〈Original paper〉

Monomeric sarcosine oxidase exhibiting high substrate affinity and thermostability

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Summary Monomeric sarcosine oxidase (Sox) is utilized in clinical creatinine assays as an index of renal function via coupling with creatininase, creatinase, and peroxidase. To improve the assay reagent, a Sox more stable than the conventional *Bacillus* and *Arthrobacter* enzymes (SoxB and SoxA, respectively) would be beneficial. A Sox homologue from the thermophilic photosynthetic bacterium *Chloroflexus aurantiacus* (SoxCa) was selected as a candidate enzyme. Highly conserved residues in the substrate-binding sites of the other enzymes were not conserved in the amino acid sequence of SoxCa. The tertiary structure of SoxCa constructed by homology modeling suggests that an unconserved glutamine residue is responsible for enhanced substrate binding. The gene encoding SoxCa was expressed in *Escherichia coli*, and SoxCa was purified to homogeneity and characterized. SoxCa exhibited an extremely high thermostability compared with SoxB. The K_m value of SoxCa for sarcosine (N-methylglycine) was 1/35 and 1/7 of the SoxB and SoxA values, respectively. Simple sequential enzyme reaction calculations for the creatinine assay demonstrated the excellent suitability of SoxCa and its high substrate affinity.

Key words: sarcosine oxidase, *Chloroflexus aurantiacus*, substrate affinity, thermostability, assay simulation

1. Introduction

Sarcosine oxidase (Sox, EC 1.5.3.1; sarcosine: oxygen oxidoreductase) is a monomeric or heterotetrameric flavoprotein that catalyzes 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 the related enzymes creatininase (Crn, EC 3.5.2.10; creatinine amidohydrolase) and creatinase (Cre, EC 3.5.3.3; creatine amidinohydrolase)[1]. Monomeric Sox is industrially important and often used with Crn, Cre, and horseradish peroxidase in enzymatic assays of creatinine and creatine in clinical settings (Fig. 1). The enzymes from organisms of the genera *Arthrobacter* (SoxA) and *Bacillus* (SoxB) are produced commercially and used in diagnostic reagents[2-4]. Both the substrate specificity and stability of SoxA have been altered using mutagenesis techniques[5-8].

Based on its relationship to the glomerular

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Received for publication July 11, 2016
Accepted for publication August 4, 2016

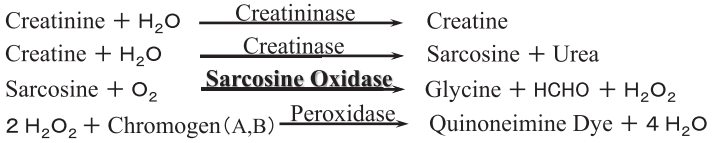

Fig. 1. Principle of enzymatic creatinine assay.

filtration rate, the creatinine concentration in serum serves as an important index value of renal function in clinical medicine. An enzymatic endpoint spectrophotometric assay was developed due to the need for a method to accurately estimate serum creatinine levels. However, both the substrate affinity and thermostability of SoxA and SoxB are insufficient for the endpoint assay. In order to enhance the accuracy of the assay and the quality of the assay reagents, these enzyme parameters must be improved.

In this study, a new Sox exhibiting higher thermostability and substrate affinity than the conventional enzymes was identified from orthologue groups of prokaryotic genomes. This enzyme, designated SoxCa, was isolated from *Chloroflexus aurantiacus*, a thermophilic photosynthetic bacterium belonging to the green non-sulfur group. Simple sequential enzyme reaction calculations demonstrated the excellent suitability of SoxCa for the endpoint assay of creatinine.

2. Materials and Methods

Strains, plasmid, and culture conditions

Escherichia coli BL21 (DE3) and pET23b (Ap^r) were used for recombinant strain preparation and plasmid construction, respectively. Bacteria were grown in LB-broth or on LB-agar (LB-broth plus 1.5% agar) at 30 or 37°C. The antibiotic used was ampicillin (100 μg/mL).

