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Note

Purification of germinated barley α-amylase isozymes and limit-dextrinase by chromatofocusing and affinity chromatography

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α-Amylases of barley seeds are synthesized during germination, and consist of two enzymic groups with low and high isoelectric points (p*I*)[1]. These have been called α-amylase 1 (low p*I*) and α-amylase 2 (high p*I*), and each corresponds to a distinct antigen[1]. The enzymic groups have similar M_r (*ca.* 44 000)[2] and behaviour towards glycogen or Schardinger dextrins[3,4], so it is difficult to separate them, unless ion-exchange chromatography is used[5]. A classical scheme for isozyme purification involves heat treatment, glycogen precipitation, separation of α-amylases 1 and 2 by ion-exchange chromatography and possibly a final purification of each enzyme by further ion-exchange chromatography[6,7]. Chromatofocusing appeared to be a particularly efficient technique for analytical purposes, to resolve activities due to each α-amylase isozyme[8]. The method was modified here to allow a scale-up, leading to a two-step procedure for preparing purified α-amylase 1 (containing 7% of α-amylase 2) and pure α-amylase 2. This consisted of preparative-scale chromatofocusing followed by affinity chromatography on a column of cycloheptaamylose epoxy-Sepharose. Another carbohydrase enzyme, limit-dextrinase[9,10], was also separated during the same chromatofocusing process, and also was purified further by affinity chromatography using the same ligand.

EXPERIMENTAL

Plant material

Barley grains (*Hordeum vulgare* L., cv. Menuet) were allowed to germinate on water-saturated cotton covered with filter-paper at 20°C in the dark for 5 days.

Extraction

Roots and shootlets were cut off and 20 g of the grains obtained were homog-

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enized in a grinding mill with 80 ml of 0.025 *M* histidine–HCl buffer (pH 6.2) containing 10^{-4} *M* calcium chloride. The extract was centrifuged at 20 000 *g* for 30 min, then the supernatant was reduced to 10 ml in a vacuum concentrator, dialysed (molecular weight cut-off 12 000–14 000) against the histidine buffer and filtered through 0.22-μm pore membranes (Millex GV; Millipore).

Chromatofocusing

A 30-ml volume of Polybuffer Exchanger Gel PBE 94 (Pharmacia) was packed in a 30 cm × 0.9 cm I.D. column, the starting buffer being 0.025 *M* histidine–HCl (pH 6.2) containing 10^{-4} *M* calcium chloride. After application of the dialysed extract, eluent buffer (Polybuffer PB 74 diluted 10-fold with water, pH 4.5, containing 10^{-4} *M* calcium chloride) was pumped through the column at 0.3 ml/min and 3-ml fractions were collected. After 130 fractions, 1 *M* sodium chloride solution was applied to the column to elute the remaining material.

Affinity chromatography

Affinity chromatography was performed with cycloheptaamylose (Schardinger β-cyclodextrin) as the ligand[3]. Pooled fractions from chromatofocusing were pumped through a column of cycloheptaamylose epoxy-Sepharose at 0.2 ml/min. After washing with 0.02 *M* acetate buffer (pH 5.5) containing 10^{-4} *M* calcium chloride, enzyme elution was achieved with the same buffer, containing 8 mg/ml of cycloheptaamylose for α-amylases and 40 μg/ml for limit-dextrinase. For α-amylase 2 processing, the pooled fractions and wash buffer prior to the affinity column were made 0.5 *M* with sodium chloride.

Protein determination and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein content of column fractions was monitored by measuring the absorbance at 280 nm, and determined by microtitration using Pierce BCA protein reagent as described by Sorensen and Brodbeck[11]. SDS-PAGE was performed as described by King and Laemmli[12] in 15% acrylamide gels (3 mm thick).

Enzyme detection

Enzymes were detected by means of fused rocket immunoelectrophoresis[13], which gives an elution profile for each antigen depending on the amount of protein present. Antigen concentrations were assessed by means of rocket immunoelectrophoresis[14] and converted into protein amount with the final pure enzyme being used as a reference. The precipitin lines were rendered visible by Serva Violet 49 staining as described by the manufacturer.

The immune sera were those used previously by MacGregor *et al.*[15] for α-amylases and by Daussant *et al.*[16] for limit-dextrinase.

