CELL DETACHMENT BY PROLYL-SPECIFIC ENDopeptidase FROM WOLFIPORIA COCOS

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Received 2014-01-20; Received 2014-02-06; Accepted 2014-02-08

ABSTRACT

As requirements for Advanced Therapy Medicinal Product (ATMP) production differ from other production processes (e.g., therapeutic protein production), cell detachment is often a crucial step for the process success. In most cases, cell detachment is done enzymatically. Although many peptidases are established in cell culture in R&D, e.g., Trypsin as gold standard, many of them seem to be unsuitable in ATMP production processes. Therefore, the present study investigated a novel endopeptidase used in food biotechnology for its applicability in ATMP processes where cell detachment is needed. The Prolyl-specific Peptidase (PsP) is of non-mammalian origin and considered as safe for humans. PsP was purified from the supernatant of the fungus Wolfiporia cocos. The isolation and purification resulted in an enzyme solution with 0.19 U mg$^{-1}$ prolyl-specific activity. By in silico analysis it was confirmed that attachment-promoting proteins can be cleaved by PsP in a similar amount than with Trypsin. Further the proteolytic activity was determined for PsP and Trypsin by using the same enzymatic assay. Detachment with both enzymes was compared for cells used in typical therapeutic production processes namely a mesenchymal stem cell line (hMSC-TERT) as a model for a cell therapeutic, Vero and MA104 cells used for viral therapeutic or vaccine production. The cell detachment experiments were performed with comparable enzyme activities (1.6 U mL$^{-1}$). hMSC-TERT detachment was faster with PsP than with Trypsin. For Vero cells the detachment with PsP was not only faster but also more efficient. For MA104 cells the detachment rate with PsP was similar to Trypsin. For all cell types, detachment with PsP showed less influence on cell growth and metabolism compared to standard Trypsin. Thus, three cell types used in ATMP, viral therapeutics or vaccine production can be detached efficiently and gently with PsP. Therefore, PsP shows potential for cell detachment in ATMP and viral/vaccine production processes.

Keywords: Cell Detachment, Prolyl-Specific Peptidase, ATMP, Trypsin, Wolfiporia cocos

1. INTRODUCTION

In many cell cultivation processes adherent growing cells are used. Suitable growth surfaces of these cells are often made of polystyrene. Polystyrene is offered as cell culture plates for R&D or as spherical carriers for bioreactor processes (Weber et al., 2007). A precondition for cell attachment is a protein adsorption to the polystyrene. Positively charged medium proteins bind via electrostatic interactions to the negatively charged

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Science Publications

American Journal of Biochemistry and Biotechnology 10 (1): 14-21, 2014
ISSN: 1553-3468
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doi:10.3844/ajbbsp.2014.14.21 Published Online 10 (1) 2014 (http://www.thescipub.com/ajbb.toc)
polystyrene surface. The cell adherence is formed via peptide bonds between medium proteins and extracellular cell surface proteins e.g., integrins (Merten and Flickinger, 2009).

Therefore, a common method for cell detachment is proteolytic cleavage via peptidases. In cultivation processes, detachment might be necessary during the seeding train or at the end of the cultivation. The most prominent peptidase in cell culture for this purpose is mammalian Trypsin isolated from the pancreas. Trypsin (EC 3.4.21.4) is a serine-endopeptidase which cleaves peptide chains after most basic amino acids (Polgar, 2005). Despite its widespread use, detachment with Trypsin has several disadvantages, especially if the detached cells are used to produce therapeutics or are themselves an Advanced Therapy Medicinal Product (ATMPs). Strong requirements are set to those production processes particularly on the cell detachment step. First, the whole production adheres to the guidance of the American Food and Drug Administration (FDA) and the European Medicines Agency (EMEA). These guidelines hold that during production, use of raw materials from animal origin (as bovine or porcine Trypsin is) should be kept to a minimum because of ethic and safety reasons (FDA, 2007). Second, ATMPs are often produced in dynamic reactor systems and/or cells are further processed after detachment. This influences the cells as shear forces are applied. Shearing cells may decrease their viability. Therefore the cell detachment has to be as gentle as possible to achieve highly viable cells. After prolonged incubation times, Trypsin can irreversibly damage surface proteins of the cells (Canavan et al., 2005) e.g., membrane receptors and cell adhesion molecules (Wachs et al., 2003). Third, the forces responsible for cell detachment differ between dynamic (e.g., bioreactors) and static systems (e.g., T-flasks). In static systems, the enzymatic detachment is promoted by brief tapping. The tapping results in brief but strong shear forces that help to detach the cells. In contrast, tapping is not possible in dynamic systems. After enzymatic cleavage, detached cells are simply flushed out of the reactor. The resulting shear forces of the fluid flow are much weaker and more heterogeneous than those resulting from tapping. This decreases the rate of cell detachment and the subsequent harvest yield. Therefore, the enzymatic cleavage of cells grown on carriers in dynamic systems must be as efficient as possible. Trypsin, which works quite well in static systems, is too harsh for cell harvest in dynamic systems.

