Analytical Biochemistry 442 (2013) 75–82

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

A new proteinase 3 substrate with improved selectivity over human neutrophil elastase

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ARTICLE INFO

Article history:
Received 7 June 2013
Received in revised form 16 July 2013
Accepted 19 July 2013
Available online 30 July 2013

Keywords:
Neutrophil serine proteases
Intermolecular quenched substrate
Proteinase 3
Combinatorial chemistry

ABSTRACT

We report the synthesis and enzymatic studies on a new proteinase 3 intermolecular quenched substrate with enhanced selectivity over neutrophil elastase. Using combinatorial chemistry methods, we were able to synthesize the hexapeptide library with the general formula ABZ-Tyr-Tyr-Abu-X_1'-X_2'-X_3'-Tyr(3-NO_2)-NH_2 using the mix and split method. The iterative deconvolution of such a library allowed us to obtain the sequence ABZ-Tyr-Tyr-Abu-Asn-Glu-Pro-Tyr(3-NO_2)-NH_2 with a high specificity constant ($k_{cat}/K_M = 1534 \times 10^3\ M^{-1}\ s^{-1}$) and superior selectivity over neutrophil elastase and other neutrophil-derived serine proteases. Moreover, using the obtained substrate, we were able to detect a picomolar concentration of proteinase 3 (PR3). Incubation of the above-mentioned substrate with neutrophil lysate resulted in a strong fluorescent signal that was significantly reduced in the presence of a PR3 selective inhibitor.

Neutrophils are the first cells that arrive at inflammatory sites, thereby forming the first line of defense against invading microorganisms. The average life span of a neutrophil barely exceeds 6 h. On activation, these cells release multiple cytotoxic products, including reactive oxygen species, antimicrobial peptides, and proteases (serine, cysteine, and metalloproteinases). Human neutrophil elastase (HNE),[1] proteinase 3 (PR3), and cathepsin G (CG) are three hematopoietic novel serine proteases (NSPs) stored in relatively large amounts in the neutrophil cytoplasmic azurophilic granules that help to degrade engulfed microorganisms inside the phagolysosomes [1]. In 2012, the new fourth protease, called neutrophil serine proteinase 4 (NSP4), was discovered and described by a German group [2]. NSP activity in healthy humans is controlled by a cohort of associated inhibitors such as elafin, the secretory leukocyte protease inhibitor, the serpin family (α1 protease inhibitor α1 antichymotrypsin), and (nonspecifically) macroglobulins [3]. However, when NSPs escape from tight and strict control, they become invasive and destructive within a human body. Thus, much pathology is associated with uncontrolled NSP action such as chronic inflammatory lung diseases (e.g., chronic obstructive pulmonary disease, cystic fibrosis, acute respiratory distress syndrome), anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitides (mainly Wegener granulomatosis), and Papillon–Lefevre syndrome [4].

PR3 and HNE are two closely related enzymes with overlapping substrate specificities, which are different from those of CG. Despite their common/mutual function, some NSPs display a unique role in the human organism. The activity of HNE is associated with some inflammatory lung disorders [5]. PR3 is the main antigen in Wegener's granulomatosis [6], a chronic autoimmune disorder with unknown etiology. It is characterized clinically as being involved in respiratory tract and renal disease; in patient serum, a high prevalence of ANCAs is observed, mostly against PR3. This enzyme is the main target of the immune response of the human body. A high level of ANCAs induces the activation of neutrophils, leading to a PR3 boost and (self-driven) mechanism of disease.

As mentioned above, the specificities of both secreted neutrophil enzyme PR3 and HNE are similar and focused on proteolytic processing of peptides composed of amino acids with aliphatic side chains in position P_1 (according to the Schechter–Berger notation [7], considered to be the primary substrate specificity position). Interestingly, the activity of these two enzymes is manifested in different compartments; that is, PR3 is predominantly associated with the cell membrane because HNE is present in the bloodstream. Moreover, in many situations, such as α1 protease inhibitor deficiency and transgenic animals, there is a need to investigate a PR3 activity outnumbered by the HNE origin activity. Such a task is not possible using commercially available substrates. This inspired us to determine the optimal sequence of a PR3

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[1] *Abbreviations used:* HNE, human neutrophil elastase; PR3, proteinase 3; CG, cathepsin G; NSP, novel serine protease; ANCA, anti-neutrophil cytoplasmic autoantibody; ABZ, 2-aminobezoic acid; Tyr(3-NO_2), 3-nitro-l-tyrosine; DIPCI, diisopropylcarbodiimide; HOBt, hydroxybenzotriazole; TFA, trifluoroacetic acid; RP, reverse-phase; HPLC, high-performance liquid chromatography; UV, ultraviolet; MALDI–TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; t_R, retention time; MW, molecular weight.

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http://dx.doi.org/10.1016/j.ab.2013.07.028

substrate that will display strong selectivity toward its close counterpart HNE.

One method for the detection of protease activity is to introduce chemical moieties into the peptide chain of a substrate so that one serves as a donor of fluorescence and the second acts as a quencher. The presence of both in a single peptide chain results in fluorescence resonance energy transfer (FRET) [8]. On enzymatic hydrolysis of peptide bond(s) located between the donor and acceptor (when the enzyme displays affinity toward an amino acid sequence), the distance between the donor and quencher increases and a fluorescence increase is observed.

