Title

Characterization of prostaglandin $F_{2\alpha}$ 9-dehydrogenase from *Rhodotorula kratochvilovae*

Authors

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Supplement data

Table S1 Result of screening for microorganisms reducing PGE_2 to $PGF_{2\alpha}$

	Number of screened strains	$PGF_{2\alpha}$ producing activity[a]
Yeasts	109	22
Molds	261	23
Basidiomycetes	83	8
Bacteria	78	0
Actinomycetes	84	0
Lactic acid bacteria	70	0
Total	685	53

[a] The number of strains producing $PGF_{2\alpha}$ more than 5.6×10^{-2} mM (2% of substrate)

Effects of coenzymes on $PGF_{2\alpha}$ production by *R. kratochvilovae* NBRC 0389 CFE

Rhodotorula kratochvilovae NBRC 0389 (AKU 4826) was inoculated into 3 mL medium [5 % (*w*/*v*) malt extract and 0.3 % yeast extract, pH 5.5] and cultivated at 28 °C with shaking at 300 rpm overnight. The culture broth was all added into 100 mL of the same medium, and it was cultured at 28 °C with shaking at 120 rpm overnight. After cultivation, the cells were harvested by centrifugation at 3,000 rpm for 10 min and washed once with 100 mM Tris-HCl buffer (pH 8.0) or appropriate buffer for following reaction.

Cells were suspended in 100 mM Tris-HCl (pH8.0) at 33% (w/v) and disrupted by sonication with Ultrasonic Disruptor UD-211 (TOMY SEIKO, Tokyo, Japan) at Output 3 and Duty 50 for 20 min in iced water. After disruption, the lysate was centrifuged at 15,000 *g* for 10 min at 4 °C to remove cell debris. The supernatant was used as cell-free extracts (CFE).

Effects of NADH and NADPH on the $PGF_{2\alpha}$ production catalyzed by *R. kratochvilovae* NBRC 0389 were evaluated with using its CFE. Reaction mixtures (500 μL) contained 100 mM Tris-HCl (pH 8.0), 2.8 mM PGE_2, 20 % (v/v) *R. kratochvilovae* NBRC 0389 CFE, and appropriate concentrations of NADH, NADPH, glucose, and GDH. The reaction and lipids extraction were performed in the same way as the screening. Yields of PGs were quantified by HPLC.

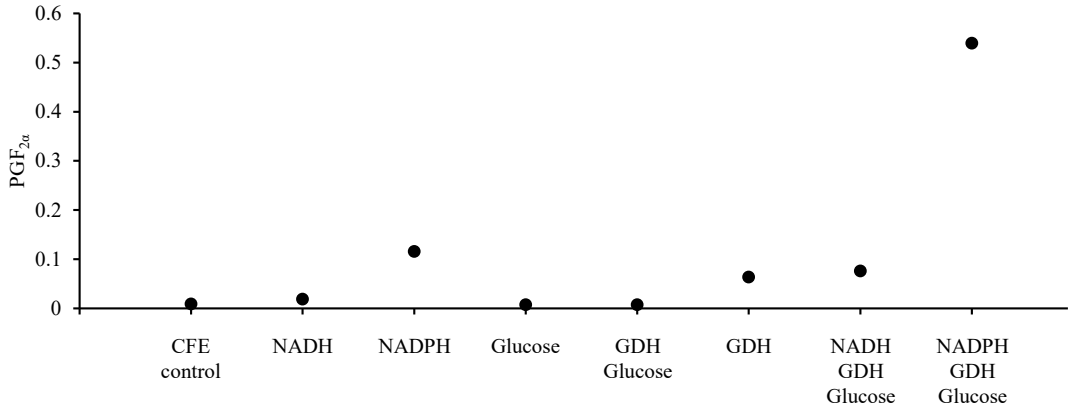


Fig. S1 *R. kratochvilovae* NBRC 0389 CFE produced $PGF_{2\alpha}$ only when there was NADPH in reaction mixture. NADH and NADPH were added at 3 mM, glucose was at 160 mM, and GDH was at 14 U/ml. n=1

Analysis of recombinant PDH activities by resting cell and cell-free extracts reactions

E. coli harboring pET-21b/His-tag-PDH was cultured in 5 mL LB medium at 28 °C with shaking at 300 rpm overnight, and the culture broth was all added into 100 mL of the same medium. The cells were cultured until OD_{600} was 0.4, and 1 mM IPTG was added to the culture. The culture medium was incubated at 28 °C with 120 rpm shaking overnight. The cells were harvested by centrifugation and washed twice with 0.75 % NaCl solution. The enzyme expression was checked by SDS-PAGE. The evaluation of the resting cell and cell-free extracts activity were performed in the same way as evaluation of PGE_2-reducing activity. The reaction time was changed to 24 hours, and the PGE_2 concentration was changed to 2.8 mM. The expression of the PDH gene was confirmed by SDS-PAGE (Fig. S2). The PDH-expressing strain was reacted with PGE_2. As a result, $PGF_{2\alpha}$ was produced about 1.5 mM in the resting cells and about 2.1 mM in cell-free extracts (Fig. S2).

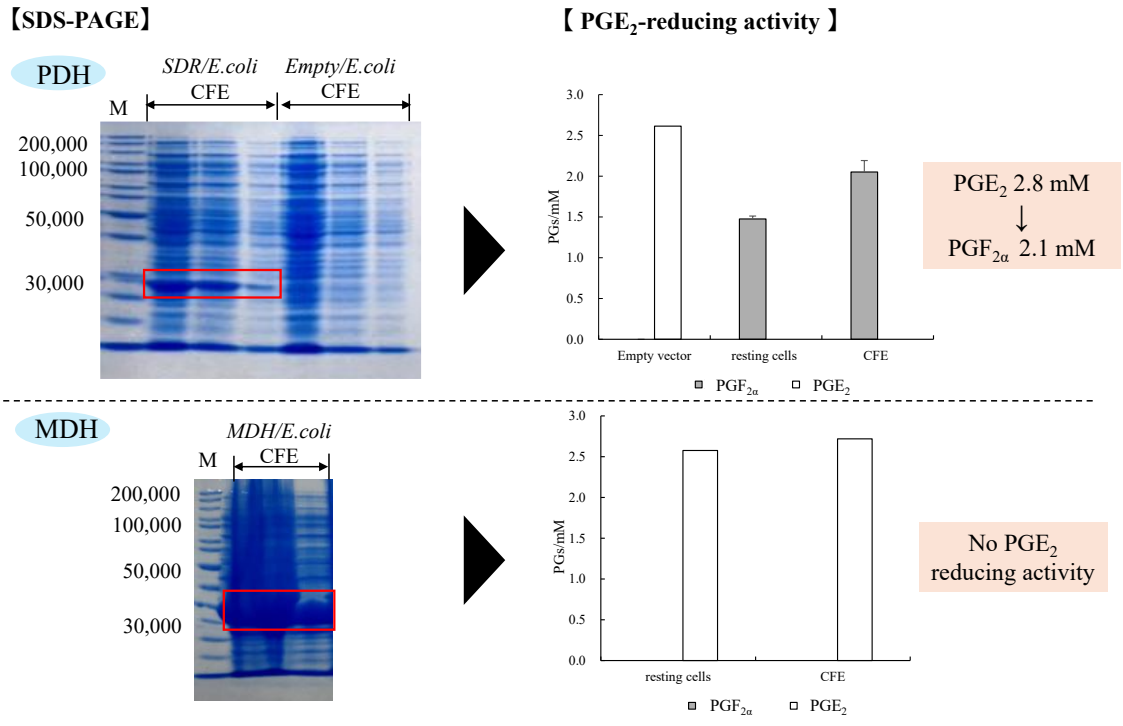


Fig. S2 SDS-PAGE analysis of cell-free extracts of the recombinant *E. coli* BL21 (DE3) expressing PDH gene and MDH gene. M: PageRuler Unstained Ptotein Ladder (thermo scientific, MA, USA). The uncropped SDS-PAGE gels are shown in Fig. S6.

