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# Cloning, *Escherichia coli* expression, purification, characterization, and enzyme assay of the ribosomal protein S4 from wheat seedlings (*Triticum vulgare*)

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ABSTRACT

S4 is a paradigm of ribosomal proteins involved in multifarious activities both within and outside the ribosome. For a detailed biochemical and structural investigations of eukaryotic S4, the wheat S4 gene has been cloned and expressed in *Escherichia coli*, and the protein purified to a high degree of homogeneity. The 285-residue recombinant protein containing an N-terminal $His_6$ tag along with fourteen additional residues derived from the cloning vector is characterized by a molecular mass of 31981.24 Da. The actual sequence of 265 amino acids having a molecular mass of 29931 Da completely defines the primary structure of wheat S4. Homology modeling shows a bi-lobed protein topology arising from folding of the polypeptide into two domains, consistent with the fold topology of prokaryotic S4. The purified protein is stable and folded since it can be reversibly unfolded in guanidinium hydrochloride, and is capable of hydrolyzing cysteine protease-specific peptide-based fluorescence substrates, including Ac-DEVD-AFC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin) and *Z*-FR-AMC (*N*-CBZ-Phe-Arg-aminomethylcoumarin).


## Introduction

One of the major objectives of ribosome research is to understand the functional roles of constituent proteins in the control and regulation of cellular protein synthesis. Studies of the past three decades however indicate that associating a particular translational activity with a specific ribosomal protein is not straightforward due to intricate protein–protein and protein–rRNA interactions in the ribosome. Ribosomal proteins often carry out extraribosomal functions [1–10], suggesting regulatory and signaling roles for them in various cellular functions. It is obvious that availability of highly pure preparations of ribosomal proteins will not only augment investigations of implicated functions, but also facilitate exploring structure–function relationships. Cloning and overexpression of a large number of ribosomal proteins of prokaryotic [11–15] and eukaryotic origins have been reported [16–23], although purification protocols of eukaryotic proteins have been described scarcely.

Here, we report on cloning, *Escherichia coli* overexpression, and complete purification of the wheat ribosomal protein S4 that has long been implicated in diverse functional activities. The protein S4 has attracted attention of many for a variety of its functional qualities. In general, it binds to rRNA [1,24,25] and plays a pivotal role in the core assembly of the smaller ribosomal subunit [26]. In *E. coli*, S4 has been shown to feedback-inhibit self-synthesis causing abrogation of translation of downstream ribosomal proteins coded by the same polycistronic mRNA [27,28]. Furthermore, S4 is an antitermination transcription factor with properties similar to NusA [29]. In humans, S4 deficiency has been associated with Turner syndrome [30,31]. In this perspective, we now find that wheat S4 is a general endoprotease capable of abrogating protein synthesis in cell-free translation systems by cleaving certain soluble factors the details of which are yet to investigate (submitted for publication). For detailed work in this direction, the S4 gene from *Triticum vulgare* has been cloned, and the overexpressed protein in *E. coli* purified to homogeneity. The recombinant protein hydrolyzes peptide based fluorogenic substrates specific for cathepsin B and L, and papain-like cysteine proteases.

## Materials and methods

### *cDNA synthesis, cloning, and generation of expression construct for wheat S4 protein*

Total RNA from 10-days old wheat seedlings was isolated using TRI Reagent (Sigma), chloroform, and isopropanol. The RNA was

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washed with 75% ethanol, dissolved in nuclease-free water, and checked for integrity by agarose gel electrophoresis. cDNA synthesis was performed using single-step cDNA synthesis kit (Abgene, USA) essentially by manufacturer's instructions. To a mixture of 25 μL 2× Master mix (Fermentas), 1 μL RT Blend enzyme, and 5 μL total RNA, 1 μL each of forward and reverse primers were added. The primers (10 picomolar each) designed by us and synthesised by Sigma were

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| *Nde1* |
| S4-F: 5′ GCCATATGGCGAGGGGTTTGAAGAA 3′ |
| S4-R: 5′ CCCTCGAGTCAGGCCTTGGCAGCTGCCT 3′ |
| *Xho1* |

where, the restriction sites are introduced to facilitate cloning into the pET28a vector, and the randomly added extra GC and CC nucleotides in the forward and reverse primers, respectively, provide support to the restriction enzymes. The total volume of the PCR reaction mixture was made up to 50 μL by adding nuclease-free water. The RT-PCR program involved an initial 30-s denaturation at 95 °C, 30-s annealing at 54 °C, and 1-min polymerization at 72 °C; to be repeated from step two for 25 cycles. Long polymerization was carried out for 10 min, and the final temperature was set at 4 °C. The amplified cDNA separated on 1% agarose gel was eluted by using the gel extraction kit from Qiagen. The purified cDNA was inserted into TA (pTZ57R/T) vector using bateriophage T4 DNA ligase (Fermentas) for 16 h at 22 °C. The cDNA was sequenced, translated into protein sequence using expasy tools, and compared by alignment with the original protein sequence using clustalW.

The TA plasmid was transformed into XL1 Blue *E. coli*, and grown on ampicilin-containing (100 $\mu g\,ml^{-1}$) LB-agar plate by streaking. To confirm the positive colonies, the recombinant vector-containing white colonies were subjected to colony PCR. The recombinant TA vector was subjected to restriction digestion between Nde1 and Xhol (Fermentas). The cleaved cDNA, isolated by elution of the agarose gel electrophoresis band, was re-inserted into pET28a (+) vector (Novagen) using bacteriophage T4 DNA ligase. The recombinant pET28a (+) vector was transformed into XL1 Blue *E. coli* cells, and grown on kanamycin (Calbiochem)-containing (50 $\mu g\,ml^{-1}$) LB-agar plate. The positive white colonies, confirmed by colony PCR, were inoculated into kanamycin-containing (50 $\mu g\,ml^{-1}$) LB medium at 37 °C. The plasmid was isolated from the overnight mini culture, and transformed into BL21 (DE3) RIL *E. coli*.

