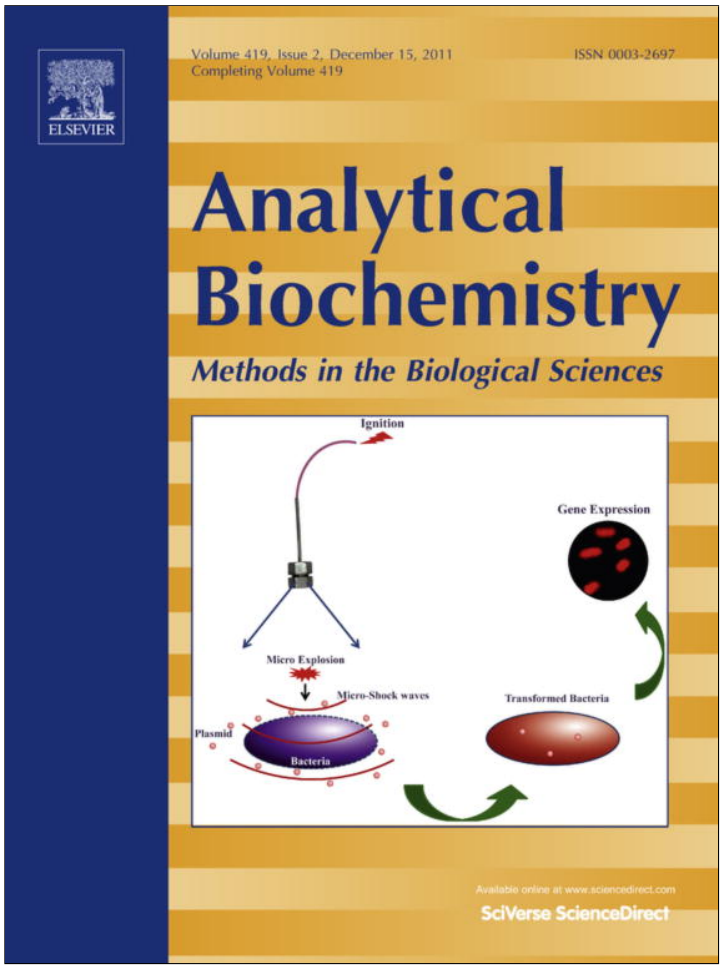

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Pluripotentialities of a quenched fluorescent peptide substrate library: enzymatic detection, characterization, and isoenzymes differentiation

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ABSTRACT

Protease inhibitors represent a major class of drugs, even though a large number of proteases remain unexplored. Consequently, a great interest lies in the identification of highly sensitive substrates useful for both the characterization and the validation of these enzyme targets and for the design of inhibitors as potential therapeutic agents through high-throughput screening (HTS). With this aim, a synthetic substrate library, in which the highly fluorescent (*L*)-pyrenylalanine residue (Pya) is efficiently quenched by its proximity with the *p*-nitro-(*L*)-phenylalanine (Nop) moiety, was designed. The cleavage between Pya and Nop leads to a highly fluorescent metabolite providing the required sensitivity. This library, characterized by a water-soluble primary sequence Ac-SGK-Pya-$(X)_n$-Nop-GGK-NH_2, X being a mixture of 10 natural amino acids (A, I, L, K, F, W, E, Q, T, P) and *n* varying from 0 to 3, was validated using enzymes belonging to the four main types of hydrolases: serine-, metallo-, cystein-, and aspartyl-proteases. The selectivity of substrates belonging to this library was evidenced by characterizing specific substrates for the isoenzymes NEP-1 and NEP-2. This library easily synthesized is of great interest for the identification and development of selective and specific substrates for still uncharacterized endoproteases.

Large scale sequencing of the human genome and of that of other organisms has shown that 1.8% of proteins belong to the group of proteases [1,2]. These enzymes are classified into four major families, i.e., serine-, metallo-, cystein-, and aspartyl-proteases based on both the involvement of specific amino acids and/or cofactors in their catalytic domains and the significant structural similarities in their active sites. As a whole, they regulate the homeostasis of their physiological endogenous substrate(s) by controlling both their production through maturation and their half-life, by inactivation. These peptide effectors 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. Consequently, inhibition or activation of these proteases could modulate the availability of these peptide messengers, thus providing possible new treatment of pathological states by restoring proper messenger peptide action. This is illustrated by the therapeutic use of angiotensin-converting enzyme (ACE)[1] inhibitors as antihypertensive agents [3], dipeptidyl peptidase (DPPIV) inhibitors in the treatment of type II diabetes [4], neprilysin (NEP) inhibitors as antisecretory agents [5], and viral protease inhibitors as antiretroviral agents [6]. The design and use of potent and selective inhibitors of any given protease, however, requires deep knowledge and understanding of its main structural and functional characteristics. 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

[1] *Abbreviations used:* Abz, *o*-aminobenzoyl; ACE, angiotensin-converting enzyme; AU, arbitrary unit; BoNT/A, botulinum neurotoxin type A; BoNT/B, botulinum neurotoxin type B; DABCYL, 4-((4-(dimethylamino) phenyl)azo)benzoic acid; DCC, dicyclohexylcarbodiimide; Dnp, 2, 4-dinitrophenyl; DPPIV, dipeptidyl peptidase; DIEA, diisopropylethylamine; Dpa, *N*-3-(2,4-dinitrophenyl)-(*L*)-2, 3-diaminopropionyl; ECE-1, endothelin-converting enzyme 1; ECE-2, endothelin-converting enzyme 2; EDANS, 5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid; Fmoc, 9-fluorenylmethoxycarbonyl; FRET, fluorescence resonance energy transfer; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HMP-resin, 4-hydroxymethylphenoxy-resin; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; HTS, high-throughput screening; ICIFQ, internal collision-induced fluorescence quenching; LCMS, liquid chromatography mass spectroscopy; MBHA-resin, methylbenzhydrylamine-resin; Mca, (7-methoxycoumarin-4-yl) acetyl; MMP, matrixin; NEP-1, neprilysin 1; NEP-2, neprilysin 2; NMP, *N*-methylpyrrolidone; Nop, (*L*)-4-nitro-phenylalanine; Pya, (*L*)-pyrenylalanine; SIM, single-ion monitoring; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; X, variable amino acid (A, I, L, K, F, W, E, Q, T, P).

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inhibitors or activators as well as in *in vivo* or *ex vivo* enzymatic assays to study their pharmacological action.

Intramolecularly quenched fluorescent peptides were developed several years ago as synthetic substrates for physiologically active peptidases. Cleavage of these substrates between the repressor and the fluorophore moieties leads to the release of highly fluorescent metabolites. Two physical mechanisms are responsible for the quenching of the fluorescent signal: the fluorescence resonance energy transfer (FRET) [7,8] and the internal collision-induced fluorescence quenching (ICIFQ) [9,10]. In recent articles, examples of such substrates, using (7-methoxycoumarin-4-yl)acetyl (Mca), 6-amino-1-naphtalenesulfonamides, Dansyl (dimethyla minonaphtalene-1 sulfonyl), *o*-aminobenzo (Abz), and 5-(2′-aminoethylamino)naphthalene sulfonic acid (EDANS) as fluorescent moieties and 2,4-dinitrophenyl (Dnp), 4-nitrophenylalanine (Nop), and 4-(4′-dimethylaminophenylazo)benzoic acid (DABCYL) as repressors have been described (reviewed in [11]). However, these fluorophore–repressor pairs must be incorporated at the N- and the C-termini of synthetic peptides, often leading to modest quenching and high background signal and, thus, low sensitivity. To circumvent this problem, the fluorophores have often been introduced on the lateral chains of functionalized amino acids, such as lysine, glutamate, or cysteine, but the size of the substituted lateral chain may hinder enzyme active-site recognition. Large libraries of fluorescent substrates have previously been developed on solid support [12], with a preference for PEGA resin [13], allowing the screening of these libraries by a protease directly on resin-bound peptide substrates [13–16]. Although these libraries allow the screening of a large number of compounds, their immobilization has been shown to alter the natural substrate cleavage site [15].

