〈Original Article〉

Screening of enzyme stabilizers using thermal shift assays on the basis of structural informations

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Summary With respect to biochemical examination, enzyme stability is one of the most important problems for long-term storages of liquid diagnostic reagents. In this study, enzyme stabilizers were screened by a simple and easy method combining thermal shift assay with X-ray structural informations. The *Bacillus* sarcosine oxidase (SoxB) was used as a model of diagnostic enzymes. We first selected some compounds, which crystallographically bound to the SoxB structure, and their related ones as candidates for stabilizers. We then evaluated several concentrations of candidates using a fluorescence-based thermal shift assay with a real-time PCR instrument. As a result, 12 hits of 19 that thermally stabilized SoxB in a concentration dependent manner were identified. The two compounds, $MgCl_2$ and $MgSO_4$, were finally chosen as SoxB stabilizers because they did not exhibit inhibitory effects for the enzyme reaction. They drastically showed the stabilizing effects for SoxB when the accelerated tests of storage periods were performed at 30 and 45℃.

Key words: Enzyme stabilizer, Thermal shift assay, Structural information, Long-term storage, Sarcosine oxidase

1. Introduction

Currently, most biochemistry diagnostic reagents for sera and urine are sold and used in a liquid state. Reagent performances should be consequently kept on a long-term basis, at least one year. Stabilities of diagnostic enzymes are especially important for the long-term storage of liquid reagents. Therefore, enzyme stabilizers that can successfully add as supplements to liquid diagnostic reagents have been desired for their ability to stably preserve. Until now, numerous stabilizer candidates have been screened and evaluated by the conventional assay-based method. The conventional method investigates thermal stability of enzymes by assaying enzyme activities, because thermostable enzymes generally exhibit long-term preservation stability. However, this method is not high-throughput and it is difficult to determine an influence on detailed temperature changes with it, as the enzymatic assays commonly use the working solutions of complicated compositions and are time-consuming.

In this study, we newly developed a simple and easy method for screening enzyme stabilizers meant for diagnostic use. It combined a fluorescence-based thermal shift assay with X-ray structural information.

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The thermal shift assay (TSA) measures protein thermal stability using a fluorescent-protein binding dye[1-3]. The protein is mixed with the dye and heated. Hydrophobic parts of the protein are exposed and bind the dye as it unfolds, resulting in a significant increase in fluorescence emission detected by the real-time PCR system. Whether the protein is stabilized or destabilized by the ligand against thermal denaturation can also be simply evaluated. Thus, TSA is capable of optimizing conditions that enhance protein stability, as well as to identifying ligands that bind and confer structural stability to a protein of interest. The analysis of protein-ligand binding was originally carried out by differential scanning calorimetry[4, 5]. However, TSA is high-throughput, cost-effective, and time-saving, compared with differential scanning calorimetry.

We used the *Bacillus* monomeric sarcosine oxidase (SoxB)[6, 7] as a model of diagnostic enzymes for verifying the availability of the new method. Monomeric sarcosine oxidase (EC 1.5.3.1; sarcosine:oxygen oxidoreductase) is a flavoprotein that catalyses the oxidative demethylation of sarcosine (N-methylglycine) to yield glycine, formaldehyde, and hydrogen peroxide. This enzyme is involved in the bacterial metabolism of creatinine with two related enzymes, creatininase and creatinase[8], and is also industrially important, having use with creatininase and creatinase for the enzymatic assay of creatinine in clinical settings[9, 10].

In this paper, the TSA-based screening of enzyme stabilizers was indicated to be useful as an alternative method for the research and development of biochemistry diagnostic reagents.

2. Materials and methods

1) Materials

The SoxB used was from Asahi Kasei Pharma (Tokyo) as reported previously[7]. Since the X-ray crystal structures of SoxB have been solved[6], it was desirable as a model for screening stabilizers on the basis of structural informations.

Compounds used as candidates of stabilizers were purchased from Nacalai Tesque (Kyoto) or Yashima Pure Chemicals (Osaka).

