Supplementary Material

Details of the purification procedure

The expression vector was transformed into *E. coli* Tuner(DE3) cells (Novagen) which were grown in 1 l LB-media containing 30 µg/ml kanamycin to OD ~0.6, cooled on ice for 10 min, induced with 50 µM IPTG and incubated overnight at 16°C. The cell pellet after centrifugation at 5000 g was re-suspended in 10 ml buffer A (20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 40 mM imidazole, pH 8.0, 10% (v/v) glycerol) per g of wet cell pellet, to which 1 µg/ml DNAseI (Roche 10104159001), $MgCl_2$ to a final concentration of 10 mM, and a protease inhibitor tablet (Roche 11873580001) were added. The cell suspension was lysed using a continuous cell disruptor (1.35 kBar, Constant Systems Ltd.). The cell lysate was incubated on ice for 20 min before centrifugation (Beckman, JA-20, 20,000 rpm, 30 min, 4 °C) and then filtered through a 20 µm syringe driven filter. The filtered supernatant liquid was loaded on a 5 ml HisTrap column (GE Healthcare), equilibrated with buffer A and eluted using a gradient from 0% to 70% buffer B (buffer A with 500 mM imidazole). Fractions containing *Hv*SSI (identified by SDS-PAGE) were pooled, concentrated in a 30 kDa Vivaspin filter to reduce volume, and injected on a Superose 12 gel filtration column equilibrated with buffer C (20 mM Tris/HCl, pH 8.0, 500 mM NaCl, 10% (v/v) glycerol). Fractions containing *Hv*SSI were pooled and concentrated to 9.6 mg/ml (as measured by A_{280}, extinction coefficient $\varepsilon = 114250\ M^{-1}cm^{-1}$) in buffer C, divided into aliquots, and flash frozen in liquid nitrogen. For *Ta*SSI, *Os*SSI and the mutants "cat-dom", "rice-like", *Hv*SSI_C126S, *Hv*SSI_C506S and *Hv*SSI_F538A, the gel filtration purification step was substituted with an anion-exchange purification step using HiTrap-Q-FF cartridges (GE Healthcare). The proteins were eluted using a gradient between buffer D (20 mM Tris/HCl, pH 8.0, 50 mM NaCl, 10% (v/v) glycerol) and buffer C (as buffer D with 500 mM NaCl). The purity of the resulting protein preparations was assessed by SDS-PAGE gel electrophoresis.

Enzymatic synthesis of ADP-glucose

The α and β-subunits of ADP-glucose pyrophosphorylase (Uniprot entries P30524 and P55238) were obtained as synthetic genes, subcloned, expressed in *E. coli* and purified by methods similar to those used for starch synthase I. A 6 ml scale reaction contained 5% glycerol, 100 mM Tris/HCl, pH 8.5, 20 mM $MgCl_2$, 1 mM DTT, 20 mM ATP (Sigma A3377) and 20 mM glucose-1-phosphate (Sigma G7000). Proteins (α- and β-subunit) were each added to a final concentration of 0.04 mg/ml. The reaction was filtered through a 20 µm syringe driven filter and incubated in the dark with mild shaking at room temperature for 20 hours. The reaction develops a white precipitate overtime. The reaction products were centrifuged (1 min, 4000 g), the supernatant was diluted 2-fold in water and loaded into a DEAE Sephacel column equilibrated with 30 mM ammonium acetate. ADP-glucose was eluted with a linear gradient to 600 mM ammonium acetate. Fractions containing the product were pooled, the solvent removed in a rotary evaporator and the residue was re-dissolved in

water and lyophilized 3 times. 20 mg of pure product (ADP-glucose, di-ammonium salt) were obtained and purity was assessed by NMR.

Analysis of MOS produced in the mother liquor and bound to the crystal.

