# Substrate specificity of monomeric and dimeric α-sarcin

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**Abstract The substrate specificity of monomeric and dimeric forms of α-sarcin was investigated by membrane blotting procedures. Dimeric α-sarcin fails to inactivate ribosomes as well as to hydrolyze mini-stem-loop RNA, whereas monomeric α-sarcin catalyzes both substrates. Both monomeric and dimeric α-sarcin are effective ribonucleases that are displayed by in situ RNA-impregnated gel electrophoresis. The same purine base specificity was detected for both dimeric and monomeric forms. α-Sarcin is also an effective deoxyribonuclease to supercoiled DNA. The action of α-sarcin as deoxyribonuclease and ribonuclease is inhibited by the presence of SDS ($3.5\times10^{-6}$ M); the inhibition on ribonuclease, but not on deoxyribonuclease, is reversible if the proteins are renatured.**

*Key words:* Ribotoxin; Ribosomes; Stem-loop RNA; Supercoiled DNA; Membrane blotting hydrolysis

## 1. Introduction

α-sarcin, a major type II ribotoxin, is secreted by the filamentous fungus *Aspergillus giganteus* [1]. The structure and function of α-sarcin and several related ribotoxins have been characterized [2-4]. The protein is known for its specific ribonucleolytic activities [5]. It hydrolyzes the large ribosomal RNA [6-8]. The action is specific; only one phosphodiester bond in the sarcin domain of 23S–28S rRNA is hydrolyzed [7,8]. It also cleaves a mini-stem-loop RNA that mimics the sarcin domain [9-11]. At higher concentrations, the protein can act as a ribonuclease [12]. It carries purine-specific endonuclease activity to single- and double-stranded RNA [12,13]. Besides the ribonuclease activity, α-sarcin has previously been documented to have deoxyribonucleolytic activity [12]. Cleavage of supercoiled DNA by α-sarcin and other related ribotoxins were reported recently [14].

The dimeric form of α-sarcin is constantly observed and is a unique characteristic among type II ribotoxins [2,15-17]. The formation of dimer is persistent even in analysis by SDS-containing polyacrylamide gel electrophoresis with reducing agent. Intra-disulfide bond linkage of dimer formation was proposed but the claim has never been proven [2,17]. Neither the nature nor the function of the dimeric form of α-sarcin has been determined yet. In this study, we have taken advantage of the protein-binding property of the PVDF membrane to separate dimers of α-sarcin from its monomeric forms. The substrate specificity of both forms was examined. Similar and dissimilar modes of action of dimeric and monomeric α-sarcin have been reported.

## 2. Materials and methods

### 2.1. Separation of dimer and monomer of α-sarcin on PVDF membrane

Monomeric and dimeric α-sarcin were separated by electrophoresis on a 15% polyacrylamide gel containing SDS and electrophoretically transferred onto PVDF membrane (Micron Separation Inc., MA, USA) [18]. The localization of monomers and dimers on the membrane was visualized by Ponceau S staining (Merck Chem. Co.). Dimers and monomers were cut out from blotted membrane and washed extensively with distilled water. They were stored for future use. The amounts of protein on the membrane were determined by amino acid analysis according to previous procedures [19]. The molar ratio of monomer and dimer (7 to 1) on the membrane was quantitatively calculated.

In an exchange experiment, the electrophoretically blotted PVDF membrane that contains either monomer or dimer was incubated with [$^{125}$I]α-sarcin in TKM buffer (20 mM Tris-HCl, pH 7.6; 50 mM KCl; and 5 mM $MgCl_2$) containing 0.05% β-mercaptoethanol. The iodinated [$^{125}$I]α-sarcin was prepared by the IODO-GEN method with [$^{125}$I]NaI [20] (Pieres Chem. Co., Rockford, USA). After a period of incubation, the membrane strips were rinsed with water, and their radioactivities were measured. The same procedures were carried out with BSA blocked blank PVDF and blank PVDF strips. Both strips were used as the control.

