Purification and properties of a catalase from potato tubers
(Solanum tuberosum)

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Catalase from potato tubers (Solanum tuberosum) has been purified to homogeneity. The purified enzyme had a low specific activity (approx. 3000 units/mg of protein) and a marked tendency to form aggregates. Polyacrylamide gel electrophoresis in the presence of SDS revealed a single 56 kDa peptide. The apparent molecular weight of the native protein was estimated to 224 kDa. Some homology was found between the N-terminal sequences of potato, Ipomea batatas, and bovine liver catalases. The enzyme exhibited optical absorbance maxima at 280, 403, 500, 535, and 620 nm, and its epr spectrum was characteristic of a hemoprotein with high-spin ferric iron. The pseudo first order rate constant was invariant for H2O2 concentrations ranging from 8 to 30 mM, but decreased rapidly at higher concentrations. Cyanide and azide were inhibitors, and 2-mercaptoethanol was a much more efficient inhibitor than for other catalases. No NADPH could be detected in the potato catalase; this was the first search for this dinucleotide in a plant catalase, and its absence in the potato enzyme could be tentatively related to its lower propensity to form the compound II state.

Key words: catalase; hydrogen peroxide; NADPH; potato; Solanum tuberosum

Introduction

Many studies have been devoted to catalase (EC 1.11.1.6), and this ubiquitous enzyme has been purified in a variety of instances from animal tissues, plants and microorganisms [1,4]. Some years ago, the discovery by Kirkman and Gaetani [5] of tightly bound molecules of NADPH to bovine liver catalase led to new insights in the physiological properties of this enzyme. Crystallographic data by Fita and Rossmann [6] confirmed the presence of four NADPH molecules per catalase tetramer. This dinucleotide was not in close contact with the enzyme hemic sites and was suspected to have a role distinct from the main function of catalase, i.e. the dismutation of two hydrogen peroxide molecules to dioxygen and water. For instance two fungal catalases from Penicillium vitale and Aspergillus niger were seemingly devoid of NADPH [5,7] despite the fact that, in the former case, the structure of the enzyme was known in great detail [8].

Later evidence showed that the function of NADPH in bovine liver catalase was to rescue this
enzyme against its spontaneous inactivation in the compound II stage induced by hydrogen peroxide [9,10]. We have found that a bacterial catalase purified from _Proteus mirabilis_ PR displayed great similarities to the mammalian enzyme and behaved much in the same way, indicating that NADPH was also present [9,11].

Few data are available for catalase from plant origin, that has been purified only in few instances: spinach leaves [12,13], lentil leaves [14], cucumber cotyledons [15], germinating pumpkin cotyledons [16], sweat potato root microbodies [17,18], and _Zantedeschia aethiopica_ leaves [19].

In this report we describe an easy method for the purification of catalase from potato tuber peroxisomes, as well as some properties of this enzyme. A search for NADPH in the pure enzyme was carried using the same method as formerly applied to _Proteus mirabilis_ catalase.

**Materials and Methods**

**Materials**

Potato tubers (_Solanum tuberosum, _L.)_ were obtained from a local market. Beef liver catalase was purchased as a crystalline suspension in water from Boehringer-Mannheim (FRG, catalogue number 106828). Pure proteins used as molecular weight standards were from Boehringer-Mannheim and Pharmacia. Protein standards for electrofocusing were from Biorad. Protein standards for electrolysis were from Sigma. Hydrogen peroxide and common salts (reagent grade) were from Merck (Darmstadt, FRG). AcA 34 Ultrogel was purchased from IBF-Pharmindustrie, France.

