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Isolation and characterization of catalase from *Penicillium chrysogenum*

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ABSTRACT

Catalase from a crude preparation of *Penicillium chrysogenum* was isolated in a single chromatographic step by immobilized metal ion affinity chromatography (IMAC) on Cu(II)–Chelating Sepharose Fast Flow. A chromatographically and electrophoretically homogeneous enzyme was obtained in 89% yield. IMAC was found to be superior to ion-exchange, hydrophobic interaction, size-exclusion and concanavalin A affinity chromatography. Analytical and preparative chromatography gave essentially the same chromatograms. Isoelectric point, molecular weight (by ultracentrifugation), amino acid composition, carbohydrate content and subunit organization were determined. The apparent Michaelis–Menten constant, K_M, and the azide competitor constant, K_i, were calculated and found to be 59 μM and 6.1 μM, respectively.

INTRODUCTION

Catalase is a key enzyme in all aerobic cells and the enzyme from various sources is widely used in biochemical and bioindustrial processes. Cultural liquid from *Penicillium chrysogenum* is reported to be a suitable source for industrial production of catalase combining the high output of biosynthesis, up to 8000 U/ml cultural liquid or 400 U/mg protein, with excretion of the enzyme into the medium. Chaga *et al.* [1–3] investigated the biosynthesis and the partial purification of this catalase, but little has been published on its purification and characterization.

In a previous paper [4], we showed that immobilized metal ion affinity chromatography (IMAC) can be used as a simple, yet highly efficient, method for the purification of animal enzymes. On an industrial scale, however, microbial sources of enzymes are preferable to animal sources, so purification techniques with a high capacity and selectivity for enzymes from bacteria, yeasts and moulds are required. With this in mind, we investigated the purification of catalase from *P. chrysogenum*.

EXPERIMENTAL

Starting material

The crude enzyme was prepared according to the methods of Chaga [2,3]. Ultrafiltration of the culture liquid was followed by "negative adsorption" using a complex of $CuSO_4$ and $K_4Fe(CN)_6$ and the crude enzyme was precipitated with $(NH_4)_2SO_4$ and isopropanol. The final precipitate was desalted and lyophilized.

Preliminary studies

Preliminary studies involving chromatography of the catalase on ion-exchange chromatographic (IEC), hydrophobic interaction chromatographic (HIC), size-exclusion chromatographic (SEC), IMAC and biospecific concanavalin A (Con A)–Sepharose columns. Only IMAC showed promise for one-step purification.

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Purification procedure

Columns for IMAC (analytical, 1.6 × 0.9 cm I.D., and preparative, 5 × 3.2 cm I.D.) were prepared by pouring in degassed slurries in deionized water of Chelating Sepharose Fast Flow (Pharmacia–LKB Biotechnology, Uppsala, Sweden) and the gel beds were settled by opening of the outlets. The gels were charged with four bed volumes of 20 m*M* $CuSO_4$ solution. Excess copper ions were removed by washing with deionized water. The columns were equilibrated with ten bed volumes of 50 m*M* sodium phosphate buffer (pH 7.3) containing 1 *M* NaCl. For the analytical protocol 25 mg of lyophilized crude enzyme were dissolved in 5 ml of the equilibration buffer and for the preparative protocol 1 g of the crude enzyme was dissolved in 100 ml of the buffer. The samples were applied to the columns and the non-adsorbed material was removed by washing with the equilibration buffer. The columns were washed with 50 m*M* sodium phosphate–0.5 *M* NaCl (pH 6.0), then the catalase eluted with 20 m*M* sodium acetate (pH 5.5). Finally, the columns were washed with 50 m*M* sodium phosphate–1.0 *M* NaCl (pH 7.3) containing 0.2 *M* imidazole. Flow-rates of 0.5 and 8 ml/min, respectively, were kept constant throughout the analytical and preparative procedures.

The relative protein concentration was monitored by detection of the absorbance at 280 nm utilizing a Model 2238 Uvicord SII and recorded on a Model 2210 recorder (Pharmacia–LKB Biotechnology). During the analytical run 2-ml fractions were collected.

