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A lysozyme and magnetic bead based separation method of intact bacteria

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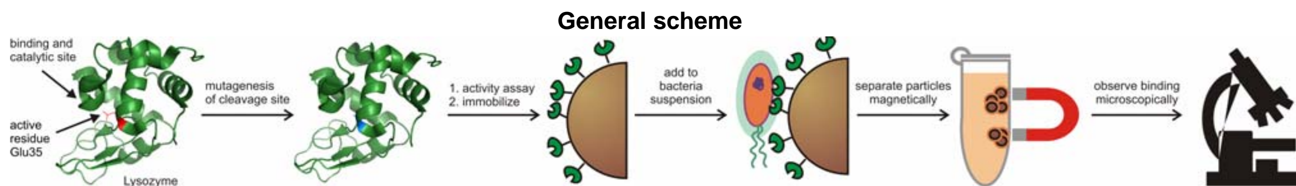
Scientific Background and Objectives

A fundamental objective in hygienic surveillance (e.g. in food production, water conditioning) is to detect and identify bacterial pathogens. Most hygienically relevant bacteria are able to change and adapt their physiology as a response to environmental stress. Some of these bacteria are alive, but no longer cultivable (VBNC). So, standardized methods for pathogen detection fail. Complex matrices or low bacterial densities limit the application of specific and sensitive nucleic acid based methods. This project aims to develop a new molecular biology platform, based on the specific binding property of lysozyme to cell wall components of bacteria, for their separation and enrichment to enable a subsequent specific pathogen detection by nucleic acid based methods.

To bind and separate *Eubacteria*, lysozyme has to be inactivated in its muramidase activity by site-directed mutagenesis, while specific and effective binding to bacteria should be retained at wild type level. Furher, the recombinant lysozyme has to be immobilized on a solid surface (linker technology), and its affinitiy to prokaryotic cell wall targets should be determined.

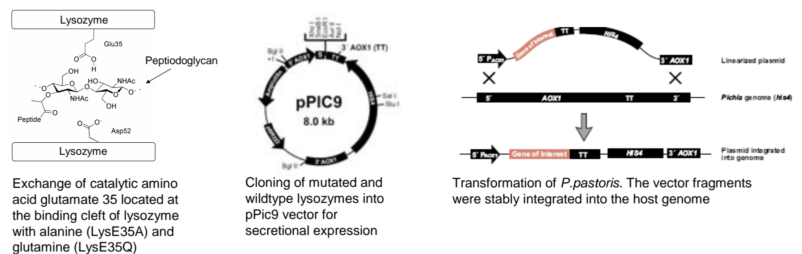
General scheme



Results

Site directed mutagenesis and cloning

To inhibit bacteria lysis the catalytic amino acid glutamate at position 35 was exchanged by alanine (LysE35A) or glutamine (LysE35Q) by site directed mutagenesis. The mutated fragments and the wildtype reference cDNA were cloned into *Pichia pastoris* expression vector pPic9 for secretional expression. After linearization and transformation the plasmids got integrated into the host genome.



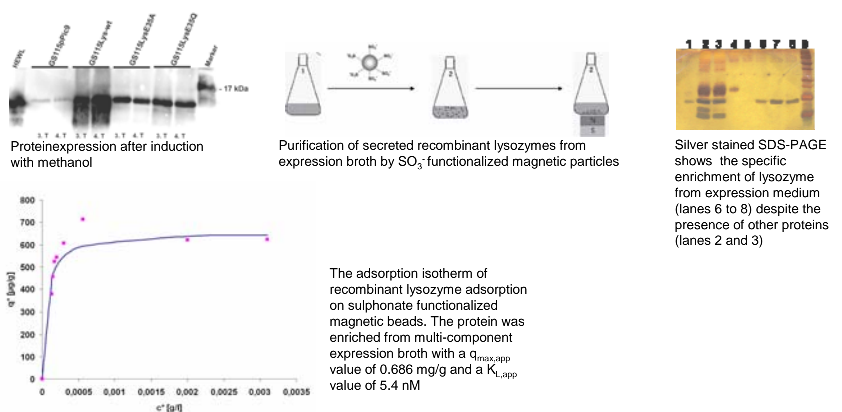
Exchange of catalytic amino acid glutamate 35 located at the binding cleft of lysozyme with alanine (LysE35A) and glutamine (LysE35Q)

Cloning of mutated and wildtype lysozymes into pPic9 vector for secretional expression

Transformation of *P.pastoris*. The vector fragments were stably integrated into the host genome

Lysozyme expression and direct purification from expression broth

After growth of the recombinant yeast cells on glycerol containing medium, the expression was induced by methanol addition. The highest lysozyme expression was detected after four days. For specific enrichment of the target lysozyme from the expression broth, sulfonate functionalized nano-scale magnetic beads were used. The enrichment process was very specific after increasing the pH of the medium to 8.5, closer to the lysozyme isoelectric point at pH 11. A direct capture of target protein from medium was possible, but the adsorption isotherm revealed $q_{max,\ app}$ and $K_{L,app}$ values of only 1% and 0.6% of the qmax and K_L values of single component adsorption of commercial lysozyme respectively.



Proteinexpression after induction with methanol

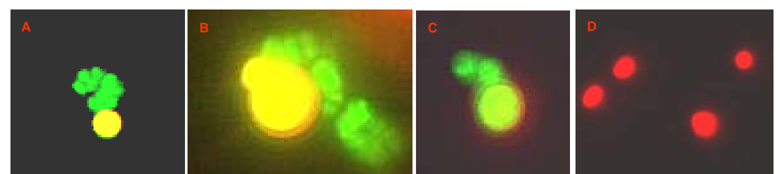
Purification of secreted recombinant lysozymes from expression broth by SO_3^- functionalized magnetic particles

Silver stained SDS-PAGE shows the specific enrichment of lysozyme from expression medium (lanes 6 to 8) despite the presence of other proteins (lanes 2 and 3)

The adsorption isotherm of recombinant lysozyme adsorption on sulphonate functionalized magnetic beads. The protein was enriched from multi-component expression broth with a $q_{max,app}$ value of 0.686 mg/g and a $K_{L,app}$ value of 5.4 nM

Proof of principle: capture of reference bacteria with recombinant lysozyme coated magnetic beads

After purification of lysozymes from expression medium they got biotinylated for immobilization on streptavidin functionalized magnetic beads carrying a fluorescent dye tag. These constructs were used for separation of stained *Micrococcus luteus* from medium. The beads coated with recombinant lysozymes showed a significantly higher bacteria capture rate than lysozyme-free magnetic beads.



Capture of *Micrococcus luteus* (green) with lysozme functionalized magnetic beads (yellow).
A: wildtype lysozyme functionalized bead **B**: LysE35A functionalized bead **C**: LysE35Q functionalized bead **D**: lysozyme- free magnetic beads.
For visualization The beads were stained with Alexa Fluor® 594 and the bacteria were stained with Syto9™