RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF BARLEY MALT α-AMYLASE ON CYCLOBOND COLUMNS

CYNTHIA A. HENSON*

U.S.D.A. Agricultural Research Service, Cereal Crops Research Unit, Department of Agronomy, University of Wisconsin, 1575 Linden Dr., Madison, WI 53706 (U.S.A.)

and

JULIE M. STONE

Department of Agronomy, University of Wisconsin, 1575 Linden Dr., Madison, WI 53706 (U.S.A.)

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SUMMARY

A procedure for separation of α- and β-amylases was developed which results in their complete resolution in less than 20 min. A cyclodextrin stationary phase column was equilibrated in 10 mM phosphoric acid at pH 7.0. Purified barley malt α- or β-amylases, mixtures of both, or crude malt extracts were injected. β-Amylase did not bind to the column and was rapidly eluted with water or buffer. α-Amylases specifically bind to the immobilized dextrin. Optimal elution of α-amylase was achieved with a 10-ml gradient from 0 mg/ml β-cyclodextrin (cycloheptaamylose) in buffer to 12 mg/ml β-cyclodextrin in 15% aqueous methanol, followed by flushing with 20 ml of 12 mg/ml β-cyclodextrin in 15% aqueous methanol. Elution buffer containing β-cyclodextrin at pH values from 6.0 to 7.0 was not as effective in eluting α-amylase as was cyclodextrin in aqueous methanol. Inclusion of methanol in the gradient resulted in enhanced recoveries of α-amylase. α-Amylase did not bind to the column at pH values higher than 7.0. This procedure should be useful for rapid separation of plant α- and β-amylases, separation of pullulanases or debranching enzymes from other carbohydrases, and purification of α-amylases.

INTRODUCTION[a]

The use of high-performance liquid chromatography (HPLC) has, within the last decade, become commonplace in protein chemistry. The rapid separations achievable with HPLC combined with the power of affinity chromatography can result in separation and purification of proteins in a few days.

Affinity chromatography of plant amylases was accomplished in 1933 using

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starch as the enzyme-specific ligand[1]. Cyclohexaamylose-bonded Sepharose, to which sweet potato β-amylase specifically adsorbs[2], as well as cycloheptaamylose[3] and glycogen[4] bound to Sepharose have been used successfully for purification of cereal amylases. Although these resins provide a very powerful tool in protein purification, the bonded ligand responsible for the biospecificity bleeds off the column with repeated use.

Commercially available cyclodextrin-bonded silica HPLC resins which are stable in both aqueous and organic mobile phases have been recently developed for use in separation of optical isomers [e.g., D,L-amino acids; ($\pm$)-barbital derivatives] and structural isomers (e.g., α- and β-naphthoflavones; o, m, p-xylenes)[5,6]. These columns are available with cyclohexaamylose, cycloheptaamylose or cyclooctaamylose chemically bonded to a spherical silica gel support through a non-nitrogenous (i.e. amines or amides) spacer arm.

We report here the use of a commercially available affinity column (Cyclobond I) for the separation and purification of carbohydrases commonly found in plant tissues —including α-amylase, β-amylase, and pullulanase (debranching enzyme). The main advantages of using HPLC over conventional open column chromatography are that separations of various carbohydrases can be achieved in 20 min rather than 6–8 h and that very stable bonded phases for separation are available.

EXPERIMENTAL

Enzyme sources

Preparation of purified α-amylases. Barley (*Hordeum vulgare* cv. Morex) malt was ground with a VirTis homogenizer in extraction buffer (40 mM Tris, pH 8.0, containing 1 mM CaCl$_2$) with a ratio of 5 ml buffer/1 g malt. The extract was centrifuged at 6000 g for 40 min (4°C). Supernatants were heated at 70°C for 15 min to inactivate β-amylase. After clarification by centrifugation at 15 000 g for 15 min, heated supernatants were chromatofocused on a Polybuffer exchanger 94 column (71 × 1.5 cm) equilibrated with 25 mM imidazole, pH 7.4. Elution was with a 1:10 dilution of Polybuffer 74, pH 4.0. α-Amylase activities were detected as described before[7]. The fractions containing high pI α-amylase activities were pooled, dialyzed against 40 mM Tris, pH 8.0 containing 1 mM CaCl$_2$, and then concentrated with an Amicon YM-10 membrane.