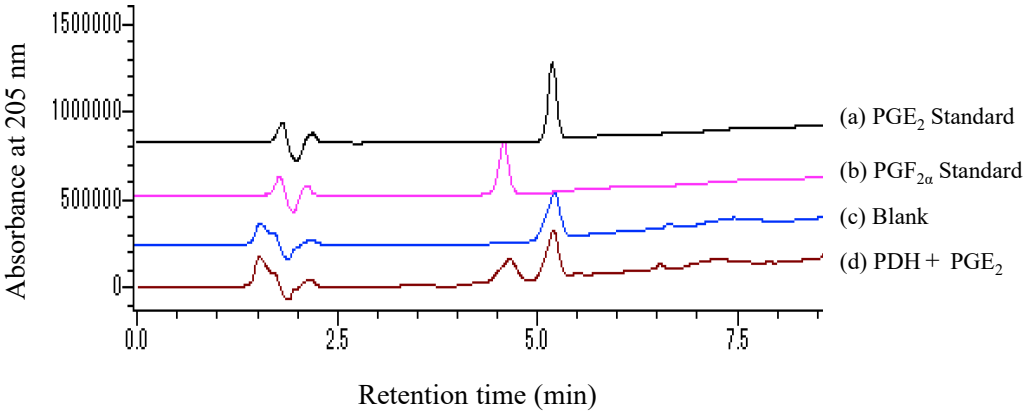


Fig. S3 HPLC chromatograms of enzymatic reaction of PDH. The chromatograms showed PGE_2 standard (**a**), $PGF_{2\alpha}$ standard (**b**), reaction solution without PDH (c), and reaction solution containing PDH (**d**). The reaction solution consisted of 20 mM KPB (pH 8), 5 mM PGE_2, 2 mM NADPH, 100 mM glucose, and 15 U/mL GDH, and the reaction was performed at 28 °C for 2 h with shaking at 200 rpm.

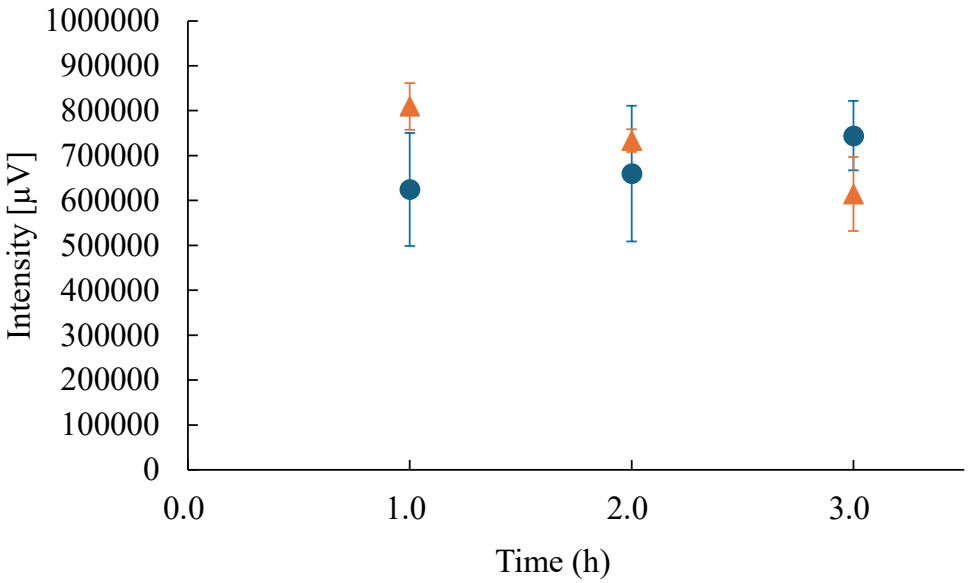
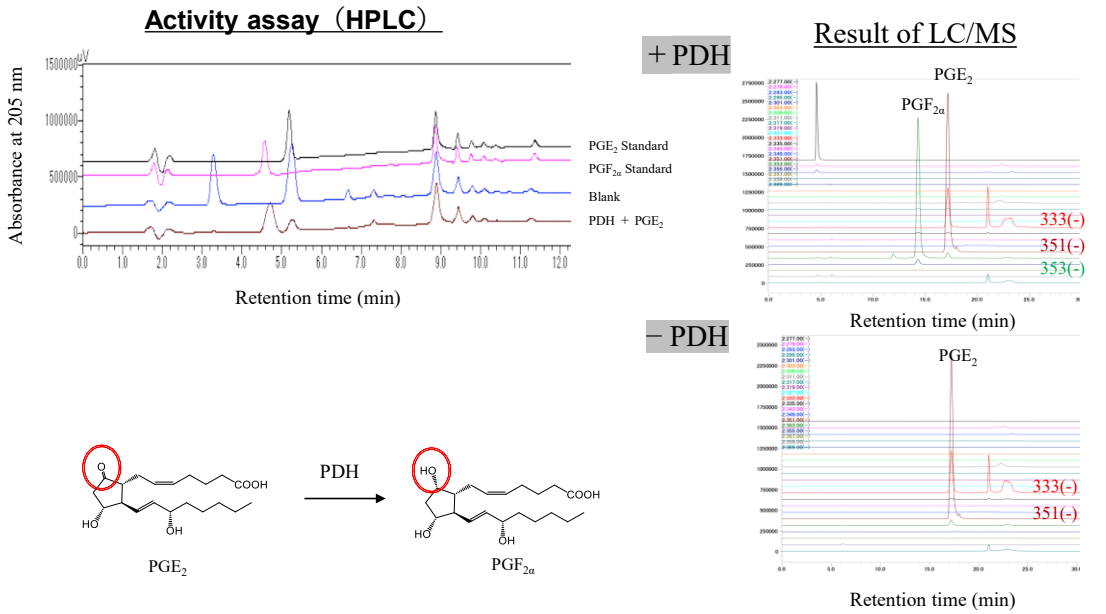
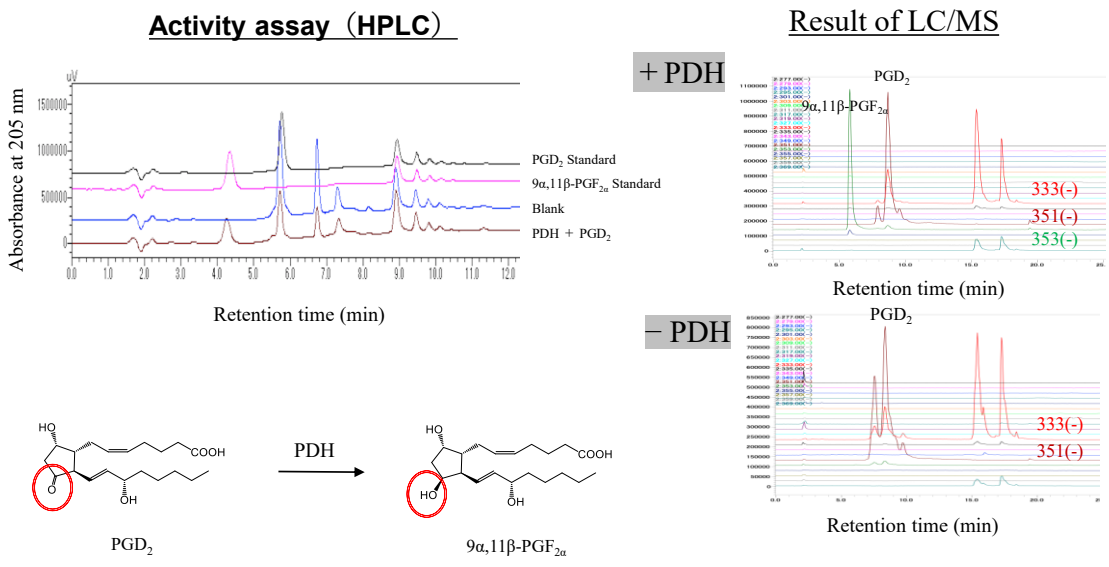