Assay for catalase activity

The catalase activity was determined according to the method described by Beers and Sizer [5]. The method is based on the determination of the rate of disappearance of H_2O_2, as measured at 240 nm. Both a Pye Unicam Model 8700 UV–VIS and an LKB Ultrospec II spectrophotometer were used for these assays.

Purity index

A purity index, R_z, the ratio of the absorbances at 405 and 280 nm ($R_z = A_{405}/A_{280}$), was used as an indicator of the purity of the enzyme in solution [6,7].

Tests for homogeneity

Chromatography. The purified enzyme was analysed for homogeneity by IEC on a Mono Q HR5/5 column and by SEC on a Superose 6 column (Pharmacia–LKB Biotechnology).

Electrophoresis. Electrophoretic assays were performed with Phast System (Pharmacia–LKB Biotechnology on Phast Gel gradient 8–25 using Phast Gel sodium dodecyl sulphate (SDS) and native buffer strips according to the manufacturer's instructions.

Characterization of the enzyme properties

Amino acid analysis. The amino acid composition and content of glucosamine were determined after hydrolysis in 6 *M* HCl for 24 and 68 h. Cysteic acid was determined after oxidation with performic acid.

N-Terminal sequence analysis. The N-terminal sequence analysis was performed according to Edman [8] on a solid-phase sequenator.

Carbohydrate analysis. The total neutral sugar content was determined by the phenol–sulphuric acid method [9] using D-mannose as a standard. The composition of the neutral sugars was determined according to Theander and Westerlund [10]. The hydrolysis with trichloroacetic acid was performed at 121°C for 90 min instead of 90°C for 16 h.

Molecular weight determination. The molecular weight of the enzyme was determined by a sedimentation experiment in an MSE ultracentrifuge, analytical model, at 7500 rpm in equilibrium medium, 0.2 *M* sodium acetate–0.04 *M* acetic acid, for 72 h.

Inhibition of catalase with sodium azide. The azide competitor constant, K_i, of NaN_3 as an inhibitor of the *P. Chrysogenum* catalase was determined spectrophotometrically by the method of Dixon and Webb [11].

Determination of isoelectric point. The isoelectric point of the enzyme was determined by isoelectric focusing (IEF) and by chromatofocusing. IEF was performed with Phast System using Phast Gel IEF 3–9, according to manufacturer's instructions. The chromatofocusing was performed on PBE 94 gel (Pharmacia–LKB Biotechnology). A 6.5 × 0.5 cm I.D. column filled with PBE 94 was equilibrated with 25 m*M* piperazine–HCl (pH 5.5). A sample of *ca.* 0.5 mg of enzyme in 0.5 ml of the equilibration buffer was loaded on to the column. After washing with three column volumes of the equilibration

buffer, a pH gradient was formed with Polybuffer 74 (Pharmacia–LKB Biotechnology) diluted tenfold at a flow-rate of 9 ml/h.

RESULTS AND DISCUSSION

Purification

In previous studies elaborate schemes were used for the purification of this enzyme. Eriksson *et al.* [12] used high-performance methods including high-performance IEC on diethylaminohydroxypropyl–agarose gel [13] and high-performance HIC on pentyl–agarose gel [14]. The final recovery was 72%; the purity factor was not reported.

In order to develop a purification procedure feasible for large-scale isolation, preliminary investigations on SEC, IEC, HIC and affinity chromatography on Con A–Sepharose were carried out. In contrast to IMAC on Cu^{2+}–Chelating Sepharose Fast Flow these methods could not achieve a one-step isolation of the enzyme, nor could they improve the purification of catalase after IMAC. Chromatography of the enzyme on Cu^{2+}–Chelating Sepharose Fast Flow (Fig. 1a and b) was promising from the initial studies and by optimizing the conditions a protocol which gave a high-purity catalase in good yields was obtained.

