(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 August 2017 (17.08.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/137459 A1

(51) **International Patent Classification**:
C12N 9/24 (2006.01)

(21) **International Application Number**:
PCT/EP2017/052792

(22) **International Filing Date**:
8 February 2017 (08.02.2017)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:

16154712.0	8 February 2016 (08.02.2016)	EP
16154739.3	8 February 2016 (08.02.2016)	EP
16173595.6	8 June 2016 (08.06.2016)	EP
16173599.8	8 June 2016 (08.06.2016)	EP
16206867.0	23 December 2016 (23.12.2016)	EP

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2017/137459 A1

(54) **Title**: ENZYMES FOR TRIMMING OF GLYCOPROTEINS

(57) **Abstract**: The invention concerns fusion proteins, wherein two endoglycosidases are fused, possibly via a linker. The fusion enzymes according to the invention have structure (1): EndoX-$(L)_p$-EndoY (1), wherein EndoX is an endoglycosidase, EndoY is an endoglycosidase distinct from EndoX, L is a linker and p is 0 or 1. Such fusion enzymes capable of trimming glycoproteins comprising at least two distinct glycoforms in a single step. The invention further concerns the use of the fusion enzyme according to the invention for trimming glycoproteins. In another aspect, the invention relates to the process of production of the fusion enzyme. In a further aspect, the inventions concerns a process for trimming glycoproteins, comprising trimming the glycoprotein with a fusion enzyme according to the invention, to obtain a trimmed glycoprotein.

Enzymes for trimming of glycoproteins

Field of the invention

The present invention is in the field of enzymatic hydrolysis of oligosaccharides, more in particular to the trimming of glycoproteins. The invention relates to improved enzymes for such trimming to liberate the core GlcNAc and to a process for trimming of glycoproteins using the enzymes according to the invention.

Background of the invention

Glycoproteins exist in many glycosylated variants, or glycoforms, which can differ substantially in their biochemical properties and (biological) functions. Glycans are structurally diverse, incorporating a wide range of monosaccharide residues and glycosidic linkages.

Many therapeutic proteins are glycoproteins, and although some are purified from natural sources, the majority are recombinantly expressed. The choice of expression system heavily influences the glycosylation. There have been notable efforts in controlling the glycosylation of glycoprotein production systems motivated by the impact on in vivo functionality. For example, monoclonal antibodies with engineered glycosylation display enhanced pharmacokinetics and effector function. Glycopeptides offer intriguing possibilities in the development of anticancer vaccines given their ability to stimulate both humoral and cellular immunity. Additionally, the HIV glycan shield is an effective target for antibody neutralization and an emerging target for vaccine design.

On the other hand, removal of N-glycans from glycoproteins provides complementary therapeutic opportunities. Deglycosylation of IgG significantly decreases binding of antibodies to Fc-gamma receptors, thereby avoiding aspecific uptake of antibodies by *e.g.* macrophages or megakaryocytes, which may lead to thrombocytopenia. The latter biological phenomenon is responsible for the dose-limiting toxicity (DLT) of Kadcyla®, an antibody-drug-conjugate to treat HER2-upregulated breast cancer. Selective deglycosylation of antibodies in vivo affords opportunities to treat patients with antibody-mediated autoimmunity. Removal of high-mannose glycoform in a recombinant therapeutic glycoprotein may be beneficial, since high-mannose glycoforms are known to compromise therapeutic efficacy by aspecific uptake by endogenous mannose receptors and leading to rapid clearance, as for example described by Gorovits and Krinos-Fiorotti, *Cancer Immunol. Immunother.* **2013**, *62*, 217-223 and Goetze et al, *Glycobiology* **2011**, *21*, 949-959 (both incorporated by reference). In addition, Van de Bovenkamp et al, *J. Immunol.* **2016**, *196*, 1435-1441 (incorporated by reference) describe how high-mannose glycans can influence immunity. It was described by Reusch and Tejada, *Glycobiology* **2015**, *25*, 1325-1334 (incorporated by reference), that inappropriate glycosylation in monoclonal antibodies could contribute to ineffective production from expressed Ig genes. In some cases, a carbohydrate addition sequence generated by either V region rearrangement or somatic hypermutation may result in an antibody that cannot be properly folded and secreted, as described by Gala and Morrison, *J. Immunol.* **2004**, *172*, 5489-5494 (incorporated by reference).

An additional advantage of deglycosylated therapeutic proteins is the much facilitated batch-to-

batch consistency and significantly improved homogeneity, which is highly advantageous for regulatory approval.

A highly useful and straightforward approach to obtain deglycosylated recombinant proteins, thereby offering a route to improving the efficacy of therapeutic antibodies and other N-glycoproteins, is by enzymatic removal of glycans. Fortuitously, endoglycosidases have been discovered that are able to cleave N-glycans, which offers the possibility of selective removal from a recombinant glycoprotein. Endoglycosidases have further found use in the preparation of conjugates from glycoproteins, by selectively liberating the core GlcNAc moieties upon trimming, followed by bioconjugation. Another field of use of endoglycosidases is mass spectrometry, one of the key analytical tools for characterizing (therapeutic) proteins, including glycoproteins and monoclonal antibodies in particular. By enzymatic cleavage of the complex and heterogeneous glycan from the protein, mass spectrometric analysis is significantly facilitated.

Bioconjugation is the process of linking two or more molecules, of which at least one is a biomolecule and the other molecule(s) may be referred to as “target molecule” or “molecule of interest”. Many different compounds have been found useful to be conjugated to glycoproteins. For example, the modulation of protein structure and function by covalent modification with a chemical probe for detection and/or isolation has evolved as a powerful tool in proteome-based research and biomedical applications. Fluorescent or affinity tagging of proteins is key to studying the trafficking of proteins in their native habitat, and vaccines based on protein-carbohydrate conjugates have gained prominence in the fight against HIV, cancer, malaria and pathogenic bacteria. PEGylation of proteins or attachment of a protein to serum albumin are useful strategies to enhance the pharmacokinetic profile by reducing clearance rates, whereas functionalization of a carrier protein such as a monoclonal antibody with a toxic payload is a promising strategy in the targeted treatment of disease (in particular cancer).

In general, two strategic concepts can be recognized in the field of bioconjugation: (a) conjugation based on a native functional group (in other words: a functional group already present in the biomolecule of interest, such as for example a thiol, an amine, an alcohol or a hydroxyphenol unit) or (b) a two-stage process involving engineering of one (or more) unique reactive groups into a biomolecule prior to the actual conjugation process.

The first approach typically involves a reactive amino acid side-chain in a protein (*e.g.* cysteine or lysine), or a functional group in a glycan (*e.g.* amine, aldehyde) or nucleic acid (*e.g.* purine or pyrimidine functionality or alcohol). As summarized inter alia in G.T. Hermanson, *“Bioconjugate Techniques”*, Elsevier, 3rd Ed. **2013**, incorporated by reference. Most prominently, cysteine-maleimide conjugation stands out for protein conjugation by virtue of its high reaction rate and chemoselectivity. However, when no free cysteine is available for conjugation, as in many proteins, other methods are often required, each suffering from its own shortcomings especially in terms of site-specificity. Moreover, a general disadvantage of protein conjugates obtained via alkylation with maleimides is that in general the resulting succinimide conjugates can be unstable due to the reverse of alkylation, *i.e.* a retro-Michael reaction.

An elegant and broadly applicable solution for bioconjugation involves the two-stage approach.

Although more laborious, two-stage conjugation via engineered functionality typically leads to higher selectivity (site-specificity) than conjugation on a natural functionality. Besides that, full stability can be achieved by proper choice of construct. Typical examples of a functional group that may be imparted onto the biomolecule include (strained) alkyne, (strained) alkene, norbornene, tetrazine, azide, phosphine, nitrile oxide, nitrone, nitrile imine, diazo compound, carbonyl compound, (O-alkyl)hydroxylamine and hydrazine, which may be achieved by either chemical or molecular biology approach. Each of the above functional groups is known to have at least one reaction partner, in many cases involving complete mutual reactivity. For example, cyclooctynes react selectively and exclusively with 1,3-dipoles, strained alkenes with tetrazines and phosphines with azides, leading to fully stable covalent bonds.

An efficient route towards the introduction of engineered functionalities such as azides into specifically glycoproteins is via selective functionalization of the glycans present on the glycoprotein. All recombinant antibodies, generated in mammalian host systems, contain the conserved N-glycosylation site on the asparagine residue at or close to position 297. These glycans are always formed as a complex mixture of isoforms, see e.g. Figures 1 and 2, consisting of a highly heterogeneous mixture of complex, hybrid and high-mannose glycans. Trimming of these glycans by an endoglycosidase leaves only the core GlcNAc moiety (attached to N297), optionally fucosylated at the 6-OH group. The liberated core GlcNAc provides a suitable anchor point for target molecules, providing a product with a much higher homogeneity in comparison to products obtained by conjugation to terminal sugar moieties present in the original glycan structure. A downside of this approach, however, is that different glycans may require different endoglycosidases, each with their own optimal conditions, such that multiple enzymatic treatments may be required for proper and complete trimming of the glycoprotein. For example, EndoH is known to trim high-mannose and hybrid glycoforms, but not complex type glycans, while EndoS is able to trim complex type glycans and to some extent hybrid glycan, but not high-mannose forms. EndoF2 is able to trim complex glycans (but not hybrid), while endoF3 can only trim complex glycans that are also 1,6-fucosylated. Another endoglycosidase, EndoD is able to hydrolyze Man_5 (M5) glycan only. An overview of specific activities of different endoglycosidases is disclosed in Freeze *et al.* in *Curr. Protoc. Mol. Biol.*, **2010**, 89:17.13A.1-17, incorporated by reference herein.

Yamamoto *et al.* disclose in *Glycoconjugate J.* **2005**, 22, 35-42, incorporated by reference herein, a chimeric construct of EndoD and EndoBH, which was completely inactive. The chimeric construct was designed to investigate the homology of both endoglycosidases in trimming of glycans. In the context of glycoprotein conjugation, WO 2007/095506 and WO 2008/029281 disclose that trimming of the glycan can take place with EndoH, thereby hydrolysing a GlcNAc-GlcNAc glycosidic bond and liberating a GlcNAc for enzymatic introduction of GalNAz. Van Geel *et al.* disclose in *Bioconjugate Chem.* **2015**, *26*, 2233, incorporated by reference herein, that transfer of a range of azido-modified galactose moieties to the core GlcNAc residue of a monoclonal antibodies, obtained by trimming with an endoglycosidase, followed by attachment of a toxic payload by means of copper-free click chemistry, is an efficient method to obtain antibody-drug conjugates with a demonstrated improved efficacy and safety profile versus marketed drug Kadcyla®.

As a product of recombinant DNA technology, fusion proteins have been developed as a class of novel biomolecules with multi-functional properties. By genetically fusing two or more proteins or protein domains together, a fusion protein product is generated that may display similar or distinctly different functions as those of the component moieties. Fusion proteins have found applications in purification strategies, immobilization, imaging, and biopharmaceuticals. For example, many protein drugs are fused to Fc domains of antibodies, such as Fc-immunoglobulin G1 (Fc-IgG1), or to carrier proteins such as human serum albumin (HSA) or transferrin (Tf) to extend their plasma half-lives and to achieve enhanced therapeutic effects. Several fusion proteins drugs including Enbrel® (tumour necrosis factor/Fc-IgG1), Ontak® (interleukin-2/diphtheria toxin), Orencia® (cytotoxic T-lymphocyte antigen-4/Fc-IgG1), Amevive® (leukocyte function antigen-3/Fc-IgG1), Arcalyst® (interleukin-1 receptor extracellular domain/ Fc-IgG1), and Nplate® (thrombopoietin/Fc-IgG1) have been approved by the FDA for therapeutic application. One relevant example of a fusion protein of an endoglycosidase can be found in Warren et al., *Prot. Eng. Design Select.* **2005**, *18*, 497–501 (incorporated by reference), disclosing a fusion of carbohydrate binding domain (CBM) to EndoF1 or PNGaseF.

The successful construction of a recombinant fusion protein requires the component proteins, but also the linkers may play an important role. Linkers may be short or long, flexible or rigid, and of cleavable or non-cleavable nature. In some cases, the linker may increase stability or folding, improve expression or biological activity, or alter pharmacokinetics. Typical nature of linkers known in the art are oligomers of glycine, *e.g.* G_8, oligomers of GGGGS, oligomers of EAAAK and variants thereof. A recent overview of linkers for fusion proteins can be found in Chen et al., *Adv. Drug Deliv. Rev.* **2013**, *65*, 1357-1369, incorporated herein by reference.

Summary of the invention

The invention concerns fusion proteins, wherein two endoglycosidases are fused, possibly via a linker. The fusion enzymes according to the invention are conveniently capable of trimming glycoproteins comprising at least two distinct glycoforms in a single step. All glycans of glycoproteins, which cannot be removed by a single conventional enzyme, are completely trimmed to liberate the core GlcNAc by the fusion enzyme according to the invention. Surprisingly, both activities of the fusion enzyme function smoothly at the optimal pH of one of the endoglycosidases, while the other endoglycosidase may normally require a different pH to operate optimally. Moreover, it was found that the activity of a particular endoglycosidase in a fusion protein can display a higher trimming efficiency compared to the same endoglycosidase as a single enzyme. The invention further concerns the use of the fusion enzyme according to the invention for trimming glycoproteins. In another aspect, the invention relates to the process of production of the fusion enzyme. In a further aspect, the inventions concerns a process for trimming glycoproteins, comprising trimming the glycoprotein with a fusion enzyme according to the invention, to obtain a trimmed glycoprotein.

Description of the figures

Figure 1 shows exemplary glycans of high-mannose type (Man_3 (M3), Man_5 (M5) and Man_9 (M9)),

complex type (biantennary), bisected type, triantennary and tetraantennary type.

Figure 2 shows an antibody comprising a glycan on each heavy chain. The most typical complex glycosylation patterns of a recombinant antibody are G1F, G0F and G1. Some mannose-type glycosylation may also be present (M5) and in some cases a hybrid type glycan (*e.g.* M5G1).

Figure 3 shows the results of trimming of high-mannose glycoprotein RNAseB in Tris pH 7.5 by endoglycosidases EndoS, EndoS2 and EndoSH. Concentration series: 0.025, 0.125, 0.125 (duplo), and 0.25 mg/mL. Upper band = intact RNaseB, lower band = trimmed RNaseB.

Figure 4 shows a plot of percentage conversion (trimming) of cAC10 at pH 6 by endoglycosidases EndoS, EndoS2 and EndoSH as obtained in Example 14. Trendline for EndoSH: $y = 2.0784x + 1.027$ ($R^2 = 0.9982$). Trendline for EndoS: $y = 2.0103x$ ($R^2 = 0.9838$). Trendline for EndoS2: $y = 1.0297x - 0.1622$ ($R^2 = 0.9998$).

Figure 5 shows a plot of percentage conversion (trimming) high-mannose trastuzumab at pH 6 by endoglycosidases EndoS, EndoS2 and EndoSH as obtained in Example 15.

Detailed description of the invention

Definitions

The verb “to comprise”, and its conjugations, as used in this description and in the claims is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded.

In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there is one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

The general term “sugar” is herein used to indicate a monosaccharide, for example glucose (Glc), galactose (Gal), mannose (Man) and fucose (Fuc). The term “sugar derivative” is herein used to indicate a derivative of a monosaccharide sugar, i.e. a monosaccharide sugar comprising substituents and/or functional groups. Examples of a sugar derivative include amino sugars and sugar acids, e.g. glucosamine ($GlcNH_2$), galactosamine ($GalNH_2$) N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), sialic acid (Sia) which is also referred to as N-acetylneuraminic acid (NeuNAc), and N-acetylmuramic acid (MurNAc), glucuronic acid (GlcA) and iduronic acid (IdoA).

The term “nucleotide” is herein used in its normal scientific meaning. The term “nucleotide” refers to a molecule that is composed of a nucleobase, a five-carbon sugar (either ribose or 2-deoxyribose), and one, two or three phosphate groups. Without the phosphate group, the nucleobase and sugar compose a nucleoside. A nucleotide can thus also be called a nucleoside monophosphate, a nucleoside diphosphate or a nucleoside triphosphate. The nucleobase may be adenine, guanine, cytosine, uracil or thymine. Examples of a nucleotide include uridine diphosphate (UDP), guanosine diphosphate (GDP), thymidine diphosphate (TDP), cytidine diphosphate (CDP) and cytidine monophosphate (CMP).

The term “protein” is herein used in its normal scientific meaning. Herein, polypeptides comprising about 10 or more amino acids are considered proteins. A protein may comprise natural, but also unnatural amino acids.

Proteins and enzyme included mutants thereof. For example, “endoglycosidase” includes both native (wild-type) endoglycosidases and mutant endoglycosidases, as long as the endoglycosidase activity is substantially maintained. A domain having an amino acid sequence that is different from a wild-type amino acid sequence is herein referred to as a mutant domain. The mutation may e.g. comprise a single amino acid change (a point mutation), but also multiple amino acid changes (e.g. of 1 to 10, preferably of 1 to 6, more preferably of 1, 2, 3 or 4, even more preferably of 1 or 2 amino acids), or a deletion or insertion of one or more (e.g. of 1 to 10, preferably of 1 to 6, such as 1, 2, 3 or 4, preferably of 1 or 2) amino acids. Alternatively, larger deletions or insertions can be applied to the enzyme. For example, truncated endoglycosidase D (deletion of 599 amino acids from its C-terminal portion) has been found to retain its endoglycosidase activity (Yamamoto *et al.* in *Glycoconjugate J.* **2005**, 22, 35-42). The skilled person is aware of the possibilities in this respect, and as long as the endoglycosidase activity is substantially retained the enzyme can contain any type of mutation.

The term “glycoprotein” is herein used in its normal scientific meaning and refers to a protein comprising one or more monosaccharide or oligosaccharide chains (“glycans”) covalently bonded to the protein. A glycan may be attached to a hydroxyl group on the protein (O-linked-glycan), *e.g.* to the hydroxyl group of serine, threonine, tyrosine, hydroxylysine or hydroxyproline, or to an amide function on the protein (*N*-glycoprotein), *e.g.* asparagine or arginine, or to a carbon on the protein (*C*-glycoprotein), *e.g.* tryptophan. A glycoprotein may comprise more than one glycan, may comprise a combination of one or more monosaccharide and one or more oligosaccharide glycans, and may comprise a combination of N-linked, O-linked and C-linked glycans. It is estimated that more than 50% of all proteins have some form of glycosylation and therefore qualify as glycoprotein. Examples of glycoproteins include PSMA (prostate-specific membrane antigen), CAL (candida antartica lipase), gp41, gp120, EPO (erythropoietin), antifreeze protein and antibodies.

The term “glycan” is herein used in its normal scientific meaning and refers to a monosaccharide or oligosaccharide chain that is linked to a protein. The term glycan thus refers to the carbohydrate-part of a glycoprotein. The glycan is attached to a protein via the C-1 carbon of one sugar, which may be without further substitution (monosaccharide) or may be further substituted at one or more of its hydroxyl groups (oligosaccharide). A naturally occurring glycan typically comprises 1 to about 10 saccharide moieties. However, when a longer saccharide chain is linked to a protein, said saccharide chain is herein also considered a glycan. A glycan of a glycoprotein may be a monosaccharide. Typically, a monosaccharide glycan of a glycoprotein consists of a single N-acetylglucosamine (GlcNAc), glucose (Glc), mannose (Man) or fucose (Fuc) covalently attached to the protein. A glycan may also be an oligosaccharide. An oligosaccharide chain of a glycoprotein may be linear or branched. In an oligosaccharide, the sugar that is directly attached to the protein is called the core sugar. In an oligosaccharide, a sugar that is not directly attached to the protein and is attached to at least two other sugars is called an internal sugar. In an oligosaccharide, a

sugar that is not directly attached to the protein but to a single other sugar, *i.e.* carrying no further sugar substituents at one or more of its other hydroxyl groups, is called the terminal sugar. For the avoidance of doubt, there may exist multiple terminal sugars in an oligosaccharide of a glycoprotein, but only one core sugar. The end of an oligosaccharide that is directly attached to the protein is called the reducing end of a glycan. The other end of the oligosaccharide is called the non-reducing end of a glycan. A glycan may be an O-linked glycan, an N-linked glycan or a C-linked glycan.

In an O-linked glycan a monosaccharide or oligosaccharide glycan is bonded to an O-atom in an amino acid of the protein, typically via a hydroxyl group of serine (Ser) or threonine (Thr). For O-linked glycans, a wide diversity of chains exist. Naturally occurring O-linked glycans typically feature a serine or threonine-linked α-O-GalNAc moiety, further substituted with galactose, sialic acid and/or fucose. The hydroxylated amino acid that carries the glycan substitution may be part of any amino acid sequence in the protein.

In an N-linked glycan a monosaccharide or oligosaccharide glycan is bonded to the protein via an N-atom in an amino acid of the protein, typically via an amide nitrogen in the side chain of asparagine (Asn) or arginine (Arg). For N-linked glycans, a wide diversity of glycans exist. Naturally occurring N-linked glycans feature an asparagine-linked β-N-GlcNAc moiety, in turn further substituted at its 4-OH with β-GlcNAc, in turn further substituted at its 4-OH with β-Man, in turn further substituted at its 3-OH and 6-OH with α-Man, leading to the glycan pentasaccharide $Man_3GlcNAc_2$. The core GlcNAc moiety may be further substituted at its 6-OH by α-Fuc. The pentasaccharide $Man_3GlcNAc_2$ is the common oligosaccharide scaffold of nearly all *N*-linked glycoproteins and may carry a wide variety of other substituents, including but not limited to Man, GlcNAc, Gal and sialic acid. The asparagine that is substituted with the glycan on its side-chain is typically part of the sequence Asn-X-Y, with X being any amino acid but proline and Y being either serine or threonine.

In a C-linked glycan a monosaccharide or oligosaccharide glycan is bonded to a C-atom in an amino acid of the protein, typically to a C-atom of tryptophan (Trp).

The term “antibody” is herein used in its normal scientific meaning. An antibody is a protein generated by the immune system that is capable of recognizing and binding to a specific antigen. An antibody is an example of a glycoprotein. The term antibody herein is used in its broadest sense and specifically includes monoclonal antibodies, polyclonal antibodies, dimers, multimers, multispecific antibodies (e.g. bispecific antibodies), antibody fragments, and double and single chain antibodies. The term “antibody” is herein also meant to include human antibodies, humanized antibodies, chimeric antibodies and antibodies specifically binding cancer antigen. The term “antibody” is meant to include whole antibodies, but also antigen-binding fragments of an antibody, for example an antibody Fab fragment, $F(ab')_2$, Fv fragment or Fc fragment from a cleaved antibody, a scFv-Fc fragment, a minibody, a diabody or a scFv. Furthermore, the term includes genetically engineered antibodies and derivatives of an antibody. Antibodies, fragments of antibodies and genetically engineered antibodies may be obtained by methods that are known in the art. Typical examples of antibodies include, amongst others, abciximab, rituximab, basiliximab, palivizumab,

infliximab, trastuzumab, alemtuzumab, adalimumab, tositumomab-I131, cetuximab, ibrituximab tiuxetan, omalizumab, bevacizumab, natalizumab, ranibizumab, panitumumab, eculizumab, certolizumab pegol, golimumab, canakinumab, catumaxomab, ustekinumab, tocilizumab, ofatumumab, denosumab, belimumab, ipilimumab and brentuximab.

A "linker" is herein defined as a moiety that connects two or more elements of a compound. For example, the fusion enzyme according to the invention may contain a linker that connects the two endoglycosidase units. In the context of the fusion enzymes according to the present invention, linkers typically contain at least one amino acid and most preferably consist of one or more amino acids.

A "bioconjugate" is herein defined as a compound wherein a biomolecule is covalently connected to a target molecule via a linker. A bioconjugate comprises one or more biomolecules and/or one or more target molecules. The linker may comprise one or more spacer moieties. A target molecule may be an active substance, a reporter molecule, a polymer, a solid surface, a hydrogel, a nanoparticle, a microparticle or a biomolecule.

The term "fusion enzyme" herein refers to an enzyme wherein the amino acid sequences of two or more enzymes that originally belonged to separate enzymes are joined together, optionally via a linker. Fusion enzymes are known in the art and may be created by the joining of two or more genes that originally code for separate enzymes. Translation of this gene results in a single polypeptide with functional properties derived from each of the original enzymes.

Fusion enzyme

In a first aspect, the invention concerns a fusion enzyme comprising two endoglycosidases, optionally connected via a linker. The fusion enzyme according to the invention may be represented by structure (**1**):

$$\text{EndoX}–(\text{L})_p–\text{EndoY} \quad (\mathbf{1})$$

Herein, EndoX and EndoY are both individually an endoglycosidase, L is a linker and p is 0 or 1. In the context of the present invention, "fusion enzyme" may also be referred to as "fusion protein". The fusion enzyme according to the invention is preferably an end-to-end fusion, either direct or via a linker L.

Endoglycosidase

Endoglycosidase are known in the art as enzymes that cleave oligosaccharides between two glycosidic bonds, as such releasing them from either glycoproteins, glycopeptides or glycolipids. Such oligosaccharides are typically referred to as glycans. In the context of the present invention, "Endo" refers to endoglycosidase. Endoglycosidases hydrolyse the bond between two sugar units in an oligosaccharide or polysaccharide, but not between the core sugar unit, which is directly bound to the peptide part of a glycoprotein, and the amino acid it is connected to. Endoglycosidases typically hydrolyse the bond between the two core N-acetylglucosamine (GlcNAc) residues in N-linked glycans, thus leaving the core GlcNAc residue connected to the peptide part of the glycoprotein.

In the context of the present invention, the term endoglycosidase encompasses all members of the family of endoglycosidase that releases oligosaccharides from glycoproteins, glycopeptides or glycolipids. Endoglycosidase may also cleave polysaccharide chains between residues that are not the terminal residue, although releasing oligosaccharides from conjugated protein and lipid molecules is more common.

In the context of the present invention, the term endoglycosidase encompasses both the native endoglycosidases or truncated endoglycosidases and mutants thereof, as long as the endoglycosidase activity is substantially retained. In other words, the amino acid sequence of EndoX and EndoY may comprise a different amino acids sequence compared to the native endoglycosidase. In one embodiment, the amino acid sequence of EndoX and EndoY comprise a mutant. In one embodiment, the amino acid sequence of EndoX and EndoY do not comprise a mutant. In one embodiment, the amino acid sequence of EndoX and EndoY comprise a truncated sequence. In one embodiment, the amino acid sequence of EndoX and EndoY do not comprise a truncated sequence. When looking at the sequence of EndoX and EndoY individually, it is preferred that each of EndoX and EndoY has at least 80% sequence identity with the corresponding native amino acid sequence of the catalytic domain of the endoglycosidase, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with the corresponding native amino acid sequence. Most preferably, each of EndoX and EndoY has 100% sequence identity with the corresponding amino acid sequence of the catalytic domain of the endoglycosidase. Alternatively or additionally, it is preferred that each of EndoX and EndoY has at least 80% sequence similarity with the corresponding native amino acid sequence, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence similarity with the corresponding native amino acid sequence of the catalytic domain of the endoglycosidase. Most preferably, each of EndoX and EndoY has 100% sequence similarity with the corresponding native amino acid sequence of the catalytic domain of the endoglycosidase.

Sequence identity and similarities can be readily calculated by known methods and/or computer program methods known in the art such as BLASTP publicly available from NCBI and other sources (BLAST Manual, Altschul, S., *et al.*, NCBI NLM NIH Bethesda, MD 20894; Altschul, S., *et al.*, *J. Mol. Biol.* **215**:403-410 (1990), incorporated by reference.

Glycans that can be cleaved by glycosidases exist in various glycoforms, which are generally grouped in three types: high-mannose, complex and hybrid. All three types have a □1,4-N,N'-diacetylchitobiose ($GlcNAc_2$) core, connected to a mannose trisaccharide (Man_3). The core GlcNAc may optionally be fucosylated, but this is not always the case. High-mannose glycans contain at least 2 further mannose residues, typically resulting in 5 to 9 mannose residues. Complex glycans have one or more sugar monomers, not being mannose, connected to two of the mannose residues of the central Man_3 unit. These further sugar monomers are typically selected from GlcNAc,

galactose (Gal), and sialic acid (Neu5Ac). Complex glycans exist in bi-, tri- and tetraantennary forms, depending on the number of (oligo)saccharide(s) that are connected to the central Man_3 unit. Hybrid glycans have high-mannose type oligosaccharide connected to one of the mannose residue of the central Man_3 unit, and a complex type oligosaccharide connected to the other mannose residue. An overview of the glycan types is given in Figure 1. Even within a specific glycan type, many possibilities exist, increasing the heterogenicity of glycoproteins. For example, biantennary complex glycans attached to the N297 residue of antibodies may exists in several distinct glycoforms, including but not limited to G1, G1F, G0F and SG1F, as depicted in Figure 2.

Endo-β-N-acetylglucosaminidases (ENGases, also referred to endoglycosidases or Endos), typically hydrolyse the N-glycan of a glycoprotein at the β-1,4-glycosidic bond of the core chitobiose. These enzymes, found mainly in GH18 and GH85 in the CAZy classification (Lombard *et al*. in *Nucleic Acids Res*. **2014**, *42*, D490, incorporated by reference) are widely distributed from bacteria to animals and are involved in various biological functions such as glycan metabolism or bacterial pathogenesis (Karamanos, *Adv. Biochem*. **2013**, 1, 81). Endoglycosidases are typically specific for hydrolysis of one or two glycan types. Some are specific for hydrolysis of high-mannose glycans (e.g. EndoA, EndoD, EndoT), while others in addition to high-mannose also cleave hybrid glycans (e.g. EndoF1, EndoH). Endoglycosidases specific for hydrolysis of complex glycans also exist in several variants. An overview of different activities is disclosed in Freeze *et al*. in *Curr. Protoc. Mol. Biol.*, **2010**, 89:17.13A.1-17, incorporated by reference herein. EndoS cleaves biantennary, triantennary and tetraantennary complex glycans, but its activity is nearly exclusively limited to antibodies, in particular the heavy chain of IgG.

EndoX and EndoY are two distinct endoglycosidases, which are preferably individually selected from the group consisting of EndoA, EndoBi, EndoBH, EndoBT, EndoCE, EndoD, EndoE, EfEndo18A, EndoF1, EndoF2, EndoF3, EndoH, EndoLL, EndoM, EndoOm, EndoS, and EndoT. These endoglycosidases and their amino acid sequences are known to the skilled person. Here below, some preferred amino acid sequences for specific endoglycosidases are given.

In a preferred embodiment, EndoS has at least 80 %, preferably at least 90 %, more preferably at least 95 % sequence identity with SEQ ID No.4 or SEQ ID No.5, most preferably EndoS has 100 % sequence identity with SEQ ID No.4 or SEQ ID No.5. In one embodiment, EndoS has SEQ ID No.4 or SEQ ID No.5. Preferably, EndoS has the indicated sequence identities with SEQ ID No.4.

