Supplemental Figures

A family AA5_2 carbohydrate oxidase from *Penicillium rubens* displays functional overlap across the AA5 family

Filip Mollerup, Ville Aumala, Kirsti Parikka, Yann Mathieu, Harry Brumer, Maija Tenkanen and Emma Master

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FgrGaOx PwiAA5_2	224 - <mark>H D M F C</mark> P <mark>G</mark> I S - 232 220 - H D M F C S G M S - 228	240 - V V T <mark>G G</mark> - 244 236 - L V T <mark>G G</mark> - 240	270 - <mark>R G Y Q</mark> S <mark>S</mark> A <mark>T</mark> - 277 266 - R G Y Q A S A T - 273
Cgr RaOx	484 - <mark>H D M F C</mark> P <mark>G</mark> M N - 492	477 - V N <mark>G G</mark> - 481	530 - <mark>RGYQ</mark> SSVT-537
Cgr AlcOx	69 - <mark>H D M F C</mark> P <mark>G</mark> T S - 77	85 - I V T <mark>G G</mark> - 89	118 - <mark>RGYQ</mark> S <mark>S</mark> C <mark>T</mark> - 125
FgrGaOx	286 - <mark> G G S</mark> W S <mark>G</mark> - 292	332 - D N H A W L F G W K - 341	345 - <mark>v</mark> f <mark>q</mark> a <mark>g p s</mark> t a <mark>m n w y</mark> - 357
PwiAA5_2	282 - <mark> G G S W N G</mark> - 288	329 - D S H G W L F G W K - 338	342 - V F Q G G P S K N M N W Y - 354
Cgr RaOx	546 - <mark> G G S</mark> Y T <mark>G</mark> - 552	599 - <mark>D</mark> N <mark>H</mark> AWLYA <mark>WK</mark> - 607	612 - V F Q A G P S K N M N W Y - 624
Cgr AlcOx	134 - <mark> G G S</mark> F S <mark>G</mark> - 140	167 - <mark>D</mark> S <mark>H</mark> A W L W S <mark>W K</mark> - 185	189 - <mark>V</mark> LQAGPSKK <mark>MNWY</mark> - 201
FgrGaOx	394 - <mark>G K I</mark> L T F <mark>G G</mark> - 401	492 - RVYHSISLLPD-503	507 - V F N <mark>G G G G L</mark> C G - 516
PwiAA5 2	387 - <mark>GKI</mark> ITF <mark>GG</mark> - 394	481 - R V Y H S I S L L L P D - 500	504 - V F N <mark>G G</mark> S <mark>G L</mark> G V - 513
Cgr RaOx	655 - <mark>GKI</mark> FAA <mark>GG</mark> - 663	752 - <mark>R N Y H S</mark> T G <mark>L L </mark> L P <mark>D</mark> - 763	767 - V M N <mark>G G G G L</mark> C Y - 776
Cgr AlcOx	234 - <mark>G K I</mark> F T Y <mark>G G</mark> - 241	331 - <mark>R</mark> N <mark>Y H S</mark> T A <mark>L L</mark> M A <mark>D</mark> - 344	346 - I W S <mark>G G </mark> G <mark>G L</mark> C G - 355
FgrGaOx PwiAA5_2 CgrRaOx CgrAlcOx	580 - T H T V N T D Q R R I P L - 593 577 - T H T V N T D Q R R I S L - 590 853 - T H S I D T D Q R R I P L - 866 420 - T H T V N T D Q R R I P L - 433		
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POCS93 GAOA_GIBZA

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Alignment of CAZy family AA5_2 Fusarium spp.