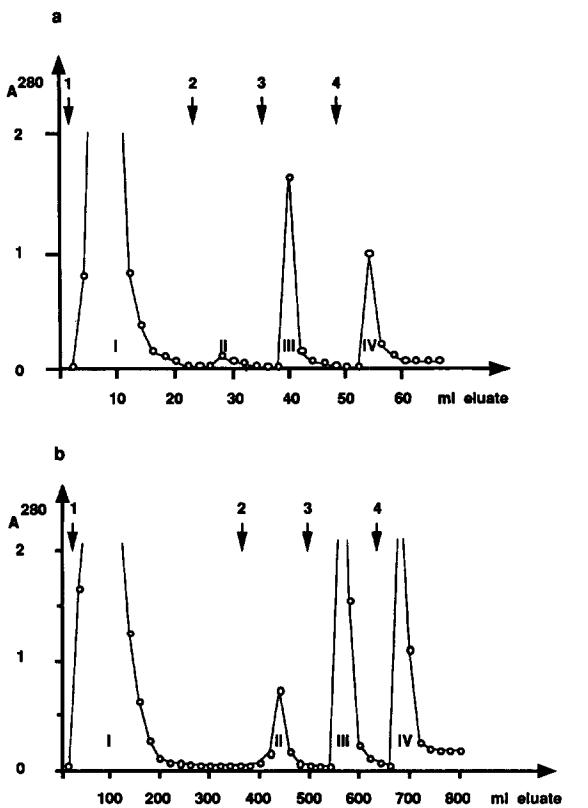

Fig. 1. Chromatography of crude preparation of catalase from *Penicillium chrysogenum* on Chelating Sepharose Fast Flow–Cu^{2+}. 1 = 50 m*M* $NaPO_4$–1 *M* NaCl (pH 7.3); 2 = 50 m*M* $NaPO_4$–0.5 *M* NaCl (pH 6.0); 3 = 20 m*M* sodium acetate (pH 5.5); 4 = 50 m*M* sodium phosphate–1 *M* NaCl–0.2 *M* imidazole (pH 7.3). (a) Analytical run; (b) preparative run.

In Cu(II)–IMAC, imidazole is frequently used for desorption of proteins [15] but in this instance proved to be unsatisfactory, giving a purity index of less than 0.6. This did not increase after desalting or dialysis against deionized water, indicating some kind of interaction between the enzyme and imidazole. It is known that protein adsorption to chelated metal ions, such as Cu^{2+}, Zn^{2+} and Ni^{2+}, is quantitatively dependent on the type of salt and concentration [16] and also the pH, and in this instance both the salt concentration and pH were changed simultaneously. High purity was achieved only with a stepwise decrease in these parameters.

The purification of the *P. chrysogenum* catalase is presented in Fig. 1. Much of the protein from the crude preparation was eluted in the equilibration buffer, but washing with 50 m*M* sodium phosphate–0.5 *M* sodium chloride (pH 6.0) was necessary to remove the contaminants. When applied in this buffer, however the catalase was only adsorbed in low amounts. The catalase-containing fraction was desorbed from the column by lowering the pH and ionic strength simultaneously to 20 m*M* sodium acetate (pH 5.5). The Cu(II) adsorbent was regenerated by washing with 50 m*M* sodium phosphate–1.0 *M* sodium chloride (pH 7.3) containing 0.2 *M* imidazole, which removed impurities and remaining catalase. Finally, the adsorbent was depleted of Cu^{2+} ions by washing with 0.2 *M* EDTA solution (pH 7.0). This protocol was used for both the analytical and the preparative runs (Fig. 1a and b). The catalase specific activity was determined and similar results were obtained for both runs (Table I).

The purity index of the enzyme in water was calculated to be 1.04 and the homogeneity of the material in peak III (Fig. 1a and b) was analysed by IEC on Mono Q and SEC on Superose 6 (Fig. 2a and b).

TABLE I

RECOVERY OF CATALASE ACTIVITIES IN ANALYTICAL AND PREPARATIVE CHROMATOGRAPHY EXPRESSED AS A PERCENTAGE OF APPLIED ACTIVITY

Peak No.	Analytical run	Preparative run
I	0	0
II	1.5	1
III	93	89
IV	4	7

Gel electrophoresis was performed with the Phast System and showed a single band. The molecular weight was calculated to be 280 000 (Fig. 3).

