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Commercial Production and Separation of Catalase Produced by *Micrococcus* sp.

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Abstract

A *Micrococcus* sp. producing catalase was isolated from soil, and a commercial-scale cultivation and purification of catalase were conducted. The maximum catalase activity was about 103 BU/mL obtained after 46 hr of cultivation in a 30 L fermenter containing 2% glucose, 2% peptone, 4% yeast extract, and 0.5% NaCl. Soybean sauce, CSL (corn steep liquor), and yeast extract were also studied as media substitutes in the media 30 L fermenter. The optimum medium components for the production catalase were found to be 2% glucose, 4% soybean sauce, and 16% CSL. In a 18 kL fermenter, the stationary phase in the cell growth and maximum catalase activity (112 BU/mL) were reached after 46 hr of cultivation, which was the same result as in the 30 L fermenter. The catalase activity was purified with over 17 folds in four steps with a 33.6% yield. From 104,250 mg of protein after cell lysis, 1,966 mg of the purified enzyme with a specific activity of 192.7 kBU/mg was obtained. The residual activity with the addition of 10% NaCl exhibited more than 100%. The use of just NaCl produced a higher residual activity than combination of bencol (benzyldimethyl ammoniumcholride) and PG (propyleneglycol).

Key words: catalase, *Micrococcus* sp. commercial production

INTRODUCTION

The fast growth in the field of biotechnology along with the rapid commercialization of protein products have led to an increase in the demand for efficient, large-scale protein purification techniques. Techniques used in research laboratories (e.g. chromatography, electrophoresis, and affinity purification) are excellent for producing small quantities of protein. However, these processes are extremely difficult to scale-up, thereby restricting the scale of production. In addition to scale-up problems, these techniques also require complex instrumental support to run efficiently and only give a low throughput of the product at an extremely high cost (1). Ultrafiltration (UF) processes are more cost effective and can be fine-tuned to achieve a high productivity and product purity (2,3). UF processes are also much easier to scale-up in comparison to chromatography and electrophoresis. Furthermore, UF modules are easy to clean and operate, and quite compact in design.

The commercial scale production of enzymes has drawn great interest from in the industrial, medical, and analytical application fields. Catalase is an example of an enzyme with various useful applications in industry. Catalase (EC 1.11.1.6) is well known for its ability to decompose hydrogen peroxide into water and dioxygen (4,5). Hydrogen peroxide is a powerful oxidant that is used as a bleaching agent or microbicide in the paper, food, textile, and semiconductor industries. However, because H_2O_2 is a very toxic substance, it needs to be decomposed before disposal. Catalases are already commercially available for use as catalysts in the destruction of H_2O_2, however, they are relatively expensive and not readily recoverable from a reaction mixture for recycling (6). Therefore, the issue of reducing the commercial-scale production costs of catalase is very important.

According the purpose of this research was to investigate commercial-scale cultivation and purification in an industrial environment to improve the cell cultivation and consequently the intracellular enzyme productivity using *Micrococcus* sp. The purification of the catalase was conducted using UF, plus the stability of the catalase, which is an important factor for practical use, was also investigated.

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MATERIALS AND METHODS

Bacterial strain and medium

Micrococcus sp. producing catalase was isolated from soil. The preliminary morphological (cells spherical 0.5 ~2.0 μm in diameter, occurring mostly in pairs, tetrads or irregular clusters) and biological characteristics (aerobic, catalase- and oxidase-positive, acid without gas produced from glucose) of the strain coincided with those of *Micrococcus* sp. The strain was cultured in a basal medium containing 2% glucose, 2% peptone, 4% yeast extract, and 0.5% NaCl (The incubation of the strain was carried out at 30°C for 70 hr. The bacterial growth was monitored at OD_{600} (DU-65 Spectrophotometer, Beckman, Fullerton, USA) (7).

