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Sucrose phosphorylase from *Alteromonas mediterranea*: structural insight into the regioselective α -glucosylation of (+)-catechin

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Abstract

Flavonoids glycosylation at different positions is paramount to solubility and modulation of bioactivities. Sucrose phosphorylases, through transglycosylation reactions, are interesting enzymes that can transfer glucose from sucrose, the donor substrate, onto polyphenols to form glycoconjugates. Here, we report for the first time the structural and enzymatic properties of sucrose phosphorylase from the marine bacteria *Alteromonas mediterranea* (AmSP). We characterized and investigated the transglucosylation capacity of two new variants of the enzyme on (+)-catechin and their propensity to catalyse its regioselective glucosylation. AmSP-Q353F and

24 AmSP-P140D were shown to catalyse the regiospecific glucosylation of (+)-catechin using sucrose
25 as donor substrate. While AmSP-WT was devoid of synthetic activity, each of its two single mutant
26 provided high yields of specific regioisomers: 89% of (+)-catechin-4'-O- α -D-glucopyranoside (CAT-
27 4') for AmSP-P140D and 92% of (+)-catechin-3'-O- α -D-glucopyranoside (CAT-3') for AmSP-Q353F.
28 The novel compound CAT-4' was fully characterized by NMR and mass spectrometry. We used
29 molecular docking simulations on structural models of the glucosyl-enzyme intermediate to
30 explain this regioselectivity. We showed that AmSP-P140D preferentially binds (+)-catechin in a
31 mode that favours glucosylation on its hydroxyl group in position 4' (OH-4') while the binding
32 mode of the flavonoid in AmSP-Q353F favoured glucosylation on its hydroxyl group in position 3'
33 (OH-3').

34 Keywords: marine microbial enzymes, regioselectivity, biocatalysis, (+)-catechin, sucrose-
35 phosphorylase, *Alteromonas mediterranea*

36 1. Introduction

37 Oceans cover more than 70% of Earth's surface and provides a unique environment to marine
38 bacteria (*i.e.* high salinity, high pressure, low temperature and special lighting conditions). For
39 decades, enzymes have been isolated and purified from terrestrial microorganisms, animals and
40 plants. With the advent of biotechnology, there has been a growing interest and demand for
41 enzymes with novel properties and robust biocatalysts. Due to its complexity, the marine
42 environment represents a great opportunity for exploration of new enzymes and molecules [1, 2].
43 Marine enzymes are capable of being active under extreme conditions, which provide
44 competitiveness and efficiency to different industrial processes [3, 4]. Among those, sucrose-
45 phosphorylases (SPs) from the Glycoside Hydrolase family 13 subfamily 18 (GH13_18, EC 2.4.1.7)
46 attract biotechnological interest as biocatalysts. Requiring only a cheap and abundant donor, SPs
47 can perform transglucosylation reaction by transferring glucose from sucrose to an acceptor to

48 yield α -glucosylated products with a retaining mechanism via a β -glucosyl-enzyme intermediate.
49 Particularly, *Bifidobacterium adolescentis* SP (*BaSP*-WT) and its mutants have been studied for
50 biocatalytic synthesis of rare disaccharides [5, 6] and α -glucosylation of polyphenols [7, 8, 9, 10].
51 To date, the only documented structures of sucrose phosphorylase in the literature are from
52 *Bifidobacterium adolescentis* [11]. Other SPs, from *Leuconostoc mesenteroides*, *Streptococcus*
53 *mutans*, *Lactobacillus acidophilus* and *Thermoanaerobacterium thermosaccharolyticum*, have also
54 been studied for the glucosylation of phenolic compounds [12, 13, 14]. Glucosylation of this type
55 of molecules increases their solubility in water and their bioavailability in health, nutraceuticals
56 and cosmetics applications [15, 16]. Controlling the regioselectivity of this glucosylation is also at
57 stake for the synthesis of new compounds. We recently documented the activity of two variants of
58 *BaSP*-WT with respect to their ability to transfer regioselectively a glucose moiety onto (+)-
59 catechin as an acceptor substrate [10]. *BaSP*-Q345F and *BaSP*-P134D/Q345F glucosylated (+)-
60 catechin on hydroxyl groups in position 3' (OH-3') and 5 (OH-5) with obtainment of three
61 glucosylated regioisomers: (+)-catechin-3'-O- α -D-glucopyranoside (CAT-3'), (+)-catechin-5-O- α -D-
62 glucopyranoside (CAT-5) and (+)-catechin-3',5-O- α -D-diglucopyranoside (CAT-3',5), with a ratio of
63 51:25:24 for *BaSP*-Q345F and 82:9:9 for *BaSP*-P134D/Q345F.

64 *Alteromonas mediterranea*, also known as *Alteromonas macleodii* "Deep Ecotype" or AltDE, is an
65 aerobic Gram-negative and mesophilic marine bacterium from the genus of *Proteobacteria* which
66 was first isolated at a depth of 1000 m in the Eastern Mediterranean Sea in 2005 [17, 18, 19]. Wild
67 type form of *AmSP* (*AmSP*-WT) shares 52% of global sequence identity with *BaSP*-WT. Sequence
68 alignments revealed that both enzymes possess highly conserved regions corresponding to the
69 loop A (*BaSP*-WT: ³³⁶AAASNLDLYQ³⁴⁵, *AmSP*-WT: ³⁴⁴AAASNLDLYQ³⁵³) and loop B (*BaSP*-WT:
70 ¹³²YRPRP¹³⁶, *AmSP*-WT: ¹³⁸FRPRP¹⁴²) of the catalytic site [20]. In a preliminary screening using
71 homology modelling and molecular docking, we identified that the catalytic cavity of the glucosyl-
72 intermediate of *AmSP*-WT could potentially host a polyphenolic acceptor compound like (+)-

73 catechin. We thus characterized *AmSP*-WT from a structural and functional perspective. Towards
74 this end, the crystallographic structure of *AmSP*-WT was for the first time recently determined
75 [Goux *et al.*, in preparation]. In the present work, we further analysed the structural features of
76 *AmSP*-WT and investigated the enzymatic properties of two variants of the enzyme towards their
77 propensity to catalyse the regioselective transglucosylation of (+)-catechin. P140D and Q353F
78 mutations, homologous to mutations P134D and Q345F of *BaSP*-WT, displayed a single transfer
79 reaction product for each enzyme: (+)-catechin-4'-O- α -D-glucopyranoside (CAT-4') for *AmSP*-
80 P140D and CAT-3' for *AmSP*-Q353F. To explain the striking enzymatic activities of those variants,
81 we provide in-depth structural insights by docking simulations and modelling. Our results
82 interestingly broaden the available chemo-enzymatic synthetic tools for the efficient regioselective
83 α -glucosylation of polyphenols.

84 2. Materials and methods

85 2.1. Vector construction and proteins

86 *AmSP*-WT and its variants were expressed as C-terminally hexahistidine-tagged proteins, allowing
87 affinity purification by standard protocols. *AmSP*-WT gene (UniProt: S5AE64_9ALTE) was ordered
88 from Genscript already cloned in a pET28b vector. *E. coli* BL21(DE3) competent cells (Novagen)
89 were transformed with pET28b_ *AmSP*-WT. Clones were selected using LB-agar medium
90 supplemented with 25 μ g/mL kanamycin and confirmed by Sanger sequencing (Eurofins
91 Genomics). Variants *AmSP*-P140D, *AmSP*-Q353F and *AmSP*-P140D/Q353F were obtained by site-
92 directed mutagenesis. Proteins were produced, purified and characterized as previously described
93 [10].

