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Research paper

Consensus mutagenesis reveals that non-helical regions influence thermal stability of horseradish peroxidase

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Abstract

The enzyme horseradish peroxidase has many uses in biotechnology but a stabilized derivative would have even wider applicability. To enhance thermal stability, we applied consensus mutagenesis (used successfully with other proteins) to recombinant horseradish peroxidase and generated five single-site mutants. Unexpectedly, these mutations had greater effects on steady-state kinetics than on thermal stability. Only two mutants (T102A, T110V) marginally exceeded the wild type's thermal stability (4% and 10% gain in half-life at 50 °C respectively); the others (Q106R, Q107D, I180F) were less stable than wild type. Stability of a five-fold combination mutant matched that of Q106R, the least-stable single mutant. These results were perplexing: the Class III plant peroxidases display wide differences in thermal stability, yet the consensus mutations failed to reflect these natural variations. We examined the sequence content of Class III peroxidases to determine if there are identifiable molecular reasons for the stability differences observed. Bioinformatic analysis validated our choice of sites and mutations and generated an archetypal peroxidase sequence for comparison with extant sequences. It seems that both genetic variation and differences in protein stability are confined to non-helical regions due to the presence of a highly conserved alpha-helical structural scaffold in these enzymes.

Keywords: Ancestral protein; Consensus concept; Protein stabilisation; Recombinant horseradish peroxidase

1. Introduction

The peroxidases (E.C. 1.11.17) are a ubiquitous subset of enzymes found throughout the animal and plant kingdoms [1]. The enzyme from horseradish roots (*Armoracia rusticana*; HRP) is the most widely studied example, due mainly to its many diverse uses in biotechnology [2]. Although moderately stable, the availability of a stabilized form of HRP would increase its applicability still further. Previous stabilisation studies have focused on the plant-derived protein, with several reports describing chemical procedures such as crosslinking [3–6], surface modification [7–9], attachment of PEG [10] and modification of carbohydrate residues [11]. Immobilisation of HRP [12,13] and addition of stabilising reagents [14,15] have also led to enhanced stability. To date, random mutagenesis has been used to genetically alter recombinant HRP stability [16,17] while yeast cell surface display has been used to select recombinant HRP variants with altered enantioselectivities [18]. There have been no reported attempts to stabilise recombinant HRP via site-directed mutagenesis.

It is not necessary to examine a protein's three-dimensional structure in order to identify stabilizing substitutions. Using sequence information alone, Steipe et al. [19] successfully predicted stabilizing mutations in a V_H antibody domain with

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzthioline-6-sulfonic acid); δ-ALA, delta aminolevulinic acid; DMF, dimethylformamide; DMSO, dimethylsulfoxide; HRP, horseradish peroxidase isoenzyme C; HRPA2, horseradish peroxidase acidic isoenzyme A2; GnCl, Guanidine Hydrochloride; IPMDH, 3-isopropylmalate dehydrogenase; LB, Luria–Bertani medium; MeOH, methanol; ML, maximum likelihood; MRCA, most recent common ancestor; NCBI, National Centre for Bioinformatic Information; PAML, phylogenetic analysis using maximum likelihood; PEG, polyethylene glycol; RZ, reinheitszahl (purity number A_{403}/A_{280}); SBP, soybean peroxidase; $t_{½app}$, apparent half-life; v/v, volume per volume; w/v, weight per volume.

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>60% frequency. This ''Consensus'' alignment procedure allows the identification of key stabilising residues in a protein structure [20]. The approach postulates that conserved residues in the sequence alignments of related proteins are more stabilising than non-conserved residues [21] and that a set of amino acid sequences of homologous, mesophilic enzymes contains sufficient information to allow rapid design of a thermostabilised, fully functional enzyme [22]. The chances of a deleterious mutation are reduced, since the replacement amino acid has already proven its evolutionary fitness at that position.

A consensus peroxidase protein sequence was developed *''in silico''* from aligned sequences of Class III secretory plant peroxidases downloaded from NCBI utilising custom-built bioinformatic software that scores the most frequent amino acid in a particular position [23]. Using this consensus sequence, five HRP residues were mutated to the corresponding consensus residue. The mutant proteins were expressed, purified and characterised in terms of their stabilities and steady-state ABTS kinetics.

Only two mutants registered marginal stability gains over wild type recombinant HRP, in sharp contrast to previous consensus studies with other proteins [19–22,24–26]. This unexpected outcome prompted us to undertake an evolutionary analysis of the Class III peroxidases and to generate a hypothetical archetypal peroxidase sequence. Comparison of extant sequences with our hypothetical archetypal peroxidase reveals an interesting insight into plant peroxidase structure, function and evolution.

