A PROMISING STOCHASTIC APPROACH FOR ENGINEERING THE ENZYME hGSTA1-1 BY IDENTICAL REPEATED MOTIFS

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ABSTRACT
The design of supramolecular enzyme architectures on molecular level (so called "bottom up fabrication) is considered as a new trend in synthetic biotechnology. Enzyme nanostructures are effective materials for the development of assembled structures, with customized characteristics that can be fabricated [1,2]. In the same vein, protein engineering is a promising approach for the design and creation of new enzymes with innovative catalytic and structural features [3]. GSTs are multi-functional enzymes of high-importance for a number of biotechnological applications. They are considered as good model systems for engineering studies, due to their modular catalytic and binding features [4]. This work was inspired by "lego'' chemistry, aiming to design and develop self-assembled novel protein scaffolds into complex-structures, by exploiting the modularity capabilities of human GSTA1-1 enzyme (hGSTA1-1) through a stochastic approach, based on the principles of higher order and geometrically irregular assembly [5]. Surprisingly and in contrast with the complex topology of the globular enzyme hGSTA1-1, a motif with regularized interactions was found. Therefore, GST can be redesigned by the addition of identical repeated motifs.

INTRODUCTION
Cytosolic glutathione transferases (GSTs, EC 2.5.1.18) catalyze the conjugation of the tripeptide GSH to electrophiles resulting in the formation of the corresponding GSH conjugates. Human GSTs comprise a multi-member family of enzymes that are grouped into different classes (Alpha, Mu, Omega, Pi, Sigma, Theta and Zeta), based on amino acid sequence similarity and evolution relationships [6]. These enzymes play essential role in detoxification, metabolism, and transport or sequestration of endogenous or xenobiotic compounds. Rational and random approaches of protein engineering reinforce the promiscuity of GSTs both in terms of specificity and catalytic activity, creating new variants with new or altered properties [7]. The engineering of xenobiotic metabolizing enzymes is promising, as molecular tools to manipulate the detoxification of drugs, agrochemicals, and pollutants [8].

The design and creation of complex functional biomaterials, based on protein building blocks is considered as a challenging research area in synthetic biotechnology, as this approach can provide protein scaffolds with novel supramolecular architectures beyond Nature's portfolio [9-11]. GSTs modular architecture, convince them as very promising and adaptable scaffolds for protein engineering purposes. Manipulation of GSTs' structure through a few hot spot mutations can enhance thermostability, selectivity and catalytic properties. This the first time that a GST has been manipulated by repeated motifs, expanding with this direction the modularity of this globular protein.

MATERIAL AND METHODS
Based on the analysis of the crystal structure of hGSTA1-1 (PDB identification code:1K3Y) [12], the residues Lys140 and Ser141 were selected as potential hot-spots for step-by-step site saturation mutagenesis. Six mutated enzymes were designed and constructed: hGSTA1-1H (K140H), hGSTA1-12H (K140H, S141H), hGSTA1-1S (with the motif KVLH before the mutation K140H), hGSTA1-1GS

(with the repetitive motif KVLH), hGSTA1-1H(E136H, K140H) and hGSTA1-1(E136H). These GST genes were cloned in 5EXP-CT TOPO vector and expressed in *E. coli*. The recombinant enzymes were purified by affinity chromatography and their catalytic and structural properties were studied.

RESULTS

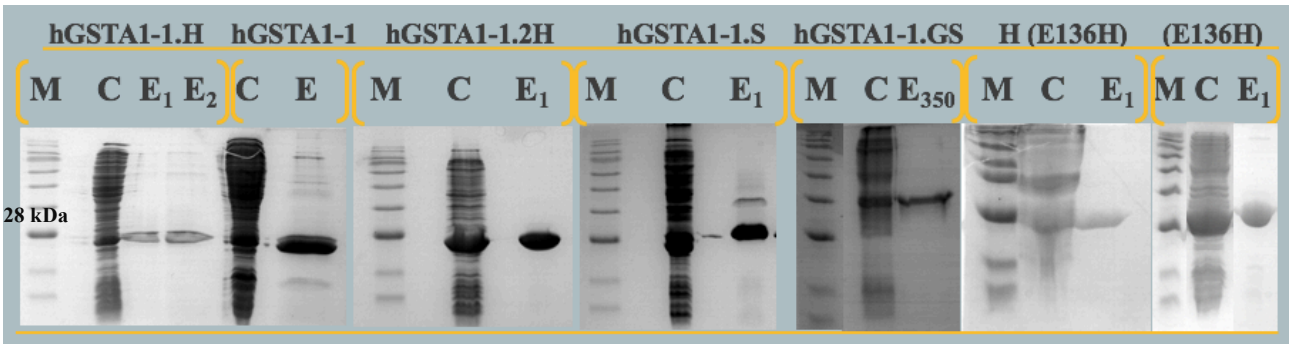

Figure 1. *SDS-PAGE analysis of hGSTA1-1 and its mutants' after purification by affinity chromatography, where M: marker, C: cell crude, E: elution.*

