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Highly Specific Substrates of Proteinase 3 Containing 3-(2-Benzoxazol-5-yl)-L-alanine and Their Application for Detection of This Enzyme in Human Serum

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A set of benzoxazolyl-L-alanine derivates along with the MCA moiety (donors of fluorescence) were introduced into a proteinase 3 (PR3) substrate with a C-terminal ANB-NH_2 that serves as a fluorescence acceptor. Five substrates with general formula X-Tyr-Tyr-Abu-ANB-NH_2 were synthesized, and their kinetic parameters against proteinase 3 were determined. The highest k_{cat}/K_M value, 1.5×10^6 M^{-1} s^{-1}, was obtained for (Pyr)-Box-Ala-Tyr-Tyr-Abu-NH_2 where (Pyr)Box-Ala stands for *N*-methylpyrrole benzoxazole-L-alanine. Titration of this peptide with proteinase 3 resulted in measurable fluorescence at an enzyme amount equal to 29 pmol. This substrate was selected used to detect quantifiable levels of proteinase 3 in serum samples, including those of normal subjects. For all c-ANCA-positive samples (diagnosed Wegener granulomatosis), a significant increase of PR3 concentration was observed. Wegener granulomatosis is a severe autoimmune disease causing inflammation of the blood vessels. Our results clearly show that this substrate can be used for the construction of a very reliable, inexpensive, and easy to use diagnostic test for PR3 determination.

Proteinase 3 (PR3) is a neutrophil serine protease localized within azurophilic cytoplasmic granules of polymorphonuclear neutrophils (PMNs). Along with cathepsin G (CG) and neutrophil elastase, proteinase 3 is released upon leukocyte activation in the inflammatory site.[1] Historically, all neutrophil serine proteases (NSP) are described as elastin-degrading enzymes;[2] however, recent data reveal its contribution to several physiologically important processes such as control of cytokine activity (e.g., tumor necrosis factor-a [TNF-a], interleukin 1b [IL-1b]),[3] platelet activation,[4] and antimicrobial activity.[5] Current work reveals that only PR3 is involved in the regulation of cell proliferation[6] and apoptosis.[7]

Moreover, PR3 is known as the principal target antigen of antineutrophil cytoplasm antibodies (c-ANCA) that are formed in patients with Wegener's granulomatosis (WG).[8,9] This severe disease leads to destruction of small blood vessels within the inflammatory tract caused by necrotizing neutrophils. The neutrophils appear to play a dual role in WG so that they are targets of autoimmunity and are also involved in causing tissue injury. PR3 becomes the pathogenic factor that boosts the progress of the disease. Data provided by Uriarte and Brachemi[10,11] indicate that the presence of c-ANCA activates the neutrophil population, leading to increased secretion of PR3. The secreted enzyme can be bound to the leukocyte membrane or released directly into the bloodstream.

Recently, we reported a selective fluorogenic−chromogenic PR3 substrate, ABZ-Tyr-Tyr-Abu-ANB-NH_2,[12] containing a donor (ABZ, 2-aminobenzoic acid) and acceptor (ANB-NH_2, amide of 5-amino-2-nitrobenzoic acid) of fluorescence. Using this compound as a lead structure, we designed more efficient and specific substrates of PR3 which could be applied for determination of this enzyme in humans. Since there are no diagnostic tests available for direct determination of PR3 in serum (however, the procedure for c-ANCA determination is common

