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Short communication

Stability properties of an ancient plant peroxidase

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ABSTRACT

Plant (Class III) peroxidases have numerous applications throughout biotechnology but their thermal and oxidative stabilities may limit their usefulness. Horseradish peroxidase isoenzyme C (HRPC) has good catalytic turnover and is moderately resistant to heat and to excess (oxidizing) concentrations of hydrogen peroxide. In contrast, HRP isoenzyme A2 (HRP A2) has better oxidative but poorer thermal stability, while soybean peroxidase (SBP) displays enhanced thermal stability. Intrigued by these variations amongst closely related enzymes, we previously used maximum likelihood methods (with application of Bayesian statistics) to infer an amino acid sequence consistent with their most recent common ancestor, the 'Grandparent' (GP). Here, we report the cloning and expression of active recombinant GP protein in *Escherichia coli*. GP's half-inactivation temperature was 45 °C, notably less than HRP C's, but its resistance to excess H_2O_2 was 2-fold greater. This resurrected GP protein enables a greater understanding of plant peroxidase evolution and serves as a test-bed to explore their ancestral properties.

1. Introduction

Benner postulated that the fully resolved evolutionary relationship of a protein family allows the determination of mechanisms of extant protein evolution (e.g. visual pigment proteins and ancestral sequence reconstruction) [1,2]. This approach allows direct study of the emergence of functions of modern-day proteins and a better understanding of evolutionary processes governing the evolution of protein stability, function and structure [1–6]. The strategy for resurrecting ancient genes/proteins in six main steps has been reviewed [4]. Such 'paleomolecular biochemistry' can potentially recapture biological functions/processes that may have been lost over time. Rediscovery of such 'lost' functions may be beneficial in biomedical or industrial applications today.

Horseradish peroxidase (HRP) and soybean peroxidase (SBP) are extant Class III plant secretory peroxidases that are used extensively throughout biotechnology [7]. However, stability issues (thermal and oxidative) limit their operational potential/efficiency.

Although HRP A2, HRP C and SBP are closely related [8], their enzymatic stabilities differ considerably. Of these three proteins, SBP is the most thermostable, and HRP A2 the least thermostable [9–12]. Their stabilities towards their primary substrate, H_2O_2, vary in an apparently inverse pattern with their thermal stabilities [See Supplementary material 1].

The sequence of the most recent common ancestor (MRCA) of HRP A2, HRP C and SBP (Fig. 1a) has been inferred by a Bayesian maximum likelihood approach [12]. The ancient peroxidase sequence, "Grandparent" (GP), is ~110 million years old (Cretaceous period of the Mesozoic era [13]). Resurrecting this ancient enzyme permits direct study of the molecular evolution of extant plant peroxidases.

Here we describe the successful expression of active GP *de novo*, and its thermal and oxidative stabilities. Compared with its extant counterparts, GP exhibits a moderate thermal stability with an increased tolerance to H_2O_2; the latter may be useful in industrial and biomedical applications.

2. Materials and methods

2.1. Materials

Commercial HRP A2, HRP C and food grade SBP were obtained from Biozyme, Sigma–Aldrich and Quest International respectively.

Abbreviations: BCA, bicinchoninic acid; GP, grandparent; HRP, horseradish peroxidase; IPTG, isopropyl β-D-1-thiogalactopyranoside; MRCA, most recent common ancestor; MYA, million years ago; Ni–NTA, nickel–nitriloacetic acid; RZ, Reinheitszahl number; SBP, soybean peroxidase; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; TMB, 3,3′,5,5′-tetramethylbenzidine dihydrochloride.