1 ASAPIG-SAISRNNWAVTCDSAQSGNECNKAIDGNKDTFWHTFYGAN--GDPKPPHTYTI

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gi 10/226 gb AAA16228 gi 225414058 gb AbG08 gi 5287414056 gb AbG08 gi 558761175 gb AhA90 gi 517324299 emb CCT7 gi 517324673 emb CCT7 AIR07394.1 AJE27924.1	1 1 1 1 1	ASAPIG-SAISRNNWAVTCDSAQSGNSCNKAIDCNKDITWHTFYGANGDPKPHTYTI AAPPVGSNDIDRKGWTVKCSSQASNFPCGRAIDCDNNTWQTPYGTTNTPPPHTITI AAPPVGSNDIDRKGWTVKCSSQASNFPCGRAIDCDNNTWQTPYGTTNTPPPHTITI ASAPIG-TAINKDKWRVTCDSQHBCDCSKAIDCRDCTWHTNMAAGGTNDPKPHTITI ASAPIG-TAISRDKWKVTCDSQHBCDCSKAIDCRDCTWHTNWAAGGTNDPKPHTITI ASAPIG-SAISRNWAVTCDSQHBCDCSKAIDCRNTWHTNWAAGGTNDPKPHTITI ASAPIG-SAISRNWAVTCDSQHBCDCSKAIDCRNTWHTNWAAGGTNDPKPHTITI ASAPIG-SAISRNWAVTCDSQHSCHCNKAIDCNKDTFWTTYGGTTNTPPPHTITI ASAPIG-SAISRNWAVTCDSQHBCDCSKAIDCRNTWHTNWAAGGTNDPKPHTITI ASAPIG-SAISRNWAVTCDSQHSCHCNKAIDCNKDTFWTTYGGTTNTPPPHTITI ASAPIG-SAISRNWAVTCDSQHSCHCNKAIDCNKDTFWTTYGGTTNTPPPHTITI	5 5 5 5 5 5 5 5 5 5 5
POCS93 GAOA GIBZA gi 167226 gb AAA16228 gi 295414058 gb ADG08 gi 5287414056 gb ADG08 gi 558761175 gb AHA90 gi 517324299 emb CCT7 gi 517324673 emb CCT7 AIR07394.1 AJE27924.1	58 58 58 60 58 60 58 58 58	DMKTTQNVNGLSMLPRQDGNQNGWIGRHEVYLSSDGTNWGSPVASGSWFADSTTKYSNFE DMKTTQNVNGLSMLPRQDGNQNGWIGRHEVYLSSDGTNNGSPVASGSWFADSTTKYSNFE DMKQTQVVSGLQITPRQDGNTRNNIGHEVVLSSDGTNNGKPVAFGTWGDKYPWTINFE DMKSGNVSGLQITPRQDGSDHGWIGRHEVILSSDGTNNGKPVAFGTWGDKYPWTINFE DMKSGNVSGLSULFRQDGSDHGWIGRHEVILSSDGTNNGKPVAFGTWGDKYPWTINFE DMKSGSUNGLSULFRQDGSDHGWIGRHEVILSSDGTNNGKPVAFGTWGDKYPWTINFE DMKSGLSULFRQDGSDHGWIGRHEVILSSDGTNNGKPVAFGTWGDKYPWTINFE DMKSGLSULFRQDGSDHGWIGRHEVILSSDGTNNGKPVAFGTWGDKYPWTINFE DMKSGNVAGLSULFRQDGSDHGWIGRHEVILSSDGTNGSPVAFGTWGDKYPWTINFE DMKSGNVAGLSULFRQDGSDHGWIGRHEVILSSDCKTNGSPVAFGTWGDXYDTWYSDNTEKYSNFE DMKQTQVVSGLQITPRQDGNTRNWIGRHEVILSSDCKTNGSPVAFGTWGDKYPWTINFE DMKQTQVVSGLQITPRQDGNTRNWIGRHEVILSSDCKTNGSPVAFGTWGDKYPWTINFE	11 11 11 11 11 11 11 11 11
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POCS93 GAOA GIBZA gi 167226 gb AAA16228 gi 295414058 gb ADG08 gi 295414056 gb ADG08 gi 5587617175 gb AHA90 gi 517324299 emb CCT7 AIR07394.1 AJE27924.1	177 178 178 178 179 178 179 177 178	EPTSGRVLMWSSYRNDAFGGSPGGITLTSSWDPSTGIVSDRTVTVTKHDHCPGISMD EPTSGRVLMWSSYRNDAFGGSPGGITLTSSWDPSTGIVSDRTVTVTKHDMCPGISMD EPVSGRVVIWSAYRDAFQGTPRGGFTLTSIWDPTNVISNRWSNNHDMCPGISMD EPVSGRVVIWSAYRDAFQGTPRGGFTLTSIWDPTNVISNRWSNNHDMCPGISMD EPTSGRVLWSSYRNDAFGGSPGGVTLTSIWDPTNVISNRWSNNHDMCPGISMD