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APPLICATION OF HIGH-PERFORMANCE CHROMATOGRAPHIC AND ELECTROPHORETIC METHODS TO THE PURIFICATION AND CHARACTERIZATION OF GLUCOSE OXIDASE AND CATALASE FROM *PENICILLIUM CHRYSOGENUM*

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SUMMARY

The high resolving power of the preparative and analytical high-performance chromatographic and electrophoretic methods recently developed in this laboratory for the separation of biopolymers has been demonstrated by the purification and characterization of glucose oxidase and catalase from *Penicillium chrysogenum*. Crude glucose oxidase was purified to homogeneity in one step by high-performance hydrophobic-interaction chromatography (HIC) on a pentylagarose column. Crude catalase was purified by a combination of HIC and high-performance anion-exchange chromatography on 3-diethylamino-2-hydroxypropylagarose. The homogeneity of the enzymes was monitored by high-performance electrophoresis and free zone electrophoresis.

The p*I* values of these two enzymes determined by isoelectric focusing in the high-performance electrophoresis apparatus were 4.2 and 6.5, respectively. Their molecular weights were determined by high-performance molecular sieve chromatography on an agarose column. Glucose oxidase has a molecular weight of 175000 and probably consists of two identical subunits, as sodium dodecyl sulphate polyacrylamide gel electrophoresis gave a molecular weight of around 72000. The molecular weight of catalase, which is probably composed of non-identical subunits, as indicated by sodium dodecyl sulphate electrophoresis, is around 320000. Some other characteristics of these two enzymes were also investigated, *e.g.*, electrophoretic mobility, pH stability and optimum pH.

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INTRODUCTION

Agarose has been widely used for the separation of proteins by classical, low-pressure chromatography. However, as agarose beads can be made very rigid they can be used also for high-performance liquid chromatography (HPLC), including hydrophobic-interaction, ion-exchange, molecular sieve and affinity chromatography. High-performance electrophoresis (HPE) is a technique developed during the last few years for the separation of high- and low-molecular-weight compounds. In this paper we demonstrate the great potential of these high-performance separation methods by the purification and analysis of glucose oxidase (E.C. 1.1.3.4) and catalase (E.C. 1.11.1.6) from *Penicillium chrysogenum*.

Glucose oxidase is of practical importance, as it can be used for the determination of the glucose content of body fluids and foods[1]. Catalase is used often for the determination and destruction of hydrogen peroxide.

Glucose oxidase has been purified from different fungi. The most studied fungal glucose oxidase originates from *Aspergillus niger*[2–5], although glucose oxidase from *Penicillium amagasakiense*[6], *P. purpurogenum*[7], *P. notatum*[8], *P. vitale*[9] and *P. chrysogenum*[10] have also been purified. The glucose oxidases from *Aspergillus* and *Penicillium* share many properties[11]: they are all glycoproteins, and their reaction mechanisms are virtually identical. Their molecular weight (MW) is around 150 000, and they contain two flavine adenine dinucleotide (FAD) molecules. However, they differ with regard to carbohydrate content and amino acid composition[11]. The *Penicillium* enzyme is extracellular, whereas the *Aspergillus* enzyme is intracellular. The immunological reactivities of glucose oxidase from *Aspergillus* and *Penicillium* indicate distinct structural differences between the two enzymes[11].

Catalases from fungi have higher molecular weights than have those of mammalian origin[12], although their three-dimensional structures are similar. As with glucose oxidase, the catalase from *A. niger* is the most studied fungal enzyme. It is a tetramer with a molecular weight of 323 000[13] or 385 000[14]. Catalase from *P. vitale*[15] is reported to be a tetramer with a subunit molecular weight of 75 000–80 000.