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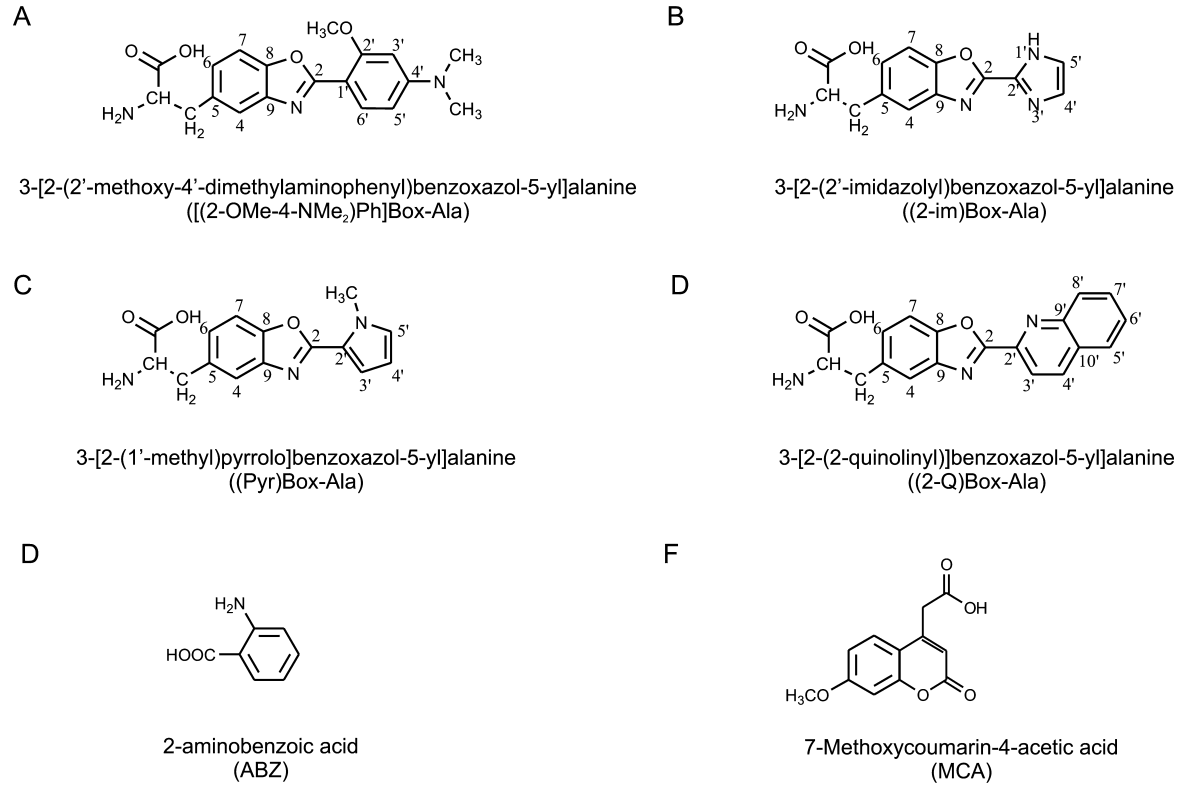

Figure 1. Chemical structures of 3-(2-benzoxazol-5-yl)alanine derivatives, ABZ, and MCA that are introduced on the N-termini of peptides.

Table 1. Physicochemical and Biochemical Characteristics of Peptides with General Formula X-Tyr-Tyr-Abu-ANB-NH_2

sequence where $X =$	MW calcd/ obtained	t_R [min][a]	F_1/F_0	$\lambda_{ext}/\lambda_{em}$ [nm]	ETE* [%]	k_{cat} [s^{-1}]	K_M [μM]	k_{cat}/K_M [$s^{-1} \times M^{-1}$] $\times 10^{-4}$
ABZ[12]	689.4/690.3	20.45	7.4	325/400	86	5.9 ± 0.3	31.4 ± 3.2	18.8 ± 0.2
MCA	808.3/809.3	25.08	5.2	315/410	80	1.1 ± 0.1	737.3 ± 94.5	1.4 ± 0.1
[(2-OMe-4-NMe_2)Ph]Box-Ala (**1**)	929.4/930.4	20.42	11.8	340/400	91	1.2 ± 0.1	201.4 ± 18.2	6.2 ± 0.5
(2-im)Box-Ala (**2**)	846.3/847.3	12.49	14.1	310/360	93	7.2 ± 0.3	163.2 ± 9.7	44.1 ± 0.4
(Pyr)Box-Ala (**3**)	859.3/860.2	19.94	10.2	314/360	90	18.1 ± 2.1	8.5 ± 1.3	154.2 ± 17.1
(2-Q)Box-Ala (**4**)	907.3/908.2	22.12	8.6	315/390	88	3.5 ± 0.2	875 ± 69.8	4.0 ± 0.1

[a] HPLC analysis indicated *ETE-energy transfer efficiency.

and widely used), we sought PR3 substrate(s) that could be applied for the construction of a reliable, inexpensive test for use in medical practice. In the synthesized peptide substrates, the N-terminal ABZ moiety was replaced by a set of benzoxazolyl-L-alanine (Box-Ala) derivates (shown in Figure 1). These derivates proved to be efficient donors of fluorescence as substrates of cysteine proteinases.[13] Box-Ala and ANB-NH_2 at N- and C-termini of the synthesized peptides served as donor and acceptor, respectively. These substituents allow fluorescence resonance energy transfer (FRET) to occur within the peptides. FRET peptides are commonly used as fluorogenic substrates for biochemical characterization of proteases. Energy transfer occurs between donor and acceptor groups located within the peptide backbone. The key factor is that energy transfer (quenching) depends on the distance between the donor and acceptor groups. Upon enzymatic proteolysis, the ANB-NH_2 is released, the distance between the donor and acceptor increases, and a boost in fluorescence is observed. Low molecular weight chromogenic/fluorogenic substrates are commonly used for determination of proteinases, even as a noncovalent complex with its cognate inhibitor (i.e., PR3 α-PI).[14] Bieth and others proved that such complexes are disrupted in the presence of a large excess of substrate.[15]

