Purification and Comparison of Peroxisomal and Cytosolic Catalases from a Methanol-Grown Yeast, *Kloeckera* sp. 2201

By

Mitsuyoshi UEDA*, Sabiha MOZAFFAR* and Atsuo TANAKA*

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Abstract

Catalases were purified from a peroxisome-containing particulate fraction and a cytosolic fraction of methanol-grown *Kloeckera* sp. 2201 cells after subcellular fractionation. No difference was observed between the enzymes in the behaviours on column chromatographies, molecular mass of the subunits (M, 62,000 daltons), and terminal amino acids, alanine. In addition, similar patterns were obtained with the peroxisomal and cytosolic enzymes on sodium dodecylsulfate/polyacrylamide slab-gel electrophoresis of the peptide fragments prepared by partial digestion with *Staphylococcus aureus* V 8 protease and papain. These results indicate that cytosolic catalase, even if functional, essentially has identical properties with the peroxisomal one in spite of the different subcellular distribution.

1. Introduction

The level of catalase, one of the important constituents of peroxisomes (microbodies), increases inducibly, and the enzyme is specifically localized in the organelles in harmony with their development. These phenomena offer an excellent model to investigate the biogenesis and development of subcellular organelles.[1)]

In spite of the fact that catalase is a typical marker enzyme of peroxisomes, a part of catalase activity is usually recovered in the cytosolic fraction, even if various subcellular fractionations are carried out carefully. This cytosolic catalase has been considered by some investigators to be the enzyme leaked from fragile peroxisomes. However, in one type of cells from Zellweger syndrome patients, which was discovered as a peroxisome-deficient hereditary disease,[2)] most of the catalase activity appeared in the cytosolic fraction.[3)] Furthermore,

* Department of Industrial Chemistry

most of the catalase is localized in cytosol of propionate-grown *Candida tropicalis* cells in spite of the appearance of peroxisomes.[4] These results indicate the possibility of the presence of extra-peroxisomal catalase, although its function in the cytosol has not yet been elucidated. We have also observed th activity of catalase in the cytosolic fraction prepared from methanol-grown *Kloeckera* sp. cells.[5, 6] Therefore, it is interesting and important to compare the properties of the enzymes present in peroxisomes and cytosol.

This paper describes the purification of peroxisomal and cytosolic catalases from methanol-grown *Kloeckera* sp. 2201. The results showed that both enzyme molecules were indistinguishable in their properties.

2. Materials and methods

Cultivation of yeast.

Kloeckera sp. 2201 (known as a strain of *Candida boidinii*) was cultivated aerobically at 30 °C in a medium containing 1% (v/v) of methanol as the sole source of carbon and energy.[7] Cells were harvested at the mid-exponential growth phase (48 h of cultivation).

Subcellular fractionation.

Protoplasts of methanol-grown cells were prepared in a lysis medium consisting of 40 mM potassium phosphate buffer (pH 7.2), 2 M sorbitol, 0.1 mg/ml of dithiothreitol and 24 μg/ml of Zymolyase 100 T (Kirin Brewer Co., Tokyo, Japan) at 30° C.[5] The protoplasts thus obtained were suspended in a solution (solution A) composed of 50 mM potassium phosphate buffer (pH 7.2), 2 M sorbitol, 0.2 mM phenylmethylsulfonyl fluoride and 0.5 mM EDTA, and homogenized with a teflon homogenizer for 10 min. under cooling on ice. The homogenate was centrifuged at 3,000 x g for 10 min. to separate the heavy particles (P_1) and the supernatant (S_1). The S_1 fraction was subjected to centrifugation again at 20,000 x g for 15 min. to obtain a particulate fraction (P_2) and a supernatant fraction (S_2). The S_2 fraction was used as a crude extract for the preparation of cytosolic catalase. The P_2 fraction was suspended in a 50 mM potassium phosphate buffer (pH 7.2) containing 0.2 mM phenylmethylsulfonyl fluoride, and was treated by a Braun cell homogenizer for 30 s followed by centrifugation at 20,000 x g for 20 min. The supernatant so obtained, P_2-S fraction, was employed as the source of peroxisomal catalase.

