

SUPPLEMENTAL DATA

Shifting the substrate scope of dimeric pyranose oxidase from monosaccharide to glycoside preference through oligomeric state modification

Anja Kostelac^{1,2#}, Enikő Hermann^{1,2,3#}, Clemens Peterbauer¹, Chris Oostenbrink^{3,4}, Dietmar Haltrich¹

#Shared first authorship

1 Department of Food Science and Technology, BOKU University, Vienna, Austria

2 Doctoral Programme BioToP - Biomolecular Technology of Proteins, BOKU University, Vienna, Austria

3 Department of Material Science and Life Sciences, BOKU University, Vienna, Austria

4 Christian Doppler Laboratory for Molecular Informatics in the Biosciences, BOKU University, Vienna, Austria

Abbreviations:

IMAC, immobilized metal affinity chromatography

KaPOx, pyranose oxidase from *Kitasatospora aureofaciens*

MtCarA, FAD-dependent C-glycoside 3-oxidase from *Microbacterium trichothecenolyticum*

PcPOx, pyranose oxidase from *Phanerochaete chrysosporium*

PsPOx, pyranose oxidase from *Pseudarthrobacter siccitolerans*

ScPOx, pyranose oxidase from *Streptomyces canus*

SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SEC, size exclusion chromatography

SEC-LS, Size-exclusion chromatography-light scattering

TmPOx, pyranose oxidase from *Trametes multicolor*

		10	20	30	40	50	60	
KaPOx	1	MI-----	-----	-----	-----	---TRYDRTL	VVGSGPVGAT	19
ScPOx	1	MT-----	-----	-----	-----	--HTPRTDVL	IVGSGIMGSL	20
KaPOx_xal	1	MI-----	-----	-----	-----	---TRYDRTL	VVGSGPVGAT	19
KaPOx_xalh	1	MI-----	-----	-----	-----	---TRYDRTL	VVGSGPVGAT	19
ScPOx_al	1	MT-----	-----	-----	-----	--HTPRTDVL	IVGSGIMGSL	20
ScPOx_alh	1	MT-----	-----	-----	-----	--HTPRTDVL	IVGSGIMGSL	20
PsPOx	1	MS-----	-----	-----	-----G	HRYPAAVDVA	IVGSGPTASA	23
MtCarA	1	MS-----	-----	-----	-----T	RVYPAQVDVA	IVGSGPAGAT	23
TmPOx	1	MSTSSSDPFF	NFAKSSFRSA	AAQKASASSL	PPLPGPDKKV	PGMDIKYDVV	IVGSGPIGCT	60

Oligomerization loop

		70	80	90	100	110	120	
KaPOx	20	FARTLVES--	GREVLMVDAG	AQLSP-RPGE	HLKNAYIYQH	NTNLFASIIR	GHLHLLSVPT	76
ScPOx	21	VARLLRRSDP	ALHITMADGG	SPIGG-VPGR	HLHDL-----	D-----	-----	55
KaPOx_xal	20	FARTLVES--	GREVLMVDAG	AQLSP-RPGE	HLKNAYIYQH	NT-----	-----	58
KaPOx_xalh	20	FARTLVES--	GREVLMVDAG	AQLSP-RPGE	HLKNAYIYQH	NT-----	-----	58
ScPOx_al	21	VARLLRRSDP	ALHITMADGG	SPIGG-VPGR	HLHDL-----	DDNLFASIIR	-HLHLLSVPT	73
ScPOx_alh	21	VARLLRRSDP	ALHITMADGG	SPIGG-VPGR	HLHDL-----	DDNLFASIIR	-HLHLLSVPT	73
PsPOx	24	YARILSEAP	GATIAMFEVG	PTVSN-PPGA	HVKNI--EDP	DS-----R	SLAQRASEGP	73
MtCarA	24	YARILSERAS	SATIAMFEVG	PTVSD-PPGA	HVKNI--ADA	DE-----R	AHAQRRSEGP	73
TmPOx	61	YARELVGA--	GKVMAMFDIG	EIDSGLKIGA	HKKNTVEYQK	NIDKFNVIQ	GQLMSVSPVP	118

Arm domain

Insertion-1 domain

Insertion-1 domain

(corresponding to oligomerization loop)

(corresponding to arm domain)

		130	140	150	160	170	180	
KaPOx	77	SARAEALVDP	AAMAEELGSNR	SSARNAENPD	ODPYRNLSAA	AACYA-----	-----	121
ScPOx	55	-----P	DLWSRYNEKV	ATGIQGMVTG	AEVVRDVAGS	LPDLTPGMFH	ALAFGEDAEA	107
KaPOx_xal	58	-----P	DLWSRYNEKV	ATGIQGMVTG	AEVVRDVAGS	LPDLA-----	-----	94
KaPOx_xalh	58	-----P	DLWSRYNEKV	ATGIQGMVTG	AEVVRDVAGS	LPDLA-----	-----	94
ScPOx_al	74	SARAEALVDP	AAMAEELGSNR	SSARNAENPD	ODPYRNLSAA	AACYTPGMFH	ALAFGEDAEA	133
ScPOx_alh	74	SARAEALVDP	AAMAEELGSNR	SSARNAENPD	ODPYRNLSAA	AACYTPGMFH	ALAFGEDAEA	133
PsPOx	74	GAGAA---TV	NSPGAVKSGE	RRARPGTYLL	QDGYA-----	---FPGED--G	-----	111
MtCarA	74	HARED---DD	RVGGIVKSAQ	RRARPGTYLL	ESGYQ-----	---ADGED--G	-----	111
TmPOx	119	NT---LVVD	LSPTSWQAST	FFVRNGSNPE	QDPLRNLSGQ	AVTRV-----	-----	160

		190	200	210	220	230	240	
KaPOx	121	-----V	GGMGTHWTGA	TPR---HHP	VLERYDGISD	Q-EWDGLYGE	AERLLRV SAR	167
ScPOx	108	MPQAALAWNA	GGMGVHWTAA	TPW---PAG	D-EVFDFGDP	D-AWAADLDT	ARRLLAVTPA	161
KaPOx_xal	94	-----V	GGMGTHWTGA	TPR---HHP	VLERYDGISD	Q-EWDGLYGE	AERLLRV SAR	140
KaPOx_xalh	94	-----V	GGMGTHWTGA	TPR---HHP	VLERYDGISD	Q-EWDGLYGE	AERLLRV SAR	140
ScPOx_al	134	MPQAALAWNA	GGMGVHWTAA	TPW---PAG	D-EVFDFGDP	D-AWAADLDT	ARRLLAVTPA	187
ScPOx_alh	134	MPQAALAWNA	GGMGVHWTAA	TPW---PAG	D-EVFDFGDP	D-AWAADLDT	ARRLLAVTPA	187
PsPOx	112	MPVAAMSSNV	GGMAAHWTAA	CPR---PGG	K-ERIPFLP-	--DLEELLND	ADRLLGVTTH	163
MtCarA	112	LPVAAFSSNV	GGMAAHWTGA	CPR---PND	S-ERIGFLDE	TGELDELLSE	GERLLGVTTD	166
TmPOx	160	-----V	GGMSTHWTCA	TPRFDRQRP	LLVKDDADAD	DAEWDRLYTK	AESYFQTGTD	211