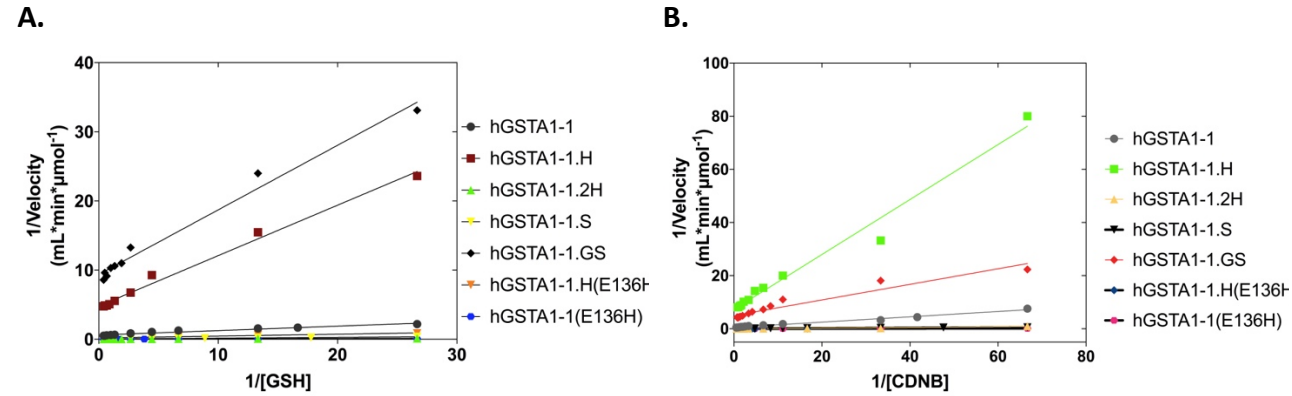

Figure 2. *Kinetic analysis (Lineweaver-Burk) of hGSTA1-1 and its mutants, using GSH as a variable substrate and CDNB at a fixed concentration (**A**) and CDNB as variable substrate and GSH at a fixed concentration (**B**). Experiments were performed in triplicate.*

