

Enzymatic Antibody Processing

The current invention is directed to a method for enzymatic downstream processing of recombinantly produced immunoglobulins. In more detail the current invention is directed to a method for modification of the (α 1,3)glycosidically bound galactose content of full length immunoglobulins or immunoglobulin Fc-parts after an affinity chromatography by an enzymatic treatment.

Background of the Invention

Polypeptides obtained from eukaryotic cells are produced as glycosylated polypeptides. The glycostructures are attached to the amino acid backbone as post-translational enzymatic modification.

The glycosyltransferases are recognized as a functional family of estimated 250-300 different intracellular, membrane-bound enzymes that participate in the coordinate biosynthesis of the glycostructures of polypeptides, including glycoproteins, proteoglycans and glycolipids. The glycosyltransferases are classified into groups based on their nucleotide monosaccharide donor specificity. For example, the galactosyltransferases are the subset of glycosyltransferases that use UDP-galactose as the activated monosaccharide donor whereas the sialyltransferases use CMP-sialic acid and the fucosyltransferases use GDP-fucose (Shaper, N.L., et al., J. Mamm. Gland Biol. Neopl. 3 (1998) 315-324).

The modification of alpha-galactosyl epitopes on various mammalian cells is of particular interest, since as much as 1% of circulating IgG antibodies in humans interact with this oligosaccharide residue. This natural antibody, designated "anti-Gal", was previously found to bind to terminal Gal(α 1,3)Gal(β 1,4)GlcNAc-R on biochemically defined glycolipids (Galili, U., et al., J. Exp. Med. 162 (1985) 573-582; Galili, U., et al., J. Exp. Med. 165 (1987) 693-704). Measurement of the binding of radiolabeled Bandeiraea (Griffonia) simplicifolia IB4 lectin to the various nucleated cells suggests that cells binding anti-Gal express 10^6 to 3.5×10^7 alpha-galactosyl epitopes, most of which, based on the anti-Gal specificity, seem to have the structure of Gal(α 1,3)Gal(β 1,4)GlcNAc-R. The absence of these epitopes from human cells results from diminished activity of the enzyme (α 1,3)galactosyltransferase (Galili, U., et al., J. Biol. Chem. 263 (1988) 17755-17762).

The synthesis of the Gal(α 1,3)epitope in the Golgi apparatus of cells of murine origin (Cummings, R.D. and Mattox, S.A., J. Biol. Chem. 263 (1988) 511-519; Blake, D.A., and Goldstein, I.J., J. Biol. Chem. 256 (1981) 5387-5393; Elices, M.J., Blake, D.A., and Goldstein, I.J. J. Biol. Chem. 261 (1986) 6064-6072),
5 leporine origin (Basu, M., and Basu, S., J. Biol. Chem. 248 (1973) 1700-1706; Betteridge, A., and Watkins, W.M., Eur. J. Biochem. 132 (1983) 29-35), porcine origin and bovine origin (Blanken, W.M., and Van den Eijnden, D.H., J. Biol. Chem. 260 (1985) 12927-12934) has been demonstrated to be catalyzed by the enzyme (α 1,3)galactosyltransferase.

10 In polypeptides which are intended for application to humans the presence of (α 1,3)glycosidically bound terminal galactose residues should be minimized as this glycostructure will elicit a response by the human immune system. This can be achieved, for example, by the time-consuming development of cell lines for the recombinant production of the therapeutic polypeptide which do not introduce
15 (α 1,3)glycosidically bound terminal galactose residues in the glycostructures of the therapeutic polypeptide. With chromatographic method generally used in the downstream processing of the crude polypeptide the Gal(α 1,3)-containing glycostructures cannot be removed.

In EP 0 255 153 a process for producing a-galactosidase capable of decreasing the
20 galactose content of galactomannans by splitting off 1,6 linked alpha-D-galactopyranosyl units attached to a main chain of 1,4 linked beta-D-mannopyranosyl units is reported. A method for clinical examination based on the structures of immunoglobulin G-linked oligosaccharides is reported in EP 0 698 793. In EP 1 878 747 glyco-engineered antibodies are reported. Selective marking
25 of immunoglobulin glycans is reported in WO 2007/071347. In WO 1997/016064 methods and compositions for the reduction of xenotransplantation rejection are reported. Antibody preparations with substantially homogeneous and unsialylated glycoforms, such as G0 and G2, which are prepared by enzymatic treatment, expression under certain conditions, use of particular host cells, and contact with
30 serum, are reported in WO 2007/024743.

In WO 2008/057634 polypeptides with enhanced anti-inflammatory and decreased cytotoxic properties and relating methods are reported. Proteolysis resistant antibody preparations are reported in WO 2007/024743.

Summary of the Invention

It has been found that an (α 1,3)galactosidases from plant origin, e.g. from green coffee beans (EC 3.2.1.22), can be used to selectively remove (α 1,3)glycosidically bound terminal galactose residues from the oligosaccharide at amino acid Asn297 in an immunoglobulin CH2 domain. (α 1,3)galactosidases from non-plant origin were found to have (β 1,4)galactosidases side reactivity and/or were less or not-reactive with tri- or tetra-antennary oligosaccharides.