2) Thermal shift assay

TSAs were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). In a typical experiment, total 25 μL of mixtures containing 2.5 μL of SYPRO Orange 50x concentrate (Life Technologies, Carlsbad, CA, USA, diluted from 5000x concentrate stock in DMSO), 1.25 μL of 1000 mmol/L HEPES buffer (pH 7.5), 10 μL of 1.0 mg/mL SoxB, and various concentrations of candidate compounds were mixed on ice in a 8-well PCR tube or 96-well PCR plate, and fluorescence was measured from 15℃ to 95℃ in 0.5℃/10 sec steps (excitation, 450-490 nm; detection, 560-580 nm). Main measurements were carried out in duplicate. Data evaluation and melting temperature determination were performed using the Bio-Rad CFX Manager software.

3) Enzyme assay

Sarcosine used as a substrate was purchased from Nacalai Tesque. The enzyme assay was based on the measurement of hydrogen peroxide produced during the oxidation of substrate. The 4-aminoantipyrine peroxidase system was used for the enzyme assay as described previously[11-13]. The assay mixture finally contained 100 mmol/L sarcosine, 0.49 mmol/L 4-aminoantipyrine, 2.1 mmol/L phenol, 50 mmol/L Tris-HCl (pH 8.0), and 5 U/mL horseradish peroxidase. An enzyme solution (0.05 mL) was incubated with an assay mixture (1.0 mL) at 37℃, and the appearance of quinoneimine dye formed by coupling with 4-aminoantipyrine, phenol, and horseradish peroxidase was measured at 500 nm against the blank by spectrophotometry. One unit of activity was defined as the formation of 1 μmol of hydrogen peroxide (0.5 mmol of quinoneimine dye) per minute at 37℃ and pH 8.0. Reaction mixtures containing several concentrations of substrate solution were used to determine the K_m and V_{max} (k_{cat}) values.

4) Structural informations

The structural informations of SoxB and several ligands were obtained from Protein Data Bank (PDB ID: 1el5, 1el7, 1el8, 1el9, 1eli, 1l9c, 1l9d, 1l9e, 2gb0,

2gf3, 3qse). The program Pymol was used for molecular visualization.

3. Results and discussion

1) Selection of stabilizer candidates

From information regarding SoxB tertiary structures, phosphate ion, chloride ion, pyrrole-2-carboxylate, (methylseleno)acetate, (methyltelluro)acetate, (methylthio)acetate, 2-froic acid, N,N-dimethylglycine, and imidazole were clearly recognized to be ligands. The binding sites of ligands are represented in Fig. 1.

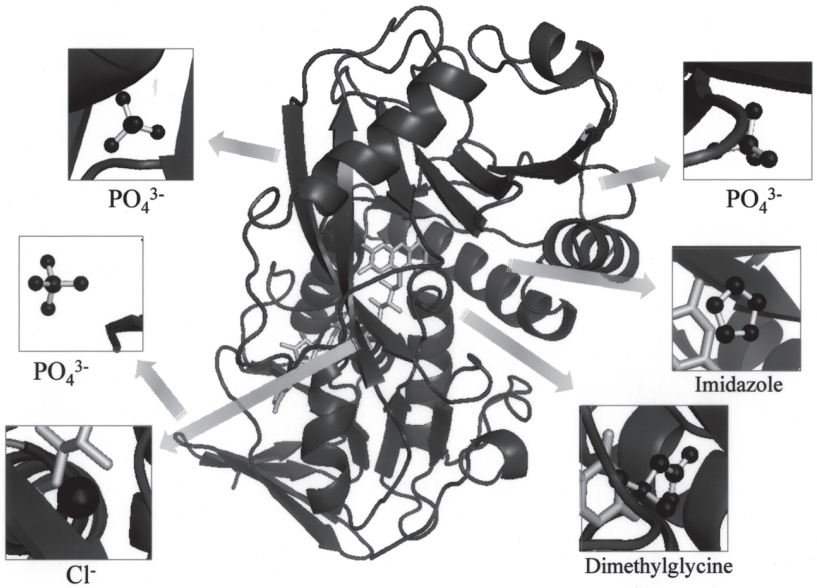

Fig. 1 Tertiary structure of SoxB and ligand binding sites. SoxB structure is shown by a ribbon model, and ligands are shown by ball and stick drawing. Binding sites of substrate analogues, such as pyrrole-2-carboxylate, are almost the same position as that of dimethylglycine.

