Substrate and mechanistic investigation of the tannases *Cb*Tan1 and 2 – serine hydrolases active on gallotannins

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Background

Tannases (tannin acyl hydrolases, EC 3.1.1.20) are serine α/β-hydrolases which act on gallotannin ester bonds [1]. **Tannins** are complex secondary metabolites that inhibit the growth of microbes and are enriched in bark and wood. Organisms that express tannases can survive tannin-rich microenvironments.

We recently expressed and characterized the biochemical activity of three tannases from the soil/gut bacterium ***Clostridium butyricum*** (*Cb*Tan1–3) [2], which was the first example of 3 tannases characterized from the same organism. Each enzyme displayed different substrate preferences, which suggested they have differing biological functions.

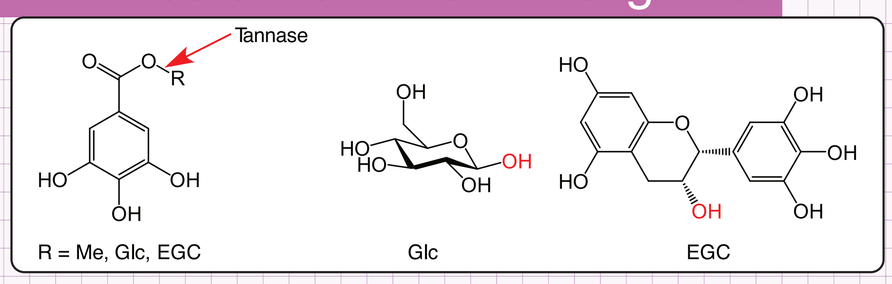
Tannases are phylogenetically categorized into subtypes A and B based on the absence or presence (respectively) of an acidic residue in the canonical Ser-His-Asp catalytic triad. *Cb*Tan1 contains an amidic residue (Gln) while *Cb*Tan2 and 3 contain a catalytic Asp, making these useful enzymes for investigating the mechanistic differences between subtypes of tannases.

*Cb*Tan1 contains a long, possibly uncoiled domain which may be a cap domain. *Cb*Tan2 contains a hairpin-style β-sheet cap which is probably involved in substrate binding. The cap of each enzyme is inserted into a different part of the α/β-hydrolase fold. It is currently unclear how the cap of each tannase impacts its preferred substrates.

Research questions

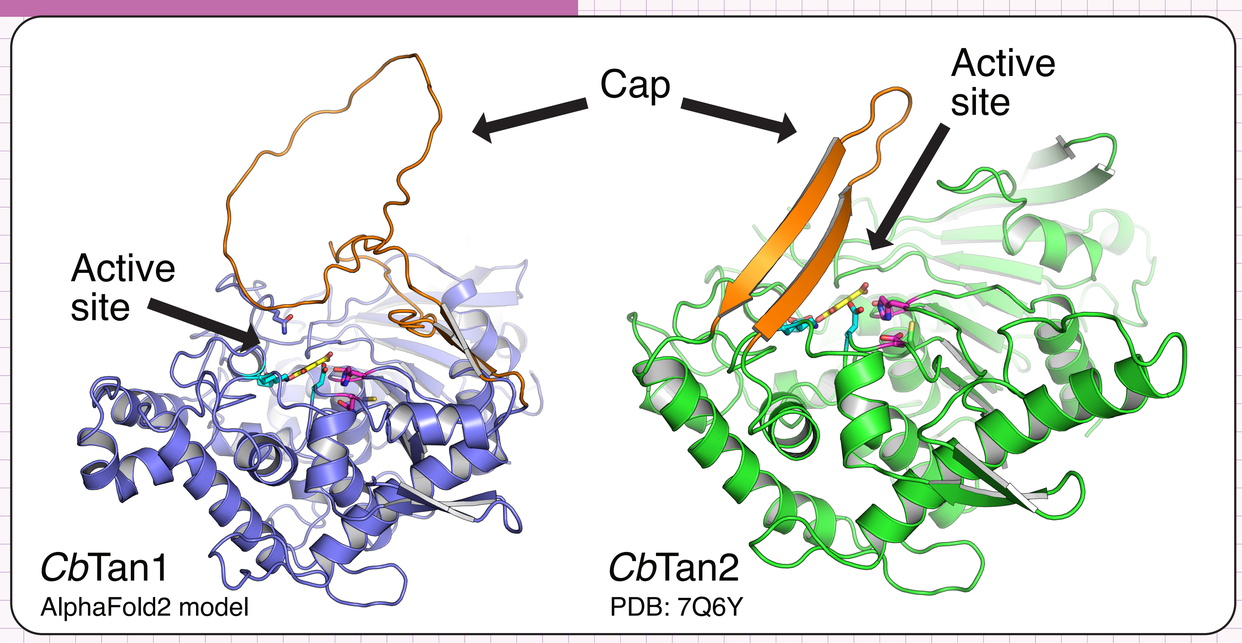
- What role does the cap domain play in tannase **substrate binding?**
- Can the enzyme activity of subtype A tannases (eg, *Cb*Tan1) be improved by **"restoring" the catalytic acidic residue?**

