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Note

Rapid separation of α-amylases from barley by ion-exchange high-performance liquid chromatography on non-porous columns

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α-Amylase (EC 3.2.1.1) is an important enzyme in biology and industry because of its central role in the hydrolysis of starch[1]. Multiple forms of α-amylase are found in many organisms due to the presence of isoenzymes[2] and possible post-translational modifications of the proteins[3]. The different forms of α-amylase in plants, especially the cereals wheat and barley, have been extensively studied because of their importance in hydrolysis of starch during germination, and the functional significance of these enzymes in the industrial utilisation of the grain[4].

Isoelectric focusing[5,6] is widely used to separate α-amylases but is difficult to quantify. α-Amylases from wheat and barley have been separated by ion-exchange chromatography on DEAE-cellulose[7,8] CM-cellulose[9] and CM-Sepharose[10] and more recently by chromatofocusing[5,11,12]. However, these separation techniques have been generally less resolving than electrophoretic methods and have been very slow. Omichi and Ikenaka[13] recently reported high-performance liquid chromatography (HPLC) of human salivary α-amylases using gel permeation, ion-exchange and chromatofocusing colums. However their study used colums containing porous media. Ion-exchange columns based upon non-porous packing materials have become available for protein separation[14]. These materials allow much faster flow-rates permitting more rapid separations.

This paper reports rapid separation of α-amylases from barley by high-performance ion-exchange chromatography on non-porous media.

MATERIALS AND METHODS

Sources of samples

Barley (cv. Grimmett) was partially malted by steeping and germination for 48 h as described previously[15]. The grain was immediately frozen by immersion in liquid nitrogen and then freeze-dried. The dry grain was ground using a laboratory mill 3100 (Falling Number) to pass a 0.8-mm screen and stored at −18°C.

Extraction of α-amylases

α-Amylase was extracted by stirring 7.5 g of ground barley in 75 ml of 50 m*M* sodium malate buffer pH 5.2, 2 m*M* calcium chloride, 50 m*M* sodium chloride, 3 m*M*

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sodium azide for 10 min at 4°C. In some extracts 2 m*M* phenylmethylsulphonyl fluoride was included. The supernatant was collected by centrifugation at 2000 *g* for 10 min at 4°C and concentrated by precipitation with 80% saturation ammonium sulphate. The precipitate was resuspended in 10 ml of 5 m*M* Tris buffer pH 8.6 containing 1 m*M* calcium chloride and stored frozen as 1-ml aliquots.

The sample was diluted (1 ml in 3 ml) filtered through a 0.45-μm membrane and de-salted on a 10-ml column of Bio-Gel P6 in 5 m*M* Tris pH 8.6, 1 m*M* calcium chloride.

High-performance liquid chromatography

The HPLC system was programmable with two pumps, a mixer, injection system, controller, variable-wavelength UV detector, fraction collector and a data station (Bio-Rad Model 402 gradient system). The system had a flow path of titanium rather than stainless steel.

Anion-exchange colums

Two anion-exchange columns were used, a Bio-Gel TSK DEAE-5-PW (diethylaminoethyl groups bound to G 5000 PW support) column of 75 × 7.5 mm I.D. and a non-porous MA7P cartridge of 30 × 4.6 mm I.D. Both columns were equilibrated in 5 m*M* Tris pH 8.6, 1 m*M* calcium chloride before sample injection.

α-Amylase assay

α-Amylase was assayed, following dilution with the extraction buffer, using dye-labelled starch substrate tablets (Pharmacia)[16]. Protein was monitored at 280 nm.

Isoelectric focusing

Isoelectric focusing was performed on Ampholine PAG plate gels pH 3.5–9.5 (LKB). Gels were run for 1.5 h at 30 W. The pH gradient was determined using p*I* markers and staining with Coomassie Blue.

α-Amylase was detected using Procion Red MX 2B starch impregnated paper[17]. Procion Red MX-2B React Red 1 (Polysciences) was reacted with soluble starch [American Society of Brewing Chemists (ASBC) for diastatic power][18]. Dyed starch was separated from unreacted dye by gel filtration on a 84 × 1.9 cm column of Fractogel TSK HW-40 (S) (Merck). Filter paper sheets (Whatman No. 4) 12 × 25 cm were soaked in a 0.5 mg/ml solution of Procion Red MX 2B starch containing 50 m*M* sodium chloride, 2 m*M* calcium chloride, 50 m*M* sodium maleate buffer pH 5.2, 3 m*M* sodium azide. The dry papers were pressed against the isoelectric focusing gels and incubated at 20°C for 30 min. The reaction was stopped by flooding the gel and paper with 1.0 *M* hydrochloric acid. α-Amylase bands appeared as white areas on a pink background. Amylases were also detected by incubating the isoelectric focusing gels in a solution of 1% soluble starch (ASBC for diastatic power) in 50 m*M* sodium chloride, 2 m*M* calcium chloride, 50 m*M* sodium maleate buffer pH 5.2, 3 m*M* sodium azide at 40°C for 20 min. Amylase bands were revealed as colourless bands on a blue background by staining with 0.04% iodine, 0.4% potassium iodide.