Enzyme assay and protein estimation.

Catalase activity was measured spectrophotometrically according to the previous paper.[8] The protein was determined according to Lowry *et al.*[9]

Electrophoresis.

Polyacrylamide slab-gel electrophoresis in the presence of sodium dodecylsulfate was performed as described previously.[10]

Estimation of molecular mass of the subunits.

The molecular mass of the subunits of purified catalases was determined by electrophoresis on sodium dodecylsulfate/polyacrylamide slab-gel containing 10% acrylamide. A molecular mass kit containing phosphorylase b (94,000 daltons), bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), trypsin inhibitor (20,100 daltons), and α-lactalbumin (14,400 daltons) was used as a standard.

Analysis of amino terminus.

The purified enzyme was dialyzed against deionized water and lyophilized. Amino terminal analysis was performed by the dansylation method of Gray.[11]

Analysis of peptide fragments.

After the enzyme was dissociated into subunits on 10% polyacrylamide slab-gel in the presence of sodium dodecylsulfate, the gel area corresponding to the subunit was excised and electrophoresed with *Staphylococcus aureus* V 8 protease or papain as described by Cleveland *et al.*,[12] except that 12.5 % acrylamide gels were used as the digesting gels. Peptide fragments were detected by the silver staining method (Technical Manual I, Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan).

Chemicals

Hydroxyapatite (Hypatite) HCA 100-S was kindly donated by Mitsui Toatsu Chemicals (Tokyo, Japan). Sephacryl S-300, DEAE-Sepharose, and a molecular mass kit were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and phenylmethylsulfonyl fluoride from Sigma (St. Louis, MO, USA). Other chemicals were also purchased from commercial sources.

3. Results

Purification of peroxisomal and cytosolic catalases

Peroxisomal and cytosolic catalases were purified from the peroxisome-containing particulate fraction (P_2) and the cytosolic fraction (S_2), respectively. Since peroxisomes of methanol-grown yeast cells are very fragile, it is usually difficult to recover peroxisomal catalase in a high yield after subcellular fractionation. Use of 2.0 M sorbitol in the lysis solution and in solution A, instead of 0.8 M sorbitol for the former and 0.65 M sorbitol for the latter employed before,[5] improved the recovery of catalase in the P_2 fraction from 15 % to 30 %. Solubilization of catalase from peroxisomes was another problem. Treatment of the P_2 fraction with a Braun cell homogenizer enabled us to solubilize the enzyme successfully (yield, 80 %) compared with the case of the osmotic disruption of peroxisomes (yield, 60 %).

The peroxisomal and cytosolic catalases were purified by ammonium sulfate fractionation (40-70% saturation) followed by the successive column chromatographies with buffers containing 0.2 mM phenylmethylsulfonyl fluoride; 1) hydroxyapatite column (2.8 × 18 cm) chromatography with a linear concentration gradient of potassium phosphate buffer prepared from 20 mM and 500 mM potassium phosphate buffer (pH 7.2); 2) Sephacryl S-300 column (2.2 × 85 cm) chromatography with 50 mM potassium phosphate buffer (pH 7.2); 3) DEAE-Sepharose column (2.2 × 22.5 cm) chromatography with a linear concentration gradient of KCl prepared from 50 mM potassium phosphate buffer (pH 7.2) and the same buffer containing 0.4 M KCl. In the case of cytosolic catalase, the S_2 fraction was extensively dialyzed against 20 mM potassium phosphate buffer (pH 7.2) containing 0.2 mM phenylmethylsulfonyl fluoride to remove sorbitol before