The capacity of Cu^{2+}–Chelating Sepharose Fast Flow to adsorb catalase from the crude preparation was determined by frontal analysis according to ref. 17. The crude enzyme in equilibration buffer was fed into the column and the eluate was analysed for catalase activity. The dynamic binding capacity is given by $C(V_M - V_0)$, where C is the concentration of enzyme, 1.5 mg/ml, in the crude mixture, 7–8 mg protein/ml, and V_M and V_0 are the breakthrough

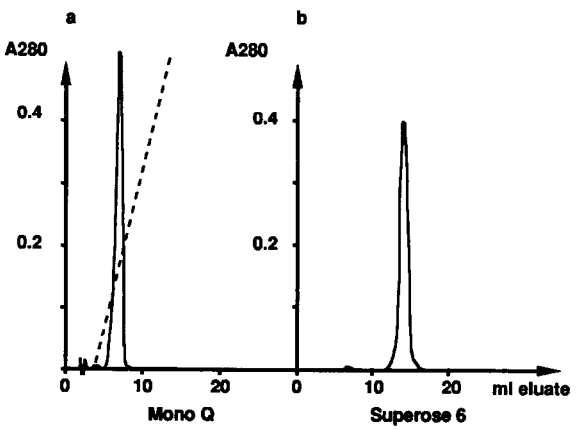

Fig. 2. (a) Chromatography of catalase (peak III from Fig. 1) on Mono Q HR 5/5. A 500-μl sample (*ca.* 1 mg of enzyme) in 20 m*M* Tris–HCl (pH 7.5) was applied to the column. The enzyme was eluted in a linear gradient of NaCl from 0 to 0.8 *M* at a flow-rate of 1.0 ml/min. (b) Chromatography of catalase (peak III from Fig. 1) on Superose 6, HR 10/30. A 200-μl sample (*ca.* 0.7 mg of enzyme) in 20 m*M* Tris–HCl–0.1 *M* NaCl (pH 7.5) was applied to the column. The enzyme was chromatographed at a flow-rate of 0.2 ml/min.

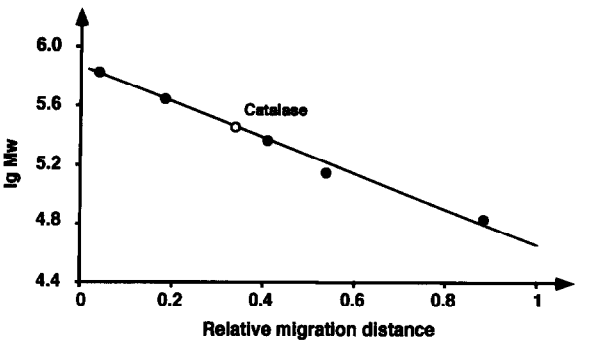

Fig. 3. Electrophoresis of the purified native catalase on Phast Gel gradient 8–25. Filled circles, represent calibration proteins, HMW-kit (Pharmacia-LKB Biotechnology). lg Mw = log molecular weight.

elution volumes of the enzyme in the presence and absence of metal ions, respectively. The capacity for binding of catalase from *P. chrysogenum* in a crude preparation was calculated to be 7.0 mg per ml of Cu^{2+}–Chelating Sepharose Fast Flow.

The catalase from *P. chrysogenum* was purified in a single step from crude material with a specific activity of 6500 U/mg protein to an electrophoretically homogeneous preparation with a specific activity of 35 000 U/mg. The recovery was higher than 85% (Table I), which demonstrates the high capacity and resolution of IMAC.