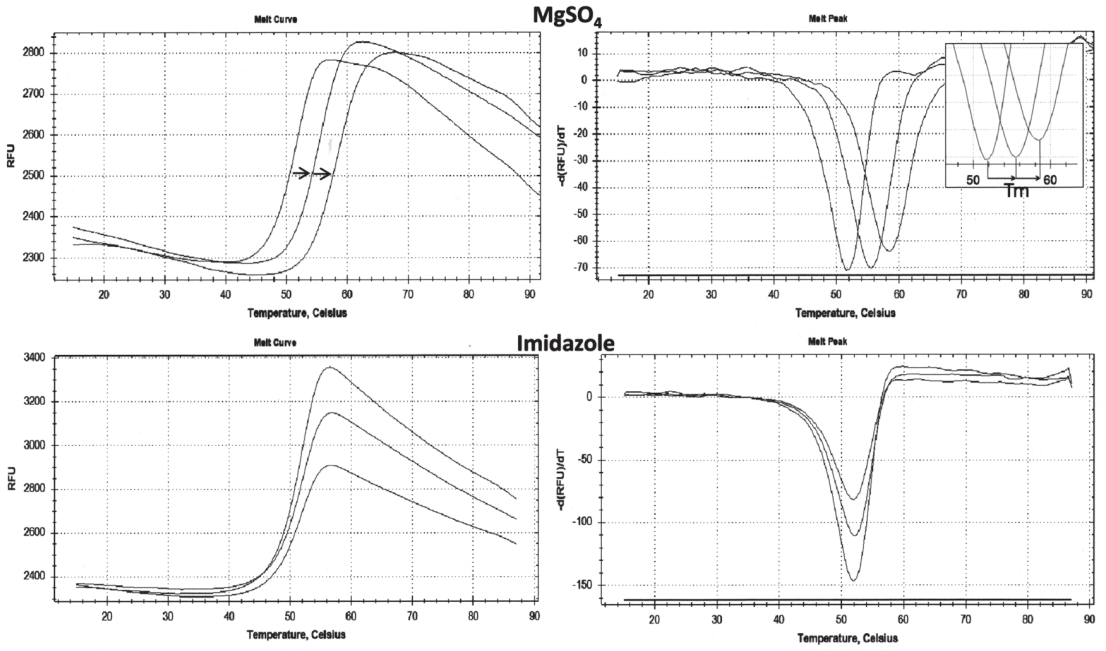

Fig. 2 Denaturation curves and differential peaks of fluorescence-based thermal shift assays. Compound concentrations used in assays were 0, 5, and 50 mmol/L, respectively.

Seven compounds, NaH_2PO_4, Na_2HPO_4, NaCl, KCl, $MgCl_2$, N,N-dimethylglycine, and imidazole, were then selected as available candidates of enzyme stabilizers. Other compounds related to the ligands were also selected as additional candidates. They were dipolyphosphate ($Na_4P_2O_7$), tripolyphosphate ($Na_5P_3O_{10}$), tetrapolyphosphate ($Na_6P_4O_{13}$), polyphosphoric acid, riboflavin, glycine, and N,N,N-trimethylglycine (betaine). Five salts, $MgSO_4$, Na_2SO_4, $(NH_4)_2SO_4$, $NaNO_3$, and KNO_3, were also selected as they included acid ions such as the phosphate salts.

Toal of 19 compounds were thus selected as stabilizer candidates, and were subsequently provided for screening with TSA.

2) Thermal shift assays

The candidates of SoxB stabilizers were evaluated at different compound concentrations by using the fluorescence-based thermal shift assays, as described in the Materials and Methods section. Each denaturation curve was generated, the melting temperature (Tm) was determined, and changes in Tm induced by prospective binding ligands could be calculated. The mean Tm for SoxB was 51.7℃ with 0.46% of within-run CV (n=8) and 51.6℃ with 0.43% of between-run CV (n=8) when no ligands were present. It could be confirmed that Tm estimations by TSAs were highly reproducible. As examples of experimental results, the denaturation curves and differential peaks of $MgSO_4$ and imidazole are shown in Fig. 2. A typical stabilizer like $MgSO_4$ elicited dose-response of increased Tm, whereas a non-stabilizer like imidazole did not induce thermal stability even at a high concentration.