94 2.2. *Transglucosylation studies*

95 Reactions were carried out in 50 mM 3-morpholinopropane-1-sulfonic acid (MOPS)-NaOH solution
96 at pH 8.0 in a total volume of 1 mL. Reaction mixture containing 10 mM (+)-catechin in DMSO (1
97 eq., 100 μ L), 10% DMSO (100 μ L, (v/v)), 80 mM sucrose in H₂O (8 eq., 100 μ L) was incubated with a
98 final concentration of 10 μ M of purified enzyme at 25°C under slight agitation. Enzymatic synthesis
99 was monitored by thin layer chromatography (TLC) for 48h. TLC plates were developed in solution
100 composed of ethyl acetate/methanol/cyclohexane/water (6.75:1.35:1:0.9, v/v/v/v) with 0.1%
101 formic acid (v/v). Products were visualized using a UV lamp at 254 nm and revealed with vanillin-
102 sulphuric acid reagent.

103 2.3. *Purification and analysis of glucosylated (+)-catechin*

104 After centrifugation (12 000 x g, 20 min), 10 μ L of the supernatant was analysed by analytical HPLC
105 at 280 nm on a C-18 column (Interchim, 5 μ m, 250 x 4.6 mm, US5C18HQ-250/046) with an
106 isocratic flow of 80% H₂O (v/v), 0.1% formic acid (v/v) and 20% MeOH (v/v), 0.1% formic acid (v/v)
107 for 20 min. Then, remaining supernatant was purified by HPLC at 280 nm on a C-18 column
108 (Interchim, 5 μ m, 250 x 21.2 mm, US5C18HQ-250/212) with a gradient system (solvent A: H₂O
109 HCOOH 0.1%; solvent B: MeOH, HCOOH 0.1%; t₀ min = 70/30, t₁₀ min = 70/30, t₇₀ min = 10/90).
110 Products were identified by NMR ¹H and ¹³C in MeOD or DMSO-d₆ (400 Hz, 256 scans). For
111 characterization of CAT-3', CAT-5 and CAT-3',5, see [10].

112 2.4. *Molecular modelling of variants of AmSP-WT*

113 Glucosyl-enzyme intermediate 3D-models were built for AmSP-Q353F and AmSP-P140D using the
114 following procedure and the Rosetta software [22]. Glucosylated-aspartyl 192 residue from chain
115 A of crystal structure of BaSP-WT (PDB: 2GDV-A) was inserted into the crystal structure AmSP-WT

116 (PDB: 7ZNP) that served as initial template for both variants. As this glucosylated aspartyl is a non-
117 standard residue, it was absent from the database of the Rosetta software. Using Pymol3, the
118 initial coordinates of this modified residue were retrieved. While this residue (D192) and the
119 glucose moiety (BGC) are covalently linked in the crystal structure, the Pymol software considered
120 them as two distinct residues. Thus, they were merged them into a single non-standard residue,
121 which was called with a new ID, DGC. Associated charges and rotamers were calculated for this
122 new residue using the Rosetta software. All those data were merged a single file that we added
123 into the Rosetta database (Section 2.7).

124 With the DGC residue ready to be used, glucosyl-intermediates were built for the two variants of
125 AmSP-WT. From the crystal structure (PDB: 7ZNP), using Rosetta the native aspartyl residue in
126 position 203 was mutated by the glucosylated-aspartyl DGC residue together with either the
127 P140D or Q353F mutation. For each variant (AmSP-Q353F or AmSP-P140D), a sample of 50
128 conformers was generated thanks to the program Backrub from Rosetta suite, with 10 000 tries. In
129 parallel, 12 conformers of (+)-catechin were also generated using the Mercury software (CCDC)
130 [23] from the crystal structure OZIDOR of (+)-catechin.

131 2.5. Docking analysis of binding mode of (+)-catechin in the catalytic pocket

132 All docking experiments were performed with AutoDock Vina using the glucosyl-intermediates and
133 (+)-catechin conformers built above. Docking perimeter was limited to the residues of the active
134 site of the enzyme. Each of the 12 conformers of (+)-catechin were docked on every conformer of
135 the two variants. This amounts to a total of 600 (50x12) docking experiments for each variant of
136 the enzyme. Only the productive poses that could lead to a glucosylation of (+)-catechin were
137 selected. To do so, docking poses were filtered using the following distance constraints: distances
138 within 3.0 Å between any oxygen of (+)-catechin and the anomeric carbon atom C1 of the glucosyl
139 moiety were assessed. Docking scores were compiled for these productive poses and compared

140 between the two variants. R statistics software package was used to perform the boxplots
141 analysis.

142 3. Results

143 3.1. *Highly conserved structural features and potential activity of AmSP-WT*

144 Through structural comparison of *BaSP*-WT (PDB: 1R7A, 2GDV), *BaSP*-Q345F (PDB: 5C8B), and our
145 recently determined structure of *AmSP*-WT (PDB: 7ZNP), we identified the conserved residues
146 likely involved in the reaction mechanism and potential substrate interactions (Table S1). The -1
147 subsite of SPs, also called donor site, has an optimal topology for binding glucose and is conserved
148 between *BaSP*-WT and *AmSP*-WT (Figure 1). The configuration of the two catalytic residues
149 involved in the reaction mechanism are also almost identical (Figure 1A, in blue): a glutamyl
150 residue acts as a general acid/base catalyst (E243 for *AmSP*-WT and E232 for *BaSP*-WT) and an
151 aspartyl residue performs the nucleophilic attack (D203 for *AmSP*-WT and D192 for *BaSP*-WT). The
152 third member of the catalytic triad (D301 for *AmSP*-WT and D290 for *BaSP*-WT) stabilises the
153 transition state with a strong hydrogen bond and presents also an identical configuration in the
154 catalytic site. The structural elements that were shown to stabilize the glucosyl moiety in *BaSP*-WT
155 by non-polar contacts between a hydrophobic platform (F53/F156 for *BaSP*-WT) and the
156 hydrophobic C3-C4-C5 part of glucose are also conserved in *AmSP*-WT (F56/F167). The acceptor or
157 +1 site of SPs is mainly shaped by two highly dynamic loops (Figure S1), which were shown to
158 adopt different conformations based on the progress of the reaction mechanism: one
159 conformation is the “donor binding mode” or closed conformation and the other is the “acceptor
160 binding mode” or open conformation where an arginyl residue (R135 in *BaSP*-WT) is thought to
161 enable the enzyme to outcompete water as an acceptor through strong electrostatic interactions.
162 When we compare the apoenzyme of *BaSP*-WT (PDB: 1R7A) with our newly obtained apoenzyme

163 of AmSP-WT (PDB: 7ZNP), a striking difference in the positioning of loop A is noticed. The enzyme
164 has already an opened conformation and is in the “acceptor binding mode” with Y352 residue
165 pointing inside the active site (Figure 1, in magenta) and R141 pointing outside (Figure 1, in
166 orange). This open conformation was also observed for BaSP-WT crystallized with the end-product
167 of the reaction after hydrolysis of glucose (PDB: 2GDV, chain B). Moreover, crucial conserved
168 residues involved in binding of both phosphate and fructose, Y196 and H234 for BaSP-WT vs. Y207
169 and H245 for AmSP-WT, are in the same conformational positions thus allowing sucrose
170 phosphorylase activity (Figure 1B, in cyan)[21]. In BaSP-WT, Y132 is located at the entrance of the
171 active site and contributes to sucrose specificity thanks to hydrophobic interactions with Y196 and
172 F206. In AmSP-WT, the aromatic structure is conserved with the replacement of the tyrosinyl
173 moiety by a phenylalanyl residue in position 138 (Figure 1B, in orange).

174 3.2. Determination of the apparent kinetic parameters

175 Interestingly, the P140D and Q353F mutations did not alter the enzyme stability, as evidenced by
176 unchanged melting temperature (T_m) of 43°C (Figure S2, Table S2). The apparent kinetic
177 parameters for sucrose at 25°C with and without 20% DMSO were determined. For each condition,
178 variants present a decrease of catalytic efficiency towards sucrose of one order of magnitude
179 compared to the wild-type (Table 1). A loss in specificity for sucrose was observed for AmSP-
180 Q353F with an increasing of the K_m value from 1 mM for AmSP-WT to 5 mM for AmSP-Q353F. With
181 DMSO, AmSP-P140D displayed a higher specificity for sucrose with a K_m value similar to the wild-
182 type, resulting in a catalytic efficiency increasing by almost 2-fold compared to the results
183 obtained without DMSO. For AmSP-Q353F, with 20% of DMSO, we observed no change for the
184 specificity for sucrose and the turnover number.

