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Voltammetric and atomic force microscopy characterization of chymotrypsin, trypsin and caspase activities of proteasome

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ABSTRACT

Proteasome is a multicatalytic enzyme complex responsible for proteolysis of damaged proteins and an important target for drug discovery in the pharmaceutical industry. Development of fast and economic strategies for detection of proteasome activity and inhibition is a topic of intensive research. The activity of the 20S proteasome was investigated by voltammetry and atomic force microscopy. The hydrolysis of peptide bonds was studied in incubated solutions of proteasome with oligopeptide sequences specific to each chymotrypsin, trypsin and caspase activity of proteasome, before and after inhibition with epoxomicin. The time-dependence of the proteolysis and the effect of substrate and inhibitor concentrations on the rate of enzymatic reaction were investigated. Different interaction mechanisms were characterized and enzyme kinetic parameters determined. The adsorption patterns of reaction mixture components were characterized by atomic force microscopy in order to understand the processes when saturation of enzyme catalytic centres occurs for high substrate concentrations.

1. Introduction

One of the most important function of biomolecules is to act as catalysts in order to increase the rate of chemical reactions within a living cell [1]. Although nucleic acids (ribozymes and deoxyribozymes) are capable of catalysing some reactions [2,3] most biological reactions are catalyzed by enzymes. Cells contain thousands of different enzymes, and their activities determine the multitude of chemical reactions that take place in order to maintain the proper functioning and sustain life. Structural and conformational damages to proteins frequently occur and this represents the main cause of abnormal functioning which consequently can lead to undesired medical anomalies [4].

Proteasome is a multicatalytic enzyme system responsible for keeping the balance between proper-functioning and damaged proteins [5,6]. Essentially, proteasome function is to degrade unneeded or damaged proteins by catalysing the hydrolysis of peptide bond in long polypeptide chains which are broken down into shorter oligopeptides or even amino acid residues [7]. Proper proteasome activity is essential for normal cellular functioning and deregulated proteasome action was observed in many malignancies [8]. In this context, fast, economic and reliable methodologies or strategies for detection of proteasome and to assess its activity are necessary for understanding the mechanisms of action, the enzyme kinetic and for development of inhibitors with potential medical applications [9].

Commonly, the in vitro measurement of proteasome catalytic activities is based on the use of fluorogenic peptides composed typically from a short amino acids sequence with C-terminally attached fluorescent probe [10], such as 2-naphtylamine, 7-amino-4-methylcoumarin and 4-methoxy-2-naphtylamine [11]. The proteasome cleaves these substrates between the last amino acid and the probe, resulting in the release of the fluorescent molecule [12]. Similarly, a bioluminescent assay which enables the measurement of proteasome activities directly from cultured cells was reported [13]. On the other hand, the assembly of the proteasome was investigated by atomic force microscopy [14] while surface plasmon resonance was employed for development of methods for quantification of proteasome [15] and X-rays crystallography was used to study proteasome-inhibitor complexes [16].

Electrochemical methods are advantageous due to their fast response and the possibility to detect compounds at concentrations

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below nanomole level [17,18]. Voltammetry allows discriminating compounds through their redox potential values and represent one of the most economic strategies for detection of biological interactions [19]. Electrochemical methods were used for detection and assessment of the activity of different enzymes [20] including some proteases such as thrombin [21], β-secretase [22] or trypsin [23]. Referring strictly to proteasome, voltammetry was used for quantification of proteasome [24] or of adenosine triphosphate through self-assembly of proteasome particles [25], but there is no report on the use of voltammetry for assessing proteasome activity.

In the present work voltammetry was used to assess proteasome activity. The kinetic of the proteasome catalyzed-proteolysis was investigated before and after inhibition of proteasome with epoxomicin [26] and using several substrate specific to each chymotrypsin, trypsin and caspase activity. The interaction mechanisms between substrates and proteasome were characterized by voltammetry and atomic force microscopy was also used in order to understand the adsorption processes at electrode surface. The results are important in the context of development of fast and economic methodologies/strategies for detection of proteasome, its activity and inhibitors.

2. Experimental

2.1. Materials and reagents

Proteasome 20S human, proteasome substrates: Suc-LLVY-AMC of chymotrypsin, Ac-RLR-AMC and Boc-LRR-AMC of trypsine, and Z-LLE-AMC of caspase activity, epoxomicin inhibitor from Enzo Life Sciences and 4-amino 7-methylcoumarin (AMC) from Sigma-Aldrich, were used without further purification.

Stock solutions of substrates and inhibitor in DMSO and of 20S proteasome in proteasome assay buffer were prepared and kept at +4 °C until further utilisation. Solutions of different concentrations of enzyme, substrates or inhibitor were obtained by dilution of the appropriate volume in the proteasome assay buffer.

