Journal of Biotechnology 255 (2017) 57–65

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Evaluation of a biomimetic 3D substrate based on the Human Elastin-like Polypeptides (HELPs) model system for elastolytic activity detection

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ARTICLE INFO

Keywords:
Biomimetic substrate
3D matrix
elastin
elastolytic activity assay

ABSTRACT

Elastin is a fibrous protein that confers elasticity to tissues such as skin, arteries and lung. It is extensively cross-linked, highly hydrophobic and insoluble. Nevertheless, elastin can be hydrolysed by bacterial proteases in infectious diseases, resulting in more or less severe tissue damage. Thus, development of substrates able to reliably and specifically detect pathogen-secreted elastolytic activity is needed to improve the *in vitro* evaluation of the injury that bacterial proteases may provoke.

In this work, two human biomimetic elastin polypeptides, HELP and HELP1, as well as the matrices derived from HELP, have been probed as substrates for elastolytic activity detection. Thirty strains of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients were analyzed in parallel with standard substrates, to detect proteolytic and elastolytic activity. Results point to the HELP-based 3D matrix as an interesting biomimetic model of elastin to assess bacterial elastolytic activity *in vitro*.

Moreover, this model substrate enables to further elucidate the mechanism underlying elastin degradation at molecular level, as well as to develop biomimetic material-based devices responsive to external stimuli.

1. Introduction

Elastin, the main constituent of the elastic fibers of connective tissues, is present in the arteries and it is particularly abundant in the large blood vessels such as the aorta and in connective tissue of lungs, ligaments, skin, bladder, cartilage, and eye. It is formed by tropoelastin chains, covalently linked by lysyl oxidase and assembled in a resistant network with desmosine and isodesmosine bonds (Vrhovski and Weiss, 1998). The structure of the protein, mainly composed of few hydrophobic aminoacids, is characterized by the presence of two principal domains: the cross-linking domain, a hydrophilic region characterized by the presence of lysines embedded in stretches of alanine and the hydrophobic domain, rich in non-polar residues, in particular valine (V), proline (P), alanine (A) and glycine (G). The hydrophobic residues are typically present in tetra-, penta- and hexa-repetitions, such as -VPGG-, -VPGVG- and -VAPGVG-. These repeated sequences are the main responsible for the unique protein properties (Vrhovski and Weiss, 1998). The behavior of the hydrophobic motifs of elastin has been studied using synthetic polypeptides, known as elastin-like polypeptides (ELPs), which are based on the -VPGVG- pentapeptidic repeat characterizing the bovine homologue (Urry et al., 1984; Urry et al., 1987).

HELP (Human elastin-like polypeptides) recombinant proteins, which are a subset of the ELP family, are based on the hexapeptidic variant of the motif found among the hydrophobic domains of primate tropoelastin (Ciofani et al., 2014). We have produced two biopolymers, named HELP and HELP1, based on these sequences but with different primary structures (Bandiera, 2010).

HELP is a biomimetic macromolecule that maintains the regular alternation of the hydrophobic domains with the cross-linking ones characterizing elastin. HELP1 is more similar to the ELPs described in the literature (Chilkoti et al., 2006), being constituted only by the hydrophobic repeated region, without the cross-linking domains (Bandiera, 2010).

Looking at natural structures as a model for biotechnologically produced materials has been gaining increasing importance. Development of artificial matrices inspired by nature represents a biomimetic approach that leads to realization of substitutes that mimic the native tissue and maintain its feature, especially referring to its functionality. These materials allow setting up experimental arrangements, where interactions between protein components and the microenvironment reliably resemble their physiological behavior.

Abbreviations: ELP, elastin-like polypeptides; HELP, human elastin like polypeptides

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http://dx.doi.org/10.1016/j.jbiotec.2017.06.006
Received 25 November 2016; Received in revised form 28 March 2017; Accepted 9 June 2017
Available online 15 June 2017
0168-1656/

Due to the presence of the cross-linking domains, HELP can form hydrogel matrices (Bandiera, 2011).

Such HELP matrices have been successfully assayed for elastolytic degradation in our lab (Bandiera et al., 2014).

Enzymes with elastase activity are found in animals as well as in plants and bacteria (Morihara and Tsuzuki, 1967; Bieth, 1986). They play key roles in several diseases such as infections, inflammation, pulmonary emphysema, cystic fibrosis, atherosclerosis and chronic wounds (Reid and Sallenave, 2001; Russell et al., 2002; Kapui et al., 2003; Yasmin McEniery et al., 2005).

Human elastin biomimetic substrates could be very helpful not only to evidence the enzymatic activity but also to gain a deeper knowledge about the mechanisms of elastin degradation.

In this work, we set up assays of bacterial elastolytic activity, by using three novel substrates modeled after human elastin, i.e. HELP, HELP1 and the HELP matrix, and compared their performance with the standard substrates (azocasein, gelatin and Elastin-Congo red), which are based on partially fragmented elastin of animal origin. Assays based on the use of human elastin-like molecules for elastolytic activity detection have not been reported so far.

These tests were performed on thirty *P. aeruginosa* clinical strains isolated from cystic fibrosis patients.

Differences were observed in the degradation pattern of either HELP or HELP1 polypeptides and could be clearly assigned to their diversity in the primary structure.

Our results point to a significant property of the 3D HELP matrix consisting in a different susceptibility to proteolysis compared to the other tested substrates, thus opening the way to a new approach for prediction of the *in vivo* elastolytic potential of bacterial proteases.

