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Expression and Functional Analysis of a Predicted AtsG Arylsulphatase Identified from *Mycobacterium tuberculosis* Genomic Data

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Sulphatase family enzymes hydrolyse the sulphate ester, found on the pathogens cell surface and playing an important role for host–pathogen interaction. The AtsG, homologue of arylsulphatase, predicted in the *Mycobacterium tuberculosis* genomic data, was successfully expressed in *Escherichia coli*. The recombinant AtsG protein exhibited hydrolysis of *para*-nitrophenyl sulphate and *para*-nitrocatechol sulphate, and binding affinity to the heparin–sepharose resin. This is the first report of molecular evidence for an arylsulphatase activity of the AtsG protein. The maximum activity was detected at pH 8.0 and 37°C. As EDTA completely inhibited this activity, a divalent cation was required for the activity.

Key words: arylsulphatase, heparin–sepharose resin binding affinity, *Mycobacterium tuberculosis*, *para*-nitrocatechol sulphate, *para*-nitrophenyl sulphate.

Abbreviations: IPTG, isopropyl-1-thio-β-D-galactopyranoside; ORF, open reading frame; pNP, *para*-nitrophenol; pNC, *para*-nitrocatechol; pNPS, *para*-nitrophenyl sulphate; pNCS, *para*-nitrocatechol sulphate.

It is well known that conjugation and removal of sulphate to and from the primary and secondary metabolites can radically modify their biological properties. Naturally sulphated molecules serve as extracellular traffic and modulators of cell–cell interactions (*1*), and sulphate esters play roles to protect the host (*2*). For mycobacteria, it is known that sulphated compounds are produced and that some molecules correlate to *Mycobacterium tuberculosis* virulence (*3*, *4*). In *M. tuberculosis*, *M. bovis* BCG, *M. avium* and *M. leprae*, some sulphated molecules bound to hemagglutinin provide affinity for heparin sulphate on the epithelial cells, and this affinity promotes mycobacterial adherence to the host epithelial cells prior to infection (*5*, *6*).

Conjugation and removal of the sulphate group, which plays an important role in biological processes, are regulated by two large sulphate metabolic enzyme families, sulphotransferase catalysing the biosynthesis of sulphate ester compounds, and sulphatase catalysing the hydrolysis of sulphate esters (or *N*-sulphates) to an alcohol (or amine) and free sulphate. Therefore, it can be considered that sulphotransferases and sulphatases regulate the balance of sulphate modification in components of most biological processes.

The activity of arylsulphatase (arylsulphate sulphohydrolase, EC 3.1.6.1), a subgroup within the sulphatase family, was detected in the cell lysate of *M. avium, M. smegmatis, M. marinum*, and *M. tuberculosis* (*7*, *8*). In the *M. tuberculosis* genome, six ORFs, Rv0711 (AtsA), Rv3299c (AtsB), Rv0663 (AtsD), Rv3076 (AtsF), Rv0296c (AtsG) and Rv3796 (AtsH), were identified as sulphatase homologues through similarity search. Among these, only the AtsG showed significant similarity to the eukaryotic sulphatase, i.e. 24% identity with the murine sulphamidase (heparan *N*-sulphatase, EC 3.10.1.1), which catalyses the hydrolysis reaction of *N*-sulphate ester linkage from sulphated glycosaminoglycans, heparin sulphate, and heparan sulphate (*1*). The AtsG homologues are also identified from *M. smegmatis*, *M. avium*, *M. leprae*, *Pseudomonas aeruginosa*, *Rhizobium meliloti and Mus musculus*. Despite the widespread distribution of the AtsG homologues from microorganisms to eukaryotes, their functions, actual physiological substrates, and biological roles remain undefined. Therefore, this ORF was chosen as a target for heterologous expression and functional analyses. This study showed that the recombinant AtsG protein produced from the ORF Rv0296c of *M. tuberculosis* H37Rv possesses arylsulphatase activity.

For construction of AtsG expression vector, the AtsG region of *M. tuberculosis* H37Rv was amplified with two primers, a 5′-primer, 5′-GGGAATTCCATATGGTGACGAGTGAGCGTGCCACA-3′, and a 3′-primer, 5′-CCCAAGCTTCTAGCTGCAGTGTTCGTCGATGCCGC-3′ (*Nde*I and *Hind*III sites are shown by underline), which were designed from the nucleotide sequences of the 5′ and 3′ regions of the ORF Rv0296c. The *Nde*I and *Hind*III digested PCR fragment was cloned into plasmid vector pET-15b (Novagen, Darmstadt, Germany) and sequence was confirmed for any mutation. The recombinant AtsG protein with His-tag at the N-terminus was expressed in

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E. coli strain BL21(DE3) codon plus RIL cells and purified by affinity chromatography with His-Bind Resin (Novagen, Madison, WI, USA) followed by gel filtration with a Superdex 200 column (Hiload 16/60). The recombinant AtsG protein was successfully expressed and purified to homogeneity and the apparent molecular mass, detected by 8% sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE), was ~52 kDa. Approximately 40 mg of pure recombinant AtsG protein was obtained from 1 l of *E. coli* culture and was used for following analyses of arylsulphatase activity and heparin–sepharose resin binding affinity.

