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Research report

Bacterial enzymes effectively digest Alzheimer's β-amyloid peptide

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ABSTRACT

Aggregated β-amyloid peptides play key roles in the development of Alzheimer's disease, and recent evidence suggests that microbial particles, among others, can facilitate their polymerization. Bacterial enzymes, however, have been proved to be beneficial in degrading pathological fibrillar structures in clinical settings, such as strepto-kinases in resolving blood-clots. The purpose of this study was to investigate the ability of bacterial substances to effectively hydrolyze β-amyloid peptides. Degrading products of several proteinases from *Bacillus pumilus* were evaluated using MALDI-TOF mass-spectrometry, and their toxicity was assessed *in vitro* using cell-culture assays and morphological studies. These enzymes have proved to be non-toxic and were demonstrated to cleave through the functional domains of β-amyloid peptide. By yielding inactive fragments, proteinases of *Bacillus pumilus* may be used as candidate anti-amyloid agents.

1. Introduction

Alzheimer's disease (AD) is a slowly progressive primary neurodegenerative disorder. Its histo-pathological hallmark features, the senile plaques, are extra-cellular deposits of β-amyloid-peptide (βAP) and impaired cellular brain structures associated with inflammatory elements such as microglia. Although a number of hypotheses exist around the patho-mechanism of AD, there is perhaps no single causative agent. It is also dubious if plaques are responsible for and actively involved in the degenerative process, or just innocent by-products formed during the course of the disease that is triggered by yet unknown other factors. According to the amyloid-theory, soluble monomeric βAP is a physiological peptide with several vital functions in the brain (Wu et al., 1995; Schulz, 1996; Huber et al., 1997; Kamenetz et al., 2000), and aggregation is key to its neuro-toxicity (Pike et al., 1993; Dumery et al., 2001), however it is not clearly established what causes polymerization.

Converging lines of evidence suggest that bacterial and viral particles might play a role in cerebral amyloidosis seen in AD, and bacterial endotoxins, among others, have been demonstrated to promote βAP fibrillo-genesis (Reis et al., 2010; Asti and Gioglio, 2014). Neuro-infections may also lead to global cerebral inflammation similar to that is seen in AD brains. In order to facilitate trans-migration of mono-nuclear leukocytes from the periphery into the central nervous system (CNS), inflammatory mediators such as cytokines enhance the permeability of blood-brain barrier (BBB) (de Vries et al., 1996). Similarly, *Helicobacter pylori* also releases products that could induce break-down of the BBB (Kountouras et al., 2014). Other micro-organisms such as *Herpes simplex* virus type 1 (HSV1) and *Chlamydia pneumoniae* are postulated to be risk factors for AD (Itzhaki et al., 2004).

In addition to their role in infection, bacteria are also known for their therapeutic effects. Microbial proteinases are widely used as medicinal agents, such as the thrombo-lytic drugs staphylo- and strepto-kinase. Bacterial proteases have several advantages over similar products of animal origin: they are secreted into the medium and can easily undergo purification, they have high stability with low patho-genicity and toxicity, and cultivation of the micro-organisms is also more cost effective. Their role in βAP metabolism, however, is not yet characterized.

βAP is continuously produced in low concentrations, and is rapidly turned over in the normal brain: reduced catabolism may

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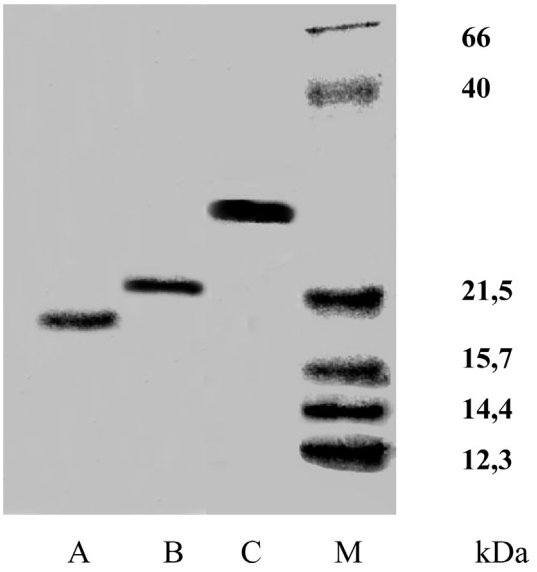