General DNA manipulation

Isolation of plasmids, cleavage of DNA with restriction enzymes, and ligation of DNA with T4 DNA ligase were carried out as described previously[1]. Transformation of *E. coli* was carried out according to the competent-cell method. Polymerase chain reaction (PCR) was performed under standard conditions. A KOD-Plus Mutagenesis kit (Toyobo Co., Ltd., Osaka, Japan) was used for site-directed mutagenesis.

Recombinant plasmid construction

The gene encoding a Sox homologue of *C. aurantiacus* (KEGG database entry name: Caur_3716) was artificially synthesized by the manufacturer (Takara Bio Inc., Shiga, Japan). The codon usage was adapted to the codon bias of *E. coli* genes. In addition, regions of very high (>80%) or very low (<30%) GC content were avoided where possible. The 1173-bp synthesized DNA was ligated between the *Nde* I and *Bam* HI sites of pET23b, and the recombinant construct was designated pET-SoxCa. An additional His-tag sequence was then introduced into the C-terminus of SoxCa by inverse PCR using the mutagenesis kit and the following primers: 5'-GGATCCCACCACCACCACCACCA CTGAGA-3' (sense primer, corresponding to the sequence that encodes the 6×His-tag plus spacer peptide GSHHHHHH and the stop codon) and 5'-ACCTGCCTGCTGTGCACGAAAACGCAG-3' (antisense primer, complementary to the sequence that encodes the C-terminal peptide LRFRAQQAG). This construct was designated pET-SoxCaHT. The DNA sequences of the constructs were verified by sequencing. The pET-SoxCa and pET-SoxCaHT carriers were induced to produce the gene product by the addition of isopropyl-L-D-thiogalactopyranoside (IPTG) to the culture medium.

Enzyme purification

E. coli BL21 (DE3) (pET-SoxCaHT) was grown in LB medium (500 mL) containing 100 μg/mL of ampicillin at 37°C for 2 h, to an optical density of 0.6 at 590 nm. The culture was then supplemented with 0.1 mmol/L IPTG and 5% glycerol, and the cells were grown for an additional 15 h at 37°C to stationary phase. Cells were harvested by

centrifugation and re-suspended in 20 mmol/L potassium phosphate buffer (pH 7.5). Crude extract was prepared by sonication of the cells following centrifugation. The supernatant was incubated at 65°C for 10 min and centrifuged to remove proteins from the host cells. Ammonium sulfate was added to the supernatant to 50% saturation. The precipitate collected by centrifugation, which included SoxCa, was dissolved in 20 mmol/L potassium phosphate buffer (pH 7.5), incubated at 55°C for 5 min, and then centrifuged. The supernatant was then loaded onto a His GraviTrap immobilized metal affinity chromatography column (GE Healthcare, Uppsala, Sweden). SoxCa was eluted using 20 mmol/L potassium phosphate buffer (pH 7.5) containing 500 mmol/L imidazole and 500 mmol/L NaCl. Pooled fractions exhibiting Sox activity were finally purified to homogeneity by gel filtration with a Cosmosil 5Diol-300-II column (Nacalai Tesque, Kyoto, Japan) on an AKTA Explorer 10S HPLC system (GE Healthcare) using 20 mmol/L potassium phosphate (pH 7.5) for elution. The enzymatic properties of the purified product were then characterized.

Enzyme assay and characterization

Compounds used were purchased from Nacalai Tesque. The enzyme assay was based on the measurement of hydrogen peroxide produced during substrate oxidation. A 4-aminoantipyrine peroxidase system was used for the enzyme assay, as described previously[2]. The final assay mixture 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 of horseradish peroxidase. Enzyme solution (35 μL) was incubated with the assay mixture (1000 μL) at 37°C, and the amount of quinoneimine dye formed by the coupling of 4-aminoantipyrine, phenol, and horseradish peroxidase was measured spectrophotometrically at 500 nm against a sample blank. One unit of activity was defined as the formation of 1 μmol of hydrogen peroxide (0.5 μmol of quinoneimine dye) per minute at 37°C and pH 8.0. Reaction mixtures consisting of various concentrations of sarcosine solution were used to determine the K_m and k_{cat} values. Molecular weights were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration on a Cosmosil 5Diol-300-II column.