EXPERIMENTAL

Materials

Horseradish peroxidase and *o*-dianisidine were purchased from Sigma (St. Louis, MO, U.S.A.) and hydrogen peroxide from E. Merck (Darmstadt, F.R.G.). The molecular weight standard proteins for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (myosin, MW 200 000; β-galactosidase, 116 000; phosphorylase B, 92 500; bovine serum albumin, 66 200; and ovalbumin, 45 000) and the isoelectric point marker standards (human carbonic anhydrase, p*I* 6.50; bovine carbonic anhydrase, 6.00; β-lactoglobulin, 5.10; and phycocyanin, 4.65) were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Pharmalyte solutions (pH 3–10) were purchased from Pharmacia (Uppsala, Sweden). The molecular weight standard proteins for molecular sieve chromatography were thyroglobulin (MW 669 000), ferritin (440 000) and catalase (beef liver, 232 000) from Pharmacia, human serum albumin (66 400) from Kabi Vitrum (Stockholm, Sweden) and ovalbumin (43 000) from Sigma. All other chemicals used were of analytical-reagent grade.

The chromatographic system, including Model 2150 HPLC pumps, Model 2152 controllers, Model 2138 and 2158 UV detectors, a Model 2220 recording integrator and a Model 2210 recorder, was supplied by LKB (Bromma, Sweden). Loop injectors were purchased from Rheodyne (Berkeley, CA, U.S.A.). A DMS 100 spectrophotometer from Varian (Walnut Creek, CA, U.S.A.) was used for the enzyme activity measurements.

The agarose beads, the ion exchangers and the amphiphilic gels used for hydrophobic interaction chromatography were prepared in this laboratory as described in refs. 16, 17 and 18, respectively.

Methods

Purification. The starting material was partially purified extracts of glucose oxidase and catalase, prepared according to the method described in refs. 10 and 19. The procedure is outlined below. The *Penicillium chrysogenum* culture was constructed by ultrafiltration and the filtrate was precipitated by a complex of copper sulphate and potassium hexacyanoferrate(II). The sediment was dissolved in 0.1 *M* phosphate buffer (pH 7.2) and precipitated with isopropanol. The sediment was used for further purification of catalase as described below.

For the purification of glucose oxidase, the same sediment was used for fractionation on an Amberlite IRA-400 column. The active fraction was precipitated with acetone and used for further purification of glucose oxidase as described herein.

The final purification of the glucose oxidase from the above preparation was effected by high-performance hydrophobic interaction chromatography on a column of pentylagarose[18]. Elution was effected with a negative salt gradient of ammonium sulphate.

Catalase was prepared from the partially purified preparation by a combination of ion-exchange chromatography on 3-diethylamino-2-hydroxypropyl (DEAHP) agarose[17] and hydrophobic interaction chromatography on pentylagarose[18].

Absorbance measurements were made at 280 nm and enzymatic activity was measured in all fractions.

Enzyme activity measurements, determination of optimum pH and pH stability measurements. Glucose oxidase activity measurements were made according to the method of Keston[20] (1 U catalyses the formation of 1 μmol of hydrogen peroxide per minute at 25°C), and the catalase activity measurements were carried out as described by Beers and Sizer[21] (1 U catalyses the transformation of 1 μmol of hydrogen peroxide per minute at 25°C). Measurements of optimal pH were made in buffers ranging from pH 3.5 to 9 (for pH 3.5–6 sodium acetate was used and for pH 5.5–9, sodium phosphate was used). To determine the pH stability of the two enzymes, assays were performed at pH 7 after incubation of the enzymes at room temperature for 3 h in buffers with pH values ranging from 3.5 to 9. The activity values at pH 7 did not change during these 3 h for either of the two enzymes.

Determination of molecular weights of the enzymes and their subunits. The native enzyme molecular weights were determined by molecular sieve chromatography on a 5% agarose column[22] in 0.05 *M* sodium phosphate buffer (pH 6.5). Subunit molecular weights were determined by discontinuous SDS-PAGE. The electrophoresis system was essentially that described by Neville[23]. The concentration of the sepa-

ration gel was T = 11.1% and C = 1% (for the definition of T and C, see ref. 24). The SDS-PAGE gels were stained with Coomassie Brilliant Blue.

