

PFOMT Crystallization Screening – BBZ L - 01.02.2014

PFOMT and variants from Expression of pET28a(+) with N-terminal His6-Tag and Thrombin cleavage site in *E.coli* BL21(DE3).

Concentrated and rebuffered into 10 mM Tris-HCl pH 7.5 with centrifugal concentrators.

Absorption at 280 nm (A280):

Variant	A280 (triplets)	Mean(A280)	Abs 0.1% (=1 g/l) [by PROTPARAM]	c (mg/mL), reduced
Wildtype	5.178	5.19	0.714 (reduced) 0.709 (ox.)	7.27
	5.208			
	5.176			
Y51K N202W	5.252	5.33	0.852 (red.) 0.848 (ox.)	6.26
	5.387			
	5.367			

3 different crystallization conditions per Buffer-well:

- a) 250 µM MgCl₂, 250 µM SAH, 250 µM Ferulic Acid (wildtype PFOMT)
- b) 250 µM MgCl₂, 250 µM SAE, 250 µM Eriodictyol (wildtype PFOMT)
- c) 250 µM MgCl₂, 250 µM SAH, 250 µM Ferulic Acid (PFOMT Y51K N202W)

Stock solutions:

1. 25 mM Ferulic Acid in 20% DMSO
2. 25 mM Eriodictyol in 20% DMSO
3. 25 mM MgCl₂

(fällt aus, unvollständig gelöst)

Prepared protein solutions for crystallization:

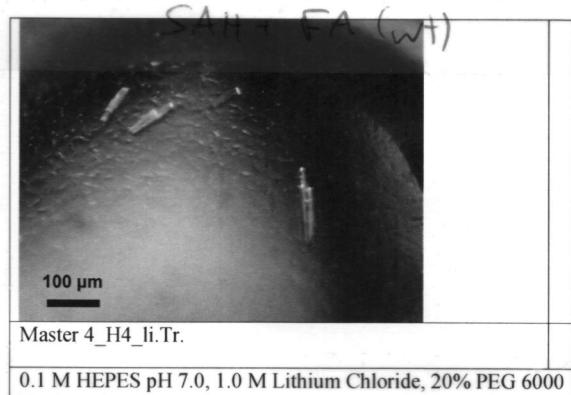
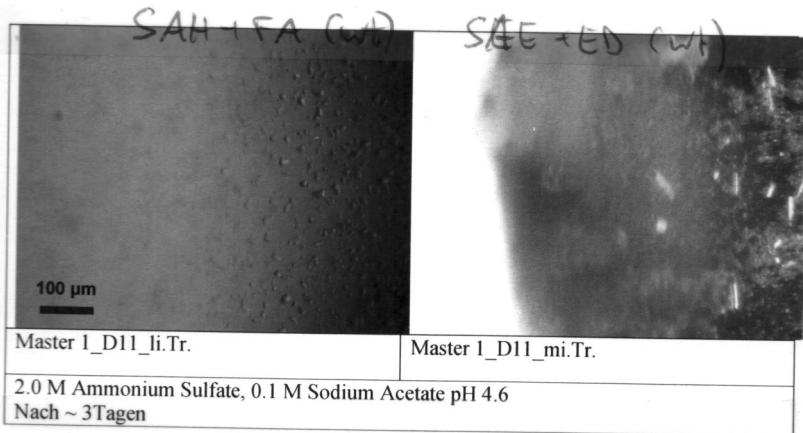
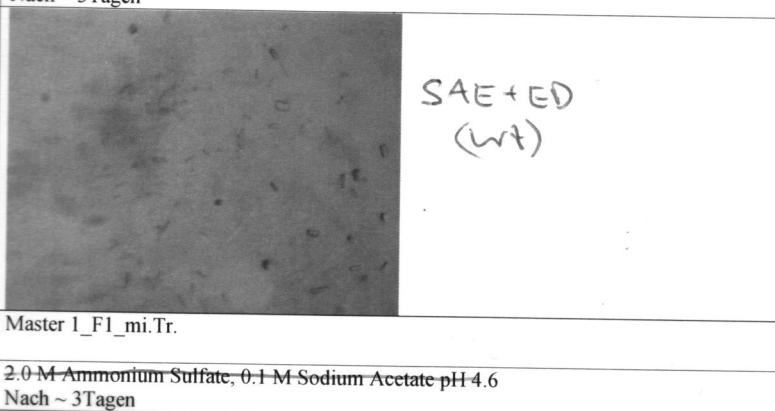
A) left	B) center	C) right
4 µl 5 mM SAH	11 µl 1.83 mM SAE	4 µl 5 mM SAH
0.8 µl 25 mM MgCl ₂	0.8 µl 25 mM MgCl ₂	0.8 µl 25 mM MgCl ₂
0.8 µl 25 mM ferulic acid	0.8 µl 25 mM eriodictyol	0.8 µl 25 mM ferulic acid
75 µl 7.27 mg/ml PFOMT wt	75 µl 7.27 mg/ml PFOMT wt	75 µl 6.26 mg/ml PFOMT YK NW
6.77 mg/ml PFOMT	6.22 mg/ml PFOMT	5.83 mg/ml PFOMT



→ aussichtsreiche
Bedingungen

Master 1_F1_li.Tr. Kristallform 1	Master 1_F1_li.Tr. Kristallform 2
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30% PEG MME 2000, 0.1 M Na Acetate pH 4.6, 0.2 M Ammonium Sulfate
Nach ~ 3 Tagen



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OKOKAU

Buch 3 S 200

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SAMS Aktivität

17,5 µl 100mM L-methionin

35 µl 10 x SAMS Buffer

35 µl 50mM ATP (fish liver)

140 µl ddH₂O

70 µl SAMS (1:5 VD; 1.4 µg/ml)

→ bei 30°C inkubiert

→ nach 0, 5, 10, 15, 20 & 25 min

je 50 µl entnommen & mit 100 µl Phosphatreakz 2

abgestoppt (S.u.) → 3 min inkubiert und A=620nm gemessen
(MTP)

Phosphatreakz 2: 1 Vol. 6mg/ml Malachitgrün in
 3 Vol. 10mM Ammoniummolybdat in
 2.2M HCl gegeben und 30min

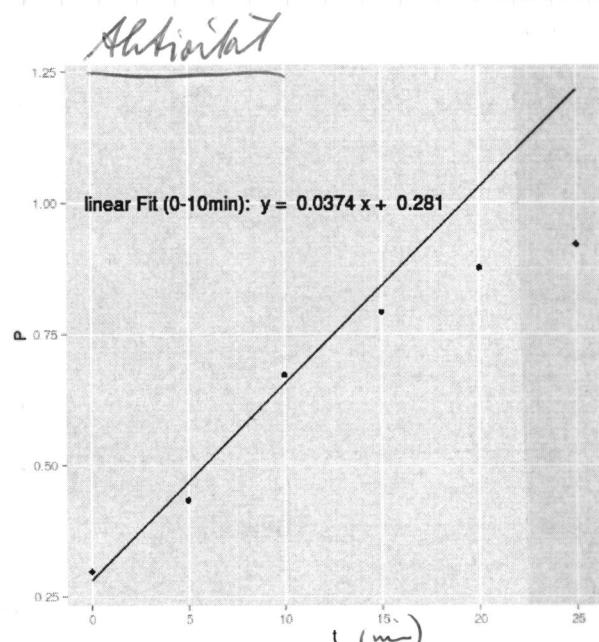
@ RT inkubiert

→ lila wird blau
 aber trübe wird

$$\text{Aktivität (mU/ml)} = \frac{0.0374}{0.006425} \times 25$$

(aus D/M's
Protokoll)

$$= 146 \frac{\text{mU}}{\text{ml}}$$



SAE-Sephadex (60 ml)WEB230

6 ml SAMs-Puffer

250 µg ATP

6 ml D/L-Ethanol

4.1 ml SAM (@ 145 µM/ml)

63.9 ml H₂O

→ 18 h @ 30°C / 80 rpm inkubiert

→ + 270 µl 10 M Essigsäure

→ mit N₂ eingeschüttet & in N₂-Lyophilisiert

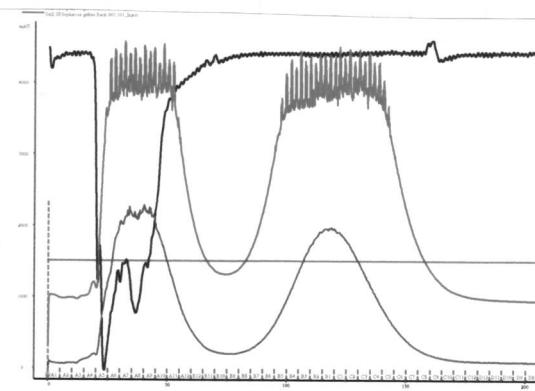
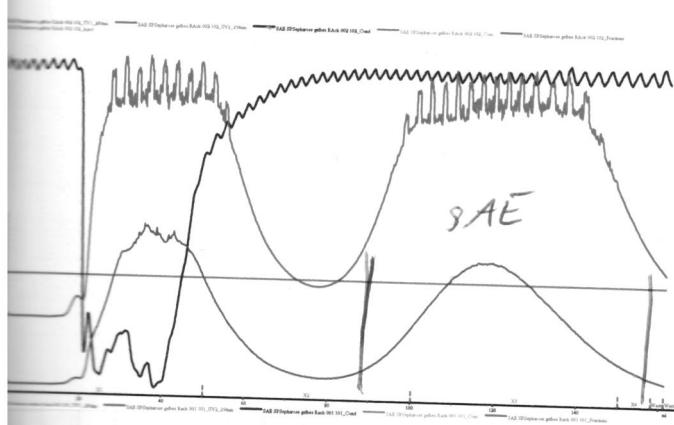
→ 1x mit 12 ml 73% EtOH extrahiert

und weiter mit 3 ml 73% EtOH extrahiert

- ~~überstand abseihen~~→ SP-Sepharose 1EX Chromatografie wie in Ditt's
Protokoll

NICORN 5.31 (Build 743)

result file: c:\...\WEB\SAE SP Sepharose gelbes RAck 002



zweiter Peak gepolt → in N₂ eingeschüttet & lyophilisiert

- in ~ 6 ml H₂O aufnehmen
- 1:50 VF (2 µl = 98 µl H₂O)
- A²⁶⁰ nm gemessen → A²⁶⁰ = 0.245 (@ 1mm)
- A²⁶⁰ = 0.245 × 10 mm × 50 (VF) = ~~7.98~~ 122,5
- $\frac{122,5}{15400 \text{ cm}^{-1} \text{ M}^{-1}} = 7.95 \cdot 10^{-3} \text{ M} = 7.95 \text{ mM}$
- auf 1 mM verdünne (add H₂O)

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PFOMT Mutagenese

K157D (30.01.14)

Mastermix

2.5 µl Pfu Ultra Buffer
 + 0.75 µl PFOMT-K157D-fw
 & -rev
 0.5 µl Pfu Polymerase
 0.5 µl dNTP-Mix (QIAGEN Kit)
 15 µl ddH₂O

Auszüge

je 11.5 µl lös.
 + (A) 0.25 µl pET2b(PFOMT Y51K
 (B) —————— Y51R

PCR-Programm

siehe Buch 3, S. 21

→ Nach PCR

→ (L) → 1.5 µl FD-Buffer (green)
 + 1 µl QPCR-Fast Digest
 + 31.5 µl ddH₂O

→ Thaw QPCR / 20 µl @ 65°C

→ 1 µl in DNA transformiert

→ in-N. @ 37°C

→ Colony PCR

→ Sequenzierungswürze

→ Buch 3 S. 197

Colony PCR
 PFOMT Y51K M157D
 Y51R K157D



Mutagenere PFOMT7 Rens

(A) pET28a (+) PFOMT

- K157 S ⑦
- D154 G ②
- Δ D154 ③

Mastermix (87.5 μl total)

- 1.75 μl dNTPs (QC-kit)
- 8.75 μl Pfu Buffer + MgSO₄

1.75 μl Pfu DNA Polymerase

57.5 μl H₂OAusarbeiten

- 9.75 μl Mastermix
- + 3 μl Prime - mix
- + 0.25 μl 50 μg/μl Plasmid (A, B, C)

PCR-Programm

siche Buch 3/5.21

- verdaut mit Dpn I
- 1-5 μl in DH5α transformiert
- @37°C / 12h Nacht inkubiert
- wenige Kolonien
- bei positive Colony - PCR → ⇒ Plasmid prep & Sequenzierung
- alle ~~positive~~ Xantenbe wurde korrekt sequenziert
- ~~positive~~ transformation in BL21 (DE3) & Expression

Expansion PFOA1 Variante

(WEB228)

→ Expansion in 50 - 100 ml ZYP 5052 Autoinduktiv medium bei 31°C über Nacht (mit Anhebung) ausgeführt

An nächste Höhe:

Variante	OD ₆₀₀
PFOA1 Y51K K157D	7.581
PFOA1 Y51R K157D	7.327
PFOA1 ΔD154	9.72
PFOA1 Y51K D154G	8.97
PFOA1 Y51R K157S	8.93
PFOA1 K157S	8.11
PFOA1 D154G	8.42
PFOA1 Y51K K157S	7.97
PFOA1 Y51K ΔD154	7.44

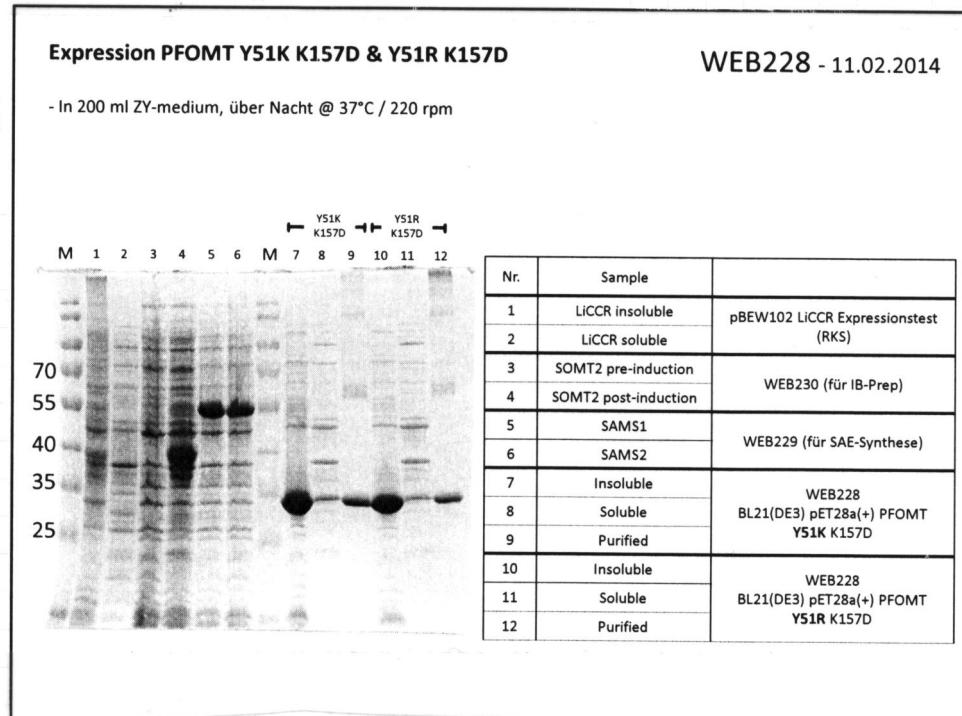
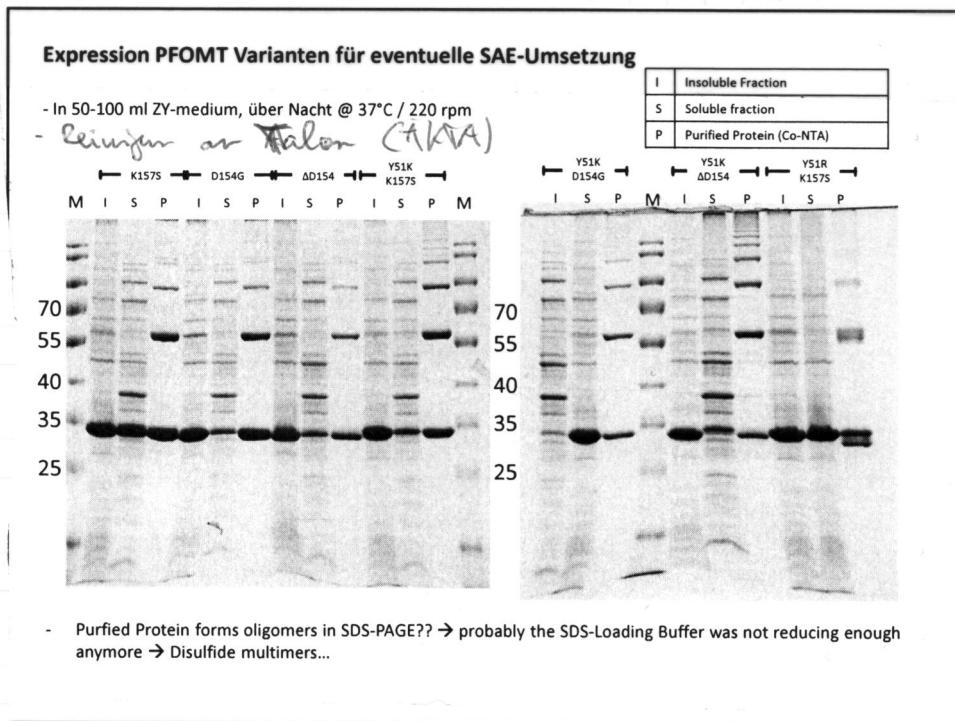
200 ml ZY
 215 ml ~~H~~ 1h hySiO₄
 4.3 ml 5052
 43 µl Trace metals
 10.75 ml NBS
 (200 µg/ml Kanamycin)

→ Absterben
 → Zelle alleletisch
 hyper & p ZKA
 (mit Titer) reing.
 → unvollständig durch
 2x Dialyse gegen
 25 mM Hepes, 150 mM NaCl,
 8% glycerol pH 7.5

nach Reinigung & Dialyse A₂₈₀:

Variante	E (1 ml)	A ₂₈₀	c (mg/ml)
Y51K K157D		4.08	
Y51R K157D		1.57	
ΔD154	0.77	0.75	1.05
Y51K D154G	0.66	0.50	0.75
Y51R K157S	0.66	0.73	1.11
K157S		1.31	
D154G		1.48	
Y51K K157S		0.97	
Y51K ΔD154		0.79	

SDS - Gel



Aktivitätsbt PFO_{MT} Variante

WT8240

8 Zeitpunkte 0, 6, 12, 18, 24, 30, 36, 40 min

→ 5 Varianten → Doppelkettensäure
→ 80 Samples

82 x 1ml

656 µl 10 x OMS - Buffer

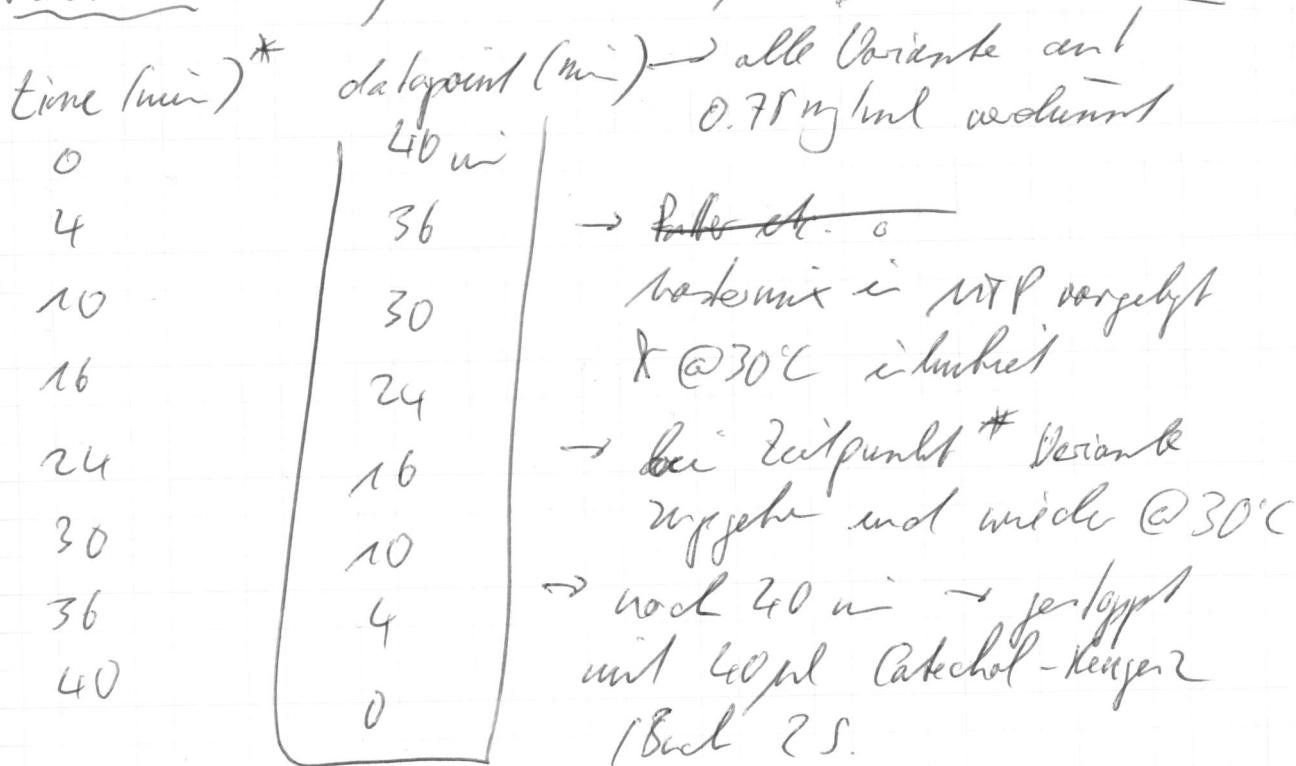
656 µl 10 mM Katalase

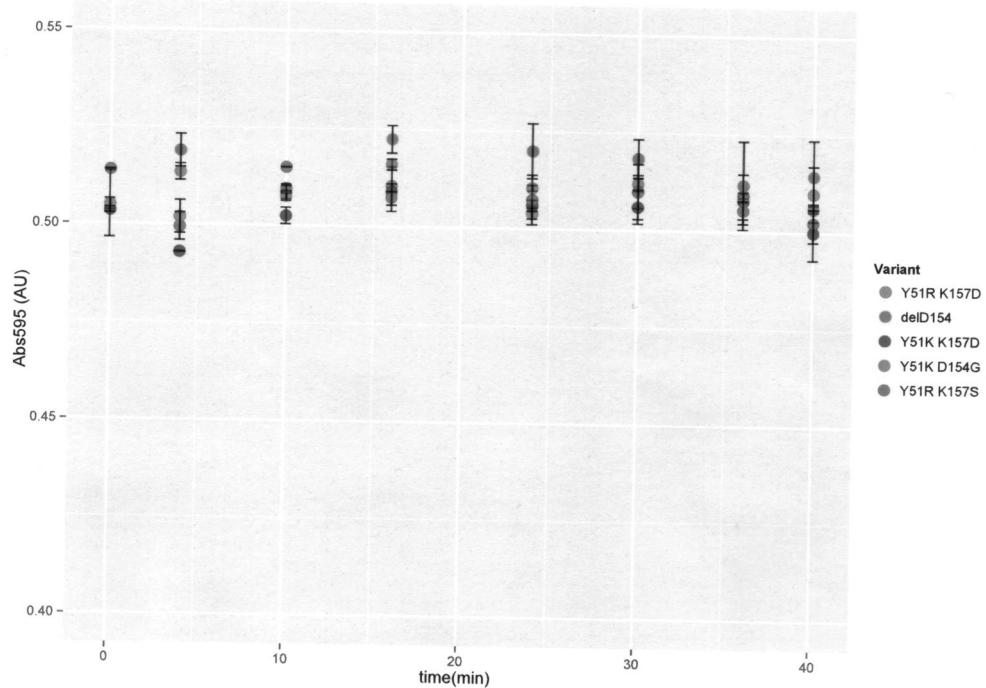
164 µl 0,1M GSH

1,804 ml 5 mM SAM (72,5%)

1,066 ml dH₂O

Reaction: 53 µl MM + 25 µl 0,75 mg/ml Variante



AktivitätsabfallAktivitätsabfall RENT Variante

→ keine messbare Aktivität bei cypdariv
Variante

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SOMT 2 IB PrepWED 23.1

- SOMT 2 expressed in BL21(DE3) pBEM104 SOMT2 (500ml?)

$$7.5 \mu\text{L} \text{ GeneGlobe} \times 0.5 \mu\text{g}/\mu\text{L}$$

$$= 3.75 \mu\text{g} \cdot 0.04 = \underline{\underline{150 \mu\text{g}}}$$

5000 bp band \rightarrow 4%

$$300 \mu\text{g}/80 \mu\text{L} = \underline{\underline{3.75 \mu\text{g}/\mu\text{L}}}$$

\rightarrow 2.5 μL Tris

SOMT 1B PrepWED 23.10.75 1B (After last wash)

\rightarrow in 5ml 1B-sol. 8ml/l

and for some

\rightarrow OD²⁶⁰ \rightarrow OD²⁸⁰ penetrator

$$A^{260} = 0.66$$

(1:10 VD in

$$A^{280} = 0.76$$

1B SM. (Ball)

$$\text{c}(\text{ug}/\text{ml}) > ((1.55 + 0.76) - (0.76 - 0.66)) \times 50 \\ = 33.8 \text{ ug/ml}$$

73 ml

WEB 73

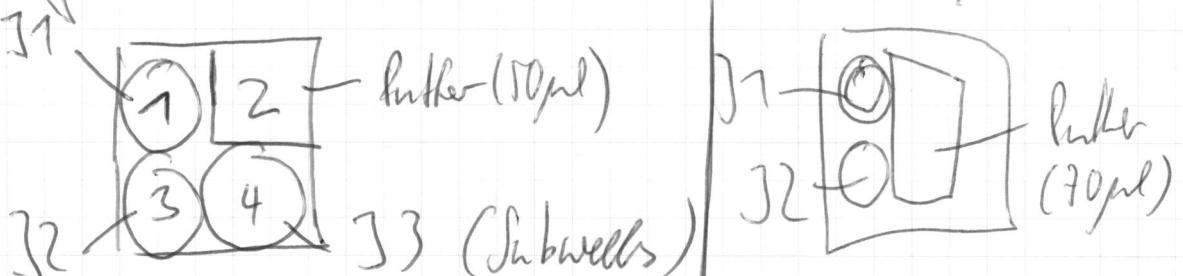
X-Tallization Screening PFOMA

an MC1 (mit Christoph Rother)
AG Stabbs

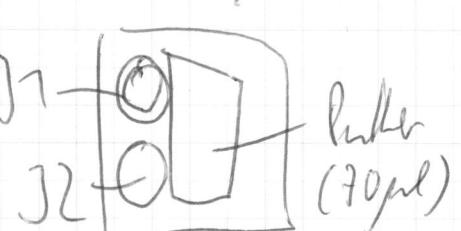
- 60 µl je Screen → 3x Probe je Platte
→ bei 120 µl Probe → 3-4 verschwundene Platten
- o Screen wt & Y51R N202W Variante
→ ungepuffert (10mM Tris pH 7.5) & konzentriert

	A280	c (mg/ml)
wt (Stab)	16.8	23.6
Y51K	3.43 (^{E(1nm)} 0.658)	5.2
Y51R N202W	6.83	8
wt	5.77	8.1

Screening Platte → 96 well mit 4 Subwells/well



4 Subwells
(3 Bodenje)



3 Subwells
(2 Bodenje)

Source Plate → 364 well

(A) J1 (120 μl volume)

6 μl 5mM SAE
 1.2 μl 25mM MgCl₂
 1.2 μl 25mM Foulasäure (FA)
 111.6 μl 8.1 mg/ml PFOA wt
 ⇒ 7.53 ^{μg}/μl Protein im Ansatz

Rigaken Server
 14.1.48.19. 158
 user: Benjamin Wengel
 pass: bw2014

(B) J2 (120 μl)

6 μl 5mM SAE
 1.2 μl 25mM MgCl₂
 1.2 μl 25mM Ethyldiethanol
 111.6 μl 8.1 mg/ml PFOA wt

(C) J3 (40 μl)

2 μl SAE (5mM)
 0.4 μl 25mM MgCl₂
 0.4 μl 25mM FA
 29 μl 8.1 mg/ml PFOA wt Y51R
 + 5 μl Buffer N202W
 ⇒ 6.3 ^{μg}/μl Ansatz

⇒ Screens angelegt mit A, B, C → Hampton Research HT
 Screens mit A, B → Jena Bioscience classic 1-4
 Jena Bioscience cryo 1-4

Blank - Code:

MP000715 HR - 3well
 MC001413 JBS 1-4 classic
 MC001415 JBS 1-4 cryo

A) J1 (120 μl Volume)

6 μl 5mM SAE

1.2 μl 25mM MgCl₂

1.2 μl 25mM Fomosasäure (FA)

111.6 μl 8.1 mg/ml PFOA wt

⇒ 7.53 ^{μg}/ml Protein im AnsatzRigaken Server

141.48.19.158

usr: Benjamin Wengel

pass: bw2014

B) J2 (120 μl)

6 μl 5mM SAE

1.2 μl 25mM MgCl₂

1.2 μl 25mM Ethyldiglycol

111.6 μl 8.1 mg/ml PFOA wt

C) J3 (40 μl)

2 μl SAE (Funk)

0.4 μl 25mM MgCl₂

0.4 μl 25mM FA

29 μl 8 ^{μg}/ml PFOA Y51R

+ 5 μl Buffer

N202W

⇒ 6.3 ^{μg}/ml in Ansatz

⇒ Screens ansetzt mit A, B, C → Hampton Research HT
 Screens mit A, B → Jena Bioscience classic 1-4
 Jena Bioscience cryo 1-4

Blank - Code:

MP000715 HR - 3well

MC001413 TBS 1-4 classic

MC001415 TBS 1-4 cryo

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~~AST~~LC MS/MS-Messung für Varianten~~Pfarr~~

WEB 234

von Renona für Dlh

Code:

- AXX-Z → $\underline{\underline{A}}$ - Substrat (L-Citrullin
T-Taurin
E-Essigsäure)
- XX - ein- oder zweistellig
→ Varianten:

<u>XX</u>	Y51K
<u>K</u>	- Y51K
<u>R</u>	- Y51R
<u>NA</u>	- Y51R N202A
<u>NY</u>	- ~ N202Y
<u>NV</u>	- ~ N202V
<u>NS</u>	- ~ N202S
<u>NW</u>	- ~ N202W

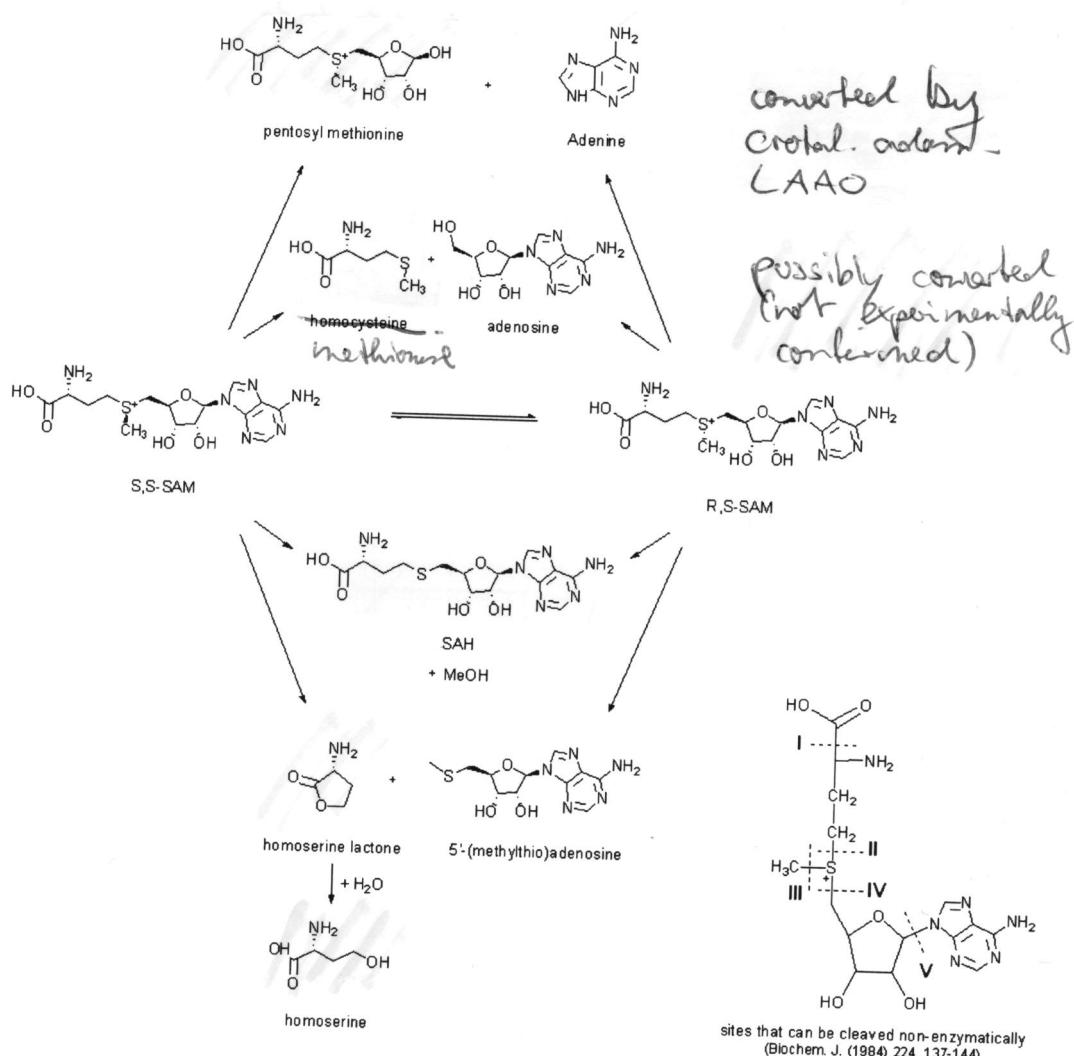
Z → Nummer entweder
1 oder 2 (Zweitau-
tosierung)

WZL

Stability of SAM in our reaction

Sul/w (0,1M Tris HCl)
1mM MgCl₂
pH 7,5

- Is SAM non-enzymatically converted to a LAAO - convertible amino acid?



Zwei Ansätze:

(A) OMT-Buffer + Caffeic acid + GSlt (Volume 500µl)

100 µl 5mM SAE
 50 µl 10x OMT-Buffer
 72,5 µl 0,1M GSlt (in 10,000)
 50 µl 10 mM Caffeic acid
 add to 500 µl ddH₂O

Im Ansatz
 10mM SAE
 1x OMT-Buffer
 0,002M GSlt
 1mM Caffeic acid

(B) OMT-Buffer

100 µl 5mM SAE
 50 µl 10x OMT-Buffer
 add to 500 µl ddH₂O

→ (A) + (B) zu je 50 µl aliquotiert, & in Flasche N₂ &
 bei -20°C gelagert

→ Ansatz für bestimmte Zeit bei 30°C inkubiert
 (aus -20°C genommen & in Kühlfach gelegt)

1 Aliquot rausgenommen @ 2
Tag 1 für Zeit (h)

10⁰⁰ Uhr

34

16⁰⁰ Uhr

28

Tag 2: Zeit (h)

13⁰⁰

7 h

15⁴⁵

4,25 h

17⁰⁰

3 h

19⁰⁰

1 h

20⁰⁰

0 h

Tag 2

11

11⁰⁰ Uhr

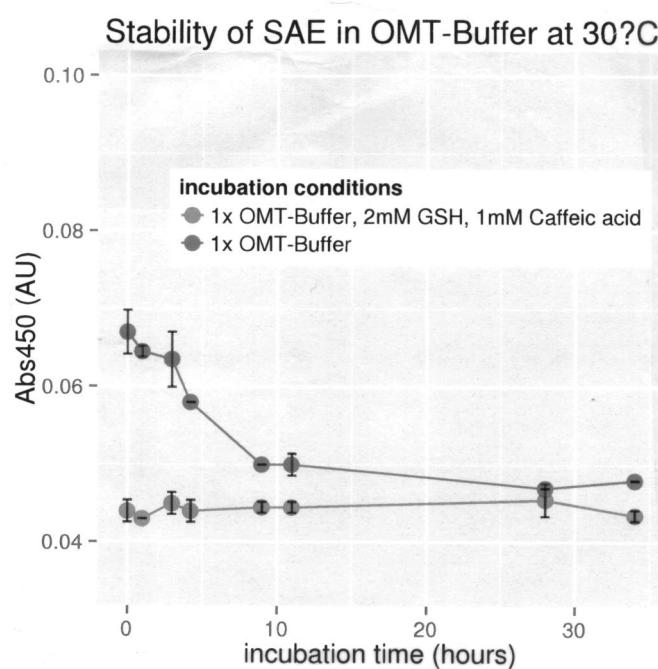
9

Tag 2

bei 20° Uhr \rightarrow je $2 \times 25 \mu\text{l}$ Probe von jeder Probe
auf MTP

$\rightarrow + 75 \mu\text{l}$ LAAO-Reagenz (5-10 U/ml HRP,
0.1 mg/ml TMB-HCl)
0.1 mg/ml LAAO

- \rightarrow 10 min @ 30°C inkubiert
- \rightarrow stoppt mit 50 μl 12 N H_2SO_4
- \rightarrow gewisse Absorption bei 450 nm



\rightarrow no change in
buffer with GSH &
Caffeic acid
 \rightarrow inhibitory effect
of GSH / CA on
LAAO?
 \rightarrow rapid degradation
of detectable SAE
at 30°C
 \rightarrow but SAE is not
degraded into other
LAAO convertible
amino acids!

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Einfluss verschiedener Substanzen
auf LAAO-Assay

WEB 243

- SA - Experimente
- (A) SAH in H₂O
 - (B) SAH in OM-buffer, CA, GST
 - (C) SAH mit 1mM SAE

A) SAH in H₂O

Endkonz. (Soll) SAH (µm)	V (ml) SAH (500µl)	V H ₂ O (ml)	A260	c(SAH) (Soll) (µM)
800	100	—	6.25	
250	50	50	3.1	
125	25	75	1.68	
50	10	90	0.67	
25	5	95	0.35	
10	2.5	97.5	0.15	
5	1	99	0.08	
0	—	100	0	

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B) SAH in 0.1M-Buf/Tr + GSH + Caffic Acid (wie OMT-Assay)

SAH (nmol)	V(SAHLGSH) (nl)	VBuf/Tr	V _{H2O}
500	10 (5nmol)	25 µl	65
250	50	↓	25
125	25	↓	50
50	10	↓	65
25	5	↓	70
10	2.5	↓	72.5
5	1	↓	74
0	0	↓	75

4x Buffer (800µl)

100µl 10x OMT Buffer
 100µl 10mM GSH Caffic Acid
 25 µl 0.1M SAH
 25 µl H₂O

C) SAH in 1mM SAE

SAH (nmol)	V(SAH+500µm) (nl)	V(5nmolSAE)	V _{H2O}
500	10µ (5nmol)	20 µl	70
250	50	↓	30
125	25	↓	55
50	10	↓	70
25	5	↓	75
10	2.5	↓	77.5
5	1	↓	79
0	0	↓	80

→ f 3x Testmix → 6 ml LAAO Reagenz

311 µl 193 µl/ml HRP

400 µl 1mg/ml LAAO

0.1ml 5mg/ml TMB-HCl

5,189 ml Parker (0,1M Trig HCl
pH 7,5)

WEB 251

Expression SOMT 2 aus

- ① C43 (DE3) pLysS pET20b SOMT2
 ② Origami (DE3) pET32a SOMT2

- in Autoinduktionsmedium (ZYP5052) [75ml] mit EK angeimpft
- über Nacht @ 37°C / 140 rpm
 - ① mit Chloramphenicol (15µ/ml) & Ampicillin (200µ/ml)
 - ② mit Tetracycline (10µ/ml), Kanamycin (50µ/ml) & streptomycin (200µ/ml)
- OD_{600nm} nach 24h ablesen

	OD ₆₀₀
①	10.44
②	4.38
- 200µl Probe abgefiltert & mit S-PER® anflocken
- für lösliche & unlösliche Fraktion

③ Kulturen gesammelt
 → je 1ml überstand abgenommen & Medium fraktioniert

C. Medium Fraction

Analysis of the medium may be instructive when performing prolonged inductions, when expecting protein export, or target protein leakage from the cells is suspected. Many recombinant proteins that are directed to the periplasm often also end up in the medium through a poorly understood "leakage" phenomenon. In most cases, target protein in the medium is due to damage of the cell envelope rather than true secretion (Stader et al., 1990).

