RESEARCH ARTICLE

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EndoSd: an IgG glycan hydrolyzing enzyme in *Streptococcus dysgalactiae* subspecies *dysgalactiae*

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Aim: The aim of this study was to identify and characterize EndoS-like enzymes in *Streptococcus dysgalactiae* subspecies *dysgalactiae* (SDSD). **Materials & methods:** PCR, DNA sequencing, recombinant protein expression, lectin blot, ultra high performance liquid chromatography analysis and a chitinase assay were used to identify *ndoS*-like genes and characterize EndoSd. **Results:** EndoSd were found in four SDSD strains. EndoSd hydrolyzes the chitobiose core of the glycan on IgG. The amino acid sequence of EndoSd is 70% identical to EndoS in *S. pyogenes*, but it has a unique C-terminal sequence. EndoSd secretion is influenced by the carbohydrate composition of the growth medium. **Conclusion:** Our findings indicate that IgG glycan hydrolyzing activity is present in SDSD, and that the activity can be attributed to the here identified enzyme EndoSd.

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Glycoproteins are involved in many biological processes, and are of fundamental importance for innate and adaptive immunity. Carbohydrate chains, or glycans, are covalently attached to specific amino acid side chains of glycoproteins. IgG is the most abundant immunoglobulin in blood and is one of the most important factors in defending the body against invading pathogens [1–3]. IgG molecules consist of two heavy chains and two light chains. The CH2 domain in the Fc region of IgG contains an *N*-linked glycan attached to asparagine 297 [4]. The stability of Fc region of IgG and interaction with Fc receptors (FcRs) is dependent on the *N*-linked glycan [5]. The *N*-linked glycan preorganizes the FcγRIIIa interface for optimal binding affinity, so the glycan plays an essential role in binding of IgG to FcRs [5–7]. Absence of the Fc-glycan in the CH2 domain of IgG diminishes binding to FcRs on leukocytes and reduces complement activation [8].

Streptococcus pyogenes, or group A *Streptococcus* (GAS) causes a broad range of diseases, such as uncomplicated pharyngitis and skin infection. It is also the cause of more severe infections, such as necrotizing fasciitis and toxic shock syndrome, as well as the noninfectious sequelae acute rheumatic fever, rheumatic heart disease, poststreptococcal glomerulonephritis, together resulting in more than 500,000 deaths worldwide each year [9]. This important human pathogen has the ability to hydrolyze the *N*-linked glycan on IgG by the secreted enzyme EndoS [10,11]. EndoS is a 108-kDa protein belonging to GH18 [12]. It is an endo-β-*N*-acetylglucosaminidase that cleaves the β1–4 linkage between the two *N*-acetylglucosamines (GlcNAcs) in the chitobiose core of the *N*-linked glycan of IgG [13,14].

KEYWORDS

- asparagine linked
- chitinases • Endo-β-*N*-acetylglucosaminidase
- glycosidase • IgG
- *Streptococcus dysgalactiae*

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It exhibits a specificity for biantennary complex type *N*-glycans and does neither cleave larger complex type glycans nor oligomannose and nor hybrid-type glycans to any major extent [15,16]. Protein–protein interactions are important for the activity of EndoS on glycans since it hydrolyzes the *N*-linked glycan only on native but not denatured IgG [17]. EndoS is a multidomain protein containing an N-terminal GH18 chitinase domain, a leucine-rich repeat domain, a hybrid Ig domain, a carbohydrate binding module and a three-helix bundle [10]. Systematic analysis of truncated human IgG and EndoS revealed that EndoS is active on isolated CH2 domains of IgG, and that C-terminally truncated EndoS is still enzymatically active against IgG glycans but at a slower rate [18].

The loss of the IgG glycan affects the binding of the Fc part of IgG to FcRs and diminishes classical complement activation [19,20]. EndoS has, therefore, been suggested to be involved in evasion of IgG mediated immune responses, but its role during human infection is still under investigation [20].

Furthermore, EndoS outside of its infectious context has been put forward as promising new type of therapeutic agent against antibody mediated autoimmunity in animal models [21,22]. EndoS is also used as a tool to analyze and/or modify glycans on antibodies [15,23–24]. In addition, EndoS has been shown to selectively deactivate serum IgG, thus allowing an EndoS-resistant engineered oligomannose monoclonal antibody to interact better with FcRs [7].

EndoS is conserved in all analyzed *S. pyogenes* serotypes except serotype M49 strains that have

a different endoglycosidase gene, *ndoS2*, in the same genomic context as *ndoS* in the other serotypes. The *ndoS2* gene also encodes an endo- β -*N*-acetylglucosaminidase, denoted EndoS2 with activity on all glycoforms of IgG, including oligomannose hybrid and bisected type glycans [11,16].

S. dysgalactiae primarily belongs to Lancefield groups C and G streptococci (GCS, GGS) and shares many structural and biochemical characteristics with *S. pyogenes* [25]. Accumulating evidence indicate that *S. dysgalactiae* can be further divided into two distinct subspecies: the β -hemolytic *S. dysgalactiae* subsp. *equisimilis* (SDSE) and the α -hemolytic *S. dysgalactiae* subsp. *dysgalactiae* (SDSD), and this has recently been confirmed by multilocus sequence analysis (MLSA) of isolates combined with phenotypic characterization [26,27]. SDSE causes a diversity of diseases in humans similar to those caused by *S. pyogenes* while SDSD infects a number of different mammalian species [28]. Prevention of diseases caused by SDSD is of commercial interest, since it causes bovine mastitis, which results in economical losses in the dairy industry [29]. Some virulence factor genes found in *S. pyogenes* are also present in SDSE strains, including the genes encoding streptokinase and streptolysin O [30].