MATERIALS AND METHODS

Chemical Synthesis. *Benzoxazolyl Alanine Derivates.* *N*-Boc-3-[2-[2-(1′-methyl)pyrrolo]benzoxazol-5-yl]-L-alanine (Boc-(Pyr)Box-Ala) and *N*-Boc-3-[2-(2-imidazolyl)benzoxazol-5-yl] -L-alanine (Boc-(2-im)Box-Ala) were synthesized from *N*-Boc-protected 3-amino-L-tyrosine methyl ester whereas *N*-Boc-3-[2-(2-quinolinyl)benzoxazol-5-yl]alanine (Boc-(2-Q)Box-Ala) and *N*-Boc-3-[2-(2′-methoxy-4′-dimethylaminophenyl)benzoxazol-5-yl]-L-alanine (Boc-[(2-OMe-4-NMe_2)Ph]Box-Ala) were synthesized from *N*-Boc-protected 3-amino-L-tyrosine, via the intermediate Schiff base, which

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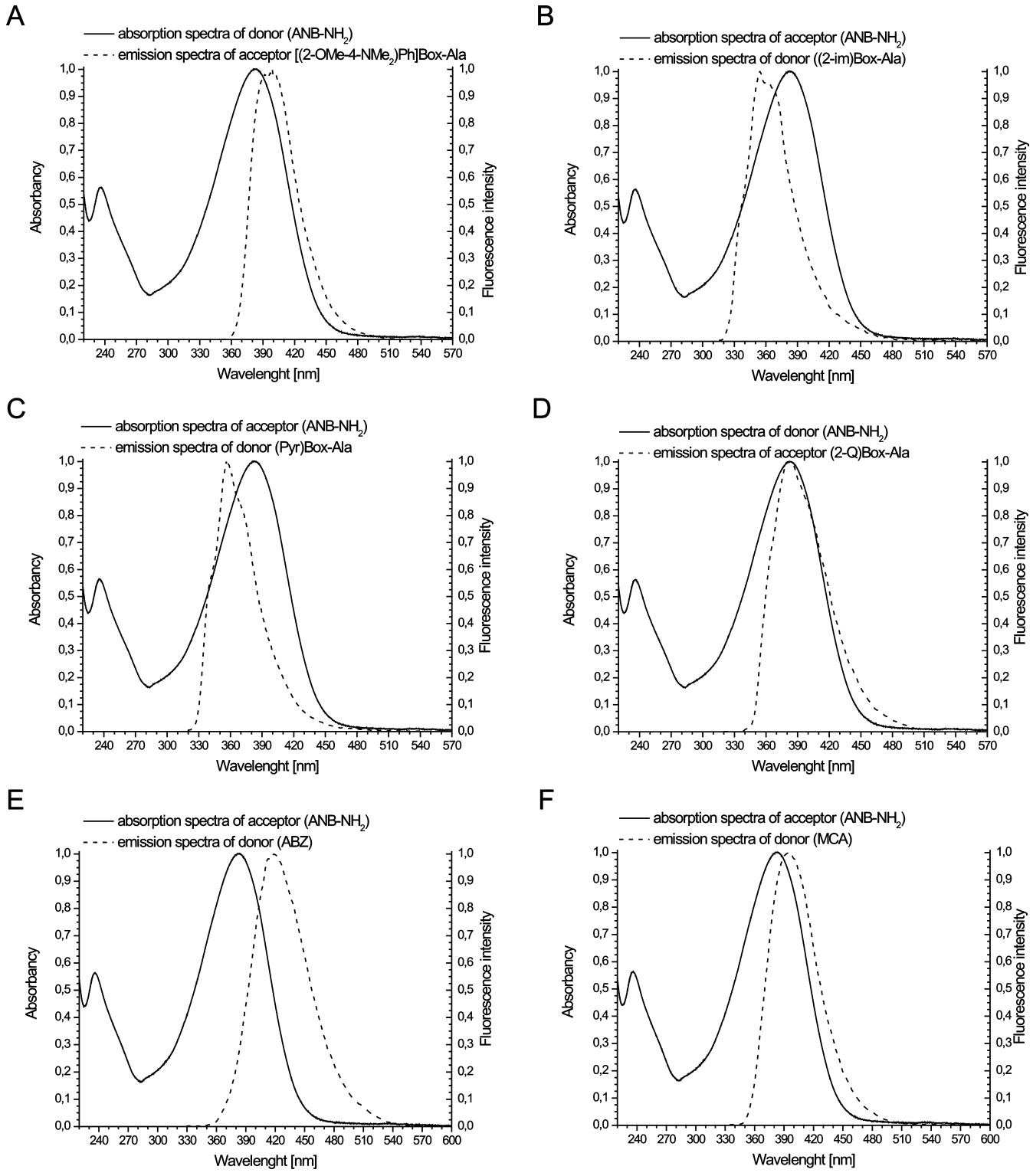