1. Add 40 ml of the culture to a pre-weighed tube and harvest the cells by centrifugation at 10,000 × g for 10 min at 4°C.
2. Carefully transfer 1 ml supernatant to a microcentrifuge tube. Avoid removing any cell pellet. The remaining medium can be saved for further assays. Place the cell pellet on ice until used to prepare the periplasmic fraction.
3. Concentrate the medium sample by either TCA precipitation or spin filter concentration, as follows:

Note: Alternatively, the ProteoExtract™ Protein Precipitation Kit (Cat. No. 539180) quickly and efficiently concentrates and prepares protein from a variety of sources.

- a) Add 100 µl (1/10 volume) 100% TCA (w/v) to 1 ml medium and vortex for 15 s.
- b) Place on ice for a minimum of 15 min.
- c) Centrifuge at 14,000 × g for 10 min.
- d) Remove and discard the supernatant.
- e) Resuspend the pellet twice with 100 µl acetone, by adding the acetone, mixing, and then spinning for 5 min (14,000 × g). Remove the acetone and acetone residue by air-drying. Allow the final pellet to air dry thoroughly (leave the tube open on the bench top or in a hood for about 60 min, or spin briefly in a Speed-Vac® evaporator (Thermo Savant)). The presence of residual acetone will make resuspension more difficult.
- f) Add 100 µl of 1X PBS (sample concentration factor = 10X) and 100 µl of 4X SDS Sample buffer (Cat. No. 70607-3).
- g) Immediately heat for 3 min at 85°C to denature the proteins and then store at -20°C until SDS-PAGE analysis.

5) 50µl Sx 3ml

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② Keine Periplasma-Faktion isoliert, da bei phys/phys Stärme die Zellwand lyse wird

D. Periplasmic Fraction

When using vectors with pelB or DsbA/C signal sequences, target proteins may be directed to the periplasmic space. The leader is necessary, but not sufficient for export into the periplasm. Cleavable signal sequences that mediate the export of *E. coli* proteins to the periplasm can be classified as either Signal Recognition Particle (SRP) dependent or SRP independent. Signal Non-SRP dependent signal sequences like those found in the PhoA and MalE promote export by a post-translational mechanism (Huber, et al., 2005). However, it is clear that translocation also can depend on the mature domain of the target protein, which is recognized by SecB, the major chaperone of export. The following osmotic shock protocol (Ausubel et al., 1989) is a simple method of preparing the periplasmic fraction from λDE3 lysogens. However, osmotic shock is not appropriate for use with host strains containing pLYS or pLYSE because T7 lysozyme damages the cell wall, resulting in disruption of the inner membrane and release of the cytoplasmic fraction.

③ Zellen lyset: (per System manual Protocoll)
- Zellpellet gesetzen in R. N₂ → aufgetaut

r.Lysozyme™ Solution and freeze/thaw treatment

This protocol isolates soluble protein using r.Lysozyme (Cat. No. 71110-3) and a freeze/thaw of the cell pellet. If the bacterial strain contains a plasmid encoding lysozyme (e.g., pLYS or pLYSE), additional lysozyme treatment is not necessary.

- If medium and periplasmic fractions are not needed, harvest cells from liquid culture by centrifugation at 10,000 × g for 10 min using a pre-weighted centrifuge tube. Decant and allow the pellet to drain, removing as much liquid as possible. Determine the wet weight of the pellet.
- Using the pellet from step 1 or from step 5 "Periplasmic Fraction", freeze the pellet completely at -20°C or -70°C.
- Completely thaw and resuspend the cell pellet by pipetting up and down or gentle vortexing in room temperature lysis buffer (50 mM Tris-HCl or NaH₂PO₄, 5% glycerol, 50 mM NaCl, pH 7-8) using 7 ml lysis buffer per gram of wet cell paste. Add protease inhibitors if desired.

Note: DO NOT add r.Lysozyme solution until a uniform cell suspension has been obtained. The freeze/thaw step ruptures the cell membrane allowing r.Lysozyme to access the cell wall. If Lysozyme is added prematurely, the immediate viscosity increase will make complete cell resuspension difficult and incomplete lysis may result.

- Add approximately 7.5 KU of r.Lysozyme per 1 ml of lysis buffer (45-60 KU/gram cell paste).

- User Protocol TE055 Rev. A, 2011-06-14 Page 1 of 2
- Optional: Add 1 µl (25 U) of Benzonase® Nuclease per 1 ml of lysis buffer used for resuspension. Benzonase is not recommended for nucleic free preparations. Other methods of reducing viscosity include shearing or precipitating the nucleic acids (Burgess, 1991).
 - Incuab the cell suspension on a shaking platform or rotating mixer at a slow setting for 10-20 min at room temperature. Longer incubation time may be required if lysis is performed at 4°C. Determine empirically.
 - Note: If Benzonase is added, the extract should not be viscous at the end of incubation.
 - Resuspend cell debris by centrifugation at 4°C for 20 min at 16,000 × g. The pellet may be used to isolate the "Insoluble Cytoplasmic Fraction" page 22.
 - Transfer the supernatant to a clean tube for analysis and/or purification. Combine an equal volume of the supernatant with 4X SDS Sample Buffer (Cat. No. 70607-3) for SDS-PAGE Analysis. Additional analysis and/or purification can be performed with the remainder of the soluble extract. Maintain clarified extracts on ice for short-term storage (a few hours) or freeze at -20°C until needed.
 - Immediately heat the sample containing SDS for 3 min at 85°C to denature proteins and store at -20°C until SDS-PAGE analysis.

Canada Germany United Kingdom and Ireland All Other Countries
78-8470 Tel 0800 100 3496 UK Telephone 0800 622935 www.merk-biosciences.com
info@merk-biosciences.de Ireland Toll Free 1800 409445 bioscience@merk-biosciences.com
customer.service@merk-biosciences.co.uk emdchemicals.com

- Zellen resuspendiert in 50ml Tris/HCl, 5% Glycerol,
(7ml Rührer/g Zellen) 50ml NaCl pH 7.1
- zu Oxygarm (DE3) füge nach 50ml Lysozyme jehe
- 15 min @ 37°C inkubiert
- 15 min @ 16000×g zentrifugiert
- Überstand abcentrifugiert und A²⁸⁰ bestimmt

	A ²⁸⁰	A ²⁶⁰ /A ²⁸⁰
① C63	3,49	1,67
② Oxygarm	5,18	1,87

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Aktivitäts test

100 µl 10x OMT-Bath

4 µl 250 mM Caffeic Acid in DMSO

200 µl 5 mM (81%) SAM

100 µg Rohproteinextrakt ($\frac{28 \mu\text{l}}{26 \mu\text{l}}$ original)ad 10 1000 µl ddH₂O

→ 1h @ 30°C inkubiert

→ 2x mit 500 µl EtOAc (+1% Formic Acid)

abwaschen → reingt, an SpeedVac abgedampft

→ an $\frac{100}{50}$ µl MeOH aufgezogene

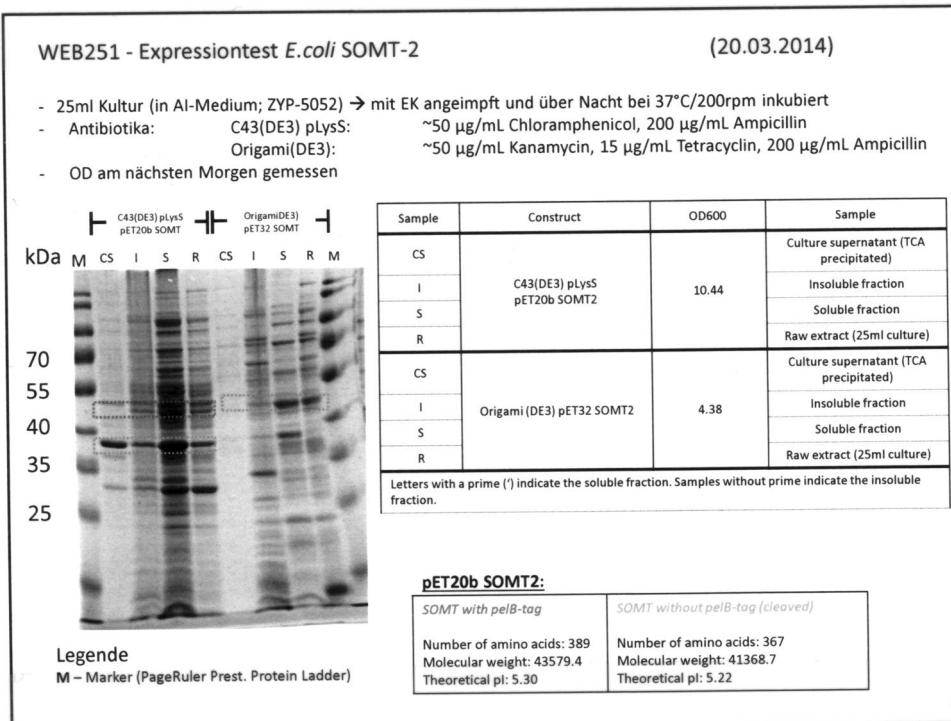
→ DC in EtOAc: Hexan 1:2 + 1% Empfohlene

b CHCl₃: Methanol: Formic Acid (85:17:1)

→ keine Aktivität

gel:

- Mediumfraktion (Origami, C43M)
- löslich & unlösliche Fraktion



WB 242

Einfluss der LAAO-Konzentration auf SAH-Oxidation

- 4 verschiedene LAAO-Reagenzien mit:

~~100 μl/ml HRP~~

- 10 U/ml HRP

- 0.083 mg/ml TMB-HCl

in 0.1 M Tris/HCl pH 7.5

→ mit verschiedenen LAAO-Konzentrationen

① + 0.01 mg/ml LAAO

② 0.05 mg/ml

③ 0.1 mg/ml

④ 0.25 mg/ml

- 1 mg/ml stock solution of LAAO in 0.1M Tris/HCl

+ 100 mM KCl

pH 7.5

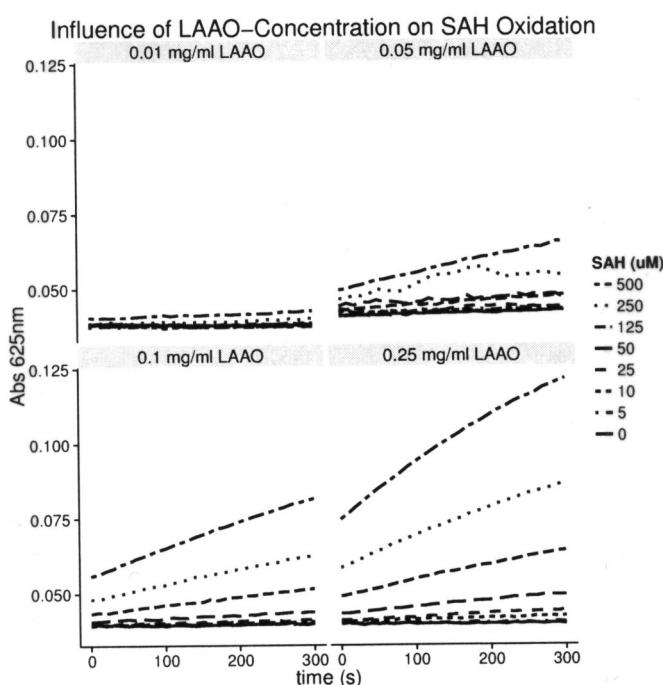
→ LAAO Konzentration

bei A²⁸⁰ bestimmt

→ 1 mg/ml

= OD²⁸⁰ von 1

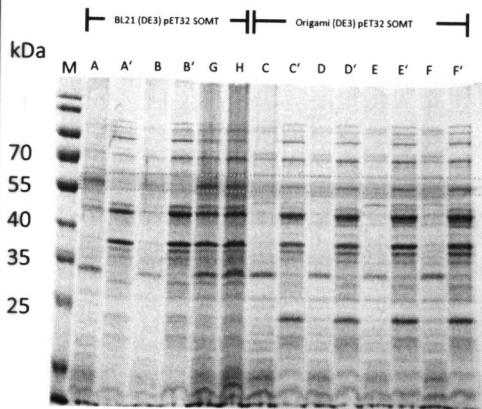
(Anwahne)



WBS247

Expressions test SOMT2

- 3 ml culture in ZYP5052 autoinduction medium
- Antibiotics: BL21(DE3) → 200 µg/mL Ampicillin
Origami (DE3) → 200 µg/mL Ampicillin, 100 µg/mL Kanamycin, 10 µg/mL Tetracyclin
- Inoculated with single colony
- growth at 30 and 37°C at 200 rpm over night



Sample	Construct	Temperature (°C)	Time (h)
A	BL21(DE3) pET32 SOMT2	30	20.5
B		37	16.5
G		30	22.5
H		37	18.5
C	Origami(DE3) pET32 SOMT2	30	20.5
D		37	16.5
E		30	22.5
F		37	18.5

Letters with a prime (') indicate the soluble fraction. Samples without prime indicate the insoluble fraction.

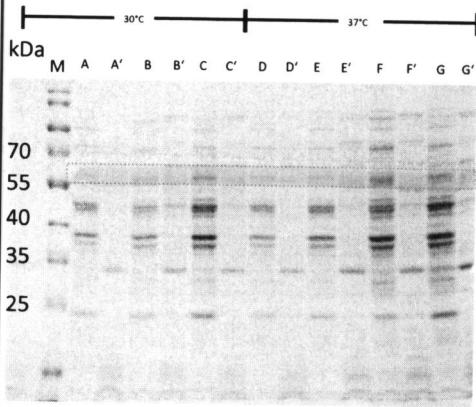
Legende

M - Marker (PageRuler Prest. Protein Ladder)

pET32a SOMT2:
SOMT with TrxA-tag

Number of amino acids: 523
Molecular weight: 58128.6
Theoretical pl: 5.36

- 3 ml culture in ZYP5052 autoinduction medium
- Antibiotics: Origami (DE3) → 200 µg/mL Ampicillin, 100 µg/mL Kanamycin, 10 µg/mL Tetracyclin
- Inoculated with single colony
- growth at 30 and 37°C at 200 rpm over night



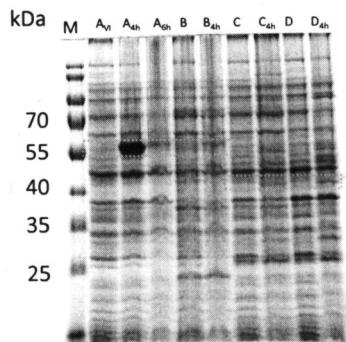
Sample	Construct	Temperature (°C)	Time (h)
A	Origami(DE3) pET32 SOMT2	30	22.5
B			25.5
C			31
D	Origami(DE3) pET32 SOMT2	37	16.5
E			18.5
F			21.5
G			27

Letters with a prime (') indicate the INSOLUBLE fraction. Samples without prime indicate the SOLUBLE fraction.

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W.B. M

- 3 ml culture in LB medium
- Antibiotics: BL21(DE3) → 200 µg/mL Ampicillin
Origami (DE3) → 200 µg/mL Ampicillin, 100 µg/mL Kanamycin, 10 µg/mL Tetracycline
C43(DE3) pLysS → 50 µg/mL Chloramphenicol, 200 µg/mL Kanamycin
C41(DE3) pLysS → 50 µg/mL Chloramphenicol, 200 µg/mL Kanamycin
- Inoculated with single colony
- growth at 37 and 200 rpm until OD₆₀₀ between 0.6 and 1
- Cooling on ice to ~30°C
- Addition of 1 mM IPTG and induction at 30°C and 200rpm

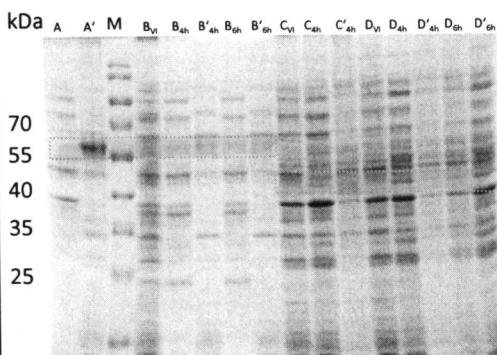


Sample	Construct	Temperature (°C)
A	BL21(DE3) pET32 SOMT	30
B	Origami (DE3) pET32 SOMT	
C	C41 (DE3) pLysS pET20b SOMT2	
D	C43 (DE3) pLysS pET20b SOMT2	

Letters with a prime (') indicate the INSOLUBLE fraction. Samples without prime indicate the SOLUBLE fraction.

Subscripts: V – pre-induction sample
4h – 4hours post IPTG induction
6h – 6 hours post IPTG induction

- Same as above but nicer



Sample	Construct	Temperature (°C)
A	BL21(DE3) pET32 SOMT	30
B	Origami (DE3) pET32 SOMT	
C	C41 (DE3) pLysS pET20b SOMT2	
D	C43 (DE3) pLysS pET20b SOMT2	

Letters with a prime (') indicate the INSOLUBLE fraction. Samples without prime indicate the SOLUBLE fraction.

Subscripts: V – pre-induction sample
4h – 4hours post IPTG induction
6h – 6 hours post IPTG induction

pET20b SOMT2: SOMT with pelB-tag

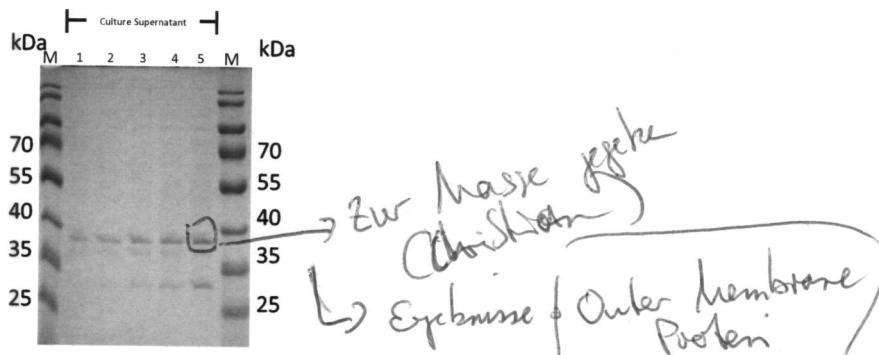
Number of amino acids: 389
Molecular weight: 43579.4
Theoretical pl: 5.30

~~WEB2511~~ WEB252

WEB252 – Reinigung von SOMT-2 aus Kulturüberstand von C43(DE3) pLysS (04.04.2014)

- 250 ml Kultur (in AI-Medium; ZYP-5052) → mit EK angeimpft (1700 Uhr) und über Nacht bei 37°C/200rpm inkubiert
- Antibiotika: ~50 µg/mL Chloramphenicol, 200 µg/mL Ampicillin
- Am nächsten Morgen Proben genommen → OD gemessen, pH im Kultur-Überstand (nach zentrifugation), von 1mL Kulturüberstand TCA-Fällung und auf 10% SDS-Gel

Sample	time (h)	Total growth time (measured 1:10 in H ₂ O)	pH (media supernatant)	TCA-Pellet size from medium (optical)	Rpm
1	10.30	17.5	4.9	-	200
2	12.30	19.5	5.435	+	200
3	14.30	21.5	6.9	++	200
4	16.30	23.5	8.0	+	240
5	18.30	25.5	9.46	+	240



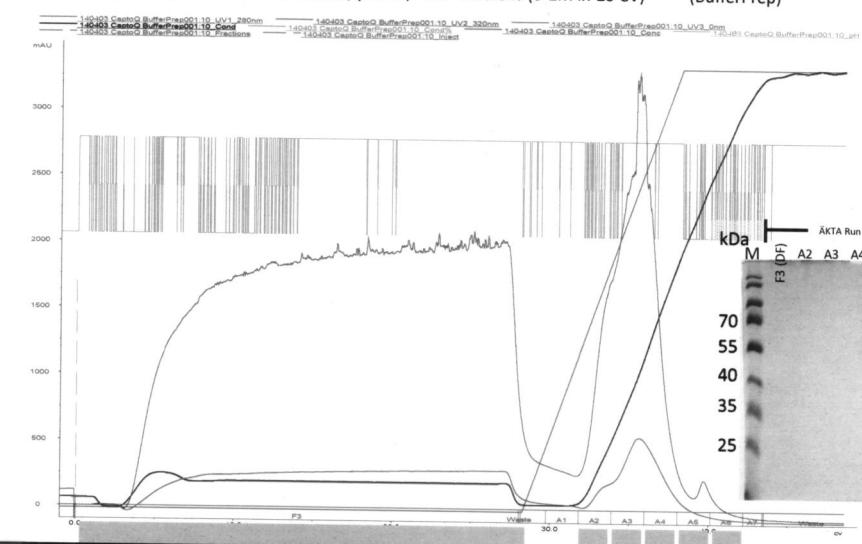
WEB252 – IEX-Run 1

(04.04.2014)

- 5 mL Kulturüberstand (21.5 h sample) + 10 mL ddH₂O + 10 mL 50 mM BisTris pH 6.5
→ ÄKTA, Anionentauscher (HiTrap Capto Q, 1ml)
- Puffer A: 20 mM BisTris/HCl, pH 6.5
Puffer B (elution): 20 mM BisTris/HCl, pH 6.5, NaCl-Gradient (0-1M in 10 CV)

(BufferPrep)

(BufferPrep)

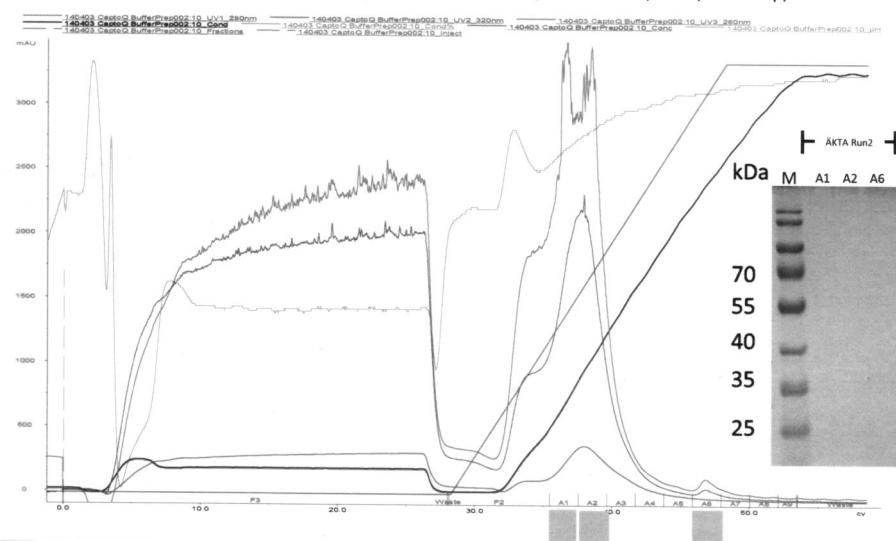
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WEB252 – IEX-Run 2

(04.04.2014)

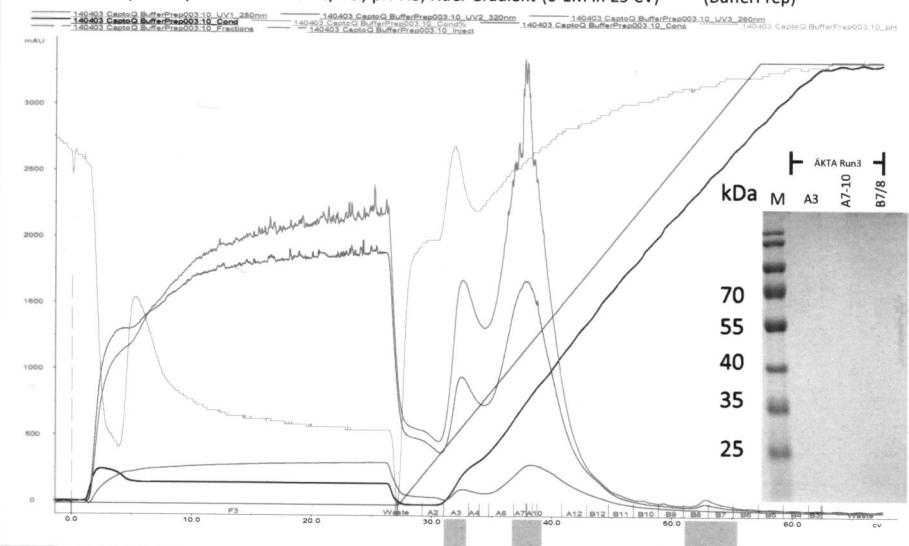
- 5 mL Kulturüberstand (21.5 h sample) + 10 mL ddH₂O + 10 mL 50 mM BisTris pH 7.5
→ ÄKTA, Anionentauscher (HiTrap Capto Q 1ml)
- Puffer A: 20 mM BisTris/HCl, pH 7.5
Puffer B (elution): 20 mM BisTris/HCl, pH 7.5, NaCl-Gradient (0-1M in 20 CV) (BufferPrep)
(BufferPrep)



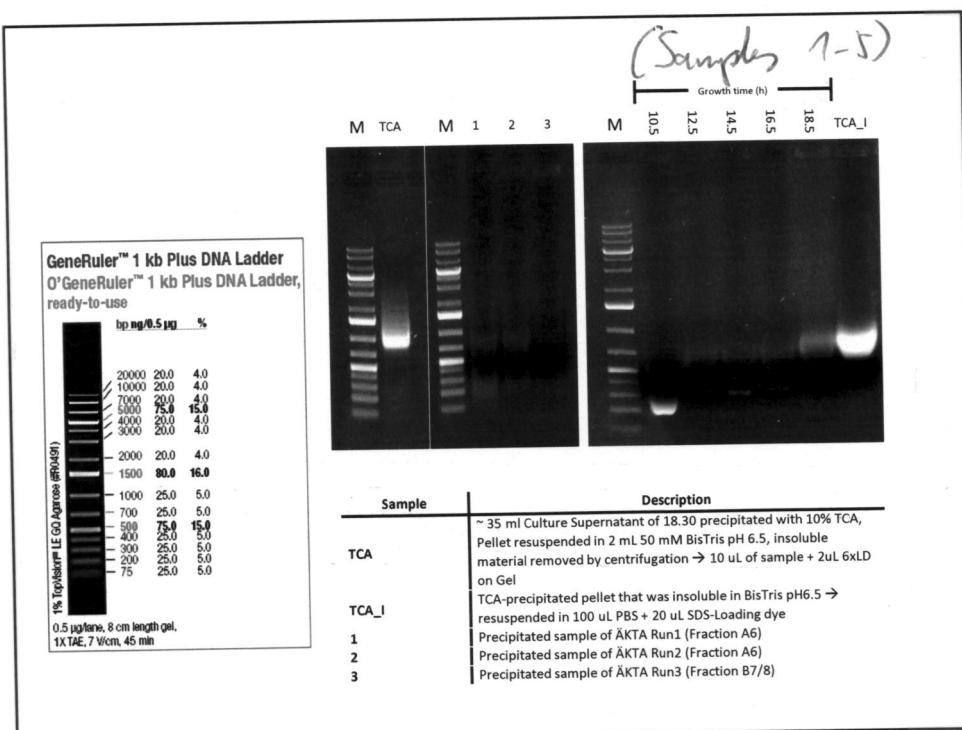
WEB252 – IEX-Run 3

(04.04.2014)

- 5 mL Kulturüberstand (23.5h sample) + 20 mL ddH₂O
→ ÄKTA, Anionentauscher (HiTrap Capto Q 1ml)
- Puffer A: 20 mM BisTris/HCl, pH 7.5
Puffer B (elution): 20 mM BisTris/HCl, pH 7.5, NaCl-Gradient (0-1M in 25 CV) (BufferPrep)
(BufferPrep)

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- kein Protein im SDS-gel trotz Pellet
 bei TCA - Fällung → wesentlich höher
 Absorption bei 260 nm als bei 280 nm
 → DNA? → Agarose gel von Röten gemacht



time	sample	OD ⁶⁰⁰ (1:10 in H ₂ O)	pH (media supernatant)	Activity	TCA-Pellet size from medium	Rpm
10.30	1	4.9	5.6	?	-	200
12.30	2	5.435	5.7	?	+	200
14.30	3	6.9	6.0	-	++	200
16.30	4	8.0	6.3	?	+	240
18.30	5	9.46	6.6	?	+	240

Füllung von ÄKTA Fraktionen:

- + 1/10 Volumen 100% TCA → 15min on ice
- 10min @ 14000xg, 4°C
- 2xwash with 200 uL Acetone
- dry & resuspended in 100 uL PBS → into 1.5 ml tube → add 20 uL SDS-Buffer
- 5 min @ 95°C → @ -20°C for SDS-PAGE

Aktivitätstest (Probe von 1430) → 500 uL Gesamtvolumen (250 uL Mediumüberstand)

30 min @ 25°C inkubiert
 2 x mit 200 uL EtOAc + 1% FA extrahiert (15s)
 ORG: Phasen VEREINIGT
 abgedampft in speedvac
 in 100 uL MeOH aufgenommen

TLC

	M	SN1	SN2	Sn3	Sn4	Sn5	M	2a1	2a2	2a6
m	Df1	1a2	1a3	1a4	1a5	1a6	M	3a3	3a7-10	3b78

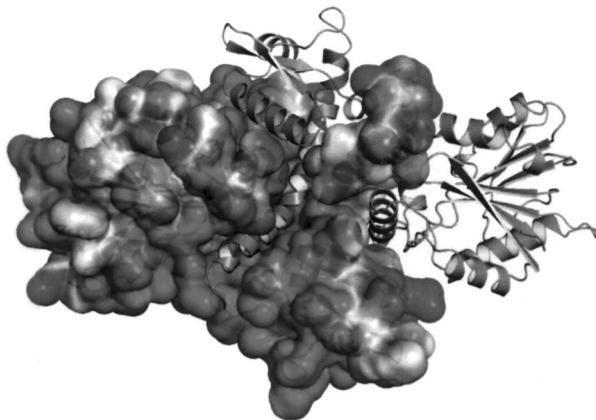
07.06.2014

HiTrap Capto Q 1ml mit 2CV 200 mM KPi, 8M Urea, 2M NaCl pH 8.5, 1 mM DTT gewaschen

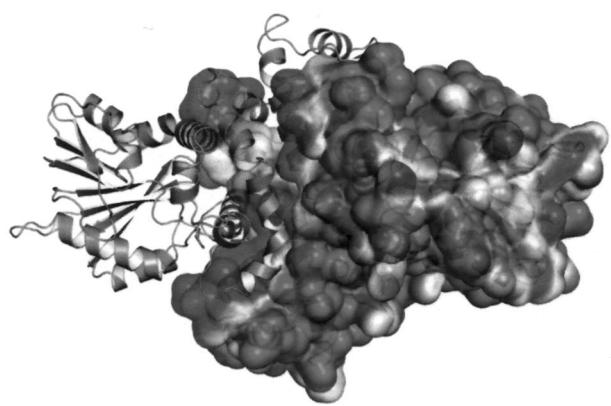
→ Eluat gefällt mit TCA → Gel

1 WEB254 – Oberflächenladung von SOMT2

- SOMT2-Modell von Phyre Server → Oberflächenladung mit APBS Tools berechnet.
- SOMT2-Dimer anhand von IOMT gemodelt (IOMT → Symexp → SOMT dupliziert → Duplicate mit IOMT Dimer aligned → IOMT Objekt gelöscht)



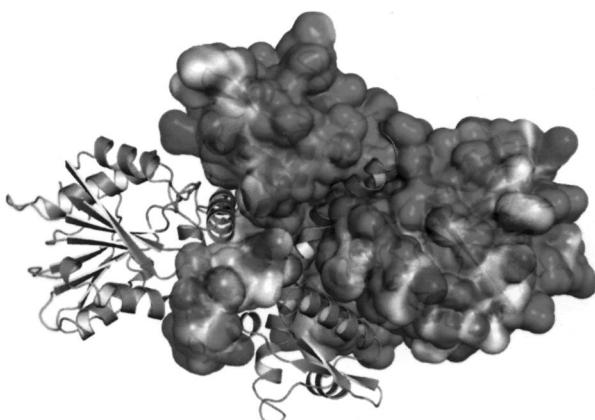
-1.000 1.000



-1.000 1.000

A) SOMT2 Dimer (model), ChainA mit Oberflächenladung, ChainB in Grün

- Oberfläche größtenteils negativ geladen (Andere Ex)
- Dimersärumpf domäne positiv geladen



-1.000 1.000

WEB255 – Biotransformation von Naringenin

250 µM Naringenin in AI-Medium (ZYP5052)

Ansätze:

- 250 µM Naringenin (aus 250 mM stock in DMSO)
- In ZYP5052-Medium
- Konstrukte:
 - o *E.coli* C43(DE3) pET20b SOMT2 (~200 µg/mL Ampicillin)
 - o *E.coli* C43(DE3) pLysS pET20b SOMT2 (~200 µg/mL Ampicillin, 50 µg/mL Chloramphenicol)
 - o *E.coli* Origami(DE3) pET32a SOMT2 (~200 µg/mL Ampicillin, 100 µg/mL Kanamycin, 10 µg/mL Tetracyclin)
 - o *E.coli* DH5α pET20b SOMT2 (~200 µg/mL Ampicillin)
- mit jeweils Einzelkolonie angeimpft
- Wachstum über Nacht @37°C und 200 rpm

No.	Sample	OD600 (harvesting time)
1	C43(DE3) + Naringenin	11.02 (11.00 Uhr)
2	C43(DE3)pLysS + Naringenin	10.82 (11.00 Uhr)
3	DH5α + Naringenin	11.68 (11.00 Uhr)
4	Origami (DE3) + Naringenin	8.8 (18.00Uhr)
5	C43(DE3) NO Naringenin	11.16

1. 1ml Cell suspension pelleted (10.000xg, RT, 3 min)
2. Cell pellet and supernatant extracted 2x with 500 µL EtOAc + 1% Formic Acid
3. Organic phases reduced in SpeedVac (45°C)
4. Residue resuspended in MeOH
5. Centrifuged at 10.000xg for 10 min
6. Analysis by TLC and HPLC

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WEB256 - Subfractionation of periplasmic fraction of C43(DE3) pET20b SOMT2

- 3 mL Kulturen in ZYP5052-Medium (Autoinduction, ~200 µg/mL Ampicillin)
- mit jeweils Einzelkolonie *E.coli* C43(DE3) pET20b SOMT2 angeimpft
- Wachstum über Nacht @37°C und 200 rpm

No.	Sample	OD600 (11.00 Uhr)	Samplevolume	B-PER
1	C43(DE3) / 08.04.14	11.165	1 mL for A) [Subfractionation]	558 µL
2	C43(DE3) / 09.04.14	12.75	1 mL	628 µL

A) Subfractionation of *E.coli* C43(DE3):

1. 1ml Suspension → 3 min @ 10.000xg
 - a. Pellet for Preparation of Periplamic Fraction
 - b. Supernatant → Medium fraction
2. Periplasmic fraction
 - a. Pellet from 1) resuspended in 30 mL (1mL for 2) 30 mM Tris-HCl, 20% sucrose, pH 8
 - b. added 60 µl (2µl for 2) 0.5 M EDTA, pH 8 (final concentration of 1 mM)
 - c. 10 min at RT on shaker (small stir bar in 1.5mL tube for 2)
 - d. Pellet at 10.000xg /4°C for 10 min
 - i. Discard supernatant
 - e. Resuspended pellet in 30 mL (1mL for 2) 5 mM ice-cold MgSO₄
 - f. On shakerplatform 10min on ice (stirring with small stir bar in 1.5mL tube for 2)
 - g. Pellet at 10.000xg /4°C for 10 min
 - i. Precipitated supernatant with TCA (add 1/10 Volume of 100% TCA)
 - ii. Used Pellet for analysis of soluble and insoluble fraction

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WEB256 – Subfractionation of periplasmic fraction of C43(DE3) pET20b SOMT2 (08.04.2014)

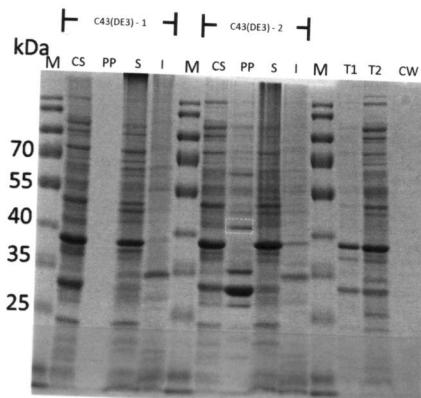
- 3 ml Kultur (in Al-Medium; ZYP-5052) → mit EK angeimpft (1700 Uhr) und über Nacht bei 37°C/200rpm inkubiert
- Antibiotika: 200 µg/mL Ampicillin
- Am nächsten Morgen Proben genommen → OD gemessen
- 1 mL Kultur laut pET-Manual fraktioniert in *medium*, *periplasmic*, *soluble* und *insoluble* fraction

Sample	date	OD ⁶⁰⁰ (measured 1:10 in H ₂ O)	Comment
C43(DE3) - 1	08.04.2014	11.165	Bad TCA precipitation (too much volume)
C43(DE3) - 2	09.04.2014	12.75	Good sample

Legende

M – Marker (PageRuler Prest. Protein Ladder)
 CS – culture supernatant (TCA precip.)
 PP – periplasmic fraction (TCA precip.)
 S – soluble fraction
 I – insoluble fraction
 T1 – TCA-precipitated Medium Fraction of C43(DE3) pLysS pET20b SOMT2 (taken up in 50 mM BisTris pH6.5)
 T2 – remaining pellet from T1 (taken up in PBS + SDS-Sample buffer)
 CW – HiTrap Capto Q 1mL column washed with 3 CV of 2 M NaCl + 8 M Urea In 200 mM KPi pH 8 (TCA precipitated, see WEB252)

All samples prepared from 1 mL culture suspension as described in the pET Manual



- Periplasma Fraktion (2) zeigt Bande bei ~ 41 kDa
- vielleicht ist das SOMT2 ?
- unsolubile die Bande → vielleicht bringt mess-peptid-Eferprint was

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U58

WEB258

Expression SOMT in C43(DE3) pET20b und Origami(DE3) pET32a in TB

Origami(DE3) pET32a SOMT2

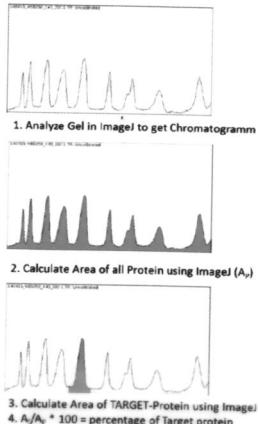
- 20 mL culture in TB-Medium (+200 µg/ml Amp, 100 µg/mL Kan, 10 µg/mL Tet)
- Inoculation with single colony
- Incubation at 37°C, 220 rpm over night
- Measured OD⁶⁰⁰
- Inoculated 75 mL culture in TB-medium with 800 µL pre-culture
- Incubation at 37°C for 1.5 h → then incubation at 30°C (see table below)

