Molecular Insights in the Cleavage of Rubber by the Latex-Clearing-Protein (Lcp) of *Streptomyces* sp. strain K30

Supplementary material

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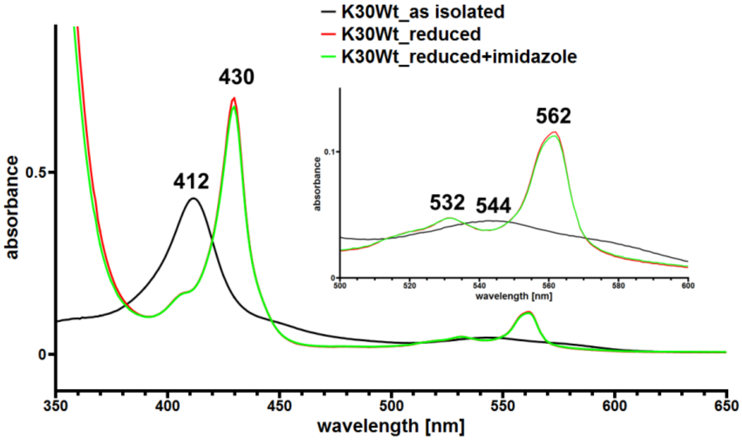
1. Alignment of 1280 DUF2236 amino acid sequences
2. UVvis spectra
3. Graphs of the oxygen consumption assays
4. HPLC chromatograms of separated polyisoprene cleavage products
5. List of amino acid sequence identifiers

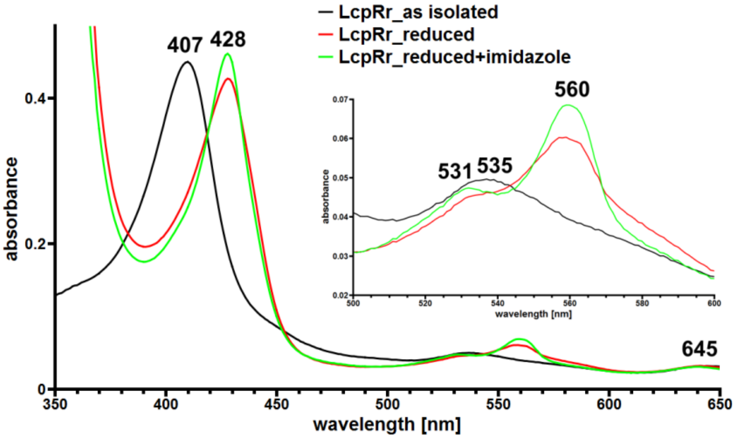
1. **Multi-sequence alignment of 1280 amino acid sequences containing the domain of unknown function DUF2236.** 6598 sequences containing the DUF2236 were accessible with the identifier IPR018713 in the interpro database (June 2016). The first 1500 prokaryotic sequences were used and sequences shorter than 250 and longer than 450 residues were removed. This led to 1280 sequences that were used for a multiple sequence alignment (Clustal Omega). The sequences of Lcp$_{K30}$, Lcp$_{Rr}$ and Lcp$_{VH2}$ are provided for comparison, numbering refers to Lcp$_{K30}$. Values of conservation > 80 % were observed for residues R164, T168 and H198. The degree of conservation of each residue is represented by the height of the column.

2. **UVvis spectra of Lcp$_{K30}$ wild type (from *Streptomyces* sp. K30), Lcp$_{Rr}$ (from *R. rhodochrous* PRK1) and the Lcp$_{K30}$ muteins purified in this study.** Black lines refer to the *as isolated* states, red lines to the states after reduction with sodium dithionite and green lines to the spectra that were recorded after the addition of 10 mM imidazole to the reduced protein and incubation for 10 to 15 minutes at room temperature. The Q-band region is enlarged in the insets for the heme-containing muteins.

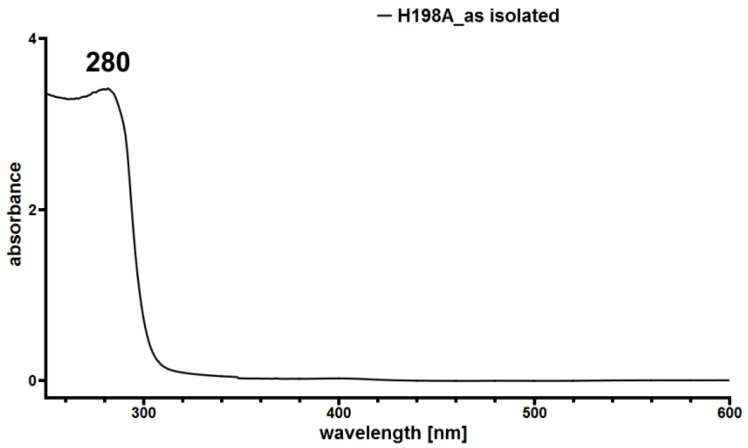
   a. **Lcp$_{K30}$ wild type.** In the *as isolated* state (black), a strong absorbance at 412 nm was observed (Soret band) as well as a 544 nm β-band. After chemical reduction (red line) a shift of the Soret band to 430 nm was detected and the Q-bands absorbed at 532 nm (β) and 562 nm (α). Addition of imidazole (green line) resulted in no increase of absorbance at 430 and 562 nm, indicating no accessibility for imidazole to the heme site and thereby suggesting a heme coordination by two axial amino acid heme ligands (closed state).