Therefore there is an interest in finding non-mammalian alternatives to Trypsin for ATMP production processes.

In the current study, an endopeptidase from the fungus Wolfiporia cocos (W. cocos) was investigated concerning its suitability for cell detachment to replace Trypsin. This endopeptidase cleaves after proline residues and was therefore named prolyl-specific endopeptidase (PsP). Endopeptidases used for cell harvest or tissue digestion are mostly serine- or metalloproteases. Their cleavage patterns are various. Common examples are dispase (Stenn et al., 1989) (EC 3.4.24.3; hydrolyzes the N-terminal peptide bonds of non-polar AA), neutrophil elastase (Harlan et al., 1981) (EC 3.4.21.37; cleaves at the carboxyside of small hydrophobic AA) or thermolysin (EC 3.4.24.27; hydrolyses after big hydrophobic AA). Collagenase (EC 3.4.24.8) another cell detachment/tissue digestion enzyme (Lasfargues and Moore, 1971) shows a cleavage pattern similar to the PsP (cleaves between proline and other AA).

The cells which should be detached in the current study are models for two types of therapeutic production processes. The two process types mainly differ in the role of the cultivated cells. In the first process type, cells are used to produce a therapeutic virus. Vero cells were used as an example for adherent cells used for therapeutic virus production (Weiss et al., 2012; 2013) where cell detachment is only crucial during the seeding train to achieve enough cell mass for virus infection. MA104 cells are adherent cells also used in vaccine production and were chosen in the current study as they are very adhesive. Therefore MA104 cells are a model for cells difficult to detach. In the second process, the cultivated cells present the final ATMP product. In the present study, hMSC-TERT, an adherent stem cell line, worked as a model cell for an ATMP product. hMSC-TERT detachment is necessary at the end of the expansion process to harvest the cells as final product (Cierpka et al., 2013).

The aim of the work was to compare the proteolytic activity of the PsP from W. cocos to that of the bovine Trypsin under the same experimental conditions. First, the activity of both enzymes was compared in silico as well as in a proteolytic assay followed by detachment studies with three different cell lines (hMSC-TERT, Vero cells, MA104 cells). The detachment kinetics of each enzyme was investigated by using the same proteolytic activities. To estimate a putative negative influence of the enzyme on the cells, the detached cells were recultivated. The growth and metabolic kinetics of these cells were compared with unaffected controls.
2. MATERIALS AND METHODS

2.1. Fungal Strain

The *Wolfiporia cocos* strain (CBS 279.55) was obtained from the Dutch “Centraalbureau voor Schimmelcultures”, Baarn, The Netherlands.

2.2. Production of the Proly-Specific Endopetidase

The Prolyl-specific endopeptidase (PsP) was expressed natively in the supernatant of *W. cocos* cultures and purified via ion exchange chromatography. In short, for preparation of precultures, 14 mm diameter agar plugs from the leading nutrition solution (30 g L\(^{-1}\) mycelial edge were transferred into 100 mL of standard column-volume 1.3 mL, 20 mg max. protein load, Bio-Schimmelcultures, Baarn, The Netherlands. Obtained from the Dutch “Centraalbureau voor Schimmelcultures”. During the main culture, samples were taken daily and the PsP activity was determined according to Szewajser-Dey et al. (1992). The reaction mixture was composed of 100 µL enzyme sample, 890 µL 50 mM citrate phosphate buffer pH 4.0 and 10 µL of an 8 mM methanolic substrate solution.