Fig. S4 The degradability of PGE_2. Blue circles and orange triangles represent KPB (pH 8) and Tris-HCl buffer (pH 9.5), respectively. Each value represents the mean ± standard deviation of three independent experiments.

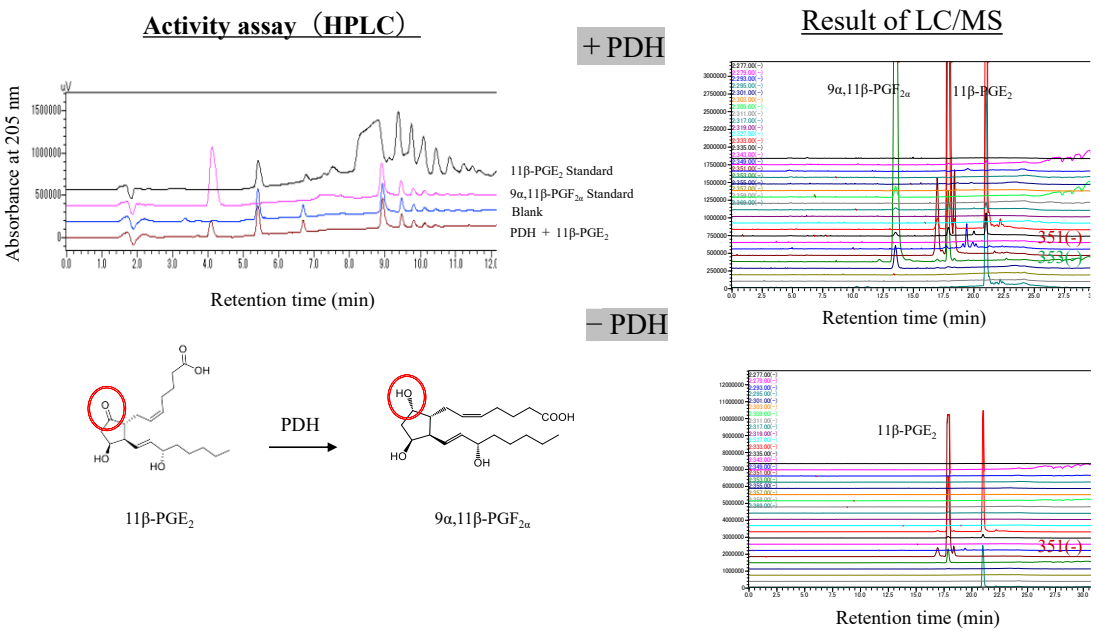
a) PGE_2


Activity assay（HPLC）
Absorbance at 205 nm
Retention time (min)
PGE2 Standard
PGF2α Standard
Blank
PDH + PGE2
+ PDH
Result of LC/MS
PGE2
PGF2α
333(-)
351(-)
353(-)
Retention time (min)
− PDH
PGE2
333(-)
351(-)
Retention time (min)
PDH
PGE2
PGF2α

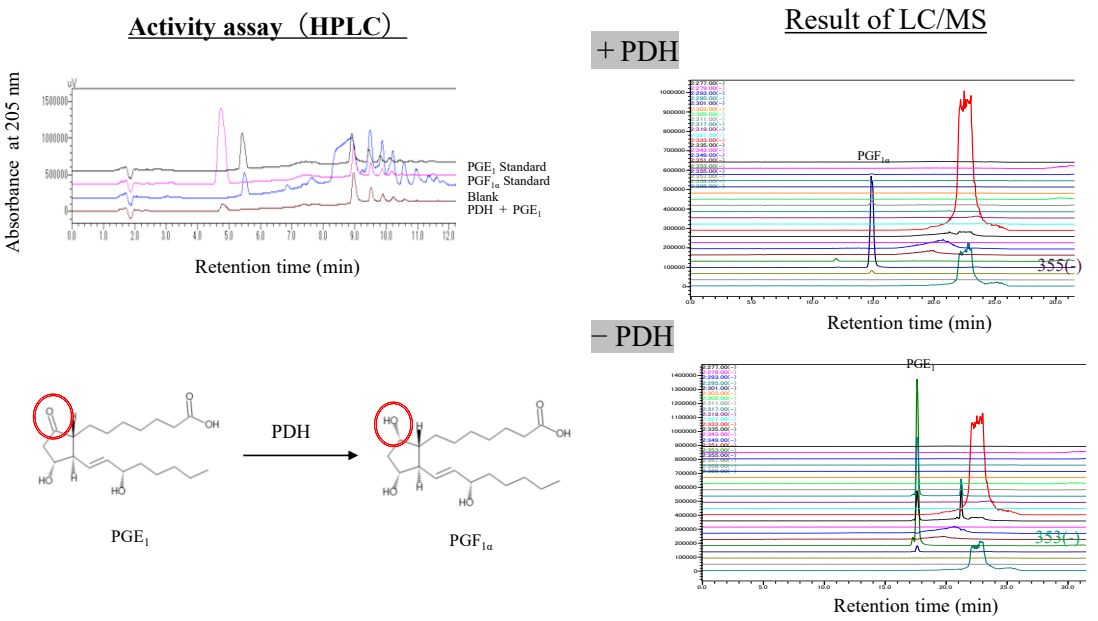
b) PGD_2


Activity assay（HPLC）
Absorbance at 205 nm
Retention time (min)
PGD2 Standard
9α,11β-PGF2α Standard
Blank
PDH + PGD2
+ PDH
Result of LC/MS
PGD2
9α,11β-PGF2α
333(-)
351(-)
353(-)
Retention time (min)
− PDH
PGD2
333(-)
351(-)
Retention time (min)
PDH
PGD2
9α,11β-PGF2α