Figure 2. Superposition of absorption spectrum of acceptor (ANB-NH_2) and emission spectrum of donors (A−F).

underwent oxidative cyclization to the heterocyclic compound in the presence of lead tetraacetate in DMSO, according to the procedure published previously.[16,17] The methyl ester was removed by saponification using 1 N NaOH in methanol. 3-Nitro-L-tyrosine methyl ester, and *N*-Boc-3-nitro-L-tyrosine methyl ester and *N*-Boc-3-nitro-L-tyrosine, were prepared according to literature procedures published in refs 18 and 19, respectively.

Peptide Synthesis. All peptides were synthesized manually by the solid-phase method using Fmoc chemistry, as described previously.[20] TentaGel S RAM (substitution 0.25 mequiv/g) (RAPP Polymere, Germany) was used as a solid support. The α-amino

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groups of amino acids were Fmoc-protected. 5-Amino-2-nitrobenzoic acid (ANB) was attached to the resin by the TBTU/DMAP method.[21] Briefly, 2 mmol of ANB was dissolved in 5 mL of DMF, and 2 mmol of TBTU was added, which was followed by the addition of 1 mmol of DMAP. The resulting solution was added to 1 g of the resin and after 30 s supplemented with 4 mmol of DIPEA. The whole mixture was stirred for 3 h. The procedure was repeated three times. The C-terminal amino acid residues were incorporated using $POCl_3$ as the coupling reagent.[21] The other amino acid derivatives were coupled by the DIPCI/HOBt method. Briefly, a mixture of N-protected amino acid, DIPCI, and HOBt (molar ratio, 1:1:1) was dissolved in DMF:NMP solution (1:1, v/v) and added to the ANB-resin. A 3-fold excess to the resin active sites was used. The *tert*-butyloxycarbonyl derivates of benzoxazolyl-L-alanine (Boc-(X)Box-Ala) were introduced into the peptide chain using prolonged coupling (4 h) under the same conditions. After completion of the synthesis, the peptides were cleaved from the resin using a TFA/phenol/triisopropylsilane/H_2O mixture (88:5:2:5, v/v).[22] Purity of individual peptides was checked on an RP-HPLC Pro Star system (Varian, Australia) equipped with a Kromasil 100 C_8 column (8 × 250 mm) (Knauer, Germany) and a UV−vis detector. A linear gradient from 20 to 80% B during 40 min was applied (A: 0.1% TFA; B: 80% acetonitrile in A). The analyzed peptides were monitored at 226 nm.

Enzymatic Studies. *UV Studies.* All UV measurements were performed using a Cary 3E spectrophotometer (Varian, Australia). Bovine β-trypsin and turkey ovomucoid third domain (OMTKY3) come from Sigma-Aldrich (Germany). Proteinase 3 and human neutrophil elastase (HNE) come from Elastin Products Co., Inc. (Owensville, MO). The concentration of bovine β-trypsin stock solution was determined by titration with NPGB. OMTKY3 was used as a mutual inhibitor of bovine β-trypsin, proteinase 3, and HNE for titration of the active form of proteinase 3 and elastase.

The stock solutions of the synthesized substrates were prepared by dissolving about 3 mg of each peptide in 250 μL of DMSO and were further diluted 2−100 times; the enzyme concentration was 2.06×10^{-9} M for PR3 and 2.8×10^{-5} M for HNE. Three to five measurements were carried out for each compound (systematic error expressed as a standard deviation never exceeded 20%). All details of kinetic studies and the method of calculating kinetic parameters, Michaelis constants (K_M), catalytic constants (k_{cat}), and specificity constants (k_{cat}/K_M) have been described in our previous papers.[23,24]

Titration Curve. A constant amount (3.3 μM) of (Pyr)Box-Ala-Tyr-Tyr-Abu-ANB-NH_2 (**3**) was added into a buffered solution of proteinase 3 in Tris-HCl, pH 7.5, supplemented with 500 mM NaCl. The amount of assayed enzyme ranged from 2.2×10^{-9} M to 1.2×10^{-10} M. The absorbance increase at 405 nm versus time was measured. All obtained values were measured against substrate concentration with no enzyme added. The threshold limit for all measurements was 3:1, expressed as signal-to-noise ratio.