**Purification of potato catalase**

The peroxisomal fraction of potato tubers was prepared from 16 kg tubers as previously described by Neuberger et al. [20]. Peroxisomes were suspended in 0.3 M Tris—HCl (pH 7.5) containing 0.75 M sucrose, 2 mM EDTA, 0.3 mM PMSF, and 0.0125 mM TLCK, then lysed by sonication in an ice bath. To the lysed peroxisome preparation (25 mg total protein), powdered ammonium sulfate was added up to 20% saturation at 0°C. The precipitate thus formed was eliminated by centrifugation and ammonium sulfate was added again to the supernatant until a 45% saturation at 0°C was reached. The precipitate contained the bulk of catalase activity. It was collected by centrifugation, dissolved in a small volume (1.5 ml) of 0.1 M Tris—HCl buffer (pH 7.5) and layered on the top of an AcA 34 Ultrogel column (1.5 × 100 cm). Elution of catalase was performed with 0.1 M Tris—HCl buffer (pH 7.5). The 3.5-ml fractions were collected at a flow rate of 12 ml h⁻¹. Active fraction were pooled and concentrated by ammonium sulfate precipitation at 85% saturation (0°C), centrifugation of the precipitate at 40 000 × _g_ for 30 min, and dissolution in 0.1 M Tris—HCl buffer (pH 7.5). A second chromatographic step was performed using the same column and conditions as above. The active fractions were pooled and concentrated by ammonium sulfate precipitation as above. The preparation was dissolved in a small volume of Tris—HCl buffer, supplemented with glycerol up to 10% (v/v), and stored at -20°C.

**Enzyme assays**

Catalase activity was assayed using the initial rate of decrease in _H₂O₂_ concentration, as measured by its absorbance at 240 nm [21]. Peroxide was 11 mM at the starting conditions. Cyanide or azide as inhibitors were supplied to the assay mixture before adding the enzyme. In the case of inhibition by thiols, catalase was pre-incubated in the presence of these agents prior to the addition of peroxide as previously specified [22]. The molar extinction of _H₂O₂_ at 240 nm was taken as 39.4 M⁻¹ cm⁻¹ [23], and used for calculations of _H₂O₂_ concentrations. The apparent first order rate constant (kₐ) was estimated in each case from the initial rate of _H₂O₂_ disappearance.

**Protein concentration**

The Bradford method [24] was used for the estimation of total protein in non-purified extracts. The Kalckar's spectrophotometric method [25] was used for purified preparations. The concentrations of pure catalases from bovine liver (BLC)
or from *P. mirabilis* PR were determined using the molar extinction coefficients at the Soret band wavelength as specified previously [11].

**Effect of pH**

The activity of potato catalase as a function of pH was determined using the following buffers: 0.2 M Na₂HPO₄-citric acid in the pH range under 6, 0.2 M sodium phosphate in the pH range close to neutrality, 0.025 M sodium borate-HCl in the pH range above 8.5. All buffer solutions were made 11 mM in H₂O₂ just prior to the addition of enzyme, and the resulting pH was determined. Catalase (0.9 µM) used for the experiment was in 30 mM Tris—HCl (pH 7.5), and added to the reaction mixture in a small volume (10 µl), with no significant alteration of the final pH. For determining the catalase stability at different pH values, the enzyme (0.3 µM) was first incubated for 3 h at 37°C in buffer in the absence of H₂O₂, the constancy of pH was checked, and the residual catalase activity was determined in the presence of substrate at pH 6.8 in the standard conditions of assay.

**Electrophoresis**

Analytical polyacrylamide gel electrophoresis was performed in the presence of SDS using the method of Laemmlli [26], in slabs 0.8 mm in thickness as previously indicated [27]. The Pharmacia Phast System was used for electrofocusing in polyacrylamide gels. The catalase stain was as earlier specified [28].

**Spectroscopy**

An Uvikon 810 spectrophotometer (Kontron, Switzerland) was used for light absorbance spectroscopy. The epr spectra were recorded using a Varian E 109 spectrometer as indicated earlier [22].