EPTSGRVLWSSYRNDAFGGSPGGVTLTSIWDPTNVISNRWSNNHDMCPGISMD EPTSGRVLWSSYRNDAFGGSPGGVTLTSIWDPTNVISNRWSNNHDMCPGISMD EPTSGRVLWSSYRNDAFGGSPGGVTLTSIWDPTNVISNRWSNNHDMCPGISMD EPTSGRVLWSSYRNDAFGGSPGGVTLTSIWDPTSGVISORTVTVTKHDMCPGISMD EPTSGRVLWSSYRNDAFGGSPGGVTLTSIWDPSGVISORTVTVTKHDMCPGISMD EPTSGRVLWSSYRNDAFGGSPGGVTLTSIWDPSGVISORTVTVTNKHDMCPGISMD	2 3 2 3 2 3 2 3 2 3 2 3 2 3 2 3 2 3 2 3
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POCS93 GAOA GIBZA gi 167226 gb AAA16228 gi 295414058 gb AbC08 gi 5287414056 gb AbC08 gi 558761175 gb Ah890 gi 517324299 emb CCT7 gi 517324673 emb CCT7 AIR07394.1 AJE27924.1	413 417 417 415 417 415 417 415 413 417	NAHIITLGEPGTSPNTVFASNGLYFARTFHTSVVLPDGSTFITGGQRRGIPFEDSTFV NAHIITLGEPGTSPNTVFASNGLYFARTFHTSVVLPDGSTFITGGQRRGIPFEDSTFV NAHVLTLDQFGAIAQTALVGNNGAGIHARVFATSVILPDGNVFITGGQSYSNPFTDTNAQ NAHVLTIDQFGAIAQTALVGNNGAGIHARVFATSVILPDGNVFITGGQSYSNPFTDTNAD NAHIITLSERGSTFKTVFASNGLYFARTFHTSVILPDGNVFITGGQSYSNPFTDTNAQ NAHIITIGEFGSTFKTVFASNGLYFARTFHTSVILPDGNVFITGGQSYSNPFTDTNAQ NAHIITIGEFGSTFKTVFASNGLYFARTFHTSVILPDGNVFITGGQSYSNPFTDTNAQ NAHIITIGEFGSTFKTVFASNGLYFARTFHTSVILPDGNVFITGGQSYSNPFTDTNAQ NAHIITIGEFGSTFKTVFASNGLYFARTFHTSVILPDGNVFITGGQSYSNPFTDTNAQ NAHIITIGEFGSTFKTVFASNGLYFARTFHTSVILPDGNVFITGGQSYSNPFTDTNAQ NAHIITIGEFGSTFKTVFASNGLYFARTFHTSVILPDGNVFITGGQSYSNPFTDTNAQ NAHIITIGEFGSTFKTVFASNGLYFARTFHTSVILPDGNVFITGGQSYSNPFTDTNAQ NAHIITIGEFGSTFKTVFASNGLYFARTFHTSVILPDGNVFITGGQSYSNPFTDTNAQ	47 47 47 47 47 47 47 47 47
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POCS93 GAOA GIBEA gi 167226 gb AAA16228 gi 295414058 gb AbG08 gi 558761175 gb AbG08 gi 558761175 gb AbG08 gi 517324299 emb CCT7 gi 517324673 emb CCT7 AIR07394.1 AJE27924.1	591 597 597 593 593 597 593 591 597	PLTLTNN-GGNSYSFQUPBDSGVALPGYMNLFUNNSAGUPSVASTIRUTO PLTLTNN-GGNSYSFQUPBDSGVALPGYMNLFUNNSAGUPSVASTIRUTO PLAITGA-GTNKYSFKIPDSGIALPGYMNLFUNNSAGUPSVASTIRUTO PLAITGA-GTNKYSFKIPDSGIALPGYMNLFUNNSAGUPSVASTIRUTO PLAITGA-GTNKYSFKIPDSGIALPGYMLFUNNSAGUPSVASTIRUTO PLAITGA-GSNSYPQUPBDGIALPGYMLFUNNSAGUPSVASTIRUTO PLAITGA-GSNSYPQUPBDGIALPGYMLFUNNSAGUPSVASTIRUTO PLAITGA-GSNSYPQUPBDGIALPGYMLFUNNSAGUPSVASTIRUTO PLAITGA-GTNKYSFRIPDBGIALPGYMLFUNNSAGUPSVASTIRUTO PLAITGA-GTNKYSFRIPDBGIALPGYMLFUNNSAGUPSVASTIRUTO PLAITGA-GTNKYSFRIPDBGIALPGYMLFUNNSAGUPSVASTIRUTO PLAITGA-GTNKYSFRIPDBGIALPGYMLFUNNSAGUPSVASTIRUTO PLAITGA-GTNKYSFRIPDBGIALPGYMLFUNNSAGUPSVASTIRUTO	63 63 64 64 64 64 64 64 63 64

