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Catalase from *Aspergillus niger* KUF-04

Yang, Ho-Suk[1*], Han-Chul Yang, and Yoshiki Tani[2]

[1] *Department of Food Technology, Korea University, Seoul 136-701, Korea*
[2] *Department of Agricultural Chemistry, Kyoto University, Kyoto, Japan*

Aspergillus niger KUF-04가 생산하는 Catalase

梁鎬錫[1*]·梁漢喆[1]·谷吉樹[2]

[1]高麗大學校 食品工學科 [2]日本 京都大學校 農化學科

Catalase from *Aspergillus niger* KUF-04 was purified by five steps including gel filtration. The overall purification gave 64-fold purified preparation, a yield of about nine percent. The enzyme showed its maximum absorption at 406 nm. The optimum pH and temperature for the enzyme activity were around pH 7.0 and 60°C, respectively. The catalase was found to be stable in the range of pH 4.0 to pH 8.3 and temperature 20°C to 60°C. However, it lost nearly all of the activity by heating at 80°C for 20 min. The activity was markedly inhibited by hydroxylamine, potassium cyanide and sodium azide.

Catalase (E.C.1.11.1.6) is one of the first enzymes isolated in a high degree of purity. The crystallization of catalase from the liver extract (1) is ranked among the early triumphs of biochemistry. The enzyme is widely distributed in microorganisms except anaerobic bacteria. For example, it exists in mammalian liver (2), erythrocytes (3), chloroplast in plant (4), bacteria (5), yeasts (6), and fungi (7). Catalases from beef liver (1), lamb liver (8), horse liver (9), human erythrocyte and bacteria were crystallized. Catalase is a ferric hemoprotein which mediates the decomposition of hydrogen peroxide. Therefore, this enzyme biologically plays an important role in freeing a living organism from the toxicity of hydrogen peroxide. Catalase is applied to determine the enzymatic activity of glucose oxidase, galactosidase and xanthine oxidase, and is used to remove glucose or oxygen from many kinds of foods with glucose oxidase. Catalase co-exists with the oxidase which produces hydrogen peroxide with molecular oxygen during enzyme reactions. *Asp. niger* produces a lot of catalase together with glucose oxidase.

Although the physical, chemical properties and the purification procedure of catalase from other sources have been studied, little has been published on the catalase from *Asp. niger*. Hence, the purification procedure and properties of catalase from *Asp. niger* KUF-04 were studied.

Materials and Methods

Chemicals

Reference catalase used in this work was purchased from Boehringer Mannheim GmbH (German). All other chemicals used were obtained from general commercial sources and used without further purification.

Microorganism and cultivation

Asp. niger KUF-04 was cultivated as the same method as described previously (10).

Preparation of cell-free extract

Key words: Catalase, purification, *Aspergillus niger*
*Corresponding author

A Cell-free extract was obtained according tc the same method as glucose oxidase (10).

Enzyme purification

The mycelial extract (2,600 m*l*) was fractionated with ammonium sulfate. The active precipitate formed by 65-85% saturation was dissolved in 50 mM potassium phosphate buffer, pH 7.0., and dialyzed against the same buffer which was changed three times. The dialyzed enzyme solution was applied tc a DEAE-cellulose column (2.5 × 55 cm) which has been equilibrated with 50 mM potassium phosphate buffer, pH 7.0. The adsorbed enzyme was eluted by stepwise increasing the concentration of the buffer solution from 50 to 150 mM. Active fractions of the eluate were concentrated from 160 m*l* to 11 m*l* in a collodion bag. The concentrated enzyme soluton was applied to a DEAE-Sephacel column (2.5 × 55 cm) which had been equilibrated with 50 mM potassium phosphate buffer, pH 7.0. The adsorbed enzyme was eluted by stepwise increasing the concentration of the same buffer with 5, 50 and 150 mM. Active fractions were concentrated from 115 m*l* to 3 m*l*. The concentrated enzyme solution was applied to a sephadex G-200 column (1.2 × 85 cm) which had been equilibrated with 50 mM potassium buffer, pH 7.0. The enzyme was eluted with the buffer.

Determination of enzyme activity

The activity of the catalase was determined by measuring the time of absorbance decrease from 0.45 to 0.40 at 240 nm in a cuvette of 1-cm path with a Hitachi 200 spectrophotometer. The standard reaction mixture (3.0 m*l*) containing 2.9 m*l* of 10 mM hydrogen peroxide in 50 mM phosphate buffer, pH 7.0 was pre-incubated at 25 °C for 5 min. The reaction was initiated by an addition of enzyme solution (0.1 m*l*).

