Development of a Novel Assay for Synthesis and Hydrolysis of Sedoheptulose 1,7-bisphosphate (SBP) *in vitro* by Combinations of Purified Fructose 1,6-bisphosphate aldolases (FBA) Proteins and Fructose 1,6-bisphosphatases (FBPase) Proteins from *Bacillus methanolicus* MGA3

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[Abstract] *Bacillus methanolicus* (*B. methanolicus*) is a Gram-positive, thermotolerant, and facultative methylotrophic bacterium that can use the one-carbon (C1) compound methanol as a source of carbon and energy (Schendel *et al.*, 1990; Arfman *et al.*, 1997; Arfman *et al.*, 1992). *B. methanolicus* uses the Ribulose monosphosphate (RuMP) cycle for the fixation of formaldehyde (Anthony, 1986; Brautaset *et al.*, 2007). In the RuMP cycle, Sedoheptulose 1,7-bisphoasphate (SBP) can be produced from erythrose 4-phosohate (E4-P) and dihydroxyacetone phosphate (DHAP) by sedoheptulose 1,7-bisphosphate aldolase (SBA) and dephosphorylated to yield sedoheptulose 7-phosphate (S7-P) by sedoheptulose 1,7-bisphosphatase (SBPase). Unfortunately, since neither E4-P nor SBP is commercially available, these compounds cannot be used directly in enzyme assays to obtain evidence for synthesis and hydrolysis of SBP. To circumvent this limitation, a coupled discontinuous enzyme assay including transketolase from *Saccharomyces cerevisiae* (*S. cerevisiae*) was used. E4-P and xylulose 5-phosphate (XU5-P) were generated from fructose 6-phosphate (F-6P) and glyceraldehyde 3-phosphate (GAP) by transketolase from *S. cerevisiae.* Aldol condensation of E-4P with DHAP to yield SBP was tested by using purified fructose 1,6-bisphosphate aldolase (FBA^C or FBA^P) from *B. methanolicus* (Stolzenberger *et al.*, 2013a). Subsequently, hydrolysis of SBP to S7-P was assayed by using purified fructose 1,6-bisphosphatase ($GlpX^C$ or $GlpX^P$) from *B. methanolicus* (Aldolases and phosphatases are not commercially available) (Stolzenberger *et al.*, 2013b).

Materials and Reagents

1. Transketolase (TKT) from *S. cerevisiae* (Sigma-Aldrich)
2. Purified proteins: Fructose 1,6-bisphosphatase ($GlpX^C$) and promiscuous fructose 1,6-bisphosphatase/Sedoheptulose 1,7-bisphosphatase ($GlpX^P$), as well as promiscuous

fructose 1,6-bisphosphate aldolase/ Sedoheptulose 1,7-bisphosphate aldolase (FBA^C and FBA^P) from *B. methanolicus* MGA3
3. Sephadex G25 gel filtration (Amersham Biosciences)
4. Factor Xa (Novagen)
5. *(1 M) 50 mM Tris-HCl (pH 7.5)
6. *(400 mM) 20 mM D-fructose 6-phosphate dipotassium salt (F6-P) (Sigma-Aldrich)
7. *(400 mM) 20 mM DL-glyceraldehyd 3-phosphate solution (GAP) (Sigma-Aldrich)
8. *(400 mM) 20 mM dihydroxyacetone phosphate dilithium salt (DHAP)
9. *(200 mM) 10 µM thiamine pyrophosphate (TPP) (Sigma-Aldrich)
10. *(40 mM) 2 mM $MnCl_2$
11. *(200 mM) 10 mM ammonium bicarbonate solution (pH 9.3)

*Note: *Concentration of stock solution are given in brackets.*

Equipment

1. Amicon Ultra-0.5 centrifugal filter (EMD Millipore)
2. LaChromUltra HPLC system (Hitachi)
3. MicroTOF-Q hybrid quadrupole/time-of-flight mass spectrometer (BD)
4. SeQuant ZIC-pHILLIC column (150 x 2.1 mm) (Merck KGaA)

Software

1. Compass software 1.3 (BD)

Procedure

A. Enzyme assays for the purified GlpX proteins *in vitro*
1. The FBA activity in the direction of SBP synthesis was done by using a discontinuous, coupled enzyme assay containing TKT (from *S. cerevisiae*), recombinant $GlpX^C$ and $GlpX^P$, as well as FBA^C and FBA^P from *B. methanolicus* as described below.
2. Because E4-P is not acquired by purchase, E4-P was generated in a pre-reaction by the TKT (5 U/mg) from F6-P and GAP.
3. Protein production and purification was done as follows:
    a. The enzymes were cloned into pET16b, which allows IPTG-inducible expression with an N-terminal decahistidyl tag and a factor Xa cleavage site in *Escherichia coli* (*E. coli*) BL21 (DE3).
    b. The induction with 0.5 mM IPTG was started at an OD_{600} of 0.5 to 0.6 in LB. The cells