A sitting drop with crystals of *Hv*SSI was prepared analogously to the one described in materials and methods with the exception that double the amount of acceptor maltotriose was used. After 15 months at 4°C a crystal of 250 × 200 × 50 µm was extracted from the drop with a polypropylene loop and dissolved in 1 µl of 70% DMSO, 30% acetic acid and 2 M 2-aminobenzamide for fluorescent labelling. Then 2 µl of 1M $NaBH_3CN$ in the same solvent was added, the solution was heated at 60°C in a water bath for three hours, and then kept at room temperature for two days. The sample was centrifuged and 27 µL of 75% acetonitrile, 25% 10 mM ammonium formate, pH 4.5 was added. To 10 µl of this sample, 40 µl of dichloromethane and 10 µl of water were added and the vial was mixed and then centrifuged. 10 µl of the top aqueous layer were mixed with 10 µl of injection buffer (75% acetonitrile, 25% 10 mM ammonium formate, pH 4.5) and transferred to a Waters complete recovery vial.

Analysis was performed on a Waters Acquity UPLC-MS system fitted with a Waters fluorescence detector (FLD) operating with excitation at 330 nm and detection at 420 nm. Separation was achieved by means of a Waters Acquity UPLC BEH Glycan 1.7 µm, 2.1 × 150 mm column and the injection volume was 5 µl. The gradient elution method used mobile phase solvent A: 10 mM ammonium formate buffer, pH 4.5 and mobile phase solvent B: acetonitrile (HPLC grade from Labscan). The initial flow rate was 0.2 ml/minute , 22% solvent A was used for the first 5 minutes and then a gradient from 22% solvent A (78% B) to 50% solvent A was run for the next 25 minutes. Then the flow was reduced to 0.1 ml/minute for the next 15 minutes during which the gradient was from 50% solvent A to 65% solvent A.

Sequence of the expressed constructs

Codon optimized gene construct used for *Hv*SSI:

CATATGGCTCGCCTGCGCCGCGTAGCACGTGGCCGTTACGTGGCAGAGTTGTCTAGGGAAGGCCCGGCAGCGCGG
CCCGCACAACAATTGGCGCCTCCTGTTGTACCGGGTTTCCTGGCCCCCCCTCCACCAGCCCCCGCGCAGAGCCCC
GCACCTACCCAGCCCCCTTTGCCTGATGCTGGCGTCGGTGAATTAGCTCCCGACCTGCTCCTTGAGGGAATTGCG
GAGGATTCTATCGATACAATTGTTGTTGCAGCTAGCGAACAAGACAGTGAAATTATGGATGCAAATGATCAGCCC
CTTGCTAAAGTAACCAGATCAATCGTGTTTGTCACAGGTGAAGCAGCTCCGTACGCAAAAAGCGGCGGCCTAGGG
GATGTCTGTGGCAGCCTGCCGATTGCCCTGGCTGCACGCGGTCATCGGGTTATGGTGGTCATGCCACGCTATCTG
AATGGAACAAGTGACAAGAACTACGCAAAAGCCTTATACACAGGTAAACATATTAAAATTCCTTGCTTCGGCGGT
TCACACGAGGTTACTTTCTTCCACGAGTACCGTGACAACGTGGACTGGGTATTTGTTGACCACCCGTCGTATCAT
CGGCCGGGTTCCCTGTATGGTGACAATTTCGGCGCGTTTGGTGATAATCAATTTCGATATACGCTGCTGTGTTAT
GCGGCGTGCGAAGCGCCACTGATCCTGGAACTGGGGGGATACATCTACGGCCAGAGCTGTATGTTTGTAGTGAAC
GATTGGCACGCCAGTCTGGTCCCGGTATTGCTTGCAGCCAAATACCGCCCCTATGGCGTCTACCGCGATAGCCGT

