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Studies of stabilization of native catalase using additives

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Abstract

Native catalase preparations isolated from *Bacillus Sp* were formulated with different additives for storage stabilization and better performance at high temperature and pH. The additives studied were: polyethylene glycol, glycerol, BSA, casein, glutaraldehyde, n-butylamine, ethylenediamine, 1.6-diaminohexane, BSA/glutaraldehyde and casein/glutaraldehyde. The glycerol and glutaraldehyde showed the best performance for long-term storage at 30°C and neutral pH. No stabilization additives were effective at pH 12, but below that pH the polyethylene glycol and glycerol appeared to be the most appropriate. Amines, polyethylene glycol and glycerol shifted the pH activity maximum of the native catalase toward more alkaline region, while glycerol were the only additive to improve the temperature profile of the enzyme.

Keywords: Catalase; Hydrogen peroxide; Thermo and alkaline stability; Additives

1. Introduction

The resistance of the catalytically active protein structure toward high temperatures, pHs and other denaturing influences is one of the most important criteria for commercialization and for industrial application of the enzymes [1,2]. The limited stability of enzymes during long-term storage has been attributed to the deleterious effects of environmental moisture and microbial contamination [3,4]. Enzymes may be easily denatured by slight change of the environmental conditions such as temperature, pressure, pH and ionic strength [5]. In general, stabilization of the enzymes could be achieved in several ways: screening for more stable ones (thermophiles, extremophiles), chemical modification, protein engineering, and immobilization or using stabilizing additives [6,7]. Increase of the thermostability of the enzymes by the addition of salts, polyols (polyethylene glycol, glycerol), dextran, bovine serum albumine, polyethylenimine, polyelectrolytes, organic osmolytes (e.g. betaine), organic solvents (isoctane, cyclohexane, chloroform, benzene), sugars (e.g. lactiol, mannitol, sorbitol, xylitol, inositol, erythritol) and others has been previously reported [8–11]. The selection of the appropriate additive depends on the nature of the enzyme. In the present research the stability of catalase enzyme was of interest in order to use this enzyme for degradation of hydrogen peroxide after bleaching of cellulose fabrics [12–14]. Even a trace amount of hydrogen peroxide could cause dyeing problems such as unevenness of color, insufficient dyeing, and shade changes between batches [15,16]. The alkali remaining in the bleaching liquor and the high temperature of the latter are expected to influence the catalase stability, due to the fact that this enzyme is most efficient in the temperature range of 20 to 50°C and pH 6–8. Thus the objective of this study was to improve the stability of native catalase obtained from *Bacillus Sp*, against aggressive environmental conditions, by means of various additives.

2. Materials and methods

2.1. Enzyme

The catalase was produced from *Bacillus sp.*, isolated from the wastewater of a local textile finishing company. The strain was deposited and identified by the German culture collection DSMZ (Braunschweig, Germany) [17].

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Table 1
Effect of additives concentration on native catalase stability, after incubation for 24 h at pH 7, and 30°C

Catalase/additives	Absolute activity (μmol/min)						
	(0.0%)	(0.2%)	(0.5%)	(0.6%)	(1.0%)	(5.0%)	(10.0%)
Catalase	8.8	—	—	—	—	—	—
Catalase/betaine	—	nd	8.3	nd	8.4	7.9	nd
Catalase/polyethylene glycol	—	nd	nd	nd	8.3	8.0	6.8
Catalase/glutaraldehyde	—	6.8	nd	5.3	na	na	na
Catalase/ethylenediamine	—	7.1	7.4	nd	7.4	7.2	nd
Catalase/n-butylamine	—	6.8	7.1	nd	7.1	7.9	nd
Catalase/1.6-diaminohexane	—	7.2	7.8	nd	7.5	7.2	nd

nd: not determined.
na: no activity.
% (w/v)—betaine, polyethylene glycol.
% (v/v)—glutaraldehyde, ethylenediamine, n-butylamine, 1.6-diaminohexane.

