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Purification of the glycoprotein glucose oxidase from *Penicillium amagasakiense* by high-performance liquid chromatography

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ABSTRACT

Fast protein liquid chromatography (FPLC) in combination with ion-exchange chromatography on a Mono Q column was used to purify glucose oxidase from *Penicillium amagasakiense* to homogeneity. Purification was performed with a mixed pH and salt gradient, with 20 m*M* phosphate buffer (pH 8.5) as starting buffer (A) and 50 m*M* acetate buffer (pH 3.6) with 0.1 *M* NaCl as elution buffer (B). Elution conditions were optimized to permit the simultaneous purification and separation of the glucose oxidase isoforms. Three peaks, each consisting of 1–2 isoforms and exhibiting a homogeneous titration curve profile, were resolved with a very flat linear gradient of 5.0–5.1% B in 40 ml. Three more peaks, each consisting of several isoforms, were eluted at 10%, 30% and 100% B. Optimization of the elution conditions and separation of the glucose oxidase isoforms was only possible because of the rapidity of each purification step and the high resolution provided by FPLC and Mono Q.

INTRODUCTION

The glycoprotein glucose oxidase (GO) (E.C. 1.1.3.4) is of considerable commercial importance [1–3]. GO catalyses the oxidation of glucose to D-glucono-1,4-lactone. The enzyme is produced by several filamentous fungi, those from *Aspergillus niger* and *Penicillium* species being commercially the most important. Despite its commercial importance, structural information about the enzyme is not available, owing to the lack of growth of crystals suitable for X-ray analysis. The large carbohydrate moiety, accounting for 10–16% of the molecular mass of GO, is believed to inhibit the crystallization process. Cleavage of the carbohydrate moiety from *A. niger* GO was shown to be an important prerequisite step for the growth of crystals suitable for X-ray diffraction analysis [4].

However, other factors, such as sample purity and homogeneity, are important criteria for crystallization, as contaminants can hinder the crystallization proces [5]. Development of purification techniques, such as fast protein liquid chromatography (FPLC), together with high-resolution liquid chromatographic resins, such as MonoBeads, has greatly enhanced protein purification procedures [6,7]. Consequently, the time required for each purification step has been greatly reduced and peak

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resolution significantly increased. Further, optimum purification conditions can be quickly established.

This paper decribes the application of FPLC to the purification of GO from *Penicillium amagasakiense*. The enzyme was purified to homogeneity on a Mono Q column, using a mixed pH and salt gradient. In addition, the procedure served to separate the multiple GO isoforms.

EXPERIMENTAL

Materials

GO from *P. amagasakiense* was purchased from Nagase Biochemicals (Osaka, Japan). All chemicals were obtained from Merck (Darmstadt, F.R.G.) and PhastGel polyacrylamide gels from Pharmacia–LKB (Uppsala, Sweden).

Methods

GO activity was assayed at 420 nm by the method of Sahm *et al.* [8] using 2,2′-azino-di(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as dye and 0.1 *M* glucose as substrate. Assays were performed in 0.1 *M* acetate buffer (pH 6) at 25°C under oxygen saturation.

Protein was determined by the method of Bradford [9] using Coomasie Brilliant Blue G reagent (Bio-Rad Labs.) with bovine serum albumin as a standard.

Polyacrylamine gel electrophoresis (PAGE) was performed either in the presence (dissociating conditions) or in the absence (non-dissociating conditions) of sodium dodecyl sulphate (SDS) on 10–15% or 8–25% gradient gels, respectively, using the Phast System (Pharmacia–LKB) according to the manufacturer's instructions [10]. Isoelectric focusing was performed in the pH range 4.0–6.5 according to Olsson *et al.* [11]. Electrophoretic titration curves were generated by performing electrophoresis perpendicular to a stable pH gradient (in the pH range 3.0–9.0) according to Haff *et al.* [12], as described by Jacobson and Skoog [13]. Gels were silver stained by the method of Butcher and Tomkins [14].