		250	260	270	280	290	300	
KaPOx	168	EFDIFSIRQHL	VTEALRREFS	EL-PDGYQVQ	SLPLAARRRR	DNPRMVHWTG	VDTVLG----	222
ScPOx	162	PIGPTKV GEL	VLDVLR RRYG	GTGPADRAPQ	PMPMAVTPTP	SGP--MPRTA	PGTIFP----	215
KaPOx_xal	141	EFDIFSIRQHL	VTEALRREFS	EL-PDGYQVQ	SLPLAARRRR	DNPRMVHWTG	VDTVLG----	195
KaPOx_xalh	141	EFDIFSIRQHL	VTEALRREFS	EL-PDGYQVQ	SLPLAARRRR	DNPRMVHWTG	VDTVLG----	195
ScPOx_al	188	PIGPTKV GEL	VLDVLR RRYG	GTGPADRAPQ	PMPMAVTPTP	SGP--MPRTA	PGTIFP----	241
ScPOx_alh	188	PIGPTKV GEL	VLDVLR RRYG	GTGPADRAPQ	PMPMAVTPTP	SGP--MPRTA	PGTIFP----	241
PsPOx	164	AFDGAFFSDL	VRERLAAVVD	QGRTPAFRVQ	PMPPLAVHRRQ	DGA--LVWSG	SDVVMG----	217
MtCarA	167	AFDASPYAGI	VRERLAAVED	AHRDADERVQ	RMPLAVHRRD	DGP--LVWSG	SDVVLG----	220
TmPOx	212	QFKESIRHNL	VLNKLTEEYK	GQ----RDFQ	QIPLAATRR-	-SPTFVWESS	ANTVFDLQNR	265

		310	320	330	340	350	360	
KaPOx	223	DLAD-GHPLF	SLLPQHLCTR	LVLDRDGTRI	AYA EVRDLNR	SETVRVVADN	YVVAAGAVLA	281
ScPOx	216	PLAQGGDPAF	TLTGTGLVTA	LV--RDAGR	TGARLRRVAD	GTESELSADT	VVVCADALRT	273
KaPOx_xal	196	DLAD-GHPLF	SLLPQHLCTR	LVLDRDGTRI	AYA EVRDLNR	SETVRVVADN	YVVAAGAVLA	254
KaPOx_xalh	196	DLAD-GHPLF	SLLPQHLCTR	LVLDRDGTRI	AYA EVRDLNR	SETVRVVADN	YVVAAGAVLA	254
ScPOx_al	242	PLAQGGDPAF	TLTGTGLVTA	LV--RDAGR	TGARLRRVAD	GTESELSADT	VVVCADALRT	299
ScPOx_alh	242	PLAQGGDPAF	TLTGTGLVTA	LV--RDAGR	TGARLRRVAD	GTESELSADT	VVVCADALRT	299
PsPOx	218	EATR-DNPQF	ELFDESLVTR	VL--VEDGTA	AGVEVQDRRS	GDTYQVAARY	VVVGADALRT	274
MtCarA	221	DITR-GNPNF	TLFDESLVTR	VL--VEDGTA	AGVVVTDVTR	GERRDVRRARF	VVVAADALRT	277
TmPOx	266	PNTDAPEERF	NLFPAVACER	VVRNALNSEI	ESLHIHDLIS	GDRFEIKADV	YVLTAGAVHN	325

		Head domain									
		Barrel shaped bottom									
		370	380	390	400	410	420				
											
KaPOx	282	PQLLHASGI-	---RP----	---AALGRY	LTEHPMAFCQ	VILLKDLVEQ	ARTDQRFGG-	327			
ScPOx	274	PQLLYASGI-	---RP----	---EALGRH	LNEHAFVTAR	VLLDLD----	-----RFG--	309			
KaPOx_xal	255	PQLLHASGI-	---RP----	---AALGRY	LTEHPMAFCQ	VILLKDLVEQ	ARTDQRFGG-	300			
KaPOx_xalh	255	PQLLHASGI-	---RP----	---AALGRY	LTEHPMAFCQ	VILDLD----	-----RFG--	290			
ScPOx_al	300	PQLLYASGI-	---RP----	---EALGRH	LNEHAFVTAR	VLLDLD----	-----RFG--	335			
ScPOx_alh	300	PQLLYASGI-	---RP----	---EALGRH	LNEHAFVTAR	VLLLKDLVEQ	ARTDQRFGG-	345			
PsPOx	275	PQLLWASGI-	---RP----	---DALGRY	LNDQAQVFA	SRL-RDVQP-	EDAPAAANGA	319			
MtCarA	278	PQLLWASGI-	---RP----	---DALGRY	LNDQAQIVFA	VRM-RDFTPV	VDADGVPQTG	323			
TmPOx	326	TQLLVNSGFG	QLGRPNPANP	PELLPSLSGY	ITEQSLVFCQ	TVMSTELIDS	VKSDMTIRGT	385			
		430	440	450	460	470	480				
											
KaPOx	327	-----	-----	-----QVARH	TTLFPDD-DL	PIE	VDDPEPN	VWIPVS-EGR	360		
ScPOx	309	-----	-----	-----	LDPDALPL	PRPGEFSTDS	LWLPCNGPSQ	337			
KaPOx_xal	300	-----	-----	-----QVARH	TTLFPDD-DL	PIPVDDPEPN	VWIPVS-EGR	333			
KaPOx_xalh	290	-----	-----	-----	LDPDALPL	PRPVDDPEPN	VWIPVS-EGR	317			
ScPOx_al	335	-----	-----	-----	LDPDALPL	PRPGEFSTDS	LWLPCNGPSQ	363			
ScPOx_alh	345	-----	-----	-----QVARH	TTLFPDD-DL	PIPGEFSTDS	LWLPCNGPSQ	379			
PsPOx	319	-----	-----	-----	-----	LSEQSGV	AWVPYT-DEA	335			
MtCarA	323	-----	-----	-----	-----	LSEYTG	TWVPFT-DM	339			
TmPOx	386	PGELTYSVTY	TPGASTNKHP	DWWNEKVKNH	MMQHQED-PL	PIPFEDPEPQ	VTTLFQ-PSH	443			
		Substrate loop									
		490	500	510	520	530	540				
											
KaPOx	361	PWHAQITR--	DAFHYGDVP	PHVDGRLIVD	LRWFGIVEPR	PDNRVTFSDT	RTDVMGMPQP	417			
ScPOx	338	PFHGQIMN--	RTYVDGAGR	PLAHS---VG	LSLYVPVESR	PQNRLVFSPG	ETDLAGLPRI	391			
KaPOx_xal	334	PWHAQITR--	DAFHYGDVP	PHVDGRLIVD	LRWFGIVEPR	PDNRVTFSDT	RTDVMGMPQP	390			
KaPOx_xalh	318	PWHAQITR--	DAFHYGDVP	PHVDGRLIVD	LRWFGIVEPR	PDNRVTFSDT	RTDVMGMPQP	374			
ScPOx_al	364	PFHGQIMN--	RTYVDGAGR	PLAHS---VG	LSLYVPVESR	PQNRLVFSPG	ETDLAGLPRI	417			
ScPOx_alh	380	PFHGQIMN--	RTYVDGAGR	PLAHS---VG	LSLYVPVESR	PQNRLVFSPG	ETDLAGLPRI	433			
PsPOx	336	PFHGQIMQLD	ASPVPLADDD	PIVPGS-IVG	LGLFCAKDLQ	REDRVAFDD	TRDSYGLPAM	394			
MtCarA	340	PFHGQVMQLD	ASPVKLADDD	PAAPGS-IVG	LGLFCAKDLQ	ASDRVAFSDS	DVDGYGMPAM	398			
TmPOx	444	PWHTQIHR--	DAFSYGAVQ	QSIDSRILIVD	WRFGRTEPK	EENKLWFSK	ITDAYNMPQP	500			
		Catalytic His									
		550	560	570	580	590	600				
											