Rato *et al.* used a microarray containing 220 virulence factor genes to compare the presence of virulence factor genes in α -hemolytic SDSD isolates associated with bovine mastitis and β -hemolytic SDSE isolated from human disease. They demonstrated that all isolates shared a fraction of these genes. Most of the bovine and human isolates carry the virulence

Table 1. List of bacteria and plasmids included in this study.				
SDSD/SDSE strains or plasmids	Characteristics	Ref/source	Lancefield antigen	Putative glycosidase
SDSD strains				
SK1226	<i>S. dysgalactiae</i> subsp. <i>dysgalactiae</i>	Horse	C	EndoSd
SK1238	<i>S. dysgalactiae</i> subsp. <i>dysgalactiae</i>	Dog	L	EndoSd
SK1240	<i>S. dysgalactiae</i> subsp. <i>dysgalactiae</i>	Pig	C	EndoSd
SK1241	<i>S. dysgalactiae</i> subsp. <i>dysgalactiae</i>	Horse	C	EndoSd
SK1242	<i>S. dysgalactiae</i> subsp. <i>dysgalactiae</i>	Dog	C	EndoS2d
SK1244	<i>S. dysgalactiae</i> subsp. <i>dysgalactiae</i>	Dog	C	EndoS2d
Plasmids				
pGEX-5X-3	GST fusion vector	GE Healthcare	N/A	N/A
pGEX-5X-3-ndoSd	Recombinant EndoSd clone	This study	N/A	N/A
pGEX-5X-3-ndoSd tail	Recombinant EndoSd tail clone	This study	N/A	N/A
pGEX-5X-3-ndoSd without tail	Recombinant EndoSd without tail clone	This study	N/A	N/A

Table 2. Oligonucleotide primers used in this study.

Primer name	Sequence 5' → 3'
ndoS/C-Forward	TGTGTTTGTGCTGCAACGTTGAT
ndoS/C-Reverse	GTAAGCCTTATAGTCTGCTTCGCTATTGAT
scrA-Forward	AAATTGTCCTTTGAAACAGGTCATGC
scrK-Reverse	GACGACGGATCAATATCAATCGGC
ndoX-Forward	AAAACGCGGACCACTATATGC
ndoX-Reverse	AAACGTTGTCCGAGGATTG
ndoS-For-BamHI	TCTGGGATCCCGGAGGAGAAGATAACTCA
ndoS-Rev-XhoI	TTATATCTCGAGCTACTTAACAAAGGACTTTACTGT
1241-Tail-For-BamHI	TAGCCTATGGGATCCATAAAGAACTGCC
1241-No-Tail.Rev.XhoI	AAGGCAGTTTCTTTATCTCGAGCATAGGCTAA

genes encoding streptolysin S, the plasminogen-binding M-like protein PAM and a collagen-like protein. Some of the *S. pyogenes* bacteriophage-associated virulence genes, such as genes encoding DNases, were detected in most of the bovine isolates. Although SDSD is an animal pathogen and rarely infects humans, they carry a number of virulence genes that have been suggested to be responsible for pathogenesis in humans [29].

The reported presence of homologs of *S. pyogenes* virulence genes in SDSD isolates, motivated us to search for the presence of homologs to EndoS in *S. dysgalactiae* subsp. *dysgalactiae*.

Materials & methods

• Bacterial isolates

A total of eight SDSD strains were analyzed in this study and six SDSD strains were chosen for further characterization of EndoS. Among these six strains, SK1242, SK1244 and SK1238 were isolates from dogs, SK1241 and SK1226 from horses and SK1240 from a pig (Table 1). These six strains belong to the same phylogenetic group, based on MLSA combined with phenotypic characterization [26].

• Bacterial growth conditions

All the strains of SDSD were cultured on blood agar plates overnight and then propagated in C-medium (0.5% Proteose Peptone, 1.5% w/v yeast extract, 10 mM K₂ PO₄, 0.4 mM MgSO₄ and 17 mM NaCl, pH 7.5) at 37°C in a 5% CO₂ atmosphere. Genomic DNA was extracted using innuPREP Bacteria DNA Kit (Analytikjena Biometra, Göttingen, Germany). Chemically competent *Escherichia coli* (*E. coli*) strains Top10 (Invitrogen, Hämeenlinna, Finland) and BL21 pLysS (Life Technologies, CA, USA) were propagated on lysogeny broth (LB) agar and used for cloning and recombinant expression. 100-μg ml⁻¹

carbenicillin was added to medium for selection in *E. coli* Top10 cells and 100-μg ml⁻¹ carbenicillin and 34-μg ml⁻¹ chloramphenicol were added for *E. coli* BL21 pLysE. All strains of *E. coli* were cultured overnight in LB at 37°C with aeration. Transformation of chemically competent *E. coli* was performed using heat shock at 42°C for 30 s followed by phenotypic expression and culturing on selective solidified LB.

• Sequencing of *ndoSd* and *ndoS2d* & phylogenetic analysis

The *ndoSd* gene was amplified from SDSD by PCR using internal primers ndoS/C-Forward, ndoS/C-Reverse and flanking primers scrA-Forward, scrK-Reverse in all of the strains. For *ndoS2d* gene ndoX-Forward and ndoX-Reverse primers were used (Table 2). Sequencing was performed using the Lightrun Sanger sequencing service at GATC Biotech (Konstanz, Germany). Primer design, sequence alignment and phylogenetic analyses were performed using the MacVector software (Macvector, NC, USA). The EndoSd sequence of SDSD SK1241 was used to search for similar endo-β-N-acetylglucosaminidases in the protein database at NCBI. ClustalW was used to align the protein sequences. Based on the protein alignment, a reconstruction of phylogenetic trees was performed using the neighbor-joining method, ignoring gaps and with uncorrected p-values. Bootstrapping with 1000 replications was used to validate the tree, and a consensus tree was generated.

