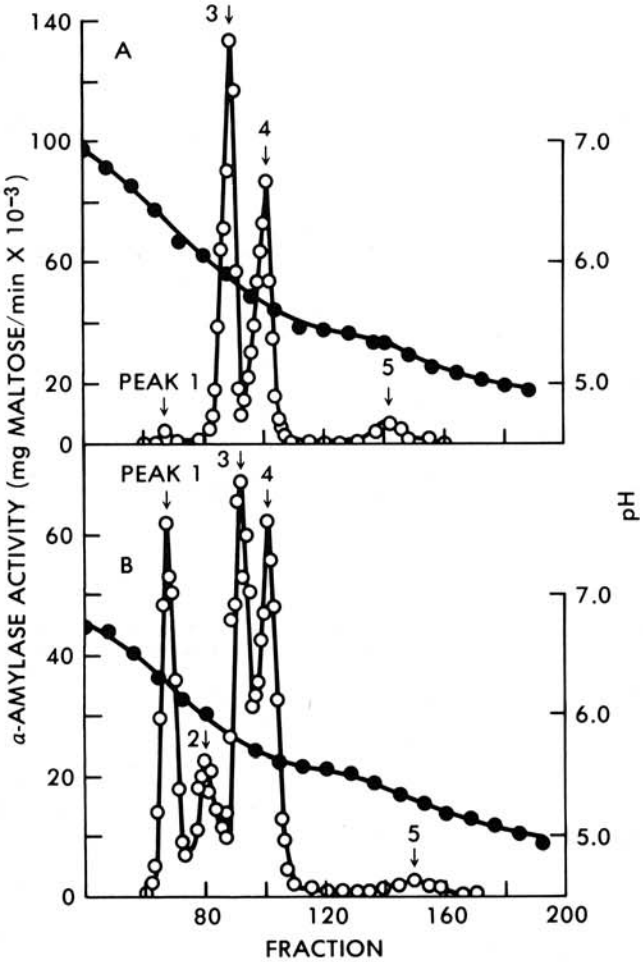

Fig. 1. Separation of green malt α-amylase by chromatofocusing (open symbols). pH gradient obtained during chromatofocusing (closed symbols). Column: K9/60. Bed height 45 cm. Elution conditions: start buffer 0.025*M* imidazole-HCl, pH 7.4; eluent buffer Polybuffer 74 diluted 10 times containing 10^{-4}*M* $CaCl_2$, pH 5.0. Flow rate 0.5 ml/min. **A,** room temperature (22°C); extract volume, 4 ml; **B,** 4°C; extract volume, 2 ml.

The major component of peak 1, as assessed by visual band intensity, was α-amylase IIb for chromatofocusing analyses at both room temperature and 4°C. Significant levels of α-amylase IIIa and b were present at room temperature and 4°C. The presence of both α-amylase II and α-amylase III components in peak 1 is surprising, since α-amylase components should elute in order of descending pI. On this basis, α-amylase III components should have eluted first because PAG-IEF analysis of green malt barley α-amylase showed that α-amylase III components had higher pIs (6.7–6.8 ± 0.2 pH units) than α-amylase II components (6.1 ± 0.2 – 6.3 ± 0.1 pH units).

To verify further the composition and purity of peak 1, pooled eluent fractions from the peak maximum were rechromatofocused. As shown in Fig. 4A, two peaks equivalent in elution pH to peaks 1 and 3 (Figs. 1A, 1B) were eluted. PAG-IEF analysis of these two peaks showed that peak 1 again was composed of a mixture of α-amylase IIIa and b and α-amylase IIb components, whereas peak 3 was composed of α-amylase IIb. These results indicate that α-amylase III components are being converted specifically to α-amylase IIb. This supports previous studies (MacGregor and Ballance 1980, MacGregor and Daussant 1981) showing that α-amylase III is converted to α-amylase II during ion-exchange chromatography, heat treatment, or kilning.