KaPOx	418	TFEYAL-SPQ	DAERQHAMMA	EMMRAATALG	GFLPGSEPRF	TAPGLPIHIA	GTIRMG-DDP	475			
ScPOx	392	RVEFGY-SET	DRALIRRALD	EVRSVAEEFG	PFDPAESTV	LPPGSSIHILT	GTVRAGVTDD	450			
KaPOx_xal	391	TFEYAL-SPQ	DAERQHAMMA	EMMRAATALG	GFLPGSEPRF	TAPGLPIHIA	GTIRMG-DDP	448			
KaPOx_xalh	375	TFEYAL-SPQ	DAERQHAMMA	EMMRAATALG	GFLPGSEPRF	TAPGLPIHIA	GTIRMG-DDP	432			
ScPOx_al	418	RVEFGY-SET	DRALIRRALD	EVRSVAEEFG	PFDPAESTV	LPPGSSIHILT	GTVRAGVTDD	476			
ScPOx_alh	434	RVEFGY-SET	DRALIRRALD	EVRSVAEEFG	PFDPAESTV	LPPGSSIHILT	GTVRAGVTDD	492			
PsPOx	395	RIHYRL-TER	DHVVLDRARQ	EIVRLGKAVG	EPL-DERPFV	LPPGASIHLYQ	GTTTMRGETDD	452			
MtCarA	399	QLHYTL-SDR	DHATIDRAKA	EIVRLGKAIG	DPL-DDRPFV	MPLGASIHLYQ	GTVRMGLADD	456			
TmPOx	501	TFDFRFPAGR	TSKEAEDMMT	DMCVMSAKIG	GFLPGSLPQF	MEPGLVILHL	GTHRMG-FDE	559			
		Catalytic Asn									
		610	620	630	640	650	660				
											
KaPOx	476	Q--SSVVDTD	SRVWGLENLY	LGGNGVIPTG	TACNPTLTSTV	AMALKAAHHL	AG-----S	526			
ScPOx	451	G--TGVCDDP	GRVWGFDNLY	LAGNGVVPTP	MAANVTLTGA	VTAVRTARAV	TA-----R	501			
KaPOx_xal	449	Q--SSVVDTD	SRVWGLENLY	LGGNGVIPTG	TACNPTLTSTV	AMALKAAHHL	AG-----S	499			
KaPOx_xalh	433	Q--SSVVDTD	SRVWGLENLY	LGGNGVIPTG	TACNPTLTSTV	AMALKAAHHL	AG-----S	483			
ScPOx_al	477	G--TGVCDDP	GRVWGFDNLY	LAGNGVVPTP	MAANVTLTGA	VTAVRTARAV	TA-----R	527			
ScPOx_alh	493	G--TGVCDDP	GRVWGFDNLY	LAGNGVVPTP	MAANVTLTGA	VTAVRTARAV	TA-----R	543			
PsPOx	453	G--ESVCSPD	SQVWQVPLGF	VAGNGVIPTA	TACNPTLTSTV	ALAVRGARKI	AEETSSLLM	510			
MtCarA	457	G--ASVCSPD	SEVWGAPGLF	VAGNGVIPTA	TACNPTLTGV	ALAVRGARHI	ADEITADL--	512			
TmPOx	560	KEDNCCVNTD	SRVFGFKNLF	LGGCGNIPTA	YGANPTLTAM	SLAIKSCYI	KQNFTPSFFT	619			
		670									
											
KaPOx	527	REARERRRTG	ADEVLAVERS	545							
ScPOx	502	T-----	-----	502							
KaPOx_xal	500	REARERRRTG	ADEVLAVERS	518							
KaPOx_xalh	484	REARERRRTG	ADEVLAVERS	502							
ScPOx_al	528	T-----	-----	528							
ScPOx_alh	544	T-----	-----	544							
PsPOx	511	SESDNR----	-----LSK	519							
MtCarA	512	-----	-----	512							
TmPOx	620	SEAQ-----	-----	623							

Figure S1. Multiple sequence alignment of the bacterial dimeric *KaPOx* (UniProt AoA1E7NAU4) [12], monomeric *ScPOx* (UniProt AoA117Q443) [11], *PsPOx* (UniProt AoAo24H8G7)[14] and *MtCarA* (UniProt AoAoM2HFA3) [10] and fungal tetrameric *TmPOx* (UniProt Q7ZA32)[18]. Mutants designed in this study are included in the alignment (*KaPOx_xal*, *KaPOx_xalh*, *ScPOx_al*, *ScPOx_alh*). Important structural and catalytical features such as oligomerization motifs, substrate loop and catalytic dyad are annotated with boxes. The amino acid region T367-L384 (*KaPOx* numbering) and the glucose binding residues D369 and Y373 are highlighter with yellow and red colour, respectively in both *KaPOx* and *KaPOx_xalh*.

Table S1. Amino acid sequences of wild type enzymes *KaPOx* and *ScPOx* and variants *KaPOx_xal*, *KaPOx_xalh*, *ScPOx_al* and *ScPOx_alh*. Structurally important segments and motifs are labelled as following: oligomerization loop, arm domain, head domain, insertion-1 domain and barrel shaped bottom.