(500 ml) were harvested 4 h after induction and washed in 20 mM Tris, 300 mM NaCl, 5 mM imidazol, and 5% (vol/vol) glycerol.

c. Pelleted cells were stored at 20 °C until protein purification.

d. Prior to lysis by French press, cells were resuspended in 20 mM Tris, 300 mM NaCl, 5 mM imidazol, and 5% (vol/vol) glycerol, and protease activity was inhibited by addition of 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM diisopropylfluorophosphate (DFP).

e. The extract was cleared by centrifugation for 1.5 h at 140,000 *x g*.

f. Peak fractions of Ni-nitrilotriacetic acid (Ni-NTA) agarose affinity chromatography eluted with 20 mM Tris, 300 mM NaCl, 100, 200, or 400 mM imidazol, and 5% (vol/vol) glycerol were pooled, and the pooled fractions were desalted using Sephadex G25 gel filtration and buffered in 50 mM Tris/HCl (pH 7.5).

g. After purification, the His tag was cleaved by factor Xa according to the manufacturer's recommendations.

4. The purified protein was buffered in 50 mM Tris-HCl (pH 7.5). The reaction mixture contained 50 mM Tris-HCl (pH 7.5), 20 mM F6-P, 20 mM GAP, 20 mM DHAP, 10 µM thiamine pyrophosphate (TPP), 2 mM $MnCl_2$ and 3 U/mg of each purified enzyme (FBA^C or FBA^P and $GlpX^C$ or $GlpX^P$).
5. The reaction was started by addition of TKT (5 U/mg). The detection of the generated products was performed via liquid chromatography–mass spectrometry (LC-MS) as described below.
6. The assay was performed at 50 °C for 45 min in volume of 1 ml. The reaction was stopped by purification of the containing enzymes using Amicon Ultra-0.5 centrifugal filter. Therefore, the reaction mixture was sampled to the Amicon® Ultra filter device and cap it and the device was centrifuged at 14,000 *x g* for approximately 30 min. Thereby the proteins were filtered out of the mixture but the remaining incredients passed. The supernatant was then free of protein and the incredients were analyzed by LCMS. The expectations and results and described in a separate section below.

B. LC-MS analysis of the products after enzyme assay

1. LC-MS data were obtained using a LaChromUltra HPLC system coupled to a microTOF-Q hybrid quadrupole/time-of-flight mass spectrometer.
2. For Ionization the mass spectrometer is equipped with an electrospray ionization (ESI) source. Separation of the samples via HPLC was carried out by a SeQuant ZIC-pHILLIC column using the solvents 10 mM ammonium bicarbonate solution (pH 9.3) as eluent A and acetonitrile as eluent B.

3. The injection volume was 2 µl, flow rate was set to 150 µl min^{-1}, and gradient elution was performed as follows: t = 0 min, 80% B; t = 30 min, 10% B; t = 35 min, 10% B; t = 40 min 80% B; t = 60 min 80% B.
4. MS detection was performed via ESI source in negative ionization mode. Nitrogen was applied as sheath, dry and collision gas.
5. For internal mass calibration a solution of formate (0.1 M) in 50 % (v/v) isopropanol was injected within each MS analysis.
6. MSMS analyses were performed by the auto MSMS mode of the microTOF-Q.

Table S1. Parameters for microTOFcontrol in full scan MS mode

Mode	Scan Mode	MS	Ion Polarity	Positive
	Mass Range	50-1000 m/z	Rolling Average	off
	Spectra Acquisition	Save Spectra	Absolute Threshold	10
	Include Profile Spectra	Always	Peak Summation Width	5 pts
	Focus	Inactive	Acquisition Rate	1.0 Hz
Source	Endplate Offset	-500 V	Dry Gas	8.0 L / min
	Capillary	+ 4500 V	Dry Temp	180 °C
	Nebulizer	2.0 bar		
Transfer	Funnel 1 RF	300.0 Vpp	ISCID Energy	0.0 eV
	Funnel 2 RF	300.0 Vpp	Hexapole RF	200.0 Vpp
Quadrupole	Ion Energy	4.0 eV	Low Mass	150.00 m/z
Collision Cell	Collision Energy	8.0 eV	Collision RF	130.0 Vpp
	Transfer Time	75.0 µs	Pre Puls Storage	5.0 µs

Table 2. Additional parameters for microTOFcontrol in MS/MS (MRM) mode

Auto MSMS	Precursor Ions	3	Threshold	6000 counts
Transfer	Funnel 1 RF	300.0 Vpp	ISCID Energy	0.0 eV
	Funnel 2 RF	300.0 Vpp	Hexapole RF	200.0 Vpp
Quadrupole	Ion Energy	4.0 eV	Low Mass	150.00 m/z
Collision Cell	Collision Energy	8.0 eV	Collision RF	130.0 Vpp
	Transfer Time	75.0 µs	Pre Puls Storage	5.0 µs

7. Raw data were analyzed using the Compass software 1.3. Automatic internal mass calibration was achieved using the HPC quadratic algorithm.
8. Identification of compounds was performed either by the specific mass to charge ratio and the retention time or by comparing the fragment ions in MSMS mode.