185 3.3. (+)-catechin transglucosylation studies

186 We assessed the ability of SPs to transfer a glucose moiety from sucrose to (+)-catechin at 25°C
187 after 24h under agitation. Observed products were purified by preparative HPLC and analysed by
188 NMR. AmSP-P140D and AmSP-Q353F, catalyse efficiently the synthesis of two different
189 regioisomers of (+)-catechin glucoside: AmSP-P140D glucosylate mostly the hydroxyl groups in
190 position 4' (OH-4') while AmSP-Q353F glucosylate the OH-3' position (Figure S5, Table S4). We
191 monitored the synthesis of glucosylated (+)-catechin products during 24h by HPLC to determine
192 conversion yields and proportion of regioisomers (Figure 2, Table 2). Interestingly, the main
193 product formed with AmSP-P140D was CAT-4' with a relative proportion of 89% while the main
194 product formed with AmSP-Q353F was CAT-3' with a relative proportion of 92%. The
195 corresponding synthetic yields (percentage of (+)-catechin that was converted into these
196 glycosylated products) was 26% and 82% respectively. These results clearly indicate that these
197 two variants are highly regioselective with respect to their transglycosylation activities on (+)-
198 catechin.

199 3.4. Structural insights into the regioselectivity of AmSP-P140D and AmSP-Q353F

200 To further understand the observed regioselectivities, we performed molecular docking
201 simulations with several conformations of the glucosyl intermediate of AmSP-Q353F and AmSP-
202 P140D. The preferred orientations of various (+)-catechin conformers in the acceptor site of the
203 glucosyl-enzyme intermediate were assessed using docking protocols implemented in Autodock
204 Vina. Docking poses were filtered for reactivity, by considering those with an oxygen of (+)-
205 catechin within 3 Å of the C1 atom of the glucosyl moiety as productive. Docking results were
206 consistent with the observed experimental regioselectivity. For AmSP-Q353F, productive poses for
207 glucosylation of (+)-catechin in OH3' position is overwhelmingly favoured energetically when

208 compared to the other hydroxyl groups (OH-4', OH-5 and in position 7) of the flavonoid (Figure
209 S6). On the other hand, for *AmSP-P140D*, the most favoured productive poses for glucosylation of
210 (+)-catechin is on OH-4' position (Figures S6). Comparison of the binding energies of the
211 productive poses of (+)-catechin towards OH-3' and OH-4' glucosylation provided in Figure 3A
212 clearly explains the observed regioselectivity. For each enzyme, structural analysis of the best
213 productive poses showed a distance of 3.0 Å between the C1 atom of the glucosyl moiety and the
214 OH-3' or OH-4' of (+)-catechin, and a distance with D203 residue of 4.0 Å (Figure 3B and 3C). For
215 *AmSP-P140D*, (+)-catechin is stabilized in the active +1 site by a network of 4 hydrogen bonds and
216 numerous hydrophobic contacts (Figure 4).

217 4. Discussion

218 In glycochemistry, the fine control of the regioselectivity is the Holy Grail in enzymatic reactions
219 catalysed by glycosyl hydrolase (GHs) such as sucrose phosphorylase. A disadvantage of GHs is
220 their moderate regioselectivity, meaning that a mixture of products is often formed when the
221 acceptor contains more than one hydroxyl group. Previously, for (+)-catechin which consists of five
222 phenolic hydroxyl groups, we generated with *BaSP-Q345F* and *BaSP-P134D/Q345F* a mixture of
223 glucosylated regioisomers: CAT-3', CAT-5 and CAT-3',5 with a ratio of 51:25:24 for *BaSP-Q345F* and
224 82:9:9 for *BaSP-P134D/Q345F*. Another drawback is the relatively low product yields. With the
225 same variants, we obtained a synthetic yield of 34%/15%/9% and 40%/5%/4%, respectively. In the
226 active site of the SPs, the -1 site is rigid to allow a high selectivity on glucose while the +1 site is
227 more flexible and can accept several types of acceptors or leaving groups. The +1 site of *AmSP-WT*,
228 is mainly shaped by two highly labile loops, loop A (³⁴⁴AAASNLDLYQ³⁵³) and loop B (¹³⁸FRPRP¹⁴²),
229 which undergo crucial conformational changes throughout the catalytic cycle suited for binding
230 either fructose or phosphate. Crystal structure of *AmSP-WT* shows a wide access channel capable
231 of accommodating naturally large polyphenolic acceptors. By site-directed mutagenesis, we

232 substituted the residue Q353 in loop B into F353 and/or the residue P140 into D140 in Loop A. We
233 obtained three variants: *AmSP-P140D*, *AmSP-Q353F* and *AmSP-P140D/Q353F*. The double variant
234 showed no improvement for the synthesis of CAT-3' with the obtainment of a mixture of products
235 at 25°C (data not shown). By engineering the residue 353 of the active site, we enhanced the
236 enzyme regioselectivity from a mixture of products to OH-3' position almost exclusively. Docking
237 studies confirmed that the most favoured pose for (+)-catechin in the catalytic +1 site of *AmSP-*
238 *Q353F* lead to the formation of CAT-3'. As seen with *BaSP-Q345F*, we hypothesized that the
239 introduction of F353 as a potential partner for π - π stacking leads to rearrangements in loop A with
240 a shift of Y352 which can stabilise (+)-catechin in the active site by hydrophobic interactions. *BaSP-*
241 *Q345F*, *BaSP-P134D/Q345F* and *AmSP-Q353F* preferentially glucosylate the OH-3' position of
242 flavonoids while ignoring the OH-4' position. This was confirmed by docking simulations which
243 highlighted that the most favoured pose for (+)-catechin in the catalytic site of *AmSP-Q353F*
244 (Figure 3 and Table 2) would lead to the regioselective formation of CAT-3'. Surprisingly, while
245 *BaSP-P140D* was not active, *AmSP-P140D* leads to the regioselective formation of CAT-4' (Figure 3
246 and Table 2). Thus, switching Q353F mutation to P140D had shift completely the regioselectivity of
247 *AmSP-WT* from CAT-3' to CAT-4'. An explanation enlightened by molecular modelling is the steric
248 hindrance caused by F138/Y207/F217 residues between *AmSP-P140D* and the polyphenol rings
249 (Figures 3B and S7). Indeed, we observed that the conformation of the acceptor site drastically
250 changed and seems to allow only an almost linear orientation of all three (+)-catechin rings.
251 Contrarily to *AmSP-Q353F*, those very strong constraints lead to the regioselective glucosylation of
252 the OH-4' position of the flavonoid with a high proportion (89%).

253 5. Conclusion

254 In this study, we provided the first report of the use of variants of sucrose phosphorylase from
255 *Alteromonas mediterranea* for the regioselective transglucosylation of (+)-catechin and the

256 synthesis of a novel compound fully characterized, (+)-catechin-4'-O- α -D-glucopyranoside (CAT-4').
257 *AmSP-Q353F* and *AmSP-P140D* are able to synthesize regioselectively compound CAT-3' and CAT-
258 4', with a proportion of 92% and 89%, respectively. With *AmSP-P140D*, we succeed to switch the
259 regioselectivity from OH-3' to OH-4'-glucosylated (+)-catechin. Mutation P140D changes drastically
260 the conformation of the acceptor site and seems to allow an almost linear alignment of the
261 glucose moiety and of all three (+)-catechin rings allowing selectively the glucosylation of the
262 position OH-4' of this flavonoid. Overall, the results described herein suggest that *AmSP-Q353F*
263 and *AmSP-P140D* are suitable for the enzymatic regioselective synthesis of polyphenolic glucosides
264 at high yields and could facilitate the synthesis of *de novo* products in OH-4' position using other
265 phenolic phytochemicals such as quercetin or kaempferol.

266 **Supporting information**

267 For details about melting curves, kinetic parameters analysis, HPLC/MS, NMR spectra, etc., please
268 see provided Electronic Supplementary Information.

