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Design and Properties of Novel Proteasome Substrates Containing a Polyglutamine Sequence

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Abstract—Fluorogenic polyglutamine-containing peptides with five and ten glutamine residues in a row, having a FRET pair of EDANS (fluorophore) and Dabcyl (quencher), are characterized using spectral and mass spectrometric methods. The possibility of their hydrolysis by the 20S proteasome is examined. The kinetic parameters (catalytic efficiency) for these substances are determined. The presence of glycine in the substrate significantly decreases the solubility of the substrate and diminishes the efficiency of hydrolysis with the proteasome.

Keywords: proteasome, fluorogenic substrates, proteasome activity, polyglutamine diseases

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Huntington's disease is caused by the presence of an elongated polyglutamine tract in the huntingtin protein, which causes many cellular changes and ultimately leads to the neurodegeneration of the striatum neurons [1]. Although many theories have been suggested, the exact mechanism through which the expression of polyglutamine-containing proteins causes cellular changes has not been proven. Recent studies have shown that the ubiquitin–proteasome system is involved in the pathogenesis of Huntington's disease and understanding this process can have therapeutic significance [2]. There is conflicting information about whether proteasome is capable of hydrolyzing the polyglutamine sequences. Several groups of researchers have studied this issue, using both synthetic peptides and protein fragments. For example, Pratt and Rechsteiner [3] showed that short substrates of fluorescein-HPHQ_{10}RR and fluorescein-GGQ_{10}RR could be hydrolyzed by the proteasome in the presence of a PA28γ activator. Venkatraman et al. [4] demonstrated by MALDI–MS that for some synthetic peptides containing 10 to 30 glutamine residues in a row (the general structure of peptides is biotin-KKQ_{10-30}KK), the 20S and 26S proteasomes from rabbit and yeast are incapable of cutting the polyglutamine chain. For peptides having 10 and 20 glutamine residues in a row, cleavage of only one glutamine close to the *N*-terminus of the peptide was observed. Jana et al. [5] created stable cell lines expressing the pIND-tNhtt-polyQ-EGFP constructs from Neuro2a mouse cells. There, the *N*-terminal fragment of huntingtin (amino acid residues of 1–90 numbered according to the conventional huntingtin sequence annotated in the PDB database), containing 16, 60, or 150 glutamine residues in a row, was attached to the *N*-terminus of the enhanced green fluorescent protein (EGFP). The authors claim that inhibition of proteasome by the treatment of cells with solutions of lactacystin and *N*-Acetyl-Leu-LeuNle-CHO (ALLN) accelerates the aggregation of mutant fragments in the cell and the formation of inclusion bodies [5]. They also suggest that proteasome is involved in the hydrolysis of the *N*-terminal huntingtin fragment containing a polyglutamine fragment. It is noted that the rate of degradation is inversely proportional to the number of repeats of glutamine in this fragment. The activity of proteasome in lysates of cells expressing proteins, having 16 and 150 glutamine residues (depending on the time of expression) was also studied. In the case of the tNhtt-150Q-EGFP protein, approximately half of the proteasome activity is observed in the insoluble fraction of the cells after 4 days of expression, that is, the proteasome is bound to protein aggregates. Using mouse MEF cells expressing exon 1 of huntingtin with glutamine residues (25 or 97 residues in a row), other authors have shown that the mammalian proteasome can completely degrade the polyQ-containing fragment of both the normal and mutant proteins [6]. For the experiment, this protein fragment was isolated and purified and then incubated with proteasome for 16 h. The resulting mixture was analyzed by LC–MS and one or more discontinuities were observed [6].

