



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-NO₂)-NH₂ ($k_{\text{cat}}/K_{\text{M}} = 934,000 \text{ M}^{-1} \text{ s}^{-1}$) and (ii) ABZ-Val-Val-Ser-Gln-Ala-Met-Gly-Tyr(3-NO₂)-NH₂ ($k_{\text{cat}}/K_{\text{M}} = 1,980,000 \text{ M}^{-1} \text{ s}^{-1}$). Both compounds were efficiently hydrolyzed by the 20S proteasome at picomolar concentrations, demonstrating significant selectivity over other proteasome entities.

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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, deubiquitylation, and translocation into the catalytic particle.

Abbreviations used: UPS, ubiquitin–proteasome system; ACC, 7-amino-4-carbamoylmethylcoumarin; AMC, 7-amino-4-methylcoumarin; FRET, fluorescent resonance energy transfer; ABZ, 2-aminobenzoic acid; Tyr(3-NO₂), 3-nitro-L-tyrosine; ANB, 5-amino-2-nitrobenzoic acid; DIPCl, *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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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 ($\beta 1$, $\beta 2$, and $\beta 5$) 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 S₁ binding pockets, in particular, by the type of amino acid residue found in position 45 in a polypeptide chain [9]. Subunit $\beta 1$ 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 $\beta 2$, 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 $\beta 5$, 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 $\beta 5$ 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 $\beta 1$, $\beta 2$, and $\beta 5$ subunits with $\beta 1i$, $\beta 2i$, and $\beta 5i$ subunits, thereby forming the so-called 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 (REG α/β and REG γ). The peptides were labeled with the C-terminal fluorogenic leaving group, 7-amino-4-carbamoylmethylcoumarin (ACC). These investigations revealed the optimal sequence for the $\beta 2$ subunit—Ac-Glu-Ala-Nle-Arg-ACC for activated form of 20S proteasome [19]. Nazif and Bogoy [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 $\beta 2$ subunit. Two peptides Ac-Tyr(Pro)-Arg-Leu-Asn-VS were identified as very efficient and $\beta 2$ -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 $\beta 2$ 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 $\beta 2$ 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 $\beta 2$ subunit appears to be a better co-target than the $\beta 1$ 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-methylcoumarin (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 aminoluciferin 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 $3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$.

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(NO₂)-NH₂) 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-NO₂)), 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-nitrobenzoic 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 (X₁'–X₃') was analyzed using the FRET substrate library with N-terminal 2-aminobenzoic acid (fluorophore) and C-terminal Tyr(3-NO₂) (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-X₄-X₃-X₂-X₁-ANB-NH₂), where in positions X₄, X₃, and X₂ the set of proteinogenic amino acid residues, except Cys, was present. In position X₁, 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-X₁'-X₂'-X₃'-Tyr(3-NO₂)-NH₂), where in positions X₁', X₂', and X₃' 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 POCl₃ 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, DIPCl, and HOBT was dissolved in DMF/NMP (1-methyl-2-pyrrolidone)/DCM (dichloromethane) solution (1:1:1, v/v/v). A 3-fold excess was applied to the active resin sites. The synthetic procedure for the library of the internally quenched ABZ/Tyr(3-NO₂) peptides was similar to that described above, starting with Fmoc protected 3-nitro-L-tyrosine.

After completing the synthesis, the peptides were cleaved from the resin using a trifluoroacetic acid (TFA)/phenol/triisopropylsilane/H₂O mixture (88:5:2:5, v/v/v/v). The purity of the synthesized peptides and the correctness of the synthesis were tested using a high-performance liquid chromatography (HPLC) system equipped with ChromNAV software. The reverse phase (RP)-HPLC method using a Kromasil 100 C8 column (4.6 × 250 mm; Knauer, Germany) was applied. UV-vis (ultraviolet-visible, at 226 nm) detection mode and fluorescence monitoring (Jasco, Japan), with the excitation wavelength set to 320 nm and the emission at 450 nm, were used. A linear gradient from 10 to 90% B within 45 min was applied (A: 0.1% TFA; B: 80% acetonitrile in A). The molecular masses of the synthesized peptides and peptide libraries were confirmed by mass spectrometry using a Biflex III MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometer (Bruker, Germany).

Enzymatic studies

For the enzymatic studies, we used a commercially available enzyme from Boston Biochem (Cambridge, MA, USA). The concentrations of human 20S proteasome used for the deconvolution of the ABZ/ANB library ranged from 1.78×10^{-9} to 8.92×10^{-11} M. For the second library, the enzyme concentration was varied from 1.42×10^{-10} to 3.57×10^{-11} M. Deconvolution of peptide libraries was carried out by an iterative solution method [26]. The lyophilizates obtained for each sublibrary were dissolved in DMSO (dimethyl sulfoxide) to a final concentration of 5 mg/ml and then diluted 10 times. Next, 20 µl of all sublibraries or peptides were pipetted into each well and supplemented with assay buffer (180 µl) and enzyme solution (10 µl). The fluorescence tests were performed with a FLUOstar Omega fluorescent microplate reader (BMG, Germany). Excitation and emission wavelengths were 320 and 410 nm, respectively, for ABZ/ANB and were 320 and 450 nm, respectively, for the ABZ/Tyr(3-NO₂) donor/acceptor pair. Enzymatic hydrolysis of the peptide was performed in 50 mM Tris-Cl (pH 8.2) supplemented with 0.02% sodium dodecyl sulfate (SDS) buffer at 37 °C and continued over 30 min.

Determination of kinetic parameters

Assay conditions for the determination of the Michaelis constants (K_M) and catalytic constants (k_{cat}) were as noted above. The specificity constants (k_{cat}/K_M) were calculated from k_{cat} and K_M values. Measurements were performed with an enzyme concentration of 8.92×10^{-11} M. Three to five measurements were carried out for each compound (systematic error expressed as a standard deviation never exceeded 20%). The calculated initial hydrolysis rates were used as a measure of the proteasome activity toward the investigated peptides. All of the details of kinetic studies and the method of calculating kinetic parameters have been described elsewhere [27].

Proteolytic cleavage pattern determination

For each sample of each respective diluted substrate (concentration 4.34×10^{-6} M), the appropriate amount of the enzyme

(8.92×10^{-11} M) was added and the solution was incubated for 5, 30, and 60 min. The progress of the proteolytic reaction was monitored by RP-HPLC. The individual peaks resulting from the hydrolysis reaction were collected and identified by mass spectrometry.