Thus, herein is reported a method for producing an immunoglobulin or immunoglobulin fragment with defined glycostructure comprising the following steps in the following order:

- providing an affinity chromatography column eluate containing the immunoglobulin or immunoglobulin fragment,
- incubating the affinity chromatography column eluate with an enzyme, which cleaves off the terminal monosaccharide residues of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment,
- applying the incubated affinity chromatography eluate to a protein A chromatography material under conditions suitable for binding of the immunoglobulin or immunoglobulin fragment to the protein A chromatography material and recovering the immunoglobulin or immunoglobulin fragment from the protein A chromatography material and thereby producing an immunoglobulin or immunoglobulin fragment with defined glycostructure.

In one embodiment the enzyme, which cleaves off the monosaccharide residue at the non-reducing end of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment, is of plant origin. In one embodiment the enzyme, which cleaves off the monosaccharide residue at the non-reducing end of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment, is selected from α -D-galactoside galactohydrolase (EC 3.2.1.22) or Melibiase. In another embodiment the enzyme is (α 1,3)galactosidase from green coffee beans (EC 3.2.1.22). In another embodiment the terminal monosaccharide residue of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment is an (α 1,3)glycosidically bound galactose residue.

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In one embodiment the incubating the affinity chromatography column eluate with an enzyme, which cleaves off the monosaccharide residue at the non-reducing end of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment, comprises the following steps:

- 5 - incubating the affinity chromatography column eluate with an (α 1,3)glycosidase,
- taking a sample from the incubation mixture,
- either
 - 10 o applying the sample to protein A coated sepharose beads and afterwards washing the beads,
 - o recovering the immunoglobulin or immunoglobulin fragment from the protein A sepharose beads,
 - o adjusting the buffer conditions for glycosidase/sialidase digest,
 - 15 o incubating the immunoglobulin or immunoglobulin fragments with a glycosidase/sialidase to cleave off all N-Glycans, and
 - o taking an aliquot of the digest prior to MALDI analysis,
- or
 - 20 o applying the sample to protein A coated magnetic beads and afterwards washing the beads,
 - o incubating the beads with a glycosidase and recovering the cleaved off oligosaccharides, and
 - o purifying the cleaved off oligosaccharides with a cation exchange chromatography,
- 25 - determining the kind and amount of the monosaccharide residue at the non-reducing end of the glycostructure in the cleaved off oligosaccharides by mass spectrometry,
- continuing the incubating until all monosaccharide residue at the non-reducing end of the glycostructures, which can be cleaved off by the enzyme, have been cleaved off.
- 30 In still a further embodiment comprises the method the following steps in the following order as first steps:
 - providing a cell comprising a nucleic acid encoding the immunoglobulin or immunoglobulin fragment,
 - cultivating the cell under conditions suitable for the expression of the
 - 35 immunoglobulin or immunoglobulin fragment,

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- recovering the immunoglobulin or immunoglobulin fragment from the cell or the cultivation medium,
- applying the immunoglobulin or immunoglobulin fragment to a protein A chromatography material under conditions suitable for binding of the immunoglobulin to the protein A chromatography material and recovering the immunoglobulin from the protein A chromatography material.

In one embodiment comprises the method the following step as final step:

- purifying the produced immunoglobulin or immunoglobulin fragment with a defined glycostructure with one to three chromatography steps.

- 10 In one embodiment the enzyme, which cleaves off the monosaccharide residue at the non-reducing end of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment, is of plant origin. In another embodiment the enzyme, which cleaves off the monosaccharide residue at the non-reducing end of the glycostructure in the CH2 domain of the immunoglobulin or
- 15 immunoglobulin fragment, is selected from α -galactosidase, β -galactosidase, mannosidase, fucosidase, or sialidase. In a further embodiment the cell is a mammalian cell. In another embodiment the mammalian cell is a hamster cell, or murine cell, or a rabbit cell, or a sheep cell, or a hybridoma cell thereof. In still a further embodiment the cell is a CHO cell, a NS0 cell, a BHK cell or a SP2/0 cell.
- 20 A further aspect as reported herein is the use of an (α 1,3)galactosidase from green coffee beans (EC 3.2.1.22) for cleaving off the terminal (α 1,3)glycosidically bound galactose residues from the glycostructures in the CH2 domain of a recombinantly produced immunoglobulin or immunoglobulin fragment.

Detailed Description of the Invention

- 25 Herein is reported that e.g. (α 1,3)galactosidases from green coffee beans (EC 3.2.1.22) can be used to selectively remove (α 1,3)glycosidically bound terminal galactose residues from the oligosaccharide attached at amino acid residue Asn297 to an immunoglobulin CH2 domain. (α 1,3)galactosidases from other sources beside plants were found to have (β 1,4)galactosidases side reactivity and/or were
- 30 less or not-reactive with tri- or tetra-antennary oligosaccharides (see Table 1).

Table 1: Comparison of (α 1,3)galactosidases from different sources

(α1,3)galactosidase from (EC 3.2.1.22)	cleave off of (α1,3)glycosidically bound galactose	cleave off of (α1,4)glycosidically bound galactose	cleave off of oligosaccharides	
			tri- and tetra- biantennary	antennary
green coffee bean	yes	no	yes	yes
Xanthomonas manihotis	yes	yes	yes	yes
Escherichia coli	yes	yes	yes	no

Thus, in one embodiment the enzyme in the methods as reported herein, which cleaves off the terminal monosaccharide residues of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment, is of plant origin. In another embodiment the enzyme in the methods as reported herein, which cleaves off the terminal monosaccharide residues of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment, is an enzyme that (i) cleaves off (α 1,3)glycosidically bound sugar residues, and (ii) does not cleave off (α 1,4)glycosidically bound sugar residues, and (iii) cleaves off the residues from bi-, tri-, and tetra-antennary oligosaccharides.

