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Research Article

Effect of Immobilized Proteases on Bacterial Growth and Cell Adhesion on Polypropylene Surfaces

Abstract

The bacterial planktonic growth and the removal of bacterial cells grown on polypropylene surface coated with covalently immobilized proteases (subtilisin Carlsberg or α-chymotrypsin) was investigated for *Enterococcus hirae*, *Staphyloccocus epidermidis* and *Eschericha coli*. Immobilization of both proteases on plasma-treated polypropylene was carried out using as cross-linking agent i) glutaraldehyde or ii) N'-diisopropylcarbodiimide and N-hydroxysuccinimide. In the presence of immobilized proteases a higher bacterial planktonic growth (up to 40 %) was observed. Instead, a different effect was observed on cell removal, and it varied according to the bacteria strain, the immobilized protease and the immobilization procedure. In particular, the presence of subtilisin in the polypropylene coating increased the cell removal of *E. hirae* by simple washing of the polypropylene surface and both subtilisin and α-chymotrypsin immobilized by N'-diisopropylcarbodiimide and N-hydroxysuccinimide favored the removal of *S. epidermidis* after sonication. No significant differences compared to the control where observed in all the other cases. In conclusion this study indicates that proteases can be an enhancer of microbial biomass (a phenomena that could be exploited for industrial fermentation) and can affect the strength of cell adhesion for some bacteria.

Abbreviations

aCT: pancreatic α-Chymotrypsin from *Bos Taurus*; SubC: Subtilisin Carlsberg from *Bacillus licheniformis*; PP: Polypropylene; DMF: N,N-Dimethylformamide; GA: Glutaraldehyde; DIC: N-N'-Diisopropylcarbodiimide; NHS: N-Hydroxysuccinimide; aCT-GA: aCT linked to PP using GA as cross-linking agent; aCT-DIC: aCT linked to PP using DIC and NHS for the cross-linking reaction; SubC-GA: SubC linked to PP using GA as cross-linking agent; SubC-DIC: SubC linked to PP using DIC and NHS for the cross-linking reaction; LB: Luria Bertani Agar; PBS: sterile Phosphate-Buffered Saline;

Introduction

Among the strategies proposed to prevent or inhibit undesired and (often) pathogenic microbial biofilm, enzyme based coatings have been developed by different research groups [1,2]. In particular, coatings containing proteases might be employed to degrade i) the proteinaceous component of the self-produced polymeric substance [3] (a major structural constituent of the biofilm made of polysaccharides, proteins, lipids and nucleic acids) and/or ii) the proteins (e.g., adhesins) involved in adhesion processes of cells to a surface to form a biofilm.

Enzyme immobilization presents some advantages compared to the use of free enzymes. For example, in the field of biocatalysis immobilization ensures the reusability of the enzymes improving the productivity of the biocatalytic process [4] and it can favorably affect operational flexibility by increasing enzyme thermal stability and selectivity [5]. This latter feature also depends on the procedure adopted for enzyme immobilization and it could be modulated for changing protease specificity toward the proteins involved in the bacterial adhesion. In addition, the confinement of enzymes on a solid surface through immobilization can be a procedure to maximize the enzyme activity just where the biofilm grows.

Herein we present how two commercial and readily available proteases (aCT and SubC), immobilized on polypropylene surface [6], affect bacterial planktonic growth and the removal of cells grown on the plastic surface itself. To this end, *Enterococcus hirae* (gram positive and it causes sepsis in humans), *Staphyloccocus epidermidis* (gram positive and opportunistic human pathogen) and *Eschericha coli* (a gram negative component of human microflora and an opportunistic human pathogen) were chosen as model bacteria.

Materials and Methods

Materials

Round PP coupons (Ø 1.3 cm) were cut from PP sheets purchased from Alfa Aesar. The enzymes aCT (54 U/mg) and SubC (8.6 U/mg) were purchased from Sigma. Analytical grade reagents were purchased from Alfa-Aesar. Bacterial strain were *Enterococcus hirae* (ATCC 10541), *Staphyloccocus epidermidis* (KTCC 1917) both Gram-positive and *Eschericha coli* (ATCC 25922, Gram-negative).