In order to find out how the proteasome hydrolyzes polyglutamine peptides, we developed new fluoro-

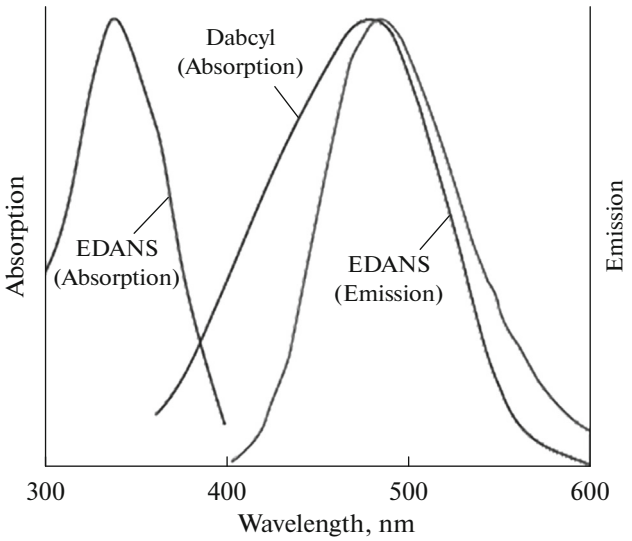

Fig. 1. Absorption and emission spectra of FRET pair of Dabcyl–EDANS [12].

genic proteasome substrates, the structure of which reflects the structure of the *N*-terminal part of the huntingtin protein. The goal of this work is the design and spectral and mass spectrometric analysis of new fluorogenic peptide substrates and testing of the possibility of their hydrolysis by the proteasome.

EXPERIMENTAL

Instruments and materials. All reagents were of cp grade or for molecular biology grade. We used tris(hydroxymethyl)aminomethane (Tris), glycerol, EDTA, dithiothreitol (DTT), Suc-Leu-Leu-Val-Tyr-AMC peptide fluorescent substrate, NaCl, $(NH_4)_2SO_4$, Na_2HPO_4, NaH_2PO_4, dimethylsulfoxide (DMSO) (Sigma, United States), and Bortezomib (LC Laboratories, United States). The Dabcyl-Lys-$(Gln)_5$-Gly-Asp-EDANS peptide was synthesized at Peptide Protein Research (United Kingdom); and the Dabcyl-Lys-$(Gln)_{10}$-Gly-Asp-EDANS and Dabcyl-Lys-$(Gln)_{10}$-Pro-Pro-Asp-EDANS peptides were synthesized in GenScript (United States).

Centrifugation was carried out using Eppendorf instruments (Germany). To determine the protein concentration, a NanoDrop-2000 analyzer (Thermo Scientific, United States) was used. Spectral characteristics were studied using a Hitachi 2800A spectrophotometer and a Cary Eclipse spectrophotometer (Varian). Kinetic fluorimetric measurements were performed using a Multilabel Reader Victor X5 microplate reader in black 96-well plates (Greiner Bio-One, Germany). All chromatographic stages were carried out using an AKTA Purifier high-speed high-performance liquid chromatograph (GE Healthcare) with columns Superose 6 10/300 GL, Superdex 200 10/300 GL, and MonoQ 5/50 (GE Healthcare).

Solutions. All solutions were prepared in distilled water or in high-purity water obtained using a Milli-Q system (Millipore).

The following buffer solutions were used for the isolation of the 20S proteasome:

Buffer A: 20 mM of Tris-HCl, 100 mM of NaCl, 1 mM of EDTA, 1 mM of DTT, and 10% of glycerol (pH 7.5);

Buffer B: 20 mM of Tris-HCl, 250 mM of NaCl, 1 mM of EDTA, 1 mM of DTT, and 20% of glycerol (pH 7.5);

Buffer D: 20 mM of Tris-HCl, 1 mM of EDTA, and 1 mM of DTT (pH 7.5);

Buffer F: 20 mM of Tris-HCl, 0.1 mM of EDTA, 1 mM of DTT, and 10% of glycerol (pH 7.8).

Procedures. The 20S proteasome was isolated according to the procedure described in [7–9]. The presence of proteasome was confirmed by immunoblotting, as described in [10, 11].