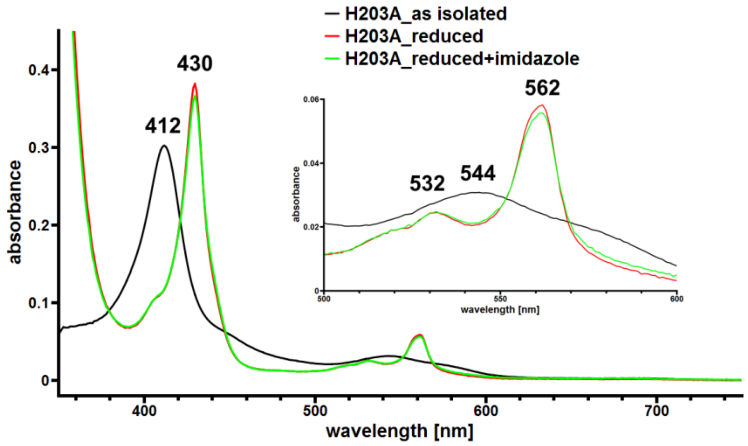
   b. **Lcp$_{Rr}$ wild type.** In the *as isolated* state (black) the Soret α band showed a maximum at 407 nm as well as a 535 nm band. After chemical reduction (red line) a shift to 428 nm occurs and the Q bands had maxima at 531 and 560 nm. A strong increase of absorbance at 430 and 560 nm after addition of imidazole (green line) was observed, indicating an open, accessible state suggesting the absence of an amino acid as a second heme ligand.



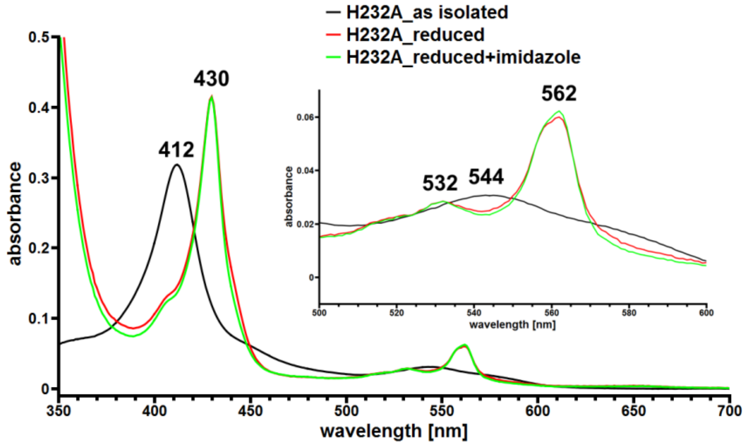
c. **Lcp$_{K30}$ mutein H198A.** In the *as isolated* state (black line) only a strong absorbance at 280 but no Soret band (absorbance around 400 nm) was observed, indicating the absence of heme.



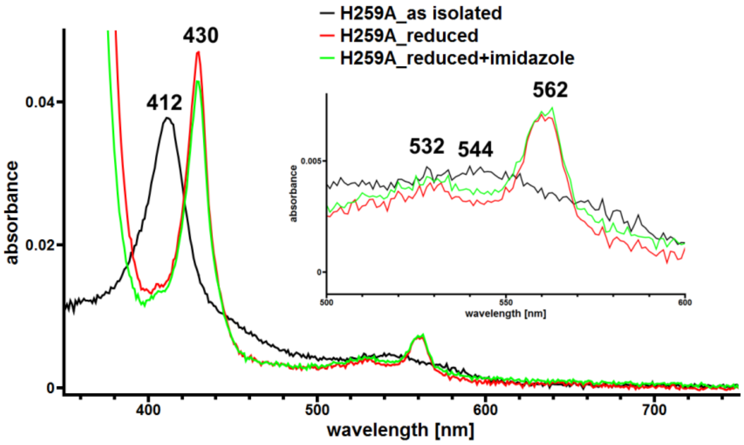
d. **Lcp$_{K30}$ mutein H203A.** Spectrum comparable to Lcp$_{K30}$ wild type



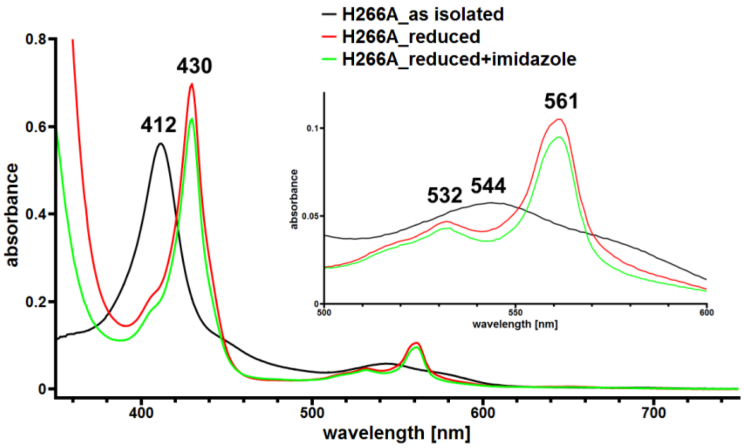
Lcp$_{K30}$ mutein H232A. Spectrum comparable to Lcp$_{K30}$ wild type.