**HPLC separation**

The apparatus used for high performance liquid chromatography was a Beckman model 330, equipped with a reverse-phase column (Ultrapore RPSC, 4.6 × 7.5 mm). The solvents used were of HPLC purity grade. The absorbance at 214 nm of the eluate was used for protein detection. The protein samples were precipitated with 10% trichloracetic acid (w/v). After 3 h at 4°C the pellet was collected by a 20 min centrifugation at 17 000 × g, and dissolved in 0.1 M Tris—HCl buffer (pH 7.9) containing 6 M urea, 0.15 M LiCl, 6 mM 2-ME. A volume of 50 µl of this solution was injected into the HPLC column. The flow rate was 1 ml min⁻¹. Elution was performed using a 10—60% gradient (45 min) of acetonitrile in 0.1% TFA. The potato catalase subunits were eluted as a single peak at an acetonitrile concentration of 48.5—49%. After HPLC separation, the protein fractions used for N-terminal sequencing were lyophilized in a Speedvac concentrator.

**N-terminal sequencing**

An aliquot of denatured pure catalase monomer (about 300 picomol) was submitted to N-terminal sequencing in duplicate, using a gas phase sequencer (Applied Biosystems, model 477A), equipped with an online PTH aminoacid analyser (Applied Biosystems, model 130A), according to Ref. [29].

**Results**

**Enzyme purification**

Differential centrifugation in Percoll media [20] has been devised as a simple method for the purification of potato tuber peroxisomes, that contained a large crystalline inclusion associated with an elevated catalase activity. Further purification of this catalase was accomplished as described in Methods. Purified catalase was recovered in 35% yield. Because of the large amount of catalase present in peroxisomes, the purification thus obtained was about only two-fold. The purification factor from crude extracts of potato tubers was much higher, but could not be estimated with precision due to the difficulty of using the spectrophotometric assay in crude extracts of grinded tubers [20]. The specific activity of the purified catalase was about 3000 units/mg of protein.

It was noticeable that the purified enzyme had a marked tendency to form aggregates on standing at elevated concentrations, i.e. above 1 mg ml⁻¹ in a dilute buffer such as 10 mM potassium phos-
phate at pH 6.8. The protein was firmly retained by a Phenyl-Sepharose gel even at diluted salt concentrations (10 mM Tris—HCl, pH 7.5), and only partially released by the same buffer containing 50% ethylene glycol (v/v), a fact suggesting that the enzyme was hydrophobic in nature. Similarly it had a marked tendency to attach to ordinary Amicon membranes used for ultrafiltration, preventing the use of this method.

**Gel electrophoresis**

The purified potato catalase migrated as a major component when analyzed by SDS-PAGE, together with weak contaminants. The polypeptide chain behaved as 56 ± 2 kDa in molecular weight. This was a somewhat smaller value compared to BLC or the catalase of *P. mirabilis* PR [21] (Fig. 1a). Using PAGE in non-denaturing conditions with 7.5% acrylamide, the purified catalase migrated as a spurious band which was best explained by the tendency of the protein to precipitate or to form aggregates. Using PAGE of native proteins in a 8—25% acrylamide gradient, catalase was recovered as a protein band near that of *P. mirabilis* PR catalase which has a molecular weight of 250 kDa [21] (Fig. 1b). This result suggested that catalase from potato microbodies was also a tetramer, with a molecular weight of about 224 ± 8 kDa. The gel permeation method using AcA34 Ultrogel for estimating the apparent molecular weight of catalase was not used here because of the marked interaction of the enzyme with the gel matrix.

Electrofocusing of the purified enzyme on ultrathin polyacrylamide gel revealed 3 or 4 components with catalase activity and an isoelectric pH close to 5.5. Several bands were also observed in the case of *P. mirabilis* catalase, although in

![Fig. 1. Electrophoretic migration of catalases from potato tuber, bovine liver and *P. mirabilis*. (a) SDS-PAGE analysis. Lane T, protein markers of known molecular weights (kilodaltons), using the Pharmacia calibration kit for low molecular weights. Lane P, purified potato catalases (5.5 µg). Lane B, BLC (2 µg). Lane R, *P. mirabilis* PR catalase (2.5 µg). (b) PAGE of native proteins in a 8—25% acrylamide gradient (Pharmacia Phast System). Lane T, protein markers of known molecular weights (kilodaltons), i.e. animal ferritin (440 kDa), l-alanine dehydrogenase (228 kDa), lactate dehydrogenase (140 kDa), alkaline phosphatase (100 kDa), bovine serum-albumin (67 kDa), ovalbumin (43 kDa). Lane R, *P. mirabilis* PR catalase (1 µg). Lane P, purified potato catalase (2 µg). Lane B, BLC (2 µg).](image-url)
Purified potato catalase (about 300 μg) was successively denatured by trichloroacetic acid and urea, then injected to the column at the arrow. Elution was performed as specified in Methods.