Limit of detection

Titration of the dropping amount of 20S proteasome in the presence of a constant concentration of substrate **1** or **15** (at concentration 4.34×10^{-6} M) was performed in assay buffer or buffered solution of human serum or urine (1:1, v/v). The fluorescence increase at 450 nm was monitored for 30 min.

Inhibitory study

A 96-well black flat-bottom microplate was used. Two inhibitors, bortezomib and epoxomicin (Merck, Germany), were used in the inhibition study at final concentrations of 1.15×10^{-6} M and 2.0×10^{-5} M, respectively. β2 inhibitor PR671A was used at a concentration of 2.12×10^{-8} M and was kindly provided by Alexei Kisselev of Dartmouth University. The proteasome concentrations ranged from 5.42×10^{-10} to 1.78×10^{-10} M. The buffered solution of proteasome was mixed with 10 µl of inhibitor solution to reach the final concentration mentioned above. The whole mixture was incubated for 30 min. After this time, the substrate solution was added at a concentration of 4.34×10^{-6} M and the fluorescence of the system was measured over time. The 320-nm excitation and 450-nm emission wavelengths were used.

Results and discussion

To optimize the N-terminal (nonprimed) region, a peptide library with the general formula ABZ-X₄-X₃-X₂-Arg/Lys-ANB-NH₂ was synthesized using the mix and split approach. All proteinogenic amino acid residues, with the exception of Cys, were introduced in the three variable positions X₂, X₃, and X₄. All assays with human 20S proteasome were conducted in the presence of its artificial activator, SDS, at a concentration of 0.02%. The deconvolution process was performed in solution via the iterative method. As the first step, the library with the fixed amino acid residues at position X₄ was screened (Fig. 1A). Because the highest fluorescence rate was observed for peptides with Val residues, this aliphatic amino acid was selected and fixed in the next library with the formula ABZ-Val-X₃-X₂-Arg/Lys-ANB-NH₂. Similarly, the most intense proteolytic breakdown was observed for substrates with Val in position X₃ (Fig. 1B). Substrates with other aliphatic residues, such as Ile, Met, and Ala, were hydrolyzed with slightly lower efficiency. A moderate fluorescence increase was recorded for amino acids with polar side chains (Ser, His, Thr, Gln, and Asn) and basic ones, whereas low cleavage rates were observed for negatively charged residues (Asp and Glu), aromatic ones (Phe, Tyr, and Trp), and Pro. The screening of the third library with the general formula ABZ-Val-Val-X₂-Arg/Lys-ANB-NH₂ revealed that Ser-containing peptides in position X₂ are the most susceptible to proteolysis (Fig. 1C). Other polar residues (Gln, Asn, and Thr) also promoted the efficient cleavage of the Arg/Lys-ANB-NH₂ scissile peptide bond. Proteolysis of substrates with Val, Met, and Ala caused a moderate fluorescence increase. Finally, the substrate with Arg residue in position P1 (ABZ-Val-Val-Ser-Arg-ANB-NH₂) displayed a much higher rate of signal intensity than its counterpart with Lys (Fig. 1D). The ratio between Arg- and Lys-containing peptides was 45.

The release of the ANB-NH₂ group was monitored at 410 nm, at each stage of deconvolution, to confirm that the peptide bond between the P1 residue (Arg or Lys) and ANB-NH₂ was uniquely cleaved.

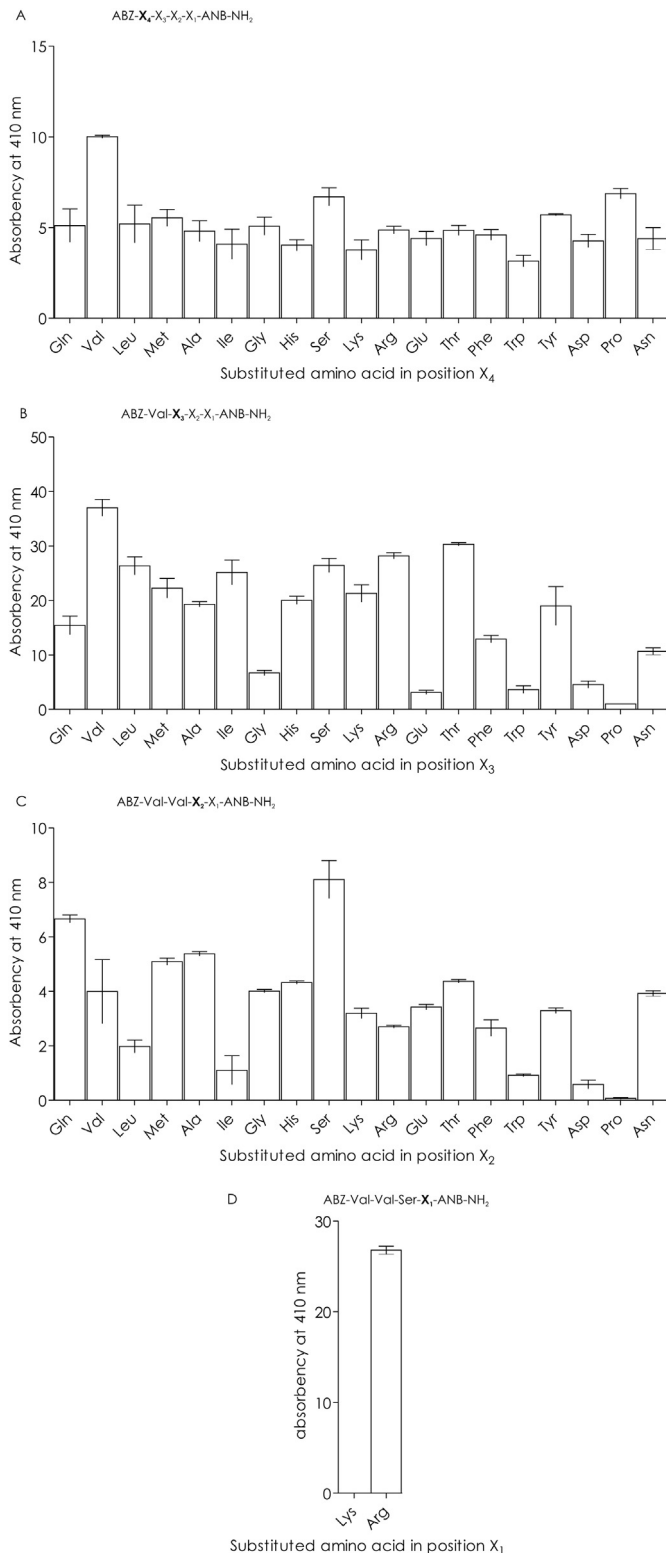


Fig. 1. Results of the deconvolution of the tetrapeptide library of human 20S proteasome substrates with the general formula $ABZ-X_4-X_3-X_2-Arg/Lys-ANB-NH_2$. Enzymatic hydrolysis of the library was performed in 50 mM Tris–Cl (pH 8.2) supplemented with 0.02% SDS buffer at 37 °C and continued over 30 min. Absorbency at 410 nm was measured as a result of ANB release. Such an approach allowed us to identify the cleaved peptide bond in the substrate sequence because only proteolysis of the Arg-ANB bond resulted in color formation.