Protease immobilization on polypropylene coupons

Round PP coupons (Ø 1.3 cm) were preventively washed with MilliQ water and acetone and dried. Next they were exposed to oxygen plasma for 20 min using a Harrick Plasma PDC-002 plasma cleaner; 740 V, 40 mA, 29.6 W) for surface functionalization [7]. Immediately after the plasma treatment two different immobilization procedures were applied. In the first procedure coupons were coated with 80 µL of protease (aCT or SubC) solution (5 mg/mL) in 20 mM phosphate buffer, pH 7.2 (buffer A), containing 0.005% (v/v) GA, and

let dry overnight at 25 °C under vacuum [8]. In a second procedure (for aCT-DIC or SubC-DIC preparation) coupons were dipped in 0.1 M, pH 3.5 MES buffer and dried at 40 °C under vacuum. Afterwards coupons were immersed in DMF containing 2 mM DIC and 5 mM NHS and kept shaken for 2 h at 150 rpm, then washed with buffer A. Each coupon was then covered with 80 µL of a 5 mg/mL of a-CT or SubC solution in the same buffer, which was allowed to react for 2 h. For both procedures the coupons were washed for 30 min with 3 mL of bi-deionized water for three times to get rid of the unbound enzyme. Control coupons were analogously prepared but using buffer a solution instead of protease solution (in Figure 1 they were indicated as GA or DIC).

Bacterial growth and analysis of cell dispersion from PP surface

Bacterial strains were recovered from glycerol stocks (-80 °C) on LB. A single colony from each agar plate was used to inoculate 5 mL of LB (0.5% yeast extract, 1% peptone, 1% NaCl). Cultures were incubated for 24 hours at 37 °C. Next, cultures were dissolved with fresh medium to reach an OD (600 nm) of 0.01 (and 0.05 in the case of S. epidermidis, because of its poor growth rate) and used for further experiments with PP coupons.

Coupons covered with immobilized proteases and control coupons were placed in wells of a 24-well microplate and 1 mL of microbial culture was added to each well. Micro plates were incubated for 24 hours at 37 °C to allow the growth of microorganisms as well as the colonization of PP coupons. Each coupon was tested in duplicate.

After incubation, coupons were transferred to wells of new 24-well micro plates and filled with 1 mL of PBS (8.0 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4 per liter; pH 7.2). Media, left behind, were used to estimating microbial growth by measuring the OD (600 nm) (Figure 1). Next, micro plates with coupons and PBS were vortex-shaken (500 rpm) for 30 seconds (to detach weakly adsorbed cells) and solutions were drawn out for OD analyses. Afterwards, again 1 mL of fresh, sterile PBS was added to each coupon and the plate was sonicated for 30 seconds to detach more strongly adsorbed cells from the coupons. Analogously solutions were drawn out and used for OD analyses. OD intensity was used as criterion to evaluate cell amount in solution.

Results and Discussion

The microbial cultures obtained from single colony after 24 hours at 37 °C in 5 mL of LB, showed an OD (600 nm) of 1.002 ± 0.029, 0.244 ± 0.011, 1.138 ± 0.018 for *E. hirae*, *S. epidermidis* and *E. coli*, respectively. These cultures were diluted (see Methods), transferred into micro plate wells and incubated at 37 °C. The resulting bacterial biomass was significantly higher when carried out in the presence of immobilized SubC (Figure 1a). In particular, with respect to the control coupons, OD increased 29%, 29% and 13% in the presence of SubC-DIC ($P<0.05$) and 24%, 43% and 17% ($P<0.05$) in the presence of SubC-GA for *E. hirae*, *S. epidermidis* and *E. coli*, respectively. Therefore, independently of the mechanism which causes the increase of biomass, the presence of SubC in the culture medium promotes the planktonic cell growth.