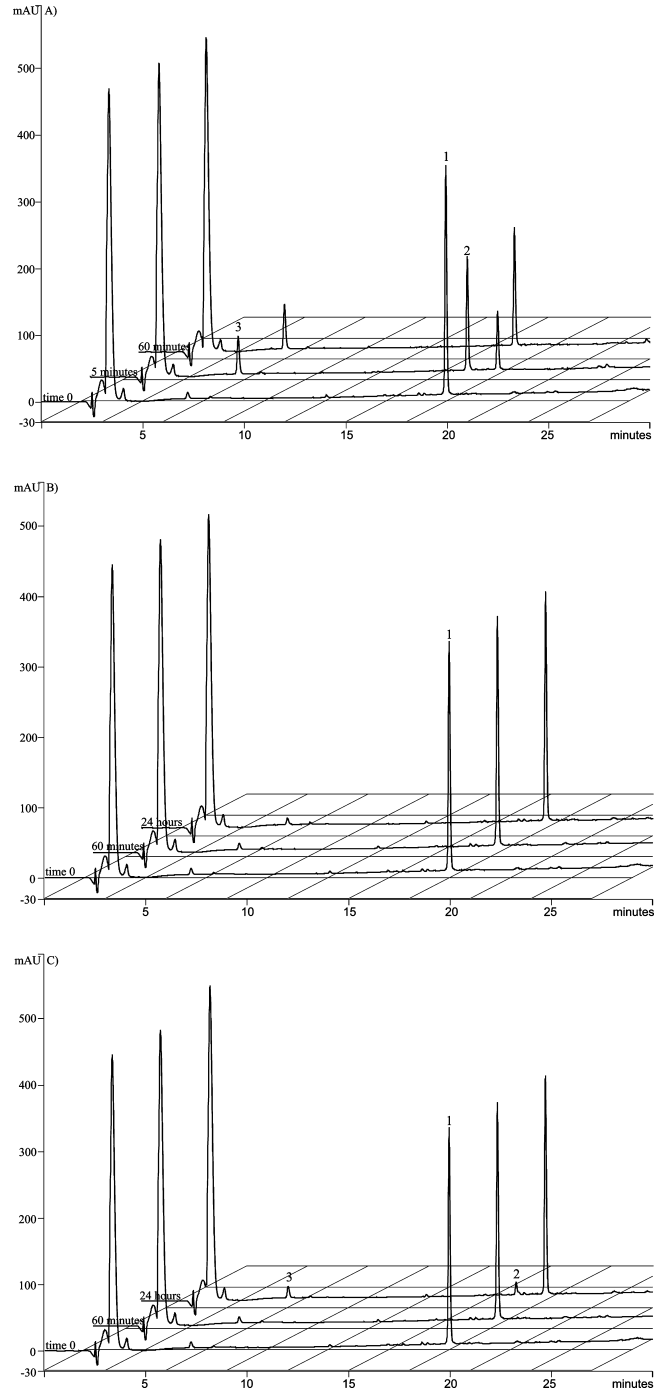

Figure 3. Proteolytic cleavage pattern of (Pyr)Box-Ala-Tyr-Tyr-Abu-ANB-NH_2 (**3**) in the presence of (A) proteinase 3, (B) cathepsin G, and (C) human neutrophil elastase. The incubation mixture was analyzed using the following time points: (A) 5 and 60 min; (B and C) 60 min and 24 h. MALDI-TOF analysis indicates that peak 1 corresponds to intact substrate, peak 2 to (Pyr)Box-Ala-Tyr-Tyr-Abu, and peak 3 to the amide of ANB.

Proteolytic Cleavage Pattern Determination. Selected substrates were mixed with a 2-fold molar excess of the enzyme in a buffer used for kinetic studies. HPLC analysis of this mixture was performed after the following incubation times: 5, 60 min in the case of proteinase 3, and 1 and 24 h in the case of cathepsin G (CG) and neutrophil elastase.