Kinetic measurements. To determine the rate of hydrolysis of the peptide substrates, a substrate solution in DMSO, mixed with Buffer D ([*S*] = 4–150 μM) was placed in a black opaque 96-well plate. A proteasome solution in Buffer D was added, and the total volume in the well was adjusted to 120 μL with Buffer D. The concentration of substrates in DMSO was determined by absorbance at a wavelength of 473 nm, using a value of the molar absorption coefficient of 32000 [L cm^{-1} mol^{-1}]. The fluorescence intensity was measured for 1 h with intervals of 30 s at an excitation wavelength of 355 nm, a fluorescence wavelength of 495 nm, and a temperature of 37°C. The measurement results were processed in the MS Excel program. To determine the fluorescence intensity I^{100} of free 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid (EDANS), the calibration measurements were performed in the EDANS concentration range from 10^{-6} to 5×10^{-4} M. The hydrolysis rate was calculated by the equation

$$V = \frac{\frac{dI}{dt}c}{(I^{100} - I^{0})},$$

where dI/dt is the change in the fluorescence intensity for the corresponding time; c is the concentration of the substrate; I^{100} is the fluorescence intensity of EDANS; and I^0 is the initial fluorescence intensity of the substrate.

Liquid chromatography–mass spectrometry. Samples of peptides were analyzed using a UPLC system (Waters) equipped with a PDA Detector and a TQD mass spectrometer (Waters). The separation was performed in an Acquity BEH C18 column (Waters). Elution was carried out with a gradient from 5 to 50% of Solvent B over 3 min at 35°C; the mobile phase flow rate was 0.5 mL/min. Solvent A was formic acid (20 mM) in a mixture of acetonitrile : water (1 : 20);

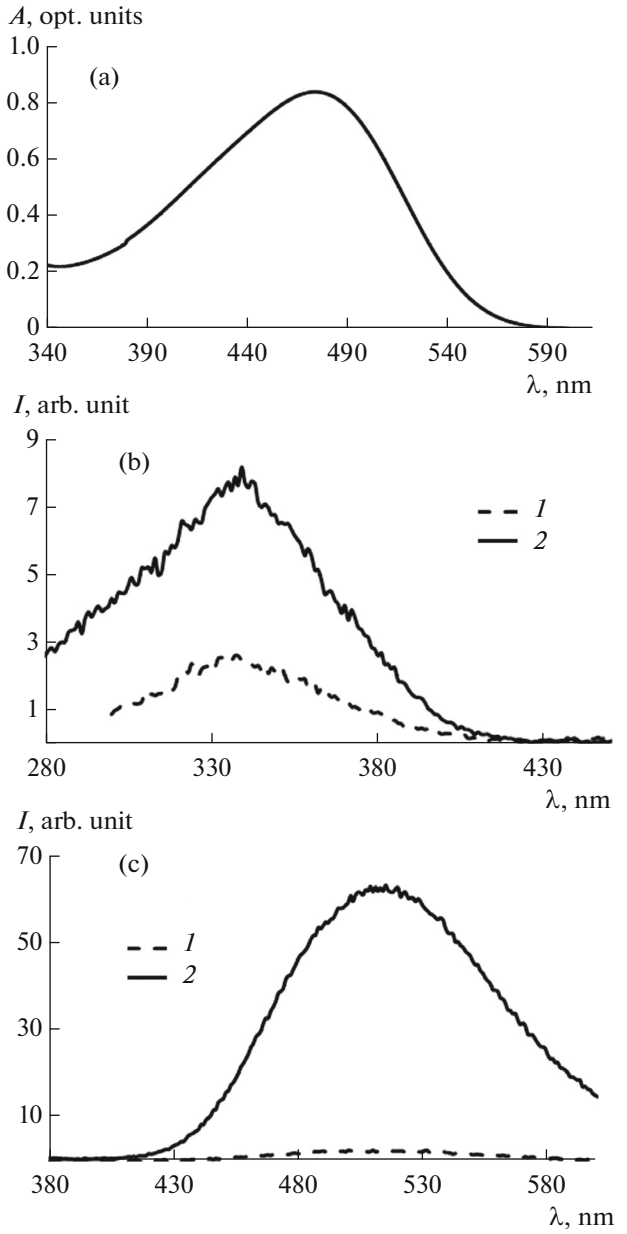

Fig. 2. Spectra of (a) absorption, (b) fluorescence excitation, and (c) fluorescence emission of Dabcyl-Lys-$(Gln)_5$-Gly-Asp-EDANS solution (*1*) before hydrolysis and (*2*) after hydrolysis with subtilisin in Tris-HCl buffer (pH 7.8) containing 1% of DMSO.