As a result, 12 hits (Na_2HPO_4, $Na_4P_2O_7$, $Na_5P_3O_{10}$, $Na_6P_4O_{13}$, $MgSO_4$, Na_2SO_4, $(NH_4)_2SO_4$, $NaNO_3$, KNO_3, NaCl, KCl, and $MgCl_2$, respectively) of 19 that thermally stabilized SoxB in a concentration-dependent manner were identified. A series of phosphate salts had a stabilizing effect except for K_2HPO_4 and polyphosphoric acid, which possibly destabilized the enzyme by lower pH (Fig. 3). Sulfate and nitrate salts were also effective. From comparison of Na_2HPO_4, Na_2SO_4, and $NaNO_3$, it seems that the stabilizing effects of acid ions are increased as the number of minus charges increases. This might be related to the interaction strength of ligand with the enzyme. Chloride ion was clearly effective, and this result corresponds to our previous study on the other sarcosine oxidase[14]. Interestingly, we found Mg^{2+} to be an effective stabilizer by comparing $MgCl_2$ with NaCl and KCl, or comparing $MgSO_4$ with Na_2SO_4 and $(NH_4)_2SO_4$. It is well-known that Mg^{2+} interacts with adenosine diphosphate, which composes the coenzyme, flavin adenine dinucleotide, of SoxB.

In particular, we selected four compounds, $MgCl_2$,

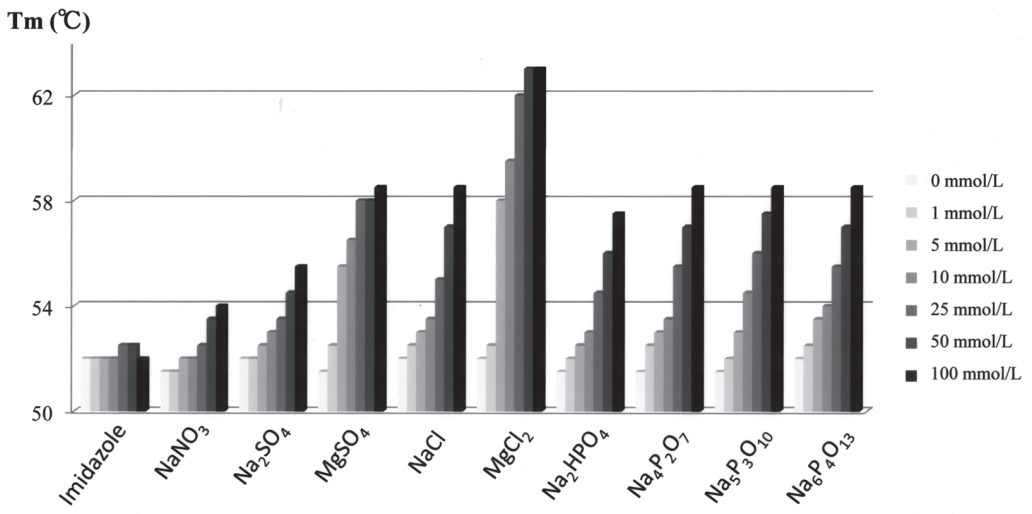

Fig. 3 Concentration-dependent Tm values of stabilizer candidates.

NaCl, $MgSO_4$, and $Na_5P_3O_{10}$, as promising stabilizers.

3) Influence of stabilizers on the enzyme reaction

Although stabilizers were screened by TSA, whether they have an influence on the enzyme reaction or not still remains unclear. The four discovered stabilizers were, therefore, evaluated an inhibition of the enzyme activity by measuring kinetic parameters. In the presence of each stabilizer, the K_m and V_{max} values of SoxB for sarcosine were estimated from Lineweaver-Burk plots (Table 1). The kinetic parameters estimated from Eadie-Hofstee plots and Hanes-Woolf plots were almost the same as those of Lineweaver-Burk plots (data not shown). In presence of 25 mmol/L $Na_5P_3O_{10}$, the catalytic efficiency (V_{max}/K_m) of SoxB was about one-third that in absence. The high concentration of NaCl (200 mmol/L) also decreased the catalytic efficiency, due to lower value of the binding affinity ($1/K_m$). In contrast, the catalytic efficiency was almost unchanged in the presence of effective concentration of $MgCl_2$, $MgSO_4$, or both.

