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A SUMMARY OF A DISSERTATION

Application of the combinatorial chemistry methods in the characteristics of the selected serine proteinases

A key element describing the proteases is their ability to recognize and hydrolyze substrates of a particular amino acid sequence. Only in recent years a variety of chemical and biochemical methods using fluorescent substrates (obtained by chemical synthesis) have been developed to identify the substrate specificity and determine the assessment of enzyme activity under various reaction conditions. Identification and optimization of these compounds gives great opportunities, inter alia, in the design of a new generation of inhibitors or biomarkers of these enzymes.

The object of my research conducted in the framework of this dissertation was proteolytic enzymes stored in cytoplasmic granules of neutrophil cells, belonging to the family of serine proteinases, common called Neutrophil Serine Proteinases (NSPs). Overexpression of these enzymes or their endogenous inhibitor deficiency can lead to serious pathological conditions in the organism.

The aim of my dissertation was to design and synthesis of fluorescent substrates based on Förster resonance energy transfer (FRET) phenomenon for three members of NSPs: proteinase 3 (PR3), human neutrophil elastase (HNE) and newly discovered fourth neutrophil serine proteinase (NSP4) using combinatorial chemistry methods. Chemical synthesis of peptide libraries was carried out with split and mix method using chemically modified polymeric resin as a solid support. Peptide sequences were optimized in nonprime positions (numbering of amino acid residues in the *N*-terminus P_1, P_2, P_3) as well as in prime positions (numbering of amino acid residues at the *C*-terminus P_1', P_2, P_3') according to the nomenclature of Schechter and Berger model. First, I analyzed *S*-subsite substrate specificity designing peptide libraries with the general formula ABZ-X_3-X_2-X_1-ANB-NH_2 (for NSP4 additional position X_4), wherein the FRET pair

were respectively 2-aminobenzoic acid (fluorescence donor) and amide of 5-amino-2-nitrobenzoic acid (fluorescence acceptor). As a result of successive steps of the iterative deconvolution in solution I received the peptide sequences that were efficiently hydrolyzed in presence of targeted NSP: for HNE – ABZ-Met-Pro-Val-ANB-NH_2, for human NSP4 – ABZ-Met-Phe-Pro-Arg-ANB-NH_2 and for mouse NSP4 – ABZ-Met-Val-Pro-Arg-ANB-NH_2. The above-mentioned compounds I used then to design substrates allowing to investigate *S*'-subsite substrate specificity of proteinases. The general formula of peptide libraries was as follows: ABZ-peptide-X_1'-X_2'-X_3'-Tyr(3-NO_2)-NH_2, where 'peptide' sequences were chosen for each NSP from optimizing nonprime position synthesis step. To design a new substrate for PR3 with increased selectivity, in which I put another three prime positions, I decided to use previously obtained in our team sequence ABZ-Tyr-Tyr-Abu-ANB-NH_2. This time as donor/acceptor fluorescence pair was used ABZ/Tyr(3-NO_2)-NH_2 (amide of 3-nitro-L-tyrosine), as this pair has shown a long-range energy transfer (more than five amino acid residues).

As a result of iterative deconvolution of the above-mentioned peptide libraries I received the following substrate sequences for each of NSPs:

- for PR3 ABZ-Tyr-Tyr-Abu-Asn-Glu-Pro-Tyr(3-NO_2)-NH_2,
- for HNE ABZ-Met-Pro-Val-Ala-Trp-Glu-Tyr(3-NO_2)-NH_2,
- for human NSP4 ABZ-Met-Phe-Pro-Arg-Thr-Leu-Gly-Tyr(3-NO_2)-NH_2,
- for mouse NSP4 ABZ-Met-Val-Pro-Arg-Ser-Ala-Tyr- Tyr(3-NO_2)-NH_2,

which have been efficiently hydrolyzed in presence of dedicated enzyme.

During the work, for the physicochemical analysis of peptide libraries as well as selected substrates I used mass spectrometry (MALDI-TOF) and high performance liquid chromatography in reverse phase (RP-HPLC). The selected substrates were tested by determination of enzyme activity and kinetic parameters (catalytic constant k_{cat}, Michaelis constant K_M and specificity constant k_{cat}/K_M). I investigated the effect of peptide chain elongation of three prime positions in substrate sequences (relative to the nonprime analogues) on the catalytic efficiency of each of the proteinases. I made the identification of cleavage pattern of the peptide bond in substrates and checked their susceptibility to enzymatic hydrolysis to all members of NSPs. Finally, I determined the influence of pH of the reaction to the activity each of enzyme.

The last step of my project included the measurements of the concentration and evaluation of enzyme activity of human NSPs using dedicated FRET substrates at the molecular level in biological samples such as: cell lysates, plasma, sera, BAL fluids (BALF, *bronchoalveolar lavage fluids*) and the like, from patients suffering from diseases associated with dysfunction of these enzymes or their endogenous alpha-1 proteinase inhibitor (α1-Pi, A1AT) deficiency.