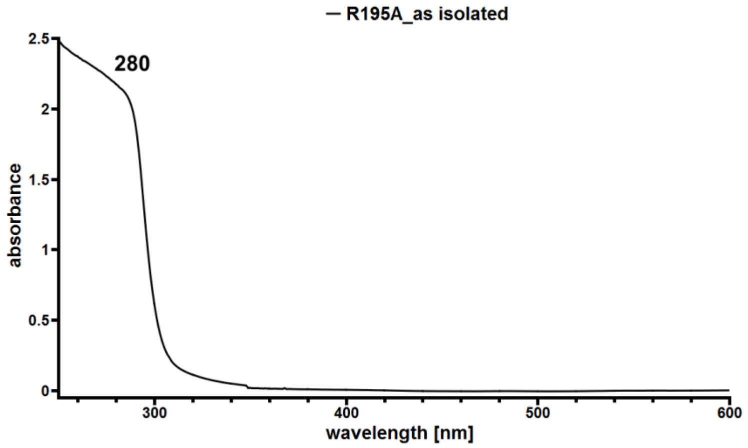
e. **Lcp$_{\mathrm{K30}}$ mutein H259A.** Spectrum comparable to Lcp$_{\mathrm{K30}}$ wild type. Due to low yield, only a small amount of the protein was used for spectroscopic characterization.



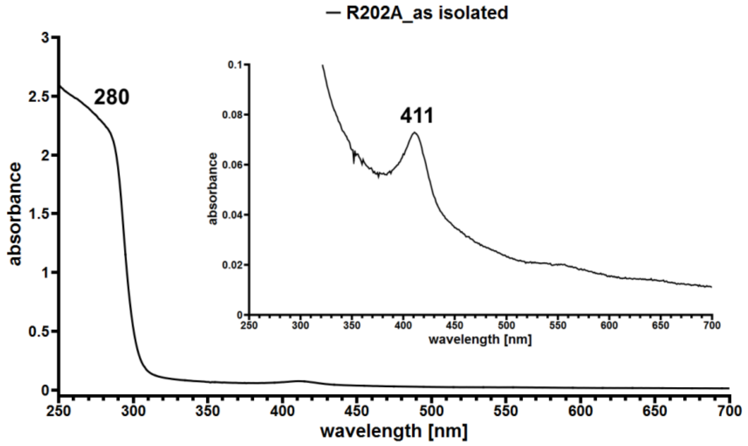
f. **Lcp$_{\mathrm{K30}}$ mutein H266A.** Spectrum comparable to Lcp$_{\mathrm{K30}}$ wild type.



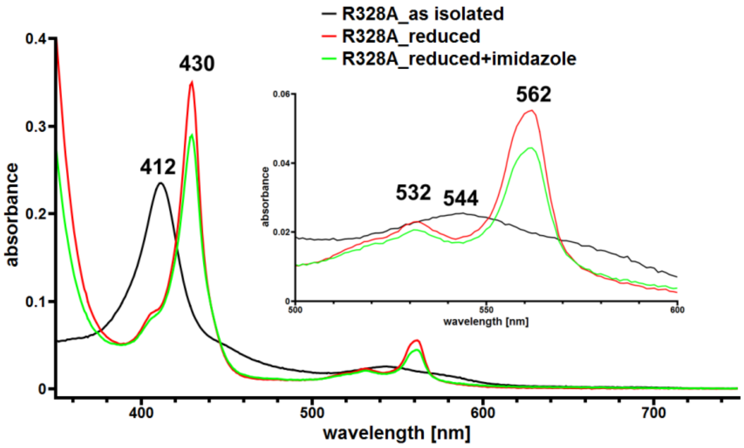
g. **Lcp$_{K30}$ mutein R195A.** In the *as isolated* state (black line) only a strong absorbance at 280 nm but no Soret band (absorbance around 400 nm) was observed, indicating the absence of heme.



h. **Lcp$_{K30}$ mutein R202A.** In the *as isolated* state (black line) a strong absorbance at 280 and a very weak Soret band (weak absorbance around 411 nm) was observed, indicating the presence of heme in only a minor fraction of mutein molecules. Due to instability of the Lcp$_{K30}$ mutein R202A no spectra after reduction or with the potential heme ligand imidazole were recorded.

