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Analysis of recombinant DNA-derived glycoproteins via high-performance capillary electrophoresis coupled with off-line matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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Abstract

This paper describes the analysis of glycoform populations of the glycoproteins ovalbumin and Desmodus salivary plasminogen activator (DSPA α1) by a combination of capillary electrophoresis (CE) and off-line matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Ovalbumin has a single N-linked glycosylation site and DSPA α1 has six sites for potential glycosylation, 2 N-linked and four O-linked. The conditions used for the electrophoretic separation of ovalbumin include a borate buffer system, together with a diamine additive such as 1,4-diaminobutane (DAB). An electropherogram of DSPA glycoforms could be obtained at pH 3.0 (phosphate buffer) using a bovine serum albumin (BSA) coated capillary. Fraction collection was performed by controlled application of pressure [5000 Pa (50 mbar)] for zone elution and MALDI-TOF-MS was performed on samples prepared by a 1:1 dilution with the UV absorbing matrix sinapinic acid. Both electrophoretic separations were successfully characterized by good quality mass spectra and distinct mass trends were observed for the collected fractions. It is likely that each of the collected fractions are still mixtures of glycoforms and explanation of relative mobilities or masses of different fractions is not possible at this stage. The ability to perform rapid off-line MALDI-TOF-MS of fractions from complex electropherograms will be a powerful tool to demonstrate product consistency in the manufacture of glycoprotein pharmaceuticals.

Keywords: Matrix-assisted laser desorption ionization; DNA; Glycoproteins; Ovalbumin; Desmodus salivary plasinogen activator

1. Introduction

At a recent workshop sponsored by the FDA the concept of a "Well Characterized Biological" was discussed in the context of reducing the regulatory burden on the biotechnology industry [1]. Thus the approval of new facilities or processes for the commercial production of pharmaceutical biopolymers can be accelerated by the demonstration of an ability to "completely" characterize these high molecular weight biosynthetic products. In addition to primary sequence determination, it has become necessary to determine secondary and tertiary structure as well as post-translational modifications such as glycosylation, phosphorylation and deamidation. Indeed, the complexity of the problems encountered in high molecular weight protein pharmaceuticals are such that multiple methods are required to gain a complete picture of the sample.

Glycoproteins are, however, among the most analytically challenging classes of biopharmaceuticals to characterize. The adverse behavior of these

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samples often may be linked to extreme sample microheterogeneity which can be attributed to the carbohydrate moieties in glycoproteins [2]. For example, it has been estimated that recombinant tissue plasminogen activator (rtPA) could contain as many as 11 500 different glycoforms and yet an extensive analytical program has resulted in this glycoprotein fitting the concept of a well characterized biological. Other examples of complex recombinant DNA-derived proteins include human erythropoietin (rHu-Epo), blood coagulation factor VIIa and a humanized monoclonal antibody raised against the human interleukin 2 receptor (Hu-anti-TAC).

The key to the characterization of complex protein samples lies in the use of appropriate combinations of the different analytical methods that analyze the sample from substantially orthogonal and independent directions. An important advantage of high-performance capillary electrophoresis (HPCE) in this application is the complementarity of the technique with reversed-phase HPLC [3,4]. The mobility of samples in free zone HPCE can be correlated with mass and charge, while retention times in HPLC are primarily related to the hydrophobicity of the sample. Therefore mixtures of variants of a polypeptide that are difficult to separate by HPLC can often be readily resolved by HPCE [5]. In the case of glycoforms, HPCE is well suited to the separation of variants with different degrees of sialic acid, which is negatively charged at neutral pH values. It has been shown that neutral glycoforms, such as mannose oligomers, can also be separated by careful selection of the separation conditions.

While HPCE is a promising approach to analysis of biopolymers that combines the instrumental convenience of HPLC, factors such as the relatively low sample capacity, the use of high ionic strength buffers and organic additives (e.g., surfactants and anticonvective agents) have prevented the routine characterization of fractions from a HPCE separation by mass spectrometry. In this context matrix- assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is a valuable technique for the off-line characterization of CE fractions due to the high sensitivity of the method and its tolerance of samples with moderate levels of buffer salts and other additives.