2.3. Prolyl-Specific Activity Assay

Prolyl specific peptidase activity was determined photometrically at 410 nm and 40°C using Z-glycyl-L-prolyl-4-nitroanilide (Sigma Aldrich) as a substrate according to Szewajser-Dey et al. (1992). The reaction mixture was composed of 100 µL enzyme sample, 890 µL 50 mM citrate phosphate buffer pH 4.0 and 10 µL of an 8 mM methanolic substrate solution.

2.4. Proteolytic Activity Assay

Proteolytic activity was determined fluorimetrically using FITC-casein (Pierce) as a substrate (Twining, 1984). The reaction mixture was composed of 100 µL enzyme and 100 µL substrate solution (10 µg mL\(^{-1}\) FITC-Casein in TBS pH 7.2). In the assay 1 unit is defined as change in RFU (485 nm/538 nm, ex/em) of 31 per min at 25°C, pH 7.2.

2.5. Cultivation of hMSC-TERT, Vero and MA104 cells

Human mesenchymal stem cells with the reverse telomerase transcriptase gene (hMSC-TERT) are a bone marrow derived cell line. hMSC-TERT cells (passage 75-85) were cultivated in T-flasks in Minimal Essential Medium (MEM), supplemented with 10% fetal calf serum, 2 mM glutamine and 1% Penicillin/Streptomycin (obtained from PAA, Velizy-Villacoublay; France). Vero cells (# CCL-81, ATCC) were isolated from African green monkey kidneys. The permanent cell line used in the current study was kindly provided by the PEI (Langen, Germany). Vero cells (passage 147) were grown in T-flasks in VPSFM medium (Invitrogen). MA 104 cells are a permanent cell line derived from an African rhesus monkey kidney. MA104 cells (passage 78) used in this study were kindly provided by MSD (Burgwedel, Germany). Cultivation was performed in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum and 1% Penicillin/Streptomycin (PAA). The cells were grown in T-flasks.

For all cells, culture conditions were set to 37°C, 5% CO\(_2\) and 95% humidity. Cells were seeded with 3,500 to 10,000 cells cm\(^{-2}\). Cells were grown until 80% confluence.

2.6. Detachment Experiments

For cell detachment experiments, the culture medium was aspirated and the cells were washed twice with PBS without Ca\(^{2+}\)/Mg\(^{2+}\) (PAA). The enzyme solution (PsP/EDTA or Trypsin/EDTA [porcine, 0.05/0.02% ready-to-use solution, PAA]) was added and the plates were incubated at 37°C, 5% CO\(_2\) and 95% humidity. After the incubation, the reaction was stopped by adding three volumes of culture medium. Detachment was analyzed...
qualitatively by microscope and quantitatively. For quantitative analysis, the detached cells were centrifuged for 5 min at 300 g and the cells were resuspended in fresh culture medium. The cell number was determined by a Neubauer improved counting chamber.

2.7. Determination of the Growth and Ground Metabolism of Detached Cells

After detachment experiments, the cells were seeded (5×10^3-1×10^4 cells cm^-2) in culture medium in 24 well-plates and grown for 5 days. Cell numbers were determined everyday by enzymatic Trypsin-EDTA harvest. Furthermore, glucose and lactate concentrations were determined and microscopic analyses were performed.

Cell growth rate was calculated as followed Equation (1):

\[ \mu = \frac{\ln(N_{t+1}) - \ln(N_t)}{t_{t+1} - t_t} \]  

\( \mu \) = Cell growth rate in h^-1  
\( N \) = Cell concentration in cells cm^-2  
\( t \) = Time in h

Glucose consumption rates were calculated after Equation 2:

\[ q_{Gc} = \frac{1}{N_{t+1}} \frac{C_{G,t+1} - C_{G,t}}{t_{t+1} - t_t} \]  

\( q_{Gc} \) = Specific glucose consumption rate in mg h^-1 10^-6 cells  
\( C_G \) = Glucose in mg

Lactate accumulation rates were calculated after Equation 3:

\[ q_{Lac} = \frac{1}{N_{t+1}} \frac{C_{L,t+1} - C_{L,t}}{t_{t+1} - t_t} \]  

\( q_{Lac} \) = Specific lactate accumulation rate in mg h^-1 10^-6 cells  
\( C_L \) = Lactate in mg

2.8. Statistical Analysis

For all quantitative data, mean and standard deviation were calculated. To compare the mean values, a t-test (two sample test with known variances) was performed. A p-value under 0.05 was deemed to indicate a statistically significant difference.