Protein	Amino acid sequence
<i>KaPOx</i>	MITRYDTLVVSGSPVGATFARTLVESGREVLMVDAGAQLSPRPGEHLKNAYIQHNTNLFASIIRGHLHLLSVPTSAEALAVDPAAMAEELGSNRSSARNAENPDQDPYRNLAAAAACYAVGGMGTHWTGATPRHHPVLERYDGISDQEWGDLGYGEAERLLRVSAREFDFSIRQHLVTEALRREFSELPDGYQVQSLPLAARRRRDNPRMVHWTGVDTVLGDLAGHPLFSLLPQHLCRTLVLDRDGTIRIAYAEVRDLNRSETVRVADNYVVAAGAVLAPQLLHAGSIRPAALGRYLTEHPMAFCQVILKDLVEQARTDQRFGGQVARHTTLFPDDDLPIPVDDPEPNVWIPVSEGRPWHAQITRDAFHYGDVPPHVDGRLIVDLRWFGIVEPRPDNRVTFSDTRTDVMGMGPQPTFEYALSPQDAERQHAMMAEMMRAATALGGFLPGSEPRFTAPGLPLHIAGTIRMGDDPQSSVVDTSRVWGLENLVGGNGVIPTGTACNPTLTSVAMALKAHHLAGSREARERRRTGADEVLAVERS
<i>ScPOx</i>	MHTHPRDVLIVGSGIMGSLVARLLRRSDPALHITMADGGSPIGGVPRHLHDLDDPDLWSRYNEKVATGIQGMYTGAEEVVDVAGSLPDLTPGMFHALAFGEDAEAMPQAAALAWNAGGMGVHWTAAATPWPAGDEVDFGDPDAWAADLDTARRLLAVTPAPIGPTKVGEVLVDLVRRLRYGGTGPADRAPQPMMAVTPTPSGMPMPRTAPGTIFPPLAQGGDPAFTLLTGTLVTALVRDAGRVTGARLRRVADGTESELSADTVVVCADALRTPQLLYASGIRPEALGRHLNEHAFVTVARVLDLDRFGLDPDALPLPRPGEFSTDLSLWPCNGSPQPFHGQIMNRTYVDGAGRPLAHSVGLSLYVPVESRPQNRVSPGETDLAGLPRIRVEFGYSETDRALIRRALDEVRSVAEEFGPDPATESTVLPPGSSSLHLTGTVRAGVTDDGTGVCDDPGRVWGFNDLYLAGNGVVPTPMAANVTLTGAVTAVRTARAVTART
<i>KaPOx_xal</i>	MITRYDTLVVSGSPVGATFARTLVESGREVLMVDAGAQLSPRPGEHLKNAYIQHNTPDLWSRYNEKVATGIQGMYTGAEEVVRDVGSLPDLAVGGMGTHWTGATPRHHPVLERYDGISDQEWGDLGYGEAERLLRVSAREFDFSIRQHLVTEALRREFSELPDGYQVQSLPLAARRRRDNPRMVHWTGVDTVLGDLAGHPLFSLLPQHLCRTLVLDRDGTIRIAYAEVRDLNRSETVRVADNYVVAAGAVLAPQLLHAGSIRPAALGRYLTEHPMAFCQVILKDLVEQARTDQRFGGQVARHTTLFPDDDLPIPVDDPEPNVWIPVSEGRPWHAQITRDAFHYGDVPPHVDGRLIVDLRWFGIVEPRPDNRVTFSDTRTDVMGMGPQPTFEYALSPQDAERQHAMMAEMMRAATALGGFLPGSEPRFTAPGLPLHIAGTIRMGDDPQSSVVDTSRVWGLENLVGGNGVIPTGTACNPTLTSVAMALKAHHLAGSREARERRRTGADEVLAVERS
<i>KaPOx_xalh</i>	MITRYDTLVVSGSPVGATFARTLVESGREVLMVDAGAQLSPRPGEHLKNAYIQHNTPDLWSRYNEKVATGIQGMYTGAEEVVRDVGSLPDLAVGGMGTHWTGATPRHHPVLERYDGISDQEWGDLGYGEAERLLRVSAREFDFSIRQHLVTEALRREFSELPDGYQVQSLPLAARRRRDNPRMVHWTGVDTVLGDLAGHPLFSLLPQHLCRTLVLDRDGTIRIAYAEVRDLNRSETVRVADNYVVAAGAVLAPQLLHAGSIRPAALGRYLTEHPMAFCQVILKDLVEQARTDQRFGGQVARHTTLFPDDDLPIPVDDPEPNVWIPVSEGRPWHAQITRDAFHYGDVPPHVDGRLIVDLRWFGIVEPRPDNRVTFSDTRTDVMGMGPQPTFEYALSPQDAERQHAMMAEMMRAATALGGFLPGSEPRFTAPGLPLHIAGTIRMGDDPQSSVVDTSRVWGLENLVGGNGVIPTGTACNPTLTSVAMALKAHHLAGSREARERRRTGADEVLAVERS
<i>ScPOx_al</i>	MHTHPRDVLIVGSGIMGSLVARLLRRSDPALHITMADGGSPIGGVPRHLHDLDDNLFASIIRGHLHLLSVPTSAEALAVDPAAMAEELGSNRSSARNAENPDQDPYRNLAAAAACYTPGMFHALAFGEDAEAMPQAAALAWNAGGMGVHWTAAATPWPAGDEVDFGDPDAWAADLDTARRLLAVTPAPIGPTKVGEVLVDLVRRLRYGGTGPADRAPQPMMAVTPTPSGMPMPRTAPGTIFPPLAQGGDPAFTLLTGTLVTALVRDAGRVTGARLRRVADGTESELSADTVVVCADALRTPQLLYASGIRPEALGRHLNEHAFVTVARVLDLDRFGLDPDALPLPRPGEFSTDLSLWPCNGSPQPFHGQIMNRTYVDGAGRPLAHSVGLSLYVPVESRPQNRVSPGETDLAGLPRIRVEFGYSETDRALIRRALDEVRSVAEEFGPDPATESTVLPPGSSSLHLTGTVRAGVTDDGTGVCDDPGRVWGFNDLYLAGNGVVPTPMAANVTLTGAVTAVRTARAVTART
<i>ScPOx_alh</i>	MHTHPRDVLIVGSGIMGSLVARLLRRSDPALHITMADGGSPIGGVPRHLHDLDDNLFASIIRGHLHLLSVPTSAEALAVDPAAMAEELGSNRSSARNAENPDQDPYRNLAAAAACYTPGMFHALAFGEDAEAMPQAAALAWNAGGMGVHWTAAATPWPAGDEVDFGDPDAWAADLDTARRLLAVTPAPIGPTKVGEVLVDLVRRLRYGGTGPADRAPQPMMAVTPTPSGMPMPRTAPGTIFPPLAQGGDPAFTLLTGTLVTALVRDAGRVTGARLRRVADGTESELSADTVVVCADALRTPQLLYASGIRPEALGRHLNEHAFVTVARVLDLDRFGLDPDALPLPRPGEFSTDLSLWPCNGSPQPFHGQIMNRTYVDGAGRPLAHSVGLSLYVPVESRPQNRVSPGETDLAGLPRIRVEFGYSETDRALIRRALDEVRSVAEEFGPDPATESTVLPPGSSSLHLTGTVRAGVTDDGTGVCDDPGRVWGFNDLYLAGNGVVPTPMAANVTLTGAVTAVRTARAVTART

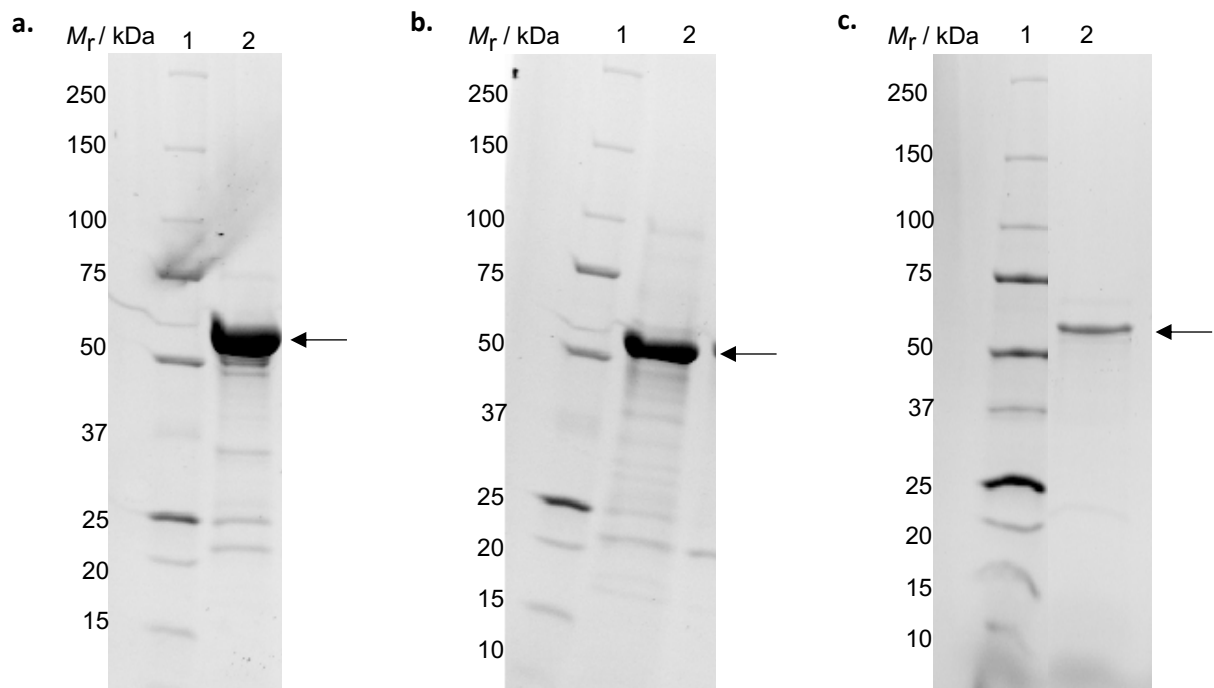


Figure S2. SDS-PAGE of pure *KaPOx_xal* and *KaPOx_xalh* in comparison with a protein ladder (BioRad, Precision Plus Protein Standard (All Blue Standards)). **a.** *KaPOx_xal* variant after purification with IMAC. 1 – protein ladder, 2 – *KaPOx_xal*. **b.** *KaPOx_xalh* variant after purification with IMAC. 1 – protein ladder, 2 – *KaPOx_xalh*. **c.** *KaPOx_xalh* variant after purification with SEC. 1 – protein ladder, 2 – *KaPOx_xalh*. Replicates not applicable. Lanes 1 and 2 in Figure S2C are spliced together.