For mapping of sequence conservation in EndoS-like proteins, we created an ClustalW alignment of our newly identified EndoSd and EndoS2d sequences together with 4 *S. pyogenes* EndoS sequences (AP1, MGAS315, MGAS6180 and MGAS9429), one *S. pyogenes* EndoS2

(NZ131), as well as one *S. equi* EndoSe sequence. Sequence conservation was calculated and mapped onto the crystal structure of EndoS (Protein Data Bank [PDB]: 4NUY) [10] using ConSurf [31,32].

• Recombinant expression of EndoSd

Recombinant *E. coli* expression and purification of EndoSd was carried out as follows. The coding sequence of *ndoSd* gene was PCR amplified from chromosomal DNA of SDS 1241 strain using the *ndoS*-For-BamHI and *ndoS*-Rev-XhoI primer (Table 2) with purified chromosomal DNA as a template. The 3205-bp *ndoSd* PCR product was digested with restriction enzymes BamHI and XhoI (Thermo Fisher Scientific, NY, USA), separated on 1% TAE-agarose gel followed by gel purification of excised fragments using NucleoSpin Gel and PCR Clean-up kit (Macherey-NAGEL GmbH, Düren, Germany). Purified *ndoSd* PCR product was ligated into BamHI and XhoI digested expression vector pGEX-5X-3 (GE Healthcare, Little Chalfont, UK) using T4 DNA ligase (Thermo Fisher Scientific) generating the pGEX-*ndoSd* plasmid (Table 1). Generated plasmid was transformed into *E. coli* Top10 chemically competent cells (Life Technologies). Colony PCR using *ndoS*-For-BamHI primer and *ndoS*-Rev-XhoI primer was used to identify plasmid with correct insert for expression of the protein. The pGEX-5X-3 with correct insert was used to transform *E. coli* BL21 generating the strain BL21(DE3) pLysE/pGEX-*ndoSd*. The transformed cells were grown at 37°C using LB medium supplemented with carbenicillin (100 µg ml⁻¹) and chloramphenicol (34 µg ml⁻¹) until the culture reached an optical density of 0.6 at 620 nm. Recombinant protein was produced by induction with 0.1-mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) (VWR International, Radnor, PA, USA) for 2 h. Cells were harvested by centrifugation and lysed using BugBuster Protein Extraction Reagent (Novagen/Merck, Darmstadt, Germany). GST-EndoSd was purified on a Glutathione Sepharose 4B column (GE Healthcare) and eluted with reduced glutathione. For amplification of the tail encoding part of the *ndoSd* gene, the 1241-Tail-For-BamHI and *ndoS*-Rev-XhoI primers were used, and amplification of *ndoSd* without tail was carried out by using *ndoS*-For-BamHI primer and 1241-No-Tail. Rev. XhoI primer (Table 2). The tail of EndoSd and EndoSd without tail were expressed in *E. coli* and purified as described above.

• Analysis of EndoSd expression

Strain SK1241 was selected as a reference strain in this experiment and was cultured in Todd Hewitt broth (THY) (Becton Dickinson, Le Pont-de-Claix, France) at 37°C with 5% CO₂ overnight. Bacterial pellets were washed with PBS twice and bacteria were diluted 1:50 in five separate tubes of chemically defined medium (CDM) [33] containing 20 µM of the cysteine protease inhibitor E-64. Glucose, galactose, sucrose, acetylglucosamine and mannose were added independently to the CDM tubes at the concentration of 0.01%. Bacteria were grown for 16 h at 37°C with 5% CO₂. Supernatants were concentrated using precipitation with 0.3-mM trichloroacetic acid (TCA) and resuspended in SDS-PAGE loading buffer and loaded onto a 10% SDS-PAGE Stain-Free gel (Bio-Rad Laboratories, CA, USA). The electrophoresis was performed at 200V for 40 min. PVDF membrane (Bio-Rad Laboratories) was used for blotting according to the manufacturer's instructions using a Trans-blot Turbo equipment (Bio-Rad Laboratories). Membrane was blocked with 5% skim milk in PBST (PBS with 0.05% Tween 20) for 20 min at room temperature and incubated with 10-µl polyclonal rabbit antiserum raised against recombinant EndoSd (Davids Biotechnologie GmbH, Germany) for 45 min at 37°C. After washing the membrane in PBST, it was incubated with 2.5 as Alexa flour 488 goat anti-rabbit IgG (H+L) (Life technologies) for 30 min at 37°C. Blot was visualized using a Chemidoc XRS (Bio-Rad Laboratories). Antiserum against recombinant EndoSd (without GST tag) was obtained using standard protocols by Davids Biotechnologie. Removal of GST tag from EndoSd was performed using Factor Xa according to the manufacturer instructions (New England BioLabs, CA, USA).