TCTACCCTGGTGATTCACAATTTAGCCCACCAAGGAGTCGAGCCAGCGTCCACGTATCCTGACCTTGGCCTGCCT
CCGGAGTGGTACGGGGCCCTCGAGTGGGTGTTTCCGGAATGGGCGCGTCGACACGCGCTGGATAAGGGGGAAGCC
GTGAACTTCCTGAAGGGTGCTGTAGTTACGGCCGATCGTATTGTGACTGTGTCCCAAGGCTACAGTTGGGAAGTG
ACCACCGCCGAAGGGGGTCAGGGCCTGAACGAACTGTTATCCAGCCGCAAGTCAGTCCTGAACGGCATTGTGAAT
GGCATCGATATCAACGACTGGAACCCTACGACGGACAAATGTCTACCGCATCATTACTCGGTTGATGATCTGTCG
GGGAAAGCGAAATGCAAAGCGGAACTGCAGCGTGAATTGGGTTTACCGGTTCGCGAGGACGTACCGTTAATAGGA
TTTATCGGGCGCCTGGATTATCAGAAAGGTATTGATTTAATCAAAATGGCGATTCCGGATCTGATGCGCGAAGAC
GTGCAATTTGTGATGTTAGGCAGTGGCGATCCGGTCTTTGAAGGCTGGATGCGCTCGACCGAATCCAGCTATAAA
GATAAATTTCGCGGATGGGTGGGATTCAGCGTCCCGGTCAGCCATCGTATCACTGCCGGCTGCGATATACTCCTC
ATGCCGTCTCGTTTTGAACCGTGCGGTTTGAACCAGTTGTATGCGATGCAGTATGGCACCGTACCGGTCGTCCAT
GGCACCGGGGGGCTTCGTGATACGGTTGAGACTTTTAATCCGTTCGGCGCGAAAGGTGAGGAAGGTACTGGTTGG
GCGTTCTCGCCGCTCACCGTTGAAAAAATGTTATGGGCGCTGCGTACGGCGATTTCGACCTTCCGTGAACATAAA
CCGTCTTGGGAGGGCCTGATGAAACGTGGTATGACCAAAGATCATACCTGGGATCATGCGGCGGAACAGTATGAG
CAGATCTTTGAATGGGCGTTTGTGGATCAGCCGTATGTCATGTAAAAGCTT

Protein sequence of the expressed construct for *Hv*SSI crystallization: Amino acid number 1 in the numbering system used in the crystal structure is bolded and underlined.

MGSSHHHHHHSSGLVPRGSHM**A**RLRRVARGRYVAELSREGPAARPAQQLAPPVVPGFLAPPPPAPAQSPAPTQPP
LPDAGVGELAPDLLLEGIAEDSIDTIVVAASEQDSEIMDANDQPLAKVTRSIVFVTGEAAPYAKSGGLGDVCGSL
PIALAARGHRVMVVMPRYLNGTSDKNYAKALYTGKHIKIPCFGGSHEVTFFHEYRDNVDWVFVDHPSYHRPGSLY
GDNFGAFGDNQFRYTLLCYAACEAPLILELGGYIYGQSCMFVVNDWHASLVPVLLAAKYRPYGVYRDSRSTLVIH
NLAHQGVEPASTYPDLGLPPEWYGALEWVFPEWARRHALDKGEAVNFLKGAVVTADRIVTVSQGYSWEVTTAEGG
QGLNELLSSRKSVLNGIVNGIDINDWNPTTDKCLPHHYSVDDLSGKAKCKAELQRELGLPVREDVPLIGFIGRLD
YQKGIDLIKMAIPDLMREDVQFVMLGSGDPVFEGWMRSTESSYKDKFRGWVGFSVPVSHRITAGCDILLMPSRFE
PCGLNQLYAMQYGTVPVVHGTGGLRDTVETFNPFGAKGEEGTGWAFSPLTVEKMLWALRTAISTFREHKPSWEGL
MKRGMTKDHTWDHAAEQYEQIFEWAFVDQPYVM

Protein sequences expressed for *Ta*SSI and *Os*SSI.

