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Charge heterogeneity study of a Fc-fusion protein, abatacept, using two-dimensional gel electrophoresis

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Medicinal products obtained by recombinant DNA technology are complex molecules and demonstrate a high degree of molecular heterogeneity. Charge heterogeneity and isoform pattern of this class of medicines, are parameters important for their quality, safety, and efficacy. In this study we report the application of two-dimensional gel electrophoresis (2-D electrophoresis) for the quality assessment, identification, charge heterogeneity and isoform pattern study of recombinant protein, CTLA4-Ig (abatacept), which has been selected as an example of the drug class, known as Fc-fusion proteins. In order to achieve an efficient separation of this complex analyte,2-D electrophoresis was optimized employing different experimental conditions regarding the selection of an immobilized pH gradient (IPG), sample pretreatment, presentation and detection procedure. Experimental datadocumented that 2-D electrophoresis is a suitable method for the assessment of identity, purity, structural integrity, isoform pattern and to monitor charge heterogeneity and post-translational glycosylation of the Fc-fusion protein, abatacept.

1. Introduction

Abatacept [USAN INN, Orencia(trade name); synonyms: CTLA4-Ig, rDNA; cytototoxic T-lymphocyte-associated-antigen-Immunoglobulin G1 fragment fusion protein, recombinant; 1-25-Oncostatin M (humanprecursor) fusion protein with CTLA4 (antigen)(human) fusion protein with immunoglobulin G1 (humanheavy chain fragment), bimolecular (146→146')-disulfide. Drug Bank ID: DB01281, CAS No: 332348-12-6] is the first representative of a new class of biopharmaceuticals known as selective T-cell co-stimulation modulators. This drug is used to treat rheumatoid arthritis in adults and juvenile idiopathic arthritis in adolescents and children (EMA 2014a; US FDA 2014a). CTLA4-Ig isaglycosylated, soluble Fc-fusion protein producedby recombinant DNA technology ingenetically engineered Chinesehamster ovary (CHO) cells.It is a homodimer consisting of two identicalsubunits,each composed of the extracellular domain (ECD) of human cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and the modified (hinge, C_H2 and C_H3 domains) sequence of the human IgG1 Fc portion, linked by one disulphide bond (Fig. 1) (EMA 2014a; Linsley et al., 1991, 1998). Each monomer polypeptide chain contains 357amino acids and two monomers are linked through disulfide bond (120, 120') to form a covalent homodimer with apparent M_r of 92.300 kDa (determined by MALDI/TOF MS) of which 15% (13.500 kDa) is composed of carbohydrates. The measured M_r isgreater than the theoretical value predicted by the cDNA-derived amino acidsequence (39.448 kDa, averageM_r of monomer, in reduced form), owing to post-translationalglycosylation(Wishart et al. 2008; EMA 2014b; US FDA 2014b). The theoretical *pI* of abatacept monomer is 5.67 (Wilkinset al. 1997).

Modifications to the original sequences were introduced to avoid unintended disulphide bridge formation and to reduce constant region-mediated biological effector functions and complement activation (EMA 2014b; Duncan and Winter 1998; Chappel et al. 1991). ECD of CTLA-4, amino acid sequence from + 1-1 to 125 ofCTLA4-Ig is responsible for interaction with CD80 and CD86 (B7) receptorsof T-lymphocyte(Linsley et al.1991, 1998; Emery 2004), whereas IgG1 portion endows prolonged circulating half life of abatacept (Isaacs1997). In addition, abatacept comprises an Fc region, andit is therefore amenable to purification by protein A chromatography during thedownstream processing(Breeceet al. 2005).

Alike other biotechnologically driven glycoproteins, CTLA4-Ig demonstrates a high degree of molecular complexity. Through the recombinant production process various forms of CTLA4-Ig can be produced, including glycoforms, conformational isomers, and primary sequence variants (US FDAb 2014).Besides posttranslational modifications, also may occur modifications resulting from subsequent degradation of the molecule. Analytical methods used for the characterization of recombinant monoclonal antibodies and fusion proteins have been broadly reviewed (Beck et al. 2013a,b). Literature data revealed that

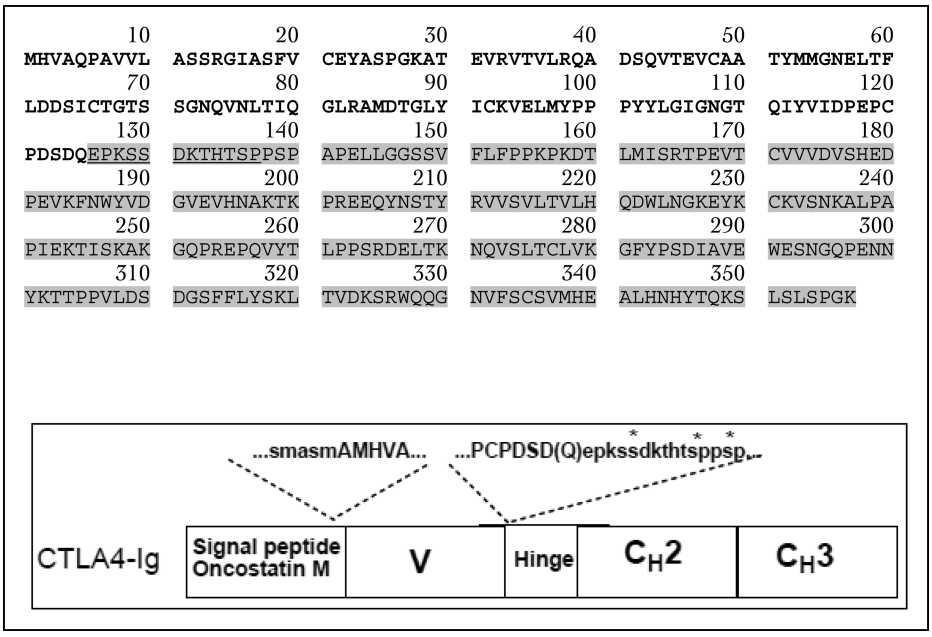

Fig. 1: Top: Amino acid sequence of abatacept. ECD of human CTLA-4 (residues 1-125) is indicated by boldface, modified sequence of the human IgG1 Fc region is highlighted, and hinge region underlined. Bottom: Construction and expression of CTLA4-Ig. A cDNA construct encoding Signal peptide (SP), CTLA-4 (V), and human IgCγ1 (Hinge, CH2 and CH3). During the construction of CTLA4-Ig, two cysteine (C) residues in the hinge region and one C residue in the CH2 region were substituted with serines (S) by mutation of the human IgG1 gene sequence (C-S, at positions 130, 136 and 139). Furthermore, glutamine (Q) was introduced between aspartic acid (D) from the ECD of CTLA-4 and glutamic acid (E) from the hinge region of the Ig HC and one proline (P) was unintentionally substituted by serine in the CH2 region (P-S, position 148). Asterisks denote C-S mutations. Junctions between CTLA-4 (capital letters) and the SP of oncostatin M, and the hinge region are presented as well. (Linsley et al. 1998).