269 **Author Contributions**

270 MG and MD wrote the original draft. MG, MD, CM, BO and JH developed the methodology. MG,
271 MD, CS and EL performed the experimental investigation and the following analysis. DT and FF
272 obtained the crystallographic structure of *AmSP-WT*. JH and BO performed the molecular and
273 docking simulations and the following analysis. CM obtained the funding, designed and directed
274 the project. All authors discussed the results and contributed to the final manuscript.

275 **Conflicts of interest**

276 There are no conflicts to declare.

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- 355

356 **Figures caption and legend**

357

358 **Figure 1: Crystallographic structures of *Ba*SP-WT and *Am*SP-WT focused on residues involved in**

359 **(A) sucrose and (B) fructose binding. (A)** In magenta: Loop A (in sticks for *Ba*SP-WT: Y344/D342, in

360 sticks for *Am*SP-WT: Y352/D350); orange: Loop B (in sticks for *Ba*SP-WT: R135, in sticks for *Am*SP-

361 WT: R141); and blue: residues of the catalytic triad (in sticks for *Ba*SP-WT: D192/E232/D290, in

362 sticks for *Am*SP-WT: D203/E243/D301). **(B)** In magenta: Loop A (in sticks for *Ba*SP-WT:

363 D342/L343/Y344/Q345, in sticks for *Am*SP-WT: D350/L351/Y352/Q353); orange: Loop B (in sticks

364 for *Ba*SP-WT: Y132/R133/P134/R135, in sticks for *Am*SP-WT: F138/R139/P140/R141); Cyan:

365 residues involved in sucrose phosphorylase activity (in sticks for *Ba*SP-WT: Y196/V233/H234, in

366 sticks for *Am*SP-WT: Y207/I244/H245).

367

368 **Figure 2: Products profile of *Am*SP-Q353F and *Am*SP-P140D using (+)-catechin as acceptor.**

369 Proportion of each regioisomers was calculated from the area under the curves obtained by

370 analytical HPLC (isocratic mode at 80% H₂O (v/v), 0.1% formic acid (v/v) and 20% MeOH (v/v), 0.1%

371 formic acid (v/v)).

372

373 **Figure 3: Structural rearrangement of *Am*SP active site and best productive poses of (+)-catechin**

374 **towards OH-3' and OH-4' for *Am*SP-P140D (B) and *Am*SP-Q353F (C) glucosyl-enzyme. (A)**

375 Comparison of binding energy of productive poses of (+)-catechin towards OH-3' and OH-4' for

376 *Am*SP-P140D and *Am*SP-Q353F glucosyl-enzyme ($\Delta G = -9.3$ kcal/mol for *Am*SP-Q353F and -9.2

377 kcal/mol for *Am*SP-P140D). In magenta: Loop A with in sticks Y352/D350/Q(F)353 residues;

378 orange: Loop B with in sticks R141/F138 and D140 for *Am*SP-P140D; blue: residues of the catalytic

379 triad with in sticks D203/D301/DGC243; and pink : (+)-catechin.

380 **Figure 4. Analysis of the interaction between (+)-catechin and AmSP-P140D.** Shown are hydrogen
381 bonds and hydrophobic contacts between the (+)-catechin substrate (denoted Kxn1) and the
382 interacting residues including the glucosylated-aspartyl (Dgc203). The diagram was obtained using
383 LigPlot Plus v.2.2 [24].

384

385

386 **Table 1: Apparent kinetic parameters for sucrose hydrolysis by AmSP and its variants.** Reactions
 387 were conducted in MOPS 50 mM pH 8.0 at 25°C with or without 20% of DMSO. Values are based
 388 on Michaelis-Menten fittings obtained with Microsoft Excel. Hanes-Woolf plots are included in the
 389 electronic supplementary information (Figures S3 and S4).

	0% DMSO			20% DMSO		
	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M
AmSP-WT	0.8 ± 0.2	120 ± 34	156.6 ± 26.2	0.91 ± 0.1	109 ± 12	124.6 ± 7.6
AmSP-P140D	1.8 ± 0.5	13 ± 2.0	7.34 ± 1.4	1.02 ± 0.4	19 ± 4.0	20.12 ± 7.1
AmSP-Q353F	4.6 ± 1.2	2 ± 0.0	0.46 ± 0.1	4.23 ± 0.8	3 ± 0.0	0.79 ± 0.1

390

391

392 **Table 2: Synthetic yields (A) and relative proportion (B) of (+)-catechin glycosylated products**
 393 **obtained with P140D and Q353F variants of AmSP.** Synthetic yields are expressed as a percentage
 394 of (+)-catechin that was converted into the corresponding glycosylated products (CAT-4': (+)-
 395 catechin-4'-O- α -D-glucopyranoside; CAT-3': (+)-catechin-3'-O- α -D-glucopyranoside; CAT-5: (+)-
 396 catechin-5-O- α -D-glucopyranoside; CAT3',5: (+)-catechin-3',5-O- α -D-diglucopyranoside). The
 397 relative proportion of each product was calculated from the area under the curves obtained by
 398 analytical HPLC at 24 h with the same conditions than for Figure S5.

Product	Synthetic yields				Proportion of each product			
	CAT-4'	CAT-3'	CAT-5	CAT-3',5	CAT-4'	CAT-3'	CAT-5	CAT-3',5
P140D	26.36%	3.21%	Traces	Traces	89.08%	10.92%	Traces	Traces
Q353F	3.49%	82.64%	2.22%	1.85%	3.93%	91.58%	2.28%	2.21%

399

400

401

Electronic Supplementary Information

Sucrose phosphorylase from *Alteromonas mediterranea*: structural insight into the regioselective α -glucosylation of (+)-catechin

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Table of Contents

16	Sucrose phosphorylase from <i>Alteromonas mediterranea</i> : structural insight into the regioselective	
17	α -glucosylation of (+)-catechin.....	1
18	Characteristics of AmSP and its variants.....	3
19	Sequence of AmSP-WT including His-tag on C-term.....	3
20	Structural homology between BaSP and AmSP.....	4
21	Determination of the apparent kinetic parameters.....	8
22	(+)-catechin transglycosylation studies.....	10
23	Synthesis yields.....	10
24	^1H and ^{13}C NMR Spectral Data of CAT-4' in DMSO.....	12
25		

26 **Characteristics of AmSP and its variants**

27 ***Sequence of AmSP-WT including His-tag on C-term***

28 1 MGSIRNGVQL ITYADRLGDG NIESLTNLLD GPLKGLFKGV HILPFYYPYD GEDAGFDPID
29 61 HTTPVDERLGD WNNIKKLGES VDIMADLIVN HMSGQSEAFD DVLKKGRESE YWPLFLTKED
30 121 VFSGNDQAEI DEQIAKVFRP RPTPFSDYE VGIETDSTET VPFWTTFTSN QIDIDVESEL
31 181 GKEYLSSILQ SFTESNVDLI RLDAAGYAIK RAGSNCFMLE ETFEFIEALS KRARTMGMQC
32 241 LVEIHSYQT QIDIAARCDS VYDFALPPLV LHTLFTK DAS ALAHWLSISP RNCFTVLDTH
33 301 DGIGIVDVGA SGDKPGLISA DAINALVEQI HVNSNGESKK ATGAAANNVD LYQVNCTYYD
34 361 ALGKDDFAYL VARAIQFFSP GIPQVYYGGL LAAHNDMELL ANTNVGRDIN RPYLTTAMVE
35 421 DAIQKPVVKG LMQILITRNE NKAFFGAFDV TYTDNTLVLS WSNMGDAASL TVDFAAMDAT
36 481 INTVSNGEES TSLGALLAH HHHHH

45 **Table S1: Conserved residues and potential substrate interaction of AmSP.** Potential substrate
 46 interaction and function of conserved residues were determined using structural homology
 47 between crystal structures of apoenzyme AmSP-WT (PDB: 7ZNP), apoenzyme BaSP-WT (1R7A) and
 48 open conformation BaSP-WT (2GDV.B). Residue numbering of AmSP derived from crystal structure
 49 7ZNP. Residue numbering of BaSP came from crystal structures 1R7A and 2GDV.B. Numbered OH-
 50 groups and C-atoms address to sucrose (apostrophe for fructosyl moiety), unless stated otherwise.
 51 ^a: residues from the -1 subsite, ^b: residues from the Loop A/+1 subsite, ^c: residues from the Loop
 52 B/+1 subsite. Approx.: Approximately the same position.