Fig A. Analysis of AA5_2 Sequences. (A) Sequence conservation between *Fgr*GaOx, *Pru*AA5_2A, *Cgr*RaOx and *Cgr*AlcOx in within 10 sequence stretches recognized for having identical amino acids in the 9 analyzed *Fusarium spp*. Yellow highlights identical amino acids throughout the four sequences of *Fgr*GaOx, *Pru*AA5_2A, *Cgr*RaOx and *Cgr*AlcOx. Amino acids that also occur in Table 1 are in bold, red positions denotes variances. The placements of the sequence segments in the structure of *Fgr*GaOx are highlighted in Figure 2C. (B) Sequence alignment of the 9 Fusarium spp. in subfamily AA5_2 used to identify the 10 conserved sequence segments and information presented in Table 1. The conserved sequence segments were defined as strings of 4 or more consecutive and conserved amino acids in alignment of the *Fusarium spp*. sequences. **S1 File:** A new family AA5_2 member displays dual activity preference

 MW, kDa 250 150	0.1	<i>Pru</i> AA5 0.25	_2A 0.5 mg/mL
100			
75			
50		•	
37			
		•	

Fig B. SDS-PAGE of pure *Pru***AA5_2A after production and purification.** The molecular weight of *Pru***AA5_2A** was estimated to be 75 kDa, indicating that the purified enzyme is glycosylated.



Fig C. Effect of temperature on *Pru***AA5_2A activity.** (A) Activity on 300 mM raffinose in 20 mM MOPS (pH 7.5). (B) Residual activity at 30 °C after 15, 30 min or 1, 2, 4 and 24 hours at 22, 30, 40, 50 and 60 °C in 20 mM MOPS (pH 7.5). n=4; error bars indicate standard deviation.





Fig D. Analysis of oxidized products by mass spectrometry. (A) Negative mode ESI-MS spectra of oxidized raffinose produced by *Cgr*RaOx -catalyzed reaction (3), and (B) by *PruAA5_2A* -catalyzed reaction. m/z 517, uronic acid; m/z 539, unoxidized raffinose (Cl⁻adduct); m/z 569, aldehyde product reacted with methanol (Cl⁻adduct); m/z 207.9, MOPS buffer.



Fig E. ¹H NMR spectra of sucrose oxidation products. (A) Analyzed in D₂O, and (B) DMSO-

d₆. Chemical shifts indicating the oxidized product formation and putative oxidation sites as hydrates (A) and aldehydes (B) marked with a ring. Due to low degree of oxidation the final structure of the oxidation product could not be determined.



Fig F. Comparative plot of *Pru***AA5_2A and** *Fgr***GaOx substrate kinetics using the calculated Michealis-Menten plot from kinetical analysis.** The activity axis (y-axis) were converted to relative activity for easy comparison. Actual kinetic parameters are present in Fig 6 for *Pru*AA5_2A and the discussion for *Fgr*GaOx. No activity on glycolaldehyde dimer was detected for *Fgr*GaOx.