Table I. Purification of Peroxisomal Catalase from *Kloeckera* sp. 2201

Fraction	Total protein (mg)	Total activity ($mmol.min^{-1}$)	Yield (%)	Specific activity ($mmol.min^{-1}.mg^{-1}$)	Purification (−fold)
P_2-S fraction	185	1,078	100	5.8	1
Ammonium sulfate (40-70 %) saturation	20	346	32	20	4
Hydroxyapatite	1.7	193	18	113	19
Sephacryl S-300	0.94	171	16	182	31
DEAE-Sepharose	0.47	103	10	219	38

Table II. Purification of Cytosolic Catalase from *Kloeckera* sp. 2201

Fraction	Total protein (mg)	Total activity ($mmol.min^{-1}$)	Yield (%)	Specific activity ($mmol.min^{-1}.mg^{-1}$)	Purification (−fold)
S_2 fraction	1,565	3,633	100	2.3	1
Ammonium sulfate (40−70%) saturation	222	1,502	41	6.8	3
Hydroxyapatite	11.8	1,156	31	98	43
Sephacryl S-300	6.6	959	26	157	68
DEAE-Sepharose	2.9	784	21	270	117

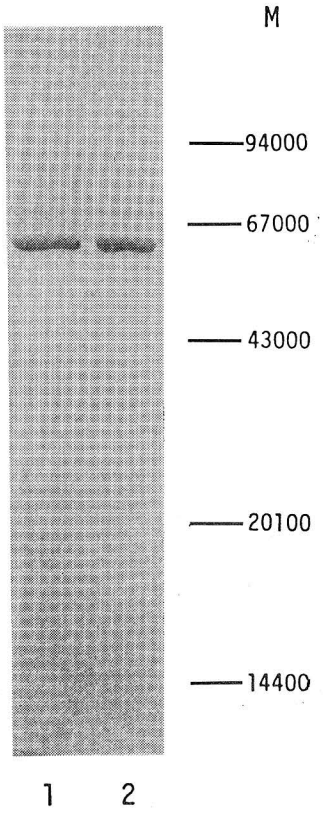

Fig. 1. Sodium dodecylsulfate/polyacrylamide slab-gel electrophoresis of peroxisomal and cytosolic catalases purified from *Kloeckera* sp. 2201. Lane 1, peroxisomal catalase (3 μg) ; lane 2, cytosolic catalase (3 μg).

the ammonium sulfate fractionation. As shown in Tables I and II, the peroxisomal enzyme was purified 38-fold from the P_2-S fraction (yield, 9.5%) and the specific activity was 219 mmol. min^{-1}.mg $protein^{-1}$. The cytosolic enzyme was purified 117-fold from the S_2 fraction (yield, 21%) and the specific activity was 270 mmol. min^{-1}.mg $protein^{-1}$.

Characterization of peroxisomal and cytosolic catalases

Single bands corresponding to the molecular mass of 62,000 daltons were obtained with peroxisomal and cytosolic catalases on sodium dodecylsulfate/