Preparation of crude malt extract. Morex malt was ground in extraction buffer. Grinding ratios of 5:1 were used when extracting pullulanase and 10:1 when extracting other carbohydrases. The homogenates were centrifuged at 12 000 g for 15 min (4°C), and then dialyzed against extraction buffer.

Purified barley malt β-amylase (No. 13440) was purchased from Serva (Westbury, NY, U.S.A.) and solubilized in extraction buffer (0.5 mg β-amylase/ml). Pullulanase (P-2138) and bovine serum albumin (BSA) (A-7906) were purchased from Sigma (St. Louis, MO, U.S.A.).

Enzyme assays

Total amylolytic activities were assayed with soluble starch as the substrate. Reducing sugars were detected with 3,5-dinitrosalicylic acid[7]. Assays using soluble starch as the substrate detect α-amylase, β-amylase, and debranching or pullulanase

type enzymes. Starch azure assays, which are specific for α-amylase in the presence of β-amylase, were conducted as described in Henson and Stone[8]. Pullulanase was assayed with 2% pullulan as the substrate. Detection was with the dinitrosalicylic acid technique used for total amylolytic assays.

Chromatography

HPLC-grade phosphoric acid, 85%, was purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Phosphoric acid was passed through a C_8 column (Aquapore Octyl RP-300) to remove impurities prior to use as a mobile-phase component. HPLC-grade methanol, ChromAR, was purchased from Mallinckrodt (Paris, KY, U.S.A.). β-Cyclodextrin (C-4767) was purchased from Sigma.

All separations were done at 25°C (FIAtron TC-50 temperature controller) using a Shimadzu LC-6A liquid chromatograph. The separation column, Cyclobond I (10 × 0.46 cm), was obtained from Advanced Separation Technologies, Whippany, NJ, U.S.A. An OH-100 Aquapore guard column (Brownlee Labs., Santa Clara, CA, U.S.A.) was used. The flow-rate was 2 ml/min. The gradient was composed of 0–100% B in 5 min followed by 13 min of 100% B. The composition of solvents A and B varied as described in the figure legends. All solvents and protein solutions were filtered through a 0.45-μm membrane prior to chromatography. The column was cleaned between injections with 100% methanol.

RESULTS AND DISCUSSION

The effects of mobile phase composition on binding and elution of amylases were investigated (Fig. 1). Effects of pH on column selectivity were determined from pH 6.0 to 8.0 in 10 mM phosphate buffer. Data in Fig. 1a–c were obtained using the same pH buffer for both equilibration and elution. A gradient of 0–12 mg β-cyclodextrin/ml equilibration buffer was used for elution (see figure legends). At low pH (6.0 and 6.5), both α- and β-amylase were tightly bound to the column (Fig. 1a and b). At high pH (7.5 and 8.0), both amylases eluted in the void volume (Fig. 1a and b). Fig. 1c shows chromatography under what was determined to be the optimal conditions for separation of α- and β-amylase. These conditions were column equilibration at pH 7.0 and elution with a gradient of β-cyclodextrin in pH 8.0 phosphate buffer. Even though this was optimal, repeated use of elution gradients at pH 8.0 was considered unsuitable because of the instability of the silica resin.

Aqueous–organic mobile phases are commonly used with cyclodextrin stationary phases and frequently result in altered selectivities or retention times. Effects of methanol addition to the β-cyclodextrin gradient, made in unbuffered water, are shown in Fig. 1d. While the retention of either α- or β-amylase was not greatly altered, the addition of an organic modifier resulted in better peak resolution (Fig. 1d) and a 28% greater recovery of the amylolytic activity. Optimal conditions for separation of α- and β-amylase were determined to be on a column equilibrated with 10 mM phosphoric acid at pH 7.0, followed by elution with a gradient of 0–100% solvent B (12 mg β-cyclodextrin/ml of 15% methanol in water) and a 13-min flush with 100% B. Under these conditions approximately 75% of the α-amylase activity applied to the column was recovered in fractions 19–22.