Herein is reported a method for producing an immunoglobulin or immunoglobulin fragment with defined glycostructure comprising the following steps:

- providing an affinity chromatography column eluate containing the immunoglobulin or immunoglobulin fragment,
- incubating the affinity chromatography column eluate with the (α 1,3)-galactosidase from green coffee beans (EC 3.2.1.22),
- applying the incubated affinity chromatography column eluate to a protein A chromatography material, optionally under conditions suitable for binding of the immunoglobulin or immunoglobulin fragment to the protein A chromatography material, and recovering the immunoglobulin or immunoglobulin fragment from the protein A chromatography material and thereby producing an immunoglobulin or immunoglobulin fragment with defined glycostructure.

Human immunoglobulins are mainly glycosylated at the asparagine residue at position 297 (Asn297) of the heavy chain CH2 domain with a core fucosylated

biantennary complex oligosaccharide (immunoglobulin amino acid residue numbering according to Kabat, see below). The biantennary glycostructure can be terminated by up to two consecutive galactose (Gal) residues in each arm. The arms are denoted (1,6) and (1,3) according to the glycoside bond to the central mannose residue. The glycostructure denoted as G0 comprises no galactose residue. The glycostructure denoted as G1 contains one or more galactose residues in one arm. The glycostructure denoted as G2 contains one or more galactose residues in each arm (Raju, T.S., *Bioprocess Int.* 1 (2003) 44-53). Human constant heavy chain regions are reported in detail by Kabat, E.A., et al., *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991), and by Brueggemann, M., et al., *J. Exp. Med.* 166 (1987) 1351-1361; Love, T.W., et al., *Methods Enzymol.* 178 (1989) 515-527. CHO type glycosylation of immunoglobulin Fc parts is e.g. described by Routier, F.H., *Glycoconjugate J.* 14 (1997) 201-207.

The term "immunoglobulin" denotes and encompasses the various forms of immunoglobulins such as human immunoglobulins, humanized immunoglobulins, chimeric immunoglobulins, or T-cell antigen depleted immunoglobulins (see e.g. WO 98/33523, WO 98/52976, and WO 00/34317). In one embodiment the antibody in the methods as reported herein is a human or humanized antibody. Genetic engineering of immunoglobulins is e.g. described in Morrison, S.L., et al., *Proc. Natl. Acad. Sci. USA* 81 (1984) 6851-6855; US 5,202,238 and US 5,204,244; Riechmann, L., et al., *Nature* 332 (1988) 323-327; Neuberger, M.S., et al., *Nature* 314 (1985) 268-270; Lonberg, N., *Nat. Biotechnol.* 23 (2005) 1117-1125.

An immunoglobulin in general comprises two so called full length light chain polypeptides (light chain) and two so called full length heavy chain polypeptides (heavy chain). Each of the full length heavy and light chain polypeptides contains a variable domain (variable region) (generally the amino terminal portion of the full length polypeptide chain) comprising binding regions which interact with an antigen. Each of the full length heavy and light chain polypeptides comprises a constant region (generally the carboxyl terminal portion). The constant region of the full length heavy chain mediates the binding of the immunoglobulin i) to cells bearing a Fc gamma receptor (FcγR), such as phagocytic cells, or ii) to cells bearing the neonatal Fc receptor (FcRn) also known as Brambell receptor. It also mediates the binding to some factors including factors of the classical complement system such as component (C1q). The variable domain of a full length

immunoglobulin's light or heavy chain in turn comprises different segments, i.e. four framework regions (FR) and three hypervariable regions (CDR). A "full length immunoglobulin heavy chain" is a polypeptide consisting in N-terminal to C-terminal direction of an immunoglobulin heavy chain variable domain (VH), an immunoglobulin constant domain 1 (CH1), an immunoglobulin hinge region, an immunoglobulin constant domain 2 (CH2), an immunoglobulin constant domain 3 (CH3), and optionally an immunoglobulin constant domain 4 (CH4) in case of an immunoglobulin of the subclass IgE. A "full length immunoglobulin light chain" is a polypeptide consisting in N-terminal to C-terminal direction of an immunoglobulin light chain variable domain (VL), and an immunoglobulin light chain constant domain (CL). The full length immunoglobulin chains are linked together via inter-polypeptide disulfide bonds between the CL-domain and the CH1 domain and between the hinge regions of the full length immunoglobulin heavy chains.

The term "immunoglobulin fragment" denotes within this application a polypeptide comprising at least the CH2 domain and the CH3 domain of a full length immunoglobulin heavy chain. An immunoglobulin fragment may also comprise additional non-immunoglobulin derived amino acid sequences.

It has been reported in recent years that the glycosylation pattern of immunoglobulins, i.e. the saccharide composition and multitude of attached glycostructures, has a strong influence on the biological properties (see e.g. Jefferis, R., *Biotechnol. Prog.* 21 (2005) 11-16). Immunoglobulins produced by mammalian cells contain 2-3 % by mass oligosaccharides (Taniguchi, T., et al., *Biochem.* 24 (1985) 5551-5557). This is equivalent e.g. in an immunoglobulin of class G (IgG) to 2.3 oligosaccharide residues in an IgG of mouse origin (Mizuochi, T., et al., *Arch. Biochem. Biophys.* 257 (1987) 387-394) and to 2.8 oligosaccharide residues in an IgG of human origin (Parekh, R.B., et al., *Nature* 316 (1985) 452-457), whereof generally two are located in the Fc-region at Asn²⁹⁷ and the remaining in the variable region (Saba, J.A., et al., *Anal. Biochem.* 305 (2002) 16-31).