### 2.2. Hydrolysis of ribosomes by membrane-bound α-sarcin

Measurements of ribosome inactivation by dimers and monomers were conducted according to the previously described procedure [21]. Rabbit reticulocyte ribosomes were used as the substrates. The assay was done in appropriate amounts of protein for monomer, dimer, and native α-sarcin — 1.2, 1.2, and $0.8\times10^{-8}$ M, respectively. α-fragments generated from large subunit 28S rRNA were scored by a composite gel.

### 2.3. Hydrolyzing mini-stem-loop RNA by membrane-bound α-sarcin

The specific ribonucleolytic activities of dimers and monomers were directly examined by their abilities to cleave the mini-stem-loop RNA that mimics sarcin domain of rat liver ribosomes. A cDNA template that carries T7 promoter and sequences complementary to the sarcin domain of 28S rRNA of rat liver ribosomes was synthesized chemically using DNA synthesizer (API 470A). The template was further purified by electrophoresis on 20% polyacrylamide gel in TE buffer (89 mM Tris-borate, pH 8.3; 1 mM EDTA). Radioactive 35-mer RNA was synthesized by T7 RNA polymerase and cDNA template procedures with $^{32}$P-labeled ribonucleotide according to procedures described previously [9]. The [$^{32}$P]35-mer was slowly renatured overnight at low temperature (4°C) before incubation with the same amount of membrane-bound monomer and dimer. The reaction was carried out at 37°C for 15 min in TE buffer. The mixtures were analyzed on a 20% polyacrylamide gel containing 7 M urea and autoradiographed at −70°C.

### 2.4. Detection of ribonuclease activity by RNA-impregnated polyacrylamide gel

RNA-impregnated SDS polyacrylamide gel electrophoresis was employed to examine the hydrolytic ability of monomeric and dimeric α-sarcin to naked RNA [22]. Purified α-sarcin was electrophoretically separated in an SDS polyacrylamide gel slab as usual except that the gel contained 2.5 mg/ml of a mixture of ribosomal RNA (prepared from total extraction of ribosomes). The RNA fragments do not interfere with the separation of proteins. After completion of electrophoresis, the RNA-impregnated gel was renatured by treating it with

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incubation buffer (10 mM Tris-HCl, pH 7.4) with 25% isopropanol, followed by four washes with incubation buffer. The gel was then incubated for 30 min at 35°C in incubation buffer with vigorous shaking and stained with 2% toluidine blue O in water. RNAs at the protein zone in the gel slab were digested into smaller fragments if the protein expressed the ribonuclease activity, and could be eluted after vigorous washing. Thus, the protein zone was white in color against a blue background (the staining of undigested large RNAs) of gel slab.

*2.5. Determination of the base specificity by membrane-bound α-sarcin*

Rat liver 5S rRNA was used as the substrate for determining the base specificity of membrane-bound α-sarcin. Rat liver 5S rRNA was isolated according to the procedures previously described [13], and labeled at the 5′ end using T4 polynucleotide kinase method with [$^{32}$P]ATP. Radioactive [$^{32}$P]5S rRNA was incubated with native α-sarcin, membrane-bound monomer and dimer in 50 μl of buffer containing 50 mM Tris/HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, and 1 μg of tRNA at 37°C for 30 min. The incubation was stopped by adding sufficient urea (containing bromophenol blue and xylene cyanol FF) to give a final concentration of 7 M of urea. Products of the digestion were analyzed by electrophoresis on 10% polyacrylamide sequencing gels. The sites of cleavage were compared from the published data [12].

*2.6. Digestion of supercoiled double-stranded DNAs with dimeric α-sarcin*

Plasmid pGEM-T from *E. coli* JM109 cells was used as the source of double-stranded supercoiled DNA. After culture cells reached the exponential growth phase in LB medium, total plasmid DNA was purified by the alkali lysis procedure. The supercoiled DNA was purified by cesium chloride–ethidium bromide gradient centrifugation.

Cleavage of supercoiled DNA by membrane-bound α-sarcin was done as follows: 0.75 μg of supercoiled DNA was incubated separately with membrane-bound dimeric and monomeric α-sarcin in a final volume of 50 μl containing 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl, at room temperature for 30 min. The amounts of protein used for monomer, dimer, and native α-sarcin were 1.2, 1.2, and $0.8\times10^{-8}$ M, respectively. The reaction was stopped by adding 20 μl of loading solution (30% Ficoll, 200 mM EDTA, 0.25% bromophenol blue and 0.25% xylene cyanol FF). The reaction mixtures were analyzed on 1% agarose gel electrophoresis under non-denaturing conditions in TAE buffer (20 mM Tris-acetate, 1 mM EDTA). DNA was visualized by ethidium bromide staining.