In this case the protein was more acidic with an isoelectric pH equal to 4.8 [21,27].

**N-terminal sequencing**

Denatured potato catalase subunits were further purified extensively by HPLC in order to eliminate the remaining impurities. An apparently homogeneous preparation of catalase polypeptide chains was thus obtained (Fig. 2). It was submitted to N-terminal sequencing. The result of this analysis was compared to the N-terminal sequences of BLC [30] and *Ipomea batatas* catalase [18] as follows:

<table>
<thead>
<tr>
<th>Potato</th>
<th>YTPSSAVDTPFL</th>
<th>NACG</th>
<th>FTV</th>
<th>VRNS</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ipomea</em></td>
<td>MDPSKYRPSPPFTTFCTNHSAPVWNNTC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLC....</td>
<td>QMKHWKEORAAOKPDLV</td>
<td>CGGDP</td>
<td>VDKL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The residues that have been framed are usually shared by catalase from other origins [31—33]. The N-terminal sequence of potato catalase has 12 residues in common with the N-terminal sequence of *Ipomea batatas* [18] provided the latter is shortened by 5 residues. Similarly, eight residues are found in common with BLC [30], provided the latter is shortened by 14 residues.

**Spectroscopic data**

The absorbance spectrum of the purified potato enzyme showed maxima at 280, 403, 500, 533, and 620 nm, with a spectral coefficient ($R_z = A_{405}/A_{280}$) equal to 0.88.

The epr spectrum of potato catalase at low temperature was characteristic of a hemoprotein with high-spin ferric iron [34]. The effective signal $g$ values were $g_x = 6.41$, $g_y = 5.45$, and the rhombicity percentage was $R = 5.9\%$ [35]. At pH below 7 a more rhombic form appeared with an $R$ value near 9%, recalling former observations with catalase from other sources [22,36].

**Enzyme activity and inhibitors**

Assuming a molecular weight of 224 kDa the specific molar rate constant for the purified potato catalase at 25°C could be estimated to $10^6$ M$^{-1}$ s$^{-1}$, and the turnover number to 11200 s$^{-1}$.

As for other known catalases [27,37] the pseudo first order rate constant remained invariant for H$_2$O$_2$ concentrations ranging from 8 to 30 mM, but at higher concentrations, it decreased rapidly (Fig. 3).

Both cyanide and azide were inhibitors of potato tuber catalase (Fig. 4). Concentrations causing 50 percent inhibition were 2 μM and 20 μM, respectively. Thiols are also known as catalase inhibitors [38]. DTE at 5 mM suppressed about only half of the potato catalase activity.

Fig. 2. HPLC separation of the potato catalase subunits. Purified potato catalase (about 300 μg) was successively denatured by trichloroacetic acid and urea, then injected to the column at the arrow. Elution was performed as specified in Methods.

Fig. 3. Effect of hydrogen peroxide concentration on the activity of purified potato catalase. The enzyme (about 10 μl of a 0.45 μM solution) was assayed with various H$_2$O$_2$ concentrations at 25°C as described in Methods.
Fig. 4. Inhibition of potato catalase by sodium azide and sodium cyanide. The catalase reaction was started by adding 10-μl aliquots of the enzyme solution (0.58 μM) to the reaction mixture (1 ml) containing 10 mM potassium phosphate (pH 6.8) and 11 mM H₂O₂, at 25°C. Variable amounts of inhibitors were used, as indicated. Initial catalase activity in the absence of inhibitor was about 5 μmol H₂O₂ min⁻¹. Data were expressed as percentages of residual activity compared to the non-inhibited reaction. ■—■, Inhibition by cyanide. □—□, inhibition by azide.

after 100 min at 25°C, but 2-ME was a much stronger inhibitor, producing the same effect at 0.05 mM after 10 min only. Complete inhibition of the enzyme (0.3 mg ml⁻¹) was observed in the presence of 5 mM 2-ME, and could not be reversed after extensive dialysis against 10 mM potassium phosphate, pH 7.