To investigate the primed binding sites of human 20S proteasome, we designed the library of FRET peptide substrates containing N-terminal ABZ as a donor of fluorescence and C-terminal Tyr(3-NO₂) as a quencher. The general formula was as follows: $ABZ-Val-Val-Ser-Arg-X_1'-X_2'-X_3'-Tyr(3-NO_2)-NH_2$. The expected scissile bond was located between Arg and the X_1' residue. In all primed positions ($X_1'-X_3'$), a set of 19 proteinogenic amino acid residues, except Cys, was introduced. Incubation of each library with the 20S proteasome was followed by monitoring of the fluorescence increase at 450 nm, reflecting the emission of liberated ABZ attached to the peptide fragment.

In position X_1' , the highest fluorescence increase was recorded for libraries with Ser, whereas this increase was slightly lower for peptides with Gly, Pro, and Ala (Fig. 2A). For the rest of the tested

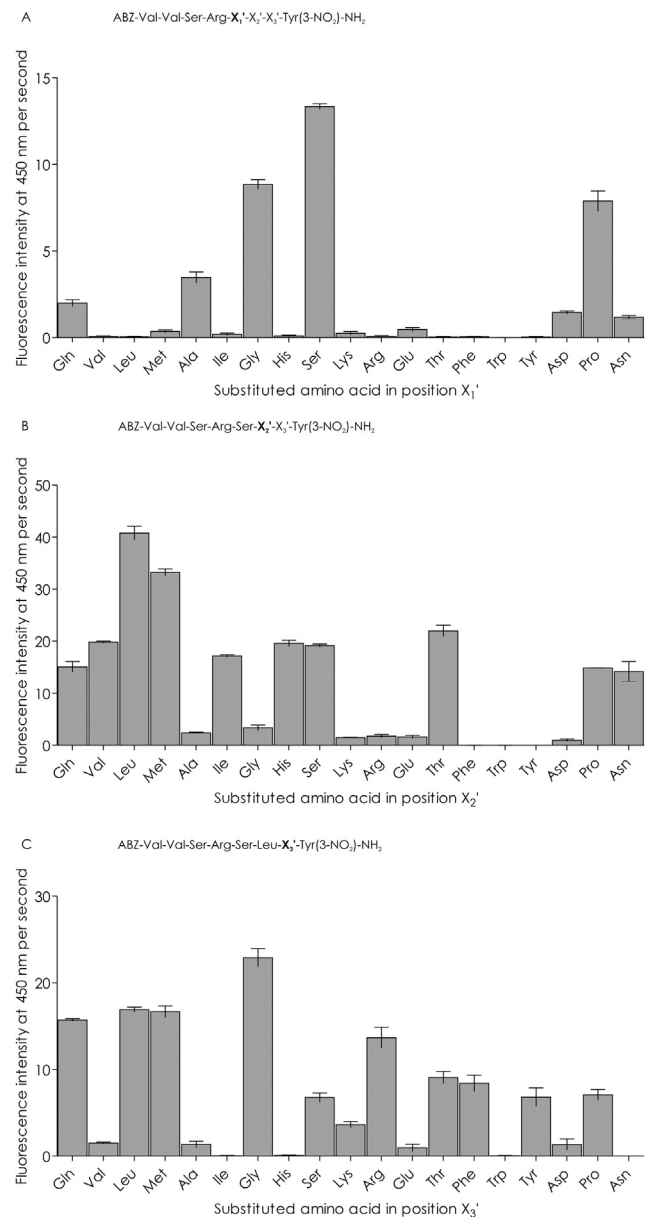


Fig. 2. Results of the deconvolution of the FRET substrate library with the general formula $ABZ-Val-Val-Ser-Arg-X_1'-X_2'-X_3'-Tyr(3-NO_2)-NH_2$, where in positions X_1' , X_2' , and X_3' a set of proteinogenic amino acids, except Cys, was present. Enzymatic hydrolysis of the peptide was performed in 50 mM Tris–Cl (pH 8.2) supplemented with 0.02% SDS buffer at 37 °C and continued over 30 min. The fluorescence of ABZ (excited at 320 nm) was monitored at 450 nm.

sublibraries, low fluorescence (Asn, Gln, Glu, and Asp) or irrelevant fluorescence was observed. Thus, Ser was incorporated into the next stage of deconvolution. Among the amino acids introduced in position X_2' , Leu yielded the highest fluorescence increase. However, it is worth noting that a considerably high substrate processing rate was also recorded for other residues such as Met, Val, Pro, Thr, Ser, Asn, and Gln. Considering position X_3' (Fig. 2C), the preference for Gly was observed. Otherwise, Trp was not favored in any of the primed positions. Finally, a peptide with the formula ABZ-Val-Val-Ser-Arg-Ser-Leu-Gly-Tyr(3-NO₂)-NH₂ was identified as the most efficiently cleaved substrate of the 20S proteasome.

In our previous study, we were able to solve the substrate preference of the chymotrypsin subunit of 20S proteasome [24]. In its prime area, the $\beta 5$ subunit of human 20S proteasome displayed a preference toward small aliphatic residues such as Ser and Ala that dominate over other residues, indicating the presence of a small cleft subsite formed on the proteasome surface. This finding is strongly manifested in position X_1' of the tested libraries. However, most of the commonly used artificial peptide substrates have a large reporter group such as coumarin or luciferin in position X_1' that, in light of our data, makes them difficult to accommodate. Analysis of positions X_2' and X_3' of the 20S proteasome reveals rather broad specificity with preference toward aliphatic (Leu) or small aliphatic (Gly) amino acid residues.