## 3. Results

*3.1. Authenticity of membrane-bound dimeric α-sarcin*

Native α-sarcin was separated on 15% polyacrylamide gel electrophoresis containing SDS, and transferred electrophoretically to PVDF membrane. Separation of dimer and monomer of α-sarcin on the membrane was visualized by staining with Ponceau S and Coomassie blue (Fig. 1A). The authenticity of dimeric α-sarcin was positively identified by Western blotting of anti-α-sarcin serum (Fig. 1A). Dimeric and monomeric α-sarcin were permanently mounted on PVDF membrane after electrophoretic blotting, and have an identical amino acid composition. The molar ratio between monomers and dimers on the membrane is 7 to 1 (monomers to dimers). The amounts of protein on the membrane were kept constant between each gel electrophoretic separation. Moreover, the amounts of protein retained on the membrane were unchanged when membranes were vigorously washed, or exchanged when membranes were placed in a solution of radioactive [$^{125}$I]α-sarcin (Table 1).

*3.2. Specificity of membrane-bound α-sarcin when ribosomes are the substrates*

The membrane-bound monomeric α-sarcin generates the specific α-fragment from ribosomes in the rabbit reticulocyte

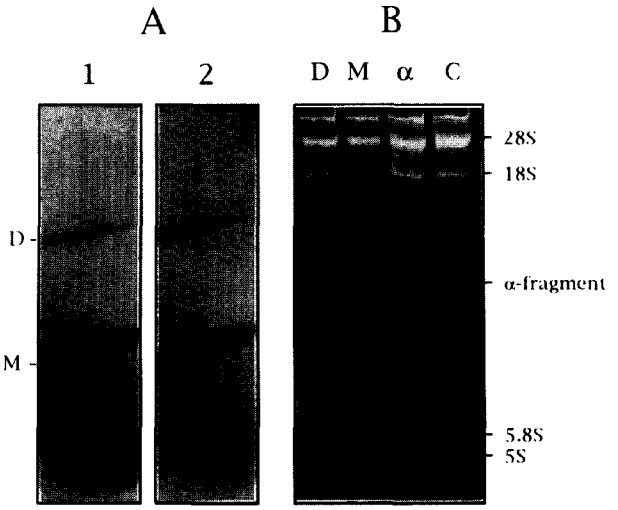


Fig. 1. Separation of dimer and monomer of α-sarcin and their effect on ribosome inactivation. Panel A shows the separation of dimeric and monomeric α-sarcin by SDS polyacrylamide gel electrophoresis (lane 1), and the corresponding positions of monomeric (M) and dimeric (D) α-sarcin on the membrane as identified by Western blot using anti-α-sarcin serum (lane 2). Panel B shows the results of ribosome inactivation by membrane-bound dimeric α-sarcin (lane D), monomeric α-sarcin (lane M), the native α-sarcin (lane α), and rabbit reticulocyte ribosomes only (lane C). The assays were done in a composite gel and stained with ethidium bromide. Positions of α-fragment, 28S, 18S, 5.8S, and 5S rRNA are indicated.

assay as demonstrated previously [22]. When membrane-bound dimeric α-sarcin was used in the same assay, the protein was unable to produce the α-fragment, even when the amounts used were comparable to the requirement of monomers for generating the α-fragment [12,21] (Fig. 1B).

*3.3. Specificity of membrane-bound α-sarcin when mini-stem-loop RNAs are the substrates*

Native α-sarcin is able to cleave specifically a 35-mer RNA that mimics the sarcin domain of the large ribosomal subunit (Fig. 2) [9-11]. The reactivities of membrane-bound dimeric and monomeric α-sarcin were therefore examined. It was found that dimeric α-sarcin can not cleave the 35-mer RNA but the monomeric form hydrolyzes the 35-mer precisely at the position where the native α-sarcin acts. Hydrolysis generates two fragments, a 21-mer and 14-mer, as shown in Fig. 2.

