The main goal of this project is the investigation of new substrates of proteolytic enzymes from family of serine and cysteine proteases by application of one bead-one compound (OBOC) peptide combinatorial libraries modified by ionization tags. The synthesis of such libraries on solid support was developed recently by the project leader [1, 2] and its applicability was tested using small model training library of -chymotrypsin substrates. Within this project we would like to optimize the proposed synthetic strategy and to investigate the proposed methodology in combinatorial analysis of new substrates of proteolytic enzymes.

The idea of sensitive detection of such libraries depends on the solid support derivatization of their components using fixed charge tag in the form of quaternary ammonium (QA) group as *N,N,N*-trialkylammonium acetyl, which increases the ionization efficiency and allows the unambiguous analysis of trace amounts of compounds obtained from a single resin bead by electrospray ionization tandem mass spectrometry (ESI-MS/MS). We plan to design and synthesize several different combinatorial libraries composed of peptides containing both proteinogenic and unnatural amino acid residues. The synthesis of relatively large and complex libraries would extend the spectrum of study and would increase the chance of finding new, previously unknown enzyme substrates. The primary structure of peptides will be identified by MS/MS analysis. Such analysis in combinatorial chemistry used to be difficult because of the low sensitivity of applied analytical techniques . We have proven the usefulness of ionization tags in the form of QA group to increase the sensitivity of detection and allow the reliable identification even of the 15×10^{-18} mole (15 attomole) of peptide using commercially available ESI-MS/MS triple quadrupole mass spectrometer [3].

The applicability of proposed methodology was previously confirmed by us in the analysis of small model peptide libraries of -chymotrypsin substrates [1, 2]. The obtained results confirmed the well-known specificity of this serine protease. Therefore, it may be concluded that properly designed libraries of peptides on the solid support may be used to discover new substrates for enzymes important for medical diagnostics. Although the knowledge of the proteolytic enzymes is expanding, still too little is known about the detailed substrate specificities of many proteolytic enzymes. The enormous importance of specific substrates or inhibitors of such enzymes in diagnostics and pharmacology makes their combinatorial studies very important. New substrates that allow for enzyme activity monitoring are investigated by many research groups. In the present project we would like to apply the proposed methodology in investigation of new substrates of important proteolytic enzymes from families of cysteine and serine proteases: catepsins, caspases and elastase. We will design and synthesize on solid support (TentaGel HL-NH_2) peptide or peptidomimetic libraries containing hundreds to thousands of components. For each enzyme the substrate peptides will be connected to the resin by carefully designed linker. The resin beads containing recognized and partially hydrolyzed peptides by enzyme will be identified by a proper color test and selected. After the peptide cleavage the sequence will be analyzed by ESI-MS/MS. To the best of our knowledge, the application of fixed charge tag in the investigations of new substrates of such important proteases like caspases, catepsins or elastase has never been described in scientific literature.

Proteolytic enzymes play key roles in many important biological processes like infections and inflammation, apoptosis, blood clotting, and cell cycle control. The misregulation of proteolysis may result in pathological changes. Therefore the investigation of new substrates of proteases which may serve in the future as specific biomarkers for enzyme activity monitoring is under study. Several methods have been developed to define the optimal substrate specificity of proteases, however, usually only the proteinogenic amino acid residues are considered for the library synthesis. Recently Dr g and co-workers [4] applied unnatural amino acid residues in combinatorial analysis, which led to the discovery of new highly active substrates of caspases, as well as provided a method of distinguishing (specific) caspases.

The OBOC peptide libraries are widely used in the investigation of new biologically active compounds [5, 6]. However, the necessity of analysis of trace amount of peptide obtained from a single resin bead (about 10^{-15} mole) is insufficient for reliable sequence analysis. Previously we invented the application of quaternary ammonium ionization tags for ultrasensitive sequencing of peptides by tandem mass spectrometry. We believe that application of this method may revolutionize combinatorial search for new substrates of proteolytic enzymes. The proposed approach will overcome all problems connected to insufficient amount of peptide released form a single resin bead and may be widely used for discovering new substrates of proteolytic enzymes. Such substrates may serve as new tools for analysis of biomarkers of several diseases and disorders.

The project involves the synthesis of several different QA-OBOC libraries containing both proteinogenic and unnatural amino acid residues. This project consists of five tasks. We plan to optimize the structure of linker binding peptide to the resin. The linker will be composed of cleavable group (i), spacer (ii) and residue modified by QA-group (side chain of Lys residue). Linkers will be optimized individually for each tested enzyme. In the next task we plan to synthesize a series of different QA-OBOC libraries (acetylated at the N-terminus) on TentaGel resins and apply them to the investigations of new substrates of proteases. The OBOC resins will be incubated with tested proteases. After enzymatic reaction the recognized peptide will be partially hydrolyzed, resulting in the appearance of free amino group. The active sequences on the resin beads will be then identified by the color test for free amino group (ninhydrin reaction, chloranil or isatine test). Then peptide samples obtained from single resin beads will be analyzed by ESI-MS/MS. Identified active sequences will be resynthesized, purified and their properties will be confirmed using enzymatic analysis in solution. Additionally we plan to synthesize several (10-20) series of fluorogenic analogs of the most active sequences and to analyze their kinetic parameters (KM, kcat or kcat/kM). The QA-OBOC libraries composed of hundreds to thousands of peptides will be synthesized according to the method described previously by project leader. The enzymatic studies will be performed in collaboration with Marcin Dr g (Faculty of Chemistry, Wroclaw University of Technology). Resynthesized substrates for analysis in solution will be purified by HPLC.

We believe that application of the proposed method may revolutionize the combinatorial search for new substrates of proteolytic enzymes. The suggested approach will overcome all problems connected to insufficient amount of peptide released form a single resin bead, opening new possibilities in discovery of new substrates of proteolytic enzymes. Such substrates may serve as new tools for analysis of biomarkers of several diseases and disorders.

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