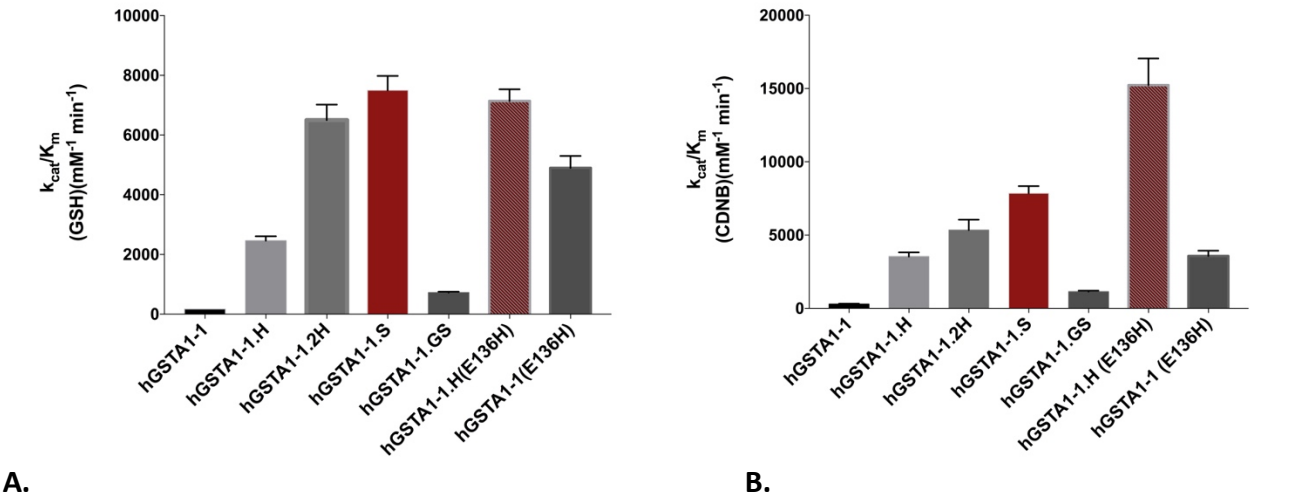

***Figure 3**. Catalytic efficiency of hGSTA1-1 and its mutants (hGSTA1-1.H, hGSTA1-1.2H, hGSTA1-1.S and hGSTA1-1.GS) expressed as K_{cat}/K_m ($mM^{-1}*min^{-1}$) for GSH (**A**) and CDNB (**B**).*

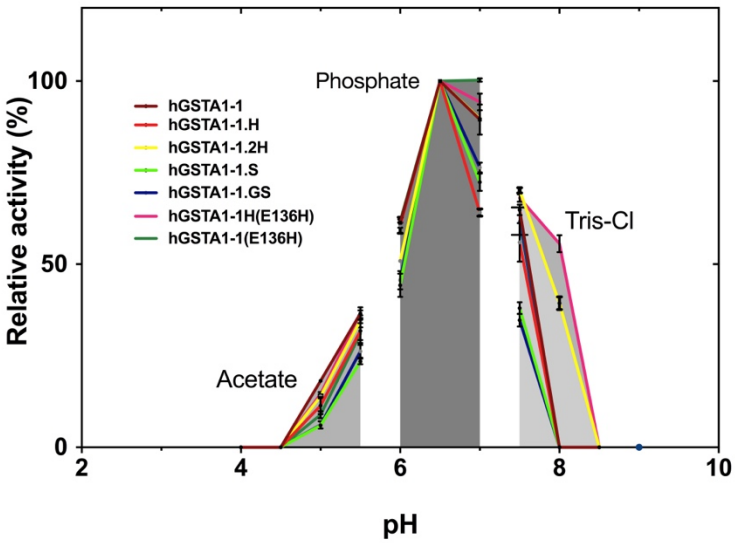

***Figure 4.** Effect of pH on hGSTA1-1 activity and its mutants. Enzyme activities were measured using the standard assay reaction at 37°C, at pH values ranging from 4 to 9, using the indicated buffers.*

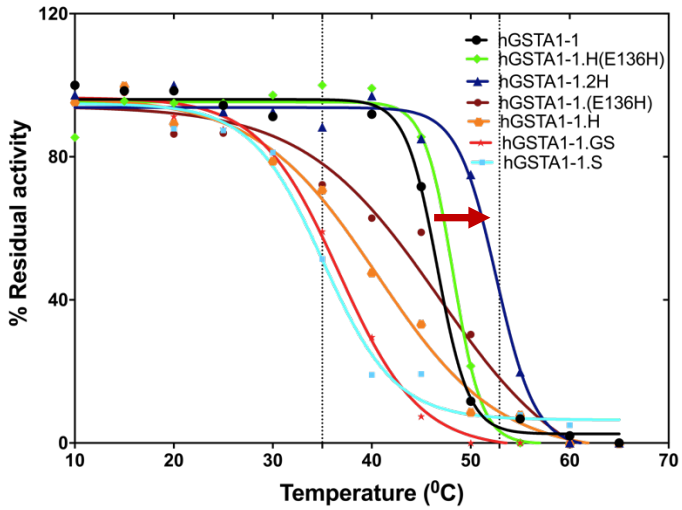

***Figure 5**. Thermal inactivation curves for hGSTA1-1 and its mutants. The residual catalytic activities were measured after heat treatment at temperatures ranging from 25 to 65°C (pH 7) for 5 min. The reported values correspond to the mean of triplicate measurements and the error bars correspond to the standard error from the mean value.*

CONCLUSION

The results showed that the mutant and "lego" enzymes appear to exhibit improved catalytic efficiency without losing their structural stability. In particular, the amino acids positions 136, 140 and 141 can be considered as hot spots for modulating the catalytic activity and thermostability of hGSTA1-1. In addition, the motif KVLH seems to be essential for the catalytic activity and a potential structural element for tailored made GSTs. The discovery and characterization of these mutants depicts the possibility that globular enzymes (e.g. GSTs) can be explored for the generation of complex architectures through protein engineering, by inserting specific repeat motifs. Therefore, it is a new way to enlarge the library of GST scaffolds of biomaterials for advanced applications.

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