*3.4. Ribonucleolytic action of α-sarcin when naked RNAs are the substrates*

The RNA-impregnated gel electrophoresis was employed for detecting the ribonuclease activity of monomeric and dimeric α-sarcin. Both dimeric and monomeric α-sarcin show extensive digestion of large fragment of RNA in gel regardless of the presence of SDS in gel (Fig. 3). A further experiment was conducted to test the degree of susceptibility of α-sarcin to SDS with respect to ribonuclease activity. It was found that α-sarcin was inactive with SDS at a concentration of $3.5\times10^{-6}$ M, but the lost action could be recovered when the protein was renatured (data not shown).

*3.5. Base specificity of ribonucleolytic action of dimeric α-sarcin*

Rat liver 5S rRNA was used as the substrate for determining the base specificity of dimeric α-sarcin. The results of

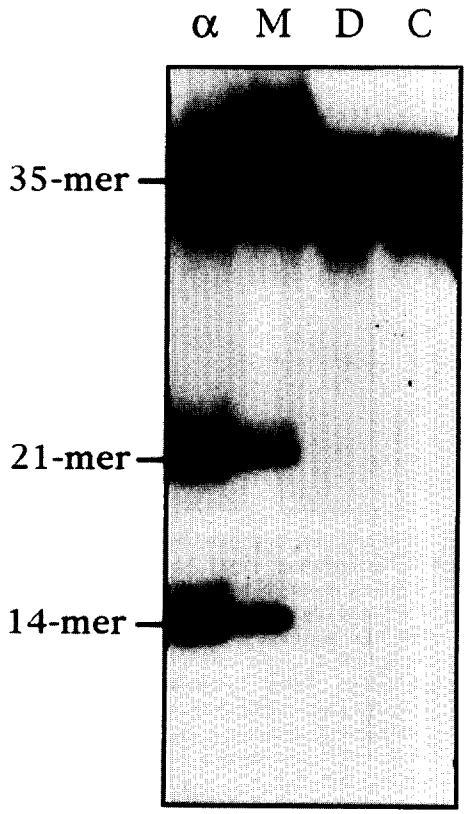


Fig. 2. Hydrolysis of a synthetic 35-mer RNA by membrane-bound α-sarcin. A 35-mer of RNA that mimics the sarcin domain of rat liver 28S RNA was synthesized and labeled uniformly with [$^{32}$P]GTP [9]. The 35 oligomer (0.26 μM) was incubated for 15 min at 37°C with purified α-sarcin (lane α), membrane-bound monomer (lane M), membrane-bound dimer (lane D), and without protein (lane C). The products of digestion were separated by electrophoresis and autoradiography. The 35 oligomer (35-mer) and two RNA fragments (21-mer and 14-mer) are marked.

digestion were analyzed by 10% polyacrylamide sequencing gels. Dimeric α-sarcin shows an identical digestion pattern to that of the native and monomeric α-sarcin (Fig. 4). The pattern is comparable to the previously published data [13]. Purine base specificity was confined for all three molecules. Again, no single- or double-stranded preference was observed.

### *3.6. Digestion of supercoiled DNA by α-sarcin and effect of SDS on digestion*

Supercoiled DNA of plasmid pGEM-T was used as the substrate. Analyses were done by incubating substrates with differently treated α-sarcin. The nicked form of supercoiled DNA was detected from plasmids treated with native α-sarcin and SDS-free membrane-bound α-sarcin (native α-sarcin immobilized on membrane). When membrane-bound dimeric or monomeric α-sarcin separated by SDS gel and electrophoretically transferred was used in the incubation of plasmid, no nicked form of supercoiled DNA or linear DNA was found. The effect of SDS on nucleolytic digestion of supercoiled DNA was further examined. The results indicate that the action of α-sarcin to supercoiled DNA can be nullified at $3.5\times10^{-6}$ M of SDS (Fig. 5). The renatured protein that was recovered from SDS treatment can not perform the deoxyribonucleolytic action on supercoiled DNA.