2.2. Enzyme activity assay

Catalase activity was measured using a UV spectrophotometer equipped with magnetic stirrer (Unicam model He λ ios- α) at 30°C, using 26 mM H_2O_2 as substrate in 50 mM potassium phosphate buffer (pH 7.0). The degradation of hydrogen peroxide was followed at 240 nm and the activity was calculated using a molar absorption coefficient for H_2O_2 of 39.4 M^{-1} cm^{-1} [18].

2.3. Effect of additives concentration on enzyme stability

Aqueous solutions with various concentration of betaine (0.5, 1, and 5% w/v), polyethylene glycol (1, 5, and 10% w/v), glutaraldehyde (0.2, and 0.6% v/v), ethylenediamine (0.2, 0.5, 1, and 5% v/v), n-butylamine (0.2, 1, and 5% v/v), and 1.6-diaminohexane (0.2, 0.5, 1, and 5% v/v) were added to 300 U/ml native catalase, pH 7.0 (phosphate buffer). The reaction mixtures were incubated at 30°C, and after 24 h the enzyme activity was assayed.

2.4. Half-life time of native and treated with different additives catalase

Samples containing 300 U/ml native catalase were treated with water solutions of respectively: polyethylene glycol (1, 5, and 10% w/v), glycerol (5, and 20% v/v), BSA (0.5, and 2% w/v), casein (1, and 5% w/v), glutaraldehyde (0.001, and 0.2% v/v), n-butylamine (0.5, and 1% v/v), ethylenediamine (0.5, and 1% v/v), 1.6-diaminohexane (0.5, and 1% v/v), BSA/glutaraldehyde (0.5% w/v/0.001% v/v), casein/glutaraldehyde (1% w/v/0.001% v/v). The preparations were incubated in phosphate buffer pH 7.0, at 4 and 30°C, for several days. The enzyme activity was assayed at proper time intervals.

All the experiments were carried out in triplicate and the results represent mean values with less than 2% of error.

3. Results and discussion

3.1. Storage stability of catalase

Various additives in different concentrations were studied in order to evaluate their effect on the catalase storage stability at pH 7.0 and 30°C (Table 1). The increase of additives concentration did not affect significantly the catalase activity at the above conditions, except for the glutaraldehyde. When glutaraldehyde, a bifunctional reagent, was added at higher concentration, the enzyme activity decreased. Possibly the reaction of the aldehyde groups with the amine groups of catalase, promoted cross-linking of the protein chains, blocking the active site of enzyme, and there from resulting in enzyme inactivation during the stabilization process.

The storage stability of native and additives treated catalase was studied both at 4 and 30°C (Table 2), to simulate refrigerated and ambient conditions. At 4°C, the highest half-life time values were obtained in the presence of polyethylene glycol (10% w/v), glycerol (20% v/v) and glutaraldehyde (0.2% v/v), while at 30°C the polyethylene glycol (5% w/v), glycerol (5, 20% v/v), glutaraldehyde (0.2% v/v) and BSA/glutaraldehyde (0.5% w/v/0.001% v/v) induced the best stabilization effect. The denaturation of the enzymes in aqueous solution proceeds through hydratation of the protein. The role of the polyols in enzyme stabilization is as a water-structure maker, which depresses the hydratation of the protein. The polyol molecules are preferentially excluded from the surface layer of the protein molecule and the water shell around the protein molecule is preserved, so that the conformation of the protein becomes more rigid [5,19].

The storage stability of native catalase treated with different additives was studied additionally at stress conditions, e.g. pH 10.0–12.0, and 60 and 70°C. For all the treatments, the residual activity was determined after 10 min incubation in the corresponding medium. The enzyme residual activity decreased with the increase of the alkalinity

Table 2
Half-life time of native catalase and catalase treated with different additives

