Quenched fluorescent peptide substrate library for peptidases characterisation, selective substrate and inhibitors design and isoenzymes differentiation.

Bernard P. Roques
Professor Emeritus University Paris Descartes,
SCO Pharmaleads SAS, 11 rue Watt, 75013 Paris, France.

Sequencing studies of human genome have shown that 1.8% of proteins belong to the group of proteases. Endogenous peptide substrate(s) of these proteases are involved in the regulation of a wide variety of crucial physiological functions such as respiration, arterial pressure, heart rhythm, intestinal transit, food consumption, pain, stress and reproduction. Inhibition or activation of these proteases is one of the most interesting domain of therapeutic, illustrated by inhibitors of angiotensin converting enzyme (ACE), HIV protease, dipeptidyl peptidase (DPPIV) etc, used in treatment of hypertension, AIDS and diabetes (II) respectively. However only a very small part of peptidases have been studied from a putative therapeutic point of view. This is essentially due to the lack of informations on the physiological role(s) of these enzymes and on their endogenous substrates. Various genomic and chemical approaches are used to take up the challenge. This requires specific tools such as high affinity and selective substrates, which can be used in high-throughput screening assays to obtain and optimize highly potent inhibitors or activators. Their development is based on a deep knowledge and understanding of the main structural and functional characteristics of any given protease.

With this aim we have designed universal and highly sensitive quenched fluorescent libraries, introducing inside peptide chains, the highly fluorescent L-Pyrenyl-alanine (Pya) (1,2) and the efficient quencher pNO2-Phenyl-alanine (NOP) fluorophores. To avoid amino-peptidase and carboxypeptidase recognition, the N and C termini of these peptide substrates were protected by acetyl and amino groups (3).

In previous libraries of fluorescent substrates the fluorophore-repressor pairs are located at the N- and C-termini of synthetic peptides often leading to modest quenching and high background signal or were immobilized on resin with one or both fluorophores at the end of amino acid side chains. These features may hinder active-site recognition.

The primary sequence of libraries is Ac-SGK-Pya-(X)n-NOP-GGK-NH2 and its complementary sequence where Pya and NOP are reversed (3,4). The selected amino acids surrounding Pya and NOP are hydrophilic providing the required aqueous solubility for enzymatic assays achieved at 37°C. “X” is a mixture of 10 representative natural a.a (A, I, L,
K, F, W, E, Q, T and P) and “n” a number from 0 to 4. Assembly of the library was carried out using the split and mix method (3) through solid phase, leading to sub-libraries of equimolar quantities of 10 peptides with all possible combinations (3). Due to the very high fluorescence emission (\(\lambda_{ex} = 340\) nm ; \(\lambda_{em} = 405\) nm) of Pya and its efficient quenching by NOP (1,2) a very large fluorescence increase (X 500 to 1000) resulting from the cleavage between Pya and NOP or NOP and Pya is observed.

The reaction was followed by fluorescence emission using a multimer plate-reader fluorimeter and rapidly stopped to isolate the most efficient substrate among the 10 peptides and its chemical characterisation was achieved by HPLC and mass analysis (3). The validation of the libraries was done by using enzymes belonging to the four main types of hydrolases : serine-, metallo-, cystein-, and aspartyl-proteases. This is schematized in the Figure for X = 2 in which each colored well represent a peptide mixture of the sub-library cleaved by the indicated peptidase. Overall, the results obtained with the different enzymes tested shows that this library provided useful information concerning the subsite preferences of a protease of unknown specificity. With the n = 3, 4 sub-libraries, more data about the subsite preferences of the tested enzymes can be obtained and allow refined active site models to be proposed (data not shown). Moreover these libraries are able to preserve all the requirements for a good adaptation of the amino-acid side chains for a given peptidase active site.

This platform designated Fluofast (4) was used to design the first highly selective substrates for iso-enzymes such as NEP2 vs NEP1 (3), ECE2 vs ECE1 (5). These substrates and metabolites are now commercially available. Fluofast allows high-throughput screening of specific inhibitors (or activators) for one or several protease at the same time.
Screening of the libraries can also discriminate closely related activities, providing new tools, i.e. specific high-affinity model substrates, to study enzymatic activities in their physiological context, *in vivo* and *ex vivo* (5). As proteases constitute a large pool of novel therapeutic targets, these tools will be essential to their validation.