Time	OD ⁶⁰⁰	B-PER	Comments
1000	6.53		Inoculation of 75 mL TB culture
1130	0.66		Incubation at 30°C and 240 rpm
1217	0.687	35	Induction (1mM IPTG) → 3 different culture aliquots (20, 30, 37°C induction) [each ~20mL]

C43(DE3) pET20b SOMT2

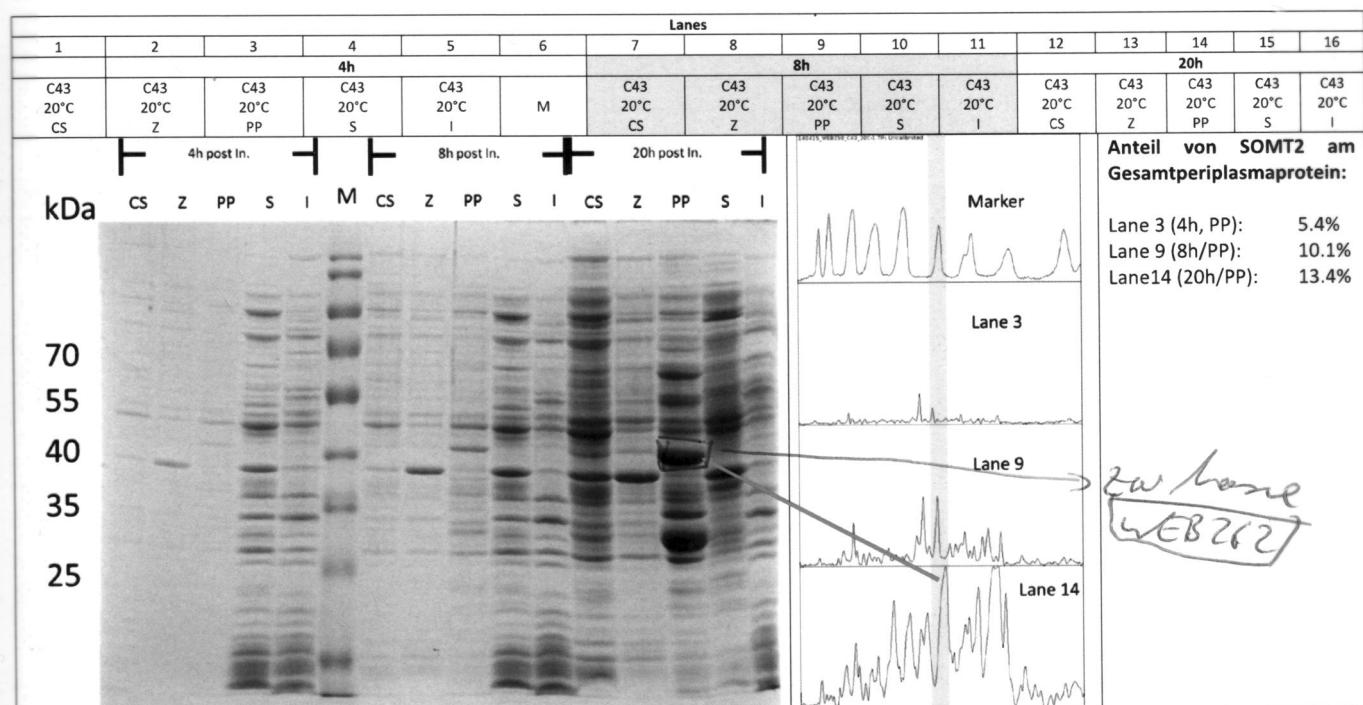
- Non-inducing 3 mL culture in LB-Medium (+200 µg/ml Amp)
- Inoculation with single colony
- Incubation at 37°C, 220 rpm over night
- Measured OD⁶⁰⁰
- Inoculated 75 mL culture in TB-medium with 800 µL pre-culture
- Incubation at 37°C for 1.5 h → then incubation at 30°C (see table below)

Time	OD ⁶⁰⁰	B-PER	Comments
1000	6.12		Inoculation of 75 mL TB culture
1130	0.4		Incubation at 30°C and 240 rpm
1217	0.7	35	Induction (1mM IPTG) → 3 different culture aliquots (20, 30, 37°C induction) [each ~20mL]

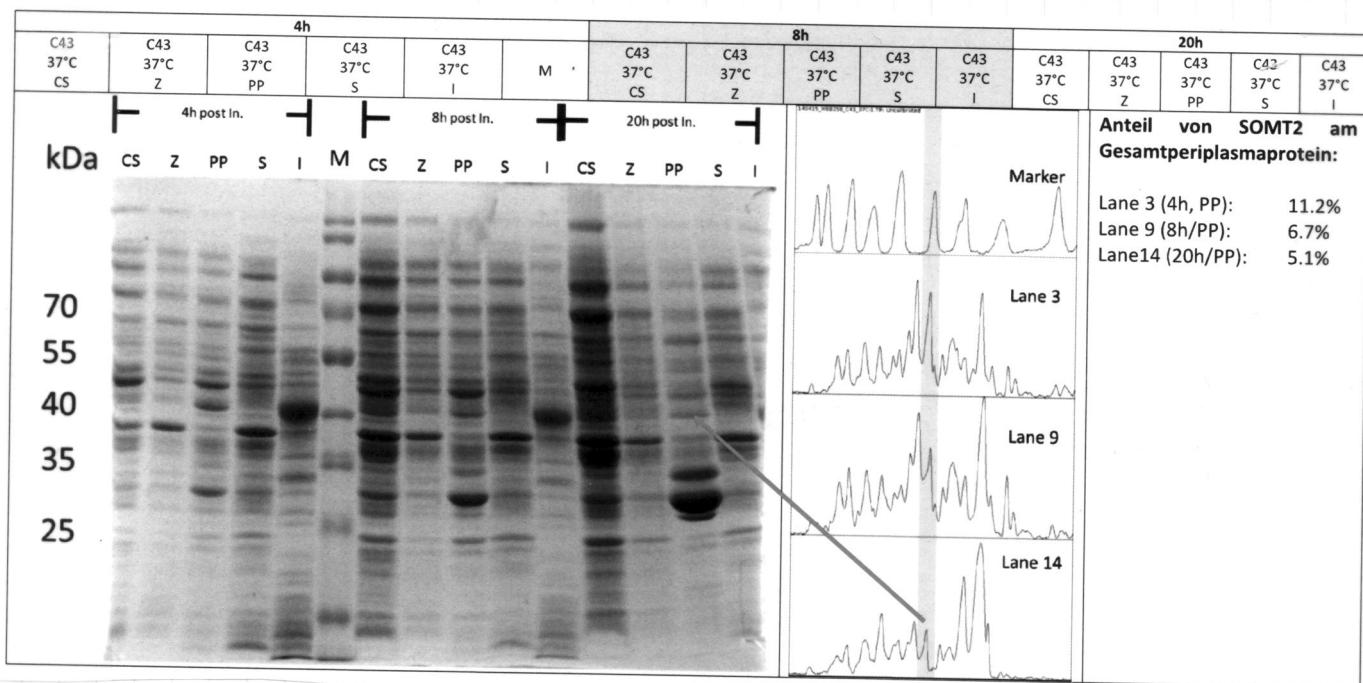
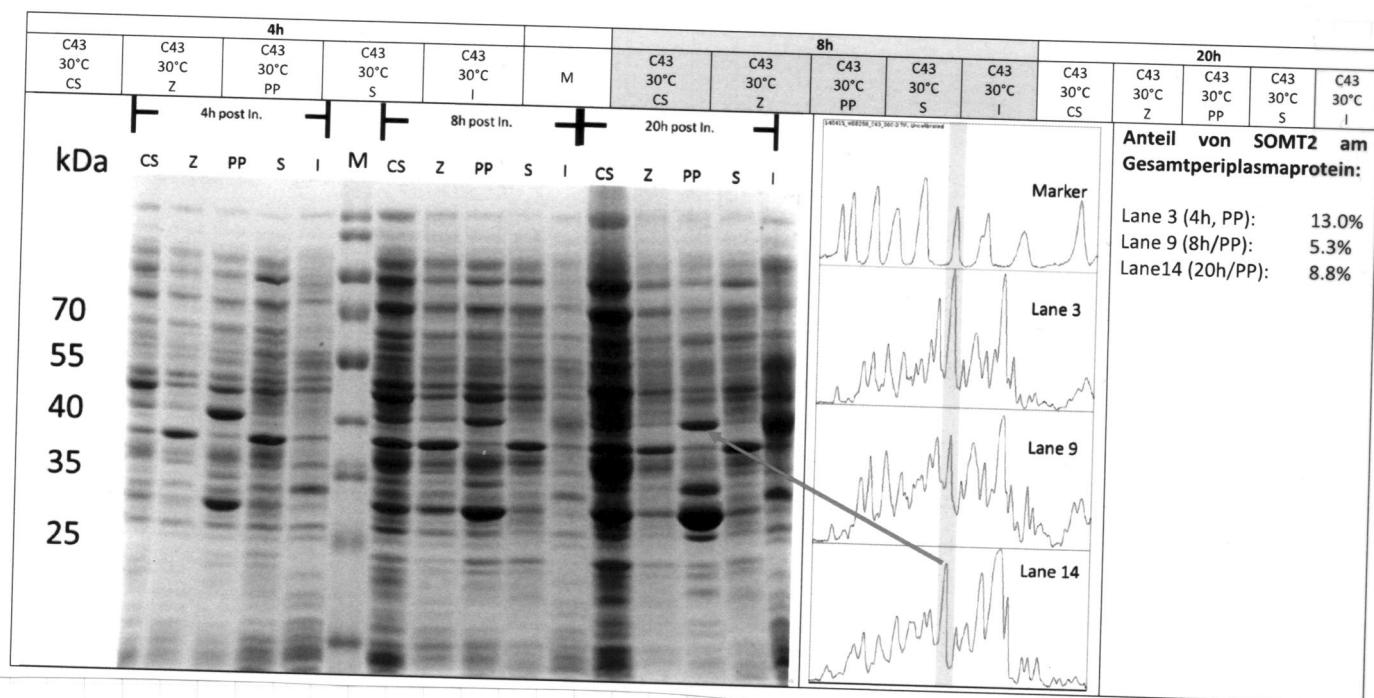
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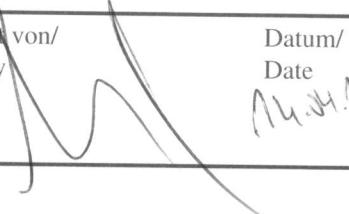
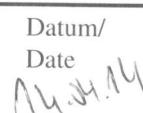
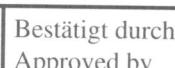
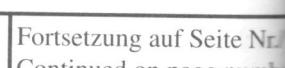
Sample	Temperature	Time	OD ⁶⁰⁰	B-PER (μ l)	Subfractionation (y/n)						Comments
					Medium [CS]	Periplasm (Sucrose) [Z]	Periplasm (cold water) [PP]	Soluble [S]	Insoluble [I]		
C43(DE3)	20	1617	2.16	108	Y	Y	Y	Y	Y		
C43(DE3)	30	1617	8.39	418	Y	Y	Y	Y	Y		
C43(DE3)	37	1617	8.34	418	Y	Y	Y	Y	Y		
Origami(DE3)	20	1617	1.21	60.5	Y	Y	Y	Y	Y		
Origami(DE3)	30	1617	1.89	94.5	Y	Y	Y	Y	Y		
Origami(DE3)	37	1617	2.13	106.5	y	y	Y	y	y		
C43(DE3)	20	2030	5.9	295	Y	Y	y	Y	Y		
C43(DE3)	30	2030	15.2	760	Y	Y	Y	Y	Y		
C43(DE3)	37	2030	14.1	705	Y	Y	Y	Y	Y		
Origami(DE3)	20	2030	1.7	85	Y	Y	Y	Y	Y		
Origami(DE3)	30	2030	2.9	145	Y	Y	Y	Y	Y		
Origami(DE3)	37	2030	9.1	455	Y	y	Y	y	y		
C43(DE3)	20	0730	25.7	1285	Y	Y	y	Y	Y		
C43(DE3)	30	0730	19.1	955	Y	Y	Y	Y	Y		
C43(DE3)	37	0730	21.7	1055	Y	Y	Y	Y	Y		
Origami(DE3)	20	0730	2.44	122	Y	Y	Y	Y	Y		
Origami(DE3)	30	0730	19.4	970	Y	Y	Y	Y	Y		
Origami(DE3)	37	0730	19.0	950	y	y	Y	y	y		

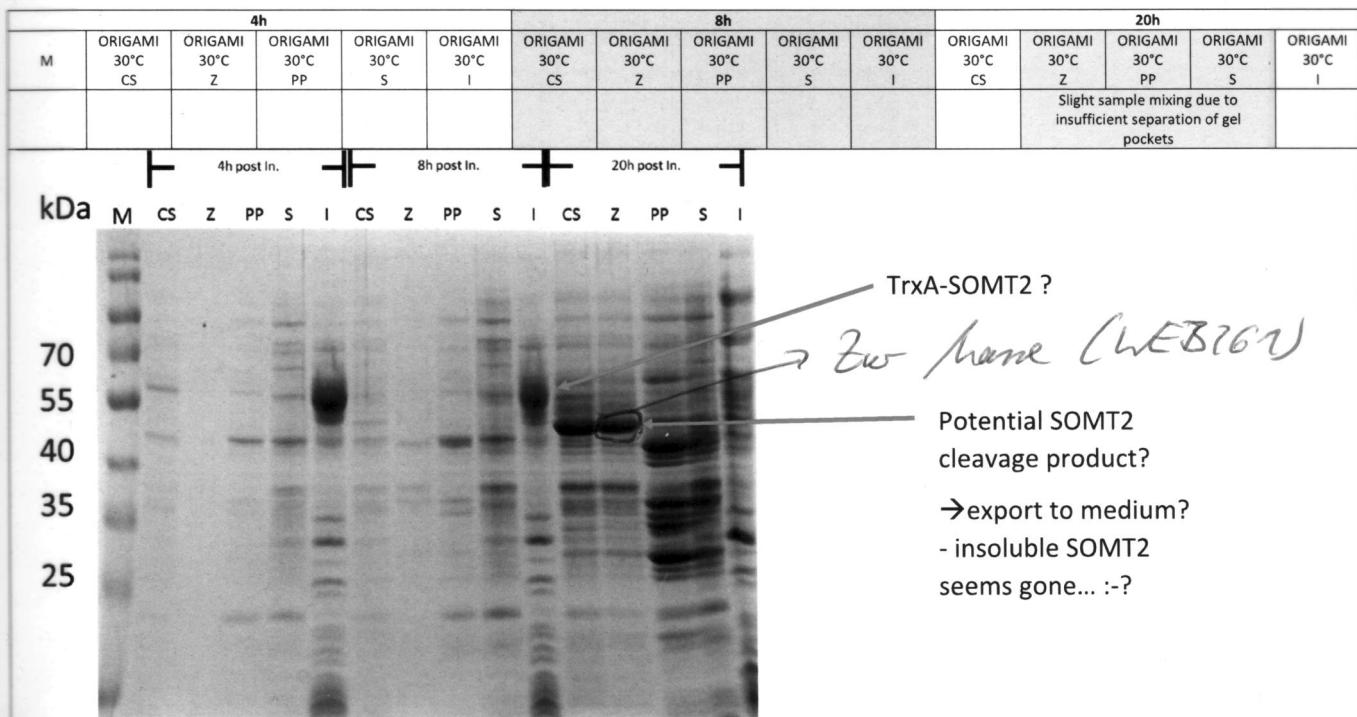
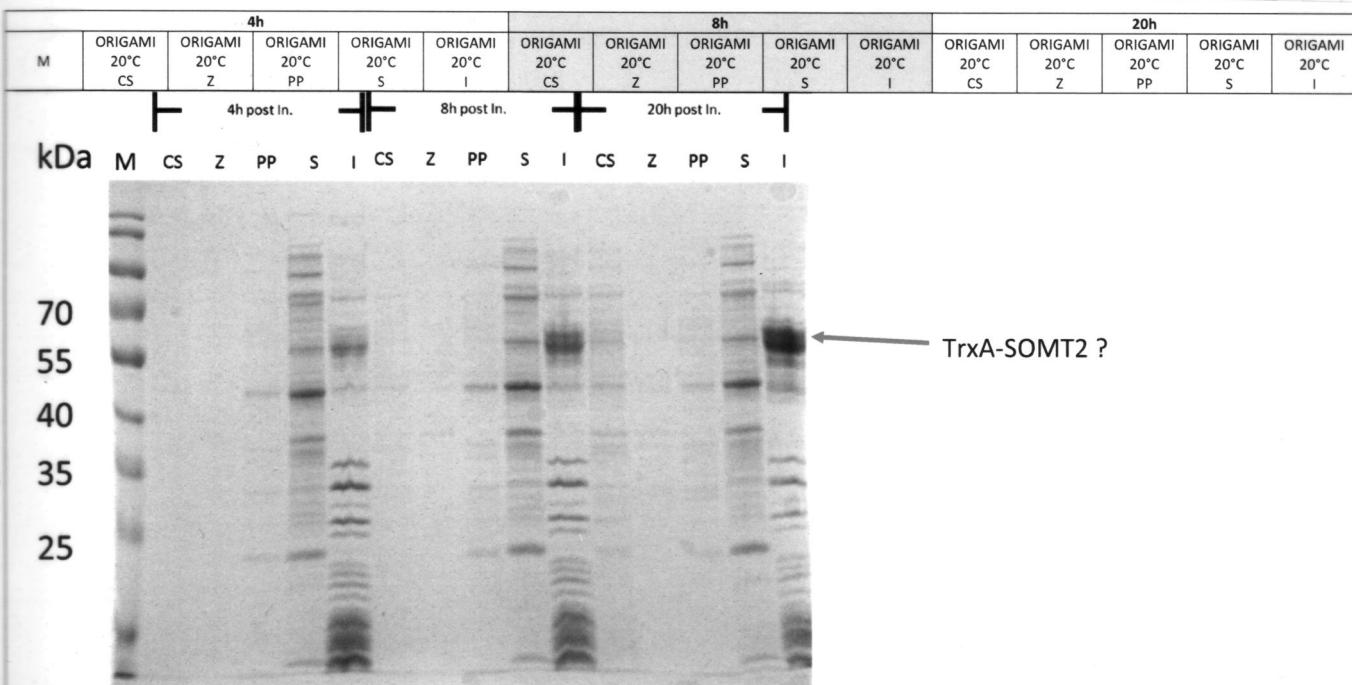
Gele



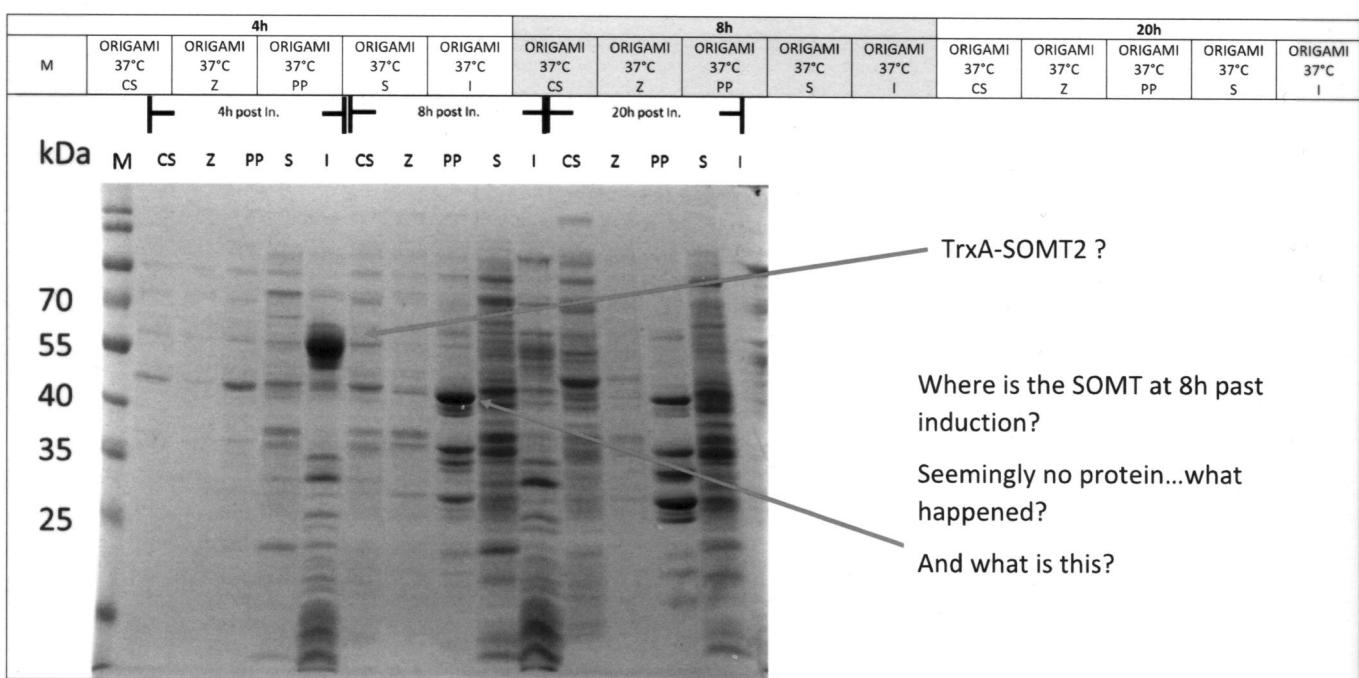
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WEB260

Test-Expression SOMT in C43(DE3) pET20b in TB ohne Antibiotikum

C43(DE3) pET20b SOMT2

- 3 mL culture in TB-Medium (+ 200 µg/mL Ampicillin)
- Inoculation with single colony
- Incubation at 37°C, 220 rpm over night
- Measured OD⁶⁰⁰
- Inoculated 50 mL culture in TB-medium with 650 µL pre-culture (to an OD⁶⁰⁰ of ~ 0.06)
- Incubation at 37°C for 1 h → then incubation at 30°C (see table below)

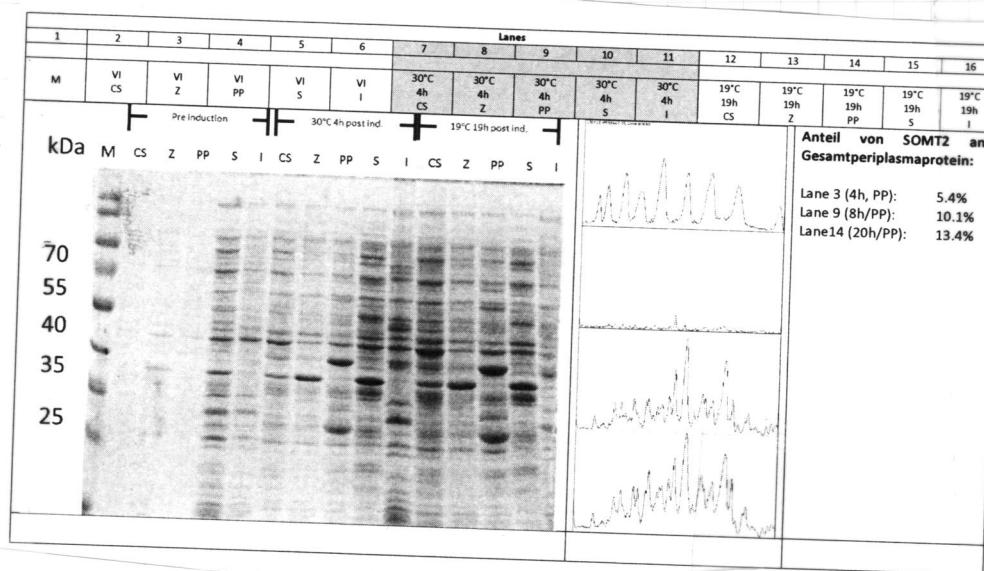
Time	OD ⁶⁰⁰	B-PER	Comments
1030	5.1		Inoculation of 50 mL TB culture
1120	0.257		Incubation at 30°C
1213	0.39		
1330	1.05	50 µL	Induction mit 1 mM IPTG → aliquotieren zu 25 mL für Inkubation @ 19 and 30°C

- Sample at 19°C was incubated while at 240rpm for 19h
- A 1 mL sample was taken for SDS-PAGE analysis

Periplasmic Preparation of the remaining culture (19°C & 19h)

- The culture was pelleted at 10.000 x g and 4°C for 10 min
- The pellet was drained from the supernatant
- Resuspended pellet in 80 mL per g of cell wet weight of 30 mM Tris-HCl, 20% D-Saccharose, 1 mM EDTA, pH 8.0 (vortexed)
- Stirred the solution slowly at RT for 10 min
- Centrifuged at 10.000 x g and 4°C for 10 min
- Drained pellet of supernatant
- Resuspended pellet **carefully** in the same volume as above in **ice-cold** 5 mM MgSO₄
- Stirred the solution slowly on ice for 10 min
- Centrifuged at 16.000 x g and 4°C for 10 min
- Filtrated the supernatant through a 50 µM mesh (CellTricks), added 1 mM PMSF
- adjusted to 20 mM BisTris-HCl pH 7.5 using a 1 M BisTris-HCl pH 7.5 solution and placed on ice
 - o !!! white insoluble material collected at the bottom of the bottle !!!
- The solution was filtrated through a 0.2 µm filter
- Afterwards the solution was subjected to Anion Exchange Chromatography using the ÄKTA system

Sample	Temperature	Time	OD ⁶⁰⁰	B-PER (µL)	Subfractionation (y/n)						Comments
					Medium [CS]	Periplasm (Sucrose) [Z]	Periplasm (cold water) [PP]	Soluble [S]	Insoluble [I]		
C43(DE3)	19	0830	19h	15.2	760	y	y	y	y	y	Pelleted remaining culture (m _{pellet} =0.9g) → Periplasma Prep
C43(DE3)	30	1730	4h	7.9	395	y	y	y	y	y	



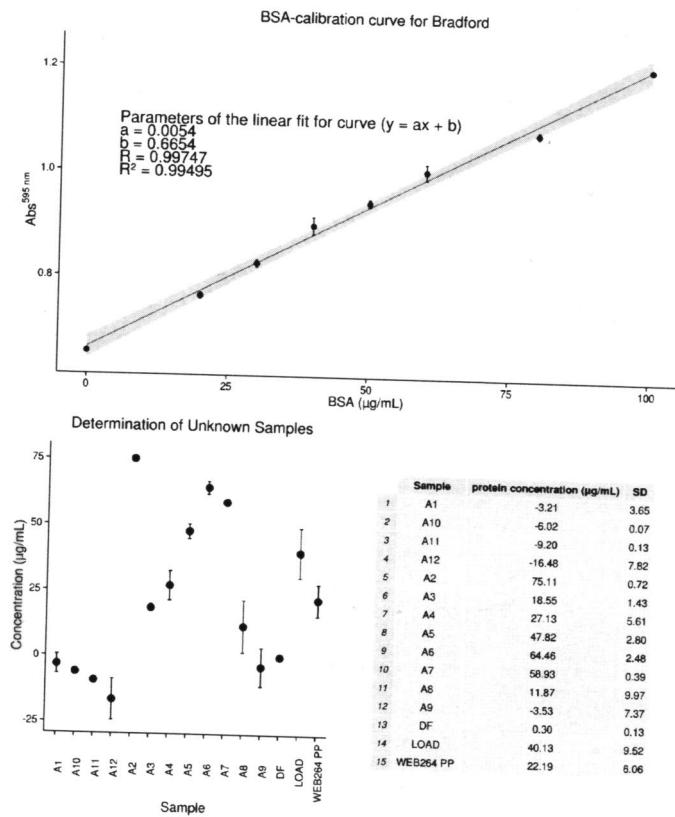
Proteinbestimmung mit Bradford in MTP

BSA-Verdünnungsreihe		
Endkonzentration (µg/mL)	µl BSA aus (Verdünnung)	µl H ₂ O
0	0	110
20	40 (100 µg/mL)	160
30	45 (100 µg/mL)	105
40	80 (100 µg/mL)	120
50	60 (100 µg/mL)	60
60	120 (100 µg/mL)	80
80	160 (100 µg/mL)	40
100	50 (2 mg/mL)	950

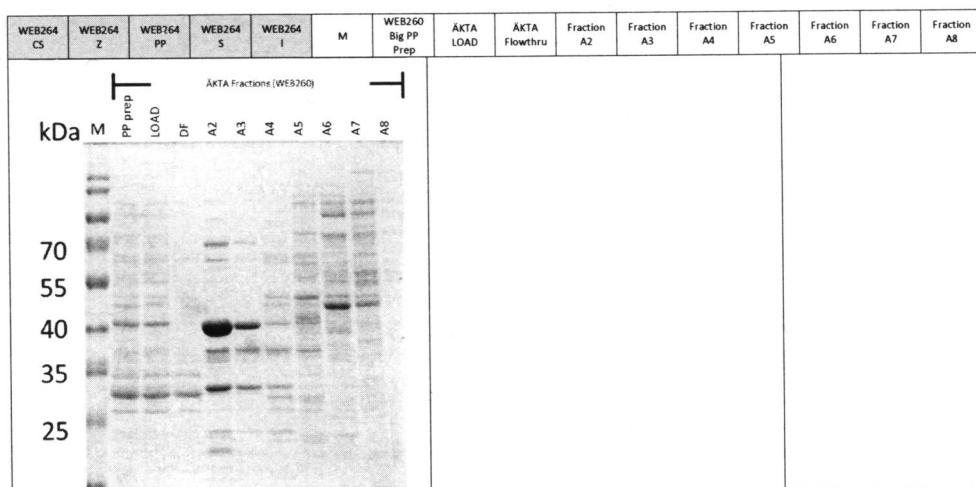
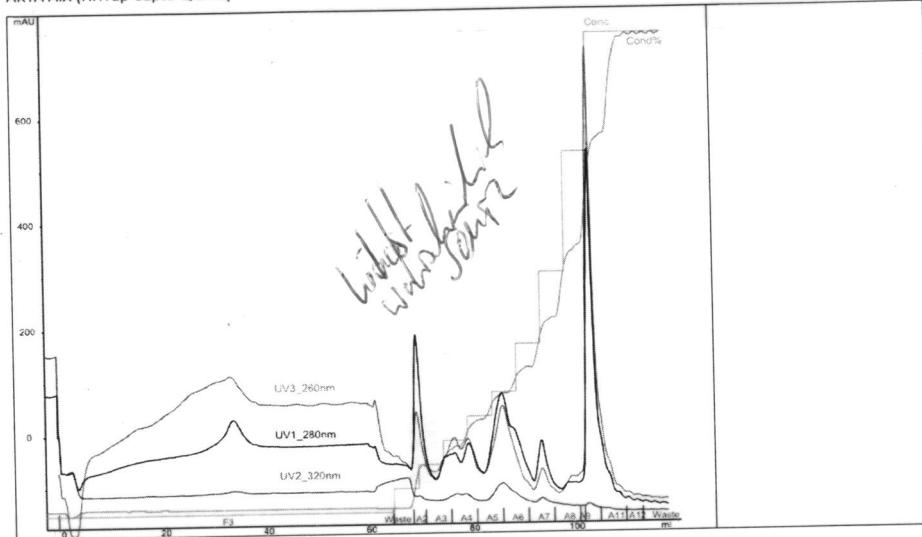
- 10 µl sample and 40 µl ddH₂O were put into MTP (1:5 dilution factor), 50 µl BSA standard was put into MTP
- 200 µl diluted Bradford reagent was added
- Incubated at RT for 5 min
- Measured absorbance at 595 nm in MTP reader

Legende	BSA Standard (µg/mL)			ÄKTA Fractions (see next page)			Sample from WEB264					
	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	A1	A1	A9	A9						
B	20	20	A2	A2	A10	A10						
C	30	30	A3	A3	A11	A11						
D	40	40	A4	A4	A12	A12						
E	50	50	A5	A5	DF (ÄKTA flowthrough [F3])	DF						
F	60	60	A6	A6	LOAD (Sample that was injected)	LOAD						
G	80	80	A7	A7	PP WEB264	PP WEB264						
H	100	100	A8	A8								

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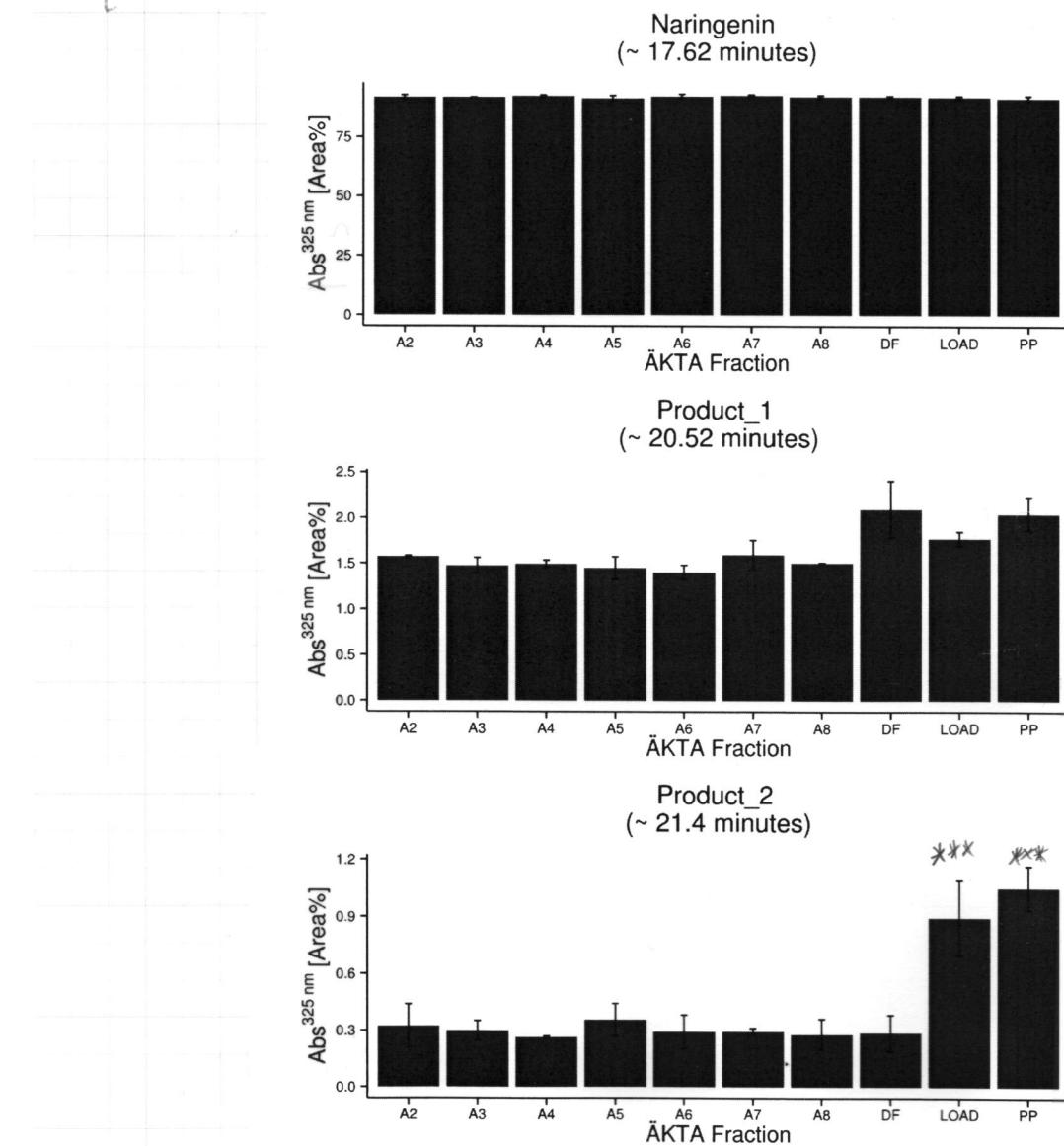
ÄKTA AIX (HiTrap Capto Q 1mL)

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- Protein concentration of every fraction was determined by Bradford Assay on MTP (1:5 dilution)
- 1 mL of every fraction precipitated with 1/10 V of 100% TCA, washed with 200 µL acetone
- Resuspended pellet in 100 µL PBS and 20 µL 6x SDS-PAGE sample buffer (5 min @ 85°C) → stored at -20°C
- Activity test with Naringenin and SAM as substrates

Activity Test using Naringenin and SAM for HPLC or GC measurements

- Based on PFOMT HPLC activity test
- In: 100 mM Tris-HCl, 1 mM MgCl₂, pH 7.5
200 µM Naringenin
250 µM SAM
- Mastermix:
440 µL 0.5 M Tris-HCl, 5 mM MgCl₂, pH 7.5
44 µL 10 mM Naringenin in MeOH
135.9 µL 5 mM SAM (81% biologisch aktiv)
ad to 1100 µL ddH₂O (480 µL ddH₂O added)
- Ansatz:
50 µL Mastermix + 50 µL Proteinprobe (Fraktion A2-A8, flowthrough, loaded sample and WEB264)
- Incubation at 30°C and 80 rpm at 2000 U/min



WEB264

Test-Expression SOMT in C43(DE3) pET20b in TB ohne Antibiotikum
C43(DE3) pET20b SOMT2

- 3 mL culture in TB-Medium (+ 200 µg/mL Ampicillin)
- Inoculation with single colony
- Incubation at 37°C, 220 rpm over night
- Measured OD⁶⁰⁰
- Inoculated 100 mL culture in TB-medium with 1.16 mL pre-culture (to an OD⁶⁰⁰ of ~ 0.06)
- Incubation at 37°C for 1.5 h → then incubation at 30°C (see table below)

Time	OD ⁶⁰⁰	B-PER	Comments
0820	5.16		Inoculation of 100 mL TB culture without AB
1000	0.44		Incubation at 30°C
1050	1.05		Induction with ~1 mM IPTG
1510	6.72	336	m _{Pellet} =1.7g

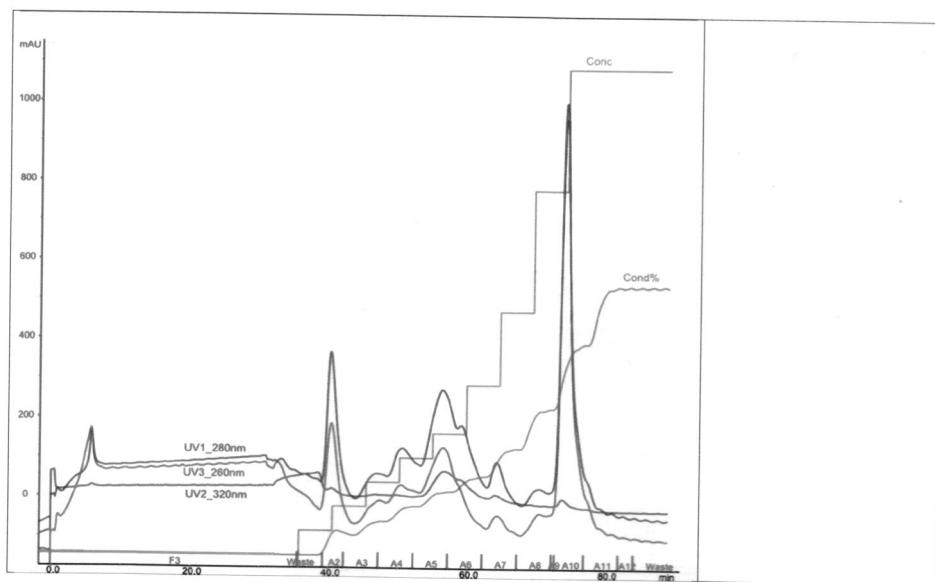
- After induction with 1 mM IPTG (@ OD⁶⁰⁰ 1.05) incubation at 30°C for 4 hours
- 1 mL sample was taken for SDS-PAGE analysis