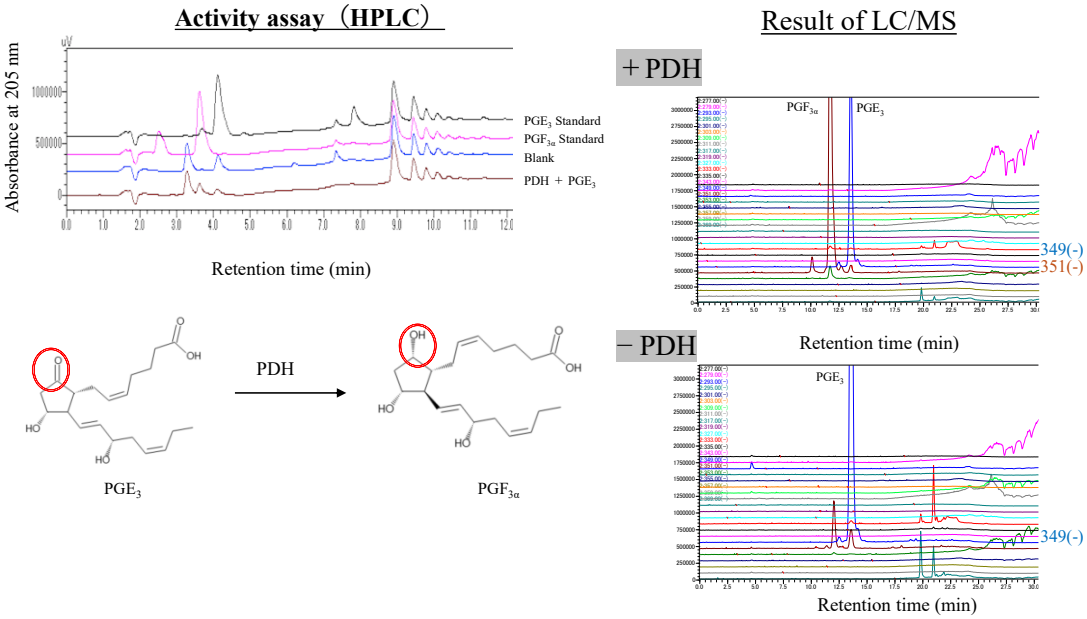
c) 11β-PGE_2



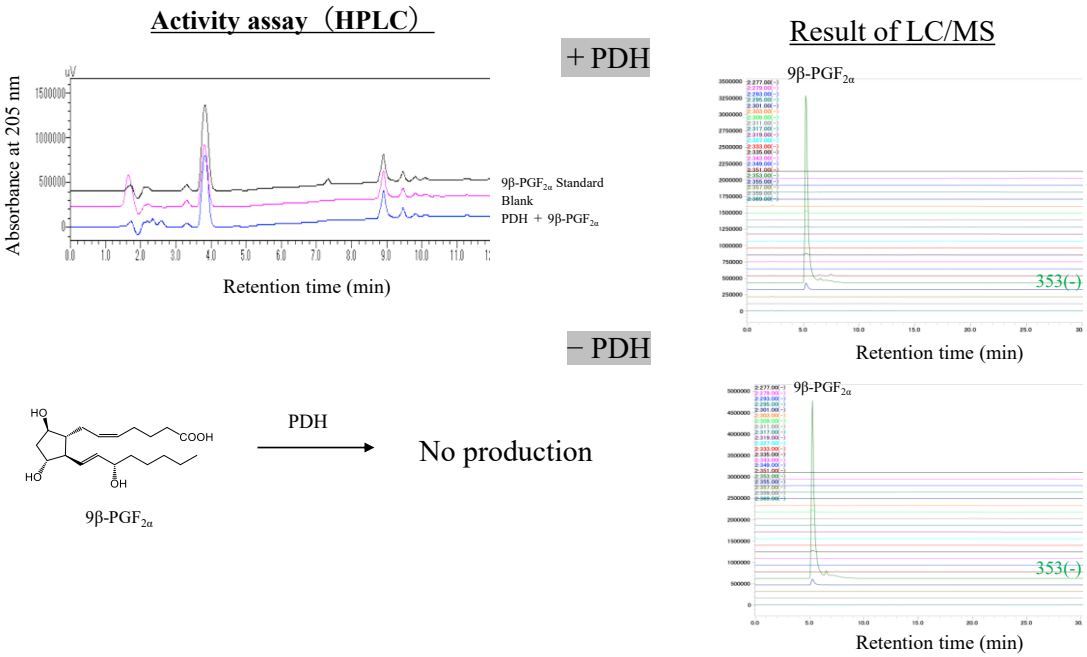
d) PGE_1



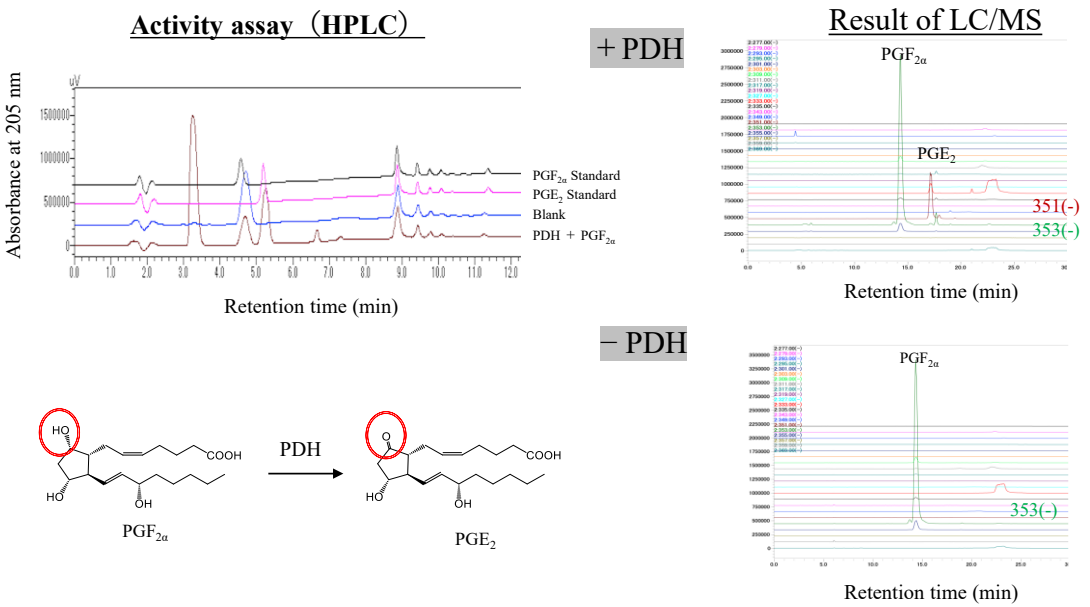
e) PGE_3



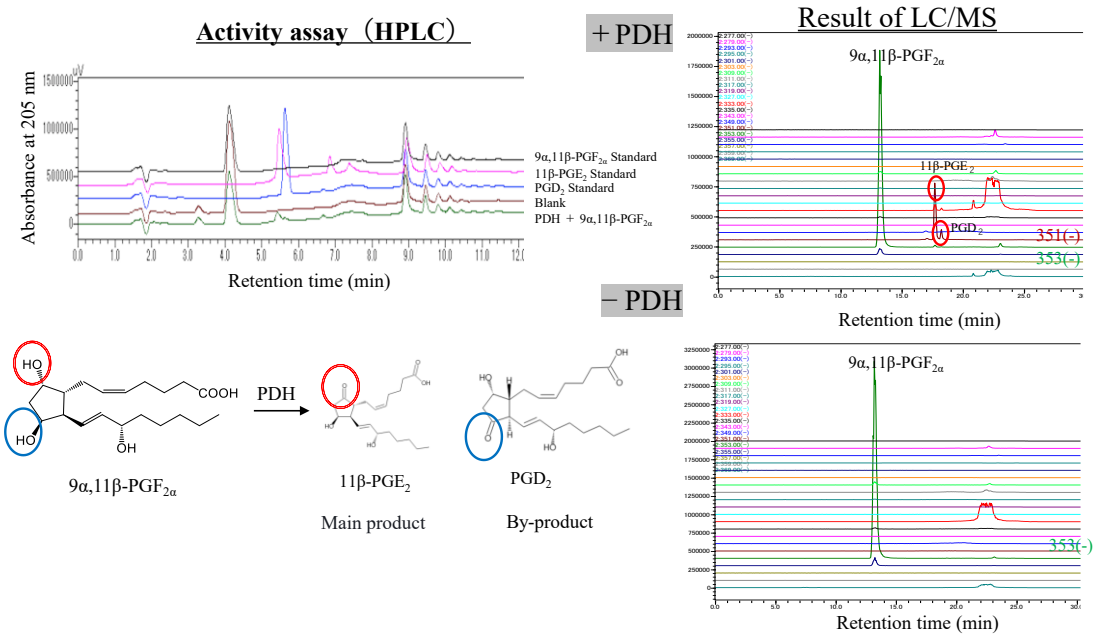
f) $PGF_{2\beta}$



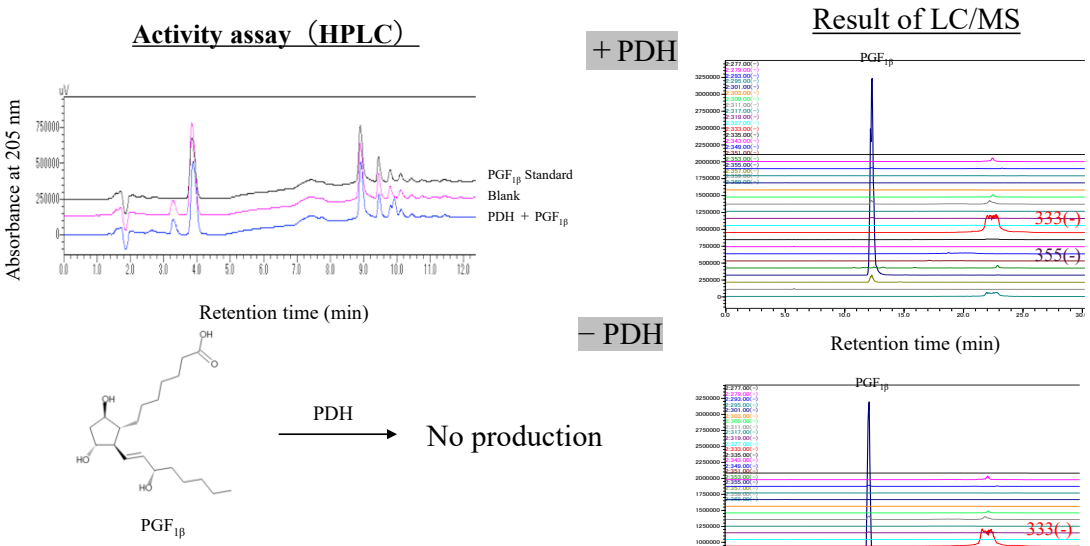
g) $PGF_{2\alpha}$



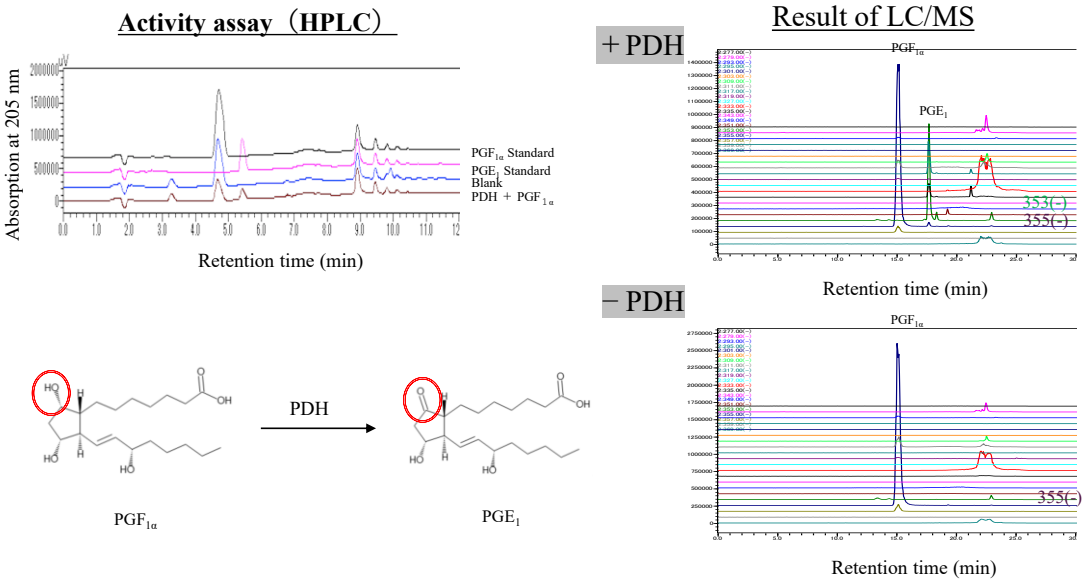
h) 9α,11β-$PGF_{2\alpha}$



i) $PGF_{1\beta}$



j) $PGF_{1\alpha}$



k) $PGF_{3\alpha}$

