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# Quantification of PEGylated proteases with varying degree of conjugation in mixtures: An analytical protocol combining protein precipitation and capillary gel electrophoresis

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ABSTRACT

PEGylation, i.e. the covalent attachment of chemically activated polyethylene glycol (PEG) to proteins, is a technique commonly used in biopharmaceutical industry to improve protein stability, pharmacokinetics and resistance to proteolytic degradation. Therefore, PEGylation represents a valuable strategy to reduce autocatalysis of biopharmaceutical relevant proteases during production, purification and storage. In case of non-specific random conjugation the existence of more than one accessible binding site results in conjugates which vary in position and number of attached PEG molecules. These conjugates may differ considerably in their physicochemical properties. Optimizing the reaction conditions with respect to the degree of PEGylation (number of linked PEG molecules) using high-throughput screening (HTS) technologies requires a fast and reliable analytical method which allows stopping the reaction at defined times.

In this study an analytical protocol for PEGylated proteases is proposed combining preservation of sample composition by trichloroacetic acid (TCA) precipitation with high-throughput capillary gel electrophoresis (HT-CGE). The well-studied protein hen egg-white lysozyme served as a model system for validating the newly developed analytical protocol for 10 kDa mPEG-aldehyde conjugates. PEGamer species were purified by chromatographic separation for calibrating the HT-CGE system. In a case study, the serine protease Savinase® which is highly sensitive to autocatalysis was randomly modified with 5 kDa and 10 kDa mPEG-aldehyde and analyzed. Using the presented TCA protocol baseline separation between PEGamer species was achieved allowing for the analysis of heterogeneous PEGamer mixtures while preventing protease autocatalysis.



## 1. Introduction

Proteases already are widely applied as industrial catalysts in food processing (e.g. manufacturing of cheese in dairy industry), in the leather industry and as laundry detergents [1]. The rapid technological progress in the field of biotechnology enables the production of proteases with novel properties and substrates causing an increased interest in proteases for diagnostic and therapeutic applications in the pharmaceutical industry [1]. The global pharmaceutical market benefits from proteases as a growing class of drugs. In 2011 twelve protease therapies had been approved by the Food and Drug Administration (FDA) [2]. For instance, the recombinant extracellular serine proteases plasmin and tissue plasminogen activator (tPA) are applied in thrombolytic therapy as a treatment of heart attacks and ischemic stroke [3]. Both cardiovascular diseases represent the major causes of death in the western world [4]. They are both caused by circulatory blockages of blood vessels consisting mainly of insoluble fibrin. Plasmin directly degrades fibrin initiating clot breakdown whereas tPA catalyzes the reaction of the precursor plasminogen to the active plasmin by cleavage of a single bond [5,6]. In the case of tPA the therapeutic application is aggravated due to a fast clearance from the blood stream resulting in an extremely short half-life of only 2–6 min [7,8]. Moreover, proteases such as enterokinase, thrombin and factor Xa are used during the production of recombinant proteins for the removal of fusion tags [9–11]. Those tags are often attached to different peptides and proteins in order to simplify their expression, purification and analytics as well as to improve their solubility [11–13]. Fusion tags often need to be cleaved from protein drugs for meeting the regulatory standards since the tag may potentially modify the biological

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activity of the target protein or induce an immune response in the patient [14].

Modern biotechnology is capable of producing recombinant proteases to an industrial scale with high production efficiencies. However, a high loss of protease activity due to autoproteolytic degradation during production, purification and storage represent a major challenge [12,15]. Different approaches to solve both short serum half-life of therapeutic proteins and autoproteolytic degradation include mutagenic variations of the amino-acid sequence [15], incorporating proteins into drug-delivery vehicles such as liposomes [8,16] or microspheres of biodegradable polymers [17] and protein conjugation [18].