Mobility determination. Mobility determinations, which also gave an indication of the homogeneity of the enzymes, were made in a free zone electrophoresis (FZE) apparatus[25]. As the buffer for both enzymes, 0.03 *M* sodium phosphate (pH 6.8) was used.

High-performance electrophoresis (HPE). High resolution and short run times are characteristic of high-performance electrophoresis[26,27]. This is achieved by the use of narrow-bore, thin-walled electrophoresis tubes, which guarantee rapid removal of the Joule heat to minimize thermal zone deformation even at high field strengths. The diameters of the capillary tubes are typically 0.05–0.2 mm, the wall thickness 0.05–0.1 mm and the length 100–200 mm. There are two versions of the HPE equipment, one for on-tube detection[26,27] and the other for off-tube detection[28,29]. The homogeneity of the purified enzymes was studied by HPE using off-tube detection[28,29] at 195 nm. Catalase and glucose oxidase were subjected to electrophoresis in 0.01 *M* sodium phosphate buffer (pH 8.0).

Isoelectric focusing. Homogeneity studies and determination of the isoelectric points of the enzymes were performed by isoelectric focusing in the HPE equipment with on-tube detection at 280 nm[30]. The p*I* values of the enzymes were determined with the aid of the p*I* markers mentioned under *Materials*. The focused protein zones were mobilized towards the cathode without impairment of the resolution by exchanging the base at the cathode for phosphoric acid[30].

Amino acid analysis. The amino acid composition was determined after hydrolysis with 6 *M* hydrochloric acid for 24 and 72 h. Half-cystine was determined as cysteic acid after oxidation with performic acid. Tryptophan was not determined.

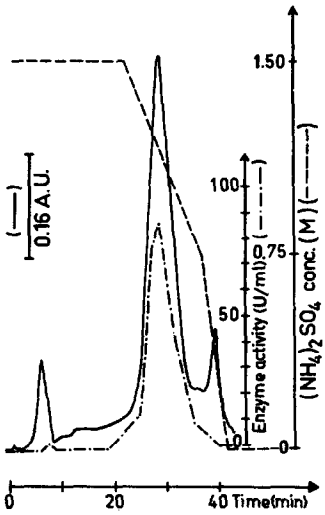
RESULTS AND DISCUSSION

Some comments on the high-performance methods used

Most of the supporting media used earlier for the HPLC of proteins were based on silica. Although silica columns can give good separations, they have some inherent drawbacks, notably low stability at high pH and non-specific interactions. We pointed out several years ago that agarose, a polysaccharide, is highly compatible with biopolymers and that it might therefore by used for the HPLC of such macromolecules[31]. In the last few years, we have successfully developed agarose gels and derivatives thereof, which are suitable for the molecular-sieve, hydrophobic interaction, ion-exchange and affininity chromatography of proteins in the HPLC mode[32–40]. The advantages of agarose-based HPLC demonstrated in our previous papers are a low level of non-specific interactions, high pH stability, simplicity of preparation and excellent resolution. The experiments described here demonstrate again that proteins can be purified and analysed rapidly and efficiently by the proper use of agarose-based HPLC methods, separately or in combination.

HPE is another high-performance method developed in this laboratory in the last few years[26–30,41–43]. The results presented here clearly demonstrate the great potential of this method for the analysis, characterization and micropreparation of proteins.

