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Purification, crystallization, and X-ray crystallographic analysis of spermidine synthase from *Kluyveromyces lactis*

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Spermidine synthase (SpdS) is an aminopropyltransferase that transfers an aminopropyl moiety from decarboxylated S-adenosylmethionine to a variety of polyamines. SpdS enzymes convert putrescine to spermidine and its byproduct methylthioadenosine. In this study, we have overexpressed an N-terminal His₆-tagged SpdS from the fungal species *Kluyveromyces lactis*; it was overexpressed, purified and crystallized to obtain X-ray diffraction data at a high resolution of 1.9 Å. The *K. lactis* SpdS crystal belongs to the space group *P*2₁2₁2₁ with the following unit cell parameters: *a* = 65.252 Å, *b* = 98.180 Å, *c* = 102.134 Å, and $\alpha = \beta = \gamma = 90^\circ$. There are two molecules in the asymmetric unit.

INTRODUCTION

Polyamines, such as putrescine, spermidine, and spermine, are synthesized by the enzymes aminopropyltransferases. Unlike other enzymes, which utilize the cofactor S-adenosylmethionine (SAM), aminopropyltransferases uniquely use decarboxylated S-adenosylmethionine (dcSAM). During this process, dcSAM facilitates the enzymatic reaction of aminopropyltransferase through the transfer of the aminopropyl moiety of dcSAM to the amino acceptor. This results in the production of a new polyamine molecule that has an additional positive charge, and the formation of methylthioadenosine (MTA) as a byproduct (Pegg and McCann, 1982; Tabor and Tabor, 1984). The formation of dcSAM occurs from S-adenosylmethionine decarboxylase (SAMDC) converting SAM to dcSAM (Fontecave et al., 2004).

Aminopropyltransferases are a family of SAM-dependent methyltransferases (MTase) that contain over 15 superfamilies and can be further subdivided into 8 classes (Schubert et al., 2003; Kozbial and Mushegian, 2005; Kaminska et al., 2010; Kimura et al., 2014). There are two major types of aminopropyltransferase: spermidine synthase (SpdS; EC 2.5.1.16) and spermine synthase (SpmS; EC 2.5.1.22) (Korolev et al., 2002). In *Escherichia coli*, the identification of only one aminopropyltransferase has been observed and it uses putrescine as the substrate for spermidine. This SpdS is also known as putrescine aminopropyltransferase (Dufe et al., 2005). The *speABCDE* genes encode several enzymes in *E. coli* that are related to the polyamine biosynthesis pathway. Above all, SpdS in *E. coli* accumulates by the expression of *SpeE* gene (Zhou et al., 2010). In contrast, yeast and mammalian cells possess both of the major aminopropyltransferases, SpdS and SpmS (Wu et al., 2007). Two aminopropyltransferases are present in *Arabidopsis thaliana* and expressed in various forms due to their

alternative splicing and relating to numerous genes (Sekula and Dauter, 2019).

In human, the general mechanisms of SpdS to form spermidine have been elucidated since 2007 (Wu et al., 2007). SpdS has a critical structural feature called the gate-keeping loop (Korolev et al., 2002; Guédez et al., 2019), which closes the entrance to the active site of SpdS when no substrate is present. The recognition of the substrate putrescine is also affected. Site-directed mutation analysis revealed that some residues in this loop are important for the substrate binding and assisting the stabilization of the active site (Lee et al., 2013).

To date, several SpdS structures of various species have been identified. However, the structural features of SpdS and its enzymatic reaction process in the fungal species have not been elucidated. The *Kluyveromyces lactis* is well known fungus that is genetically similar to *Saccharomyces cerevisiae*. In this study, we have expressed, purified, and crystallized the full-length protein, tagged with hexahistidine (His₆) at its N-terminus, to provide further specific structural features and molecular mechanism of K/SpdS.