2. MATERIALS AND METHODS

2.1. Clinical isolates and reference strains

P. aeruginosa strains were isolated at the Microbiology Unit of the University Hospital ASUITS, Trieste (Italy) from sputa of anonymous 21 cystic fibrosis patients, in September and October 2015. Seven of these patients were infected by two or three different *P. aeruginosa* isolates. Patients were attending the Institute for Maternal and Child Health, IRCCS “Burlo Garofolo”, Trieste. The isolated strains were not associated to the patient's identity. *P. aeruginosa* PAO1 was used as the reference strain (Bandiera et al., 2014) and was kindly provided from the Microbiology Laboratory of the Department of Life Science of Trieste University (dr. C. Lagatolla).

2.2. Growth conditions of bacterial isolates and sample preparation

Clinical isolates of *P. aeruginosa* were inoculated in 3 mL of 2YT broth (1% w/v yeast extract, 1.6% w/v tryptone, 1% w/v NaCl) and cultured for 18 hours at 37 °C without shaking.

After bacterial growth, the optical density at 600 nm (OD_{600}) was measured for each isolate. Cultures were centrifuged (Eppendorf 5804R) at 10,000 rpm for 5 min. After centrifugation, crude samples containing proteolytic enzymes secreted by *P. aeruginosa* isolates were prepared collecting and filtering (0.22 µm) the culture supernatants and were then aliquoted and stored at −80 °C. Cultures were normalized upon OD_{600}, then cell pellets were collected, resuspended in sample buffer (0.05 M Tris/HCl, pH 6.8; 2% w/v SDS; 10% v/v glycerol, 5% v/v 2-mercaptoethanol and 0.002% w/v bromophenol blue) and analyzed on SDS-PAGE (Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis) to confirm total protein content. The culture supernatant samples were analyzed for total exoprotease and elastase activities.

2.3. Analysis of the exoproteolytic activity of the P. aeruginosa isolates with standard methods

Azocasein assay

Total protease activity was determined by azocasein assay (Safaríik, 1987). Azocasein (3 mg/mL; Sigma-Aldrich, #A2765) was dissolved in buffer solution (50 mM Tris/HCl, pH = 7.5, 1 mM $CaCl_2$,). 25 µL of each sample were added to reaction mixture (final volume 1 mL). The supernatants of all isolates, included the PAO1 reference strain, were diluted upon the culture density (OD_{600}) values. The reaction solutions were incubated at 37 °C for 24 hours. 0.5 mL of trichloroacetic acid (10% v/v) were added to each reaction. Tubes were immediately mixed, incubated at RT for 30 min and centrifuged (10,000 x g; 20 min). The amount of azopeptides released was measured at 400 nm by a multiplate reader (Synergy H1, Bio Tek). Negative control contained fresh 2YT broth in the reaction mixture. Results were calculated as the mean ± standard deviation (SD) of three independent experiments performed in triplicate.

Zymography

Gelatin zymography for detection of protease activity was carried out as previously described (Bandiera et al., 2014). Each sample, included the reference PAO1 strain, was diluted upon normalization to the culture density (OD_{600}) value. 15 µL of bacterial supernatant were mixed with 5 µL of a non-reducing buffer and loaded in a 10% polyacrylamide gel containing 0.13% (w/v) gelatin (Sigma Aldrich, #G2500). Following electrophoresis, gel was washed to refold proteins, incubated overnight at 37 °C to restore proteolytic activity and stained under gentle shaking. Zymograms were imaged using a gel documentation system. Protease activities were visualized as negative bands against a Coomassie blue background. Molecular weight markers (LMW Markers, Amersham Biosciences) were used as reference.

Elastin-Congo red assay

Specific elastolytic activity was assessed as previously described (Dieppois et al., 2012) with minor modifications. Briefly, 500 µL of Elastin-Congo red (Sigma Aldrich, #E0502) 10 mg/mL in 50 mM Tris/HCl, pH = 7.5, 1 mM $CaCl_2$ buffer was mixed with 70 µL of bacterial supernatant previously diluted upon cell density (OD_{600}) value, incubated with shacking at 37 °C for 24 hours and then centrifuged at 12,000 rpm for 15 min to remove insoluble Elastin-Congo red. The absorption of the supernatants was recorded at 495 nm using the multiplate reader. Fresh 2YT medium was used as a negative control. Results were calculated as the mean ± standard deviation (SD) of three independent experiments performed in triplicate.

2.4. Production of HELP and HELP1 polypeptides

HELP and HELP1 recombinant biopolymers were expressed in E. coli. Briefly, the pellet obtained from 1.2 L of IPTG-induced bacterial culture was re-suspended in 400 mL of extraction buffer (50 mM Tris/HCl pH = 8, 250 mM NaCl, 0,1 mM EDTA, 0,1% Triton X-100, 1 mM PMSF) and disrupted using a high pressure homogenizer (Panda NS1001L, GEA Niro Soavi, Italy). The recovered suspension was cooled on ice, 2-mercaptoethanol was added to 20 mM and centrifuged at 10000 rpm, for 30 min at 8 °C (Beckman–Coulter, J-26 XP). Supernatant was properly diluted adding fresh extraction buffer and precipitated adding NaCl to a final concentration of 0.5 M at 37 °C. Aggregated polypeptide particles were separated by centrifugation at 7000 rpm, 37 °C for 30 min. The pellet was re-dissolved in cold water, non-soluble material was discarded after cold centrifugation and solution was precipitated again by NaCl addition and rising temperature to 37 °C. Three of these cycles yield pure recombinant protein. After the last temperature-dependent transition cycle, the purified polypeptides were lyophilized for long-term storage.