Sequence homology analysis and molecular modeling

Amino acid sequence homologies were analyzed using GENETYX software (Software Development, Tokyo, Japan). The DDBJ accession numbers of sequences were ABY36894, BAA03967, 1ZOV_A, and BAA09716 for SoxCa, SoxB, SoxBs (another *Bacillus* Sox[9]), and SoxA, respectively. The tertiary structure of SoxCa was constructed by homology modeling. The model was generated using MOE software (Chemical Computing Group Inc., Montreal, Canada), based on the structure of SoxB (PDB ID: 2gf3a). Energy minimization was applied to the model to further refine the structure. There were no outliers on the Ramachandran plot of the model. MOE and Pymol software were used for molecular visualization.

Endpoint assay simulation

Endpoint assays of creatinine with SoxCa, SoxB, and SoxA were simulated using Microsoft Excel. Changes in the amount of creatinine, creatine, sarcosine, and hydrogen peroxide were characterized based on Michaelis-Menten kinetics. Amounts and concentrations for all analytes were calculated every 0.1 s.

3. Results and Discussion

Comparison of amino acid sequences and structures

To identify a stable and analytically useful Sox, orthologues of SoxA and SoxB from various thermophiles were investigated. Enzymes of extreme thermophiles and hyperthermophiles were deemed impractical for use in clinical settings and therefore excluded from the study because they function at extremely high temperatures. An orthologue of the photosynthetic, moderate thermophile *C. auranti-acus* was finally selected. The amino acid sequence

of this orthologue, which was designated SoxCa, exhibited a relatively low degree of homology with SoxA and SoxB (estimated at 36.6 and 38.5% identity, respectively).

A multiple alignment of the sequences of SoxCa and commercially available Sox enzymes is presented in Figure 2. Although there were many consensus residues, several important residues were different only in SoxCa. This included three residues described in a previous study as important determinants of the substrate specificity of Sox[10] (i.e., F100, M245, and H269 in SoxB were substituted with T103, I247, and F274, respectively) (Fig. 2). Moreover, the sequence of SoxCa contained a glutamine residue (Q255) in one of the high-consensus regions.

To elucidate the effect of the additional residue Q255, a three-dimensional model of SoxCa was constructed by homology modeling. The model structure was based on the SoxB sequence and the X-ray crystallographic structure of the closed form (PDB ID: 2gf3a), with a high level of resolution (1.3 Å). The structures of SoxCa and SoxB superimposed well, with a root mean square deviation (RMSD) for atomic Cα positions of 1.34 Å. In contrast, superimposition of SoxB and the SoxA model constructed