Fig. 1. SDS-PAGE of the *B. pumilus* proteinase. (A) Metallo-endo-peptidase (19 kDa), (B) glutamyl-endo-peptidase (23 kDa), (C) subtilisin-like proteinase (27 kDa), (M) markers.

account for the development of AD (Savage et al., 1998). βAP is normally degraded by a number of physiological proteases, but in AD it soon becomes resistant to them as a result of structural changes associated with its polymerization into fibrils. As such, it is necessary to distinguish between enzymes that can degrade βAP only in its monomer state and those that can cleave oligomeric and/or highly aggregated forms. The purpose of this study, therefore, was to investigate the activity of various classes of bacterial enzymes against βAP.

2. Materials and methods

Full-length βAP_{1-42} (GenScript, Piscataway, NJ, USA) was dissolved in distilled water, and final concentration of 0.2 mg/mL was used in experiments. Bacterial proteo-lytic enzymes (glutamyl-endo-peptidase, subtilisin-like proteinase and metallo-proteinase) of *Bacillus pumilus* 3–19 were isolated from 2 L of culture fluid of *Bacillus subtilis* BG2036 recombinant strain as described elsewhere (Shamsutdinov et al., 2008), and were used to digest βAP. These are different classes of proteases with varying substrate specificity. Hydrolyzed amyloid-fragments were subjected to sodium-dodecyl-sulphate (SDS) poly-acryl-amide gel electro-phoresis (PAGE) according to the method of Laemmli (Laemmli, 1970), and were identified using matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometer (Vision 2000, Thermo BioAnalysis, Altrincham, UK). Data were processed using PeptideMass Fingerprint (Matrix-Science, Boston, MA, USA) and PeptideMass (www.expasy.org) programs.

Proteolytic activity of the enzymes was determined by using the following substrates: Z-Ala-Ala-Leu-pNa homogeneous substance was cleaved by subtilisin-like proteinase (Lublinskaya et al., 1982), synthetic Z-Glu-pNa homogeneous molecule was hydrolyzed by glutamyl-endo-peptidase (Rudakova et al., 2010), and asocasein (Sigma–Aldrich, St. Louis, MO, USA) was digested by metallo-proteinase (Demidyuk et al., 1994). The unit of activity was equated to the amount of enzymes hydrolyzing 1 μmol substrate in 1 min.