MALDI-TOF-MS is ideally suited for the rapid and sensitive analysis of large biomolecules ($M_r <$ 500) of relevance to the biotechnology industry [6,7] even in the presence of substantial microheterogeneity and useful spectra have been reported for samples such as the plasminogen activator DSPA [8], erythropoeitin [9], a monoclonal antibody [10], interleukin 4 receptor [11] and poly(ethylene glycol)-conjugated human urokinase [12]. The use of MALDI-TOF-MS for the characterization of fractions resulting from the analysis of intact glycoproteins by HPCE has received little use because of the difficulties described above. The combined use of two such powerful analytical methods would be of interest to the biotechnology industry because of the need for better methods for the demonstration of lot to lot consistency of different batches of a recombinant glycoprotein. In this paper, the development of an effective protocol for the analysis of glycoform populations of intact glycoproteins by a combination of HPCE and off-line MALDI-TOF-MS has been demonstrated by the successful analysis of two highly heterogeneous glycoproteins, ovalbumin and Desmodus salivary plasminogen activator (DSPA α1).

2. Experimental

2.1. Materials

Buffer components were purchased from Sigma (St. Louis, MO, USA) and were of the highest purity available. The ovalbumin was from Sigma (A-5503, Grade V: ca. 99% by agarose electrophoresis). The Desmodus rotundus salivary plasminogen activator (DSPA α1), a potential thrombolytic agent, was generously provided by Berlex Biosciences (Brisbane, CA, USA).

2.2. Capillary electrophoresis

HPCE was performed using the HP 3D CE system with diode-array detection and ChemStation software for computer control and data acquisition/analysis (Hewlett-Packard, Palo Alto, CA, USA).

For the ovalbumin work, bare fused-silica capillaries (L_{eff} 72 cm×75 μm I.D., 300 V/cm) were used with 100 m*M* borate buffer, pH 8.5 containing

3 m*M* 1,4-diaminobutane (DAB) additive [13]. For the DSPA α1 CE analyses, bovine serum albumin (BSA)-treated [14] fused-silica capillaries (L_{eff} 41 cm×50 μm I.D., 500 V/cm) were used with 100 m*M* phosphate buffer, pH 3.0. Fraction collection was performed by controlled application of pressure [5000 Pa (50 mbar)] for zone elution. The receiving vials for fraction collection contained a minimal volume of water (ca. 5 μl), sufficient to wet the end of the capillary and to avoid excessive sample dilution.

2.3. MALDI-TOF-MS

MALDI-TOF-MS was performed on a HP G2025A system. Samples were typically prepared by a 1:1 dilution with the UV-absorbing matrix sinapinic acid (HP G2055A) followed by rapid vacuum evaporation in the HP 2024A sample preparation accessory. Typically 50 to 100 laser shots were summed for each spectrum. Calibration of the mass axis was performed externally with a protein standard mixture (HP G2053A) consisting of equine cytochrome *C*, equine apomyoglobin and BSA.

3. Results and discussion

3.1. Model protein ovalbumin

The model protein chosen for investigation of the separation of glycoforms by HPCE and subsequent analysis by off-line MALDI-TOF-MS was ovalbumin. Ovalbumin has a single N-linked glycosylation site at Asn-292. Its non-glycosylated molecular mass is 42 786 and several known factors, in addition to the glycosylation site, affect its heterogeneity. There are two possible sites of phosphorylation at Ser-68 and Ser-344 and there is a 34 amino acid clip site (from the C-terminal end). In this manner ovalbumin is an excellent example of a complex protein which contains substantial post translational heterogeneity in addition to glycosylation. Such diversity of post translational modifications can greatly add to the analytical challenge, for example it was demonstrated that the charge heterogeneity introduced by the carbohydrate side-chains in a glycoprotein made it difficult to detect additional heterogeneity caused by deamidation [15].

The conditions used for the electrophoretic separation of ovalbumin developed by Oda et al. [13] include a borate buffer system, together with a diamine additive such as diaminobutane (DAB). Fig. 1 presents the electropherogram obtained for the analysis of ovalbumin using the borate buffer system and the pooled regions are indicated by the solid lines. It is thought that the individual peaks within each of the collected fractions are due to differing numbers of mannose residues. The collected fractions were mixed 1:1 with the UV-absorbing MALDI matrix and subjected to mass analysis.