multiple charged isoforms in the native CTLA4-Ig ranging from *pI* 4.5- 5.5 are detected using isoelectric focusing (*IEF*) (BMS, Orencia 2013). As expected, CTLA4-Ig is a mixture of different iso- and glycoforms of the protein and inaddition to heterogeneity derived from different glycoforms, there is N-terminal and C-terminal heterogeneity. Three N-linked glycosylation sites are confirmed by peptide mapping tooccur at Asn76, Asn108 (both at CTLA4 region) and Asn207 (Fc region) and two O-linked glycosylation sites have been identified at Ser129 and Ser139 (hinge region)(US FDAb 2014).LC–MS tryptic and endoproteinase Asp-N peptide mapping were used for the characterization of glycosylation of abatacept (Bongers et al. 2011; Lynaugh 2013). For CTLA4-Ig as for all biotechnologically driven drugs,glycosylation differences have been shown to impact the stability, pharmacokinetics, potency, and biological activities and terminal sialic acids of the carbohydrate moieties are said tobe important for pharmacokinetics of the protein (Greveet al. 1996; Flesheret al. 1994; Kim and Kim 2007; Jefferis 2009; Jones 2007; Sola and Griebenow 2009). Underivatized oligosaccharide mixture of CTLA4-Ig (abatacept) has been examinedby CE and HPAEC-PAD under normal and thermally stressed conditions (Greve et al. 1996).In addition, oligosaccharide composition of different lots of CTLA4-Ig has been examined by electrophoresis of fluorophore-conjugated carbohydrates (Flesher et al. 1994). For the demonstration of abatacept quality, numerous release testing methods have been provided by the product manufacturer: peptide mapping, to demonstrate molecular identity; capillary electrophoresis, to discriminate abatacept from the related molecule belatacept; IEF, to show the distribution of different glycoforms; IEX HPLC, monosaccharide analysis, to show the consistency of glycosylation. Bioassay has been used for the release and stability testing (US FDA 2014b). In pharmaceutical formulation, abatacept (Orencia®, Bristol-Myers Squibb) is supplied as a sterile lyophilized powder for intravenous infusion (each single-use vial provides 250 mg abatacept) and as a solution for subcutaneous injection (125 mg /mL), single dose prefilled glass syringe (BMS, Orencia 2013).

One dimensional SDS-PAGE and IEF have been widely used for the quality control of rDNA derived medicinal products; such are recombinant monoclonal antibodies (*rmAbs*) and their related products, *Fc* -fusion proteins, mainly for the characterization of their purity and structural integrity. However, SDS-PAGE and IEF performed separately provide limited information related to their heterogeneity. Since 2-DE combines separation in two dimensions, according M_r (SDS-PAGE) and isoelectric point (*pI*), more comprehensive information related to the heterogeneity of *Fc-fusion proteins* products can be obtained.

Previously we have studied the suitability of 2-D electrophoresis in combination with MALDI-TOF MS for the identification and quality assessment of recombinant monoclonal antibodies and fusion protein, abatacept (Nebija et al. 2011a,b). In this work we report the application of 2-D electrophoresis for the study of the charge heterogeneity, glycosylation and 2-D electrophoresis pattern of abatacept aiming to propose a relatively simple and straightforward method for the quality testing of this class of medicinal products in regulatory environment.

2. Investigations, results and discussion

2.1. One dimensional (1D-SDS) PAGE of abatacept

1D-SDS-PAGE analysis showed that abatacept in its reduced form migrated as a sharp band at ∼ 50 kDa, and undernon-reducing conditions migrated at ∼ 100 kDa (Fig. 2). The higher sensitivity of silver staining *versus* Coomassie staining has been documented as well: in the gel A the band of the lowest amount of abatacept (0.05 μg) is clearly visible (Lane 6). On the other hand for the Coomassie stained gel B respective lane isbarely noticeable. Furthermore, SDS-PAGE analysis was used for the study of deglycosylation efficiency. In Fig. 2 (gel C), SDS-PAGE gels of glycosylated and deglycosylated abatacept samples under reducing and non-reducing conditions has been presented. Under non-reducing conditions, M_r of glycosylated, native sample (Lane 2), estimated ∼ 100 kDa, while after N-Glycosidase F digestion dropped to ∼ 75 kDa (lanes 3 and 5). Whereas, under