Assay of catalase

The catalase activity was determined using Baker Units (8). For a standard assay, an aliquot of no more than 1.0 mL of the sample, previously diluted to contain approximately 3.5 BU (Baker Units) of catalase. A hounded mL of 1.5% hydrogen peroxide in a 50 mM phosphate buffer (pH 7.0) previously adjusted to 25°C was placed in a 200 mL beaker then catalase was added to the 200 mL beaker, which was immediately stirred for 5 to 10 s. The mixture was incubated at 25°C until the reaction was completed. After stirring vigorously for 5 s, 4.0 mL from the beaker was placed in a 50 mL Erlenmeyer flask. 5 mL of 2 N sulfuric acid was then added to the flask. Next, 5.0 mL of a 40% potassium iodide solution, freshly prepared, was added followed by 1 drop of a 1% ammonium molybdate solution. While continuing to mix the solution, titration with 0.25 N sodium thiosulfate was rapidly carried out to a colorless endpoint. A blank determination was performed with 4.0 mL of a peroxide substrate solution. One BU was defined as the amount of catalase that decomposed 264 mg of hydrogen peroxide under the conditions of the assay.

Separation of catalase

The culture broth was suspended with 800 L of tap water at pH 7.5. Then, after the addition of lysozyme (3.6 kg, 23,900 units/mg), the suspension was stirred for 60 min at 30°C and treated with perlite, $CaCl_2 \cdot 2H_2O$, and Na_2HPO_4. The suspension was passed through a filter press. The filtrate was concentrated in an UF 5/4040 (Prochem Tech Internation Inc., New York, USA) using an ultrafilter (G-50, 1,5000 cut off) to remove any substances smaller than about 15 kDa. The final concentrate was used for further studies.

Enzyme stability

The enzyme stability was determined by incubating a mixture containing the enzyme (5 BU/mL) and the solute to be tested in a 50 mM potassium phosphate buffer (pH 7.0) in stopper glass tubes at 5°C and 30°C. After mixing, the mixtures were assayed for their activity during the experimental time using a standard assay method.

RESULTS AND DISCUSSION

Cell growth and production of catalase in 30 L fermenter

A strain of *Micrococcus* sp. was cultured in a medium containing 2% glucose, 2% peptone, 4% yeast extract, and 0.5% NaCl. Figure 1 shows the growth curves for *Micrococcus* sp. at 30°C. As shown in Fig. 1, the maximum catalase activity was about 103 BU/mL obtained after 46 hr of cultivation. In the isolate, the maximum enzyme activity was obtained after the logarithmic phase. At the end of the logarithmic phase or in the early stationary phase, the enzyme activities exhibited minimal changes. In *Mycobacterium* sp. (9), the enzyme is secreted at the end of the logarithmic phase, which is similar to the current results. Romantsev et al. (7) also reported that catalase activity increases with cultivation time and exhibits the maximum activity at the end of the logarithmic phase. However, the maximum catalase activity of *Deinococcus* sp. (10) was produced in an aerobically grown culture during the stationary phase.

Effect of nitrogen substitutes on cell growth and production of catalase in 30 L fermenter

To enable biotechnological applications of the catalase

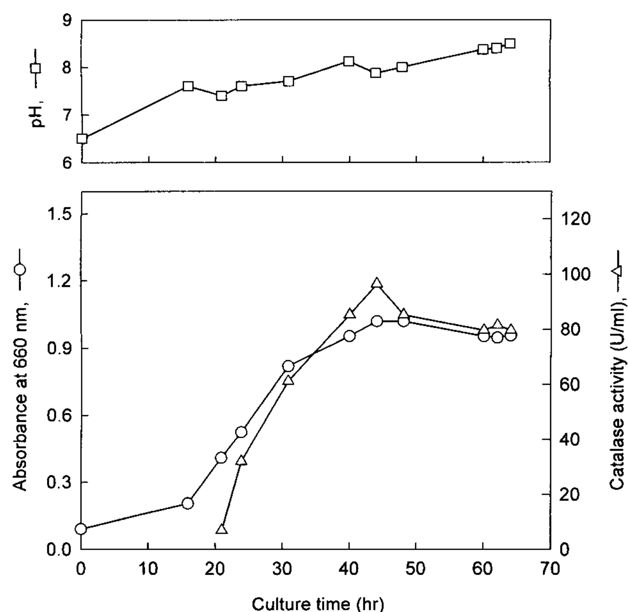

Fig. 1. Cell growth and catalase activity in 30 L fermenter. Culture media were composed with 2% glucose, 2% peptone, 4% yeast extract, and 0.5% NaCl. Cell culture was carried out with a stirring rate of 300 rpm and an aeration rate of 1 vvm at 30°C and pH 6.5.