SEQ ID No.4:

MPSIDSLHYLSENSKKEFKEELSKAGQESQKVKEILAKAQQADKQAQELAKMKIPEKIPMKPLHGPLYGGYF
RTWHDKTSDPTEKDKVNSMGELPKEVDLAFIFHDWTKDYSLFWKELATKHVPKLNKQGTRVIRTIPWRFLAG
GDNSGIAEDTSKYPNTPEGNKALAKAIVDEYVYKYNLDGLDVDVEHDSIPKVDKKEDTAGVERSIQVFEEIG
KLIGPKGVDKSRLFIMDSTYMADKNPLIERGAPYINLLLVQVYGSQGEKGGWEPVSNRPEKTMEERWQGYSK
YIRPEQYMIGFSFYEENAQEGNLWYDINSRKDEDKANGINTDITGTRAERYARWQPKTGGVKGGIFSYAIDR
DGVAHQPKKYAKQKEFKDATDNIFHSDYSVSKALKTVMLKDKSYDLIDEKDFPDKALREAVMAQVGTRKGDL
ERFNGTLRLDNPAIQSLEGLNKFKKLAQLDLIGLSRITKLDRSVLPANMKPGKDTLETVLETYKKDNKEEPA

TIPPVSLKVSGLTGLKELDLSGFDRETLAGLDAATLTSLEKVDISGNKLDLAPGTENRQIFDTMLSTISNHV
GSNEQTVKFDKQKPTGHYPDTYGKTSLRLPVANEKVDLQSQLLFGTVTNQGTLINSEADYKAYQNHKIAGRS
FVDSNYHYNNFKVSYENYTVKVTDSTLGTTTDKTLATDKEETYKVDFFSPADKTKAVHTAKVIVGDEKTMMV
NLAEGATVIGGSADPVNARKVFDGQLGSETDNISLGWDSKQSIIFKLKEDGLIKHWRFFNDSARNPETTNKP
IQEASLQIFNIKDYNLDNLLENPNKFDDEKYWITVDTYSAQGERATAFSNTLNNITSKYWRVVFDTKGDRYS
SPVVPELQILGYPLPNADTIMKTVTTAKELSQQKDKFSQKMLDELKIKEMALETSLNSKIFDVTAINANAGV
LKDCIEKRQLLKK

SEQ ID No.5:

MGSSHHHHHHSSGLVPRGSHMPSIDSLHYLSENSKKEFKEELSKAGQESQKVKEILAKAQQADKQAQELAKM
KIPEKIPMKPLHGPLYGGYFRTWHDKTSDPTEKDKVNSMGELPKEVDLAFIFHDWTKDYSLFWKELATKHVP
KLNKQGTRVIRTIPWRFLAGGDNSGIAEDTSKYPNTPEGNKALAKAIVDEYVYKYNLDGLDVDVEHDSIPKV
DKKEDTAGVERSIQVFEEIGKLIGPKGVDKSRLFIMDSTYMADKNPLIERGAPYINLLLVQVYGSQGEKGGW
EPVSNRPEKTMEERWQGYSKYIRPEQYMIGFSFYEENAQEGNLWYDINSRKDEDKANGINTDITGTRAERYA
RWQPKTGGVKGGIFSYAIDRDGVAHQPKKYAKQKEFKDATDNIFHSDYSVSKALKTVMLKDKSYDLIDEKDF
PDKALREAVMAQVGTRKGDLERFNGTLRLDNPAIQSLEGLNKFKKLAQLDLIGLSRITKLDRSVLPANMKPG
KDTLETVLETYKKDNKEEPATIPPVSLKVSGLTGLKELDLSGFDRETLAGLDAATLTSLEKVDISGNKLDLA
PGTENRQIFDTMLSTISNHVGSNEQTVKFDKQKPTGHYPDTYGKTSLRLPVANEKVDLQSQLLFGTVTNQGT
LINSEADYKAYQNHKIAGRSFVDSNYHYNNFKVSYENYTVKVTDSTLGTTTDKTLATDKEETYKVDFFSPAD
KTKAVHTAKVIVGDEKTMMVNLAEGATVIGGSADPVNARKVFDGQLGSETDNISLGWDSKQSIIFKLKEDGL
IKHWRFFNDSARNPETTNKPIQEASLQIFNIKDYNLDNLLENPNKFDDEKYWITVDTYSAQGERATAFSNTL
NNITSKYWRVVFDTKGDRYSSPVVPELQILGYPLPNADTIMKTVTTAKELSQQKDKFSQKMLDELKIKEMAL
ETSLNSKIFDVTAINANAGVLKDCIEKRQLLKK

In a preferred embodiment, EndoH has at least 80 %, preferably at least 90 %, more preferably at least 95 % sequence identity with SEQ ID No.6, most preferably EndoH has 100 % sequence identity with SEQ ID No.6. In one embodiment, EndoH has SEQ ID No.6. SEQ ID No.6:

APAPVKQGPTSVAYVEVNNNSMLNVGKYTLADGGGNAFDVAVIFAANINYDTGTKTAYLHFNENVQRVLDNA
VTQIRPLQQQGIKVLLSVLGNHQGAGFANFPSQQAASAFAKQLSDAVAKYGLDGVDFDDEYAEYGNNGTAQP
NDSSFVHLVTALRANMPDKIISLYNIGPAASRLSYGGVDVSDKFDYAWNPYYGTWQVPGIALPKAQLSPAAV
EIGRTSRSTVADLARRTVDEGYGVYLTYNLDGGDRTADVSAFTRELYGSEAVRTP

In a preferred embodiment, EndoF1 has at least 80 %, preferably at least 90 %, more preferably at least 95 % sequence identity with SEQ ID No.7, most preferably EndoF1 has 100 % sequence identity with SEQ ID No.7. In one embodiment, EndoF1 has SEQ ID No.7. SEQ ID No.7:

AVTGTTKANIKLFSFTEVNDTNPLNNLNFTLKNSGKPLVDMVVLFSANINYDAANDKVFVSNNPNVQHLLTN
RAKYLKPLQDKGIKVILSILGNHDRSGIANLSTARAKAFAQELKNTCDLYNLDGVFFDDEYSAYQTPPPSGF
VTPSNNAAARLAYETKQAMPNKLVTVYVYSRTSSFPTAVDGVNAGSYVDYAIHDYGGSYDLATNYPGLAKSG
MVMSSQEFNQGRYATAQALRNIVTKGYGGHMIFAMDPNRSNFTSGQLPALKLIAKELYGDELVYSNTPYSKD
W

In a preferred embodiment, EndoF2 has at least 80 %, preferably at least 90 %, more preferably at least 95 % sequence identity with SEQ ID No.8, most preferably EndoF2 has 100 % sequence identity with SEQ ID No.8. In one embodiment, EndoF2 has SEQ ID No.8. SEQ ID No.8:

MAVNLSNLIAYKNSDHQISAGYYRTWRDSATASGNLPSMRWLPDSLDMVMVFPDYTPPENAYWNTLKTNYVP
YLHKRGTKVIITLGDLNSATTTGGQDSIGYSSWAKGIYDKWVGEYNLDGIDIDIESSPSGATLTKFVAATKA
LSKYFGPKSGTGKTFVYDTNQNPTNFFIQTAPRYNYVFLQAYGRSTTNLTTVSGLYAPYISMKQFLPGFSFY
EENGYPGNYWNDVRYPQNGTGRAYDYARWQPATGKKGGVFSYAIERDAPLTSSNDNTLRAPNFRVTKDLIKI
MNP

In a preferred embodiment, EndoF3 has at least 80 %, preferably at least 90 %, more preferably at least 95 % sequence identity with SEQ ID No.9, most preferably EndoF3 has 100 % sequence identity with SEQ ID No.9. In one embodiment, EndoF3 has SEQ ID No.9. SEQ ID No.9:

MATALAGSNGVCIAYYITDGRNPTFKLKDIPDKVDMVILFGLKYWSLQDTTKLPGGTGMMGSFKSYKDLDTQ
IRSLQSRGIKVLQNIDDDVSWQSSKPGGFASAAAYGDAIKSIVIDKWKLDGISLDIEHSGAKPNPIPTFPGY
AATGYNGWYSGSMAATPAFLNVISELTKYFGTTAPNNKQLQIASGIDVYAWNKIMENFRNNFNYIQLQSYGA
NVSRTQLMMNYATGTNKIPASKMVFGAYAEGGTNQANDVEVAKWTPTQGAKGGMMIYTYNSNVSYANAVRDA
VKN

In a preferred embodiment, EfEndo18A has at least 80 %, preferably at least 90 %, more preferably at least 95 % sequence identity with SEQ ID No.10, most preferably EfEndo18A has 100 % sequence identity with SEQ ID No.10. In one embodiment, EfEndo18A has SEQ ID No.10. SEQ ID No.10:

ASTVTPKTVMYVEVNNHDFNNVGKYTLAGTNQPAFDMGIIFAANINYDTVNKKPYLYLNERVQQTLNEAETQ
IRPVQARGTKVLLSILGNHEGAGFANFPTYESADAFAAQLEQVVNTYHLDGIDFDDEYAEYGKNGTPQPNNS
SFIWLLQALRNRLGNDKLITFYNIGPAAANSSANPQMSSLIDYAWNPYYSTWNPPQIAGMPASRLGASAVEV
GVNQNLAAQYAKRTKAEQYGIYLMYNLPGKDSSAYISAATQELYGRKTNYSPTVPTP

These preferred sequences for the individual endoglycosidases also apply to the fusion enzyme according to the invention. Thus, for example in case EndoX is EndoS, it is preferred that the amino acid sequence of EndoS is as defined here above. The skilled person is capable of applying the sequences provided above to the fusion enzyme according to formula (**1**).

In one embodiment, the enzyme according to the invention comprises an amino acid sequence selected from SEQ ID NO:4 – SEQ ID NO:10, connected via an amino acid sequence selected from SEQ ID NO:11 and SEQ ID NO:12 to another amino acid sequence selected from SEQ ID NO:4 – SEQ ID NO:10, individually having at least 50% sequence identity, preferably at least 70%, more preferably at least 80% sequence identity with the individual SEQ IDs, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with respect to each one of SEQ ID NO:4 – SEQ ID NO:12. In a preferred embodiment, these sequence identities apply to the combination of SEQ IDs in the fusion enzyme according to the invention.

Preferably, the enzyme of the invention, having the above indicated sequence identities with respect to SEQ ID NO: 2, has EndoS and EndoH activity. Most preferably, the enzyme according to the invention has 100% sequence identity with SEQ ID NO: 2. In one embodiment, the enzyme according to the invention comprising SEQ ID NO:4 connected via SEQ ID NO:11 to SEQ ID NO:6, individually having at least 50% sequence identity, preferably at least 70%, more preferably at least 80% sequence identity with the individual SEQ IDs, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with respect to SEQ ID NO:4, SEQ ID NO:11 and SEQ ID NO:6. In a preferred embodiment, these sequence identities apply to the combination of SEQ ID NO:4, SEQ ID NO:11 and SEQ ID NO:6.

Preferably, the enzyme of the invention, having the above indicated sequence identities with respect to SEQ ID NO: 1, has EndoS and EndoH activity. Most preferably, the enzyme according to the invention has 100% sequence identity with SEQ ID NO:1. In one embodiment, the enzyme according to the invention comprising SEQ ID NO:4 connected via SEQ ID NO:12 to SEQ ID NO:6, individually having at least 50% sequence identity, preferably at least 70%, more preferably at least 80% sequence identity with the individual SEQ IDs, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with respect to SEQ ID NO:4, SEQ ID NO:12 and SEQ ID NO:6.

In a preferred embodiment, the fusion enzyme according to the invention has at least 50% sequence identity, preferably at least 70%, more preferably at least 80% sequence identity, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or most preferably 100% sequence identity with respect to any one of SEQ ID NO:1, 2 and 13 – 21. Preferred sequence IDs are selected from SEQ ID NO:1, 2, 17, 19 and 21. Most preferred sequence IDs are selected from SEQ ID NO:1, 2 and 21.

In one embodiment, the fusion enzyme according to the invention has at least 50% sequence identity, preferably at least 70%, more preferably at least 80% sequence identity, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or most preferably 100% sequence identity with respect to SEQ ID NO:1. In one embodiment, the fusion enzyme according to the invention has at least 50% sequence identity, preferably at least 70%, more preferably at least 80% sequence identity, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or most preferably 100% sequence identity with respect to SEQ ID NO:2. In one embodiment, the fusion enzyme according to the invention has at least 50% sequence identity, preferably at least 70%, more preferably at least 80% sequence identity, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or most preferably 100% sequence identity with respect to SEQ ID NO:13. In one embodiment, the fusion enzyme according to the invention has at least 50% sequence identity, preferably at least 70%, more preferably at least 80% sequence identity, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or most preferably 100% sequence identity with respect to SEQ ID NO:14. In one embodiment, the fusion enzyme

according to the invention has at least 50% sequence identity, preferably at least 70%, more preferably at least 80% sequence identity, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or most preferably 100% sequence identity with respect to SEQ ID NO:15. In one embodiment, the fusion enzyme according to the invention has at least 50% sequence identity, preferably at least 70%, more preferably at least 80% sequence identity, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or most preferably 100% sequence identity with respect to SEQ ID NO:16. In one embodiment, the fusion enzyme according to the invention has at least 50% sequence identity, preferably at least 70%, more preferably at least 80% sequence identity, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or most preferably 100% sequence identity with respect to SEQ ID NO:17. In one embodiment, the fusion enzyme according to the invention has at least 50% sequence identity, preferably at least 70%, more preferably at least 80% sequence identity, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or most preferably 100% sequence identity with respect to SEQ ID NO:18. In one embodiment, the fusion enzyme according to the invention has at least 50% sequence identity, preferably at least 70%, more preferably at least 80% sequence identity, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or most preferably 100% sequence identity with respect to SEQ ID NO:19. In one embodiment, the fusion enzyme according to the invention has at least 50% sequence identity, preferably at least 70%, more preferably at least 80% sequence identity, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or most preferably 100% sequence identity with respect to SEQ ID NO:20. In one embodiment, the fusion enzyme according to the invention has at least 50% sequence identity, preferably at least 70%, more preferably at least 80% sequence identity, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%, or most preferably 100% sequence identity with respect to SEQ ID NO:21.

In one embodiment, EndoX and EndoY are two distinct endoglycosidases and both are selected from the group consisting of EndoA, EndoBi, EndoBH, EndoBT, EndoCE, EndoD, EndoE, EfEndo18A, EndoF1, EndoF2, EndoF3, EndoH, EndoLL, EndoM, EndoOm, EndoS, and EndoT. A preferred group of endoglycosidases to be used as EndoX and EndoY consists of EndoE, EfEndo18A, EndoF1, EndoF2, EndoF3, EndoH, EndoS and EndoT, more preferably of EndoF1, EndoF2, EndoF3, EfEndo18A, EndoH and EndoS. In one embodiment, at least one of EndoX and EndoY, preferably EndoX, is selected from the group consisting of EndoF2, EndoF3 and EndoS. In one embodiment, at least one of EndoX and EndoY, preferably EndoY, is selected from the group consisting of EfEndo18A EndoF1 and EndoH.

In one embodiment, one of EndoX and EndoY is an endoglycosidase capable of cleaving a glycan of the high-mannose type, such as EndoA, EndoE, EfEndo18A, EndoF1, EndoH, EndoM, or EndoT.

Preferably, the endoglycosidase capable of cleaving a glycan of the high-mannose type is selected from the group consisting of EndoE, EfEndo18A, EndoF1, EndoH and EndoT, more preferably selected from the group consisting of EfEndo18A, EndoF1 and EndoH. Most preferably, the endoglycosidase capable of cleaving a glycan of the high-mannose type is EndoH. Preferably, the other of EndoX and EndoY is an endoglycosidase having a different activity, preferably an endoglycosidase capable of cleaving a glycan of the complex type.

In one embodiment, one of EndoX and EndoY is an endoglycosidase capable of cleaving a glycan of the complex type, such as EndoE, EndoF2, EndoF3 and EndoS. Preferably, the endoglycosidase capable of cleaving a glycan of the complex type is selected from the group consisting of EndoF2, EndoF3, and EndoS, more preferably selected from the group consisting of EndoF3 and EndoS. Most preferably, the endoglycosidase capable of cleaving a glycan of the complex type is EndoS. Preferably, the other of EndoX and EndoY is an endoglycosidase having a different activity, preferably an endoglycosidase capable of cleaving a glycan of the high-mannose type.

It is especially preferred that the fusion enzyme according to the invention contains two distinct endoglycosidases which differ in endoglycosidase activity, as two distinct endoglycosidase activities can as such be combined in a single enzyme. Thus, EndoX and EndoY preferably each have a distinct endoglycosidase activity selected from the capacity of hydrolysing high-mannose glycans, the capacity of hydrolysing complex glycans and the capacity of hydrolysing hybrid glycans, more preferably selected from the capacity of hydrolysing high-mannose glycans and the capacity of hydrolysing complex glycans. Preferably, one of EndoX and EndoY is an endoglycosidase that capable of hydrolysing high-mannose glycans, and the other endoglycosidase is capable of hydrolysing complex glycans. Preferably, the endoglycosidase that is capable of hydrolysing high-mannose glycans is also capable of hydrolysing hybrid glycans. Preferably, the endoglycosidase that is capable of hydrolysing complex glycans is capable of hydrolysing biantennary and/or triantennary complex glycans, most preferably all complex glycans.

For example, when EndoX is EndoS and EndoY is EndoH, the resulting fusion enzyme exhibits both EndoS and EndoH activity, and is capable of trimming complex glycans on glycoproteins (such as antibodies) at the core GlcNAc unit, leaving only the core GlcNAc residue on the glycoprotein (EndoS activity) as well as well as trimming (splitting off) high-mannose glycans (EndoH activity). Surprisingly, both activities of the fusion enzyme function smoothly at a pH around 7 – 8, while monomeric EndoH requires a pH in the range of 5 – 6, or even a pH of 6 to operate optimally.
In one embodiment, EndoX and EndoY are two distinct endoglycosidases that differ in optimal pH of at least 1 pH units, preferably at least 1.5 pH unit, most preferably at least 2 pH units. The skilled person is aware of the pH optimum that belongs to specific endoglycosidases. Such fusion enzymes may be active at a specific pH, which is not the optimal pH of at least one of EndoX and EndoY.

In a preferred embodiment, one of EndoX and EndoY is selected from EndoF2, EndoF3 or EndoS, and the other of EndoX and EndoY is selected from EndoD, EndoH, EndoE, EfEndo18A, EndoT or EndoF1. Preferably, EndoX is selected from EndoF2, EndoF3 or EndoS, and EndoY is selected from EndoD, EndoH, EndoE, EfEndo18A, EndoT or EndoF1. A such, the fusion enzyme is capable of hydrolysing complex glycans (EndoF2, EndoF3 and EndoS activity) as well as hydrolysing high-mannose glycans (EndoD, EndoF1, EndoH, EndoE, EfEndo18A, EndoT or EndoF1 activity). In one embodiment, EndoX is EndoS, and EndoY is preferably EndoD, EndoF1, EndoH, EndoE, EfEndo18A, EndoT or EndoF1, more preferably EndoY is EndoF1, EndoH or EfEndo18A, most preferably EndoY is EndoH. Most preferably, EndoX is EndoS and EndoY is EndoH. Alternatively, EndoX is EndoF2 and EndoY is preferably EndoD, EndoH, EndoE, EfEndo18A, EndoT or EndoF1, more preferable EndoY is EndoF1, EndoH or EfEndo18A, most preferably EndoY is EndoF1. Most preferably, EndoX is EndoF2 and EndoY is EndoF1. Alternatively, EndoX is EndoF3 and EndoY is preferably EndoD, EndoH, EndoE, EfEndo18A, EndoT or EndoF1, more preferably EndoY is EndoF1, EndoH or EfEndo18A, most preferably EndoY is EndoH. Most preferably, EndoX is EndoF3 and EndoY is EndoH.

In one embodiment, Endo X and EndoY are both individually selected from EndoF1, EndoF2, EndoF3, EfEndo18A, EndoS and EndoH. Preferably, EndoX is selected from EndoF2, EndoF3 and EndoS and EndoY is selected from EndoF1, EfEndo18A and EndoH.

In one embodiment, one of EndoX and EndoY is EndoS or EndoF3, and the other one of EndoX and EndoY is EndoF1 or EndoH. Preferably, EndoX is EndoS or EndoF3, and EndoY is EndoF1 or EndoH.

In a preferred embodiment, the fusion enzyme according to the invention is selected from the group consisting of enzymes of structure (**1**), wherein EndoX = EndoF3 and EndoY = EndoH; EndoX = EndoF3 and EndoY = EndoE; EndoX = EndoF3 and EndoY = EfEndo18A; EndoX = EndoF3 and EndoY = EndoT; EndoX = EndoF3 and EndoY = EndoF1; EndoX = EndoS and EndoY = EndoH; EndoX = EndoS and EndoY = EndoE; EndoX = EndoS and EndoY = EfEndo18A; EndoX = EndoS and EndoY = EndoT; EndoX = EndoS and EndoY = EndoF1; EndoX = EndoF2 and EndoY = EndoH; EndoX = EndoF2 and EndoY = EndoE; EndoX = EndoF2 and EndoY = EfEndo18A; EndoX = EndoF2 and EndoY = EndoT; and EndoX = EndoF2 and EndoY = EndoF1. More preferably, the fusion enzymes according to the invention is selected from the group consisting of enzymes of structure (**1**), wherein EndoX = EndoF3 and EndoY = EndoH; EndoX = EndoF3 and EndoY = EfEndo18A; EndoX = EndoF3 and EndoY = EndoF1; EndoX = EndoS and EndoY = EndoH; EndoX = EndoS and EndoY = EfEndo18A; EndoX = EndoS and EndoY = EndoF1; EndoX = EndoF2 and EndoY = EndoH; EndoX = EndoF2 and EndoY = EfEndo18A; and EndoX = EndoF2 and EndoY = EndoF1. Even more preferably, the fusion enzymes according to the invention is selected from the group consisting of enzymes of structure (**1**), wherein EndoX = EndoF3 and EndoY = EndoH; EndoX = EndoS and EndoY = EndoH; EndoX = EndoS and EndoY = EfEndo18A; EndoX = EndoS

and EndoY = EndoF1; and EndoX = EndoF2 and EndoY = EndoF1. Most preferably, the fusion enzymes according to the invention is an enzyme of structure (**1**), wherein EndoX = EndoS and EndoY = EndoH.

Linker

In the enzyme according to the invention, EndoX and EndoY are preferably linked by a linker. In case a linker is present, p = 1. In case no linker is present, p = 0. Preferably, p = 1. Linkers for fusion enzymes are known in the art, and any suitable linker may be used, including flexible and rigid linkers. Further guidance can be found in Chen *et al.*, *Adv. Drug Deliv. Rev.* **2013**, 65, 1357-1369 and *Fusion Protein Technologies for Biopharmaceuticals: Application and Challenges*, Chapter 4: *Fusion Protein Linkers: Effects on Production, Bioactivity, and Pharmacokinetics*, **2013**, John Wiley & Sons, Inc, both of which are incorporated herein in their entirety. Preferably, said linker is a flexible linker allowing the adjacent protein to move relative freely.

In one embodiment, the linker, preferably the flexible linker, is composed of amino residues and has a length of 1 to 100 amino acid residues, preferably 3 to 59, 10 to 45 or 15 to 40 amino acid residues, such as 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 amino acid residues.

In one embodiment, the linker, preferably the flexible linker, is composed of amino residues like glycine, serine, histidine and/or alanine and has a length of 3 to 59 amino acid residues, preferably 10 to 45 or 15 to 40 amino acid residues, such as 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 amino acid residues.

The linker preferably comprises one or more flexible domains, that provide flexibility to the linker. Preferably, one or two, most preferably two of such flexible domains are comprised in the linker. Such flexible domains are known in the art and are typically composed of glycine, serine and/or threonine. In one embodiment, the linker comprises at least one glycine, serine and/or threonine residue. Preferably, at least 40 % of the amino acids of the linker are selected from glycine, serine and threonine, more preferably 50 – 90 %, most preferably 70 – 85 % of the amino acids of the linker are selected from glycine, serine and threonine. In one embodiment, the linker does not comprise threonine and the above ranges apply to glycine and serine.

Specific suitable flexible domains include GS-domains (such as $(G_4S)_n$, wherein n is an integer in the range 1 – 10, preferably 1 – 6, most preferably 2 – 4), poly-G (such as G_m, wherein m is an integer in the range 1 – 30, preferably 3 – 20, most preferably 5 – 10), GSAGSAAGSGEF, EGKSSGSGSESKST, PAS linkers (Pro, Ala, Ser based linkers; see Schlapschy et al., *Protein Eng Des Sel.* **2013**, 26, 489-501, incorporated by reference) and extended recombinant polypeptide (XTEN) linkers (see Podust et al., *Protein Eng Des Sel.* **2013,** 26, 743-753, incorporated by reference). GS-domains, consisting of stretches of glycine and serine residues, are most preferred.

So, in one embodiment, the linker comprises one or more $(G_4S)_n$ domains, preferably one or two, most preferably two domains.

Alternatively or additionally, the linker may comprise one or more rigid domains, such as □-helix forming domains, such as $(EAAAK)_o$ or $A(EAAAK)_oA$ (wherein o is an integer in the range 1 – 10, preferably 2 – 5, most preferably 3 or 4), and proline-rich domains, such as $(XP)_q$ (wherein X is any amino acid, preferably selected from alanine, lysine and glutamine, and q is an integer in the range 2 – 25, preferably 5 – 17).

Optionally, the linker comprises a tag for ease of purification and/or detection as known in the art, such as an Fc-tag, FLAG-tag, poly(His)-tag, $(RP)_6R$-tag, HA-tag and Myc-tag. Such a tag may also be present elsewhere in the linker according to the invention. Thus, in one embodiment, the fusion enzyme according to the invention comprises a tag for ease of purification and/or detection, such as an Fc-tag, FLAG-tag, poly(His)-tag, $(RP)_6R$-tag, HA-tag and Myc-tag, most preferably a poly(His)-tag. In one embodiment, the fusion enzyme according to the invention comprises a linker, i.e. p = 1, and the linker comprises a tag for ease of purification and/or detection, such as an Fc-tag, FLAG-tag, poly(His)-tag, $(RP)_6R$-tag, HA-tag and Myc-tag, most preferably a poly(His)-tag. The tag may be located at the C-terminus of the linker, at the N-terminus of the linker or may be embedded in the linker with further amino acid(s) at either side of the tag. The latter conformation is preferred, especially when flexible domains are located at either side of the tag, as it brings optimal accessibility of the tag for binding to an affinity matrix.

In one embodiment, the linker has the structure $(G_4S)_{n1}(H)_r(EF)_s(G_4S)_{n2}$, wherein n1 and n2 individually are integers in the range 1 – 10, preferably 1 – 6, even more preferably 2 – 4, most preferably 3, and r is an integer in the range of 2 – 10, preferably 4 – 8, most preferably 6, and s = 0 or 1. In one embodiment, the linker has the structure $(G_4S)_3(H)_6(G_4S)_3$, i.e. wherein n1 = 3, n2 = 3, r = 6 and s = 0 (amino acids 950 to 985 of SEQ ID No. 2). In one embodiment, the linker has the structure $(G_4S)_3(H)_6EF(G_4S)_3$, i.e. wherein n1 = 3, n2 = 3, r = 6 and s = 1 (amino acids 950 to 987 of SEQ ID No. 1).

In a preferred embodiment, the linker has at least 80 %, preferably at least 90 %, more preferably at least 95 % sequence identity with SEQ ID No.11 or 12, most preferably the linker has 100 % sequence identity with SEQ ID No.11 or SEQ ID No.12. In one embodiment, the linker has SEQ ID No.11. In one embodiment, the linker has SEQ ID No.12.

SEQ ID No.11: GGGGSGGGGSGGGGSHHHHHHEFGGGGSGGGGSGGGGS

SEQ ID No.12: GGGGSGGGGSGGGGSHHHHHHGGGGSGGGGSGGGGS

The fusion enzyme according to the invention can be prepared by routine techniques known in the art, such as introducing an expression vector (e.g. plasmid) comprising the enzyme coding sequence into a host cell (e.g. *E. coli*) for expression, from which the enzyme can be isolated.

Alternatively, the enzyme is produced by transient expression in CHO. A possible approach for the preparation and purification of the fusion enzyme according to the invention is given in examples 1 – 4 and 16 – 24, and its functioning is demonstrated in examples 5, 6, 8, 13 – 15 and 25 – 37, wherein various glycoproteins, including trastuzumab and high-mannose trastuzumab, are efficiently trimmed in a single step.

Preferred fusion enzyme

In an especially preferred embodiment, the invention concerns a fusion enzyme comprising the two endoglycosidases EndoS and EndoH. In a particular example the two endoglycosidases EndoS and EndoH are connected via a linker, preferably a -$(Gly_4Ser)_3$-$(His)_6$-$(Gly_4Ser)_3$- linker. The fusion enzyme according to the invention as also referred to as EndoSH. In one embodiment, the enzyme according to the invention has at least 50% sequence identity with SEQ ID NO: 1, preferably at least 70%, more preferably at least 80% sequence identity with SEQ ID NO: 1, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with SEQ ID NO: 1. Preferably, the enzyme of the invention, having the above indicated sequence identity to SEQ ID NO: 1, has EndoS and EndoH activity. Most preferably, the enzyme according to the invention has 100% sequence identity with SEQ ID NO: 1. In one embodiment, the enzyme according to the invention has at least 50% sequence identity with SEQ ID NO: 2, preferably at least 70%, more preferably at least 80% sequence identity with SEQ ID NO: 2, such as at least 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with SEQ ID NO: 2. Preferably, the enzyme of the invention, having the above indicated sequence identity to SEQ ID NO: 2, has EndoS and EndoH activity. Most preferably, the enzyme according to the invention has 100% sequence identity with SEQ ID NO: 2.

Also encompassed are fusion enzymes of EndoS and EndoH, wherein the linker is replaced by another suitable linker known in the art, wherein said linker may be rigid or flexible. Preferably, said linker is a flexible linker allowing the adjacent protein domains to move relative freely to one another. Preferably, said flexible linker is composed of amino residues like glycine, serine, histidine and/or alanine and has a length of 3 to 59 amino acid residues, preferably 10 to 45 or 15 to 40 amino acid residues, such as 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 amino acid residues, or 20 to 38, 25 to 37 or 30 to 36 amino acid residues. Optionally, the fusion enzyme is covalently linked to, or comprises, a tag for ease of purification and or detection as known in the art, such as an Fc-tag, FLAG-tag, poly(His)-tag, HA-tag and Myc-tag. Trimming of glycoproteins is known in the art, from e.g. Yamamoto, *Biotechnol. Lett.* **2013**, 35, 1733, WO 2007/133855 or WO 2014/065661, which are incorporated herein in their entirety. The enzyme according to this embodiment exhibits both EndoS and EndoH activity, and is capable of trimming glycans on glycoproteins (such as antibodies) at the core GlcNAc unit, leaving only the core GlcNAc residue on the glycoprotein (EndoS activity) as well as well as high-mannose glycans

(EndoH activity). Surprisingly, both activities of the fusion enzyme function smoothly at a pH around 7 – 8, while monomeric EndoH requires a pH of 6 to operate optimally.