The proteasome assay buffer (PAB) pH = 7.5 containing 50 mM Tris/HCl, 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$ and 100 μM SDS was prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity $\leq 0.1\ \mu S\ cm^{-1}$).

The pH measurements were carried out with a Crison 2001 pH-meter with an Ingold combined glass electrode.

2.2. Voltammetric parameters and electrochemical cells

Voltammetric experiments were carried out using a Compact-Stat potentiostat running IviumSoft 2.471 from Ivium Technologies, The Netherlands. The measurements were performed using a three-electrode system in a one compartment V-shape electrochemical cell of 3 mL maximum capacity. The conical bottom of the electrochemical cell allowed measurements in 50 μL solution. A glassy carbon (GCE, $d = 1.0$ mm), a Pt wire, and a Ag/AgCl (3 M KCl) were used as working, auxiliary and reference electrodes, respectively.

The experimental conditions for differential pulse (DP) voltammetry were: pulse amplitude of 50 mV, pulse width of 100 ms, interval time 1 s and scan rate of $5\ mV\ s^{-1}$.

Before each measurement the GCE was cleaned by surface polishing using diamond spray, particle size 3 μM (Kemet, UK), and conditioned in buffer by recording several cyclic voltammograms until a reproducible baseline was obtained. After mechanical or chemical cleaning the GCE was rinsed thoroughly with Milli-Q water.

2.3. Atomic force microscopy

Atomic force microscopy (AFM) was performed in the acoustic AC (AAC) mode, with a PicoScan controller and a CS AFM S scanner with a scan range of 6 μm in *x*-*y* and

2 μm in *z*, from Agilent Technologies, USA. AppNano type FORT of 225 μm length, $3.0\ N\ m^{-1}$ spring constants and 47–76 kHz resonant frequencies in air (Applied NanoStructures, Inc., USA) were used. All AFM images were topographical and were taken with 512 samples/line x 512 lines and scan rates of 0.8–2.5 lines s^{-1}. When necessary, the AFM images were processed by flattening in order to remove the background slope and the contrast and brightness were adjusted.

Highly oriented pyrolytic graphite (HOPG), grade ZYB of $15 \times 15 \times 2\ mm^3$ dimensions, from Advanced Ceramics Co., USA, and mica, from Agilent Technologies, USA, were used as substrates in the AFM study, because they are atomically flat. The GCE used for the voltammetric characterization is rough and therefore unsuitable for AFM surface characterization. Furthermore, the voltammetric experiments using HOPG and GCE showed similar electrochemical behaviours. The HOPG was freshly cleaved with adhesive tape prior to each experiment and imaged by AFM in order to establish its cleanliness.

The activity of 20S proteasome on the Suc-LLVY-AMC substrate of chymotrypsin was studied onto HOPG by AAC mode AFM in air, using incubated solutions of $0.5\ \mu g\ mL^{-1}$ 20S proteasome with 10 μM Suc-LLVY-AMC in PAB, during periods of time up to 24 h. Control experiments with the Suc-LLVY-AMC substrate were also performed using 10 μM Suc-LLVY-AMC in PAB. 50 μL samples of the desired solution were placed onto the freshly cleaved HOPG for 3 min, allowing the free adsorption of the molecules. The excess of solution was removed with Millipore Milli-Q water, and the modified HOPG was dried in a sterile atmosphere.

2.4. Calibration curve

The AMC calibration curves were constructed by increasing the AMC concentration from 0 to 20 μM in the presence of decreasing concentrations of the desired substrate from 100 to 80 μM simulating the proteasome activity. Between measurements the GCE surface was always cleaned. DP voltammograms were recorded in the potential range of +0.20 V and +1.00 V. Three measurements were performed for each sample ($n = 3$).

2.5. Incubation procedure; measurement of 20S proteasome activity

Samples of 20S proteasome and the desired substrate were allowed to equilibrate for 10 min at the reaction temperature under continuous stirring at 750 rpm in an Eppendorf ThermoMixer C.

The reactions were carried out in Eppendorf test tubes and started by the addition of substrate to the 20S proteasome solution. 50 μL of the reaction mixture were collected after different time intervals and placed into the electrochemical cell where DP voltammograms were recorded. AMC released from the substrate by the specific proteasome activity were measured up to 60 min by recording the current of the AMC oxidation peak at $E_{pa} \sim +0.82$ V on the DP voltammogram. Between measurements the GCE surface was cleaned.

In order to study the inhibition of 20S proteasome by epoxomicin, similar reaction mixtures were prepared and incubated in the presence of the inhibitor.

For control experiments, similar reaction mixtures were prepared in the absence of proteasome.

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