RESULTS AND DISCUSSION

The respective activity due to each α-amylase isozyme cannot be measured in the original extract. Further, because α-amylase 2 may be partly complexed with an endogenous inhibitor ($M_r \approx 20\,000$)[17], depending on the pH and ionic strength[19],

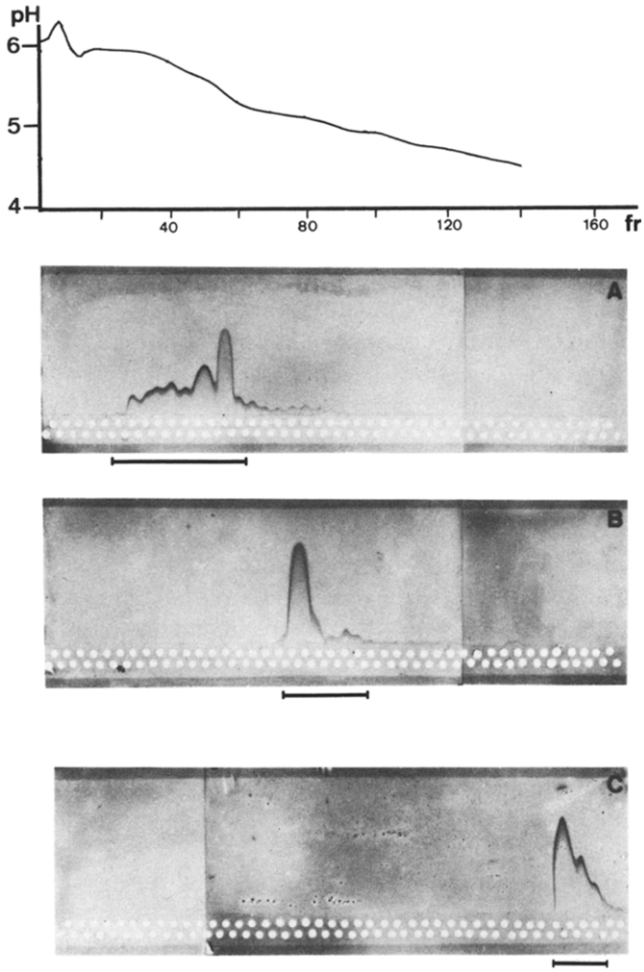

Fig. 1. Chromatofocusing elution profile and corresponding fused rocket immunoelectrophoresis results. (A) α-Amylase 2 antiserum; (B) α-amylase 1 antiserum; (C) limit-dextrinase antiserum. fr = Fraction number. Bars indicate pooled fractions.

the specific activity of the enzyme may be modified. Immunochemical methods were used to monitor the enzyme purification because they can discriminate the two α-amylase components[1]. These methods were also used for limit-dextrinase, thus avoiding the tedious determination of the activity of the enzyme[16,19].

Chromatofocusing of the crude extract

The elution profiles of the three enzymes are shown in Fig. 1. Both α-amylase components and limit-dextrinase were bound to the column following sample application at pH 6.2. α-Amylase 2 was eluted first from the column as several peaks (elution pH between 5.45 and 5.8), followed by a peak of α-amylase 1 (average elution pH 5.1), as expected from previous results[8]. Under the conditions used, all limit-

dextrinase remained bound to the gel but it was eluted thereafter with 1 *M* sodium chloride solution. An efficient separation of substantial amounts of α-amylase 1 and 2 and small amounts of limit-dextrinase was then achieved. The α-amylase 2 pool was free from α-amylase 1 and the limit-dextrinase pool was free from both amylases; only a slight contamination of the α-amylase 1 pool by α-amylase 2 was detected (Fig. 1B). The broad dispersion of α-amylase 2 was caused by the high polymorphism of the group, previously observed by gel isoelectric focusing[1]. The α-amylase 2–inhibitor complexation seemed to be decreased by chromatofocusing, as most of the inhibitor did not bind to the column, the remaining part being eluted with the very first α-amylase 2 fractions (results not shown).

Removal of amphoteric buffer from proteins after chromatofocusing proved to be difficult to achieve by precipitation, even though the proteins were at their p*I*s. Further, the absorbance of the column effluent at 280 nm showed no marked variations along the gradient, suggesting that there was continuous elution of a complex mixture of proteins. This was confirmed by SDS-PAGE analysis of chromatofocusing fraction pools. Each pool contained several other proteins in addition to the enzymes (Fig. 3, lanes 1, 2 and 3). Affinity chromatography was therefore added to the purification scheme both to improve the enzyme purification and to remove the amphoteric buffer.