RESULTS AND DISCUSSION

DEAE-Resins

Separation of α-amylases from barley on a Bio-Gel TSK DEAE-5-PW column is shown in Fig. 1. Analysis of a sample and regeneration of the column for the next sample required about 1 h. Recoveries of α-amylase activity were high (82% in the example in Fig. 1). Two main peaks of α-amylase were observed with several minor components.

Non-porous colums

Separation of α-amylases from barley on a MA7P cartridge is shown in Fig. 2. This method was much more rapid allowing higher flow-rates with total analysis times of less than 10 min at 2 ml/min and less than 5 min at 5 ml/min (Fig. 3). Separation was improved by using a shallow gradient (Fig. 4) requiring about 10 min per sample.

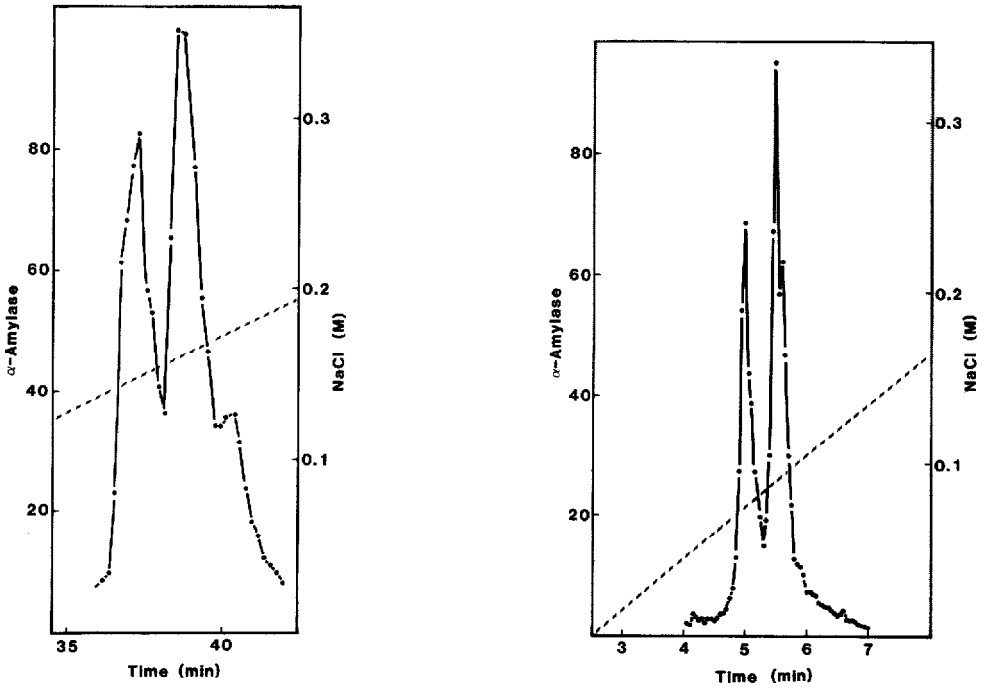

Fig. 1. Separation of barley α-amylases on a Bio-Gel TSK DEAE-5-PW anion-exchange column (75 × 7.5 mm). The sample (0.2 ml) was loaded in 5 m*M* Tris pH 8.6, 1 m*M* calcium chloride. The enzymes were eluted in a linear gradient from 0% to 80% Tris pH 8.6, 1 m*M* calcium chloride, 0.3 *M* sodium chloride between 15 min and 50 min at a flow-rate of 0.5 ml/min. Fractions collected were 0.125 ml. α-Amylase activity plotted in arbitrary relative units.

Fig. 2. Separation of barley α-amylases on a non-porous anion-exchange column (MA7P Cartridge, Bio-Rad, 30 × 4.6 mm). The sample (0.2 ml) was loaded in 5 m*M* Tris pH 8.6, 1 m*M* calcium chloride. α-Amylases were eluted in a linear gradient from 0% to 100% Tris pH 8.6, 1 m*M* calcium chloride, 0.3 *M* sodium chloride between 2.5 min and 12.5 min at a flow-rate of 2 ml/min. Fractions collected were 0.05 ml.

