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Note

Purification of β-amylase from mustard (*Sinapis alba* L.) to homogeneity by chromatofocusing and gel permeation chromatography

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Chromatofocusing, a new column chromatography method developed by Sluyterman and co-workers[1,2], allows the preparative separation of proteins according to their isoelectric points. It offers high resolution together with the capacity of the ion-exchange technique. Since a protein held on a chromatofocusing column can be eluted within an extremely narrow range of pH (0.02–0.04 pH units), this technique can also be used as an effective purification step[3]. In many cases, a high degree of protein purification has been achieved using chromatofocussing as a preliminary step[4–7]. In higher plants, although the technique of chromatofocussing has been used to resolve isozymic forms of enzyme, such as α-amylase in wheat[8] and barley[9], its effectiveness as a purification step has not been investigated.

In this paper we report the use of chromatofocusing in the purification of mustard β-amylase to homogeneity.

EXPERIMENTAL

Protein and enzyme assay

Protein was estimated either by the procedure of Lowry *et al.*[10] or by that of Sedmak and Grossberg[11]. β-Amylase was assayed essentially by the procedure described by Subbaramaiah and Sharma[12]. The pH of chromatofocusing fractions was monitored by a pH microelectrode.

Plant growth conditions and extraction of enzyme

Mustard (*Sinapis alba* L.) seedlings were grown on moist chromatographic papers in closed transparent plastic trays at 25°C under continuous white light. Cotyledons from 6-day old seedlings were harvested and homogenized in ice-cold 25 m*M* histidine–hydrochloric acid buffer pH 6.2 (1 g cotyledons per 4 ml of buffer) in a Waring blender. The homogenate was filtered through a double layer of cheesecloth and centrifuged at 16 000 *g* at 4°C. The supernatant was dialysed against histidine–hydrochloric acid buffer to eliminate anthocyanins and other low-molecular-weight substances present. The dialysate was concentrated by lyophilization prior to loading on a chromatofocusing column.

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Chromatofocusing

The chromatofocusing was performed on a PBE 94 column using polybuffer 74 (Pharmacia, Uppsala, Sweden) essentially following the procedures described[13]. The column (90 cm × 0.8 cm) was equilibrated with 25 m*M* histidine-hydrochloric acid buffer, pH 6.2. The polybuffer 74 was diluted 1:10 (v/v) with water, adjusted to pH 4.0 with 0.5 *M* hydrochloric acid and 5 ml of it were loaded onto the column. Thereafter, the sample was applied to the column followed and eluted with polybuffer 74 (pH 4.0) at the rate of 8 ml/h. Fractions of 1 ml were collected and protein, β-amylase activity and pH were estimated. The fractions containing β-amylase activity were pooled, dialyzed against 20 m*M* sodium citrate buffer pH 6.1 and concentrated by centrifugation in Amicon-Centricones at 1000 *g*.

Gel permeation chromatography (GPC)

A 120-ml volume of Sephadex G-200 gel (Pharmacia) was packed into a column (60 cm × 2.5 cm) and equilibrated with 20 m*M* sodium citrate buffer pH 6.1. The pooled β-amylase preparation from chromatofocusing was applied to the column and eluted at the rate of 15 ml/h. Fractions of 1 ml of were collected and monitored for protein and β-amylase activity.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE) of β-amylase was carried out by the procedure of Laemmli[14]. The gels were stained for protein with Coomassie brilliant blue dye.

RESULTS

A summary of the purification protocol is presented in Table I. It is evident from the results presented that the crude supernatant from mustard when subjected to chromatofocusing folowed by GPC on Sephadex G-200 yields a highly enriched β-amylase preparation. The chromatofocusing of the crude supernatant on the PBE 94 column resulted in a single-step 53-fold purification of β-amylase enzyme, with a yield greater than 50%. On chromatofocusing, mustard β-amylase was resolved into three species having isoelectric points (p*I*s) at 4.83, 4.71 and 4.58 respectively (Fig. 1). The first peak at pH 4.83 is more prominent than the other two. Although an high degree of purification of β-amylase was achieved by chromatofocusing, SDS-

TABLE I

PURIFICATION OF β-AMYLASE FROM MUSTARD COTYLEDONS

Purification stage	*Volume (ml)*	*Activity (nkat)*	*Protein (mg)*	*Specific activity (nkat/mg)**	*Yield (%)*	*Purification fold*
Crude supernatant	6	3800	100.5	37.8	100	1
Chromatofocusing	30	2220	1.1	2018	58	53
GPC	7.5	1600	0.29	5517	42	146

* A katal is defined as the moles of maltose released per second at 30°C.