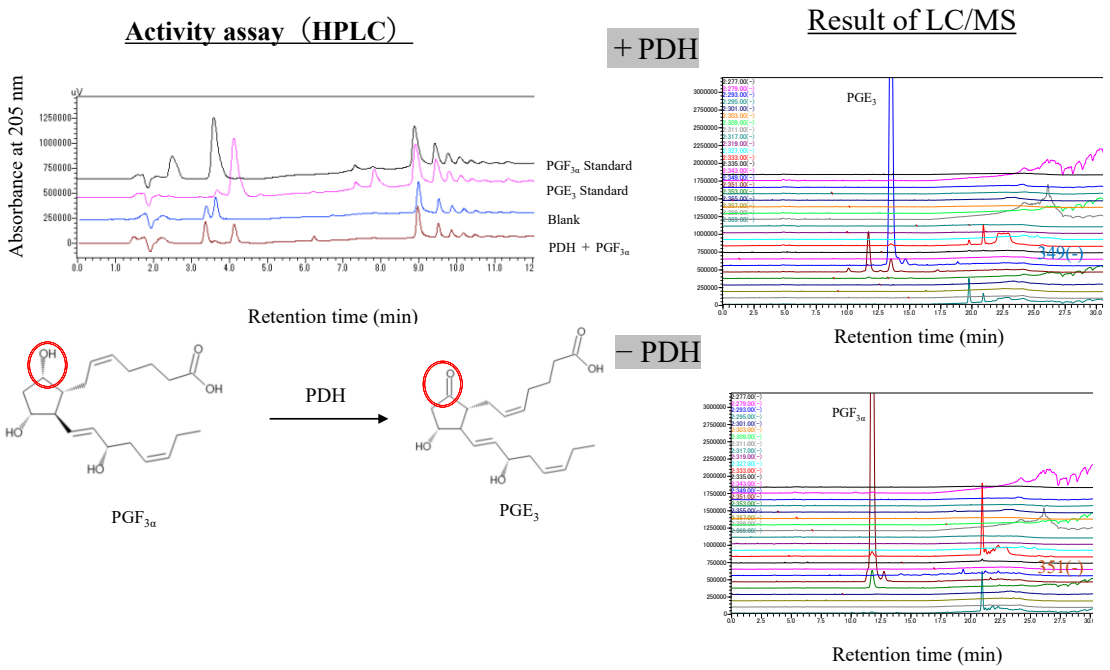
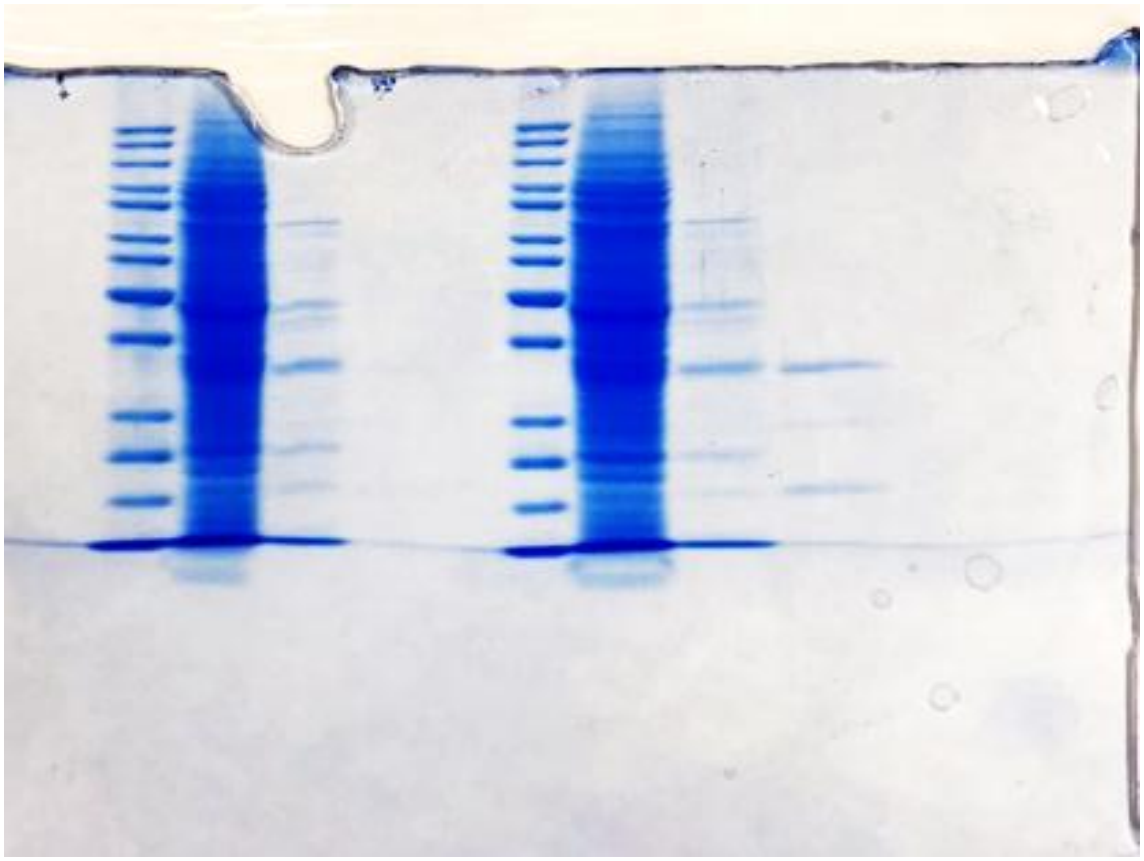


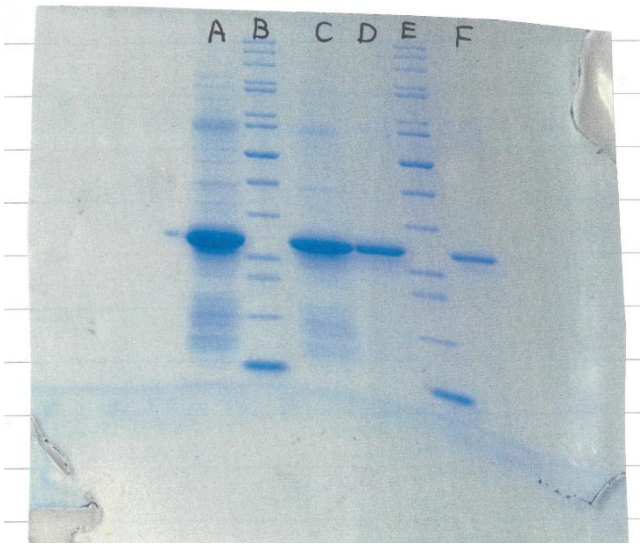
Fig. S5 HPLC chromatograms and LC-MS chromatograms of reduction products of a) PGE_2, b) PGD_2, c) 11β-PGE_2, d) PGE_1, and e) PGE_3 with PDH and dehydrogenation products of f) $PGF_{2\beta}$, g) $PGF_{2\alpha}$, h) 9α,11β-$PGF_{2\alpha}$, i) $PGF_{1\beta}$, j) $PGF_{1\alpha}$, and k) $PGF_{3\alpha}$ with PDH.

The substrate specificity of PDH was evaluated using enzyme reaction solutions. The reaction solution consisted of 7.2 μM (186 mg/L;26 U/L) PDH, 20 mM KPB (pH 8.0), 5 mM substrate, 100 mM glucose, 15 U/mL GDH, and 2 mM NADPH in the reduction reaction. The oxidation reaction consisted of 4.8 μM (= 124 mg/L (= 17 U/L) PDH, 20 mM KPB (pH 8.0), 5 mM substrate, and 5 mM $NADP^+$.