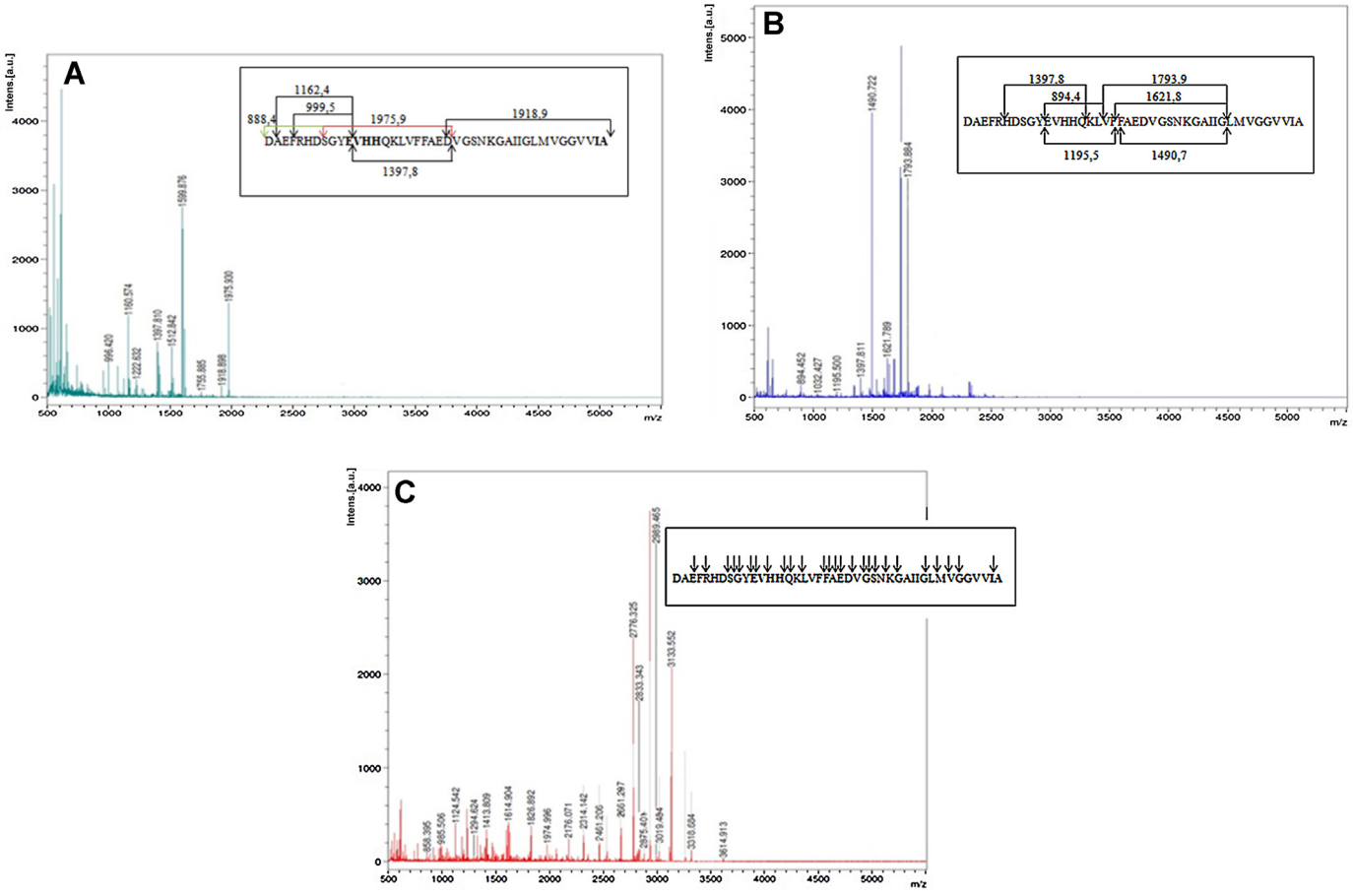

Fig. 2. Cleavage of βAP_{1-42} by *B. pumilus* proteases. (A) Glutamyl-endo-peptidase, (B) subtilisin-like protease, (C) metallo-proteinase.

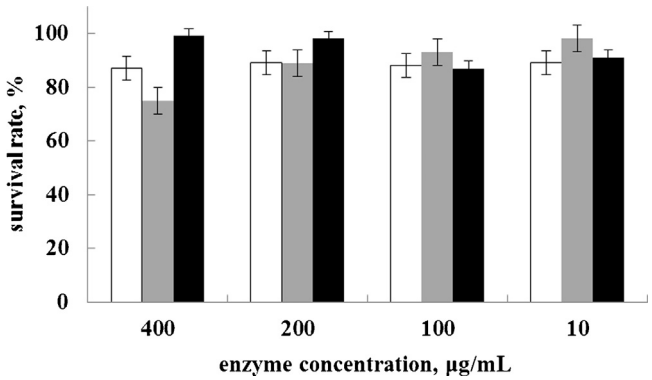

Fig. 3. Survival of SH-SY5Y cells after treatment with bacterial enzymes. Glutamyl-endo-peptidase (white), subtilisin-like proteinase (gray) and metallo-proteinase (black) were used at a concentrations of 10–400 μg/ml. Total (100%) survival of cells was defined in controls without enzyme treatment. The values are represented as mean ± SD.

Human neuro-blastoma SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and a mixture of antibiotics of penicillin and streptomycin (100 U/mL and 100 μg/mL, respectively) (Sigma–Aldrich, St. Louis, MO, USA). Cells were plated at a concentration 2×104 cells/well and incubated for 24 h at 37 °C in 5% of CO_2.