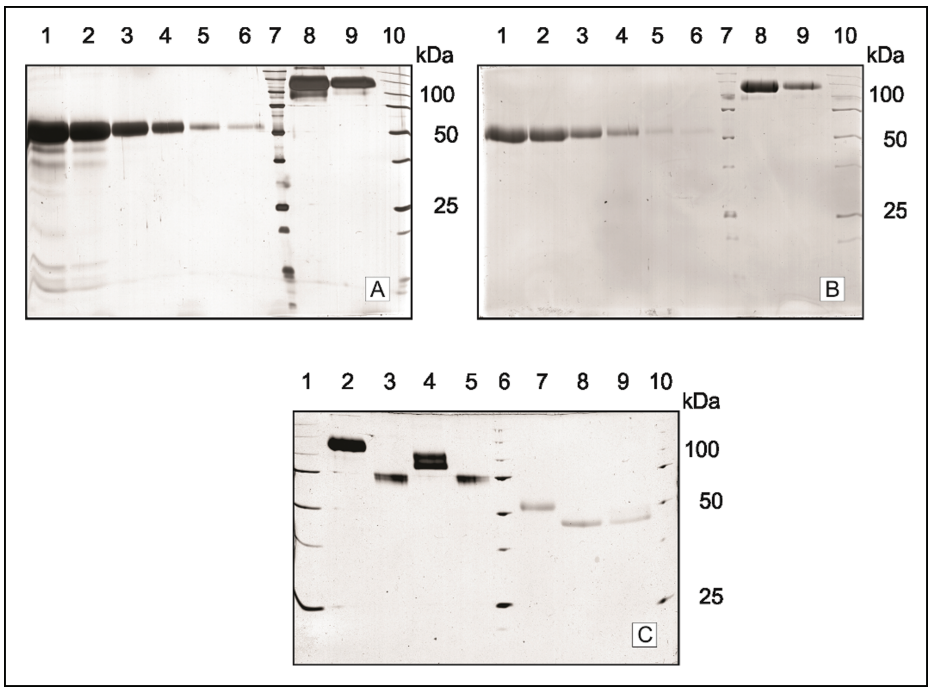

Fig. 2: Lanes 1-6: reducing conditions, sample amount: 5 μg - 0.05 μg; Lanes 7 and 10: Molecular weight standards; Lanes 8-9 non-reducing conditions, sample amount: 1 and 5 μg. C) Optimization of the deglycosylationconditions. Sample amount: 2 μg abatacept. Silver staining. Lanes 1, 6 and 10: MW Standards; Lane 2: glycosylated abatacept, non-reducing conditions; lanes 3, 4 and 5: deglycosylatedabatacept, non-reducing conditions; Lane 7: glycosylated abatacept, reducing conditions; lanes 8 and 9: deglycosylatedabatacept, reducing conditions. Optimal deglycosylation conditions were achieved adding 1 μl 2-mercaptoethanol (2-ME) into digestion mixture (lanes 3, 5 and 8). Lanes 4 and 9 are obtained when sample was deglycosylated without the addition of 2-ME.

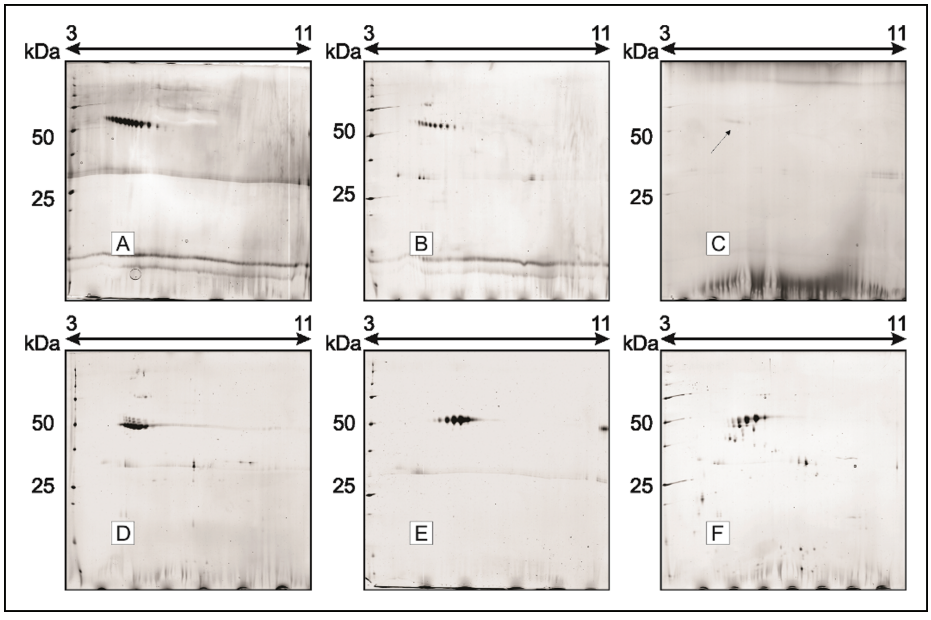

Fig. 3: A) 10 μgabatacept B) 1 μgabatacept. C) 1 μgabatacept, Coomassie staining D) N-Glycosidase F, E) Sialidase, F) N-glicosidase F and Sialidase. Gel staining: A, B, D, E and F, silver staining; C, Coomassie staining.

reducing conditions, M_r of glycosylated reduced sample (Lane 7), estimated ~50 kDa after N-deglycosylation it decreased to ~40 kDa (lane 8). Although SDS-PAGE does not make possible exact measurement of $M_r$difference between glycosylated and deglycosylated samples it enabled to notice a significant M_r difference after N-deglycosylation, suggesting that N-linked sugars are most abundant in the molecule of abatacept.In addition,migration pattern of abatacept following O-degycosilation and desialination of the native product was studied using one dimensional SDS-PAGE(data not shown). However SDS-PAGE results did not show significant differences between the corresponding M_r.

2.2. Two-dimensional, 2-D electrophoresis analysis of abatacept

For the 2-DE optimisation, electrophoresis experiments are performed using differentimmobilized*pH gradients* (*IPG strips*: 3-11 *NL*, 6-9 *L*; 4-7 *L* and 3-6 *L*). In order tohave complete view on the broader *pI* range the preliminary experiments are performed with*IPG gradient* 3-11 *NL.* As expected, 2-D electrophoresis analysis revealed that abatacept is very complex mixture ofdifferent isoforms (Fig. 3, gels A, B, and C). The estimated *pI* of this acidic glycoprotein ranged from 4.5-5.5 and $M_r > 50$ kDa, and it was resolved in more than 13 spots. As in the

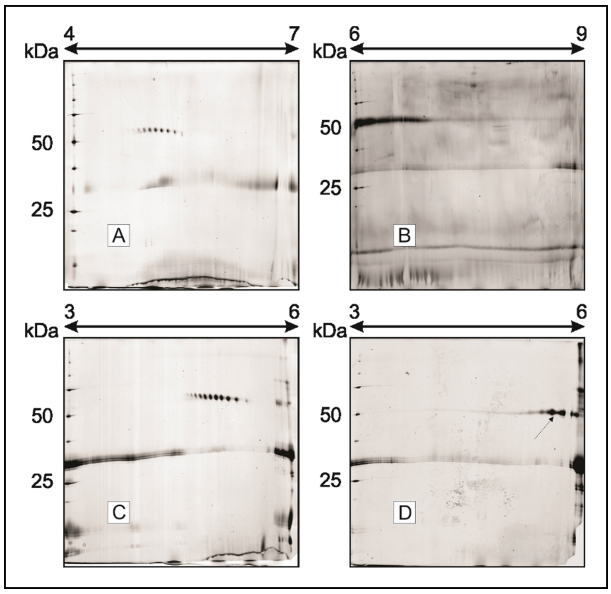

Fig. 4: Silver staining. Sample amount: 10 μgabatacept A) Immobilized pH gradient, 4-7 L. B) Immobilized pH gradient, 6-9 L C) Immobilized pH gradient, 3-6 L D) Immobilized pH gradient, 3-6 L, sample treated with sialidase. Arrow indicates the position of abatacept spots.

previous section, SDS PAGE, in Fig. 3, Gels B and C depict the higher sensitivity of silver *vs*. Coomassie staining procedure. Furthermore, experimental data revealed that the reduction of sample concentration generallybrought about enhancement in the spot resolution which can be seen comparing gels A and B. On the other hand, apart from the sample concentration, comparing to IPG gradient 3-11 *NL,* the improvement in the spot resolution was achievedemploying narrower IPGs: 3-6 *L* and 4-7 *L*, whereas, as expected from the *pI* of abatacept, the IPG 6-9 *L* brought about poorly resolved spots around *pI* 6, most likely only minor component of the abatacept protein, consequentlystudies with this IPG gradient were not pursued further (Fig. 4 gels A, B and C).