To select a new substrate(s) with enhanced selectivity, we decided to use the sequence of PR3 substrate, ABZ-Tyr-Tyr-Abu-ANB-NH_2, that had been developed by our group [9]. Our intention was to improve its selectivity, using a combinatorial approach, by adding a tripeptide prime segment (the P_1-P_1' reactive site is located in the parent compound between Abu and ANB). As a result, a library with the general formula ABZ-Tyr-Tyr-Abu-X_1'-X_2'-X_3'-Tyr(3-NO_2)-NH_2, where ABZ is 2-aminobezoic acid (the donor of fluorescence) and Tyr(3-NO_2) is 3-nitro-L-tyrosine (the acceptor), was designed and synthesized using a mix and split method. In positions $X_1' = X_2' = X_3'$, the set of 19 proteinogenic amino acid residues, except Cys, were introduced.

Materials and methods

Peptide synthesis

All peptides were synthesized manually via the solid-phase method using Fmoc chemistry, as described previously [10]. TentaGel S RAM (substitution 0.24 meq/g) (RAPP Polymere, Germany) was used as a solid support. The α-amino groups of amino acids were Fmoc protected. The Fmoc-protected amino acid residues were attached to the resin using the DIPCI/HOBt (diisopropylcarbodiimide/hydroxybenzotriazole) method. Briefly, the mixture of N-protected amino acid derivative, DIPCI and HOBt (molar ratio, 1:1:1), was dissolved in DMF/NMP/DCM (*N,N*-dimethylformamide/*N*-methylpyrrolidone/dichloromethane) solution (1:1:1, v/v/v) and added into the resin. A 3-fold excess was applied to the active resin sites. The N-terminal ABZ moiety was introduced using the aforementioned method. After completing synthesis, the peptides were cleaved from the resin using a trifluoroacetic acid (TFA)/phenol/triisopropylsilane/H_2O mixture (88:5:2:5, v/v) [11]. The purity of the peptides was checked on an RP–HPLC (reverse-phase high-performance liquid chromatography) Pro Star system (Varian, Australia) equipped with a Supelco–Discovery Bio Wide Pore C_8 column (250 × 4.6 mm, 5 μm, Sigma–Aldrich) and a UV–VIS (ultraviolet–visible) detector. A linear gradient from 10% to 90% B within 40 min was applied (A: 0.1% TFA; B: 80% acetonitrile in A). The analyzed peptides were monitored at 226 nm. Mass spectra of both the peptides synthesized and the ANB derivates were recorded using a Biflex III matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) device. Subsequently, a Jasco HPLC system with UV and fluorescent detection modes was used. A linear gradient from 10% to 80% or 10% to 70% B (for inhibition studies) within 40 min was applied (A: 0.1% TFA; B: 80% acetonitrile in A). The same HPLC column was used.

Library synthesis

For the synthesis of the library, 14.7 g of the resin was used. The library was synthesized using the split and mix method, as described previously [12,13]. The whole synthetic procedure was the same as that described in the peptide synthesis section above.

Enzymatic studies

The human enzymes PR3 and HNE came from Elastin (USA). Bovine β-trypsin and turkey ovomucoid third domain (OMTKY3) were purchased from Sigma–Aldrich (Germany). The concentration of bovine β-trypsin stock solution was determined by titration with NPGB (nitrophenol-*p*-guanidino benzoate) using burst kinetics. The concentration of each enzyme was determined using a standardized solution of common inhibitor (OMTKY3) previously titrated by a standardized solution of bovine β-trypsin, as described previously [14].

Fluorescence studies

During the studies, fluorescence spectra of all compounds were recorded in a FluoroSTAR microplate reader (BMG, Germany) equipped with a set of 12 extinction emission filters. ABZ was excited at 320 nm, and its emission was followed at 450 nm. The substrate's water solution was used at a concentration of 10^{-5} M.

Deconvolution of library

The iterative mode of deconvolution was used [14]. Briefly, constant amounts of 20 μl each of the 19 sublibraries (at a concentration of 0.3 mg/ml in Tris–HCl buffer) that differ in N-terminal amino acid residue were incubated with 10 μl of an appropriate protease in a 96-well plate. The excitation wavelength 320 nm was used. The fluorescent increase at 450 nm was followed over time. The amino acid residue that produced the highest fluorescence increase was introduced in the analyzed position. This procedure was repeated three times for the tripeptide library used in this study.

Proteolytic cleavage pattern determination

First, 1 μl of the appropriate enzyme (10^{-7} M) in experimental buffer was added to 5 μl of a solution of the selected substrate (2.73×10^{-4} M). HPLC analysis of this mixture was performed after the following incubation times: 0 min, 15 min, 1 h, and 48 h. A linear gradient from 10% to 90% B within 40 min was applied (A: 0.1% TFA; B: 80% acetonitrile in A). The analyzed peptides were monitored at 226 nm. The peaks that appeared were collected and analyzed with a MALDI–TOF device as described above.

Sensitivity curve

A constant amount of the selected peptide (2.73×10^{-4} M, 0.1 M Tris–HCl buffer) was added into a solution of the specific enzyme in appropriate buffer. The concentration of the assayed enzyme ranged from 4.31×10^{-11} to 1.73×10^{-9} M for PR3 and ranged from 1.33×10^{-8} to 1.33×10^{-7} M for HNE. The fluorescence increase at 450 nm versus time was measured. All of the obtained values were measured against a substrate solution with no enzyme added as a control. The threshold limit for all measurements was 3:1 expressed as signal-to-noise ratio.

Determination of kinetic parameters

For each peptide (**1**–**5**), the titration curve for the donor of fluorescence was obtained. Briefly, the ABZ fluorescence was measured as a function of its concentration in 0.1 M Tris–HCl buffer (pH 7.5) supplemented with 500 mM NaCl. These data were used to calculate the concentration of peptide-containing donor. Then, to an appropriately buffered PR3 solution at a level of 1.94×10^{-10} M,