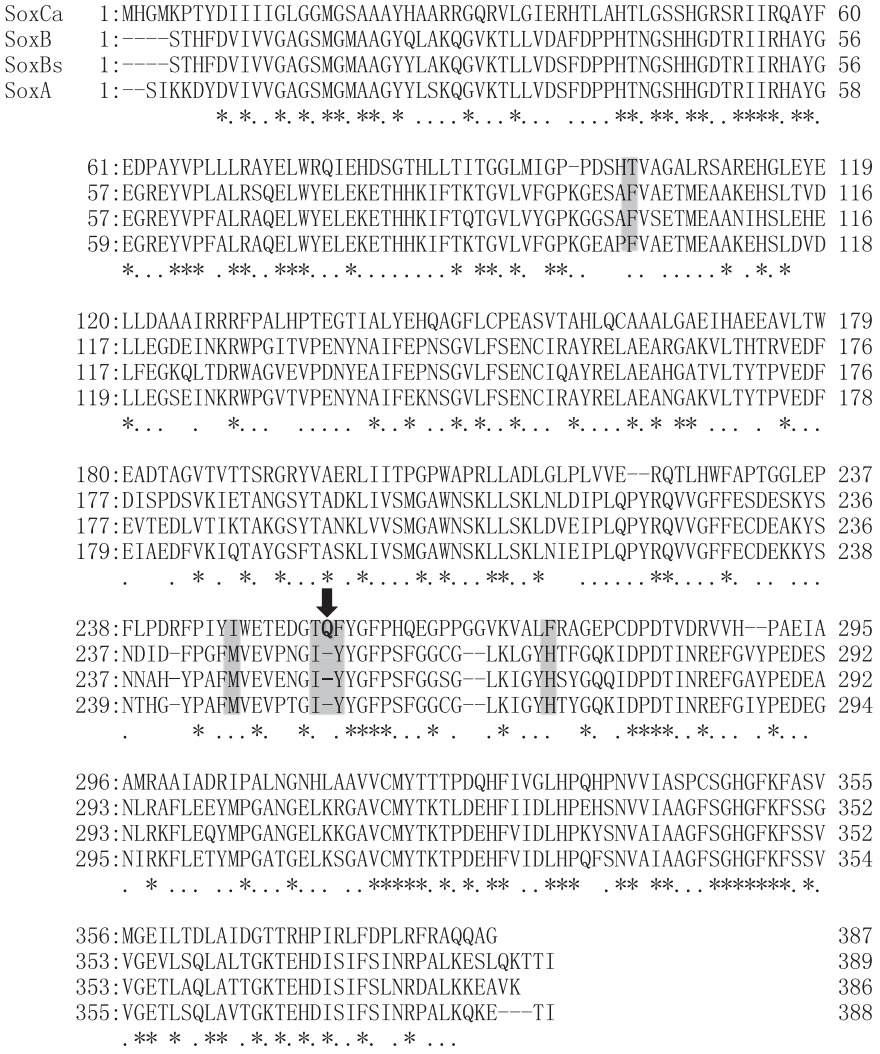

Fig. 2. Amino acid sequence alignment SoxCa and commercially available Sox enzymes. SoxB, SoxBs, and SoxA are from *Bacillus* sp. B-0618, *Bacillus* sp. NS-129, and *Arthrobacter* sp. TE1826, respectively. The additional residue Q255 of SoxCa was indicated by an arrow. The three important residues for the substrate specificity of Sox were surrounded by grey boxes.

previously yielded an RMSD for Cα positions of 1.08 Å. The structure modeled in the present study could thus enhance understanding of the structure-function relationship of SoxCa.

Close-up views of the active site structures of SoxCa and SoxB are shown in Figure 3. As a remarkable difference, the side chain of the Q255 residue in SoxCa was close to the Cα of the substrate, at a distance of 3.7 Å, suggesting that this residue plays an important role in substrate binding.

Gene expression and enzyme purification

A Sox activity of 0.20 U/mL was detected in the crude extract of a culture of recombinant *E. coli* BL21 (DE3) (pET-SoxCaHT). SoxCa was then purified to homogeneity as described in the Materials and Methods section. The purification of SoxCa is summarized in Table 1 and Figure 4. The purified preparation gave a single protein band on SDS-PAGE and exhibited an absorption spectrum characteristic of a flavoprotein. The molecular weight was estimated at 44.0 kDa by SDS-PAGE (Fig. 4). Gel filtration analyses showed that the enzyme was monomeric (data not shown). The molecular weights of SoxCa and its His-tagged derivative were calculated from the deduced amino acid sequences as 42.1 and 43.1 kDa, respectively. The molecular weight of His-tagged SoxCa agreed with the SDS-PAGE result.

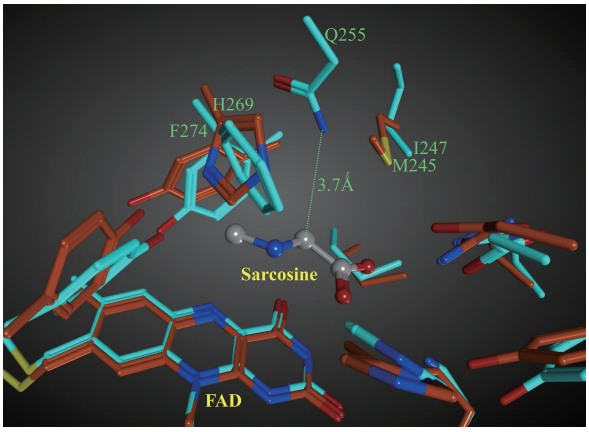

Fig. 3. Close-up views of active sites. Tertiary structures of SoxCa (light cyan) and SoxB (dark orange) were compared. In both structures, oxygens, nitrogens, and sulfurs are in red, blue, and yellow, respectively. Amino acid residues and flavin adenine dinucleotide (FAD) were shown by stick drawings. The substrate sarcosine which was predicted from the SoxB structural data[11,12] was represented by ball-and-stick.