• Liquid chromatography–tandem mass spectrometry analysis

Strain SK1241 was cultured in CDM containing 20-µl cysteine protease inhibitor E-64 and 0.01% sucrose for 16 h at 37°C with 5% CO₂ [33]. Bacterial supernatant was concentrated using 10 kDa MWCO (molecular-mass cutoff) spin column (Sartorius stedim biotech, Göttingen Germany) and resuspended to SDS-PAGE loading buffer and loaded on to a 10% Mini-Protean TGX Stain-Free gel (Bio-Rad Laboratories). 120-kDa band was cut from the gel, reduced by TCEP (Tris [2-carboxyethyl] phosphine)

(Sigma-Aldrich, MO, USA), alkylated with Iodoacetamide (Sigma-Aldrich) and digested with 100-ng Trypsin (Promega, WI, USA) and analyzed by mass spectrometry. Peptides were analyzed on a Q-Exactive plus mass spectrometer with an EasySpray electrospray ion source that was connected to an Easy-nLC 1000 system equipped with an EasySpray ES802 column (Thermo Scientific). MS/MS spectra were acquired for the 15 most abundant precursor ions each cycle using the following parameters: 400–1600 m/z , 70,000 resolving power at 200 m/z , (17,500 resolving power at 200 m/z for fragmentation). The raw data were searched with the Trans Proteomic Pipeline v4.7 [34] against a decoyed database containing all *S. dysgalactiae* sequences present in the Uniprot database, as well as the newly determined EndoSd sequences from this study. The false discovery rate was set to 1%.

• IgG glycan hydrolysis assay

For IgG glycan hydrolysis assay, 1 μg of recombinant EndoSd from strain SK1241 was incubated with 10 μg of human IgG (Calbiochem/Merck, Darmstadt, Germany) and also all subclasses of human IgG (Sigma-Aldrich) independently in PBS at 37°C for 2 h. In addition, 10- μg equine, canine and bovine IgG (Sigma-Aldrich) in PBS was incubated with the EndoSd at a ratio of 1:10, at 37°C for 2 h. The tail and EndoSd without tail were also incubated with human IgG. As a positive control PNGase F (New England Biolabs) was incubated with IgG as well. All reactions were analyzed by 10% Stain-Free SDS-PAGE (Bio-Rad Laboratories). Proteins were transferred to a PVDF membrane using a Trans-Blot Turbo (Bio-Rad Laboratories). For all samples lectin blot analysis was performed by incubating membrane with 5 $\mu\text{g ml}^{-1}$ of biotinylated Lens culinaris agglutinin (LCA) (Vector Laboratories, Burlingame, CA) in lectin buffer (10-mM Hepes, 0.15-M NaCl, 0.1% Tween 20, 0.01-mM MnCl_2 and 0.1-mM CaCl_2 , pH 7.5) for 1 h. The filters containing subclasses of human IgG were also incubated with 5 $\mu\text{g ml}^{-1}$ of biotinylated GNL (*Galanthus nivalis* lectin). Afterwards, 2.5 $\mu\text{g ml}^{-1}$ of HRP coupled to streptavidin (Vector Laboratories) was added to membrane and incubated for 1 h. The membranes were developed using Clarity™ Western ECL (Bio-Rad Laboratories) substrate and visualized using a Chemidoc XRS (Bio-Rad Laboratories).

• Chitinase assay & EndoSd substrate specificity

The fluorogenic substrate 4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide (4MU-GlcNAc) (Sigma-Aldrich) at a concentration of 0.2 mM was incubated with 0.3 MU of a chitinase from *Streptomyces griseus* (Sigma-Aldrich) as a positive control. Recombinant EndoSd from strain SK1241 and EndoS from *S. pyogenes* AP1 were incubated with 0.2-mM 4MU-GlcNAc in a total volume of 100- μl PBS. All reactions were performed at 37°C for 1 h in a black 96-well plate (Thermo Fisher Scientific). Finally, the reactions were stopped by adding 100 μl of 0.1-M glycine (pH 10). Absorbance was measured by using an ELISA reader at an excitation wavelength of 355 nm and emission wavelength of 460 nm. The experiments were carried out in five replicates. Data are presented as means $\pm$ SD. Results were statistically analyzed using an unpaired Student's *t*-test, where differences were considered significant if $p < 0.05$ (**** $p < 0.0001$).

To check the specificity of enzyme, 1 μg of recombinant EndoSd was incubated with 0.1- μl human plasma for 2 h at 37°C. The EndoSd treated plasma was subjected to SDS-PAGE and Concanavalin A (ConA) lectin blot. Untreated plasma was used as a negative control. In addition, recombinant EndoSd was incubated with purified proteins, such as Fetuin, IgM, IgE, IgA, AGP, hLF (all Sigma-Aldrich) for 2 h at 37°C and analyzed by SDS-PAGE.

• Analysis of released IgG glycans by UHPLC

N-glycans present on 100 μg of human serum IgG and bovine IgG were released overnight using dependent and 2500 U PNGase F and fluorescently labeled with 2-aminobenzamide (2-AB) using LudgerTag (Ludger, Oxfordshire, UK) [13]. Following the labeling reaction, the HILIC phase pipette tips (PhyNexus, San Jose, CA) were used to remove the excess of labeling reagents. HILIC separation of 2-AB labeled glycans was carried out on a 2.1 mm $\times$ 15 mm 1.7- μm BEH glycan column (Waters, MA, USA) and analyzed by UHPLC-FLD using Waters ACQUITY UPLC® I-class with fluorescence detection. The column temperature was kept at 40°C and the flow rate set to 0.561 ml min^{-1} using a linear gradient of 50-mM ammonium formate (pH 4.4) against acetonitrile with ammonium formate increasing from 30 to 47% over a 25-min period. An injection volume of

25- μ l sample prepared in 70% (v/v) acetonitrile was used throughout. Fluorescence detection was achieved using excitation and emission wavelengths of 330 and 420 nm, respectively. The obtained chromatograms were compared with previously published and assigned IgG glycoforms [35,36]. The sequence, composition and linkage specificities of the glycans were analyzed by exoglycosidase digestion arrays (Prozyme, CA, USA). Fluorescently labeled glycans were digested in supplied buffers at 37°C overnight using a panel of enzymes with each digestion reaction brought to a final volume of 10 μ l using double-distilled water. Digested glycans were separated from the enzyme mixtures using 10 kDa MWCO centrifugal filters (PALL, NY, USA) and separation was performed as described previously and above [37]. Nonreducing end monosaccharides were specifically removed as follows: terminal sialic acid in all linkages was removed with 5 U ml⁻¹ ABS; terminal galactose monosaccharides (β 1-4 and β 1-3) were removed using 2 U ml⁻¹ BTG and terminal GlcNAc monosaccharides were released with 50 U ml⁻¹ GUH (*Streptococcus pneumoniae* hexosaminidase).