Toxicity of the enzymes was assessed by the colori-metric 3-(4,5-dimethyl-thiazol-2-yl)-2,5-di-phenyl-tetrazolium-bromide (MTT)-assay (CellTiter96 Aqueous One Solution, Promega, Madison, WI, USA): 20 μl MTT (5 mg/mL) was added to SH-SY5Y cultures in 96 multi-wells for 60 min at 37 °C, and optical densities were performed using Infinity spectro-photometer (Biosan, Warren, MI, USA) at a wave-length of 570 nm. Viability of cells was measured at different concentrations of enzyme preparations (10, 100, 200, 400 μg/mL). Relative ratios (%) were compared to the activity of mitochondrial dehydrogenases of untreated cells.

The effect of the proteins was determined by the change of cell morphology and cell mono-layer of SH-SY5Y cultures. Glutamyl-endo-peptidase, subtilisin-like proteinase and metallo-proteinase in various (10, 100, 200, 400 μg/mL) concentrations were added after forming an 80% mono-layer of cells. Visual evaluation of changes in cell morphology was performed before and after treatment of cultures with enzymes using an inverted fluorescence microscope AxyObserver Z1.

Results are presented as arithmetic mean ± standard deviation (SD). The program *MicrosoftExcel* was used for statistical calculations. Significance of differences ($P < 0.05$) was determined by Student's criteria (*t*-test).

3. Results and discussion

Enzymes of *Bacillus pumilus* 3–19 were isolated from culture medium of *Bacillus subtilis* BG2036 recombinant strain, and were electro-phoresed (Fig. 1). Subtilisin-like proteinase and glutamyl-enodo-peptidase are both serine-proteases, but with different substrate specificity. The subtilisin-like enzyme cleaves peptide bonds at the carboxyl-groups of hydro-phobic amino-acid residues phe, tyr, leu, and also at hydro-philic amino-acids ser, cys, asn, gln (Mikhailova et al., 2007; Itskovich et al., 1997). Unlike subtilisin-like proteinase, the glutamyl-endo-peptidase is a highly specific enzyme: it cleaves at glu, asp, and cys (Shamsutdinov et al., 2008) In contrast, the metallo-peptidase of *Bacillus pumilus* 3–19 has a wide substrate specificity and low specificity (Rudakova et al., 2010).