TaSSI:
MGSSHHHHHHSSGLVPRGSHMARLRRVARGRYVAELSREGPAARPAQQQQLAPPLVPGFLAPPPPAPAQSPAPTQ
PPLPDAGVGELAPDLLLEGIAEDSIDSIIVAASEQDSEIMDAKDQPQAKVTRSIVFVTGEAAPYAKSGGLGDVCG
SLPIALAARGHRVMVVMPRYLNGSSDKNYAKALYTAKHIKIPCFGGSHEVTFFHEYRDNVDWVFVDHPSYHRPGS
LYGDNFGAFGDNQFRYTLLCYAACEAPLILELGGYIYGQNCMFVVNDWHASLVPVLLAAKYRPYGVYRDSRSTLV
IHNLAHQGVEPASTYPDLGLPPEWYGALEWVFPEWARRHALDKGEAVNFLKGAVVTADRIVTVSQGYSWEVTTAE
GGQGLNELLSSRKSVLNGIVNGIDINDWNPTTDKCLPHHYSVDDLSGKAKCKAELQKELGLPVREDVPLIGFIGR
LDYQKGIDLIKMAIPELMREDVQFVMLGSGDPIFEGWMRSTESSYKDKFRGWVGFSVPVSHRITAGCDILLMPSR
FEPCGLNQLYAMQYGTVPVVHGTGGLRDTVETFNPFGAKGEEGTGWAFSPLTVDKMLWALRTAMSTFREHKPSWE
GLMKRGMTKDHTWDHAAEQYEQIFEWAFVDQPYVM

OsSSI:
MGSSHHHHHHSSGLVPRGSHMSEQESEIMDVKEQAQAKVTRSVVFVTGEASPYAKSGGLGDVCGSLPIALALRGH
RVMVVMPRYMNGALNKNFANAFYTEKHIKIPCFGGEHEVTFFHEYRDSVDWVFVDHPSYHRPGNLYGDNFGAFGD
NQFRYTLLCYAACEAPLILELGGYIYGQKCMFVVNDWHASLVPVLLAAKYRPYGVYRDARSVLVIHNLAHQGVEP
ASTYPDLGLPPEWYGALEWVFPEWARRHALDKGEAVNFLKGAVVTADRIVTVSQGYSWEVTTAEGGQGLNELLSS
RKSVLNGIVNGIDINDWNPSTDKFLPYHYSVDDLSGKAKCKAELQEELGLPIRPDVPLIGFIGRLDYQKGIDLIK
LAIPDLMRDNIQFVMLGSGDPGFEGWMRSTESGYRDKFRGWVGFSVPVSHRITAGCDILLMPSRFEPCGLNQLYA
MQYGTVPVVHGTGGLRDTVENFNPFAEKGEQGTGWAFSPLTIEKMLWALRMAISTYREHKSSWEGLMKRGMSSDF
TWDHAASQYEQIFEWAFMDQPYVM

Primers used in mutagenesis

Actual mutated nucleotides are bolded and underlined for the forward primer.

*Hv*SSI_C126S:

Forward primer: 5'-cctaggggatgtc**a**gtggcagcctgcc-3'

Reverse primer: 5'-ggcaggctgccactgacatcccctagg-3'

*Hv*SSI_C506S:

Forward primer: 5'-cgtctcgttttgaaccg**a**gcggtttgaaccagttg-3'

Reverse primer: 5'-caactggttcaaaccgctcggttcaaaacgagacg-3'

*Hv*SSI_F538A:

Forward primer: 5'-gttgagacttttaatccg**gc**cggcgcgaaaggtgagga-3'

Reverse primer: 5'-tcctcacctttcgcgccggccggattaaaagtctcaac-3'

“rice-like” *Hv*SSI:

Forward primer: 5’-gtgccgcgcggcagccattcaagcgaacaagacagtgaaattatggatgc-3’

Reverse primer: 5’-gcatccataatttcactgtcttgttcgcttgaatggctgccgcgcggcac-3’

“cat-dom” *Hv*SSI:

Forward primer: 5’-gtgccgcgcggcagccattcaaatgatcagcccttgctaaagtaacc-3’

Reverse primer: 5’-ggttactttagcaaggggctgatcatttgaatggctgccgcgcggcac-3’

Sequence sources used in multiple sequence alignments

Hv_GBSSI: BAC41202.1 ; Hv_SSII: AAN28309.1 ; HvSSIIIa: AEL97581.1 ; HvSSIV: BAJ86666.1 ; TaSSI: CAB99209.1 ; OsSSI: ACY56162.1 ; ZmSSI: NP_001104892.1 ; AtSSI: AAF24126.1 ; StSSI: P93568.1 ; EcGS: PDB_ID 2QZS ; AtGS: PDB_ID 1RZU ; PaGS: PDB_ID 3L01, OsGBSSI: PDB_ID 3VUE.