• GenBank accession numbers

The sequences of *Streptococcus dysgalactiae* subspecies *dysgalactiae* *ndoSd* and *ndoS2d* have been submitted to GenBank under the accession numbers KT030921 (strain SK 1241), KT030922 (strain SK1240), KT030923 (strain SK1238), KT030924 (strain SK 1226), KT071706 (strain SK1242) and KT071707 (strain SK1244).

Results

• Identification of *ndoSd* genes in SDSD

In order to investigate the presence of *ndoS* and *ndoS2* genes in SDSD isolates, we set up a PCR approach based on external and internal primer pairs. The external primer pair hybridizes to the flanking genes (*scrA* and *scrK*) known from the *S. pyogenes* genomic context, while the internal primer pair (*ndoS/C*) hybridizes within the *ndoS* or *ndoS2* gene. In this experiment, six strains of SDSD were analyzed using PCR with internal and flanking primers. PCR products from strains SK1242 and SK1244 were smaller than the PCR products of strains SK1241, SK1226, SK1238, SK1240. PCR using this approach on SDSD strains SK1241, SK1226, SK1238 and SK1240 indicated that an *ndoSd* gene is located in the same genomic context as the *ndoS* gene in *S. pyogenes* where *scrA* and *scrK* genes flank the

ndoS gene. By sequencing the PCR products, the exact size and sequence of the fragments were obtained. In strains SK1241 and SK1226, the size of the *ndoSd* gene is 3204 bp and in the strains SK1240 and SK1238 it is 3159 bp. Sequencing of the PCR products confirmed that *ndoSd* genes in strains SK1241, SK1226, SK1238 and SK1240 are located in between *scrA* and *scrK* genes (**Supplementary Figure 1**). The *ndoSd* genes in strains SK1241 and SK1226 are 100% identical and in strain SK1240 is 95% identical to the former two genes. Furthermore, the encoded protein EndoSd in strain SK1241 is 75% identical to EndoS from *S. pyogenes* strain AP1 as shown by ClustalW alignments (**Supplementary Figure 2A**). The amino acid identity of EndoSd within the strains SK1241, SK1226, SK1238 and SK1240 and is 94%.

Sequencing of the generated PCR products from strains SK1242 and SK1244 showed that they did not contain an *ndoS* gene but instead have another gene between *scrA* and *scrK* similar to *ndoS2* in *S. pyogenes* serotype M49. We have denoted this gene *ndoS2d*. The sequence of the gene *ndoS2* from *S. pyogenes* NZ131 was compared with *ndoS2d* in SDSD strains. The size of *ndoS2d* gene in the strains SK1242 and SK1244 is 2544 bp. This gene is 79% identical to *ndoS2* in *S. pyogenes*. The gene *ndoS2* has to date only been found only in *S. pyogenes* serotype M49 strains. This enzyme has been shown to harbor a family 18 glycoside hydrolase domain and has glycan hydrolyzing activity on both IgG and AGP, which is a human acute phase glycoprotein [11]. **Supplementary Figure 2B** shows the alignment of EndoS2 and EndoS2d. The putative signal peptide sequences are conserved in all six strains including EndoSd and EndoS2d. In *S. pyogenes*, the protein EndoS harbors a family 18 glycoside hydrolase (chitinase) motif [11]. Here we show that EndoSd from SDSD also contain a putative chitinase family 18 active site (DxxDxDxE) (**Supplementary Figure 2**). The conserved glutamic acid in the active site of the enzyme, as well as tryptophan residues are essential for activity of EndoS [14]. These tryptophans are also present and conserved in all analyzed EndoSd sequences from SDSD (**Supplementary Figure 2**).

Alignment of the protein sequences of EndoS and EndoSd showed a significant difference at the C-termini of the proteins. EndoSd has an extra C-terminal sequence of 78 amino acids that is hereafter referred to as the C-terminal

tail. This sequence does not exist in EndoS but it is present in SDSD strains SK1241, SK1226, and SK1240, SK1238 (**Supplementary Figure 2A**). EndoSd and EndoS2d were aligned with the most similar endo- β -*N*-acetylglucosaminidases from different species and a phylogenetic tree was reconstructed (**Figure 1A**). This analysis showed that EndoSd is closely clustered with EndoS and EndoS_e. Most interesting, EndoS2d is clustered in a distinct group with EndoS₂. The phylogenetic tree also indicates that EndoSd and EndoS2d are more closely related to EndoF₂ from *Elizabethkingia* and EndoE from *Enterococcus faecalis* than to EndoF₁ and EndoF₃ from *Elizabethkingia*.

Sequence conservation among EndoS-like proteins was mapped onto the published structure of EndoS [10]. This revealed several hotspots of sequence conservation, as well regions of variability. Most of the GH18 domain, as well as the leucine-rich repeat and hybrid IgG domains are well conserved among all EndoS-like proteins. Not surprisingly, the active site shows a very high degree of sequence conservation. There are however also a few notable regions where the sequences showed a high degree of variability, namely two loops in the GH18 domain (residues 312–323 and 415–425), as well as a large proportion of the carbohydrate binding module (**Figure 1B**).