The term "glycostructure" as used within this application denotes and comprises all oligosaccharides which are attached to a specified amino acid residue in an immunoglobulin. Due to the glycosylation heterogeneity of a cell, a recombinantly produced immunoglobulin comprises not only a single, defined N- or O-linked oligosaccharide at a specified amino acid residue, but is a mixture of polypeptides

(immunoglobulin molecules) each having the same amino acid sequence but comprising differently composed oligosaccharides at the specified amino acid position. Thus, the term “glycostructure” denotes a group of oligosaccharides that are attached at a specified amino acid position of a recombinantly produced immunoglobulin, i.e. the heterogeneity of the attached oligosaccharide. The term
5 “oligosaccharide” as used within this application denotes a polymeric saccharide comprising two or more covalently linked monosaccharide units.

For the notation of the different N- or O-linked oligosaccharides in the current invention the individual sugar residues are listed from the non-reducing end to the
10 reducing end of the oligosaccharide molecule. The longest sugar chain was chosen as basic chain for the notation. The reducing end of an N- or O-linked oligosaccharide is the monosaccharide residue, which is directly bound to the amino acid of the amino acid backbone of the immunoglobulin, whereas the end of an N- or O-linked oligosaccharide, which is located at the opposite terminus as the
15 reducing end of the basic chain, is termed non-reducing end.

The term “affinity chromatography” as used within this application denotes a chromatography method which employs an “affinity chromatography material”. In an affinity chromatography polypeptides are separated based on their biological activity or chemical structure depending on the formation of electrostatic interactions, hydrophobic bonds, and/or hydrogen bonds to the chromatographical functional groups of the chromatography material. To recover the specifically bound polypeptide from the affinity chromatography material either a competitor ligand can be added or the chromatography conditions, such as pH value, polarity or ionic strength of the buffer, can be changed. Exemplary “affinity
20 chromatography materials” are metal chelating chromatography materials such as Ni(II)-NTA or Cu(II)-NTA, or immunoglobulin affinity chromatography materials such as in one embodiment of the methods as reported herein chromatography materials comprising thereto covalently linked protein A or protein G, or enzyme binding affinity chromatography materials such as chromatography materials comprising thereto covalently bound enzyme substrate analogues, enzyme
25 cofactors, or enzyme inhibitors as chromatographical functional group, or lectin binding chromatography materials such as chromatography materials comprising thereto covalently linked polysaccharides, cell surface receptors, glycoproteins, or intact cells as chromatographical functional group.
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The term “enzyme, which cleaves off the monosaccharide residue at the non-reducing end of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment” denotes an enzyme that selectively cleaves off a monosaccharide residue at the non-reducing end of a glycostructure. Such an enzyme is of plant origin. In one embodiment the enzyme is of plant origin and is selected from α -galactosidases (cleaving off of $(\alpha 1,3)$ -, $(\alpha 1,4)$ -, and/or $(\alpha 1,6)$ glycosidically bound galactose residues, β -galactosidase (cleaving off of $(\beta 1,4)$ glycosidically bound galactose residues), mannosidase (cleaving off of mannose residues), fucosidase (cleaving off of fucose residues), and sialidase (cleaving off of sialic acid residues). Exemplary α -galactosidases are $(\alpha 1,3)$ galactosidases such as EC 3.2.1.22 or EC 2.4.1.151 (see, e.g., Dabkowski, P.L., et al., Transplant Proc. 25 (1993) 2921 and Yamamoto, F., et al., Nature 345 (1990) 229-233). In one embodiment the enzyme is $(\alpha 1,3)$ galactosidase from green coffee beans (EC 3.2.1.22).

The term “defined glycostructure” denotes within this application a glycostructure in which the monosaccharide residue at each of the non-reducing ends of the glycostructure is of a specific kind and linked with a specific glycosidic bond to the rest of the glycostructure, i.e. all further monosaccharides residues have been cleaved off. The term “defined glycostructure” denotes within this application a glycostructure in which all monosaccharide residue at the non-reducing end of glycostructures of a specific kind and linked with a specific glycosidic bond to the glycostructure have been cleaved off, i.e. the glycostructures of an immunoglobulin or immunoglobulin fragment are depleted of or lack a specific terminal monosaccharide residue linked via a specific glycosidic bond to the remainder of the glycostructure. For example, if as in one embodiment the immunoglobulin or immunoglobulin fragment is incubated with an $(\alpha 1,3)$ galactosidase all the glycostructures of the immunoglobulin or immunoglobulin fragment lack $(\alpha 1,3)$ glycosidically bound galactose monosaccharide residue at the non-reducing end, i.e. the immunoglobulin or immunoglobulin fragment has a defined glycostructure which lacks $(\alpha 1,3)$ glycosidically bound terminal galactose residues.