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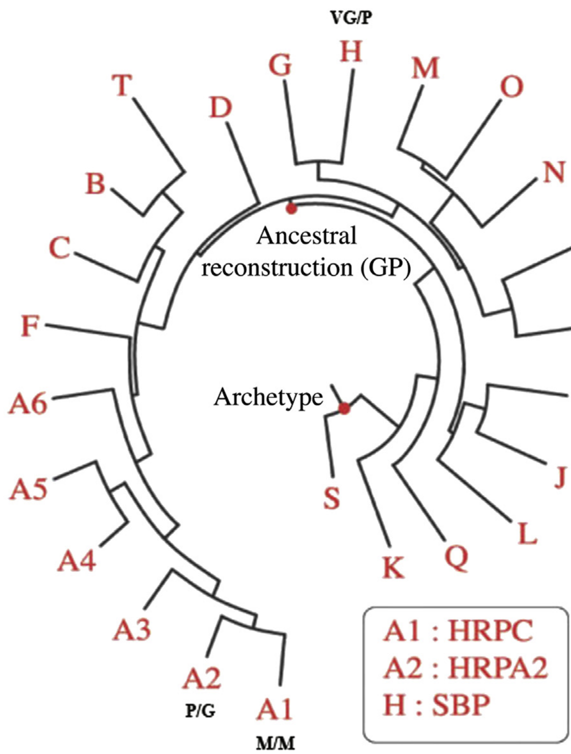


```
SBP          ----------------------------------------------------------QLT 3
HRPC         ---------------------------------------------------------MQLT 4
HRPA2        ----------------------------------------------------------QLN 3
Grandparent  MKNLFNLHMAMAFSSSSVLLLLLVLLLALIASTTFPQTGGYGGDDDDDIALASLEDAGLS 60
                                                                         *.

SBP          PTFY---RETCPN-------------------------------------------------L 14
HRPC         PTFY---DNSCPN-------------------------------------------------V 15
HRPA2        ATFY---SGTCPN-------------------------------------------------A 14
Grandparent  PNFYYKLSKSCPDNGSLLKNVSPDFDSLESGVASILTLNNNKKRNSDKYLRQQLTPEACV 120
             ..**   :**:
               A           B
SBP          FPIVFGVIFDASFTDPRIGASLMRLHFHDCFVQGCDGSVLLN--NTDTIESEQDALPNIN 72
HRPC         SNIVRDTIVNELRSDPRIAASILRLHFHDCFVNGCDASILLD--NTTSFRTEKDAFGNAN 73
HRPA2        SAIVRSTIQQAFQSDTRIGASLIRLHFHDCFVDGCDASILLD--DSGSIQSEKNAGPNAN 72
Grandparent  ESIVRSVVQQAFKSDPTIAAALLRLHFHDCFVNGCDASVLLDPDGGGNGTEEKTAPPNLN 180
               ** ..: :   :*. *.*:::*********:***.*:**:   .   .    *: *  * *
                  C                  D
SBP          S-IRGLDVVNDIKTAVENS--CPDT-VSCADILAIAAEIASVLGGGPGWPVPLGRRDSLT 128
HRPC         S-ARGFPVIDRMKAAVESA--CPRT-VSCADLLTIAAQQSVTLAGGPSWRVPLGRRDSLQ 129
HRPA2        S-ARGFNVVDNIKTALENT--CPGV-VSCSDILALASEASVSLTGGPSWTVLLGRRDSLT 128
Grandparent  LGLRGFEVIDDIKAALEAEPQCPGVSVSCADILALAARDAVVLSGGPSWEVPLGRRDGLR 240
               **: *:: :*:*:*     **  . ***:*:*::*:  :  * ***.* * ***** *
                 D'           E              F                   F'
SBP          -ANRTLANQNLPAPFFNLTQLKASFAVQGLN-TLDLVTLSGG-HTFGRARCSTFIN--RL 183
HRPC         -AFLDLANANLPAPFFTLPQLKDSFRNVGLNRSSDLVALSGG-HTFGKNQCRFIMD--RL 185
HRPA2        -ANLAGANSAIPSPFEGLSNITSKFSAVGLN-TNDLVALSGA-HTFGRARCGVFNN--RL 183
Grandparent  SAAADVANNNLPSPTFTVTVLISLFASKGLNNVTDLVALSGAGHTIGKAHCSSFSNRFRL 300
              *    **  :*:*     :..:   *   ***   ***:***. **:*: :*   : :  **
                            F''                                      G
SBP          YNFSN-TGNPDPTLNTTYLEVLRARCPQNAT-----GDNLTNLDLSTPDQFDNRYYSNLL 237
HRPC         YNFSN-TGLPDPTLNTTYLQTLRGLCPLNGN-----LSALVDFDLRTPTIFDNKYYVNLE 239
HRPA2        FNFSG-TNGPDPTLNSTLLSSLQQLCPQNGS-----ASTITNLDLSTPDAFDNNYFANLQ 237
Grandparent  YNFNNTTGNPDPSLDPTFAKQLQALCPQSGNNGPNDDTTVVNLDPGTPNTFDNSYYKNLL 360
             :**..  *. ***:*:.*   . *:  **  ...         :.::*  **   *** *: **
                      H                I          J
SBP          QLNGLLQSDQELFSTPGA--DTIPIVNSFSS-NQNTFFSNFRVSMIKMGNIGVLTGD--E 292
HRPC         EQKGLIQSDQELFSSPNAT-DTIPLVRSFAN-STQTFFNAFVEAMDRMGNITPLTGT--Q 295
HRPA2        SNNGLLQSDQELFSTLGS--ATIAVVTSFAS-NQTLFFQAFAQSMINMGNISPLTGS--N 292
Grandparent  SGKGLLQSDQELFTDPSTKPIVKAFVESFANGNQTTFFEEFARAMVKMGNIGVLTGGKRQ 420
             . :**:*******:   .:    . ..* **:. .   **. *  :* .****  ***    :

SBP          GEIRLQCNFVNG----------------------- 304
HRPC         GQIRLNCRVVNS----------------------- 307
HRPA2        GEIRLDCKKVDGS---------------------- 305
Grandparent  GEIRKNCRVVNSNSVAPDSVLVSDIKQKLVAQSKM 455
             *:** :*. *:.
```