Characterization

The amino acid and carbohydrate compositions of *P. chrysogenum* catalase in comparison with two other fungal catalases are presented in Table II. The amino acid composition of the enzyme differs significantly from these reported for *Aspergillus niger* and *Penicillium vitale* catalases [6,18,19] (Table II). *P. chrysogenum* catalase contains a smaller number of the neutral amino acids glycine and alanine but more of the aromatic amino acids phenylalanine and tryptophan. There is also a considerable difference in carbohydrate composition between these catalases. Thus catalase from *A. niger* is reported to have a total carbohydrate content of 10.2% (w/w) [6], the enzyme from *P. vitale* to have 8.2% (w/w) [19] whereas the content of the *P. chrysogenum* enzyme was determined to be 8.5% (w/w). The carbohydrate composition differs qualitatively in that *A. niger* is lacking galactose and *P. vitale* lacks glucose residues, but *P. chrysogenum* catalase contains both

TABLE II

AMINO ACID AND CARBOHYDRATE COMPOSITIONS OF CATALASES FROM DIFFERENT FUNGI

Component	Percentage of MW		
	Penicillium chrysogenum	*Penicillium vitale* [18]	*Aspergillus niger* [5,17][c]
Residue			
Aspartic acid	11.0	11.9	9.87
Glutamic acid	11.5	11.6	7.63
Proline	3.8	–	5.84
Glycine	4.1	10.0	6.65
Alanine	5.8	9.30	7.99
Valine	5.3	5.25	5.39
Methionine	1.3	1.14	1.62
Isoleucine	3.5	3.76	3.23
Leucine	7.1	7.65	5.66
Tyrosine	2.2	1.78	3.23
Phenylalanine	8.6	8.16	5.39
Lysine	4.8	4.48	5.03
Histidine	2.6	2.15	2.16
Arginine	6.5	5.31	3.95
Cysteine	0.14		1.53
Threonine	5.4	7.07	6.29
Serine	5.5	5.68	8.26
Tryptophan	2.2[a]	–[b]	–
Carbohydrate			
Mannose	6.8	+	8.1
Galactose	0.9	+	
Glucose	0.2	–	0.2
Glycosamine	0.6	+	1.9

[a] Calculated.
[b] – indicates the absence of this monosaccharide residue in the glycoprotein; + indicates the presence of this monosaccharide.
[c] Calculated from data given in ref. 17, using an MW of 385 000 [5].

glucose and galactose. The sequence of the last twenty amino acid residues at the N-terminus of the enzyme was determined and is reported in comparison with the N-terminal sequence of catalase from *P. vitale* in Table III [20].

TABLE III

AMINO ACID SEQUENCES OF CATALASES FROM *P. CHRYSOGENUM* AND *P. VITALE*

Source	Amino acid sequence (N-terminal)
P. chrysogenum	TEEFLS Q FYL NDQDVYLTS D
P. vitale	AAAQRRQNDS S VFLA IMVAA

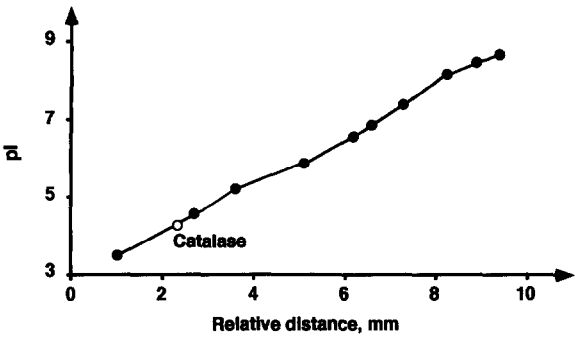

Fig. 4. Isoelectric focusing of the purified catalase on Phast Gel IEF 3–9. Filled circles represent calibration proteins, p*I* markers (Pharmacia-LKB Biotechnology).

Gel electrophoresis performed on Phast Gel gradient 8–25 revealed only one band with silver staining. The molecular weight was found to be 280 000 (Fig. 3). For a more precise determination of the molecular weight a sedimentation equilibrium experiment in the ultracentrifuge was performed. The partial specific volume was calculated according to ref. 21 to be 0.718 and the molecular weight for the enzyme was calculated to be 280 000, confirming the value found by gel electrophoresis.