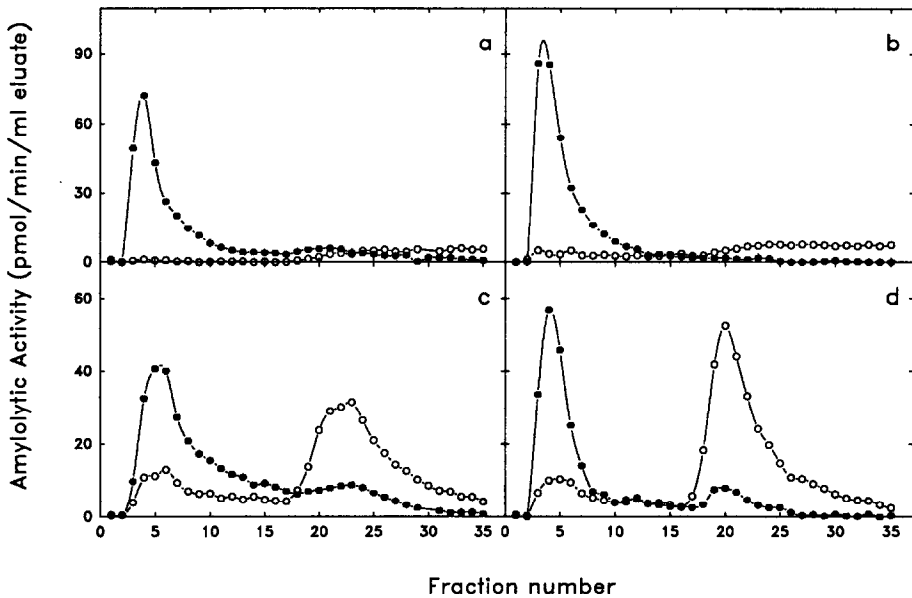Separation on Cyclobond columns is the result of formation of inclusion

Fig. 1. Chromatography of purified barley malt α- and β-amylases on a cyclodextrin-bonded HPLC column. Solvent A was 10 mM phosphoric acid at varying pH values. Solvent B was 12 mg β-cyclodextrin per ml solvent A or aqueous methanol. The gradient was 0–100% B in 5 min and was followed by 13 min flush with 100% B. (a) Purified α-amylase, solvent A was pH 6.5 (○) or 7.5 (●), solvent B was solvent A containing cyclodextrin; (b) purified β-amylase, solvent A was pH 6.5 (○) or 7.5 (●), solvent B was solvent A containing cyclodextrin; (c) purified α-amylase (○) and β-amylase (●) solvent A was pH 7.0 and solvent B was 12 mg β-cyclodextrin/ml pH 8.0 phosphate buffer; (d) same as (c) except solvent B was β-cyclodextrin made in 15% aqueous methanol rather than in solvent A.

complexes between the adsorbed compound and the hydrophobic cavity of the cyclodextrin[9]. Typical compounds which can form inclusion complexes with the hydrophobic cavity of the cyclodextrin ring range from small inorganic ions to aromatic compounds such as substituted benzene isomers and enantiomeric organometallic compounds. The decreased retention of a solute in the presence of an organic modifier is due to competition for the hydrophobic cavity of the cyclodextrin[9]. As the concentration of organic modifier increases, a solute's interaction with the cyclodextrin is decreased until it is eventually no longer retained. It is likely that the binding of at least some of the α-amylase is due to an exposed aromatic amino acid forming an inclusion complex with the cyclodextrin cavity. Indeed, it has been demonstrated that at least one non-catalytically essential tryptophanyl residue is involved in the binding of β-cyclodextrin to α-amylase[10].