The term “applying to” and grammatical equivalents thereof as used within this application denotes a partial step of a purification method in which a solution containing a substance of interest is brought in contact with a stationary phase. The solution containing the substance of interest to be purified passes through the stationary phase providing for an interaction between the stationary phase and the

substances in solution. Depending on the conditions, such as e.g. pH, conductivity, salt concentration, temperature, and/or flow rate, some substances of the solution are bound to the stationary phase and therewith are removed from the solution. Other substances remain in solution. The substances remaining in solution can be found in the flow-through. The "flow-through" denotes the solution obtained after the passage of the chromatographic device, which may either be the applied solution containing the substance of interest or the buffer, which is used to flush the column or to cause elution of one or more substances bound to the stationary phase. The substance of interest can be recovered from the solution after the purification step by methods familiar to a person of skill in the art, such as e.g. precipitation, salting out, ultrafiltration, diafiltration, lyophilization, affinity chromatography, or solvent volume reduction to obtain the substance in substantially homogeneous form.

An immunoglobulin or immunoglobulin fragment whose glycostructure can be modified in the methods as reported herein can be produced by recombinant means. Methods for recombinant production are widely known in the state of the art and comprise protein expression in eukaryotic cells with subsequent isolation of the immunoglobulin or immunoglobulin fragment and purification to a pharmaceutically acceptable purity. For the expression of the immunoglobulin or immunoglobulin fragment either a hybridoma cell or a eukaryotic cell, in which one or more nucleic acids encoding the immunoglobulin or immunoglobulin fragment have been introduced, is used. In one embodiment the eukaryotic cells is selected from CHO cells, NS0 cells, SP2/0 cells, HEK 293 cells, COS cells, PER.C6 cells, BHK cells, rabbit cells, or sheep cells. In another embodiment the eukaryotic cell is selected from CHO cells, HEK cells, or rabbit cells. After expression the immunoglobulin or immunoglobulin fragment is recovered from the cells (from the supernatant or from the cells after lysis). General methods for recombinant production of immunoglobulins are well-known in the state of the art and reported, for example, in the review articles of Makrides, S.C., *Protein Expr. Purif.* 17 (1999) 183-202; Geisse, S., et al., *Protein Expr. Purif.* 8 (1996) 271-282; Kaufman, R.J., *Mol. Biotechnol.* 16 (2000) 151-160; Werner, R.G., *Drug Res.* 48 (1998) 870-880.

Purification of immunoglobulins or immunoglobulin fragments can be performed in order to eliminate cellular components or other contaminants, e.g. other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment,

CsCl banding, column chromatography, agarose gel electrophoresis, and others well known in the art (see e.g. Ausubel, F., et al. (ed.), Current Protocols in Molecular Biology, Greene Publishing and Wiley Interscience, New York (1987)). Different methods are well established and widespread used for protein purification, such as affinity chromatography with microbial proteins (e.g. protein A or protein G affinity chromatography), ion exchange chromatography (e.g. cation exchange (carboxymethyl resins), anion exchange (amino ethyl resins) and mixed-mode exchange), thiophilic adsorption (e.g. with beta-mercaptoethanol and other SH ligands), hydrophobic interaction or aromatic adsorption chromatography (e.g. with phenyl-sepharose, aza-arenophilic resins, or m-aminophenylboronic acid), metal chelate affinity chromatography (e.g. with Ni(II)- and Cu(II)-affinity material), size exclusion chromatography, and electrophoretical methods (such as gel electrophoresis, capillary electrophoresis), as well as combinations thereof, such as affinity chromatography with microbial proteins, cation exchange chromatography and anion exchange chromatography (see e.g. Vijayalakshmi, M.A., Appl. Biochem. Biotech. 75 (1998) 93-102).

The glycostructure of a recombinantly produced immunoglobulin or immunoglobulin fragment will be determined by the employed cell line and the employed cultivation conditions. With conventional down stream processing techniques selective removal of specific glycostructures is not possible.

With the methods as reported herein an immunoglobulin or immunoglobulin fragment with defined glycostructure can be obtained in down stream processing. It has been found that for the removal of (α 1,3)glycosidically bound galactose residues at the non-reducing end of the glycostructures of immunoglobulins or immunoglobulin fragments an (α 1,3)galactosidase of plant origin, especially from green coffee beans, is only suited.

The removal of (α 1,3)glycosidically bound galactose residues during the down stream processing of a recombinantly produced immunoglobulin or immunoglobulin fragment with a method as reported herein

- provides a method for the reduction of the immunogenicity of a recombinantly produced immunoglobulin or immunoglobulin fragment,
- abolishes the need to obtain/select/use a cell line that does not produce glycostructures with a terminal (α 1,3)glycosidically bound galactose residue,

- does not change the product quality due to the additional incubation step of the immunoglobulin or immunoglobulin fragment compared to a method without the additional incubation,
- 5 - provides a method for producing an immunoglobulin or immunoglobulin fragment with a defined glycostructure during the down stream processing, i.e. after the expression is finished in vitro,
- provides the immunoglobulin with defined glycostructure with improved yield as no immunoglobulin with unwanted glycostructure is removed but all immunoglobulin is enzymatically converted to a defined glycostructure.

10 Thus, one aspect as reported herein is a method for producing an immunoglobulin or immunoglobulin fragment with defined glycostructure comprising the following step:

- incubating the immunoglobulin or immunoglobulin fragment with an enzyme, which cleaves off specifically a terminal monosaccharide residue
15 of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment.