• EndoSd hydrolyzes glycans on IgG without affecting other plasma proteins

It is well established that EndoS from *S. pyogenes* hydrolyzes glycans on the CH2 domain of IgG. Here, recombinantly expressed EndoSd was incubated with human, canine, equine and bovine IgG, and the glycan hydrolyzing activity was analyzed using SDS-PAGE and LCA lectin blot (LCA recognizes α -linked mannose). A mobility shift of the IgG heavy chain on SDS-PAGE and a corresponding lack of signal on the LCA blot was observed for all subclasses of human and also animal IgG treated with EndoSd, indicating that EndoSd can hydrolyze the *N*-glycan chain on IgG from different mammalian species (**Figure 2**). Since the human IgG₂ did not react with LCA, we used GNL (an alternative lectin) that also binds to α -linked mannose. A strong signal in the negative control and lack of signal after EndoSd treatment, confirmed the glycosidase activity (**Figure 2B**). In order to test if EndoSd is specific for the *N*-linked glycan on IgG, we treated human plasma with EndoSd and analyzed the proteins by SDS-PAGE followed by ConA lectin blotting. Apart for the

IgG heavy chain, there was no apparent shift in electrophoretic mobility of any other plasma proteins or corresponding loss of signal on the ConA blot in the EndoSd treated sample (**Figure 3A**). We further tested EndoSd activity on the purified model glycoproteins fetuin, IgM, IgE, IgA, AGP and hLF but could not observe any glycan hydrolysis (**Figure 3B**). Taken together these results indicate that EndoSd hydrolyzes human IgG in a plasma environment without any apparent effects on other glycoproteins.

To investigate the function of the C-terminal tail, human IgG was incubated with recombinant EndoSd with and without the C-terminal tail, as well as with the C-terminal tail alone. PNGase F that was used as a positive control is an amidase from *Elizabethkingia meningoseptica* that hydrolyzes bonds between *N*-acetylglucosamines of *N*-linked glycans and the asparagines they are attached to [38]. The SDS-PAGE analysis revealed a shift in the apparent mass of the heavy chain of IgG of about 4 kDa coinciding with a loss of LCA signal when IgG was incubated with EndoSd and EndoSd without the tail. IgG incubated only with the tail of EndoSd was unaffected (**Figure 3C**). This clearly indicates that hydrolysis of the *N*-linked glycan on the heavy chain of IgG had occurred and that the activity of the enzyme is not dependent on the tail.

• EndoSd is a secreted protein tightly regulated by carbohydrates in the growth medium

Since EndoSd could not be identified in the culture supernatant of bacteria grown in standard THY medium (data not shown), we hypothesized that EndoSd expression might be under catabolite control and that carbohydrate composition is important as has been described for EndoS₂ in *S. pyogenes* [11]. Therefore, to analyze EndoSd expression during growth, strain SK1241 was subcultured in chemically defined medium (CDM) supplemented with 0.01% of glucose, galactose, sucrose, *N*-acetylglucosamine or mannose independently. Based on the results of SDS-PAGE and western blot, a 120-kDa protein band in the supernatant appeared in the gel only when sucrose was added to the medium (**Figure 4**). The same pattern of EndoSd expression could also be observed in strain SK1226 (data not shown). The 120-kDa protein band was excised from the gel and subjected to trypsin digestion and protein identification by mass spectrometry. Detected peptides covered most of the predicted protein

Figure 1. Phylogenetic analysis and structural alignment of EndoSd/EndoS2d and GH18 homologs. (A) EndoSd and EndoS2d were aligned with similar proteins from different species. A phylogenetic tree was reconstructed using the neighbor-joining method, validated by bootstrapping (1000 replications) generating a consensus tree. Numbers on branching points shows the percentage of resampling trees supporting the consensus tree. Species names are indicated with the protein accession numbers in parentheses. (B) Sequence conservation among EndoS-like proteins was mapped onto the crystal structure of *S. pyogenes* EndoS (Protein Data Bank ID: 4NUY) [10]. Conserved residues are shaded red, variable residues blue. Residues for which no high-confidence conservation score could be calculated are colored in yellow. Two hot spots of variability in the chitinase domain are marked by red arrows (lower arrow: residues 312–323 and upper arrow: residues 415–425).

(Supplementary Figure 4), supporting the predicted size and secretion of EndoSd by SDSD. The results of this experiment indicate that EndoSd is a secreted enzyme that is influenced by the carbohydrate composition of the growth medium.

• **EndoSd hydrolyzes biantennary glycans on IgG but it is not a general chitinase**

Enzymatically inactive EndoS of *S. pyogenes* has been shown to bind with high affinity to the Fc part of IgG, suggesting a protein–protein interaction providing substrate specificity [39]. Based on the high degree of identity between EndoSd and EndoS, EndoSd is most likely not a general endochitinase hydrolyzing any GlcNAc–GlcNAc bond. However, to exclude a general chitinase activity of EndoSd we tested the activity of this enzyme on the well-characterized fluorogenic chitinase substrate 4MB–GlcNAc. This assay showed

that EndoSd does not have general chitinase activity as compared with a verified chitinase from *Streptomyces griseus* (Figure 5).