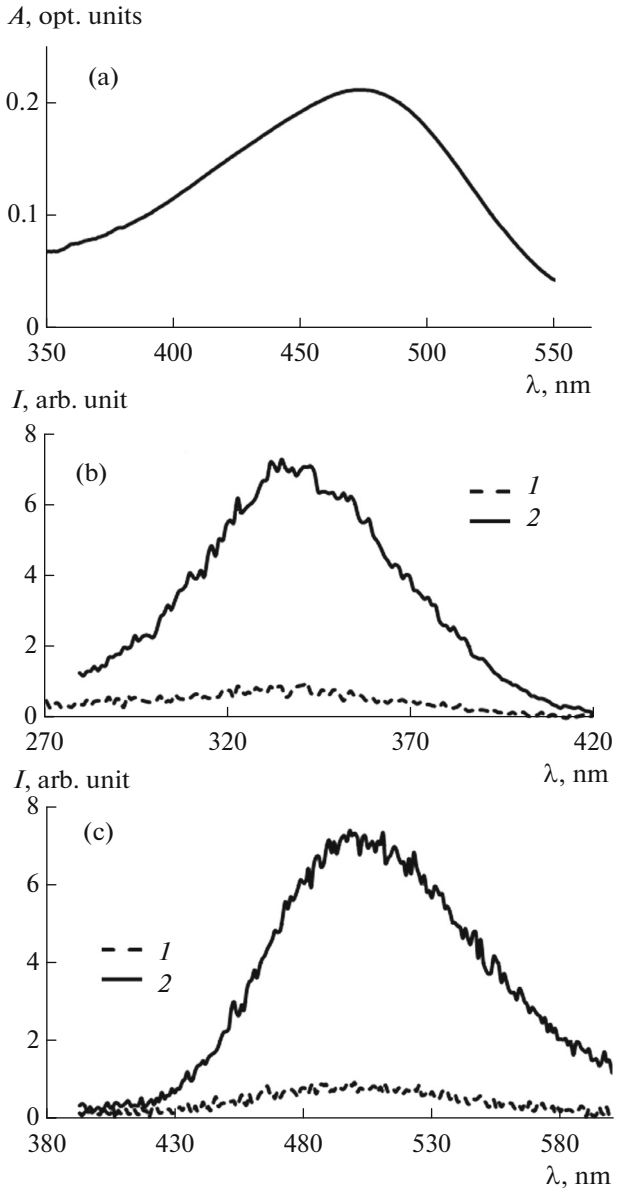

Fig. 3. Spectra of (a) absorption, (b) fluorescence excitation, and (c) fluorescence emission of Dabcyl-Lys-$(Gln)_{10}$-Gly-Asp-EDANS solution (*1*) before hydrolysis and (*2*) after hydrolysis with subtilisin in Tris-HCl buffer (pH 7.8) containing 1% of DMSO.

Solvent B was formic acid (20 mM) in acetonitrile. The mass detector worked in a positive-ion mode; the range of detectable masses was from 50 to 1500 Da; the wavelength range of the absorbance detector was 220–500 nm. The data were processed using the MassLynx software (version 4.1 SCN919).