The two compounds, $MgCl_2$ and $MgSO_4$, were finally chosen as SoxB stabilizers because they did not exhibit inhibitory effects for the enzyme reaction.

Table 1 Kinetic parameters of SoxB for sarcosine

Stabilizer (Conc.)	K_m (mM)	V_{max} (U/mg)	V_{max}/K_m (%)
None	17	34	100
$Na_5P_3O_{10}$ (25 mmol/L)	28	20	36
NaCl (200 mmol/L)	32	40	63
$MgSO_4$ (10 mmol/L)	14	31	110
$MgCl_2$ (50 mmol/L)	15	28	93
$MgSO_4 + MgCl_2$	12	25	100

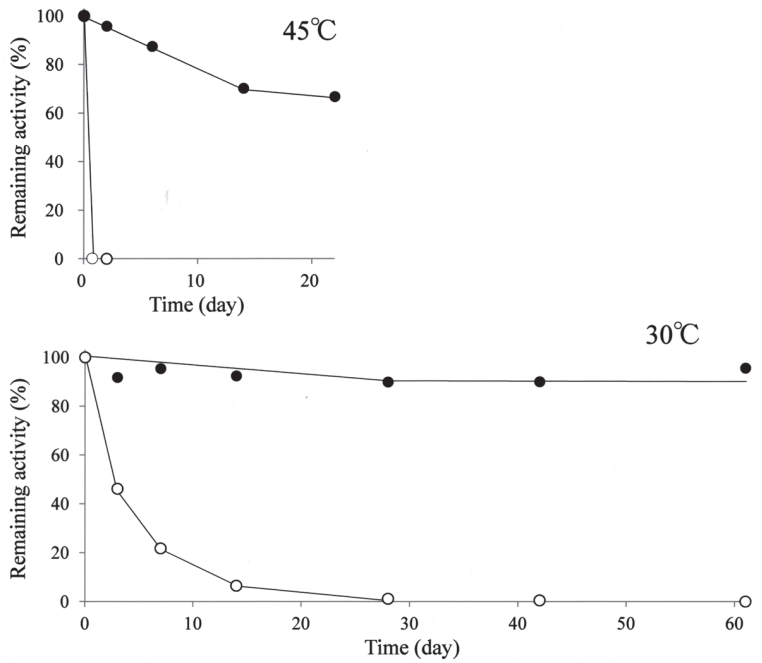

Fig. 4 Accelerated tests of SoxB in presence and absence of stabilizers. The 50 mmol/L HEPES buffer solutions (pH 7.5) containing 10 U/mL SoxB were incubated at 30 and 45℃ in presence and absence of 10 mmol/L $MgSO_4$ and 50 mmol/L $MgCl_2$, and enzyme activities were assayed at certain times. Experiments were carried out in duplicate.

4) Accelerated tests

An accelerated test of storage period is empirically effective in order to predict the long-term stability of the enzyme. We focused on the long-term thermostability at 30 and 45℃ of SoxB in presence or absence of the stabilizers screened.

As shown in Fig. 4, SoxB in the aqueous solution had lost almost total activities at 45 and 30℃ by 1 and 28 days, respectively. In contrast, the loss of enzyme activities were drastically recovered in presence of 10 mmol/L $MgSO_4$ and 50 mmol/L $MgCl_2$. The initial enzyme activities were the same in the presence and absence of the stabilizers. However, SoxB had above 60% of the initial activity in presence of the stabilizers even when it was incubated at 45℃ for 22 days. Moreover, the enzyme had above 90% of the initial activity in presence of the stabilizers when it was incubated at 30℃ for 61 days.

To improve the long-term stability of SoxB, the addition of $MgSO_4$ and $MgCl_2$ was demonstrated to be extremely promising. From the model experiments, the TSA-based screening of enzyme stabilizers on the basis of structural informations was indicated to be an easy and time-saving method, and to be useful in research and development of diagnostic enzymes. We expect this method will be expanded to an improved scheme for stabilizing enzymes.

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