In one embodiment the enzyme, which cleaves off specifically a terminal monosaccharide residues of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment, is (α 1,3)galactosidase from green
20 coffee beans. In another embodiment the terminal monosaccharide is (α 1,3)glycosidically bound galactose.

Thus, one aspect as reported herein is a method for producing an immunoglobulin or immunoglobulin fragment with defined glycostructure comprising the following steps:

- 25 - providing an affinity chromatography column eluate containing the immunoglobulin or immunoglobulin fragment,
- incubating the affinity chromatography column eluate with an enzyme, which cleaves off specifically a terminal monosaccharide residues of the glycostructure in the CH2 domain of the immunoglobulin or
30 immunoglobulin fragment,
- applying the enzymatically modified affinity chromatography column eluate to a protein A chromatography material and recovering the immunoglobulin or immunoglobulin fragment from the protein A

chromatography material and thereby producing an immunoglobulin or immunoglobulin fragment with defined glycostructure.

To monitor the progress of the enzymatic reaction an on-line determination of the glycosylation profile of the immunoglobulin or immunoglobulin fragment can be performed. Therefore, one aspect as reported herein is a method for producing an immunoglobulin or immunoglobulin fragment with defined glycostructure comprising the following steps:

- providing an affinity chromatography column eluate containing the immunoglobulin or immunoglobulin fragment,
- modifying the immunoglobulin or immunoglobulin fragment contained in the affinity chromatography column eluate by
 - a) incubating the affinity chromatography column eluate with an enzyme, which cleaves off specifically a terminal monosaccharide residues of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment,
 - b) taking a sample from the incubation mixture,
 - c) applying the sample to protein A coated magnetic beads and afterwards washing the beads,
 - d) incubating the beads with a glycosidase and recovering the cleaved-off oligosaccharides,
 - e) purifying the cleaved-off oligosaccharides with a cation exchange chromatography,
 - f) determining the kind and amount of monosaccharide residue at the non reducing end of the cleaved-off oligosaccharides by mass spectrometry, e.g. by MALDI-TOF MS,
 - g) repeating steps a) to f) until all monosaccharide residues at the non-reducing end of the glycostructure of the immunoglobulin or immunoglobulin fragment that can be cleaved off by the enzyme have been cleaved off,
- applying the enzymatically modified affinity chromatography column eluate to a protein A chromatography material, optionally under conditions suitable for binding of the contained immunoglobulin or immunoglobulin fragment to said protein A chromatography material, and recovering the immunoglobulin or immunoglobulin fragment from the protein A

chromatography material and thereby producing an immunoglobulin or immunoglobulin fragment with defined glycostructure.

It has been found that the enzyme (α 1,3)galactosidase from green coffee beans is surprisingly useful in the methods as reported herein. (α 1,3)galactosidases from a bacterial source have been evaluated in the methods as reported herein but found to be not suited. Although all these enzymes can cleave an (α 1,3)glycosidic bond in oligosaccharides only the (α 1,3)galactosidase from green coffee beans cleaves such a bond in glycostructures of immunoglobulins or immunoglobulin fragments at suitable conditions, with suitable specificity, and with suitable substrate specificity.

A further aspect as reported herein is a method for producing an immunoglobulin or immunoglobulin fragment with defined glycostructure comprising the following steps:

- providing a cell comprising a nucleic acid encoding the immunoglobulin or immunoglobulin fragment,
- cultivating the cell under conditions suitable for the expression of the immunoglobulin or immunoglobulin fragment,
- recovering the immunoglobulin or immunoglobulin fragment from the cell or the cultivation medium,
- applying the recovered immunoglobulin or immunoglobulin fragment to a protein A chromatography material and recovering the immunoglobulin from the protein A chromatography material by eluting the immunoglobulin or immunoglobulin fragment from the protein A chromatography material,
- modifying the immunoglobulin or immunoglobulin fragment contained in the affinity chromatography column eluate by
 - a) incubating the affinity chromatography column eluate with an enzyme, which cleaves off specifically a terminal monosaccharide residues of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment,
 - b) taking a sample from the incubation mixture,
 - c) applying the sample to protein A coated magnetic beads and afterwards washing the beads,
 - d) incubating the beads with a glycosidase and recovering the cleaved-off oligosaccharides,

- e) purifying the cleaved-off oligosaccharides with a cation exchange chromatography,
 - f) determining the kind and amount of the monosaccharide residue at the non-reducing end of the cleaved off oligosaccharides by mass spectrometry, e.g. by MALDI-TOF MS,
 - g) repeating steps a) to f) until all monosaccharide residues at the non-reducing end of the glycostructure of the immunoglobulin or immunoglobulin fragment that can be cleaved off by the enzyme have been cleaved off,
- 10 - applying the enzymatically modified affinity chromatography column eluate to a protein A chromatography material and recovering the immunoglobulin or immunoglobulin fragment from the protein A chromatography material and thereby producing an immunoglobulin or immunoglobulin fragment with defined glycostructure.
- 15 - optionally purifying the produced immunoglobulin or immunoglobulin fragment with a defined glycostructure with one to three additional chromatography steps.