Fig. 2 shows the spectra obtained from mass analysis of the fractions. The broad nature of both peaks in each spectrum indicates that both are glycosylated with a mass difference of ~3900 amu. This mass difference results not from a carbohydrate glycoform but corresponds to the loss of a 34 amino acids fragment from the C-terminus. This conclusion was supported by N-terminal sequencing and MALDI-TOF analysis of the starting ovalbumin sample (data not shown) Thus the combined use of HPCE and MALDI-TOF is capable of detecting a clipped form of ovalbumin despite the presence of the other post translational modifications. It should be noted that each collected fraction is still a mixture of glycoforms.

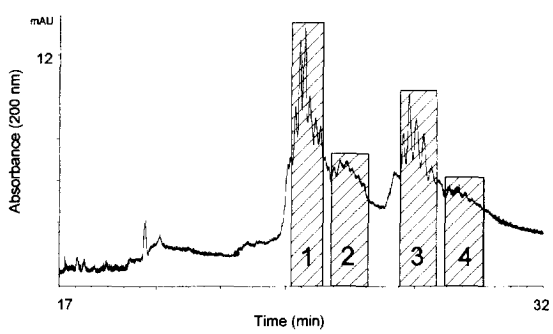

Fig. 1. Electropherogram of ovalbumin. CE conditions: 80.5 cm (effective length, 72 cm)×75 μm I.D.; applied voltage, 300 V/cm; detection at 200 nm; 100 m*M* sodium borate buffer (pH 8.5) containing 3 m*M* 1,4-diaminobutane [13]. The fractions collected for MALDI-TOF-MS are indicated as regions 1 to 4.

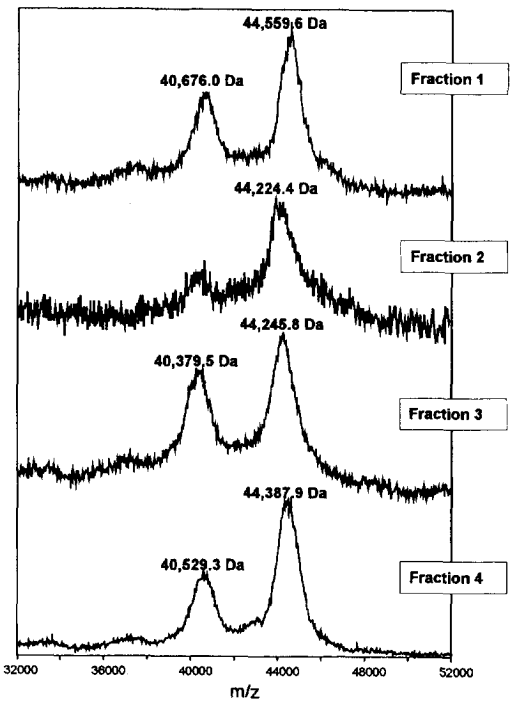

Fig. 2. MALDI-TOF spectra of fractions isolated from CE separation of ovalbumin (Fig. 1).

3.2. Desmodus salivary plasminogen activator (DSPA α1)

DSPA α1 is a glycoprotein with properties of a serine protease derived from the Desmodus rotundus (vampire bat) salivary gland [16]. DSPA is known to be heterogeneous when expressed in CHO cells with six sites for potential glycosylation, four O-linked and 2 N-linked and the number of potential glycoforms was recently estimated as being in excess of 330 000. The non-glycosylated molecular mass is 49 508 amu [16]. Since DSPA α1 is such a heterogeneous glycoprotein, it was chosen to investigate the role of HPCE and off-line MALDI-TOF as one of the approaches to developing this complex rDNA-protein to the point of a well characterized biological.

The HPCE conditions required for the resolution of the isoforms of DSPA were different from that of ovalbumin. Some of the differences may be related to the differences in isoelectric points, p*I* (pH at which the net charge is zero), between the two proteins (pH 4.6 vs. 7.8–8.2, respectively). Ovalbumin is highly charged at pH 8.5, while DSPA is not. DSPA is more stable and more highly charged at lower pH values. An electropherogram of DSPA glycoforms could be obtained at pH 3.0 (phosphate buffer) using a BSA coated capillary. The change in the pattern of the electropherogram as a function of initial sample concentration is shown in Fig. 3 (1 mg/ml vs. 10 mg/ml). For subsequent fraction collection from a single 50 μm I.D. capillary CE analysis, the higher sample concentration was used.