of *Micrococcus* sp., the cultivation cost is very important. Therefore, further research on the substitution of nitrogen sources was performed. In the basal medium, the yeast extract was substituted with soybean sauce, CSL (corn steep liquor), yeast extract (liquid type), urea, and ammonia sulphate. The soybean sauce, CSL, and yeast extract were found to significantly increase the production of the catalase (data not shown). Yeast autolysate and yeast extract are the most common nitrogen sources in fermentation. However, the high prices of these components in developing countries renders their uses on an industrial scale prohibitive. Based on a chemical analysis of soybean sauce produced from the acid-hydrolysis of soybeans, it was found to contain various amino acids and 2.56% total nitrogen. So, soybean sauce was considered as a potential nitrogen source for the growth of *Micrococcus* sp.

Fig. 2 showes the cell growth and catalase production

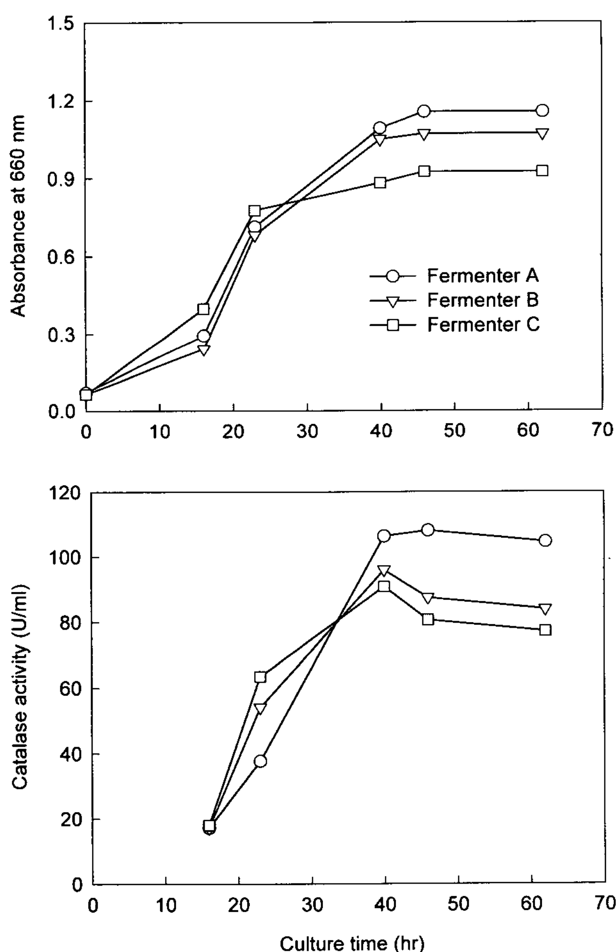

Fig. 2. Cell growth and catalase activity with various nitrogen sources in 30 L fermenter. Culture media were composed with 2% glucose, 4% soybean sauce and assigned nitrogen source as follows, Fermenter A: 16% CSL, Fermenter B: 12% CSL and 1% yeast extract, Fermenter C: 8% CSL and 2% yeast extract. Cell culture was carried out with a stirring rate of 300 rpm and an aeration rate of 1 vvm at 30°C and pH 6.5.

with various nitrogen sources in 30 L fermenters. In fermenter C (2% glucose, 4% soybean sauce, 8% CSL, and 2% yeast extract), the cell growth was faster than that in the other fermenters. After a cultivation of 20 hr, cell growth in fermenter C reached the stationary phase. However, in fermenter A (2% glucose, 4% soybean sauce, and 16% CSL) and fermenter B (2% glucose, 4% soybean sauce, 12% CSL and 1% yeast extract), the stationary phase was only reached after 46 hr and 40 hr, respectively. The maximum production of catalase in the fermenters was exhibited after 40 hr. In particular, the catalase activity (116 BU/mL) in fermenter A was higher than those of the other fermenters after 40 hr. The optimum medium composition for the production of catalase consisted of 2% glucose, 4% soybean source, and 16% CSL. It appeared that that soybean sauce was a suitable as a nitrogen source for the reduction of production costs. A mixture of soybean sauce and CSL was better the cell growth and production of catalase by *Micrococcus* sp. than a mixture of peptone (2%) and yeast extract (4%) as nitrogen sources. Also the cost of raw material cost and production was reduced as 41% and 12.8% with use of a mixture of soybean sauce and CSL, respectively.