2.3. The study of charge heterogeneity

In order to obtain the maximal information for the spot pattern complexity of abatacept sample for the charge heterogeneity study we employed IPG strips which provide maximally resolved peptide spots. The influence of glycosylation pattern in the charge heterogeneity of abatacept was studied using enzyme N-glycosidase F, for the release of N-linked glycans; O-glycosidase for O-linked glycans and the sialidase for the release of terminal sialic acids. In addition carboxypeptidase B was used for the study of lysine truncation.

Preliminary experiments indicated that strips 3-6 *L* and4-7 *L*might fulfill these requirements (Fig. 4, Gels A and C).Therefore, for the study of the influence of terminal sialic acids in charge variantcomplexity, the first IPG employed was IPG 3-6 *L*.Experimental data revealed that after sialidase digestion due to the cleavage ofterminal N-acetylneuraminic acid residues, spot complexity was substantiallyreduced, however since *pI* of resulting variants shifted toward more basic values,approaching *pI* 6, and this *pI* is the upper pH limit for the strips (3-6 *L*), the number ofspots and their position could not be precisely determined (Fig. 4, gel D). The next step was to study more suitable *pI* gradient conditions (broader *pI* range, 4-7 *L*)with additional examination of the influence of other factors such are N-linked glycansand O-linked glycans on the charge heterogeneity pattern.

In Fig. 5 Gels of abatacept under different digestion conditions using N-glycosidase F, O-glycosidase, and Sialidase are presented, and in the Table 1 a list of corresponding *pI* values obtained with control and samples treated with given enzymes is presented. Figure 5 shows that in comparison with the spots of control (gel A, glycosylated sample), spots of abatacept treated with N-glycosidase F, in gel B of the same figure, migrated at lower M_r than control sample. Their *pI* range shifted toward lower *pI* range although not in greater extent (Table). A considerable difference in $M_r$confirms that abatacept is extensively N-glycosylated molecule containing predominantly N-linked glycans. On the other hand, very small *pI* shift toward lower *pI* range after N-oligosaccharide release, suggests that N-linked glycans of abatacept could be neutral and negatively charged, however upon their cleavage, after the hydrolysis of glycoside bond with the release of oligosaccharide and ammonia, for each N-glycosylation site one free carboxylate of aspartic acid is released, therefore both effects are compensated. These results are in accordance with the published data concerning the glycosylation of this Fc protein, discussed in introductory part (Bongers et al. 2011; Lynaugh et al. 2013).

Larger difference in abatacept spot pattern is observed upon the digestion of samplewith sialidase and with two enzymes simultaneously:sialidase and oglycosidase(Fig. 5 gels C and gel D). In these instances, the *Mr* change is not so much pronounced as after digestion with N-glycosidase F, but there is a considerable change in the abatacept *pI* and its spot complexity.Namely, after sialidase digestion (Fig. 5C) *pI* range increased in order of magnitude 0.5 suggesting that O-linked abatacept glycans contain terminal N-Acetylneuraminic acid residues which are responsible for acidic nature of abatacept.However after sialidase cleavage, they are released, with concomitant release ofneutral serine residues from protein backbone therefore *pI* of asialo abatacept isshifted toward higher values. In contrast to glycosylated abatacept the spotcomplexity is substantially reduced, as well suggesting that the different degree ofsialidation and/or different structures of O-linked glycans, are major contributors toabatacept charge heterogeneity.

In order to examine the influence of O-linked glycans in the charge variantdistribution abatacept was first digested with sialidase followed by O-glycosidase (Fig. 5D). The previous desialination was needed due to the fact that in contrast to N-glycosidase F,

O-glycosidase cannot cleave modified (i.e. sialinated) O-linked-glycans. The experimental results show that O-deglycosylated (and desialinated) abatacepthas lower molecular M_r comparing with to control and desialinated sample, although still considerably higher than of N-deglycosylated abatacept form. This observation suggests that the M_r of O-glycans and their abundance is lower than of N-linked glycans which is in accordance with published data concerning the glycosylationof

Table 1: Posttranslational glycosylation of abatacept

Deglycosylation status	G	NGF	S	OG	S, NGF	OG, S, NGF
pI range	4.6 – 5.5	4.3 – 5.3	5.4 – 5.9	5.3 – 5.9	4.9 – 5.3	5.0 – 5.6