Arylsulphatase activity was monitored according to the method described by Kim *et al*. (*9*) with some modification. A 50 μl reaction mixture containing 100 mM Tris–HCl (pH 8.0), 1 mM $MgCl_2$, 10 mM *para*-nitrophenyl sulphate (pNPS) or *para*-nitrocatechol sulphate (pNCS), and 40 μg of the recombinant AtsG protein was incubated for 15 h at 37°C. After addition of 50 μl of 1 N NaOH for termination of the reaction, the amount of products, *para*-nitrophenol (pNP) and *para*-nitrocatechol (pNC), were measured at 414 and 515 nm, respectively, using the spectrophotometer ImmunoMini NJ-2300 (Inter Med Co., Tokyo, Japan). pNP or pNC production was not detected in absence of the recombinant AtsG protein. However, when the recombinant AtsG protein was added to the reaction mixture, the enzymatic dose-dependent production of pNP (open circles, Fig. 1) and pNC (open triangles, Fig. 1) was observed. These results indicated that arylsulphatase activity is present on the recombinant AtsG protein. The production rate of pNP and pNC are 1 and 0.25 μmole/μg of AtsG/15 h, respectively.

Kim *et al*. (*10*) showed that arylsulphatase has a binding affinity for heparin. Heparin–sepharose, which mimics the natural substrate of arylsulphatase, is usually used to assess the binding affinity of arylsulphatase to heparin. To determine the heparin–sepharose resin binding affinity, the recombinant AtsG protein was applied to heparin–sepharose CL-6B resin (Pharmacia, Uppsala, Sweden) column pre-equilibrated with the buffer containing 50 mM Tris–HCl (pH 7.5) and 50 mM NaCl. After washing with equilibration buffer, the bound protein was eluted with the elution buffer containing 50 mM Tris–HCl (pH 7.5) and 100, 200, 300, 400, 500, 600, 700, 800, 900 or 1000 mM NaCl. As shown in Fig. 2, the recombinant AtsG protein was eluted at the fractions in the presence of 200–500 mM NaCl, with the maximum elution at 300 mM NaCl while no AtsG protein was detected in the flow-through and wash fractions. This result indicates that the recombinant AtsG protein has binding affinity to heparin–sepharose resin and the bound protein was released from the resin at around 300 mM of NaCl. Arylsulphatase from *Pseudoalteromonas carrageenovora* and heparin sulphamidase from bovine testis are released from the heparin–sepharose resin at ~200–400 mM of NaCl (*10*,*11*). The similarity of NaCl concentration for binding onto the heparin–sepharose resin between the known arylsulphatases and the recombinant AtsG protein indicates that the recombinant AtsG protein is very close to the known arylsulphatase.

These two results, hydrolysis of arylsulphated compounds and binding affinity to the heparin–sepharose resin, indicate that the recombinant AtsG protein is actually an arylsulphatase.

To clarify the detailed feature of the recombinant AtsG protein, the effect of different pH on the AtsG enzymatic activity was measured at 37°C in the following buffers: 100 mM sodium acetate (pH 4.0, 4.5, 5.0 and 5.5), 100 mM 2-(N-morpholino) ethanesulphonic acid (pH 6.0 and 6.5), 100 mM Tris–HCl (pH 7.0, 7.5, 8.0, 8.5 and 9.0) or 100 mM Na_2CO_3/$NaHCO_3$ (pH 9.5 and 10.0). The arylsulphatase activity of AtsG protein was pH sensitive and was detected only between pH 7.5 and 8.5, with the maximum activity at pH 8.0.