Peak 2, which was eluted at 4°C (Fig. 1B), was made up of a mixture of α-amylase II and III components. α-Amylase IIa was the major component of this peak, but significant levels of α-amylase IIIa, IIb, and IIc also were present. The presence of α-amylase III components as well as of α-amylase IIb is probably due to contamination of peak 2 by peaks 1 and 3. As shown in Fig. 1B, there is considerable overlap between these peaks and, as shown by rechromatofocusing and PAG-IEF analyses, peak 1 is composed of α-amylase IIIa, b, and α-amylase IIb, whereas peak 3 is made up of α-amylase IIb. This leaves α-amylase IIa and IIc as the primary components of peak 2. It is surprising that α-amylase IIa is present in this peak, since the major proportion of this component elutes in peak 4. These results suggest that α-amylase IIc may be converting

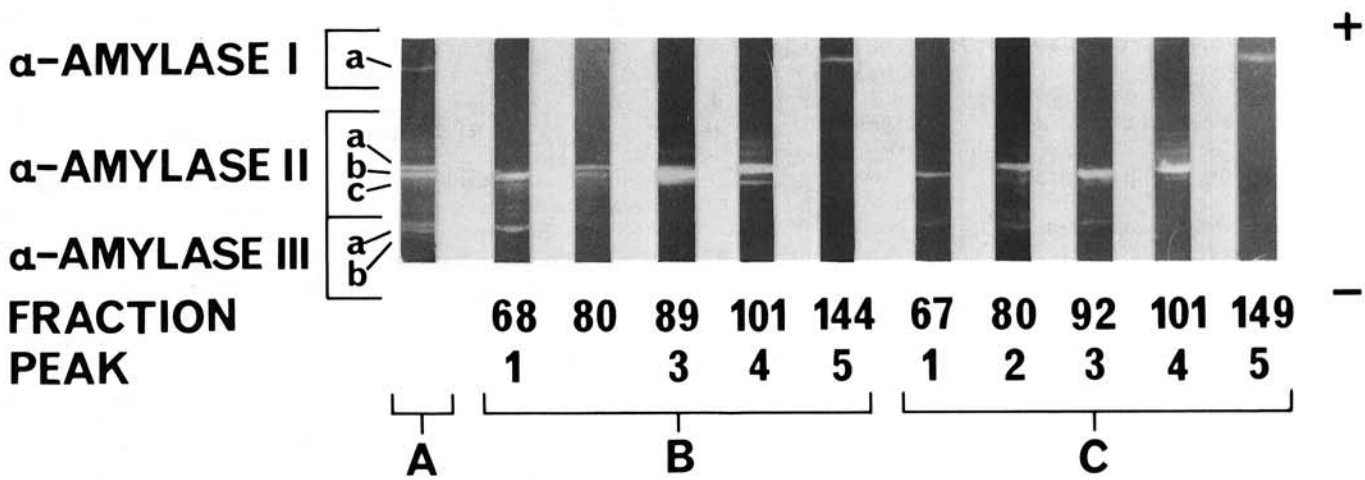

Fig. 2. Polyacrylamide gel isoelectric focusing of green malt barley α-amylases in a pH 3.5–9.5 gradient. Zymograms of: **A,** green malt barley extract; **B,** eluent fractions from room temperature (22°C) chromatofocusing analysis; **C,** eluent fractions from 4°C chromatofocusing analysis.