RESULTS AND DISCUSSION

Peptide substrates with reporter groups suitable for detection by spectral methods are a well-proven system for the study of protease hydrolysis in vitro. Peptide substrates either with fluorogenic or with fluorescent groups are most often used to increase the sensitivity. In this paper, we selected peptides Lys-Gln-Gln-Gln-Gln-Gln-Gly-Asp and Lys-Gln-Gln-Gln-Gln-Gln-Gln-Gln-Gln-Gln-Gln-Gly-Asp, containing five and ten glutamine residues in a row and a FRET pair of the fluorophore (EDANS) and the quencher (Dabcyl) at the *C*-terminus and *N*-terminus, respectively, as model substrates to study the hydrolysis of polyglutamine-containing peptides and proteins.

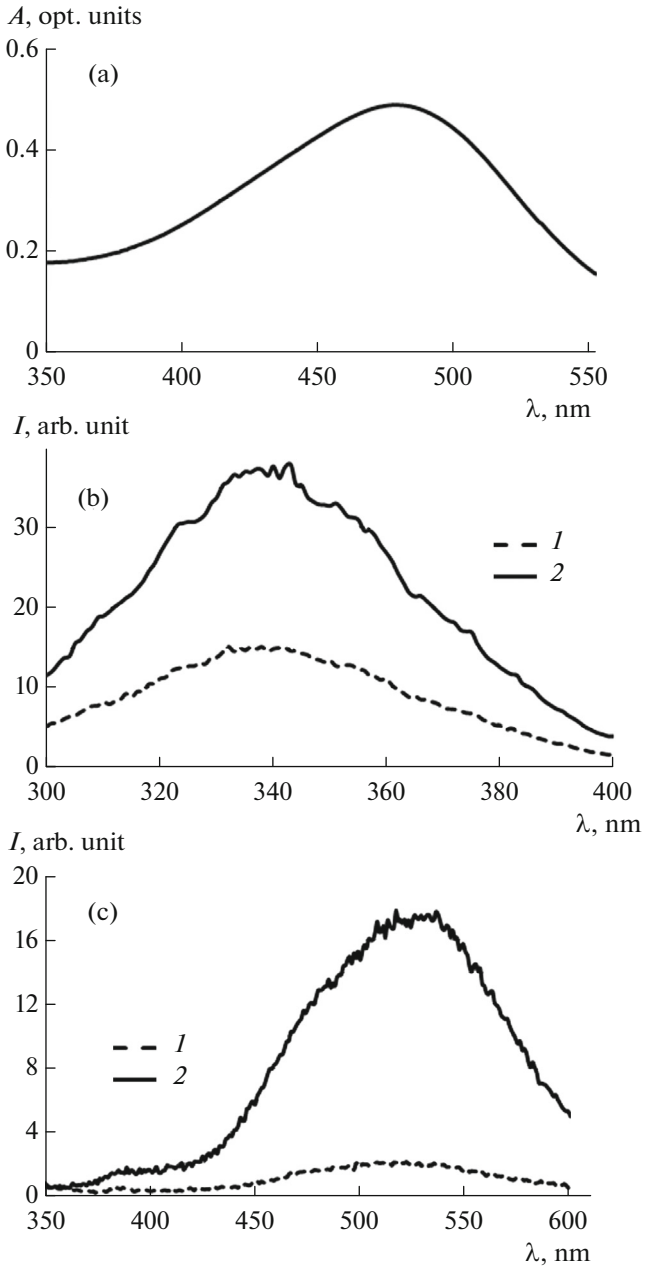

Fig. 4. Spectra of (a) absorption, (b) fluorescence excitation, and (c) fluorescence emission of Dabcyl-Lys-$(Gln)_{10}$-Pro-Pro-Asp-EDANS solution (*1*) before hydrolysis and (*2*) after hydrolysis with subtilisin in Tris-HCl buffer (pH 7.8) containing 1% of DMSO.

The absorption spectra (Dabcyl) and emission spectra (EDANS), reported in [12], have maxima at $\lambda \approx 500$ nm (Fig. 1).