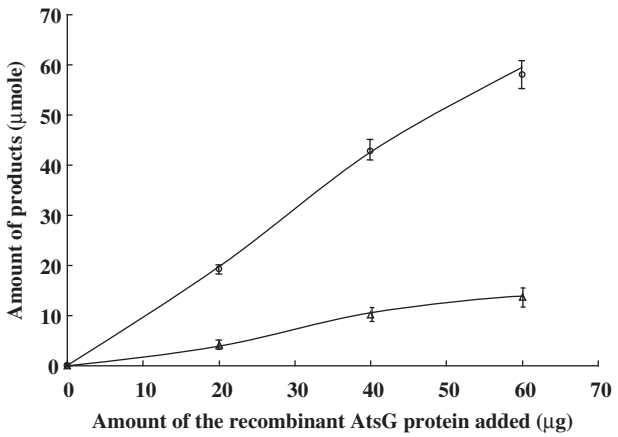

Fig. 1. **Production of pNP and pNC depending on the amount of the recombinant AtsG protein added into the reaction mixture.** The arylsulphatase reaction was carried out with the indicated amount of the recombinant AtsG protein at 37°C for 15 h. Amount of pNP and pNC produced are shown by open circles and open triangles, respectively. These experiments were triplicated.

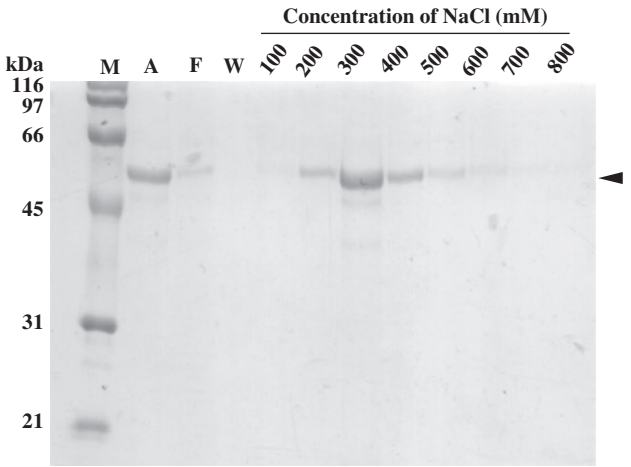

Fig. 2. **SDS–PAGE pattern of the recombinant AtsG protein eluted from the heparin–sepharose resin.** The recombinant AtsG protein was analysed on a polyacrylamide gel containing 0.1% SDS. Lane A, the recombinant AtsG protein used for the heparin–sepharose resin affinity analysis. Lane F, the flow-through fraction. Lane W, the wash fraction. Lanes 100–800, the recombinant AtsG protein eluted from the heparin–sepharose resin by elution solution containing the indicated concentration of NaCl.

The temperature effect was measured in the reaction mixture with 100 mM Tris–HCl (pH 8.0) at 20, 25, 30, 37, 40, 45, 50, 55, 60, 65, 70 and 80°C. The arylsulphatase activity of the recombinant AtsG protein was detected between 20 and 40°C, with the maximum activity at 37°C. No detection of the arylsulphatase activity at or over 45°C indicates that the recombinant AtsG protein is unstable at 45°C and pre-incubation of the recombinant AtsG protein at 55°C for 10 min leads ~60% loss of its maximum activity, revealing that activity loss by the temperature rapidly occurred in the protein.

To analyse the effect of ethylenediaminetetraacetic acid (EDTA), the reaction mixtures with the addition of different concentrations (0.1, 0.5, 1 and 5 mM) of EDTA were used. The presence of EDTA in the reaction mixture significantly inhibits the arylsulphatase activity of the recombinant AtsG protein and no activity was detected in the presence of 1 mM EDTA. Inhibition of the arylsulphatase activity by EDTA implies that the AtsG protein requires a divalent cation for its activity. Similar inhibition by EDTA was also reported for the recombinant arylsulphatase cloned from *P. carrageenovora* (*10*). Furthermore, this result is in agreement with the fact that in the crystal structure of human arylsulphatase A, Mg^{2+} was detected at the active site of the protein (*12*).

Although actual arylsulphatase activity was detected on the recombinant AtsG protein, but the detected activity was found relatively very low compared to that of the known arylsulphatases. However, all the amino acid residues predicted to be important for this activity were conserved in the AtsG protein, including the (C/S)XPXR motif sequences. The first cysteine or serine residue of this motif is predicted to undergo post- or co-translational modification, conversion to formylglycine by oxidation (*13*,*14*). The lack of this correct post- or co-translational modification during heterologous expression of the recombinant AtsG protein in *E. coli* is thought to be one of the reasons for very low activity of the recombinant AtsG protein.

The present study is the first to detect the actual arylsulphatase activity on the recombinant *M. tuberculosis* protein expressed in *E. coli*. The actual *in vivo* role of the AtsG protein remains unclear, but the success to detect the arylsulphatase activity on the recombinant AtsG protein can serve as a powerful means for the recognition of the *in vivo* role of this protein.

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CONFLICT OF INTEREST

None declared.

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