The complex electropherogram shown in Fig. 3 is consistent with the large population of glycoforms expected for a protein with six potential sites of glycosylation. Without characterization of individual components in such a complex profile, however, it is difficult for an analyst to demonstrate that the separation is consistent and not influenced by artifact formation. Fig. 4 shows the four fractions that were collected from the HPCE separation and the corresponding MALDI-TOF spectra. Table 1 gives the mass recorded by MALDI-TOF-MS of the initial sample of DSPA as well as the values obtained for the four collected fractions. The first fraction collected has a higher molecular mass than that of the starting material, while the masses determined for fractions 2 and 3 decrease relative to the unfractionated sample. Finally, fraction 4 has an intermediate value. As was observed with the CE and off-line MALDI-TOF analysis of ovalbumin, each of the collected fractions may still be mixtures of glycoforms and explanation of relative mobilities or masses of different fractions is not possible. Despite these issues due to the extreme heterogeneity of such samples, the ability to perform off-line MALDI-TOF-MS of fractions from complex electropherograms will be a powerful tool to demonstrate product consistency in the manufacture of glycoprotein pharmaceuticals.

3.3. Discussion and conclusions

The mechanism of separation of glycoforms in capillary electrophoresis is not totally characterized at this time. It appears to be not just electrophoretically mediated, but moreover a result of additional protein/surface interactions resulting in subtle but significant chromatographic selectivity [17,18]. A

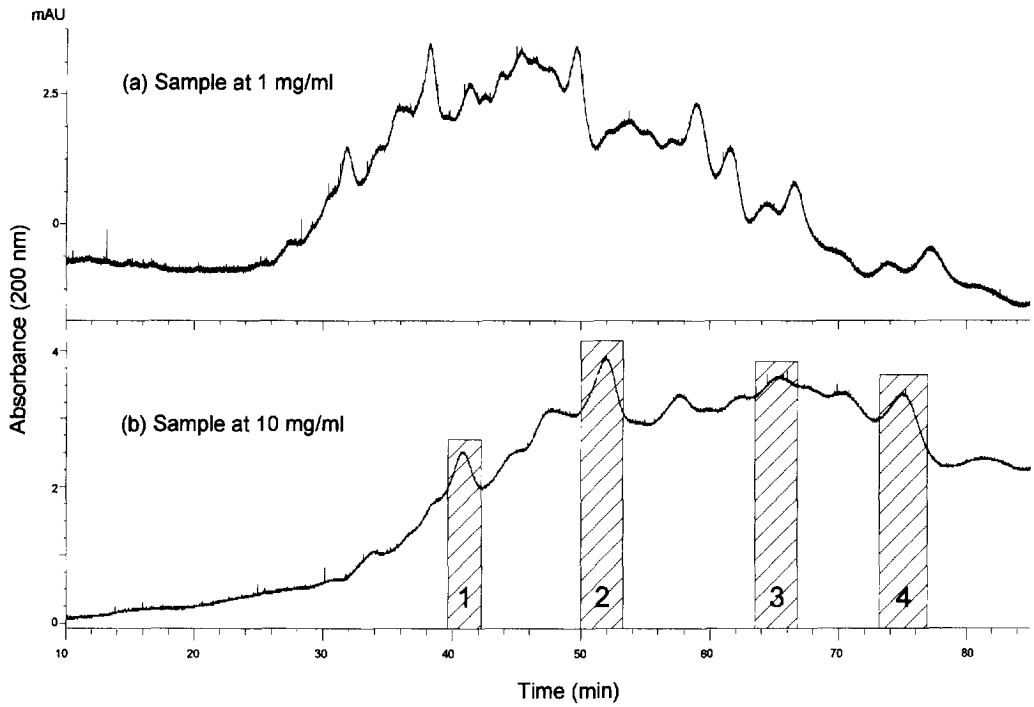

Fig. 3. Electropherogram of DSPA αI at sample loadings of (a) 1 mg/ml and (b) 10 mg/ml. CE conditions: 49.5 cm (effective length, 41 cm)×50 μm I.D. BSA treated capillary [14]; applied voltage 500 V/cm; detection at 200 nm; 100 m*M* sodium phosphate buffer (pH 3.0). The fractions collected for MALDI-TOF-MS are indicated as regions 1 to 4 in (b).

careful optimization of the separation conditions, however, can result in a high resolution separation of a variety of subtle differences in a protein sample, as can be seen in both the ovalbumin and DSPA examples. In these examples, however, a fraction can not be attributed by the analyst to a single glycoform, but rather to a mixture of glycoforms. It is thus crucial to have MALDI-TOF-MS as an off-line technique to demonstrate that the capillary electrophoresis analysis has achieved a real separation.