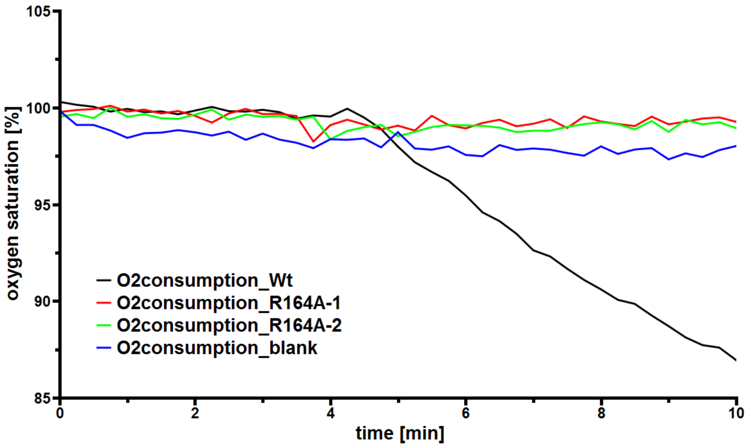


i. **Lcp$_{K30}$ mutein R328A.** Spectrum comparable to Lcp$_{K30}$ wild type. The slight decrease in absorption after addition of imidazole can be explained by partial degradation of the protein upon the addition of dithionite.

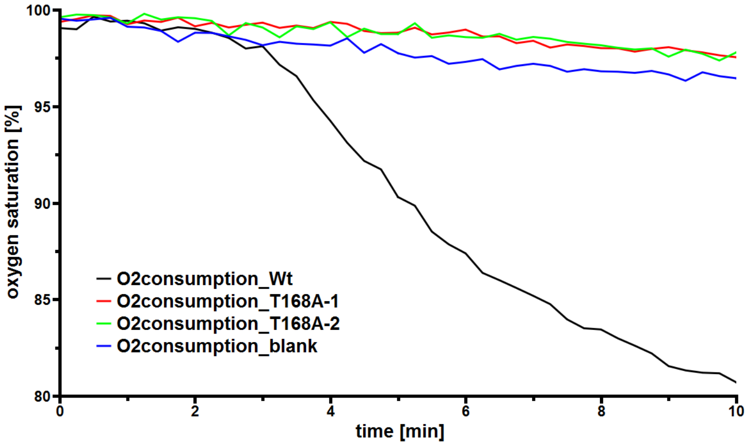


3. **Oxygen consumption assays during polyisoprene cleavage.** The degree of oxygen saturation of potassium phosphate buffer supplemented with 0.2% polyisoprene latex was recorded at 22°C in four cuvettes equipped with fluorescence sensor spots and the oxy-4-mini apparatus. After a constant oxygen concentration was recorded (3-5 min), 4 µg of the indicated purified protein (if not stated otherwise, blank without enzyme) was added. Assays with two cuvettes supplemented with the same mutein (A-1, A-2) were repeated at least three times; one representative data set is shown. Black line refers to activity of wild type Lcp$_{K30}$ in all assays.

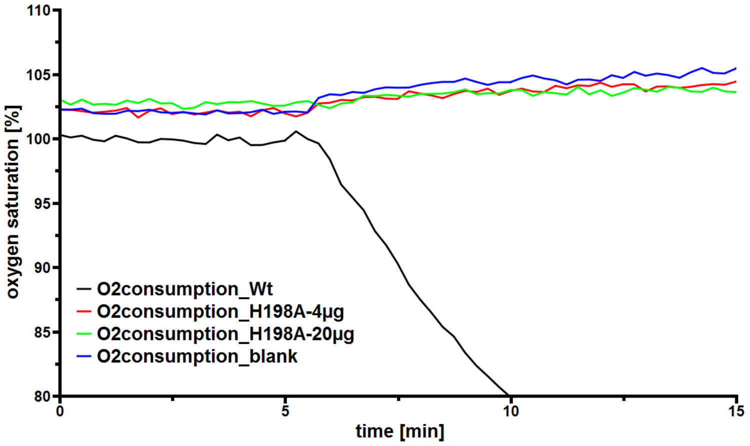
   a. **Lcp$_{K30}$ R164A,** DUF 2236 residue, red, heme containing mutein albeit no consumption of oxygen was observed, indicating inactivity of the mutein and identifying Lcp$_{K30}$ R164A as an essential residue for the catalytic mechanism. (Note: no residual activity could be shown by HPLC analysis).



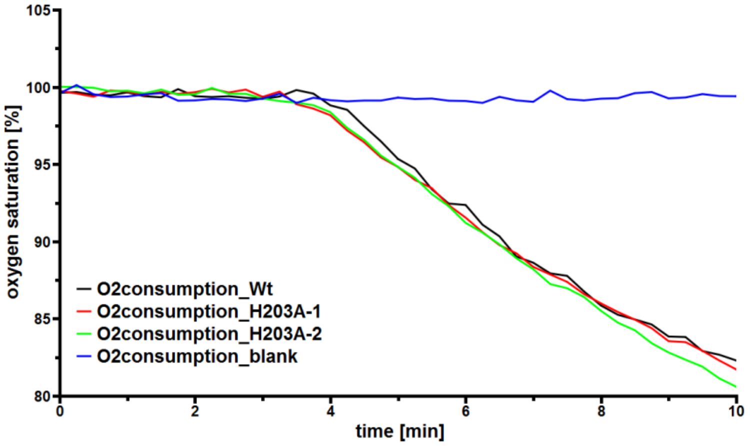
   b. **Lcp$_{K30}$ T168A,** DUF 2236 residue, red, heme containing mutein albeit no consumption of oxygen was observed, indicating inactivity or very low activity of the mutein and identifying Lcp$_{K30}$ T168A as an important residue for the catalytic mechanism. (Note: minor residual activity shown by HPLC analysis).