As previous data were obtained with partially purified high p*I* α-amylases and highly purified β-amylases, we next tested the separation of carbohydrases from crude barley malt. Using the previously defined optimal conditions, all of the malt α-amylases, both high and low p*I* isozymes, were bound to the column (Fig. 2a; open circles). The first peak of amylolytic activity detected is β-amylase free of contaminating α-amylase. This peak of activity corresponds well with the β-amylase "standard" shown in Fig. 1d. The second peak of activity detected with dinitrosalicylic
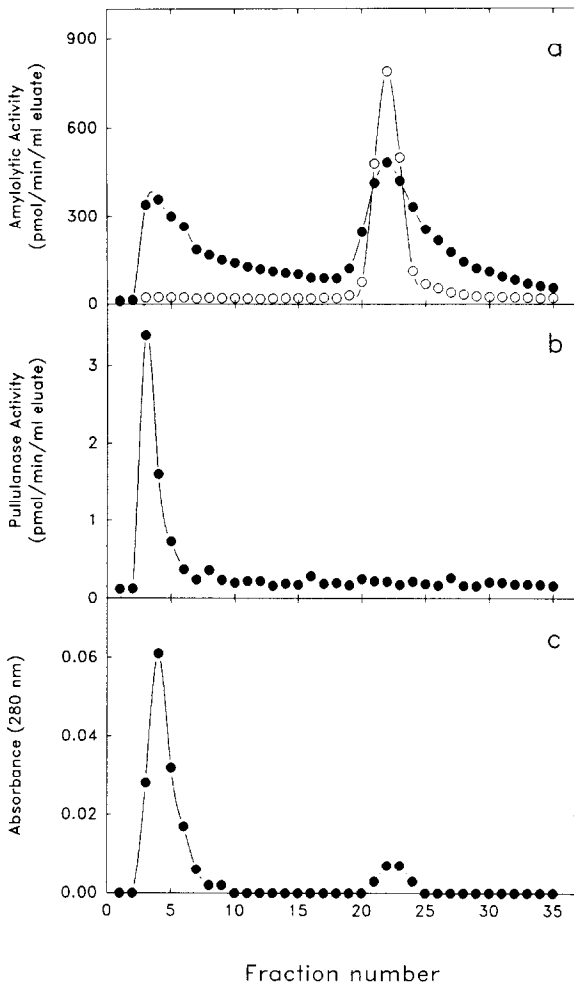
Fig. 2. Chromatography of crude barley malt extract on a cyclodextrin-bonded HPLC column. Solvent A was 10 mM phosphoric acid at pH 7.0. Solvent B was 12 mg β-cyclodextrin/ml 15% aqueous methanol. The gradient was from 0–100% B in 5 min and was followed by 13 min flush with 100% B. (a) α-Amylase activity measured with the starch azure assay (○), total amylolytic activity measured as reducing sugar production from soluble starch (●); (b) pullulanase or debranching enzyme activity; (c) elution profile of malt proteins.

acid coelutes with the α-amylase "standard" (Fig. 1d), and with the single peak of endolytic amylase activity detected by the starch azure assay. Hence, the second peak in Fig. 2a is α-amylase. The α-amylase recovered in the second peak migrated as a single band on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (Fig. 3) and thus was purified to homogeneity by chromatography of crude malt extracts on the cyclobond column.

Pullulanase, or debranching enzyme, is present in crude malt extracts in low levels although much greater activities are found in unkilned malt and in tissues of other plants. Malt pullulanase does not bind to the β-cyclodextrin ring of the
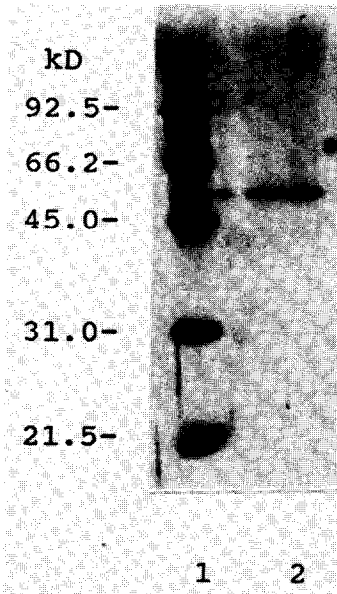
Fig. 3. SDS-PAGE[14] of crude barley malt extract proteins eluted in fractions 19–22. Lane 1 contains molecular weight standards (in order of decreasing molecular weight: phosphorylase B, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor). Lane 2 contains the α-amylase that was eluted in fractions 19–22 when a crude malt extract was injected.

Cyclobond column and is eluted in the void volume as is β-amylase (Fig. 2b). In contrast, *Enterobacter aerogenes* pullulanases eluted as three distinct peaks with k' values of 1, 2.33, and 5.33 (data not shown). The barley malt and bacterial pullulanases we have chromatographed elute prior to and separate from the α-amylases.