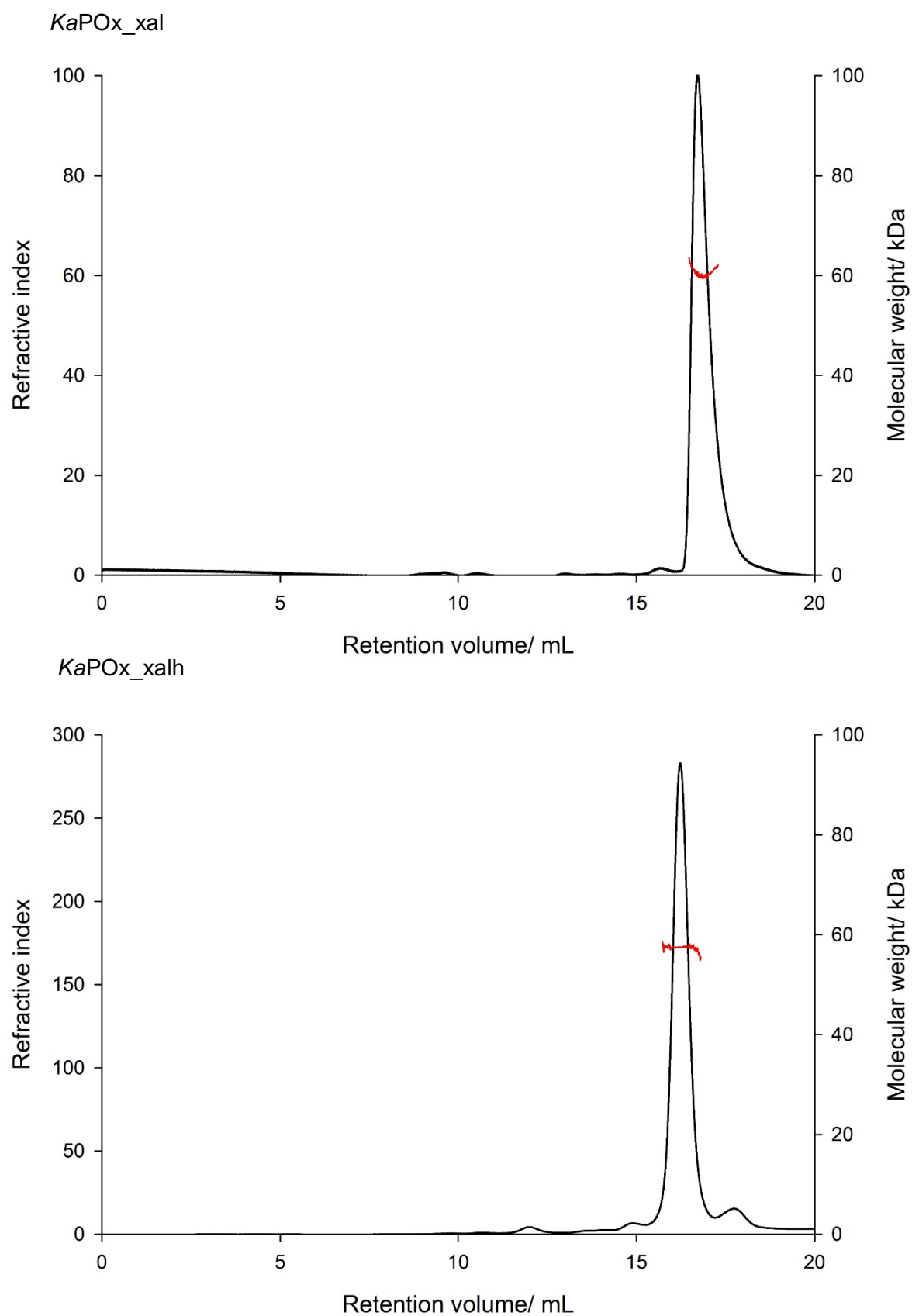


Figure S3. SEC-LS chromatograms of pure *KaPOx_xal* and *KaPOx_xalh* showing molecular weight of 61 and 57 kDa, which corresponds to monomeric state of the two mentioned variants. Replicates not applicable.

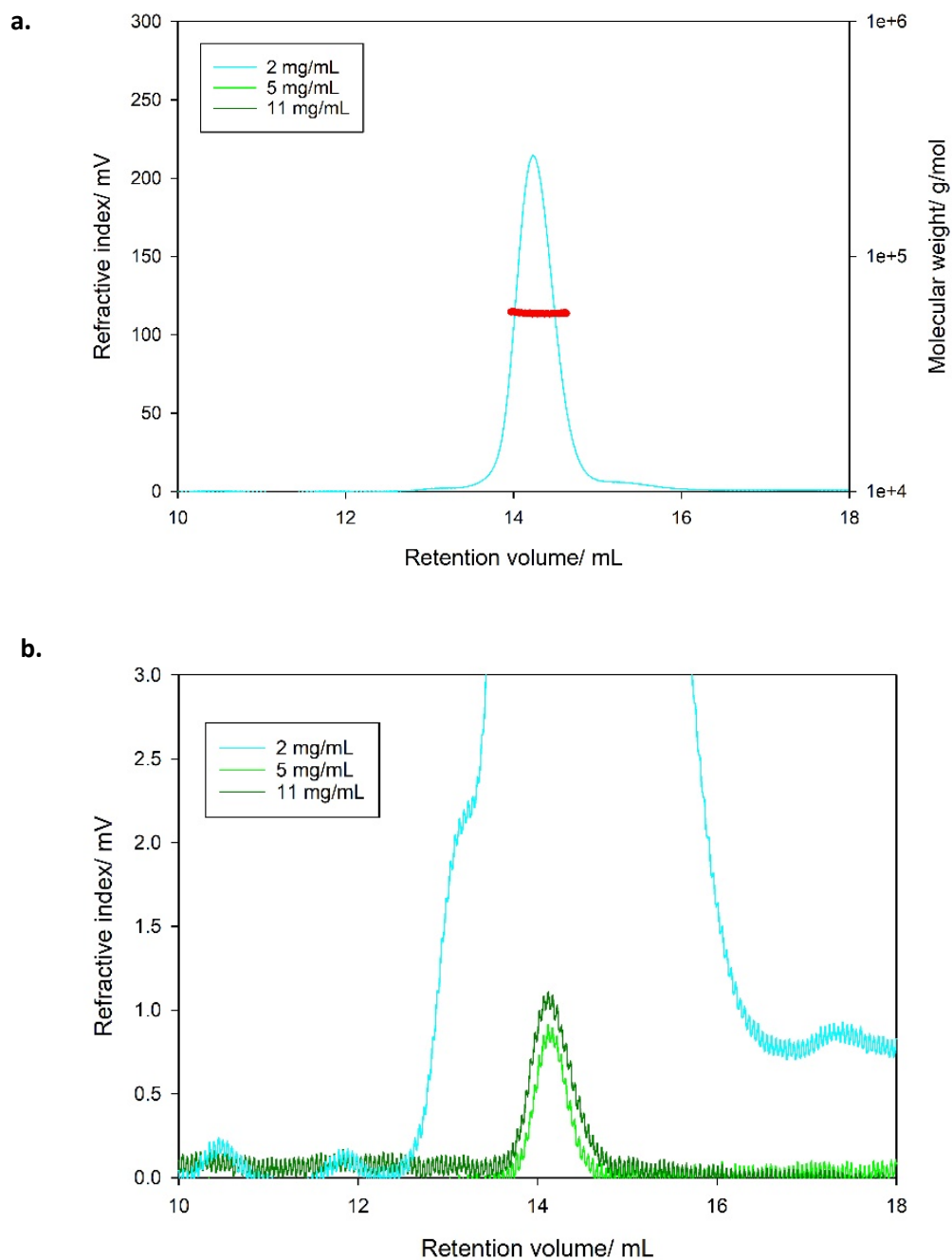


Figure S4. a. SEC-LS chromatograms of a *KaPOx_xalh* sample after purification in concentrations of 2, 5 and 11 mg/mL showing a molecular weight of 57 kDa, which corresponds to a monomeric state of the protein. **b.** Zooming in to the SEC-LS chromatograms of *KaPOx_xalh* of different concentrations. Replicates not applicable.