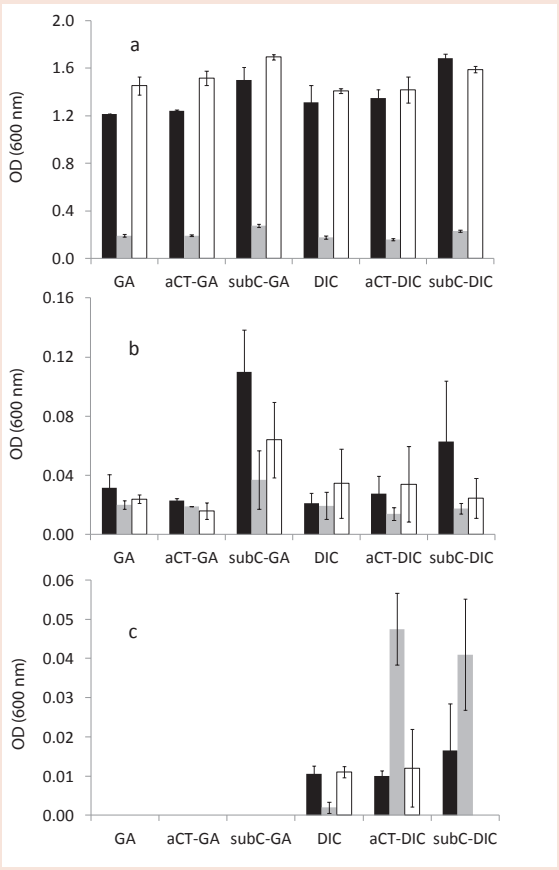

Figure 1: Optical density (OD) at 600 nm of (a) microbial cultures after 24 h at 37°C in 24-well microplates; (b) PBS solution from vortex-shaken (500 rpm, 30 seconds) coupons and (c) PBS solution from sonicated (30 seconds) coupons. PBS solution used for was added after withdrawing solution used for (b). Black bars indicate E. hirae, gray bars S. epidermidis and white bars E. coli. OD measurements were done in triplicate.

Solutions recovered from 24-wells micro plates after the first washing, showed higher OD values when recovered from wells containing SubC-GA coupons, especially in the case of *E. hirae* (Figure 1b). No significant differences were observed for all the other coupons, even though a similar trend was noted for SubC-DIC. The OD signal measured from these solutions indicates the fraction of cells weakly adsorbed on the coupons. However, it is interesting to note that there is a higher fraction of cells that adhered to the coupon in the presence of SubC. Likely, this increase depends on the higher microbial growth observed in the presence of this enzyme (Figure 1a).

Interestingly, after sonication there was not any cell release in the solutions coming from GA, aCT-GA and SubC-GA coupons, suggesting the absence of cells adhesion on these coupons. Instead, cell were released from DIC, SubC-DIC and aCT-DIC coupons (Figure 1c). In particular, in the case of *S. epidermidis* the cell release from SubC-DIC and aCT-DIC was about 20-fold higher than that observed for the control (DIC coupon). On the contrary no particular differences were observed for the other bacteria, except for *E.coli* that did not show any cell release from the SubC-DIC.

The results described suggests that the presence of GA as cross-linking agent causes a weaker adhesion of cell on the polypropylene surface because with both proteases all the cells were removed by simple washing (Figure 1b) of the coupons and no cells were observed after sonication (Figure 1c). Thus, the presence of SubC on the surface not only favours the planktonic microbial growth (Figure 1a), but also the number of cells that adhered weakly on the surface (Figure 1b).

On the basis of the OD values a stronger bacteria adhesion can be suggested on the coupons with DIC as cross-linking agent. In fact, for the complete removal of the cells a sonication step is needed (Figure 1c). Nevertheless, especially in the case of *S. epidermidis*, the presence of a protease appears to be crucial for a higher removal by washing and sonication. These results confirm the utility of proteases as biofilm dispersal, as highlighted by other research groups [9,10] and highlight the importance of the immobilization procedure to affect the enzyme capacity to weaken bacterial adhesion (Figure 1c).

Conclusion

The results herein presented indicate proteases as enhancer of microbial biomass and/or as inhibitor of cell adhesion. These properties are interesting because they can lead to higher productivity in the field of industrial fermentation and because they address the search of new enzymes (in particular in the class of hydrolases) that might play a role against biofilms. Thus, this study provides interesting information that should be taken into account for new investigations that aim at using immobilized proteases for antibiofilm purposes or as an additive for microbial growth.

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