Use

A further aspect of the invention concerns the use of the fusion enzyme according to the invention for trimming glycoproteins, preferably for trimming antibodies. Trimming may also be referred to as deglycosylation and is further defined here below, in the context of the process according to the invention. The use according to this aspect may occur in vitro or in vivo.

Process for trimming of glycoproteins

The fusion enzyme according to the invention is particularly suited for trimming of glycoproteins. Thus, in a further aspect, the invention concerns a process for the trimming of glycoproteins. The process according to this aspect may occur in vitro or in vivo. Trimming of glycoproteins is known in the art, from e.g. Yamamoto, *Biotechnol. Lett.* **2013**, 35, 1733, WO 2007/133855 or WO 2014/065661, which are incorporated herein in their entirety. Glycoproteins, such as antibodies, typically contain different glycoforms, which require different endoglycosidases to remove. The fusion enzymes of the invention are especially suitable to deglycosylate in a single step a glycoprotein having two different glycan chains. Thus, in one embodiment, the glycoprotein that is subjected to the process according to the invention comprises at least two distinct glycans, preferably two distinct glycans. Preferably, the glycoprotein comprises at least one high-mannose glycan and at least one complex glycan, more preferably the glycoprotein comprises at least one high-mannose glycan, at least one hybrid glycan and at least one complex glycan. The complex glycan may be a bi-, tri-, or tetraantennary glycan.

In an especially preferred embodiment, the glycoprotein is an antibody.

The process according to the present aspect may also be referred to as a process for modifying a glycoprotein. The process comprised contacting the glycoprotein with a fusion enzyme according to the invention, to obtain a trimmed glycoprotein. The process may also be referred to as a process for trimming a glycoprotein or deglycosylation of a glycoprotein. Trimming or deglycosylation of a glycoprotein refers to the removal of a glycan from said glycoprotein. The exact structure of the glycan that is removed may vary depending on the exact nature of the endoglycosidases that are present in the fusion enzyme, but the core GlcNAc residue is retained on the glycoprotein at all times. The skilled person will appreciate which fusion enzyme, i.e. which combination of endoglycosidases, is suitable for trimming of which glycosylation pattern of the glycoprotein.

With conventional endoglycosidases, glycoproteins containing a combination of a high-mannose glycan and a complex bi-, tri- or tetraantennary glycan would require two distinct enzymes for trimming, often requiring different buffer conditions and pH ranges. These glycoproteins can now efficiently be trimmed in a single step, without the need to apply buffer exchange to achieve the

optimal pH, with the fusion enzyme according to the invention. Thus, in one embodiment, the glycoprotein, preferably the antibody, comprises at least one high-mannose glycan and at least one complex bi-, tri- or tetraantennary glycan, more preferably at least one high-mannose glycan, at least one hybrid and/or complex bi-, tri- or tetraantennary glycan. For example, the fusion enzyme wherein one of EndoX and EndoY is selected from EndoF2, EndoF3 or EndoS, and the other of EndoX and EndoY is selected from EndoH, EndoE, EfEndo18A, EndoT or EndoF1, is suitable for trimming a glycoprotein comprising a complex N-linked complex glycan and a high-mannose glycan, to obtain a trimmed glycoprotein comprising only the optionally fucosylated core N-acetylglucosamine substituent(s).

The skilled person is aware of suitable conditions to perform the trimming of glycoproteins. For example, the process is carried out in a medium and at a temperature that is effective for trimming glycoproteins. Typically, the media and conditions that apply for one of the individual endoglycosidase enzymes are applicable. As the optimal pH of the individual endoglycosidases may differ, the process may in one embodiment be carried out at a pH which is 0.5 – 3 pH units, preferably 1 – 2 pH units, different from the optimal pH of one or both, preferably one of EndoX and EndoY. For example, in case one of EndoX and EndoY is EndoH, which has an optimal pH or 5 – 6, the process may be carried out at pH 7 – 8. In one embodiment, the trimming performed at a pH in the range of 4 – 9, preferably in the range of 6 – 8, most preferably in the range of 7 – 8. The inventors surprisingly found that the fusion enzyme according to the invention wherein EndoX = EndoS and EndoY = EndoH is fully operational at a pH above 7, whereas the functional pH range for EndoH is 5.0 to 6.0, with the optimum pH at 5.5.
Moreover, the inventors found that the activity of a particular endoglycosidase in a fusion protein can display a higher trimming efficiency compared to the same endoglycosidase as a single enzyme.

The trimming affords trimmed glycoproteins, wherein all glycan moieties present in the original glycoprotein, irrespective of their type and glycoform, are trimmed and only the optionally fucosylated core N-acetylglucosamine substituent(s) remain. Said optionally fucosylated core N-acetylglucosamine substituent is typically bonded via an N-glycosidic bond to the amide nitrogen atom in the side chain of an asparagine amino acid of the glycoprotein, such as N297 when the glycoprotein is an antibody.

The thus obtained trimmed glycoprotein can be used as deemed fit. For example, when the glycoprotein is the product of interest, the trimmed glycoprotein according to the invention is homogeneous with respect to glycosylation patterns. This can be particularly important when the glycoprotein is used as medicament, since the therapeutic efficacy and/or the toxicity may vary for different glycoforms of the glycoprotein. Such unpredictable variations in efficacy and toxicity are eradicated when the process according to the invention is utilized.

Alternatively, the trimmed glycoprotein can be used for further functionalization, such as by introduction of an optionally substituted sugar moiety is known in the art, from e.g. van Geel *et.al*, Bioconjugate Chem, **2015**, 26, 2233, incorporated by reference. The trimmed glycoprotein may be contacted with a compound of the formula S–P, wherein S is an optionally substituted sugar moiety and P is a nucleotide, in the presence of a suitable catalyst, such as a glycosyltransferase or N-acetylglycosyltransferase. The thus obtained modified glycoprotein comprises sugar moiety S connected to the non-reducing end of the trimmed glycan. Using a substituted sugar moiety S, the possibilities for further modification or functionalization of the glycoprotein via said substituent are endless. Such a sequence of reaction steps finds particular use in the preparation of bioconjugates, such as antibody-drug conjugates. Such steps are known to the skilled person, e.g. from WO 2014/065661, incorporated by reference herein.

Examples

RP-HPLC analysis of reduced monoclonal antibodies: Prior to RP-HPLC analysis samples were reduced by incubating a solution of 10 µg (modified) IgG for 15 minutes at 37 °C with 10 mM DTT and 100 mM Tris pH 8.0 in a total volume of 50 µL. A solution of 49% ACN, 49% MQ and 2% formic acid (50 µL) was added to the reduced sample. Reverse phase HPLC was performed on a Agilent 1100 HPLC using a ZORBAX Poroshell 300SB-C8 1 x 75 mm, 5 µm (Agilent Technologies) column run at 1 ml/min at 70 °C using a 16.9 minute linear gradient from 25 to 50 % buffer B (with buffer A = 90% MQ, 10% ACN, 0.1% TFA and buffer B = 90% ACN, 10% MQ, 0.1% TFA).

Mass spectral analysis of monoclonal antibodies: Prior to mass spectral analysis, IgGs were either treated with DTT, which allows analysis of both light and heavy chain, or treated with Fabricator™ (commercially available from Genovis, Lund, Sweden), which allows analysis of the Fc/2 fragment. For analysis of both light and heavy chain, a solution of 20 µg (modified) IgG was incubated for 5 minutes at 37 °C with 100 mM DTT in a total volume of 4 µL. If present, azide-functionalities are reduced to amines under these conditions. For analysis of the Fc/2 fragment, a solution of 20 µg (modified) IgG was incubated for 1 hour at 37 °C with Fabricator™ (1.25 U/µL) in phosphate-buffered saline (PBS) pH 6.6 in a total volume of 10 µL. After reduction or Fabricator-digestion the samples were washed trice with milliQ using an Amicon Ultra-0.5, Ultracel-10 Membrane (Millipore) resulting in a final sample volume of approximately 40 µL. Next, the samples were analyzed by electrospray ionization time-of-flight (ESI-TOF) on a JEOL AccuTOF. Deconvoluted spectra were obtained using Magtran software.

Example 1: Cloning of fusion protein EndoSH into (pET22B) expression vector

A pET22B-vector containing an EndoS-$(G_4S)_3$-$(His)_6$-EF-$(G_4S)_3$-EndoH (EndoSH) coding sequence (EndoSH being identified by SEQ ID NO: 1) between EcoRI-HindIII sites was obtained from Genscript. The DNA sequence for the EndoSH fusion protein consists of the encoding residues 48-995 of EndoS fused via an *N*-terminal linked glycine-serine (GS) linker to the coding residues 41-313 of EndoH. The glycine-serine (GS) linker comprises a -$(G_4S)_3$-$(His)_6$-EF-$(G_4S)_3$- format, allowing spacing of the two enzymes and at the same time introducing a IMAC-purification tag.

Example 2: E. coli expression of fusion protein EndoSH

Expression of the EndoSH fusion protein (identified by SEQ ID NO: 1) starts with the transformation of the plasmid (pET22b-EndoSH) into BL21 cells. Next step is the inoculation of 500 mL culture (LB medium + Ampilicin) with BL21 cells. When the OD600 reached 0.7 the cultures were induced with 1 mM IPTG (500 µL of 1M stock solution).

Example 3: Purification of fusion protein EndoSH from E.coli

After overnight induction at 16 °C the culture were pelleted by centrifugation. The pellet was resuspended in 40 mL PBS and incubated on ice with 5 ml lysozyme (10 mg/mL) for 30 minutes. After half an hour 5 ml 10% Triton-X-100 was added and sonicated (10 minutes) on ice. After the sonification the cell debris was removed by centrifugation (10 minutes 8000xg) followed by filtration through a 0,22 µM-pore diameter filter. Alternatively, lysis of the pellet containing EndoSH can be performed by means of French press. Here the pellet is re-suspended in 10 mL PBS/gram of pellet. The cell suspension is lysed three times under pressure (20000-25000 psi) by French press using Emulsiflex C3, Avestin. After French press the cell debris was removed by centrifugation (20 minutes 10000xg). The soluble extract/fraction was loaded onto a HisTrap HP 5 mL column (GE Healthcare). The column was first washed with buffer A (20 mM Tris buffer, 20 mM imidazole, 500 mM NaCl, pH 7.5). Retained protein was eluted with buffer B (20 mM Tris, 500 mM NaCl, 250 mM imidazole, pH 7.5, 10 mL). Fractions were analyzed by SDS-PAGE on polyacrylamide gels (12%). The fractions that contained purified target protein were combined and the buffer was exchanged against 20 mM Tris pH 7.5 and 150 mM NaCl by dialysis performed overnight at 4 °C. The purified protein was concentrated to at least 2 mg/mL using an Amicon Ultra-0.5, Ultracel-10 Membrane (Millipore). The product is stored at −80 °C prior to further use.

Example 4: CHO expression and purification of fusion protein EndoSH from CHO

EndoSH (identified by SEQ ID NO: 2) was transiently expressed in CHO K1 cells by Evitria (Zurich, Switzerland) at 20 mL scale. The supernatant, containing fusion protein EndoSH, was diluted with elution buffer (2 mL, 20 mM Tris, 500 mM NaCl, 500 mM imidazole) and binding buffer (18 mL, 20 mM Tris, 500 mM NaCl, 5 mM imidazole, pH = 7.4) to a final imidazole concentration of 10 mM. The mixture was loaded onto a Ni-NTA column (GE Healthcare) and the product was eluted following a standard elution protocol. The collected fractions (5 mL) were analysed on an SDS-PAGE (10%) gel. The faction containing product was partially concentrated (~2 mL) and dialyzed against TBS buffer. Protein concentration, determined by nanodrop analysis, was set at 0.5 mg/mL.

Example 5: Trimming of trastuzumab by EndoSH

Trastuzumab (obtained from Epirus biopharma (Utrecht, The Netherlands); 14 mg/mL) in 25 mM Tris buffer pH 8, was trimmed using a concentration of either 0.1 or 1 w/w% EndoSH. The reactions, 350 µg trastuzumab (25 µL) and the appropriate amount of EndoSH, were stirred at 37 °C and analyzed by MS analysis over time, 1 to 3 hours. Samples were subjected to Fabricator treatment

prior to analysis. Full conversions to the trimmed product, which is trimmed to the core GlcNAc sugar residue, was observed after 1 hour at 37 °C with 0.1 w/w% EndoSH.

Example 6: Trimming of high-mannose trastuzumab by fusion protein EndoSH

Trastuzumab having high-mannose glycans (obtained via transient expression in CHO K1 cells in the presence of kifunensine performed by Evitria (Zurich, Switzerland)) (14 mg/mL) in 25 mM Tris buffer pH 8, was trimmed using a concentration of either 0.1 or 1 w/w% EndoSH. The reactions, 350 µg high-mannose trastuzumab (25 µL) and the appropriate amount of EndoSH, were stirred at 37 °C and analyzed by MS analysis over time, 1 – 3 hours. Samples were subjected to Fabricator treatment prior to analysis. Full conversions to the trimmed product, which is trimmed to the core GlcNAc sugar residue, was observed after 3 hours at 37 °C with 1 w/w% EndoSH.

Example 7: Transient expression and purification of cAC10

cAC10 was transiently expressed in CHO K1 cells by Evitria (Zurich, Switzerland) at 5 L scale. The supernatant was purified using a XK 26/20 column packed with 50 mL protein A sepharose. In a single run 5 L supernatant was loaded onto the column followed by washing with at least 10 column volumes of 25 mM Tris pH 7.5, 150 mM NaCl. Retained protein was eluted with 0.1 M Glycine pH 2.7. The eluted cAC10 was immediately neutralized with 1.5 M Tris-HCl pH 8.8 and dialyzed against 25 mM Tris pH 8.0. Next the IgG was concentrated to approximately 20 mg/mL using a Vivaspin Turbo 15 ultrafiltration unit (Sartorius) and stored at −80 °C prior to further use.

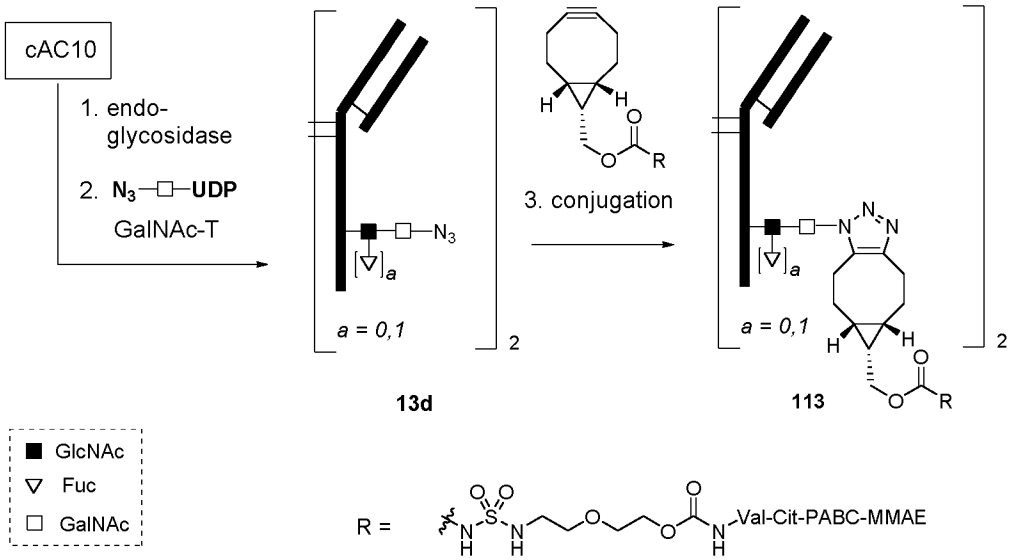
Example 8: Trimming of cAC10 by EndoSH

Glycan trimming of cAC10 (obtained via transient expression in CHO K1 cells performed by Evitria (Zurich, Switzerland)) was performed with fusion protein EndoSH. Thus, cAC10 (14.5 mg/mL) was incubated with EndoSH (1 w/w%) in 25 mM Tris pH 7.5 with 150 mM NaCl for approximately 16 hours at 37 °C. The trimmed IgG was dialyzed against 3 x 1 L of 25 mM Tris-HCl pH 8.0. Mass spectral analysis of a fabricator-digested sample showed three peaks of the Fc/2-fragment belonging to one major product (observed mass 24105 Da, approximately 80% of total Fc/2 fragment), corresponding to core GlcNAc(Fuc)-substituted cAC10, and two minor products (observed masses of 23959 and 24233 Da, approximately 5 and 15% of total Fc/2 fragment), corresponding to core GlcNAc-substituted cAC10 and core GlcNAc(Fuc)-substituted cAC10 with C-terminal lysine.

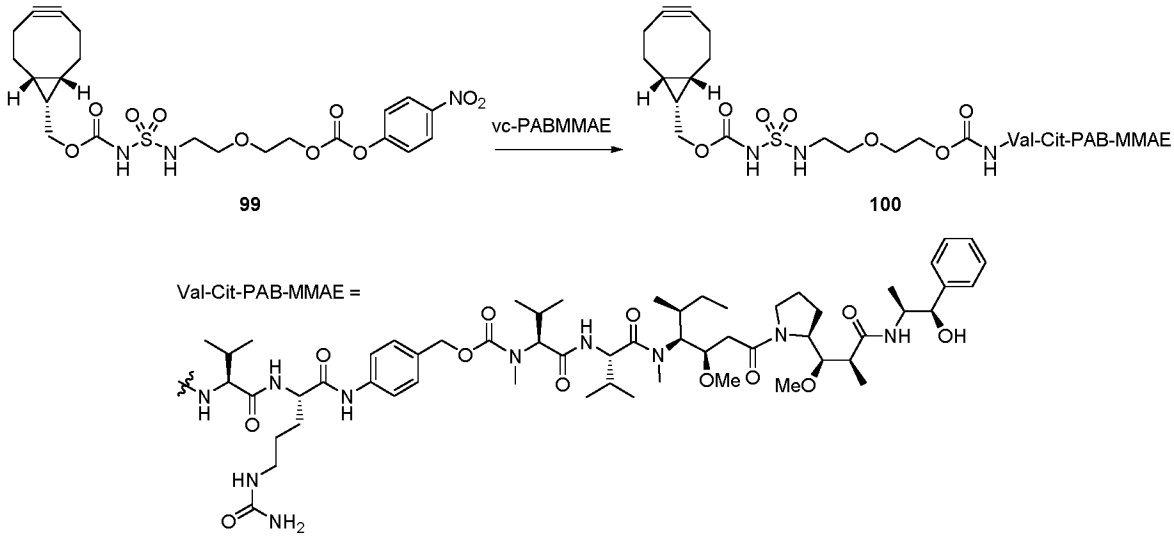
Examples 9 – 12: Preparation of cAC10 bioconjugate

To demonstrate that the antibodies trimmed by the fusion enzyme according to the invention can be further modified, antibody-drug-conjugate **113** has been prepared from the trimmed antibody of Example 8. Compound **99** was prepared via activation of compound **58** as disclosed in and prepared according to Example 50 of WO 2016/053107 (PCT/NL2015/050697). In the second step the trimmed cAC10 was converted to the azido-modified mAb **13d** through the action of His-

TnGalNAcT in the presence of 6-N_3-GalNAc-UDP (commercially available from GlycoHub) as a substrate. The preparation of the cAC10 bioconjugates is schematically depicted here below:



*Example 9: Preparation of compound **100***



A solution of compound **99** (4.7 mg, 9.0 µmol) in DMF (200 µL) was added to solid Val-Cit-PABC-MMAE (vc-PABC-MMAE, 10 mg, 8.1 µmol) followed by addition of Et_3N (3.7 µL, 2.7 mg, 27 µmol). After 23 h, 2′-(ethylenedioxy)bis(ethylamine) (1.3 µL, 1.3 mg, 8.9 µmol) in DMF was added (13 µL of 10% solution in DMF). The mixture was left for 4h and purified *via* reversed phase (C18) HPLC chromatography (30→90% MeCN (1% AcOH) in H_2O (1 % AcOH). The product was obtained as a colourless film (10.7 mg, 7.1 µmol, 87%) LCMS (ESI^+) calculated for $C_{74}H_{117}N_{12}O_{19}S^+$ ($M+H^+$) 1509.83 found 1510.59.

Example 10: Transient expression and purification of His-TnGalNAcT(33-421)

His-TnGalNAcT(33-421) (identified by SEQ ID NO: 33) was codon optimized and transiently expressed in CHO K1 cells by Evitria (Zurich, Switzerland) at 5 L scale. The supernatant was purified using a XK 16/20 column packed with 25 mL Ni sepharose excel (GE Healthcare). Each

run approximately 1.5L supernatant was loaded onto the column followed by washing with at least 10 column volumes of buffer A (20 mM Tris buffer, 5 mM imidazole, 500 mM NaCl, pH 7.5). Retained protein was eluted with buffer B (20 mM Tris, 500 mM NaCl, 500 mM imidazole, pH 7.5). The buffer of the eluted fractions was exchanged to 25 mM Tris pH 8.0 using a HiPrep H26/10 desalting column (GE Healthcare). The purified protein was concentrated to at least 3 mg/mL using a Vivaspin Turbo 4 ultrafiltration unit (Sartorius) and stored at −80 °C prior to further use.

Example 11: Glycosyltransfer of the 6-N_3-GalNAc-UDP to trimmed cAC10 under the action of TnGalNAcT

Substrate 6-N_3-GalNAc-UDP (**11d**) is used for the preparation of the modified biomolecule cAC10-(6-N_3-GalNAc)$_2$ **13d**. Trimmed cAC10 (10 mg/mL), obtained by EndoSH treatment of cAC10 as described above in Example 8, was incubated with the substrate 6-N_3-GalNAc-UDP (2.5 mM, commercially available from GlycoHub) and 0.5 mg/mL His-TnGalNAcT(33-421) (5 w/w%) in 10 mM $MnCl_2$ and 25 mM Tris-HCl pH 8.0 at 30 °C. After 3 hours the amount of His-TnGalNAcT(33-421) was increased to a final concentration of 1 mg/mL (10 w/w%) and the reaction was incubated overnight at 30 °C. Biomolecule **13d** was purified from the reaction mixture on a HiTrap MabSelect SuRe 5 ml column (GE Healthcare) using an AKTA purifier-10 (GE Healthcare). The eluted IgG was immediately neutralized with 1.5 M Tris-HCl pH 8.8 and dialyzed against PBS pH 7.4. Next the IgG was concentrated using an Amicon Ultra-0.5, Ultracel-10 Membrane (Millipore) to a concentration of 23.38 mg/mL. Mass spectral analysis of a fabricator-digested sample showed three peaks of the Fc/2-fragment belonging to one major product (observed mass 24333 Da, approximately 80% of total Fc/2 fragment), corresponding to core 6-N_3-GalNAc-GlcNAc(Fuc)-substituted cAC10, and two minor products (observed masses of 24187 and 24461 Da, approximately 5 and 15% of total Fc/2 fragment), corresponding to core 6-N_3-GalNAc-GlcNAc-substituted cAC10 and core 6-N_3-GalNAc-GlcNAc(Fuc)-substituted cAC10 with C-terminal lysine.

*Example 12: Conjugation of **13d** with **100** to obtain conjugate **113***

A bioconjugate according to the invention was prepared by conjugation of compound **100** as linker-conjugate to modified biomolecule **13d** as biomolecule. To a solution of cAC10(azide)$_2$ (**13d**) (287 µL, 6.7 mg, 23.38 mg/ml in PBS pH 7.4) was added PBS pH 7.4 (133 µL) and compound **100** (27 µL, 10 mM solution in DMF). The reaction was incubated at rt overnight followed by purification on a Superdex200 10/300 GL (GE Healthcare) on an AKTA Purifier-10 (GE Healthcare). Mass spectral analysis of the fabricator-digested sample showed one major product (observed mass 25844 Da, approximately 80% of total Fc/2 fragment), corresponding to the conjugated Fc/2 fragment. RP-HPLC analysis of the reduced sample indicated an average DAR of 1.88.

Example 13: Comparison of trimming efficiency of EndoS, EndoS2 and EndoSH on RNaseB at different concentrations

First, enzyme dilutions of the three enzymes (EndoS and EndoS2 from Genovis, Lund, Sweden; EndoSH as obtained in Example 3) are prepared to obtain stocks solutions with 0.25 mg/mL (dil 1), 0.125 mg/mL (dil 2) and 0.025 mg/mL (dil 3). Next, 12 vials were loaded with 2.5 µL RNase B (5

mg/mL) followed by 0.5 µL of dilution 1-3 (dil 2 in duplo) for each enzyme. The reactions were incubated for 30 minutes followed by addition of 36 µL water. Of these diluted solutions 6 µL was added to 6 µL sample buffer for SDS-page analysis. Twelve samples were loaded on SDS-page gel (4 per enzyme) and run for 70 min, stained in colloidal coomassie overnight, and finally de-stained in water (see Figure 3 for resulting gel). Conversion percentages were calculated based on scanning of SDS-PAGE gel with regular flatbed scanner and quantification with a software tool (CLIQS v1.1).

Table 1. Percentages trimming (conversion) of RNaseB upon treatment with different endoglycosidases at different enzyme concentrations.

[E] mg/mL	EndoS2	EndoS	EndoSH
0.25	0	0	45
0.125	0	0	53
0.125	0	0	50
0.025	0	0	66

Example 14: Comparison of trimming efficiency of EndoS, EndoS2 and EndoSH on cAC10

cAC10 (4 mg, 20 mg/mL in Tris pH 8.0) was treated with Fabricator™ (Genovis, Lund, Sweden ,4 µL, 66 U/µL) for 1h at 37 °C. Next, cleaved cAC10 was buffer exchanged to Tris pH 6.0 (50 mM, 3 ×) using an Amicon Ultra-0.5, Ultracel-10 Membrane (Merck Millipore) to a concentration of 20 mg/mL. Subsequent, three reactions containing each cAC10 (8.3 mg/mL) and an endoglycosidase (EndoS and EndoS2 from Genovis, Lund, Sweden; EndoSH as obtained in Example 3) at 0.83 µg/mL in Tris pH 6.0 50 mM were started. Samples of 2 µL were taken after 15 min and 35 min, diluted with 70 µL MiliQ and directly analysed by electrospray ionization time-of-flight (ESI-TOF) on a JEOL AccuTOF. Conversion percentages were calculated based the intensities of the trimmed and untrimmed mass peaks (see Figure 4 for the plot).

Table 2. Percentages trimming (conversion) of cAC10 upon treatment with different endoglycosidases at different timepoints.

Time	EndoS2	EndoS	EndoSH
0 min	0	0	0
15 min	15	24	34
35 min	36	73	73

Example 15: Comparison of trimming efficiency of EndoS, EndoS2 and EndoSH on high-mannose trastuzumab

High-mannose trastuzumab (1.3 mg, 8.8 mg/mL in Tris pH 8.0), obtained through expression of trastuzumab in the presence of kifunensine, was treated with Fabricator™ (3 µL, 66 U/µL) for 1h at 37 °C. Next, cleaved high-mannose trastuzumab was buffer exchanged to Tris pH 6.0 (50 mM, 3 ×) using an Amicon Ultra-0.5, Ultracel-10 Membrane (Merck Millipore) to a concentration of 20

mg/mL. Three reactions were started containing each high-mannose-trastuzumab (10 mg/mL) and an endoglycosidase (EndoS and EndoS2 from Genovis, Lund, Sweden; EndoSH as obtained in Example 3) at 4.4 µg/mL in Tris pH 6.0 50 mM. Samples of 2 µL were taken after 30, 60 and 120 min, diluted with 70 µL MiliQ and directly analysed by electrospray ionization time-of-flight (ESI-TOF) on a JEOL AccuTOF. Conversion percentages were calculated based the intensities of the trimmed and untrimmed mass peaks (see Figure 5 for the plot).

Table 3. Percentages trimming (conversion) of high-mannose trastuzumab upon treatment with different endoglycosidases at different timepoints.

Time	EndoS2	EndoS	EndoSH
0 min	0	0	0
30 min	14	14	27
60 min	25	14	40
120 min	33	15	79

These experiments show that EndoSH It is more efficient in trimming high-mannose trastuzumab and cAC10 then EdnoS2, and EndoSH allows from trimming of other glycoproteins (e.g. RNAseB) which is not possible with EndoS2 since the activity is restricted to the N297 site. Thus, if an antibody, e.g. a monoclonal antibody, has some undesirable high-mannose on a different N-glycosylation site, EndoSH would be able to trim this whereas EndoS2 cannot.

Example 16: Cloning of fusion proteins into expression vector

A pET22B-vector containing either EndoF3-$(G_4S)_3$-$(His)_6$-EF-$(G_4S)_3$-EfEndo18A (EndoF3-EfEndo18A), coding sequence EndoF3-EfEndo18A being identified by SEQ ID NO: 13; or EndoF2-$(G_4S)_3$-His_6-EF-$(G_4S)_3$-EfEndo18A (EndoF2-EfEndo18A), coding sequence EndoF2-EfEndo18A being identified by SEQ ID NO: 14; or EndoS-$(G_4S)_3$-His_6-EF-$(G_4S)_3$-EfEndo18A (EndoS-EfEndo18A), coding sequence EndoS-EfEndo18A being identified by SEQ ID NO: 15; or EndoF3-$(G_4S)_3$-His_6-EF-$(G_4S)_3$-EndoF1 (EndoF3-EndoF1), coding sequence EndoF3-EndoF1 being identified by SEQ ID NO: 16; or EndoF2-$(G_4S)_3$-His_6-EF-$(G_4S)_3$-EndoF1 (EndoF2-EndoF1), coding sequence EndoF2-EndoF1 being identified by SEQ ID NO: 17; or EndoS-$(G_4S)_3$-His_6-EF-$(G_4S)_3$-EndoF1 (EndoS-EndoF1), coding sequence EndoS-EndoF1 being identified by SEQ ID NO: 18; or EndoF3-$(G_4S)_3$-His_6-EF-$(G_4S)_3$-EndoH (EndoF3-EndoH), coding sequence EndoF3-EndoH being identified by SEQ ID NO: 19; or EndoF2-$(G_4S)_3$-His_6-EF-$(G_4S)_3$-EndoH (EndoF2-EndoH), coding sequence EndoF2-EndoH being identified by SEQ ID NO: 20, between the NdeI-HindIII sites was obtained from Genscript, Piscataway, USA.

The DNA sequence for the EndoF3-EfEndo18A fusion protein consists of the encoding residues 40-329 of EndoF3 fused via an *N*-terminal linked glycine-serine (GS) linker to the coding residues 42-314 of EfEndo18A. The DNA sequence is identified by SEQ ID NO: 22. The glycine-serine (GS) linker comprises a -$(G_4S)_3$-$(His)_6$-EF-$(G_4S)_3$- format, allowing spacing of the two enzymes and at the same time introducing a IMAC-purification tag.