The following substrates were synthesized at a commercial company:

$$\text{Dabcyl–Lys–}(\text{Gln})_5\text{–Gly–Asp–EDANS}, \quad (1)$$

$$\text{Dabcyl–Lys–}(\text{Gln})_{10}\text{–Gly–Asp–EDANS}, \quad (2)$$

$$\text{Dabcyl–Lys–}(\text{Gln})_{10}\text{–Pro–Pro–Asp–EDANS}. \quad (3)$$

To verify that the substrates correspond to the declared structure, their spectral properties (absorption, excitation, and emission spectra) were first studied. A Hitachi 2800A spectrophotometer and a Varian Cary Eclipse spectrofluorimeter were used for measuring the spectra of both initial substrates and substrates completely hydrolyzed with subtilisin. The spectra are presented in Figs. 2–4. An emission wavelength of 500 nm was selected to measure the excitation spectra. For the emission spectra, an excitation wavelength of 340 nm was recorded.

Certain conclusions can be made based on the given spectra.

(1) As follows from the absorption spectra, the maximum concentration in the aqueous buffer solution containing 1% of DMSO differs by several orders of magnitude for the three substrates. The highest concentration is achieved for the shortest substrate, and the lowest one is for the Dabcyl-KQ_{10}GD-EDANS substrate (which is probably due to the presence of glycine in the amino acid sequence).

(2) Fluorescence in the initial substrate is strongly quenched and flared during hydrolysis; the ratio of the emission intensity at the maximum of the spectrum after hydrolysis to the intensity at the same wavelength before hydrolysis depends on the length of the peptide, that is, on the distance between the fluorophore and the quencher, which confirms the presence of a resonant energy transfer.

(3) The shape of the spectrum and the position of the maxima are almost independent of the composition and length of the peptide. All the data obtained on the spectral characteristics of the substrates are summarized in Table 1. In the next stage, the FRET substrates were characterized by chromatography–mass spectrometry, which leads to the conclusion that the substrates are of a high degree of purity (Fig. 5).

Table 1. Spectral characteristics of substrates containing fluorophore-quenching pair and 5 or 10 glutamine residues in a row

Substrate	λ_{max} of optical absorption, nm	λ_{max} of fluorescence excitation, nm	λ_{max} of fluorescence emission, nm	I_{100}/I_0 (hydrolysis/initial)
Dabcyl-KQ_5GD-EDANS	473	335/339	514/514	12.8
Dabcyl-KQ_{10}GD-EDANS	473	342/338	500/500	8.05
Dabcyl-KQ_{10}PPD-EDANS	477	336/342	517/518	7.9