The process of the deconvolution of the first nonprimed library revealed a chemical structure of the substrate that differs from the commonly used commercially available substrate from Promega, Boc-LRR-AMC, where AMC is a highly fluorescent coumarin derivative. In this work [24], the substrate (Boc-LRR-AMC) has a relatively low specificity constant ($k_{\text{cat}}/K_M = 23 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$), mainly due to its high K_M value (57 μM) and low k_{cat} value (1.3 s^{-1}), because the peptide ABZ-Val-Val-Ser-Arg-ANB-NH₂ displayed higher k_{cat} and lower K_M values, resulting in a 20-fold increase in the specificity parameter. There are numerous reports describing the influence of reporter molecules present on either N or C terminals of peptide probes on their interactions with target enzymes. An excellent example of such phenomena was provided by Narawane and coworkers [28], who demonstrated that N- and C-terminal FRET groups (ABZ and EDDnp (N-(2,4-dinitrophenyl)

ethylenediamine)) significantly contribute to enzymatic hydrolysis of internally quenched peptides. They designed and chemically synthesized a new substrate specific for proteinase 3 (PR3) with excellent kinetic parameters [28]. Recently, the same group designed methyl ketone-based inhibitors for PR3 that benefit from the presence of N- and C-terminal FRET groups and secure high inhibitory potency toward PR3 [29].

To identify the location of the cleavage site in the substrate, RP-HPLC with a fluorescent detector was used to monitor the emission of the unquenched ABZ group attached to the cleaved peptide fragment. In addition, mass spectrometry analysis was performed. As shown in Fig. 1SA–F of the online supplementary material, on incubation of 20S proteasome with FRET peptides comprising an ABZ/Tyr(3-NO₂)-NH₂ pair, a single and highly fluorescent peptide (ABZ-Val-Val-Ser-Arg-OH; retention time 8.56 min and m/z 580.1) was observed. Any unwanted or shifted cleavage sites would result in multiple peaks with different retention times. Fig. 1S indicates that, at each stage of the deconvolution process, during the first 30 min the cleavage occurs solely between Arg and the amino acid in position X_1' .

Moreover, the primary sequence ABZ-Val-Val-Ser- X_1 -Ser-Leu-Gly-Tyr(3-NO₂)-NH₂ was used to test different structural derivatives of the Arg residue in position X_1 (position P1 in Schechter and Berger notation) (Fig. 3).

Among the 13 peptides (see Table 1S in supplementary material), only 2 (with homoarginine (7) and with 4-guanidino phenylalanine (13)) were cleaved by human 20S proteasome. However, none of them displayed such a high cleavage rate as the parental peptide 1 with Arg (Fig. 4).

At first glance, this finding was rather disappointing due to the dramatic reduction of cleavage rate for peptides with the modified position X_1 . However, such results provide additional proof that the scissile bond is located between Arg and Ser (see Fig. 2S in supplementary material). The obtained peptides were tested as potential competitive inhibitors of 20S proteasome $\beta 2$ subsite; however, only 2 of them (compound 6 with EPA and compound 9 with AGP in position P1) were able to slightly affect (up to 25%) the rate of cleavage of substrate 1 (see Fig. 4S). An increasing concentration of peptide 6 or 9 did not yield enhancement of the

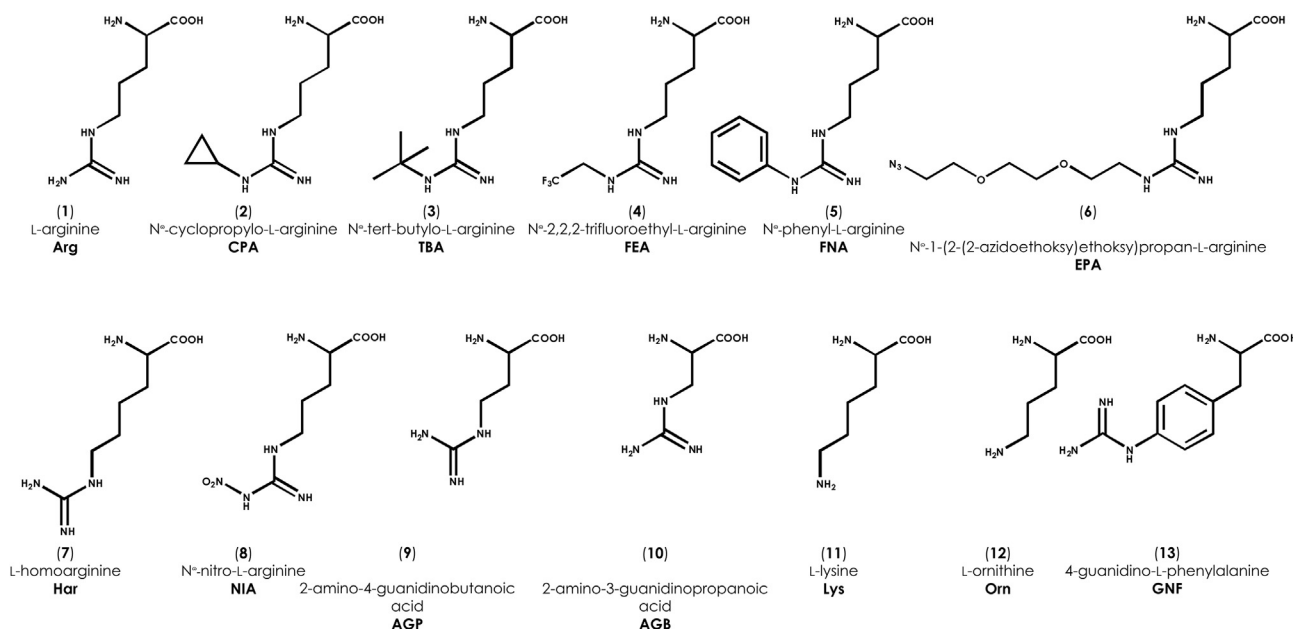


Fig. 3. Chemical structure of the set of Arg derivatives introduced in position X_1 of quenched peptides with the formula ABZ-Val-Val-Ser- X_1 -Ser-Leu-Gly-Tyr(3-NO₂)-NH₂.