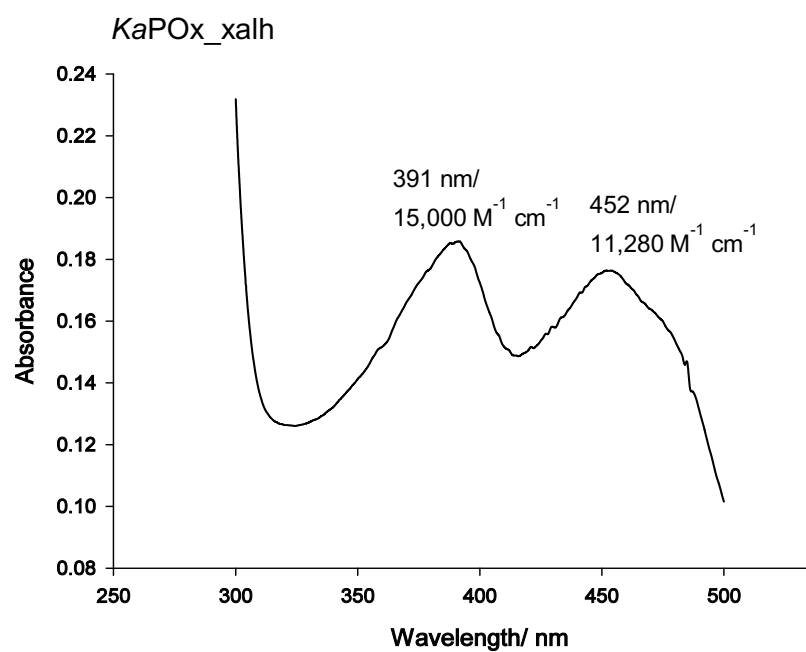
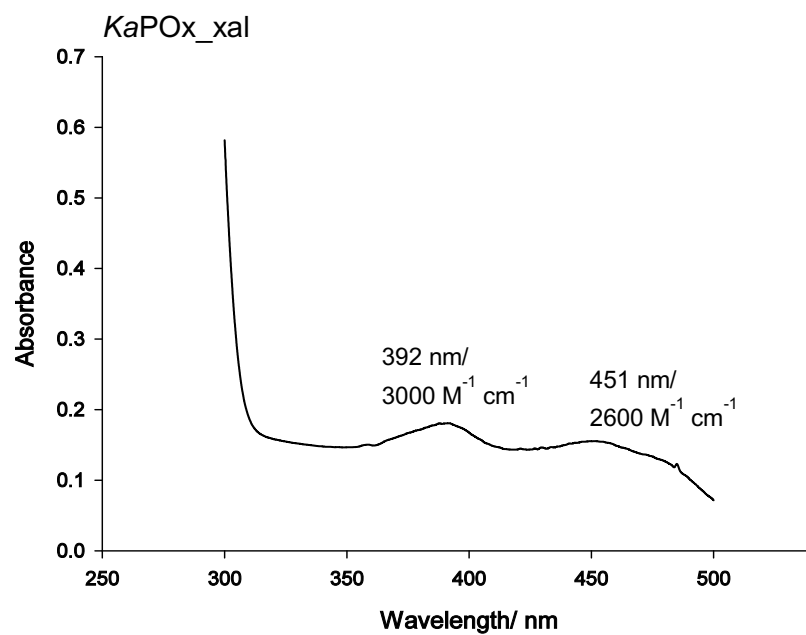
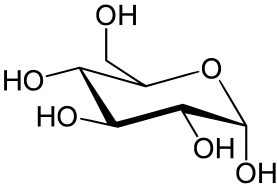
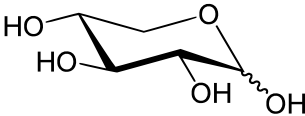
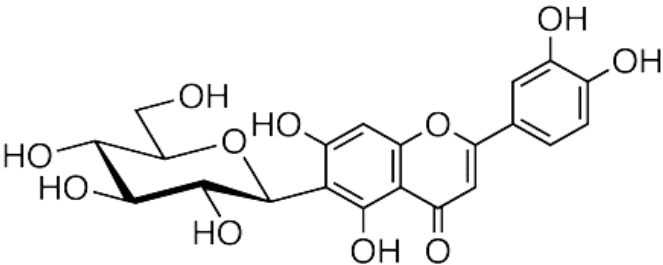
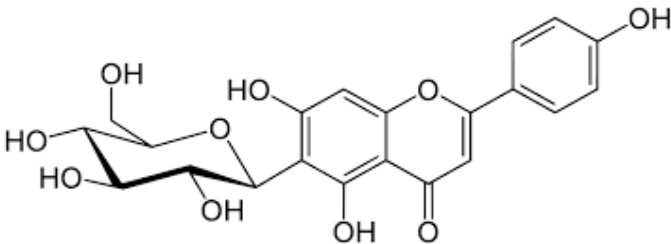
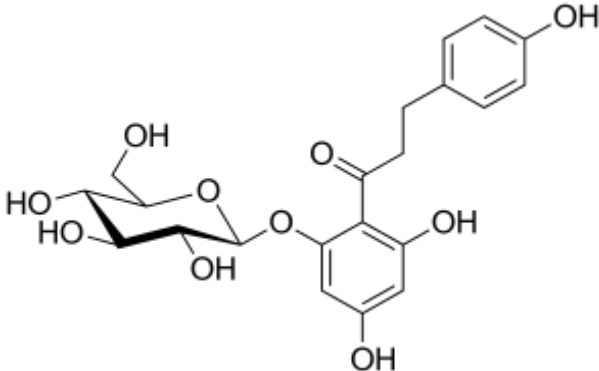


Figure S5. UV/Vis absorption spectra (300-500 nm) of *KaPOx_xal* and *KaPOx_xalh* protein samples after purification. Lambda maxima and associated extinction coefficients for the curve between 350 and 500 nm are shown. Replicates not applicable.