Tannase substrates investigated



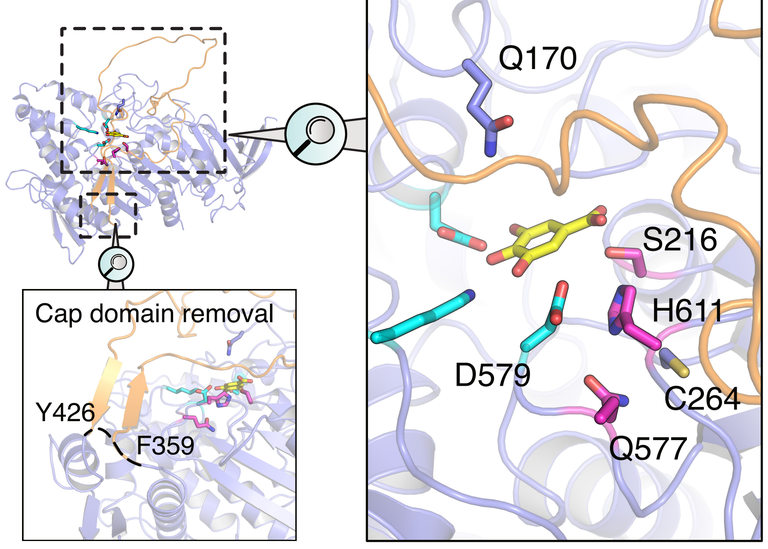
Left: General structure of gallotannins. The red arrow indicates the ester bond broken by tannase enzymes. **Right:** Structure of gluco (Glc) and epigallocatechin (EGC) moieties. The hydroxyl groups shown in red are linked to the gallate moiety.

Tannase overall structure



Mutagenesis sites and activity determination

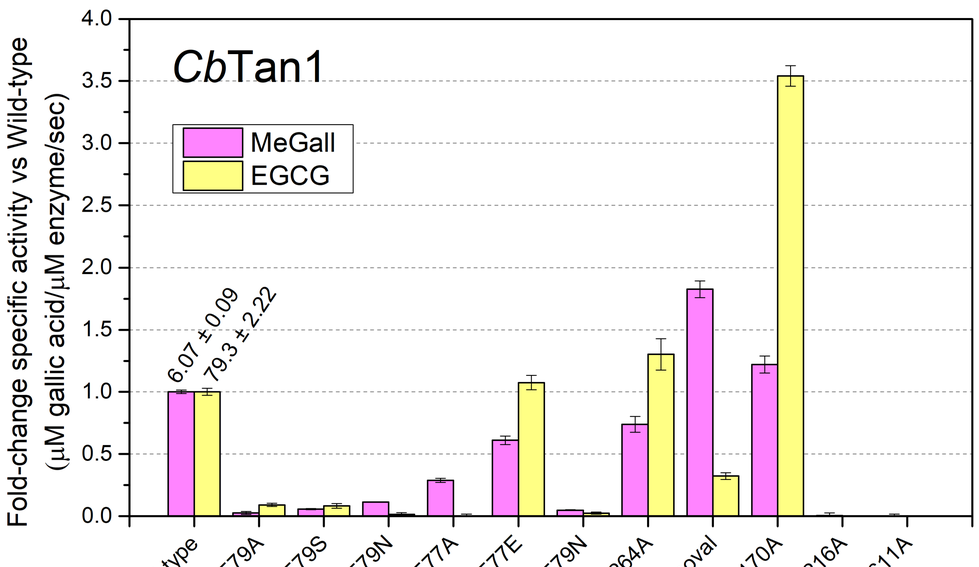
*Cb*Tan1



AlphaFold2 model shown.

Gallate (yellow) is superimposed from PDB: 4J0H.

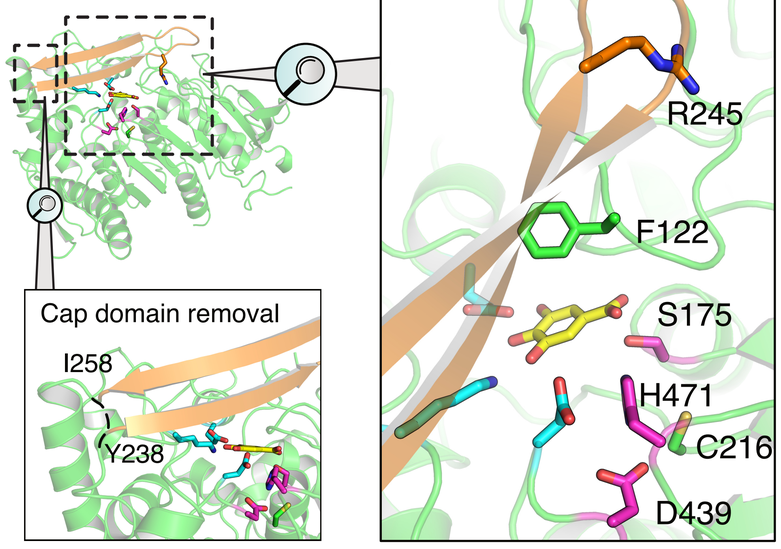
Rationale	Residues
Changing subtype from A to B	Q577 → A,E
Gallate binding / possible His H-bond donor	D579 → A,S,N
Electrostatic interaction near active site	Q170 → A
Cys near catalytic Ser, His	C264 → A
Catalytic residues	S216 → A H611 → A
Cap domain removal	aa 359–426