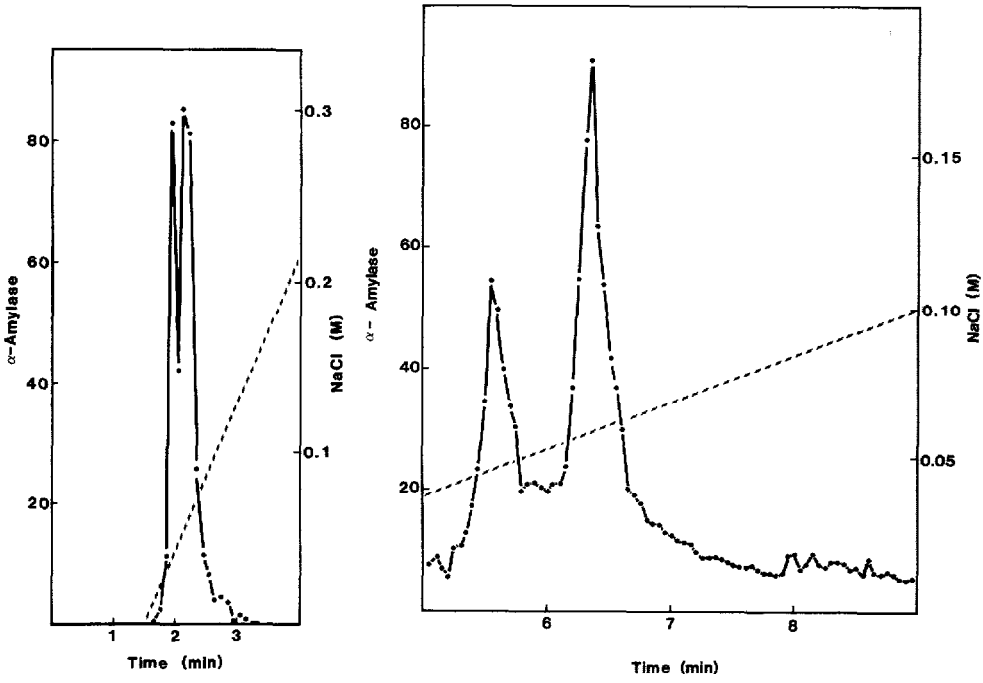

Fig. 3. Rapid separation of barley α-amylases on a MA7P cartridge. Conditions as for Fig. 2 except the gradient was between 1.5 min and 5 min, the flow-rate was 5 ml/min and fractions collected were 0.5 ml.

Fig. 4. Separation of barley α-amylases with a shallower gradient. Conditions were the same as in Fig. 2 with the gradient extended over twice the volume.

Recoveries of α-amylase activity were higher (90% in the example in Fig. 2) than with the DEAE-column. Most protein (more than 90% as assessed by the A_{280}) did not bind to these columns and was eluted before the gradient commenced. This resulted in a ten-fold purification of the α-amylase if all the fractions containing α-amylase were bulked.

Identity of α-amylases separated

The production of multiple forms of α-amylase by proteolytic degradation during isolation has been reported[3]. Inclusion of the protease inhibitor, phenylmethylsulphinyl fluoride, had very little effect on the elution profile. However, the isolation procedure used (4°C followed by ammonium sulphate precipitation) may protect against proteolysis and only two main components were detected. α-Amylase components reported may at least partially arise by proteolytic degradation of the two main isoenzymes as suggested by evidence produced by Hayes *et al.*[3] using electrophoretic analysis. The activity of α-amylase is apparently not always reduced by proteolytic action[3]. This retention of activity may explain the failure of many investigators to take steps to reduce proteolytic activity and as a result the very large number of forms of α-amylase often reported. Some heterogeneity can also be explained by the formation of α-amylase–inhibitor complexes[19]. The elution order and relative amounts

suggest that the first peak eluted may correspond to α-amylase III (a complex between α-amylase II and an inhibitor), the second, and largest peak, may be due to α-amylase II and the last minor component may be α-amylase I (Fig. 1).

Isoelectric focusing indicated that these samples contained two main bands of α-amylases between p*I* 6.7 and 5.5 with a minor band around p*I* 4.8. These correspond to α-amylases III (p*I* 6.1–6.9), II (p*I* 5.7–6.4) and I (p*I* 4.4–5.2). The proportion of α-amylase I is very low in barley. Chromatofocusing suggests that α-amylase I represents only 2.1% of total activity[5]. The major bands separated by HPLC must correspond to α-amylase II and III. Very small amounts of α-amylase (about 2.3% of total activity) probably corresponding to α-amylase I, were eluted in small peaks at higher salt concentrations (0.08–0.1 *M* NaCl, Fig. 4).