Catalase/additives	Concentrations of additives	Half-life time (days)	
		4°C	30°C
Catalase	300 U/ml	14	11
Catalase/polyethylene glycol	1% (w/v)	5	22
	5% (w/v)	7	36
	10% (w/v)	39	32
Catalase/glycerol	5% (v/v)	84	42
	20% (v/v)	102	77
Catalase/BSA	0.5% (w/v)	15	20
	2% (w/v)	14	19
Catalase/casein	1% (w/v)	11	21
	5% (w/v)	10	4
Catalase/glutaraldehyde	0.001% (v/v)	6	22
	0.2% (v/v)	180	60
Catalase/ethylenediamine	0.5% (v/v)	11	23
	1% (v/v)	21	21
Catalase/n-butylamine	0.5% (v/v)	12	10
	1% (v/v)	12	15
Catalase/1.6-Diaminohexane	0.5% (v/v)	11	7
	1% (v/v)	12	6
Catalase/casein/glutaraldehyde	1% (w/v)/0.001% (v/v)	10	3
Catalase/BSA/glutaraldehyde	0.5% (w/v)/0.001% (v/v)	11	43

and the temperature of the reaction mixture (Table 3). At pH 10.0 and 11.0 the polyethylene glycol and the glycerol were the only additives to improve the enzyme stability. However, at pH 12.0 the catalase did not present any significant residual activity independently on the additives treatment. Unfolding of the protein structure takes place under thermal stress, due to disruption of the hydrogen bonds and the other interactions, responsible for the maintenance of the tertiary enzyme structure. The precise orientation of the residual groups in the protein backbone is lost, and interaction of the water molecules with the newly exposed regions occurred, resulting in protein denaturation. The high capability of

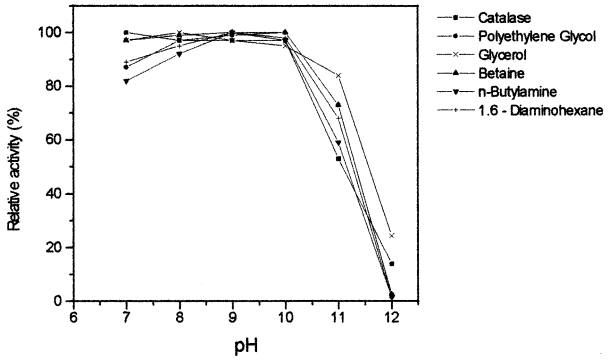

Fig. 1. pH profile of catalase activity in the rpesence of aditives (incubation for 24 h, at 30°C)

polyols to form hydrogen bonds should play the most important role in catalase stabilization, increasing the degree of organization of water molecules [8,9].

3.2. Effect of alkalinity on enzyme activity

The pH of the environment has a considerable influence on the performance of any enzyme. We studied the pH dependent behaviour of native catalase in the presence of different additives (in concentrations 1% (v/v) or (w/v), see Table 1) in the pH range from 7.0 to 12.0 (Fig. 1). Enzyme/additives preparations were incubated at 30°C for 24 h, in phosphate buffer set to the desired pH. The maximum in the pH profile of enzyme activity - pH 7, shifted toward more alkaline pH - 9.0 and 10.0. It should be noticed that the pH maximum for maintaining stability might differ considerably from that for maximum activity. The presence of additives usually has a variety of effects on the surface of protein molecule as well as on the state of ionization of the enzyme. It may result in changes in the relationship between pH and stability or activity. The use of additives at different pHs, probably modifies the charge of protein surface and consequently changes the isoelectric constant of the sur-

Table 3
Stability of native catalase and catalase treated with different additives at alkaline pHs and temperatures of 60 and 70°C

Catalase/additives	Concentrations	Temperature (°C)	Residual activity % (10 min incubation)		
			pH 10.0	pH 11.0	pH 12.0
Catalase	300 U/ml	60	87.9	23.5	0.0
		70	4.8	0.0	0.0
Catalase/polyethylene glycol	1% (w/v)	60	98.8	48.7	0.6
		70	76.1	14.6	0.3
Catalase/glycerol	5% (v/v)	60	92.9	64.5	0.9
		70	53.5	3.1	0.0
Catalase/glutaraldehyde	0.2% (v/v)	60	10.0	1.3	0.0
		70	3.2	0.2	0.0
Catalase/ethylenediamine	0.5% (v/v)	60	1.3	0.0	0.0
		70	0.0	0.0	0.0
Catalase/n-butylamine	0.5% (v/v)	60	56.5	3.2	0.0
		70	0.0	0.0	0.0