Enzyme	Specific activity (μM gallic acid/μM enzyme/sec) MeGall	EGCG
***Cb*Tan1**	6.07 ± 0.09	79.3 ± 2.22
***Cb*Tan2**	64.02 ± 3.54	4.93 ± 1.30

- Cap domain appears to control binding of larger substrates
- Modifying subtype (Q577E) does not improve catalysis

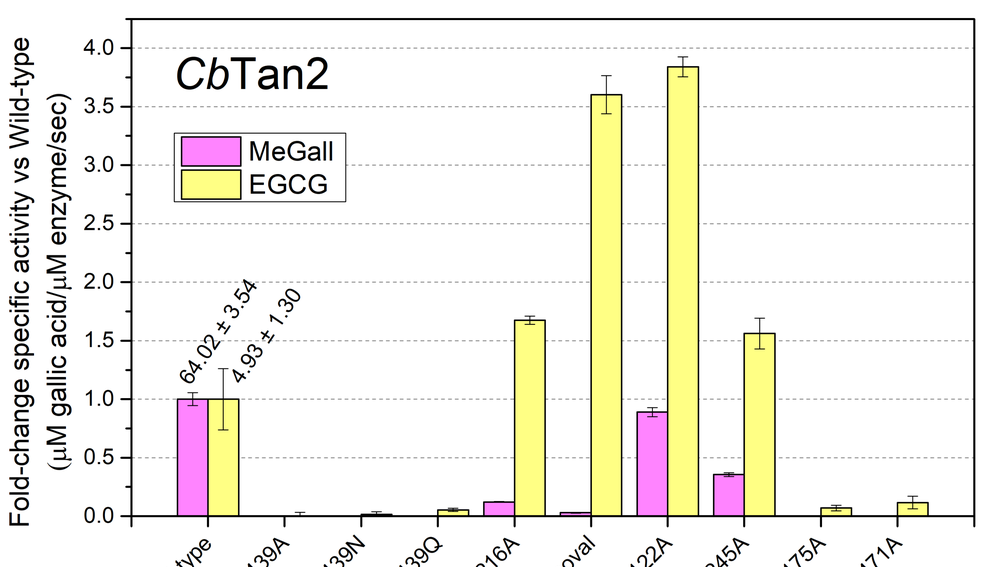
*Cb*Tan2



PDB: 7Q6Y. AlphaFold2 model shown for orange cap, and loop with F122.

Gallate (yellow) is superimposed from PDB: 4J0H.

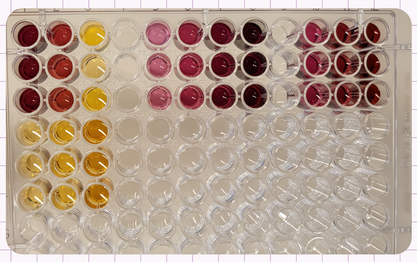
Rationale	Residues
Changing subtype from B to A	D439 → A,N,Q
Pi stacking on loop near active site	F122 → A
Electrostatic interaction under cap	R245 → A
Cys near catalytic Ser, His	C216 → A
Catalytic residues	S175 → A H471 → A
Cap domain removal	aa 238–258



- Cap domain and C216 important for smaller substrates
- Changing subtype abolishes activity

Activity measurement

- Activity determined using 1 mM substrate using the rhodanine assay [3], in triplicates with 5 min reaction time
- Absorbance at 520 nm measured



Key findings

- The role of the catalytic acidic/amide residue is nontrivial in tannases - subtypes require further mechanistic investigation
- *Cb*Tan1 well adapted for EGCG; can be engineered to improve activity
- *Cb*Tan2 well adapted for MeGall; EGCG activity can be improved

Future directions

- Characterization with β-glucogallin substrate
- Crystal structures of substrate-bound tannases

References

1. de las Rivas, B.; Rodríguez, H.; Anguita, J.; Muñoz, R. Bacterial tannases: classification and biochemical properties. *Applied Microbiology and Biotechnology* **2019**, *103* (2), 603-623
2. Ristinmaa, A. S.; Coleman, T.; Cesar, L.; Langborg Weinmann, A.; Mazurkewich, S.; Brändén, G.; Hasani, M.; Larsbrink, J. Structural diversity and substrate preferences of three tannase enzymes encoded by the anaerobic bacterium *Clostridium butyricum*. *Journal of Biological Chemistry* **2022**, *298* (4)
3. Sharma, S.; Bhat, T.; Dawra, R. A spectrophotometric method for assay of tannase using rhodanine. *Analytical Biochemistry* **2000**, *279* (1), 85-89