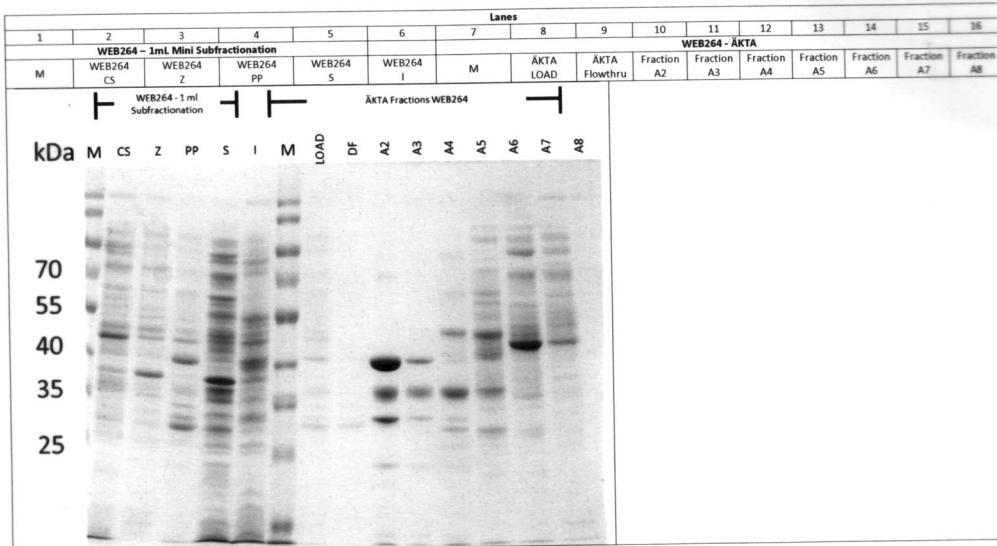
Periplasmic Preparation of the remaining culture

- The culture was pelleted at 10.000 x g and 4°C for 10 min
- The pellet was drained from the supernatant
- added 80 mL per g of cell wet weight of 30 mM Tris-HCl, 20% D-Saccharose, 1 mM EDTA, pH 8.0 (~ 130 mL)
- the solution was shaken at 200 rpm in a shaking incubator at room temp for 10 min
- Centrifuged at 10.000 x g and 4°C for 10 min
- Drained pellet of supernatant
- Resuspended pellet **carefully** (glass rod) in the same volume as above in **ice-cold** 5 mM MgSO₄
- Stirred the solution slowly on ice for 10 min
- Centrifuged at 16.000 x g and 4°C for 10 min
- Filtrated the supernatant through a 50 µm mesh (CellTricks), added 1 mM PMSF and placed on ice → PMSF (in propanol) gives a precipitate
- Added 500 µl 10 mg/mL DNase I → incubated on ice for 30 min
- ÄKTA

m(50mLGreiner)=13.82g

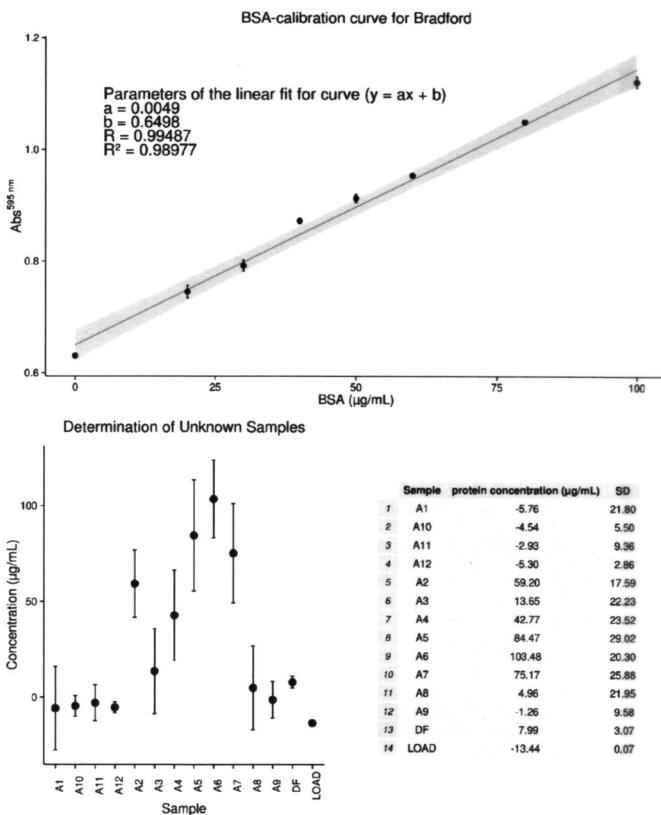
m(JA10 Tubes)=69.1g+-0.1





Bradford ur Bestäigung der Fraktionen

	1	2	3	4	5	6	7	8	9	10	11	12
A							0	0	A1	A1	A9	A9
B							20	20	A2*	A2	A10	A10
C							30	30	A3*	A3	A11	A11
D							40	40	A4*	A4	A12	A12
E							50	50	A5*	A5	DF (AKTA flowthrough [F3])	DF
F							60	60	A6*	A6	LOAD (Sample that was injected)	LOAD
G							80	80	A7*	A7		
H							100	100	A8*	A8		



WEB266

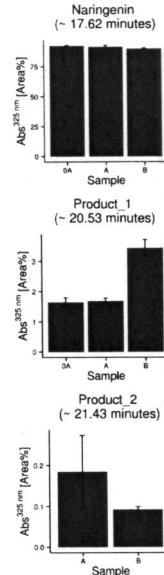
Concentration of SOMT2 in 50 mM BisTris pH 7.5

- Fractions A2 of WEB264 and WEB260 were mixed (from storage at 4°C)
- Concentration & re-buffering of fractions in a centrifugal filter membrane in 50 mM BisTris pH 7.5
- Rebuffering 3x with 15 ml 50 mM BisTris pH 7.5

Activity Test using Naringenin and Daidzein for HPLC or GC measurements

- Based on PFOMT HPLC activity test
- In: 100 mM Tris-HCl, 1 mM MgCl₂ pH 7.5
200 μM Naringenin / Daidzein
250 μM SAM
- **Mastermix I (A):**
40 μl 0.5 M Tris-HCl, 5 mM MgCl₂, pH 7.5
4 μl 10 mM Naringenin in MeOH
12.3 μl 5 mM SAM (81% biologisch aktiv)
100 μl 0.33 mg/mL SOMT
ad to 200 μl ddH₂O (43.7 μl ddH₂O added)
- **Mastermix II (B):**
40 μl 0.5 M Tris-HCl, 5 mM MgCl₂, pH 7.5
4 μl 10 mM Naringenin in MeOH
12.3 μl 5 mM SAM (81% biologisch aktiv)
100 μl 0.33 mg/mL SOMT
0.5 μl 1 M MgSO₄
ad to 200 μl ddH₂O (43.7 μl ddH₂O added)
- **Mastermix III (C):**
40 μl 0.5 M Tris-HCl, 5 mM MgCl₂, pH 7.5
4 μl 10 mM Daidzein in MeOH
12.3 μl 5 mM SAM (81% biologisch aktiv)
100 μl 0.33 mg/mL SOMT
ad to 200 μl ddH₂O (43.7 μl ddH₂O added)
- **Blank I (B1 A):**
40 μl 0.5 M Tris-HCl, 5 mM MgCl₂, pH 7.5
4 μl 10 mM Naringenin in MeOH
12.3 μl 5 mM SAM (81% biologisch aktiv)
100 μl 50 mM Bis-Tris pH 7.5
ad to 200 μl ddH₂O (43.7 μl ddH₂O added)
- **Blank II (B1 C):**
40 μl 0.5 M Tris-HCl, 5 mM MgCl₂, pH 7.5
4 μl 10 mM Daidzein in MeOH
12.3 μl 5 mM SAM (81% biologisch aktiv)
100 μl 50 mM Bis-Tris pH 7.5
ad to 200 μl ddH₂O (43.7 μl ddH₂O added)

- Aliquoted each reaction into 100 μl (double determination)
- Incubation at 30°C and 80 rpm at 2000 Uhr



WEB267

Test-Expression SOMT in C43(DE3) pET20b in TB ohne Antibiotikum

C43(DE3) pET20b SOMT2

- 3 mL culture in TB-Medium (+ 200 µg/mL Ampicillin)
- Inoculation with single colony
- Incubation at 37°C, 220 rpm over night
- Measured OD⁶⁰⁰
- Inoculated 100 mL culture in TB-medium with 1.38 mL pre-culture (to an OD⁶⁰⁰ of ~ 0.06)
- Incubation at 37°C for 1.5 h → then incubation at 30°C (see table below)

Time	OD ⁶⁰⁰	B-PER	Comments
0900	4.34		Inoculation of 100 mL TB culture without AB
1010	0.314		@ 30°C incubation
1105	0.645		Induction using 1 mM IPTG
1600	5.64		

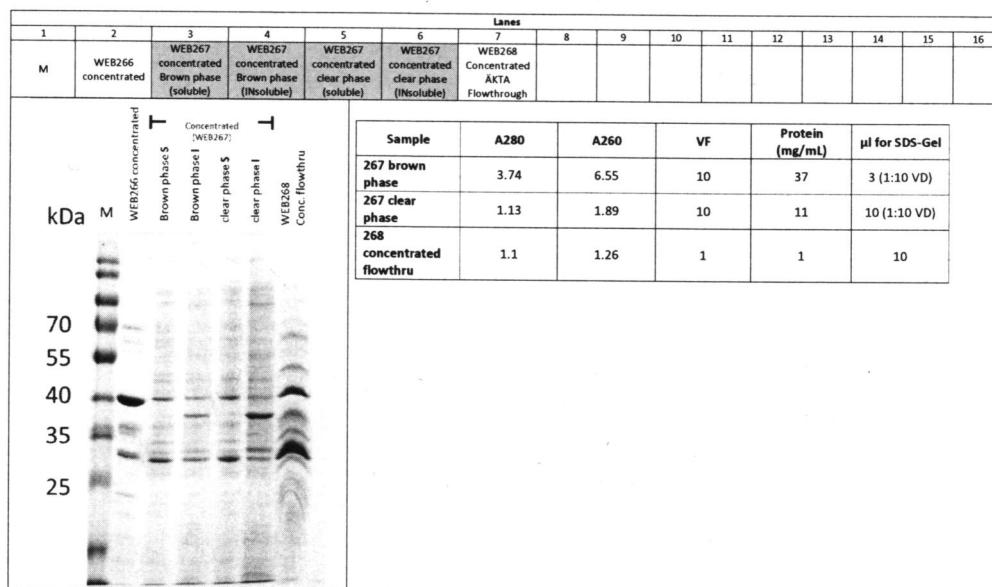
- After induction with 1 mM IPTG (@ OD⁶⁰⁰) incubation at 30°C for 4 hours
- 1 mL sample was taken for SDS-PAGE analysis

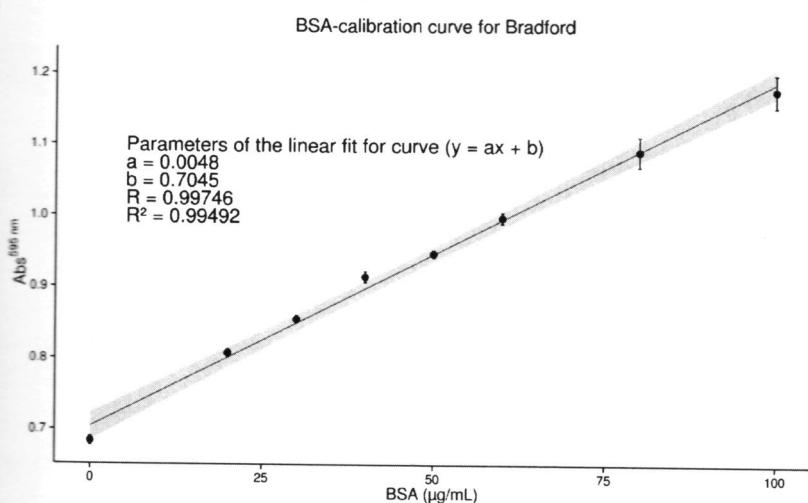
Periplasmic Preparation of the remaining culture

- The culture was pelleted at 10.000 x g and 4°C for 10 min
- The pellet was drained from the supernatant
- added 80 mL per g of cell wet weight of 30 mM Tris-HCl, 20% D-Saccharose, 1 mM EDTA, pH 8.0 (~ 130 mL)
- the solution was shaken at 200 rpm in a shaking incubator at room temp for 10 min
- Centrifuged at 10.000 x g and 4°C for 10 min
- Drained pellet of supernatant
- Resuspended pellet **carefully** (glass rod) in the same volume as above in **ice-cold** 5 mM MgSO₄
- Stirred the solution slowly on ice for 10 min
- Centrifuged at 16.000 x g and 4°C for 10 min
- Filtrated the supernatant through a 50 µM mesh (CellTricks), added 1 mM PMSF
- Adjusted to 20 mM BisTris pH 7.5 using 1 M BisTris pH 7.5
- concentrated using centrifugal filters at 4°C
 - o ran out of time (stored the periplasmic prep and the until then concentrated solution at 4°C) **[30.04.2014]**
 - o measured protein concentration using Bradford after two days **[02.05.2014]**
- concentrated the remaining periplasmic preparation and rebuffered into 50 mM BisTris pH 7.5 using the spin filters
- → phase separation (brown lower phase and clear upper phase)
- Volume of the concentrate: ~300 µl of lower phase and ~100µl of upper phase
- Some aggregate visible → centrifuged to remove aggragat → stored pellet for SDS-PAGE

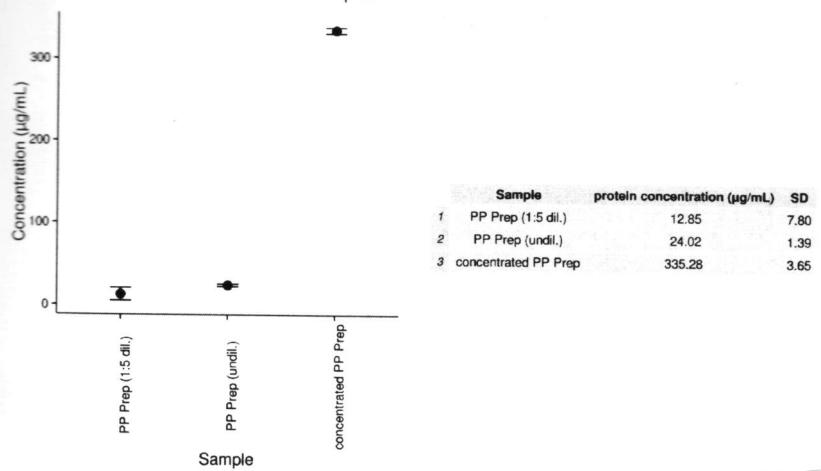
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Measuring protein concentration using Bradford (02.05.2014)							BSA-Standard			Samples			
	1	2	3	4	5	6	7	8	9	10	11	12	
A	0	0	PP Prep 1	PP Prep 2									
B	20	20	PP Prep 1 VF 5	PP Prep 2 VF 5									
C	30	30	Conc. PP Prep 1 VF 5	Conc. PP Prep 2 VF 5									
D	40	40											
E	50	50											
F	60	60											
G	80	80											
H	100	100											





Determination of Unknown Samples



WEB268

Test-Expression SOMT in C43(DE3) pET20b in TB ohne Antibiotikum

C43(DE3) pET20b SOMT2

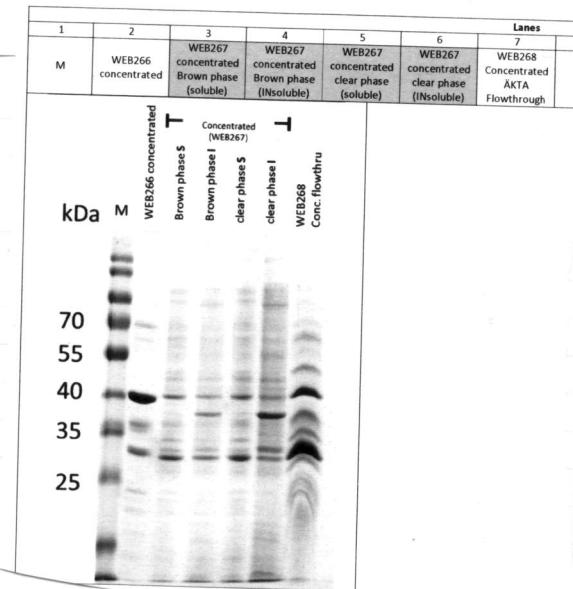
- 15 mL culture in LB-Medium (+ 200 µg/mL Ampicillin)
- Inoculation with single colony
- Incubation at 37°C, 220 rpm over night
- Measured OD⁶⁰⁰
- Inoculated 2 x 500 mL culture in TB-medium with 6 mL pre-culture (to an OD⁶⁰⁰ of ~ 0.06)
- Incubation at 37°C for 1 h → then incubation at 30°C (see table below)

Time	OD ⁶⁰⁰	B-PER	Comments
0915	5.0		Inoculation of 2 x 500 mL TB culture without AB
1100	0.495 0.45		@ 30°C
1130	0.576 0.597		+ 1 mM IPTG
1600	8.25		Harvesting time → weight of pellet: 13.79 g

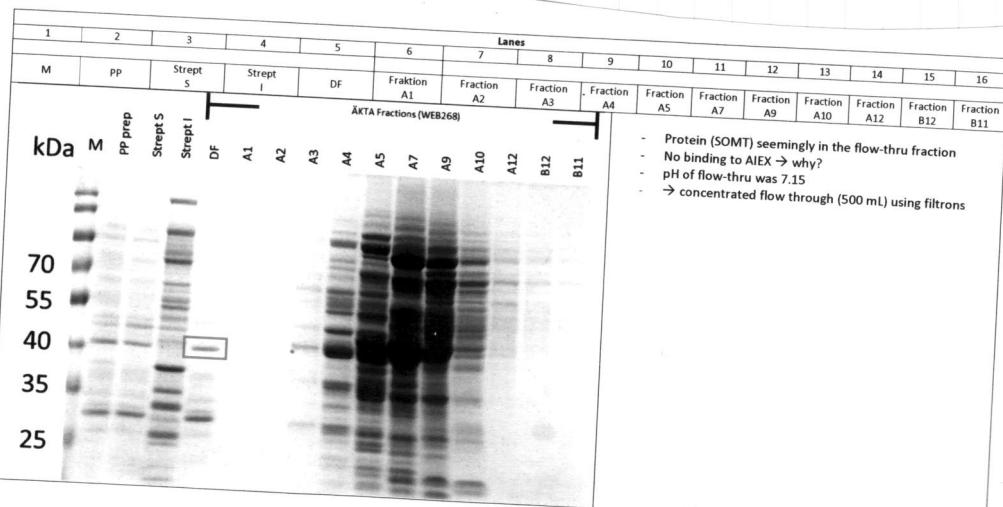
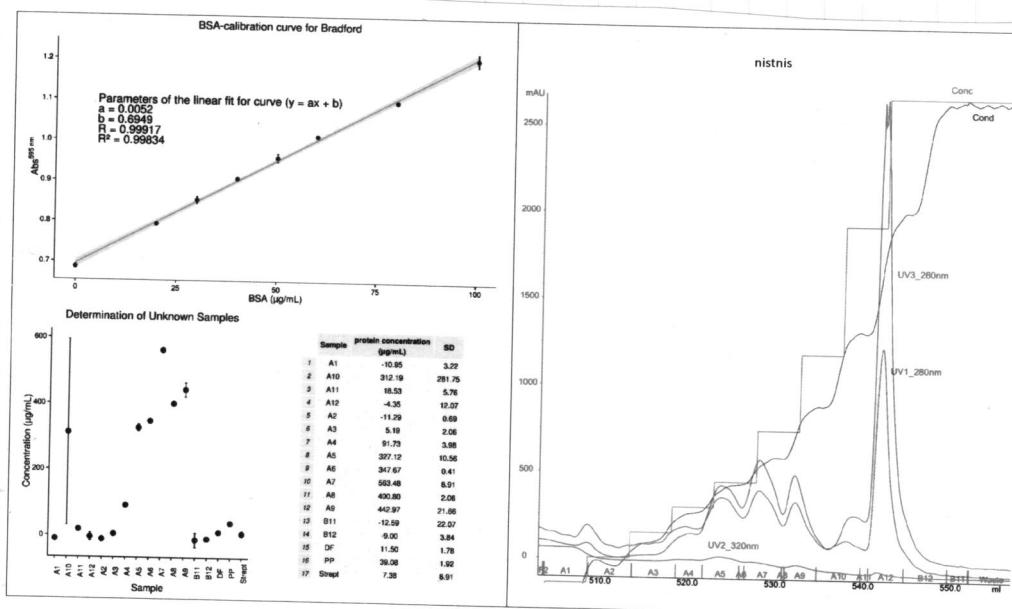
- After induction with 1 mM IPTG (@ OD⁶⁰⁰) incubation at 30°C for 4 hours
- 1 mL sample was taken for SDS-PAGE analysis

Periplasmic Preparation of the remaining culture

- The culture was pelleted at 10.000 x g and 4°C for 10 min (two aliquots)
- The pellet was drained from the supernatant → the added weight of the cell pellets was 13.79 g
- added ~ 25 mL per g of cell wet weight of 30 mM Tris-HCl, 20% D-Saccharose, 1 mM EDTA, pH 8.0 (~ 300 mL total),
- the solution stirred at ~300 rpm at room temp for 18 min and 2 min @ 500 rpm
- Centrifuged at 10.000 x g and 4°C for 10 min
- Drained pellet of supernatant [1 mL SDS-PAGE sample from supernatant]
- Added 500 mL ice-cold 5 mM MgSO₄ to pellet and stirred on ice at 399 rpm for 10 min
- Stirred the solution slowly on ice for 10 min
- Centrifuged at 16.000 x g and 4°C for 10 min
- Filtrated the supernatant through a 50 µm mesh (CellTricks) [no PMSF!]
- Adjusted to 20 mM BisTris pH 7.5 using 1 M BisTris pH 7.5 [1 mL sample for SDS-PAGE]
- Precipitated DNA and DNA binding proteins using Streptomycin sulfate
 - o Prepared 20% stock solution in ddH₂O
 - o Stirred periplasmic fraction on ice and slowly added Streptomycin sulfate until 1% (w/v)
 - o Let stay on ice for 20 min
 - o Centrifuged @ 4°C, 10.000 x g for 10 min
 - o Filtrated through 0.2 µm filter [1 mL sample for SDS-PAGE]
- ÄKTA (Capto Q) → Müll



Measuring protein concentration using Bradford (.05.2014)							BSA-Standard			Samples			
	1	2	3	4	5	6	7	8	9	10	11	12	
A	0	0	PP	PP	A6	A6	B11	B11					
B	20	20	Strept	Strept	A7	A7							
C	30	30	DF	DF	A8	A8							
D	40	40	A1	A1	A9	A9							
E	50	50	A2	A2	A10	A10							
F	60	60	A3	A3	A11	A11							
G	80	80	A4	A4	A12	A12							
H	100	100	A5	A5	B12	B12							



WEB272

Versuchsplanung SOMT refolding screen

Factors

- Arginine
- pH
- divalent cation (Mg^{2+} , Ca^{2+})
- ionic strength (NaCl, KCl)
- glycerin
- Redox system
- Cyclodextrin
- SAH
- temperature

Faktor/Parameter	Symbol	Setting		Unit
		-1	+1	
pH	A	5.5	9.5	-
Arginine	B	0	0.5	M
Glycerin	C	0	10	%
div. Cation	D	1 mM EDTA	2 mM $CaCl_2$, $MgCl_2$	mM
Ionic strength (NaCl/KCl, each)	E	Low (10 mM NaCl, 0.5 mM KCl)	High (250 mM NaCl, 10 mM KCl)	mM
Redox	F	5 mM DTT	1 mM GSH/ 0.2 mM GSSG	mM
Cyclodex	G	0	0.03	M
SAH	H	0	0.5	mM
Buffer	-	50 mM Mes / Borate		
Temperature	-	22°C (RT)		
Time	-	4h		

Stocks to prepare:

- 0.1 M Mes pH 5.5 [A1B1]
- 0.1 M Mes, 1 M Arginine pH 5.5 [A1B2]
- 0.1 M Borate pH 9.5 [A2B1]
- 0.1 M Borate, 1 M Arginine pH 9.5 [A2B2]
- 200 mM $MgCl_2$, 200 mM $CaCl_2$ [C1]
- 0.5 M EDTA [C2]
- 2.5 M NaCl, 100 mM KCl [D1]
- 0.1 M NaCl, 5 mM KCl [D2]
- 1M DTT [E1]
- 50 mM GSH, 10 mM GSSG [E2]
- 5 mM SAH [F1]
- Glycerin [G]
- 120 mM a-Cyclodex [H]

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Buffers to prepare:

No.	Buffer (1 mL each)	Add: (μl)													
		A1B1	A1B2	A2B1	A2B2	C1	C2	D1	D2	E1	E2	F	G	H	H ₂ O
1	pH 9.5 0.5 M Arg 10% Glycerin Low Ionic EDTA DTT SAH	-	-	-	500	-	2	-	10	5	-	100	100	-	283
2	pH 5.5 Low Ionic EDTA DTT	500	-	-	-	-	2	-	10	5	-	-	-	-	483
3	pH 9.5 10% Glycerin Low Ionic MgCa GSH:GSSG CycloDex	-	-	500	-	10	-	-	10	-	20	-	100	250	110
4	pH 5.5 0.5 M Arg 10% Glycerin High Ionic EDTA GSH:GSSG CycloDex	-	500	-	-	-	2	10	-	-	20	-	100	250	118
5	pH 9.5 0.5 M Arg High Ionic EDTA GSH:GSSG	-	-	-	500	-	2	10	-	-	20	-	-	-	468
6	pH 5.5 0.5 M Arg High Ionic MgCa DTT CycloDex SAH	-	500	-	-	10	-	10	-	5	-	100	-	250	125
7	pH 9.5 0.5 M Arg MgCa DTT CycloDex	-	-	-	500	10	-	-	10	5	-	-	-	250	225
8	pH 5.5 10% Glycerin High Ionic EDTA DTT CycloDex SAH	500	-	-	-	-	2	10	-	5	-	100	100	250	33

9	pH 9.5 10% Glycerin High Ionic MgCa DTT	-	-	500	-	10	-	10	-	5	-	-	100	-	375
10	pH 5.5 EDTA GSH:GSSG CycloDex SAH	500	-	-	-	-	2	-	10	-	20	100	-	250	118
11	pH 9.5 High Ionic MgCa GSH:GSSG SAH	-	-	500	-	10	-	10	-	-	20	100	-	-	360
12	pH 5.5 0.5 M Arg 10% Glycerin MgCa GSH:GSSG SAH	-	500	-	-	10	-	-	10	-	20	100	100	-	260

200ml (7):

50 mM Borate

m (g)

0.309 g

0.5M Arg

10.53 g

2 mM MgCl₂0.5 ml 400 mM MgCl₂2 mM CaCl₂100 μl 2M CaCl₂

5 mM DTT

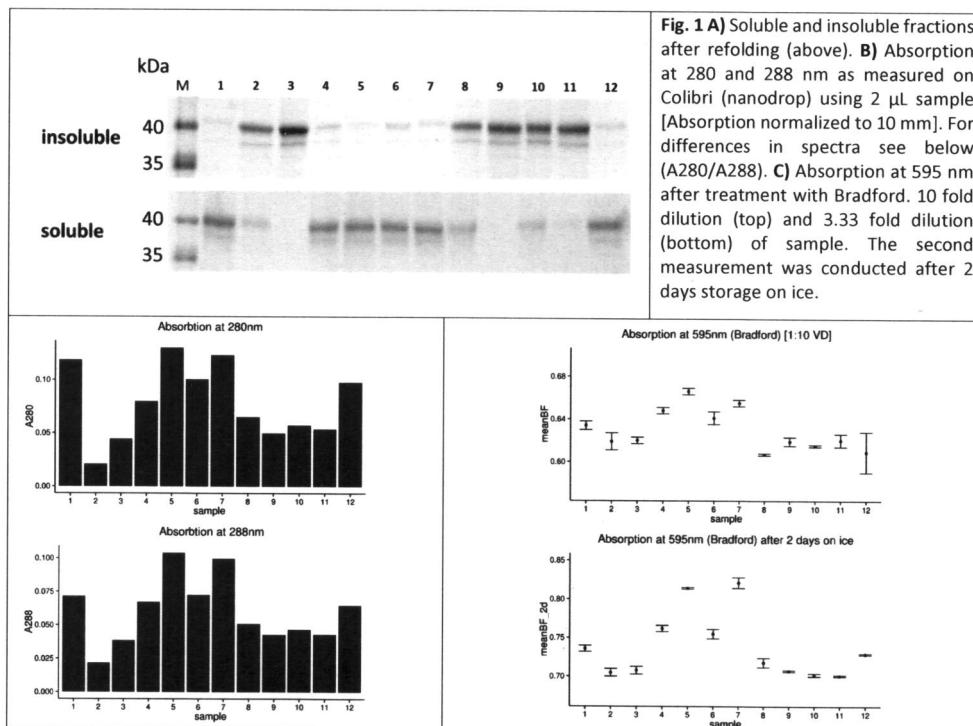
500 μl 1M DTT

30 mM α-Cyclodextrin

2.92 g

1.1 Refolding screen (8 Factor, 12 run design)

- Mixed 1 mL of each refolding buffer (see above)
- Quickly added and mixed 50 μ L 1 mg/mL SOMT2 in IB-solubilization buffer (~50 μ g/mL final protein concentration, 286 mM Gdm-HCl) – short vortex boost (1s)
- Incubated at RT for 1h → then at 4°C over night (1900 to 1030)
- **1)** Centrifuged at 10.000 x g and 4°C for 10 min to remove insoluble material
 - o Washed pellet twice with 200 μ L Acetone and once with 400 μ L MeOH:Acetone 1:1
 - o Took up dry pellet in 100 μ L PBS and 20 μ L 6x SDS Loading Buffer → 10 μ L for SDS-PAGE (**insoluble**)
- Took 100 μ L sample of the supernatant for SDS-PAGE analysis
 - o precipitated using 10 μ L 10% TCA → wash and take up in 10 μ L PBS + 2 μ L SDS-PAGE loading buffer (**soluble**)



1.5 Supplemental Figures

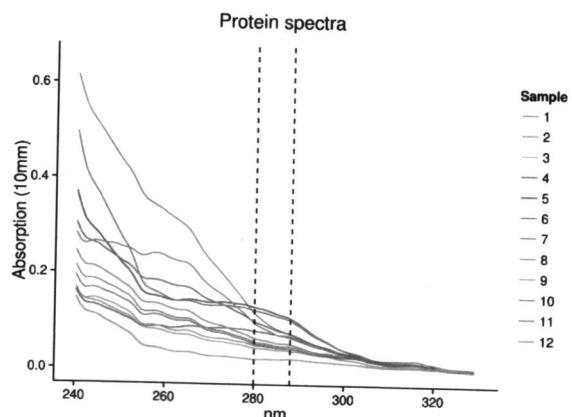
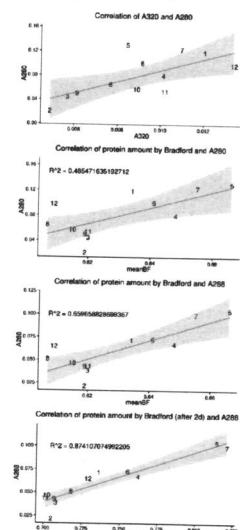


Fig 2. A) Correlation of A³²⁰ and A²⁸⁰, Bradford and A²⁸⁰ and Bradford and A²⁸⁸. The correlation between protein determination by Bradford and A²⁸⁰ should ideally be 1. Measuring Bradford with a lower dilution helps to increase the correlation (due to the fact that Bradford has a higher detection limit than A²⁸⁰). Some UV absorbing component (maybe SAH) interferes with the protein measurement at A²⁸⁰ → moved to A²⁸⁸, which should also be correlated to protein amount. **B)** UV-VIS-Spectra of the samples as measured by nanodrop (normalized to 10 mm pathlength).

- 2) Concentrated soluble supernatant using 0.5 mL spin filters (10k MWCO)
 - o Add 500 µl sample → spin 10 min @ 14000 x g
 - o Add remaining 400 µl sample → spin 10 min @ 14.000 x g
 - o Add 500 µl 0.05 M BisTris pH 7.5 → spin
 - o Add 500 µl 0.05 M BisTris pH 7.5 → spin until Volume ~ 100 µl
 - o Weighed out empty collection tubes (see below) → collected sample by inversion of spin columns and centrifugation for 2 min @ 1000 x g
 - o Weighed out collection tubes with sample → calculated volume needed for 100 mg (100 µl) sample volume → added buffer until 100 µl

sample	weight(tube) [g]	weight(afterrecovery) [g]	recovery amount [mg]	add buffer [µl]
1	1,152	1,206	54,0	46,000
2	1,151	1,191	40,0	60,000
3	1,147	1,184	37,0	63,000
4	1,148	1,193	45,0	55,000
5	1,150	1,188	38,0	62,000
6	1,148	1,193	45,0	55,000
7	1,157	1,195	38,0	62,000
8	1,145	1,181	36,0	64,000
9	1,133	1,173	40,0	60,000
10	1,142	1,180	38,0	62,000
11	1,147	1,183	36,0	64,000
12	1,145	1,204	59,0	41,000

- o Use the rest to measure UV-VIS-Spectrum & Bradford (see Fig. 1 and Fig. 2)
 - Bradford (5µl sample - Doppelbestimmung)
 - After 2 days → another Bradford with the remaining sample (15 µl Doppelbestimmung)
- o Use 50 µl for activity test (Naringenin, standard activity test)

Step	Samplename (X_...) X = runnumber
1 - insoluble material	X
1 - soluble fraction (TCA precip)	X_A
2 -	

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1.2 Activity Test (after rebuffering concentration)

- Measured Protein spectrum on nanodrop (200-350nm)
- **Mastermix (700 µl):**
 - 280 µl 0.5 M Tris-HCl, 5 mM MgCl₂, pH 7.5
 - 28 µl 10 mM Naringenin in MeOH
 - 86.4 µl 5 mM SAM (81% biologisch aktiv)
 - 7 µl 1M MgSO₄
 - ad to 700 µl ddH₂O (298.6 µl ddH₂O added)
- Add 50 µl Mastermix and 50 µl refolded Proteinfraction (1-12)
- Incubate at 30°C , 80rpm (1900 until 0845)
- Added 1 µl 10 mM AC-9 and extracted twice with 500 µl EtOAc + 1% FA
- Dried organic phase in speedvac
- Redissolved in 100 µl MeOH for HPLC analysis

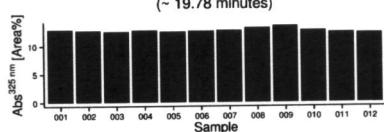
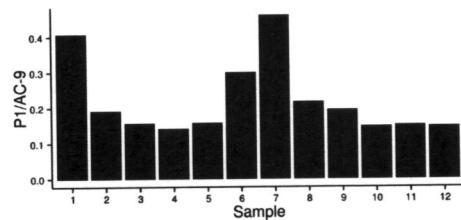
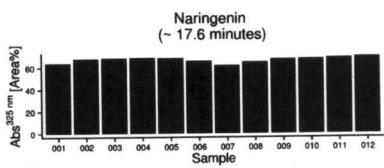
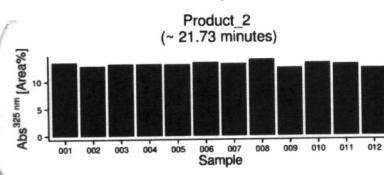
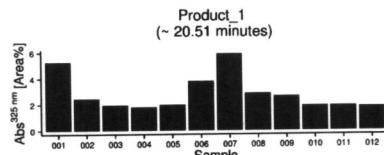
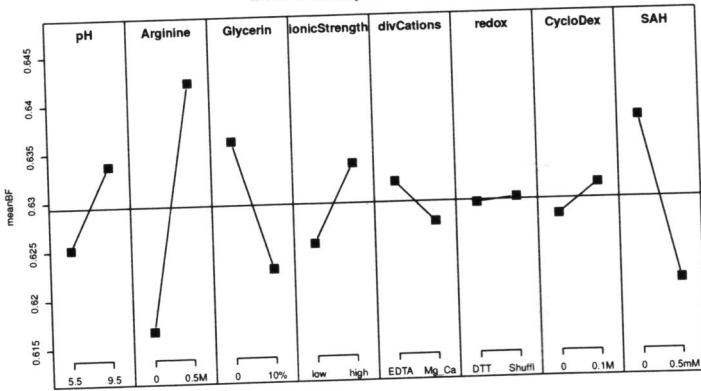


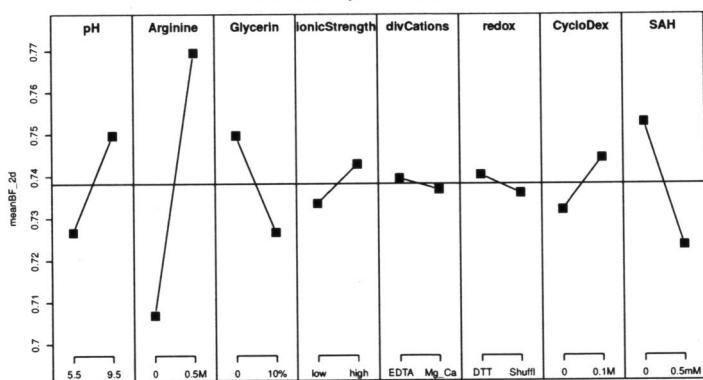
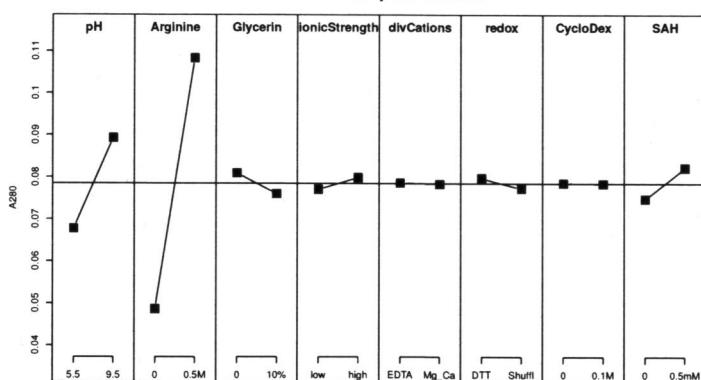
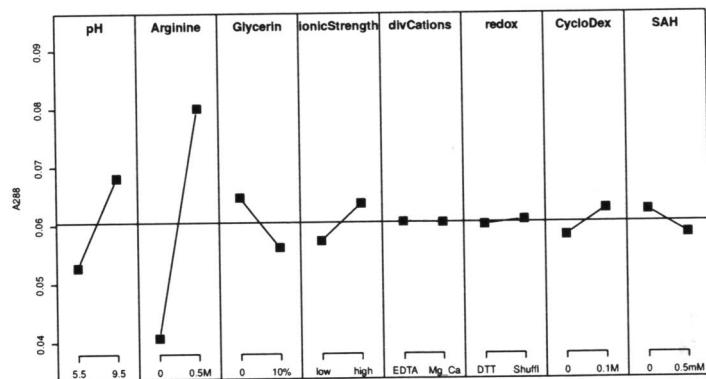
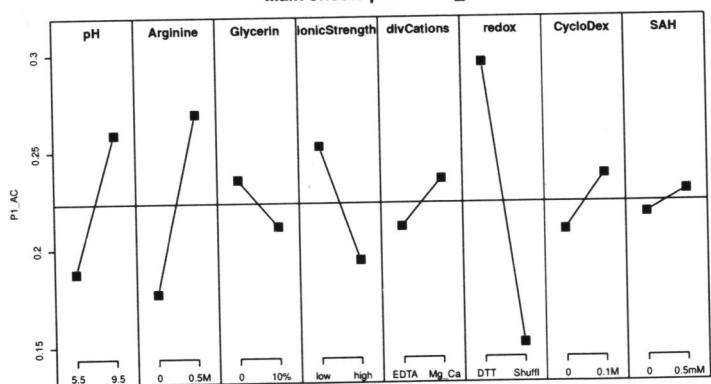
Fig. 2. A) Results of the integration of the HPLC runs.
Area% of each compound. **B)** above – Divided the
absolute integration result of Product 1 (20.51 min) by
the integration results of the internal standard
(Anthracene-9-carboxylic acid [AC-9]). Sample 1 and 7
show the most activity.