AmSP	BaSP	Conserved Conformation 1R7A/2GDV.B	Potential substrate interaction/remarks	Potential function
Asp203 ^a	Asp192	Yes	Hydrogen bond with OH6	catalytic nucleophile
Glu243 ^a	Glu232	Yes	Hydrogen bond with OH1 and OH1'	general acid/base catalyst
Asp301 ^a	Asp290	Yes	Hydrogen bond with OH2	transition state stabiliser
Phe56 ^a	Phe53	Yes	hydrophobic/ π interaction with C3-C4-C5; Cation- π interaction with oxocarbenium ion-like transition state	hydrophobic platform
Phe167 ^a	Phe156	Yes	hydrophobic/ π interaction with C6 and C1'	hydrophobic platform
His91 ^a	His88	Approx.	Hydrogen bond with OH6	binding of glycosyl moiety
Arg201 ^a	Arg190	Yes	Hydrogen bond with OH2	binding of glycosyl moiety
His300 ^a	His289	Yes	Hydrogen bond with OH2 and OH3	binding of glycosyl moiety
Asp53 ^a	Asp50	Yes	Hydrogen bond with OH4	binding of glycosyl moiety
Arg407 ^a	Arg399	Yes	Hydrogen bond with OH3 and OH4	binding of glycosyl moiety
Gln171	Gln160	Approx.	Hydrogen bond with OH6	binding of glycosyl moiety
Ala204	Ala193	Yes	hydrophobic interaction with C6 and C1'	binding of glycosyl/fructosyl moiety
Leu351 ^b	Leu341	No	hydrophobic interaction with C6 and C6'	binding of glycosyl/fructosyl moiety
Tyr207	Tyr196	Approx.	hydrophobic/ π interaction with C1'	involved in fructose binding
Asp350 ^b	Asp342	No/Approx.	Hydrogen bond with OH4'	involved in fructose binding
Gln353 ^b	Gln345	Yes	Hydrogen bond with O3' and O6'	involved in fructose binding
Phe138 ^c	Tyr132	Approx.	at the entrance of active site; hydrophobic surroundings	Specificity for fructose
Pro140 ^c	Pro134	Approx.	in vicinity of C4-OH of fructose	involved in the binding of the fructose- bound phosphate group
Tyr207	Tyr196	Approx.	CE2-atom close to C1 of fructose	Specificity for fructose/phosphate
His245	His234	Yes	in vicinity of C3 and C3-OH of fructose	Specificity for fructose/phosphate
Arg141 ^c	Arg135	No/Approx.	Hydrogen bond with phosphate O; Specificity for fructose/phosphate	involved in phosphate binding
Leu351 ^b	Leu343	No/Yes	in between important residues Asp342 and Tyr344	Specificity for phosphate
Tyr352 ^b	Tyr344	No/Yes	Hydrogen bond with phosphate O	involved in phosphate binding
Ile244	Val233	Yes	next to the acid/base catalyst; side chain turned away from active site	unknown

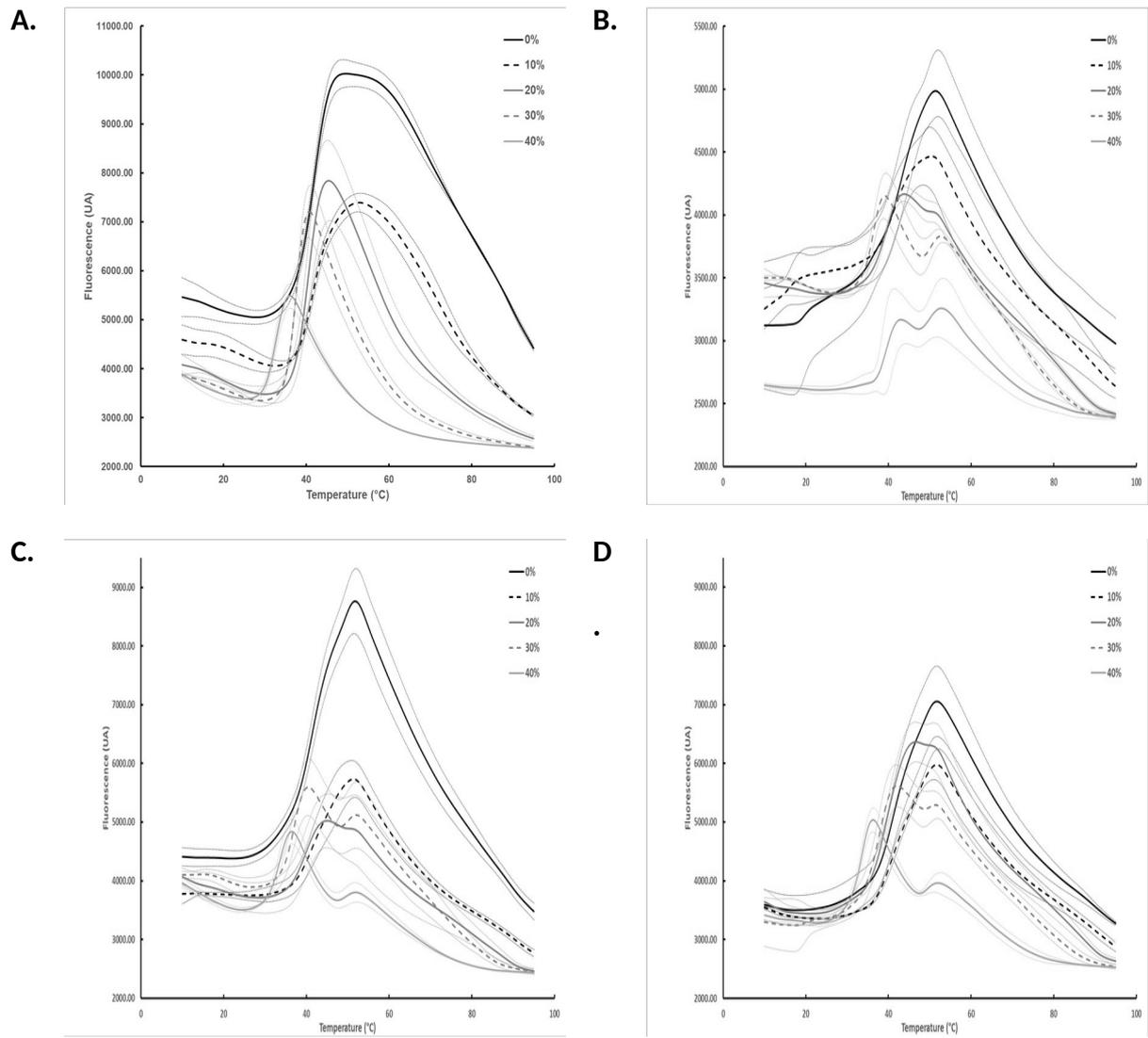
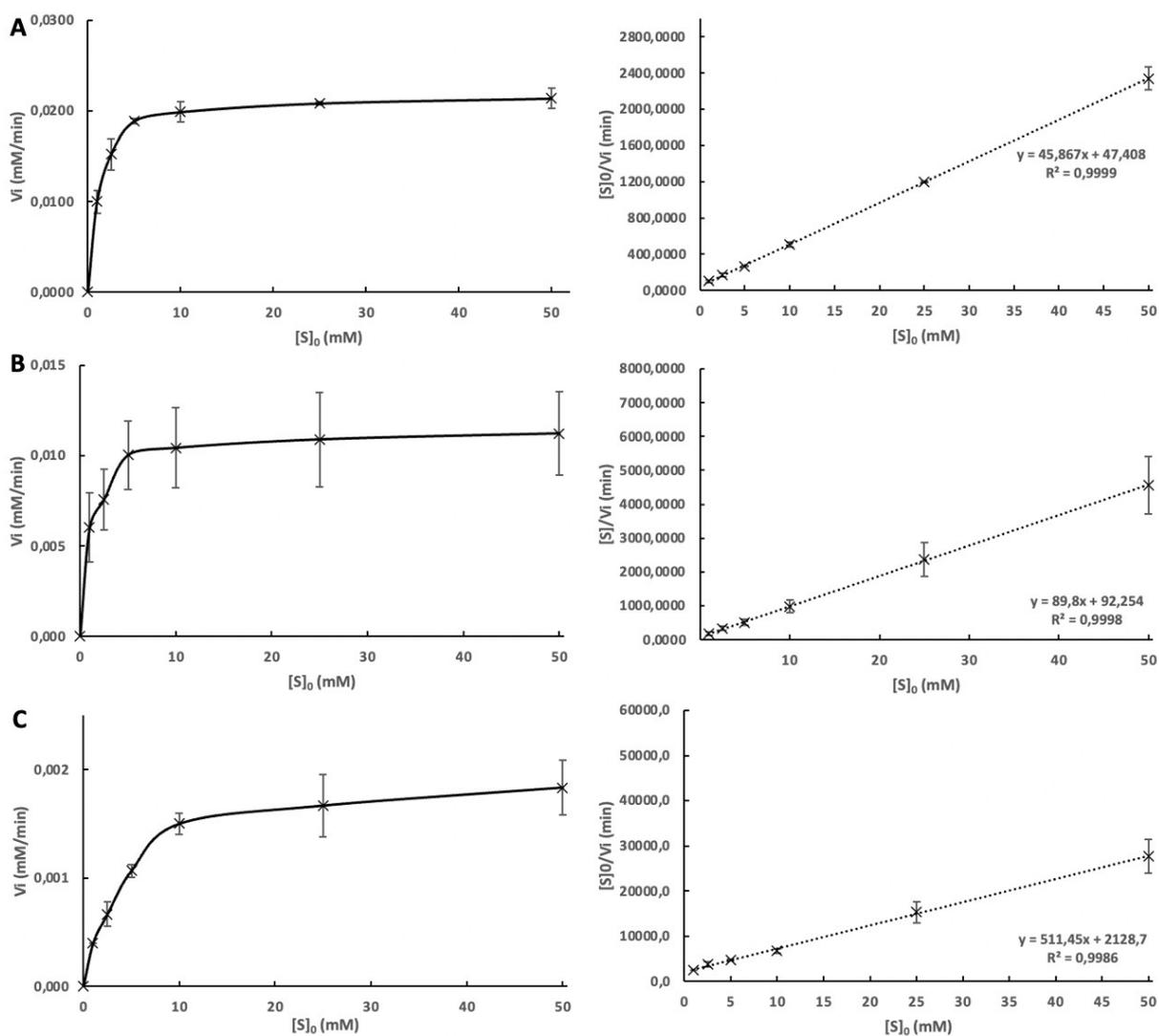