Affinity chromatography of chromatofocusing pools

Limit-dextrinase from malted barley has been shown to complex with cyclohexaamylose[20]. Cereal α-amylases are known to bind to cycloheptaamylose at a non-

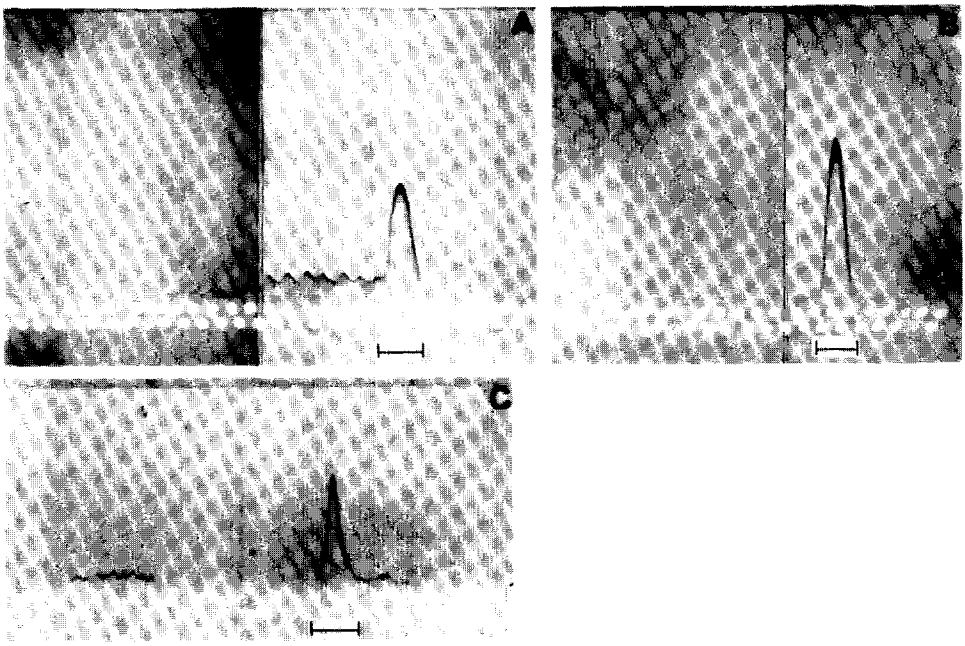

Fig. 2. Fused rocket immunoelectrophoresis of fractions from affinity chromatography. Symbols as in Fig. 1.

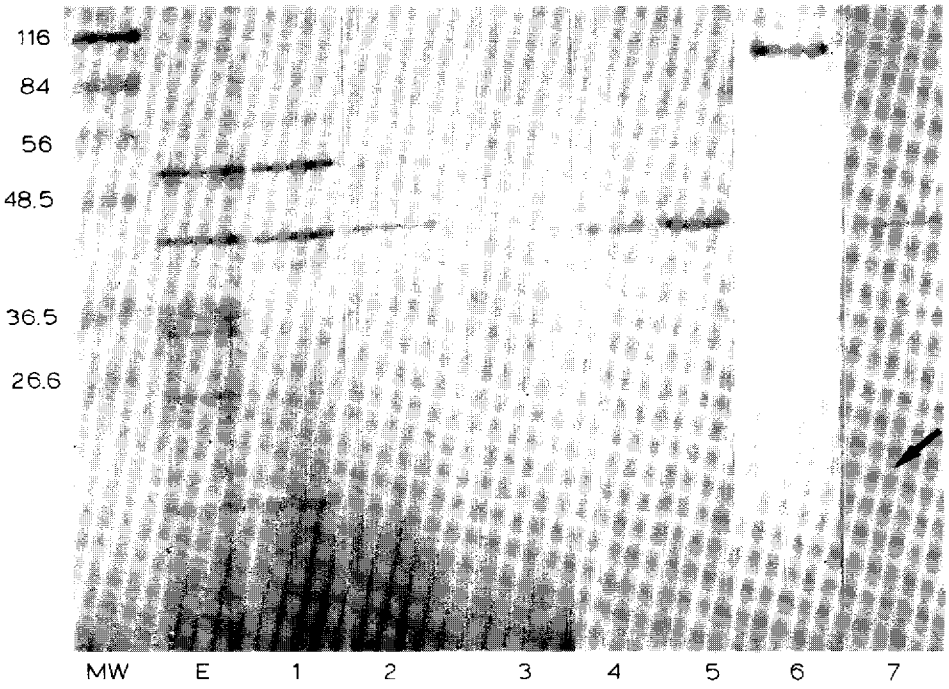

Fig. 3. SDS-PAGE of chromatographic fractions. MW = Molecular weight markers; E = extract; 1 = α-amylase 2 chromatofocusing pool; 2 = α-amylase 1 chromatofocusing pool; 3 = limit-dextrinase chromatofocusing pool; 4 = α-amylase 2 affinity peak; 5 = α-amylase 1 affinity peak; 6 = limit-dextrinase affinity peak; 7 = α-amylase 2 affinity peak (processed without sodium chloride). The arrow indicates α-amylase inhibitor.