→ vermutlich
Zwischenprodukt
von AC-9

Main effects plot for meanBF



Main effects plot for meanBF_2d**Main effects plot for A280****Main effects plot for A288****Main effects plot for P1_AC**

Refinement (Front)

PFL3K-1P-95~~19~~-ccp4.mtz

~~no differences~~

loop region N44 - Y51
V78 - G82

'no density'
loop N140 - E145

K157 - Y160

B171ASN → Sx

D 155 E
D 58

check ASN 177
A X AA 16
B 50
D 465 B Phe 15

chain C
6 30

* Results X-Hallizatio

Xtal

140515-PFOMT
(MC001415-B10.1)

140514-PFOMT
(MC001415-C3.2)

140515-PFOMT-B
(MC001415-B5.1)

140519-PFOMT
(MC001415-B5.2)

140520-PFOMT
(MC001415-C3.1)

140520-PFOMT-D

Comments

- unclear ligand (small) ~~is~~ in active site (maybe phosphate, sulfate etc?)
- see above
- problems with Phony & map
→ bad data?
- no ligand in active site
→ ~~was~~ possibly Sulphate or place holder (in place of ~~the~~ carbonyl of ~~the~~)
- no ligand → maybe just water, sulfate etc.

MT-Assay "Quick & Dirty"

WEB284

Quick&Dirty MT Screen

MT-Reaction (50 µl)

- 0.1 M Tris pH 7.5
- 10 mM MgCl₂
- 200 µM ED
- 250 µM SAM
- 2.5 µg PFOMT
- 10 µg MTAN
- 30 min @ 30 °C

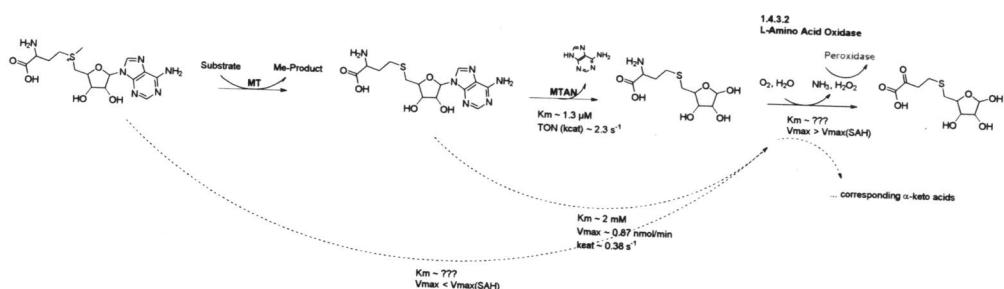
6xMastermix OMT (300 µL)

- 30 µl 1 M Tris pH 7.5
- 6 µl 10 mM ED
- 12 µl 250 mM MgCl₂
- 18.5 µl 5 mM SAH (81%)
- (15 µl 1 mg/ml PFOMT)
- 71 µl 1.41 mg/mL MTAN
- 147.5 µl H₂O

Reaction	PFOMT (µg/Rxn)	PFOMT – stock	Add 2.5 µl PFOMT
1	0	0	2.5 µl
2	0.01	0.004 mg/mL	2.5 µl
3	0.1	0.04 mg/mL	2.5 µl
4	1	0.4 mg/mL	2.5 µl
5	2.5	0.8 mg/mL	2.5 µl

LAAO-Reaction-Mix (800µl)

- 10 U/ml HRP 41.5 µl 193 U/ml HRP
- 0.1 mg/mL TMB 16 µl 5 mg/mL TMB
- 0.1 mg/mL LAAO 68.9 µl 1.16 mg/mL LAAO
- 0.673 mL 0.1 M Tris-HCl pH 7.5
- Add 100 µl LAAO-Mix to 50 µl OMT Reaction
- Incubate for 30 min at RT
- Stop with 50 µl 12 N H₂SO₄
- no color reaction ⊗ → maybe inhibition by flavonoid?? → separate flavonoid and SAM/SAH etc.



Tetrahydrofyl bromide

$$\varepsilon(\text{Dimer}) = 53\,000 \text{ m}^{-1} \text{ cm}^{-1} = 59\,000 \text{ m}^{-1} \text{ mm}^{-1}$$

Höhe im Well bei 200μm

$$h = \frac{200 \text{ mm}^2}{55 \cdot (3.3 \text{ mm})^2} = 5.85 \text{ mm}$$

$$E = \varepsilon \cdot c \cdot d$$

$$E = 59\,000 \text{ m}^{-1} \text{ min}^{-1} \cdot 1 \cdot 10^{-6} \text{ M}^{1/2} \cdot 5.85 \text{ mm}$$

$$= 0.0345 \quad (\text{TmB})$$

$$0.345 \quad (20 \mu\text{m})$$

$$3.45 \quad (100 \mu\text{m})$$

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1.1 Test Chromabond

1.1.1 Quick Test using LAAO-Mix and L-Methionine

- 100 µl C18 Chromabond Kartusche mit 1 mL MeOH und 1 mL H₂O äquilibriert
- *Sample solutions:*
 - 100 µM L-Met in ddH₂O
 - 100 µM L-Met + 0.2 mM Eriodictyol in ddH₂O
 - 1 mL of each sample solutions was extracted with SPE columns (just passed 1 mL sample through column)
 - Samples:
 - A1 - 100 µM Met, 0.2 mM ED passed through C18 column
 - B1 - 100 µM Met, 0.2 mM ED **without** C18 column
 - A2 - 100 µM Met passed through C18 column
 - B2 - 100 µM Met **without** C18 column
- *Reactions:* (A – with C18 Treatment, B – without Treatment)
 - Added 75 µl LAAO-Mix to 25 µl sample
 - Incubation for ~ 10 min at RT
 - Stopped with 50 µl 12 N H₂SO₄

A1	A2
B1	B2

- Strong color reaction of all samples, except the one not treated with C18 columns → inhibition of LAAO or HRP by flavonoids → flavonoids can be separated from hydrophilic SAM/SAH by C18 columns

1.1.2 Test with OMT Reaction

Mastermix:

200 µl 0.5 M Tris, 5 mM MgCl₂ pH 7.5
 20 µl 10 mM ED
 61 µl 5 mM SAM
 236 µl MTAN
 (1 µl 24 mg/mL PFOMT) – omitted until next step
 482 µH₂O

- 500 µl MM + (A) 5 µl 24 mg/mL PFOMT
- 500 µl MM + (B) 5 µl PFOMT Buffer
- Incubate reaction 10 min @ 30 °C
- Condition 100 µL C18 column with 1) 1 mL MeOH and 2) 1 mL H₂O
- Add and immediately collect 500 µl sample on H₂O-conditioned C18 & wash with 500 µL H₂O (H₂O Fraction)
 Wash with 500 µl MeOH and collect elution (org. Fraction)
- Use 50 µl of the aqueous sample and add 100 µl LAAO-Mix →
- incubate 30 min @ RT → stop with 50 µl 12 N H₂SO₄ → Wells on plate: A3 = (B, **without PFOMT**), A4 = (A, **with PFOMT**)
 → color reaction on sample treated with PFOMT !!!

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zurück

WEB282

Gefi-Trials with SOMT

- Refolding of SOMT as in WEB278 Buffer 7

Refolding Buffer:	per 50 mL
- 50 mM Borate pH 9.5	0.309 g Boric Acid
- 0.5 M Arginine	10.53 g L-Arginine monohydrochloride
- 2 mM MgCl ₂	
- 2 mM CaCl ₂	
- 30 mM α-Cyclodextrin	2.92 g
- 5 mM DTT	0.5 mL 1M DTT

ATTENTION: forgot to add 10 mM NaCl and 0.5 mM KCl

Adjust pH to 9.5 using NaOH pellets (at least 30).

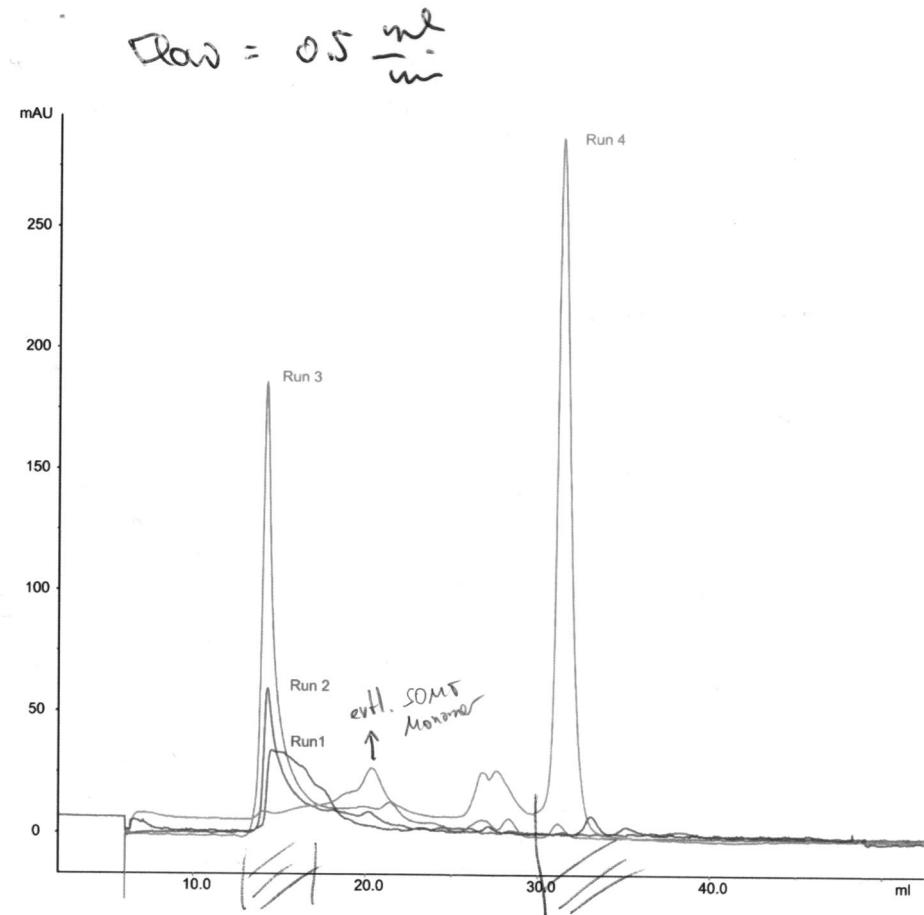
1.1 Refolding:

- Diluted solubilized SOMT-IB to 1 mg/mL (A₂₈₀ = 1) using solubilization Buffer (6 M GdmCl, 100 mM Tris-HCl pH 8)
- flash dilution (final GdmCl concentration ~280 mM, 50 µg/mL SOMT at final conc.):
 - dropwise addition of 2.5 mL 1 mg/mL SOMT to 50 mL of refolding buffer in 50 mL Tube
 - incubation at RT for 1 h
 - incubation at ~4°C for 4 hours
 - Centrifuged for 20 min @ 20.000 x g to remove insoluble material
 - Filtered thruh 50 µm mesh
 - Concentrated & rebuffered using centrifugal concentrator (MWCO 10000)
 - Rebuffering Buffer 50 mM Borate, 0.5 M Arginine, 2 mM MgCl₂, 2 mM CaCl₂, pH 9.5

1.2 ÄKTA Gel Filtration Runs (Superdex 200 10/300 GL)

RUN	Sample	loop	Running-Buffer
1	1.66 mg/mL SOMT (in 50 mM BisTris pH 7.5)	100 µl	50 mM BisTris pH 7.5
2	0.98 mg/mL SOMT in 10 mM KP _i pH 8 from CD measurements	100 µl	10 mM KP _i pH 8
3	~3mg/mL SOMT in 50 mM Tris, 250 mM NaCl, pH 8	100 µl	50 mM Tris 250 mM NaCl pH 8
4	1.85 mg/mL SOMT in Refolding buffer 7 (see above)	100 µl	50 mM Borate-NaOH 500 mM Arginine 2 mM MgCl ₂ 2 mM CaCl ₂ pH 9.5

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Ausdrucksvolumen \Rightarrow Salze etc.
 \Rightarrow Oligosse, Aggregate etc.
 \Rightarrow intrazellel. Zeil

WEB280 - X-Linking SOMT mit EDC

1.1 Concentration dependence

1. 1 mg/mL → 20 µl 1 mg/mL SOMT
2. 0.1 → 20 µl 1 mg/mL SOMT + 180 µl KPi
3. 0.01 mg/mL → 20 µl SOMT + 1980 µl KPi

- Treated with 5 mM EDC-HCl
- EDC-HCl
 - o Soluble up to 100 mg/mL H₂O (523 mM)
 - o 0.5 M Stock solution of EDC-HCl (95.5 mg/mL) in 10 mM KPi pH 8

Table 1. Crosslinking of SOMT oligomers via EDC

Sample	Protein [µl]	EDC [µl]	EDC Stock
1 mg/ml	20	0.2 µl	0.5 M
0.1	200	2 µl	0.5 M
0.01	2000	20 µl	0.5 M

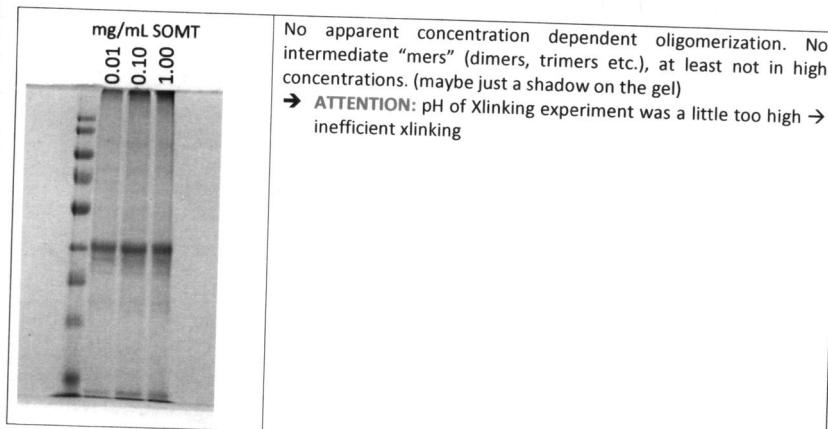
- Added 5 mM EDC-HCl solution and protein in 10 mM KPi pH 8.0
- Incubated at 25°C for 1h
- Quenched reaction by adding 0.1 M Tris-HCl pH 7.5

Table 2. Quenching of EDC coupling reaction by Tris-HCl Buffer

Sample	Volume	Add 1 M Tris-HCl [µl]
1 mg/ml	20.2 µl	2 µl
0.1	202 µl	20 µl
0.01	2020 µl	200 µl

- Precipitated protein from dilute solution with 1/10 Vol 100 % TCA (on ice 15 min)
- Redissolved in 22 µl PBS
- One half of each X-linking reaction was treated with DTT and reducting loading dye, the other wasnt

Y. Kimata-Ariga et al./Biochemical and Biophysical Research Communications 434 (2013) 867–872



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lysozym

BRS667

1 10 μl

2 ~

3 ~

2 μl (0.166 M)

1 μl (21 mM)

1 μl (21 mM)

1 Labelling Lysozym mit BRS667

1.1 Lysozyme

- 147 resis (129 resis without signal peptide)
- 6 lysines without signal peptide
- MW = 14313.1 g/mol
- 50 μg → 3.49 nmol protein → 20.9 nmol lysines

1.2 Reaction

- Dissolve 5 mg/mL Lysozyme in 200 mM KPi, 8 M Urea, pH 8.5
- 10 μl Lysozyme (50 μg)
- Heat 5 min @ 95°C and cool after on ice
- Add 1 μl 0.166 M BRS667 (166 nmol → ~ 8 fold excess)
- Incubate 30 min on ice
- Add 10 μl 1M Lysine

*(ant 100μl und PB-für
Oberfläche für SDS-PAGE anfüllen)*

Excess of BRS regarding Lysines	BRS667	nmoles of BRS
8	1 μl 0.166 M	166
1	1 μl 21 mM	21

4 μg Protei / Lane

→ 8 μl + 2 μl SDS -
Buffer

J
dann
0.5 μl / μl

WEB299

Versuchsplanung SOMT refolding screen II

Factors

- Arginine
- pH
- divalent cation (Mg^{2+} , Ca^{2+})
- ionic strength (NaCl, KCl)
- glycerin
- Redox system
- DES
- Detergent
- temperature

Faktor/Parameter	Symbol	Setting		Unit
		-1	+1	
pH	A	5.5	8.5	-
Arginine	B	0	0.5	M
Glycerin	C	0	10	%
div. Cation	D	1 mM EDTA Low (10 mM NaCl, 0.5 mM KCl)	2 mM $CaCl_2$, $MgCl_2$ High (250 mM NaCl, 10 mM KCl)	mM
Ionic strength (NaCl/KCl, each)	E	5 mM DTT	1 mM GSH/ 0.2 mM GSSG	mM
Redox	F	0	25	M
DES	G	0	0.5	mM
Tween-80	H			
Buffer	-	50 mM Mes / Tris		
Temperature	-	22°C (RT)		
Time	-	4h		

Stocks to prepare:

- 0.1 M Mes pH 5.5 [A1B1]
- 0.1 M Mes, 1 M Arginine pH 5.5 [A1B2]
- 0.1 M Tris pH 8.5 [A2B1]
- 0.1 M Tris, 1 M Arginine pH 8.5 [A2B2]
- 200 mM $MgCl_2$, 200 mM $CaCl_2$ [C1]
- 0.5 M EDTA [C2]
- 2.5 M NaCl, 100 mM KCl [D1]
- 0.1 M NaCl, 5 mM KCl [D2]
- 1M DTT [E1]
- 50 mM GSH, 10 mM GSSG [E2]
- 5 mM Tween-80 [F1]
- Glycerin [G]
- DES (PCH) [H]

Buffers to prepare:

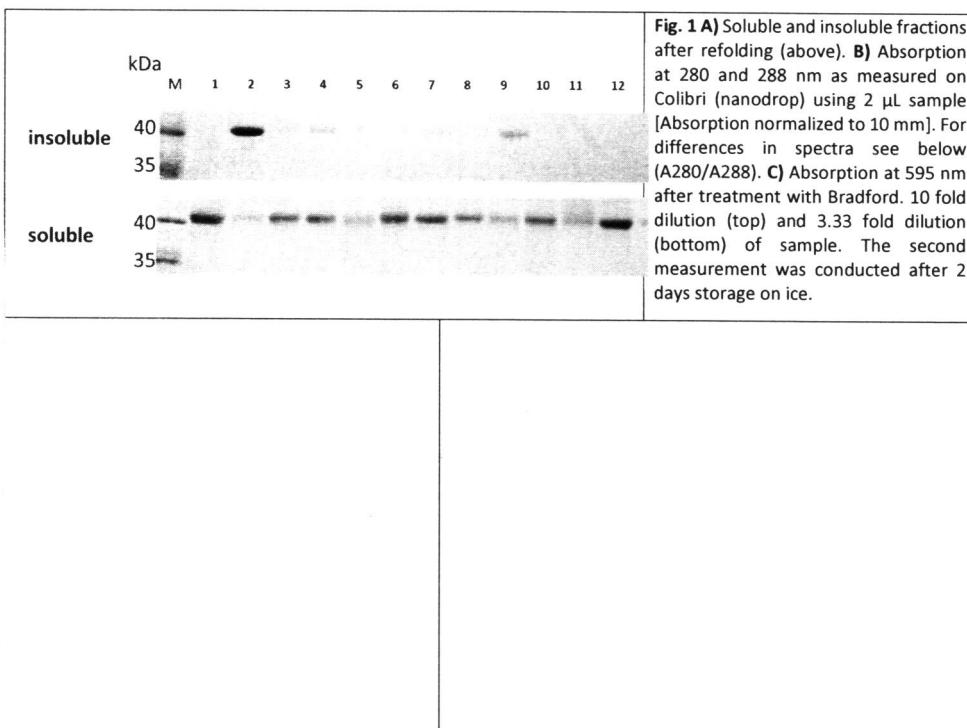
No.	Buffer (1 mL each)	Add: (μ L)													
			A1B1	A1B2	A2B1	A2B2	C1	C2	D1	D2	E1	E2	F	G	H
1	pH 9.5 0.5 M Arg 10% Glycerin low ionic EDTA DTT Tween	-	-	-	500	-	2	-	10	5	-	100	100	-	283
2	pH 5.5 low ionic EDTA DTT	500	-	-	-	-	2	-	10	5	-	-	-	-	483
3	pH 9.5 10% glycerin Low ionic MgCa GSH:GSSG DES	-	-	500	-	10	-	-	10	-	20	-	100	250	110
4	pH 9.5 0.5 M Arg 10% Glycerin High ionic EDTA GSH:GSSG DES	-	500	-	-	-	2	10	-	-	20	-	100	250	118
5	pH 9.5 0.5 M Arg High ionic EDTA GSH:GSSG	-	-	-	500	-	2	10	-	-	20	-	-	-	468
6	pH 9.5 0.5 M Arg High ionic MgCa DTT DES Tween	-	500	-	-	10	-	10	-	5	-	100	-	250	125
7	pH 9.5 0.5 M Arg Low ionic MgCa DTT DES	-	-	-	500	10	-	-	10	5	-	-	-	250	225
8	pH 5.5 10% Glycerin High ionic EDTA DTT DES	500	-	-	-	-	2	10	-	5	-	100	100	250	33

Tween															
9	pH 9.5 10% Glycerin High ionic MgCa DTT	-	-	500	-	10	-	10	-	5	-	-	100	-	375
10	pH 5.5 EDTA GSH:GSSG Low ionic DES Tween	500	-	-	-	-	2	-	10	-	20	100	-	250	118
11	pH 9.5 High ionic MgCa GSH:GSSG Tween	-	-	500	-	10	-	10	-	-	20	100	-	-	360
12	pH 5.5 0.5 M Arg 10% Glycerin MgCa Low ionic GSH:GSSG Tween	-	500	-	-	10	-	-	10	-	20	100	100	-	260

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1.1 Refolding screen (8 Factor, 12 run design)

- Mixed 1 mL of each refolding buffer (see above)
- Quickly added and mixed 50 µl 1 mg/mL SOMT2 in IB-solubilization buffer (~50 µg/mL final protein concentration, 286 mM Gdm-HCl) – short vortex boost (1s)
- Short incubation at RT → then Incubated at 5°C over night (1900 to 1030)
- 1) Centrifuged at 10.000 x g and 4°C for 10 min to remove insoluble material
 - o Washed pellet twice with 200 µl Acetone and once with 400 µl MeOH:Acetone 1:1
 - o Took up dry pellet in 100 µl PBS and 20 µl 6x SDS Loading Buffer → 10 µl for SDS-PAGE (**insoluble**)
- Took 100 µl sample of the supernatant for SDS-PAGE analysis
 - o precipitated using 10 µl 10% TCA → wash and take up in 10 µl PBS + 2 µl SDS-PAGE loading buffer (**soluble**)



- 2) Concentrated soluble supernatant using 0.5 mL spin filters (10k MWCO)
 - 2,4,12 – old columns (used on WEB272)
 - o Add 500 µl sample → spin 10 min @ 14000 x g
 - o Add remaining 400 µl sample → spin 10 min @ 14.000 x g
 - o Add 500 µl 0.1 M Tris pH 8.5, 20% Glycerol, 150 mM NaCl → spin
 - o Add 500 µl 0.1 M Tris pH 8.5, 20% Glycerol, 150 mM NaCl → spin until Volume ~ 100 µl
 - o Weighed out empty collection tubes (see below) → collected sample by inversion of spin columns and centrifugation for 2 min @ 1000 x g
 - o Weighed out collection tubes with sample → calculated volume needed for 100 mg (100 µl) sample volume → added buffer until 100 µl

1.2 Activity Test (after rebuffering concentration)

- Measured Protein spectrum on nanodrop (200-350nm)
- **Mastermix (700 µl):**
 - 280 µl 0.5 M Tris-HCl, 10 mM MgCl₂, pH 7.5
 - 28 µl 10 mM Naringenin in MeOH
 - 95.2 µl 5 mM SAM (73.5% biologisch aktiv)
 - 14 µl 1M DTT
 - ad to 700 µl ddH₂O (282.8 µl ddH₂O added)
- Add 50 µl Mastermix and 50 µl refolded Proteinfraktion (1-12)
- Incubate at 30°C, 80rpm (1700 until 1000)
- Added 1 µl 10 mM AC-9 and extracted twice with 500 µl EtOAc + 1% FA
- Dried organic phase in speedvac
- Redissolved in 100 µl MeOH for HPLC analysis

2 Results

2.1 Protein concentration by UV/VIS

The UV/VIS-Spectrum was measured on a Colibri Picodrop (blank against Storagebuffer) and exportes as CSV for analysis with R.

Firstly we need to load required packages:

```
library(reshape2)
library(ggplot2)
```

Now load the data from the CSV.

```
UVVIS <- read.table("ProtA280-P_web299_2014-07-30.csv", sep = ";",
                     dec = ",", skip = 17, header = TRUE,
                     na.strings = c("", " ", "\n"),
                     blank.lines.skip = TRUE)

UVVIS[14] <- NULL
names(UVVIS) <- c("nm", 1:12)
save(UVVIS, file = "UVVIS_Spectra.Rda")
spectra <- UVVIS
```

or load the Rda file later..

Melt down the spectra into a long format (all absorption values in one column).

```
UVVIS[1:4,1:5]

##      nm     1     2     3     4
## 1 224.8 2.870 0.6606 1.274 1.344
## 2 225.7 2.797 0.6407 1.231 1.297
## 3 226.7 2.709 0.6203 1.186 1.247
## 4 227.6 2.616 0.5978 1.138 1.195

spectra_long <- melt(UVVIS, id.vars = "nm")
spectra_long[1:5,]

##      nm variable value
## 1 224.8       1 2.870
## 2 225.7       1 2.797
## 3 226.7       1 2.709
## 4 227.6       1 2.616
## 5 228.6       1 2.513
```

Find the highest absorption at 280 nm and to use for the legend later on...

```
A280 <- sort(spectra[round(spectra$nm, 0) == 280, 2:13])
A280.1 <- paste(names(A280), "-", round(A280, 3), "AU")
```

This is the code for the plot (Fig. 1):

```
ggplot(spectra_long, aes(x = nm, y = value, color = variable)) + geom_line() +
  theme_classic(base_size = 14, base_family = "Helvetica") +
  theme(strip.background = element_rect(color = "transparent", fill = "#E6E6E6",
                                         size = 0),
        strip.text.x = element_text(size = 14, hjust = 0.5, vjust = 0.5),
        legend.key = element_rect(color = "transparent"),
        plot.title = element_text(vjust = 2, size = 16),
        panel.grid.minor = element_blank(),
        panel.grid.major = element_blank(),
        panel.background = element_blank(),
        plot.background = element_blank(),
        axis.text = element_text(size = 11)) +
  ylab("Absorption (10mm)") +
  scale_colour_discrete(name = "Sample - A280", breaks = c(names(A280)),
                        labels = c(A280.1)) +
  xlim(240, 330) + ylim(0, 1) + geom_vline(xintercept = 280, linetype = "dashed")
```

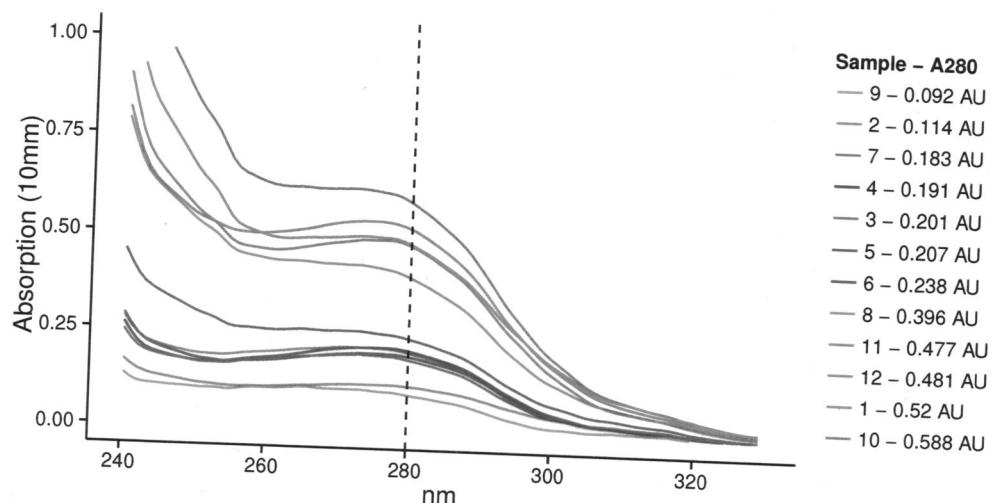


Figure 1: Spectra of all the rebuffered and refolded Samples. The legend is ordered from lowest to highest absorption at 280 nm (dashed line indicates 280 nm).

2.2 Activity Test - HPLC results

```

library(stringr)
library(plyr)
library(ggplot2)
library(gridExtra)
library(reshape2)

ext<-".txt"
sep<="\t"
dir<-"media/IPB//IPB_Y/Experimente/WEB299 - Refolding DoE///HPLC"

# get wanted files in directory
file_list <- grep(paste(ext, "$", sep=""), list.files(dir), value=TRUE)
total <- NULL
#rm(ext, sep, dir)

for (file in file_list){

  ##read data table (csv format)
  AreaData<-read.table(file=paste(dir,file, sep="/"), header=TRUE,
                        sep="\t", skip=7, quote="", dec=",")

  ##read run information (ID, filename etc.)
  ID<-as.vector(scan(file=paste(dir,file, sep="/"), sep="\n", what=character(),
                      nlines=5, quote=""))

  ##define regexp for extraction of experiment name, variant, product etc. from sample ID (eg. WEB211_Tatzenföhn_Y5IRN20W_2)
  #regexp <- "[[:alnum:]]+"
  a <- str_extract_all(ID[2], regexp)

  expmatrix <- a

  ## find sample ID and filename and put into a data frame with the length of AreaData DF
  a<-data.frame(rep(ID[2], nrow(AreaData)),
                 ID[6],
                 matrix(unlist(expmatrix), nrow = 1))
  colnames(a)<- c("SampleID", "Filename", "Experiment", "Fraction")

  AreaData<-cbind(AreaData, a)
  total<-rbind(total, AreaData)
}

rm(AreaData, file, file_list, regexp, a, expmatrix, ID)

sub<-subset(total, Area.>0.01)

# breaks<-c(seq(from=0,to=25,by=0.15))
# sub$bin <- cut(sub$Retention.Time, breaks)
# sub <- dplyr::summarise(sub, .by=Identified.as, Fraction, meanRT=mean(Retention.Time), meanA=mean(Area), sdA=sd(Area))

peaks <- vector("list", length=0)

peaks[["Naringenin"]]<- subset(sub, Identified.as == "Naringenin")
peaks[["AC-9"]]<- subset(sub, Identified.as == "anthracene-9-carboxylic acid")
peaks[["Peak_1"]]<- subset(sub, Identified.as == "Peak 1")
peaks[["Peak_2"]]<- subset(sub, Identified.as == "Peak 2")
peaks[["Peak_4"]]<- subset(sub, Identified.as == "Peak 4")
peaks[["Peak_5"]]<- subset(sub, Identified.as == "Peak 5")

rm(total, breaks)

PL <- vector("list", length=0)

#PL[["Title"]]<- textGrob(label="in vitro methylation of Naringenin using SMT2", gp=gpar(fontsize=14))

INTDATA <- data.frame(Fraction=c(1:12))
for(i in 1:length(peaks)) {

  NAME <- names(peaks)[i]
  tmp <- list(peaks[[i]]$meanA)
  names(tmp)<-NAME
  INTDATA <- cbind(INTDATA, tmp)

  PL[[NAME]]<- ggplot(data=peaks[[i]], aes(x=Fraction, y=meanA)) +
    geom_bar(stat="identity") +
    theme_classic(base_size = 14, base_family = "Helvetica") +
    theme(strip.background=element_rect(color="transparent", fill = "#E6E6E6", size = 0),
          strip.text.x = element_text(size = 14, hjust = 0.5, vjust = 0.5),
          legend.key = element_rect(color="transparent"),
          plot.title = element_text(vjust=2, size=16),
          panel.grid.minor = element_blank(),
          panel.grid.major = element_blank(),
          panel.background = element_blank(),
          plot.background = element_blank(),
          axis.text = element_text(size=9)) +
    geom_errorbar(peaks[[i]], mapping=aes(ymax=meanA+sdA, ymin=meanA-sdA, width=0.1))+
    labs(title=paste(NAME, "\n", round(mean(peaks[[i]]$meanRT), 2), "minutes"))+
    xlab("Sample") +
    ylab(parse(text=expression(Abs~{325~nm}~"[Area]")))

}

args <- c(PL, list(ncol=1))
Plots <- do.call(arrangeGrob, args)
ggsave(filename="Test.pdf", Plots, width=6, height=10)

```

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```

ggsave(filename="Test.png", Plots, width=6, height=10)

INTDATA <- cbind(INTDATA, as.numeric(INTDATA$Peak_1/INTDATA$'AC-9'))
INTDATA <- cbind(INTDATA, as.numeric(INTDATA$Peak_2/INTDATA$'AC-9'))
INTDATA <- cbind(INTDATA, as.numeric(INTDATA$Peak_4/INTDATA$'AC-9'))
INTDATA <- cbind(INTDATA, as.numeric(INTDATA$Peak_5/INTDATA$'AC-9'))
INTDATA <- cbind(INTDATA, as.numeric(INTDATA$Naringenin/INTDATA$'AC-9'))
names(INTDATA)[8:12]<-c("P1_AC", "P2_AC", "P4_AC", "P5_AC", "Naringenin_AC")

```

2.2.1 If the HPLC data has already been processed

Load the processed data. This contains the area for each peak, as determined by the HPLC program. Furthermore the Area relative to the internal standard (Anthracene-9-carboxylic acid) is included.

```

load("HPLC.Rda")
INTDATA

##      Fraction Naringenin   AC-9 Peak_1 Peak_2 Peak_4 Peak_5 P1_AC P2_AC
## 1        1223879 620228 693113 208252 32202 44354 1.1175 0.3358
## 2        1409942 634857 670432 137346 9466 39188 1.0560 0.2163
## 3        741386 636199 738882 265117 108275 19701 1.1614 0.4167
## 4        1311445 637850 687497 160390 16736 30613 1.0778 0.2515
## 5        1159872 635758 716372 244191 33925 44354 1.1268 0.3841
## 6        988461 623506 664867 259262 43788 39188 1.0663 0.4158
## 7        694961 641379 709688 298485 93470 19701 1.1065 0.4654
## 8        1318177 624957 638849 144343 13413 30613 1.0222 0.2310
## 9        936359 629538 689957 274175 57488 44354 1.0960 0.4355
## 10       1251477 598790 621821 159520 17717 39188 1.0385 0.2664
## 11       955192 718524 686722 275554 66028 19701 0.9557 0.3835
## 12       731088 640893 661359 316508 94440 30613 1.0319 0.4939
##          P4_AC  P5_AC Naringenin_AC
## 1        0.05192 0.07151     1.973
## 2        0.01491 0.06173     2.221
## 3        0.17019 0.03097     1.165
## 4        0.02624 0.04799     2.056
## 5        0.05336 0.06977     1.824
## 6        0.07023 0.06285     1.585
## 7        0.14573 0.03072     1.084
## 8        0.02146 0.04898     2.109
## 9        0.09132 0.07045     1.487
## 10       0.02959 0.06545     2.090
## 11       0.09189 0.02742     1.329
## 12       0.14736 0.04777     1.141

```

To plot the data as bar-plots, we need to melt it down.