Figure S2: Melting curves of AmSP and its variants in MOPS-NaOH 50 mM pH 8.0 with DMSO from 0% to 40%. (A) AmSP-WT, (B) AmSP-P140D, (C) AmSP-Q353F, (D) AmSP-P140D/Q353F

54 **Table S2: Melting temperature of AmSP and its variants.** Values, given in °C, were obtained by
 55 calculating the first derivative $-(dRFU)/dT$ of the melting curves.

	WT	P140D	Q353F	P140D/Q353F
H₂O	42.0 ± 0.0	42.8 ± 0.3	42.0 ± 0.9	42.2 ± 0.3
NPI-5	42.7 ± 0.3	42.5 ± 0.0	42.2 ± 0.3	41.5 ± 0.0
NPI-250	38.7 ± 0.3	38.2 ± 0.3	38.3 ± 0.3	37.3 ± 0.3
MOPS pH 7.0	44.8 ± 0.6	45.2 ± 0.3	44.0 ± 0.0	43.7 ± 0.3
pH 7.0 10%D	42.5 ± 0.0	42.2 ± 0.3	41.8 ± 0.3	42.3 ± 0.3
pH 7.0 20%D	39.5 ± 0.0	39.0 ± 0.0	39.2 ± 0.3	39.5 ± 0.0
pH 7.0 30%D	36.3 ± 1.0	36.3 ± 0.3	36.5 ± 0.5	36.7 ± 0.3
pH 7.0 40%D	31.3 ± 0.3	32.5 ± 0.0	32.7 ± 0.3	33.2 ± 0.3
MOPS pH 8.0	42.0 ± 0.0	43.0 ± 0.0	41.5 ± 0.0	41.2 ± 0.3
pH 8.0 10%D	41.3 ± 0.3	41.3 ± 0.4	41.5 ± 0.5	40.8 ± 1.0
pH 8.0 20%D	40.5 ± 0.0	39.3 ± 0.6	39.8 ± 0.3	39.5 ± 0.5
pH 8.0 30%D	37.5 ± 0.5	39.0 ± 0.7	37.0 ± 0.0	38.0 ± 0.5
pH 8.0 40%D	32.7 ± 0.3	39.5 ± 1.4	33.7 ± 0.3	33.7 ± 0.3
Citrate	45.5 ± 0.0	43.8 ± 0.4	43.5 ± 0.0	43.3 ± 0.3
HEPES	43.5 ± 0.3	44.0 ± 0.0	42.2 ± 0.6	42.2 ± 0.6
HEPES NaCl DTT	44.8 ± 0.6	45.0 ± 0.0	43.7 ± 0.8	44.3 ± 0.3