G = Glycosylated; NGF = N-Glycosidase F; S = Sialidase; OG = O-Glycosidase

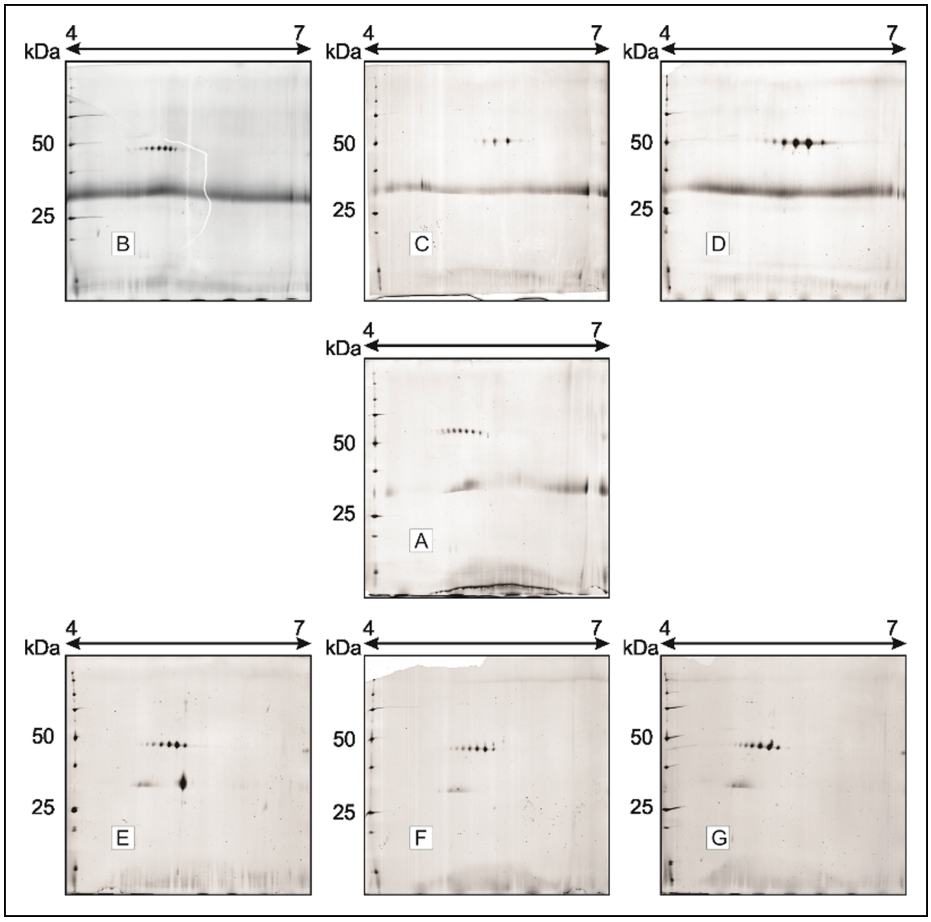

Fig. 5: Immobiized pH gradient 4-7 L. Sample amount: abatacept (10 μg). Silver staning. A) Glycosylated sample, control; B) N-glycosidase F; C) Sialidase; D)Sialidase and O-glycosidase; E) N-glycosidase F and sialidase; F) N-glycosidase F, Sialidase and O-glycosidase; G) N-glycosidase F, Sialidase and O-glycosidase. Gels F and G were obtained from samples deglycosylated under slightly different conditions.

CTLA4-Ig:firstly, in the molecule of abatacept, there are two O-glycosylation sites and three N-glycosylation sites, and, secondly, the M_r of N-linked glycans is higher than of O-liked glycans. In relation to N-linked glycans, the simplest glycan population is that on the Asn207 (Fc region) containing neutral biantennary complex-type core-fucosylated N-glycans, which are typical for rmAbs produced by mammalian cell culture. On the other hand, the two N-glycosylation sites, Asn76, Asn108, (CTLA4 domain), show highly diverse microheterogeneous populations of sialylated and neutral forms of bi-, tri-, and tetra-antennary complex-type core-fucosylated glycans with variable numbers of terminal galactose. Literature data reveiled that O-linked glycans of abatacept are heterogenous, neutral, monosialidated and disialidated species of considerably lower M_r than N-linked glycans (Bongers et al. 2011; Lynaugh2013).

In addition, Fig. 5D shows that the *pI* range ofO-deglycosylated and desialinated abatacept has moved toward higher *pI* values, comparing with gel pattern of desialinated abatacept (Fig. 5C). In order to cleave both, N-linked glycans and N-Acetyl neuraminic acid termini fromabatacept molecule N-glycosidase F and sialidase were used simultaneously

(Fig. 5 gel E). For the removal of N-linked and O-linked glycans, abatacept sample was initially desialinated and subsequently N and O-deglycosylated using slightly differentdeglycosylation procedures which gave similar results (Fig. 5, gels F and G).

Figure 5 E shows that asialo and N-deglycosylated abatacept has a lower molecular mass that control sample with additional reduction in chargeheterogeneity pattern. Its *pI* range however is similar to control provided that spotsbegin at higher pI values, but end at the approximately same pI values as control.

Very small difference in pI might be consequence of two opposite processes duringthe N-deglycosylation and desialination. In the first instance pI is decreased due toaspartic acid release, and in the second, *pI* increases due to removal of negatively-charged sialic acid species (Table). On the basis of experimental datadesialination resulted preponderant.

In Fig. 5 (gels F and G) electrophoresis migration patterns of sample digested simultaneously with three enzymes, N-glycosidase F, O-glycosidase and sialidase has been presented. In these gels, larger difference in $M_r$betweenresulting enzymatically treted abatacept and control sample has been observed, confirming the theadditive effect in the $M_r$reduction of all three enzymes. Chargeheterogeneity is substantially reduced, and the *pI* ranged from is similar to the protein from 5.0 – 5.6.

Comparing to gradient conditions employing IPGs 3-6 L and 4-7 *L, s*imilar results are obtained after the application of IPG 3-11 *NL.* Corresponding gelsare presented inFig. 3 (gels D, E and F). In this case the *pI* gradient is broader and non-linear. As in previous cases (3-6 *L* and 4-7 *L*) comparing to native samples, N-deglycosylated abatacept displayed similar spot pattern complexity (Fig. 3, gel D). The *pI* shift was rather small however the M_r difference comparing to native sample is considerable. Desialinated less acidic abatacept, displayed reduced spot complexity comparing to control sample and $M_r$decrease was very small, (Fig. 3 gel E). Finally, abatacept sample digested simultaneously with N-glycosidase F and sialidase displayed difference in *pI* range with considerable $M_r$and spot complexity reduction (Fig. 3 gel F).

In conclusion, one- dimensional SDS-PAGE under reducing and non-reducing conditions and 2-D electrophoresis pattern of abatacept have been studied, focusing on the examination of

different factors influencing migration behavior of this complex molecule. Aiming the estimation of M_r and *pI* range, examination of charge heterogeneity and the influence of the posttranslational modifications in the isoform distribution, 1-SDS-PAGE and 2-DE experiments were run under different conditions. Experimental data documented that SDS-PAGE and 2-D electrophoresis are suitable techniques for the assessment of therapeutic glycoproteins, however for their comprehensive quality study, orthogonal strategies applying various complementary techniques are needed, in which planar electrophoresis represents a significant component.