Purification

Purification was performed with a Pharmacia FPLC unit equipped with two P-500 pumps, an LCC-500 controller and an LKB 2238 Univord SII ultraviolet monitor, fitted with a 280-nm filter. Chromatograms were recorded with an LKB 2210 two-channel recorder. Samples were collected with a FRAC-100 fraction collector. GO was dissolved in and dialysed against the starting buffer and applied to a Mono Q HR 5/5 column pre-equilibrated with the appropriate buffer. All samples and buffers were filtered through 0.22-μm Millex-GV_{13} (Millipore) or cellulose acetate (Sartorius) filters before use. GO-containing fractions were pooled, desalted and concentrated as described previously [4]. Sample purity and homogeneity were assessed electrophoretically. Unless stated otherwise, a flow-rate of 2 ml/min was used.

RESULTS AND DISCUSSION

Following the successful crystallization of the purified and deglycosylated *A. niger* GO [4], similar success was envisaged with the *P. amagasakiense* GO. However,

despite its purification to homogeneity by native and SDS-PAGE [15], the deglycosylated enzyme could not be crystallized, probably owing to its isoelectric heterogeneity [16]. Therefore, a reduction in the number of its isoforms before deglycosylation was considered important.

Ion-exchange chromatography on a Mono Q column was used for the purification of *P. amagasakiense* GO. The influence of the steepness of the salt gradient on GO elution at different pH values was initially tested. The resolution of the GO isozymes was poor under all conditions tested.

However, the resolution was significantly improved by using a mixed pH and salt gradient, with 20 m*M* phosphate buffer (pH 8.5) as starting buffer and 100 m*M* acetate buffer (pH 3.6) with 200 m*M* NaCl as elution buffer (Fig. 1A). The resolution of the three peaks was further improved by a flattening of the linear gradient from 0–25% B in 15 ml to 5–10% B in 15 ml (Fig. 1B).

A major improvement in the resolution of the GO isoforms was achieved with 50 m*M* acetate buffer (pH 3.6) and 100 m*M* NaCl as elution buffer (Fig. 2a). Minor changes in the slope of the gradient dramatically affected the elution profile of GO. Thus, a flattening of the linear gradient from 5–10% B in 15 ml to 5.0–5.1% B in 12 ml permitted the resolution of three sharp peaks (Fig. 2b). Optimum resolution was achieved with a linear gradient of 5.0–5.1% B in 40 ml at a flow-rate of 1 ml/min (Fig.