Enzymatic properties

Various enzymatic properties of SoxCa were investigated. As expected, the thermal stability of SoxCa was much higher than that of SoxB (Fig. 5). When SoxA was kept at 60°C for 10 min, it was almost completely denatured, as previously described[2]. Therefore, the thermal stability of SoxA was much lower than that of SoxCa. The stability of the Sox enzyme used significantly impacts the quality of the creatinine and creatine assay reagents. Generally, thermostable enzymes exhibit excellent long-term stability in refrigerated storage. SoxCa might be more suitable for practical use than conventional enzymes. The pH profile of SoxCa is also shown in Figure 5. The optimum pH was esti-

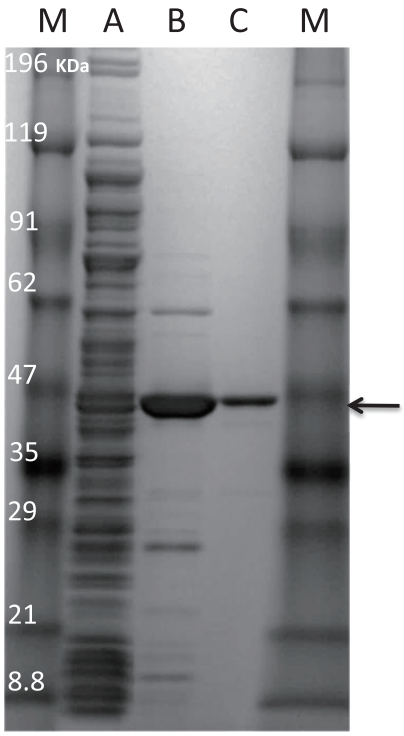

Fig. 4. SDS-PAGE analysis. A: Crude extract of *E. coli* BL21(DE3)(pET-SoxCaHT); B: purification by an immobilized metal affinity chromatography; C: the purified protein; M: molecular weight marker. Arrow indicates SoxCa.

Table 1 Purification of SoxCa

Step	Protein(mg)	Activity(U)	Yield(%)
Crude extract	1100	10	100
Heat treatment (60˚C, 10min)	960	9.1	90
Ammonium sulfate	83	9.4	92
Heat treatment (55˚C, 5min)	46	13	130
Affinity chromatography	1.5	6.8	68
HPLC	1.1	5.5	55