The DNA sequence for the EndoF2-EfEndo18A fusion protein consists of the encoding residues 46-335 of EndoF2 fused via an *N*-terminal linked glycine-serine (GS) linker to coding residues 42-314 of EfEndo18A. The DNA sequence is identified by SEQ ID NO: 23. The glycine-serine (GS) linker comprises a -$(G_4S)_3$-$(His)_6$-EF-$(G_4S)_3$- format, allowing spacing of the two enzymes and at the same time introducing a IMAC-purification tag.

The DNA sequence for the EndoS-EfEndo18A fusion protein consists of the encoding residues 48-995 of EndoS fused via an *N*-terminal linked glycine-serine (GS) linker to the coding residues 42-314 of EfEndo18A. The DNA sequence is identified by SEQ ID NO: 24. The glycine-serine (GS) linker comprises a -$(G_4S)_3$-$(His)_6$-EF-$(G_4S)_3$- format, allowing spacing of the two enzymes and at the same time introducing a IMAC-purification tag.

The DNA sequence for the EndoF3-EndoF1 fusion protein consists of the encoding residues 40-329 of EndoF3 fused via an *N*-terminal linked glycine-serine (GS) linker to the coding residues 51-339 of EndoF1. The DNA sequence is identified by SEQ ID NO: 25. The glycine-serine (GS) linker comprises a -$(G_4S)_3$-$(His)_6$-EF-$(G_4S)_3$- format, allowing spacing of the two enzymes and at the same time introducing a IMAC-purification tag.

The DNA sequence for the EndoF2-EndoF1 fusion protein consists of the encoding residues 46-335 of EndoF2 fused via an *N*-terminal linked glycine-serine (GS) linker to the coding residues 51-339 of EndoF1. The DNA sequence is identified by SEQ ID NO: 26. The glycine-serine (GS) linker comprises a -$(G_4S)_3$-$(His)_6$-EF-$(G_4S)_3$- format, allowing spacing of the two enzymes and at the same time introducing a IMAC-purification tag.

The DNA sequence for the EndoS-EndoF1 fusion protein consists of the encoding residues 48-995 of EndoS fused via an *N*-terminal linked glycine-serine (GS) linker to the coding residues 51-339 of EndoF1. The DNA sequence is identified by SEQ ID NO: 27. The glycine-serine (GS) linker comprises a -$(G_4S)_3$-$(His)_6$-EF-$(G_4S)_3$- format, allowing spacing of the two enzymes and at the same time introducing a IMAC-purification tag.

The DNA sequence for the EndoF3-EndoH fusion protein consists of the encoding residues 40-329 of EndoF3 fused via an *N*-terminal linked glycine-serine (GS) linker to the coding residues 41-313 of EndoH. The DNA sequence is identified by SEQ ID NO: 28. The glycine-serine (GS) linker comprises a -$(G_4S)_3$-$(His)_6$-EF-$(G_4S)_3$- format, allowing spacing of the two enzymes and at the same time introducing a IMAC-purification tag.

The DNA sequence for the EndoF2-EndoH fusion protein consists of the encoding residues 46-335 of EndoF2 fused via an *N*-terminal linked glycine-serine (GS) linker to the coding residues 41-313 of EndoH. The DNA sequence is identified by SEQ ID NO: 29. The glycine-serine (GS) linker comprises a -$(G_4S)_3$-$(His)_6$-EF-$(G_4S)_3$- format, allowing spacing of the two enzymes and at the same time introducing a IMAC-purification tag.

The DNA sequence for the EndoS-EndoH fusion protein consists of the encoding residues 48-995 of EndoS fused via an *N*-terminal linked glycine-serine (GS) linker to the coding residues 41-313 of EndoH. The DNA sequence is identified by SEQ ID NO: 30. The glycine-serine (GS) linker comprises a -$(G_4S)_3$-$(His)_6$-EF-$(G_4S)_3$- format, allowing spacing of the two enzymes and at the same time introducing a IMAC-purification tag.

The DNA sequence for the His_6-EndoS-EndoH fusion protein consists of the encoding residues 48-995 of EndoS directly fused (i.e. no (GS) linker) to the coding residues 41-313 of EndoH. The DNA sequence is identified by SEQ ID NO: 31. The His_6-tag allows for purification by means of IMAC-purification.

The DNA sequence for the encoding residues 33-421 of His_6-TnGalNAcT with a *N*-terminal His_6-Tag is identified by SEQ ID NO: 32. The His_6-tag allows for purification by means of IMAC-purification.

Example 17: Small scale E. coli expression of fusion protein

Expression of the fusion proteins EndoF3-EndoF1 (SEQ ID NO: 16), EndoS-EndoF1 (SEQ ID NO:18), EndoF2-EndoH (SEQ ID NO:20) starts with the transformation of the plasmid into BL21(DE3) cells. Next step is the inoculation of 50 mL culture (LB medium + ampilicin; 100 µg/ml) with BL21(DE3) cells. In case of His_6-EndoS-EndoH kanamycin (50 µg/mL) was used. When the OD_{600} reached a value of 0.5-0.7 the cultures were induced with 1 mM IPTG (50 µL of 1M stock solution). Expressions of EndoF3-EndoF1 (SEQ ID NO:16) and EndoF2-EndoH (SEQ ID NO: 20) were repeated on large scale as described in Examples 19 and 21.

Example 18: Small scale purification of fusion protein from E.coli by NiNTA

After overnight induction at 16 °C the cultures of expressions EndoF3-EndoF1 (SEQ ID NO: 16), EndoS-EndoF1 (SEQ ID NO: 18), EndoF2-EndoH (SEQ ID NO: 20) were pelleted by centrifugation. The pellets were re-suspended in 3-8 mL PBS and sonicated by Sonopuls Mini20, Bandelin (using microtip MS 2.5) at 70% (3 x 1 min) on ice. After the sonication the cell debris was removed by centrifugation (10 min 10000x g). The soluble extract was loaded onto a hand-made Ni sepharose column (obtained from ThermoFisher Scientific and Ni sepharose from GE Healthcare). The column was first washed with buffer A (20 mM Tris buffer, 5 mM imidazole, 500 mM NaCl, pH 7.5). Retained protein was eluted with buffer B (20 mM Tris, 500 mM NaCl, 250 mM imidazole, pH 7.5, 5 mL). Fractions were analyzed by SDS-PAGE on polyacrylamide gels (12%). The fractions that contained purified target protein were combined and the buffer was exchanged against TBS pH 7.5 by dialysis performed overnight at 4 °C. The yields are shown in Table 4. The proteins were snap-frozen and stored at −80 °C prior to further use.

Table 4. Yields for the small-scale purifications of fusion proteins from E. coli by NiNTA.

Protein	Yield (mg)
EndoF3-EndoF1	0,36
EndoS-EndoF1	2,7
EndoF2-EndoH	0,39

Example 19: Large scale E. coli expression of fusion protein

Expression of the fusion proteins EndoF3-EndoF1 (SEQ ID NO:16), EndoF2-EndoH (SEQ ID NO: 20), EndoS-EfEndo18A (SEQ ID NO: 15), EndoF2-EndoF1 (SEQ ID NO: 17) and EndoF3-EndoH

(SEQ ID NO: 19) started with the transformation of the plasmid into BL21(DE3) cells. Next step was the inoculation of 500 mL culture (LB medium + ampilicin; 100µg/ml) with BL21(DE3) cells. When the OD_{600} reached 0.5-0.7 the cultures were induced with 1 mM IPTG (500 µL of 1M stock solution).

Example 20: Large scale purification of fusion protein from E. coli by NiNTA

After overnight induction at 16 °C the cultures of proteins EndoS-EfEndo18A (SEQ ID NO: 15), EndoF2-EndoF1 (SEQ ID NO: 17), EndoF3-EndoH (SEQ ID NO: 19), EndoF3-EndoF1 (SEQ ID NO:16) and EndoF2-EndoH (SEQ ID NO: 20) were pelleted by centrifugation. The pellets were re-suspended in 10 mL PBS/gram of pellet. The cell suspension is lysed three times under pressure (20000-25000 psi) by French press using Emulsiflex C3, Avestin. After French press the cell debris was removed by centrifugation (20 minutes 10000xg). The soluble extract was loaded onto a HisTrap HP 5 mL column (GE Healthcare). The column was first washed with buffer A (20 mM Tris buffer, 5 mM imidazole, 500 mM NaCl, pH 7.5). Retained protein was eluted with buffer B (20 mM Tris, 500 mM NaCl, 250 mM imidazole, pH 7.5, ~10 mL). Fractions were analysed by SDS-PAGE on polyacrylamide gels (12%). The fractions that contained purified target protein were combined and the buffer was exchanged against Tris pH 7.5 by dialysis performed overnight at 4 °C. The yields are shown in the table 5 below. The proteins were snap-frozen and stored at −80 °C prior to further use.

Table 5. Yields for the large-scale purifications of fusion proteins from E. coli by NiNTA.

Protein	Yield (mg)
EndoS-EfEndo18A	55
EndoF2-EndoF1	22,1
EndoF3-EndoH	28
EndoF3-EndoF1	5,1
EndoF2-EndoH	3,5

Example 21: Purification of fusion protein from E. coli by SEC

For EndoF3-EndoF1 (SEQ ID NO:16), EndoF2-EndoF1 (SEQ ID NO: 17), EndoF3-EndoH (SEQ ID NO: 19) and EndoF2-EndoH (SEQ ID NO: 20) the NiNTA-purification, which is described in example 20, was followed by size-exclusion chromatography (SEC) to isolate the monomer. A Superdex 75 10/300 GL was installed on the Akta Purifier. The column was rinsed with MilliQ (20mL) followed by equilibration with TBS pH 7.5 (25 mL, 0.8 mL/min). Approximately 1 - 3 mg of NiNTA-purified protein was loaded and run with 0.8 mL/min using TBS pH 7.5. The monomer protein was collected and fractions were analysed by SDS-PAGE on polyacrylamide gels (12%) or by mass

on AccuTOF. The yields are shown below in table 6. The proteins were snap-frozen and stored at −80 °C prior to further use.

Table 6. Overview of the amount of NiNTA-purified endoglycosidase fusion protein which was loaded onto the SEC-column and the yields for the monomer fraction.

Protein	Amount loaded (mg)	Yield (mg)
EndoF2-EndoF1	1,75	0,10
EndoF3-EndoH	0,80	0,02
EndoF3-EndoF1	2,30	0,79
EndoF2-EndoH	1,40	0,12

Example 22: Cloning of fusion protein His_6-EndoS-EndoH (without GS-linker) into pET28B expression vector

A pET28B-vector containing His_6-EndoS-EndoH (His_6-EndoSH *without GS-linker*) coding sequence His_6-EndoS-EndoH being identified by SEQ ID NO: 21, between the NcoI-HindIII sites was obtained from Genscript Piscataway, USA.

The DNA sequence for the His_6-EndoSH fusion protein encodes a *N*-terminal linked IMAC-purification tag and a thrombin cleavage site fused to the coding residues 48-995 of EndoS fused to the coding residues 41-313 of EndoH.

Example 23: Small scale E. coli expression of fusion protein His_6-EndoS-EndoH (without GS-linker)

Expression of the fusion protein His_6-EndoS-EndoH (SEQ ID NO: 21) starts with the transformation of the plasmid into BL21(DE3) cells. Next step is the inoculation of 50 mL culture (LB medium + kanamycin; 50 µg/ml) with BL21(DE3) cells. When the OD_{600} reached a value of 0.5 the culture was induced with 1 mM IPTG (50 µL of 1M stock solution).

Example 24: Small scale purification of fusion protein from E.coli by NiNTA

After overnight induction at 16 °C the culture of the expression in Example 23 was pelleted by centrifugation. The pellet was re-suspended in 7 mL PBS and sonicated by Sonopuls Mini20, Bandelin (using microtip MS 2.5) at 70% (3 x 1 min) on ice. After the sonication the cell debris was removed by centrifugation (10 min 10000x g). The soluble extract was loaded onto a hand-made Ni sepharose column (obtained from ThermoFisher Scientific and Ni sepharose from GE Healthcare). The column was first washed with buffer A (20 mM Tris buffer, 5 mM imidazole, 500 mM NaCl, pH 7.5). Retained protein was eluted with buffer B (20 mM Tris, 500 mM NaCl, 250 mM imidazole, pH 7.5, 5 mL). Fractions were analysed by SDS-PAGE on polyacrylamide gels (12%). The fractions that contained purified target protein were combined and the buffer was exchanged against TBS pH 7.5 by dialysis performed overnight at 4 °C. The yield after dialysis is 9 mg. The product was snap-frozen and stored at −80 °C prior to further use.

Example 25: Comparison of trimming efficiency of EndoSH, EndoF3-EndoH, EndoS-EfEndo18A and EndoF2-EndoF1 on high-mannose trastuzumab

High-mannose trastuzumab (0.7 mL, 6.0 mg, 8.8 mg/mL in Tris pH 8.0), was treated with Fabricator™ (9 µL, 50 U/µL) for 1h at 37 °C. Next, Fabricator™-digested high-mannose trastuzumab was divided into three equal portions and buffer exchanged to 50 mM sodium citrate pH 4.5 with 150 mM NaCl, 50 mM Tris.HCl pH 6.0 with 150 mM NaCl and 50 mM Tris.HCl pH 7.5 with 150 mM NaCl, respectively. Buffer exchange was performed using an Amicon Ultra-0.5, Ultracel-10 Membrane (Merck Millipore) and samples were concentrated to a final concentration of 10 mg/mL. For EndoSH (identified by SEQ ID NO: 1), EndoF3-EndoH (identified by SEQ ID NO: 19), EndoS-EfEndo18A (identified by SEQ ID NO: 15) and EndoF2-EndoF1 (identified by SEQ ID NO: 17) dilution series of 10, 50 and 250 nM in each of the above-mentioned reaction buffers were prepared. The reactions were started by adding 2 µL of Fabricator™-digested high-mannose trastuzumab (10 mg/mL) to 2 µL of the diluted endoglycosidase fusion protein in the corresponding buffer, resulting in a final concentration of 5 mg/mL Fabricator™-digested IgG (67 µM Fc/2-fragment) with 5, 25 and 125 nM endoglycosidase. The reactions were incubated for 60 minutes at 37 °C. Reactions were quenched by addition of 16 µL 1x Laemmli sample buffer without 2-mercaptoethanol followed by incubation for 5 minutes at 95 °C. Samples (5 µL/sample) were loaded on SDS-page gel and run for 70 min (20 mA), stained in colloidal coomassie overnight, and finally de-stained in water. Conversion percentages were calculated based on scanning of SDS-PAGE gel with regular flatbed scanner and quantification with a software tool (CLIQS v1.1).

Table 7. Percentages trimming (conversion) of Fabricator™-digested high-mannose trastuzumab upon treatment of various endoglycosidase fusion proteins at pH 4.5, 6.0 and 7.5.

pH	Enzyme (nM)	EndoSH	EndoF3-EndoH	EndoS-EfEndo18A	EndoF2-EndoF1
4.5	5	23*	26*	26*	26*
	25	35	31	38	26*
	125	60	60	61	83
6.0	5	27*	25*	27*	28*
	25	36	37	31	32
	125	64	57	69	61
7.5	5	24*	26*	24*	26*
	25	29*	31	31	30
	125	61	53	58	58

Conversion was calculated using the following formula: conversion (%) = 100 × ($Fc/2_{trimmed}$) / ($Fc/2_{trimmed}$ + $Fc/2_{glycosylated}$). *Note: Results are difficult to interpret accurately due to background signal at the height of untreated Fabricator™-digested high-mannose trastuzumab. No background correction was applied for conversion quantification.

Example 26: Comparison of trimming efficiency of EndoSH, EndoF3-EndoH, EndoS-EfEndo18A and EndoF2-EndoF1 on trastuzumab

Trastuzumab (obtained from Epirus biopharma (Utrecht, The Netherlands); 287 µL, 6.0 mg, 21 mg/mL in Tris pH 8.0), was treated with Fabricator™ (9 µL, 50 U/µL) for 1h at 37 °C. Next, Fabricator™-digested trastuzumab was divided into three equal portions and buffer exchanged to 50 mM sodium citrate pH 4.5 with 150 mM NaCl, 50 mM Tris.HCl pH 6.0 with 150 mM NaCl and 50 mM Tris.HCl pH 7.5 with 150 mM NaCl, respectively. Buffer exchange was performed using an Amicon Ultra-0.5, Ultracel-10 Membrane (Merck Millipore) and samples were concentrated to a final concentration of 10 mg/mL. For EndoSH (identified by SEQ ID NO: 1), EndoF3-EndoH (identified by SEQ ID NO: 19), EndoS-EfEndo18A (identified by SEQ ID NO: 15) and EndoF2-EndoF1 (identified by SEQ ID NO: 17) dilution of 50 and 250 nM in each of the above-mentioned buffers were prepared. The reactions were started by adding 5 µL of Fabricator™-digested trastuzumab (10 mg/mL) to 5 µL of the diluted endoglycosidase fusion protein in the corresponding buffer, resulting in a final concentration of 5 mg/mL Fabricator™-digested IgG (67 µM Fc/2-fragment) with 25 and 125 nM endoglycosidase. The reactions were incubated for 60 minutes at 37 °C. For each reaction a sample (4 µL) was taken and added to 16 µL 1x Laemmli sample buffer without 2-mercaptoethanol and incubated for 5 minutes at 95 °C. Samples (5 µL/sample) were loaded on SDS-page gel and run for 70 min (20 mA), stained in colloidal coomassie overnight, and finally de-stained in water. Conversion percentages were calculated based on scanning of SDS-PAGE gel with regular flatbed scanner and quantification with a software tool (CLIQS v1.1).

Table 8. Percentages trimming (conversion) of Fabricator™-digested trastuzumab upon treatment of various endoglycosidase fusion proteins at pH 4.5, 6.0 and 7.5.

pH	Enzyme (nM)	EndoSH	EndoF3-EndoH	EndoS-EfEndo18A	EndoF2-EndoF1
4.5	25	29*	29*	29*	29*
	125	28*	40	29*	50
6.0	25	72	28*	77	28*
	125	85	31	84	42
7.5	25	81	27*	82	26*
	125	87	31	86	41

Conversion was calculated using the following formula: conversion (%) = 100 × ($Fc/2_{trimmed}$) / ($Fc/2_{trimmed}$ + $Fc/2_{glycosylated}$). *Note: Results are difficult to interpret accurately due to background signal at the height of untreated Fabricator™-digested trastuzumab. No background correction was applied for conversion quantification.

Example 27: Trimming of trastuzumab by EndoF3-EndoH

To demonstrate that complete conversion can be achieved for trimming of trastuzumab by EndoF3-EndoH (identified by SEQ ID NO: 19), Fabricator™-digested trastuzumab (4 µL, 40 µg, 10 mg/mL

in 50 mM sodium citrate pH 4.5 with 150 mM NaCl) was incubated with 50 mM sodium citrate pH 4.5 with 150 mM NaCl (8.73 µL) and EndoF3-EndoH (7.27 µL, 4 µg, 0.55 mg/mL in 50 mM sodium citrate pH 4.5 with 150 mM NaCl) for 60 minutes at 37 °C. Mass spectral analysis of a fabricator-digested sample showed one main peak of the Fc/2-fragment (observed mass 24139 Da, approximately 95% of total Fc/2 fragment), corresponding to core GlcNAc(Fuc)-substituted trastuzumab.

Example 28: Trimming of trastuzumab by EndoF2-EndoF1

To demonstrate that complete conversion can be achieved for trimming of trastuzumab by EndoF2-EndoF1 (identified by SEQ ID NO: 17), Fabricator™-digested trastuzumab (4 µL, 40 µg, 10 mg/mL in 50 mM sodium citrate pH 4.5 with 150 mM NaCl) was incubated with 50 mM sodium citrate pH 4.5 with 150 mM NaCl (11.06 µL) and EndoF2-EndoF1 (4.94 µL, 4 µg, 0.81 mg/mL in 50 mM sodium citrate pH 4.5 with 150 mM NaCl) for 60 minutes at 37 °C. Mass spectral analysis of a fabricator-digested sample showed one main peak of the Fc/2-fragment (observed mass 24139 Da, approximately 95% of total Fc/2 fragment), corresponding to core GlcNAc(Fuc)-substituted trastuzumab.

Example 29: Comparison of trimming efficiency of EndoSH, EndoF3-EndoF1, EndoS-EndoF1, EndoF2-EndoH on high-mannose trastuzumab

High-mannose trastuzumab (0.7 mL, 6.0 mg, 8.8 mg/mL in Tris pH 8.0), was treated with Fabricator™ (9 µL, 50 U/µL) for 1h at 37 °C. Next, Fabricator™-digested high-mannose trastuzumab was divided into three equal portions and buffer exchanged to 50 mM sodium citrate pH 4.5 with 150 mM NaCl, 50 mM Tris.HCl pH 6.0 with 150 mM NaCl and 50 mM Tris.HCl pH 7.5 with 150 mM NaCl, respectively. Buffer exchange was performed using an Amicon Ultra-0.5, Ultracel-10 Membrane (Merck Millipore) and samples were concentrated to a final concentration of 10 mg/mL. EndoSH (identified by SEQ ID NO: 1), EndoF3-EndoF1 (identified by SEQ ID NO: 16), EndoS-EndoF1 (identified by SEQ ID NO: 18) and EndoF2-EndoH (identified by SEQ ID NO: 20) were diluted to a concentration of 250 nM in each of the above-mentioned reaction buffers. The reactions were started by adding 2 µL of Fabricator™-digested high-mannose trastuzumab (10 mg/mL) to 2 µL of the endoglycosidase fusion protein (250 nM) in the corresponding buffer, resulting in a final concentration of 5 mg/mL Fabricator™-digested IgG (67 µM Fc/2-fragment) with 125 nM endoglycosidase. The reactions were incubated for 60 minutes at 37 °C. Reactions were quenched by addition of 16 µL 1x Laemmli sample buffer without 2-mercaptoethanol followed by incubation for 5 minutes at 95 °C. Samples (5 µL/sample) were loaded on SDS-page gel and run for 70 min (20 mA), stained in colloidal coomassie overnight, and finally de-stained in water. Conversion percentages were calculated based on scanning of SDS-PAGE gel with regular flatbed scanner and quantification with a software tool (CLIQS v1.1).

Table 9. Percentages trimming (conversion) of Fabricator™-digested high-mannose trastuzumab upon treatment of various endoglycosidase fusion proteins (125 nM) at pH 4.5, 6.0 and 7.5.

pH	EndoSH	EndoF3-EndoF1	EndoS-EndoF1	EndoF2-EndoH
4.5	61	23*	60	33
6.0	69	19*	73	28*
7.5	67	17*	72	25*

Conversion was calculated using the following formula: conversion (%) = 100 × ($Fc/2_{trimmed}$) / ($Fc/2_{trimmed}$ + $Fc/2_{glycosylated}$). *Note: Results are difficult to interpret accurately due to background signal at the height of untreated Fabricator™-digested high-mannose trastuzumab. No background correction was applied for conversion quantification.

Example 30: Comparison of trimming efficiency of EndoSH, EndoF3-EndoH and EndoS-EfEndo18A, EndoF2-EndoF1 on trastuzumab

Trastuzumab (obtained from Epirus biopharma (Utrecht, The Netherlands); 287 µL, 6.0 mg, 21 mg/mL in Tris pH 8.0), was treated with Fabricator™ (9 µL, 50 U/µL) for 1h at 37 °C. Next, Fabricator™-digested trastuzumab was divided into three equal portions and buffer exchanged to 50 mM sodium citrate pH 4.5 with 150 mM NaCl, 50 mM Tris.HCl pH 6.0 with 150 mM NaCl and 50 mM Tris.HCl pH 7.5 with 150 mM NaCl, respectively. Buffer exchange was performed using an Amicon Ultra-0.5, Ultracel-10 Membrane (Merck Millipore) and samples were concentrated to a final concentration of 10 mg/mL. EndoSH (identified by SEQ ID NO: 1), EndoF3-EndoF1 (identified by SEQ ID NO: 16), EndoS-EndoF1 (identified by SEQ ID NO: 18) and EndoF2-EndoH (identified by SEQ ID NO: 20) were diluted to a concentration of 250 nM in each of the above-mentioned reaction buffers. The reactions were started by adding 5 µL of Fabricator™-digested trastuzumab (10 mg/mL) to 5 µL of the endoglycosidase fusion protein (250 nM) in the corresponding buffer, resulting in a final concentration of 5 mg/mL Fabricator™-digested IgG (67 µM Fc/2-fragment) with 125 nM endoglycosidase. The reactions were incubated for 60 minutes at 37 °C. For each reaction, a sample (4 µL) was taken and added to 16 µL 1x Laemmli sample buffer without 2-mercaptoethanol and incubated for 5 minutes at 95 °C. Samples (5 µL/sample) were loaded on SDS-page gel and run for 70 min (20 mA), stained in colloidal coomassie overnight, and finally de-stained in water. Conversion percentages were calculated based on scanning of SDS-PAGE gel with regular flatbed scanner and quantification with a software tool (CLIQS v1.1).

Table 10. Percentages trimming (conversion) of trastuzumab upon treatment with different endoglycosidases (125 nM) at pH 4.5, 6.0 and 7.5.

pH	EndoSH	EndoF3-EndoF1	EndoS-EndoF1	EndoF2-EndoH
4.5	22*	19*	23*	20*
6.0	77	16*	79	16*
7.5	80	14*	83	13*

Conversion was calculated using the following formula: conversion (%) = 100 × ($Fc/2_{trimmed}$) / ($Fc/2_{trimmed}$ + $Fc/2_{glycosylated}$). *Note: Results are difficult to interpret accurately due to background signal at the height of untreated Fabricator™-digested trastuzumab. No background correction was applied for conversion quantification.

Example 31: Comparison of trimming efficiency of EndoS-EndoH fusion proteins with and without linker on high-mannose trastuzumab

High-mannose trastuzumab (0.7 mL, 6.0 mg, 8.8 mg/mL in Tris pH 8.0), was treated with Fabricator™ (9 µL, 50 U/µL) for 1h at 37 °C. Next, Fabricator™-digested high-mannose trastuzumab was divided into three equal portions and buffer exchanged to 50 mM sodium citrate pH 4.5 with 150 mM NaCl, 50 mM Tris.HCl pH 6.0 with 150 mM NaCl and 50 mM Tris.HCl pH 7.5 with 150 mM NaCl, respectively. Buffer exchange was performed using an Amicon Ultra-0.5, Ultracel-10 Membrane (Merck Millipore) and samples were concentrated to a final concentration of 10 mg/mL. For EndoSH (identified by SEQ ID NO: 1) and His_6-EndoSH (His_6-EndoS-EndoH without GS-linker; identified by SEQ ID NO: 21) dilution series of 10, 50 and 250 nM in each of the above-mentioned reaction buffers were prepared. The reactions were started by adding 2 µL of Fabricator™-digested high-mannose trastuzumab (10 mg/mL) to 2 µL of the diluted endoglycosidase fusion protein in the corresponding buffer, resulting in a final concentration of 5 mg/mL Fabricator™-digested IgG (67 µM Fc/2-fragment) with 5, 25 and 125 nM endoglycosidase. The reactions were incubated for 60 minutes at 37 °C. Reactions were quenched by addition of 16 µL 1x Laemmli sample buffer without 2-mercaptoethanol followed by incubation for 5 minutes at 95 °C. Samples (5 µL/sample) were loaded on SDS-page gel and run for 70 min (20 mA), stained in colloidal coomassie overnight, and finally de-stained in water. Conversion percentages were calculated based on scanning of SDS-PAGE gel with regular flatbed scanner and quantification with a software tool (CLIQS v1.1).

Table 11. Percentages trimming (conversion) of Fabricator™-digested high-mannose trastuzumab upon treatment with EndoS-EndoH fusion proteins with and without linker at pH 4.5, 6.0 and 7.5.

pH	Enzyme (nM)	EndoSH (with GS-linker)	His_6-EndoSH (without GS-linker)
4.5	5	23*	23*
	25	35	31
	125	60	63
6.0	5	27*	28*
	25	36	42
	125	64	66
7.5	5	24*	28*
	25	29*	38
	125	61	63

Conversion was calculated using the following formula: conversion (%) = 100 × ($Fc/2_{trimmed}$) / ($Fc/2_{trimmed}$ + $Fc/2_{glycosylated}$). *Note: Results are difficult to interpret accurately due to background signal at the height of untreated Fabricator™-digested high-mannose trastuzumab. No background correction was applied for conversion quantification.

Example 32: Comparison of trimming efficiency of EndoS-EndoH fusion proteins with and without linker on trastuzumab

Trastuzumab (obtained from Epirus biopharma (Utrecht, The Netherlands); 287 µL, 6.0 mg, 21 mg/mL in Tris pH 8.0), was treated with Fabricator™ (9 µL, 50 U/µL) for 1h at 37 °C. Next, Fabricator™-digested trastuzumab was divided into three equal portions and buffer exchanged to 50 mM sodium citrate pH 4.5 with 150 mM NaCl, 50 mM Tris.HCl pH 6.0 with 150 mM NaCl and 50 mM Tris.HCl pH 7.5 with 150 mM NaCl, respectively. Buffer exchange was performed using an Amicon Ultra-0.5, Ultracel-10 Membrane (Merck Millipore) and samples were concentrated to a final concentration of 10 mg/mL. For EndoSH (identified by SEQ ID NO: 1) and His_6-EndoSH (EndoS-EndoH without GS-linker; identified by SEQ ID NO: 21) dilution series of 2, 10, 50 and 250 nM in each of the above-mentioned buffers were prepared. The reactions were started by adding 5 µL of Fabricator™-digested trastuzumab (10 mg/mL) to 5 µL of the diluted endoglycosidase fusion protein in the corresponding buffer, resulting in a final concentration of 5 mg/mL Fabricator™-digested IgG (67 µM Fc/2-fragment) with 1, 5, 25 and 125 nM endoglycosidase. The reactions were incubated for 60 minutes at 37 °C. For each reaction, a sample (4 µL) was taken and added to 16 µL 1x Laemmli sample buffer without 2-mercaptoethanol and incubated for 5 minutes at 95 °C. Samples (5 µL/sample) were loaded on SDS-page gel and run for 70 min (20 mA), stained in colloidal coomassie overnight, and finally de-stained in water. Conversion percentages were calculated based on scanning of SDS-PAGE gel with regular flatbed scanner and quantification with a software tool (CLIQS v1.1).

Table 12. Percentages trimming (conversion) of Fabricator™-digested trastuzumab upon treatment with EndoS-EndoH fusion proteins with and without linker at pH 4.5, 6.0 and 7.5.

pH	Enzyme (nM)	EndoSH	EndoSH, no linker
4.5	1	30	28*
	5	29*	29*
	25	29*	29*
	125	28*	29*
6.0	1	36	35
	5	45	46
	25	72	73
	125	85	83
7.5	1	34	40
	5	47	51

	25	81	80
	125	87	84

Conversion was calculated using the following formula: conversion (%) = 100 × ($Fc/2_{trimmed}$) / ($Fc/2_{trimmed}$ + $Fc/2_{glycosylated}$). *Note: Results are difficult to interpret accurately due to background signal at the height of untreated Fabricator™-digested trastuzumab. No background correction was applied for conversion quantification.