Notes

1. The assay was performed to detect the synthesis and hydrolysis of SBP *in vitro* by combinations of purified FBA proteins and FBPase proteins from *B. methanolicus* MGA3. In the SBPase variant of the RuMP cycle, SBP is produced from E4-P and DHAP by SBA and dephosphorylated to yield S7-P by SBPase. Unfortunately, since neither E4-P nor SBP is commercially available, these compounds cannot be used directly in enzyme assays to obtain evidence for synthesis and hydrolysis of SBP. To circumvent this limitation, a coupled discontinuous enzyme assay including transketolase from *S. cerevisiae* was used.
2. Various combinations of substrates and enzymes were tested (Figure 1). No evidence for instabilities of the sugar phosphates at 50 °C was obtained when the substrates were incubated without enzymes.

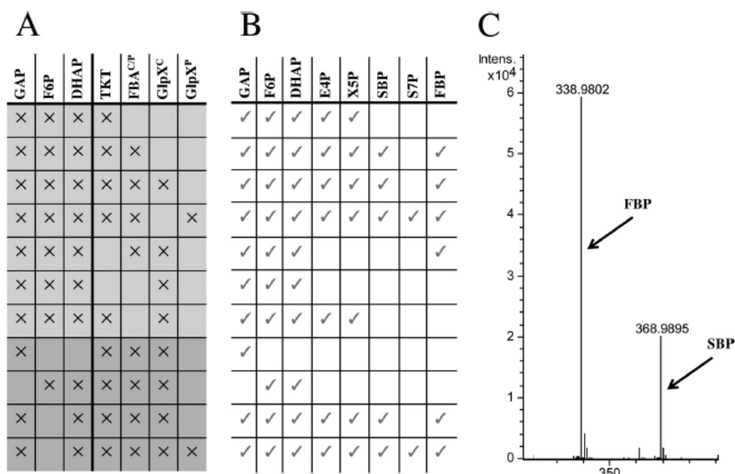

Figure 1. Determination of the sugar phosphate intermediates of the RuMP cycle using liquid chromatography-mass spectrometry (LC-MS). Shown is the experimental setup and determination of the sugar phosphate intermediates of the RuMP cycle using liquid chromatography-mass spectrometry (LC-MS). For the identification of sedoheptulose 1,7-bisphosphate and fructose 1,6-bisphosphate, MS/MS was used. (A) Scheme of the substrate and enzyme combinations used in the assay. X indicates presence in the assay. (B) Presence (check marks) or absence of the indicated sugar phosphates, as detected by LC-MS/MS analysis. (C) LC-MS spectra of FBP and SBP.

3. E4-P and xylulose 5-phosphate (XU5-P) were generated from F-6P and GAP by transketolase from *S. cerevisiae* (Figure 1, line 1). Aldol condensation of E-4P with DHAP to yield SBP could be detected for both purified FBA^C or FBA^P from *B. methanolicus* (Figure 1, line 2). LC-MS/MS analysis confirmed the identity of SBP, which showed the expected mass shift of 30 Da (-CHOH-) compared to FBP (Figure 1, C). Thus, these results indicated that both FBAs from *B. methanolicus* are active as SBAs *in vitro*. Subsequently, hydrolysis of SBP to S7-P was assayed by using purified $GlpX^C$ or $GlpX^P$ from *B. methanolicus*. Hydrolysis of SBP and formation of S7-P occurred only when $GlpX^P$, but not $GlpX^C$, was added (Figure 1, line 3, 4). Thus, for the major FBPase of *B. methanolicus*, $GlpX^C$, evidence for SBPase activity could not be obtained *in vitro*, while $GlpX^P$ showed activity as an SBPase.

Abbreviations

Enzymes

FBA	fructose-bisphosphate aldolase (EC 4.1.2.13)
TKT	transketolase (EC 2.2.1.1)
GlpX	fructose-bisphosphatase (EC 3.1.3.1)
SBA	sedoheptulose 1,7-bisphosphate aldolase (EC 4.1.2.x)
SBPase	sedoheptulose 1,7-bisphosphate (EC 3.1.3.37)

Metabolites

F6-P	fructose-6-phosphate
FBP	fructose-1,6-bisphosphate
GAP	glyceraldehyde 3-phosphate
DHAP	dihydroxyacetone phosphate
E4-P	erythrose 4-phosphate
SBP	sedoheptulose 1,7-bisphosphate
S7-P	sedoheptulose-7-phosphate
Ri5-P	ribose 5-phosphate
X5P	xylulose 5-phosphate
Ru5P	ribulose 5-phosphate

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