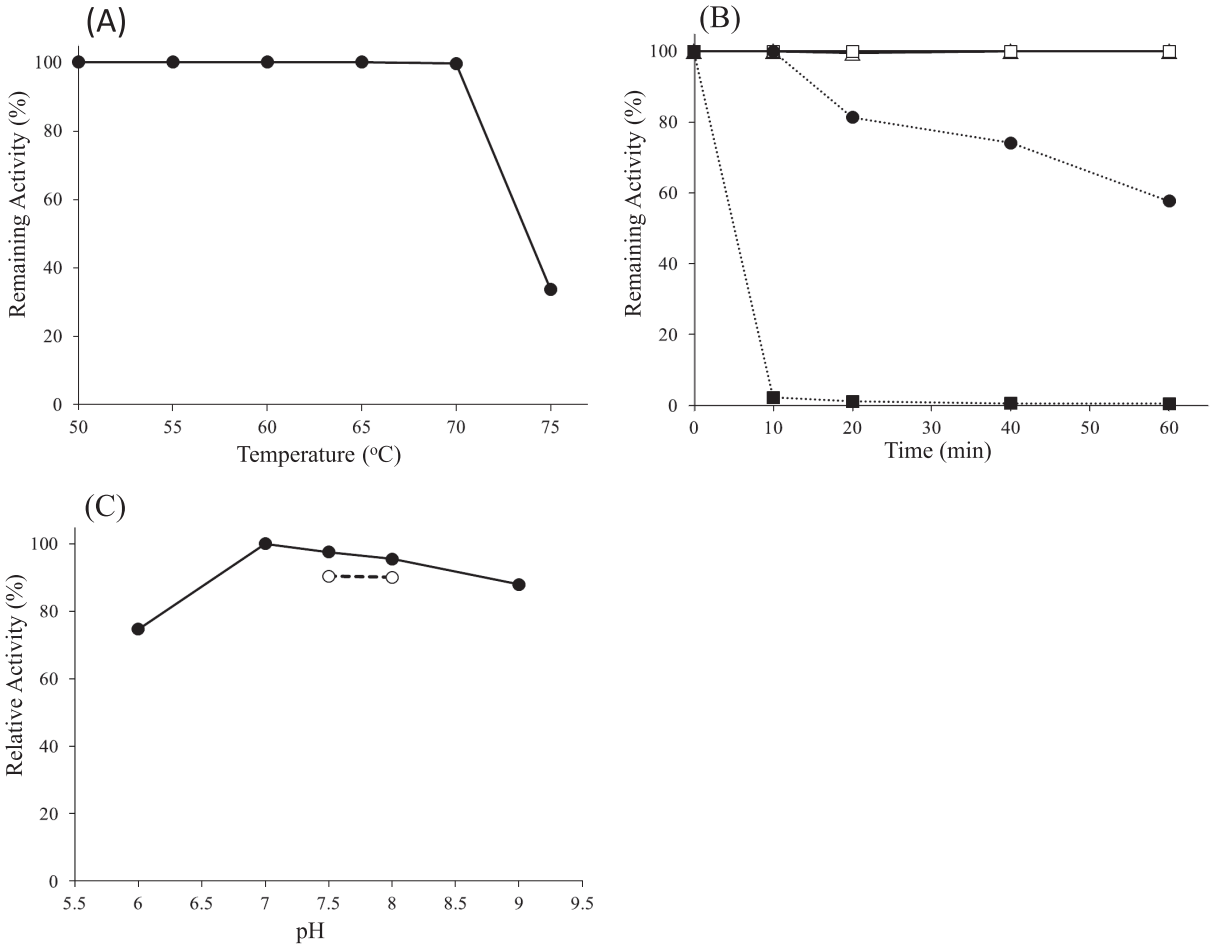

Fig. 5. Enzymatic properties. (A) Effect of temperature on the stability of SoxCa. The enzyme solutions (5 U/mL) were incubated for 1 hour at the indicated temperatures, and the remaining activities were measured as described in the Materials and Methods section. (B) Comparison of thermal stabilities between SoxCa and SoxB. 5 U/mL of each enzyme solution was used for incubation. Stabilities of SoxCa and SoxB were shown by solid and dashed lines, respectively. The incubation temperature was 50°C (filled circles), 55°C (open and filled squares), or 65°C (open triangles). (C) Effect of pH on the activity of SoxCa. The reaction mixtures contained 50 mmol/L potassium phosphate (filled circle and solid line) and Tris-HCl (open circle and dashed line) buffers at various pH values. The Sox activity of the enzyme solution used was 0.05 U/mL at 37°C and pH 8.0.

mated as almost neutral (7.0-8.0).

The K_m value of SoxCa for sarcosine was calculated as 0.49 mmol/L. This value was 1/35 and 1/7 that of SoxB and SoxA, respectively (Table 2). The high substrate affinity of SoxCa (which was predicted by comparisons of the active site structures

Table 2 Comparison of kinetic parameters

Enzyme	K_m for sarcosine (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (%)
SoxCa	0.49	3.7	100
SoxB	17	24	18
SoxA	3.6	14	51

described in this paper) was demonstrated experimentally. The catalytic efficiency (k_{cat}/K_m) of SoxCa was 5.3 and 1.9 times higher than that of SoxB and SoxA, respectively (Table 2). Further structural investigations using site-directed mutagenesis are now in progress in order to determine whether the high substrate affinity and catalytic efficiency of SoxCa are associated with residue Q255.