Activity (unit/m*l* enzyme extract) =

$$\frac{1.7 \times 13.1}{\text{time(sec)} \times \text{enzyme extract (m}l\text{)}}$$

Results and Discussion

Enzyme purification

The mycelial extract (2,600 m*l*) was prepared as described previously (10). The precipitate, (2,600 g × 30min) collected by centrifugation, was dissolved in a small amount of 50 mM potassium phosphate buffer, and dialyzed against the same buffer. The dialyzed enzyme was purified 2.6-fold as compared with the original cell-free extract. The concentrated enzyme solution was applied to a DEAE-Sephacel column. The adsorbed enzyme was eluted showing three peaks in 5, 50 and 150 mM phosphate buffer. The first two peaks had a small quantity of catalase, but the last one contained most of the catalase activity. The first two peaks were eluted more broadly than the third. The last one eluted showed a symmetric protein peak. The protein peak was coincided with almost exactly with the enzyme activity. The enzyme purity was increased by 17.6-fold through these steps. The combined enzyme solution from DEAE-Sephacel column chromatography was applied to a Sephadex G-200 column. A summary of the purification results is given in Table 1. The specific activity of the finally purified enzyme was increased by 64-fold as compared with that of mycelial extract. When companing each step of the purification process, the chromatography by DEAE-Sephacel Sephadex G-200 was most effective in obtaining the purified enzyme.

Absorption spectra

Fig. 1. shows absorption spectra of the catalase purified from *Asp. niger* KUF-04. The maximum absorption was shown at 406 nm. Most porphyrins have a colour and show a specific absorption spectrum at around 400 nm called a Soret band. The purified catalase of this work was also expressed the typical Soret band. The absorption maxima of all catalase from bakers' yeast (11), spinach leaf (12) and *Asp. niger* (13)'were reported to be at 408, 500 and 630 nm, respectively. Catalases from other sources were also expressed almost the same absorption spectra as that from *Asp. niger* KUF-04. The absorption spectrum at 406 nm was very sharp in comparison with other peaks.

Effect of protein concentration on catalase activity

The initial reaction velocity was linear up to 0.4 mg protein per m*l* showing the activity of 1,200 u g/m*l*. When the concentration of protein was higher than 0.4 mg/m*l*, the activity was saturated

Table 1. Summary of catalase purification.

Fraction	Total[a] activity (units)	Total[b] protein (mg)	Specific activity (unit/mg)	Activity (%)	Fold
Cell-free extract	369,740	2,600	139	100.0	1.0
Ammonium sulfate(65-85%)	169,262	418	404	46.1	2.9
DEAE-Cellulose	108,262	299	362	29.3	2.6
DEAE-Sephacel	58,784	24	2,449	15.9	17.6
Sephadex G-200	35,667	4	8,917	9.6	64.2

a) Activity was measured by spectrophotometry at 240 nm.
b) The amount of protein was estimated by the absorbance at 280 nm, assuming $E_{cm}^{1\%} = 10.1$

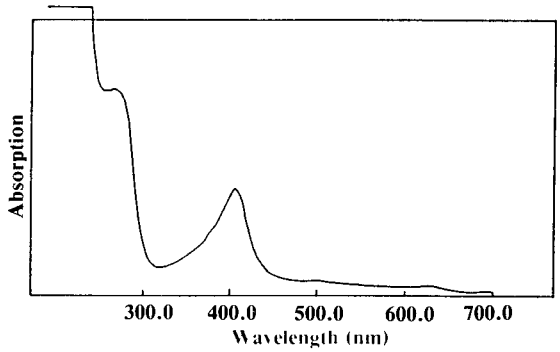

Fig. 1. Absorption spectrum of catalase
Absorption spectra were recorded with solution of enzyme in 50 mM of potassium phosphate buffer, pH 7.0

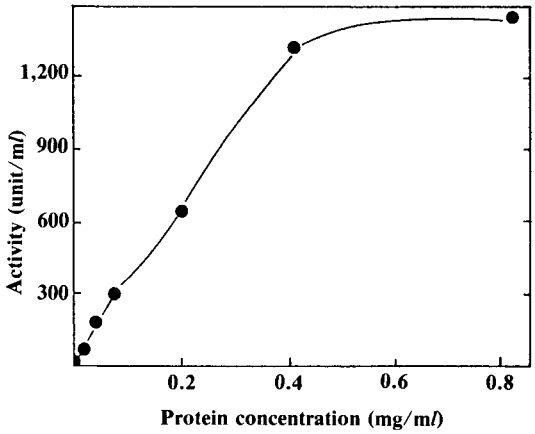

Fig. 2. Effect of protein concentration on catalase activity

as shown in Fig. 2.