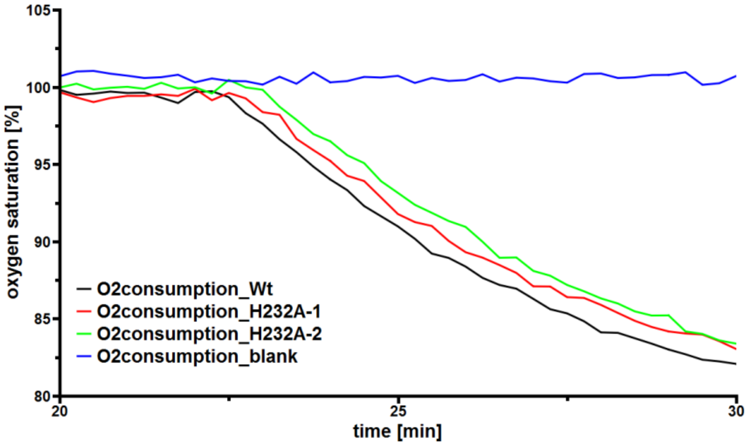
c. **Lcp$_{K30}$ H198A,** colorless mutein containing no heme group. No consumption of oxygen was observed for 4 and 20 µg enzyme , indicating inactivity of the mutein. (Note: no residual activity could be shown by HPLC analysis).



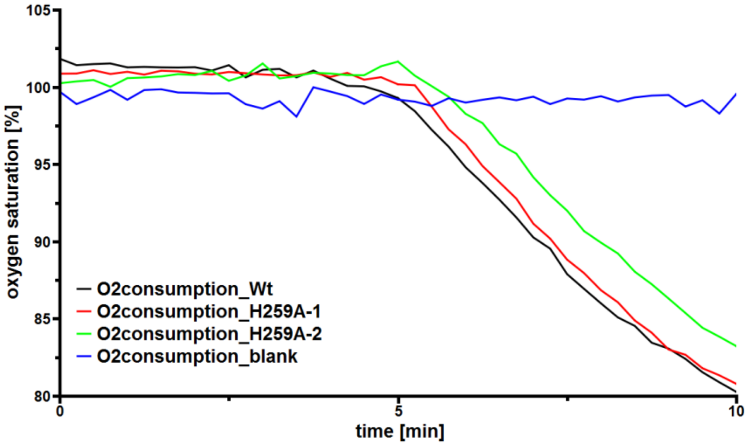
d. Lcp$_{K30}$ H203A, red, heme containing mutein showing consumption of oxygen comparable to Lcp$_{K30}$ wild type indicating no importance for the catalytic mechanism.



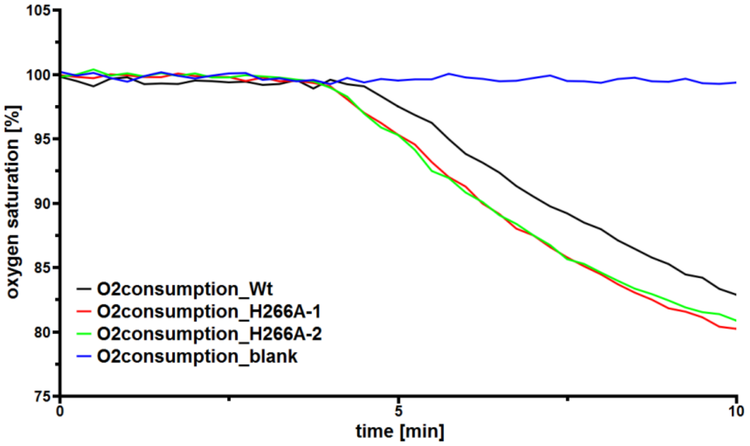
e. **Lcp$_{K30}$ H232A,** red, heme containing mutein showing consumption of oxygen comparable to Lcp$_{K30}$ wild type indicating no importance for the catalytic mechanism.



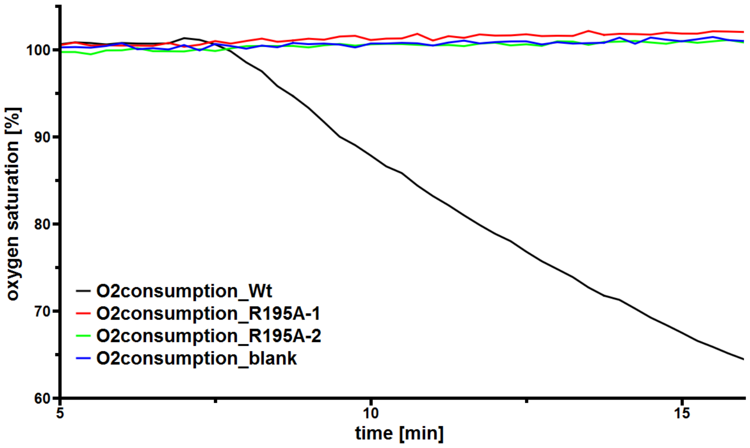
f. **Lcp$_{K30}$ H259A,** red, heme containing mutein showing consumption of oxygen comparable to Lcp$_{K30}$ wild type indicating no importance for the catalytic mechanism.