Cell growth and catalase production in 18 kL fermenter

For the large-scale production of catalase, the above results were applied to a 18 kL fermenter. Figure 3 shows

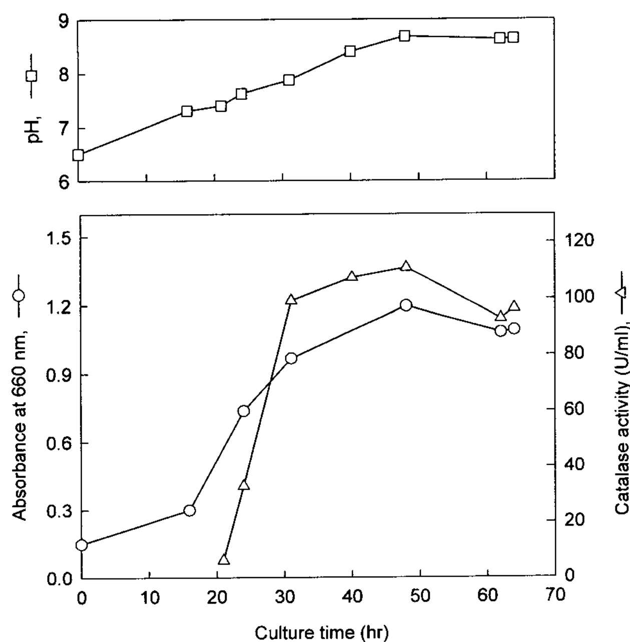

Fig. 3. Cell growth rate and catalase activity in 18 kL fermenter. Culture media were composed with 2% glucose, 4% soybean sauce, and 16% CSL. Cell culture was carried out with a stirring rate of 300 rpm and an aeration rate of 1 vvm at 30°C and pH 6.5.

the cell growth and catalase production in the 18 kL fermenter. The stationary phase in cell growth was reached after a cultivation of 46 hr, which was the same result as in the 30 L fermenter. The catalase activity exhibited 112 BU/mL after a cultivation of 40 hr, which was similar to the result in Fig. 2. The medium used in the 18 kL fermentation was the same as that used in the 30 L fermentation.

Separation of catalase

For the practical use of catalase, large-scale separation is also important along with the production conditions of catalase. A scheme for the large-scale purification of catalase is shown in Fig. 4. The step of cell recovery with centrifuge or filtration was omitted because it increased the production cost. Therefore, the cell lysis was directly carried out in the cell broth after the dilution of broth with tap water.

A filter aid is necessary for the filtration of the biocatalyst slurry to avoid the formation of a slimy layer of cells and cell debris on the surface of the filter cloth, which can cause a dramatic decrease in the filtration rate. Of the many filter aids available (11), diatomaceous earth and expanded perlite are the most suitable as they are cheap, inert, and stable up to pH 9. Accordingly, perlite was selected as the filter aid for the preliminary experiments. Ultrafiltration (UF) offers a better performance in retention, resulting in a higher quality the UF-permeate compared to that of the filtrate. When ultrafiltration was applied, the catalase activity was purified over four steps with a 33.6% yield (Table 1). From 27 kL of cell lysis (41.7 BU/ml), 1.4 kL of the purified enzyme with 270.6 BU/ml was obtained. The catalase activity was purified over 17.8 fold (Table 1). From 104,250 mg of protein after cell lysis, 1,966 mg of the purified enzyme with a specific activity of 192.7 kBU/mg was obtained.

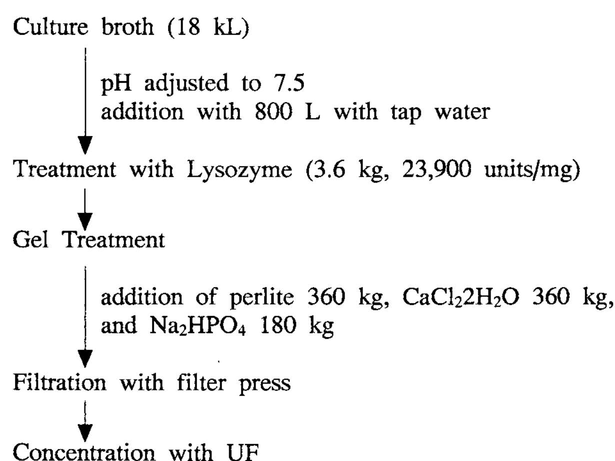

Fig. 4. A schematic diagram for the purification of catalase from *Micrococcus* sp.