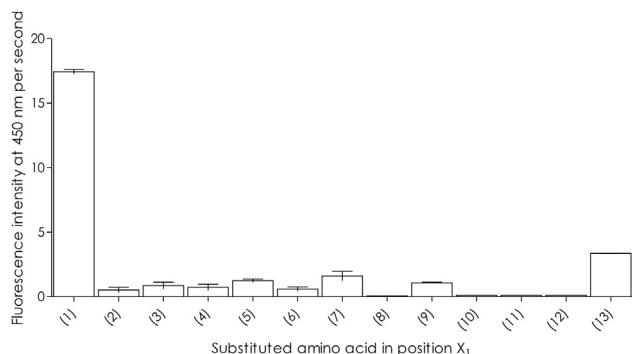


Fig. 4. Cleavage rates of peptides with the general formula ABZ-Val-Val-Ser-X₁-Ser-Leu-Gly-Tyr(3-NO₂)-NH₂ incubated with human 20S proteasome. Enzymatic hydrolysis of the peptide was performed in 50 mM Tris-Cl (pH 8.2) supplemented with 0.02% SDS buffer at 37 °C and continued over 30 min.

proteasome inhibition. For 2 of the peptides, a slight increase in proteasome activity was observed. None of the remaining compounds was unable to inhibit trypsin-like proteasome activity of the enzyme.

In the case of all inactive substrates, the lack of hydrolysis was confirmed by mass spectrometry and HPLC studies (data not shown).

Inhibitory study

To prove that ABZ-Val-Val-Ser-Arg-Ala-Met-Gly-Tyr(3-NO₂)-NH₂ (**14**) is cleaved exclusively by the trypsin-like active site of human 20S proteasome, a highly selective inhibitor of the β 2 subunit (PR671A [30]) with a C-terminal vinyl sulfone warhead was used. As shown in Fig. 5, only the trypsin-like subsite was significantly inhibited (up to 95%). Instead, both remaining proteolytic sites, the chymotrypsin- and caspase-like sites, were still active and able to hydrolyze their specific substrates. This result indicates that the substrate **14** is hydrolyzed solely by the β 2 subsite.

The peptide **15** with the non-proteinogenic GNF residue in position P1 was subjected to the same experiment, and its hydrolysis was also found to be strictly connected to the activity level of the trypsin-like proteasome subunit.

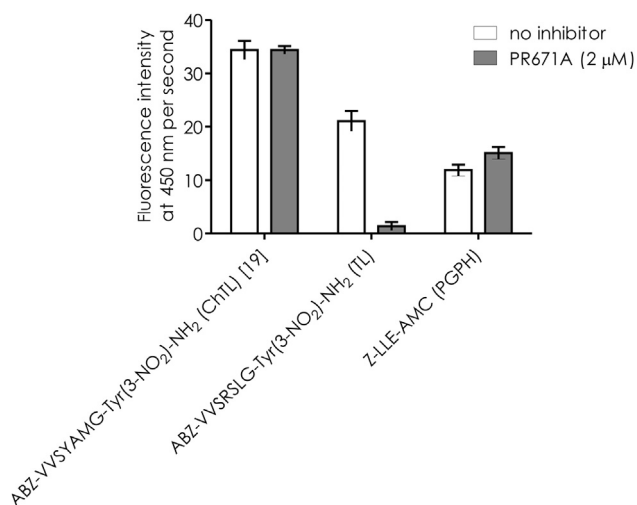


Fig. 5. Effect of inhibition of the 20S trypsin-like subunit on the cleavage rates of fluorogenic substrates. Enzymatic hydrolysis of the peptide was performed in 50 mM Tris-Cl (pH 8.2) supplemented with 0.02% SDS buffer at 37 °C and continued over 30 min.

Subsequently, the effect of the inhibition of other proteasomal subunits (chymotrypsin- or caspase-like) on the cleavage rates of substrates **1** and **15** (ABZ-Val-Val-Ser-GNF-Ala-Met-Gly-Tyr(3-NO₂)-NH₂) was investigated. Deactivated in this way, proteasomal subunits did not influence the processing of substrate **1** (data not shown).

Further modification of substrate **1** by replacing the several structural analogs of Arg proved that the β 2 subunit of 20S proteasome is able to recognize and cleave peptide bonds after positively charged residues with substantial but much lower efficiency than that of the starting peptide. However, only 1 substrate (**13**) with non-proteinogenic amino acid (4-guanidine phenylalanine) in the tested position displayed a moderate cleavage rate as the parental substrate **1** (15–20% of the rate recorded for substrate **1**).

Our result seems to be consistent with the findings presented by Geurink and coworkers [30], who described that replacing Arg with non-natural amino substituted phenylalanine derivatives leads to an improved potency of the inhibitors of the trypsin-like subunit. Moreover, it turned out that, in addition to the basic group in position P1, the phenyl ring also contributes to the inhibitory potency.

Kinetics

At this stage of our research, two peptide variants that differ in their C-terminal parts were synthesized: ABZ-Val-Val-Ser-Arg-Ser-Leu-Gly-Tyr(NO₂)-NH₂ and ABZ-Val-Val-Ser-Arg-Ala-Met-Gly-Tyr(NO₂)-NH₂ (variable sequences were underline). The rationale of such a synthesis was the results of our previous experiments yielding the optimization of internally quenched peptides selectively cleaved by the β 5 subunit (chymotrypsin-like) of eukaryotic 20S proteasome [24] (see Fig. 3S in supplementary material). Kinetic parameters are presented in Table 1. All of the obtained peptides were assayed against three eukaryotic proteasome entities: 20S, 26S, and 20S immunoproteasome. In that study, the substrate ABZ-Val-Val-Ser-Tyr-Ala-Met-Gly-Tyr(3-NO₂)-NH₂ (P1 residue in bold) was cleaved specifically and efficiently ($k_{cat}/K_M = 9.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) by the β 5 subunit. When the X₁ Tyr residue was replaced with phenylalanine substituted in the *para* position by a guanidine group (GNF), yielding ABZ-Val-Val-Ser-GNF-Ala-Met-Gly-Tyr(3-NO₂)-NH₂, such a peptide became resistant to the β 5 subunit but was cleaved by the trypsin-like subunit of proteasome (**15**) ($k_{cat}/K_M = 198 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$). Initially, we linked this result with GNF properties that enabled the formation of multiple contacts within the protease pocket. Surprisingly, peptide ABZ-Val-Val-Ser-GNF-Ser-Leu-Gly-Tyr(3-NO₂)-NH₂ (**13**) displayed an extremely low cleavage rate when incubated with eukaryotic 20S proteasome (Fig. 6). We had no explanation for these data. To further analyze this phenomenon, another peptide (**14**) was synthesized in the prime area where the Ala-Met-Gly (chymotryptic prime fragment from Ref. [19]) was present. Again, the proteolysis rate of this compound was very low (0.1% of peptide **1**). This indicated the crucial significance of the peptide fragment located in the prime area of all the substrates, which was clearly manifested in the difference between the proteolysis rates observed for pairs (**13** and **15** or **1** and **14**) (Fig. 6). Developed peptides have outstanding kinetic parameters as compared with commercially used proteasome substrate (Boc-Leu-Arg-Arg-AMC; $k_{cat}/K_M = 23,000 \text{ M}^{-1} \text{ s}^{-1}$). The discrepancy between the commercial peptide and compound **15** is 50 times ($k_{cat}/K_M = 1,980,000 \text{ M}^{-1} \text{ s}^{-1}$), and for compound **1** ($k_{cat}/K_M = 934,000 \text{ M}^{-1} \text{ s}^{-1}$) the factor is 24. It is clearly visible that a peptide fragment occupying the P1' to P3' subsites strongly decreases the K_M value, indicating the increase in affinity toward the β 2 subunit.