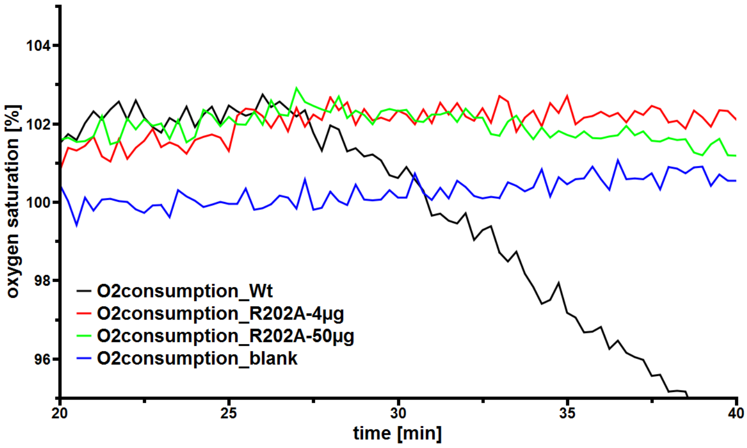
g. **Lcp$_{K30}$ H266A,** red, heme containing mutein showing consumption of oxygen comparable to Lcp$_{K30}$ wild type indicating no importance for the catalytic mechanism.



h. **Lcp$_{K30}$ R195A,** instable, colorless mutein not containing a heme group and no consumption of oxygen was observed, indicating inactivity of the mutein. (Note: no residual activity could be shown by HPLC analysis).

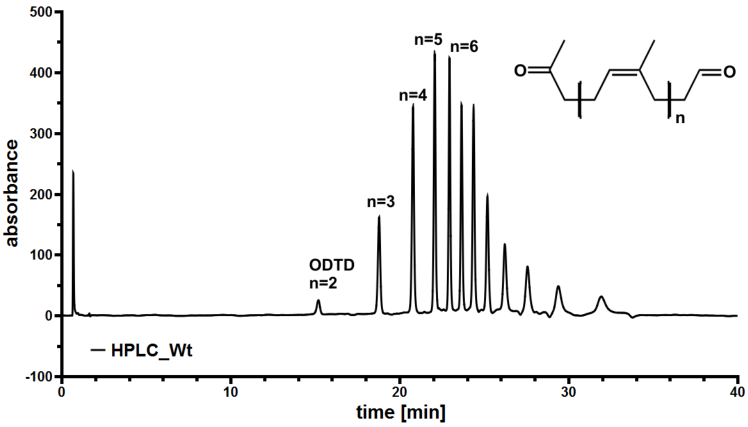


i. **Lcp$_{K30}$ R202A,** instable mutein. Only a few molecules of the mutein protein contained a heme group as revealed by a very weak Soret band. Only very low consumption of oxygen was observed even for 50µg enzyme. (Note: a minor residual activity could be shown by HPLC analysis for 50 µg enzyme).

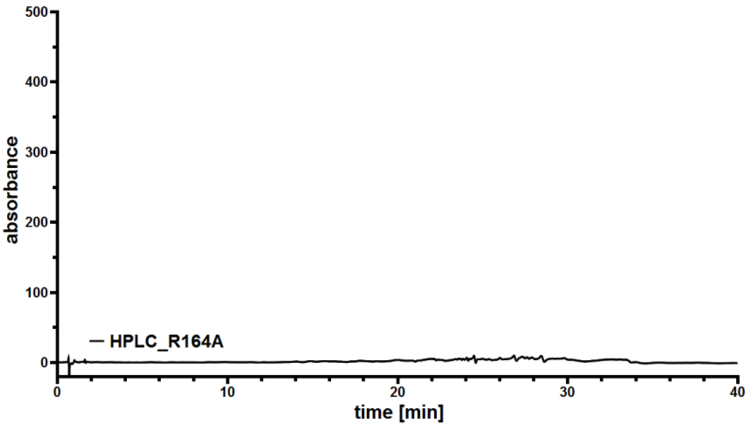


4. **HPLC chromatograms of Lcp$_{K30}$-generated cleavage products.** 4 µg of each enzyme was incubated in 1 ml potassium phosphate buffer supplemented with 0.2% latex for 2 hours at 22°C (for R164A and R195A, 25µg, for H198A 20 µg were incubated for 16 hours, in case of R202A, 50µg were used for 16 hours). The reaction mixtures were extracted with ethyl acetate after incubation and the solvent was evaporated. The formed cleavage products were dissolved in 100 µl methanol and analyzed by HPLC (RP8 column and methanol:water gradient from 50 to 100 % methanol, detection at 210nm). A blank without enzyme was prepared and was subtracted from the chromatogram with protein. Lcp$_{K30}$ wild type served as positive control. For the wild type, the chemical formula of the formed oligo-isoprenoids with varying number (n=2 to 6) of isoprene units is shown.