Example 33: Comparison of trimming efficiency of EndoSH, EndoF3-EndoH and EndoS-EfEndo18A, EndoF2-EndoF1 on cAC10

cAC10 (300 µL, 6.0 mg, 20.1 mg/mL in Tris pH 8.0), obtained by as described above in Example 7, was treated with Fabricator™ (9 µL, 50 U/µL) for 1h at 37 °C. Next, Fabricator™-digested cAC10 was divided into three equal portions and buffer exchanged to 50 mM sodium citrate pH 4.5 with 150 mM NaCl, 50 mM Tris.HCl pH 6.0 with 150 mM NaCl and 50 mM Tris.HCl pH 7.5 with 150 mM NaCl. Buffer exchange was performed using an Amicon Ultra-0.5, Ultracel-10 Membrane (Merck Millipore) and samples were concentrated to a final concentration of 10 mg/mL. Example 26 showed an optimal pH for the trimming of complex-type glycans of pH 7.5 for EndoSH (identified by SEQ ID NO: 1), pH 4.5 for EndoF3-EndoH (identified by SEQ ID NO: 19), pH 7.5 for EndoS-EfEndo18A (identified by SEQ ID NO: 15) and pH 4.5 for EndoF2-EndoF1 (identified by SEQ ID NO: 17). For each of the above mentioned fusion proteins a dilution series was prepared of 5, 50 and 500 nM in the reaction buffer with the optimal pH as mentioned above. The reactions were started by adding 5 µL of Fabricator™-digested cAC10 (10 mg/mL) to 5 µL of the diluted endoglycosidase fusion protein in the corresponding buffer, resulting in a final concentration of 5 mg/mL Fabricator™-digested IgG (67 µM Fc/2-fragment) with 2.5, 25 and 250 nM endoglycosidase fusion protein. The reactions were incubated for 60 minutes at 37 °C. For each reaction, a sample (4 µL) was taken and added to 16 µL 1x Laemmli sample buffer without 2-mercaptoethanol and incubated for 5 minutes at 95 °C. Samples (5 µL/sample) were loaded on SDS-page gel and run for 70 min (20 mA), stained in colloidal coomassie overnight, and finally de-stained in water. Conversion percentages were calculated based on scanning of SDS-PAGE gel with regular flatbed scanner and quantification with a software tool (CLIQS v1.1).

Table 13. Percentages trimming (conversion) of Fabricator™-digested cAC10 upon treatment of various endoglycosidase fusion proteins at an optimal pH specific for each fusion protein.

Enzyme concentration (nM)	EndoSH (at pH 7.5)	EndoF3-EndoH (at pH 4.5)	EndoS-EfEndo18A (at pH 7.5)	EndoF2-EndoF1 (at pH 4.5)
2.5	27*	70	26*	71
25	100	71	100	71
250	100	70	100	73

Conversion was calculated using the following formula: conversion (%) = 100 × ($Fc/2_{trimmed}$) / ($Fc/2_{trimmed}$ + $Fc/2_{glycosylated}$). *Note: Results are difficult to interpret accurately due to background signal at the height of untreated Fabricator™-digested cAC10. No background correction was applied for conversion quantification.

Example 34: Comparison of trimming efficiency of EndoSH, EndoF3-EndoH and EndoS-EfEndo18A, EndoF2-EndoF1 on RNAseB

A stock solution of RNaseB (2 mg/mL) was prepared in 50 mM sodium citrate pH 4.5 with 150 mM NaCl and in 50 mM Tris.HCl pH 6.0 with 150 mM NaCl. Example 26 showed an optimal pH for the trimming of high-mannose glycans of pH 6.0 for EndoSH (identified by SEQ ID NO: 1), pH 6.0 for EndoF3-EndoH (identified by SEQ ID NO: 19), pH 6.0 for EndoS-EfEndo18A (identified by SEQ ID NO: 15) and pH 4.5 for EndoF2-EndoF1 (identified by SEQ ID NO: 17). For each of the above mentioned fusion proteins a dilution series was prepared of 10, 50 and 250 nM in the reaction buffer with the optimal pH as mentioned above. The reactions were started by adding 5 µL of RNase B (2 mg/mL in the corresponding buffer) to 5 µL of the diluted endoglycosidase fusion protein in the optimal reaction buffer as mentioned above. This results in a final concentration of 1 mg/mL RNase B with 5, 25 and 125 nM endoglycosidase fusion protein. The reactions were incubated for 60 minutes at 37 °C. For each reaction, a sample (4 µL) was taken and added to 16 µL 1x Laemmli sample buffer without 2-mercaptoethanol and incubated for 5 minutes at 95 °C. Samples (5 µL/sample) were loaded on SDS-page gel and run for 70 min (20 mA), stained in colloidal coomassie overnight, and finally de-stained in water. Conversion percentages were calculated based on scanning of SDS-PAGE gel with regular flatbed scanner and quantification with a software tool (CLIQS v1.1).

Table 14. Percentages trimming (conversion) of RNase B upon treatment of various endoglycosidase fusion proteins at the optimal pH value specific for each fusion protein.

Enzyme concentration (nM)	EndoSH (at pH 6.0)	EndoF3-EndoH (at pH 6.0)	EndoS-EfEndo18A (at pH 6.0)	EndoF2-EndoF1 (at pH 4.5)
5	34	37	52	43
25	100	100	100	100
125	100	100	100	100

Conversion was calculated using the following formula: conversion (%) = 100 × ($RNaseB_{trimmed}$) / ($RNaseB_{trimmed}$ + $RNaseB_{glycosylated}$).

Example 35: Comparison of trimming efficiency of EndoSH, EndoF3-EndoH and EndoS-EfEndo18A, EndoF2-EndoF1 on fibrinogen

Fibrinogen from human plasma (commercially available from Sigma), which contains one glycosylation-site on the alpha-, beta- and gamma-chain, was dissolved to a final concentration of

10 mg/mL in 50 mM Tris.HCl pH 6.0 with 150 mM NaCl and in 50 mM Tris.HCl pH 7.5 with 150 mM NaCl by rotating at 300 rpm at 37 °C for 15 minutes. Fibrinogen could not be dissolved in 50 mM sodium citrate pH 4.5 with 150 mM NaCl using the above-mentioned procedure. Example 26 showed an optimal pH for the trimming of complex-type glycans of pH 7.5 for EndoSH (identified by SEQ ID NO: 1), pH 4.5 for EndoF3-EndoH (identified by SEQ ID NO: 19), pH 7.5 for EndoS-EfEndo18A (identified by SEQ ID NO: 15) and pH 4.5 for EndoF2-EndoF1 (identified by SEQ ID NO: 17). For EndoSH and EndoS-EfEndo18A a dilution series was prepared of 5, 50 and 500 nM in 50 mM Tris.HCl pH 7.5 with 150 mM NaCl, which is the optimal reaction buffer for these enzymes. For EndoF3-EndoH and EndoF2-EndoF1 a dilution series was prepared of 5, 50 and 500 nM in 50 mM Tris.HCl pH 6.0 with 150 mM NaCl, which is the most optimal pH in which fibrinogen can be solubilized. The reactions were started by adding 5 µL of fibrinogen (10 mg/mL) to 5 µL of the diluted endoglycosidase fusion protein in the corresponding buffer, resulting in a final concentration of 5 mg/mL fibrinogen with 2.5, 25 and 250 nM endoglycosidase fusion protein. The reactions were incubated for 60 minutes at 37 °C. For each reaction, a sample (4 µL) was taken and added to 16 µL 1x Laemmli sample buffer with 2-mercaptoethanol and incubated for 5 minutes at 95 °C. Samples (5 µL/sample) were loaded on SDS-page gel and run for approximately 120 min (20 mA), stained in colloidal coomassie overnight, and finally de-stained in water. Conversion percentages were calculated for the beta- and gamma-chain based on scanning of SDS-PAGE gel with regular flatbed scanner and quantification with a software tool (CLIQS v1.1).

Table 15. Percentages trimming (conversion) of fibrinogen upon treatment of various endoglycosidase fusion proteins at the optimal pH value specific for each fusion protein.

Fibrinogen chain	Enzyme concentration (nM)	EndoSH (at pH 7.5)	EndoF3-EndoH (at pH 6.0)	EndoS-EfEndo18A (at pH 7.5)	EndoF2-EndoF1 (at pH 6.0)
beta-chain	2.5	9*	10*	1*	23
	25	9*	10*	10*	34
	250	8*	8*	7*	54
gamma-chain	2.5	5*	7*	6*	13
	25	5*	4*	5*	25
	250	4*	6*	4*	50

Conversion was calculated separately for the beta- and gamma-chain using the following formula: conversion (%) = 100 × ($fibrinogen_{trimmed}$) / ($fibrinogen_{trimmed}$ + $fibrinogen_{glycosylated}$). *Note: Results are difficult to interpret accurately due to background signals at the height of untreated fibrinogen. No background correction was applied for conversion quantification.

Example 36: Comparison of trimming efficiency of fusion proteins EndoSH and EndoF3-EndoH with individual proteins EndoS, EndoF3 and EndoH on trastuzumab

Trastuzumab (obtained from Epirus biopharma (Utrecht, The Netherlands); 287 µL, 6.0 mg, 21 mg/mL in Tris pH 8.0), was treated with Fabricator™ (9 µL, 50 U/µL) for 1h at 37 °C. Next, Fabricator™-digested trastuzumab was divided into three equal portions and buffer exchanged to 50 mM sodium citrate pH 4.5 with 150 mM NaCl, 50 mM Tris.HCl pH 6.0 with 150 mM NaCl and 50 mM Tris.HCl pH 7.5 with 150 mM NaCl, respectively. Buffer exchange was performed using an Amicon Ultra-0.5, Ultracel-10 Membrane (Merck Millipore) and samples were concentrated to a final concentration of 10 mg/mL. EndoSH (identified by SEQ ID NO: 1), EndoF3-EndoH (identified by SEQ ID NO: 19), EndoS (commercially available from Genovis, Lund, Sweden), EndoF3 (commercially available from Sigma-Aldrich, EU) and EndoH (commercially available from New England Biolabs, Ipswich, USA) were diluted to 50 and 500 nM in each of the above-mentioned buffers. The reactions were started by adding 5 µL of Fabricator™-digested trastuzumab (10 mg/mL) to 5 µL of the diluted endoglycosidases in the corresponding buffer, resulting in a final concentration of 5 mg/mL Fabricator™-digested IgG (67 µM Fc/2-fragment) with 25 and 250 nM endoglycosidase. The reactions were incubated for 60 minutes at 37 °C. For each reaction a sample (4 µL) was taken and added to 16 µL 1x Laemmli sample buffer without 2-mercaptoethanol and incubated for 5 minutes at 95 °C. Samples (5 µL/sample) were loaded on SDS-page gel and run for 70 min (20 mA), stained in colloidal coomassie overnight, and finally de-stained in water. Conversion percentages were calculated based on scanning of SDS-PAGE gel with regular flatbed scanner and quantification with a software tool (CLIQS v1.1).

Table 16. Percentages trimming (conversion) of Fabricator™-digested trastuzumab upon treatment of various endoglycosidases and endoglycosidase fusion proteins at pH 4.5, 6.0 and 7.5.

pH	Enzyme (nM)	EndoSH	EndoF3-EndoH	EndoS	EndoF3	EndoH
4.5	25	21*	21*	22*	21*	21*
	250	27*	50	29*	21*	19*
6.0	25	68	23*	76	24*	21*
	250	83	39	82	22*	19*
7.5	25	77	26*	82	15*	21*
	250	100	36	100	12*	20*

Conversion was calculated using the following formula: conversion (%) = 100 × ($Fc/2_{trimmed}$) / ($Fc/2_{trimmed}$ + $Fc/2_{glycosylated}$). *Note: Results are difficult to interpret accurately due to background signal at the height of untreated Fabricator™-digested trastuzumab. No background correction was applied for conversion quantification.

Example 37: Comparison of trimming efficiency of fusion proteins EndoSH and EndoF3-EndoH with individual proteins EndoS, EndoF3 and EndoH on RNaseB

A stock solution of RNaseB (2 mg/mL) was prepared in 50 mM sodium citrate pH 4.5 with 150 mM NaCl, in 50 mM Tris.HCl pH 6.0 with 150 mM NaCl and in 50 mM Tris.HCl pH 7.5 with 150 mM NaCl. EndoSH (identified by SEQ ID NO: 1), EndoF3-EndoH (identified by SEQ ID NO: 19), EndoS (commercially available from Genovis, Lund, Sweden), EndoF3 (commercially available from Sigma-Aldrich, EU) and EndoH (commercially available from New England Biolabs, Ipswich, USA) were diluted to a concentration of 50 nM in each of the above-mentioned buffers. The reactions were started by adding 5 µL of RNase B (2 mg/mL) to 5 µL of the diluted endoglycosidase fusion protein (50 nM) in the corresponding reaction buffer, resulting in a final concentration of 1 mg/mL RNase B and 25 nM endoglycosidase. The reactions were incubated for 60 minutes at 37 °C. For each reaction, a sample (4 µL) was taken and added to 16 µL 1x Laemmli sample buffer without 2-mercaptoethanol and incubated for 5 minutes at 95 °C. Samples (5 µL/sample) were loaded on SDS-page gel and run for 70 min (20 mA), stained in colloidal coomassie overnight, and finally de-stained in water. Conversion percentages were calculated based on scanning of SDS-PAGE gel with regular flatbed scanner and quantification with a software tool (CLIQS v1.1).

Table 17. Percentages trimming (conversion) of RNase B upon treatment of various endoglycosidases and endoglycosidase fusion proteins (25 nM) at pH 4.5, 6.0 and 7.5.

pH	EndoSH	EndoF3-EndoH	EndoS	EndoF3	EndoH
4.5	74	75	0	0	30
6.0	70	100	0	0	39
7.5	42	48	0	0	33

Conversion was calculated using the following formula: conversion (%) = 100 × ($RNaseB_{trimmed}$) / ($RNaseB_{trimmed}$ + $RNaseB_{glycosylated}$).

Sequences

Sequence identification of fusion protein EndoS-EndoH *(or EndoSH) as expressed in E coli (SEQ. ID NO: 1):*

```
  1 MPSIDSLHYL SENSKKEFKE ELSKAGQESQ KVKEILAKAQ QADKQAQELA
 51 KMKIPEKIPM KPLHGPLYGG YFRTWHDKTS DPTEKDKVNS MGELPKEVDL
101 AFIFHDWTKD YSLFWKELAT KHVPKLNKQG TRVIRTIPWR FLAGGDNSGI
151 AEDTSKYPNT PEGNKALAKA IVDEYVYKYN LDGLDVDVEH DSIPKVDKKE
201 DTAGVERSIQ VFEEIGKLIG PKGVDKSRLF IMDSTYMADK NPLIERGAPY
251 INLLLVQVYG SQGEKGGWEP VSNRPEKTME ERWQGYSKYI RPEQYMIGFS
301 FYEENAQEGN LWYDINSRKD EDKANGINTD ITGTRAERYA RWQPKTGGVK
351 GGIFSYAIDR DGVAHQPKKY AKQKEFKDAT DNIFHSDYSV SKALKTVMLK
401 DKSYDLIDEK DFPDKALREA VMAQVGTRKG DLERFNGTLR LDNPAIQSLE
451 GLNKFKKLAQ LDLIGLSRIT KLDRSVLPAN MKPGKDTLET VLETYKKDNK
```

501 EEPATIPPVS LKVSGLTGLK ELDLSGFDRE TLAGLDAATL TSLEKVDISG
551 NKLDLAPGTE NRQIFDTMLS TISNHVGSNE QTVKFDKQKP TGHYPDTYGK
601 TSLRLPVANE KVDLQSQLLF GTVTNQGTLI NSEADYKAYQ NHKIAGRSFV
651 DSNYHYNNFK VSYENYTVKV TDSTLGTTTD KTLATDKEET YKVDFFSPAD
701 KTKAVHTAKV IVGDEKTMMV NLAEGATVIG GSADPVNARK VFDGQLGSET
751 DNISLGWDSK QSIIFKLKED GLIKHWRFFN DSARNPETTN KPIQEASLQI
801 FNIKDYNLDN LLENPNKFDD EKYWITVDTY SAQGERATAF SNTLNNITSK
851 YWRVVFDTKG DRYSSPVVPE LQILGYPLPN ADTIMKTVTT AKELSQQKDK
901 FSQKMLDELK IKEMALETSL NSKIFDVTAI NANAGVLKDC IEKRQLLKKG
951 GGGSGGGGSG GGGSHHHHHH EFGGGGSGGG GSGGGGS*APA PVKQGPTSVA*
1001 *YVEVNNNSML NVGKYTLADG GGNAFDVAVI FAANINYDTG TKTAYLHFNE*
1051 *NVQRVLDNAV TQIRPLQQQG IKVLLSVLGN HQGAGFANFP SQQAASAFAK*
1101 *QLSDAVAKYG LDGVDFDDEY AEYGNNGTAQ PNDSSFVHLV TALRANMPDK*
1151 *IISLYNIGPA ASRLSYGGVD VSDKFDYAWN PYYGTWQVPG IALPKAQLSP*
1201 *AAVEIGRTSR STVADLARRT VDEGYGVYLT YNLDGGDRTA DVSAFTRELY*
1251 *GSEAVRTP*

(linker is underlined, EndoH sequence is denoted in italics)

Sequence identification of fusion protein EndoS-EndoH *(or EndoSH) as expressed in CHO (SEQ. ID NO: 2):*

1 MPSIDSLHYL SENSKKEFKE ELSKAGQESQ KVKEILAKAQ QADKQAQELA
51 KMKIPEKIPM KPLHGPLYGG YFRTWHDKTS DPTEKDKVNS MGELPKEVDL
101 AFIFHDWTKD YSLFWKELAT KHVPKLNKQG TRVIRTIPWR FLAGGDNSGI
151 AEDTSKYPNT PEGNKALAKA IVDEYVYKYN LDGLDVDVEH DSIPKVDKKE
201 DTAGVERSIQ VFEEIGKLIG PKGVDKSRLF IMDSTYMADK NPLIERGAPY
251 INLLLVQVYG SQGEKGGWEP VSNRPEKTME ERWQGYSKYI RPEQYMIGFS
301 FYEENAQEGN LWYDINSRKD EDKANGINTD ITGTRAERYA RWQPKTGGVK
351 GGIFSYAIDR DGVAHQPKKY AKQKEFKDAT DNIFHSDYSV SKALKTVMLK
401 DKSYDLIDEK DFPDKALREA VMAQVGTRKG DLERFNGTLR LDNPAIQSLE
451 GLNKFKKLAQ LDLIGLSRIT KLDRSVLPAN MKPGKDTLET VLETYKKDNK
501 EEPATIPPVS LKVSGLTGLK ELDLSGFDRE TLAGLDAATL TSLEKVDISG
551 NKLDLAPGTE NRQIFDTMLS TISNHVGSNE QTVKFDKQKP TGHYPDTYGK
601 TSLRLPVANE KVDLQSQLLF GTVTNQGTLI NSEADYKAYQ NHKIAGRSFV
651 DSNYHYNNFK VSYENYTVKV TDSTLGTTTD KTLATDKEET YKVDFFSPAD
701 KTKAVHTAKV IVGDEKTMMV NLAEGATVIG GSADPVNARK VFDGQLGSET
751 DNISLGWDSK QSIIFKLKED GLIKHWRFFN DSARNPETTN KPIQEASLQI
801 FNIKDYNLDN LLENPNKFDD EKYWITVDTY SAQGERATAF SNTLNNITSK
851 YWRVVFDTKG DRYSSPVVPE LQILGYPLPN ADTIMKTVTT AKELSQQKDK
901 FSQKMLDELK IKEMALETSL NSKIFDVTAI NANAGVLKDC IEKRQLLKKG
951 GGGSGGGGSG GGGSHHHHHH GGGGSGGGGS GGGGS*APAPV KQGPTSVAYV*
1001 *EVNNNSMLNV GKYTLADGGG NAFDVAVIFA ANINYDTGTK TAYLHFNENV*

1051 *QRVLDNAVTQ IRPLQQQGIK VLLSVLGNHQ GAGFANFPSQ QAASAFAKQL*
1101 *SDAVAKYGLD GVDFDDEYAE YGNNGTAQPN DSSFVHLVTA LRANMPDKII*
1151 *SLYNIGPAAS RLSYGGVDVS DKFDYAWNPY YGTWQVPGIA LPKAQLSPAA*
1201 *VEIGRTSRST VADLARRTVD EGYGVYLTYN LDGGDRTADV SAFTRELYGS*
1251 *EAVRTP*

(linker is underlined, EndoH sequence is denoted in italics)

Sequence of His_6-TnGalNAcT(33-421) *as expressed in E coli (SEQ. ID NO: 3):*

1 MGSSHHHHHH SSGLVPRGSH MSPLRTYLYT PLYNATQPTL RNVERLAANW PKKIPSNYIE
61 DSEEYSIKNI SLSNHTTRAS VVHPPSSITE TASKLDKNMT IQDGAFAMIS PTPLLITKLM
121 DSIKSYVTTE DGVKKAEAVV TLPLCDSMPP DLGPITLNKT ELELEWVEKK FPEVEWGGRY
181 SPPNCTARHR VAIIVPYRDR QQHLAIFLNH MHPFLMKQQI EYGIFIVEQE GNKDFNRAKL
241 MNVGFVESQK LVAEGWQCFV FHDIDLLPLD TRNLYSCPRQ PRHMSASIDK LHFKLPYEDI
301 FGGVSAMTLE QFTRVNGFSN KYWGWGGEDD DMSYRLKKIN YHIARYKMSI ARYAMLDHKK
361 STPNPKRYQL LSQTSKTFQK DGLSTLEYEL VQVVQYHLYT HILVNIDERS

Sequence identification of fusion protein EndoF3-EfEndo18A *as expressed in E coli (SEQ. ID NO: 13):*

1 MATALAGSNG VCIAYYITDG RNPTFKLKDI PDKVDMVILF GLKYWSLQDT
51 TKLPGGTGMM GSFKSYKDLD TQIRSLQSRG IKVLQNIDDD VSWQSSKPGG
101 FASAAAYGDA IKSIVIDKWK LDGISLDIEH SGAKPNPIPT FPGYAATGYN
151 GWYSGSMAAT PAFLNVISEL TKYFGTTAPN NKQLQIASGI DVYAWNKIME
201 NFRNNFNYIQ LQSYGANVSR TQLMMNYATG TNKIPASKMV FGAYAEGGTN
251 QANDVEVAKW TPTQGAKGGM MIYTYNSNVS YANAVRDAVK NGGGGSGGGG
301 SGGGGSHHHH HHEFGGGGSG GGGSGGGGSA *STVTPKTVMY VEVNNHDFNN*
351 VGKYTLAGTN QPAFDMGIIF AANINYDTVN KKPYLYLNER VQQTLNEAET
401 QIRPVQARGT KVLLSILGNH EGAGFANFPT YESADAFAAQ LEQVVNTYHL
451 DGIDFDDEYA EYGKNGTPQP NNSSFIWLLQ ALRNRLGNDK LITFYNIGPA
501 AANSSANPQM SSLIDYAWNP YYSTWNPPQI AGMPASRLGA SAVEVGVNQN
551 LAAQYAKRTK AEQYGIYLMY NLPGKDSSAY ISAATQELYG RKTNYSPTVP
601 TP

(linker is underlined, EfEndo18A sequence is denoted in italics)

Sequence identification of fusion protein EndoF2-EfEndo18A *as expressed in E coli (SEQ. ID NO: 14):*

1 MAVNLSNLIA YKNSDHQISA GYYRTWRDSA TASGNLPSMR WLPDSLDMVM
51 VFPDYTPPEN AYWNTLKTNY VPYLHKRGTK VIITLGDLNS ATTTGGQDSI
101 GYSSWAKGIY DKWVGEYNLD GIDIDIESSP SGATLTKFVA ATKALSKYFG
151 PKSGTGKTFV YDTNQNPTNF FIQTAPRYNY VFLQAYGRST TNLTTVSGLY
201 APYISMKQFL PGFSFYEENG YPGNYWNDVR YPQNGTGRAY DYARWQPATG
251 KKGGVFSYAI ERDAPLTSSN DNTLRAPNFR VTKDLIKIMN PGGGGSGGGG

301 SGGGGSHHHH HHEFGGGGSG GGGSGGGGSA *STVTPKTVMY VEVNNHDFNN*
351 VGKYTLAGTN QPAFDMGIIF AANINYDTVN KKPYLYLNER VQQTLNEAET
401 QIRPVQARGT KVLLSILGNH EGAGFANFPT YESADAFAAQ LEQVVNTYHL
451 DGIDFDDEYA EYGKNGTPQP NNSSFIWLLQ ALRNRLGNDK LITFYNIGPA
501 AANSSANPQM SSLIDYAWNP YYSTWNPPQI AGMPASRLGA SAVEVGVNQN
551 LAAQYAKRTK AEQYGIYLMY NLPGKDSSAY ISAATQELYG RKTNYSPTVP
601 TP

(linker is underlined, EfEndo18A sequence is denoted in italics)

Sequence identification of fusion protein EndoS-EfEndo18A *as expressed in E coli (SEQ. ID NO: 15):*

1 MPSIDSLHYL SENSKKEFKE ELSKAGQESQ KVKEILAKAQ QADKQAQELA
51 KMKIPEKIPM KPLHGPLYGG YFRTWHDKTS DPTEKDKVNS MGELPKEVDL
101 AFIFHDWTKD YSLFWKELAT KHVPKLNKQG TRVIRTIPWR FLAGGDNSGI
151 AEDTSKYPNT PEGNKALAKA IVDEYVYKYN LDGLDVDVEH DSIPKVDKKE
201 DTAGVERSIQ VFEEIGKLIG PKGVDKSRLF IMDSTYMADK NPLIERGAPY
251 INLLLVQVYG SQGEKGGWEP VSNRPEKTME ERWQGYSKYI RPEQYMIGFS
301 FYEENAQEGN LWYDINSRKD EDKANGINTD ITGTRAERYA RWQPKTGGVK
351 GGIFSYAIDR DGVAHQPKKY AKQKEFKDAT DNIFHSDYSV SKALKTVMLK
401 DKSYDLIDEK DFPDKALREA VMAQVGTRKG DLERFNGTLR LDNPAIQSLE
451 GLNKFKKLAQ LDLIGLSRIT KLDRSVLPAN MKPGKDTLET VLETYKKDNK
501 EEPATIPPVS LKVSGLTGLK ELDLSGFDRE TLAGLDAATL TSLEKVDISG
551 NKLDLAPGTE NRQIFDTMLS TISNHVGSNE QTVKFDKQKP TGHYPDTYGK
601 TSLRLPVANE KVDLQSQLLF GTVTNQGTLI NSEADYKAYQ NHKIAGRSFV
651 DSNYHYNNFK VSYENYTVKV TDSTLGTTTD KTLATDKEET YKVDFFSPAD
701 KTKAVHTAKV IVGDEKTMMV NLAEGATVIG GSADPVNARK VFDGQLGSET
751 DNISLGWDSK QSIIFKLKED GLIKHWRFFN DSARNPETTN KPIQEASLQI
801 FNIKDYNLDN LLENPNKFDD EKYWITVDTY SAQGERATAF SNTLNNITSK
851 YWRVVFDTKG DRYSSPVVPE LQILGYPLPN ADTIMKTVTT AKELSQQKDK
901 FSQKMLDELK IKEMALETSL NSKIFDVTAI NANAGVLKDC IEKRQLLKKG
951 GGGSGGGGSG GGGSHHHHHH EFGGGGSGGG GSGGGGS*AST VTPKTVMYVE*
1001 VNNHDFNNVG KYTLAGTNQP AFDMGIIFAA NINYDTVNKK PYLYLNERVQ
1051 QTLNEAETQI RPVQARGTKV LLSILGNHEG AGFANFPTYE SADAFAAQLE
1101 QVVNTYHLDG IDFDDEYAEY GKNGTPQPNN SSFIWLLQAL RNRLGNDKLI
1151 TFYNIGPAAA NSSANPQMSS LIDYAWNPYY STWNPPQIAG MPASRLGASA
1201 VEVGVNQNLA AQYAKRTKAE QYGIYLMYNL PGKDSSAYIS AATQELYGRK
1251 TNYSPTVPTP

(linker is underlined, EfEndo18A sequence is denoted in italics)

Sequence identification of fusion protein EndoF3-EndoF1 *as expressed in E coli (SEQ. ID NO: 16):*

1 MATALAGSNG VCIAYYITDG RNPTFKLKDI PDKVDMVILF GLKYWSLQDT

51 TKLPGGTGMM GSFKSYKDLD TQIRSLQSRG IKVLQNIDDD VSWQSSKPGG
101 FASAAAYGDA IKSIVIDKWK LDGISLDIEH SGAKPNPIPT FPGYAATGYN
151 GWYSGSMAAT PAFLNVISEL TKYFGTTAPN NKQLQIASGI DVYAWNKIME
201 NFRNNFNYIQ LQSYGANVSR TQLMMNYATG TNKIPASKMV FGAYAEGGTN
251 QANDVEVAKW TPTQGAKGGM MIYTYNSNVS YANAVRDAVK NGGGGSGGGG
301 SGGGGSHHHH HHEFGGGGSG GGGSGGGGSA *VTGTTKANIK LFSFTEVNDT*
351 NPLNNLNFTL KNSGKPLVDM VVLFSANINY DAANDKVFVS NNPNVQHLLT
401 NRAKYLKPLQ DKGIKVILSI LGNHDRSGIA NLSTARAKAF AQELKNTCDL
451 YNLDGVFFDD EYSAYQTPPP SGFVTPSNNA AARLAYETKQ AMPNKLVTVY
501 VYSRTSSFPT AVDGVNAGSY VDYAIHDYGG SYDLATNYPG LAKSGMVMSS
551 QEFNQGRYAT AQALRNIVTK GYGGHMIFAM DPNRSNFTSG QLPALKLIAK
601 ELYGDELVYS NTPYSKDW

(linker is underlined, EndoF1 sequence is denoted in italics)

Sequence identification of fusion protein EndoF2-EndoF1 *as expressed in E coli (SEQ. ID NO: 17):*

1 MAVNLSNLIA YKNSDHQISA GYYRTWRDSA TASGNLPSMR WLPDSLDMVM
51 VFPDYTPPEN AYWNTLKTNY VPYLHKRGTK VIITLGDLNS ATTTGGQDSI
101 GYSSWAKGIY DKWVGEYNLD GIDIDIESSP SGATLTKFVA ATKALSKYFG
151 PKSGTGKTFV YDTNQNPTNF FIQTAPRYNY VFLQAYGRST TNLTTVSGLY
201 APYISMKQFL PGFSFYEENG YPGNYWNDVR YPQNGTGRAY DYARWQPATG
251 KKGGVFSYAI ERDAPLTSSN DNTLRAPNFR VTKDLIKIMN PGGGGSGGGG
301 SGGGGSHHHH HHEFGGGGSG GGGSGGGGSA *VTGTTKANIK LFSFTEVNDT*
351 NPLNNLNFTL KNSGKPLVDM VVLFSANINY DAANDKVFVS NNPNVQHLLT
401 NRAKYLKPLQ DKGIKVILSI LGNHDRSGIA NLSTARAKAF AQELKNTCDL
451 YNLDGVFFDD EYSAYQTPPP SGFVTPSNNA AARLAYETKQ AMPNKLVTVY
501 VYSRTSSFPT AVDGVNAGSY VDYAIHDYGG SYDLATNYPG LAKSGMVMSS
551 QEFNQGRYAT AQALRNIVTK GYGGHMIFAM DPNRSNFTSG QLPALKLIAK
601 ELYGDELVYS NTPYSKDW