In this last decade, high-performance methods for the separation and analysis



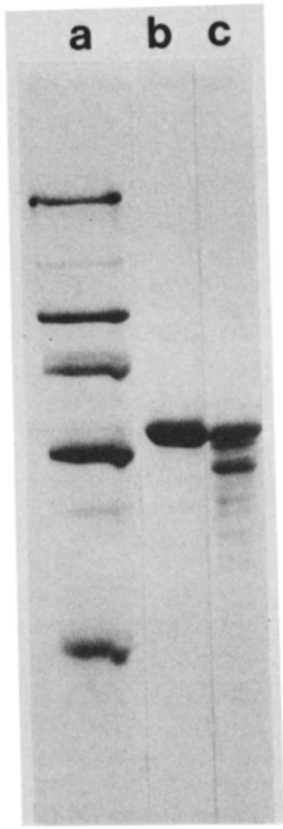


Fig. 1. Purification of the crude glucose oxidase product by high-performance hydrophobic interaction chromatography on pentylagarose. Column dimensions, 4.5 × 0.6 cm I.D., bead diameter, 5–7 μm; sample amount, 0.7 mg; buffer, 0.05 *M* sodium phosphate (pH 6.5); elution with a negative salt gradient from 1.5 to 0 *M* ammonium sulphate; flow-rate, 0.2 ml min^{-1}; fraction volume, 0.2 ml. The thin, full line represents absorption measurements at 280 nm.

Fig. 2. SDS-PAGE of glucose oxidase. (a) Standard proteins (see *Materials*) from top, 200, 116, 92, 66 and 45 kilodalton; (b) glucose oxidase purified by chromatography on the pentylagarose column (Fig. 1); (c) glucose oxidase before chromatography (the crude material).

of proteins have attracted great interest. However, the advantages of these methods can be fully utilized only when the separation mechanism and the characteristics of the methods are well understood. If properly used, the high-performance methods can simplify and speed up the purification and characterization of proteins, which is often a difficult and cumbersome problem, particularly when classical fractionation methods are employed.

Glucose oxidase

The glucose oxidase preparation was highly purified by hydrophobic interaction chromatography (Fig. 1) in a yield of 84% (according to enzymatic activity measurements). The enzyme was homogeneous upon analysis by SDS-PAGE (Fig.

Fig. 3. High-performance ion-exchange chromatography of glucose oxidase on DEAHP-agarose. Sample, active fraction (main peak) from the experiment in Fig. 1. Column dimensions, 5.6 × 0.6 cm I.D.; buffer, 0.03 *M* Tris–HCl (pH 7.6); bead diameter, 5–10 μm; elution with a 30-min gradient from 0 to 0.3 *M* sodium acetate; flow-rate, 0.2 ml min^{-1}. The figure 17.26 in the chromatogram refers to the elution time in minutes.

Fig. 4. Isoelectric focusing of glucose oxidase in the HPE apparatus. Sample, dialysed glucose oxidase from the pentylagarose column (Fig. 1); dimensions of the glass tube, 110 mm × 0.05 mm I.D. × 0.19 mm O.D.; carrier ampholytes, 2.5% (v/v) Pharmalyte (pH 3–10). Focusing was performed at 3000 V for 7 min with 0.02 *M* phosphoric acid as anolyte and 0.02 *M* sodium hydroxide as catholyte. The on-tube detection was carried out at 280 nm. Mobilization of the focused protein zones towards the cathode was accomplished at 4000 V with 0.02 *M* phosphoric acid in both electrode vessels.

2), ion-exchange chromatography (Fig. 3), isoelectric focusing (Fig. 4) and FZE (Fig. 5). The glucose oxidase seems to be a dimer with an approximate molecular weight of 175000 and a subunit molecular weight of 72000, as determined by molecular sieve chromatography and SDS-PAGE, respectively. These findings are in agreement with results obtained for *P. vitale* glucose oxidase[9], which was reported to be a dimer with an estimated subunit molecular weight of 74000, and also with results obtained for *A. niger* glucose oxidase[4], which indicated a subunit molecular weight of 80000. However, *P. amagasakiense*[6] glucose oxidase was reported to be a tetramer with a subunit molecular weight of 45000.