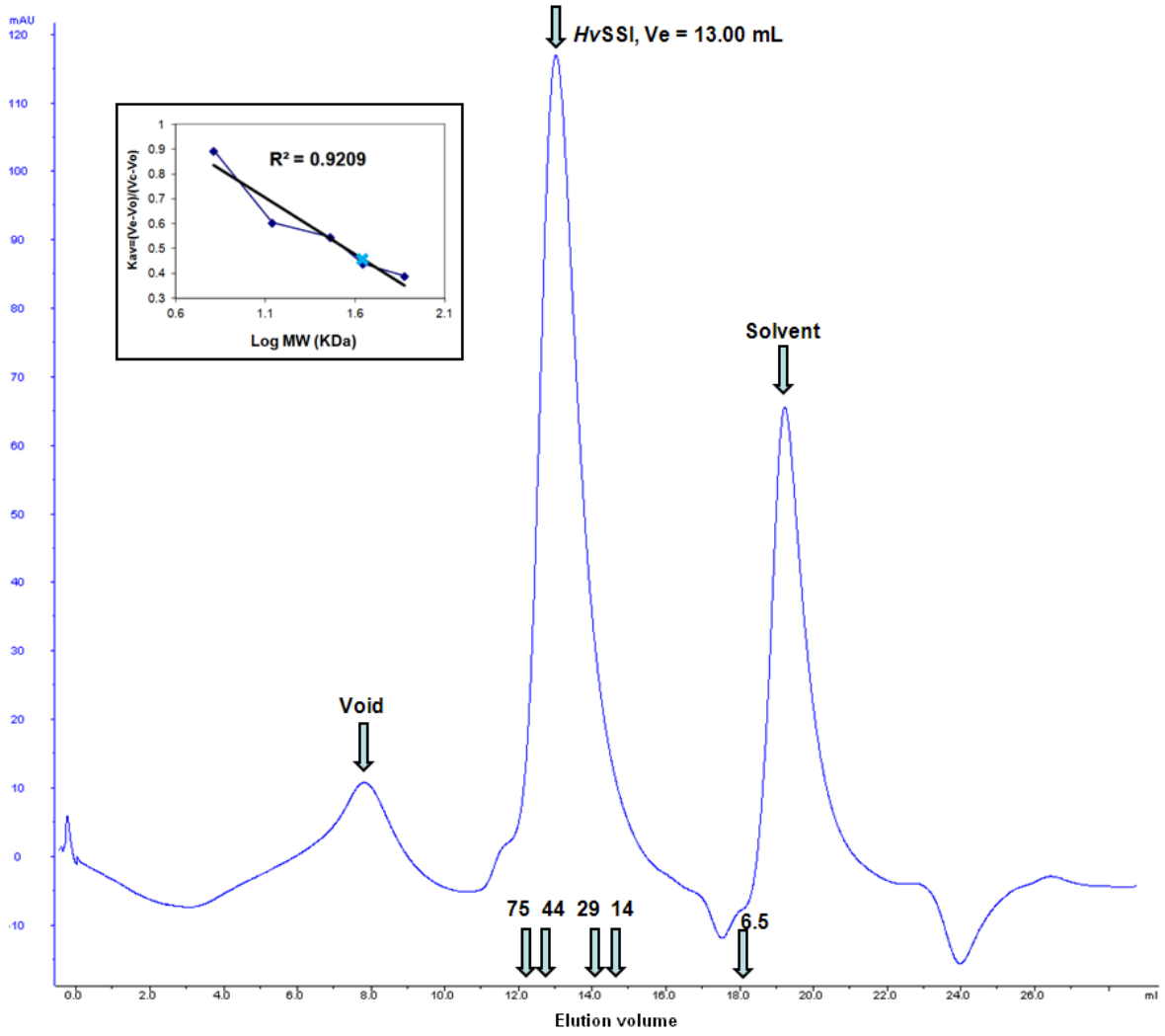

Figure S1. Size exclusion chromatography of *Hv*SSI. Size exclusion chromatography profile of *Hv*SSI run on a Superdex 12 column. SSI was injected in 0.1 ml at approximately 1 mg/ml. The running buffer was 10% glycerol, 20 mM Tris HCl, pH 8.0, with 500 mM NaCl and 5 mM DTT. The flow rate was 0.4 ml/min. For column calibration, equivalent runs were made with the following standards (molecular weight in kDa): apoprotinin (6.5), ribonuclease A (13.7), carbonic anhydrase (29), ovalbumin (44), canalbumin (75); and blue dextran 2000 was used to determine the void volume. The elution volumes of the standards are indicated by arrows in the graph, where the *Hv*SSI and solvent peaks are also indicated. A linear calibration curve (R=0.92, inset) yields an apparent molecular weight (blue cross) of 44.7 kDa for *Hv*SSI, below the theoretical mass of 69.8 kDa for our construct.

Figure S2. Sequence and structural alignment of *Hv*SSI. Sequence alignment of *Hv*SSI with other structures mentioned in the main text: *Os*GBSSI, *Ec*GS and *At*GS. Secondary structure is highlighted in color with pink for β-strands and blue for α-helices. Residues of *Hv*SSI absent from the final model are marked with gray background. Key residues of *Hv*SSI mentioned in the main text: Cys126, Cys506 and Phe538 are bolded and underlined and highlighted with green arrows. Strict residue conservation is depicted with asterisks, while double and single dots represent decreasing degrees of similarity.