(linker is underlined, EndoF1 sequence is denoted in italics)

Sequence identification of fusion protein EndoS-EndoF1 *as expressed in E coli (SEQ. ID NO: 18):*

1 MPSIDSLHYL SENSKKEFKE ELSKAGQESQ KVKEILAKAQ QADKQAQELA
51 KMKIPEKIPM KPLHGPLYGG YFRTWHDKTS DPTEKDKVNS MGELPKEVDL
101 AFIFHDWTKD YSLFWKELAT KHVPKLNKQG TRVIRTIPWR FLAGGDNSGI
151 AEDTSKYPNT PEGNKALAKA IVDEYVYKYN LDGLDVDVEH DSIPKVDKKE
201 DTAGVERSIQ VFEEIGKLIG PKGVDKSRLF IMDSTYMADK NPLIERGAPY
251 INLLLVQVYG SQGEKGGWEP VSNRPEKTME ERWQGYSKYI RPEQYMIGFS
301 FYEENAQEGN LWYDINSRKD EDKANGINTD ITGTRAERYA RWQPKTGGVK
351 GGIFSYAIDR DGVAHQPKKY AKQKEFKDAT DNIFHSDYSV SKALKTVMLK
401 DKSYDLIDEK DFPDKALREA VMAQVGTRKG DLERFNGTLR LDNPAIQSLE
451 GLNKFKKLAQ LDLIGLSRIT KLDRSVLPAN MKPGKDTLET VLETYKKDNK

501 EEPATIPPVS LKVSGLTGLK ELDLSGFDRE TLAGLDAATL TSLEKVDISG
551 NKLDLAPGTE NRQIFDTMLS TISNHVGSNE QTVKFDKQKP TGHYPDTYGK
601 TSLRLPVANE KVDLQSQLLF GTVTNQGTLI NSEADYKAYQ NHKIAGRSFV
651 DSNYHYNNFK VSYENYTVKV TDSTLGTTTD KTLATDKEET YKVDFFSPAD
701 KTKAVHTAKV IVGDEKTMMV NLAEGATVIG GSADPVNARK VFDGQLGSET
751 DNISLGWDSK QSIIFKLKED GLIKHWRFFN DSARNPETTN KPIQEASLQI
801 FNIKDYNLDN LLENPNKFDD EKYWITVDTY SAQGERATAF SNTLNNITSK
851 YWRVVFDTKG DRYSSPVVPE LQILGYPLPN ADTIMKTVTT AKELSQQKDK
901 FSQKMLDELK IKEMALETSL NSKIFDVTAI NANAGVLKDC IEKRQLLKKG
951 GGGSGGGGSG GGGSHHHHHH EFGGGGSGGG GSGGGGS*AVT GTTKANIKLF*
1001 SFTEVNDTNP LNNLNFTLKN SGKPLVDMVV LFSANINYDA ANDKVFVSNN
1051 PNVQHLLTNR AKYLKPLQDK GIKVILSILG NHDRSGIANL STARAKAFAQ
1101 ELKNTCDLYN LDGVFFDDEY SAYQTPPPSG FVTPSNNAAA RLAYETKQAM
1151 PNKLVTVYVY SRTSSFPTAV DGVNAGSYVD YAIHDYGGSY DLATNYPGLA
1201 KSGMVMSSQE FNQGRYATAQ ALRNIVTKGY GGHMIFAMDP NRSNFTSGQL
1251 PALKLIAKEL YGDELVYSNT PYSKDW

(linker is underlined, EndoF1 sequence is denoted in italics)

Sequence identification of fusion protein EndoF3-EndoH *as expressed in E coli (SEQ. ID NO: 19):*

1 MATALAGSNG VCIAYYITDG RNPTFKLKDI PDKVDMVILF GLKYWSLQDT
51 TKLPGGTGMM GSFKSYKDLD TQIRSLQSRG IKVLQNIDDD VSWQSSKPGG
101 FASAAAYGDA IKSIVIDKWK LDGISLDIEH SGAKPNPIPT FPGYAATGYN
151 GWYSGSMAAT PAFLNVISEL TKYFGTTAPN NKQLQIASGI DVYAWNKIME
201 NFRNNFNYIQ LQSYGANVSR TQLMMNYATG TNKIPASKMV FGAYAEGGTN
251 QANDVEVAKW TPTQGAKGGM MIYTYNSNVS YANAVRDAVK NGGGGSGGGG
301 SGGGGSHHHH HHEFGGGGSG GGGSGGGGS*A PAPVKQGPTS VAYVEVNNNS*
351 MLNVGKYTLA DGGGNAFDVA VIFAANINYD TGTKTAYLHF NENVQRVLDN
401 AVTQIRPLQQ QGIKVLLSVL GNHQGAGFAN FPSQQAASAF AKQLSDAVAK
451 YGLDGVDFDD EYAEYGNNGT AQPNDSSFVH LVTALRANMP DKIISLYNIG
501 PAASRLSYGG VDVSDKFDYA WNPYYGTWQV PGIALPKAQL SPAAVEIGRT
551 SRSTVADLAR RTVDEGYGVY LTYNLDGGDR TADVSAFTRE LYGSEAVRTP

(linker is underlined, EndoH sequence is denoted in italics)

Sequence identification of fusion protein EndoF2-EndoH *as expressed in E coli (SEQ. ID NO: 20):*

1 MAVNLSNLIA YKNSDHQISA GYYRTWRDSA TASGNLPSMR WLPDSLDMVM
51 VFPDYTPPEN AYWNTLKTNY VPYLHKRGTK VIITLGDLNS ATTTGGQDSI
101 GYSSWAKGIY DKWVGEYNLD GIDIDIESSP SGATLTKFVA ATKALSKYFG
151 PKSGTGKTFV YDTNQNPTNF FIQTAPRYNY VFLQAYGRST TNLTTVSGLY
201 APYISMKQFL PGFSFYEENG YPGNYWNDVR YPQNGTGRAY DYARWQPATG
251 KKGGVFSYAI ERDAPLTSSN DNTLRAPNFR VTKDLIKIMN PGGGGSGGGG
301 SGGGGSHHHH HHEFGGGGSG GGGSGGGGS*A PAPVKQGPTS VAYVEVNNNS*

351 MLNVGKYTLA DGGGNAFDVA VIFAANINYD TGTKTAYLHF NENVQRVLDN
401 AVTQIRPLQQ QGIKVLLSVL GNHQGAGFAN FPSQQAASAF AKQLSDAVAK
451 YGLDGVDFDD EYAEYGNNGT AQPNDSSFVH LVTALRANMP DKIISLYNIG
501 PAASRLSYGG VDVSDKFDYA WNPYYGTWQV PGIALPKAQL SPAAVEIGRT
551 SRSTVADLAR RTVDEGYGVY LTYNLDGGDR TADVSAFTRE LYGSEAVRTP

(linker is underlined, EndoH sequence is denoted in italics)

Sequence identification of fusion protein His_6-EndoS-EndoH (EndoS-EndoH without GS-linker) as expressed in E coli (SEQ. ID NO: 21):

1 MGSSHHHHHH SSGLVPRGSH MPSIDSLHYL SENSKKEFKE ELSKAGQESQ
51 KVKEILAKAQ QADKQAQELA KMKIPEKIPM KPLHGPLYGG YFRTWHDKTS
101 DPTEKDKVNS MGELPKEVDL AFIFHDWTKD YSLFWKELAT KHVPKLNKQG
151 TRVIRTIPWR FLAGGDNSGI AEDTSKYPNT PEGNKALAKA IVDEYVYKYN
201 LDGLDVDVEH DSIPKVDKKE DTAGVERSIQ VFEEIGKLIG PKGVDKSRLF
251 IMDSTYMADK NPLIERGAPY INLLLVQVYG SQGEKGGWEP VSNRPEKTME
301 ERWQGYSKYI RPEQYMIGFS FYEENAQEGN LWYDINSRKD EDKANGINTD
351 ITGTRAERYA RWQPKTGGVK GGIFSYAIDR DGVAHQPKKY AKQKEFKDAT
401 DNIFHSDYSV SKALKTVMLK DKSYDLIDEK DFPDKALREA VMAQVGTRKG
451 DLERFNGTLR LDNPAIQSLE GLNKFKKLAQ LDLIGLSRIT KLDRSVLPAN
501 MKPGKDTLET VLETYKKDNK EEPATIPPVS LKVSGLTGLK ELDLSGFDRE
551 TLAGLDAATL TSLEKVDISG NKLDLAPGTE NRQIFDTMLS TISNHVGSNE
601 QTVKFDKQKP TGHYPDTYGK TSLRLPVANE KVDLQSQLLF GTVTNQGTLI
651 NSEADYKAYQ NHKIAGRSFV DSNYHYNNFK VSYENYTVKV TDSTLGTTTD
701 KTLATDKEET YKVDFFSPAD KTKAVHTAKV IVGDEKTMMV NLAEGATVIG
751 GSADPVNARK VFDGQLGSET DNISLGWDSK QSIIFKLKED GLIKHWRFFN
801 DSARNPETTN KPIQEASLQI FNIKDYNLDN LLENPNKFDD EKYWITVDTY
851 SAQGERATAF SNTLNNITSK YWRVVFDTKG DRYSSPVVPE LQILGYPLPN
901 ADTIMKTVTT AKELSQQKDK FSQKMLDELK IKEMALETSL NSKIFDVTAI
951 NANAGVLKDC IEKRQLLKKA *PAPVKQGPTS VAYVEVNNNS MLNVGKYTLA*
1001 DGGGNAFDVA VIFAANINYD TGTKTAYLHF NENVQRVLDN AVTQIRPLQQ
1051 QGIKVLLSVL GNHQGAGFAN FPSQQAASAF AKQLSDAVAK YGLDGVDFDD
1101 EYAEYGNNGT AQPNDSSFVH LVTALRANMP DKIISLYNIG PAASRLSYGG
1151 VDVSDKFDYA WNPYYGTWQV PGIALPKAQL SPAAVEIGRT SRSTVADLAR
1201 RTVDEGYGVY LTYNLDGGDR TADVSAFTRE LYGSEAVRTP

(N-terminal sequence including His-tag and thrombin cleavage site is underlined, EndoH sequence is in italics)

Sequence identification of DNA encoding for fusion protein EndoF3-EfEndo18A *as expressed in E coli (SEQ. ID NO: 22):*

ATGGCTACAGCGCTGGCTGGTTCTAACGGGGTCTGCATCGCGTATTACATCACCGATGGGCGTAATCCGACG
TTCAAATTGAAAGACATCCCGGATAAAGTAGACATGGTAATTCTTTTTGGTCTTAAGTATTGGTCATTGCAG

GATACAACCAAATTGCCAGGGGGTACTGGTATGATGGGTTCGTTTAAATCCTACAAGGACCTGGACACCCAG
ATTCGTAGTCTTCAAAGCCGTGGAATCAAAGTGTTGCAGAACATTGACGACGACGTCTCATGGCAGTCCTCG
AAGCCGGGTGGGTTCGCTTCCGCCGCTGCTTACGGGGATGCTATTAAGAGTATCGTAATTGATAAGTGGAAG
CTGGACGGGATTAGCTTGGATATTGAGCATTCGGGGGCTAAACCCAACCCTATCCCAACTTTTCCTGGATAT
GCCGCGACAGGATATAATGGCTGGTATTCAGGATCTATGGCAGCCACGCCTGCCTTTCTTAATGTTATCTCA
GAGCTTACTAAATACTTTGGTACAACGGCACCGAATAATAAGCAACTTCAGATTGCTTCGGGTATTGACGTA
TATGCCTGGAATAAAATCATGGAGAACTTTCGTAATAACTTCAACTACATCCAATTACAGTCATACGGAGCT
AATGTCTCTCGTACTCAACTTATGATGAATTACGCAACGGGAACTAATAAAATTCCCGCCTCTAAAATGGTT
TTCGGCGCCTACGCAGAGGGTGGCACTAACCAGGCAAATGACGTGGAGGTCGCCAAGTGGACACCTACGCAG
GGCGCAAAGGGCGGTATGATGATCTATACTTACAATTCGAACGTGAGCTATGCAAATGCGGTTCGCGACGCA
GTGAAAAATGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTCACCACCACCACCACCAC
GAATTCGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGCTTCAACCGTAACCCCTAAA
ACGGTTATGTACGTAGAAGTAAATAACCACGATTTCAACAATGTCGGGAAATACACTCTTGCCGGTACTAAT
CAGCCGGCGTTCGATATGGGTATTATTTTTGCCGCCAACATCAATTATGACACCGTCAATAAGAAACCATAC
CTGTACTTGAACGAGCGCGTACAGCAAACACTGAATGAAGCGGAGACGCAGATCCGTCCGGTCCAGGCACGT
GGAACGAAGGTTTTGCTTTCCATCTTGGGTAATCACGAAGGCGCAGGATTTGCCAATTTTCCTACGTATGAG
TCGGCGGACGCTTTCGCCGCGCAACTTGAGCAGGTTGTCAATACGTACCATTTAGACGGGATTGATTTCGAT
GATGAGTACGCCGAGTACGGAAAAAACGGGACCCCTCAGCCGAACAACTCATCCTTCATCTGGTTACTGCAA
GCTCTTCGCAACCGTCTGGGAAATGATAAACTTATCACTTTCTACAACATTGGCCCGGCAGCCGCTAACAGC
AGCGCAAACCCTCAAATGTCATCTTTGATTGACTATGCCTGGAATCCCTATTATTCGACATGGAACCCCCCA
CAAATTGCAGGTATGCCTGCCTCCCGCCTGGGGGCTTCTGCGGTTGAAGTGGGCGTTAACCAGAATCTTGCA
GCACAGTATGCCAAGCGTACTAAGGCTGAGCAGTATGGAATCTATCTGATGTACAATCTGCCAGGAAAAGAT
TCTAGCGCTTATATCTCAGCAGCGACTCAGGAGCTGTATGGGCGCAAGACGAACTATAGCCCCACGGTCCCG
ACTCCGTGATAA

Sequence identification of DNA encoding for fusion protein EndoF2-EfEndo18A *as expressed in E coli (SEQ. ID NO: 23):*

ATGGCGGTAAACCTTAGTAATCTTATCGCTTATAAAAATAGTGACCATCAGATCAGTGCGGGATATTACCGT
ACATGGCGTGACAGCGCCACAGCCAGTGGTAATCTTCCTAGTATGCGTTGGTTGCCAGACTCATTGGACATG
GTAATGGTATTCCCAGACTATACTCCTCCGGAAAATGCGTATTGGAACACACTGAAGACTAACTACGTACCA
TACCTGCATAAGCGTGGCACGAAAGTTATTATCACATTGGGGGACCTTAACTCTGCAACGACCACGGGAGGG
CAAGATTCTATTGGGTATTCATCGTGGGCCAAAGGAATCTATGATAAATGGGTGGGCGAGTATAATCTTGAT
GGAATCGATATTGACATCGAATCGTCACCGTCCGGTGCGACCTTAACGAAGTTTGTTGCGGCAACAAAAGCG
TTGTCAAAGTATTTTGGACCAAAGAGTGGGACAGGCAAGACCTTTGTATACGATACCAATCAGAATCCGACT
AATTTCTTTATCCAAACTGCCCCACGCTACAACTACGTATTTCTTCAAGCATACGGGCGCTCGACCACTAAT
CTGACGACGGTCTCTGGATTATACGCCCCCTATATTTCAATGAAACAATTTCTGCCCGGCTTCTCTTTTTAC
GAAGAAAACGGTTACCCAGGTAATTATTGGAATGATGTGCGTTACCCCCAGAACGGTACAGGCCGTGCCTAC
GACTACGCGCGCTGGCAGCCCGCCACGGGAAAAAAAGGAGGGGTGTTCAGTTATGCCATCGAGCGCGACGCC
CCTCTTACATCGTCAAACGACAATACCCTGCGTGCGCCTAACTTTCGTGTAACGAAGGACTTAATCAAAATT
ATGAATCCTGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTCACCACCACCACCACCAC
GAATTCGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGCTTCAACCGTAACCCCTAAA

ACGGTTATGTACGTAGAAGTAAATAACCACGATTTCAACAATGTCGGGAAATACACTCTTGCCGGTACTAAT
CAGCCGGCGTTCGATATGGGTATTATTTTTGCCGCCAACATCAATTATGACACCGTCAATAAGAAACCATAC
CTGTACTTGAACGAGCGCGTACAGCAAACACTGAATGAAGCGGAGACGCAGATCCGTCCGGTCCAGGCACGT
GGAACGAAGGTTTTGCTTTCCATCTTGGGTAATCACGAAGGCGCAGGATTTGCCAATTTTCCTACGTATGAG
TCGGCGGACGCTTTCGCCGCGCAACTTGAGCAGGTTGTCAATACGTACCATTTAGACGGGATTGATTTCGAT
GATGAGTACGCCGAGTACGGAAAAAACGGGACCCCTCAGCCGAACAACTCATCCTTCATCTGGTTACTGCAA
GCTCTTCGCAACCGTCTGGGAAATGATAAACTTATCACTTTCTACAACATTGGCCCGGCAGCCGCTAACAGC
AGCGCAAACCCTCAAATGTCATCTTTGATTGACTATGCCTGGAATCCCTATTATTCGACATGGAACCCCCCA
CAAATTGCAGGTATGCCTGCCTCCCGCCTGGGGGCTTCTGCGGTTGAAGTGGGCGTTAACCAGAATCTTGCA
GCACAGTATGCCAAGCGTACTAAGGCTGAGCAGTATGGAATCTATCTGATGTACAATCTGCCAGGAAAAGAT
TCTAGCGCTTATATCTCAGCAGCGACTCAGGAGCTGTATGGGCGCAAGACGAACTATAGCCCCACGGTCCCG
ACTCCGTGATAA

Sequence identification of DNA encoding for fusion protein EndoS-EfEndo18A *as expressed in E coli (SEQ. ID NO: 24):*

ATGCCGTCAATCGATTCGCTGCATTATCTGAGCGAAAACTCTAAAAAAGAATTTAAAGAAGAACTGAGCAAA
GCGGGCCAGGAATCTCAAAAAGTTAAAGAAATCCTGGCAAAAGCTCAGCAAGCCGATAAACAGGCACAAGAA
CTGGCTAAAATGAAAATTCCGGAAAAAATCCCGATGAAACCGCTGCATGGTCCGCTGTACGGCGGTTATTTC
CGTACCTGGCACGATAAAACGTCAGACCCGACCGAAAAAGACAAAGTCAACTCGATGGGCGAACTGCCGAAA
GAAGTGGATCTGGCTTTTATTTTCCATGATTGGACCAAAGACTACTCTCTGTTTTGGAAAGAACTGGCAACG
AAACACGTTCCGAAACTGAACAAACAGGGTACGCGTGTCATTCGTACCATTCCGTGGCGCTTCCTGGCTGGC
GGTGATAATTCAGGCATCGCGGAAGACACCTCGAAATATCCGAACACGCCGGAAGGTAATAAAGCGCTGGCC
AAAGCAATCGTCGATGAATACGTGTACAAATACAATCTGGACGGCCTGGATGTGGACGTTGAACATGATTCA
ATTCCGAAAGTGGATAAAAAAGAAGACACCGCCGGCGTGGAACGTTCGATCCAGGTTTTTGAAGAAATTGGT
AAACTGATCGGCCCGAAAGGTGTTGATAAAAGCCGTCTGTTCATCATGGATTCTACCTATATGGCCGACAAA
AATCCGCTGATTGAACGCGGTGCACCGTACATCAACCTGCTGCTGGTCCAGGTGTATGGCAGCCAAGGTGAA
AAAGGCGGTTGGGAACCGGTGTCTAACCGTCCGGAAAAAACCATGGAAGAACGCTGGCAGGGCTACTCAAAA
TATATTCGTCCGGAACAATACATGATCGGCTTTTCGTTCTATGAAGAAAACGCGCAGGAAGGTAATCTGTGG
TACGATATTAATAGTCGCAAAGATGAAGACAAAGCCAACGGCATTAATACCGATATCACGGGTACCCGTGCG
GAACGCTATGCCCGTTGGCAGCCGAAAACCGGCGGTGTTAAAGGCGGTATTTTTAGCTACGCGATCGATCGT
GACGGTGTCGCCCATCAGCCGAAAAAATACGCAAAACAAAAAGAGTTCAAAGATGCTACCGACAACATCTTC
CACAGCGATTACAGTGTCTCCAAAGCGCTGAAAACCGTGATGCTGAAAGATAAATCTTACGATCTGATCGAC
GAAAAAGATTTTCCGGACAAAGCGCTGCGCGAAGCCGTTATGGCACAGGTCGGCACCCGCAAAGGTGACCTG
GAACGTTTTAATGGCACGCTGCGCCTGGATAACCCGGCCATTCAGAGCCTGGAAGGTCTGAATAAATTCAAA
AAACTGGCACAACTGGACCTGATTGGCCTGAGCCGTATCACCAAACTGGATCGCTCTGTGCTGCCGGCCAAC
ATGAAACCGGGTAAAGACACGCTGGAAACCGTTCTGGAAACCTACAAAAAAGATAACAAAGAAGAACCGGCA
ACGATCCCGCCGGTGTCTCTGAAAGTTTCCGGCCTGACCGGTCTGAAAGAACTGGATCTGAGCGGCTTTGAC
CGTGAAACGCTGGCAGGTCTGGATGCGGCCACGCTGACCAGTCTGGAAAAAGTTGATATTTCCGGCAATAAA
CTGGACCTGGCGCCGGGTACCGAAAACCGCCAGATTTTTGATACGATGCTGAGTACCATCTCCAACCATGTT
GGCAGCAATGAACAGACCGTCAAATTCGACAAACAAAAACCGACGGGCCACTACCCGGATACGTATGGTAAA
ACCAGCCTGCGTCTGCCGGTCGCCAACGAAAAAGTGGATCTGCAGTCTCAACTGCTGTTTGGCACGGTTACC

AATCAGGGTACCCTGATTAACAGCGAAGCAGATTACAAGGCTTACCAAAACCATAAAATCGCGGGTCGCTCA
TTTGTGGATTCGAACTACCACTACAACAACTTCAAAGTTAGTTACGAAAACTACACCGTTAAAGTCACGGAT
TCCACCCTGGGCACCACGACCGATAAAACGCTGGCCACCGACAAAGAAGAAACCTACAAAGTCGATTTCTTT
AGCCCGGCAGACAAAACGAAAGCGGTGCATACCGCCAAAGTGATTGTTGGCGATGAAAAAACCATGATGGTG
AACCTGGCTGAAGGTGCGACGGTTATCGGCGGTTCCGCAGACCCGGTTAACGCTCGCAAAGTCTTTGATGGC
CAGCTGGGTAGTGAAACCGATAATATTTCCCTGGGTTGGGACTCAAAACAGTCGATTATCTTCAAACTGAAA
GAAGACGGCCTGATCAAACACTGGCGTTTCTTTAACGATAGTGCCCGCAATCCGGAAACGACCAACAAACCG
ATTCAGGAAGCATCCCTGCAAATCTTCAACATCAAAGATTACAACCTGGACAATCTGCTGGAAAACCCGAAT
AAATTCGATGACGAAAAATACTGGATCACGGTGGATACCTATAGCGCGCAGGGCGAACGTGCTACGGCGTTT
AGTAACACCCTGAACAATATTACGTCCAAATACTGGCGTGTGGTTTTCGATACCAAAGGTGACCGCTATAGC
TCTCCGGTCGTGCCGGAACTGCAGATTCTGGGCTATCCGCTGCCGAATGCTGATACGATCATGAAAACCGTG
ACGACCGCGAAAGAACTGTCACAGCAAAAAGATAAATTCTCGCAGAAAATGCTGGACGAACTGAAAATTAAA
GAAATGGCTCTGGAAACCAGCCTGAACAGTAAAATTTTCGATGTTACGGCGATCAATGCTAACGCTGGTGTG
CTGAAAGACTGTATTGAAAAACGCCAACTGCTGAAAAAAGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGC
GGCGGCGGCTCTCACCACCACCACCACCACGAATTCGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGCGGC
GGCGGCTCTGCTTCAACCGTAACCCCTAAAACGGTTATGTACGTAGAAGTAAATAACCACGATTTCAACAAT
GTCGGGAAATACACTCTTGCCGGTACTAATCAGCCGGCGTTCGATATGGGTATTATTTTTGCCGCCAACATC
AATTATGACACCGTCAATAAGAAACCATACCTGTACTTGAACGAGCGCGTACAGCAAACACTGAATGAAGCG
GAGACGCAGATCCGTCCGGTCCAGGCACGTGGAACGAAGGTTTTGCTTTCCATCTTGGGTAATCACGAAGGC
GCAGGATTTGCCAATTTTCCTACGTATGAGTCGGCGGACGCTTTCGCCGCGCAACTTGAGCAGGTTGTCAAT
ACGTACCATTTAGACGGGATTGATTTCGATGATGAGTACGCCGAGTACGGAAAAAACGGGACCCCTCAGCCG
AACAACTCATCCTTCATCTGGTTACTGCAAGCTCTTCGCAACCGTCTGGGAAATGATAAACTTATCACTTTC
TACAACATTGGCCCGGCAGCCGCTAACAGCAGCGCAAACCCTCAAATGTCATCTTTGATTGACTATGCCTGG
AATCCCTATTATTCGACATGGAACCCCCCACAAATTGCAGGTATGCCTGCCTCCCGCCTGGGGGCTTCTGCG
GTTGAAGTGGGCGTTAACCAGAATCTTGCAGCACAGTATGCCAAGCGTACTAAGGCTGAGCAGTATGGAATC
TATCTGATGTACAATCTGCCAGGAAAAGATTCTAGCGCTTATATCTCAGCAGCGACTCAGGAGCTGTATGGG
CGCAAGACGAACTATAGCCCCACGGTCCCGACTCCGTGATAA

Sequence identification of DNA encoding for fusion protein EndoF3-EndoF1 *as expressed in E coli (SEQ. ID NO: 25):*

ATGGCTACAGCGCTGGCTGGTTCTAACGGGGTCTGCATCGCGTATTACATCACCGATGGGCGTAATCCGACG
TTCAAATTGAAAGACATCCCGGATAAAGTAGACATGGTAATTCTTTTTGGTCTTAAGTATTGGTCATTGCAG
GATACAACCAAATTGCCAGGGGGTACTGGTATGATGGGTTCGTTTAAATCCTACAAGGACCTGGACACCCAG
ATTCGTAGTCTTCAAAGCCGTGGAATCAAAGTGTTGCAGAACATTGACGACGACGTCTCATGGCAGTCCTCG
AAGCCGGGTGGGTTCGCTTCCGCCGCTGCTTACGGGGATGCTATTAAGAGTATCGTAATTGATAAGTGGAAG
CTGGACGGGATTAGCTTGGATATTGAGCATTCGGGGGCTAAACCCAACCCTATCCCAACTTTTCCTGGATAT
GCCGCGACAGGATATAATGGCTGGTATTCAGGATCTATGGCAGCCACGCCTGCCTTTCTTAATGTTATCTCA
GAGCTTACTAAATACTTTGGTACAACGGCACCGAATAATAAGCAACTTCAGATTGCTTCGGGTATTGACGTA
TATGCCTGGAATAAAATCATGGAGAACTTTCGTAATAACTTCAACTACATCCAATTACAGTCATACGGAGCT
AATGTCTCTCGTACTCAACTTATGATGAATTACGCAACGGGAACTAATAAAATTCCCGCCTCTAAAATGGTT
TTCGGCGCCTACGCAGAGGGTGGCACTAACCAGGCAAATGACGTGGAGGTCGCCAAGTGGACACCTACGCAG

GGCGCAAAGGGCGGTATGATGATCTATACTTACAATTCGAACGTGAGCTATGCAAATGCGGTTCGCGACGCA
GTGAAAAATGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTCACCACCACCACCACCAC
GAATTCGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGCGGTAACCGGGACAACGAAG
GCTAACATCAAACTTTTTAGTTTTACAGAGGTAAACGACACTAATCCGTTGAACAATCTGAACTTTACCTTA
AAAAACTCGGGAAAACCCTTAGTAGATATGGTAGTGTTATTTTCCGCGAACATTAACTATGACGCGGCCAAC
GATAAGGTCTTCGTATCGAATAATCCGAACGTACAGCATCTTTTGACCAATCGTGCGAAGTACCTTAAGCCG
TTACAAGACAAGGGGATCAAGGTGATTTTGTCAATCTTAGGGAACCATGATCGCTCCGGGATCGCCAATTTG
AGTACGGCTCGTGCGAAGGCATTTGCTCAGGAACTGAAGAATACTTGCGATTTGTATAATTTAGACGGGGTA
TTCTTTGATGATGAGTACTCTGCTTACCAAACGCCACCGCCGAGCGGCTTCGTGACACCCAGTAATAACGCC
GCAGCTCGCCTTGCTTATGAAACAAAGCAGGCTATGCCAAACAAGCTGGTCACGGTGTACGTCTATTCCCGC
ACTTCGAGTTTTCCCACAGCGGTAGACGGGGTCAACGCCGGGTCCTACGTAGACTATGCGATTCATGACTAC
GGTGGCTCATACGACTTGGCTACTAATTATCCGGGGTTGGCTAAGTCTGGGATGGTGATGTCTAGTCAGGAG
TTTAACCAGGGCCGTTACGCGACTGCACAAGCATTGCGCAACATTGTGACCAAGGGCTATGGAGGCCACATG
ATCTTTGCCATGGACCCCAATCGTTCTAATTTCACGTCAGGGCAACTGCCCGCACTGAAGCTGATTGCCAAG
GAGCTTTACGGGGATGAGCTTGTGTACAGCAACACTCCTTACAGTAAGGATTGGTGATAA

Sequence identification of DNA encoding for fusion protein EndoF2-EndoF1 *as expressed in E coli (SEQ. ID NO: 26):*

