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Preparation and evaluation of dual-enzyme microreactor with co-immobilized trypsin and chymotrypsin

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ABSTRACT

The preparation of capillary microfluidic reactor with co-immobilized trypsin and chymotrypsin with the use of a low-cost commercially available enzymatic reagent (containing these proteases) as well as the evaluation of its usefulness in proteomic research were presented. The monolithic copolymer synthesized from glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) was used as a support. Firstly, the polymerization conditions were optimized and the monolithic bed was synthesized in the fused silica capillary modified with 3-(trimethoxysilyl)propyl methacrylate (γ-MAPS). The polymer containing epoxy groups was then modified with 1,6-diaminohexane, followed by the attachment of glutaraldehyde and immobilization of enzymes. The efficiency of the prepared monolithic *Immobilized Enzyme Microreactor* (μ-IMER) with regard to trypsin activity was evaluated using the low-molecular mass compound (Nα-benzoyl-L-arginine ethyl ester, BAEE). The activities of both enzymes were investigated using a macromolecular protein (human transferrin, Tf) as a substrate. In the case of BAEE, the reaction product was separated from the substrate using the capillary liquid chromatography and the efficiency of the reaction was determined by the peak area of the substrate. The hydrolysis products of transferrin were analyzed with MALDI-TOF which allows for the verification of the prepared enzymatic system applicability in the field of proteomic research.

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1. Introduction

Enzymatic reactions, due to their high specificity and selectivity, constitute a very attractive solution in many branches of industry as well as a valuable analytical tool [1,2]. On the laboratory scale, the preparation of microfluidic reactors with enzymes immobilized on the solid, monolithic support is one of the ways of using enzymes [3,4]. Such an approach combines the advantages of miniaturization, monolithic beds as a supports, and enzymes as environmentally friendly, specific, and selective biocatalysts. Additionally, the microfluidic μ-IMER may be easily connected to a subsequent capillary system which enables its coupling with separation and detection techniques. Such a system prevents the reaction products from being contaminated or lost. With regard to the application and the type of an immobilized biocatalyst (e.g. glucose oxidase [5], lipase [6], acetylcholinesterase [7], l-asparaginase [8]), the microfluidic reactors enable carrying out highly specific reactions with minimal manual handling, reagent consumption, and time-effectively.

μ-IMERs can be particularly useful in proteomic research, e.g. for the identification of proteins with PMF technique (*Peptide Mass Fingerprinting*). The PMF is based on the initial digestion of a protein of interest by a specific enzyme and a subsequent MS analysis of the resulting polypeptides. The pattern of peaks recorded on the MS spectrum is finally compared with the theoretical masses available in proteomic databases [9]. Trypsin is the most common of the proteolytic enzymes immobilized in microfluidic monolithic reactors. It is also widely discussed in available literature [10–16]. However, it is possible to immobilize some other proteases for protein digestion e.g. chymotrypsin [17,18], papain [19–21], or pepsin [22,23].

It is important to notice that the application of proteolytic enzymes for protein digestion is not limited only to the qualitative analysis in proteomic research involving the use of one, highly specific enzyme. It may also be employed in protein quantification and the investigation of the post-translational modifications, e.g.

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glycosylation [24]. In both cases, the fragmentation of the proteins to low-molecular weight polypeptides is the main aim of the proteolysis. Then, the quantitative analysis of the target protein may be performed on the basis of one specific peptide formed during enzymatic digestion, e.g. by AQUA analysis, with the use of labeled internal standards [25]. Such a fragment, selected as a representative, cannot occur in other polypeptides. The situation may be caused by missed cleavage sites. It is directly related to the efficiency of proteolysis. On the other hand, for research focused on the protein glycosylation, it is important to obtain short glycopeptide chains. Taking into account the fact that the digestion with trypsin usually leads to obtaining long glycopeptides with several glycosylation sites, it is more reasonable to use the enzyme which acts on more amino acid residues or to apply a mixture of nonspecific endoproteases for glycosylated protein digestion. For such a purpose, the preparation of a pronase-immobilized reactor was justified [26]. Tryptic digestion is also limited in the case of *Laser-Induced Fluorescence* (LIF) detection due to the slow reaction between a fluorescent reagent and resulting polypeptides, and, on the other hand, because the derivatization of an intact protein limits the trypsin activity to an arginine residue "cleave sites". Such a situation, in turn, leads to obtaining a small number of fragments. The above mentioned problems justify the immobilization of chymotrypsin for digestion of protein in the case of the microreactor coupling with CE-LIF [27]. The same issue is also important in the case of MS detection because tryptic digestion of a protein containing a small number of arginine and lysine residues may lead to obtaining a few fragments which are too long for the MS analysis. In such a case, the proteins can be digested by several proteases of different activities to improve the protein fragmentation and to obtain a greater number of fully sequenced peptides. Such an approach can result in the higher sequence coverage and is particularly important when the reliable identification of one target protein is needed. The creation of an online system composed of two coupled microreactors (chymotrypsin- and trypsin-based) is one of the ways to avoid classical tandem reactions performed in solutions with dissolved enzymes [28]. Such a system allowed obtaining higher sequence coverage for β-casein (70%) when compared to single-enzyme reactors with immobilized trypsin (14%) or chymotrypsin (57%) in reaction time of 20.8 min. In a comparison, the classical digestions performed in solutions for 18 h and employing these enzymes resulted in the sequence coverage of 21% and 45% for trypsin and chymotrypsin, respectively.

