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Development of immobilized-pepsin microreactors coupled to nano liquid chromatography and tandem mass spectrometry for the quantitative analysis of human butyrylcholinesterase☆

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ABSTRACT

Human butyrylcholinesterase is a serine hydrolase that reacts with organophosphorus compounds (OP) to form stable adducts. These adducts are valuable biomarkers for OP exposure and can be analyzed by liquid chromatography-tandem mass spectrometry (LC–MS/MS) after a preliminary digestion step in solution. However, this digestion step is time-consuming and cannot be directly coupled with LC–MS set ups. Therefore, the aim of this work was to develop pepsin-based immobilized enzyme microreactors (IMERs) for the rapid digestion of human butyrylcholinesterase (HuBuChE). Various IMERs were synthesized by grafting different amounts of pepsin on a CNBr-sepharose gel and the grafting yield was measured by a bicinchoninic acid assay (BCA). A sensitive nanoLC–MS/MS method was developed to evaluate the digestion yields of HuBuChE on IMERs which was made possible by a synthetic peptide which was used as a calibrant. The digestion was optimized by studying the impact of different parameters such as the digestion time, the temperature and the amount of pepsin grafted on IMER. This optimization allowed HuBuChE to be digested with-in 20 min without pretreatment and with digestion yields up to 20%. The repeatability of the IMER synthesis and HuBuChE digestion was highlighted with the characterization of 3 similar IMERs. Finally, the digestion yields of HuBuChE were higher with IMERs when compared to a typical in solution digestion.

1. Introduction

Human butyrylcholinesterase (HuBuChE) is a serine esterase glycoprotein present in plasma and is made up of four 85 kDa subunits [1,2]. HuBuChE catalyzes the hydrolysis of esters including acetylcholine, aspirin, cocaine or heroin [3]. It can also be used as a biomarker for exposure to organophosphorus compounds (OPs) such as sarin [4–7], soman [5,6,8], tabun [6,8,9], or VX [5,10]. These nerve agents covalently bind to acetylcholinesterase (AChE) and HuBuChE and irreversibly inhibit their activity. Inhibition of AChE activity induces the accumulation of acetylcholine within nerve synapses, leading to paralysis and respiratory dysfunction which can result in death [11,12]. Because of their high degree of toxicity, these nerve agents can be used as chemical warfare agents [4]. Thus, the retrospective detection of OP exposure after a chemical weapons attack is of the utmost importance. It provides a reliable diagnosis leading to appropriate medical countermeasures, as well as confirming the alleged use of these chemical warfare agents which are prohibited under the terms of the Chemical Weapons Convention [13]. HuBuChE is usually the preferred biomarker of OP exposure over AChE because it can form adducts with a broad range of OPs, is 10 times more abundant than AChE in the plasma (40–80 nM) and can persist in the blood longer than AChE [14]. The binding of OP nerve agents on the 198 serine residue of the HuBuChE active site allows retrospective detection of HuBuChE-OP adducts in blood for as long as 16 days when urinary metabolites are no longer detectable [15].

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Protein determination is commonly performed by liquid chromatography coupled to mass spectrometry analysis (LC/MS). However, ionization of intact proteins produces multiply charged ions envelopes, leading to a very complex mass spectrum. In order to avoid such problems, the detection of HuBuChE-OP adducts is usually performed after a preliminary digestion step. This digestion produces small peptide fragments that are then analyzed by liquid chromatography tandem mass spectrometry (LC–MS/MS).

The digestion of HuBuChE-OP is typically achieved in solution with trypsin [16], chrymotrypsin [6] or pepsin [17,18], commonly over 2 h. Generally, to decrease lengthy digestion time of proteins, the enzyme/protein ratio can be increased. However, as the enzyme is free in solution, autoprotolysis can occur thus generating peptides that can interfere during the mass spectrometry analysis. To overcome this drawback, the proteolytic enzyme can be immobilized on a functionalized solid support [19]. The resulting immobilized enzyme reactors (IMERs) allow for an efficient digestion and low digestion time while minimizing sample contamination during handling and avoiding autoprotolysis. In addition, as the stability of the enzyme is enhanced, immobilized enzyme reactors can be reused thus decreasing the cost of the digestion step[19]. IMERs have been synthesized on various formats for the digestion of proteins including pre-columns [20–25], membranes [26,27], capillaries [28–30], pipette tips [31], disks [32,33] and using various supports such as silica particles [34], silica monolith [34,35], agarose [36–39], graphene [40], or synthetic polymers such as monolithic polymethacrylate derivatives [31,41–43] and polystyrene-divinylbenzene particles (Poroszyme) [21–23,30,44,45].

Performances of the IMERs depend on many factors including the technique used for enzyme immobilization (adsorption, encapsulation or covalent linkage), digestion conditions and the nature of the functionalized immobilization support [46]. The ideal support should be biologically inert, easily activated, homogeneous with high surface area and should favor a good accessibility of the substrate [47]. It should also present good mechanical and chemical stability over a wide range of pH, pressure, temperature and solvent conditions [48,49]. Finally, this support should be hydrophilic to avoid non-specific hydrophobic interactions with proteins or peptides. A study comparing the performance of IMERs made with 3 different precolumn-format supports [36], highlighted the importance of the immobilization support for the efficient digestion of cytochrome C on a trypsin-based IMER. Between CNBr-activated Sepharose (Seph-CNBr), NHS-activated Sepharose and glutaraldehyde-activated silica, Seph-CNBr appeared to be the best immobilization support in terms of capacity and digestion yield [36].