Endpoint assay simulation

To examine the influence of the substrate affinity of Sox on the enzymatic creatinine assay, we simulated endpoint assays using SoxCa, SoxB, and SoxA (Fig. 6). The kinetic parameters of each

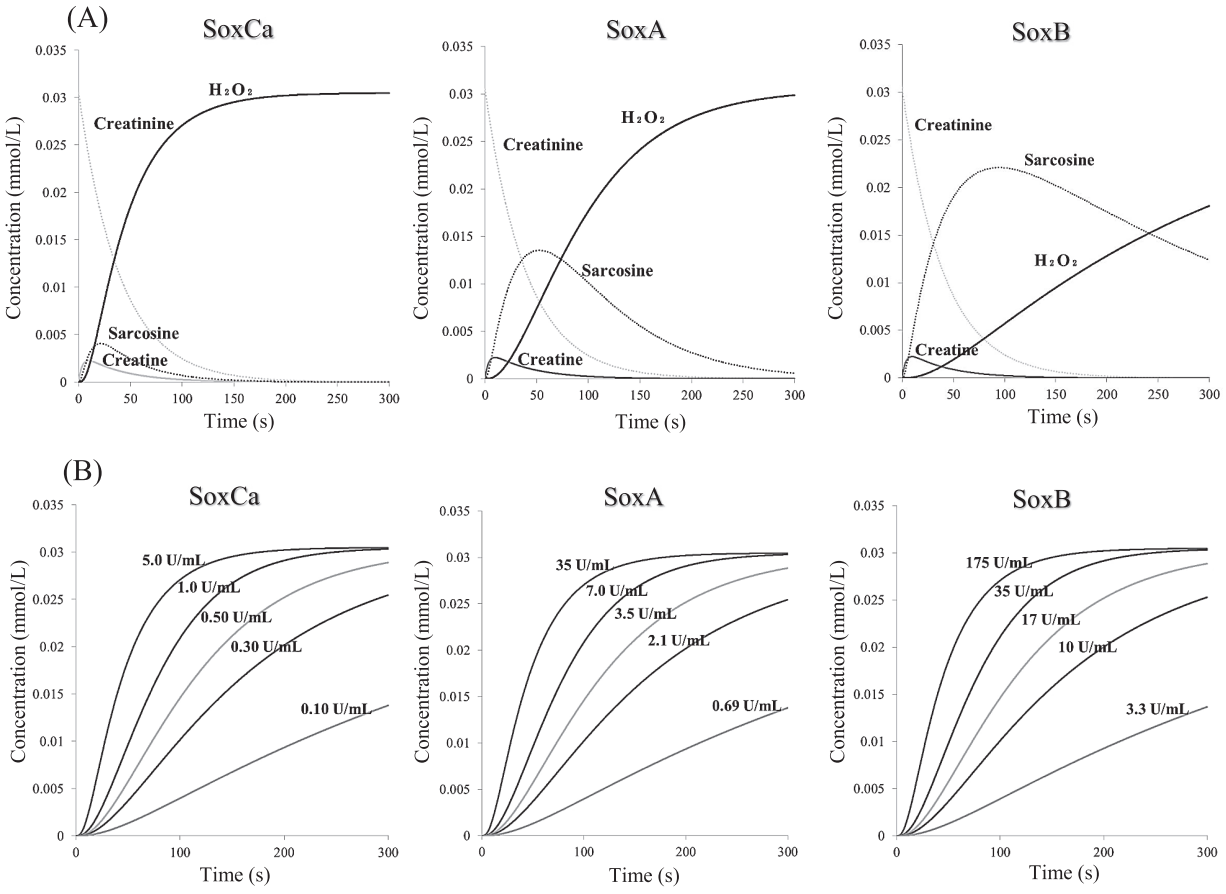

Fig. 6. Simulation of endpoint assays of creatinine with SoxCa, SoxA, and SoxB. (A) It was assumed that the assay reagent 1 contained 100 U/mL of Cre with 4.5 mmol/L of K_m and 5 U/mL of each Sox (210 µL), the assay reagent 2 contained 200 U/mL of Crn with 32 mmol/L of K_m (70 µL), and 10 mg/dL of the creatinine sample (10 µL) were mixtured and incubated at 37°C for 5 minutes. The time courses of creatinine, creatine, sarcosine, and hydrogen peroxide were, then, compared to those of SoxA and SoxB. (B) The time courses of endpoint assays simulated as concentrations of hydrogen peroxide were compared to each other. It was assumed that the assay reagents contained various concentrations of Sox were used without changing other assay conditions.