3. RESULTS

3.1. Suitability of the PsP for Cell Detachment

In general, it was questionable if the PsP is suitable for a cell detachment approach. In order to support this, two points were considered. The first point is that the optimum activity of this endopeptidase is naturally at pH 4.0. Conditions for cell detachments are typically at pH around 7 to guarantee high survival of the cells. Nonetheless, the PsP had enough proteolytic activity at pH 7 to detach cells as shown in preliminary experiments.

The second point is that enough peptidase-specific cleavage sites in the attachment-promoting proteins need to be available. When cells are grown in serum-containing media, mainly the serum proteins are responsible for the attachment of adherent cells to cell culture surfaces. Cell adhesion is promoted by binding of these proteins to the cell culture plastic. Afterwards, the cell surface integrins bind these molecules (Merten and Flickinger, 2009). In consequence, for cell detachment these molecules have to be mechanically disrupted or cleaved e.g., by peptidases. Trypsin is used as a standard enzyme for cell detachment. Trypsin’s cleavage site is determined by basic amino acids (Lys, Arg and modified Cys). In contrast, PsP is a prolyl-specific peptidase. Using the amino acid sequences of common proteins in bovine serum the percentage of cleavage sites either for Trypsin or for PsP was analyzed in silico (Table 1).

Based on the availability of cleavage sites within the sequences of attachment-promoting proteins, a cell detachment with PsP should be possible.

3.2. Proteolytic Activity of PsP Compared to Trypsin

For a comparison of PsP with other standard detachment enzymes, it is required to use comparable enzymatic activities in the experiments. For Trypsin at least three different definitions of activity units have been published (TAME, BAEE, USP/NF) which differ in substrate and experimental setup (Hummel, 1959; Kezdy and Bender, 1965; Lin et al., 1969; Schwert and Takenaka, 1955). Therefore, a peptidase assay with Trypsin and PsP was performed under the same experimental conditions. In the current study, fluorescein-labeled casein was used as a substrate for assessing protease activity (Twining, 1984). The assay has similarity to the DMC-U assay which uses dimethyl-casein as a substrate (Lin et al., 1969). As shown in Table 2 PsP and Trypsin have, when averaged, the same number of cleavage sites for casein substrates (p = 0.75) indicating good comparability of this assay.
Using the proteolytic cleavage assay, the purified PsP fraction had a proteolytic activity of 1.6 U mL$^{-1}$. The Trypsin solution had an activity of 100 U mL$^{-1}$. For the comparability of the study PsP and Trypsin solutions with a proteolytic activity of 1.6 U mL$^{-1}$ were used for all subsequent cell detachment experiments.

3.3. Cell detachment of Different Adherent Cell Lines from Polystyrene Surfaces

To investigate the detachment properties of PsP, it was first proven if a cell detachment was generally possible for all three cell types. Due to the low enzyme concentrations, a complete detachment at 37°C took 30 min. During this period the detachment kinetics were measured. None of the three cell types showed a decrease in viability after 30 min of detachment using either PsP or Trypsin (porcine, PAA).

3.4. hMSC-TERT

For hMSC-TERT, it was observed that cell detachment using the PsP was faster than detachment with Trypsin.