In order to design high-affinity substrates for the development of easy and sensitive enzymatic assays, we have previously proposed the use of the highly fluorescent synthetic amino acid (*L*)-pyrenylalanine [17] (Pya) and (*L*)-*p*-nitro-phenylalanine (Nop) as a new fluorophore–repressor pair [18]. Both synthetic residues can be incorporated within a peptide sequence at any position by standard solid-phase synthesis and are generally recognized by the peptidase active subsites, despite their large side chains [19]. However, because the quenching through intramolecular collision is dependent on the distance between the Pya and the Nop moieties, they should not be separated by more than 3 to 4 amino acids to ensure maximal quenching and optimal sensitivity. This distance, for an extended conformation of the substrate, however short, remains compatible with the mean size of almost all protease active sites, as shown by crystallographic analysis of protease–inhibitor complexes [20–23]. Accordingly, the Pya/Nop fluorophore/repressor pair has successfully been incorporated in highly selective and sensitive substrates designed for the study of endothelin-converting enzyme (ECE-1) [18,24], botulinum neurotoxin type B (BoNT/B) [25,26], and neurotoxin type A (BoNT/A) [27,28] enzymatic activities.

To further extend this concept to the four major families of hydrolases and to provide an unbiased approach appropriate to substrate identification, we report here the design of a library of peptides containing Pya as fluorophore and Nop as quencher moieties, separated by a short sequence of natural amino acids, allowing their binding within any type of protease active site. The amino acids were chosen to be representative of the different types of residues (acidic, basic, hydrophobic, hydrophilic, and aromatic), and for their ease of introduction by a solid-phase method. The cysteinyl residue was eliminated to avoid disulfide bridge formation. The maximal number of amino acids between Pya and Nop has here been limited to 3. The Pya and Nop residues were flanked by small flexible sequences containing a lysine residue to enhance water solubility and to facilitate mass detection. With the aim of targeting endopeptidase activities and avoiding amino peptidase and carboxy peptidase recognition, the N- and C-terminal residues of the substrates were protected by acetyl and amide groups, respectively. The primary sequence of the designed library is defined as Ac-SGK-Pya-$(X)_n$-Nop-GGK-NH_2, where X is a mixture of 10 among the 20 possible natural amino acids (A, I, L, K, F, W, E, Q, T, and P), and n a number from 0 to 3.

Well-characterized enzymes belonging to each of the four main families of proteases were tested and shown to efficiently hydrolyze specific sublibraries. These sublibraries can then be used to identify high-affinity fluorescently quenched peptides by LCMS. The characterized most efficient substrate can then be synthesized to provide a useful high-throughput assay for inhibitor or activator screening. It could also be used to selectively study the enzymatic *in vivo* or *ex vivo* activities of the target protease. Moreover, we have recently shown that this library could also be used to evidence subtle differences between the active sites of ECE-1/ECE-2 isoenzymes and to propose a selective substrate for ECE-2 [29]. The same type of experiment was performed here with the aim to differentiate NEP-1/NEP-2 active sites, providing structural information on their respective subsite specificity as well as a selective substrate for NEP-2.

Materials and methods

Reagents

Fmoc-protected amino acids, piperidine, *N*-methylpyrrolidone, dichloromethane, dicyclohexylcarbodiimide, 1-hydroxybenzotriazole, and *O*-(7-azabenzotriazol-1-yl) 1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were purchased from Applied Biosystems (Courtaboeuf, France). Fmoc-(*p*-nitro)-(*L*)-phenylalanine and MBHA resin (0.73 mmol/g) were from Novabiochem (Laüfelfingen, Switzerland) and Fmoc-(*L*)-pyrenylalanine (Pya), prepared as previously described [17], was from Polypeptide Laboratories (Strasbourg, France). Trifluoroacetic acid was from SDS-Carlo Erba (France). Triisopropylsilane and the MMP substrate Mca-PLGL-Dpa-AR-NH_2 were from Sigma–Aldrich (Strasbourg, France).

Peptide synthesis

Assembly of the library (Fig. 1) was carried out using the split and mix method [30]. First, a large amount of Nop-GGK-MBHA-resin was prepared using a large scale (10×0.25 mmol, 10×342 mg) of MBHA resin on a ABI 433 automated synthesizer (Applied Biosystems) coupled to a programmable absorbance detector ABI 785A and HBTU/HOBt as coupling reagents. The peptidyl-resin was divided in 11 sublibraries and one sublibrary (~0.25 mmol) was extended through SPPS by introduction of the sequence Ac-SKGPya, leading to the substrate corresponding to $n = 0$. The 10 other samples were coupled with 1 mmol (4 eq of amino acid per equivalent of resin) of either Fmoc-N-protected A, I, L, K, F, W, E, Q, T or P, respectively. This coupling reaction and all the following steps were performed on a Symphony apparatus (a 12 reactor peptide synthesizer from Protein Technology Inc., USA) and HBTU/DIEA in NMP as coupling reagents. At the end of the coupling reaction (45 min) and after deprotection of the N-terminal amino acid, these 10 sub-sublibraries were mixed and split in 11 equal new sub-sublibraries, each of them containing the same ratio of the 10 selected amino acids. One was extended with the Ac-SKG-Pya sequence, giving a sublibrary of 10 peptides with one residue between Pya and Nop ($n = 1$). The 10 other sub-sublibraries were coupled separately with either Fmoc-N-protected A, I, L, K, F, W, E, Q, T or P. At the end of the coupling reaction followed by deprotection of the N-terminus, each sub-sublibrary was again separated in 11 samples, one of them being extended by Ac-SKG-Pya and corresponding to one of the 10

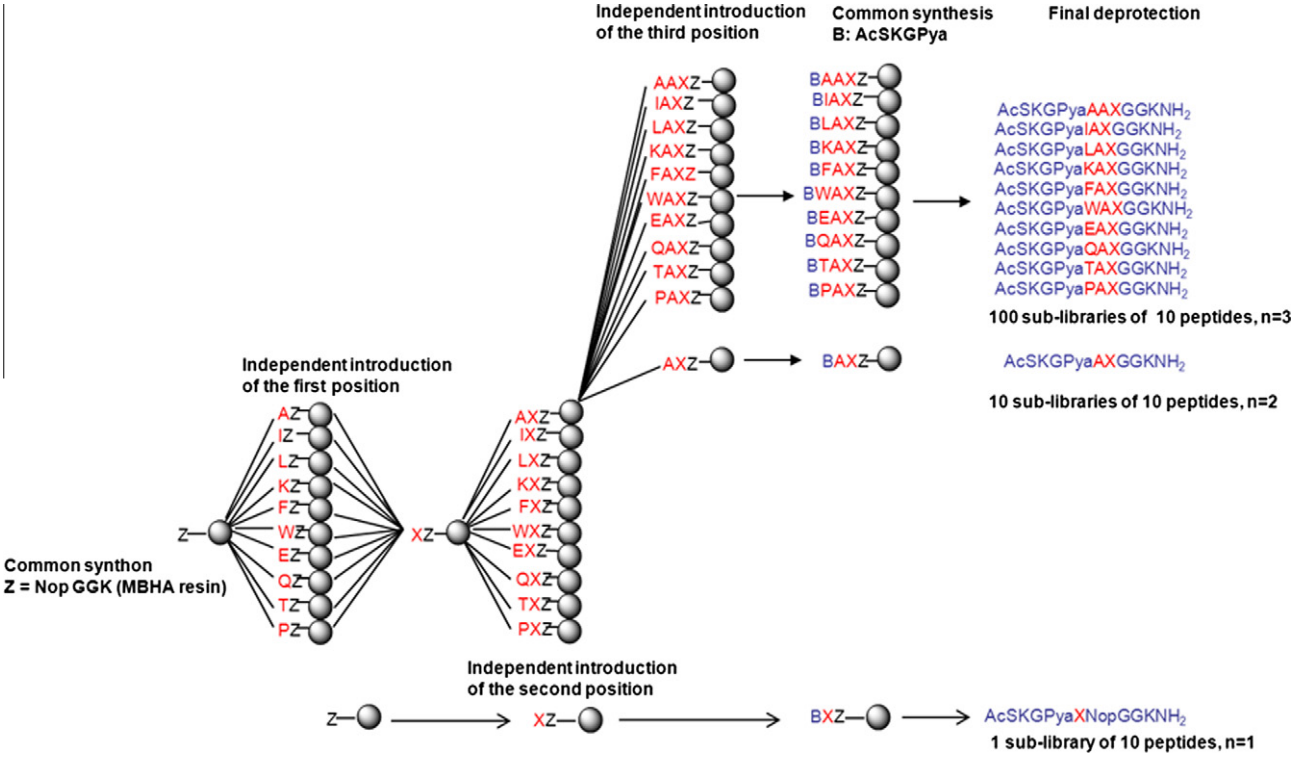

Fig.1. Synthesis of intramolecularly quenched fluorescent substrate library. Assembly of the library was performed by a split and mix method according to the SPPS technique using standard Fmoc chemistry. Each amino acid was coupled independently to avoid deletion. Common primary sequence of the library is Ac-SKG-Pya-$(X)_n$-Nop-GGK-NH_2, where X = Ala, Ile, Leu, Lys, Phe, Trp, Glu, Gln, Thr, or Pro and n = 0, 1, 2, or 3 residues. (*L*)-Pyrenylalanine (Pya) and *p*-nitro-(*L*)-phenylalanine (Nop) were used as fluorescent and quencher moieties, respectively. Pya and Nop are flanked by two short flexible sequences enhancing the solubility of the substrates and the detection of the metabolites by both HPLC and mass spectroscopy. Only one position was allowed to vary. The library consists of 1 peptide for n = 0 and110 sublibraries of 10 different peptides.

n = 2 sublibraries (two residues between Pya and Nop). The 10 other samples were coupled with one of the 10 selected amino acids and then extended by Ac-SKG-Pya to finalize the sublibraries with n = 3 sublibraries.