### *Protein Expression and Purification*

A starter culture was grown by inoculating a single colony from the petri plate containing pET28a (+) S4 vector in BL21 (DE3) RIL cells into 50 $\mu g\,ml^{-1}$ kanamycin-containing LB media. Growth was allowed overnight at 37 °C shaking the culture constantly at180 rpm. A secondary culture was made in 50 $\mu g\,ml^{-1}$ kanamycin-containing LB media from the starter culture in a 1:25 ratio. Cells were grown at 37 °C in LB media to a $A_{600}$ of 0.5, and protein expression was induced with 0.5 mM IPTG at 30 °C. Following two hours of post-induction growth under constant shaking at 180 rpm cells were harvested by centrifuging the culture at 10,000 rpm for 10 min. The cell pellet (∼1.89 g wet weight per liter of culture) was washed with PBS, and resuspended in 1:5 W/V buffer A (50 mM Tris, 0.2 M NaCl, 5 mM β-mercaptoethanol, 5 mM imidazole, 1% glycerol, pH 8.0, 5 °C), and stirred for 15 min at ∼5 °C. Cells were disrupted by pulsed sonication, and the lysate was spun at 15000 rpm for 15 min. The soluble fraction was loaded onto a Ni–NTA-His bind column (Novagen) equilibrated with buffer A at 5 °C. The column was washed with 300 mL of buffer B (50 mM Tris, 0.2 M NaCl, 50 mM imidazole, pH 8.0, 5 °C) until the $A_{280}$ reached 0.001. The second wash was done with 50 mL of buffer C (50 mM Tris, pH 8.0, 5 °C) and the protein was eluted with buffer D (150 mM imidazole in 50 mM Tris, pH 8.0, 5 °C). The protein-containing fractions were pooled and dialyzed exhaustively against 20 mM HEPES, pH 7.0 or 50 mM Tris, pH 8.0. Protein concentration was estimated by Lowry and Bradford methods, and purity was checked by 10% to 15% SDS/PAGE gel electrophoresis. Often the purified protein is checked further by anti His-tag probing of the SDS/PAGE-separated sample transferred onto a PVDF membrane.

### *Estimation of extinction coefficient of the native protein*

A highly homogeneous S4 preparation, initially in buffer D, was extensively dialyzed against 3 mM Tris buffer at pH 7.4 so as to minimize the concentration of buffer and solvent components that often reduce the accuracy of Lowry's method. The protein concentration and corresponding UV spectra were used to determine the extinction coefficient. The reported value is the average of at least three independent determinations using different preparations.

### *Mass spectrometry and homology sequence analysis*

For MALDI *m*/*z* spectra, 2 μL of the purified protein solution (∼21 μg) was mixed with 2 μL of 2% TFA and 2 μL of the matrix solution (2,5-dihydroxyacetophenone with 10 mM di-ammonium citrate). Mass measurements were performed on a Autoflex III mass spectrometer (Bruker Daltonics) in the positive linear mode of operation. About 1000 single spectra were added to a sum spectrum. Masses of tryptic peptides subjected to MS–MS analysis were used in Mascot search program (Matrix Science) for protein identification from primary sequence databases. Homology sequence analysis was performed by BLAST search.

### *Fluorescence measurement of guanidinium hydrochloride (GdnHCl)-induced equilibrium unfolding*

Samples for equilibrium unfolding were prepared by mixing appropriate volumes of stock solutions of native and unfolded protein prepared in 20 mM HEPES, pH 7. The former contained no denaturant and the latter was 6 M in GdnHCl. The initial volumes of the two stock solutions were identical and they were sufficiently uniform in S4 content. Therefore, samples prepared by mixing these two stocks were identical in terms of protein content (∼1 μM). This procedure of samples preparation also provides a test for reversibility of the folding-unfolding equilibrium. The samples were incubated for 5 h at 23 °C, and fluorescence spectra in the 300–390 nm region were measured at the same temperature by 280-nm excitation.

### *Assay for S4 enzyme activity by cleavage of peptide-based substrates*

Hydrolysis of two peptide substrates, Ac-DEVD-AFC or Ac-LEHD-AFC (*N*-acetyl-XEXD-7-amino-4-trifluoromethylcoumarin) and *Z*-FR-AMC (*N*-CBZ-Phe-Arg-aminomethylcoumarin), were used for enzyme assay. The S4 solution in 50 mM Tris, pH 7 or 20 mM HEPES, pH 7, contained in the fluorescence cuvette and equilibrated at 37 °C in the cell holder of the fluorometer, was combined with a very small volume of Ac-XEXD-AFC dissolved in DMSO and mixed manually. *Z*-FR-AMC hydrolysis assay was set up identically, and the buffer was 20 mM HEPES, pH 7. Time-base fluorescence emission due to the released AFC fluorophore was recorded in a FluoroMax 3 or Fluoromax 4P instrument (Jobin–Yvon). Typical dead-time of these measurements is ∼10–15 s. Excitation wavelengths were 400 and 360 nm for probing hydrolysis of AFC and AMC, and the corresponding emission wavelengths were 490 and 460 nm.