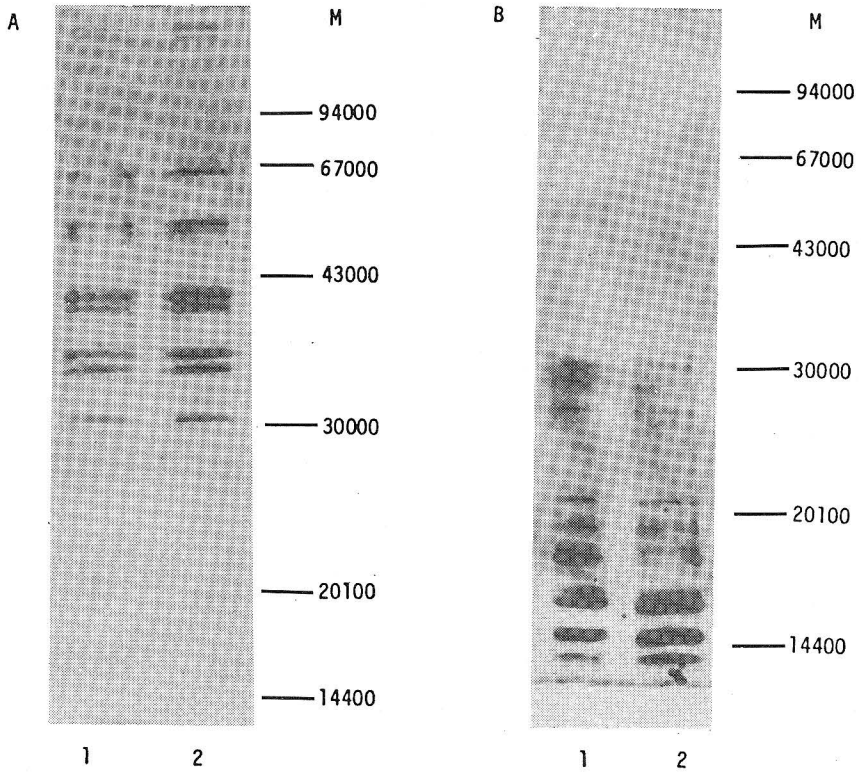

Fig. 2. Limited proteolysis of peroxisomal and cytosolic catalases from methanol-grown *Kloeckera* sp. 2201 with proteases on sodium dodecylsulfate/polyacrylamide slab-gels (12.5%). Peptides were stained with silver staining reagents. (A), Peptide maps of the respective enzymes (5 μg each) treated with *Staphylococcus aureus* V8 protease (25 ng each) ; (B), peptide maps of the respective enzymes (10 μg each) treated with papain (12 ng each) . Lane 1, peroxisomal catalase; lane 2, cytosolic catalase.

polyacrylamide slab-gel electrophoresis (Fig. 1), the molecular mass of the subunits of the peroxisomal and cytosolic enzymes coinciding with that of catalase purified from the methanol-grown whole cells.[13]

Dansylation reaction showed that alanine occupied the amino-terminal positions of both peroxisomal and cytosolic catalases.

Sodium dodecylsulfate/polyacrylamide slab-gel electrophoresis of the peptide fragments prepared by partial digestion with *Staphylococcus aureus* V 8 protease and papain revealed similar patterns with the peroxisomal and cytosolic enzymes (Fig. 2). These results together with those of the molecular mass determination strongly indicate that peroxisomal and cytosolic catalases are essentially identical polypeptides.

The K_m value of the respective enzymes for hydrogen peroxide was almost the same (about 25 mM), and was comparable to that of the catalase purified from whole cells.[13]

4. Discussion

Biogenesis and development of peroxisomes seems to be closely related to the regulation of the synthesis, transportation and localization of peroxisomal enzymes. Catalase in a methanol-utilizing yeast *Kloeckera* sp. 2201, which has characteristic peroxisomes,[14] has two specific features. The first is that this enzyme possesses a peroxidatic activity of physiological significance to oxidize methanol to formaldehyde with hydrogen peroxide formed in the first step of methanol oxidation by alcohol oxidase.[15] The second feature is that the enzyme together with alcohol oxidase constitutes a kind of crystalloids in peroxisomes.[16] During subcellular fractionation of the yeast cells, we have detected the activity of catalase not only in peroxisomes but also in the cytosolic fraction. However, there was no difference in the molecular mass of the subunits, the terminal amino acids, and the peptide fragments between the peroxisomal and cytosolic enzymes. In the preceeding paper,[13] we have proved immunochemically that only one type of catalase is present in the whole cells of methanol-grown *Kloeckera* sp. 2201. These results strongly indicate that the cytosolic catalase is essentially identical to the peroxisomal enzyme, although it is unclear whether the cytosolic enzyme is derived from disintegrated peroxisomes or originally present in the cytosol, and whether the enzyme is functionable in cytosol or not. The absence of a so-called precursor-type polypeptide of catalase and another catalase encoded by the different gene, so far as examined by the mRNA-dependent translation system[17] and the analysis of DNA sequence encoding the

enzyme,[18,19] was also demonstrated with alkane-grown *Candida tropicalis*.

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