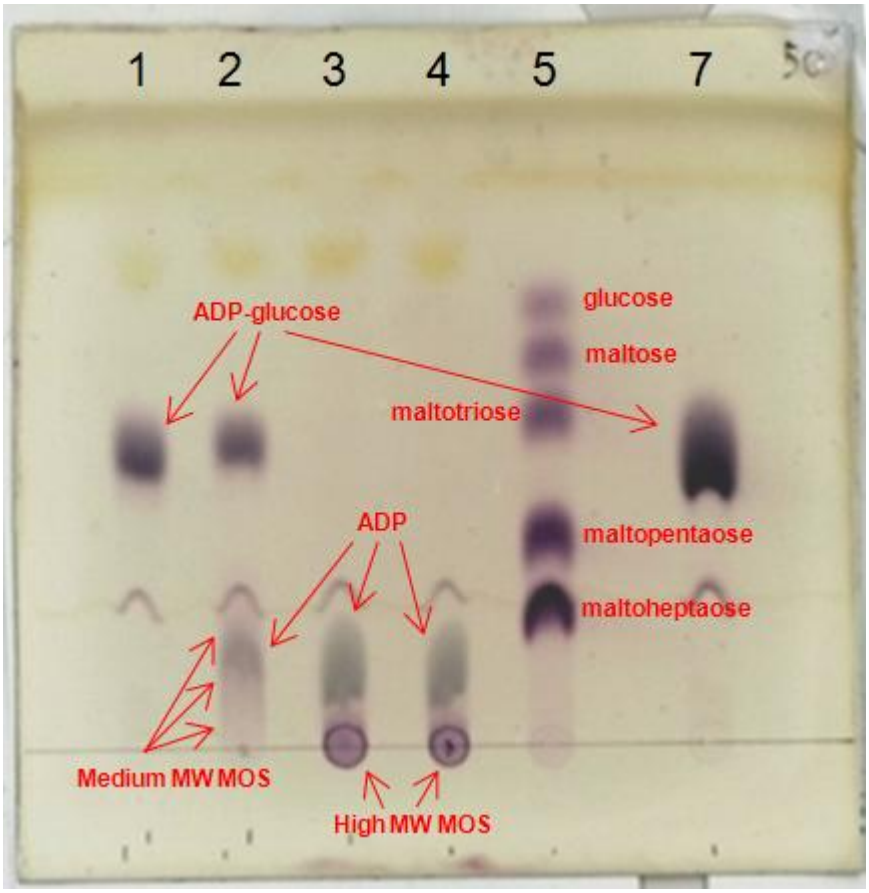

Figure S3. TLC of maltooligosaccharide production. TLC analysis of a crystal protein stock reaction mixture. Protein stock and buffer conditions are as described in the text for crystallization, with 100 µM maltotriose and 3.3 mM ADP-glucose. Lanes 1, 2, 3 and 4 are after 0, 1, 7 and 14 days of 4°C incubation. Lane 5 has 1 mM standards of glucose (top), maltose, maltotriose, maltopentaose and maltoheptaose. Lane 7 has 10 mM ADP-glucose. The initial maltotriose is at the detection limit and partially overlapping with the ADP-glucose band, hence it cannot be clearly distinguished. The TLC system used lacks resolution for maltooligosaccharides larger than DP 7. The thin layer chromatography (TLC) assay was performed with a protocol adapted from (Robyt & Mukerjea, 1994). It was carried out on a TLC Silica gel 60 plate (Merck), loading 1 µl of aqueous solution per lane. Running solvent was ACN:EtOAc:PrOH:H_20 in 85:20:50:50 proportions. Development was with a 5% H_2SO_4 solution in EtOH containing 0.5% 1-Naphtol followed by charring. Hexoses (glucose) color purple under