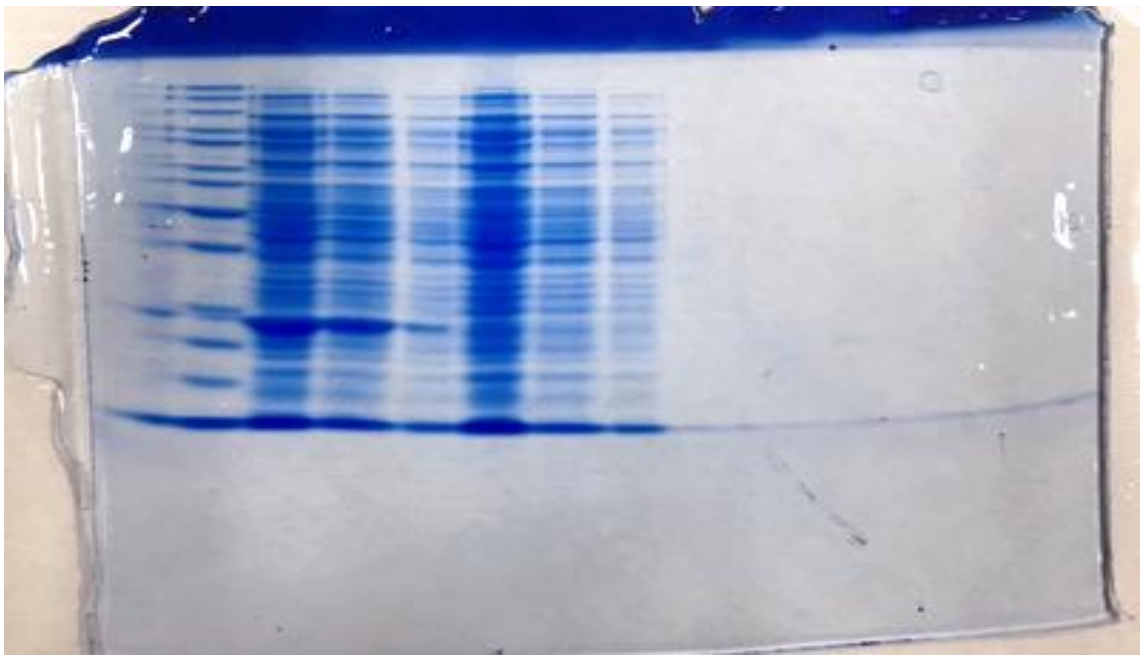
a)



b)



c)



d)

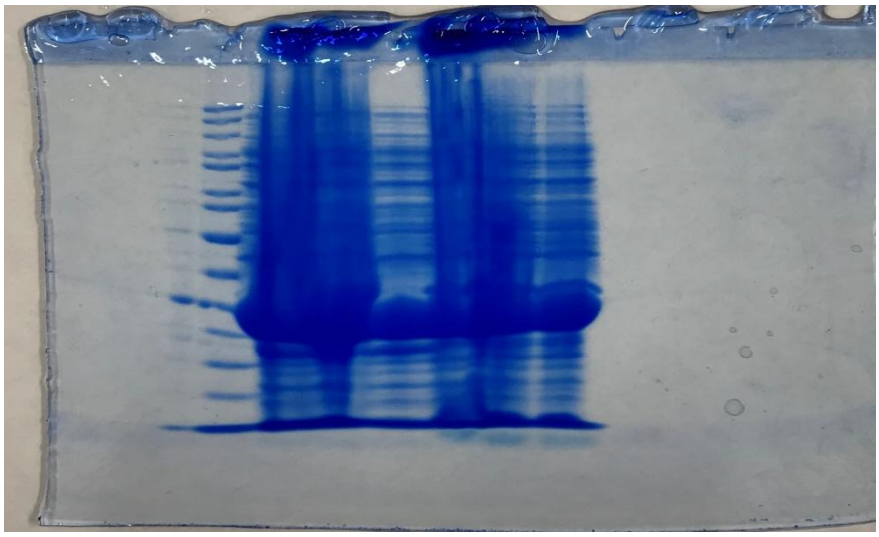
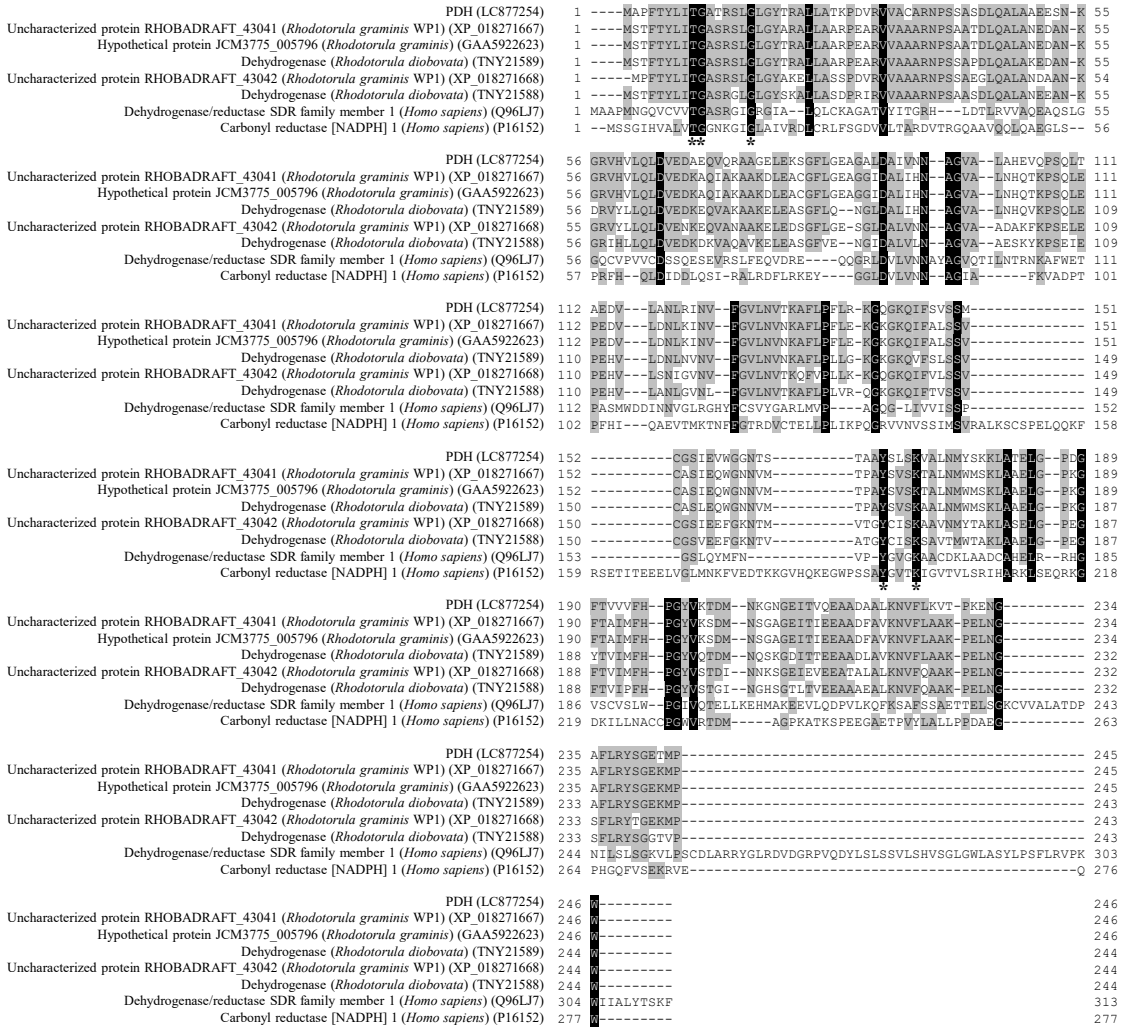


Fig. S6 The uncropped SDS-PAGE gels of the figures. a) Fig.2a), b) Fig. 2b), c) Fig. S2 PDH, d) Fig. S2 MDH

133 a)


PDH (LC877254)
Uncharacterized protein RHOBADRAFT_43041 (Rhodotorula graminis WP1) (XP_018271667)
Hypothetical protein JCM3775_005796 (Rhodotorula graminis) (GAA5922623)
Dehydrogenase (Rhodotorula diobovata) (TNY21589)
Uncharacterized protein RHOBADRAFT_43042 (Rhodotorula graminis WP1) (XP_018271668)
Dehydrogenase (Rhodotorula diobovata) (TNY21588)
Dehydrogenase/reductase SDR family member 1 (Homo sapiens) (Q96LJ7)
Carbonyl reductase [NADPH] 1 (Homo sapiens) (P16152)