RESULTS AND DISCUSSION

The genes encoding K/SpdS (NCBI ID: XP_451945) were amplified from *Kluyveromyces lactis* genomic DNA and subsequently cloned into the pET28a vector for N-terminal His₆-tagging (Table 1). K/SpdS was expressed using 4 L of culture medium of *E. coli* BL21 (DE3) Star. Following sonication, histidine-affinity chromatography on an Ni-NTA resin was performed. Following this, the eluted sample was loaded onto a gel filtration column for size-exclusion chromatography (SEC). In the SEC peak profiles, K/SpdS had a single and symmetric peak at an elution volume of 73.0 ml, which indicates a molecular

weight of approximately 66.0 kDa. As the calculated molecular weight of monomeric *K*SpdS is 33.2 kDa, this indicated that *K*SpdS exists as a dimer in solution (Figure 1A). The purified protein was subsequently concentrated to 40 mg/ml using ultrafiltration filters, which yielded a high purity of over 95% on SDS-PAGE analysis, which was sufficient to be used for sparse-matrix crystal screening (Figure 1B).

Initial protein crystal screening for *K*SpdS (10 mg/ml) was performed at 20°C using approximately 600 distinct sparse-matrix crystal screening solutions of the Wizard 1/2, Salt Rx 1/2, PEG/Ion, Index 1/2, Shotgun 1, and Crystal Screen 1/2 kits. To improve the crystals, additional screening using the Hampton Research additive and detergent screening kits was performed. Within 1 day, the plate-shaped crystals of *K*SpdS appeared, with the average crystal size being 200 × 50 × 50 μm (Figure 2). The best plate-shaped crystals were obtained in the presence of 9.2% (v/v) Tacsimate™ pH 5.0, 16.5% (w/v) PEG 3350, and 2.5% (v/v) 1-butanol at 20°C (Table 2).

The most promising crystals were selected from the optimized crystallization conditions for X-ray diffraction analysis. Cryo-protectants contained 30% glycerol to minimize radiation damage. The raw data from the X-ray diffraction for *K*SpdS were collected at a resolution of 1.9 Å (Figure 3). The peaks collected from several consecutive diffraction images were indexed and integrated using Scalepack in the HKL2000 software package. The integrated data was then processed to refine the electron density map by applying a scale factor. The unit cell of *K*SpdS crystals belonged to a primitive orthorhombic in the Bravais lattice, and its space group was $P2_12_12_1$, with the following unit cell parameters: $a = 65.252$, $b = 98.180$, $c = 102.134$ Å, and $\alpha = \beta = \gamma = 90^\circ$. Both the unit cell volume (V_M) and Matthews' coefficient were 654315.49 and 2.31 Å³/Da, respectively. This data indicates that there are two molecules in the asymmetric unit, containing a 46.74% solvent content (Matthews, 1968). The

dimeric state is in agreement with the SEC profile detailed in Figure 1A.

To interpret the electron density map and to obtain the phase information, molecular replacement was performed using an Auto MR software in the Phenix crystallographic software package (Adams et al., 2010). *Hs*SpdS (PDB ID: 2O06) was used for the initial search model. Currently, preliminary structure refinement and model building are in progress using the PHENIX.refine and Wincoot softwares (Emsley and Cowtan, 2004; Adams et al., 2010). The detailed crystallography information is summarized in Table 3.

FIGURE 1 | Size exclusion chromatography profile and SDS-PAGE analysis of spermidine synthase (SpdS) from *Kluyveromyces lactis* (*K*SpdS). The SEC profile and SDS-PAGE analysis of the N-terminal His₆-tagged *K*SpdS. SDS-PAGE demonstrating the purification process, including affinity chromatography and the SEC steps. The molecular weights of the standard size markers are shown on the left-hand side, and the test lanes are shown on the right-hand side. (A) The protein size of *K*SpdS is approximately 66 kDa. (B) In lanes 8-15, the calculated molecular weight was 33.2 kDa. Each lane is as follows: lane 1, protein size marker (low range); lane 2, cells induced with IPTG; lane 3, insoluble fraction; lane 4, soluble fraction; lane 5, unbound fraction from His-affinity chromatography; lanes 6 and 7, elution fractions from His-affinity chromatography with 20 mM and 250 mM imidazole, respectively; and lanes 8-15, SEC elution fractions. The size of the target protein is indicated by the black arrowhead.