The amino acid composition of glucose oxidase, shown in Table I, is in agreement with that reported for other glucose oxidases[2,9,28]. The main difference in the amino acid composition between the different glucose oxidases from *Penicillium*[9,44]

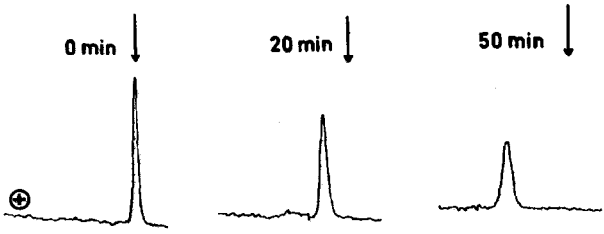


Fig. 5. Free zone electrophoresis of glucose oxidase fractions obtained from the experiment shown in Fig. 1. Buffer, 0.03 *M* sodium phosphate (pH 6.8); sample, 10 μl of a 0.02% solution dialysed against the electrophoresis buffer; I.D. of revolving electrophoresis tube, 3 mm; current, 5.0 mA; voltage, 1000 V; temperature, 18°C. The scans were made at the times indicated. The arrow indicates the position of the starting zone.

TABLE I

AMINO ACID COMPOSITION OF THE GLUCOSE OXIDASE FROM *PENICILLIUM CHRYSOGENUM*

Amino acid	*Concentration (mol.-%)*	*Amino acid*	*Concentration (mol.-%)*
Cys	0.6	Val	8.0
Asx	11.6	Ile	4.7
Met	2.2	Leu	8.5
Thr	6.6	Tyr	3.7
Ser	6.2	Phe	3.6
Glx	10.8	His	2.1
Pro	5.1	Lys	5.4
Gly	9.6	Arg	3.2
Ala	8.6	Trp	Not determined

and *Aspergillus*[2,44] is the number of lysines, which is reported to be double for the *Penicillium* enzymes. Glucose oxidase from *P. chrysogenum* has a lysine content similar (in mole-%) to that of the other *Penicillium* glucose oxidases.

The mobility of glucose oxidase was $-7.3 \cdot 10^{-5}$ cm^2 s^{-1} V^{-1} in 0.03 *M* sodium phosphate (pH 6.8) at 18°C. The isoelectric point was 4.2 as determined by isoelectric focusing in the HPE apparatus. This value is slightly below that reported for glucose oxidase from *A. niger* (4.35)[45]. Fig. 6a and b shows the pH optimum and the pH stability, respectively. The enzyme has a pH optimum of 5–6, which is higher than that of the *P. amagasakiense* glucose oxidase (pH 4–5.5)[44]. The data for glucose oxidase are summarized in Table II.

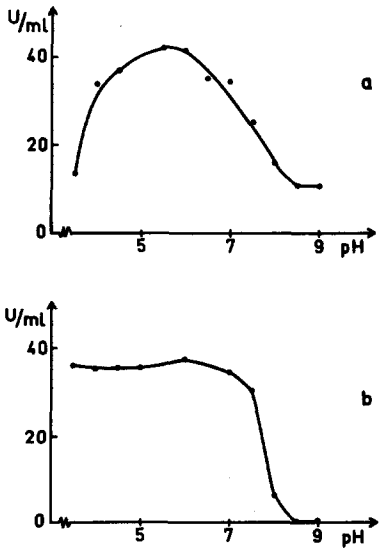


Fig. 6. (a) Determination of optimum pH of glucose oxidase; (b) effect of pH on glucose oxidase stability. Glucose oxidase was incubated in buffers of different pH for 3 h. The residual activity was then measured by the standard assay at pH 7.0.