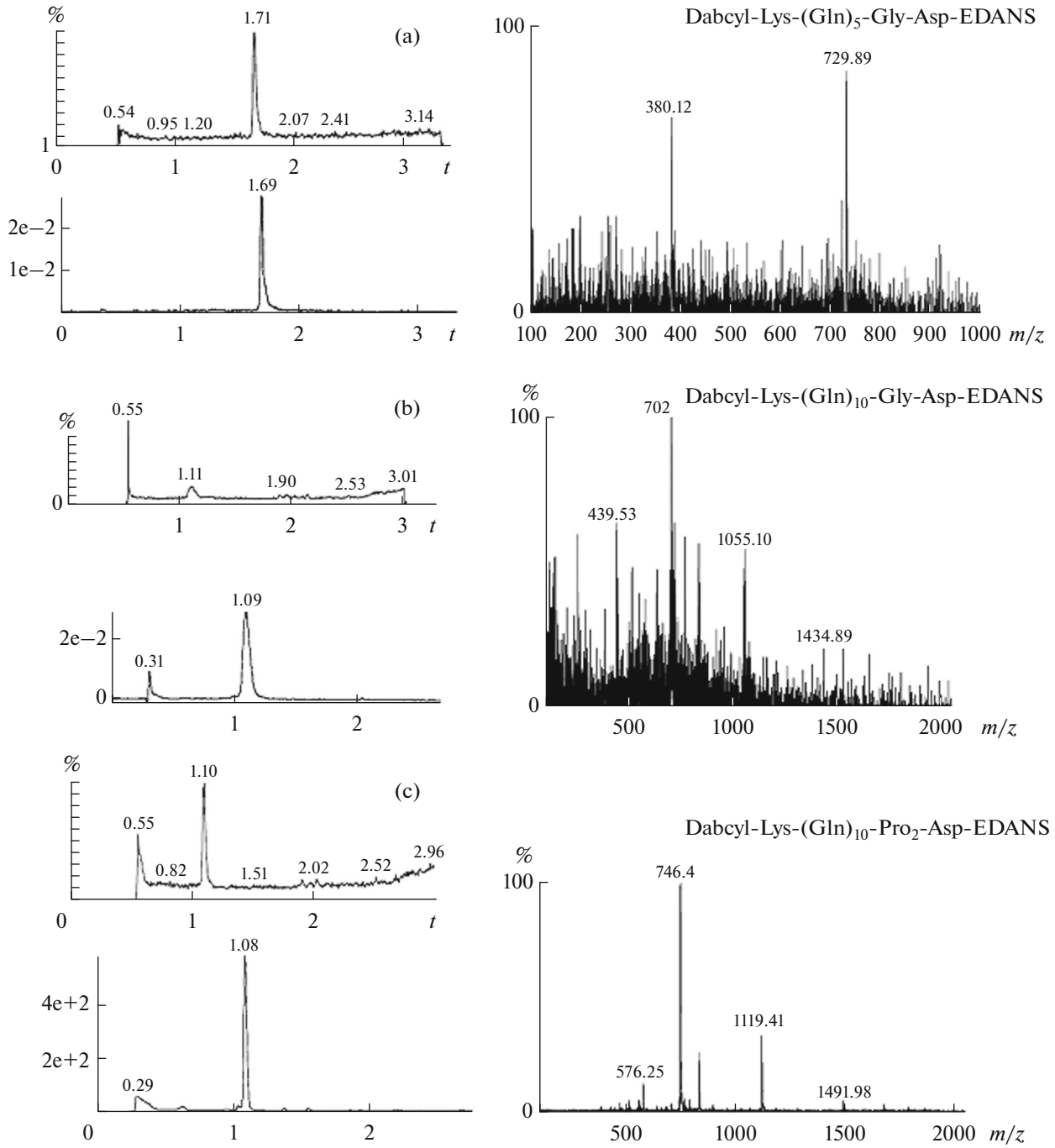

Fig. 5. (left column) Chromatograms recorded by (top) mass detection and (bottom) absorbance measurements and (right column) mass spectra of main chromatographic peak for (a) Dabcyl-KQ_5GD-EDANS, (b) Dabcyl-KQ_{10}GD-EDANS, and (c) Dabcyl-KQ_{10}RPD-EDANS.

Table 2. Kinetic parameters V_{max}/K_M of hydrolysis of various substrates by 20S proteasome

Substrate	V_{max}/K_M 10^4, min^{-1}
Dabcyl-KQ_5GD-EDANS (1)	4.1 ± 0.4
Dabcyl-KQ_{10}GD-EDANS (2)	1.8 ± 0.3
Dabcyl-KQ_{10}PPD-EDANS (3)	7.9 ± 0.9

It should be noted that only one peak of the substance was recorded in all the chromatograms. The weight, determined from the mass spectrum of this peak, corresponds to the weight of the starting compound taking into account the charge ($Z = 2$).

We also tested the possibility of the hydrolysis of these three substrates with the 20S proteasome. A 96-well