Moreover, substrate **15** displayed the highest selectivity toward trypsin-like subunit of 20S proteasome, which is manifested by a

Table 1
Kinetic parameters of selected internally quenched substrates.

Number	Substrate	Proteasome entity	V_{\max} ([mol/s] $\times 10^{-10}$)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($[\text{M}^{-1} \text{s}^{-1}] \times 10^4$)
14	ABZ-VVS-Arg-AMG-Tyr(3-NO ₂)-NH ₂	20S	Low rate	ND	ND	ND
		26S	Low rate	ND	ND	ND
		20Si	Low rate	ND	ND	ND
1	ABZ-VVS-Arg-SLG-Tyr(3-NO ₂)-NH ₂	20S	14.71 ± 1.11	17.65 ± 3.15	16.48 ± 1.21	78.47 ± 5.10
		26S	4.39 ± 0.21	43.02 ± 4.3	4.92 ± 0.23	11.44 ± 0.6
		20Si	5.44 ± 0.69	17.98 ± 5.16	6.1 ± 0.80	33.92 ± 6.35
15	ABZ-VVS-GNF-AMG-Tyr(3-NO ₂)-NH ₂	20S	7.51 ± 0.59	4.24 ± 0.87	8.41 ± 0.57	198.31 ± 27.54
		26S	3.25 ± 0.17	2.09 ± 0.30	3.64 ± 0.19	17.43 ± 1.54
		20Si	0.44 ± 0.05	10.61 ± 2.83	0.49 ± 0.06	4.64 ± 0.66
13	ABZ-VVS-GNF-SLG-Tyr(3-NO ₂)-NH ₂	20S	Low rate	ND	ND	ND
		26S	Low rate	ND	ND	ND
		20Si	Low rate	ND	ND	ND
16	ABZ-VVS-Arg-ANB-NH ₂	20S	15.80 ± 0.62	7.59 ± 1.31	2.94 ± 0.28	38.92 ± 5.72
		26S	17.85 ± 1.19	32.15 ± 2.34	5.23 ± 0.89	16.28 ± 3.87
		20Si	11.25 ± 2.84	19.27 ± 1.14	1.98 ± 0.48	10.27 ± 2.11

Note. Low rate: rate below 0.01 mol/s; ND: not determined.

higher value of the specificity constant ($k_{\text{cat}}/K_M = 2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$). In the cases of 26S proteasome and 20S immunoproteasome, these values are 10 and 50 times lower, respectively.

A similar but less pronounced effect is observed for peptide **1** with Arg residue in position P₁, whereas the short peptide **16** is less specific with a 3- to 4-fold differentiation factor obtained for 20S proteasome and its immune counterpart. In light of the data presented in Fig. 5, it is worth underlining that the obtained substrates (**1** and **15**) exclusively interact only with the $\beta 2$ 20S proteasome subunit. Incubation of the proteasome with a selective inhibitor of the trypsin-like subunits dramatically reduced substrate proteolysis (>95%). In contrast, the application of a selective inhibitor of the chymotrypsin-like subunit (carfilzomib at 10 nM concentration) did not influence the cleavage rate of either substrate **1** or substrate **15**. In all systems, the substrate was cleaved into two fragments: ABZ-Val-Val-Ser-Arg-OH and Ser-Leu-Gly-Tyr(NO₂)-NH₂ (peptide **1**) and ABZ-Val-Val-Ser-GNF-OH and Ala-Met-Gly-Tyr(3-NO₂)-NH₂ (peptide **15**) (data not shown).

Limit of detection

Titration of the dropping amount of 20S proteasome in the presence of a constant concentration of substrate **1** or **15** revealed that an observable fluorescent increase (3:1 signal-to-noise ratio) was detected when $5.12 \times 10^{-12} \text{ M}$ (in the assay with peptide **1**) and $1.23 \times 10^{-13} \text{ M}$ (in the assay with peptide **15**) of the 20S proteasome was used. The dose–response curve remains linear up to $4.23 \times 10^{-8} \text{ M}$ for peptide **1** and up to $2.36 \times 10^{-9} \text{ M}$ for peptide **15**.

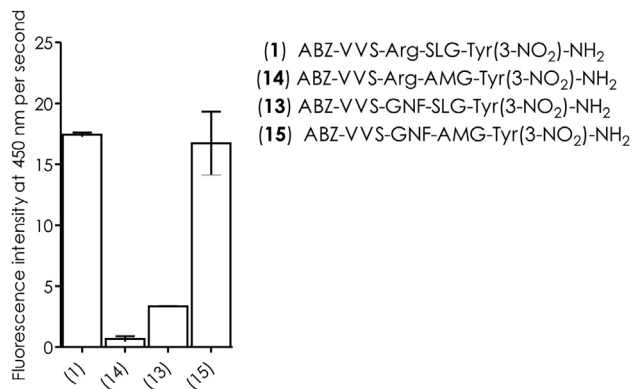


Fig. 6. Velocity of proteolysis of the peptides tested mediated by human 20S proteasome. Enzymatic hydrolysis of the peptide was performed in 50 mM Tris–Cl (pH 8.2) supplemented with 0.02% SDS buffer at 37 °C and continued over 30 min.

This again indicated an efficient interaction of the studied substrates with the enzyme (Fig. 7). The obtained limit of detection is at least one order of magnitude lower than that obtained for a commercially available fluorogenic substrate [22] with a value of approximately 0.1 $\mu\text{g/ml}$ ($\sim 1 \times 10^{-12} \text{ M}$).