Table 1
Molecule exchange on membrane-bound forms of α-sarcin

| Membrane bound proteins | % of [$I^{125}$]α-sarcin on PVDF membrane[a] |
|---|---|
| Monomer | 5.0 |
| Dimer | 7.6 |
| Blank membrane (BSA-blocked)[b] | 6.4 |
| Blank membrane (non-blocked)[c] | 100 |

[a]Experiments were conducted as follows: PVDF membrane-bound monomeric and dimeric α-sarcin were identified with $^{125}$I-labeled α-sarcin (total $6.4\times10^4$ cpm) for 1 h followed by consecutive TKM buffer washes. The percentage was calculated on the basis of total cpm of non-blocked blank membrane.[b]Blank PVDF membrane that was soaked with BSA.[c]Blank PVDF membrane only.

## 4. Discussion

α-sarcin and its related ribotoxins characteristically form dimers even under denaturing conditions [2,15-17]. In solution, a constant ratio is found between the monomeric and dimeric forms. Therefore, there is no way to differentiate the hydrolyzing action of the monomer from that of the dimer with regard to their substrate specificity. Previously, we have demonstrated that membrane-bound monomeric α-sarcin was capable of hydrolyzing ribosomes [21]. Hence, the possible action of the dimeric form of α-sarcin could be examined when bound to membranes. Prior to determining the action of membrane-bound dimeric α-sarcin, the formation of dimers on membranes was carefully evaluated. The identity of the dimer from its position on the membrane was first confirmed by Western blot using anti-α-sarcin antibody. The amino acid composition of membrane-bound dimer is identical to that of membrane-bound monomer as well as to that of native sarcin protein (data not shown). Moreover, the results of exchange experiments (Table 1) indicated that there was no loss of protein when radioactive membrane-bound dimer was washed

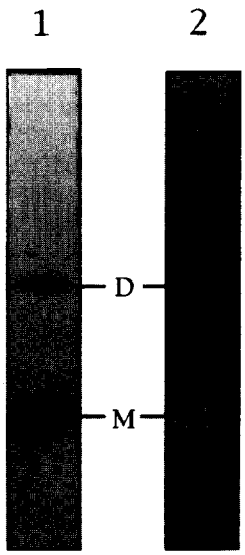


Fig. 3. In situ digestion of naked RNA by α-sarcin. RNA-impregnated polyacrylamide gel electrophoresis was carried out according to [22]. Lane 1 is the separation of the dimer (D) and monomer (M) of α-sarcin in RNA-impregnated SDS gel electrophoresis, and lane 2 illustrates the expressions of ribonucleolytic activity (shown in white) of dimeric (D) and monomeric (M) in gel. The background of lane 2 is undigested RNA that stained with toluidine blue O.

vigorously, no exchange of molecule had taken place on the membrane when membrane-bound dimer was saturated with radioactive labeled α-sarcin or vice versa. All data indicate that dimers do not dissociate into the monomeric form on the membrane, and both forms are firmly bound on the membrane. The legitimacy of dimer formed on PVDF membrane is, therefore, established.

The activity of membrane-bound α-sarcin on substrates of ribosomes, mini-stem-loop RNA, naked RNA, and supercoiled DNA was determined. Membrane-bound dimeric α-sarcin is incapable of digesting ribosomes and of hydrolyzing mini-stem-loop RNA that mimics the sarcin domain of large subunit rRNA, whereas membrane-bound monomeric α-sarcin does positively digest both substrates. Thus the action of ribosome inactivation that results in inhibition of translation,