Effect of hydrogen peroxide concentration on enzyme activity

Table 2. shows the effect of hydrogen peroxide concentration as substrate on the enzyme reaction when the enzyme concentration was fixed. The enzyme activity with the same protein concentration was proportionated to the concentration of hydrogen peroxide.

Effect of pH on enzyme activity

The activity of the catalase was measured at various pHs (Fig. 3). The optimum pH of the enzyme was found to be between 6.0 and 8.3. Chance (14) reported that the activity of catalase from horse or human erythrocytes was constant within the experimental error from pH 4.0 to pH 8.5 and fell off along a curve at approximately pH 3.0. Scott and Hammer (15) also reported that the optimum pH of *Asp. niger* enzyme was not shown in a typical bell curve; the activity was practically constant over the pH range of 2.8-8.0. Tony and Kaplan (11) reported that the optimum pH of catalase from bakers' yeast had two peaks, a broad one the pH 6.0 to 7.0 and the other around pH 9.5. Generally, catalase

Table 2. Effect of substrate concentration for the reaction of catalase activity.

Absorbance (H_2O_2) at 240 nm	Relative enzyme activity(%)	Enzyme concentration (mg/ml as protein)
0.45	100	100
0.40	92	91
0.35	78	76
0.30	65	68
0.25	51	49
0.20	39	35
0.15	24	21

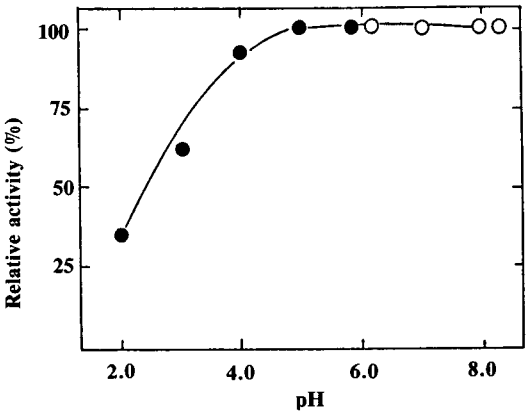

Fig. 3. Effect of pH on the catalase activity
Sodium acetate buffer was used from pH 2.0 to 6.0 and potassium phosphate buffer from pH 6.0 to 8.5.
● – ● , Sodium acetate buffer
○ – ○ , Potassium phosphate buffer

has a broad range of pH for the optimum activity.

Effect of temperature on enzyme activity

The enzyme activity was determined at different temperatures under the standard assay condition. As shown in Fig. 4, the optimum temperature was between 50 and 70 °C, and did not show a sharp response to the temperatures.

Effect of pH on enzyme stability

The pH stability of catalase was studied using different buffers. An enzyme solution at various pHs was incubated 25 °C for 15 min, and the remaining activity was assayed under the standard assay condition. It was found that the enzyme was considerably stable over the pH range of 4.0 to 8.3, but unstable below than pH 4.0 (Fig. 5). Approximately 30% of the activity remained at pH 2.0.

Effect of temperature on enzyme stability

The heat stability of catalase was tested with increasing temperature from 20 to 80 °C. The enzyme in 50 mM potassium phosphate buffer, pH 7.0, was incubated for 20 min at different temperatures. It was then cooled to room temperature and the remaining activity was assayed under the standard assay condition. The enzyme was found to be quite stable below 70 °C, whereas completely inactive at 80 °C for 20 min. (Fig. 6). Scott and Hammer (15) reported that *Asp. niger* catalase lost 85% of its activity by heating at 65 °C whereas beef liver catalase became completely inactive at the same temperature. The catalase from *Asp. niger* KUF-04 was thermally more stable than that reported by Scott and Hammer (15).