To elucidate the IgG glycan specificity of EndoSd in strain SK1241, we treated human and bovine IgG with this enzyme and analyzed the released glycans by UHPLC. For comparison, glycan profiles of both human and bovine IgGs generated by PNGase F were used. The UHPLC analysis of released glycan from IgG confirmed that the cleavage site of EndoSd is identical to that of EndoS (Figure 6) [18]. EndoSd was found to hydrolyze all major glycoforms of both human and bovine IgG. However, there was a notable absence of structures containing a bisecting GlcNAc among the glycans released from human IgG by EndoSd (such structures are largely absent on bovine IgG). Nevertheless, exoglycosidase sequencing of this glycan pool

Figure 2. Glycan hydrolyzing activity of recombinant EndoSd on animal IgG and subclasses of human IgG. (A) Human, canine, equine and bovine IgG were incubated with recombinant EndoSd from strain SK1241 and EndoS at 37°C for 2 h and analyzed by 10% SDS-PAGE and LCA lectin blot. Both the stained gel and LCA blot show the heavy chain of IgG at approximately 55 kDa. **(B)** Subclasses of human IgG were incubated with recombinant EndoSd at 37°C for 2 h and analyzed on 10% SDS-PAGE, and subsequent LCA and GNL blot. PBS: Phosphate-buffered saline.

Figure 3. EndoS hydrolyzes human IgG and has no major activity on other plasma and purified glycoproteins. (A) Human plasma was incubated with recombinant EndoS at 37°C for 2 h and analyzed on 10% SDS-PAGE, and subsequent ConA blot. The arrow indicates a 150-kDa band that corresponds to intact IgG. (B) Purified glycoproteins: Fetuin, IgM, IgE, IgA, AGP and hLF were incubated with recombinant EndoS and PNGase F at 37°C for 2 h and analyzed on 10% SDS-PAGE. (C) Human IgG was incubated with recombinant EndoS with and without tail, PNGase F, and EndoS at 37°C for 2 h and analyzed on 10% SDS-PAGE, and subsequent LCA blot. ConA: Concanavalin A; PBS: Phosphate-buffered saline.

revealed the presence of a small amount of glycans with a bisecting GlcNAc, but the modification could not be attributed to any specific glycoforms (Figure 6 & Supplementary Figure 3).

Discussion

Glycosylation is a major post-translational protein modification, and almost all the key proteins of the immune defense are glycosylated [2]. IgG is an important glycoprotein in the adaptive immune defense that orchestrates a number of vital cellular and humoral effector functions [41].

EndoS from *S. pyogenes* has the ability to hydrolyze the glycan on the Fc region of all subclasses of human IgG and influences their binding to FcRs to varying extent [42,43]. This activity of EndoS promotes bacterial survival in human blood *in vitro* [14,44]. Previous studies have indicated that both humans and animals have antibody titers against EndoS suggesting exposure to similar protein epitopes in both humans and animals [20,45].

A homolog to EndoS (70% identity), EndoSe has been discovered in *Streptococcus equi* subsp.

Figure 4. EndoSd is tightly regulated by carbohydrates in the growth medium. Strain SK1241 was cultured in CDM with the cysteine protease inhibitor E-64 and one of the carbohydrates glucose, sucrose, mannose, GlcNAc or galactose. Concentrated supernatants were analyzed by 10% SDS-PAGE and Western blot using polyclonal rabbit antiserum raised against recombinant EndoSd. Recombinant EndoSd with GST tag is approximately 140 kDa and EndoSd expressed in strain SK1241 is 120 kDa.

CDM: Chemically defined medium.

equi and subsp *zooepidemicus* [46]. EndoS_e is functionally similar to EndoS, and participates in evasion of the host immune system. The activity of this enzyme has been determined using lectin blots of IgG and through the inhibition of IgG binding to FcRs on macrophages. It has been suggested that EndoS_e is the result of horizontal gene transfer from GAS to *S. equi* [46].

The functional and phylogenetic analysis of EndoSd presented here suggests that also SDSD might have acquired these genes by horizontal gene transfer (Figure 1A). The phylogenetic analysis also suggests that EndoSd and EndoS2d have the same ancestor as EndoS and EndoS2d do. Although *ndoSd* and *ndoS2d* are located in the same genomic context in the genome of SDSD,

Figure 5. EndoSd is not a general chitinase. EndoSd from strain SK1241, EndoS and a chitinase from *S. griseus* was incubated with the fluorescent substrate 4MU-GlcNAc for 1 h and fluorescence was measured at 355/460 nm. The experiments were performed using five replicates. Data are presented as mean $\pm$ SD.

**** Indicates statistically significant differences ($p < 0.05$) between chitinase + 4MU-GlcNAc and EndoSd + 4MU-GlcNAc.

PBS: Phosphate-buffered saline.

they are clustered in two distinct groups in the phylogenetic tree (**Figure 1A**). This confirms that *ndoSd* is not conserved in all isolates of SDSD. Gherardi *et al.* suggested that the recent increase in virulence factors traits in SDSE indicates horizontal gene transfer from related streptococci, such as *S. pyogenes* and *S. agalactiae* [47].

Sequence alignments of EndoS-like proteins revealed a much higher degree of sequence conservation in the catalytic domain at the N-terminus. A notable exception is a loop consisting of residues 312–323. This loop is absent in other GH18 glycoside hydrolases and has been suggested to mediate interaction with IgG. Indeed, deletion of this loop affects EndoS binding to IgG [10]. However, on a primary sequence level, it is poorly conserved in EndoSd and mostly absent in EndoS2 sequences. The C-terminus of EndoS contains several regions of variability (**Figure 1B**). These regions harbor a carbohydrate binding module that was suggested to mediate the primary interaction between EndoS and its substrate IgG [10]. Analysis of truncated EndoS mutants further showed that the C-terminus is not required for activity. However, C-terminally truncated EndoS mutants exhibited a reduced enzymatic activity, especially toward glycoforms modified with a bisecting GlcNAc [18]. As EndoS, EndoS2 and EndoSd show somewhat different glycan substrate specificities and vary the most in the C-terminal domains; it is, therefore, tempting to speculate that the C-terminus with the carbohydrate binding module mediates glycoform specificity in EndoS-like proteins.