56 **Determination of the apparent kinetic parameters**

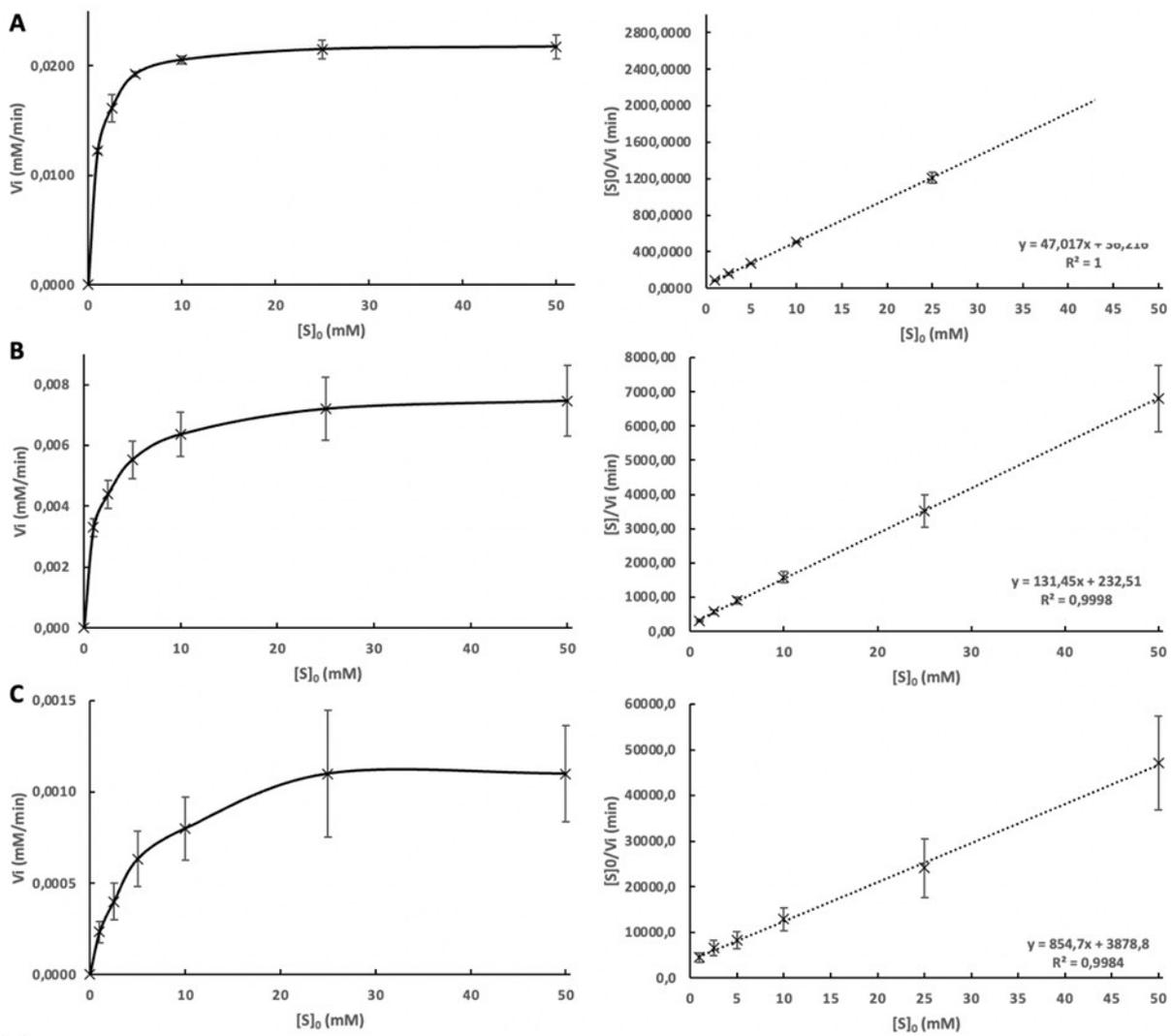


57

58 **Figure S3: Kinetics and Hanes-Woolf plots of sucrose hydrolysis with DMSO 20% (v/v) by (A)**

59 **AmdSP-WT, (B) AmdSP-P140D, (C) AmdSP-Q353F. Data were obtained by glucose titration (10 μ M**

60 **AmdSP-Q353F, 0.5-240 mM saccharose in MOPS-NaOH 50 mM pH 8.0 at 25°C).**



61

62 **Figure S4: Kinetics and Hanes-Woolf plots of sucrose hydrolysis by (A) AmSP-WT, (B) AmSP-**

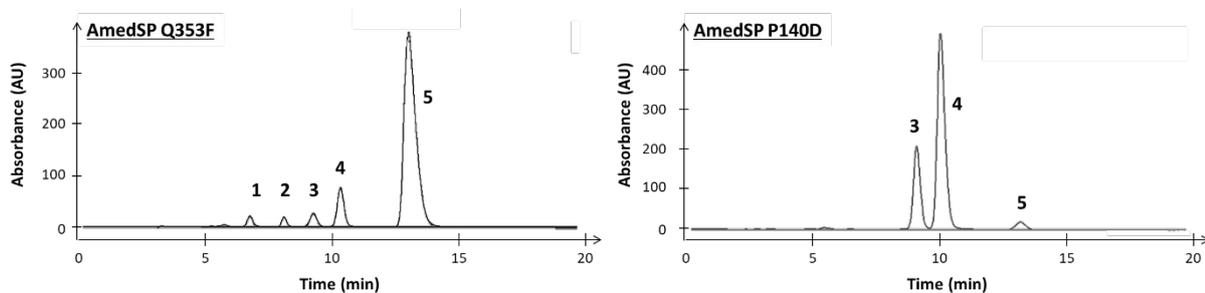
63 **P140D, (C) AmSP-Q353F.** Data were obtained by glucose titration (10 μ M AmSP - Q353F , 0.5 -

64 240 mM saccharose in MOPS-NaOH 50 mM pH 8.0 at 25°C)

65

66 (+)-catechin transglycosylation studies

67 Synthesis yields



68

69 **Figure S5: HPLC chromatogram of a 24 h reaction medium of (+)-catechin glucosylation by**

70 **AmSP-WT variants.** Peak 1: CAT-5, peak 2: CAT-3',5, peak 3: CAT-4', peak 4: (+)-catechin, peak 5:

71 CAT-3'. Unassigned peaks correspond to impurities and degradation of (+)-catechin. (10 μ M

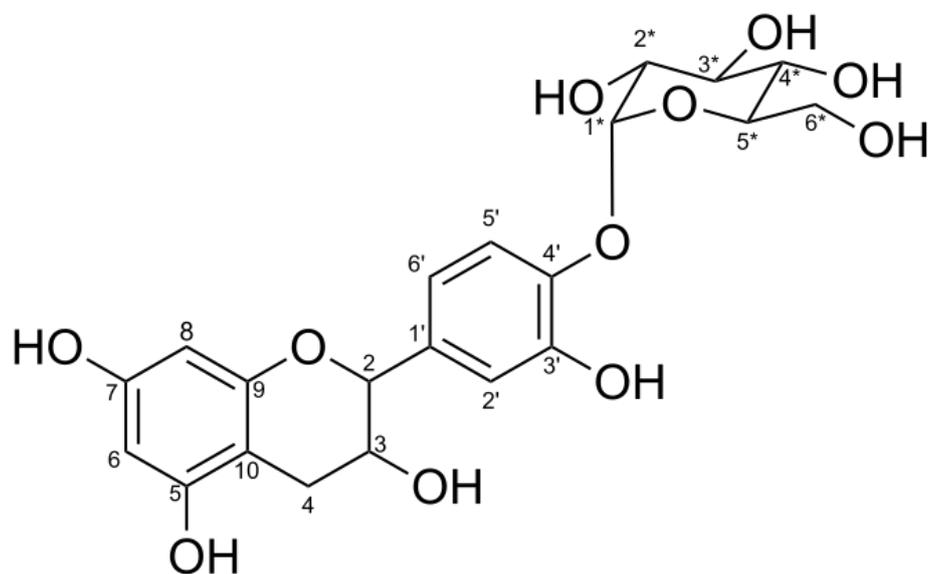
72 enzyme, 10 mM (+)-catechin, 80 mM sucrose, 20% DMSO (v/v) in MOPS 50 mM pH 8.0 at 25°C for

73 24 h). HPLC conditions: isocratic mode at 80% H₂O (v/v), 0.1% formic acid (v/v) and 20% MeOH

74 (v/v), 0.1% formic acid (v/v).

76 ^1H and ^{13}C NMR Spectral Data of CAT-4' in DMSO

77



78

79

80 MS (ESI positive):

81 Ion Formula: $\text{C}_{21}\text{H}_{23}\text{O}_{11}$

82 m/z calculated: 451.1239

83 m/z experimental: 451.1240

84 error [ppm]: -0.2

85

86 ^1H NMR (DMSO- d_6 , δ): 7.14 (d, $^3J_{5-6} = 8.3$ Hz, 1H, H_5), 6.80 (d, $^3J_{2-6} = 2.0$ Hz, 1H, H_2), 6.72 (dd, $^3J_{6-2}$

87 = 2.0 Hz, $^3J_{6-5} = 8.3$ Hz, 1H, H_6), 5.91 (d, $^3J_{6-8} = 2.2$ Hz, 1H, H_6), 5.71 (d, $^3J_{8-6} = 2.2$ Hz, 1H, H_8), 5.17 (d,

88 $^3J_{1*-2*} = 3.6$ Hz, 1H, H_{1*}), 4.55 (d, $^3J_{2-3} = 7.4$ Hz, 1H, H_2), 3.85 (td, $^3J_{3-2} = 7.4$ Hz, $^3J_{3-4a} = 5.3$ Hz, $^3J_{3-4b} =$

89 8.4 Hz, 1H, H_3), 3.71-3.65 (m, $^3J_{3*-2*} = 9.7$ Hz, , 1H, H_{3*}), 3.65-3.62 (m, 1H, H_{6a*}), 3.55-3.49 (m, 1H,

90 H_{5*}), 3.55-3.49 (m, 1H, H_{6b*}), 3.33 (dd, $^3J_{2*-1*} = 3.6$ Hz, $^3J_{2*-3*} = 9.7$ Hz, 1H, H_{2*}), 3.21-3.17 (m, 1H, H_{4*}),

91 2.65 (dd, $^3J_{4a-3} = 5.3$ Hz, $^2J_{4a-4b} = 16.0$ Hz, 1H, H_{4a}), 2.36 (dd, $^3J_{4b-3} = 8.4$ Hz, $^2J_{4b-4a} = 16.3$ Hz, 1H, H_{4b}).