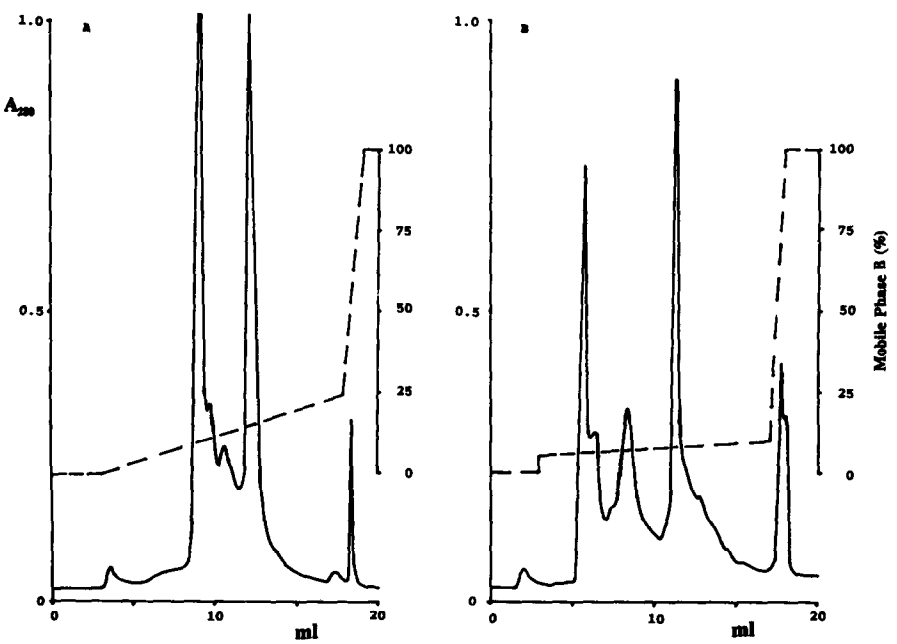

Fig. 1. Influence of a mixed pH and steepness of the salt gradient on the separation of *Penicillium amagasakiense* GO isoforms. GO was applied to a Mono Q column in 20 m*M* potassium phosphate buffer (pH 8.5) (buffer A) and eluted with 100 m*M* sodium acetate buffer (pH 3.6)–0.2 *M* NaCl (buffer B) with a gradient of (A) 0–25% B in 15 ml and (B) 5–10% B in 15 ml.

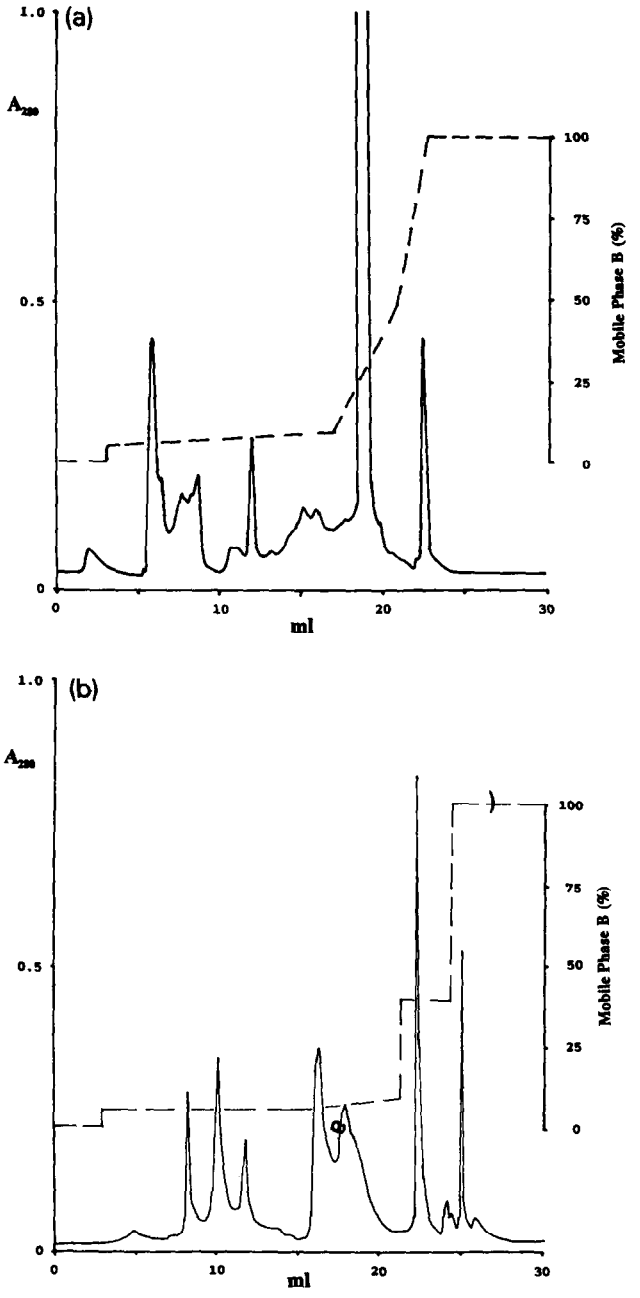

Fig. 2. Influence of a mixed pH and steepness of the salt gradient on the separation of *Penicillium amagasakiense* GO isoforms. GO was applied to a Mono Q column in 20 m*M* potassium phosphate buffer (pH 8.5) (buffer A) and eluted with 50 m*M* sodium acetate buffer (pH 3.6)–0.1 *M* NaCl (buffer B) with a gradient of (a) 5–10% B in 15 ml, 10–50% B in 4 ml and 50–100% B in 2 ml and (b) 5.0–5.1% B in 12 ml, 5.1–10% B in 6 ml then in stepwise increments at 30% B and 100% B.