catalytic site, whereas cereal β-amylases do not show any binding[4]. It seemed likely, therefore, that affinity chromatography on cycloheptaamylose would provide a specific technique for eliminating other barley proteins and also Polybuffer from the α-amylase and limit-dextrinase preparations. As the affinity of limit-dextrinase for the ligand is known to be much lower[20], different conditions were used for elution. Each enzyme was indeed eluted in a concentrated form as a sharp peak from the affinity column (Fig. 2). It can be seen that some of the limit-dextrinase did not bind to the column, and that some α-amylase 2 was eluted during the washing step. However, these losses represented only a small proportion of the total enzymes.

Evaluation of the purification procedure

The final purified samples were analysed by SDS-PAGE. Only one band of protein was detected in each sample (Fig. 3, lanes 4, 5 and 6), indicating that significant purification of the enzymes had been achieved by the affinity step.

Affinity chromatography of a-amylase 2 using classical conditions (*i.e.*, without adding sodium chloride) resulted in a faint additional band of $M_r \approx 20\,000$ together with the amylase (Fig. 3, lane 7). This indicated the presence of α-amylase inhibitor, accounting for about 10% of the protein in the fraction. Addition of 0.5 *M* sodium

TABLE I

PURIFICATION OF α-AMYLASE ISOZYMES

The amounts were determined using the Pierce BCA assay (with bovine serum albumin as the standard), except for α-amylases in the extract and chromatofocusing pools, for which the immunoassay was used.

Material	*Total protein (μg)*	*α-Amylase (μg)*
Extract (after dialysis)	83 000	6100 (α-amylase 2) 900 (α-amylase 1)
Chromatofocusing pools		
α-Amylase 2	N.D.*	1430
a-Amylase 1	N.D.*	470
Affinity peaks		
α-Amylase 2	740	
α-Amylase 1	260	

* Not determinable, owing to Polybuffer interference.

chloride to the applied sample and wash appeared to dissociate the α-amylase 2–inhibitor complex and elute the inhibitor, because after this treatment only one band was seen at $M_r \approx 44\,000$ (Fig. 3, lane 4).

The α-amylase 1 band was located at a slightly higher molecular weight than α-amylase 2 (Fig. 3, lane 5). Rocket immunoelectrophoresis revealed that the fraction contained about 7% of α-amylase 2, which is not visible in Fig. 3 (lane 5) because the molecular weights of the two enzymes are so similar.

The limit-dextrinase had a molecular weight of 102 000 (Fig. 3, lane 6), a value in agreement with previous reports concerning barley and malt enzyme[20].

Antigen amounts were determined in the crude extract (after dialysis) and in the final purified fractions (Tables I and II). The results show that α-amylase 2, α-amylase 1 and limit-dextrinase accounted for 7%, 1% and 0.1%, respectively, of the total proteins in the dialysed extract (*i.e.*, proteins of $M_r \geqslant 14\,000$). As the experimental protocol used in this study was designed primarily for α-amylase isozyme purification, limit-dextrinase was obtained here only as a by-product. It is a very minor component of the extract (Table II), and larger amounts of grains and more specific conditions of germination and purification are required in order to obtain larger amounts[21].

TABLE II

PURIFICATION OF LIMIT-DEXTRINASE

Total proteins were determined with the Pierce BCA assay, and limit-dextrinase in the extract and chromatofocusing pool by means of the immunoassay.

Material	*Total proteins (μg)*	*Limit-dextrinase (μg)*
Extract (after dialysis)	83 000	90
Chromatofocusing pool	4600	50
Affinity peak	30	

CONCLUSION

Owing to its good flow-rate and high resolving power, chromatofocusing is a simple and efficient technique for the separation of α-amylase isozymes and limit-dextrinase from extracts of germinating barley. Further purification of these enzymes can be achieved by means of affinity chromatography, leading to pure α-amylase 2 and limit-dextrinase. A further step would be needed to obtain pure α-amylase 1.

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