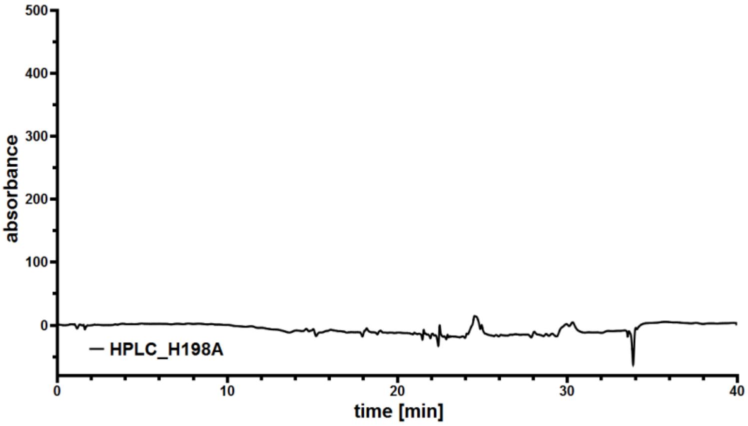
   a. **Lcp$_{K30}$ wild type,** red, heme containing protein producing oligo-isoprenoids with varying number of isoprene units (n=2 to 6) resulting in a typical pattern that indicated a random (endo) cleavage mechanism.



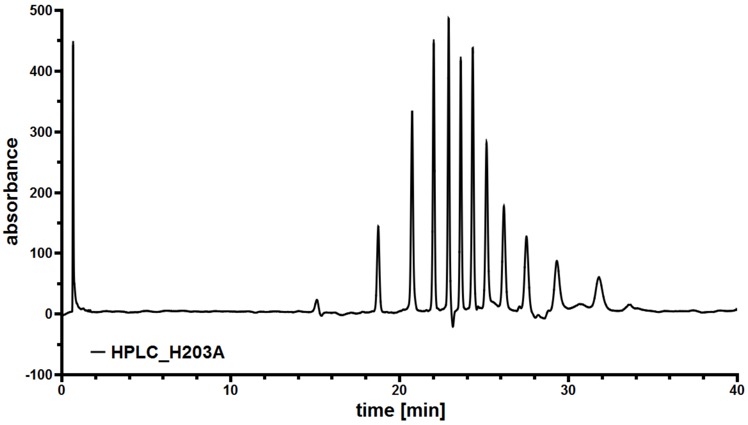
   b. **Lcp$_{K30}$ R164A,** DUF2236 residue, red, heme containing. 25 µg were incubated for 16 hours but no cleavage products could be observed identifying R164A as an essential residue for the catalytic reaction.



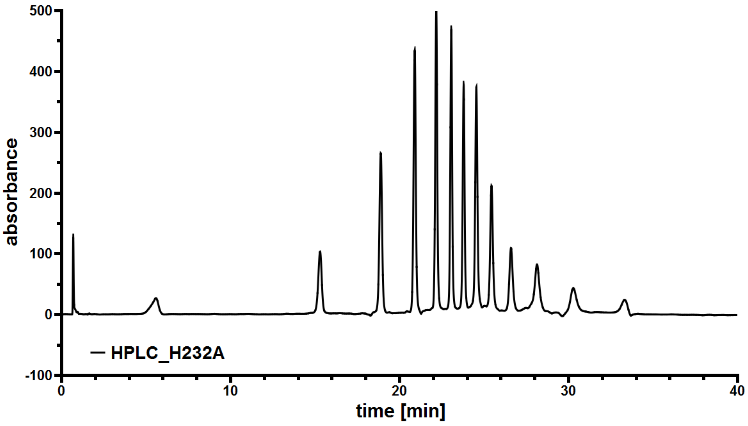
c. **Lcp$_{K30}$ H198A,** DUF2236 residue, colorless, containing no heme. 20 µg were incubated for 16 hours but no cleavage products could be observed identifying H198A as an essential residue for the catalytic reaction.



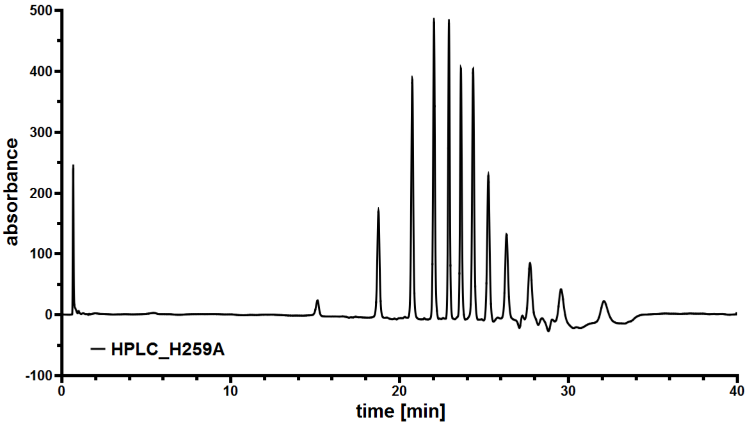
d. **Lcp$_{K30}$ H203A**, red, heme containing mutein showing a pattern of separated polyisoprene cleavage products similar to Lcp$_{K30}$ wild type indicating no importance for the catalytic mechanism.