TABLE 1 | Protein production information

Source organism	<i>Kluyveromyces lactis</i>
DNA source	Genomic DNA
Forward primer* (5'–3')	AAGGCATATGTCAGAAATTAACACATCCTA
Reverse primer* (5'–3')	AAGGCTCGAGTCACAGGTCCAATTCTTTAG
Cloning vector	pET28a
Expression host	<i>Escherichia coli</i> BL21 Star (DE3)
Complete amino acid sequence of the construct produced** (N–C)	MGSSHHHHHHSSGLVPRGSHMSELTHTPTVD GWFREISDTMWPGQAMTLRVEKILHHEKSKY QDVLVFKSTDYGNVLVDNAIQVTERDEFSYQ EMIAHLALNSHPNPKKVLVIGGGDGGVLREIV KHDSVQEAWLCDIDEAVIRVSKEYLPEMAKSY SHPKVKTHIGDGFQFLRDYQNTFDVIITDSSDP EGPAASLFQSSYFELLNGALTEKGIVSTQAES MWIHLPIKELKKACKEVFPTVGAYTTIPTTYPT GQIGFMVCSKDANVDVTKPLRSISEEEEEAKY RYYNKKVHEASFVLPTWAKELDL

*Underlined nucleotide sequence indicates restriction-enzyme sites. **Underlined amino acid sequence indicates an N-terminal Histidine tag.

FIGURE 2 | Crystals of the K/SpdS protein. The diffracted crystals were obtained from the condition of 9.2% (v/v) Tacsimate™ pH 5.0, 16.5% (w/v) PEG 3350, and 2.5% (v/v) 1-butanol.

TABLE 2 | Crystallization

Method	Vapor diffusion
Plate type for screening	96-well sitting-drop crystallization plate, Art Robbins Instruments
Plate type for optimization	24-well plate, Hyundai Micro
Temperature (°C)	20
Protein concentration (mg/ml)	10
Composition of protein solution	20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM DTT
Composition of reservoir solution	9.2% v/v Tacsimate™ pH 5.0, 16.5% (w/v) PEG 3350 and 2.5% (v/v) 1-butanol
Volume and ratio of drop	2 μ L, 1:1
Volume of reservoir (μ L)	500

METHODS

Preparation of K/SpdS expression construct

The gene encoding K/SpdS (NCBI ID: XP_451945) was amplified from *K. lactis* genomic DNA (Korean Collection for Type Cultures, Republic of Korea) using polymerase chain reaction (PCR). The amplified fragments were digested at 37°C for 4 h with *Nde*I and *Xho*I restriction enzymes (R006S and R007S, respectively; Enzynomics, Republic of Korea). The digested fragments were ligated with the pET28a vector using T4 ligase (M0202S; Roche, Germany) overnight at 18°C. Subsequently, the vector was transformed into the *E. coli* strain DH5 α using kanamycin (AppliChem, USA) as a selection marker. Transformants were confirmed by colony PCR. All oligonucleotide primers used in the study were purchased from Cosmogenetech, Inc (Republic of Korea), and the primer sequences are listed in Table 1.

Purification of recombinant proteins

The plasmids encoding the K/SpdS protein were transformed into *E. coli* strain BL21 (DE3) Star. Cells were cultured in Luria-Bertani medium containing 50 mg/L kanamycin (AppliChem,

FIGURE 3 | Representative diffraction pattern of a K/SpdS crystal. Diffraction image of a K/SpdS crystal. The black circles and bold texts represent the resolution ranges.

TABLE 3 | Data collection and processing

K/SpdS	
Diffraction source	Beamline 5C, PAL
Wavelength (Å)	0.9796
Temperature (°C)	–180
Detector	ADSC Quantum 270 CCD
Crystal-to-detector distance (mm)	270
Rotation range per image (°)	1
Total rotation range (°)	360
Exposure time per image(s)	1
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
a, b, c (Å)	65.252, 98.180, 102.134
α , β , γ (°)	90, 90, 90
Resolution range (Å)	50.0–1.9
Total no. of reflections	676985
No. of unique reflections	52396
Completeness (%)	100 (100) ^a
Multiplicity	12.9
<i>I</i> / σ <i>I</i>	33.0 (4.9)
<i>R</i> _{merge} (%) ^b	13.5 (45.2)
<i>CC</i> _{1/2}	0.996 (0.965)

^aThe numbers in parentheses are statistics from the highest resolution shell.
^b $R_{\text{merge}} = \sum |I_{\text{obs}} - I_{\text{avg}}| / I_{\text{obs}}$, where I_{obs} is the observed intensity of individual reflections and I_{avg} is averaged over symmetry equivalents.