A protein elution profile ($A_{280\,nm}$) from a crude malt injection is shown in Fig. 2c. The majority of the protein eluted near the void volume, as did β-amylase. The actual void volume, calculated from the elution of bovine serum albumin (Fig. 4), was 3.0 ml.
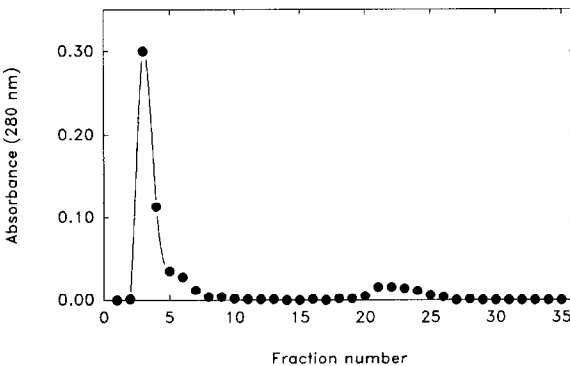

Fig. 4. Chromatography of BSA on cyclodextrin-bonded HPLC column. Elution conditions were the same as in Fig. 2.

BSA was selected as a void volume marker as it is similar in molecular weight (66 000) to both malt α- and β-amylase (45 000 and 65 000 daltons, respectively)[11,12]. Additionally, BSA was not expected to bind to the carbohydrate ligand. Over 98% of the protein absorbing at 280 nm eluted in the first six fractions. A slight increase in absorbance was also detected in fractions 21–24. Apparently, this preparation of BSA contains some protein which, based on its ability to specifically bind β-cyclodextrin, either contains a carbohydrate binding site or has exposed aromatic amino acid residues capable of binding the Cyclobond resin. The void volume value is in excellent agreement with the value (2.95 ml) determined by injecting water while pumping a mixed mobile phase and detecting the peak via a change in refractive index[13]. Setting $V_0 = 3.0$, then $k' = 1.33$ for purified β-amylase (Fig. 1d) and 1.67 when separated from a crude malt mixture (Fig. 2a). Similarly, $k' = 6.67$ for partially purified α-amylase (Fig. 1d) and 7.33 in a crude malt extract (Fig. 2a).

In conclusion, the use of cycloheptaamylose-bonded HPLC columns results in complete separation of malt α-amylases from β-amylases and pullulanase or de-branching enzyme in less than 20 min. The stability of this affinity resin is a significant advantage over the amine-coupled resins commonly used in open column chroma-tography. We have injected over 300 crude plant samples onto this column with no detectable loss of selectivity or binding capacity. The application of cyclodextrin-bonded phases for separation of other carbohydrate binding proteins or other proteins with exposed aromatic amino acid residues remains to be exploited.

REFERENCES

1 O. Holmbergh, *Biochem. Z.*, 258 (1933) 134–140.
2 P. Vretblad, *FEBS Lett.*, 47 (1974) 86–89.
3 M. P. Silvanovich and R. D. Hill, *Anal. Biochem.*, 73 (1976) 430–433.
4 R. Tkachuk, *FEBS Lett.*, 52 (1975) 66–68.
5 D. W. Armstrong, W. DeMond, A. Alal, W. L. Hinze, T. E. Riehl and K. H. Bui, *Anal. Chem.*, 57 (1985) 234–237.
6 D. W. Armstrong, *J. Liq. Chromatogr.*, 7 (1984) 353–376.
7 C. A. Henson, S. H. Duke and W. L. Koukkari, *Plant Cell Physiol.*, 27 (1986) 233–242.
8 C. A. Henson and J. M. Stone, *J. Cereal Sci.*, 8 (1988) 39–46.
9 D. W. Armstrong and W. DeMond, *J. Chromatogr. Sci.*, 22 (1984) 411–415.
10 R. M. Gibson and B. Svensson, *Carlsberg Res. Commun.*, 51 (1986) 295–308.
11 F. Kerhardy, I. Hara-Nishimura, M. Nishimura and J. Daussant, *Electrophoresis*, 8 (1987) 144–148.
12 C. Lauriere, M. Lauriere and J. Daussant, *Physiol. Plant.*, 67 (1986) 481–483.
13 D. W. Armstrong, W. DeMond and B. P. Czech, *Anal. Chem.*, 57 (1985) 481–484.
14 U. K. Laemmli, *Nature (London)*, 227 (1970) 680–685.