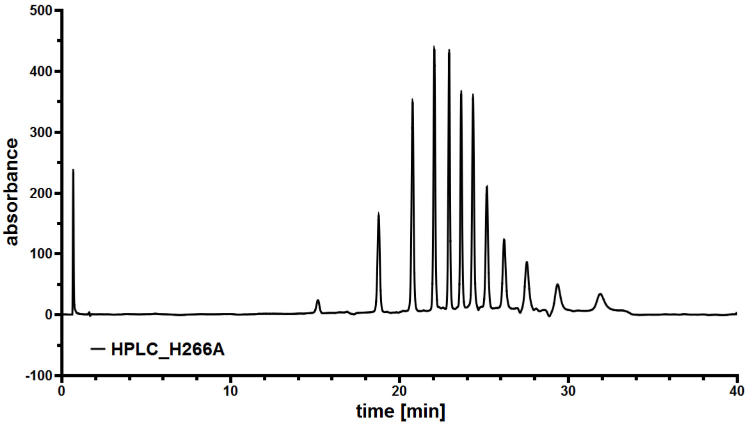
e. **Lcp$_{K30}$ H232A,** red, heme containing mutein showing a pattern of separated polyisoprene cleavage products similar to Lcp$_{K30}$ wild type indicating no importance for the catalytic mechanism.



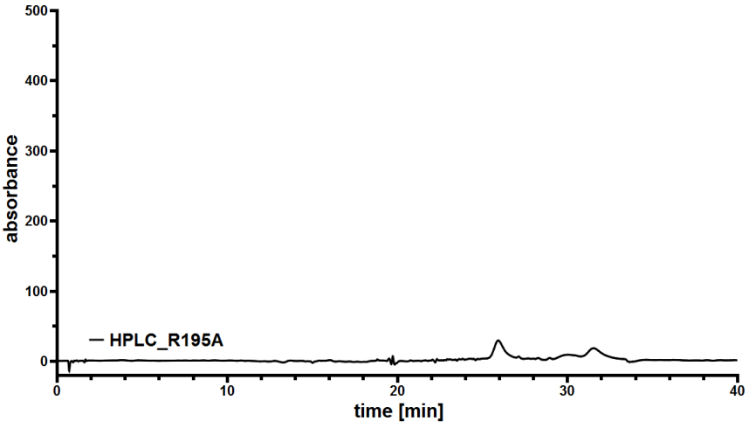
f. **Lcp$_{K30}$ H259A,** red, heme containing mutein showing a pattern of separated polyisoprene cleavage products similar to Lcp$_{K30}$ wild type indicating no importance for the catalytic mechanism.



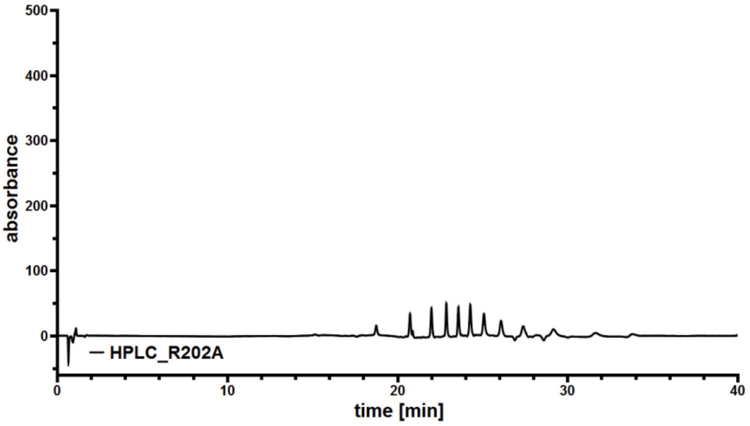
g. **Lcp$_{K30}$ H266A,** red, heme containing mutein showing a pattern of separated polyisoprene cleavage products similar to Lcp$_{K30}$ wild type indicating no importance for the catalytic mechanism.



h. **Lcp$_{K30}$ R195A,** colorless, instable mutein containing no heme. 25 µg were incubated for 16 hours but no cleavage products could be observed.



i. **Lcp$_{K30}$ R202A,** instable mutein. Only a few molecules of the mutein contained a heme group as revealed a very weak Soret band. 50 µg were incubated for 16 hours and a very low yield of cleavage products with the same pattern as observed for Lcp$_{K30}$ wild type could be observed being in agreement with the observed instability of the mutein.



5. List of amino acid sequence identifiers used for the multiple sequence alignments.

1280 identifiers of the sequences containing the DUF2236 used for Figure S1.

LcpK30/1-407
LcpRR/1-408
LcpVH2/1-407
Q3L8N0/1-407
P9WLH1/1-255
P9WLH0/1-255
P64958/1-255
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A0A0G2ZL99/1-335
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A0A0G2ZE11/1-380
A0A0G2J9M8/1-299
A0A0G2J8W2/1-365
A0A0G2J5Q8/1-448
A0A0G2ID28/1-425
A0A0G2F455/1-413
A0A0G2ENY3/1-425
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A0A0E3TQ83/1-327
A0A0E3TP53/1-366
A0A0E3TN40/1-370
A0A0E3MP23/1-289
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495 sequences homologue to Lcp$_{K30}$ used for Figure 1

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