### Table 1. Comparison of protease cleavage sites in the amino acid sequence of attachment-promoting proteins in bovine serum

| Attachment-promoting proteins in bovine serum | UniProtKB AC number | Cleavage sites [%] | Prolyl-specific endopeptidase |
|----------------------------------------------|---------------------|--------------------|-----------------------------|
| Vitronectin                                   | Q3ZBS7              | Arg 7.6, Lys 3.4, Pro 7.1 | Trypsin                     |
| Fibronectin                                   | P07589              | Arg 5.2, Lys 3.2    | Pro 7.7                     |
| Laminin                                      | O19174              | Arg 6.0, Lys 3.6    | Pro 7.1                     |
| Collagen type I A1/A2                        | P02453 / P02465     | Arg 4.9/5.3, Lys 3.6/3.0, Arg 5.1 | Trypsin Pro 22.9/20.3 |
| Collagen type IV                             | Q29442              | Lys 2.9            | Pro 18.8                    |

Table 1. Comparison of protease cleavage sites in the amino acid sequence of attachment-promoting proteins in bovine serum

a. [http://www.uniprot.org/](http://www.uniprot.org/)

### Table 2. Comparison of protease cleavage sites in the amino acid sequence of proteins in casein Hammarsten. Analysis was done with ExPASy-Peptide Cutter.

| Bovine casein component | UniProtKB AC number | Number of cleavage sites |
|-------------------------|---------------------|--------------------------|
| α-casein S1             | P02662              | 20.0                     |
| α-casein S2             | P02663              | 30.0                     |
| β-casein                | P02666              | 16.0                     |
| κ-casein                | P02668              | 13.0                     |
| Average                 |                     | 19.8                     |

Table 2. Comparison of protease cleavage sites in the amino acid sequence of proteins in casein Hammarsten. Analysis was done with ExPASy-Peptide Cutter.

### Table 3. Growth rate, glucose consumption rates and lactate accumulation rates of detached cells after 48h recultivation. Experiments were performed as described in 2.7

|                  | μ [h$^{-1}$] | q_{Gluc} [mg*h$^{-1}$*10$^6$ cells] | q_{Lac} [mg*h$^{-1}$*10$^6$ cells] |
|------------------|-------------|-------------------------------------|-------------------------------------|
| hMSC-TERT        |             |                                     |                                     |
| Detached with PsP| 0.031±0.004 | 0.063±0.003                         | -0.060±0.007                        |
| Detached with Trypsin | 0.028±0.004 | 0.051±0.004*                        | -0.059±0.007                        |
| Unaffected control | 0.033±0.011 | 0.076±0.008                         | -0.069±0.020                        |
| Vero cells       |             |                                     |                                     |
| Detached with PsP| 0.022±0.003 | 0.175±0.013*                        | -0.161±0.007                        |
| Detached with Trypsin | 0.020±0.003 | 0.147±0.019*                        | -0.140±0.009                        |
| Unaffected control | 0.025±0.004 | 0.219±0.014                         | -0.162±0.010                        |
| MA104 cells      |             |                                     |                                     |
| Detached with PsP| 0.016±0.003 | 0.082±0.004*                        | -0.106±0.004                        |
| Detached with Trypsin | 0.017±0.003 | 0.066±0.003*                        | -0.085±0.004                        |
| Unaffected control | 0.018±0.004 | 0.120±0.012                         | -0.099±0.007                        |

n = 3, * p≤0.05 (comparison of PsP or Trypsin treatment with control)
After 10 min enzyme incubation, 55±13% of the cells were detached with PsP, whereas only 19±3% of the cells were detached with Trypsin (Fig. 1, left). This was also observed microscopically. After 20 min of PsP treatment, only 8±1% of the cells were attached to the cell culture plastic, whereas after Trypsin treatment 47±16% of the cells were still attached to the surface (Fig. 1, right).

The growth analysis of the detached cells showed comparable growth rates for cells detached with PsP and Trypsin to that of an unaffected control (p≥0.6, Table 3). This indicated that hMSC-TERT cells were not influenced in their normal growth after enzyme treatment. Interestingly, glucose consumption rates were decreased for enzyme-treated hMSC-TERT. \( q_{\text{Gl}} \) of cells detached with PsP were lower, but not significant different from the control (p = 0.13). Cells detached with Trypsin showed a higher and more significant decrease of 33±9% (p<0.05).

3.5. Vero Cells

PsP treatment detached Vero cells faster than Trypsin treatment. After 10 min of incubation with PsP, 57±21% of the Vero cells were detached. For Trypsin only 38±2% of the cells were detached under the same experimental conditions. Moreover, detachment with PsP was more efficient. With PsP, 100% of the cells could be detached, whereas for Trypsin, 80% of the cells were detached at the maximum (Fig. 1, left).