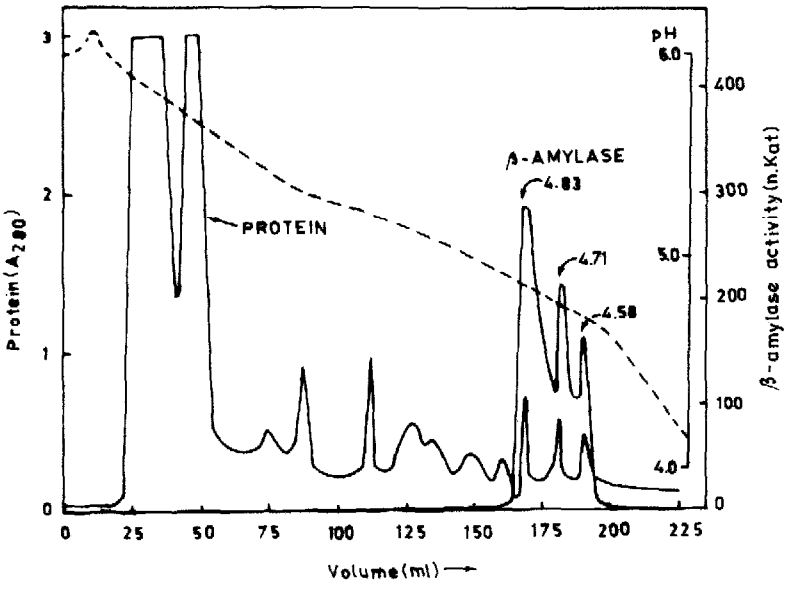

Fig. 1. Elution profile of mustard β-amylase on PBE 94. The elution conditions, pH (– – –), protein and β-amylase activity monitoring were as described in the Experimental section.

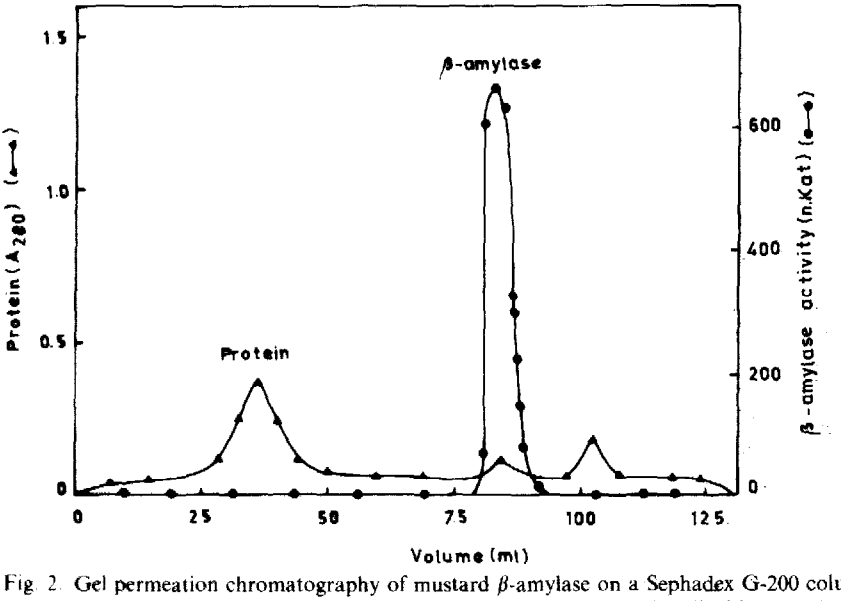

Fig. 2. Gel permeation chromatography of mustard β-amylase on a Sephadex G-200 column. The fractions were monitored for protein (▲) and β-amylase activity (●) as described in Experimental.