For each sublibrary, a ~0.25 mmol scale was used to obtain from 10 to 20 mg of final material. They were independently analyzed by LCMS, to obtain both an optimal separation of the different peaks and a determination of the corresponding peptide weights, allowing the attribution of each HPLC signal to a single peptide substrate of the sublibrary (for an example, see Fig. 2).

Enzymatic assays

Neprilysin-1 (EC 3.4.24.11), purified from rabbit kidney [31] (0.146 mg/ml, activity = 345.1 nmol of DGPA/mg/min) was used in 50 mM Tris–HCl buffer, pH 7.4, at 200 ng/ml. Recombinant, secreted forms of human neprilysin-1 and -2, from R&D Systems, were used at 100 ng/ml in 50 mM Tris–HCl buffer, pH 7.4, and at 100 mM Hepes, pH 7.2, respectively.

MMP-9 (EC 3.4.24.35) from R&D systems was activated with 1 mM *p*-aminophenylmercuric acetate for 4 h at 37 °C and then used at 100 ng/ml in 50 mM Tris–HCl buffer, pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij 35. Active human recombinant MMP-7 (EC 3.4.24.23) from Calbiochem was used at 100 ng/ml in Tris buffer 50 mM, pH 7.4, $CaCl_2$ 10 mM, NaCl 150 mM. Porcine gastric mucosa pepsin (EC 3.4.23.1), purchased from Sigma, was used at 100 ng/ml in sodium citrate buffer 50 mM, pH 3.0. Carica papaya papain (EC 3.4.22.2) (25 mg/ml) from Sigma was used at 25 μg/ml in 50 mM MES-OH, pH 6.0 containing 2 mM EDTA and 2 mM dithiothreitol (DTT). Porcine pancreas Kallikrein (EC 3.4.21.35) from Sigma (40 U/mg) was diluted 20 times in 20 mM Tris–HCl buffer, pH 8.7. Bovin pancreas trypsin (EC 3.4.21.4) (1 mg/ml), from Sigma, was used at 1 μg/ml in 50 mM Tris–HCl buffer, pH 8.0, containing 1 mM $CaCl_2$. HIV-1 protease (retropepsin EC 3.4.23.16), from Calbiochem, was used at 5 μg/ml in 1 M sodium acetate buffer, pH 4.7, with NaCl 1 M, EDTA 1 mM, DTT 1 mM, and BSA 1 mg/ml. Using these conditions, hydrolysis of the substrate sublibraries by each enzyme was studied in parallel by fluorescence and by HPLC coupled to either fluorescence detection or mass spectrometry.

Each sublibrary containing equimolar quantities of 10 different peptides was used at a concentration of 100 μM (i.e., 10 μM of each peptide), while the pure Ac-SKG-PyaNop-GGK-NH_2 peptide (corresponding to n = 0) was used at a final concentration of 10 μM. All the reactions were performed in half 96-well low binding black plates in a final volume of 100 μl at a temperature of 37 °C, for times varying, depending on the enzyme tested, between 10 and 30 min, in order to remain as close as possible to initial rate conditions. Direct fluorescent monitoring of substrate hydrolysis was performed using a multiwell plate-reader fluorimeter (Berthold Series Twinkle LB 970 coupled to Mikrowin 2000 software) with excitation at 340 nm, emission at 405 nm, and lamp energy at 10,000. For subsequent HPLC and mass analysis, the reactions were stopped by the addition of 10 μl of 6 N HCl. Every experiment was performed at least twice independently, in duplicate.

The fluorescent signal obtained as a result of the cleavage of the commercial nonspecific MMP-substrate (7-methoxycoumarin-4-yl) acetyl-PLGL-(3-[2,4-dinitrophenyl]-2,3-diamino-propionyl)-AR-NH_2 was compared to that obtained using Ac-SKG-Pya-Pro-Leu-Gly-Leu-Nop-GGK-NH_2 using MMP-9. This assay was performed under the above described conditions, but using 10 μM of either substrate. Fluorescence excitation and emission spectra of Mca-substrate showed maxima at 324 and 393 nm, respectively [32].

Spectrophotometric measurements

Ultraviolet absorption spectra were recorded on a Shimadzu spectrophotometer UV mini 1240. The peptides were studied at 10 μM in Hepes buffer 50 mM, pH 7. Spectrofluorimetric spectra of purified peptides were measured using a Perkin-Elmer LS50B fluorimeter (λex = 343 nm) equipped with a thermostated cell holder for a comparison of the fluorescence spectra of a substrate and of its fluorescent metabolite. The fluorimetric properties of various sublibraries and of particular metabolites were studied under the same experimental conditions as those described for the enzymatic assays, i.e., using a Twinkle plate fluorimeter (Berthold) with excitation and emission wavelengths at 340 and 405 nm, respectively.

HPLC analysis and fluorescence detection

Enzymatic assays were analyzed by HPLC (Shimadzu Prominence LC-20AB) using both UV and fluorescence detectors. For UV measurements, two wavelengths were used, 210 and 343 nm, this later wavelength corresponding to the λmax of Pya-containing peptides [17,33]. For fluorescence HPLC detection, the excitation wavelength was 343 nm and the emission spectra wavelength was 377 nm. The samples (100 μl) were injected on a Phenomenex Kinetex C18 column (2.6 μm, 100 Å, 2.1 × 100 mm). Elution was carried out for 30 min using a gradient of eluent B (eluent A, H_2O, 0.1% TFA; eluent B, CH_3CN, 0.1% TFA) at a flow rate of 0.2 ml/min.

LCMS analysis

For each 10 peptide sublibrary tested, the masses of both the substrates and the metabolites were determined by an Agilent Quadripole 6120 spectrometer using positive electrospray ion source in a mass range of 250 to 1500 amu. Electrospray voltage was set at 3 kV, nozzle voltage at 32 V and nozzle temperature at 350 °C. Data acquisition and data evaluation were performed using Chemstation instrument manufacturer's software (Agilent). The spectrometer was coupled to an LC 1200 liquid chromatography series system from Agilent. Sample preparation was identical to that described above for fluorescent studies and 3 μl of sample was injected on a Phenomenex Kinetex C18 column (2.6 μm, 100 Å, 2.1 × 100 mm). Elution was carried out for 30 min using a 10–40% gradient of eluent B (eluent A, H_2O (0.1% TFA; eluent B, CH_3CN (0.1% TFA) at a flow rate of 0.2 ml/min).

HPLC analyses and mass spectroscopy were carried out under almost similar conditions to characterize metabolites. The retention times of the substrates and fluorescent metabolites, depending essentially on the presence of the Pya moiety, were generally very close, whereas the Nop-containing metabolites, being less hydrophobic, eluted more rapidly. Mass analysis was required to identify both the position of each substrate in the chromatograms of the different sublibraries and the primary sequence of the formed metabolites. It was carried out with a spectrometer possessing an orthogonal electrospray ionization source, which increases sensitivity and reduces source contamination. Moreover, all potential substrate and metabolite masses were calculated and their presence or lack was thereof verified using the SIM mode. In that case, only specific m/z ratio corresponding to a given substrate or metabolite was selected. These data reflect the decrease of substrates and/or increase of specific metabolite(s) as a result of enzyme hydrolysis. However, when the cleavage sites or the formed metabolites were difficult to characterize, a complete deconvolution of the tested 10-peptide sublibrary was achieved and the cleavage of the 10 peptides tested independently.