The determination of the isoelectric point for this catalase was performed by IEF on the Phast System and chromatofocusing and showed good agreement, with p*I* values of 4.3 and 4.2, respectively (Figs. 4 and 5).

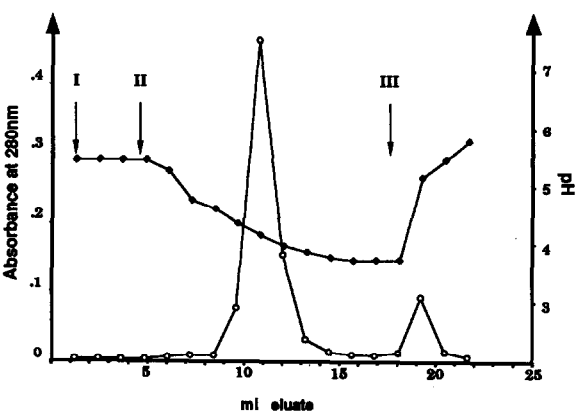

Fig. 5. Chromatofocusing of the purified catalase on PBE 94 with Polybuffer 74. I = 25 m*M* piperazine–HCl (pH 5.5); II = Polybuffer–HCl diluted tenfold (pH 4.0); III = 25 m*M* piperazine–HCl (pH 5.5) containing 1 *M* NaCl.

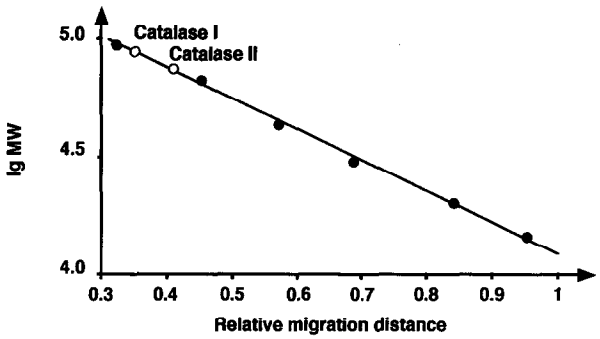

Fig. 6. SDS electrophoresis of the purified catalase on Phast Gel gradient 8–25 and SDS buffer strips. Lanes: 1 = LMW-kit (Pharmacia–LKB Biotechnology); 2 = purified enzyme. Filled circles represent calibration proteins, LMW-kit (Pharmacia-LKB Biotechnology).

The subunit organization of the enzyme was investigated by SDS electrophoresis with Phast System on Phast Gel gradient 8–25. The results confirmed those published in ref. 12 (Fig. 6). The presence of two different subunits (Fig. 6) is an interesting phenomenon also reported for a catalase from the bacterium *Vitreoscilla* [22]. This is distinctly different from *A. niger* and *P. vitale* catalases, which consists of four identical subunits [18,19]. This could be interpreted as an impure enzyme preparation, but all attempts to increase the purity of the enzyme did not improve the result as judged from electrophoresis of the native enzyme (electrophoresis run with sample overloading). Moreover, when the enzyme was studied for its stability against denaturation as in the pretreatment of samples for SDS electrophoresis, the enzyme was found to remain in its native state even after treatment at 60°C in a solution containing 1% of SDS and 2.5% of 2-mercaptoethanol (not shown).

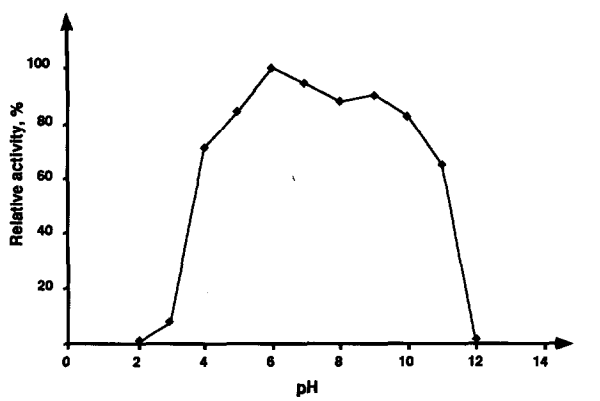

Fig. 7. pH optimum of catalase. Relative activity (% of maximum activity) *versus* pH.