S. pyogenes secretes EndoS in the late stationary growth phase when grown in the fairly rich C medium [13]. By contrast, EndoSd could not be detected under these conditions. It has been shown that bacteria modulate transcription of carbohydrate utilization genes in response to alteration in environmental conditions under infections, and that carbohydrate consumption and virulence factor production can be linked [48]. Here, expression of EndoSd was found to be dependent on carbohydrate availability in the medium as only a limited amount of EndoSd were detected when mannose or GlcNAc was supplemented (**Figure 4**). The presence of glucose and galactose inhibits bacterial expression of EndoSd and sucrose induces expression. It was previously found that expression of EndoS2 in *S. pyogenes* serotype M49 is tightly regulated by a mechanism sensitive to the presence of carbohydrates. Serotype M49 strains express EndoS2

during starvation, and this enzyme can be found in C medium only after addition of sucrose [11]. The gene *scrA* and *scrK* surrounding EndoSd are a part of a sucrose utilization operon. It was, therefore, not surprising to find an increase of EndoSd expression when sucrose was added to the culture medium. It is obvious that there is a clear correlation between expression of EndoSd and carbon sources in the medium even though the regulatory network are at present obscured.

Hydrolyzing activity of EndoS has been shown on all subclasses of human IgG and also on animal IgGs [14]. EndoSd can also hydrolyze all subclasses of human IgG and several animal IgGs in a fashion similar to EndoS (**Figure 2**). Incubation of EndoSd with human plasma and purified glycoproteins showed that it has glycosidase activity on IgG without any major activities on other glycoproteins (**Figure 3A & B**).

UHPLC analysis of EndoSd released glycans showed that this enzyme is able to hydrolyze all major glycoforms on both human and bovine IgG. With one notable exception, in contrast to EndoS [16], EndoSd only released minute amount of glycans modified with a bisecting GlcNAc (**Figure 6**). Previous studies have shown that EndoS lacking its carbohydrate binding domain was considerably slower in releasing structures with bisecting GlcNAc as compared with other glycoforms, indicating that these structures might be more resistant to endoglycosidases [18]. As bovine IgG is almost completely devoid of glycans with a bisecting GlcNAc (**Figure 6**) [35], this limitation in substrate specificity is unlikely to hinder EndoSd efficacy in its natural host. It does, however, open up new applications for this enzyme: a difference in the hydrolysis rate of high-mannose structures between EndoS and EndoS2 has been exploited to establish an easy, chromatography-based assay to determine the amount of high-mannose glycans on recombinant antibody therapeutics [16]. A similar approach for the quantification of glycans with a bisecting GlcNAc could be set up using EndoSd. Furthermore, EndoSd in combination with an engineered monoclonal antibody modified with bisecting GlcNAc could also be used as an alternative strategy for receptor refocusing [7].

Lastly, EndoS has been shown to be a potential therapeutic in many models of autoimmune disease [20,22]. There might however be difficulties in using a bacterial product as a therapeutic agent, most notably potential toxic side effects and immunogenicity. While a range of animal studies

Figure 6. EndoSd releases most major glycoforms from human and bovine IgG. Hydrophilic interaction liquid chromatography separation of 2-AB-labeled glycans released from human and bovine IgG by EndoSd and PNGase F, respectively, analyzed by ultra high performance liquid chromatography with fluorescence detection. The EndoSd profile shows the released biantennary glycans from IgG and the cleavage site of the glycan chain. Monosaccharide identities are shown to the right and conform to the standard colors and shapes as devised by the Consortium for Functional Glycomics [40].

have shown that EndoS is well tolerated and can be administered repeatedly [20,22], it might nevertheless be of advantage to have several antigenically distinct versions of EndoS available, which could

be used sequentially to avoid pre-existing immunity, as well as loss of efficacy after prolonged treatment due to raised antibody titers against EndoS. EndoSd fulfills these criteria and might therefore

be an interesting candidate as autoimmunity therapeutic.

Conclusion

It has been firmly established that the *N*-linked glycans on IgG are instrumental in antibody-mediated immune responses [49]. Therefore, enzymes with activity on these glycans could be developed as pharmaceuticals against antibody mediated autoimmune diseases. Toward that end, EndoS has been successful as experimental treatment in a number of animal models of antibody mediated autoimmunity, including arthritis, systemic lupus erythematosus, multiple sclerosis, autoimmune kidney disease and autoimmune blistering skin diseases [20,22,50–56]. This work, which is based on the functional similarities between EndoSd from SDS and EndoS from *S. pyogenes* emphasize the close evolutionary relationship between the species and contributes to a deeper understanding of the biology of immune evasion by bacterial pathogens.

Future perspective

Analysis and modification of IgG glycans are of great interest for development of antibody based pharmaceuticals. In this study, we identified two new enzymes EndoSd/EndoS2d in SDS that have the potential to be developed into pharmaceuticals against autoimmunity in themselves, and for glycan analysis of antibodies for research purposes or during development of antibody based drugs.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/fmb.16.14

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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EXECUTIVE SUMMARY

- *ndoS*-like genes were found in six *Streptococcus dysgalactiae* subspecies *dysgalactiae* strains in the same genomic context as *ndoS* in *S. pyogenes*.
- EndoSd is a secreted enzyme and has a unique C-terminal sequence consisting of 78 amino acids.
- EndoSd hydrolyzes the conserved *N*-linked glycans on the heavy chain of animal and human IgG.
- Expression on EndoSd is dependent on carbohydrate availability in the culture medium.
- EndoSd hydrolyzes IgG biantennary glycans in the chitobiose core on IgG.
- EndoSd and EndoS2d are clustered in two distinct phylogenetic groups.

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