Results

Synthesis

The synthesis of the library was carried out by solid-phase peptide synthesis (SPPS) on MBHA resin, using the split and mix method [30]. This methodology is schematically depicted in Fig. 1, and described in detail in under Materials and methods.

For the libraries corresponding to $n = 2$ and 3, a partial deconvolution of the mixtures is directly performed on the resin to facilitate the analysis of the enzymatic degradation: only one X position, over the two or three possible ones, is a mixture of 10 amino acids, the others containing a defined amino acid chosen between the selected residues. This means that each sublibrary contains 10 peptides that differ by a single amino acid at one selected position (Fig. 1). Consequently, the library consists of a single peptide for $n = 0$, one sublibrary of 10 peptides for $n = 1$, 10 sublibraries of 10 peptides when $n = 2$, and 100 sublibraries of 10 peptides when $n = 3$. Thus 1111 peptides were obtained for the complete library.

Spectroscopic properties of a given substrate of the library (UV, fluorescence)

The UV spectra of the Ac-SKGPya-NopGGK-NH_2 ($n = 0$) substrate and of its fluorescent metabolite Ac-SKGPya displayed a maximal absorption wavelength at 343 nm, characteristic of the Pya moiety, with ε mol of 27,600 and 35,700 L cm^{-1} mol^{-1}, respectively (Fig. 3).

The fluorescent emission spectra, recorded on a Perkin-Elmer LS50B fluorimeter, of Ac-SKGPya after excitation at λex = 343 nm showed two intense absorption peaks at 377 and 398 nm. Under the same conditions, the emission spectrum of the substrate revealed that the fluorescence of the pyrenylalanine was practically entirely quenched by Nop, as expected from their spatial proximity [26]. The ratio between the fluorescence intensities of the metabolite and the substrate, at 377 nm, was $F_{0(M)}/F_{0(S)} = 997.8/8.54 = 116$ (Fig. 3).

Fluorimetric characteristics of the library

In the ICIFQ method, the fluorescence quenching is due to intramolecular collisions between the fluorophore (Pya) and the repressor (Nop). Therefore, the sensitivity of the substrate will be dependent on the spatial proximity between the fluorophore and the repressor as well as on the conformational flexibility of the peptide sequence governing the number of fluorophore–repressor collisions. Moreover, sensitivity will also be a function of the intensity of the fluorescent signal emitted by the released metabolite.

To address these issues, we determined the fluorimetric parameters of the smallest substrate Ac-SKGPya-NopGGKNH_2 ($n = 0$) and of its fluorescent metabolite Ac-SKGPya and then compared these values to those obtained with the various peptide sublibraries ($n = 1$, 2, or 3). Basal fluorescence (F_0), measured on a multiwell plate-reader fluorimeter (Berthold Series Twinkle LB 970 (λex = 340 nm; λem = 405 nm), of the various peptide sublibraries in Hepes buffer, pH 7 (10 μM), was then determined and compared. These experiments showed that while the $n = 0$ peptide displayed an F_0 value of 56 AU, a significant increase could be observed with the $n = 1$ sublibrary with an F_0 of 340 AU. For the $n = 2$ sublibraries, a small decrease in the later F_0 could be observed, with values of about 200 ± 9 AU, whereas these values decreased again to 150 ± 5 AU for the $n = 3$ sublibraries (data not shown). These results suggest that the basal fluorescence of a given peptide and of the peptide sublibraries is dependent on the populations of conformers in which Pya and Nop are in close proximity. Three exceptions were

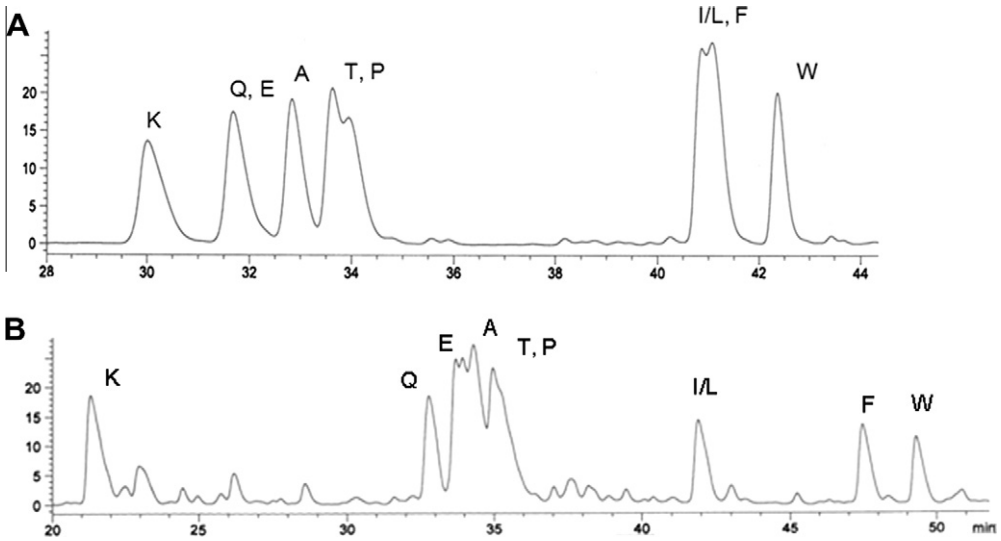

Fig.2. LCMS analysis of peptide sublibraries. (A) LCMS analysis of Ac-SKG-PyaEXNop-GGKNH$_2$ sublibrary. Each component of the Ac-SKG-Pya-EX-Nop-GGK-NH$_2$ 10 peptide sublibrary was identified by LCMS analysis on a Phenomenex Kinetex C18 column 100 × 2.1 mm, 100 Å, 2.6 μm, using a 10–40% CH_3CN(0.1% HCOOH)/H_2O(0.1% HCOOH) elution gradient in 60 min, at a 0.2 ml/min flow rate, on an electrospray Agilent 1200 mass spectrometer. (B) LCMS analysis of Ac-SKG-PyaXNop-GGK-NH$_2$ sublibrary. The experiment was performed as in panel A.

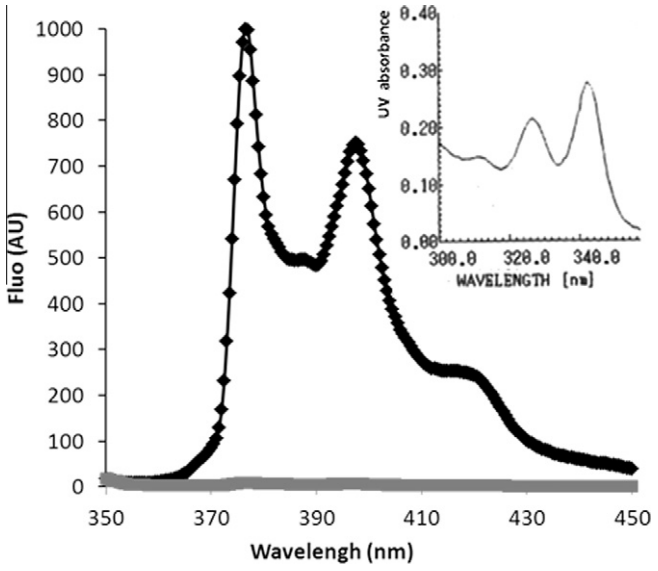

Fig.3. Spectra of Ac-SKG-PyaNop-GGK-NH$_2$ and its Ac-SKG-Pya metabolite. Fluorescence emission spectra of the substrate (1 μM) (gray) and fluorescent metabolite (1 μM) (black) were obtained using a Perkin Elmer LS970B fluorimeter, λex = 343 nm, Slot ex = 15; Slot em = 2.5. Insert: Ultraviolet spectrum of Ac-SKG-PyaNop-GGK-NH$_2$. The UV spectrum of the substrate (10 μM in 50 mM Hepes, pH 7) was recorded on a Shimadzu 1240 UV mini spectrophotometer.

found: first, every sublibrary containing a tryptophan residue displayed a basal fluorescence inferior to 90 AU whatever the nature of the other surrounding residues. This is probably due to the additional fluorescence quenching between Trp and Pya. Moreover, the sublibraries, containing Lys or Pro residues, showed significantly greater basal fluorescence than those in the corresponding series, probably due to charge repulsion and/or Pro-dependent unfavorable conformations for fluorescence quenching (data not shown).