The identity of peaks was investigated by isoelectric focusing following concentration and de-salting of fractions from HPLC using Unicep-10 ultrafiltration cartridges (Bio-Rad). The results confirmed the predicted identities for the peaks but indicated that some peaks included a mixture of different forms of α-amylase. The first peak (5.5–6 min, Fig. 4) had a major band at p*I* 6.4 (α-amylase III); the second peak (6–6.5 min, Fig. 4) at p*I* 6.15 (α-amylase II) and the third (7.5–8 min, Fig. 4) at p*I* 4.8 (α-amylase I). However, small amounts of α-amylase III were detected in fractions collected between 6 and 7 min (Fig. 4).

DISCUSSION

Chromatography on non-porous anion-exchange columns provides a rapid and quantitative method for the analysis of multiple forms of α-amylase. The method may also have potential in the rapid purification of α-amylases. Affinity chromatography on cycloheptaamylose-Sepharose[20] and copper-iminodiacetic acid-Sepharose[21] has been used to purify α-amylases. Chromatofocusing has been used to separate isoenzymes in the purification of barley α-amylases[22]. The very rapid ten-fold purification on non-porous ion exchange columns reported here may be a useful step in any purification of α-amylase. Non-porous columns have several advantages, high flow rates are possible with good resolution and equilibration with solvents is rapid allowing fast recycling to the starting solvent for repeated analyses[23–25].

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REFERENCES

1 J. F. Robyt and W. J. Whelan, in T.A. Radley (Editor), *Starch and its Derivatives*, Chapman and Hall, London, 1968, p. 423.
2 A. H. D. Brown and J. V. Jacobsen, *Genet. Res., Camb.*, 40 (1982) 315.
3 B. Hayes, M. A. Harmey and H. Byrne, *J. Inst. Brew.*, 94 (1988) 253.
4 R. D. Hill and A. W. MacGregor, *Adv. Cereal Sci. Technol.*, 9 (1988) 217.
5 A. W. MacGregor, B. A. Marchylo and J. E. Kruger, *Cereal Chem.*, 65 (1988) 326.
6 B. A. Marchylo, L. J. Lacroix and J. E. Kruger, *Can. J. Plant Sci.*, 60 (1980) 433.

7 J. E. Kruger and R. Tkachuk, *Cereal Chem.*, 46 (1969) 219.
8 B. Marchylo, J. E. Kruger and G. N. Irvine, *Cereal Chem.*, 53 (1976) 157.
9 A. W. MacGregor, D. E. LaBerge and W. O. S. Meredith, *Cereal Chem.*, 48 (1971) 490.
10 E. Bertoft, C. Andtfolk and S. E. Kulp, *J. Inst. Brew.*, 90 (1984) 298.
11 B. A. Marchylo and A. W. MacGregor, *Cereal Chem.*, 60 (1983) 311.
12 B. A. Marchylo, A. W. MacGregor and J. E. Kruger, *J. Inst. Brew.*, 91 (1985) 161.
13 K. Omichi and T. Ikenaka, *Anal. Biochem.*, 168 (1988) 332.
14 D. Josic, W. Hofmann and W. Reutter, *J. Chromatogr.*, 371 (1986) 43.
15 R. J. Henry, *J. Sci. Food Agric.*, 42 (1988) 333.
16 R. J. Henry, D. J. Martin and A. B. Blakeney, *J. Cereal Sci.*, 5 (1987) 155.
17 L. Galleschi and J. M. Chapman, *Phytochemistry*, 24 (1985) 352.
18 W. F. Dudman and C. T. Bishop, *Can. J. Chem.*, 46 (1968) 3079.
19 C. A. Henson and J. M. Stone, *J. Cereal Sci.*, 8 (1988) 39.
20 M. P. Silvanovich and R. D. Hill, *Anal. Biochem.*, 73 (1976) 430.
21 V. Zawistowska, K. Sangster, J. Zawistowiski, J. Langstaff and A. D. Friesen, *Cereal Chem.*, 65 (1988) 414.
22 D. Lecommandeur, A. W. MacGregor and J. Daussant, *J. Chromatogr.*, 441 (1988) 436.
23 D. J. Burge, J. K. Duncan, L. C. Dunn, L. Cummings, C. J. Siebert and G. S. Ott, *J. Chromatogr.*, 353 (1986) 425.
24 D. J. Burke, J. K. Duncan, C. Siebert and G. S. Ott, *J. Chromatogr.*, 359 (1986) 583.
25 J. K. Duncan, A. J. Chen and C. J. Siebert, *J . Chromatogr.*, 397 (1987) 3.