Since MALDI-TOF-MS can detect peaks with an accuracy of 0.1% (50 mass units for a 50 kDa molecule), the mass differences demonstrated in the examples presented are significant. Additionally, in these examples, the differences can be attributed solely to structural variations and is not confounded by the anomalous behavior of glycoproteins often encountered in other analytical techniques such as sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE). For SDS–PAGE, the accuracy at best is in the order of several hundreds of mass units and SDS–PAGE is known to often provide spurious molecular mass determinations for glycoproteins [2].

In contrast also to SDS–PAGE, not only is the information content of the CE-MALDI-TOF analysis superior but analysis times of under an hour are typical for the complete separation-to-mass determination. The approach used here, which allows collection of a single fraction of a few nanoliters into a few microliters of a receiving solution for subsequent MALDI-TOF mass analysis, is also compatible with limited sample availability.

Future work will be directed to refinement of the CE separation of glycoforms followed by MALDI-TOF mass analysis. Additionally, we are developing a glycoprotein "fingerprinting" method based on the use of sequential glycosidase digestions in addition to the analysis of released glycan pools to permit the analyst to more fully characterize the carbohydrate moieties present at different sites in a given glycoprotein.

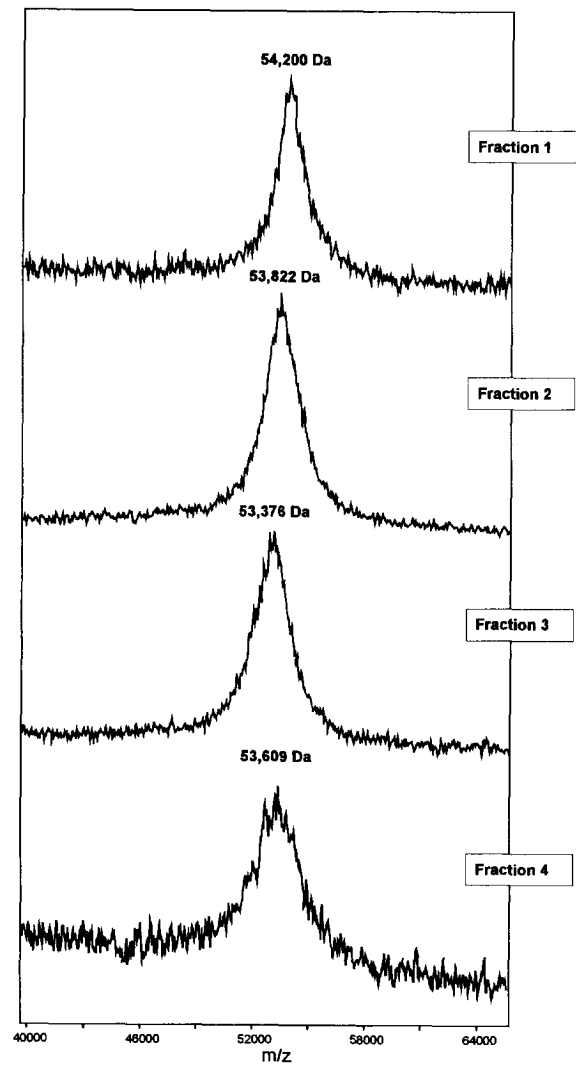

Fig. 4. MALDI-TOF spectra of fractions isolated from CE separation of DSPAα1 (Fig. 3(b)).

Table 1
Molecular mass determination of DSPA α1 CE fractions by MALDI-TOF-MS with external standard mass calibration (0.1% mass accuracy)

CE fraction	Mass by MALDI-TOF
1	54 200
2	53 822
3	53 376
4	53 609
Starting material	53 432

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