TABLE II

DATA FOR GLUCOSE OXIDASE AND CATALASE

Parameter	*Glucose oxidase*	*Catalase*
Molecular weight	175 000	320 000
Subunit molecular weight	72 000	77 000 and/or 74 000
Isoelectric point	4.2	6.5
Optimum pH	5–6	7
pH stability	3.5–7.5	4–9
Mobility in 0.03 *M* sodium phosphate, pH 6.8 (18°C) ($cm^2\ s^{-1}\ V^{-1}$)	$-7.3 \cdot 10^{-5}$	$-0.5 \cdot 10^{-5}$

Catalase

The final steps in the purification of catalase were a combination of ion-exchange chromatography (Fig. 7) and hydrophobic interaction chromatography (Fig. 8) with yields of 97% and 74%, respectively (according to enzymatic activity measurements). Fig. 9 shows the isoelectric focusing of this highly purified catalase and Fig. 10 an HPE experiment in a polyacrylamide gel. The molecular weight of the native enzyme was estimated at 320000 by molecular sieve chromatography on a column of 5% agarose (in the absence of SDS). In SDS-PAGE (Fig. 11), this highly purified catalase gave two bands, corresponding to molecular weights of 77000 and

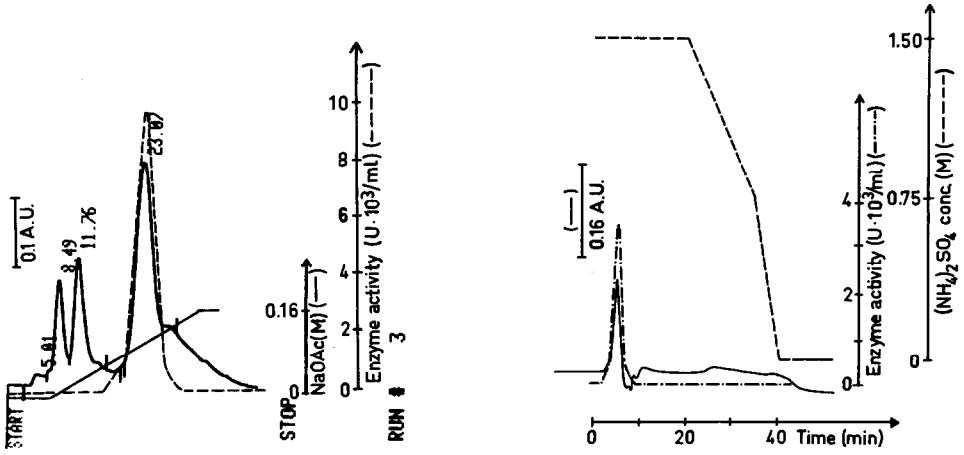


Fig. 7. High-performance ion-exchange chromatography of a crude extract of catalase on DEAHP-agarose. Column dimensions, 5.6 × 0.6 cm I.D.; bead dimensions, 5–10 μm; sample amount, 1 mg; buffer, 0.03 *M* Tris–HCl (pH 7.6); elution with a 25-min salt gradient from 0 to 0.16 *M* sodium acetate; flow-rate, 0.2 ml min^{-1}; fraction volume, 0.2 ml.

Fig. 8. High-performance hydrophobic interaction chromatography of catalase on pentylagarose. Sample, catalase fractions from the experiment shown in Fig. 7. Column dimensions, 4.5 × 0.6 cm I.D.; buffer, 0.05 *M* sodium phosphate (pH 6.5); elution with a negative salt gradient from 1.50 to 0 *M* ammonium sulphate; flow-rate, 0.2 ml min^{-1}; fraction volume, 0.2 ml.

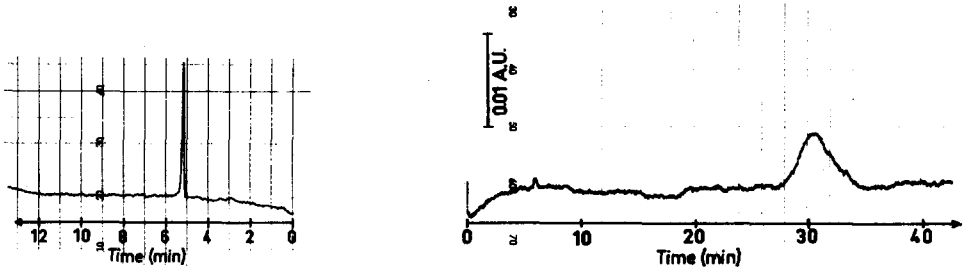


Fig. 9. Isoelectric focusing of catalase in the HPE apparatus. Sample, dialysed catalase from the pentyl-agarose column (Fig. 8). Conditions similar to those in Fig. 4.