3A). The three GO peaks each consisted of a maximum of two isoforms (Fig. 3B). Further, these samples exhibited a homogeneous profile in the pH range 3–9 on an electrophoretic titration curve (Fig. 4). The homogeneous *P. amagasakiense* GO isoform which eluted as peak 1 at 5% B has been successfully crystallized, with the crystals diffracting to at least 2.2 Å resolution [17]. Detailed analysis of the properties of each isoform, especially protein and carbohydrate composition and stability, is currently in progress.

Peaks eluted at or above 10% B were heterogeneous with respect to both their isoelectric forms and titration curve profiles. This heterogeneity, in contrast to that of *A. niger* GO [4], could not be eliminated or reduced by deglycosylation. This implies that, in contrast to the *A. niger* GO [18], variations in the carbohydrate content

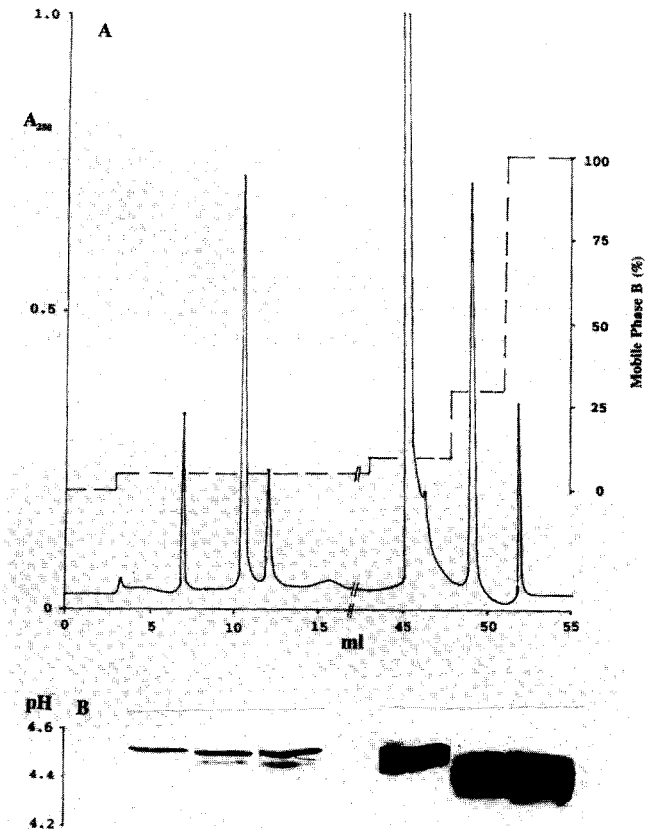

Fig. 3. Separation of *Penicillium amagasakiense* GO isoforms (A) on a Mono Q column and (B) by isoelectric focusing. GO was applied to a Mono Q column in 20 m*M* potassium phosphate buffer (pH 8.5) (buffer A) and eluted with 50 m*M* sodium acetate buffer (pH 3.6)–0.1 *M* NaCl (buffer B) with a linear gradient of 5.0–5.1% B in 40 ml, then in stepwise increments at 10% B, 30% B and 100% B. Isoelectric focusing was performed in the pH range 4.0–6.5, as described under Experimental.