USA) at 37°C to reach an optical density at 600 nm (OD_{600}) of approximately 0.6 (Ambrothia, Republic of Korea). Following induction with 0.3 mM isopropyl β -D-1-thiogalactopyranoside (IPTG; Calbiochem, Germany), the cells were further grown for an additional 16 h at 20°C. The cultured cells were harvested by centrifugation at 4000 rpm, 4°C for 20 min. The cell pellet was resuspended in a buffer containing 20 mM Tris (pH 8.0; Sigma-Aldrich, USA), 250 mM NaCl (AppliChem), 5% glycerol (Affymetrix, USA), 0.2% Triton X-100 (Sigma-Aldrich), 10 mM β -mercaptoethanol (BioBasic, Canada), and 0.2 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). Cells were disrupted by ultrasonication (VCX-500/750, Sonics, USA) with 3-s pulse-on and 3-s pulse-off cycles continuously for 15 min. Cell debris was removed by centrifugation at 13,000 rpm for 40 min, and the supernatant was bound to Ni-NTA agarose (Qiagen, Germany) at 7°C for 90 min. After washing with His-binding buffer (300 mM NaCl, 50 mM Tris, pH 8.0) that contained 5 mM imidazole (Sigma-Aldrich), bound proteins were eluted using His-elution buffer (200 mM NaCl, 50 mM Tris, pH 8.0) containing 250 mM imidazole (Sigma-Aldrich). The purified protein underwent SEC using a HiPrep 16/60 Sephacryl S-300 HR column (GE Healthcare, Canada), and the buffer eluent contained 20 mM Tris (pH 7.5), 150 mM NaCl, and 2 mM dithiothreitol (DTT; Calbiochem). Following SEC, proteins were stored at -80°C until required for the crystallization trials. The purity of the proteins was evaluated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 15% acrylamide gel, where a single band corresponding to the calculated molecular weight of the target protein was observed.

Crystallization and improvements

The crystallization trials were performed at 20°C using a sitting-drop vapor diffusion method in 96-well sitting-drop plates (Art Robbins Instruments, USA). Approximately 600 different conditions from sparse-matrix screening solution kits were tested to identify optimal crystallization conditions. The kits used included PEG/Ion (HR2-126 and -098), Index (HR2-144), Salt Rx 1/2 (HR2-107 and -109), and Crystal Screen 1/2 (HR2-110 and -112) from Hampton Research (USA), besides Wizard 1/2 (CS-311, Jena Bioscience, Germany) and SG1 Screen (MD1-88, Molecular Dimensions, UK). The crystals of K/SpdS grew within 1 day in drops containing equal volumes (1 μ L) of protein sample (10 mg/ml in 150 mM NaCl, 2 mM DTT, and 20 mM Tris, pH 7.5) and reservoir solution (9.2% v/v Tacsimate™ pH 5.0, 16.5% w/v PEG 3350). To improve the crystals, additional screening was performed using the additive (HR2-428, Hampton Research) and detergent (HR2-406, Hampton Research) screening kits. The optimized crystallization condition was 9.2% v/v Tacsimate™ pH 5.0, 16.5% (w/v) PEG 3350, and 2.5% (v/v) 1-butanol.

Data collection and processing

Prior to data collection, 30% glycerol was added to the reservoir solutions as a cryoprotectant, and the crystals were flash-cooled

in liquid nitrogen. The diffraction datasets were collected at 100 K on beamline 5C of the Pohang Accelerator Laboratory (PAL, Republic of Korea) using a Quantum 270 CCD detector (USA). Diffraction data were processed using the HKL-2000 software (HKL Research Inc, USA) suite. Experimental electron density maps were obtained by molecular replacement methods using Phenix software version 1.9 and interpreted using the WinCoot program (Phenix Software International, USA), and HsSpdS (PDB code 2O06) was used as the search model.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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