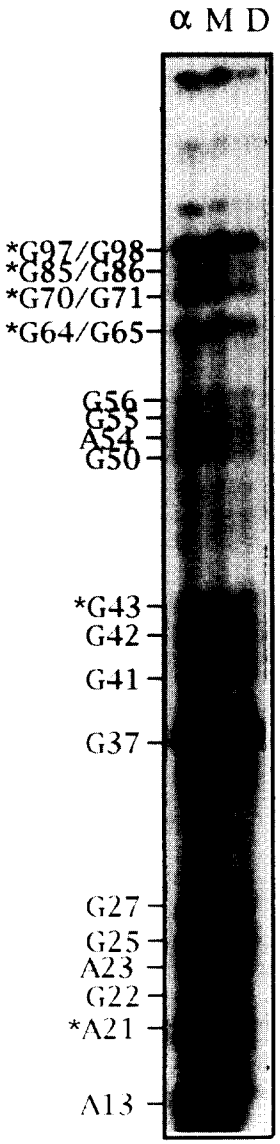


Fig. 4. Effect of membrane-bound α-sarcin on 5S rRNA. Rat liver 5S rRNA (5′ end $^{32}$P-labeled) was incubated with native α-sarcin (α), membrane-bound monomer (M), and dimer (D). The products of the digestion were analyzed by electrophoresis on 10% polyacrylamide sequencing gels. The sites of cleavage on 5S rRNA were identified and marked according to published data [13]. Bases of double-stranded or single-stranded ribonucleotide are marked with or without asterisk, respectively.

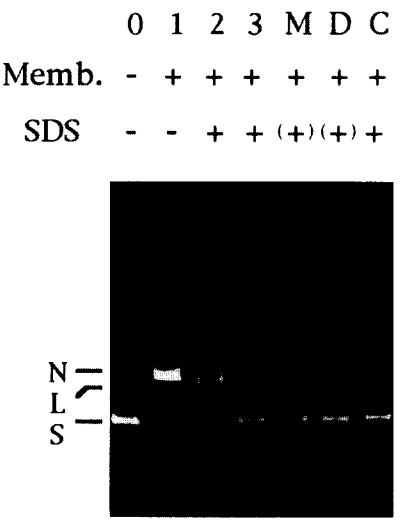


Fig. 5. Effect of SDS on deoxyribonucleolytic action of α-sarcin on supercoiled DNA. Deoxyribonucleolytic activity of α-sarcin on supercoiled double-stranded pGEM-T DNA was carried out according to the following procedures. Each lane contained 0.75 μg of pGEM-T DNA with 0.5 μg of α-sarcin treated with or without SDS before it was immobilized on PVDF membrane. Lane 0, pGEM-T only; lane 1, with native α-sarcin; lanes 2 and 3, α-sarcin treated with $0.7\times10^{-6}$ M and $3.5\times10^{-6}$M of SDS, respectively; lanes M and D, membrane-bound monomeric and dimeric α-sarcin obtained from SDS gel separation, respectively; and lane C, blank membrane strip only. N, nicked circular DNA; L, linear DNA; and S, supercoiled DNA; (+), renatured protein recovered from the SDS treatment.

as well as the hydrolysis of secondary structure of stem-loop RNA is mainly due to the activity of the monomeric form of α-sarcin. However, dimeric α-sarcin, like the majority of known dimeric ribonucleases [23], is an effective ribonuclease as demonstrated by in situ RNA-impregnated gel. In fact, membrane-bound dimers and monomers of α-sarcin have the same purine base specificity as demonstrated previously for the native α-sarcin [12].

Much to our surprise, when supercoiled DNAs were the substrates, membrane-bound dimer and monomer were incapable of digesting supercoiled DNAs, whereas native α-sarcin has clearly displayed its action and produced the nicked circular and linear forms of DNA as reported by others [14]. Since our experiments were conducted with membrane-bound materials and treatment with SDS, the effects of membrane or SDS could cause inactivation of the protein. Our experimental data clearly rule out the possibility that the membrane has this effect (Fig. 5), but registered the influence of SDS. The activities of α-sarcin on supercoiled DNA and on naked RNA are both inhibited by SDS at a concentration of $3.5\times10^{-6}$ M. However, renatured α-sarcin recovered from SDS treatment is active only as a ribonuclease, and no deoxyribonucleolytic activity was found. The difference in renaturation could illustrate that the action centers for ribonuclease and deoxyribonuclease are at separate domains in the protein.

This study has indicated that dimers do not participate in the ribotoxic action of ribosome-inactivation and of stem-loop RNA digestion. The study also implies that formation of dimers could inhibit the ribosome inactivation of monomeric α-sarcin. Since ribosome inactivation is known to account for the cytotoxic effect [24], it is possible that dimer formation could play a biological role in preventing the suicidal action in

ribotoxin-producing cells. The regulation of the formation of dimers is currently under investigation.

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