ATGGCGGTAAACCTTAGTAATCTTATCGCTTATAAAAATAGTGACCATCAGATCAGTGCGGGATATTACCGT
ACATGGCGTGACAGCGCCACAGCCAGTGGTAATCTTCCTAGTATGCGTTGGTTGCCAGACTCATTGGACATG
GTAATGGTATTCCCAGACTATACTCCTCCGGAAAATGCGTATTGGAACACACTGAAGACTAACTACGTACCA
TACCTGCATAAGCGTGGCACGAAAGTTATTATCACATTGGGGGACCTTAACTCTGCAACGACCACGGGAGGG
CAAGATTCTATTGGGTATTCATCGTGGGCCAAAGGAATCTATGATAAATGGGTGGGCGAGTATAATCTTGAT
GGAATCGATATTGACATCGAATCGTCACCGTCCGGTGCGACCTTAACGAAGTTTGTTGCGGCAACAAAAGCG
TTGTCAAAGTATTTTGGACCAAAGAGTGGGACAGGCAAGACCTTTGTATACGATACCAATCAGAATCCGACT
AATTTCTTTATCCAAACTGCCCCACGCTACAACTACGTATTTCTTCAAGCATACGGGCGCTCGACCACTAAT
CTGACGACGGTCTCTGGATTATACGCCCCCTATATTTCAATGAAACAATTTCTGCCCGGCTTCTCTTTTTAC
GAAGAAAACGGTTACCCAGGTAATTATTGGAATGATGTGCGTTACCCCCAGAACGGTACAGGCCGTGCCTAC
GACTACGCGCGCTGGCAGCCCGCCACGGGAAAAAAAGGAGGGGTGTTCAGTTATGCCATCGAGCGCGACGCC
CCTCTTACATCGTCAAACGACAATACCCTGCGTGCGCCTAACTTTCGTGTAACGAAGGACTTAATCAAAATT
ATGAATCCTGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTCACCACCACCACCACCAC
GAATTCGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGCGGTAACCGGGACAACGAAG
GCTAACATCAAACTTTTTAGTTTTACAGAGGTAAACGACACTAATCCGTTGAACAATCTGAACTTTACCTTA
AAAAACTCGGGAAAACCCTTAGTAGATATGGTAGTGTTATTTTCCGCGAACATTAACTATGACGCGGCCAAC
GATAAGGTCTTCGTATCGAATAATCCGAACGTACAGCATCTTTTGACCAATCGTGCGAAGTACCTTAAGCCG
TTACAAGACAAGGGGATCAAGGTGATTTTGTCAATCTTAGGGAACCATGATCGCTCCGGGATCGCCAATTTG
AGTACGGCTCGTGCGAAGGCATTTGCTCAGGAACTGAAGAATACTTGCGATTTGTATAATTTAGACGGGGTA
TTCTTTGATGATGAGTACTCTGCTTACCAAACGCCACCGCCGAGCGGCTTCGTGACACCCAGTAATAACGCC
GCAGCTCGCCTTGCTTATGAAACAAAGCAGGCTATGCCAAACAAGCTGGTCACGGTGTACGTCTATTCCCGC
ACTTCGAGTTTTCCCACAGCGGTAGACGGGGTCAACGCCGGGTCCTACGTAGACTATGCGATTCATGACTAC
GGTGGCTCATACGACTTGGCTACTAATTATCCGGGGTTGGCTAAGTCTGGGATGGTGATGTCTAGTCAGGAG

TTTAACCAGGGCCGTTACGCGACTGCACAAGCATTGCGCAACATTGTGACCAAGGGCTATGGAGGCCACATG
ATCTTTGCCATGGACCCCAATCGTTCTAATTTCACGTCAGGGCAACTGCCCGCACTGAAGCTGATTGCCAAG
GAGCTTTACGGGGATGAGCTTGTGTACAGCAACACTCCTTACAGTAAGGATTGGTGATAA

Sequence identification of DNA encoding for fusion protein EndoS-EndoF1 *as expressed in E coli (SEQ. ID NO: 27):*

ATGCCGTCAATCGATTCGCTGCATTATCTGAGCGAAAACTCTAAAAAAGAATTTAAAGAAGAACTGAGCAAA
GCGGGCCAGGAATCTCAAAAAGTTAAAGAAATCCTGGCAAAAGCTCAGCAAGCCGATAAACAGGCACAAGAA
CTGGCTAAAATGAAAATTCCGGAAAAAATCCCGATGAAACCGCTGCATGGTCCGCTGTACGGCGGTTATTTC
CGTACCTGGCACGATAAAACGTCAGACCCGACCGAAAAAGACAAAGTCAACTCGATGGGCGAACTGCCGAAA
GAAGTGGATCTGGCTTTTATTTTCCATGATTGGACCAAAGACTACTCTCTGTTTTGGAAAGAACTGGCAACG
AAACACGTTCCGAAACTGAACAAACAGGGTACGCGTGTCATTCGTACCATTCCGTGGCGCTTCCTGGCTGGC
GGTGATAATTCAGGCATCGCGGAAGACACCTCGAAATATCCGAACACGCCGGAAGGTAATAAAGCGCTGGCC
AAAGCAATCGTCGATGAATACGTGTACAAATACAATCTGGACGGCCTGGATGTGGACGTTGAACATGATTCA
ATTCCGAAAGTGGATAAAAAAGAAGACACCGCCGGCGTGGAACGTTCGATCCAGGTTTTTGAAGAAATTGGT
AAACTGATCGGCCCGAAAGGTGTTGATAAAAGCCGTCTGTTCATCATGGATTCTACCTATATGGCCGACAAA
AATCCGCTGATTGAACGCGGTGCACCGTACATCAACCTGCTGCTGGTCCAGGTGTATGGCAGCCAAGGTGAA
AAAGGCGGTTGGGAACCGGTGTCTAACCGTCCGGAAAAAACCATGGAAGAACGCTGGCAGGGCTACTCAAAA
TATATTCGTCCGGAACAATACATGATCGGCTTTTCGTTCTATGAAGAAAACGCGCAGGAAGGTAATCTGTGG
TACGATATTAATAGTCGCAAAGATGAAGACAAAGCCAACGGCATTAATACCGATATCACGGGTACCCGTGCG
GAACGCTATGCCCGTTGGCAGCCGAAAACCGGCGGTGTTAAAGGCGGTATTTTTAGCTACGCGATCGATCGT
GACGGTGTCGCCCATCAGCCGAAAAAATACGCAAAACAAAAAGAGTTCAAAGATGCTACCGACAACATCTTC
CACAGCGATTACAGTGTCTCCAAAGCGCTGAAAACCGTGATGCTGAAAGATAAATCTTACGATCTGATCGAC
GAAAAAGATTTTCCGGACAAAGCGCTGCGCGAAGCCGTTATGGCACAGGTCGGCACCCGCAAAGGTGACCTG
GAACGTTTTAATGGCACGCTGCGCCTGGATAACCCGGCCATTCAGAGCCTGGAAGGTCTGAATAAATTCAAA
AAACTGGCACAACTGGACCTGATTGGCCTGAGCCGTATCACCAAACTGGATCGCTCTGTGCTGCCGGCCAAC
ATGAAACCGGGTAAAGACACGCTGGAAACCGTTCTGGAAACCTACAAAAAAGATAACAAAGAAGAACCGGCA
ACGATCCCGCCGGTGTCTCTGAAAGTTTCCGGCCTGACCGGTCTGAAAGAACTGGATCTGAGCGGCTTTGAC
CGTGAAACGCTGGCAGGTCTGGATGCGGCCACGCTGACCAGTCTGGAAAAAGTTGATATTTCCGGCAATAAA
CTGGACCTGGCGCCGGGTACCGAAAACCGCCAGATTTTTGATACGATGCTGAGTACCATCTCCAACCATGTT
GGCAGCAATGAACAGACCGTCAAATTCGACAAACAAAAACCGACGGGCCACTACCCGGATACGTATGGTAAA
ACCAGCCTGCGTCTGCCGGTCGCCAACGAAAAAGTGGATCTGCAGTCTCAACTGCTGTTTGGCACGGTTACC
AATCAGGGTACCCTGATTAACAGCGAAGCAGATTACAAGGCTTACCAAAACCATAAAATCGCGGGTCGCTCA
TTTGTGGATTCGAACTACCACTACAACAACTTCAAAGTTAGTTACGAAAACTACACCGTTAAAGTCACGGAT
TCCACCCTGGGCACCACGACCGATAAAACGCTGGCCACCGACAAAGAAGAAACCTACAAAGTCGATTTCTTT
AGCCCGGCAGACAAAACGAAAGCGGTGCATACCGCCAAAGTGATTGTTGGCGATGAAAAAACCATGATGGTG
AACCTGGCTGAAGGTGCGACGGTTATCGGCGGTTCCGCAGACCCGGTTAACGCTCGCAAAGTCTTTGATGGC
CAGCTGGGTAGTGAAACCGATAATATTTCCCTGGGTTGGGACTCAAAACAGTCGATTATCTTCAAACTGAAA
GAAGACGGCCTGATCAAACACTGGCGTTTCTTTAACGATAGTGCCCGCAATCCGGAAACGACCAACAAACCG
ATTCAGGAAGCATCCCTGCAAATCTTCAACATCAAAGATTACAACCTGGACAATCTGCTGGAAAACCCGAAT

AAATTCGATGACGAAAAATACTGGATCACGGTGGATACCTATAGCGCGCAGGGCGAACGTGCTACGGCGTTT
AGTAACACCCTGAACAATATTACGTCCAAATACTGGCGTGTGGTTTTCGATACCAAAGGTGACCGCTATAGC
TCTCCGGTCGTGCCGGAACTGCAGATTCTGGGCTATCCGCTGCCGAATGCTGATACGATCATGAAAACCGTG
ACGACCGCGAAAGAACTGTCACAGCAAAAAGATAAATTCTCGCAGAAAATGCTGGACGAACTGAAAATTAAA
GAAATGGCTCTGGAAACCAGCCTGAACAGTAAAATTTTCGATGTTACGGCGATCAATGCTAACGCTGGTGTG
CTGAAAGACTGTATTGAAAAACGCCAACTGCTGAAAAAAGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGC
GGCGGCGGCTCTCACCACCACCACCACCACGAATTCGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGCGGC
GGCGGCTCTGCGGTAACCGGGACAACGAAGGCTAACATCAAACTTTTTAGTTTTACAGAGGTAAACGACACT
AATCCGTTGAACAATCTGAACTTTACCTTAAAAAACTCGGGAAAACCCTTAGTAGATATGGTAGTGTTATTT
TCCGCGAACATTAACTATGACGCGGCCAACGATAAGGTCTTCGTATCGAATAATCCGAACGTACAGCATCTT
TTGACCAATCGTGCGAAGTACCTTAAGCCGTTACAAGACAAGGGGATCAAGGTGATTTTGTCAATCTTAGGG
AACCATGATCGCTCCGGGATCGCCAATTTGAGTACGGCTCGTGCGAAGGCATTTGCTCAGGAACTGAAGAAT
ACTTGCGATTTGTATAATTTAGACGGGGTATTCTTTGATGATGAGTACTCTGCTTACCAAACGCCACCGCCG
AGCGGCTTCGTGACACCCAGTAATAACGCCGCAGCTCGCCTTGCTTATGAAACAAAGCAGGCTATGCCAAAC
AAGCTGGTCACGGTGTACGTCTATTCCCGCACTTCGAGTTTTCCCACAGCGGTAGACGGGGTCAACGCCGGG
TCCTACGTAGACTATGCGATTCATGACTACGGTGGCTCATACGACTTGGCTACTAATTATCCGGGGTTGGCT
AAGTCTGGGATGGTGATGTCTAGTCAGGAGTTTAACCAGGGCCGTTACGCGACTGCACAAGCATTGCGCAAC
ATTGTGACCAAGGGCTATGGAGGCCACATGATCTTTGCCATGGACCCCAATCGTTCTAATTTCACGTCAGGG
CAACTGCCCGCACTGAAGCTGATTGCCAAGGAGCTTTACGGGGATGAGCTTGTGTACAGCAACACTCCTTAC
AGTAAGGATTGGTGATAA

Sequence identification of DNA encoding for fusion protein EndoF3-EndoH *as expressed in E coli (SEQ. ID NO: 28):*

ATGGCTACAGCGCTGGCTGGTTCTAACGGGGTCTGCATCGCGTATTACATCACCGATGGGCGTAATCCGACG
TTCAAATTGAAAGACATCCCGGATAAAGTAGACATGGTAATTCTTTTTGGTCTTAAGTATTGGTCATTGCAG
GATACAACCAAATTGCCAGGGGGTACTGGTATGATGGGTTCGTTTAAATCCTACAAGGACCTGGACACCCAG
ATTCGTAGTCTTCAAAGCCGTGGAATCAAAGTGTTGCAGAACATTGACGACGACGTCTCATGGCAGTCCTCG
AAGCCGGGTGGGTTCGCTTCCGCCGCTGCTTACGGGGATGCTATTAAGAGTATCGTAATTGATAAGTGGAAG
CTGGACGGGATTAGCTTGGATATTGAGCATTCGGGGGCTAAACCCAACCCTATCCCAACTTTTCCTGGATAT
GCCGCGACAGGATATAATGGCTGGTATTCAGGATCTATGGCAGCCACGCCTGCCTTTCTTAATGTTATCTCA
GAGCTTACTAAATACTTTGGTACAACGGCACCGAATAATAAGCAACTTCAGATTGCTTCGGGTATTGACGTA
TATGCCTGGAATAAAATCATGGAGAACTTTCGTAATAACTTCAACTACATCCAATTACAGTCATACGGAGCT
AATGTCTCTCGTACTCAACTTATGATGAATTACGCAACGGGAACTAATAAAATTCCCGCCTCTAAAATGGTT
TTCGGCGCCTACGCAGAGGGTGGCACTAACCAGGCAAATGACGTGGAGGTCGCCAAGTGGACACCTACGCAG
GGCGCAAAGGGCGGTATGATGATCTATACTTACAATTCGAACGTGAGCTATGCAAATGCGGTTCGCGACGCA
GTGAAAAATGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTCACCACCACCACCACCAC
GAATTCGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGCCCCGGCCCCGGTGAAGCAG
GGGCCGACCTCGGTGGCCTACGTCGAGGTGAACAACAACAGCATGCTCAACGTCGGCAAGTACACCCTGGCG
GACGGAGGCGGCAACGCCTTCGACGTAGCCGTGATCTTCGCGGCGAACATCAACTACGACACCGGCACGAAG
ACGGCCTACCTGCACTTCAACGAGAACGTGCAGCGCGTCCTTGACAACGCTGTCACGCAGATACGGCCGTTG

CAGCAACAGGGCATCAAGGTCCTCCTCTCGGTGCTCGGCAACCACCAGGGCGCCGGGTTCGCGAACTTCCCC
TCACAGCAGGCGGCTTCGGCGTTCGCGAAGCAGCTCTCGGACGCCGTGGCGAAGTACGGCCTCGACGGCGTC
GACTTCGACGACGAATACGCCGAGTACGGCAACAACGGCACCGCGCAGCCCAACGACAGTTCGTTCGTGCAC
CTGGTGACGGCACTGCGCGCGAACATGCCCGACAAGATCATCAGCCTCTACAACATCGGCCCGGCCGCGTCC
CGCCTGTCGTACGGCGGTGTCGACGTCTCCGACAAGTTCGACTACGCCTGGAATCCCTACTACGGCACCTGG
CAGGTCCCCGGCATCGCACTGCCCAAGGCGCAGCTGTCGCCGGCGGCCGTCGAGATCGGCCGGACCTCACGG
AGCACCGTCGCCGACCTCGCCCGTCGCACCGTCGACGAGGGGTACGGCGTCTATCTGACGTACAACCTCGAC
GGCGGCGATCGCACCGCCGACGTCTCCGCGTTCACCAGGGAGCTGTACGGCAGCGAGGCGGTCCGGACGCCG
TGATAA

Sequence identification of DNA encoding for fusion protein EndoF2-EndoH *as expressed in E coli (SEQ. ID NO: 29):*

ATGGCGGTAAACCTTAGTAATCTTATCGCTTATAAAAATAGTGACCATCAGATCAGTGCGGGATATTACCGT
ACATGGCGTGACAGCGCCACAGCCAGTGGTAATCTTCCTAGTATGCGTTGGTTGCCAGACTCATTGGACATG
GTAATGGTATTCCCAGACTATACTCCTCCGGAAAATGCGTATTGGAACACACTGAAGACTAACTACGTACCA
TACCTGCATAAGCGTGGCACGAAAGTTATTATCACATTGGGGGACCTTAACTCTGCAACGACCACGGGAGGG
CAAGATTCTATTGGGTATTCATCGTGGGCCAAAGGAATCTATGATAAATGGGTGGGCGAGTATAATCTTGAT
GGAATCGATATTGACATCGAATCGTCACCGTCCGGTGCGACCTTAACGAAGTTTGTTGCGGCAACAAAAGCG
TTGTCAAAGTATTTTGGACCAAAGAGTGGGACAGGCAAGACCTTTGTATACGATACCAATCAGAATCCGACT
AATTTCTTTATCCAAACTGCCCCACGCTACAACTACGTATTTCTTCAAGCATACGGGCGCTCGACCACTAAT
CTGACGACGGTCTCTGGATTATACGCCCCCTATATTTCAATGAAACAATTTCTGCCCGGCTTCTCTTTTTAC
GAAGAAAACGGTTACCCAGGTAATTATTGGAATGATGTGCGTTACCCCCAGAACGGTACAGGCCGTGCCTAC
GACTACGCGCGCTGGCAGCCCGCCACGGGAAAAAAAGGAGGGGTGTTCAGTTATGCCATCGAGCGCGACGCC
CCTCTTACATCGTCAAACGACAATACCCTGCGTGCGCCTAACTTTCGTGTAACGAAGGACTTAATCAAAATT
ATGAATCCTGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTCACCACCACCACCACCAC
GAATTCGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGCCCCGGCCCCGGTGAAGCAG
GGGCCGACCTCGGTGGCCTACGTCGAGGTGAACAACAACAGCATGCTCAACGTCGGCAAGTACACCCTGGCG
GACGGAGGCGGCAACGCCTTCGACGTAGCCGTGATCTTCGCGGCGAACATCAACTACGACACCGGCACGAAG
ACGGCCTACCTGCACTTCAACGAGAACGTGCAGCGCGTCCTTGACAACGCTGTCACGCAGATACGGCCGTTG
CAGCAACAGGGCATCAAGGTCCTCCTCTCGGTGCTCGGCAACCACCAGGGCGCCGGGTTCGCGAACTTCCCC
TCACAGCAGGCGGCTTCGGCGTTCGCGAAGCAGCTCTCGGACGCCGTGGCGAAGTACGGCCTCGACGGCGTC
GACTTCGACGACGAATACGCCGAGTACGGCAACAACGGCACCGCGCAGCCCAACGACAGTTCGTTCGTGCAC
CTGGTGACGGCACTGCGCGCGAACATGCCCGACAAGATCATCAGCCTCTACAACATCGGCCCGGCCGCGTCC
CGCCTGTCGTACGGCGGTGTCGACGTCTCCGACAAGTTCGACTACGCCTGGAATCCCTACTACGGCACCTGG
CAGGTCCCCGGCATCGCACTGCCCAAGGCGCAGCTGTCGCCGGCGGCCGTCGAGATCGGCCGGACCTCACGG
AGCACCGTCGCCGACCTCGCCCGTCGCACCGTCGACGAGGGGTACGGCGTCTATCTGACGTACAACCTCGAC
GGCGGCGATCGCACCGCCGACGTCTCCGCGTTCACCAGGGAGCTGTACGGCAGCGAGGCGGTCCGGACGCCG
TGATAA

Sequence identification of DNA encoding for fusion protein EndoS-EndoH *(or EndoSH) as expressed in E coli (SEQ. ID NO: 30):*

ATGCCGTCAATCGATTCGCTGCATTATCTGAGCGAAAACTCTAAAAAAGAATTTAAAGAAGAACTGAGCAAA
GCGGGCCAGGAATCTCAAAAAGTTAAAGAAATCCTGGCAAAAGCTCAGCAAGCCGATAAACAGGCACAAGAA
CTGGCTAAAATGAAAATTCCGGAAAAAATCCCGATGAAACCGCTGCATGGTCCGCTGTACGGCGGTTATTTC
CGTACCTGGCACGATAAAACGTCAGACCCGACCGAAAAAGACAAAGTCAACTCGATGGGCGAACTGCCGAAA
GAAGTGGATCTGGCTTTTATTTTCCATGATTGGACCAAAGACTACTCTCTGTTTTGGAAAGAACTGGCAACG
AAACACGTTCCGAAACTGAACAAACAGGGTACGCGTGTCATTCGTACCATTCCGTGGCGCTTCCTGGCTGGC
GGTGATAATTCAGGCATCGCGGAAGACACCTCGAAATATCCGAACACGCCGGAAGGTAATAAAGCGCTGGCC
AAAGCAATCGTCGATGAATACGTGTACAAATACAATCTGGACGGCCTGGATGTGGACGTTGAACATGATTCA
ATTCCGAAAGTGGATAAAAAAGAAGACACCGCCGGCGTGGAACGTTCGATCCAGGTTTTTGAAGAAATTGGT
AAACTGATCGGCCCGAAAGGTGTTGATAAAAGCCGTCTGTTCATCATGGATTCTACCTATATGGCCGACAAA
AATCCGCTGATTGAACGCGGTGCACCGTACATCAACCTGCTGCTGGTCCAGGTGTATGGCAGCCAAGGTGAA
AAAGGCGGTTGGGAACCGGTGTCTAACCGTCCGGAAAAAACCATGGAAGAACGCTGGCAGGGCTACTCAAAA
TATATTCGTCCGGAACAATACATGATCGGCTTTTCGTTCTATGAAGAAAACGCGCAGGAAGGTAATCTGTGG
TACGATATTAATAGTCGCAAAGATGAAGACAAAGCCAACGGCATTAATACCGATATCACGGGTACCCGTGCG
GAACGCTATGCCCGTTGGCAGCCGAAAACCGGCGGTGTTAAAGGCGGTATTTTTAGCTACGCGATCGATCGT
GACGGTGTCGCCCATCAGCCGAAAAAATACGCAAAACAAAAAGAGTTCAAAGATGCTACCGACAACATCTTC
CACAGCGATTACAGTGTCTCCAAAGCGCTGAAAACCGTGATGCTGAAAGATAAATCTTACGATCTGATCGAC
GAAAAAGATTTTCCGGACAAAGCGCTGCGCGAAGCCGTTATGGCACAGGTCGGCACCCGCAAAGGTGACCTG
GAACGTTTTAATGGCACGCTGCGCCTGGATAACCCGGCCATTCAGAGCCTGGAAGGTCTGAATAAATTCAAA
AAACTGGCACAACTGGACCTGATTGGCCTGAGCCGTATCACCAAACTGGATCGCTCTGTGCTGCCGGCCAAC
ATGAAACCGGGTAAAGACACGCTGGAAACCGTTCTGGAAACCTACAAAAAAGATAACAAAGAAGAACCGGCA
ACGATCCCGCCGGTGTCTCTGAAAGTTTCCGGCCTGACCGGTCTGAAAGAACTGGATCTGAGCGGCTTTGAC
CGTGAAACGCTGGCAGGTCTGGATGCGGCCACGCTGACCAGTCTGGAAAAAGTTGATATTTCCGGCAATAAA
CTGGACCTGGCGCCGGGTACCGAAAACCGCCAGATTTTTGATACGATGCTGAGTACCATCTCCAACCATGTT
GGCAGCAATGAACAGACCGTCAAATTCGACAAACAAAAACCGACGGGCCACTACCCGGATACGTATGGTAAA
ACCAGCCTGCGTCTGCCGGTCGCCAACGAAAAAGTGGATCTGCAGTCTCAACTGCTGTTTGGCACGGTTACC
AATCAGGGTACCCTGATTAACAGCGAAGCAGATTACAAGGCTTACCAAAACCATAAAATCGCGGGTCGCTCA
TTTGTGGATTCGAACTACCACTACAACAACTTCAAAGTTAGTTACGAAAACTACACCGTTAAAGTCACGGAT
TCCACCCTGGGCACCACGACCGATAAAACGCTGGCCACCGACAAAGAAGAAACCTACAAAGTCGATTTCTTT
AGCCCGGCAGACAAAACGAAAGCGGTGCATACCGCCAAAGTGATTGTTGGCGATGAAAAAACCATGATGGTG
AACCTGGCTGAAGGTGCGACGGTTATCGGCGGTTCCGCAGACCCGGTTAACGCTCGCAAAGTCTTTGATGGC
CAGCTGGGTAGTGAAACCGATAATATTTCCCTGGGTTGGGACTCAAAACAGTCGATTATCTTCAAACTGAAA
GAAGACGGCCTGATCAAACACTGGCGTTTCTTTAACGATAGTGCCCGCAATCCGGAAACGACCAACAAACCG
ATTCAGGAAGCATCCCTGCAAATCTTCAACATCAAAGATTACAACCTGGACAATCTGCTGGAAAACCCGAAT
AAATTCGATGACGAAAAATACTGGATCACGGTGGATACCTATAGCGCGCAGGGCGAACGTGCTACGGCGTTT
AGTAACACCCTGAACAATATTACGTCCAAATACTGGCGTGTGGTTTTCGATACCAAAGGTGACCGCTATAGC
TCTCCGGTCGTGCCGGAACTGCAGATTCTGGGCTATCCGCTGCCGAATGCTGATACGATCATGAAAACCGTG
ACGACCGCGAAAGAACTGTCACAGCAAAAAGATAAATTCTCGCAGAAAATGCTGGACGAACTGAAAATTAAA

GAAATGGCTCTGGAAACCAGCCTGAACAGTAAAATTTTCGATGTTACGGCGATCAATGCTAACGCTGGTGTG
CTGAAAGACTGTATTGAAAAACGCCAACTGCTGAAAAAAGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGC
GGCGGCGGCTCTCACCACCACCACCACCACGAATTCGGCGGCGGCGGCTCTGGCGGCGGCGGCTCTGGCGGC
GGCGGCTCTGCCCCGGCCCCGGTGAAGCAGGGGCCGACCTCGGTGGCCTACGTCGAGGTGAACAACAACAGC
ATGCTCAACGTCGGCAAGTACACCCTGGCGGACGGAGGCGGCAACGCCTTCGACGTAGCCGTGATCTTCGCG
GCGAACATCAACTACGACACCGGCACGAAGACGGCCTACCTGCACTTCAACGAGAACGTGCAGCGCGTCCTT
GACAACGCTGTCACGCAGATACGGCCGTTGCAGCAACAGGGCATCAAGGTCCTCCTCTCGGTGCTCGGCAAC
CACCAGGGCGCCGGGTTCGCGAACTTCCCCTCACAGCAGGCGGCTTCGGCGTTCGCGAAGCAGCTCTCGGAC
GCCGTGGCGAAGTACGGCCTCGACGGCGTCGACTTCGACGACGAATACGCCGAGTACGGCAACAACGGCACC
GCGCAGCCCAACGACAGTTCGTTCGTGCACCTGGTGACGGCACTGCGCGCGAACATGCCCGACAAGATCATC
AGCCTCTACAACATCGGCCCGGCCGCGTCCCGCCTGTCGTACGGCGGTGTCGACGTCTCCGACAAGTTCGAC
TACGCCTGGAATCCCTACTACGGCACCTGGCAGGTCCCCGGCATCGCACTGCCCAAGGCGCAGCTGTCGCCG
GCGGCCGTCGAGATCGGCCGGACCTCACGGAGCACCGTCGCCGACCTCGCCCGTCGCACCGTCGACGAGGGG
TACGGCGTCTATCTGACGTACAACCTCGACGGCGGCGATCGCACCGCCGACGTCTCCGCGTTCACCAGGGAG
CTGTACGGCAGCGAGGCGGTCCGGACGCCGTGATAA

Sequence identification of DNA encoding for fusion protein His_6-EndoS-EndoH *(EndoS-EndoH without GS-linker) as expressed in E coli (SEQ. ID NO: 31):*

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGCCGTCAATC
GATTCGCTGCATTATCTGAGCGAAAACTCTAAAAAAGAATTTAAAGAAGAACTGAGCAAAGCGGGCCAGGAA
TCTCAAAAAGTTAAAGAAATCCTGGCAAAAGCTCAGCAAGCCGATAAACAGGCACAAGAACTGGCTAAAATG
AAAATTCCGGAAAAAATCCCGATGAAACCGCTGCATGGTCCGCTGTACGGCGGTTATTTCCGTACCTGGCAC
GATAAAACGTCAGACCCGACCGAAAAAGACAAAGTCAACTCGATGGGCGAACTGCCGAAAGAAGTGGATCTG
GCTTTTATTTTCCATGATTGGACCAAAGACTACTCTCTGTTTTGGAAAGAACTGGCAACGAAACACGTTCCG
AAACTGAACAAACAGGGTACGCGTGTCATTCGTACCATTCCGTGGCGCTTCCTGGCTGGCGGTGATAATTCA
GGCATCGCGGAAGACACCTCGAAATATCCGAACACGCCGGAAGGTAATAAAGCGCTGGCCAAAGCAATCGTC
GATGAATACGTGTACAAATACAATCTGGACGGCCTGGATGTGGACGTTGAACATGATTCAATTCCGAAAGTG
GATAAAAAAGAAGACACCGCCGGCGTGGAACGTTCGATCCAGGTTTTTGAAGAAATTGGTAAACTGATCGGC
CCGAAAGGTGTTGATAAAAGCCGTCTGTTCATCATGGATTCTACCTATATGGCCGACAAAAATCCGCTGATT
GAACGCGGTGCACCGTACATCAACCTGCTGCTGGTCCAGGTGTATGGCAGCCAAGGTGAAAAAGGCGGTTGG
GAACCGGTGTCTAACCGTCCGGAAAAAACCATGGAAGAACGCTGGCAGGGCTACTCAAAATATATTCGTCCG
GAACAATACATGATCGGCTTTTCGTTCTATGAAGAAAACGCGCAGGAAGGTAATCTGTGGTACGATATTAAT
AGTCGCAAAGATGAAGACAAAGCCAACGGCATTAATACCGATATCACGGGTACCCGTGCGGAACGCTATGCC
CGTTGGCAGCCGAAAACCGGCGGTGTTAAAGGCGGTATTTTTAGCTACGCGATCGATCGTGACGGTGTCGCC
CATCAGCCGAAAAAATACGCAAAACAAAAAGAGTTCAAAGATGCTACCGACAACATCTTCCACAGCGATTAC
AGTGTCTCCAAAGCGCTGAAAACCGTGATGCTGAAAGATAAATCTTACGATCTGATCGACGAAAAAGATTTT
CCGGACAAAGCGCTGCGCGAAGCCGTTATGGCACAGGTCGGCACCCGCAAAGGTGACCTGGAACGTTTTAAT
GGCACGCTGCGCCTGGATAACCCGGCCATTCAGAGCCTGGAAGGTCTGAATAAATTCAAAAAACTGGCACAA
CTGGACCTGATTGGCCTGAGCCGTATCACCAAACTGGATCGCTCTGTGCTGCCGGCCAACATGAAACCGGGT
AAAGACACGCTGGAAACCGTTCTGGAAACCTACAAAAAAGATAACAAAGAAGAACCGGCAACGATCCCGCCG
GTGTCTCTGAAAGTTTCCGGCCTGACCGGTCTGAAAGAACTGGATCTGAGCGGCTTTGACCGTGAAACGCTG