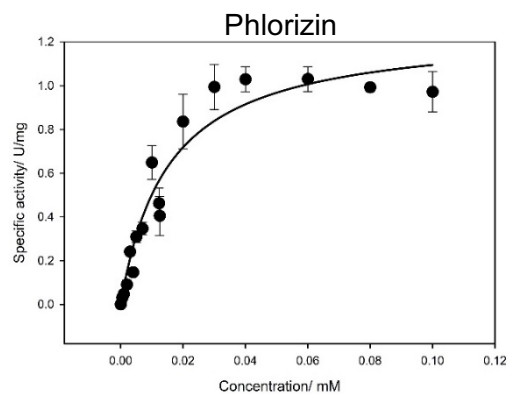
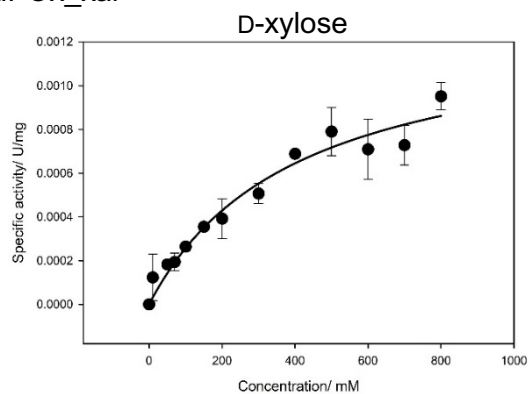
Table S2. Biochemical properties of the wild type *KaPOx* and its variants *KaPOx_xal* and *KaPOx_xalh*, together with information on overexpression as well as thermostability and oligomeric state (from **Figure S3**).

Protein	Number of amino acids	Molecular weight of monomer/ kDa	<i>E. coli</i> expression strain	Induction system	Yield per 1 L culture/ mg	Thermostability (T_m) / °C	Oligomeric state
<i>KaPOx</i>	556	61.2	T7 Express	Lactose, 20 h, 20°C	13.3	53*	Dimer
<i>KaPOx_xal</i>	531	58.7	T7 Express	Lactose, 20 h, 18°C	4.8	42	Monomer
<i>KaPOx_xalh</i>	515	56.9	T7 Express	Lactose, 20 h, 18°C	0.5	42	Monomer

Table S3. Structures of substrate that were oxidized during the initial screening.

Substrate	Structure
D-glucose	
D-xylose	
Isoorientin	
Isovitexin	
Phlorizin	

KaPOx_xal



KaPOx_xalh

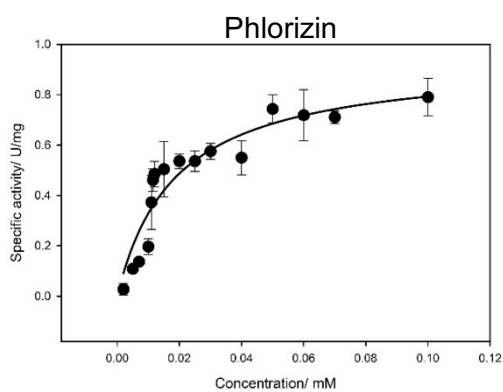
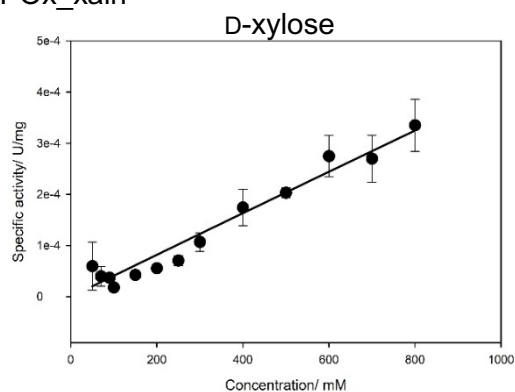


Figure S6. Michaelis-Menten curves for the variants *KaPOx_xal* and *KaPOx_xalh*. Raw data are represented with a black dot, accompanied by the fitting of the data to the Michaelis-Menten function represented by a black line. The measurements were performed in triplicates and at 30°C in 50 mM Tris-HCl, pH=7.5. Data points shown are the mean of three independent measurements $\pm$ standard deviation.

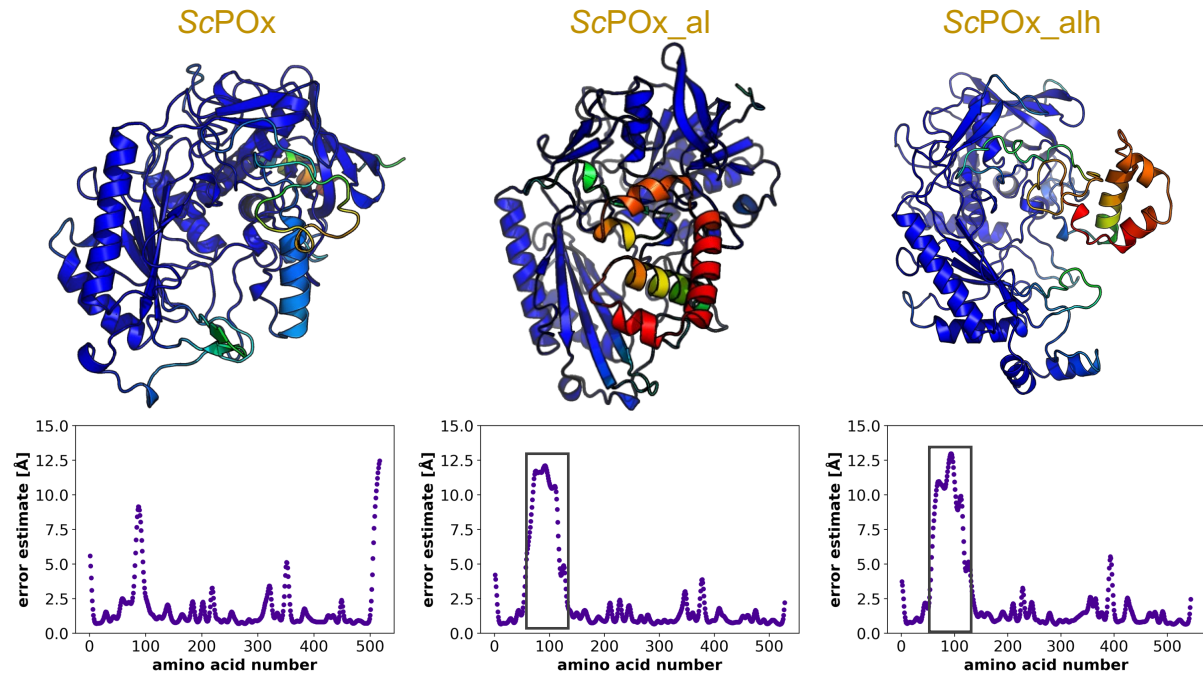


Figure S7. Error estimates in Å of each amino acid in RoseTTAFold [24] models of ScPOx and its variants. The structures are colored as a spectrum from blue to red, blue being lower error estimates. The added N57-Y117 oligomerization loop and arm domain (ScPOx_al and ScPOx_alh numbering) (black box) show high error estimates, and also not in a conformation to form dimers.

Table S4. Putative glucose binding residues in *KaPOx* and the *KaPOx_xalh* variant based on structural alignment of the wild-type *KaPOx* AlphaFold structure to the fungal *PcPOx* containing 3-deoxy-3-fluoro-D-glucose (PDB 4MIG) [25] and *ScPOx*. Residues in italics have a different orientation in structural models compared to *PcPOx* and wild-type *KaPOx*.