Cleavage of a peptide within a sublibrary releases a fluorescent metabolite in which the Pya fluorophore will be located in different positions. Indeed, it can either be positioned at the C-terminal end of the metabolite or be followed by one, two, or three amino acids, depending on the size of *n*. To determine the influence of these amino acids on the fluorescent signal of Pya, the basal fluorescence of various metabolites (1 μM) in a Hepes buffer solution at 37 °C was measured.

Thus, the fluorescent signal of the shortest possible fluorescent metabolite Ac-SKGPya was found to be 2430 AU while Ac-SKGPyaPLG, for instance, emitted a fluorescent signal of 1440 AU (data not shown). In all cases, a highly significant difference in the intensities of the fluorescent signal emitted by the substrates and metabolites was evidenced, thus providing high sensitivity to this new library of quenched peptides.

LCMS and fluorescence detection

HPLC analysis of each sublibrary, with UV detection at 343 nm (maximum absorption of Pya) detects the peaks corresponding to the 10 peptides for the libraries with *n* = 1 and 2, which differ only by one amino acid (Figs. 2A and B). A similar type of elution profile was obtained with all sublibraries: the first eluted peak corresponding to the X = K, followed by a series of peptides in which X corresponds to neutral, hydrophilic, or small hydrophobic residues (A, E, T, Q, and P) and finally the large aromatic or hydrophobic residues (I, L, F, and W) (Fig. 2). Mass spectrum analysis allowed the attribution of a single peptide for each peak except in the case of X = L/I since they share the same isotopic mass.

After enzyme degradation, chromatograms obtained with the fluorescence HPLC detector revealed a large increase of fluorescence due to the formation of a series of metabolites, possessing the unquenched Pya moiety, whose retention times are almost identical to those of the corresponding substrates. Every potentially generated metabolite was searched for using the predetermined isotopic mass monitoring (SIM mode). The nonfluorescent metabolites, containing Nop residues, having shorter retention times were detected by UV at 210 nm and characterized by the same method.

Enzyme hydrolysis

The 111 peptides corresponding to the sequence Ac-SKGPya-$(X)_n$-NopGGK-NH$_2$, with *n* = 0, 1, and 2, were tested using well-

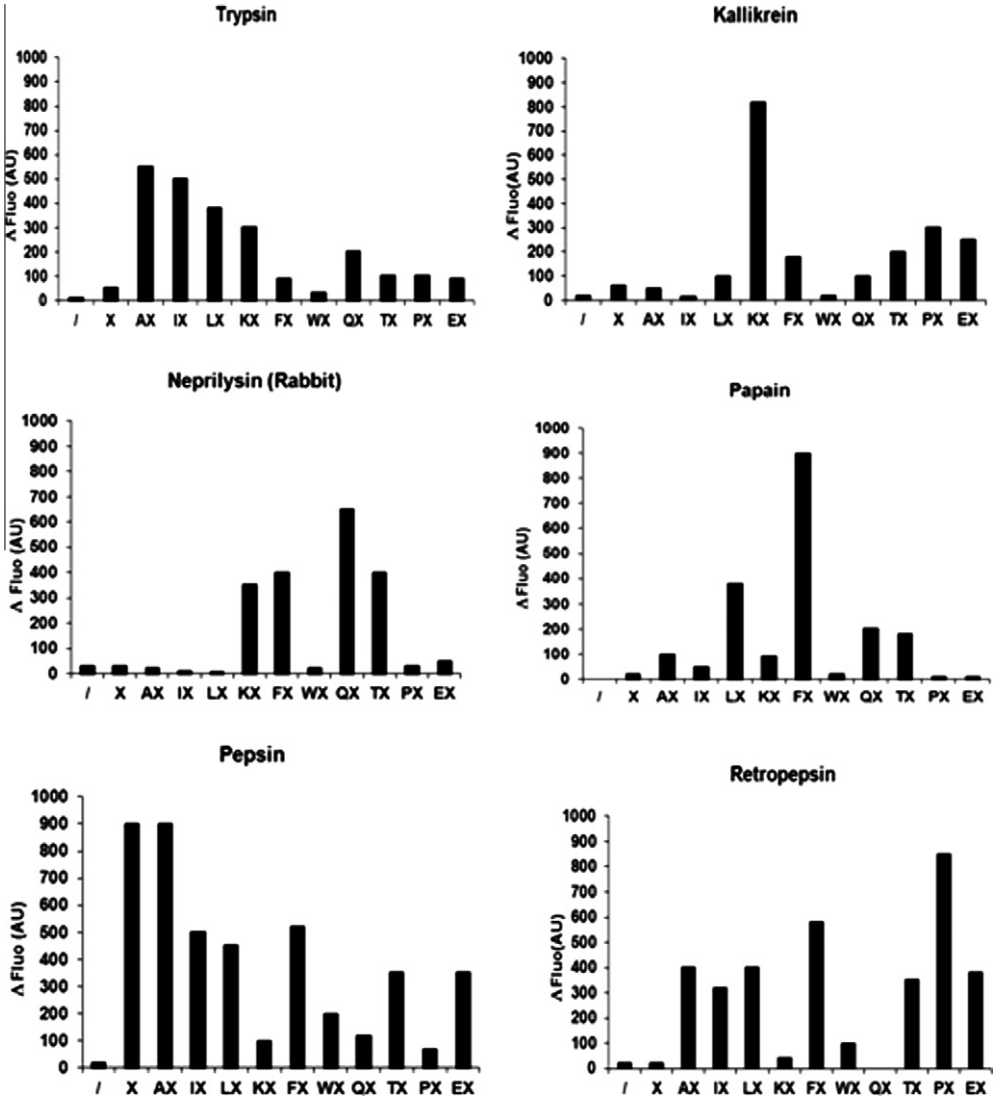

Fig.4. Peptidase hydrolysis of quenched fluorigenic substrate sublibraries. Enzymes belonging to the four main families of proteases were studied (serine protease, trypsin; kallikrein; zinc metallopeptidase, neprilysin; cysteine protease, papain; aspartyl protease, pepsin; retropepsin). Enzymatic assays were carried out in 96 half-well microplates as described under Materials and methods and results obtained with the $n = 0$, 1, or 2 sublibraries are presented. Horizontal axis represents the name of the sublibrary, "/" symbolizes no (0) residues between Ac-SKGPya and NopGGK-NH_2, X symbolizes a mixture of equimolar amount of 10 amino acids between Ac-SKGPya and NopGGKNH_2, and AX, IX, LX, KX, FX, WX, QX, TX, PX, EX correspond to a known residue placed after Ac-SKGPya followed by a mixture of equimolar amounts of 10 amino acids and then NopGGK-NH_2. Vertical axis is the delta of fluorescence obtained by subtracting the signal obtained after incubation of the sublibrary with heat-denatured enzyme from the total value of fluorescence measured after enzymatic hydrolysis, in arbitrary units (AU) of fluorescence (λex = 340 nm and λem = 405 nm). The histograms presented are from a single representative experiment (no signal was measured with "/" and papain and with QX and retropepsin).

known enzymes belonging to the four main types of hydrolases (Fig. 4).

The first interesting point is that every enzyme tested was able to cleave at least one or several sublibraries, bringing to light both the very large adaptability of this library and the broad specificity of most of these hydrolases (Fig. 4). Under the conditions used herein, the chosen enzymes did not cleave the peptide corresponding to $n = 0$, whereas the $n = 1$ sublibrary was efficiently cleaved only by pepsin, an aspartic protease. The 10 sublibraries of $n = 2$ peptides revealed distinct enzymatic profiles for each class as well as for each enzyme within these classes. Indeed, different profiles were obtained for the two serine proteases tested, i.e., trypsin and kallikrein, as well as by two aspartic proteases, pepsin and retropepsin. The sublibraries were also differentially degraded by neprilysin-1 (rabbit kidney enzyme), a metalloprotease, as well as by papain, a cysteine protease (Fig. 4).