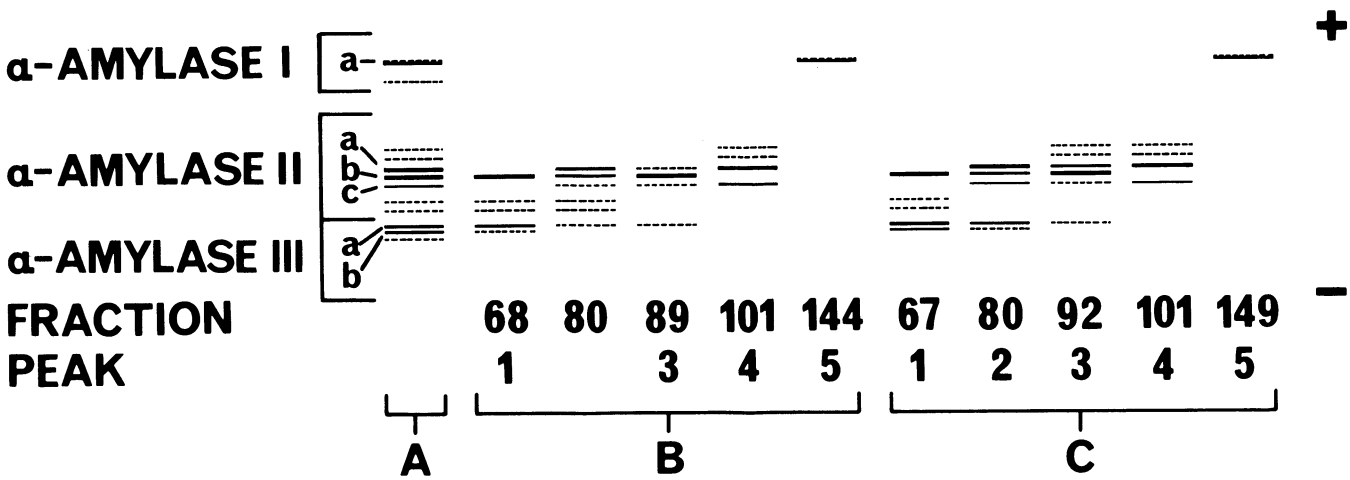

Fig. 3. Polyacrylamide gel isoelectric focusing of green malt barley α-amylases in a pH 3.5–9.5 gradient. Line drawings of: **A,** green malt barley extract; **B,** eluent fractions from room temperature (22°C) chromatofocusing analysis; **C,** eluent fractions from 4°C chromatofocusing analysis.

to α-amylase IIa.

Rechromatofocusing of peak 2 yielded four peaks. The major portion of the activity was present as peak 4, as shown in Fig. 4B. Low levels in peak 2 were present, and peaks 1 and 3 were barely detectable. PAG-IEF analysis showed that peak 1 was comprised of α-amylase IIIa, b and IIb, and peak 3 of α-amylase IIb. Peak 2 appeared to be comprised of α-amylase IIa with no trace of α-amylase IIc. As with α-amylase III components, α-amylase IIc appears to be converted to α-amylase IIb. Peak 4 was composed primarily of α-amylase IIa, with only a trace of α-amylase IIc detectable.

The conversion of α-amylase III components and α-amylase IIc during chromatofocusing also may be temperature sensitive. As shown in Fig. 1A and B, relative peak height decreases substantially for peaks 1 and 2 when chromatofocusing is performed at room temperature. This decrease may be partially due to the conversion of α-amylase III components to α-amylase IIb and α-amylase IIc to α-amylase IIa at 22°C. Although peak 2 was not detected at room temperature, PAG-IEF analysis of eluent fractions at the peak 2 elution pH (as determined in Fig. 1B) detected trace levels of peak 2 α-amylase components (Figs. 2B and 3B). Previous studies (MacGregor and Ballance 1980, MacGregor and Daussant 1981) have shown that α-amylase III components are converted to α-amylase II components when subjected to extreme temperatures associated with kilning and heat treatment. Results from this study show that this conversion is facilitated during chromatofocusing at much lower temperatures. Peak 3 was composed primarily of α-amylase IIb in both the room temperature (Figs. 2 and 3B) and 4°C analyses (Figs. 2 and 3C). Only trace levels of α-amylase IIa, c, and α-amylase IIIa were detectable in the room temperature analysis, which is indicative of the excellent resolution achieved by chromatofocusing. Slightly higher levels of α-amylase IIa were detected in peak 3 at 4°C.

Rechromatofocusing of pooled peak 3 eluent fractions yielded one peak with the same elution pH (Fig. 4C). PAG-IEF analysis showed that this peak was composed of α-amylase IIb. α-Amylase III components were not detected. Thus, the conversion of α-amylase IIb back to α-amylase III components does not appear to occur.

α-Amylase IIa was the major component of peak 4 at 4°C and room temperature. However, low levels of α-amylase IIc also were detectable. Rechromatofocusing of pooled peak 4 eluent fractions yielded a single peak with comparable elution pH (Fig. 4D). PAG-IEF analysis of this peak revealed that α-amylase IIa was the major constituent, but traces of α-amylase IIc also were present. This suggests that α-amylase IIb may be converted to a limited extent to α-amylase IIc. Further work will be necessary to determine the extent of this interconversion.

