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Novel internally quenched substrate of the trypsin-like subunit of 20S eukaryotic proteasome

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ABSTRACT

This article describes the synthesis, using combinatorial chemistry, of internally quenched substrates of the trypsin-like subunit of human 20S proteasome. Such substrates were optimized in both the nonprime and prime regions of the peptide chain. Two were selected as the most susceptible for proteasomal proteolysis with excellent kinetic parameters: (i) ABZ-Val-Val-Ser-Arg-Ser-Leu-Gly-Tyr(3-NO_2)-NH_2 (k_{cat}/K_M = 934,000 M^{-1} s^{-1}) and (ii) ABZ-Val-Val-Ser-GNF-Ala-Met-Gly-Tyr(3-NO_2)-NH_2 (k_{cat}/K_M = 1,980,000 M^{-1} s^{-1}). Both compounds were efficiently hydrolyzed by the 20S proteasome at picomolar concentrations, demonstrating significant selectivity over other proteasome entities.

The ubiquitin–proteasome system (UPS) is the major proteolytic pathway in eukaryotic cells that controls various biological processes, including cell cycle, circadian rhythm, and nerve conduction or transcription [1,2]. One of the key biomolecules of the UPS is a fully functional proteasome that is also referred to as 26S proteasome. The 26S proteasome (~2500 kDa) is an ATP-dependent, multifunctional proteolytic complex composed of a catalytic core particle—the 20S proteasome (~700 kDa)—capped at each end by two regulatory particles called 19S proteasomes (~900 kDa) or PA700 [3,4]. The 19S regulatory particle is responsible for recognition of the polyubiquitinated proteins (substrates) and their unfolding, deubiquitinylation, and translocation into the catalytic particle.

The 20S proteasome has a cylinder-like structure and consists of 28 subunits that form four rings [5,6]. The outer rings are composed of seven different α subunits, whereas the inner ones are built by seven different β subunits. All α subunits are proteolytically inactive. Only three of the β subunits (β1, β2, and β5) have active centers (catalytic sites) [7]. Each of these β subunits possesses an N-terminal threonine residue (Thr1) that is essential for the proteolytic activity of the enzyme. The hydroxyl group of the threonine side chain is responsible for the nucleophilic attack on the carbonyl carbon of the peptide bond to be cleaved [8]. The cleavage preference of the active β subunits is determined solely by the construction of the proteasomal S_1 binding pockets, in particular, by the type of amino acid residue found in position 45 in a polypeptide chain [9]. Subunit β1 has Arg45 in the binding pocket, which interacts preferentially with acidic amino acids (Glu and Asp). Thus, this subunit provides caspase-like or peptidyl-glutamyl peptide hydrolyzing activity. Subunit β2, with Gly residue at position 45 and Glu53, accepts large and positively charged amino acid residues such as Lys and Arg. This subunit is responsible for trypsin-like activity. Subunit β5, with Met45 in the binding pocket, displays mainly chymotrypsin-like activity [8,10] and cleaves peptide bonds on the carboxyl side of hydrophobic amino acid. However, it has been shown that the β5 subunit is also able to cleave bonds after small neutral and branched chain amino acids [11].

A special form of the core proteasome is synthesized by substituting the proteolytically catalytic β1, β2, and β5 subunits with β1i, β2i, and β5i subunits, thereby forming the so-called

Abbreviations used: UPS, ubiquitin–proteasome system; ACC, 7-amino-4-carbamoylmethylcoumarin; AMC, 7-amino-4-methylocoumarin; FRET, fluorescent resonance energy transfer; ABZ, 2-aminobenzoic acid; Tyr(3-NO_2), 3-nitro-L-tyrosine; ANB, 5-amino-2-nitrobenozoic acid; DIPCI, *N,N′*-diisopropylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, dimethylformamide; TBTU, *N,N,N′,N′*-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate; DMAP, 4-dimethylaminopyridine; DIPEA, *N,N*-diisopropylethylamine; TFA, trifluoroacetic acid; RP-HPLC, reverse phase high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PR3, proteinase 3.

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immunoproteasome [12]. The immunoproteasome is induced by cell treatment with interferon-γ [13].

In general, the concentration of proteasomes may vary from cell line to cell line and from tissue to tissue [14,15]. Proteasome concentrations in peripheral blood (so-called circulating proteasome concentration) are elevated in patients with certain types of malignant diseases. For example, in the case of breast cancer, the mean values range from 486 to 2138 ng/ml, depending on the tumor stage [16]. In patients with multiple myeloma, the concentration range is even wider—from 108.5 to 5181.6 ng/ml [17]. On the other hand, chemotherapy may significantly decrease the level of proteasome [18]. That is why more and more sensitive and specific assays are necessary to assess the proteasome concentration and profile its activity.

In this study, we focused our attention on the trypsin-like specificity of the human 20S proteasome. To date, the detailed substrate preferences of this subunit have been examined in only a few articles [19,20]. Harris and coworkers [19] applied a positional scanning combinatorial library to define the substrate specificity of the human 20S proteasome in the presence or absence of 11S proteasome activators (REGα/β and REGγ). The peptides were labeled with the C-terminal fluorogenic leaving group, 7-amino-4-carbamoylmethylcoumarin (ACC). These investigations revealed the optimal sequence for the β2 subunit—Ac-Glu-Ala-Nle-Arg-ACC for activated form of 20S proteasome [19]. Nazif and Bogyo [20] generated a library of tetrapeptide vinyl sulfone with Asn residue in position P1. The library screening indicated that a positively charged basic residue or Ser at position P3 and Pro or Tyr at position P4 are responsible for the higher inhibitory activity and specificity of the β2 subunit. Two peptides Ac-Tyr(Pro)-Arg-Leu-Asn-VS were identified as very efficient and β2-selective inhibitors. Further research has suggested that favorable interactions between the P3 residue and the large S3 binding pocket determine the inhibitor selectivity [20].