3. Experimental

3.1. Isoelectric focusing

An aliquot of rehydration solution (1 ml, urea, 8 M; thiourea, 2 M; CHAPS, 4%; Triton-X100, 0.5%; bromophenol blue, 0.005%) was cautiously thawed and just prior to use was mixed with 5 mg/ml dithiothreitol-DTT and 5 µl/ml IPG buffer of selected pH interval (GE Healthcare Bio-Sciences AB Uppsala, Sweden). In 340 µl of this solution, 10 µl
(1–5 µg/µl) of sample solution were mixed and shortly vortexed. The mixture was allowed to stay 1 h at room temperature followed by centrifugation (15,000 g, 5 min) to remove insoluble components. Strip Holders were put onto the cooling plate/electrode contact area of the IPGphor strip holder platform (IPGPhorTM IEF system, GE Healthcare, Biosciences AB, Uppsala, Sweden). Prepared samples were applied onto commercially available Immobiline DryStrip gels (3-6 *L*, 4-7 *L*, 6–9 *L*, and 3–11 *NL*, length 18 cm ± 2 mm, GE Healthcare Bio-Sciences AB Uppsala, Sweden) by *in-gel* rehydration (Rabilloud et al. 1994).To ensure that the rehydrated IPG strip gels do not dry out during rehydration and the focusing process they are overlaid with 1-1.5 ml Immobiline DryStrip Cover Fluid and covered with plastic closure. The temperature was set to 20 °C and the current limited to 50 µA/IPG strip. Focusing was run to a total 72 kVh.

3.2. Second dimension, SDS-PAGE

Subsequent to IEF, the second-dimension, SDS-PAGE, was performed run on a vertical system (PROTEAN® II Xi Cell for vertical electrophoresis, 20 cm × 20 cm, Bio-Rad Laboratories, USA). Focused IPG strips (gel-side up) were equilibrated twice, each time for 15 min, in equilibration tray by gently shaking on a rocker. The first equilibration step was performed in 5 ml equilibration buffer (tris(hydroxymethyl)aminomethane 0.05 M, urea 6 M, glycerol 30%, sodium dodecyl sulfate 2%, bromphenol blue 0.005%) containing 1% DTT, followed by the second equilibration in 5 ml equilibration buffer containing 2.5% iodoacetamide (Görg1998). Molecular mass standards (Precision Plus Unstained ProteinTM Standards, Bio-Rad Laboratories, USA) were applied to a section of filter paper and placed at the anodic end of the SDS-PAGE gel. The equilibrated IPG strips were embedded on top of the vertical SDS gel and overlaid with molten agarose solution (Agarose Serva Standard low EEO, research grade (Serva Electrophoresis GmbH, Heidelberg, Germany). Second dimension electrophoresis run (lab made gels 12.5% T, homogenous, 2.6% C; 200 mm × 200 mm × 1 mm) was performed under constant current of 45 mA/gel, electrophoresis time about 3.5 h. The run was terminated when the bromophenol blue dye has migrated off the lower end of the gel.

3.3. One-dimensional SDS gel electrophoresis

SDS-PAGE was performed according standard procedures (Laemmli 1970), using a mini-gel system Mini-PROTEAN® 3 Cell (8 cm *X* 7.3 cm; Bio-Rad Laboratories, USA). The samples were placed briefly on ice until ready for use, then dissolved in sample Laemmli buffer (tris(hydroxymethyl)aminomethane, 0.25 M; glycerol, 20 %; sodium dodecyl sulfate, 8 %; bromphenol blue (sol 1 %), 0.02 %) in which just prior to use are added 6.2 mg/ml DTT. The reduction/denaturation of sample was performed by heating the sample and DTT-containing sample buffer solution in termocycler, 10 min at 95 °C. *Silver staining* was performed according to Blum et al. 1987. *Coomassie staining* (Coomassie Brilliant Blue G 250, Serva Electrophoresis GmbH, Heidelberg Germany) was performed according to the manufacturer's instructions. All chemicals used were reagent and HPLC grade. Gels were scanned (Biorad Densitometer GS 710) and analyzed with PDQuest 6.2.1. (Bio-Rad Laboratories, USA).

3.4. Enzymatic deglycosylation

Solution of the pharmaceutical preparation (20 µl) of abatacept (Orencia®), 250 mg/10 ml, was dialyzed against MilliQ-water (3 days, 4 °C) using cellulose dialysis tube (cut off 12,000–14,000 Da, Japan Medical Science, Tokyo, Japan). After dialysis, solution was freeze dried and lyophilized and sample of pharmaceutical preparation (0.5 mg), was dissolved in 49 µl phosphate buffer (pH 7.5) to obtain a final concentration of 500 µg/50 µl. Deglycosylation of abatacept was performed according to the procedures described in the literature (slightly modified) (Kamoda et al. 2004; Schlagset al. 2002). Briefly, 15 µl of sample solution (150 µg abatacept) were mixed with 1 µl 2-mercaptoethanol (2-ME), the required volume of enzyme(s) (PNGase F, Sialidase, O-Glycosidase) and phosphate buffer pH 7.5 to obtain total volume of 100 µl and the mixture was incubated at room temperature for 24 h.
PNGase F (N-Glycosidase F) of *Flavobacterium meningosepticum*, recombinant, from *E. coli*, 100 U/100 µl, (Roche Diagnostics GmbH, Mannheim, Germany) was used for the cleavage of N-linked oligosaccharides, (15 U/150 µg rmAb). Neuraminidase (sialidase) from *Arthrobacter ureafaciens* 1 U/100 µl, 30 mU/150 µl rmAb (Roche Diagnostics GmbH, Mannheim, Germany) was used for the cleavage of sialic acids and O-glycosidase from *Diplococcus pneumoniae* (Roche Diagneostics GmbH, Mannheim, Germany) for the removal of O-linked oligosaccharides. Carboxypeptidase B, 750 U/ml, from pig pancreas (Roche Diagnostics GmbH, Mannheim, Germany) was used for the study of lysine truncation (0.75 U/150 µg rmAb). After digestion samples were stored at − 80 °C.

References

Beck A, Diemer H, Ayoub D, Debaene F, Wagner-Rousset E, Carapito C, Van Dorsselaer A,Sanglier-Cianferani S (2013) Analytical characterization of biosimilar antibodies and Fc-fusion proteins.Trends Anal Chem 48: 81-95.

Beck A, Wagner-Rousset E, Ayoub D, Van Dorsselaer A, Sanglier-Cianférani S (2013) Characterization of therapeutic antibodies and related products. Anal Chem 85:715–736.

Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant-proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8: 93–99.

Bongers J, Devincentis J, Jinmei F, Huang P, Kirkley DH, Leister K, Liu P, Ludwig R, Rumney K, Tao L, Wu W, Russell RJ (2011) Characterization of glycosylation sites for a recombinant IgG1 monoclonal antibody and a CTLA4-Ig fusion protein by liquid chromatography–mass spectrometry peptide mapping. JChromatogr A 1218: 8140– 8149.

Breece TN, Fahrner RL, Gorrell JR, Lazzareschi KP, Lester PM, Peng D (2005) Protein purification. United States, Genentech, Inc. United States Patent 6870034. Retrieved from. http://www.freepatentsonline.com/6870034.html, September, 2014.