Analysis of the fluorescent degradation products of the sublibraries by LCMS was then performed and these results are reported in detail for two examples, i.e., kallikrein and pepsin. Fig. 4 shows that kallikrein preferentially cleaved the Ac-SKG-Pya-KX-Nop-GGK-NH_2 sublibrary. Analysis of the latter by HPLC coupled to fluorescent detection reveals that a single fluorescent metabolite is formed Ac-SKG-Pya-K ($[M + H]^+ = 732.8$). This result suggests that the major cleavage site is located between the K and the X residues. Moreover, the detection of the two nonfluorescent metabolites, K-Nop-GGK-NH_2 ($[M + H]^+ = 747.6$) and T-NopGGK-NH_2 ($[M + H]^+ = 500.2$), revealed that Ac-SKG-Pya-KK-Nop-GGK-NH_2 and Ac-SKG-Pya-KT-Nop-GGK-NH_2 were the two substrates cleaved. These results are consistent with previous data demonstrating that this enzyme has a relatively restricted selectivity, cleaving peptides containing a bulky hydrophobic residue in the P_2 position (according to the nomenclature of Schechter and Burger [34]), a basic residue in P_1 [35], and a basic or hydrophilic residue in P_1' [36]. Moreover, Pya is well accepted into the S_2 subsite of Kallikrein.

Pepsin [37] was shown to be the only enzyme able to significantly cleave the $n = 1$ sublibrary (Fig. 4). The LCMS analysis of

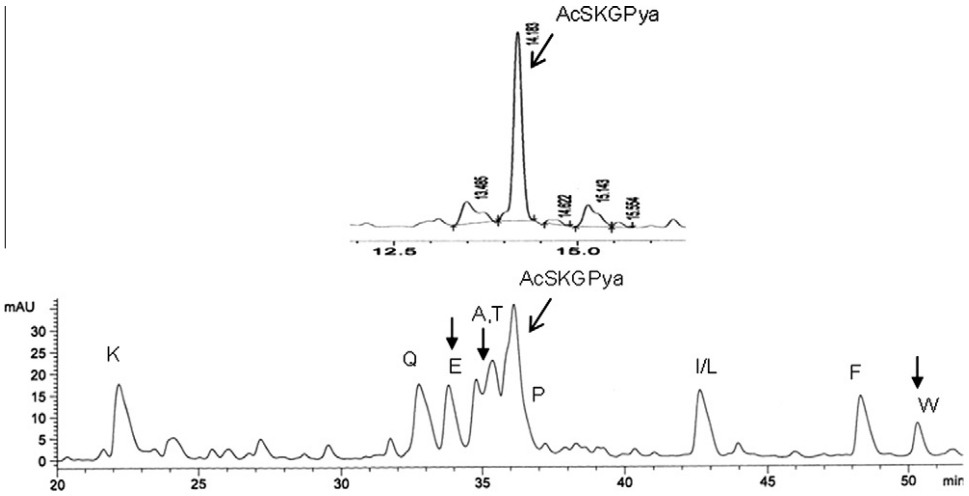

Fig.5. LCMS analysis of Ac-SKG-PyaXNop-GGK-NH_2 incubated with pepsin. Analysis by LCMS of the Ac-SKG-Pya-X-Nop-GGK-CO-NH_2 peptide sublibrary (100 μM) incubated for 1 h at 37 °C with 5 μg/ml of pepsin in a final volume of 100 μl of sodium citrate buffer 50 mM, pH 3.0. Identification of the different peaks was performed by LCMS. Top: Ac-SKG-Pya-X-Nop-GGK-NH_2 (100 μM) was incubated with native pepsin (5 μg/ml) for 1 h at 37 °C. At the end of the reaction, fluorescence was measured using a plate-reader fluorimeter and 5 μl of the sample, after 100 fold dilution, was analyzed by HPLC coupled with fluorescence detection (λex = 343 nm and λem = 377 nm) on an ACE C_{18} column 250 × 4.6 mm, 5 μm, 100 Å using a 10–90% CH_3CN (0.1% TFA)/H_2O (0.1% TFA) elution gradient in 30 min, at a 1 ml/min flow rate. A single fluorescent metabolite corresponding to Ac-SKGPya (attributed by LCMS) was detected. Down-pointing arrows (↓) identify peaks decreasing in intensity.

the cleavage products of this sublibrary revealed the presence of a single major fluorescent metabolite Ac-SKG-Pya and various nonfluorescent peptides, corresponding to cleavages at the Pya↓W, Pya↓A and Pya↓E bonds in a decreasing order of magnitude (Fig. 5).

Likewise, trypsin, papain, retropepsin, and neprilysin displayed hydrolytic profiles (not shown) corresponding to their respective specificities: trypsin hydrolyzed various substrates between K and Nop moieties, in agreement with its preference for a basic amino acid in P_1 position [38]. Papain preferentially cleaved the -Pya-FX-Nop- sublibrary between X and Nop when X was K, as expected from the high selectivity of this protease for an F residue in P_2 and R/K in P_1 positions [39]. Retropepsin preferentially hydrolyzed the sublibrary containing the -Pya-PX-Nop- sequence at the Pya↓P scissile bond, with X corresponding to I, L, and F. This result is consistent with the preference of retropepsin for the cleavage of the sequence Tyr (Phe)/Pro-Ile found at the junction of MA/CA, p6/PR, and PR/RT in the Gag polyprotein [40]. Finally, neprilysin-1 (rabbit kidney enzyme) efficiently cleaved four sublibraries, i.e., -Pya-KX-Nop-, -Pya-FX-Nop-, -Pya-QX-Nop-, and -Pya-TX-Nop-, between X and Nop, leading to various fluorescent metabolites but only one nonfluorescent fragment, Nop-GGK-NH_2. These results confirmed the preference of neprilysin for an aromatic residue in P1′ and a small residue in P_2' [41–43].

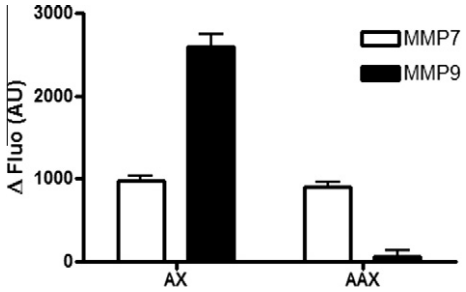

Fig.6. Analysis of different peptide sublibraries incubated with MMP-7 and MMP-9. The two peptide sublibraries (100 μM) corresponding to Ac-SKGPya-AX-NopGGK-NH_2, –Pya-AAX-Nop–, were incubated with MMP-7 (100 ng/ml) (white) or MMP-9 (100 ng/ml) (black) for 60 min at 37 °C. At the end of the reaction, the fluorescent signal was measured as described under Materials and methods (λex = 340 nm and λem = 405 nm).

Among zinc-dependent metallopeptidases, matrixin MMPs represent a very particular family of enzymes with a relatively broad specificity. Therefore, it was interesting to test some of the Pya/Nop sublibraries using these enzymes. Taking into account the consensus cleavage sequence of MMPs, Gly (Ala)↓Leu the sublibraries -Pya-AX-Nop-, and -Pya-AAX-Nop- were tested using two MMPs, recombinant MMP-7 and MMP-9. Both enzymes cleaved the Pya-AX-Nop sublibrary at the A–X bond, when X was either Leu or Ile (Fig. 6)): indeed a single fluorescent metabolite Ac-SKG-Pya-A ($[M + H]^+$ = 675.6) and a single nonfluorescent fragment L(I)-GGK-NH_2 ($[M + H]^+$ = 565.6) were observed, evidencing that this sublibrary was similarly cleaved by both MMPs. In contrast the -Pya-AAX-Nop- sublibrary was found to be cleaved only by MMP-7. The LCMS analysis of the cleavage products reveals a unique cleavage leading to the production of the fluorescent metabolite Ac-SKG-Pya-A $[M + H]^+$ = 675.6 issued from the cleavage of the Ac-SKG-Pya-A↓AW-Nop-GGK-NH_2 substrate (Fig. 6), bringing to light unsuspected differences in the specificities of the S_2' subsites of these proteases.

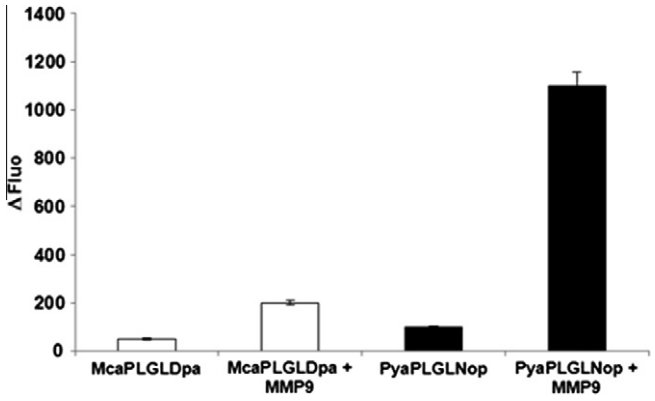

Fig.7. Comparison of the fluorescent signal induced by MMP-9 cleavage of Ac-SKG-PyaPro-Leu-Gly-Leu-Nop-GGK-NH_2 or Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2. Comparison of the fluorescent signal measured after MMP-9 cleavage of the commercial Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (McaPLGLDpa) substrate compared with that of Ac-SKGPyaPLGLNopGGK-NH_2 (PyaPLGLNop). LCMS analysis confirmed the nature of the scissile bond, between the Gly and the Leu residues in both substrates.