Fig. 10. HPE in a T = 3%, C = 4% polyacrylamide gel of purified catalase from the pentylagarose column (Fig. 8). Capillary tube, 160 × 0.2 mm I.D. Electrophoresis conditions, 2000 V and 85 μA. The flow-rate during the off-tube detection (at 195 nm) was 0.08 ml min^{-1}.

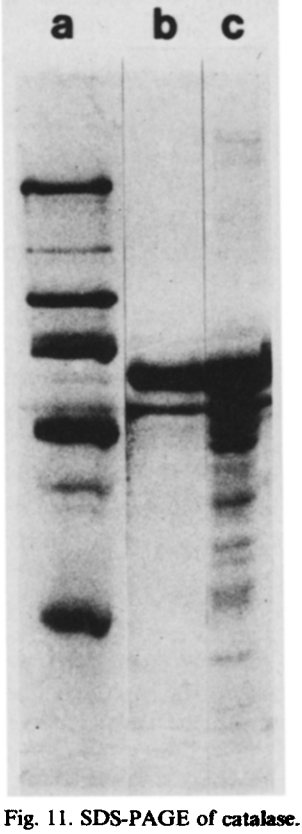


Fig. 11. SDS-PAGE of catalase. (a) Standard proteins (see *Materials*) from top, 200, 116, 92, 66 and 45 kilodalton; (b) purified fraction from the pentylagarose column (Fig. 8); (c) crude catalase.

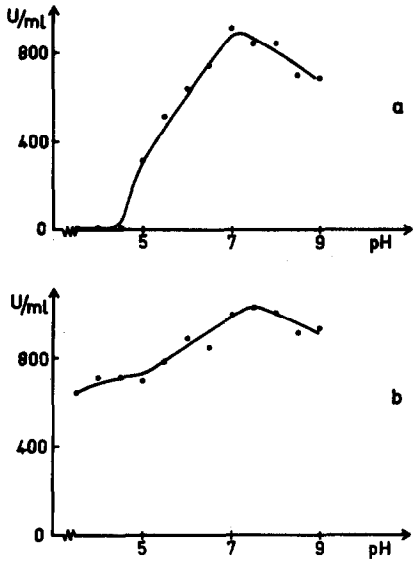


Fig. 12. (a) Determination of optimum pH of catalase; (b) effect of pH on catalase stability. Catalase was incubated in buffers of different pH for 3 h. The residual activity was measured by the standard assay at pH 7.0.

74 000. Taken together, these findings suggest that the catalase from *P. chrysogenum* consists of four subunits of which there are two types, differing in molecular weight. This enzyme therefore differs from the catalases of *A. niger*[13,14] and *P. vitale*[12,15], which are reported to consist of four identical subunits.

When the above HPE experiments (Fig. 10) with catalase were repeated with glucose oxidase, material was either not eluted or eluted in the form of a very broad peak. This may indicate that the enzyme aggregated on coming into contact with the polyacrylamide gel, which is not an uncommon phenomenon, as has been recently reported[46]. Catalase migrated very slowly towards the anode (at pH 6.8) in the mobility determination experiment. The isoelectric point should therefore be slightly below pH 6.8. Using p*I* markers, an approximate p*I* value of 6.5 was obtained by high-performance isoelectric focusing. Catalase has an optimum pH of 7 and a high pH stability at neutral and alkaline pH, as shown in Fig. 12. The data for catalase are summarized in Table II.

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