Effect of metal ions and various reagents on enzyme activity

The effect of various metal ions and reagents on the enzyme activity was examined. Among the reagents tested, Cu^{2+}, Hg^{2+}, Ag^{+}, KCN, NaN_3, cysteine, hydrazine, and especially hydroxylamine inhibited the activity greatly as shown in Table 3 and 4. Hagihara *et al.* (16) reported that the activity of

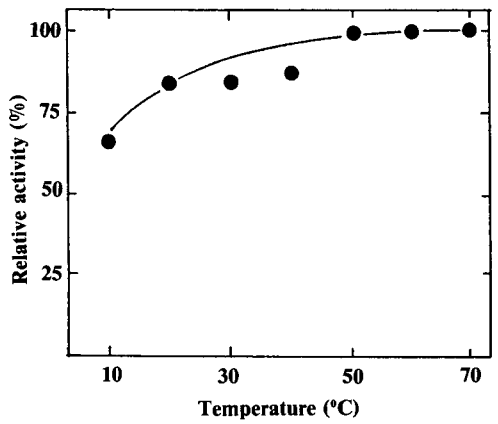

Fig. 4. Effect of temperature on the catalase activity
The temperatures were balanced for 10 min at the conditions.

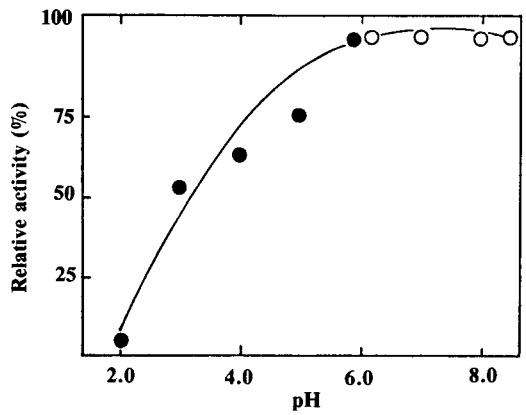

Fig. 5. Effect of pH on the catalase stability
● – ● , Sodium acetate buffer
○ – ○ , Potassium phosphate buffer

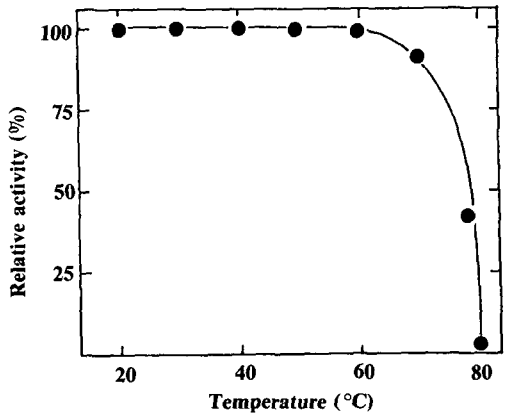

Fig. 6. Effect of temperature on the catalase stability.

Table 3. Effect of metal ions on catalase activity.

Metal ions	Relative activity(%)
None	100
$ZnCl_2$	93
$LiCl_2$	100
$CuSO_4 \cdot 5H_2O$	84
$MnSO_4 \cdot 4\text{-}6H_2O$	100
$NaHSO_3$	125
$MgSO_4 \cdot 7H_2O$	100
$NiCl_2 \cdot 6H_2O$	100
$BaCl_2 \cdot 2H_2O$	92
$CoCl_2 \cdot 6H_2O$	92
$HgCl_2$	21
$AgNO_3$	75

The concentration of each metal ions in the assay mixture was 1.0 mM.

Table 4. Effect of various reagents on catalase activity.

Reagent	Relative activity(%)
None	100
EDTA	100
Iodoacetate	93
Hydroxylamine	0
Semicarbazide	90
Hydrazine	85
Cysteine	79
KCN	43
NaN_3	18
NaF	100

The concentration of each reagents in the assay mixture was 1.0 mM.

catalase was strongly inhibited by cyanide, fluoride, azide and hydroxylamine at the concentration of 10^{-6}M, 10^{-1}M, 0.5×10^{-3}M and 2×10^{-3}M, respectively. Catalase from *Asp. niger* KUF-04 was also inhibited by the reagents described by Hagihara *et al.* Especially, hydroxylamine, azide and Hg^{2+} inhibited the activity more than that of other reagents tested.

요 약

Aspergillus niger KUF-04에서 얻은 catalase는 gel 여과를 포함하여 5단계를 걸쳐 정제하였으며, 9% 회수율로 64배 정제되었다.

본 효소는 406, 503, 625 nm에서 흡광을 나타내었으며, 특히 406 nm에서 뚜렷한 흡수대를 보여주었다. 이 효소 활성의 최적 pH와 온도는 각각 7.0과 60℃이었다. 이 효소는 pH 4.0과 8.0 사이에서 안정했으며 열에 대한 안정성은 20℃에서 60℃까지는 안정했으나, 80℃에서 20분간 반응시켰을 때 효소의 활성이 전부 소실되었다.

이 효소의 활성은 주로 hydroxylamine, potassium cyanide, sodium azide에 의해 저해되었다.

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