Protein conjugation denotes the linkage of synthetic polymers to a native protein in order to alter its physicochemical properties [19]. The attached molecules shield the protein surface which results in an enhanced stability towards proteases and a reduced recognition of the protein by the immune system [18,20,21]. The covalent attachment of chemically activated polyethylene glycol (PEG) to proteins is by this means one of the most commonly used technique in biopharmaceutical industry. PEG is especially interesting as a modifier for biopharmaceuticals since it is approved by the FDA for use in pharmaceuticals. It proved to show little toxicity and immunogenicity and it is eliminated from the body by the kidneys or in the feces [18]. The use of the hydrophilic polymer PEG as modifier results in an increased hydrodynamic radius of the protein in solution [22]. This effect engenders both an increased protein solubility and a decreased clearance from the blood stream. Several proteins, reaction mechanisms and conditions have been studied following first pioneering studies on PEGylated albumin and bovine liver catalase in the 1970s by the group of Abuchowski [23,24]. Most commonly amino coupling PEG reagents binding to the N-terminus or to surface lysines, such as PEG-aldehyde are used [21]. The existence of more than one accessible binding site results in conjugates varying in position and number of attached PEG molecules. Those conjugates can significantly differ in their physicochemical properties [25]. The PEGylation reaction and thereby the conjugate mixture can be controlled through reaction conditions like the PEG to protein ratio, reaction time, temperature and solution pH. The interactions between these factors, however, make it difficult to reproduce protein batches in terms of Quality-by-Design (QbD) [26]. One of the key tools within the QbD-framework to gain a deeper process understanding are high throughput screenings (HTS) coupled with design of experiments (DoE). Since speed and throughput of experimental facilities are constantly increasing appropriate high-throughput compatible analysis techniques of high resolution are crucial to determine the experimental outcome. The quantification of PEGylation processes remains a challenge due to poor spectrophotometric properties of PEG [27]. The increased molecular mass of protein conjugates promotes size exclusion chromatography (SEC) for quantitative separation of protein PEGamers. However, most SEC based assays are limited by a maximum throughput of a few samples per hour. Approaches to increase the throughput of SEC include interlaced injection of samples, the parallel operation of two columns and multivariate evaluation of chromatographic data with poor resolution [28]. Especially protein conjugation with low molecular weight PEG molecules lead to poor separation of individual PEGamers by SEC. Capillary gel electrophoresis (CGE) represents an alternative to SEC with a reduced processing time of approximately 60 s per sample [29]. Due to the microfluidic measurement principle, CGE provides a high peak resolution. As SEC, CGE separates proteins by molecular weight and therefore the conjugates by the number of attached PEG molecules [30].

This work demonstrates the potential of high-throughput capillary gel electrophoresis (HT-CGE) for the quantification of PEGylated proteins with varying degrees of conjugation. In the first part of this study the applicability of the standard HT-CGE protocol is shown for structural stable proteins using the model protein lysozyme from hen egg-white. For proteins that are subject to structural degradation an improved analytical protocol is presented which combines TCA (trichloroacetic acid) precipitation and high-throughput capillary gel electrophoresis. As an example the model serine protease Savinase® was chosen which could not be analyzed with the standard protocol due to high autocatalysis rates triggered by high temperatures used for denaturation during HT-CGE sample preparation. The benefit of integrating TCA precipitation into the analytical protocol is the immediate inactivation of the proteases and the resulting preservation of the sample composition. Moreover, TCA is assumed to precipitate all proteins in solution whereas salts and other buffer components remain in solution. TCA precipitation hence reduces the concentration of critical components from the sample [31] which otherwise may alter electrophoretic mobility of proteins in HT-CGE. The validated analytical method was subsequently used to determine the PEGamer composition for PEGylated Savinase® mixtures.

## 2. Materials and methods

### 2.1. Overview of workflow

The methods applied in this work are summarized schematically in Fig. 1. After producing PEGylated proteins via batch reactions a chromatographic purification was performed to isolate pure PEGamer (molecules with a varying number of attached PEG molecules) fractions. The purification of PEGylated lysozyme was performed by a two-step chromatography process of SEC followed by CEX. Savinase® was purified by a single CEX step (Fig. 1A). In this study a distinction is made between native proteins (non-PEGylated), proteins with one attached PEG molecule (mono-PEGylated) and proteins with two attached PEG molecules (di-PEGylated). Proteins with more than two attached PEG molecules (poly-PEGylated) are taken into consideration using a mass balance only. Each purified PEGamer fraction was subsequently used to determine a linear calibration curve for HT-CGE by linking the fluorescence signal to the PEGamer concentration (Fig. 1B). For the standard protocol the PEGamer fractions were thereby used without further treatment. For the TCA protocol samples were precipitated with trichloroacetic acid before HT-CGE analysis. The described HT analytic was then applied to PEGylated protein samples with an unknown mixture composition (Fig. 1C).

### 2.2. Preparation of buffer and protein solutions

All solutions were prepared with ultrapure water provided by a PURELAB Ultra water purification system (ELGA Labwater, Germany). The used buffer substances were sodium acetate trihydrate (Sigma–Aldrich, St. Louis, MO, USA) and acetic acid (Merck, Germany) for pH 5 and sodium phosphate monobasic dihydrate (Sigma–Aldrich, USA) as well as di-sodium hydrogen phosphate dihydrate (Merck, Germany) for pH 6.2, pH 7.2 and pH 8.2. The buffer capacity was set to 25 mM for all buffers. Sodium chloride (NaCl) included in the SEC running buffer and the CEX elution buffer was purchased from Merck (Germany). pH adjustment within a range of ±0.05 units was performed using a five-point calibrated pH-meter HI-3220 (Hanna Instruments, USA) with a SenTix® 62 pH electrode (Xylem Inc., USA). For pH correction hydrochloric acid and sodium hydroxide were obtained from Merck (Germany). Buffers were used at the earliest one day after preparation and repeated pH verification. All buffers were filtered using a 2 μm cellulose-acetate filter (Sartorius, Germany) and degassed for chromatographic purposes. Protein solutions were prepared using Savinase® (Protease