these conditions while pentoses (ADP) appear gray. ADP-glucose has an intermediate color. After seven days the MOS are too large to leave the origin spot and the reaction stops due to ADP-glucose depletion.

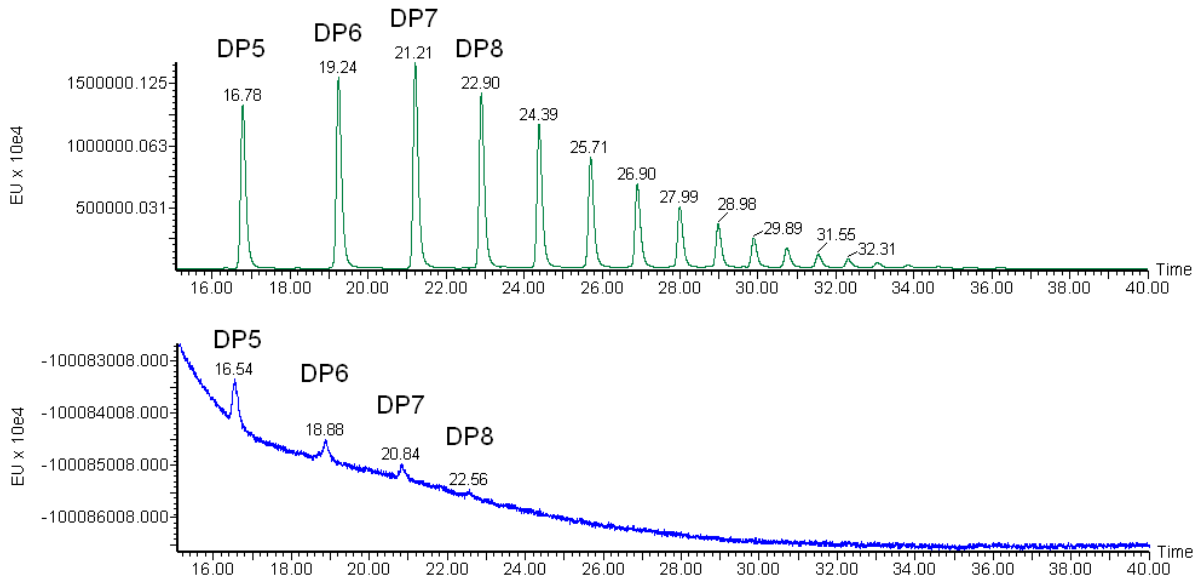

Figure S4. Profile of MOS produced in the mother liquor and bound to the crystal. Top: HPLC profile of a ladder of labelled MOS for reference. Bottom: HPLC profile of the labelled mixture from the dissolved *Hv*SSI crystal. Peaks corresponding to maltopentaose, maltohexaose, maltoheptaose and maltooctaose are labelled as DP 5 through 8. Although the background of unused fluorescent label is very high, peaks for species from maltopentaose to maltooctaose can be clearly identified. Larger species might be present below the detection limit. The expected slow reversibility of the reaction, and the slow decomposition of ADP-glucose mean that larger species could have formed initially and been slowly hydrolyzed over time. However, these peaks show that reaction occurred with the original maltotriose and that maltopentaose and larger MOS are present in the crystal.