```
INTmelt <- melt(INTDATA[,c(1,8:12)], id = "Fraction")
```

and then plot it. Basically like this:

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```
ggplot(data=INTmelt, aes(x=as.factor(Fraction), y=value)) +
  geom_bar(stat="identity") + facet_wrap(~variable, scales = "free", ncol = 1)....
```

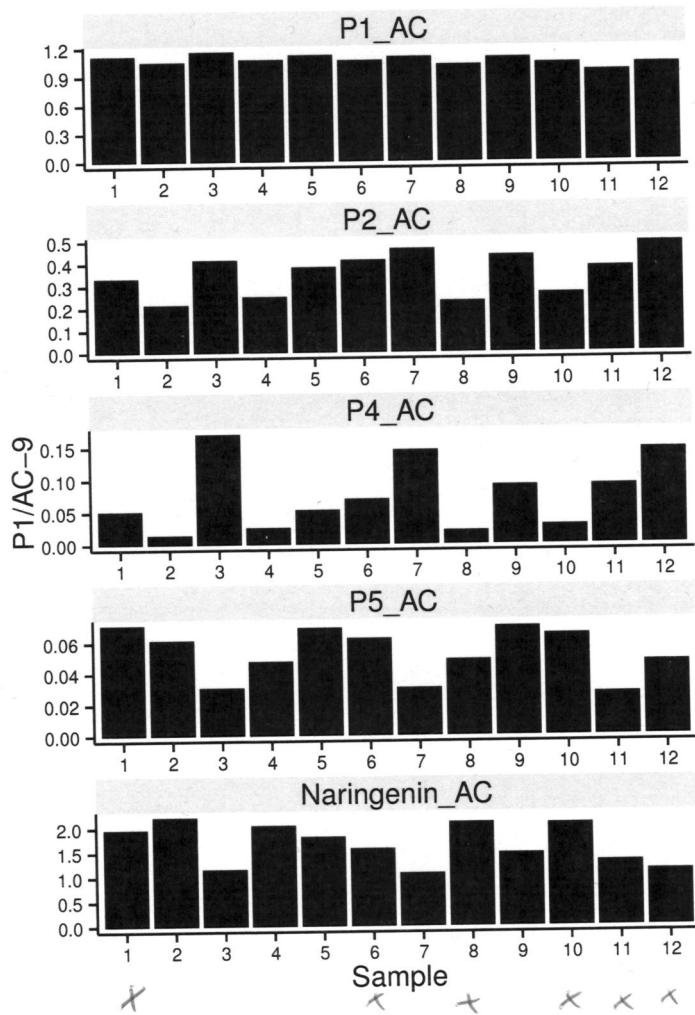


Figure 2: Relative peak areas, as measured by HPLC at 325nm

This looks pretty messed up. The chromatograms show multiple peaks, but it is not clear, which peaks are true products. So I plotted the relative peak areas of each Peak against Naringenin, to see if there is any correlation.

```
require(plyr)

tmp <- INTmelt[grepl(pattern = "(_AC)", x = INTmelt$variable), ]
tmp <- melt(data = INTDATA, id.vars = c("Fraction", "Naringenin_AC"))
```

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```

## select only the relative data
tmp <- tmp[grep(pattern = "(_AC)", x = tmp$variable), ]

## Calculate the correlation
correlation <- ddply(tmp, .variables = .(variable),
                      function(x) {c(Rsquared = (cor(x$Naringenin_AC, x$value))^2)})

correlation

##   variable Rsquared
## 1      P1_AC  0.01229
## 2      P2_AC  0.84072
## 3      P4_AC  0.92675
## 4      P5_AC  0.36518

```

So this is what the plot looks like:

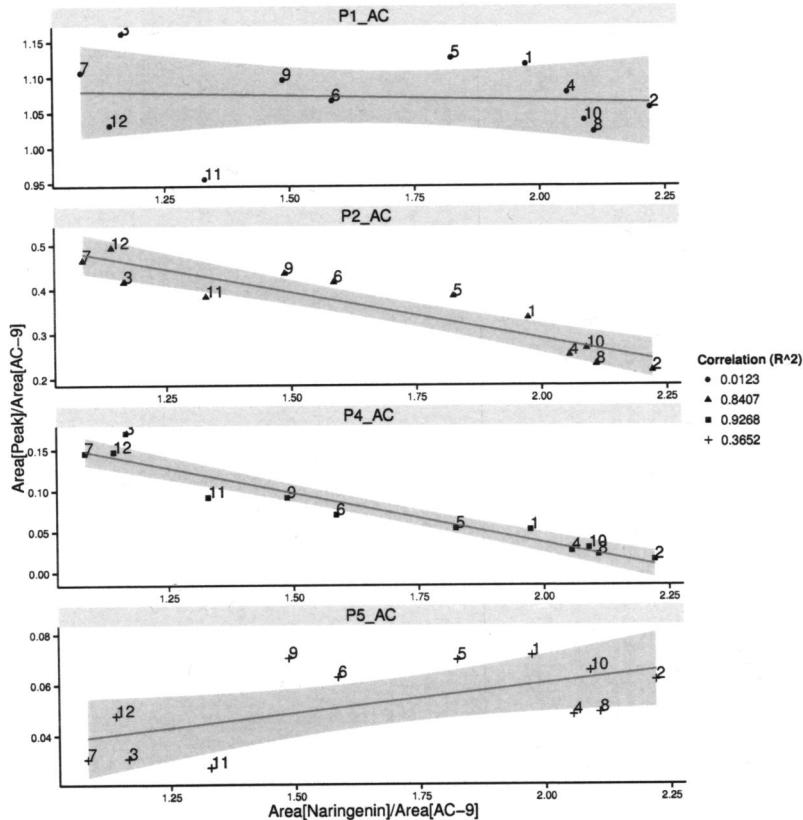


Figure 3: Correlation of the different peak areas to the peak area of Naringenin (the substrate). As the substrate concentration decreases, the concentration of products should increase. This is the case for Peak 2 and Peak 4.

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By the looks of it the Area of Peak 2 and Peak 4 negatively correlates with the Area of Naringenin. As Naringenin decreases, these compounds seem to increase.

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2.3 Fractional design table with response variables

```
library(xtable)

load("SOMT2_Refolding2_UV.rda")
print(xtable(SOMT2_Refolding2[,1:8], caption = "Factorial design table."))

```

	pH	Arginine	Glycerin	ionicStrength	divCations	redox	DES	Tween80
1	9.5	0.5M	10%	low	EDTA	DTT	0	0.5mM
2	5.5	0	0	low	EDTA	DTT	0	0
3	9.5	0	10%	low	Mg_Ca	Shuffling	0.1M	0
4	5.5	0.5M	10%	high	EDTA	Shuffling	0.1M	0
5	9.5	0.5M	0	high	EDTA	Shuffling	0	0
6	5.5	0.5M	0	high	Mg_Ca	DTT	0.1M	0.5mM
7	9.5	0.5M	0	low	Mg_Ca	DTT	0.1M	0
8	9.5	0	10%	high	EDTA	DTT	0.1M	0.5mM
9	5.5	0	10%	high	Mg_Ca	DTT	0	0
10	5.5	0	0	low	EDTA	Shuffling	0.1M	0.5mM
11	9.5	0	0	high	Mg_Ca	Shuffling	0	0.5mM
12	5.5	0.5M	10%	low	Mg_Ca	Shuffling	0	0.5mM

Table 1: Factorial design table.

	uv	P1_AC	P2_AC	P4_AC	P5_AC	Naringenin_AC
1	0.52	1.12	0.34	0.05	0.07	1.97
2	0.11	1.06	0.22	0.01	0.06	2.22
3	0.20	1.16	0.42	0.17	0.03	1.17
4	0.19	1.08	0.25	0.03	0.05	2.06
5	0.21	1.13	0.38	0.05	0.07	1.82
6	0.24	1.07	0.42	0.07	0.06	1.59
7	0.18	1.11	0.47	0.15	0.03	1.08
8	0.40	1.02	0.23	0.02	0.05	2.11
9	0.09	1.10	0.44	0.09	0.07	1.49
10	0.59	1.04	0.27	0.03	0.07	2.09
11	0.48	0.96	0.38	0.09	0.03	1.33
12	0.48	1.03	0.49	0.15	0.05	1.14

Table 2: Response values. **uv** - Absorption at 280 nm, **X_AC** - relative Area of the corresponding Peak (1-5, or Naringenin). This is the area of the peak divided by the area of anthracene-9-carboxylic acid in the corresponding run.

2.3.1 Main Effects plots

```
require("gridExtra")
require("DoE.base")
require("DoE.wrapper")
```

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```
require("FrF2")
```

```
SOMT2_AllData <- SOMT2_Refolding2
RES <- response.names(SOMT2_AllData)
```

```
MEPlot(SOMT2_AllData, abbrev=6, select=c(1:8), response=RES[1])
```

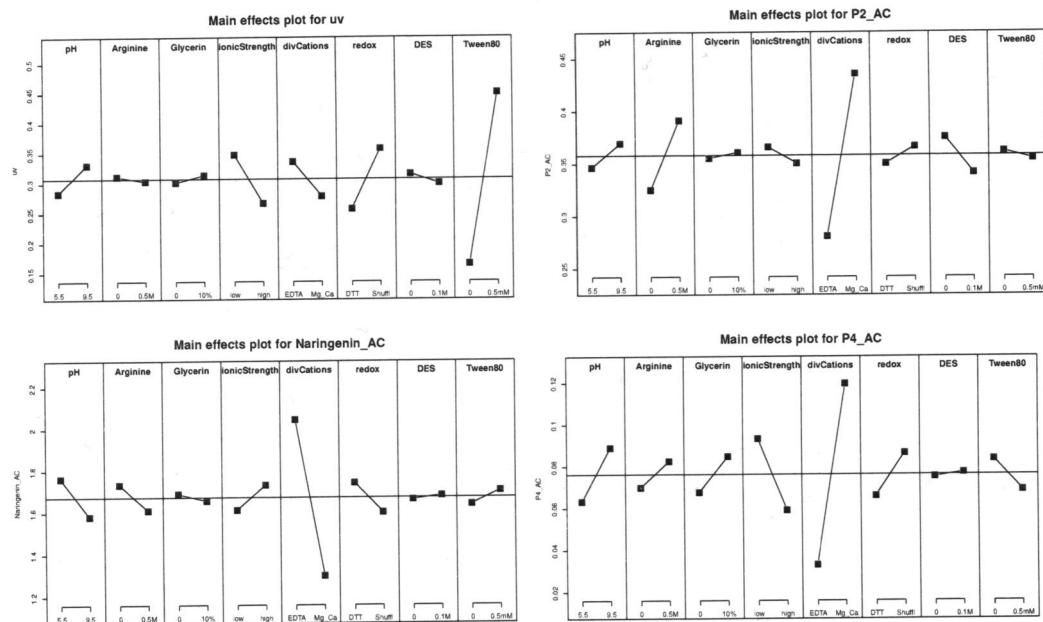


Figure 4: Main effects plots for UV(280 nm), Peak 2, Peak 4 and Naringenin.

salt stock (100x) (100ml)
200 mM CaCl_2 & MgCl_2
 1 M NaCl
 50 mM KCl
 2.2 g CaCl_2 (H_2O frei)
 1.5 g MgCl_2 (H_2O frei)
 5.84 g NaCl
 0.37 g KCl

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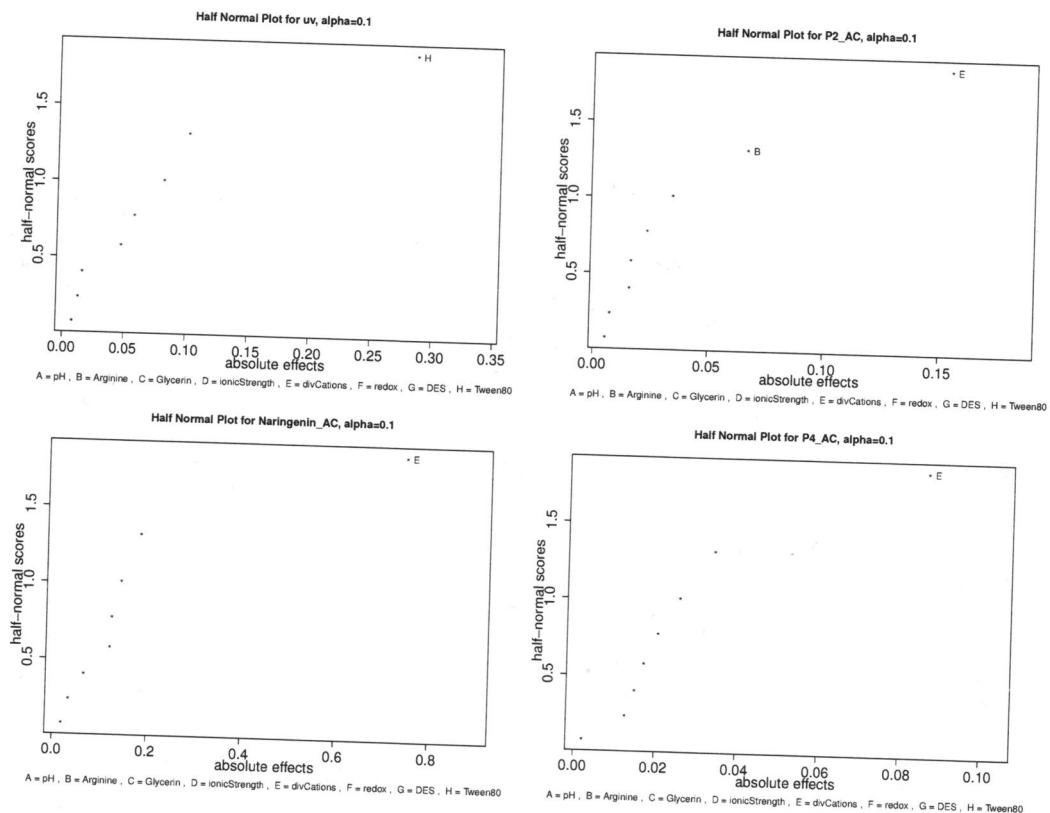


Figure 5: Half-normal (Daniels) plots for UV(280 nm), Peak 2, Peak 4 and Naringenin. The plot illustrates, which factor significantly influences the corresponding response variable. All named points significantly influence the response. The significance level was set of 0.1.

WEB301 - SOMT Refolding 50 mL

Benjamin Weigel

August 4, 2014, Halle

1 Description

for the sake of simplicity a protein solution with an absorption of $A^{280nm} = 1$ contains 1 mg/mL protein

- Experiment WEB299 showed Tween-80 as main factor effecting the refolding of SOMT (measured using A_{280})
- the refolding buffers which showed highest activity of Naringenin methylation contained divalent cations (Mg^{2+} and Ca^{2+}), as well as low salt concentration and glycerin
- addition of arginine and high pH during refolding also seemed beneficial
- based on these observation another refolding trial in 50 mL centrifuge tubes was conducted

	pH	Arginine	Glycerin	ionicStrength	divCations	redox	DES	Tween80
1	8.5	0.5M	10%	low	EDTA	DTT	0	0.5mM
2	5.5	0	0	low	EDTA	DTT	0	0
3	8.5	0	10%	low	Mg_Ca	Shuffling	0.1M	0
4	5.5	0.5M	10%	high	EDTA	Shuffling	0.1M	0
5	8.5	0.5M	0	high	EDTA	Shuffling	0	0
6	5.5	0.5M	0	high	Mg_Ca	DTT	0.1M	0.5mM
7	8.5	0.5M	0	low	Mg_Ca	DTT	0.1M	0
8	8.5	0	10%	high	EDTA	DTT	0.1M	0.5mM
9	5.5	0	10%	high	Mg_Ca	DTT	0	0
10	5.5	0	0	low	EDTA	Shuffling	0.1M	0.5mM
11	8.5	0	0	high	Mg_Ca	Shuffling	0	0.5mM
12	5.5	0.5M	10%	low	Mg_Ca	Shuffling	0	0.5mM

Table 1: Fractional factorial design table of WEB299.

2 Experimental

- use of 2 different refolding buffers from the fractional factorial WEB299 (Buffer 3 and 12, see Tab. 1)

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- additionally used buffer 12, but at pH of 8.5 using 50 mM Tris\HCl

Refolding using:

- 50 mL refolding buffer
- addition of 2.5 mL 1 mg/mL SOMT in IB-solubilization buffer
- denatured SOMT is added at RT, then the tubes were put at 5°C
- incubated over night (from 1500 to 0900)

Buffer 3	Buffer 12	Buffer 12 @ pH 8.5
2.5 mL 1M Tris/HCl pH 8.5	25 mL 0.1 M Mes, 0.5 M Arginine-HCl pH 5.5	25 mL 0.1 M Tris/HCl, 0.5 M Arginine-HCl pH 8.5
12.5 mL XoCH	30.6 l Tween-80	30.6 l Tween-80
	5 mL Glycerin	
	0.5 mL 0.2 M MgCl ₂ , 0.2 M CaCl ₂ , 1 M NaCl, 50 mM KCl	
	1 mL 50 mM GSH, 10 mM GSSG	

Table 2: Recipes for refolding buffers. **XoCH** - Xylitol/Cholin chlorid/H₂O (1:2:3)**2.1 Next morning: workup if refolding reactions**

- 1 mL of each refolding reaction was concentrated and rebuffed as described in WEB299
- the rest of refolding reactions 12 and 12@8.5 were concentrated and rebuffed into 0.1 M Tris/HCl pH 8.5, 20% (V/V) glycerol, 150 mM NaCl using Merck Millipore Centrifugal Concentrators (10.000 Da MWCO)
- refolding reaction in buffer 3 was directly loaded onto a HiTrap Talon FF 1 mL affinity column, which was equilibrated with refolding buffer 3 (omitted DES & GSH:GSSG for equilibration)

2.1.1 Rebuffering & Concentration on 1 mL scale

- concentration in microcentrifuge tube concentrators (10kDa MWCO) [see WEB299]
- rebuffed into 0.1 M Tris/HCl, 150 mM NaCl, 20 % (V/V) glycerol
→ filled up retenate with approx. 450 µl storage buffer thrice
- centrifugation at 14000 x g for 10 min each

	weight _{before} [g]	weight _{after} [g]	add Buffer [μl]
3	1.157	1.217	60
12	1.145	1.202	57
12@8.5	1.133	1.248	11.5

Table 3: Approximation of retenate from concentration in microcentrifuge tube centrifugal concentrators through mass difference of empty tube and tube with retenate.(as described in Amicon Ultra - 0.5 mL Centrifugal Filters [Ultracel 10K])

2.1.2 Analytical Gel-filtration chromatography using ÄKTA

Concentrated Refolding Reaction in Buffer 12 was injected onto analytical gel filtration column.

Run 1

Column: Superdex 200 10/300 GL

Buffer: 0.1 M Tris/HCl pH 8.5, 150 mM NaCl

Sample amount: 100 μl 0.74 mg/mL protein (in refolding buffer 12) [100 μl loop]

The fraction A2 was concentrated using 15 mL tube spin concentrator to a volume of about 80 l. The protein concentration was 0.97 mg/mL.

Run 2

Column: Superdex 200 10/300 GL

Buffer: 0.1 M Tris/HCl pH 8.5, 150 mM NaCl

Sample amount: 100 μl 2.67 mg/mL protein (in refolding buffer 12@8.5) [100 μl loop]

The fraction A2 and A3 were pooled and concentrated using 15 mL tube spin concentrators to a volume of about 500 l. The protein concentration was 0.51 mg/mL.

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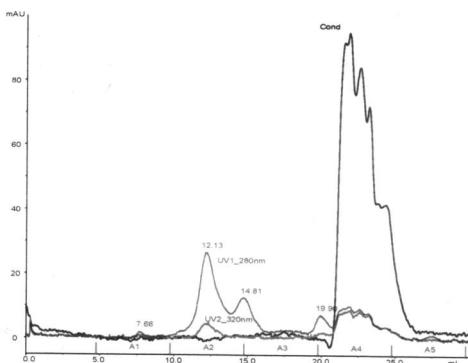


Figure 1: ÄKTA Analytical gel filtration Run 1.

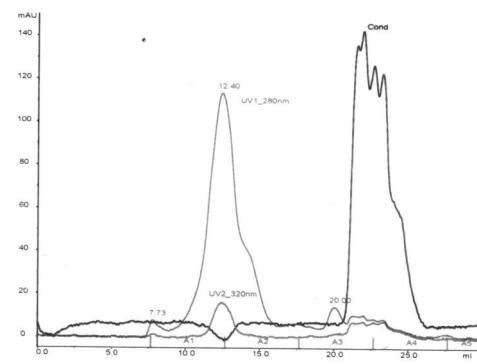


Figure 2: ÄKTA Analytical gel filtration Run 2.

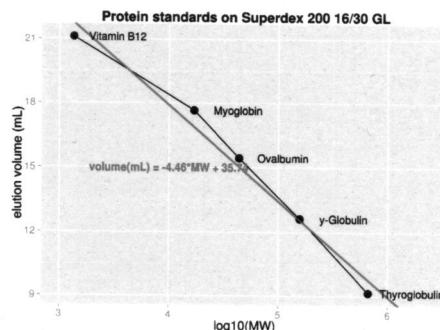


Figure 3: Gel filtration standard run.

The peak eluting at around **14.81 mL** could be a **monomer** of SOMT (calculated mass from standards is 49297 Da). The large peak eluting pretty early (**12.13 / 12.4 mL**) could be a SOMT **tetramer** (171073 - 196662 Da from calculation).

2.1.3 Concentration of 50 mL refolding reactions

- concentrated in 50 mL tube centrifugal concentrators (10kDa MWCO)
- the concentrate was dialyzed against 0.1 M Tris/HCl, 150 mM NaCl, 20 % (V/V) glycerol for 1 hour at RT

2.1.4 Affinity Chromatography using Talon FF 1mL column (Co²⁺-NTA)

Column: Superdex 200 10/300 GL

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Wash buffer

50 mM Tris/HCl pH 8.5
 10% (V/V) glycerol
 10 mM NaCl
 0.5 mM KCl
 2 mM CaCl₂ and 2 mM MgCl₂

Elution buffer

50 mM Tris/HCl pH 7.5
 10% (V/V) glycerol
 0.5 M NaCl
 2.5 mM imidazole

Sample amount: 50 mL refolding reaction 3

Method:

- equilibrated using wash buffer
- applied sample at 1 mL/min
- washed with 5 CV of wash buffer
- eluted with 5 mL of elution buffer
- concentrated and rebuffered into 0.1 M Tris/HCl, 150 mM NaCl pH 8.5 using centrifugal concentrators
- A²⁸⁰ = 0.47

2.2 Activity test**Reaction conditions**

0.1 M Tris/HCl, pH 7.5
 2 mM MgCl₂
 0.1 mM Naringenin
 0.25 mM SAM
 5 mM DTT
 50 µl SOMT (ca. 10 µg)

Mastermix (10x):

280 µl 0.5 M Tris/HCl, 10 mM MgCl₂ pH 7.5
 28 µl 10 mM Naringenin in MeOH
 95.2 µl 5 mM SAM (73.5% biologically active)
 7 µl 1M DTT
 ad to 700 µl ddH₂O (282.8 µl)

→ incubation at 30°C and 100 rpm for specified time

	Sample	concentration [mg/mL]	incubation	protein-solution [µl]	H ₂ O [µl]	Protein [µg]
1	3[1mL]	0.13	üN	50	-	6.5
2	12[1mL]	0.33	üN	50	-	16.5
3	12@8.5[1mL]	0.42	üN	50	-	21
4	3 (after elution & rebuffering)	0.47	1h	21	29	10
5	12	1.17	0, 1h, üN	8.6	41.4	10
6	12@8.5	0.36	1h	28	22	10
7	Gefi Run 1	0.97	1h	10.3	39.7	10
8	Gefi Run 2	0.51	1h	19.6	30.4	10

Table 4: Samples for activity testing.

WEB285 - LAAO Kinetiken für SAM, SAH, Methionin

1.1 Reaction solutions

LAAO-Reaction-Mix (prepare fresh)		Final Volume: 4500 µl
Add ...		Final concentration
233 µl - 193 U/mL HRP		10 U/mL HRP
90 µl - 5 mg/mL TMB-HCl		0.1 mg/mL TMB-HCl
0.852 mL - 1.32 mg/mL LAAO		0.25 mg/mL LAAO
3325 µl Buffer (0.1 M Tris-HCl pH 7.5)		

1.2 Substrate concentrations

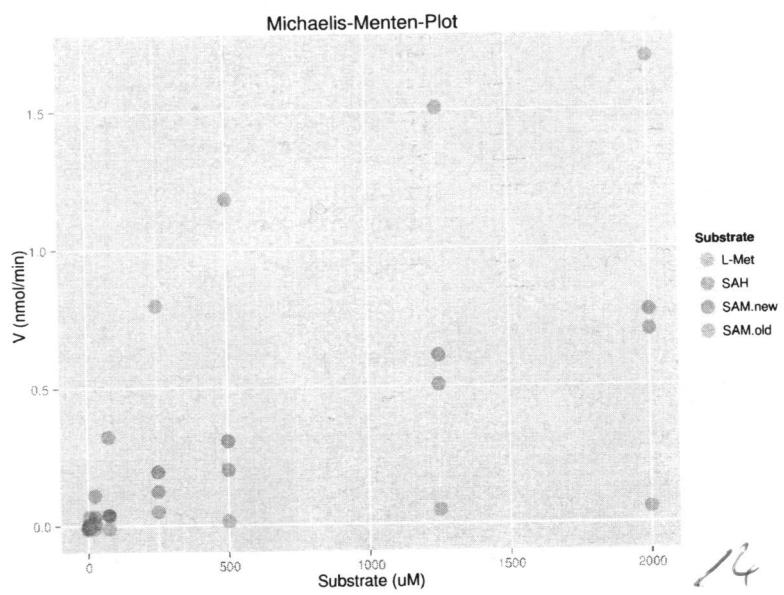
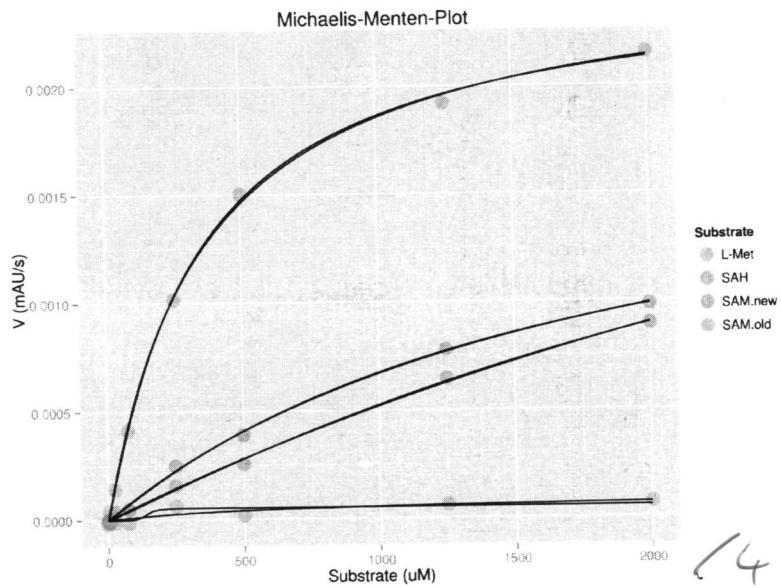
	1	2	3	4	5	6
	SAH		SAM		L-Met	
A	0	0	0	0	0	0
B	5	5	5	5	5	5
C	25	25	25	25	25	25
D	75	75	75	75	75	75
E	250	250	250	250	250	250
F	500	500	500	500	500	500
G	1250	1250	1250	1250	1250	1250
H	2000	2000	2000	2000	2000	2000

1.3 Pipetting

- Stock solution (SAM, SAH, L-Met) are 2 mM in ddH₂O

Final concentration [µM]	Stock solution [µl]	ddH ₂ O [µl]
0	-	100
5	1 (500 µM)	99
25	5 (500 µM)	95
75	15 (500 µM)	85
250	12.5	87.5
500	100	300
1250	62.5	37.5
2000	100	-

→ Attention → substrate concentrations in the actual assays are 4-fold lower (25 µl substrate + 75 µl (1A0 solution))



1.3.1 EcoMTAN (recombinant enzyme with N-term His6)

Molecular weight: 26517.2 Da
 Number AA: 252

2 µM MTAN → 53 mg/L = 53 µg/mL

Ribosylhomocysteine production	
Final concentration	
5.64 mM HCY	357 µl 7.9 mM SAH
2 µM MTAN	40 µl 1.4 mg/mL MTAN
0.1 M Tris-HCl pH 7.5	50 µl 1M Tris-HCl pH 7.5
Incubate at RT for 1h	Ad to 500 µl

LAAO-Reaction with even more substrate

Substrate and Concentration (µM)	Substrate-concentration in assay	Wells	VD in Water
5 mM Methionine	1.25 mM	AB7	20
5 mM SAH		CD7	1:1.58
5 mM SAM		EF7	0
5 mM RHyc		GH7	1:1.58

WEB285 - Calculations

Benjamin Weigel

August 7, 2014

1 Calculation

The initial velocities of curves obtained from the MTP-Reader are fitted using a linear fit function.

```
velo<-function (t, P, limlow=t[1], limup=t[length(t)])
{
  df<-data.frame(t, P)
  names(df)<-c("time", "P")
  fit<-lm(P ~ time, data=subset(subset(df, time>=limlow), time<=limup))
  return(as.numeric(fit$coefficients))
}
```

The values thus-obtained have the unit $\frac{AU}{s}$ and need to be converted to $\frac{nmol}{min}$. This is achieved by rearranging the Lambert-Beer formula:

$$E = \varepsilon \cdot c \cdot d$$

$$c = \frac{E}{\varepsilon \cdot d}$$

$$v = \frac{c}{t} = \frac{\left(\frac{E}{\varepsilon \cdot d}\right)}{t} = \frac{E}{t} \cdot \varepsilon^{-1} \cdot d^{-1}$$

$$\frac{E}{t} = v_{AU}$$

Figure 1: Calculation of the concentration (velocity) thru Lambert-Beers Law

The extinction coefficient of the yellow charge transfer complex of 3,3',5,5'-tetramethylbenzidine (TMB) is $\varepsilon_{652nm} = 39000 M^{-1} cm^{-1}$. The path length of a 100 and 150 μl solution in an MTP are 2.922 and 4.384 mm respectively.

```
require(xtable)
require(plyr)

load("/media//IPB//IPB_Y//Experimente//WEB285//Kinetics_WEB285.Rda")

in <- ddply(kin, .(Substrate, c_uM), summarize,
```

$$v = \frac{v_{AU}}{\varepsilon \cdot d} = \frac{v_{AU}}{39000 M^{-1} cm^{-1} \cdot 0.292 cm}$$

↳
 ↳
 ↳
 ↳ multiply by volume
 ↳ produces 0.5 mol charge transfer complex per mole reduced H₂O₂

operation	unit
× 60	[M · s ⁻¹]
× 1000	[mM · min ⁻¹]
× 1000	[μM · min ⁻¹]
× 1000	[nM · min ⁻¹]
× 1 · 10 ⁻⁴	[nmol · min ⁻¹ = U]
× 2	[nmol · min ⁻¹ = U]

Figure 2: calculation of velocity in $\frac{nmol}{min}$, or U from $\frac{AU}{s}$.

$$v = v / (39000 * 0.292) * 1000000000 * 60 * 0.0001 * 2$$

```
kinetic <- ddply(kin, .(Substrate), function(x){c(MM=fitMM(x$c_uM, x$v),
Hill=fitHill(x$c_uM, x$v))})
```

Michaelis-Menten Equation → $v = \frac{v_{max} \cdot [S]}{K_m + [S]}$

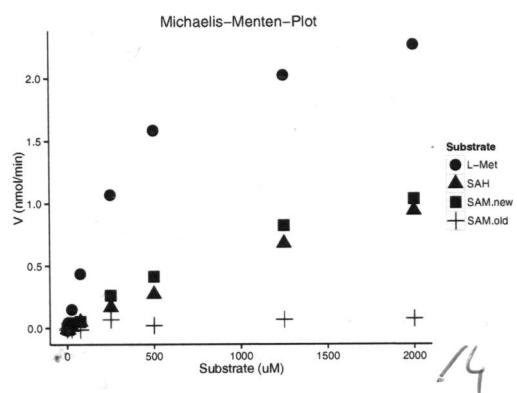
$$v_{max} = k_{cat} \cdot E_0$$

Hill-Equation → $v = \frac{v_{max} \cdot [S]^h}{K_{0.5}^h + [S]^h}$

	substrate	MM. v_{max} [$\frac{nmol}{min}$]	MM. K_m [μM]	Hill. v_{max} [$\frac{nmol}{min}$]	Hill. $K_{0.5}$ [μM]	Hill.h
1	L-Met	2.6710	368.0921	2.5958	343.0549	1.0549
2	SAH	3.5180	5421.0413	2.5866	3333.6642	1.0798
3	SAM.new	1.9811	1806.4376	1.8350	1551.3848	1.0443
4	SAM.old	0.0935	608.4634	0.0558	127.1097	56.7976

Table 1: Kinetic parameters obtained from fitting the progress curves using Hill and Michaelis-Menten models.

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Figure 3: Fitting functions for Kinetic models
(below) and v/S-Plots.

```
#####
#Calculate Kinetic Data from v/S-data
#####
require("minpack.lm")
## Michaelis-Menten Model
fitMM<-function (S, v, Km=100, Vmax=1) ## use starting parameters for Km & Vmax
{
  fit<-nls(formula=v ~ Vmax*S/(Km + S), data=data.frame(S, v),
           start=list(Vmax=Vmax, Km=Km))
  return(coef(fit))
}

## Hill Model
## use starting parameters for Km & Vmax & h
fitHill<-function (S, v, K0.5=600, Vmax=0.001, h=0.8)
{
  fit<-nlsLM(formula=v ~ Vmax*S^h/(K0.5^h + S^h), data=data.frame(S, v),
             start=list(Vmax=Vmax, K0.5=K0.5, h=h))
  return(coef(fit))
}
```

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WEB285-288

Benjamin Weigel

August 19, 2014

1 Introduction

The experiments WEB285, WEB286, WEB287 and WEB288 are all part of a complex.

All experiments were conducted as described in the LAAO SOP. The concentrations of the stock substrate solutions used were 0, 5, 25, 75, 250, 500, 1250, 2000 and 5000 μM . However, because the assay is started by addition of 75 μl LAAO-assay solution to 25 μl substrate, the substrate concentration in the final assay is 4 fold lower.

Thus, the actual concentrations in the assay were 0, 1.25, 6.25, 18.75, 62.5, 125, 312.5, 500 and 1250 μM of substrate.

All experiments were done in duplicates.

WEB285

First experiment using L-methionine, SAH, a new stock of SAM and an old stock (which had been previously used) of SAM as substrates. Only 0 to 500 μM .

WEB286

Used S-ribosyl-L-homocysteine (RHCy, 0 to 1250 μM) and L-Met, SAH, SAM (1250 μM) as substrate. **S-ribosyl-L-homocysteine** was prepared enzymatically using methyl-\thioadenosyl-nucleosidase from *E. coli* (EcoMTAN) as described by Zhu *et al.* [1]

EcoMTAN

- frozen at -20°C
- buffer: 50 mM KPi, 10 % (V/V) pH 7
- stock dates to early 2012

RHCy preparation

- 10 mM SAH
- 2 μM ~ 53 $\frac{\mu\text{g}}{\text{mL}}$ EcoMTAN (*pfs*, 26517.2 Da)
- incubated for 2 hours at 25°C
- used directly as a '10 mM RHCy solution' for LAAO reactions

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WEB287

I guess I measured some crap here ... probably checking about the SAM at high concentrations ($2000 \mu\text{M}$), which seemed to be less of a substrate than the days before :-?

WEB288 - Wrapper functions, concatenate all the data ...

All the data collected in the first three experiments was merged into one dataset and then analyzed as follows ...

References

- [1] ZHU, J., DIZIN, E., HU, X., WAVREILLE, A.-S., PARK, J., AND PEI, D. S-Ribosylhomocysteinase (LuxS) is a mononuclear iron protein. *Biochemistry* 42, 16 (Apr. 2003), 4717–26.

WEB288 - Optimized Calculations for LAAO Assay & Spectramax Import

Benjamin Weigel

August 18, 2014

1 Wrapper function for Spectramax import

Spectramax import important meta-information inside the ASCII files. Those metainformation include Plate and Wellnames. To make access to the raw data easier inside of the R environment, I wrote a small wrapper function to import Spectramax exported ASCII files. It still needs the earlier functions to import the Spectramax data.

```
require(ggplot2)
require(iterators)
library(plyr)
#source("~/media//IPB//IPB_Y/Packages/SpectraMaxImport/SM_ascii_Import.R")
#source("~/media//IPB//IPB_Y/Packages/SpectraMaxImport/DFlong.R")
source("~/Dokumente/R Projects/Packages/SpectraMaxImport/SM_ascii_Import.R")
source("~/Dokumente/R Projects/Packages//SpectraMaxImport//DFlong.R")
```

The function demands three arguments. The raw ASCII file containing the data exported by the Spectramax software [RAW], the Layout of the MTP read by the Platereader in CSV-format [LAY] and a working directory [WD].

	Well	Substrate	c_uM	rep	Plate	timePOINTS	lmlow	lmup
1	A9	SAM	5000	1	Plate#1	0.50	120	150
2	A10	SAM	5000	2	Plate#1	0.50	120	150
3	B9	SAM	5000	1	Plate#1Copy	1.00	110	125
4	B10	SAM	5000	2	Plate#1Copy	1.00	110	125
5	C9	SAM	5000	1	Plate#1Copy#2	1.50	120	150
6	C10	SAM	5000	2	Plate#1Copy#2	1.50	120	150
7	D9	SAM	5000	1	Plate#1Copy#3	2.00	120	150
8	D10	SAM	5000	2	Plate#1Copy#3	2.00	120	150
9	E9	SAM	5000	1	Plate#1Copy#4	2.50	120	150
10	E10	SAM	5000	2	Plate#1Copy#4	2.50	120	150
11	F9	SAM	5000	1	Plate#1Copy#5	3.00	120	150
12	F10	SAM	5000	2	Plate#1Copy#5	3.00	120	150
13	G9	SAM	5000	1	2000uM	3.50	5	25

Table 1: Example of a CSV containing the positional data and information about what each well contains. Two columns named **Plate** and **Well** are mandatory and need values in each row. Other columns are optional and for the description of the well.

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```

platewrap <- function(RAW, LAY, WD){
  setwd(WD)
  df<-fetchSMdata(RAW)

  ##### Conditions on Plate
 conds<-read.table(file=LAY, sep=",", header=TRUE, stringsAsFactors=FALSE,
  comment.char="", row.names=NULL)

  ##### PlateData for Kinetics #####
  for (pl in df$Plates){
    if(exists("DF") == FALSE) {
      names(pl$data)<-paste(pl$PlateName, names(pl$data), sep=".")
      DF<-pl$data
      DF<-DF[, colSums(is.na(DF))<nrow(DF)]
      DF<-data.frame(rearrangeMTP(DF,
        NAMES=c("time", "temp", "Abs", "Position")),
        stringsAsFactors=FALSE)
    } else {
      names(pl$data)<-paste(pl$PlateName, names(pl$data), sep=".")
      tmp<-pl$data
      tmp<-tmp[, colSums(is.na(tmp))<nrow(tmp)]
      tmp<-data.frame(rearrangeMTP(tmp,
        NAMES=c("time", "temp", "Abs", "Position")),
        stringsAsFactors=FALSE)
      DF<-rbind(DF, tmp)
    }
  }
  rm(tmp)

  ##### wrapper function for ddply to split positional information
  ##### into two different columns
  exNames <- function(POS){
    tmp<-t(data.frame(strsplit(POS, split=".[]")))
    colnames(tmp)<-c("Plate", "Well")
    return(tmp)
  }
  ##### end wrapper

  DF<-ddply(DF, .(time, temp, Abs, Position), function(x){exNames(x$Position)})
  DF <- transform(DF,
    Abs=as.numeric(Abs),
    time=as.numeric(time))

  DF<-ddply(DF, .(time, temp, Abs, Position),
    function(x){conds[conds$Well == x$Well & conds$Plate==x$Plate,]})

  return(DF)
}

```

In the beginning the raw data is read into a list by the function *fetchSMdata()*. Afterwards the condition CSV is read. In the following *for()*-loop checks if the object *DF* exists and extracts the information of each Plate contained within *df\$Plates*. The names of each column containing all values collected within the well and corresponding plate are expanded to contain the plate name, as well as the well name to make an assignment of wells

from multiple plates possible.

Then a dataframe containing the *data* dataframe from inside the *pl* object is created. After that every NA value is removed. Lastly the dataframe is converted to long format with *rearrangeMTP()*. All Plates and data are reassembled into one data.frame.

```
for (pl in df$Plates){
  if(exists("DF") == FALSE) {
    names(pl$data)<-paste(pl$PlateName, names(pl$data), sep=". ")
    DF<-pl$data
    DF<-DF[, colSums(is.na(DF))<nrow(DF)]
    DF<-data.frame(rearrangeMTP(DF,
                                NAMES=c("time", "temp", "Abs", "Position")),
                    stringsAsFactors=FALSE)
  } else {
    names(pl$data)<-paste(pl$PlateName, names(pl$data), sep=". ")
    tmp<-pl$data
    tmp<-tmp[, colSums(is.na(tmp))<nrow(tmp)]
    tmp<-data.frame(rearrangeMTP(tmp,
                                NAMES=c("time", "temp", "Abs", "Position")),
                    stringsAsFactors=FALSE)
    DF<-rbind(DF, tmp)
  }
}
rm(tmp)
```

The positional information which was just put into one column like *PLATE.WELL* is now being split up again into two separate columns. The columns containing numerical values are transformed into *numeric*. Finally, the dataframe is expanded by the columns from the CSV which contain additional information. This is achieved by matching Plate and Well. The resulting dataframe is returned.

```
exNames <- function(POS){
  tmp<-t(data.frame(strsplit(POS, split=".[ ]")))
  colnames(tmp)<-c("Plate", "Well")
  return(tmp)
}

DF<-ddply(DF, .(time, temp, Abs, Position), function(x){exNames(x$Position)})
DF <- transform(DF,
               Abs=as.numeric(Abs),
               time=as.numeric(time))

DF<-ddply(DF, .(time, temp, Abs, Position),
           function(x){conds[conds$Well == x$Well & conds$Plate==x$Plate,]})

return(DF)
```

2 Final Evaluation of LAAO Assay

Because the data were collected on two consecutive days those data are first read and then added together in one dataframe.

