Understanding fructan breakdown by key members of the human gut microbiota

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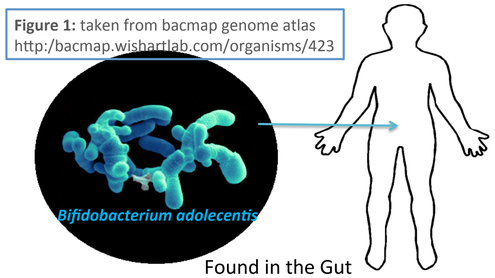


Figure 1: taken from bacmap genome atlas http:/bacmap.wishartlab.com/organisms/423

Introduction

Inulin is a chain of fructose extending from a sucrose molecule all with β-2-1 linkages.

- *Bifidobacteria* are common members of our gut microbiota.
- They are known to be health promoting and utilise some of the complex carbohydrates (glycans) we take in in our diet that we are unable to break down in exchange for many benefits such as production of vitamins and educating our immune system.
- Fructans are plant glycans common in the diet that are used by some health promoting members of the microbiota including Bifidobacteria spp. While many species can use short chain fructans (fructo-oligosaccharides; FOS), *Bifidobacterium adolescentis* can use both FOS and longer chain fructans like inulin (see above).
- Fructans are broken down by glycoside hydrolase enzymes from family 32 (GH32s) and in this project we wanted to investigate the substrate preferences of the two GH32 enzymes encoded by *B. adolescentis* to see if this could provide insight into the organism's ability to use both long and short chain fructans.

Carlett Ramirez-Farias, et al. (2009) 'Effect of inulin on the human gut microbiota: stimulation of Bifidobacterium adolescentis and Faecalibacterium prausnitzii' *British Journal of Nutrition,* 101 pp. 541-550

Aims

Most species of *Bifidobacteria* use short chain FOS from our diets. However, *Bifidobacterium adolescentis* is able to utilise both FOS and inulin. We wanted to look into to this in more detail and see how *B. adolescentis* may do this.

Methods and Materials

I researched *B. adolescentis* as well as the FOS-only users *B.longum, B.animalis* and *B. breve* to see how their family 32 glycoside hydrolases differed between species. While the FOS using Bifido's had a single GH32, *B. adolescentis* had two GH32s which differ quite substantially.

BLAST search results:

BAD_1150 GH32

BAD_1150 has query cover of 93% with BAD_1325 and 27% identity
BAD_1150 has 100% query cover with *B. longum* GH32 and 84% identity
BAD_1150 has 99% query cover with *B. animalis* GH32 and 72% identity
No signal peptide

BAD_1325 GH32

BAD_1325 has 80% query cover with *B. longum* and 28% identity
BAD_1325 has 69% query cover with *B. animalis* and 33% identity
BAD_1325 has 80% query cover with BAD_1150 and 27% identity
No signal peptide

Blast searches show that **BAD_1150 GH32 conserved in other Bifidobacteria**, whereas **BAD_1325 GH32 is divergent to other Bifidobacterial spp enzymes.**

Both *B. adolescentis* GH32s were amplified from the genomic DNA and cloned into *E. coli* pET vectors for high level expression (**Figure 2**).

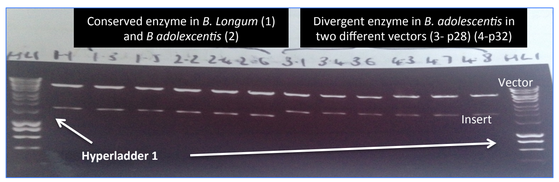


Figure 2: Agarose gel showing both *B. adolescentis* GH32s (lower bands) cloned into pET expression vector (upper band). Lanes at either end are markers.

Purifying the recombinant GH32 enzymes

After expressing the His-tagged GH32 enzymes in *E. coli*, I purified the proteins using metal affinity chromatography (**Figure 3**). The pure protein obtained was then dialysed into 20mM sodium phosphate buffer pH7.0, to assays against different putative fructan substrates (**Figure 4**).

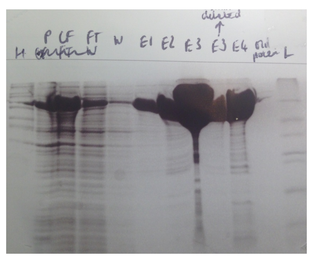


Figure 3: SDS-PAGE protein purification gel of BAD_1325 GH32. The lanes on the left are the cell free extract (CF), flow through (FT) and wash (W). The lanes labelled E1-E4 are the pure protein eluted from the metal affinity column. The conserved enzyme BAD_1150, was purified in the same way. H and L are high and low molecular weight markers.

The Substrates

Various different size fructans found in our diet were used in the different reactions against both BAD_1325 and BAD_1150.