Recently, for protein digestion, a dual-microreactor with co-immobilized trypsin and chymotrypsin has been prepared with the use of a monolithic support on which trypsin was bound and nanoparticles used as a support for chymotrypsin [29]. In this case, a sample of proteins isolated from the rat liver were digested with dual-enzymatic system which allowed for the identification of 620 individual proteins while the total number of proteins identified with two single protease digestions was 606.

The immobilization of the enzymes on the polymeric support containing glycidyl methacrylate (GMA) as a functional monomer may be performed by the aminolysis of the epoxy groups and then activation with dialdehyde. Research data [30,31] confirmed the fact that the greater the distance between the support surface and immobilized enzyme molecules is created, the higher efficiency of enzymatic reactions is achieved. It justifies the immobilization of trypsin onto membranes [32] or magnetic nanoparticles [33] functionalized with hexanediamine. It was also proved that papain immobilized on the GMA-co-EDMA monolith through hexanediamine chain exhibits activity higher than in the case of a reaction involving hydrolysis of an epoxy ring and oxidation with $NaIO_4$ (despite higher immobilization capacity in the second case) [19]. This is probably due to the fact that the spacer arm prevents the enzyme from changing its conformation (by minimized interaction between the support surface and biomolecules) and reduces the steric hindrances, thereby enhancing the formation of enzyme-substrate complexes.

For this reason, such a technique was used in the present research. In contrast to the previously mentioned approach [29], the simultaneous immobilization of both trypsin and chymotrypsin reduces the preparation stages and amount of reagents required, i.e. one type of material is used as a support and one enzymatic preparation is used during immobilization, which is more preferable from economical and practical points of view. Similar methodology was used during the modification of a classical size monolithic silica column modified with epoxy groups (100 mm × 4.6 mm i.d.) by directly bonding two enzymes (without the spacer arm) using, however, two individual separate enzyme preparations [34]. Due to the fact that enzymatic reagents containing one particular enzyme of high purity are expensive, the verification of low-cost reagents with regard to their potential applications is economically favorable.

Taking into account all the above-mentioned factors and numerous applications of proteolytic digestion, the main aim of this research was to prepare and evaluate a microfluidic reactor with two enzymes immobilized on a monolithic GMA-*co*-EDMA support using a low-cost, commercially available reagent (containing trypsin and chymotrypsin). The first stage of our research involved the selection of appropriate polymerization conditions (including porogen solvent composition and polymerization temperature) to obtain the support of good permeability, homogenous structure, and relatively high surface area. The activity of immobilized trypsin was evaluated with its commercially available substrate (BAEE) which enables the establishment of the optimal conditions for the hydrolysis reaction to be established. The activities of the both immobilized enzymes were tested by the digestion of transferrin which is known to be a protein relatively resistant to trypsin [35,36]. Therefore, the digestion in a dual-enzyme microreactor was compared with a classical digestion method (as a reference) in which a high-purity trypsin was used. The eluate from the microreactor and the post-reaction mixture were analyzed with MALDI-TOF MS.

2. Material and methods

2.1. Materials

Fused-silica capillaries (150 μm i.d. × 375 μm o.d.) were purchased from, CM Scientific Ltd. (Silsden, United Kingdom), 3-(trimethoxysilyl) propyl methacrylate (γ-MAPS), glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), 1-dodecanol, cyclohexanol, 1,6-hexanediamine, glutaraldehyde, sodium cyanoborohydride, benzamidine, Nα-benzoyl-L-arginine ethyl ester (BAEE), Trypsin Gold, Mass Spectrometry Grade (used for transferrin digestion in a solution), human transferrin (used as a model protein), and trifluoroacetic acid (TFA) were purchased from Sigma–Adrich (Steinheim, Germany). The enzyme preparation used during the microreactors synthesis was obtained from Biological Industries (trypsin: 365.47 USP-u/mg, chymotrypsin: 151.23 USP-u/mg). The storage solution (containing sodium azide) used for flushing and storage of the microreactors was purchased from MACS Miltenyi Biotec (MEDianus Sp.z o.o., Kraków, Poland). Acetone, toluene, methanol, dichloromethane, sodium hydroxide, sodium bicarbonate, sodium phosphate monobasic dihydrate, ammonium bicarbonate (all of analytical grade), acetonitrile (HPLC ultra gradient grade) were purchased from Polskie Odczynniki Chemiczne (POCh, Gliwice, Poland). Deionized water was obtained from the Mili-Q ultrapure water producing system (Millipore, Bedford, MA, USA). Azobisisobutyronitrile (AIBN) and all the

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