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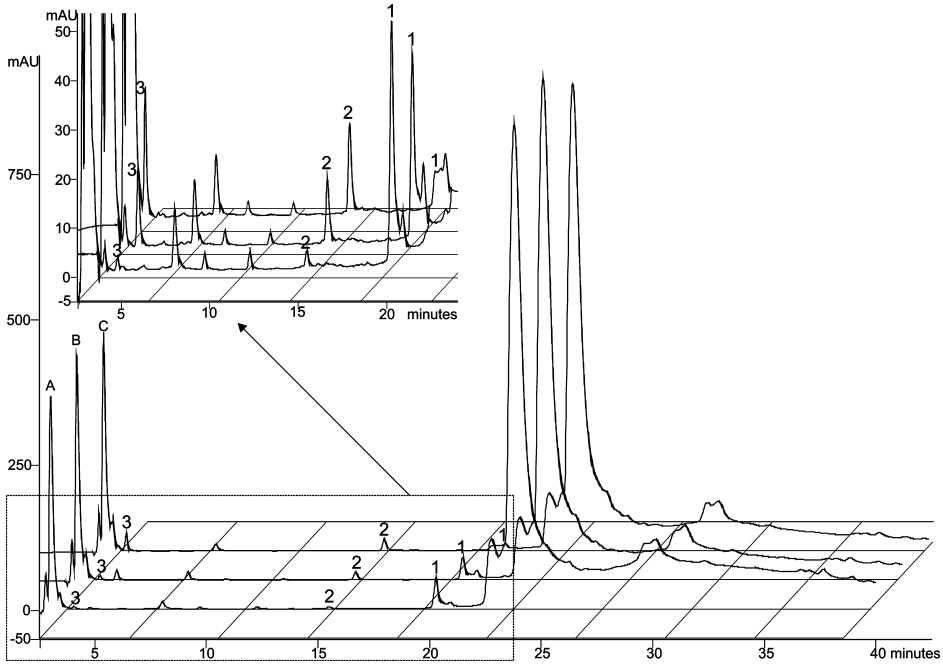

Figure 4. Incubation of substrate (Pyr)Box-Ala-Tyr-Tyr-Abu-ANB-NH_2 (**3**) in human serum: (A) time 0, (B) 1 h after incubation, (C) 24 h after incubation. MALDI-TOF analysis indicates that peak 1 corresponds to intact substrate, peak 2 to (Pyr)Box-Ala-Tyr-Tyr-Abu, and peak 3 to the amide of ANB. The signals with retention times between 22 and 26 min originate from serum.

Mass Spectrometry Analysis. Mass spectra were recorded using a Biflex III MALDI TOF mass spectrometer (Bruker, Germany) and α-cyano-4-hydroxycinnamic acid as a matrix.

Serum Stability Assay. A 500 μL amount of human serum from a Wegener-negative donor was mixed with 500 μL of 0.2 M Tris HCl buffer pH 8.3 and placed into an Eppendorf tube, followed by the addition of 10 μL of an appropriate substrate at a concentration of 3 mg/mL. After 1 and 24 h, a 50 μL sample of the mixture was analyzed by HPLC. An RP-HPLC Pro Star system (Varian, Australia) equipped with a Kromasil 100 C_8 column (8 × 250 mm) (Knauer, Germany) and a UV−vis detector was used. A linear gradient from 10 to 90% B during 45 min was applied (A: 0.1% TFA; B: 80% acetonitrile in A). The analyzed peptides were monitored at 226 nm.

Additionally, human serum was supplemented with 10 μL of NHE or PR3 stock (0.1 mg/mL) solution. The activity of the mixture was later evaluated using appropriate substrate(s).

Fluorescence Studies. The fluorescence assay was performed using a FluoroMax 4 fluorometer (Horiba Instruments, Inc., Ann Arbor, MI). For selected substrates, fluorescence increase and energy transfer efficiency were determined according to eqs 1 and 2, respectively, where F_0 is the fluorescence of the intact peptide and F_1 is the fluorescence of the product after complete hydrolysis. F_0 and F_1 values were calculated after the subtraction of buffer autofluorescence (F_{buff}) from both the initial (F_{ini}) and final fluorescence (F_{max}).

$$\frac{F_{max} - F_{buff}}{F_{init} - F_{buff}} = \frac{F_1}{F_0} \quad (1)$$

$$\text{energy transfer efficiency (\%)} = \left(1 - \frac{F_0}{F_1}\right) \times 100\% \quad (2)$$

Measurement of PR3 Activity in Human Serum. The human sera that come from both c-ANCA-negative (32) and -positive patients (12) with diagnosed WG were assayed using (Pyr)Box-Ala-Tyr-Tyr-Abu-ANB-NH_2 (**3**). A Nalgene 96-well plate was used. Into each well, 200 μL of serum was mixed with 150 μL of buffer (100 mM Tris-HCl, pH 7.5, supplemented with 500 mM NaCl) followed by 3 μM (1 μL) of substrate. The fluorescence emission at 360 nm was measured up to 180 s. Each sample was run in triplicates.