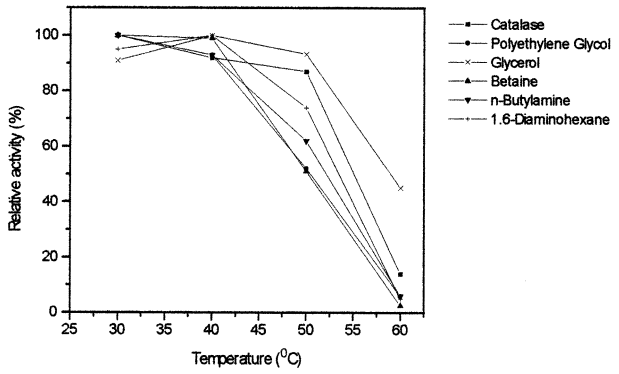

Fig. 2. Temperatufre profile of catalase activity in the prsence of additives (incubation for 24 h, at pH 7.0)

rounding. In this case, the additives with positive charge bind to the carboxylic groups of protein and the complex acquires positive charge. To reestablish electroneutrality, the amine groups on protein surface bind the hydroxide anion of water. In this way, the protein becomes more stable at alkaline pH. Our experimental results showed that from all studied additives the most appropriate for catalase stabilization against alkaline conditions was the glycerol. At pH 11.0 the glycerol treated enzyme retained more than 30% of activity compared to the native catalase. Improvement of the enzyme stability, however to a lower extent, was noticed with the other additives as well. As a whole, the pH range of enzyme activity was widened using additives.

3.3. Effect of the temperature on catalase activity

The effect of the temperature on the activity of native and additives (in concentration 1% (v/v) or (w/v), see Table 1) treated catalase was studied within the temperature range of 30 to 60°C (Fig. 2). The enzyme/additives preparations were incubated in 50 mM phosphate buffer pH 7.0, for 24 at the corresponding temperature. The temperature profiles of enzyme activity revealed that the use of betaine, 1.6-diaminohexane and glycerol led to a shift of the activity optimum from 30 to 40°C. However, at 50 and 60°C only the addition of glycerol improved the catalase activity. Compared to control measurements at 60°C, the activity of the glycerol treated catalase increased by 31%. The stabilizing effect of the glycerol against thermal denaturation of the enzyme might be explained with the preservation of the water shell around the protein molecule. In the case of the other additives, the decrease of activity above 40°C, in comparison to the native enzyme, might be explained with the occurrence of cross-linking of the protein molecule. The cross-linking of the molecules from one side could stabilize the enzyme rendering the protein structure stiffer. The linkers normally provoke change in the conformation of the protein molecule during the cross-linking itself. On the other hand the number of possible conformations of the polypeptide molecule increases when the linker is longer, i.e. polyethylene glycol. The thermally induced movement also causes a number of conformations of the chains, and probably the accumulation of all the displacements of the molecules impeded the accessibility of the active center of the enzyme. In other words inactivation, without denaturation of the protein occurred. Another explanation could be that the cross-linking limits the thermal movement of the molecules and greater stress, which could not be uniformly distributed along the protein chains, is accumulated in the polypeptide structure, leading to its disruption. In our experiment the enzyme was denaturated, which fact was confirmed by the irreversible loss of activity, and therefore the second mechanism was more reliable.

4. Conclusions

This study evaluates the stabilization efficiency of various additives on the biocatalyst activity of native catalase. The experiments revealed that the additives, even in low concentration (up to 1% w/v, or v/v) provided better long-term storage stability at neutral pH and 30°C, without significant loss in enzyme activity. The polyols appeared to be appropriate for short-time storage stabilization of catalase subjected to extreme environmental conditions – both high alkalinity and temperature. The application of additives widened the pH and temperature profile of enzyme activity, and shifted the activity optimum toward the region of more alkaline pH and higher temperature. From all studied additives the best stabilizing performance was induced by the glycerol. From the above considerations it could be suggested that the stabilized catalase could be potentially applied for degradation of residual peroxide in textile bleaching baths, thereby providing water and energy savings.

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