Fig. 3. SDS-PAGE of β-amylase purified by chromatofocusing and GPC. The gels were stained for protein by Coomassie brilliant blue.

PAGE of pooled β-amylase fractions revealed a few other protein species in addition to β-amylase polypeptide (MW 58 000)[15]. However, on subjecting the above enriched β-amylase fractions to GPC on Sephadex G-200 (Fig. 2) a homogeneous β-amylase preparation was obtained, as ascertained by SDS-PAGE of the pooled active β-amylase fractions (Fig. 3). The specific activity of purified β-amylase was 5517 nkat/mg protein, with an overall purification of 146-fold and 42% yield (Table I).

DISCUSSION

Traditionally, β-amylases from various higher plant species have been purified by conventional methods employing a combination of ammonium sulphate and acetone precipitation, gel permeation and ion-exchange chromatography[16–19]. These methods not only require a prolonged purification but also give a lower yield of purified enzyme. In contrast, chromatofocusing of β-amylase is a rapid procedure, which yields a 53-fold purification in a single step. Furthermore it can be applied

directly to crude supernatant, thereby eliminating the need for a preliminary ammonium sulphate or acetone precipitation step.

In the present study, although chromatofocusing in a single step resulted in an high degree of purification, it did not yield a homogeneous β-amylase preparation. Since a crude supernatant was directly used to load the chromatofocusing column, proteins with isoelectric points identical to β-amylase were also present in the enzyme fractions. These contaminating proteins could be separated by including a GPC step, which separated proteins according to their molecular masses, leading to purification of β-amylase to homogeneity. This two-step purification of β-amylase can be completed in a single day. In contrast, a conventional purification protocol employing ammonium sulphate and acetone precipitation, GPC and ion-exchange chromatography of β-amylase from mustard yielded after 5 days a non-homogeneous β-amylase preparation with 42-fold purification and 20% yield (data not shown).

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REFERENCES

1 L. A. AE. Sluyterman and O. Elgersma, *J. Chromatogr.*, 150 (1978) 17.
2 L. A. AE. Sluyterman and J. Wijdenes, *J. Chromatogr.*, 150 (1978) 31.
3 L. Svederberg, T. Laas and D. Low, *Protides Biol. Fluids*, 29 (1981) 955.
4 L. Kiss, S. Gál, B. L. Toth-Martinez and I. Petrikovics, *J. Chromatogr.*, 262 (1983) 379.
5 W. Welte, H. Hüdig, T. Wacker and W. Kreuz, *J. Chromatogr.*, 259 (1983) 341.
6 H. Bloemandal, M. Willemsen, G. Gorencwoud and P. Oomen, *FEBS Lett.*, 180 (1985) 181.
7 N. D. Vincent and P. Cummins, *Eur. J. Biochem.*, 148 (1985) 135.
8 B. A. Marchylo and J. E. Kruger, in J. E. Kruger and D. E. La Berge (Editors), *Proceedings of Third International Symposium on Post-harvest Sprout Damage in Cereals*, West View Press, Boulder, CO, 1983.
9 B. A. Marchylo and A. W. MacGregor, *Cereal Chem.*, 60 (1983) 311.
10 O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
11 J. J. Sedmak and S. E. Grossberg, *Anal. Biochem.*, 79 (1976) 544.
12 K. Subbaramaiah and R. Sharma, *J. Biochem. Biophys. Methods*, 10 (1985) 315.
13 *Chromatofocusing with Polybuffer and PBE*, Pharmacia Fine Chemicals, Uppsala, 1982.
14 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
15 K. Subbaramaiah, *Ph.D. Dissertation*, University of Hyderabad, 1987.
16 G. W. Chapman, J. E. Pallas and J. Mendicino, *Biochim. Biophys. Acta*, 276 (1972) 491.
17 S. Aibara, H. Yamashita and Y. Morita, *Agric. Biol. Chem.*, 42 (1978) 179.
18 K. Okamoto and T. Akazawa, *Agric. Biol. Chem.*, 42 (1978) 1379.
19 A. Gertler and Y. Birk, *Biochem. J.*, 95 (1965) 621.