```
setwd("~/Dokumente/R Projects/Experimente/WEB288")
source("DeriveWrapper.R")
source("wrapper_Plates.R")
source("fitModel.R")

df1 <- platewrap(RAW="test data/140617_WEB285.txt",
                  LAY="test data/WEB288_PlateLayout.csv", WD=getwd())
df2 <- platewrap(RAW="../WEB286/RAW/140618_WEB286.txt",
                  LAY="test data/WEB288_PlateLayout.csv", WD=getwd())

DF <- rbind(df1, df2)
```

	time	temp	Abs	Position	Well	Substrate	c_uM	rep	Plate	lmlow	lmup
1	0.00	26.3	0.04	Plate#3.C11	C11	SAM.old	25	1	Plate#3	0	100
2	0.00	26.3	0.04	Plate#3.B12	B12	SAM.old	5	2	Plate#3	0	100
3	0.00	26.3	0.04	Plate#3.B11	B11	SAM.old	5	1	Plate#3	0	100
4	0.00	26.3	0.04	Plate#3.A12	A12	SAM.old	0	2	Plate#3	0	100
5	0.00	26.3	0.04	Plate#3.A11	A11	SAM.old	0	1	Plate#3	0	100
6	0.00	26.3	0.04	Plate#3.C12	C12	SAM.old	25	2	Plate#3	0	100
7	0.00	26.3	0.05	Plate#3.F11	F11	SAM.old	500	1	Plate#3	0	100
8	0.00	26.3	0.05	Plate#3.D11	D11	SAM.old	75	1	Plate#3	0	100
9	0.00	26.3	0.05	Plate#3.H12	H12	SAM.old	2000	2	Plate#3	30	100
10	0.00	26.3	0.05	Plate#3.G11	G11	SAM.old	1250	1	Plate#3	30	100

Table 2: Example of the dataframe DF as obtained by the methods so far.

The raw data are fitted according to the borders set in the columns $lmlow$ and $lmup$.

```
kinetic <- ddply(DF, .(Substrate, c_uM, rep), function(x){
  c(v=velo(t=x$time, P=x$Abs, limlow=unique(x$lmlow), limup=unique(x$lmup))[2])
})
kinetic$c_uM <- kinetic$c_uM/4
kinetic <- ddply(kinetic, .(Substrate, c_uM), summarize, meanv = mean(v), sdv = sd(v))

kinetic <- ddply(kinetic, .(Substrate, c_uM, meanv, sdv), function(x){
  c(
    nmolmin=x$meanv/(39000*0.292)*1000000000*60*0.0001*2,
    nmolsd=x$sdv/(39000*0.292)*1000000000*60*0.0001*2)
})
```

The resulting V-S data is beridded of (really small, but) negative linear fit values and fitted using various models including Michaelis-Menten (1), Hill (2) and a simple Substrate inhibition (3) models.

Michalis-Menten (hyperbola) fit

$$v = \frac{v_{max} \cdot [S]}{K_m + [S]} \quad (1)$$

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$$v_{max} = k_{cat} \cdot E_0$$

Hill-Equation

$$v = \frac{v_{max} \cdot [S]^h}{K_{0.5}^h + [S]^h} \quad (2)$$

Substrate Inhibition model

$$v = \frac{v_{max} \cdot [S]}{K_m + [S] + \frac{S^2}{K_{SI}}} \quad (3)$$

```
kinparam <- ddply(kinetic, .(Substrate), function(x){
  c(
    MM=tryCatch(coef(fitMM(x$c_uM, x$nmolmin)),
                 error=function(e){return(c(0, 0))}),
    Hill=tryCatch(coef(fitHill(x$c_uM, x$nmolmin, K0.5=500, Vmax=0.01, h=1)),
                  error=function(e){return(c(0, 0))}),
    SubInh=tryCatch(coef(fitSubInh(x$c_uM, x$nmolmin)),
                   error=function(e){return(c(0, 0))})
  )})
```

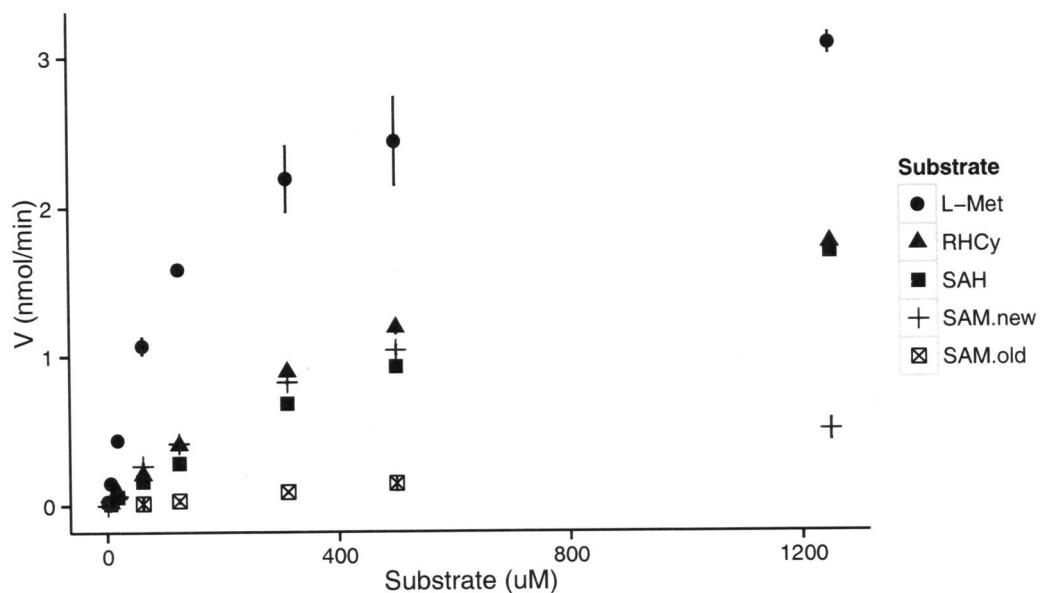


Figure 1: V/S-Plot of different substrates

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	Substrate	concentration [μM]	v [$\frac{\text{nmol}}{\text{min}}$]	sd [$\frac{\text{nmol}}{\text{min}}$]
2	L-Met	1.2500	0.0266	0.0114
3	L-Met	6.2500	0.1486	0.0045
4	L-Met	18.7500	0.4352	0.0075
5	L-Met	62.5000	1.0695	0.0551
6	L-Met	125.0000	1.5838	0.0104
7	L-Met	312.5000	2.1897	0.2176
8	L-Met	500.0000	2.4352	0.2906
9	L-Met	1250.0000	3.0853	0.0683
12	RHCy	6.2500	0.0198	0.0033
13	RHCy	18.7500	0.0828	0.0076
14	RHCy	62.5000	0.2065	0.0183
15	RHCy	125.0000	0.4032	0.0142
16	RHCy	312.5000	0.8925	0.0164
17	RHCy	500.0000	1.1886	0.0417
18	RHCy	1250.0000	1.7544	0.0506
21	SAH	6.2500	0.0109	0.0025
22	SAH	18.7500	0.0581	0.0273
23	SAH	62.5000	0.1608	0.0042
24	SAH	125.0000	0.2819	0.0119
25	SAH	312.5000	0.6786	0.0179
26	SAH	500.0000	0.9249	0.0273
27	SAH	1250.0000	1.6941	0.0009
29	SAM.new	1.2500	0.0001	0.0037
30	SAM.new	6.2500	0.0316	0.0037
31	SAM.new	18.7500	0.0620	0.0162
32	SAM.new	62.5000	0.2624	0.0194
33	SAM.new	125.0000	0.4120	0.0313
34	SAM.new	312.5000	0.8219	0.0060
35	SAM.new	500.0000	1.0358	0.0313
36	SAM.new	1250.0000	0.4966	0.0695
41	SAM.old	62.5000	0.0134	0.0366
42	SAM.old	125.0000	0.0302	0.0117
43	SAM.old	312.5000	0.0873	0.0064
44	SAM.old	500.0000	0.1421	0.0453

Table 3: Initial velocities, as fitted using the lower and upper fit limit. Negative values for initial velocities were omitted.

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Substrate	V_{max}^{MM} [$\frac{\text{nmol}}{\text{min}}$]	K_M^{MM} [μM]	V_{max}^{Hill} [$\frac{\text{nmol}}{\text{min}}$]	$K_{0.5}^{Hill}$ [μM]	h_{Hill}	V_{max}^{SubInh} [$\frac{\text{nmol}}{\text{min}}$]	K_M^{SubInh} [μM]	K_{Si}^{SubInh} [μM]
1 L-Met	3.2932	141.3721	3.6755	192.0820	0.8278	4.0810	-1919.5020	45.6480
2 RHCy	2.6750	644.3013	2.3857	501.6767	1.1209	0.8436	-462.0116	29.3656
3 SAH	3.5814	1396.7408	3.8318	1590.7232	0.9712	0.6164	-422.8719	31.6529
4 SAM,new	0.8712	97.4619	0.7888	97.9585	2.3777	0.6812	-514.3461	27.8463
5 SAM,old	0.0000	0.0000	0.6727	1434.0135	1.2498	-3.5212	-13598.5932	310.9211

Table 4: Kinetic parameters obtained from fitting the v-S data obtained from the progress curves. Only values ≥ 0 were used for fitting.

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WEB303 - Analytical Gefi with reduced refolded Protein

Benjamin Weigel

August 6, 2014, Halle

1 Description

- from WEB301 experiments with analytical gel filtration it seems as if SOMT elutes in tetrameric, as well as monomeric form
 - however those runs were under **non-reducing** conditions
 - ↪ re-run a sample eluted from the WEB301 GPC run under **reducing** conditions

2 Experimental

2.1 Protein sample

Sample: concentrated eluate from pooled fractions A2 and A3 of **WEB301.Run2**

Treatment: added approximately 10 mM DTT to sample → let incubate on ice for approx. 1h

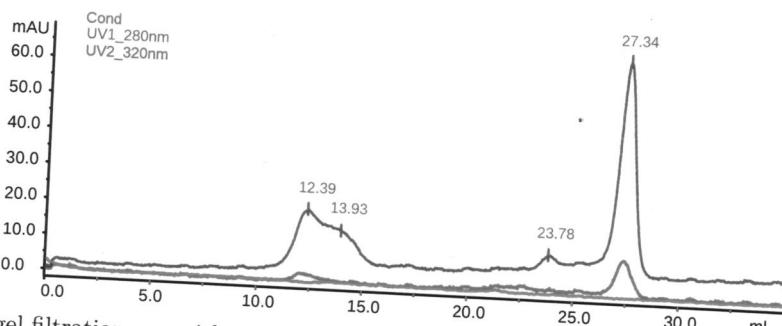
2.2 ÄKTA Run conditions (Fig. 1a)

ÄKTA column: Superdex 200 10/300 GL

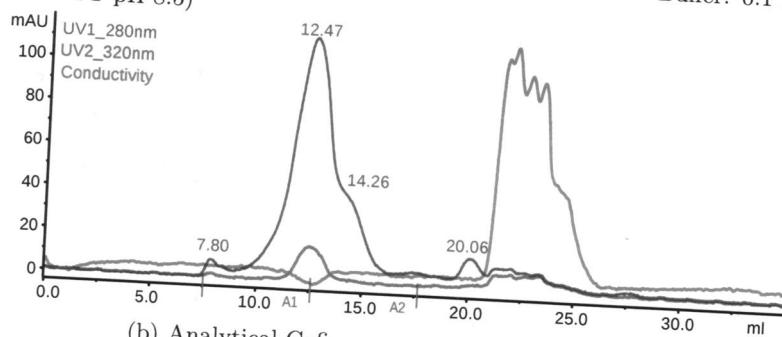
Running buffer: 0.1 M Tris/HCl, 150 mM NaCl, **1 mM DTT**, pH 8.5

Sample: 100 µl of the sample from **2.1** (xx mg/mL) [100 µl loop]

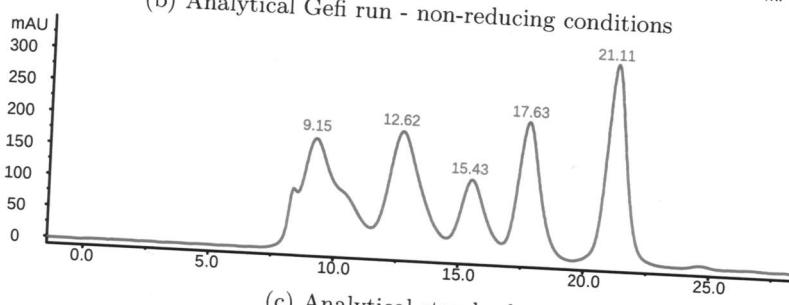
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(a) Analytical gel filtration run with protein that was subjected to gel filtration before (WEB301 Run2, see Fig. 1b). The protein was treated with 10 mM DTT prior to the run. Buffer: 0.1 M Tris/HCl, 150 mM NaCl, 1 mM DTT pH 8.5)



(b) Analytical Gefi run - non-reducing conditions



(c) Analytical standards

Figure 1: ÄKTA runs

Determination of the apo-PFOMT structure

Benjamin Weigel

September 2, 2014

1 Introduction

The three dimensional structure of PFOMT could be determined in 2008 by Kopycki and co-workers in the group of Thomas Vogt [1]. However, the enzyme could only be co-crystallized with the bound cosubstrate S-adenosyl-homocysteine (SAH). Trials, to also co-crystallize or soak a flavonoid substrate were unsuccessful.

Furthermore, the structure only shows the N-terminally truncated enzyme. It was hypothesized, that the N-terminus has the function of a lid and is somewhat flexible.

Martin Dippe (DIM) was able to obtain variants with a changed rediospecificity (3' to 4') towards the flavonoid substrate. This made us even more interested in obtaining structures with bound flavonoid substrates. DIM was also able to produce a SAM-synthase, which enabled us to synthesize donor substrates different from SAM. This way, the methyl side chain could be altered to ethyl, propyl and even butyl.

Additionally, I could show through ITC measurements that were conducted in the winter of 2013, that the PFOMT wildtype could bind SAH, SAM and SAE with similar affinities ($K_D \sim 2 \mu\text{M}$). However, the enthalpic term ΔH of the Gibbs Free Energy (Equation (1)) was getting much lower with growing chain lengths ($\Delta H_{SAH} > \Delta H_{SAM} > \Delta H_{SAE}$).

$$\Delta G = \Delta H - T\Delta S \quad (1)$$

During ITC measurements using SAE there was almost no heat detectable anymore. However, since the K_D stayed the same this must be due to entropic interactions.

2 Aim

There were several goals included within this project.

- try to obtain structures with bound SAM, SAE ...
- try to obtain structures with bound flavonoids to explain changed regiospecificity
- try to obtain an apo PFOMT structure (apo = without)

3 Experimental

3.1 Cloning

The construct pQE30 PFOMT was mutagenized using the primers PFOMT_A107_fw and *_rv to remove an NdeI cleavage site inside the PFOMT coding sequence. Afterwards, the *PFOMT* gene was amplified by PCR from the construct using the primers PFOMT_fw and PFOMT_rv

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(Table 1). The gene was then cloned into a pET28a-(+) vector using the EcoRI and NdeI cleavage sites.

Table 1: Primer table

primer	sequence (5'→3')
PFOMT_A107_fw	cagagaggcCtagagattggcttgc
PFOMT_A107_rv	gcaaggccaaatctcataGgcctctcg
PFOMT_fw	CATATGGATTTCCTGTGATGAAGCAGGTC
PFOMT_rv	GAATTCAATAAAGACGCCTGCAGAAAGTG

3.2 Protein Expression and Purification

The PFOMT gene was expressed using *E.coli* BL21(DE3) cells in ZYP5052 auto-induction medium according to Studier [2]. Therefore, 250 mL of ZYP5052 medium containing 200 µg/mL kanamycin were inoculated using a single colony of BL21(DE3) containing the pET28 PFOMT construct and incubated over night at 37°C and 220 rpm.

The cells were collected by centrifugation at 10.000 x g and 4°C for 10 minutes. The cells were resuspended in 20 mL lysis buffer (50 mM Tris/HCl, 500 mM NaCl, 10% glycerol, 2.5 mM imidazole, 1% Triton X-100, pH 7.4) per gram of wet cells. Lysozyme was added and incubated at room temperature for 15 minutes, after which the cells were lysed by ultra-sonication. The lysate was centrifuged at 10.000 x g, 4°C for 15 minutes to remove cell debris.

The cleared lysate was injected onto a HiTrap Talon FF 1mL column, which had been equilibrated using washing buffer, using an ÄKTA FPLC system. The protein was eluted using elution buffer. For desalting the eluate was dialyzed two times against 100 times the volume of storage buffer for 1 hour at RT each.

Washing buffer

50 mM Tris/HCl
500 mM NaCl
10% glycerol
2.5 mM Imidazole
pH 7.4

Elution buffer

50 mM Tris/HCl
500 mM NaCl
10% glycerol
250 mM Imidazole
pH 7.4

Lysis buffer

50 mM Tris/HCl
500 mM NaCl
10% glycerol
2.5 mM Imidazole
1% Triton X-100
pH 7.4

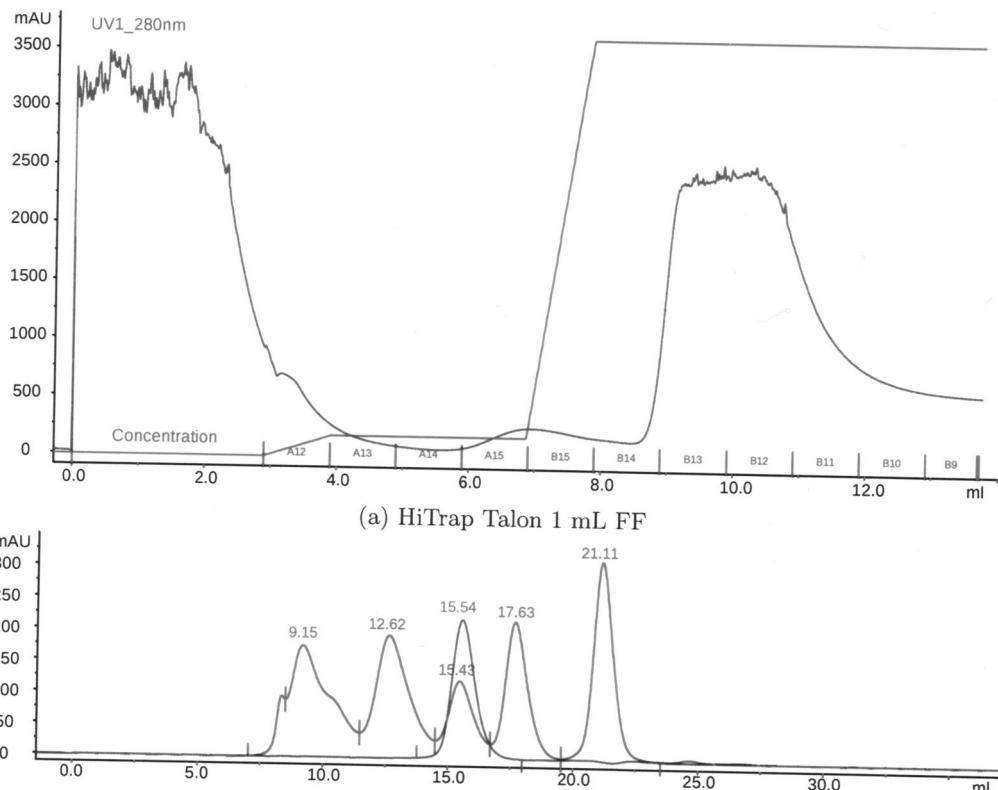
Storage buffer
25 mM HEPES
150 mM NaCl
5% glycerol
pH 7.4

The protein was concentrated and rebuffered into 10 mM Tris/HCl pH 7.5 using Amicon Ultracel (10 kDa MWCO) centrifugal concentrators. The resulting concentrate was centrifuged at 14000 x g and 4°C to remove any insoluble material and aggregates.

3.3 Crystallization screening in MTP format

A crystallization screening was conducted to determine crystallization conditions best suited for the PFOMT protein. Therefore three different protein solutions were prepared to be used with

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(b) Analytical gel filtration run of PFOMT (blue trace) using a Superdex 200 10/300 GL. Protein standards (red trace; Thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B₁₂ (1350 DA)) were injected to approximate the molecular weight of PFOMT.

Figure 1: ÄKTA runs.

different crystallization buffers.

The protein solutions consisted of ~250 μM protein and equimolar amounts of MgCl₂, as well as substrates (SAE, eriodictyol) or products (SAH, ferulic acid). The exact amounts are listed below.

Crystallization was carried out in 96-well microtiter plates, which contained either 3 or 4 subwells. One subwell was a buffer subwell, whereas the other subwells were elevated and contained the crystallization samples in a sitting-drop type fashion. The 3-subwell plates contained 70 μl buffer, the 4-subwell plates contained 50 μl buffer. The proportion of protein solution to crystallization buffer was 1 (equal volumes). The final volume of the drop was 0.2-0.3 μl . The crystallization temperature was 11°C.

Solution A (J1)

0.25 mM SAE
0.25 mM MgCl₂
0.25 mM ferulic acid
7.53 $\frac{\text{mg}}{\text{ml}}$ PFOMT wt (0.262 mM)

Solution B (J2)

0.25 mM SAE
0.25 mM MgCl₂
0.25 mM eriodictyol
7.53 $\frac{\text{mg}}{\text{ml}}$ PFOMT wt (0.262 mM)

Solution C (J3)

0.25 mM SAE
0.25 mM MgCl₂
0.25 mM ferulic acid
6.3 $\frac{\text{mg}}{\text{ml}}$ PFOMT Y51R N202W (0.219 mM)

The crystallization screens used were the *Hampton Research HT*, *Jena Bioscience 1-4* and *Jena Bioscience 1-4 Cryo* Kits, which included a variety of crystallization buffers with mainly PEGs or ammonium sulfate as precipitants. The Jena Bioscience kits were only tested on solution A and B.

Plate codes (as loaded into Rigaku robot):

- MP000715 - Hampton research (96-(three-subwell) plate)
- MC001413 - Jena Biosciences classic 1-4 (96-(two-subwell) plate)
- MC001415 - Jena Biosciences cryo 1-4 (96-(two-subwell) plate)

3.3.1 Results

Obtained crystals under multiple conditions (see p. 5). Most notably, crystals grew under a lot of conditions containing ammonium sulfate as precipitant. The crystals started to show early (\sim 3 days into the trial (e.g. Figure 2, Figure 8) into the trial for some conditions. There were crystals growing in the Jena Bioscience cryo kits, but these crystals only started to appear late (3 weeks to 2 months into the trial (e.g. Figure 7).

The crystals with the most even edges and surfaces were obtained by using ammonium sulfate as precipitant. These crystals showed low mosaicity and diffracted up to 1.8 Å on the home source (AG Stubbs, Biochemtech Uni-Halle).

3.4 Data collection and structure solving

Data was collected at Beamline 1 of AG Stubbs (CCD Saturn 944, Varimax VHF).

The crystallization buffer was tested for cryoprotective activity prior to freezing the crystal. If the buffer was not cryoprotective, cryoprotectant was added to the crystallization buffer (usually 20-30% (v/v)). Cryoprotectant was either glycerol (with ammonium sulfate as precipitate) or propylenglycol.

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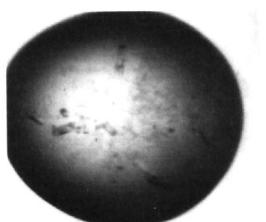


Figure 2: MC001413 well C12.1 after 3 days. Brittle, intergrown crystals. Buffer: 0.2 M CaCl₂, 0.1 M Tris/HCl pH 8.5, 20% (w/v) PEG-4000

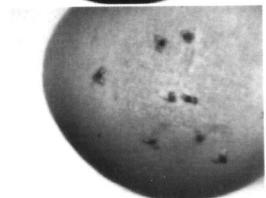


Figure 3: MC001413 well D4.1 after 92 days. Intergrown crystals, less brittle than Crystals in C12. Buffer: 0.2 M CaCl₂, 0.1 M Tris/HCl pH 8.5, 25% (w/v) PEG-4000

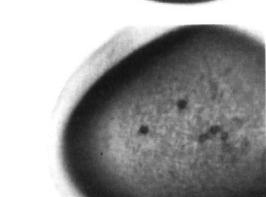


Figure 4: MC001413 well D4.2 after 92 days. Spherulites. Buffer: 0.2 M CaCl₂, 0.1 M Tris/HCl pH 8.5, 25% (w/v) PEG-4000

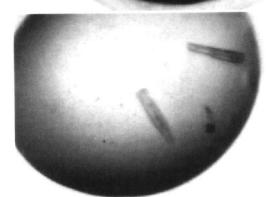


Figure 5: MC001413 well G10.1 after 64 days. Very brittle, rough crystals. Buffer: 0.1 M LiCl, 0.1 HEPES/NaOH pH 7.5, 25% (w/v) PEG-6000



Figure 6: MC001415 well B10.1 after 64 days. Nice crystals. Buffer: 1.8 M (NH₄)₂SO₄, 6 % (v/v) 2-propanol, 20 % (v/v) glycerol

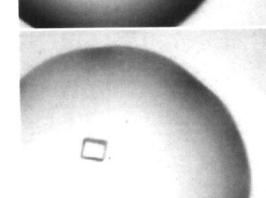


Figure 7: MC001415 well B5.3 after 64 days. Nice crystals. Buffer: 2 M (NH₄)₂SO₄, 20 % (v/v) glycerol

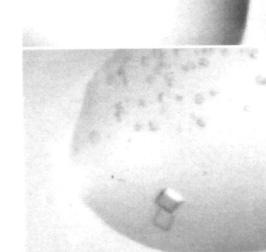


Figure 8: MC000715 well C8.3 after 64 days. Nice crystals. Buffer: 2 M (NH₄)₂SO₄

3.4.1 Results

The crystals, which crystallized from ammonium sulfate, possessed the orthorhombic space group $P_{21}2_12_1$. Contrary to the published structure (PDB: 3C3Y) these crystals contained two dimers in a unit cell.

3.4.1.1 Dataset - 140519_PFOMT

This dataset was solved using molecular replacement with pdb:3C3Y as a template. The phases could be obtained relatively easily. The structure could be solved to a resolution of XX Å. The unit cell was twice as large as that of pdb:3C3Y and contained two homodimers (Fig. 9a).

Table 2: Crystal parameters of dataset 140519_PFOMT

	initial	final	length (Å)	angle (°)
R_{work}	0.2125	0.2119	a	85.96
R_{free}	0.2472	0.2470	b	127.75
RMSD _{bondlength}			c	128.97
RMSD _{bondangle}			mosaicity	0.396
RMSD _{chiralcenter}				

Upon building the structure it became obvious, that significant shifts in the structure of PFOMT were present in this data. Furthermore, none of the ligands supplied with the protein solution could be found in the active site. Only a sole sulfate ion was present in the active site. This ion was located at the position, where the carboxylic acid function of SAH is located in the previously published structure.

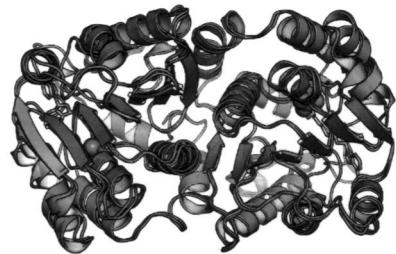
Most notably however, the N-terminus seemed to be structured, since the electron density was sufficient to describe the N-terminus up to the His₆-tag.

The N-terminus of apo-PFOMT is positioned like a lid atop the active site (Fig. 10a). Possibly it is involved in substrate binding. To what extent remains to be shown (ITC of truncated versions?).

The active site is closed off and the spaces otherwise occupied by SAM are now taken up by some amino acid side chains, which have shifted significantly (11b). The hydrophobic and very bulky side chain of Phe80 (loop B) is rotated towards the active site, possibly excluding solvent from entering. Asp156 on loop D also has moved a lot and is now reaching into the active site and in a position suitable for H-bonding with Asp102 on loop C (distance O—O is 2.5 Å).

However, Phe103 has shifted outward and only orients itself for π - π -stacking once binding occurs.

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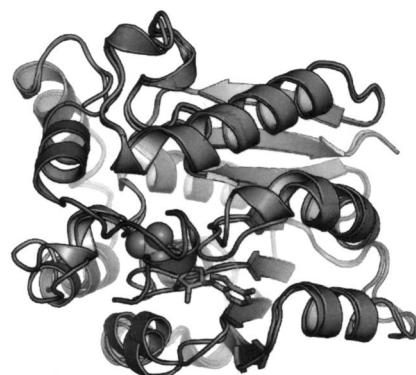
(a) The unit cell of *apo*-PFOMT contains two dimers and is twice as large as 3C3Y on side b (Table 2).

(b) The homodimers (catalytically active unit) of 3C3Y (olive) and *apo*-PFOMT (orange) aligned. In the apo form an ammonium sulfate (red and yellow spheres) is located in the pocket, where the carboxylic acid group of SAH (pinkish) is located in the 3C3Y structure.

Figure 9: The overall structure of *apo*-PFOMT in comparison with the previously published structure.



↻ x
90°



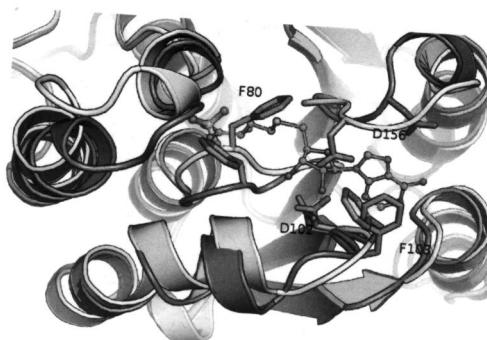
(a) The N-terminus of *apo*-PFOMT consists of an α -helix (red), which fits like a lid onto the active site. The thrombin binding site and part of the His₆-tag (magenta) are also defined by electron density.

(b) The figure 10a rotated by 90 degrees around the x-axis.

Figure 10: The active site and N-terminus of *apo*-PFOMT (orange) trim=150 0 150 50, clip, superposed with 3C3Y (olive). The bound ligands are SAH (cyan sticks), sulfate (yellow and red spheres).



(a) Flexible loops around the active site. The parts of the apo-PFOMT structure (olive), that showed a notable difference towards 3C3Y (orange) are colored in blue. The N-terminus of apo-PFOMT was omitted for convenience.



(b) The major structural changes in the active site are shown (3C3Y – orange, apo-PFOMT – olive). The active site is closed off by some residues, which would clash with SAH (cyan ball and stick). Phe80 has adapted a position, where it points into the active site to just about where the sulfur of SAH would be positioned. **Maybe this residue helps stabilize the cationic charge at the sulfur?** The residue Asp156 has shifted considerably and is positioned for H-bonding with Asp102. In the 3C3Y structure Asp102 makes H-bonds to the hydroxy-groups 2 and 3 of the ribose moiety of SAH. Phe103 is involved in SAM(H) binding through π - π -stacking.

Figure 11: View os the active site of the 3C3Y and apo-PFOMT structures. N-termini of apo-PFOMT have been omitted for easier viewing.

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APPENDIX

Pipetting for crystallization screens

Solution A (J1)

6 μ l 5 mM SAH
 1.2 μ l 25 mM MgCl₂
 1.2 μ l 25 mM ferulic acid
 111.6 μ l 8.1 $\frac{mg}{ml}$ PFOMT wt

Solution B (J2)

6 μ l 5 mM SAE
 1.2 μ l 25 mM MgCl₂
 1.2 μ l 25 mM eriodictyol
 111.6 μ l 8.1 $\frac{mg}{ml}$ PFOMT wt

Solution C (J3)

2 μ l 5 mM SAH
 0.4 μ l 25 mM MgCl₂
 0.4 μ l 25 mM ferulic acid
 28 μ l 8 $\frac{mg}{ml}$ PFOMT Y51R N202W

References

- [1] J. G. Kopycki, D. Rauh, A. a. Chumanovich, P. Neumann, T. Vogt, and M. T. Stubbs. Biochemical and structural analysis of substrate promiscuity in plant Mg²⁺-dependent O-methyltransferases. *J. Mol. Biol.*, 378(1):154–64, Apr. 2008. ISSN 1089-8638. doi: 10.1016/j.jmb.2008.02.019. URL <http://www.ncbi.nlm.nih.gov/pubmed/18342334>.
- [2] F. W. Studier. Protein production by auto-induction in high-density shaking cultures. *Protein Expr. Purif.*, 41(1):207–234, May 2005. ISSN 10465928. doi: 10.1016/j.pep.2005.01.016. URL <http://linkinghub.elsevier.com/retrieve/pii/S1046592805000264>.

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WEB306 - MTAN Activity measurement for DIM

Benjamin Weigel

September 3, 2014

1 Introduction

- need for activity of MTAN for Paper (Integrative On-Column Biocatalysis, DIM)
- activity measurement in assay buffer (0.1 M Tris/HCl, 0.2 M KCl, 20 mM MgCl₂, pH 7.5)

MTAN

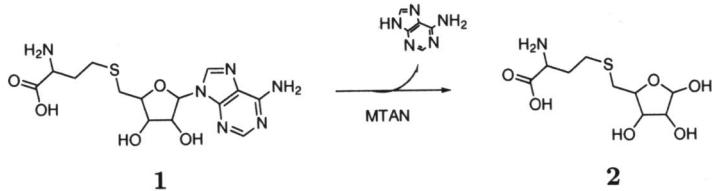


Figure 1: Reaction catalyzed by MTAN. MTAN cleaves SAH (**1**) by hydrolyzing the glycosidic bond between ribose and adenine.

Data (off BRENDa & ProtParam)

- K_M for SAH $\sim 1.3 \mu\text{M}$
- TON $\sim 2.3 \text{ s}^{-1}$
- 252 AA, 26517 kDa (recombinant in pET28)
- theoretical pI 5.82
- absorption difference between adenine & SAH
 $\rightarrow \Delta\varepsilon_{276} = -1.4 \text{ mM}^{-1} \text{ cm}^{-1}$

2 Experimental

- Calibration curve Adenine (HPLC)
- measurements (4 time points, 0, 5, 10, 20, 30 min)

2.1 Assay

Assay buffer: 0.1 M Tris/HCl, 0.2 M KCl, 20 mM MgCl₂, pH 7.5

Substrate Solution: 10 mM SAH in 50 mM HCl

1

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Reaction Mix (200 μL):

5 mM SAH	126.5 μL 7.9 mM SAH
2 μM ~ 53 $\frac{\mu\text{g}}{\text{mL}}$ MTAN in assay buffer	7.6 μL 1.4 $\frac{\mu\text{g}}{\text{mL}}$ MTAN (omit until start of reaction)
	20 μL 1 M Tris/HCl
	10 μL 4 M KCl
	10 μL 0.4 M MgCl ₂
	25.9 μL ddH ₂ O

Reaction:

1. add MTAN to reaction solution
2. incubate at 25°C for 0, 1, 2, 5 and 10 min
3. take 10 μL aliquots for analysis
4. add 80 μL H₂O & 10 μL TCA (100%, v/v), vortex for 30 s & incubate @ 4° for 10 min
5. centrifuge at 14.000 x g and 4° for 10 min
6. transfer supernatant to HPLC-vial for analysis

2.2 HPLC Method**Column:****Buffer A:** H₂O, 0.2% TFA**Buffer B:** Acetonitrile, 0.2% TFA**Gradient:**

0% B → 50% B
 50% B → 100% B
 100% B for 10 min

5% B for 4 min
 5% B → 20% B
 20% B → 200% B
 200% B → 5% B → Hold for 6 min

2.3 Nanodrop Method

Calculated activity like by using the differences in the extinction coefficients of SAH and Adenine (see p.1 for $\Delta\varepsilon$). [1]

Problem: Measuring the MTAN reaction under high substrate concentrations (5 mM) is problematic in standard cuvettes ($d = 1 \text{ cm}$), because of the high extinction coefficient of SAH ($\varepsilon_{260} = 15400 \text{ M}^{-1}\text{cm}^{-1}$). Concentrations that high would mean an absorption of $E = 15400 \text{ M}^{-1}\text{cm}^{-1} \cdot 5 \cdot 10^{-3} \text{ M} \cdot 1 \text{ cm} = 77$, which would be hugely impractical.

Solution: The nanodrop has a pathlength of 1 mm, or 0.2 mm, and is able to handle an absorption of up to 75 (10 mm equivalent) according to the manual.

Setup:

1. started the reaction as above (Section 2.1)
2. added 0.4 μL of MTAN to 9.6 μL Mastermix
3. quickly mixed and pipetted 2 μL solution ($\cong 0.112 \mu\text{g}$ MTAN) onto Nanodrop (Pathlength setup - short [0.2 mm])
4. read a spectrum every minute for 10 minutes and then at 15 minutes
5. exported spectra as CSV and read into R for analysis

Anaylsis:

1. fit data points at 276 nm versus time (Progress curve, slope unit is min^{-1})
2. use R with *propagate* package for Taylor expansion error propagation to calculate specific activity ($\frac{U}{mg}$) and standard deviation

$$E = \varepsilon \cdot d \cdot c \quad (1)$$

$$\frac{\Delta E}{\Delta t} = \varepsilon \cdot d \cdot \frac{\Delta c}{\Delta t} \quad (2)$$

$$\frac{\Delta c}{\Delta t} = \frac{\Delta E}{\varepsilon} \quad (3)$$

References

- [1] J. Zhu, E. Dizin, X. Hu, A.-S. Wavreille, J. Park, and D. Pei. S-Ribosylhomocysteinase (LuxS) is a mononuclear iron protein. *Biochemistry*, 42(16):4717–26, Apr. 2003. ISSN 0006-2960. doi: 10.1021/bi034289j. URL <http://www.ncbi.nlm.nih.gov/pubmed/12705835>.

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1 Data Analysis

- UV/VIS

	time [min]	A ₂₇₆ (1 mm)
1	0.00	2.34
2	1.00	2.32
3	2.00	2.31
4	3.00	2.28
5	4.00	2.26
6	5.00	2.24
7	6.00	2.22
8	7.00	2.20
9	8.00	2.18
10	9.00	2.17
11	10.00	2.15
12	15.00	2.06

Table 1: MTAN data from progress curve.

A simple linear fit of the absorption versus time to calculate the slope. From the slope the specific activity in $\frac{U}{mg}$ is calculated via an error propagation.

$$\frac{\Delta c}{\Delta t} \cdot V \cdot \frac{1}{d \cdot E_0} = \frac{\Delta E_{276}}{-1.4 \text{ mM}^{-1} \text{ cm}^{-1}} \cdot 2 \cdot 10^{-6} \text{ l} \cdot 1000 \frac{\mu\text{mol}}{\text{mmol}} = \frac{U}{mg} \quad (1)$$

	$\frac{U}{mg}$
1st order mean	2.4039
2nd order mean	2.4039
1st order sd	0.0437
2nd order sd	0.0437
2.5% CI	2.3190
97.5% CI	2.4899

Table 2: Results of the first and second order Taylor Expansion to calculate the specific activity of MTAN from the data above (Tab. 1)

2 Code

2.1 Error Propagation

```
fit <- lm(Abs ~ time.min, a276) # calculate U/mg
EXPR <- expression((a/-1.4 * 10 * 2e-6 * 1000)/0.000112)
tmp <- data.frame(summary(fit)$coefficients[2, 1:2])
```

1

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HPLC

$$Area = c_{adenine} \cdot a + y_0 \quad (1)$$

$$c_{ade} = \frac{Area + y_0}{a} \quad (2)$$

$$\frac{U}{mg} = \frac{\frac{\mu\text{mol}}{\text{min}}}{mg} = \frac{\frac{mM}{\text{min}} \cdot 1000 \frac{\mu M}{mM} \cdot l}{mg} \quad (3)$$

$$\frac{U}{mg} = \frac{\frac{\mu\text{mol}}{\text{min}}}{mg} = \frac{\frac{mM}{\text{min}} \cdot 1000 \frac{\mu M}{mM} \cdot 1 \cdot 10^{-4} l}{0.56 \cdot 10^{-3} mg} \quad (4)$$