For the purification of immunoglobulins or immunoglobulin fragments, which have been produced e.g. by cell cultivation methods, generally a combination of different chromatography steps can be employed. Normally a protein A affinity chromatography can be followed by one or two additional separation steps. In one embodiment the additional chromatography steps are a cation and an anion exchange chromatography step or vice versa. The final purification step is a so called "polishing step" for the removal of trace impurities and contaminants like aggregated immunoglobulins, residual HCP (host cell protein), DNA (host cell nucleic acid), viruses, or endotoxins. In one embodiment the final purification step is an anion exchange chromatography in flow-through mode.

General chromatographic methods and their use are known to a person skilled in the art. See for example, Chromatography, 5th edition, Part A: Fundamentals and Techniques, Heftmann, E. (ed), Elsevier Science Publishing Company, New York, (1992); Advanced Chromatographic and Electromigration Methods in Biosciences, Deyl, Z. (ed.), Elsevier Science BV, Amsterdam, The Netherlands, (1998); Chromatography Today, Poole, C. F., and Poole, S. K., Elsevier Science Publishing Company, New York, (1991); Scopes, Protein Purification: Principles and Practice (1982); Sambrook, J., et al. (ed), Molecular Cloning: A Laboratory Manual, Second

Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989; or Current Protocols in Molecular Biology, Ausubel, F. M., et al. (eds), John Wiley & Sons, Inc., New York.

5 At the time the invention was made a fusion protein consisting of an N-terminal mutated or non-mutated IL-15 part and a C-terminal Fc part was available in sufficient quantities in our laboratory. This fusion protein has been used as an example and should not be construed to limit the scope of the invention which is defined by the appended claims. Thus, in one embodiment the immunoglobulin or immunoglobulin fragment is a fusion protein of an interleukin-15 part and an Fc
10 part of human origin of SEQ ID NO: 1 or 2. Such a molecule is reported in example 1 and SEQ ID NO: 3 and 4 (with murine Fc part) of WO 2005/100394.

The following examples, figures and sequences are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures
15 set forth without departing from the spirit of the invention.

Description of the Sequence Listing

SEQ ID NO:1 Nucleic acid sequence of human mutated interleukin 15/Fc.

SEQ ID NO:2 Amino acid sequence of human mutated interleukin 15/Fc.

Description of the Figures

20 **Figure 1** Total ion chromatogram of the reference sample of a mutated IL15FC-Fc fusion polypeptide (upper part) and of the incubation with the (α 1,3)galactosidase from Xanthomonas manihotis (lower part). It can be seen that this enzyme has also (β 1,4)galactosidase activity.

25 **Figure 2** Total ion chromatogram of the reference sample of a mutated IL15FC-Fc fusion polypeptide (upper part) and of the incubation with the (α 1,3)galactosidase from Escherichia Coli (lower part). It can be seen that (α 1,3)glycosidically bound galactose was only removed at biantennary oligosaccharides and that this enzyme has
30 also (β 1,4)galactosidase activity.

Figure 3 Total ion chromatogram of the reference sample of a mutated IL15FC-Fc fusion polypeptide (upper part) and of the incubation with the (α 1,3)galactosidase from green coffee beans (lower

part). It can be seen that (α 1,3)glycosidically bound galactose was removed completely and that this enzyme has no (β 1,4)galactosidase activity.

Example 1

5 Preparation of purified interleukin-15/Fc fusion protein

The interleukin-15/Fc fusion protein has been prepared in accordance with the data and methods reported in the international patent applications WO 1997/041232, WO 2005/100394 and WO 2005/100395.

Example 2

10 Cleavage of (α 1,3)glycosidically bound galactose residues

Digestion with α -Galactosidase

The samples were adjusted to the corresponding buffer conditions by dialysis (see Table 2) and thereafter incubated with the enzyme.

For example:

15 40 ml of the interleukin-15/Fc fusion protein solution with a concentration of about 1 mg/ml were digested with 1.5 mL of (α 1,3)galactosidase (204 U/ml) over night (16 hours) at 25°C.

Table 2: Enzymatic digestion conditions

(α1,3)galactosidase from (EC 3.2.1.22)	buffer conditions	amount of interleukin-15/Fc fusion protein	amount of (α1,3)galactosidase	conversion conditions
green coffee beans	100 mM sodium citrate/phosphate (pH 6.0) *	100 μ g protein solution	1 U	37°C, 18 hours
Xanthomonas manihotis	50 mM sodium acetate 5 mM CaCl ₂ pH 5.5	100 μ g protein solution	840 U	37°C, 18 hours

(α 1,3)galactosidase from (EC 3.2.1.22)	buffer conditions	amount of interleukin-15/Fc fusion protein	amount of (α 1,3)galactosidase	conversion conditions
Escherichia coli	250 mM sodium phosphate, pH 6.5 *	100 μ g protein solution	1 U	37°C, 18 hours

*according to the manufacturers' manual

Small scale purification with Protein A

The protein A column was equilibrated with 25 mM Tris (hydroxymethyl) aminomethane buffer (TRIS) containing 25 mM sodium chloride and 5 mM EDTA at pH 7.2. The samples were applied to the column, the column was washed with equilibration buffer and the fusion protein was eluted with 100 mM Citrate Buffer pH 3.6.

Sample preparation for mass spectrometry

Samples containing 100 μ g of the interleukin-15/Fc fusion protein were buffer exchanged by means of centricons to 2 mM TRIS-HCl, pH 7.0. 50 μ l of the interleukin-15/Fc fusion protein were digested with 1 μ l of N-glycosidase F and 1 μ l sialidase to cleave the glycans from the protein and to eliminate sialic acid moieties.