92

93 ¹³C NMR (DMSO-d₆, δ) : 156.5 (C₇), 156.2 (C₅), 155.2 (C₁₀), 147.1 (C_{4'}), 144.7 (C_{3'}), 134.7 (C_{1'}), 118.2
 94 (C_{6'}), 117.3 (C_{5'}), 114.8 (C_{2'}), 100.4 (C_{1*}), 99.0 (C₉), 95.2 (C₆), 93.8 (C₈), 80.7 (C₂), 73.8 (C_{5*}), 73.1 (C_{3*}),
 95 72.0 (C_{2*}), 70.0 (C_{4*}), 66.3 (C₃), 60.7 (C_{6*}), 27.8 (C₄).

96 **Table S4: Comparison of ¹H and ¹³C NMR Spectrum data of CAT-4' and (+)-catechin in DMSO.**

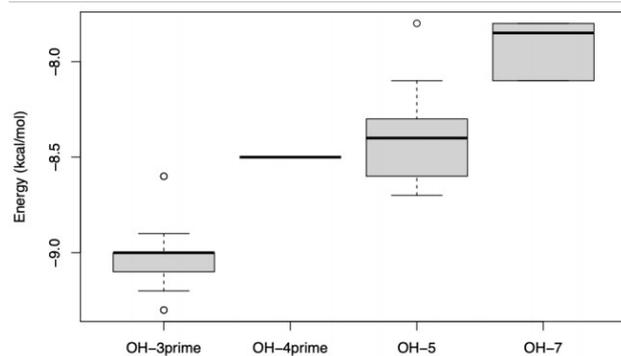
97 Multiplicity abbreviations for ¹H NMR: d = doublet, dd = doublet of doublets, td = pseudo triplet of
 98 doublets, m = multiplet. For characterization of CAT-3', CAT-5 and CAT-3',5 please see [10].

POSITION	CATECHIN δ ¹ H; J (HZ)	CAT-4' δ ¹ H; J (HZ)	CAT-4' δ ¹³ C
1	X	X	X
2	4.48 (d 7.4)	4.55 (d 7.4)	80.67
3	3.82 (td 8.1, 7.4, 5.2)	3.85 (td 8.4, 7.4, 5.33)	66.26
4	2.67 (dd 16.1, 5.2) 2.35 (dd 16.0, 8.1)	2.65 (dd 16.0, 5.3) 2.36 (dd 16.3, 8.4)	27.84
5	X	X	156.21
6	5.89 (d 2.2)	5.91 (d 2.2)	95.22
7	X	X	156.51
8	5.69 (d 2.2)	5.71 (d 2.2)	93.78
9	X	X	98.95
10	X	X	155.17
1'	X	X	134.68
2'	6.73 (d 1.9)	6.80 (d 2.0)	114.79
3'	X	X	144.71
4'	X	X	147.06
5'	6.69 (d 8.1)	7.14 (d 8.3)	117.26
6'	6.60 (dd 8.1, 1.9)	6.72 (dd 8.3, 2.0)	118.16
1*	X	5.17 (d 3.6)	100.40
2*	X	3.33 (dd 9.7, 3.6)	72.02
3*	X	3.71-3.65 (m)	73.06
4*	X	3.21-3.17 (m)	69.96
5*	X	3.59-3.55 (m)	73.75
6*	X	3.65-3.62 (m) 3.55-3.49 (m)	60.73

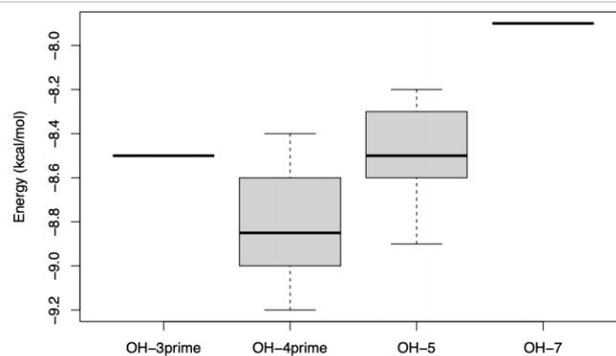
99

100

AmSP-Q353F

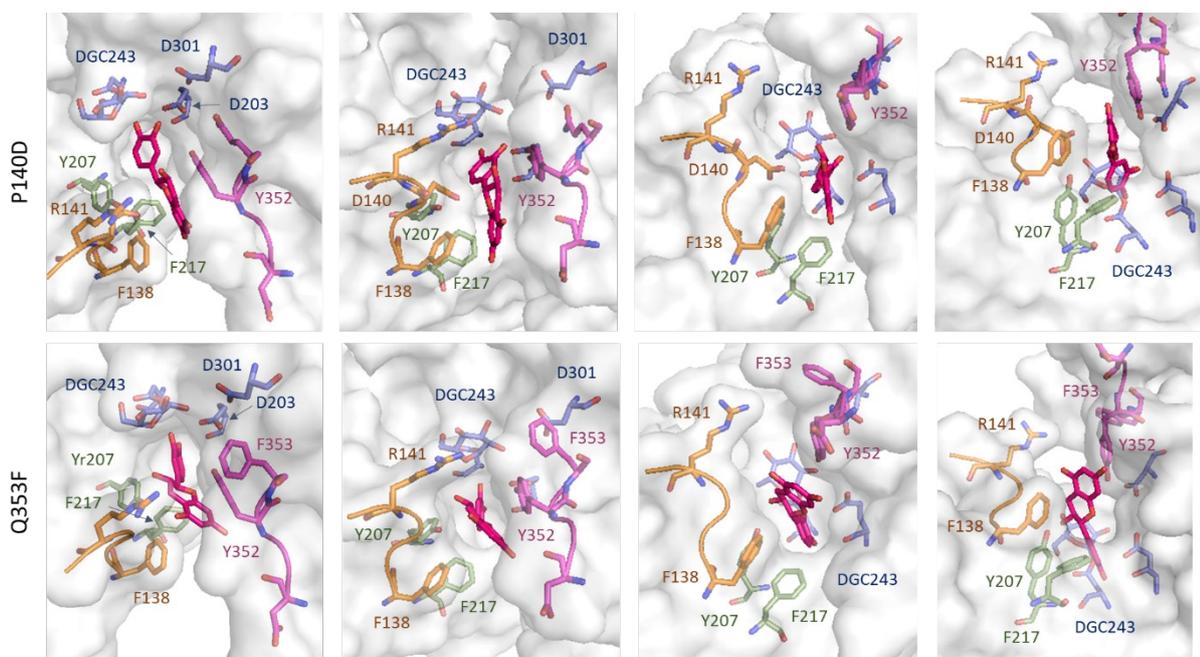


AmSP-P140D



101

102 **Figure S6: Statistical analysis of the binding energies of productive poses for glucosylation of (+)**
103 **catechin in OH-3', OH-4', OH-5 and OH-7 positions with Q353F and P140D.** Molecular docking
104 poses were filtered by considering those with an oxygen of (+)-catechin within 3 Å of the C1 atom
105 of the glucosyl moiety as productive (see sections 2.4, 2.5 and 3.4 in main text for additional
106 details).



107

108 **Figure S7: Different views of the position of the best productive poses of (+)-catechin in the**
 109 **active site of AmSP-P140D and AmSP-Q353F.** In magenta: Loop A with in sticks
 110 Y352/F350/Q(F)353 residues; orange: Loop B with in sticks R141/F138 and D140 for AmSP-P140D;
 111 blue: residues of the catalytic triad with in sticks D203/D301/DGC243; green: aromatic residues
 112 involved in steric clash with in sticks Y207 and F217; and pink: (+)-catechin.

113

114