Residue in <i>PcPOx</i>	Residue in <i>KaPOx</i>	Residue in <i>KaPOx_xalh</i>	Residue in <i>ScPOx</i>
Q454	Q365	Q322	Q342
D458	D369	<i>D326</i>	R346
Y462	Y373	<i>Y330</i>	<i>D350</i>
A551	P462	P419	S436
H553	H464	H421	H438
N596	N507	N464	N482

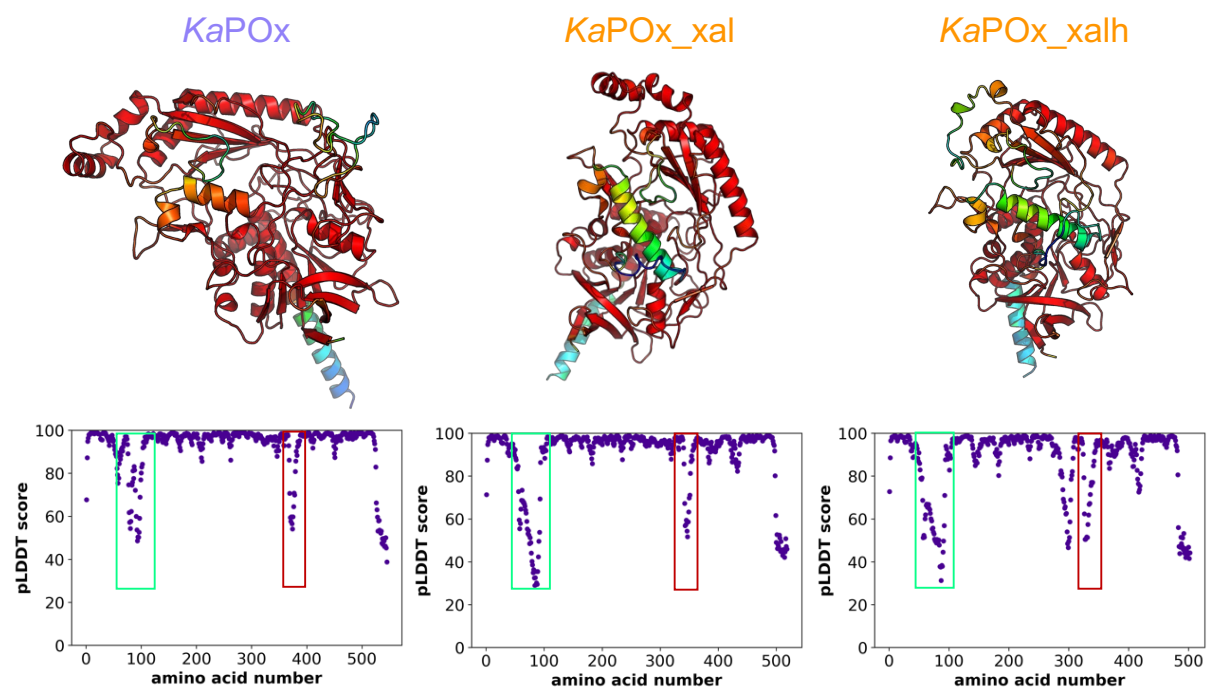


Figure S8. AlphaFold [29] models of *KaPOx* and its variants, colored in rainbow colors from red to blue (high to low) according to the pLDDT (reliability score) assigned to each amino acid, and pLDDT scores plotted against the amino acid numbers for each variant. The substrate recognition loop is marked by a red rectangle, the oligomerization loop/insertion-1 domain with a green rectangle in all plots.

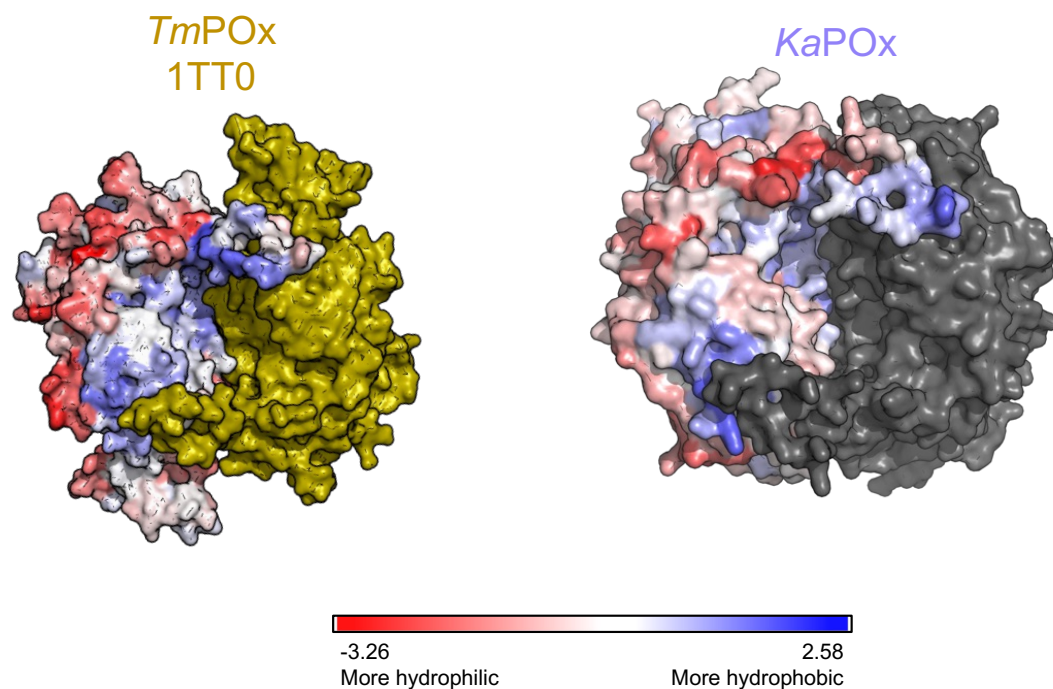


Figure S9. Comparison of the hydrophobic tetramerization surfaces of *TmPOx* (PDB 1TT0) [20] and a wild-type *KaPOx* model [12]. Tetramerization occurs through the hydrophobic surface near the hydrophobic patch and on the hydrophobic side of the arm domain in *TmPOx*. Both these regions are more hydrophilic in *KaPOx* in comparison.

Table S5. List of primers used in this study to re-clone ScPOx_al and ScPOx_alh.

Primer	Purpose	Sequence (5' - 3')
pD441_F	Forward primer to linearize pD441 plasmid between the <i>Sma</i> I and <i>Hind</i> III restriction site	TGACAGCTCGTACCAAGCTTTAATT GGTTGTAACACTGACCCCTATTTG TTTATTTTTCTAAATACATTCAAATA TGTATCC
pD441_R	Reverse primer to linearize pD441 plasmid between the <i>Sma</i> I and <i>Hind</i> III restriction site	CCCGGGCCCCCTGGAACAG
pNIC_F	Forward primer to linearize pNIC-CTHO plasmid between the <i>Afl</i> II restriction site and TEV site	GCAGAGAACCTCTACTTC
pNIC_R	Reverse primer to linearize pNIC-CTHO plasmid between the <i>Afl</i> II restriction site and TEV site	AGTATATCTCCTTCTTAAGGTAAAA C
pD441_alh_F	Forward primer to amplify the <i>scpox_alh</i> gene from pET-21a and re-clone to pD441	TTCTGTTCCAGGGGCCCCGGGATGA CCCATACCCCGCGC
pD441_alh_R	Reverse primer to amplify the <i>scpox_alh</i> gene from pET-21a and re-clone to pD441	AAGCTTGGTACGAGCTGTCAC
pNIC_alh_F	Forward primer to amplify the <i>scpox_alh</i> gene from pET-21a and re-clone to pNIC-CTHO	CCTTAAGAAGGAGATATACTATGA CCCATACCCCGCGC
pNIC_alh_R	Reverse primer to amplify the <i>scpox_alh</i> gene from pET-21a and re-clone to pNIC-CTHO	TGGAAGTAGAGGTTCTCTGCGGTA CGAGCTGTCACTGCAC
pNIC_al_F	Forward primer to amplify the <i>scpox_al</i> gene from pET-21a and re-clone to pNIC-CTHO	CCTTAAGAAGGAGATATACTATGA CCCATACCCCGCGTAC
pNIC_al_R	Reverse primer to amplify the <i>scpox_al</i> gene from pET-21a and re-clone to pNIC-CTHO	TGGAAGTAGAGGTTCTCTGCGGTA CGGGCGGTCACTGC