Importantly, the trypsin-like proteasome β2 subunit has been recognized to be a co-target for anticancer drug development. Recently, Mirabella and coworkers [21] demonstrated that administration of cell-permeable peptide epoxyketone inhibitors of the β2 subunit increases the sensitivity of multiple myeloma cell lines to well-known inhibitors of chymotrypsin-like activity such as bortezomib and carfilzomib. Based on these results, the authors suggested that the β2 subunit appears to be a better co-target than the β1 subunit with caspase-like activity.

Several substrates are available to measure the trypsin-like activity of the proteasome. The most commonly used ones have C-terminal scissile bond between Arg and 7-amino-4-methylocoumarin (AMC)—Ac-Leu-Arg-Arg-AMC and Boc-Leu-Arg-Arg-AMC. Both substrates displayed significant selectivity able to measure the trypsin-like specificity. More sensitive in terms of signal detection is peptide substrate conjugated with amino-luciferin moiety [22]. Chiba and coworkers [23] used Suc-Phe-Leu-Arg-coumarylamido-4-methanesulfonic acid for assaying trypsin-like activity. However, the above-described substrates display a low value of specificity constant at a level of 3×10^5 M^{-1} s^{-1}.

Despite being different in terms of their primary structures, all of these substrates were designed to interact only with the non-primed proteasome binding sites (S1–S3). Recently, we reported the library synthesis, screening, and biochemical characterization of a novel and unique peptide substrate (ABZ-Val-Val-Ser-Phe-Ala-Met-Gly-Tyr(NO_2)-NH_2) specific for the chymotrypsin-like subunit [24]. The assay is based on the fluorescent resonance energy transfer (FRET) principle because the substrates have N-terminal 2-aminobenzoic acid (ABZ), which is a donor of fluorescence, and C-terminal 3-nitro-L-tyrosine (Tyr(3-NO_2)), which works as a quencher. The hydrolysis of such substrates enables the detection of a fluorogenic product [24]. The FRET substrates were optimized in both nonprimed and primed regions and displayed higher (up to 10-fold) values of specificity constants than commercially available substrates [24].

In the current study, we employed a similar approach to select and characterize the substrates specific for the trypsin-like subunit of the human 20S proteasome. At first, the substrate N-terminal (nonprimed) region was investigated. The library of internally quenched tetrapeptides with N-terminal 2-aminobenzoic acid (fluorophore) and C-terminal 5-amino-2-nitrobenozoic acid (ANB, quencher) was prepared and screened to find the most efficient and selective substrate for the human 20S proteasome. Then, the C-terminal (primed) region (X_1'–X_3') was analyzed using the FRET substrate library with N-terminal 2-aminobenzoic acid (fluorophore) and C-terminal Tyr(3-NO_2) (quencher).

Materials and methods

Peptide synthesis

The chromogenic/fluorogenic peptide libraries and individual substrates were synthesized manually on a solid resin support using the "split and mix" method [25]. TentaGel S RAM (substitution = 0.25 meq/g; RAPP Polymere, Germany) was used as the solid support. For the peptide synthesis, the Fmoc/tBu approach was taken. The coupling reaction was carried out by using an equimolar mixture of the protected amino acid derivative, *N*,*N*′-diisopropylcarbodiimide (DIPCI), and 1-hydroxybenzotriazole (HOBt). All of the synthesized peptides contained the amide moiety at their C termini.

Synthesis of peptide libraries

The peptide libraries were synthesized using the split and mix method. Initially, 17.7 g of the solid support (TentaGel S RAM) was used to synthesize the first library (i.e., ABZ-X_4-X_3-X_2-X_1-ANB-NH_2), where in positions X_4, X_3, and X_2 the set of proteinogenic amino acid residues, except Cys, was present. In position X_1, either Arg or Lys was introduced. A 3-fold molar excess of amino acid was used for the coupling. The second library (i.e., ABZ-Val-Val-Ser-Arg-X_1'-X_2'-X_3'-Tyr(3-NO_2)-NH_2), where in positions X_1', X_2', and X_3' the set of proteinogenic amino acid residues, except Cys, will be present, was synthesized on 15.2 g of the above-mentioned resin.

The synthesis of the ANB-based library was initiated by the deprotection of the amino groups of the resin with 20% piperidine in dimethylformamide (DMF) and the coupling of ANB using a mixture of *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate (TBTU)/4-dimethylaminopyridine (DMAP). The resin was washed twice with *N*-methylmorpholine. Next, 2 equivalents of ANB were dissolved in 60 ml of DMF and 2 equivalents of TBTU were added, followed by 1 equivalent of DMAP. The resultant solution was added to the resin, and after 30 s 4 equivalents of *N*,*N*-diisopropylethylamine (DIPEA) were added. The whole mixture was stirred for 3 h. The solution was filtered off, and the resin was washed with DMF. The procedure was repeated three times. Then, the first amino acid residue was coupled using a special method. The amino acid derivative (a 9-fold excess was applied to the active resin sites) was dissolved in pyridine (10 ml pyridine to 1 g peptidyl resin). The whole solution was mixed until a temperature of −15 °C was reached, and then 9 equivalents of $POCl_3$ were added. The mixture was successively stirred for 20 min at −15 °C, for 20 min at room temperature, and for 6 h in an oil bath at 40 °C. After deprotection with 20% piperidine in DMF, the peptide chain was elongated as follows: an equimolar mixture of a protected amino acid derivative, DIPCI, and HOBt was dissolved in DMF/NMP (1-

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