In some instances thiols have been shown to produce reversible effects on the absorption spectrum of catalase that are indicative of the compound II state [2], due to the slow generation of hydrogen peroxide by the spontaneous thiol autoxidation [38]. This was the case of BLC and P. mirabilis PR catalase [9–11]. These spectral changes were reversed by minute amounts of NADPH (4 mol/mol of enzyme). The spectral changes produced by 10 mM DTE on potato catalase spectrum were only very slight, with a 5% decrease in the Soret band after an incubation period as long as 3 h at 30°C. There was no clear indication of any compound II accumulation in this instance. Other spectral changes were not observed after adding NADPH (8 mol/mol of enzyme or above), even after 1 h of further incubation. This result was not due to reactivation of the enzyme by internal NADPH [11] since the direct search for this dinucleotide was negative (below 0.3 mol/mol of enzyme).

**Effect of pH**

Figure 5A shows the pH dependence of the catalase activity in the purified preparation. A broad optimum was obtained in the pH 6–8 range, as found with catalase from other origins [17,27,37]. The stability of the enzyme as a function of pH is shown by Fig. 5B. No significant decrease of activity was observed after 3 h at 3°C in the pH range from 6.2 to 7.5.

**Discussion**

Because potato tuber peroxisomes contain a high level of catalase this enzyme can be easily prepared in pure state using a simple procedure. It was found to be similar in size to BLC, appearing as a tetramer of 56 kDa subunits. According to known data, this situation holds for most plant...
catalases, as in cucumber cotyledons [15], in germinating pumpkin cotyledons [16], in leaves of *Zantedeschia aethiopica* [19], or in *Zea mays* that contains three genetically distinct isozymes [39]. The spinach catalase is at variance with this general model, having only two 55 kDa subunits [13]. N-terminal sequencing of the potato catalase revealed a clear homology with the same sequence in the *Ipomea batatas* catalase [18].

Some properties of the potato tuber catalase may be judged as classical if compared to BLC, according to its absorbance, epr spectra, pH optimum, and inhibition by azide or cyanide [2,4]. However, the Soret band displayed by the potato enzyme was slightly shifted to shorter wavelength (403 nm vs. 405 nm), and the $R_z$ index ($A_{405}/A_{300}$) was somewhat higher than in the case of the *Ipomea batatas* catalase (0.88 instead of 0.67) [17].

Other properties of potato catalase were clearly distinct compared to known catalases. Firstly, the protein seemed more hydrophobic on the basis of its tight binding to phenyl sepharose gel and its tendency to form aggregates in aqueous solution. Secondly, the specific activity of the enzyme was about one order of magnitude lower compared to that of BLC [2,3]. This was possibly related to its greater sensitivity to elevated concentrations of $H_2O_2$ (up to 30 mM). Thirdly, the potato catalase appeared much more sensitive to 2-mercaptoethanol than usually seen for others [22,38]. Finally, the interaction between catalase and NADPH had never been looked for in catalase from higher plants. Here we have shown that potato catalase is apparently devoid of NADPH, a property shared by catalase from *Penicillium vitale* or *Aspergillus niger* [5,7]. It had been noticed that the *A. niger* enzyme did not show any occurrence of compound II state [40], an inactive form produced after single electron reduction of compound I [2]. A similar situation has been met here in the case of potato catalase. NADPH is considered as a possible rescuer of catalase when blocked in the compound II state [10,11]. We suggest therefore that a correlation should exist in catalase between its ability to bind NADPH and its propensity to form the compound II state. More data about catalases of various origins are needed to check this hypothesis.

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**References**