α-Amylase Ia was the major component in peak 5 (Figs. 2B,C

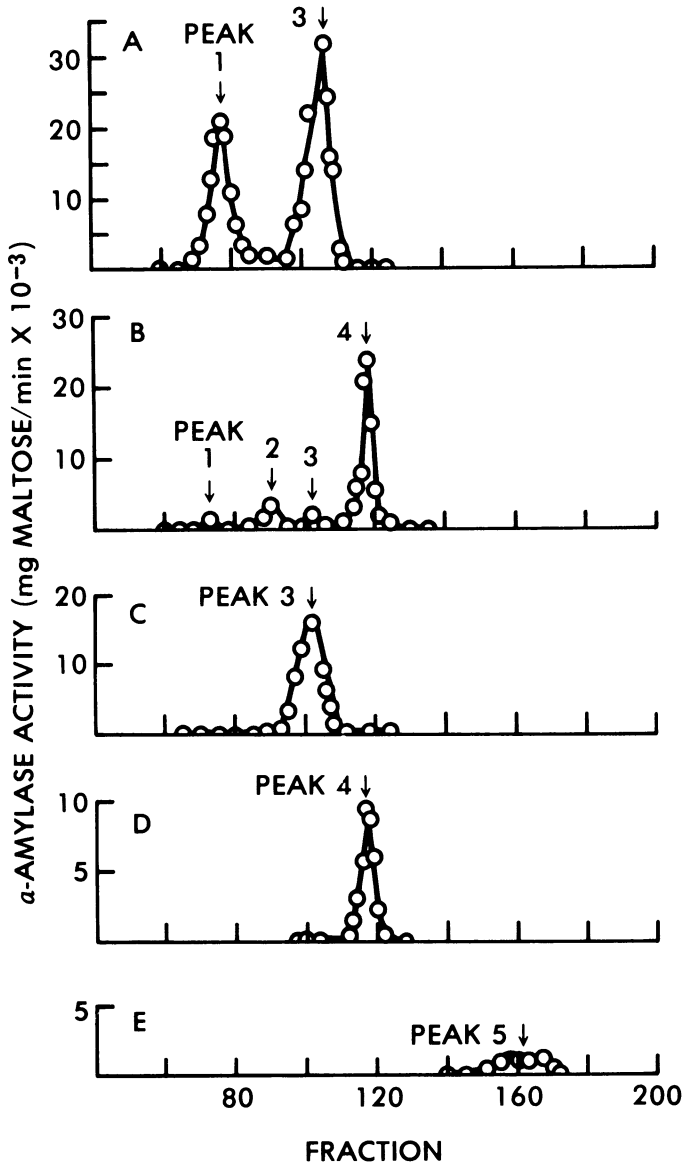

Fig. 4. Rechromatofocusing analysis of peaks 1–5 eluted during chromatofocusing of green malt barley α-amylase at 4°C. Column: K9/60. Bed height 45 cm. Elution conditions: start buffer, 0.025*M* imidazole-HCl; pH 7.4; eluent buffer, Polybuffer 74 diluted 10 times containing $10^{-4}M$ $CaCl_2$, pH 5.0. Flow rate 0.5 ml/min; temperature 4°C; sample volume 10 ml; **A** (peak 1); **B** (peak 2); **C** (peak 3); **D** (peak 4); **E** (peak 5).

TABLE I
Proportion of α-Amylase Components Following Chromatofocusing

Chromatofocusing Temperature (°C)	Percent Recovery After Chromatofocusing		
	α-Amylase III[a] (peak 1)	α-Amylase II[a] (peaks 2,3,4)	α-Amylase I[a] (peak 5)
4	23.1	74.0	2.9
22	2.6	92.3	5.2

[a] As percent total activity recovered from column.

and 3B,C). Rechromatofocusing of pooled peak 5 eluent fractions yielded a single peak of comparable elution pH (Fig. 4E). α-Amylase Ia also was the major constituent of this peak, as shown by PAG-IEF analysis. Thus, α-amylase Ia does not appear to be converted to other α-amylase components.

Chromatofocusing analysis at 4°C (Table I) showed that only 23.1% of total green malt α-amylase activity is associated with α-amylase III components. In contrast, previous quantitative isoelectric focusing studies indicated that 89% of total α-amylase activity in green malted barley is contributed by α-amylase III components (MacGregor and Daussant 1981). This apparent disparity in results may reflect a difference in stability of α-amylase III components during isoelectric focusing as compared to chromatofocusing. Also, these results signify that the proportion of activity attributable to the α-amylase III fraction is dependent on the separatory technique used. Chromatofocusing analysis at room temperature further decreased the contribution of the α-amylase III fraction to 2.6% (Table I). This result is comparable to previously reported quantitative isoelectric focusing analysis of heat-treated α-amylase from green barley malt (MacGregor and Ballance 1980). This further emphasizes the decreased stability of the α-amylase III fraction during chromatofocusing at room temperature.

