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Q1 Evolution of the active site of human glutathione transferase A2-2 for enhanced activity of dietary isothiocyanates

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ABSTRACT

Background: Organic isothiocyanates (ITCs) are produced by plants, in which they are released from glucosinolates by myrosinase. ITCs are generally toxic and serve as a chemical defense against herbivorous insects and against infections by microorganisms. In mammalian tissues subtoxic concentrations of ITCs can provide protective effects against cancer and other diseases partially by induction of glutathione transferases (GSTs) and other detoxication enzymes. Thus, human consumption of edible plants rich in ITCs is presumed to provide health benefits. ITCs react with intracellular glutathione to form dithiocarbamates, catalyzed by GSTs. Formation of glutathione conjugates is central to the biotransformation of ITCs and leads to a route for their excretion. Clearly, the emergence of ITC conjugating activity in GSTs is essential from the biological and evolutionary perspective.
Methods: In the present investigation an active-site-focused mutant library of GST A2-2 has been screened for enzyme variants with enhanced ITC activity.
Results: Significantly superior activities were found in 34 of the approximately 2000 mutants analyzed, and the majority of the superior GSTs featured His and Gly residues in one of the three active-site positions subjected to mutagenesis.
Conclusions: We explored the propensity of GSTs to obtain altered substrate selectivity and moreover, identified a specific pattern of mutagenesis in GST for enhanced PEITC detoxification, which may play an important role in the evolution of adaptive responses in organisms subjected to ITCs.
General significance: The facile acquisition of enhanced ITC activity demonstrates that this important detoxication function can be promoted by numerous evolutionary trajectories in sequence space.

1. Introduction

Organic isothiocyanates are natural products of wide-ranging interest [1]. They are synthesized and stored as glucosinolates in plants and are released by the action of myrosinase when the plant tissue is damaged. The electrophilic isothiocyanates are toxic and serve to protect plants against invading organisms such as insects and microorganisms. Organic isothiocyanates are detoxified by conjugation with glutathione, which converts the electrophile to a dithiocarbamate in a conjugation reaction catalyzed by glutathione transferases (GSTs) abundantly occurring in the cells of most aerobic organisms. The plant tissues protect themselves against organic isothiocyanates by multiple GSTs, which are expressed in all tissues ranging from roots to leaves [2]. However, herbivores such as insects mount a similar response by producing GSTs as their resistance factors [3]. The biosynthetic pathways to the glucosinolates and isothiocyanates originate from amino acid derivatives and result in large variety of isothiocyanate analogues. From an evolutionary perspective it is therefore of interest to investigate how GST activities can evolve to meet the novel challenges arising from emerging plant isothiocyanates.

It is noteworthy that GST activity with organic isothiocyanates is widely occurring. In the study of two GSTs from cyanobacteria the only prominent activity identified was that with PEITC [4]. In an evolutionary experiment, isothiocyanate activity readily emerged from a GST mutant library [5]. Most higher plants and animals investigated express

Abbreviations: GST, glutathione transferase; GSH, glutathione; AITC, allyl isothiocyanate; BITC, benzyl isothiocyanate; EA, ethacrynic acid; PEITC, phenethyl isothiocyanate; PITC, propyl isothiocyanate; CDNB, 1-chloro-2,4-dinitrobenzene; Non, nonenal; BN, butylnitrite; AD, Δ^5-androstene-3,17-dione; EA, ethacrynic acid; pNPA, p-nitrophenyl acetate; Diiodoethane, 1,2-diiodoethane; CuOOH, cumene hydroperoxide; MD, molecular dynamics

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GSTs with isothiocyanate activity. It would appear that there is a close physiological connection between isothiocyanates and GSTs.

The present investigation was designed to explore the propensity of GSTs to evolve altered substrate selectivity for isothiocyanates, which may play an important role in adaptive responses in plants and their cognate herbivores. Three point mutations in the active site of a human GST were investigated in different combinations and the resulting activities with representative GST substrates and alternative isothiocyanate substrates were investigated.

2. Results

2.1. Construction and quality of the focused library of GST A2*E

The H-site of GSTs is responsible for the binding of various electrophilic substrates and thus is hypervariable among different GST species. The H-site of human GST A2*E is composed of 13 amino acid residues, including G10, I12, S14, E104, L107, L108, S110, F111, M208, L213, S216, F220, and F222. Identification of key residues for activity enhancement for a desired substrate is therefore not trivial. However, our previous attempt to enhance GST activity with azathioprine, as a spin-off indicated that three residues (107, 108 and 222) might be important for GSH conjugation with PEITC [6]. Therefore, a focused library targeting these three positions was revisited for isothiocyanate activities. The library stochastically encoded Arg, Asn, Asp, Cys, Gly, His, Ile, Leu, Phe, Ser, Tyr, or Val at each of positions 107 and 108 based on introduction of the NDT codon degeneracy. At position 222 His, Leu, Phe, Pro, Ser, or Tyr was encoded via YHC degeneracy.