Another interesting point was to compare the sensitivity of the new fluorophore–repressor pair Pya-Nop with that of the commercially available, nonselective fluorescent substrate of MMPs containing the methoxycoumarin fluorescent moiety Mca-PLGL-Dpa-AR-NH_2 [32]. With this aim we synthesized the Ac-SKGPya-PLGL-NopGGK-NH_2 peptide containing the same cleavable sequence. As shown in Fig. 7, the Pya-containing substrate was hydrolyzed by MMP-9 giving rise to 1136 fluorescent AU, while under the same enzymatic conditions, the Mca-containing substrate led only to 204 fluorescent AU. Thus, hydrolysis of the Pya-containing substrate was characterized by an 18-fold increase of the signal while hydrolysis of the Mca-substrate gave only a 5-fold increase (Fig. 7). LCMS analysis of the enzymatic reaction products was carried out to confirm that the hydrolysis of Ac-SKGPyaPLGLNopGGK-NH_2 led to the generation of the expected metabolites. Indeed, the identification of both Ac-SKGPyaPLG-OH ($[M + H]^+ = 871.8$) and LNopGGK-NH_2 ($[M + H]^+ = 565.6$) cleavage products shows that the introduction of the Pya/Nop pair did not modify substrate binding and hydrolysis within the active site of the enzyme. This result also emphasizes the high sensitivity of the Pya/Nop fluorophore/repressor pair, even when separated by four residues.

Finally, the $n = 2$ sublibraries were screened in parallel using two closely related proteases, i.e., neprilysin-1 (NEP-1) and neprilysin-2 (NEP-2). As shown in Fig. 8A, almost every tested sublibrary was cleaved by both enzymes. Recombinant NEP-1 was more efficient than recombinant NEP-2, for the cleavage of almost every sublibrary except -EX-, which appeared exclusively cleaved by NEP-2 (Fig. 8A). Analysis of the cleavage products formed after incubation of the -EX- sublibrary with NEP-2 showed a significant

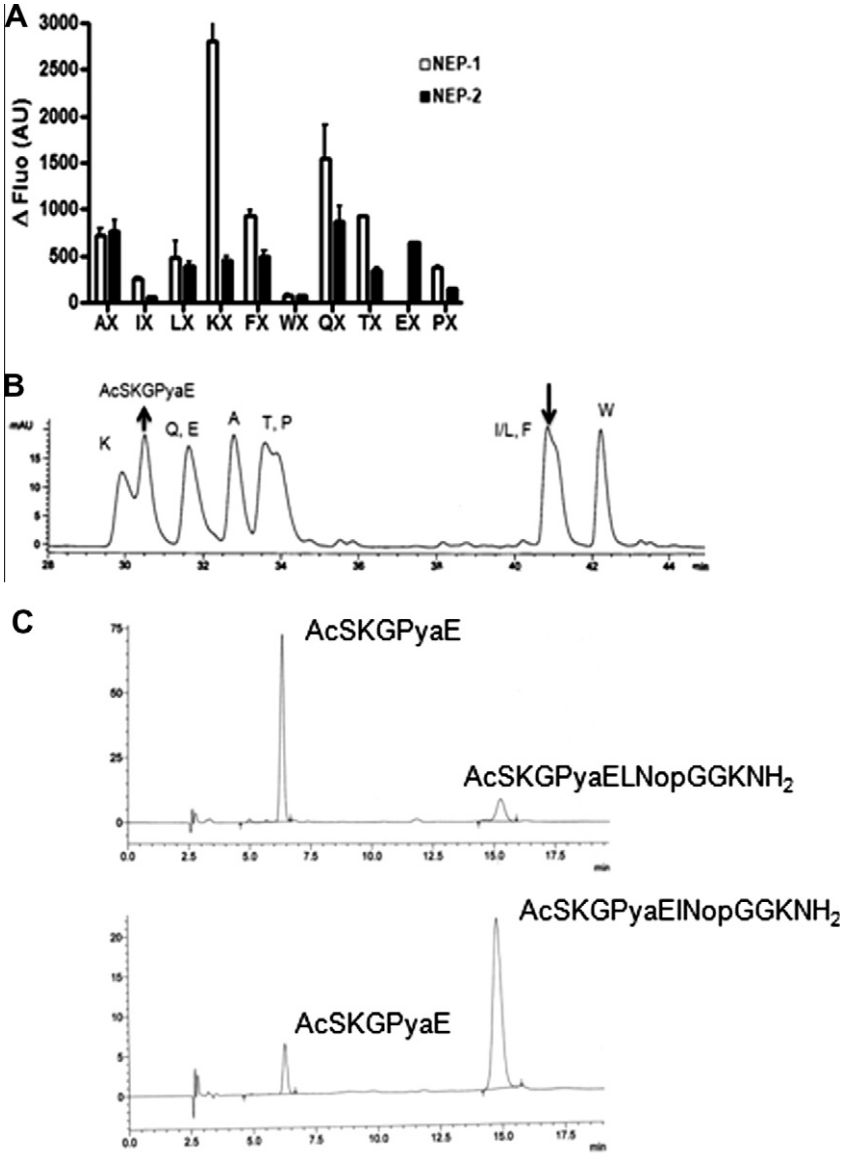

Fig.8. NEP-1 and NEP-2 enzymatic activities. (A) Comparative NEP-1 and NEP-2 enzymatic activities of the Ac-SKG-Pya-$(X)_n$-Nop-GGK-NH_2 $n = 2$ peptide sublibraries. Recombinant NEP-1 or NEP-2 at 100 ng/ml was incubated for 30 min at 37 °C with each sublibrary (100 μM) in a final volume of 100 μl as described under Materials and methods (λex = 340 nm and λem = 405 nm). (B) LCMS analysis of the Ac-SKG-Pya-EX-Nop-GGK-NH_2 peptide sublibrary. Analysis by LCMS of the Ac-SKG-Pya-EX-Nop-GGK-NH_2 peptide library (100 μM) incubated at 37 °C with 10 ng/ml of NEP-2 in a final volume of 100 μl as described under Materials and methods. The attribution of the unique fluorescent metabolite (AcSKGPyaE) was established by LCMS. Arrows point to peaks decreasing (↓) or increasing (↑) in intensity. (C) Comparative HPLC analysis of the enzymatic reaction of Ac-SKG-Pya-El-Nop-GGK-NH_2 and Ac-SKG-Pya-EL-Nop-GGK-NH_2 with NEP-2. The two peptides were incubated with NEP-2 (100 ng/ml) at 37 °C. At the end of the reaction, fluorescence was measured using a plate fluorimeter. A single fluorescent metabolite corresponding to AcSKGPyaE (attributed by LCMS) was detected, and its evaluated quantity shows that the first peptide was the most efficiently cleaved.

decrease in the peaks corresponding to -EI- and/or -EL-, and the apparition of a single fluorescent peak whose molecular weight ($[M + H]^+ = 733.8$) corresponds to Ac-SKGPyaE, indicating that the cleavage site is between E and I/L (Fig. 8B). The two peptides, Ac-SKGPya-EI-Nop-GGK-NH_2 and Ac-SKGPya-EL-Nop-GGK-NH_2, were synthesized separately and tested as NEP-2 substrates. Fig. 8C shows that the second peptide is significantly better than the former. It could be concluded, from these data, that NEP-2 recognized preferentially a hydrophobic aliphatic residue on S_1' and an aromatic one in S_2' positions, respectively, whereas NEP-1 preferred an aromatic residue in S_1' and a small residue in S_2' positions, respectively. Moreover, the glutamate residue E does not seem essential for NEP-2 recognition but seems inappropriate in the S_2 position of NEP-1.