Serum Preparation and c-ANCA Determination. The serum samples come from 5 mL of blood drawn directly from the vein into the sterile tube. After 20−30 min (blood clotting), each sample was centrifuged at 4000 rpm for 5 min at RT. The supernatant was collected and stored at 4 °C.

c-ANCA presence was determined by two methods: indirect immunofluorescence (IFT) using ethanol-fixed neutrophils and anti c-ANCA antibodies (EUROIMMUN AG, Germany) and an anti-PR3 ELISA kit that comes from ORGENTEC Diagnostika GmbH, Germany.

RESULTS AND DISCUSSION

For all 3-(2-benzoxazol-5-yl)-L-alanine derivatives, 7-methoxycoumarin-4-ylacetic acid (MCA) and 2-aminobenzoic acid (ABZ) (used as a references[12]), the emission and absorption spectra were collected. In each case, overlap of the emission spectrum of the donors and the absorption spectrum of the acceptor (ANB-NH_2) (see Figure 2) was observed. For each donor−acceptor pair, the wavelength of the excitation and emission was determined (see Table 1).

The substitution of ABZ by MCA and (X)Box-Ala at the N-terminus of the previously designed PR3 substrate ABZ-Tyr-Tyr-Abu-ANB-NH_2[12] results in a series of peptides displaying FRET. The physicochemical properties of the obtained compounds are summarized in Table 1. In all cases the energy transfer efficiency was high, ranging from 80 (for MCA derivate) to 93% for (2-im)Box-Ala-containing peptides. All synthesized compounds possess outstanding spectral properties

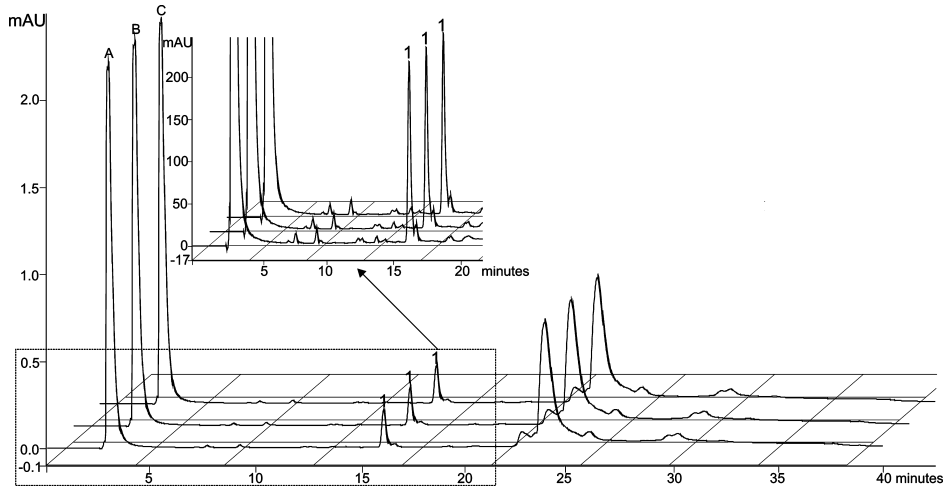

Figure 5. Incubation of substrate (Pyr)Box-Ala-Tyr-Tyr-Ser-ANB-NH_2 in human serum: (A) time 0, (B) 1 h after incubation, (C) 24 h after incubation. MALDI-TOF analysis indicates that peak 1 corresponds to intact substrate. The signals with retention times between 22 and 26 min originate from serum.

and could be utilized in the enzymatic assay. The determined values of the specificity parameter (k_{cat}/K_M) obtained for PR3 depend strongly on the introduced N-terminal benzoxazolyl alanine moiety. For the reference substrate ABZ-Tyr-Tyr-Abu-ANB-NH_2, k_{cat}/K_M reached almost 2×10^5 M^{-1} s^{-1},[12] whereas the analogue with MCA exhibited a value more than 1 order of magnitude lower. Among the four substrates containing Box-Ala derivates, two (compounds **2** and **3**) displayed a higher specificity parameter. The most active substrate, **3**, with (Pyr)Box-Ala exhibited an almost 8-fold increase, 1.5×10^6 M^{-1} s^{-1}, due to an increase of both catalytic constant (k_{cat}) (3-fold increase) and affinity (K_M) (4-fold increase) of the substrate toward the enzyme. This is not the case for compounds **1** and **4** where this parameter (K_M) was affected significantly (decrease of affinity 8 and 30 times, respectively). There is a correlation between specificity constant and the size of the chemical group introduced to Box-Ala (see Figure 1); small heterocyclic moieties (pyrrole, imidazole) optimize the specificity constant. The results presented above clearly indicate that substrate **3** is the most efficient PR3 substrate; therefore, in further experiments we focused our attention on this substrate.