2.2. Sequence divergence of A2*E variants with enhanced PEITC activity

Roughly 2000 variant colonies as well as the parent enzyme A2*E colony were picked individually into 96-well plates for growth and lysate preparation. A screening method based on UV/Vis spectroscopy was developed for identifying activity-enhanced variants from the mutant library. By lowering the PEITC concentration to 33 μM, while maintaining the GSH concentration constant at 1 mM, the assay was optimized to distinguish variants with improved enzyme properties from GST A2*E, the starting enzyme. The mean activity of the parent enzyme was 8.24 ($10^{-3}\Delta A_{274}$/min) with a S.D. value of 1.30 ($10^{-3}\Delta A_{274}$/min). Of the enzyme variants investigated 18.9% (355/1881) exhibited improved activity with PEITC, i.e., above the cut-off value of mean + 3 S.D. for the parent enzyme (Fig. 1a).

2.3. Sequence diversity among the high-activity variants

The 34 variants showing activity >20 ($10^{-3}\Delta A_{274}$/min) from the initial screening were sequenced (Fig. 1a), all of which had defined sequences without any spurious mutation introduced during PCR amplification. The variants in the winning pool after screening exhibited high molecular diversity. G and H were over-represented at position 107 and complemented with F and S from the 12 amino acids encoded by NDT randomization. Positions 108 and 222 were highly diversified, with the occurrence of 10 out of 12 possible amino acids encoded by NDT degeneracy and 5 out of 6 using codon YHC, respectively (Fig. 1b). The functional enrichment of G and H at position 107 was then subjected to determination of statistical significance using a binomial test. Both G and H were overwhelmingly dominant with P values of $2.4 * 10^{-10}$ and $2.3 * 10^{-8}$, respectively. To shed light on the over-presentation of G and H in terms of physico-chemical properties at position 107, different quantitative descriptors of amino acids were used for analysis [7–9]. But none of them could explain the under-representation of alternative residues encoded by NDT randomization in this position. It is noteworthy that all GSTs that were highly active with PEITC harbored at least two substitutions at the three targeted positions (Table 1). Q4

2.4. Characterization of the most active variants

The six variants with the highest elevated activity with PEITC were then subjected to specific activity determinations with various substrates in order to obtain an overall picture of enzyme performance in the multi-dimensional chemical-reaction space [6,10]. All of them showed increased specific activity with PEITC by a factor from 8.1 to 17.1 fold as compared to the parent GST A2*E (Fig. 2a). The specific activities with CDNB, butylnitrite and CuOOH, relative to A2*E, changed modestly within in the range from 0.26 to 1.69 fold, while the differences relative to A2*E in the remaining substrates varied up to 15 fold. It was interesting to find that all the selected variants displayed enhanced activities with nonenal and EA, considering the fact that the molecular structures of nonenal and EA are quite different from those of PEITC. On the other hand, both nonenal and EA are alkenes, which undergo additional reactions with the sulfur of glutathione, a chemical transformation initiated by nucleophilic attack on an sp^2-hybridized carbon, with mechanistic similarities to the ITC reaction.

A set of isothiocyanate compounds occurring in human diets was then used for more detailed steady-state kinetic studies of the enzyme variants. Three naturally-occurring isothiocyanates besides PEITC (in watercress and garden cress) were used for characterization, namely, BITC (in red cabbage), AITC (in cabbage, mustard, and horseradish) and PITC (in rapeseeds) [11]. For the reaction with PEITC, all the selected variants displayed >30 fold increases in catalytic efficiencies (Fig. 2b). The enhancement in performance stemmed from both increases in k_{cat} and decreases in K_M values. HLP, the most active variant with PEITC, exhibited catalytic efficiency of 625 $mM^{-1}\cdot s^{-1}$, with a 43-fold increase compared to that of the parent enzyme. The k_{cat} of HLP with PEITC increased from 1.71 to 14.8 s^{-1}, while K_M was lowered from 0.12 to

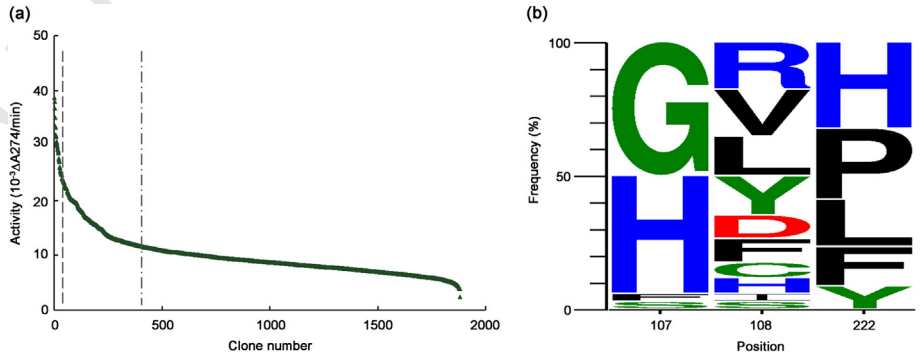

Fig. 1. (a). Activity values of crude bacterial cell lysates containing GST A2*E variants in twenty 96-well microtiter plates in descending order of PEITC activity. Variants exhibiting activity above 12.1 ($10^{-3}\Delta A_{274}$/min) (left of the dot-dashed line vertical line) are considered as variants with improved PEITC activity and variants with activity above 20 ($10^{-3}\Delta A_{274}$/min) (left of the dashed vertical line) were taken for sequence analysis. (b). A WebLogo [56] representation of substitution patterns in the three randomized positions among the high-activity GST variants. The font height of the letter is proportional to the frequency of amino acid residue appearance at each corresponding position.