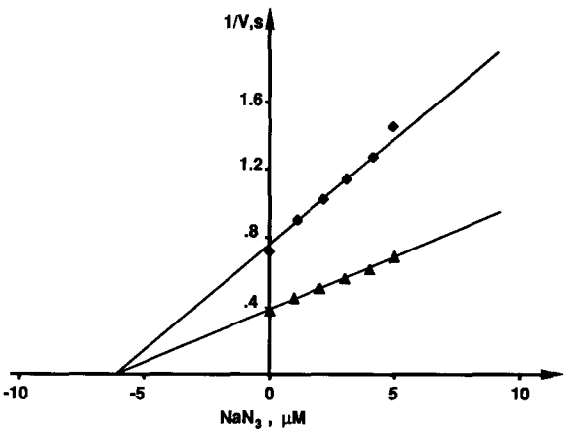

Fig. 8. Dixon plot of the inhibition of catalase from *P. chrysogenum* with NaN_3 performed in 0.1 *M* sodium phosphate buffer, pH 6.5, at substrate concentrations: ◆ = 10 m*M* H_2O_2; ▲ = 20 m*M* H_2O_2.

The apparent Michaelis–Menten constant, K_M, of the enzyme was calculated to be 59 m*M* H_2O_2. The enzyme was found to be active in a broad range of pH from 3.5 to 11.0 with an optimum of 6.0 (Fig. 7). The specific activity of the *P. chrysogenum* catalase was calculated to be 35 000 U/mg protein at an H_2O_2 concentration of 18 m*M*. The inhibition effect of sodium azide as investigated according to Dixon and Webb [11] for two different concentrations of substrate (Fig. 8) indicates that the azide is a non-competitive inhibitor of *P. chrysogenum* catalase with K_i = 6.1 m*M*. UV–VIS spectra were run and showed a typical high-spin iron(III) haeme spectrum with absorption maxima at 405 nm. The

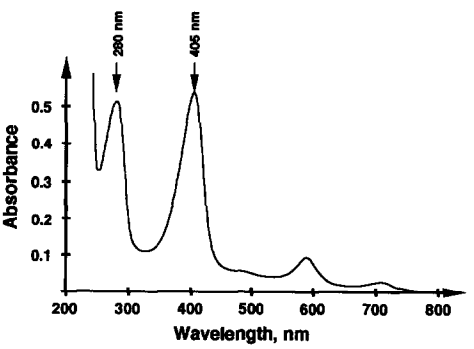

Fig. 9. UV–VIS spectrum of catalase from *Penicillium chrysogenum*.

TABLE IV

COMPARISON OF PROPERTIES OF CATALASES FROM DIFFERENT FUNGI

Species	p*I*	MW	Subunit composition	R_z	K_M, H_2O_2 (m*M*)	K_i, NaN_3 (m*M*)	Specific activity (U/mg)
Penicillium chrysogenum	4.3	280 000	$\alpha_2\beta_2$	1.05	59	6.1	35000
Aspergillus niger [6,17]	6.5	385 000	α_4	0.8	280	475	8500[a]
Cilindrocarpon didymum [24]	5.5	215 000	–	–	–	–	1500

[a] Data from Merck.

purity factor, R_z (UV–VIS spectrum in Fig. 9), being the relationship between absorbances at 405 nm (haeme group) and 280 nm (mainly tryptophan), was found to be 1.04.

A comparison of the properties of catalases from bacteria, fungi, plants and animals showed that the molecular size and subunit organization are similar. The catalases are large enzymes with molecular weights ranging from 200 000 to 400 000 and containing four subunits, with the exceptions of an enzyme from *Klebsiella pneumoniae* [23], with two subunits, and from porcine erythrocytes [7], reported to be a dimeric form of the enzyme with eight subunits. The efficiencies of the enzymes differ so that the specific activities vary from 1500 U/mg for the fungus *Cilindrocarpon didymum* enzyme [24] 68 100 U/mg for the enzyme from *Bacillus subtilis* [25,26]. Also the K_M and K_i (NaN_3) values vary considerably. *P. chrysogenum* catalase is in the middle of the range for most of the variables investigated. It is, however, the most acidic enzyme of the reported catalases (p*I* = 4.3). A comparison of the properties of catalases from fungi is given in Table IV.