Analysis of buffered solution of human serum and buffered human urine supplemented with human 20S proteasome indicates (see Fig. 7) that for substrate **1** the limit of detection is 2-fold higher ($6.34 \times 10^{-12} \text{ M}$) for human serum and it maintains the same level in buffered urine as compared with the experiment with buffer alone. Substrate **15** incubation with the mentioned biological

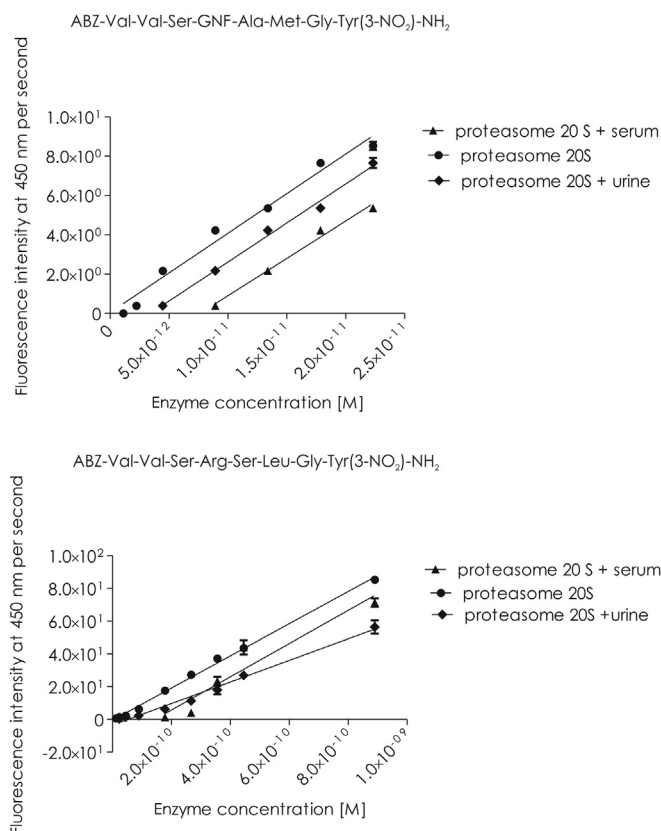


Fig. 7. Titration of peptides **15** and **1** against decreasing amounts of human 20S proteasome or buffered 2-fold diluted serum or urine solution supplemented by the same amount of 20S proteasome. Enzymatic hydrolysis of the peptide was performed in 50 mM Tris–Cl (pH 8.2) supplemented with 0.02% SDS buffer at 37 °C and continued over 30 min.

material results in an 8 times increase above the parameter, reaching 9.84×10^{-13} M for serum, and is not change in buffered urine. Analysis of whole blood incubation with the above compounds results in huge fluorescent background of blood itself, causing such measurement to be unacceptable.

Substrates developed in this study are highly sensitive, with the values of specificity constants reaching nearly 1×10^6 M⁻¹ s⁻¹ (substrate **1**) and nearly 2×10^6 M⁻¹ s⁻¹ (substrate **15**). Such parameters in combination with the low detection limit, which is superior to coumarin-based compound (Boc-Leu-Arg-Arg-AMC), makes them suitable probes for detection of eukaryotic 20S proteasome in enzymatic studies when low proteasome activity needs to be detected. Significant selectivity of the synthesized labeled peptides, especially substrate **15**, toward different proteasome entities allowed discriminating among activities of individual proteasome forms. Such profiling could be useful in assessment of the particular molecule participation in biological systems.

In conclusion, we have successfully developed and optimized novel and highly sensitive fluorogenic substrates of 20S proteasome. The X₁ modification and inhibitor study complement one another and allowed us to draw the conclusion that, using combinatorial chemistry, we were able to develop selective internally quenched peptide substrates optimized in the prime area. Their excellent kinetic parameters (k_{cat}/K_M reaching nearly 2×10^6 M⁻¹ s⁻¹) placed them among the most sensitive proteasome substrates. Such compounds appeared to be highly selective toward a particular proteasome entity and could be used for monitoring the trypsin-like specificity of the 20S proteasome in enzymatic studies. In addition, this could be applied for the design of novel kinds of proteasome inhibitors by conversion of the X₁–X_{1'} peptide bond into its steric analog.

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Appendix A. Supplementary data

Supplementary material for this article is available in the online version at <http://dx.doi.org/10.1016/j.ab.2015.08.019>.