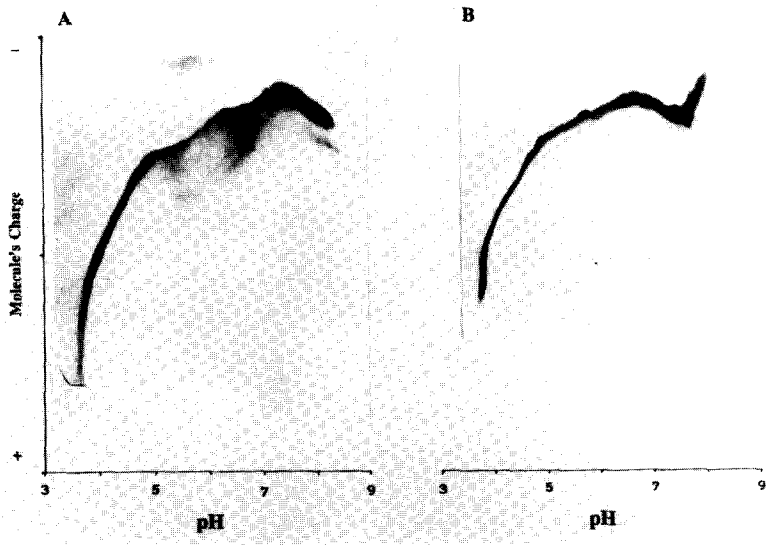

Fig. 4. Electrophoretic titration curve of *Penicillium amagasakiense* GO (A) before and (B) after purification (peaks 1–3) under the conditions as in Fig. 3. The electrophoretic titration curve was generated by prefocusing the ampholytes across a Phast IEF gel [10], then by running electrophoresis perpendicular to the pH gradient [12,13]. The sample components migrate towards the anode or cathode according to their net charge at any particular pH [6].

probably do not contribute to the heterogeneity of the *P. amagasakiense* GO. A reduction in heterogeneity is being attempted by further modifications of the buffer composition and elution conditions.

REFERENCES

1 M. Röhr, C. P. Kubicek and J. Kominek, in H.-J. Rehm and G. Reed (Editors), *Biotechnology*, Vol. 3, Verlag Chemie, Weinheim, 1983, p. 455.
2 A. P. F. Turner, I. Karube and G. S. Wilson (Editors), *Biosensors. Fundamentals and Applications*, Oxford University Press, Oxford, 1987.
3 R. D. Schmid and I. Karube, in H.-J. Rehm and G. Reed (Editors), *Biotechnology*, Vol. 6b, Verlag Chemie, Weinheim, 1988, p. 317.
4 H. M. Kalisz, H.-J. Hecht, D. Schomburg and R. D. Schmid, *J. Mol. Biol.*, 213 (1990) 207.
5 R. Riegé and V. Mikol, *TIBTECH*, 7 (1989) 277.
6 *FPLC Ion Exchange and Chromatofocusing*, Pharmacia Laboratory Separation Division, Uppsala, 1985.
7 P. M. Hammond and M. D. Scaven, *Int. Ind. Biotechnol.*, 8 (1988) 23.
8 H. Sahm, H. Schütte and M.-R. Kula, *Methods Enzymol.*, 89 (1982) 424.
9 M. M. Bradford, *Anal. Biochem.*, 72 (1972) 248.
10 *Pharmacia Phast System System Guide*, Pharmacia Laboratory Separation Division, Uppsala.
11 I. Olsson, U. Axio-Fredriksson, M. Degerman and B. Olsson, *Electrophoresis*, 9 (1988) 16.
12 L. A. Haff, L. G. Fägerstam and A. R. Barry, *J. Chromatogr.*, 266 (1983) 409.
13 G. Jacobson and B. Skoog, in M. J. Dunn (Editor), *Electrophoresis '86*, VCH, Weinheim, 1986, p. 516.
14 L. A. Butcher and J. K. Tomkins, *Anal. Biochem.*, 148 (1985) 384.
15 J. Hendle, H. M. Kalisz and R. D. Schmid, unpublished results.
16 H. M. Kalisz, J. Hendle and R. D. Schmid, *Gesellschaft Deutscher Chemiker 22. Hauptversammlung Bonn, 18–22 September, 1989 —Programm und Kurzreferate*, VCH, Weinheim, 1989, p. 182.
17 J. Hendle, H.-J. Hecht, H. M. Kalisz, D. Schomburg and R. D. Schmid, in preparation.
18 S. Hayashi and S. Nakamura, *Biochim. Biophys. Acta*, 657 (1981) 40.