Fig. 1. 'Grandparent' peroxidase (GP) phylogenetic position and sequence alignment with extant enzymes. (a) Phylogenetic location of ancestral plant peroxidase (GP) on the reduced phylogeny of Class III plant peroxidases, adapted from Ref. [12]. Stabilities of extant enzymes are in the order thermal/oxidative, where VG: very good, G: good, M: moderate, P: poor. (b) Amino acid sequence alignment of GP, HRP isozymes C and A2, and of SBP. Alpha-helices are indicated by the bars above the alignment and the capital letters refer to the helix nomenclature in Supplementary reference [S27]. See also Supplementary material 6.

Escherichia coli XL 10 Gold recombinant host cells were from Stratagene, restriction enzymes from New England Biolabs and Ni–NTA resin from Qiagen. Other reagents were from Sigma Aldrich.

2.2. Amino acid sequence alignments and in silico 3D modelling

The amino acid sequences of GP, HRP isozymes C and A2, and of SBP were aligned using CLUSTAL [14]. Homology modelling of GP's 3D structure was attempted using the first approach mode in SWISS-MODEL [14] with chain A of *Arabidopsis thaliana* A2 (Uniprot Q42578; PDB identifier 1PA2) as the structural template. The total sequence length of GP is 455 residues while the *A. thaliana* sequence (structural template) is 335 residues in length. Therefore, the first 100 residues of the GP sequence could not be homology-modelled using the available 3D model and so this approach was not pursued further.

2.3. Bacterial strain and plasmids

The GP gene sequence was commercially synthesized by GENEART AG, Germany (codon usage optimized for prokaryotes). The pGP expression plasmid comprised a pGSLink vector containing the synthesized GP gene sequence with a N-terminal pectate lyase (PelB) leader sequence [15] and a C-terminal $(His)_6$ tag for purification.

The PelB leader- and GP gene-sequences were inserted into the pGSLink expression vector via *Nco* I-*Not* I-*BamH* I and *Not* I-*Bgl* II cloning respectively to generate the pGP expression plasmid. A single pGP-transformed colony was used to inoculate 10 mL LB broth/100 μg/mL ampicillin, and grown overnight at 37 °C, 220 rpm. A 250 mL conical flask (containing 100 mL LB broth/100 μg/mL ampicillin/2% w/v glucose/2 mM $CaCl_2$) was inoculated with 1 mL of the overnight culture. The fresh culture was incubated at 37 °C, 220 rpm until $OD_{600\ nm} \approx 0.6$, then centrifuged at 10,000× *g* for 10 min at 4 °C, the supernatant discarded and pellet resuspended in 100 mL LB broth/100 μg/mL ampicillin/2 mM $CaCl_2$. Further incubation was at 30 °C for up to 18 h, followed by centrifugation at 4700× *g* for 5 min. Cell pellets were stored at −20 °C until lysis. (Supernatant samples showed no peroxidase activity.)

Cell pellets were re-suspended in 10 mL 50 mM sodium phosphate pH 7.5, sonicated on ice (40% amplitude; 90 s with 6 s pulses: Branson Digital Sonifer®) and centrifuged at 10,000× *g* for 10 min at 4 °C. Cleared lysates were filtered through 0.45 μm, applied to Ni–NTA resin (50 mM sodium phosphate buffer pH 7.5) and tumble-mixed at 4 °C for ≥1 h to permit binding. The mixture was then poured into 1.5 × 20 cm column, the settled resin washed with 50 mM sodium phosphate pH 7.5 and bound protein eluted with 50 mM sodium acetate pH 4.5. Eluted fractions were dialysed overnight at 4 °C in 5 L of 50 mM sodium phosphate pH 7.5. All samples were subjected to SDS-PAGE. Purified fractions were concentrated using Amicon Centricon® concentrators (Millipore), filter sterilized and stored at 4 °C for study.

2.4. Biochemical characterization

HRPC and HRP A2 were dissolved in 50 mM sodium phosphate pH 7.5 to 1 mg/mL. Food grade SBP was dissolved in 50 mM sodium phosphate pH 7.5 to 1 mg/mL, mixed for 20 min at room temperature and centrifuged (4000× *g* for 15 min). Supernatant (containing SBP activity) was retained. Protein was quantified using the bicinchoninic acid (BCA) assay; final working concentration was 0.1 mg/mL. 3,3′,5,5′-Tetramethylbenzidine dihydrochloride (TMB) was used to determine peroxidase activity at room temperature [16], with A_{620} recorded after 7 min.