Stability of catalase

To prevent the loss of catalase activity, PG, bencol, and NaCl were added to the purified enzyme (Fig. 5). After 20 days of storage at 30 and 5°C, the control exhibited 88% and 47% of residual activity. A temperature dependent inactivation of the catalase was also observed at 30°C. However, with the addition of PG, bencol or NaCl, the residual activity of the catalase became higher than that of the control. In particular, the residual activity with the addition of 10% NaCl was more than 100%. The addition of just NaCl produced a higher residual activity than the addition of a combination.

The optimum concentration of NaCl required to stabilize the catalase was 10% (Fig. 6). At a lower concentration of NaCl, the residual activity became lower than that with 10% NaCl, while at a higher concentration of NaCl the activity decreased significantly. Generally, the catalase of

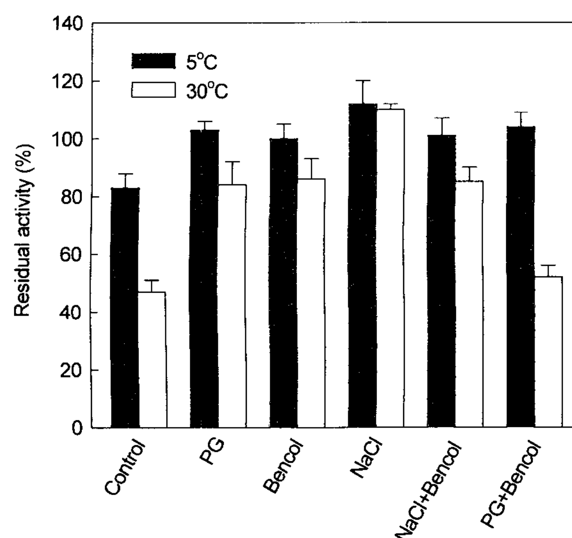

Fig. 5. Residual activities of catalase with the addition of NaCl, PG, or bencol. 10% NaCl, 10% PG, and/or 0.1% of bencol were added to the purified enzyme. Residual activity was assayed after 20 days at the storage temperature of 5°C and 30°C.

Table 1. Separation of catalase from *Micrococcus* sp.

Purification step	Volume (kL)	Protein (mg)	Activity (kBU)	Specific activity (kBU/mg)	Purification fold	Yield (%)
Cell lysis	27	104,250	1,125,900	10.8	1.0	100.0
Gel treatment	29	36,592	698,900	19.1	1.8	62.1
Filtration	30	12,364	549,000	44.4	4.1	48.8
Concentration	1.4	1,966	378,840	192.7	17.8	33.6

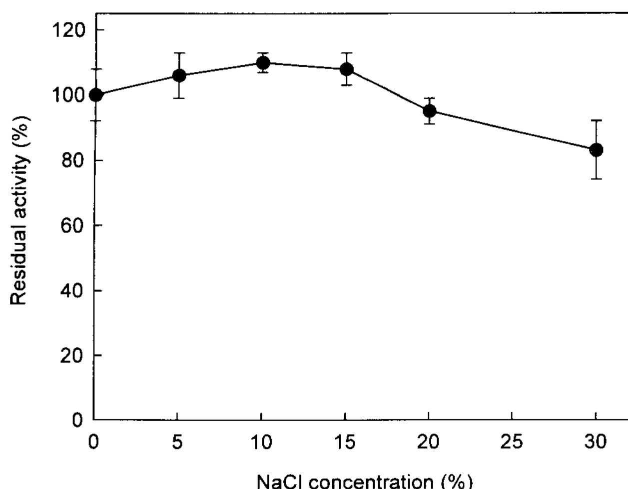

Fig. 6. Residual activities of catalase with various NaCl concentrations.

a microorganism such as yeast is located in the organelles such as peroxisomes (12), which have membrane structures composed of hydrophobic materials surrounded by hydrophilic components. This suggests that catalase may be stable in a hydrophobic state (13). As such, the addition of NaCl or PG can increase the hydrophobicity of the catalase. The effect of salts on an enzyme cannot be easily described by a general theory. The classical hydrophobic effect hypothesis approach assumes that the active enzyme structure is mainly maintained by hydrophobic forces. Salting-out solutes are excluded from the hydration shell surrounding the protein, thereby increasing the hydrophobicity of the water, whereas in the presence of salting-in solutes, there is a binding favoring unfolding. The specific interactions of ions with amino acid chains should explain the specific ion effect; however, extensive studies over the past three decades have led to the conclusion that the macro-effects induced in proteins by additives are the direct consequence of their preferential interactions with the protein (14-16).