GCAGGTCTGGATGCGGCCACGCTGACCAGTCTGGAAAAAGTTGATATTTCCGGCAATAAACTGGACCTGGCG
CCGGGTACCGAAAACCGCCAGATTTTTGATACGATGCTGAGTACCATCTCCAACCATGTTGGCAGCAATGAA
CAGACCGTCAAATTCGACAAACAAAAACCGACGGGCCACTACCCGGATACGTATGGTAAAACCAGCCTGCGT
CTGCCGGTCGCCAACGAAAAAGTGGATCTGCAGTCTCAACTGCTGTTTGGCACGGTTACCAATCAGGGTACC
CTGATTAACAGCGAAGCAGATTACAAGGCTTACCAAAACCATAAAATCGCGGGTCGCTCATTTGTGGATTCG
AACTACCACTACAACAACTTCAAAGTTAGTTACGAAAACTACACCGTTAAAGTCACGGATTCCACCCTGGGC
ACCACGACCGATAAAACGCTGGCCACCGACAAAGAAGAAACCTACAAAGTCGATTTCTTTAGCCCGGCAGAC
AAAACGAAAGCGGTGCATACCGCCAAAGTGATTGTTGGCGATGAAAAAACCATGATGGTGAACCTGGCTGAA
GGTGCGACGGTTATCGGCGGTTCCGCAGACCCGGTTAACGCTCGCAAAGTCTTTGATGGCCAGCTGGGTAGT
GAAACCGATAATATTTCCCTGGGTTGGGACTCAAAACAGTCGATTATCTTCAAACTGAAAGAAGACGGCCTG
ATCAAACACTGGCGTTTCTTTAACGATAGTGCCCGCAATCCGGAAACGACCAACAAACCGATTCAGGAAGCA
TCCCTGCAAATCTTCAACATCAAAGATTACAACCTGGACAATCTGCTGGAAAACCCGAATAAATTCGATGAC
GAAAAATACTGGATCACGGTGGATACCTATAGCGCGCAGGGCGAACGTGCTACGGCGTTTAGTAACACCCTG
AACAATATTACGTCCAAATACTGGCGTGTGGTTTTCGATACCAAAGGTGACCGCTATAGCTCTCCGGTCGTG
CCGGAACTGCAGATTCTGGGCTATCCGCTGCCGAATGCTGATACGATCATGAAAACCGTGACGACCGCGAAA
GAACTGTCACAGCAAAAAGATAAATTCTCGCAGAAAATGCTGGACGAACTGAAAATTAAAGAAATGGCTCTG
GAAACCAGCCTGAACAGTAAAATTTTCGATGTTACGGCGATCAATGCTAACGCTGGTGTGCTGAAAGACTGT
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GAGGTGAACAACAACAGCATGCTCAACGTCGGCAAGTACACCCTGGCGGACGGAGGCGGCAACGCCTTCGAC
GTAGCCGTGATCTTCGCGGCGAACATCAACTACGACACCGGCACGAAGACGGCCTACCTGCACTTCAACGAG
AACGTGCAGCGCGTCCTTGACAACGCTGTCACGCAGATACGGCCGTTGCAGCAACAGGGCATCAAGGTCCTC
CTCTCGGTGCTCGGCAACCACCAGGGCGCCGGGTTCGCGAACTTCCCCTCACAGCAGGCGGCTTCGGCGTTC
GCGAAGCAGCTCTCGGACGCCGTGGCGAAGTACGGCCTCGACGGCGTCGACTTCGACGACGAATACGCCGAG
TACGGCAACAACGGCACCGCGCAGCCCAACGACAGTTCGTTCGTGCACCTGGTGACGGCACTGCGCGCGAAC
ATGCCCGACAAGATCATCAGCCTCTACAACATCGGCCCGGCCGCGTCCCGCCTGTCGTACGGCGGTGTCGAC
GTCTCCGACAAGTTCGACTACGCCTGGAATCCCTACTACGGCACCTGGCAGGTCCCCGGCATCGCACTGCCC
AAGGCGCAGCTGTCGCCGGCGGCCGTCGAGATCGGCCGGACCTCACGGAGCACCGTCGCCGACCTCGCCCGT
CGCACCGTCGACGAGGGGTACGGCGTCTATCTGACGTACAACCTCGACGGCGGCGATCGCACCGCCGACGTC
TCCGCGTTCACCAGGGAGCTGTACGGCAGCGAGGCGGTCCGGACGCCGTGATAA

Sequence identification of DNA encoding for His_6-TnGalNAcT(33-421) *as expressed in CHO (SEQ. ID NO: 32):*

ATGAATTTTGGACTGAGGCTGATTTTCCTGGTGCTGACCCTGAAAGGCGTCCAGTGTCATCACCATCACCAT
CACTCCCCGCTTCGCACATATCTTTACACTCCATTATACAATGCCACCCAGCCCACACTCAGAAACGTCGAG
AGGCTGGCAGCTAACTGGCCAAAGAAGATCCCTAGTAATTATATAGAAGATAGCGAAGAGTATAGCATCAAG
AATATTTCTTTGAGCAACCACACAACTAGAGCATCTGTGGTACATCCTCCTTCCTCTATCACCGAAACGGCA
AGCAAACTGGATAAGAATATGACCATCCAAGACGGCGCCTTTGCTATGATTAGCCCGACGCCCTTGCTTATC
ACCAAATTGATGGATAGCATCAAATCTTATGTTACTACCGAGGATGGGGTTAAGAAAGCCGAAGCCGTCGTA
ACTCTCCCCCTCTGTGATAGCATGCCTCCTGACCTTGGTCCTATTACTCTTAACAAAACCGAGCTCGAGCTC
GAATGGGTTGAGAAAAAGTTCCCTGAGGTCGAGTGGGGTGGACGTTATAGTCCCCCCAACTGCACAGCTAGG
CATCGCGTAGCAATCATAGTCCCGTACCGAGACAGACAGCAACACCTGGCAATCTTCTTAAATCACATGCAC

CCCTTCCTGATGAAACAGCAGATCGAATATGGCATCTTTATCGTGGAGCAGGAAGGAAACAAGGACTTTAAC
CGTGCGAAACTTATGAACGTCGGCTTTGTTGAAAGTCAAAAACTCGTTGCCGAGGGATGGCAGTGTTTCGTT
TTTCATGACATAGACCTGCTCCCACTGGACACTAGAAACCTCTATAGCTGCCCGAGACAGCCACGCCACATG
AGCGCTTCCATTGACAAACTTCACTTTAAGCTGCCTTACGAAGACATCTTCGGTGGCGTGTCAGCCATGACT
CTGGAACAGTTCACCCGAGTGAATGGATTTTCAAATAAATACTGGGGATGGGGGGGAGAGGACGACGATATG
AGTTATCGGCTTAAGAAAATCAACTACCATATTGCAAGATATAAAATGTCCATCGCCCGATACGCCATGTTG
GACCACAAGAAGTCAACACCCAATCCTAAGCGGTACCAATTACTCTCACAGACCTCAAAGACATTCCAGAAA
GACGGGCTGAGCACCCTGGAATATGAGCTGGTGCAAGTCGTTCAATATCATCTGTATACTCACATCCTGGTT
AATATTGACGAGAGGTCCTGATAA

(signal sequence for secretion is underlined)

Sequence identification of His_6-TnGalNAcT(33-421) *as expressed in CHO (SEQ. ID NO: 33):*

HHHHHHSPLRTYLYTPLYNATQPTLRNVERLAANWPKKIPSNYIEDSEEYSIKNISLSNHTTRASVVHPPSS
ITETASKLDKNMTIQDGAFAMISPTPLLITKLMDSIKSYVTTEDGVKKAEAVVTLPLCDSMPPDLGPITLNK
TELELEWVEKKFPEVEWGGRYSPPNCTARHRVAIIVPYRDRQQHLAIFLNHMHPFLMKQQIEYGIFIVEQEG
NKDFNRAKLMNVGFVESQKLVAEGWQCFVFHDIDLLPLDTRNLYSCPRQPRHMSASIDKLHFKLPYEDIFGG
VSAMTLEQFTRVNGFSNKYWGWGGEDDDMSYRLKKINYHIARYKMSIARYAMLDHKKSTPNPKRYQLLSQTS
KTFQKDGLSTLEYELVQVVQYHLYTHILVNIDERS

Claims

1. Fusion enzyme of structure (**1**):

$$EndoX–(L)_p–EndoY \quad (\mathbf{1})$$

wherein EndoX is an endoglycosidase, EndoY is an endoglycosidase distinct from EndoX, L is a linker and p is 0 or 1.

2. Enzyme according to claim 1, wherein EndoX and EndoY are individually selected from the group consisting of EndoA, EndoBi, EndoBH, EndoBT, EndoCE, EndoD, EndoE, EfEndo18A, EndoF1, EndoF2, EndoF3, EndoH, EndoLL, EndoM, EndoOm, EndoS, and EndoT, preferably selected from the group consisting of EndoF1, EndoF2, EndoF3, EfEndo18A, EndoH and EndoS.
3. Enzyme according to claim 1 or 2, wherein the endoglycosidases represented by EndoX and EndoY have distinct endoglycosidase activity.
4. Enzyme according to any one of claims 1 – 3, wherein EndoX is EndoF2, EndoF3 or EndoS, preferably EndoS.
5. Enzyme according to any one of claims 1 – 4, wherein EndoY is EndoF1, EndoD, EndoE, EndoH, EfEndo18A or EndoT, preferably EndoH.
6. Enzyme according to any one of claims 1 – 5, wherein EndoX and EndoY individually have at least 80 % sequence identity with any one of SEQ ID NOs 4 – 10.
7. Enzyme according to any one of claims 1 – 6, having at least 50 % sequence identity with any one of SEQ IDs No. 1, 2 or 13 – 21.
8. Enzyme according to any one of claims 1 – 7, wherein p = 0.
9. Enzyme according to any one of claims 1 – 7, wherein p = 1 and L is composed of amino residues and has a length of 1 to 100 amino acid residues.
10. Enzyme according to claim 9, wherein the linker has the sequence $(G_4S)_{n1}(H)_r(EF)_s(G_4S)_{n2}$, wherein n1 and n2 individually are integers in the range 1 – 10, r is an integer in the range of 2 – 10 and s = 0 or 1.
11. Use of the fusion enzyme according to any one of claims 1 – 10 for trimming a glycoprotein.
12. Use according to claim 11, wherein the glycoprotein is an antibody.
13. Process for trimming a glycoprotein, by contacting the glycoprotein with a fusion enzyme according to any one of claims 1 – 10.
14. Process according to claim 13, wherein the glycoprotein comprises at least one high-mannose glycan and at least one complex glycan.
15. Process according to claim 14, wherein the glycoprotein further comprises at least one hybrid glycan.
16. Process according to any one of claims 13 – 15, wherein the glycoprotein is an antibody.
17. Process according to any one of claims 13 – 16, wherein the contacting is performed at a pH which is 0.5 – 3 pH units, preferably 1 – 2 pH units, different from the optimal pH of one or both of EndoX and EndoY.

Fig. 1

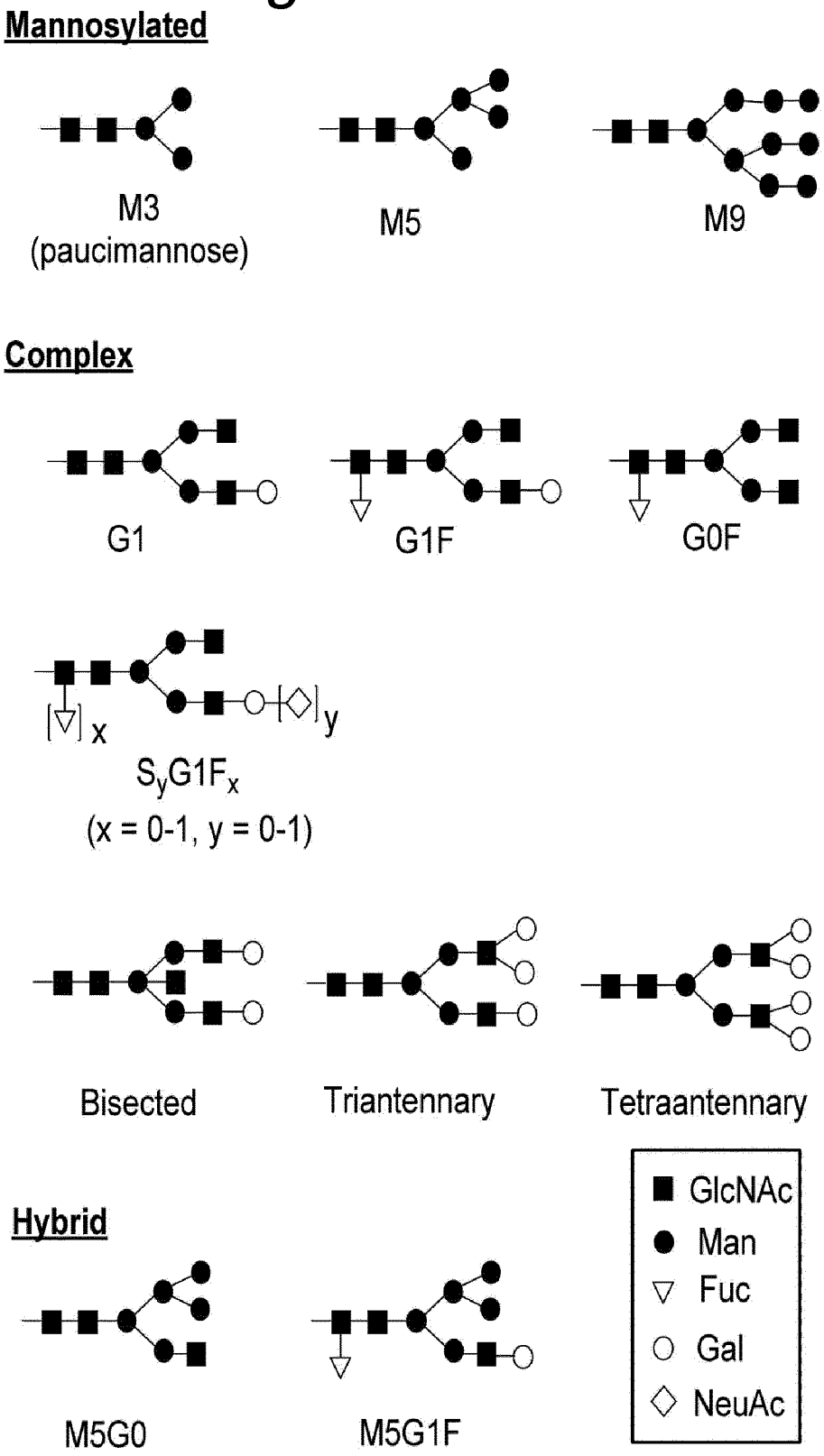

Mannosylated
M3
(paucimannose)
M5
M9
Complex
G1
G1F
G0F
x
y
S_yG1F_x
(x = 0-1, y = 0-1)
Bisected
Triantennary
Tetraantennary
Hybrid
M5G0
M5G1F
GlcNAc
Man
Fuc
Gal
NeuAc

Fig. 2

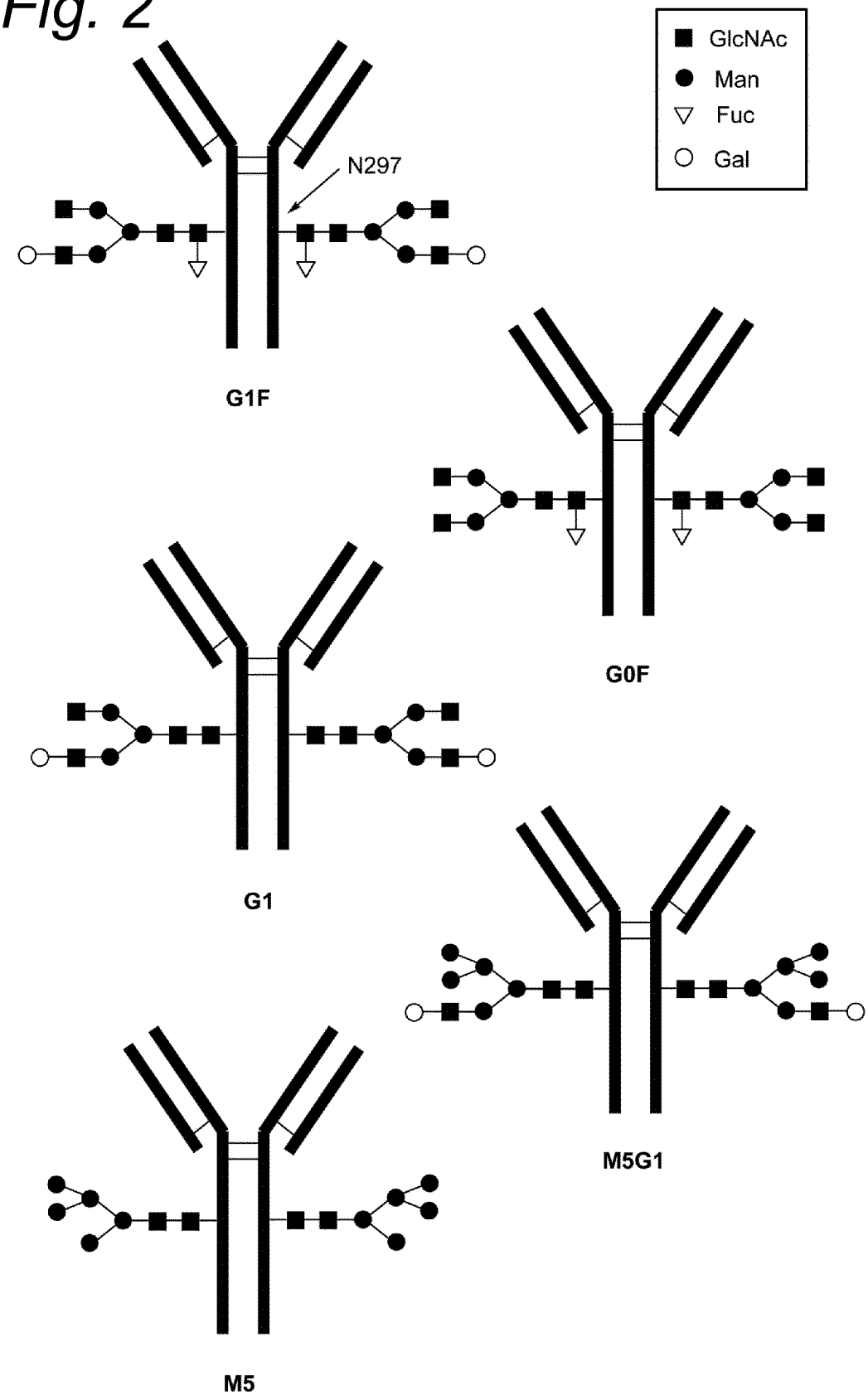


Fig. 3

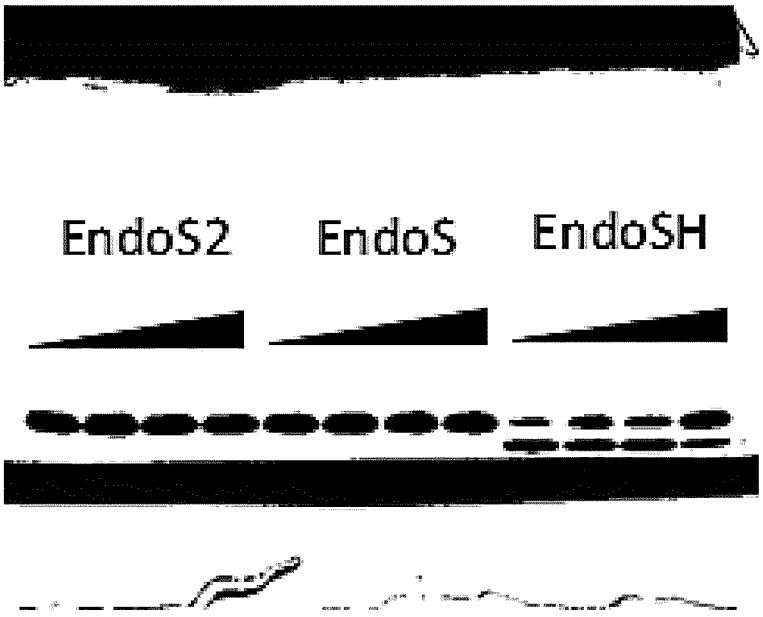
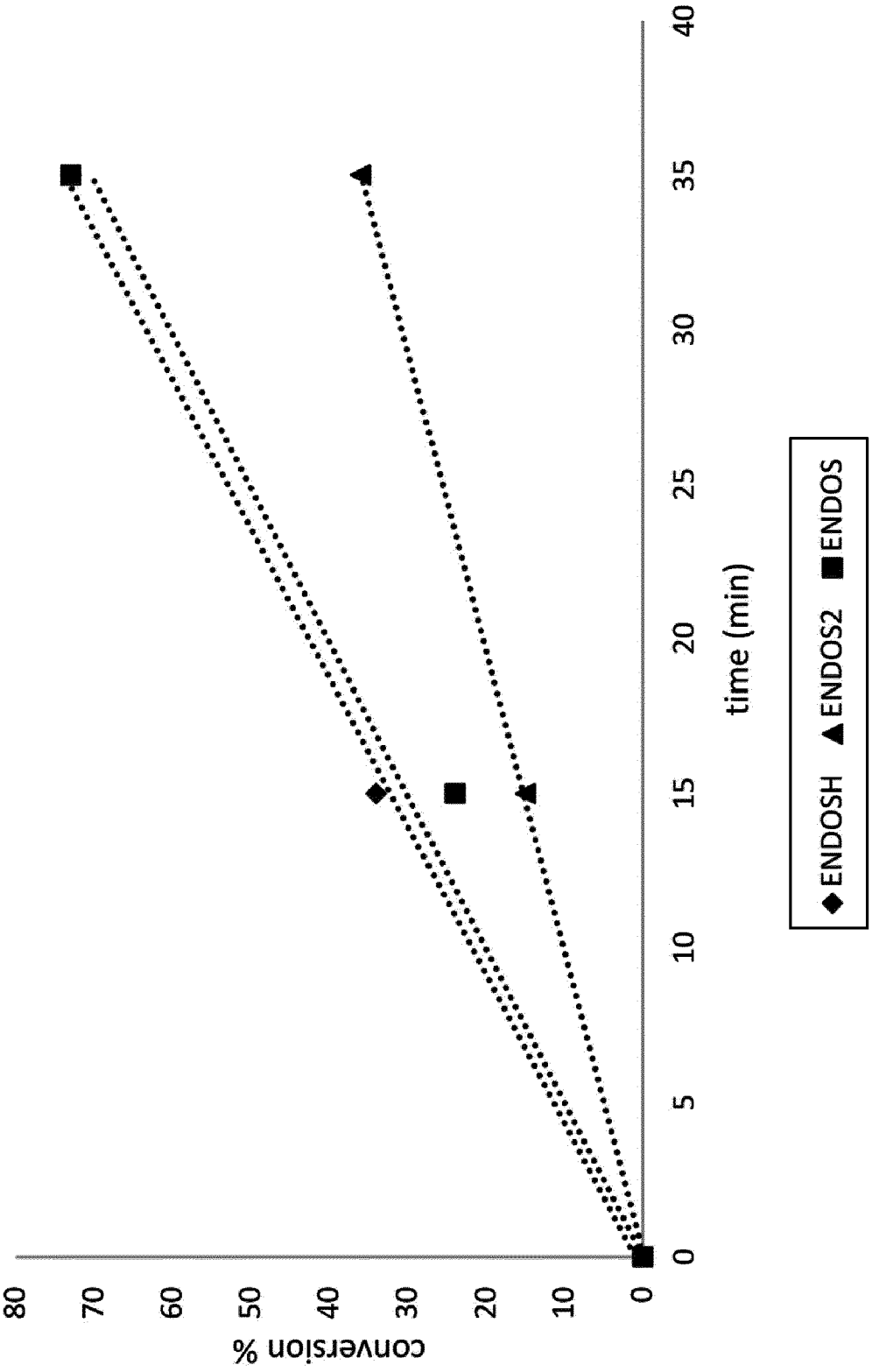


Fig 4


conversion %
0
10
20
30
40
50
60
70
80
time (min)
0
5
10
15
20
25
30
35
40
ENDOSH
ENDOS2
ENDOS

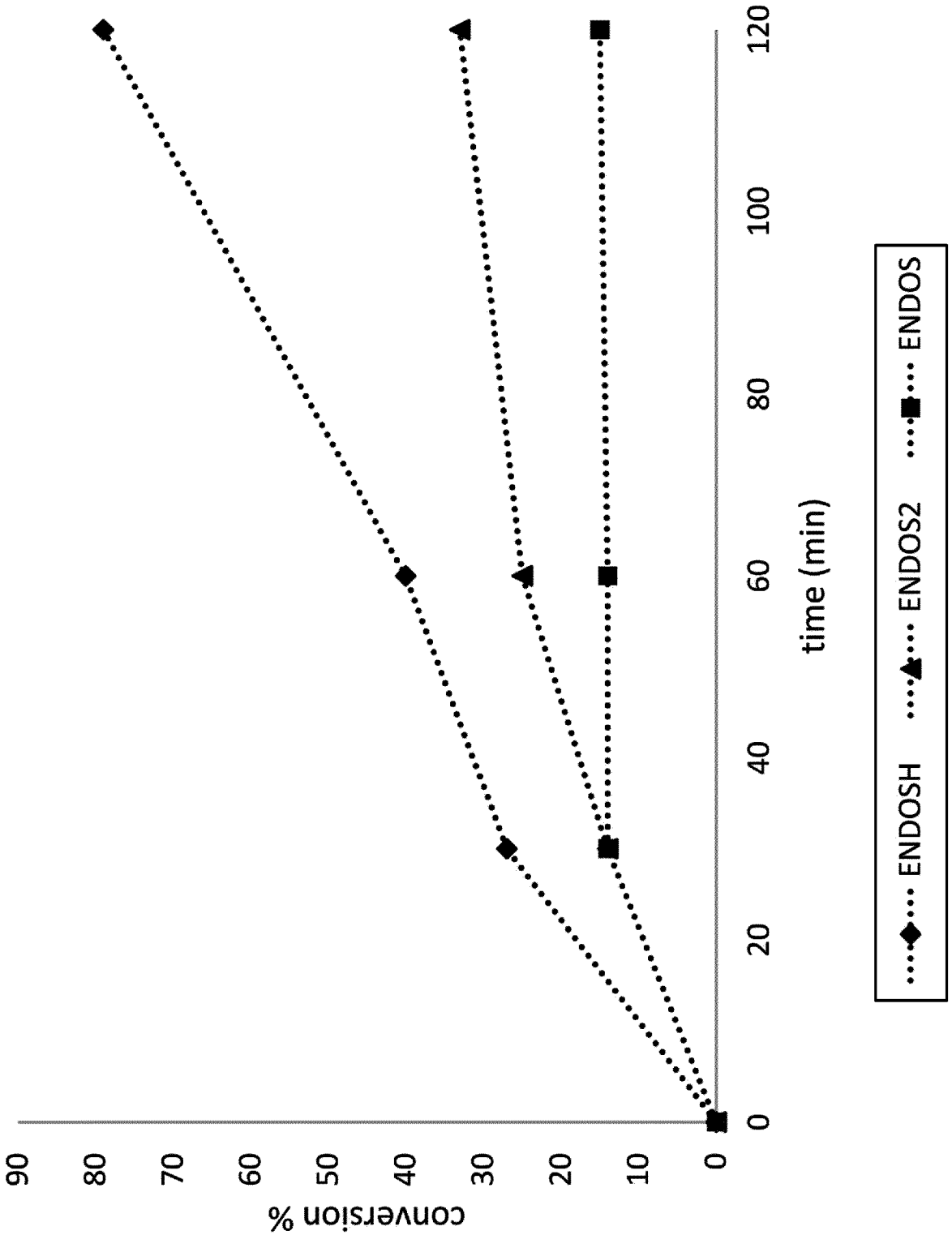

conversion %
0
10
20
30
40
50
60
70
80
90
time (min)
0
20
40
60
80
100
120
ENDOSH
ENDOS2
ENDOS

Fig 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/052792

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/24
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PING LU ET AL: "Construction and characterization of a bifunctional fusion enzyme of Bacillus -sourced beta-glucanase and xylanase expressed in Escherichia coli", FEMS MICROBIOLOGY LETTERS, vol. 261, no. 2, 1 August 2006 (2006-08-01), pages 224-230, XP055373432, GB ISSN: 0378-1097, DOI: 10.1111/j.1574-6968.2006.00367.x the whole document ----- -/--	1,3,8-10

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 May 2017	24/07/2017

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Smalt, Rolf

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JUNTAO SUN ET AL: "Construction and characterization of a fusion beta-1,3-1,4-glucanase to improve hydrolytic activity and thermostability", BIOTECHNOLOGY LETTERS, SPRINGER NETHERLANDS, DORDRECHT, vol. 33, no. 11, 7 July 2011 (2011-07-07), pages 2193-2199, XP019957899, ISSN: 1573-6776, DOI: 10.1007/S10529-011-0676-7 the whole document	1,3,8-10
A	SATOSHI YAMAMOTO ET AL: "Mutational studies on endo-[beta]-N-acetylglucosaminidase D which hydrolyzes core portion of asparagine-linked complex type oligosaccharides", GLYCOCONJUGATE JOURNAL, KLUWER ACADEMIC PUBLISHERS, BO, vol. 22, no. 1-2, 1 February 2005 (2005-02-01), pages 35-42, XP019207049, ISSN: 1573-4986, DOI: 10.1007/S10719-005-0847-7 the whole document	1-3,5,8
A	EP 0 769 550 A2 (TAKARA SHUZO CO [JP]) 23 April 1997 (1997-04-23) the whole document	1-17
A	WO 2009/141599 A1 (UNIV YORK [GB]; FAIRBANKS ANTONY [NZ]; HEIDECKE CHRISTOPH [DE]; MOIR J) 26 November 2009 (2009-11-26) the whole document	1-17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2017/052792

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-17(partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-17(partially)

A fusion enzyme consisiting of endogylcosidase EndoA, optionally a linker, and a distict endoglycosidase, and its uses in a process for trimming a glycoprotein.

2-17. claims: 1-17(partially)

Subject-matter as defined for invention 1, but limited to the respective endoglycosidases EndoBi, EndoBH, EndoBT, EndoCE, EndoD, EndoE, EfEndo18A, EndoF1, EndoF2, EndoF3, EndoH, EndoLL, EndoM, EndoOm, EndoS and EndoT.

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18. claims: 1-17(partially)

Subject-matter as defined for invention 1 above, but limited to fusions proteins that do not include any of the endoglycosidases EndoA, EndoBi, EndoBH, EndoBT, EndoCE, EndoD, EndoE, EfEndo18A, EndoF1, EndoF2, EndoF3, EndoH, EndoLL, EndoM, EndoOm, EndoS and EndoT.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2017/052792

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0769550 A2	23-04-1997	CA 2187386 A1	28-04-1997
		CN 1153820 A	09-07-1997
		EP 0769550 A2	23-04-1997
		US 5985637 A	16-11-1999
WO 2009141599 A1	26-11-2009	NONE	