enzyme with respect to sarcosine were used in the simulations. Simple sequential enzyme reaction calculations demonstrated the excellent suitability of SoxCa for the endpoint assay of creatinine. When equal Sox activity was used in the reaction, the SoxCa-containing reagent quickly reached an endpoint, as evidenced by the high rate of hydrogen peroxide production. The estimated minimum amount of SoxCa required was markedly lower than that of the other enzymes. To obtain the same results, approximately 35 or 7 times the amount of SoxCa would be required compared with SoxB or SoxA, respectively, in agreement with the K_m values of the enzymes (Fig. 6).

Thus, SoxCa is the first diagnostic enzyme selected by *in silico* analysis with both high predicted stability and substrate affinity. The use of this enzyme as a diagnostic reagent would therefore increase the reliability of creatinine determinations.

References

1. Nishiya Y, Toda A, and Imanaka T: Gene cluster for creatinine degradation in *Arthrobacter* sp. TE1826. Mol Gen Genet, 257: 581-586, 1998
2. Nishiya Y and Imanaka, T: Cloning and sequencing of the sarcosine oxidase gene from *Arthrobacter* sp. TE1826. J Ferment Bioeng, 75: 239-244, 1993
3. Wagner MA and Jorns MS: Monomeric sarcosine oxidase: 2. Kinetic studies with sarcosine, alternate substrates, and a substrate analogue. Biochemistry, 39: 8825-8829, 2000
4. Nishiya Y, Yamamoto K, Kawamura Y, and Emi S: Development of creatinine-degrading enzymes for application to clinical assays [Jpn]. Nippon Nogeikagaku Kaishi, 75: 857-862, 2001
5. Nishiya Y and Imanaka T: Alteration of substrate specificity and optimum pH of sarcosine oxidase by random and site-directed mutagenesis. Appl Environ Microbiol, 60: 4213-4215, 1994
6. Nishiya Y, Zuihara S, and Imanaka T: Active site analysis and stabilization of sarcosine oxidase by the substitution of cysteine residues. Appl Environ Microbiol, 61: 367-370, 1995
7. Nishiya Y and Kishimoto T: Alteration of L-proline oxidase activity of sarcosine oxidase and a structural interpretation. J Anal Bio-Sci, 33: 161-166, 2010
8. Nishiya Y: Altered substrate affinity of monomeric sarcosine oxidase by the mutation of phenylalanine-103 or histidine-348. Int J Anal Bio-Sci, 1: 21-26, 2013
9. Nagata K, Sasaki H, Hua M, Oka M, Kubota K, Kamo M, Ito K, Ichikawa T, Koyama Y, Tanokura M: Crystal structure of monomeric sarcosine oxidase from *Bacillus* sp. NS-129 reveals multiple conformations at the active-site loop. Proc Jpn Acad Ser B, 81: 220-224, 2005
10. Nishiya Y and Abe Y: Comparison of the substrate specificity of L-pipecolate oxidase and bacterial monomeric sarcosine oxidase, and structural interpretation of the enzymes. Int J Anal Bio-Sci, 3: 55-62, 2015
11. Wagner MA, Trickey P, Chen Z, Mathews FS, and Jorns MS: Monomeric sarcosine oxidase: 1. Flavin reactivity and active site binding determinants. Biochemistry, 39: 8813-8824, 2000
12. Kommoju P-R, Chen Z, Bruckner RC, Mathews FS, and Jorns MS: Probing oxygen activation sites in two flavoprotein oxidases using chloride as an oxygen surrogate. Biochemistry, 50: 5521-5534, 2011