Cell growth and basic metabolic rates of the detached Vero cells were similar to that of the control. Only \( q_{\text{Gl}} \) of
the enzyme-treated cells showed a slight decrease (Table 3). This decrease was similar for Vero cells detached with Trypsin (33±12%) and for those detached with PsP (20±7%) (p = 0.2).

3.6. MA104 Cells

MA104 cells are strong adherent growing cells when compared to the other two cell types. This was shown in the detachment kinetics as after 10 min incubation time only 13±5% cells were detached (Fig. 1, left hand). After longer incubation times, MA104 cell detachment kinetics and the efficiency of PsP and Trypsin were similar.

Concerning their growth rates, the rates of the detached cells were comparable to that of the untreated control (Table 3). The qGlc of the detached cells were lower than the untreated control. Cells detached with PsP showed a 32±5% decrease in qGlc, while cells treated with Trypsin showed a 45±5% decrease.

4. DISCUSSION

The results showed that PsP could detach severeral cell types. Although detachment behaviour differed between the three cell types investigated. For hMSC-TERT cells detachment with PsP was faster and more efficient when compared to porcine Trypsin. Both detachment enzymes influenced the basic cell metabolism of hMSC-TERT. Nonetheless, hMSC-TERT remained rather unaffected after PsP detachment than after that of Trypsin detachment. Detachment of Vero cells with PsP was more efficient, but not more gentle compared to Trypsin. The favorability of PsP for MA104 cell detachment was not as clear as previously shown for the other two cell types. Detachment efficiency and cell behavior after detachment with PsP and Trypsin was more or less the same.

Cell detachment is a crucial step in each ATMP/vaccine production process. With increasing interest in cell therapeutics and therapeutic virus (as ATMPs), the search for novel peptidases for cell detachment purposes is resumed. As Trypsin has been the gold standard for decades in R&D cell detachment, this is not the case for cell detachment in clinical/industrial ATMP production processes. This has to do with the fact that requirements differ. For example for hMSC-TERT ATMP production, it is not only important to efficiently detach cells, but also to keep them viable (and undifferentiated). This gets challenging when production takes places in dynamic systems (stirred tank reactors (Cierpka et al., 2013; Elseberg et al., 2012), fixed bed-systems (Weber et al., 2010a; 2010b; 2010c) and the harvested cells are further processed (e.g., bead-to-bead transfer, cell encapsulation (Freimark et al., 2010). Trypsin is unsuitable for hMSC-TERT detachment out of dynamic systems and for detachment of further processed cells. Trypsin does not efficiently detach the cells or keep the cells viable (Salzig et al., 2013). Moreover, Trypsin is often of animal origin which should be avoided in ATMP production. This problem can be easily solved by using recombinant trypsin variants as applied by many manufactures (e.g., Trypsin, recombinant, Roche; recombinant Trypsin, Invitrogen; TrypZean, Sigma-Aldrich; rTrypsin, Novozymes). As consequence, an ideal peptidase for cell detachment need to be (a) of non-animal origin/safe, (b) very efficient but (c) very gentle to the cells.

The current study showed that PsP, a novel endopeptidase from W. cocos, might fulfill all of these requirements. The enzyme is of non-animal origin and is already being used in food biotechnology. Therefore it can be assumed as safe for humans. The PsP is efficient but at the same time very gentle to several cells types used in ATMP production processes. Compared to Trypsin, PsP is advantageous concerning the detachment yield and the quality of the detached cells. As PsP can currently only be isolated native from W. cocos, it should be encouraged to produce the enzyme recombinant. This would result in higher concentrated enzyme solutions and also allow a higher purity of the enzyme by using affinity tags. As it is of high importance to investigate cell detachment out of dynamic systems, further studies should be performed to analyze the potential of the PsP in dynamic ATMP production processes.

5. ACKNOWLEDGEMENT

hMSC-TERT were kindly provided by M. Kassem, University of Southern Denmark. The authors would like to thank the Hessen State Ministry of Higher Education, Research and the Arts for the financial support within the Hessen initiative for supporting scientific and economic excellence (LOEWE-Program) as well as the Federal ministry of Economics and Technology.

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