This study has shown that chromatofocusing can quantitatively separate individual α-amylase components from green malted barley, and for this reason the technique will prove very useful in the study of these enzymes. α-Amylase III components have been shown to convert to a specific α-amylase II component (α-amylase IIb) under chromatofocusing conditions. In addition, evidence suggests that an α-amylase II component (α-amylase IIc) can convert to another α-amylase II component (α-amylase IIa). The nature of the α-amylase III and α-amylase IIc components remains unknown but they may be α-amylase II protein complexes. Further investigations will be necessary to elucidate the nature of these components.

ACKNOWLEDGMENTS

The authors thank S. Gowan for her excellent technical assistance and J. E. Kruger for the use of laboratory facilities and his helpful comments.

LITERATURE CITED

BETTNER, R. E., MEREDITH, W. O. S., and ANDERSON, J. A. 1962. Page 5 in: Proceedings of the American Society of Brewing Chemists. The Society, Minneapolis, MN.

FRYDENBERG, O., and NIELSEN, G. 1965. Amylase isozymes in germinating barley seeds. Hereditas 54:123.

KRUGER, J. E., and TIPPLES, K. H. 1981. Modified procedure for use of the Perkin-Elmer model 191 Grain Amylase Analyzer in determining low levels of α-amylase in wheats and flours. Cereal Chem. 58:271.

MacGREGOR, A. W. 1977. Isolation, purification, and electrophoretic properties of an α-amylase from malted barley. J. Inst. Brew. 83:100.

MacGREGOR, A. W., and BALLANCE, D. 1980. Quantitative determination of α-amylase enzymes in germinated barley after separation by isoelectricfocusing. J. Inst. Brew. 86:131.

MacGREGOR, A. W., and DAUSSANT, J. 1981. Isoelectricfocusing and immunochemical analysis of germinated barley α-amylases after freeze-drying and kilning. J. Inst. Brew. 87:155.

MacGREGOR, A. W., LaBERGE, D. E., and MEREDITH, W. O. S. 1971. Separation of α- and β-amylase enzymes from barley malt by ion-exchange chromatography. Cereal Chem. 48:490.

MacGREGOR, A. W., THOMPSON, R. G., and MEREDITH, W. O. S. 1974. α-Amylase from immature barley: purification and properties. J. Inst. Brew. 80:181.

MARCHYLO, B. A., and KRUGER, J. E. 1983. Separation of wheat α-amylase isoenzymes by chromatofocusing. Proceedings of the Third International Symposium on Preharvest Sprouting Damage in Cereals, 1982. J. E. Kruger and D. E. LaBerge, eds. Westview Press, Boulder, CO.

MARCHYLO, B. A., LaCROIX, L. J., and KRUGER, J. E. 1980. α-Amylase isoenzymes in Canadian wheat cultivars during kernel growth and maturation. Can. J. Plant Sci. 60:433.

PHARMACIA FINE CHEMICALS. 1980. Chromatofocusing with Polybuffer™ and PBE. Pharmacia, Uppsala, Sweden.

SLUYTERMAN, L. A. AE., and ELGERSMA, O. 1978. Chromatofocusing: Isoelectric focusing on ion-exchange columns. I. General principles. J. Chromatogr. 150:17.

SLUYTERMAN, L. A. AE., and WIJDENES, J. 1978. Chromatofocusing: Isoelectric focusing on ion-exchange columns. II. Experimental verification. J. Chromatogr. 150:31.

SLUYTERMAN, L. A. AE., and WIJDENES, J. 1981a. Chromatofocusing: III. The properties of DEAE-agarose anion exchanger and its suitability for protein separations. J. Chromatogr. 206:429.

SLUYTERMAN, L. A. AE., and WIJDENES, J. 1981b. Chromatofocusing: IV. Properties of an agarose polyethyleneimine ion exchanger and its suitability for protein separations. J. Chromatogr. 206:441.

Van ONCKELEN, H., and VERBEEK, R. 1969. La formation des isozymes de l'α-amylase durant la germination de l'orge. Planta 88:255.

[Received October 8, 1982. Accepted March 18, 1983]