Bristol-Mayers Squibb (2013) Orencia: Product monograph. pp.1–63. Control No.: 166203. Last Revised: 07 October 2013, Retrieved from http://www.bmscanada.ca/static/products/en/pm_pdf/ORENCIA_EN_PM.pdf, September, 2014.

Chappel MS, Isenman DE, Everett M, Xu YY, Dorrington KJ, Klein MH (1991) Identification of the Fc gamma receptor class I binding site in human IgG through the use of recombinant IgG1/IgG2 hybrid and point-mutated antibodies. Proc Natl Acad Sci USA 88:9036–9040.

Duncan AR, Winter G (1998). The binding site for C1q on IgG. Nature 332:738–740.

Emery P, Klareskog L, Davis JC, Westhovens RR (2004) Selective Co-stimulation Modulators: Addressing unmet needs in Rheumathoid Arthritis Management. 6(4 supplement):1, Medscape General MedicineTM, Published Online, Section 1–13. Retrieved from: http://www.medscape.com/viewarticle/496108, September, 2014.

European Medicines Agency (2014a). Product information. Orencia - EMEA/H/C/000701 -II/0077. Retrieved from http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000701/WC500048935.pdf, September 2014.

European Medicines Agency (2014b). Orencia. EPAR. Scientific Discussion. Retrieved from http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000701/WC500048938.pdf, September, 2014.

Flesher AR, Marzowski J, Wang WC, Raff HV (1994) Fluorophore-labeled carbohydrate analysis of immunoglobulin fusion proteins: Correlation of oligosaccharide content with in vivo clearance profile. Biotechnol Bioengin 46: 399–407.

Görg A, Postel W, Günther S (1998) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 9:531–546.

Greve KF, Hughes DE, Karger BL (1996) Capillary electrophoretic examination of underivatized oligosaccharide mixtures released from