Discussion

The aim of this work was to develop a substrate library for the detection and characterization of protease activities and differentiation of their eventual isoforms with a high sensitivity and selectivity, using the concept of intramolecularly quenched fluorescent substrate. One of the first advantages of this library is its easy synthesis, since both the fluorophore and the repressor moieties are introduced in the peptide sequence by a solid-phase method, like natural amino acids, and large quantities of the sublibraries could be obtained. Moreover specific substrates are very useful for both *in vitro* and *in vivo* studies. The use of the Pya/Nop fluorophore/repressor pair represents a technological progress thanks to its high fluorescent quenching factor [26]. Another interest of the library lies in the SKG and GGK flanking sequences providing water solubility, enhancing mass spectrometry detection, and affording sufficient length for recognition of peptidases with extended active sites. Nevertheless one limitation of this approach lies in the case of allosteric-like enzymes which are characterized by exosites located more or less distantly from the catalytic site. Exosite binding generally confers very high specificity to physiological substrates. This is illustrated by clostridial enzymes such as tetanus and botulinum toxins which cleave only one substrate belonging to the group of exocytosis complex [17,28]. It is, however, interesting to observe that even in these selective peptidases, the active site is sufficiently large to accommodate the Pya-Nop sequence leading to very sensitive substrates [18,24–29]. Moreover, short peptide substrates containing the Pya/Nop pair and unable to bind known exosites of BoNT/A were nevertheless found to possess Michaelis constants (K_m) many hundred-fold superior to that of its only physiological substrate, SNAP-25 [28].

The chosen amino acid diversity, reduced here to 10 representative amino acids, gives rise to clean, crude sublibrary mixtures, thus simplifying LCMS analysis, which can be performed without additional purification. A complete library made of the 20 natural amino acids could be synthesized, thus avoiding a putative bias due to an eventual very high preference of a protease for an amino acid side chain or a combination of several of them absent in the present sublibrary. However the expected high difficulty of the analysis by LCMS of such a complete library could generate other types of bias.

The efficiency of a hydrolase is related to its affinity also dependent, for a given peptide-substrate, to the dissociation from the enzyme active site of the formed metabolites. The binding affinity of endogenous peptides toward a physiological enzyme is rarely optimized, as evidenced by their low K_m. This suggests that the interactions of their different side chains have not been selected for a thermodynamically perfect binding to the S_n–S_n' subsites. The broad selectivity of enzymes observed *in vitro* disappears *in vivo* owing to the closeness between their distribution and that of their natural substrate [43].

As illustrated in Fig. 4, screening of the library ($n = 1$ and 2) with every enzyme tested, whatever its family, allowed the identification of one or more fluorescently quenched substrates for each of them.

Nevertheless in all families of hydrolases, there are some examples of highly specific enzymes as illustrated by the serine proteases involved in coagulation [44]. On the other hand several proteases have at least one subsite with a strict requirement for a given type of amino acid side chain (e.g., acidic, basic, and aromatic) as shown with chymotrypsin.

Accordingly, the identified substrates fit with the expected enzymatic selectivity, despite the presence of the bulky fluorophore Pya. For example, screening of the 12 sublibraries using kallikrein not only showed that the -KX- sublibrary was the best cleaved, but that, within this sublibrary, the fluorescent signal could be attributed to the cleavage of two peptides, Ac-SKG-PyaK↓K/TNop-GGK-NH_2 whose sequence are consistent with the kallikrein selectivity. Indeed, this peptidase has been shown to preferentially cleave peptides containing a bulky hydrophobic residue in P_2 position, here Pya, a basic residue in P_1 [35] and a basic or hydrophilic residue in P_1' [36]. As observed for kallikrein, the known preference of pepsin for extended substrates containing aromatic residues in P_1 [37] was also confirmed by this screening, as the preferred substrate was found to be Ac-SKG-Pya↓W-Nop-GGK-NH_2.

Screening of the $n = 2$ sublibraries using two closely related proteases, endothelin-converting enzymes 1 and 2 (ECE-1 and 2), has recently led to the successful identification of the first highly specific substrate for the latter protease [29]. This suggests that the library could unravel subtle differences between closely related proteases or isoenzymes. This is further confirmed here by the differences that can be evidenced between the soluble human recombinant NEP-1, devoid of its transmembrane domain, and the native form of NEP purified from rabbit. Indeed, soluble NEP-1 was found to preferentially degrade the -KX- sublibrary (Fig. 8A), whereas the native rabbit enzyme preferentially cleaved the -QX- sublibrary (Fig. 4). This result could reflect either conformational changes in the 3D structure of the NEP-1 active site resulting from its altered processing or species-specific differences of the human and rabbit enzymes [45].

Moreover, in order to confirm our previous study using ECEs, the hydrolytic properties of recombinant NEP-1 and NEP-2 toward the sublibraries corresponding to $n = 2$ were compared. NEP-2 [46], also designated SEP [47] or NL1 [48], displays 66% overall amino acid sequence similarity with NEP-1. This sequence similarity reaches 86% if one compares their active sites only [49], and there are no known model substrates and enzymatic assays which can discriminate their respective enzymatic activities. Interestingly, the analysis of the preferential sublibraries cleaved by these two enzymes suggests significant differences between their active sites. Indeed, the results show that NEP-2 prefers a hydrophobic aliphatic side chain in S_1' and an aromatic side chain in S_2' whereas NEP-1 preferred an aromatic residue in S_1' and a small residue in S_2' positions, respectively [50,51]. These results further confirm the previously published 3D modeling of the NEP-2 active site [49] and also bring to light the discriminative binding of an acidic amino in the S_1 position of NEP-2, but not NEP-1, thus providing interesting information on the putative differences between the active sites of these two proteases. This should aid in the design of a specific high-affinity substrate and *in vitro* and in *vivo* enzymatic assay of NEP-2 (article in preparation).

Overall, the results obtained with the different enzymes tested suggest that this library can provide useful information concerning the subsite preferences of a protease of unknown specificity. With

the $n = 3$ sublibraries, 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).

This shows that this library is able to preserve all the requirements for a good adaptation of one or several amino acid side chains for a given peptidase active site. As noted in the Introduction and supported by crystallographic studies and computer modeling assisted with site-directed mutagenesis [19], the active sites of about all hydrolases characterized by a high flexibility and subsequent adaptability are large enough to easily bind the Pya-Nop pair. The results reported in this paper pertain to the screening of the newly developed library and represent only the initial steps in the complete characterization of a new substrate for a given enzyme, i.e., identification of a hit sublibrary yielding a fluorescent signal, and of the cleaved peptide(s) therein mainly responsible for the signal. Once a potentially selective and/or high-affinity substrate is revealed in a sublibrary, its complete enzymatic characterization then remains to be performed: linearity of the fluorescence increase as a function of enzyme concentration, kinetic parameters, eventual role of the flanking sequences Ac-SKG and GGK-NH_2, and optimization of the variable amino acids (Lys versus Arg, Orn, homoArg, for example).

Finally, in order to expand the field of application of this novel library, improvements can be proposed. First, the peptide length could be extended to $n = 4$, as suggested by the results obtained with the MMP substrates, in which no loss of sensitivity was observed. This could be useful to better characterize the enzymatic activities of proteases with extended substrate recognition sequences as well as of those which do not accept aromatic residues within their active sites. Second, we have previously shown that the relative positions of the fluorophore/repressor pair can be of importance for substrate binding and cleavage, as, for instance, in the cases of Botulinum neurotoxin B [25,26] and neurotoxin A [27,28] the latter preferring reversed positions for Pya and Nop [28]. Thus, the synthesis of the same library but based on the reversed Ac-SKGNop-X(n)-Pya-GGK-NH_2 sequence could also broaden its applications. Finally, use of the library could be extended to carboxypeptidases or dipeptidylcarboxypeptidases [24]. As an example of a substrate for a dipeptidylcarboxypeptidase, the peptide Ac-S-K-G-Pya-KF-Nop-P was shown to be a very efficient substrate for ACE with a specific cleavage between F and Nop (unpublished result).

Conclusion

During the past 20 years, an increasing number of sophisticated libraries have been described for the screening of protease activities [9,11,12,52–55]. In the present study, we demonstrate that a library constituted by a relatively restricted but representative diversity of amino acids, and containing the extremely efficient fluorophore/repressor pair Pya/Nop, is able to provide novel information concerning uncharacterized enzymatic activities of proteases, including subsite preferences. The selected substrates become useful probes for 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*. As proteases constitute a large pool of novel therapeutic targets, these tools will be essential to their development and validation.

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