Due to the high specificity of its cleavages, trypsin has been frequently used for the digestion of HuBuChE [16,17]. Yet, HuBuChE digestions with trypsin require pretreatments (carboxymethylation and deglycosylation) and induce large peptide-OP adducts (29 amino acids) that can excessively fragment in the mass spectrometer ion source and result in an insensitive detection. In the case of HuBuChE digestion by pepsin, pretreatment of the enzyme is not necessary and the digestion leads to smaller peptides including the nonapeptide FGESAGAAS on which OPs bind [17]. Moreover, digestion by pepsin can be directly coupled to a preliminary selective extraction step by immobilized antibodies (immunoextraction) in order to remove other interfering proteins from real plasma thus improving the selectivity and the sensitivity of the whole analytical method. Additionally, the acid conditions used for pepsin digestion (pH 2.2) has been shown to be compatible with the elution of proteins such as cytochrome C from antibodies [50].

Recently, Carol-Visser et al. developed an on-line set-up that consists of a direct coupling of a sarin-intoxicated HuBuChE digestion using a commercial pepsin-IMER precolumn (Poroszyme® 30 × 2.1 mm i.d.) with LC–MS/MS analysis [7]. Efficiency of the digestion was determined by comparing the intensities of the HuBuChE specific peptide obtained after digestion on IMER to those obtained after a digestion in solution. In this study, intensity of adduct nonapeptide derived from on-line pepsin digestion was about 70% of that obtained after in-solution digestion. However, no information was provided on Poroszyme® column digestion yields or on the grafting yields of pepsin in the reactor. Apart from a study on the development of trypsin-based microreactors related to cytochrome C digestion [36], quantification of digestion yields on IMERs have not been evaluated. In addition, rather than using commercial IMERs, it appears interesting to control the synthesis of IMERs to be able to choose an appropriate grafting support and control the amount of immobilized enzymes.

Although many developments were made on trypsin microreactors [51], few studies deal with the development of pepsin-based IMERs. Moreover, none have been specifically developed for HuBuChE digestion despite the ability of pepsin to digest HuBuChE as already mentioned above. Thus, the aim of this study was the development and characterization of laboratory-made pepsin-based IMERs for the digestion of HuBuChE. Different supports were prepared by grafting different amounts of pepsin on cyanogen bromide (CNBr)-activated Sepharose and were packed in precolumns (20 × 2 mm i.d.). Digestions on pepsin-based IMERs were optimized with the evaluation of several key parameters such as the digestion time, temperature and the amount of pepsin grafted on the IMERs. This evaluation was based on the quantification of a specific nonapeptide that was quantified in the digest by nanoLC–MS/MS. The repeatability of the digestion and IMER synthesis were assessed with the preparation of 3 similar microreactors. Finally, digestion yields obtained on IMERs were compared to those obtained by digestion in solution.

2. Experimental

2.1. Chemicals

Pepsin from porcine gastric mucosa, sodium hydrogen phosphate (Na_2HPO_4), sodium azide (NaN_3), sodium chloride (NaCl) and cyanogen bromide-activated Sepharose 4 B were purchased from Sigma Aldrich (Sigma-Aldrich, Saint-Quentin Fallavier, France). Potassium dihydrogen phosphate (KH_2PO_4), di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4(2H_2O)$), sodium acetate (CH_3CO_2Na), acetic acid (CH_3COOH), formic acid (HCOOH), glycine, hydrochloric acid and acetonitrile (ACN) were purchased from VWR (Fontenay-sous-Bois, France). Sodium hydroxide was purchased from Merck (Darmstadt, Germany). HPLC-grade acetonitrile was ordered from Carlo Erba (Val de Reuil, France). High purity water was obtained from a Milli-Q purification system (Millipore, Saint Quentin en Yvelines, France).

Two different sources of HuBuChE, obtained by the purification of human serum, were used: $HuBuChE_1$ (480 μg of lyophilized HuBuChE, 50 U/mg, B4186) was purchased from Sigma Aldrich and $HuBuChE_2$ (480 μg of lyophilized HuBuChE with 0.02 mol L^{-1} ammonium bicarbonate, 160 U/mg, MBS173030) was purchased from MyBioSource (San Diego, California, USA). Synthetic nonapeptide (FGESAGAAS) was obtained from Proteogenix (Schiltigheim, France). BCA protein assay reagents were obtained from Thermo Fisher Scientific.

The phosphate buffer saline solution (PBS, pH 7.4) consisted of 0.01 mol L^{-1} of both Na_2HPO_4 and KH_2PO_4 and 0.15 mol L^{-1} of NaCl. The PBS-azide solution is a solution of PBS with 0.1% (w/w) of NaN_3. HuBuChE solutions (480 mg L^{-1}) were prepared in PBS-azide. Pepsin solutions were prepared in saline sodium acetate solution (CH_3CO_2Na, 0.1 mol L^{-1}, pH 5.8, NaCl 0.5 mol L^{-1}).