immunoglobulin G antibodies and CTLA4Ig fusion protein.J Chromatogr A 749:237–245.
Greve KF, Hughes DE, Richberg P, Kats M, Karger BL (1996) Liquid chromatographic and capillary electrophoretic examination of intact and degraded fusion protein CTLA4Ig and kinetics of conformational transition. J Chromatogr A 723: 273–284.
Isaacs JD (1997) Immunoadhesins for immunomodulation of autoimmune and rheumatic disease. Rheumatology 36:305–307.
Jefferis R (2009) Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. Trends Pharmacol Sci 30:356–362.
Jones AJ, Papac DI, Chin EH, Keck R, Baughman SA, Lin YS, Kneer J, Battersby JE (2007) Selective clearance of glycoforms of a complex glycoprotein pharmaceutical caused by terminal N-acetylglucosamine is similar in humans and cynomolgus monkeys. Glycobiology17:529–540.
Kamoda S, Nomura C, Kinoshita M, Nishiura S, Ishikawa R, Kakehi K, Kawasaki N, Hayakawa T (2004) Profiling analysis of oligosaccharides in antibody pharmaceuticals. J Chromatogr A 1050: 211–216.
Kim HJ, Kim HJ (2007) The glycosylation and pharmacokinetics of CTLA4Ig produced in rice cells. BiolPharmBull 30: 1913–1917.
Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 227: 680–685.
Linsley PS, Brady W, Urnes M, Grosmaire LS, Damle NK, Ledbetter JA (1991) CTLA-4 is a second receptor for the B cell activation antigen B7. J Exp Med 174: 561–569.
Linsley PS, Ledbetter JA, Damle NK, Brady W (1998) CTLA4Ig fusion proteins.United States Patent. Nr. 5844095.
Lynaugh H, Li H, Gong B (2013) Rapid Fc glycosylation analysis of Fc fusions with IdeS and liquid chromatography mass spectrometry.MAbs 5:641–645.
Nebija D, Kopelent-Frank H, Urban E, Noe CR, Lachmann B (2011) Comparison of two-dimensional gel electrophoresis patterns and MALDI-TOF MS analysis of therapeutic recombinant monoclonal antibodies trastuzumab and rituximab. JPharm Biomed Anal 56:684–691.
Nebija D, Urban E, Stessl M, Noe CR, Lachmann B (2011) 2-DE and MALDI-TOF-MS analysis of therapeutic fusion protein abatacept. Electrophoresis 32:1438–1443.
Rabilloud T, Valette C, Lawrence JJ (1994) Sample application by in-gel rehydration improves the resolution of two-dimensional electrophoresis with immobilized pH gradients in the first dimension. Electrophoresis 15: 1552–1558.
Schlags W, Lachmann B, Walther M, Kratzel M, Noe CR (2002) Two-dimensional electrophoresis of recombinant human erythropoietin: a future method for the European Pharmacopoeia? Proteomics 2: 679–682.
Sola RJ, Griebenow K (2009) Effects of glycosylation on the stability of protein pharmaceuticals. J Pharm Sci 98: 1223–1245.
US. Food and Drug Administration (2014a). Orencia. Label Information. Retrieved from http://www.accessdata.fda.gov/drugsatfda_docs/label/2013/125118s171lbl.pdf, September 2014.
U.S. Food and Drug Administration (2014b). Orencia. Drug Approval Package. Chemistry Review(s). Retrieved from http://www.accessdata.fda.gov/drugsatfda_docs/nda/2005/125118_S0000_ChemR.pdf, September 2014.
Wilkins MR, Lindskog I, Gasteiger E, Bairoch A, Sanchez JC, Hochstrasser DF, Appel RD (1997) Detailed peptide characterization using PEPTIDEMASS–a World-Wide-Web-accessible tool. Electrophoresis 18:403–408.
Wishart DS, Knox C, Guo AC, Cheng D, Shrivastava S, Tzur D, Gautam B, Hassanali M (2008) DrugBank: a knowledgebase for drugs, drug actions and drug targets. Nucleic Acids Res 36:D901–6.
Beck A, Diemer H, Ayoub D, Debaene F, Wagner-Rousset E, Carapito C, Van Dorsselaer A,Sanglier-Cianferani S (2013) Analytical characterization of biosimilar antibodies and Fc-fusion proteins.Trends Anal Chem 48: 81–95.
Beck A, Wagner-Rousset E, Ayoub D, Van Dorsselaer A, Sanglier-Cianférani S (2013) Characterization of therapeutic antibodies and related products. Anal Chem 85:715–736.
Blum H, Beier H, Gross HJ (1987) Improved silver staining of plant-proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8: 93–99.
Bongers J, Devincentis J, Jinmei F, Huang P, Kirkley DH, Leister K, Liu P, Ludwig R, Rumney K, Tao L, Wu W, Russell RJ (2011) Characterization of glycosylation sites for a recombinant IgG1 monoclonal antibody and a CTLA4-Ig fusion protein by liquid chromatography–mass spectrometry peptide mapping. JChromatogr A 1218: 8140– 8149.
Breece TN, Fahrner RL, Gorrell JR, Lazzareschi KP, Lester PM, Peng D (2005) Protein purification. United States, Genentech, Inc. United States Patent 6870034. Retrieved from. http://www.freepatentsonline.com/6870034.html, September, 2014.
Bristol-Mayers Squibb (2013) Orencia: Product monograph. pp.1–63. Control No.: 166203. Last Revised: 07 October 2013, Retrieved from http://www.bmscanada.ca/static/products/en/pm_pdf/ORENCIA_EN_PM.pdf, September, 2014.
Chappel MS, Isenman DE, Everett M, Xu YY, Dorrington KJ, Klein MH (1991) Identification of the Fc gamma receptor class I binding site in human IgG through the use of recombinant IgG1/IgG2 hybrid and point-mutated antibodies. Proc Natl Acad Sci USA 88:9036–9040.
Duncan AR, Winter G (1998). The binding site for C1q on IgG. Nature 332:738–740.
Emery P, Klareskog L, Davis JC, Westhovens RR (2004) Selective Co-stimulation Modulators: Addressing unmet needs in Rheumathoid Arthritis Management. 6(4 supplement):1, Medscape General MedicineTM, Published Online, Section 1–13. Retrieved from: http://www.medscape.com/viewarticle/496108, September, 2014.
European Medicines Agency (2014a). Product information. Orencia - EMEA/H/C/000701 -II/0077. Retrieved from http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/000701/WC500048935.pdf, September 2014.
European Medicines Agency (2014b). Orencia. EPAR. Scientific Discussion. Retrieved from http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000701/WC500048938.pdf, September, 2014.
Flesher AR, Marzowski J, Wang WC, Raff HV (1994) Fluorophore-labeled carbohydrate analysis of immunoglobulin fusion proteins: Correlation of oligosaccharide content with in vivo clearance profile. Biotechnol Bioengin 46: 399–407.
Görg A, Postel W, Günther S (1998) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 9:531–546.
Greve KF, Hughes DE, Karger BL (1996) Capillary electrophoretic examination of underivatized oligosaccharide mixtures released from immunoglobulin G antibodies and CTLA4Ig fusion protein.J Chromatogr A 749:237–245.
Greve KF, Hughes DE, Richberg P, Kats M, Karger BL (1996) Liquid chromatographic and capillary electrophoretic examination of intact and degraded fusion protein CTLA4Ig and kinetics of conformational transition. J Chromatogr A 723: 273–284.
Isaacs JD (1997) Immunoadhesins for immunomodulation of autoimmune and rheumatic disease. Rheumatology 36:305–307.
Jefferis R (2009) Recombinant antibody therapeutics: the impact of glycosylation on mechanisms of action. Trends Pharmacol Sci 30:356–362.
Jones AJ, Papac DI, Chin EH, Keck R, Baughman SA, Lin YS, Kneer J, Battersby JE (2007) Selective clearance of glycoforms of a complex glycoprotein pharmaceutical caused by terminal N-acetylglucosamine is similar in humans and cynomolgus monkeys. Glycobiology17:529–540.
Kamoda S, Nomura C, Kinoshita M, Nishiura S, Ishikawa R, Kakehi K, Kawasaki N, Hayakawa T (2004) Profiling analysis of oligosaccharides in antibody pharmaceuticals. J Chromatogr A 1050: 211–216.
Kim HJ, Kim HJ (2007) The glycosylation and pharmacokinetics of CTLA4Ig produced in rice cells. BiolPharmBull 30: 1913–1917.
Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 227: 680–685.
Linsley PS, Brady W, Urnes M, Grosmaire LS, Damle NK, Ledbetter JA (1991) CTLA-4 is a second receptor for the B cell activation antigen B7. J Exp Med 174: 561–569.
Linsley PS, Ledbetter JA, Damle NK, Brady W (1998) CTLA4Ig fusion proteins.United States Patent. Nr. 5844095.
Lynaugh H, Li H, Gong B (2013) Rapid Fc glycosylation analysis of Fc fusions with IdeS and liquid chromatography mass spectrometry.MAbs 5:641–645.
Nebija D, Kopelent-Frank H, Urban E, Noe CR, Lachmann B (2011) Comparison of two-dimensional gel electrophoresis patterns and MALDI-TOF MS analysis of therapeutic recombinant monoclonal antibodies trastuzumab and rituximab. JPharm Biomed Anal 56: 684–691.
Nebija D, Urban E, Stessl M, Noe CR, Lachmann B (2011) 2-DE and MALDI-TOF-MS analysis of therapeutic fusion protein abatacept. Electrophoresis 32:1438–1443.
Rabilloud T, Valette C, Lawrence JJ (1994) Sample application by in-gel rehydration improves the resolution of two-dimensional electrophoresis with immobilized pH gradients in the first dimension. Electrophoresis 15: 1552–1558.

Schlags W, Lachmann B, Walther M, Kratzel M, Noe CR (2002) Two-dimensional electrophoresis of recombinant human erythropoietin: a future method for the European Pharmacopoeia? Proteomics 2: 679–682.

Sola RJ, Griebenow K (2009) Effects of glycosylation on the stability of protein pharmaceuticals. J Pharm Sci 98: 1223–1245.

US. Food and Drug Administration (2014a). Orencia. Label Information. Retrieved from http://www.accessdata.fda.gov/drugsatfda_docs/label/2013/125118s171lbl.pdf, September 2014.

U.S. Food and Drug Administration (2014b). Orencia. Drug Approval Package. Chemistry Review(s). Retrieved from http://www.accessdata.fda.gov/drugsatfda_docs/nda/2005/125118_S0000_ChemR.pdf, September 2014.

Wilkins MR, Lindskog I, Gasteiger E, Bairoch A, Sanchez JC, Hochstrasser DF, Appel RD (1997) Detailed peptide characterization using PEPTIDEMASS–a World-Wide-Web-accessible tool. Electrophoresis 18:403–408.

Wishart DS, Knox C, Guo AC, Cheng D, Shrivastava S, Tzur D, Gautam B, Hassanali M (2008) DrugBank: a knowledgebase for drugs, drug actions and drug targets. Nucleic Acids Res 36: D901–6.