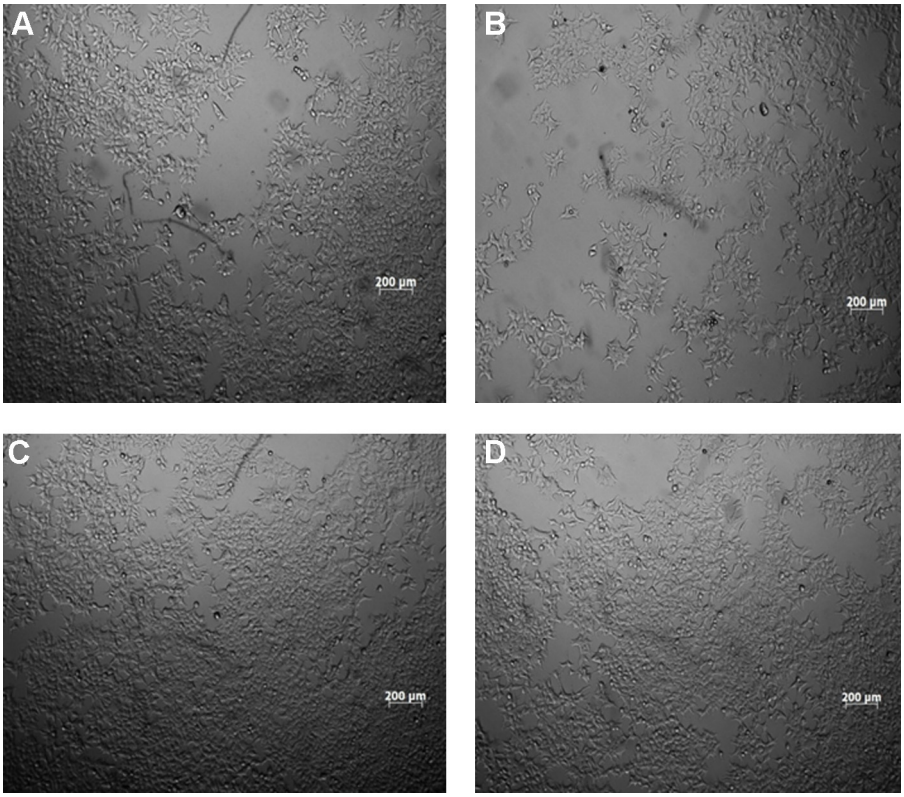

Fig. 4. Morphology of human neuro-blastoma cells after incubation with enzymes (400 μg/mL). (A) Control, (B) glutamyl-endo-peptidase, (C) subtilisin-like protease, (D) metallo-proteinase.

Digestion products of βAP were analyzed using MALDI-TOF mass-spectrometry. Having glu and asp-specificity, the glutamyl-endo-peptidase cleaved amyloid at asp^{1}–ala^{2}, glu^{3}–phe^{4}, asp^{7}–ser^{8}, glu^{11}–val^{12}, glu^{22}–asp^{23}, asp^{23}–val^{24} (Fig. 2A). Subtilisin-like protease hydrolyzed βAP at carboxyl groups of hydro-phobic amino-acids (phe^{4}–arg^{5}, tyr^{10}–glu^{11}, leu^{17}–val^{18}, phe^{19}–phe^{20}, phe^{20}–ala^{21}, leu^{34}–met^{35}), as well as at the bond formed by hydro-philic amino-acids gln^{15}–lys^{16} (Fig. 2B). Metallo-proteinase demonstrated low amino-acid specificity (Fig. 2C).

The 11-amino acid fragment $βAP_{25–35}$ has been proposed to be the functional domain of the full-length $βAP_{1–42/43}$ required for both neuro-trophic and neuro-toxic effects, of which $βAP_{31–35}$ is crucial for its cyto-toxic properties (Yankner et al., 1990; Qi and Qiao, 2001; Ashenafi et al., 2005). Micro-environment, concentration and specific amino-acid sequences at its structure (glu-val-his-his and val-ile) define the aggregation state of βAP (Esteras-Chopo et al., 2005). Amyloid polymerization also depends on the presence of metal ions, and βAP is known to have several high and low-affinity metal-binding sites (Syme and Viles, 2006). It is known that Zn^{2+}-ions induce dimerization of these metal-binding domains, including the glu^{11}–his^{14} area (Bush et al., 1994; Opazo et al., 2002).

Subtilisin-like proteinase and metallo-peptidise was able to cleave through and therefore inactivate the active center of amyloid. One of the metal-binding sites (glu^{11}–his^{14}) of βAP was also hydrolyzed by glutamyl-endo-peptidase at glu^{11}–val^{12}, and by metallo-proteinase at 3 positions (glu^{11}–val^{12}, val^{12}–his^{13}, his^{13}–his^{14}), however subtilisin-like proteinase did not splice within this important site.

Viability studies have confirmed that the enzymes had no toxic effects *in vitro* (Fig. 3). Glutamyl-endo-peptidase and metallo-proteinase did not reduce survival rate of SH-SY5Y cells, and subtilisin-like proteinase lowered culture viability up to 25% only in maximal concentration of 400 μg/mL. Visual observation of SH-SY5Y cells after treatment with bacterial proteins revealed that cell morphology and cell mono-layer were not affected (Fig. 4).

Evaluating agents that prevent βAP aggregation or clear amyloid-polymers is a hot topic in AD research. Various physiological proteases can metabolize βAP is the human body, however these natural enzymes are ineffective against aggregated amyloid-species. An amino-peptidase, isolated from Streptomyces species KK565 has been demonstrated to cleave the N-terminal amino-acid residues of $βAP_{1–42}$, thereby preventing the aggregation of its monomers (Yoo et al., 2010). It was also reported that natto-kinase of *Bacillus subtilis natto* can hydrolyze βAP (Hsu et al., 2009). In this study we have demonstrated that proteases of *Bacillus pumilus* 3–19 are non-toxic and are also capable of inactivating polymerization sites, therefore might serve as a spring-board to develop anti-aggregation drugs.

Conflict of interest

The authors declare no conflict of interest.

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