134

135

b)

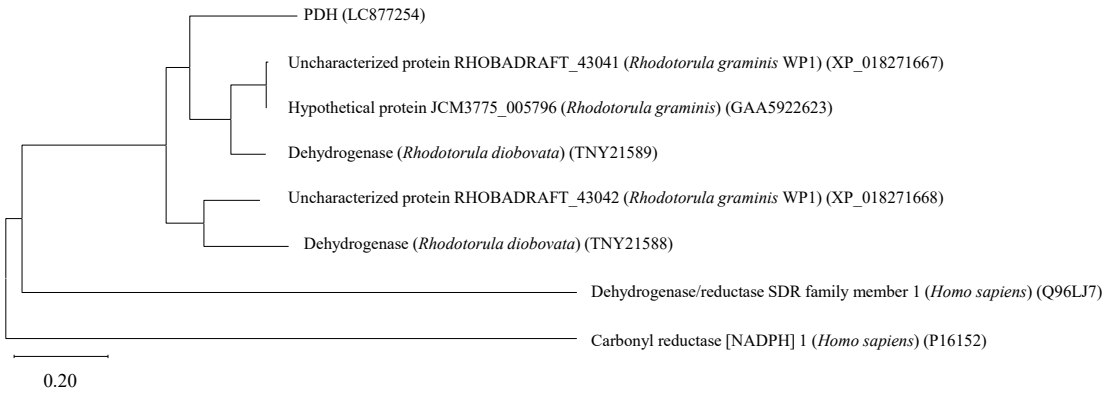


Fig. S7 Sequence analysis of PDH. a) Sequence alignment of PDH, selected sequences of similar proteins, human carbonyl reductase 1, and human dehydrogenase/reductase SDR family member 1. typical cofactor-binding motif TGXXX[AG]XG and an active-site motif YXXXK are shown by asterisks. Multiple sequence alignment was performed using the MUSCLE algorithm implemented in MEGA. The phosphate group of NADPH was predicted to bind to T12, R13, and R37 of PDH by AlphaFold 3 (Abramson 2024). b) Phylogenetic tree analysis by the maximum likelihood method.

Reference

Abramson J, Adler J, Dunger J, Evans R, Green T, Pritzel A, Ronneberger O, Willmore L, Ballard AJ, Bambrick J, Bodenstein SW, Evans DA, Hung CC, O'Neill M, Reiman D, Tunyasuvunakool K, Wu Z, Žemgulytė A, Arvaniti E, Beattie C, Bertolli O, Bridgland A, Cherepanov A, Congreve M, Cowen-Rivers AI, Cowie A, Figurnov M, Fuchs FB, Gladman H, Jain R, Khan YA, Low CMR, Perlin K, Potapenko A, Savy P, Singh S, Stecula A, Thillaisundaram A, Tong C, Yakneen S, Zhong ED, Zielinski M, Žídek A, Bapst V, Kohli P, Jaderberg M, Hassabis D, Jumper JM (2024) Accurate structure prediction of biomolecular interactions with AlphaFold 3. Nature 630:493–500. doi:10.1038/s41586-024-07487-w

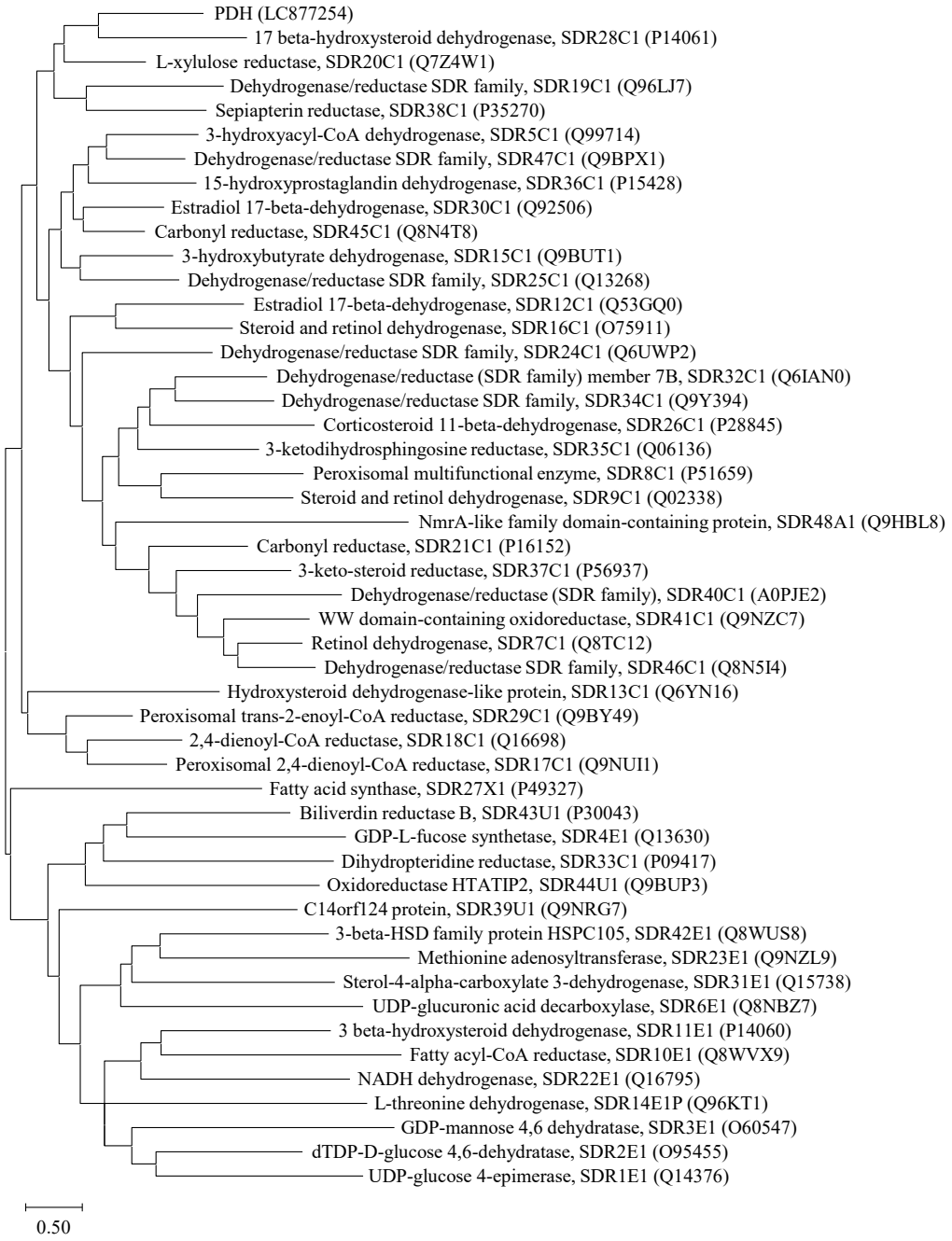


Fig. S8 Phylogenetic tree analysis of PDH and human 48 SDRs. PDH was compared with 48 kinds of SDR (Persson 2009). Multiple sequence alignment was performed using the MUSCLE algorithm implemented in MEGA. Phylogenetic analysis was performed using the maximum likelihood method.