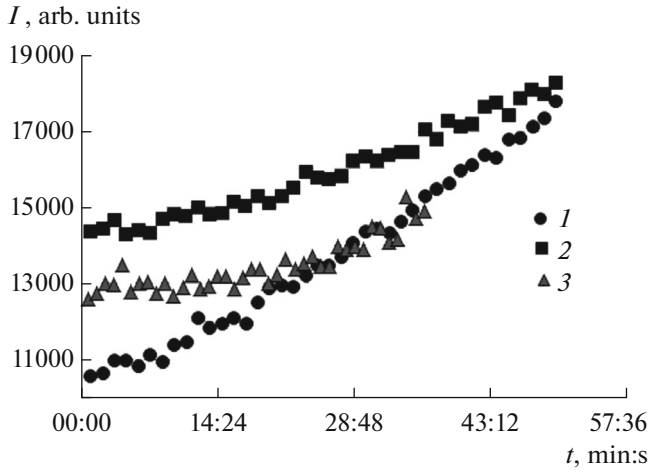

Fig. 6. Fluorescence versus time in hydrolysis of fluorogenic substrates with proteasome: (*1*) Dabcyl-KQ_5GD-EDANS, (*2*) Dabcyl-KQ_{10}GD-EDANS, and (*3*) Dabcyl-KQ_{10}PRD-EDANS.

opaque black low-absorption black plate (Greiner Bio-One, Germany) was used to reduce the amount of consumables. Fluorescence was measured using a 2030 Multilabel Reader Victor X5 microplate reader (PerkinElmer, United States). Examples of the dependence of the fluorescence intensity of the characteristic kinetic curves on time are shown in Fig. 6. The initial segment (the lag time) characteristic for such dependences is noted, which is always present in the hydrolysis of peptide substrates by the proteasome. Note that during a typical measurement time (1 h), spontaneous hydrolysis of the substrates was not observed.

The dependence of the rate of hydrolysis with the 20S proteasome on the concentration for all three fluorogenic substrates was studied. A typical curve is shown in Fig. 7. Processing of the kinetic data on the hydrolysis of substrates at the same proteasome concentration enabled the calculation of the kinetic parameters of catalytic efficiency (Table 2).

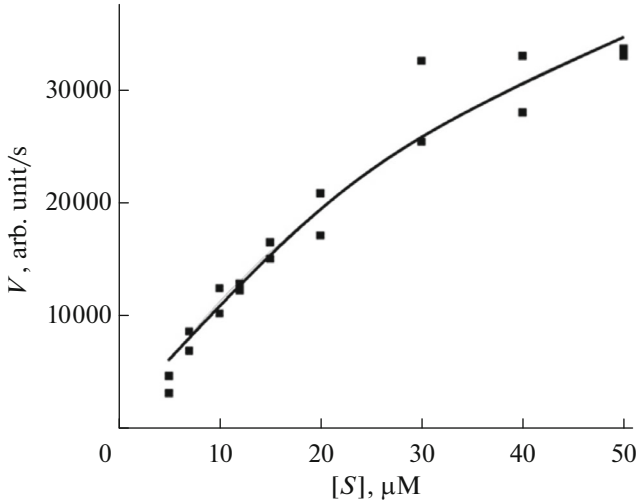

Fig. 7. Hydrolysis rate of Dabcyl-KQ_5GD-EDANS fluorogenic substrate by 20S proteasome as function of substrate concentration.

It is seen that the V_{max}/K_M ratios for Dabcyl-KQ_{10}PPD-EDANS (Substrate 3) are the highest, and they are closer to the values for Dabcyl-KQ_5GD-EDANS (Substrate 1) than to those for Dabcyl-KQ_{10}GD-EDANS (Substrate 2). As noted earlier, Substrate 1 is sufficiently soluble and its maximum concentration in a buffer containing 1% of DMSO is approximately 150 μM, while for Substrate 2, the maximum concentration under the same conditions does not exceed 50 μM, although, for Substrate 3, it reaches 120 μM. The catalytic efficiency in the case of Substrate 2 is much lower than that for Substrate 3, which is also most likely due to the low solubility of the former. According to our data, it is also possible to estimate the Michaelis constant for Substrate 1 as 30–50 μM, which agrees well with the Michaelis constants obtained earlier [13, 14] for other substrates of the 20S proteasome. Based on the results, it can be concluded that the proteasome is capable of hydrolyzing substrates containing polyglutamine fragments, while the enzyme prefers longer substrates.

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