PR3 is released from azurophile granules along with HNE and CG. Prolonged (up to 24 h) incubation of compound **3** with these proteases results in absence of proteolysis. These data indicate that peptides in the presence of these three neutrophil proteases (HNE, CG, PR3) are selectively hydrolyzed by PR3 (see Figure 3).

A sensitivity assay was performed for substrate **3**. The following equation was obtained $Y = -9.417 + 2.66e^{+11} \times X$ with $R = 0.997$. The results obtained reveal that fluorescence of substrate (Pyr)Box-Ala-Tyr-Tyr-Abu-ANB-NH_2 could be easily detected at an amount of proteinase 3 as low as 2.9×10^{-11} M, which is 10-fold lower than that for ABZ-containing peptide **6** reported previously by our group.[12]

Because peptide **3** undergoes selective proteolysis, it was subjected to a serum stability assay which may contain other enzyme(s) affecting its stability, but this would not allow accurate determination of PR3 in body fluids. HPLC and further mass spectrometry analysis of obtained fragments indicate that only cleavage corresponding to proteolysis of the bond between Abu and ANB-NH_2 is observed (Figure 4). Incubation of peptide (Pyr)Box-Ala-Tyr-Tyr-Ser-ANB-NH_2 (previously determined as unhydrolyzed by PR3) under the same conditions resulted in no observable degradation (Figure 5) or boost in fluorescence (data not shown). This indicates that compound **3** can be utilized as a tool to monitor the proteinase 3 activity in human serum.

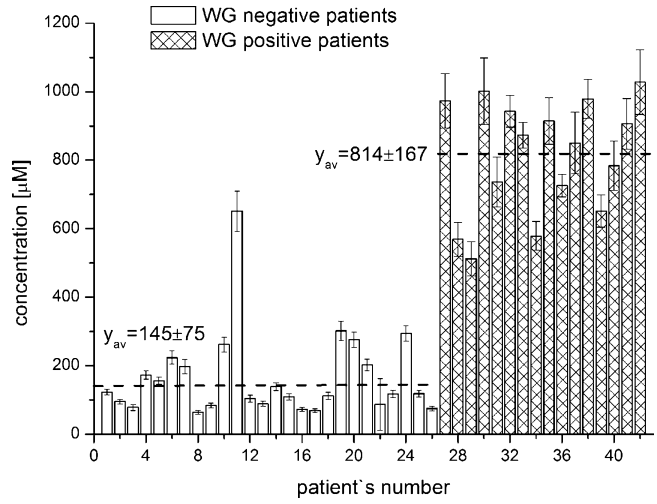

Figure 6. Analysis of results from 42 serum patients (30 negative and 12 positive ANCA samples).

Additionally, the selectivity of the substrate (**3**) was confirmed by measurements of the chromophore release in serum which was supplemented with 1 μL of 0.34 μM HNE or PR3. For each serum sample, the activity of the appropriate proteinase was measured using an NHE-specific substrate (Suc-Ala-Ala-Pro-Val-*p*NA) or PR3 (substrate **3**). For the HNE- and PR3-supplemented serum samples, there was a significant increase of the absorbance (data not shown). Serum sample supplementation was made in two independent experiments; one for HNE and the second for PR3.

We determined PR3 activity in 42 samples that originated from healthy (non-Wegener) (30 patients) and Wegener's diagnosed patients. The results of this assay are presented in Figure 6. Using substrate **3**, we detected measurable fluorescence in all analyzed samples. Based on the titration curve, the range of the PR3 concentration in all human serum samples of healthy patients was

from 64.6 to 301.9 μM but with one sample at 651.5 μM. In the serum of c-ANCA-positive patients, we observed a significant increase in PR3 activity that varied from 512.5 to 1028.0 μM. The obtained values of proteinase 3 concentration are in good agreement with data provided by Schnabel et al.[25] using an immunochemical (ELISA) method. However, these authors did not observe a correlation between c-ANCA and PR3 in the tested material. The immunochemical method used only detects the free PR3; thus, several types of antibodies are needed to detect free PR3, PR3−inhibitor complex, and PR-c-ANCA complex. This shortcoming of the Schnabel et al.[25] method is not a factor in our study because the low molecular weight chromogenic/fluorogenic compounds (in excess) used will dissociate to the noncovalent species.

In conclusion, a sensitive and selective substrate of proteinase 3 with new molecular probe was developed that serves as a fluorophore. This peptide displays dual chromogenic and fluorogenic properties and enabled us to detect as low as 28.7 pmol proteinase 3. Moreover, this substrate was successfully applied for determination of the PR3 level in human serum obtained from WG-negative and WG-positive patients. We believe that such a substrate could be utilized in preliminary Wegener's diagnosis.

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