Ion exchange resin AG 50W-X8 was suspended in water and was shaken several times. After settlement of the resin the water was discarded. This was done three times. 900 μ l of the suspension were transferred to a MICRO Bio-Spin Chromatography Column and centrifuged for 1 minute.

The digested samples were applied to the column and the columns were centrifuged for 1 min. The purified glycans were collected in the flow-through. The obtained solutions were diluted 1:1 with sDHB matrix solution (sDHB in 125 μ l ethanol and 125 μ l 10 mM sodium chloride in water).

Alternatively, an aliquot of 1 μ l digest were mixed with 1 μ l of DHB matrix solution (10 mg DHB in 10 mM sodium chloride in water) and quickly dried using high vacuum to get homogenous spots for MALDI-TOF analysis.

Mass Spectrometry

Calibration spectra were acquired with a MALDI-TOF Mass Spectrometer Voyager DE Pro from Applied Biosystems in the Reflectron Mode. The Acceleration Voltage was set to 20000 V; Grid Voltage was 76% and the Mirror
5 Voltage Ratio 1.12. The Extraction Delay Time was set to 110 ns. The Acquisition mass range was from 1000 to 5000 Da, the Laser Intensity was 2460 and the Laser Rep Rate 20.0 Hz.

Patent Claims

1. Method for producing an immunoglobulin or immunoglobulin fragment with defined glycostructure comprising the following steps:

- 5
- providing an affinity chromatography column eluate containing the immunoglobulin or immunoglobulin fragment,
 - incubating the affinity chromatography column eluate with an enzyme of plant origin, which cleaves off the monosaccharide residue at the non-reducing end of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment,
 - 10 - applying the incubated affinity chromatography eluate to a protein A chromatography material and recovering the immunoglobulin or immunoglobulin fragment from the protein A chromatography material and thereby producing an immunoglobulin or immunoglobulin fragment with defined glycostructure.

- 15
2. Method according to claim 1, characterized in that the enzyme, which cleaves off the monosaccharide residue at the non-reducing end of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment, is (α 1,3)galactosidase (EC 3.2.1.22) from green coffee beans.

- 20
3. Method according to any one of claims 1 or 2, characterized in that the incubating the affinity chromatography column eluate with an enzyme, which cleaves off the monosaccharide residue at the non-reducing end of the glycostructure in the CH2 domain of the immunoglobulin or immunoglobulin fragment, comprises the following steps:

- 25
- incubating the affinity chromatography column eluate with an (α 1,3)glycosidase,
 - taking a sample from the incubation mixture,
 - either
 - o applying the sample to protein A coated sepharose beads and afterwards washing the beads,

- 22 -

- recovering the immunoglobulin or immunoglobulin fragment from the protein A sepharose beads,
 - adjusting the buffer conditions for glycosidase/sialidase digest, and
 - 5 ○ incubating the immunoglobulin or immunoglobulin fragments with a glycosidase/sialidase to cleave of all N-Glycans,
- or
- applying the sample to protein A coated magnetic beads and afterwards washing the beads,
 - 10 ○ incubating the beads with a glycosidase and recovering the cleaved off oligosaccharides, and
 - purifying the cleaved off oligosaccharides with a cation exchange chromatography,
- 15 - determining the kind and amount of the monosaccharide residue at the non-reducing end of the glycostructure in the cleaved off oligosaccharides by mass spectrometry,
- continuing the incubating until all monosaccharide residue at the non-reducing end of the glycostructure, which can be cleaved off by the enzyme, have been cleaved off.
- 20 4. Method according to any one of the preceding claims further comprising the following steps as first steps:
 - providing a cell comprising a nucleic acid encoding the immunoglobulin or immunoglobulin fragment,
 - cultivating the cell,
 - 25 - recovering the immunoglobulin or immunoglobulin fragment from the cell or the cultivation medium,

- applying the immunoglobulin or immunoglobulin fragment to a protein A chromatography material and recovering the immunoglobulin from the protein A chromatography material.
5. Method according to any one of the preceding claims further comprising the following step as final step:
- purifying the produced immunoglobulin or immunoglobulin fragment with a defined glycostructure with one to three chromatography steps.
6. Method according to any one of claims 1 and 3 to 5, characterized in that said enzyme, which cleaves off the monosaccharide residue at the non-reducing end of the glycostructure in the CH2 domain of said immunoglobulin or immunoglobulin fragment is selected from α -galactosidase, β -galactosidase, mannosidase, fucosidase, sialidase.
7. Method according to any one of claims 4 to 6, characterized in that the cell is a mammalian cell.
8. Method according to claim 7, characterized in that the mammalian cell is a hamster cell, murine cell, a rabbit cell, a sheep cell, or a hybridoma cell thereof.
9. Method according to any one of claims 7 or 8, characterized in that the cell is a CHO cell, a NS0 cell, a BHK cell or a SP2/0 cell.
10. Method according to any one of the preceding claims characterized in that the immunoglobulin or immunoglobulin fragment comprises an amino acid sequence corresponding to amino acid residues 18 to 364 of SEQ ID NO:02.
11. Use of an (α 1,3)galactosidase from green coffee beans (EC 3.2.1.22) for cleaving off the terminal (α 1,3)glycosidically bound galactose residues from the glycostructures attached to the amino acid asparagine of the CH2 domain of a recombinantly produced immunoglobulin or immunoglobulin fragment.