2. Materials and methods

2.1. Materials

All reagents were purchased from Sigma Aldrich and were of analytical grade or higher. The pQE60 vector was purchased from Qiagen (Valencia, CA); XL 10 Gold cells and QuickChange™ Mutagenesis Kit were purchased from Stratagene (La Jolla, CA). The HRP gene was a generous gift from Prof. Frances H. Arnold (Caltech, CA).

2.1.1. Cloning

Based on refs. [27,28], the HRP gene was directionally cloned into the pQE60 vector as a fusion with the pectate lyase (PelB) leader sequence [29] (preceding the HRP's N-terminus) and a hexa-histidine purification tag (at the C-terminus), to generate plasmid pBR_I [30].

2.1.2. Bacterial strains and plasmids

E. coli XL 10 Gold was used as host strain to express the HRP protein. The plasmid pBR_I, carrying the HRP gene coding for the HRP fusion protein, was used for expression and site directed mutagenesis.

2.1.3. Recombinant DNA techniques

All DNA manipulations were carried out by standard techniques [31]. Site directed mutagenesis was carried out as described in ref. [32] utilising the QuickChange™ method. Mutant primers were supplied by MWG-Biotech (Germany). Mutants were confirmed by commercial di-deoxy sequencing (Fusion Antibodies, Belfast, Northern Ireland).

2.1.4. Expression and purification

A single cell transformed with pBR_I (or mutant derivative) was grown in 10 mL LB medium containing 100 μg/mL ampicillin and 2% w/v glucose until the $OD_{600\ nm}$ reached 0.4; the cells were removed via centrifugation at 2000 × *g* for 5 min and resuspended in fresh LB (500 mL) supplemented with 100 μg/mL ampicillin, 1 mM δ-ALA and 2 mM $CaCl_2$. The cells were then allowed to grow at 30 °C, 220 rpm for 16 h. Following overnight expression, the cells were centrifuged at 2000 × *g* for 5 min and the supernatant was treated with 50% w/v ammonium sulphate for 2 h at room temperature. The cells were periplasmically lysed [33] and the periplasmic contents were similarly treated with 50% w/v (with respect to the initial supernatant volume) ammonium sulphate. Proteins precipitated by ammonium sulphate from both the culture supernatant and the periplasmic preparation were collected via centrifugation, resuspended in 50 mM phosphate buffer pH 7.5, pooled and dialysed versus the same buffer overnight at 4 °C. Sodium chloride (1 M) and GnCl (200 mM) were added to the dialysed fractions (10 mL total volume), and these latter were purified via nickel affinity chromatography at room temperature. Sodium acetate (25 mM, pH 4.5) was utilised to elute the bound HRP. The eluted HRP was again dialysed versus 50 mM phosphate buffer pH 7.5 overnight at 4 °C, after which the protein was concentrated (Amicon-Plus 20 concentrator tubes; 2 mL final volume), filter sterilised and stored at 4 °C. These procedures led to typical expression values of 0.086 mg HRP per litre of culture medium. Purified HRP (specific activity 0.58 μmol min^{-1} mg^{-1}, RZ value 1.1) gave a single band on a 12% polyacrylamide gel (not shown).

2.1.5. Enzyme assay and characterisation

The stability parameters of recombinant HRP and mutant variants were determined as described for plant HRP [3,9] except that thermoinactivation time courses used 50 °C. Samples were removed periodically onto ice and their residual activities determined upon re-warming to room temperature; this procedure gives apparent half-life, $t_{½app}$). A constant protein concentration of 0.1 mg/mL was used for all thermoinactivations to control for possible effects of protein concentration on stability. The substrate ABTS (2,2′-azino-bis(3-ethylbenzthioline-6-sulfonic acid)) gives steady-state kinetics, permitting estimation of the apparent kinetic parameters, V_m/E and K_m' [34,35].

2.1.6. Generation of consensus and ancestral sequences

2.1.6.1. Approach 1. The *peroxidase consensus sequence* was generated *in silico* by the 'Protein Parser' software [23]. One hundred fully confirmed peroxidase protein sequences were downloaded from the NCBI homepage [http://www.ncbi.nlm.nih.gov/], using the following search terms ''((((((Peroxidase) AND (plant))) NOT (precursor)) NOT (putative)) NOT