References

- [1] A.P. Arrigo, K. Tanaka, A.L. Goldberg, W.J. Welch, Identity of the 19S “proteasome” particle with the large multifunctional protease complex of mammalian cells (the proteasome), *Nature* 331 (1988) 192–194.
- [2] J. Imai, H. Yashiroda, M. Maruya, I. Yahara, K. Tanaka, Proteasomes and molecular chaperones: cellular machinery responsible for folding and destruction of unfolded proteins, *Cell Cycle* 2 (2003) 585–590.
- [3] F. Kopp, K.B. Hendil, B. Dahlmann, P. Kristensen, A. Sobek, W. Uerkvitz, Subunit arrangement in the human 20S proteasome, *Proc. Natl. Acad. Sci. U. S. A.* 94 (1997) 2939–2944.
- [4] A.J. Marques, R. Palanimurugan, A.C. Matias, P.C. Ramos, R.J. Dohmen, Catalytic mechanism and assembly of the proteasome, *Chem. Rev.* 109 (2009) 1509–1536.
- [5] M. Groll, L. Ditzel, J. Löwe, D. Stock, M. Bochtler, H.D. Bartunik, R. Huber, Structure of 20S proteasome from yeast at 2.4 Å resolution, *Nature* 386 (1997) 463–471.
- [6] K. Tanaka, T. Mizushima, Y. Saeki, The proteasome: molecular machinery and pathophysiological roles, *Biol. Chem.* 393 (2012) 217–234.
- [7] S. Jäger, M. Groll, R. Huber, D.H. Wolf, W. Heinemeyer, Proteasome β-type subunits: unequal roles of propeptides in core particle maturation and a hierarchy of active site function, *J. Mol. Biol.* 291 (1999) 997–1013.
- [8] L.R. Dick, A.A. Cruikshank, L. Grenier, F.D. Melandri, S.L. Nunes, R.L. Stein, Mechanistic studies on the inactivation of the proteasome by lactacystin: a central role for clasto-lactacystin β-lactone, *Biochemistry* 35 (1996) 3899–3908.
- [9] L. Borissenko, M. Groll, Diversity of proteasomal missions: fine tuning of the immune response, *Biol. Chem.* 388 (2007) 947–955.
- [10] M. Verdoes, B.I. Florea, G.A. von de Marel, H.S. Overkleef, Chemical tools to study the proteasome, *Eur. J. Org. Chem.* 20 (2009) 3301–3313.
- [11] M. Groll, W. Heinemeyer, S. Jäger, T. Ullrich, M. Bochtler, D.H. Wolf, R. Huber, The catalytic sites of 20S proteasomes and their role in subunit maturation: a mutational and crystallographic study, *Proc. Natl. Acad. Sci. U. S. A.* 96 (1999) 10976–10983.
- [12] K. Tanaka, M. Kasahara, The MHC class I ligand-generating system: roles of immunoproteasomes and the interferon-γ-inducible proteasome activator PA28, *Immunol. Rev.* 163 (1998) 161–176.
- [13] H.J. Fehling, W. Swat, C. Laplace, R. Kuhn, K. Rajewsky, U. Muller, H. von Boehmer, MHC class I expression in mice lacking the proteasome subunit LMP-7, *Science* 265 (1994) 1234–1237.
- [14] K.J. Rodgers, R.T. Dean, Assessment of proteasome activity in cell lysates and tissue homogenates using peptide substrates, *Int. J. Biochem. Cell Biol.* 35 (2003) 716–727.
- [15] E.S. Lightcap, T.A. McCormack, C.S. Pien, V. Chau, J. Adams, P.J. Elliott, Proteasome inhibition measurements: clinical application, *Clin. Chem.* 46 (2000) 673–683.
- [16] O. Hoffmann, M. Heubner, T. Anlasik, M. Winterhalter, B. Dahlmann, S. Kasimir-Bauer, R. Kimmig, J. Wohlschlaeger, S.U. Sixt, Circulating 20S proteasome in patients with non-metastasized breast cancer, *Anticancer Res.* 31 (2011) 2197–2201.
- [17] C. Jakob, K. Egerer, P. Liebis, S. Türkmen, I. Zavrski, U. Kuckelkorn, U. Heider, M. Kaiser, C. Fleissner, J. Sterz, L. Kleeberg, E. Feist, G.R. Burmester, P.M. Kloetzel, O. Sezer, Circulating proteasome levels are an independent prognostic factor for survival in multiple myeloma, *Blood* 109 (2007) 2100–2105.
- [18] T. Tilgner, S. Temparis, L. Combaret, D. Taillandier, M.N. Pouch, M. Cervek, D.M. Cardenas, T. Le Bricon, E. Debiton, S.E. Samuels, J.C. Madelmont, D. Attaix, Chemotherapy inhibits skeletal muscle ubiquitin–proteasome-dependent proteolysis, *Cancer Res.* 62 (2002) 2771–2777.
- [19] J.L. Harris, P.B. Alper, J. Li, M. Rechsteiner, B.J. Backes, Substrate specificity of the human proteasome, *Chem. Biol.* 8 (2001) 1131–1141.
- [20] T. Nazif, M. Bogoy, Global analysis of proteasomal substrate specificity using positional-scanning libraries of covalent inhibitors, *Proc. Natl. Acad. Sci. U. S. A.* 98 (2001) 2967–2972.
- [21] A.C. Mirabella, S.L. Pletnev, B.I. Downey, T.B. Florea, M. Shabaneh, M. Britton, M. Verdoes, D.V. Filippov, H.S. Overkleef, A.F. Kisselev, Specific cell-permeable inhibitor of proteasome trypsin-like sites selectively sensitizes myeloma cells to bortezomib and carfilzomib, *Chem. Biol.* 18 (2011) 608–618.
- [22] Promega, Proteasome-Glo Chymotrypsin-like, Trypsin-like, and Caspase-like Cell-Based Assays [technical bulletin], <http://www.promega.com/-/media/Files/Resources/Protocols/Technical%20Bulletins/101/Proteasome-Glo%20Chymotrypsin-Like%20Trypsin-Like%20and%20Caspase-Like%20Cell-Based%20Assays%20Protocol.pdf>.
- [23] K. Chiba, E. Sato, M. Hoshi, Detection of in vivo proteasome activity in a starfish oocyte using membrane-impermeant substrate, *J. Biochem.* 122 (1997) 286–293.
- [24] N. Gruba, M. Wysocka, M. Brzezińska, D. Dębowski, M. Siernczyk, E. Gorodkiewicz, T. Guszcz, C. Czaplowski, K. Rolka, A. Lesner, Bladder cancer detection using a peptide substrate of the 20S proteasome, <http://dx.doi.org/10.1111/febs.13786>.
- [25] A. Furka, F. Sebastyén, M. Asgedom, G. Dibó, General method for rapid synthesis of multicomponent peptide mixtures, *Int. J. Pept. Protein Res.* 37 (1991) 487–493.
- [26] R.A. Houghton, C. Pinilla, S.E. Blondelle, J.R. Appel, C.T. Dooley, J.H. Cuervo, Generation and use of synthetic peptide combinatorial libraries for basic research and drug discovery, *Nature* 354 (1991) 84–86.
- [27] M. Wysocka, A. Lesner, N. Gruba, B. Korkmaz, F. Gauthier, M. Kitamatsu, A. Łęowska, K. Rolka, Three wavelength substrate system of neutrophil serine proteinases, *Anal. Chem.* 84 (2012) 7241–7248.
- [28] S. Narawane, A. Budnjo, C. Grauffel, B.E. Haug, N. Reuter, In silico design, synthesis, and assays of specific substrates for proteinase 3: influence of fluorogenic and charged groups, *J. Med. Chem.* 57 (2014) 1111–1115.
- [29] A. Budnjo, S. Narawane, C. Grauffel, A.S. Schillinger, T. Fossen, N. Reuter, B.E. Haug, Reversible ketomethylene-based inhibitors of human neutrophil proteinase 3, *J. Med. Chem.* 57 (2014) 9396–9408.
- [30] P.P. Geurink, W.A. van der Linden, A.C. Mirabella, N. Gallastegui, G. de Bruin, A.E. Blom, M.J. Voges, E.D. Mock, B.I. Florea, G.A. van der Marel, C. Driessen, M. van der Stelt, M. Groll, H.S. Overkleef, A.F. Kisselev, Incorporation of non-natural amino acids improves cell permeability and potency of specific inhibitors of proteasome trypsin-like sites, *J. Med. Chem.* 56 (2013) 1262–1275.