We conclude that *P. chrysogenum* culture liquid is an excellent source for the production of catalase. This enzyme has a higher specific activity than other commercially available catalases from bovine liver or *A. niger* culture medium. The isolation procedure reported here gives stable enzyme preparations with purity >99%.

REFERENCES

1 S. Chaga, T. Sapundzieva and A. Kraevska, *Khranit. Promst. Nauka*, 3 (1987) 75–78.
2 S. Chaga, *Tr. Nauchno-Issled. Inst. Konserv. Prom. Plovdiv*, 8 (1971) 105–113.
3 S. Chaga, personal communication, 1975.
4 G. Chaga, L. Andersson, B. Ersson and J. Porath, *J. Biotechnol. Appl. Biochem.*, 11 (1989) 424–431.
5 R. F. Beers and I. W. Sizer, *J. Biol. Chem.*, 195 (1952) 133–140.
6 K. Kikushi-Torii, S. Hayashi, H. Nanamoto and S. Nakamura, *J. Biochem.*, 92 (1982) 1449–1456.
7 A. Takeda and T. Samejma, *J. Biochem.*, 82 (1977) 1025–1033.
8 P. Edman and G. Begg, *Eur. J. Biochem.*, 1 (1967) 80–91.
9 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Reber and F. Smith, *Anal. Biochem.*, 28 (1956) 350–356.
10 O. Theander and E. Westerlund, *J. Agric. Food Chem.*, 34 (1986) 133–140.
11 M. Dixon and E. Webb, in *Enzymes*, Academic Press, New York, 3rd ed., 1979.
12 K.-O. Eriksson, I. Kourtera, K. Yao, J.-L. Liao, F. Kilár, S. Hjertén and G. Chaga, *J. Chromatogr.*, 397 (1987) 239–249.
13 K. Yao and S. Hjertén, *J. Chromatogr.*, 385 (1987) 87.
14 S. Hjertén, K. Yao, K.-O. Eriksson and B. Johansson, *J. Chromatogr.*, 359 (1986) 99.
15 L. Andersson, E. Sulkowsky and J. Porath, *Bioseparation*, 2 (1991) 15–22.
16 J. Porath and B. Olin, *Biochemistry*, 22 (1983) 1621–1630.
17 N. Ramadan and J. Porath, *J. Biochem.*, 321 (1985) 93–104.
18 A. Gruft, R. Ruan and J. Traunor, *Can. J. Biochem.*, 56 (1978) 916–919.
19 L. V. Gudkova, M. F. Guliy, R. T. Degtjar and O. V. Pisarevich, *Ukr. Biokhim. Zh.*, 57 (1985) 29.
20 B. K. Vainshtein, W. R. Melik-Adamyan, V. V. Barynin, K. S. Bartels, I. Fita and M. G. Rossmann, *J. Mol. Biol.*, 188 (1986) 49–61.
21 E. Cohen and J. Edsall (Editors), *Amino Acids and Peptides as Ions and Dipolar Ions*, Rheinold, New York, 1943.
22 J. J. Abrams and D. A. Webster, *Arch. Biochem. Biophys.*, 279 (1990) 54–59.
23 I. Goldberg and A. Hochman, *Biochim. Biophys. Acta*, 991 (1989) 330–336.
24 S. Lartillot, P. Kedziora and A. Athias, *Prep. Biochem.*, 18 (1988) 241–246.
25 P. C. Loewen and J. Switala, *Biochem. Cell Biol.*, 65 (1987) 939-947.
26 P. C. Loewen and J. Switala, *Biochem. Cell Biol.*, 66 (1988) 707–714.