```
library(propagate)
library(xtable)

setwd("/home//bweigel/Dokumente/R Projects/Experimente/WEB306 - MTAN Kinetic/HPLC/")
load("propagate_HPLC.RDa")
names(Ade)[5] <- "mean"
print(xtable(tmp))
```

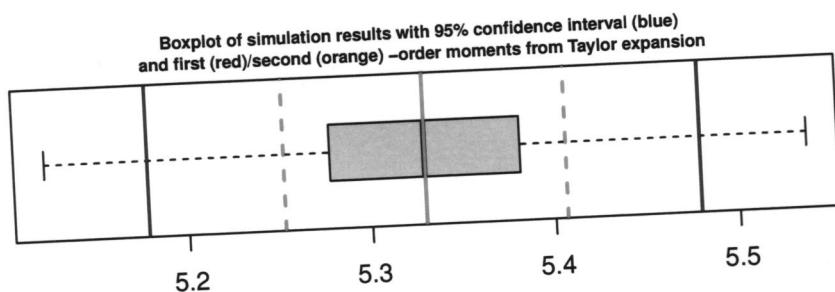
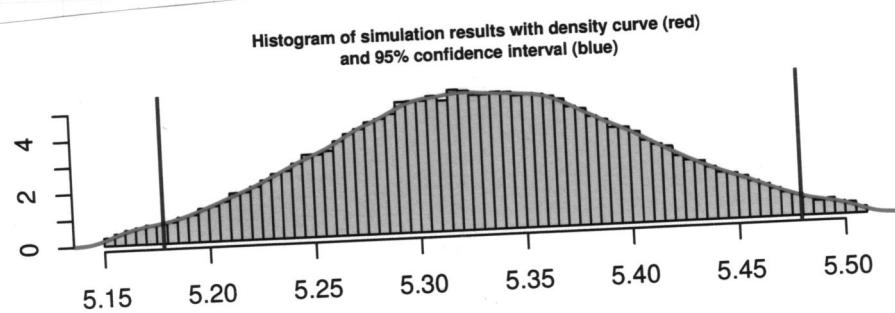
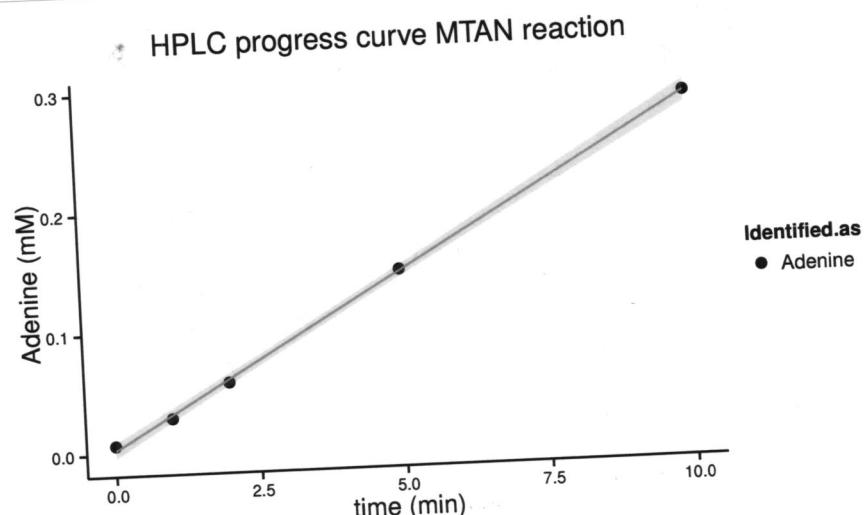
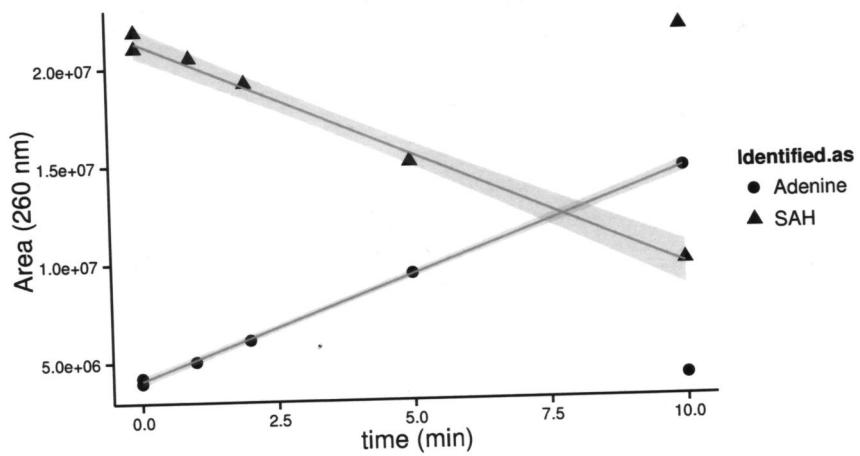
	a
1	37085348.33
2	3412529.62

```
EXPR <- expression(a * 0.1 /0.53e-3)
fit <- lm(Ade$mean ~ Ade$sample)
tmp <- data.frame(summary(fit)$coefficients[2, 1:2])
colnames(tmp) <- c("a")
rownames(tmp) <- NULL

PRO <- propagate(EXPR, tmp, type = "stat", do.sim=TRUE, verbose = TRUE)
```

	$\frac{U}{mg}$
1st order mean	5.3296
2nd order mean	5.3296
1st order sd	0.0769
2nd order sd	0.0769
2.5% CI	5.1790
97.5% CI	5.4807

Table 1: Results of the first and second order Taylor Expansion to calculate the specific activity of MTAN from the data above (Tab. ??)

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1 WEB298 - NADES Xtallization with 4 different Proteins

1.1 Proteins

1.1.1 Trypsin stock solution

- ~90 mg/mL Trypsin in 10 mg/mL benzamidine, 3 mM CaCl₂

1.1.2 Lysozyme stock solution

- 75 mg/mL Lysozyme in 0.1 M NaOAc pH 4.6

1.1.3 Proteinase K stock solution

- 23.9 mg/mL Proteinase K in 25 mM Tris-HCl pH 7.5

1.1.4 Lipase B (Candida cylindrica)

- ~6 mg/mL Lipase B in ddH₂O

	1	2	3	4	5	6
A	0.1 M NaOAc pH 4.5 50	0.1 M NaOAc pH 5.5	0.1 M Na-Citrate pH 6.5	0.1 M HEPES pH 7.0	0.1 M HEPES pH 7.5	0.1 M Tris pH 8.5
B	40					
C	30					
D	20					

- 2µl Enzyme solution + 1 µl reservoir buffer

Deckglässchen von oben	
2 - Lysozym	1 - Trypsin
4 -	3 - CCLB

Deckglässchen von unten	
1 - Trypsin	2 - Lysozym
3 - CCLB	4 - Proteinase K

WEB298 - NADES Xtallization Factorial

Benjamin Weigel

September 1, 2014

Lysozyme

Lysozyme crystallizes in every of the conditions tested, from low to high pH and PCh fraction. The morphology of the crystals changes slightly at neutral pH, when the PCh concentration is decreased. The number of crystals per drop also varies, from over 50 smaller crystals in A1 to 14 larger crystals in well D6.

Trypsin

In the screening trypsin only crystallizes at pH 8.5 and 30% (v/v) PCH. However, this could be a pipetting error ...

In some conditions, mainly at PCh fractions of 50%, there is lots of amorphous precipitate (e.g. A1-A5). However, the propensity of trypsin to form precipitate decreases with increasing pH. Whereas at pH 4.5 there is precipitate at every PCh concentration, no or only very light precipitate was observed at pH 8.5. High concentrations of PCh also increase precipitate.

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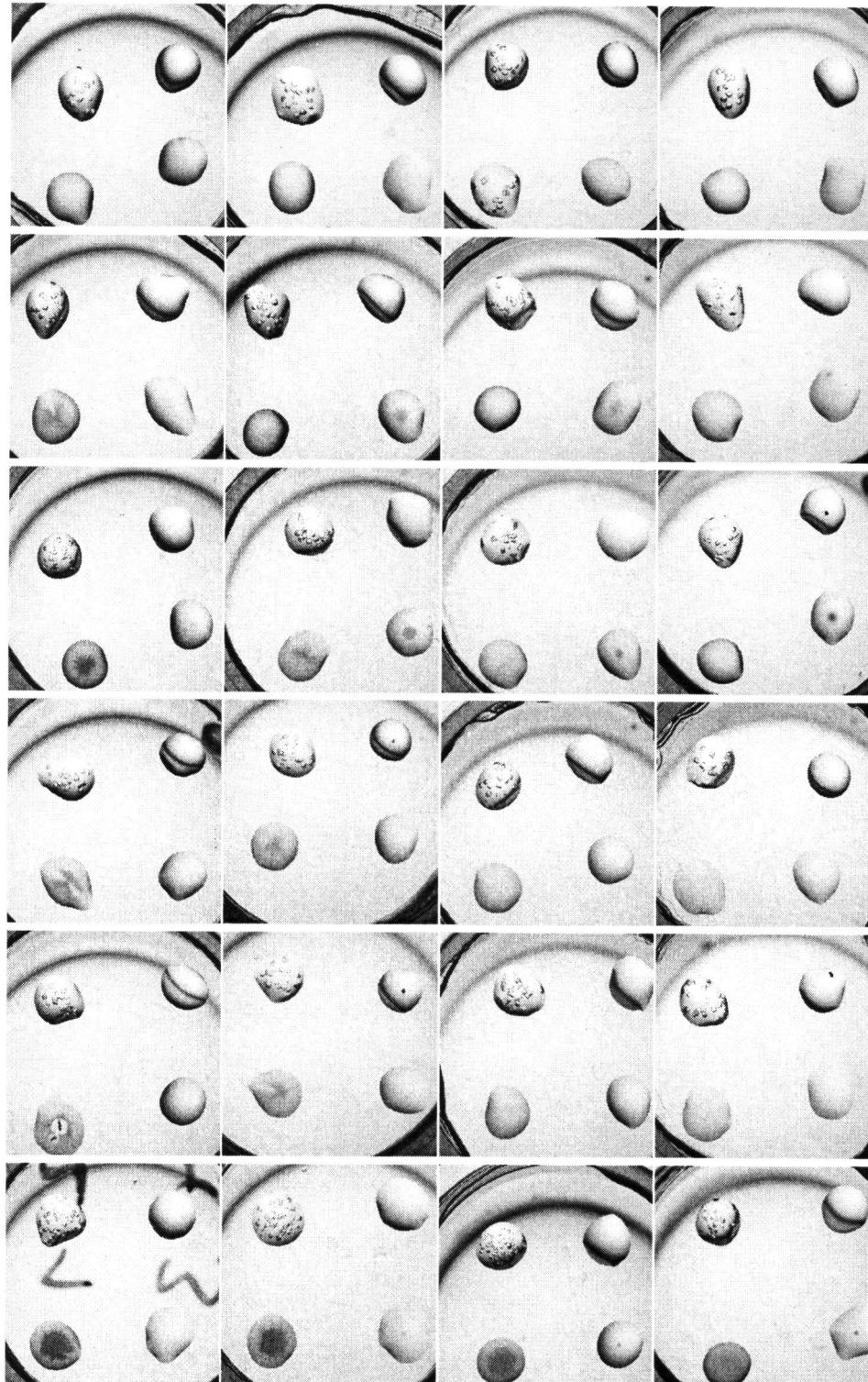


Figure 1: NADES crystallization trial using four different model proteins in a 24-well plate. The NADES (1,2-propanediol/cholin chloride/water 1:1:1, PCH) fraction was varied per row (top (A) to bottom (D) – 50, 40, 30, 20 % v/v). The pH was varied per column (left (1) to right (6) – 4.5 (NaOAc), 5.5(NaOAc), 6.5(Na citrate), 7(HEPES), 8.5(Tris), 0.1 M each). The drops from left to right, top to bottom are bovine trypsin ($\sim 90 \frac{mg}{mL}$), egg white lysozyme ($75 \frac{mg}{mL}$), *Candida cylindrica* lipase B ($\sim 6 \frac{mg}{mL}$), proteinase K ($23.9 \frac{mg}{mL}$). Setup with 2 μ L of protein and 1 μ L of reservoir buffer. Pictures were taken after 31 days at 4°C.

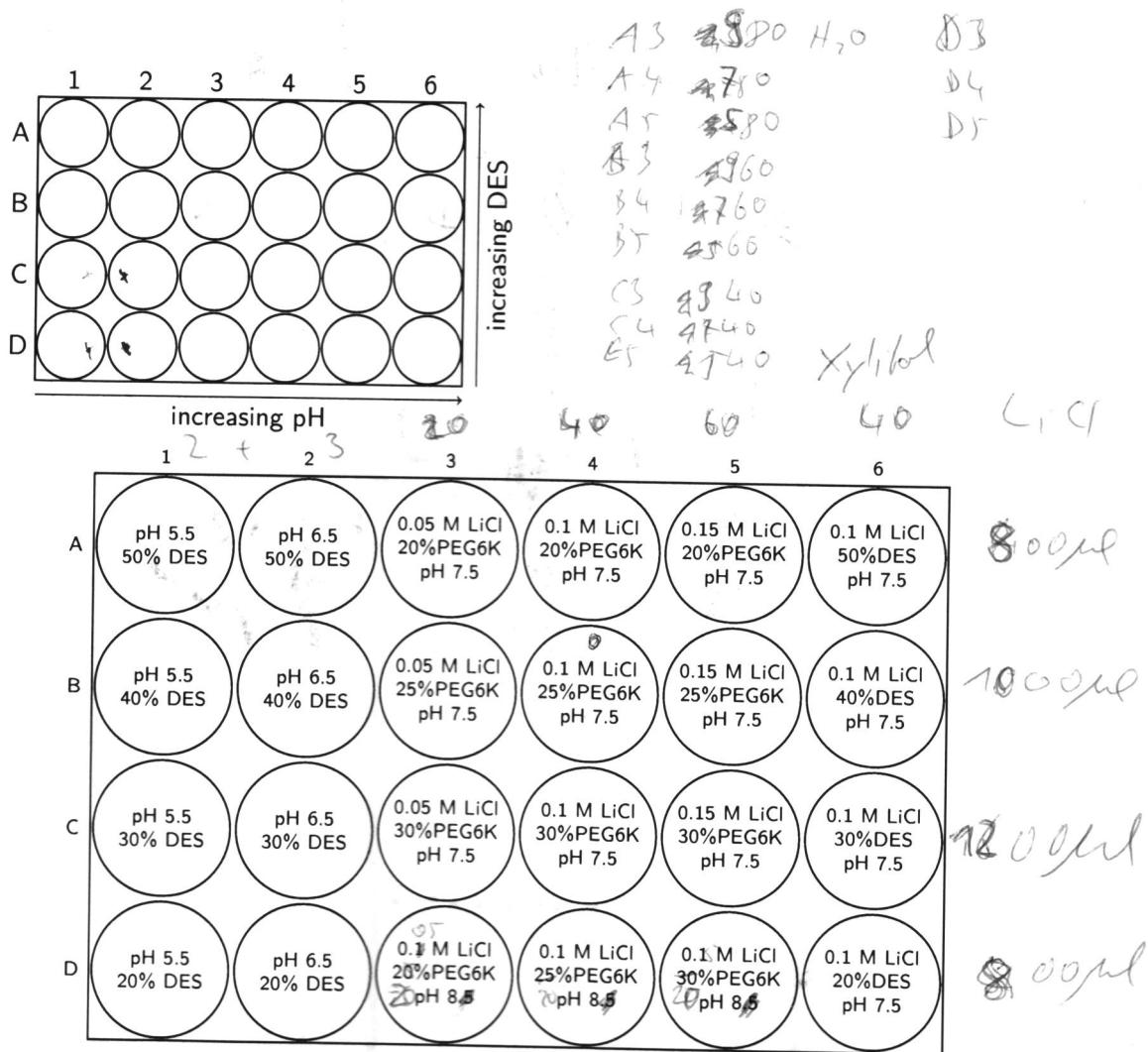
ded

Prot & with 173
Lip with 82

WEB328 - PFOMT Xtallization

Benjamin Weigel

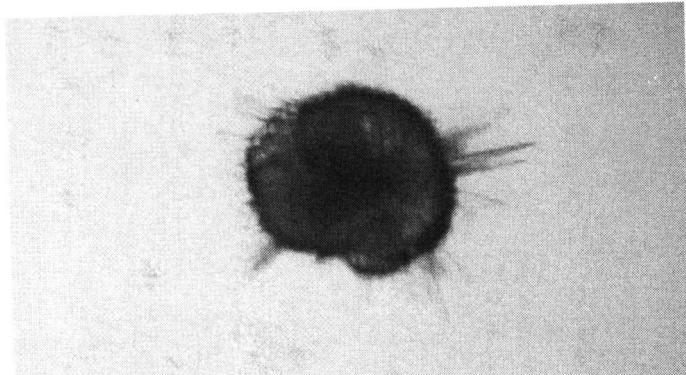
12th December 2014



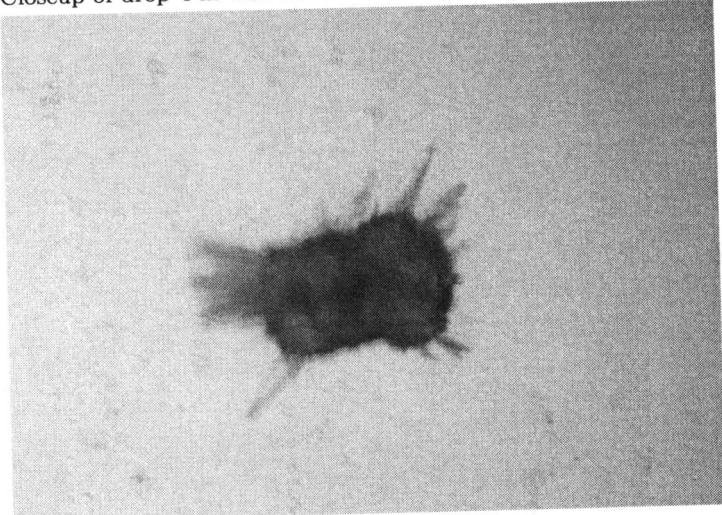
A3: 0.5 → buffer is 0.1 M Hepes

0.1M LiCl	40 μl	SM LiCl
0.05M LiCl	20 μl	SM LiCl
0.15M LiCl	60 μl	LiCl

200 ml 1M HCl



(a) Closeup of drop 4 in well 2B. 0.1 M NaOAc pH 5.5, 40 % PCH (v/v).

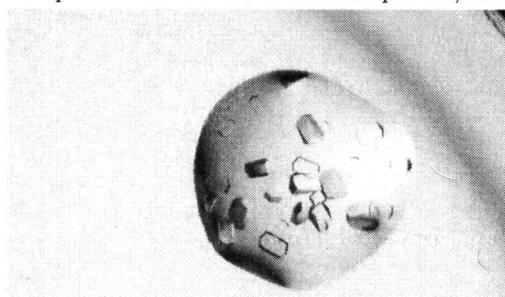


(b) Closeup of drop 4 in well 3B. 0.1 M Na citrate pH 6.5, 40 % PCH (v/v).

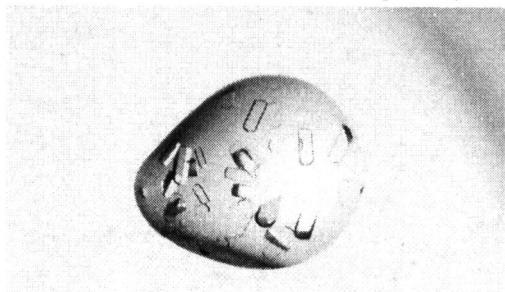
Figure 2: Closeup pictures of objects that appeared in proteinase K drop. Intergrown plate-like crystals?



(a) Closeup of drop 2 in well 4A. 0.1 M HEPES pH 7.0, 50 % PCH (v/v).



(b) Closeup of drop 2 in well 4C. 0.1 M HEPES pH 7.0, 30 % PCH (v/v).

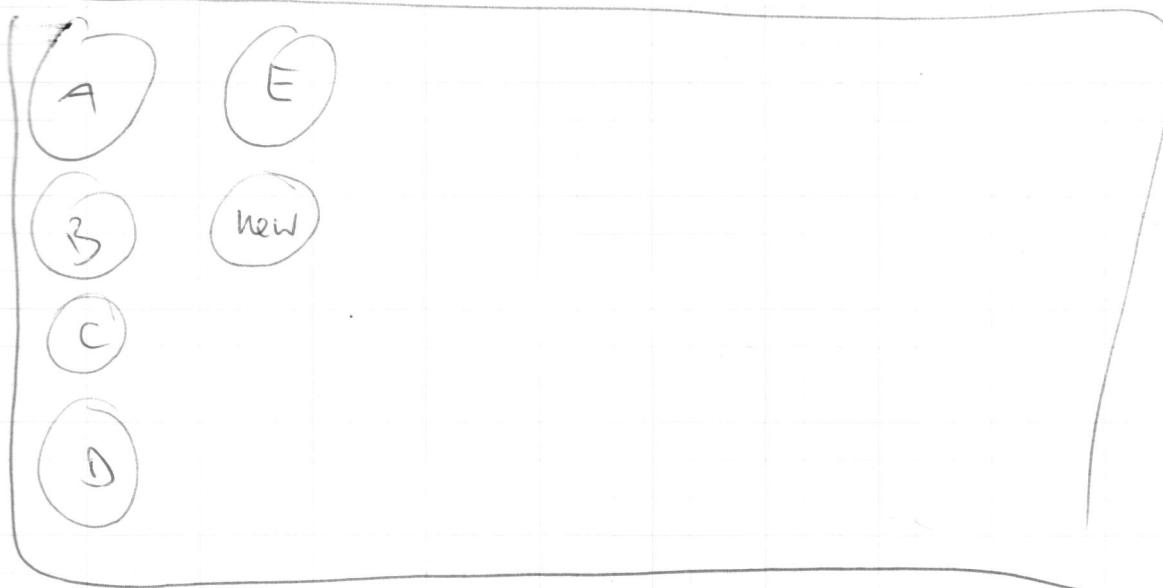


(c) Closeup of drop 2 in well 4D. 0.1 M HEPES pH 7.0, 20 % PCH (v/v).

Figure 3: Changes in the morphology of lysozyme crystals at neutral pH. The orthorhombic crystals become platelike (longer and flatter). The same phenomenon occurs at pH 7.5, but not at higher or lower pH. It cannot be excluded, that buffer substance (HEPES) has an influence.

WEB 325

PFOMT Xballization



A:E → from previous experiment (2013/2014)

new → PFOMT new (250ml HgCl_2 , EO, SAE,
PFOMT)

→ in 2 M Ammonium alkali

setup



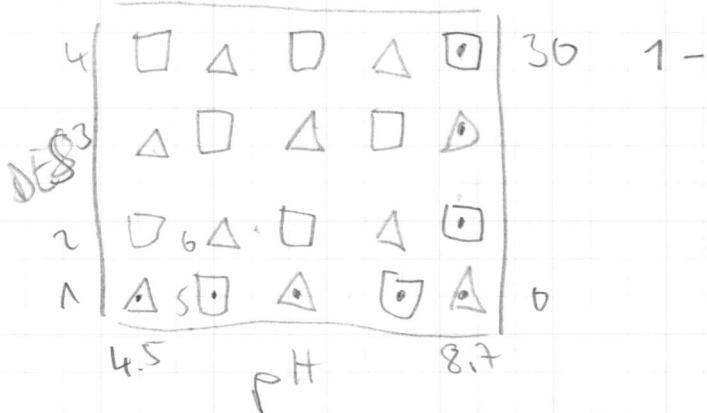
- | | |
|---------|-------------|
| 1 - 1:1 | prot : bulk |
| 2 - 2:1 | prot : bulk |
| 3 - 3:1 | |
| 4 - 1:2 | |

PFOA Assay

pH →



buffer blank - buffer 1:20 (80µl + 20µl H₂O)



23.07.15

Phenylpropanoate-Seminar

- in Vib rot -2 → C(HS)

Ü

- Kapillarelektrophorese
Parallel? ?

28.02.15

(342)

Stocks (10mM) in DMSO

~~new~~ isoferulic acid, m-coumaric acid

mit WT:

Quercetin ~~WT~~

A

isoferulic acid

B

m-coumaric acid

C

mit Variante ~~NB02W~~ NB02W Y51R

Nanji D

Assay ~~at~~ without addition of γ -Cl-PtH₂S

Run A

50 mM MTT pH 9, 2.5 mM GS4
 0.4 mM substrate
 0.5 mM SAM
 20 μ g PFOMT
 in 100 μ l

50 μ l buffer (100 μ l)
 4 μ l 10mM substrate
 2 μ l 0.1M GS4
 13.6 μ l 5mM SAM
 2 μ l 1mg/ml enzyme
 9.9 μ l H₂O

Mix
200 μ l buffer (A)

~~40 μ l~~
 20 μ l GS4
 54.4 μ l SAM
 39.6 μ l H₂O

→ 76 μ l Mixture + 10 μ l Enz
 + 4 μ l Substrate

Stepf:

100 µl Rer + 30 µl (10) fct in 50% AcN)

344 344

	mg	H_2O	pH	Enzym	Substrate	SAM/SAE
A	X	9.9	8.6	✓	iso-fenol	SAM
B	X	299	8.6	X	n	SAM
C	X	19	10	✓	n	SAM
D	X	9.9	8.6	✓	caffic acid	SAE
E	X	9.9	10	✓	n	SAE
F	✓	8.9	8.6	✓	iso-f.	SAM

solubility Phenolic acid in buffer (13-16)

200 µl buffer } MMT pH 7.6 DES 0-30
 + FA (saturating) } ~ shake @ 1400 rpm IRT for 1h
 → HPLC of supernatant
 molar extinction coeff (Sigma - Aldrich) 10mM pH
 (L-200)
 $E^{\text{mM}}(\text{e}16\text{H}) = 18.6 \text{ (322 nm)} \text{ at } 18.6 \text{ molar}^{-1} \text{ cm}^{-1}$

Gauss function

$$f(x) = \frac{1}{\sqrt{2\pi}\sigma} e^{-\frac{1}{2}(\frac{x-\mu}{\sigma})^2}$$

$$C e^{-\frac{1}{2}(\frac{x-\mu}{\sigma})^2}$$

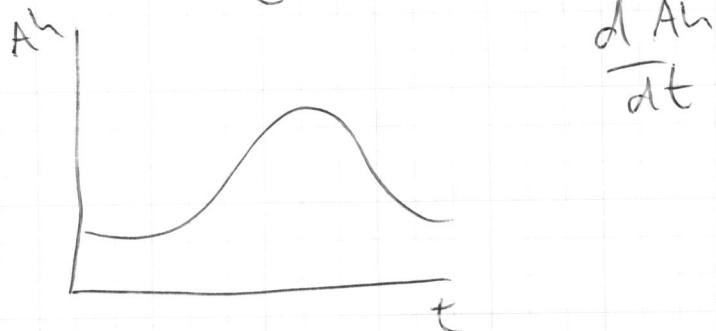
$\log h$

$$\ln y = \ln C + -\frac{1}{2}(\frac{x-\mu}{\sigma})^2 / \sigma \Rightarrow$$

$$\ln C = -\frac{1}{2}(x-\mu)^2$$

$$\ln C = -\frac{1}{2}x^2 - 2x\cancel{\mu} + \mu^2$$

$$E = \epsilon \cdot C \cdot d$$



multiplier

ITC 33P / 345

03.03.15

Bücher: MMT Malat / Mes Nis pH 7.6

Experimente:

	Cell	Spiegel
1	PROMT	Mg^{2+}
2/3	PROMT (+Mg)	SAT
4/5	PROMT (+Mg)	FA
6	PROMT + FA	SAT
7	PROMT + SAT	FA
8	PROMT	SAT
9	PROMT + SAT	CA
10	PROMT	SAE
11	PROMT + SAE	CA

PROMT page Bührer ist diskutiert
 nach Diskuss: (50mm MMT pH 7.6 für min. 1h
 $\rightarrow \text{Abs}^{280} = 2.48 \rightarrow C = 3.49 \frac{\text{mg}}{\text{ml}}$ und $1.5 \frac{\text{mg}}{\text{ml}}$ oed.
 1:2.33
 430 μl Prof + 50 μl Puffer

FTCPreparation of ITC

- cleaned with Detergent (Lab soap solution) (50°C / 30min)
- rinsed with Water

Experiment 1

Cell:
PFOMT (48.7 μM)
7.6 mg/ml

Syringe:
LyCl,
anhydrous
(0.476 mg / 5 ml)

Experiment 2

Cell:
PFOMT (48.7 μM) STH (820 μM)
7.6 mg/ml

Experiment 3

Cell:
residue
of Ex 2

Syringe:
1 ml LyCl,

Exp. 4

Cell
400 μl PFOMT
+ 40 μl STH
10 min @ RT
→ in cell
~~LyCl~~

Syringe:
500 μl ferbic
acid

$$\text{Expt. c. id} = \frac{E_{320} - E_{410}}{E_{320} + E_{410}} = \frac{67.07}{0.8225} \text{ mAU}$$

* is 1.00 → 1.00 pH 7.6 water
→ $\lambda_{\text{max}} = 286, 310$ ($320 \rightarrow 67.07$)

$$\frac{E_{320} - E_{410}}{E_{320} + E_{410}} = \frac{74.75 \text{ mAU}}{1.1} = 67.5 \text{ mAU}$$

Exp 5Spritz cell50 μM PFOMT, 500 μM MgCl_2 , 100 μM SAH

concentrated PFOMT + 3.76 ml/well

→ 8 solution: 500 μM MgCl_2

citrulline acid (500 μM)

 $\lambda_{\text{max}} = 285 \text{ nm}$ $\epsilon^{312} = 11200 \text{ M}^{-1} \text{ cm}^{-1}$ 50 μM PFOMT

$$\begin{aligned} A^{312} &= 5.71 \\ A^{285} &= 6.04 \end{aligned} \rightarrow 503.8 \mu\text{M}$$

Exp. 6Cell

50 μM PFOMT

500 μM MgCl_2

100 μM SAH

Gai Thf

Spritz
spritz
cellulase

1 ml

1 ml 1 M MgCl_2

187 μl 520 μl

433 μl PFOMT

3.76 ml

368 μl buffer

Exp. 7Cell

50 μM PFOMT

500 μM MgCl_2

100 μM SAH

gai Thf

Spritz

500 μM citrulline acid

Spritz

500 μM di-citrulline acid

 $\lambda_{\text{max}} = 310, 285$ $A^{310} = 8.18$ $A^{285} = 8.88$ Spritz

500 μM fumaryl acid

 $\lambda_{\text{max}} = 286, 310$ $A^{286} = 5.00$ $A^{310} = 5.04$

Exp 8

Cell
 100 μM SA1T
 500 μM MgCl₂

Syringe
 500 μM citric acid

Exp 9

Cell
 50 μM MgCl₂

500 μM citric acid

15-03-06

Exp 10

20 °C

Cell
 500 μM MgCl₂
 50 μM PFOMT
 100 μM SA1T
 100 μl 700 mM THF
 340 μl PFOMT (2.94 mM)
 37 μl 50 mM MgCl₂
 253 μl Buffer

Syringe
 500 μM MgCl₂
 500 μM citric acid
 (50 μl 5 mM CT
 250 μl 1 mM MgCl₂
 200 μl Buffer)

Exp 11

Same as Exp 10, but @ 30°C

Exp 12

42.3

Cell

(50) μM PFOMT
 (500) μM MgCl₂
 (100) μM Citric acid

422.8

170 μl PFOMT

84.6

3.5 μl 50 mM MgCl₂

7 μl 5 mM Citric acid

163.5 μl Buffer

Syringe

500 μM SA1T
 500 μM MgCl₂

143 μl 700 mM THF
 2 ml 50 mM MgCl₂

57 μl Buffer

Exp 13
repeated
with
shaker
interval

Cell 1.37 ml
50 μl Pfront 47.9 ml
500 μl MgCl_2
350 μl
3.5 μl 500 μl MgCl_2
167.4 μl $299 \frac{\text{ml}}{\text{ml}}$ Pfront
779.1 μl Buffer

Syringe +
500 μl Cafferic acid / 433 μl
500 μl MgCl_2
 $A^{312} = 4.85$

3
144 μl
153

Exp 14

Cell
remainder of Exp 13

40.1 μl Pfront

500 μl MgCl_2
53.11 μl Cafferic acid

Exp 15

Cell
500 μl MgCl_2 1.32 ml
50 μl Pfront
46 μl

Syringe
500 μl CA
~~500 μl MgCl_2~~
 $A^{312} = 4.55$
406 μl

analytikjena | Biometra

Cell
Pfront
Pfront-Mg

Syringe
CA
CA + Mg

Possible Substrates for non-catecholic methylation

- iso-fenolic acid
 hesperitin
 isorhamnetin
 diosmetin

} Wild type

OKA (OK)
 9.85
 9.98
 10.33
 9.57

Chrysophanol
 homoverdicyanol
 fenolic acid
 rhamnetin

} Variant

9.24
 10.06
 9.98
 8.87

12.08.15

WTB346

Reaktion

0.5 µl 10 mM Glucon
 0.17 µl H₂O
 25 µl buffer (100 mM)
 2 µl 10 mM Substrate
 1.25 µl 0.1 M GSH
 6.73 µl 5 mM SAM (81%)
 10 µl 1 mg/ml PFOAT
~~+ 0.5 µl 100 mM MgCl₂~~
 add to 50 µl H₂O

@ pH 7.5

↓
72 samples↓ (Mn⁷)
80 x M_M2 ml Mn⁷ pH 7.5
 100 µl 0.1 M GSH
 490 µl 5 mM SAM
~~400 µl 0.1 M MgCl₂~~

77 + 18 = 95

samples

↓ (Mn⁸)100 x M_M2.5 ml Mn⁸ pH 8.6
 175 µl 0.1 M GSH
 613 µl SAM

+ Mg

~~+ Mn⁷~~
 1.785 ml Mn⁷
 200 µl MgCl₂ (0.1 M)
 20 µl 100 mM Glucon
 4.8 µl ddH₂O

- Mg

1.785 ml Mn⁷
 200 µl H₂O
 20 µl 10 mM R
 4.8 ml H₂O

Mg

4.439 ml
 1295 ml Mn⁸
 200 µl MgCl₂
 70 µl 10 mM R
 4.8 ml H₂O

1.943 ml Mn⁸
 300 µl H₂O
 30 µl 10 mM R
 2.7 µl H₂O

1.52 ml 1.52 ml
 1200 µl 200 µl
 175 µl WT 175 µl Var

WEB 342 /pH-Optimization + My
mit HPLC

- pH 5.4 to 10 (6 times 12 points)
- ~~2 Substrates~~ Caffeic acid, Acetate
- 3 timepoints 0, 3, 6, 12 h

8x { pH
2 substrates
2 + my
4 times } AGT 128

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WEB 346

- general trend in conversion (as measured by JAH) product
-
- wild type shows left conversion (usually)
 - activity generally lower without Fe^{2+}
 - activity lower at lower pH

UHS samples

346-A-3 (Hep. / pH 7.5 / + 1g / wt)
A-4 HED

~~spont kinfuz~~

(12ml)

Curve Buch 3 S. 161)

(500ml)

- dialysiert 1 x 500 ml 0.05 M MMT pH 7.6
 (2 h @ RT) + 10 ml FBS

- 3 x geben 500 ml 0.05 M MMT pH 7.6
 (2 x 2 h @ RT)
 (1 x 16 h @ 4°C)

LAAO Assay - Oxidation of *L*-amino acids

Benjamin Weigel

August 19, 2014

1 Introduction

L-amino acid oxidases convert *L*-amino acids to α -keto acids via oxidative deamination of the amino-group using O_2 and water. Byproducts of this reaction are ammonia and hydrogen peroxide. The hydrogen peroxide can be detected via horseradish peroxidase (HRP), which uses H_2O_2 to oxidize a chromogenic substrate. The detected quantity is a developing color.

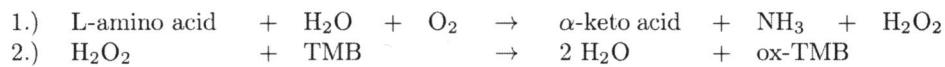


Figure 1: Formal reactions catalyzed by LAAO (1) and HRP (2).

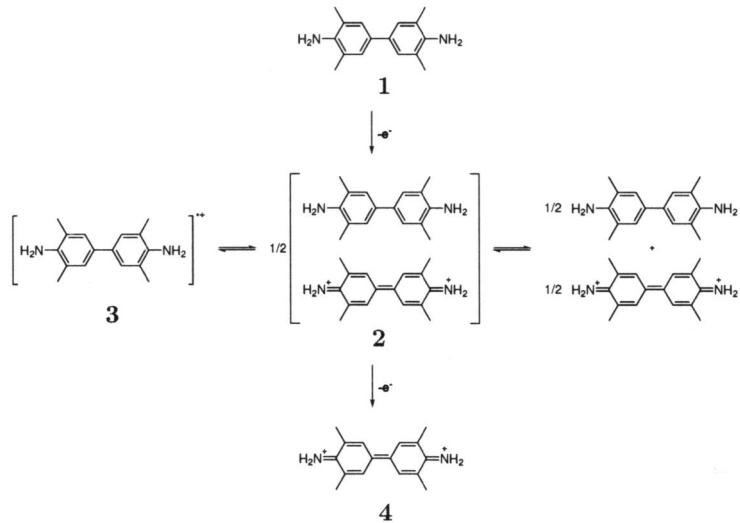


Figure 2: The two electron oxidation of 3,3',5,5'-tetramethylbenzidine (**1**). The first electron is used for the oxidation of TMB to the charge transfer complex (**2**), which has a blue color. The charge transfer complex **2** exists in rapid equilibrium with the radical cation **3**. Upon another one electron transfer, (**2**) is oxidized to the TMB diimine (**4**). The protonated form of (**4**) is highly yellow-colored at low pH. [1]

1

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If the TMB (**1**) is in excess during its oxidation by HRP and H₂O₂, all electrons are first used to oxidize **1** once, to the charge transfer complex (**2**). This proceeds, until half (1/2) a mole of H₂O₂ relative to the moles of TMB is used up [1]. Reduction of half a mole of H₂O₂ to H₂O produces one mole of electrons (e⁻).

To obtain the protonated form of the diimine it is necessary to add acid.

2 Experimental

2.1 Buffers & Solutions

Prepare the following buffers by weighing in the needed amount of each substance. Use Tris free base and adjust pH by titrating with HCl.

Assay buffer

0.1 M Tris/HCl
pH 7.5

LAAO storage buffer

0.1 M Tris/HCl
150 mM KCl
pH 7.5

Prepare the following stock solutions:

- 200 U/mL HRP in assay buffer
- 1 mg/mL LAAO in LAAO storage buffer
- 5 mg/mL 2,2',4,4'-tetramethyl benzidine hydrochloride (TMB-HCl) in ddH₂O

Attention!

Confirm concentration of LAAO by measuring A²⁸⁰ (a 1 mg/mL solution of LAAO has an absorption of 1).

The enzyme solutions are stable at 4°C for a few weeks. DO NOT freeze the LAAO solution!

Store the TMB solution in darkness at -20°C.

The stock solutions and buffers are used to prepare the assay solution. The assay solution contains both enzymes (LAAO and HRP), as well as the chromogenic substrate tetramethylbenzidine (TMB).

Dilute the stock solutions to the concentrations specified. Use assay buffer to fill to volume.

Assay solution

10 U/mL HRP
0.25 mg/mL LAAO
0.1 mg/mL TMB-HCl
in 0.1 M Tris/HCl, pH 7.5

The reaction takes place in MTP format. 25 µl of substrate is put into the well. The reaction is started by addition of 75 µl assay solution and incubated at 30°C.

Continuous assay

After addition of the assay solution the developing blue color can be followed by absorption measurements at 652 nm.

The molar extinction coefficient of the charge transfer complex is $\varepsilon_{ctc}^{652nm} = 39000 M^{-1} cm^{-1}$

Endpoint measurements

The reaction is stopped after 10-20 minutes by addition of 50 µl 12 N H₂SO₄. The absorption is then measured at 450 nm.

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The yellow colored diimine has a molar extinction coefficient of $\epsilon_{diimine}^{450nm} = 59000 M^{-1} cm^{-1}$

References

- [1] P. Josephy, T. Eling, and R. Mason. The horseradish peroxidase-catalyzed oxidation of 3, 5, 3', 5'-tetramethylbenzidine. Free radical and charge-transfer complex intermediates. *J. Biol. Chem.*, 257:3669–3675, 1982.

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