In conclusion, from an economic point of view, commercial-scale cultivation using nitrogen substitutes and separation of catalase can offer new perspectives for industrial processes, since important savings can be made in production costs. Furthermore, the addition of NaCl can significantly increase the stability of the catalase activity, thereby enabling it to use in the biotechnology industry.

REFERENCES

1. Shah NK, Upadhay CM, Nehete PM, Kothari RM, Hegde MV. 1990. An economical, upgraded, stabilized and efficient preparation of α-amylase. *J Biotechnol* 16: 97-108.
2. Ghosh R, Cui ZF. 2000. Protein purification by ultrafiltration with pre-treated membrane. *J Membr Sci* 167: 47-53.
3. Saksena S, Zydney AL. 1994. Effect of solution pH and ionic strength on the separation of albumin from immunoglobulins (IgG) by selective filtration. *Biotechnol. Bioeng* 43: 960-968.
4. Farr SB, Kogoma T. 1991. Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. *Microbiol Rev* 55: 561-585.
5. Yumoto I, Iwata H, Sawabe T, Ueno K, Ichise N, Matsuyama H, Okuyama H, Kawasaki K. 1999. Characterization of a facultatively psychrophilic bacterium, *Vibrio rumoiensis* sp. nov., that exhibits high catalase activity. *Appl Environ Microb* 65: 67-72.
6. Yumoto I, Yamazaki K, Kawasaki K, Ichise N, Morita N, Hoshino T, Okuyama H. 1998. Isolation of *Vibrio* sp. S-1 exhibiting extraordinarily high catalase activity. *J Ferment Bioeng* 85: 113-116.
7. Romantsev FE, Prozorovskij V, Andrianova LE. 1983. Isolation from *Micrococcus* sp. of homogenous heme-containing catalase and a crystalline protein possessing a catalase activity. *Biokhimiya* 48: 2023-2027.
8. Committee on Food Institute of Medicine. 1996. *Food chemical codex*. 4th ed. National Academic Press, Washignton DC, U.S.A.
9. Rafii F, Lunsford P, Hehman G, Cerniglia CE. 1999. Detection and purification of a catalase-peroxidase from *Mycobacterium* sp. Pyr-1. *FEMS Microbiol* 173: 285-290.
10. Soung NK, Lee YN. 2000. Iso-catalase profiles of *Deinococcus* sp. *J Biochem Mol Biol* 33: 412-416.
11. Akers RJ, Ward AS. 1977. Liquid filtration theory and filtration pretreatment. In *Chemical processing and engineering*. Orr C, ed. Marcel Dekker, New York. Vol 10, p 169-250.
12. Tanaka A, Yasuhara S, Osumi M, Fukui S. 1977. Immobilization of yeast microbodies by inclusion with photo-crosslinkable resins. *Eur J Biochem* 80: 193-197.
13. Lida T, Maruyama D, Fukunaga K. 2000. Stabilization of entrapped catalase using photo-crosslinked resin gel for use in wastewater containing hydrogen peroxide. *J Chem Technol Biotechnol* 75: 1026-1030.
14. Arakawa T, Timasheff SN. 1984. Mechanism of protein salting-in and salting-out by divalent cation salts: Balance between hydration and salt binding. *Biochemistry* 23: 5912-5923.
15. Ben-Naim A. 1995. Solvent effects on protein stability and protein association. In *Protein-solvent interaction*. Gregory RB, ed. Marcel Deckker, New York. p 387-420.
16. Timasheff SN. 1995. Preferential interactions of water and cosolvents with proteins. In *Protein-solvent interaction*. Gregory RB, ed. Marcel Deckker, New York. p 445-482.

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