1. Sucrose 

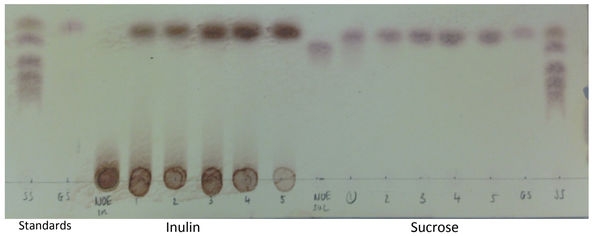
2. Kestose 

3. Kestotetraose 

4. Inulin (long chain - see above)

B. adolescentis GH32s are both fructosidases

Using thin-layer chromatography (TLC) I showed that both GH32s were fructosidases that released fructose from the chain end of a range of different fructans (**Figure 4**).



Time points: 1=5 min, 2= 10 min, 3= 30 min, 4=60 min and 5= Overnight

Figure 4: TLC showing fructose is only product released from sucrose and inulin by BAD_1325 GH32.

Linked Assays

Kinetics were carried out on different fructans using a linked assay (Megazyme). Fructose is the product to be evaluated and is measured by the rise in NADPH causing an increase in absorbance at 340nm. NADPH is stoichiometric to the amount of fructose produced. This is due to a series of reactions involving hexokinase converting fructose to fructose-6-phosphate (F-6-P) followed by phosphoglucose isomerase converting F-6-P to glucose-6-phosphate (G-6-P), then finally glucose-6-phosphate dehydrogenase converting G-6-P to NADPH along with other products.

The reactions enabled us to see the measure Km and kcat for the two different enzymes with the different substrates, enabling us to see if there was any preference of substrate depending on the enzyme- the conserved or the divergent.

Figure 5: Sucrose vs 0.01uM BAD_1150

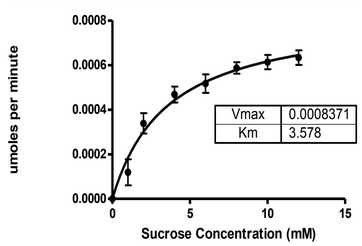


Figure 6: Sucrose vs 0.01uM BAD_1325

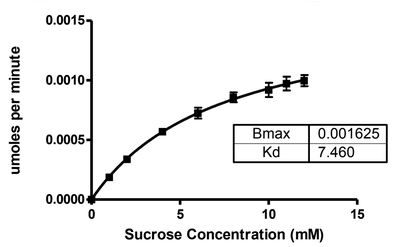


Figure 7: Inulin vs 0.01uM BAD_1150

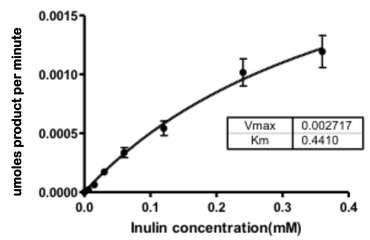


Figure 8: Inulin vs 0.01uM BAD_1325

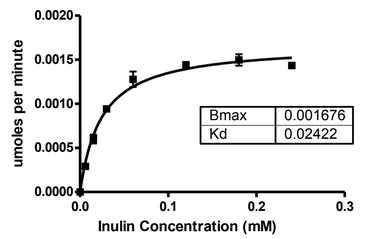


Figure 9: Kestotetraose vs 0.01uM BAD_1150

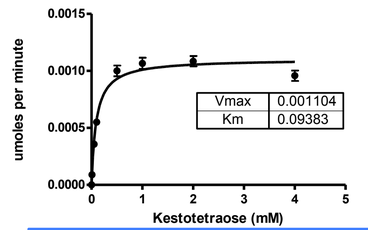
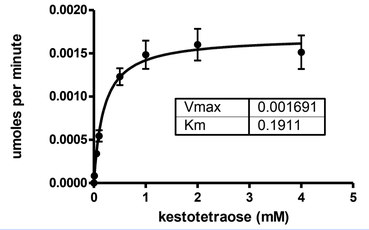


Figure 10: Kestotetraose vs 0.01uM BAD_1325



Results

The kinetics show that conserved GH32 BAD_1150 has a preference for shorter chain fructans (kestotetraose) and the divergent GH32 BAD_1325 has a preference for longer chain fructans (inulin). This is apparent from the kcat/Km values.

Table 1 - BAD_1150 kinetic data

Substrate	Km (mM)	kcat min-1	kcat/km min-1/mM
Sucrose	3.6	167	47
Inulin	0.44	543	1232
Kestotetraose	0.09	221	2353

Table 2 - BAD_1325 kinetic data:

Substrate	Km (mM)	kcat min-1	kcat/km min-1/mM
Sucrose	7.5	325	44
Inulin	0.024	335	13840
Kestotetraose	0.19	338	1770

Crystal Trials: We obtained crystals of BAD_1325 GH32 (**Figure 11**). However, when they were sent to the syncrotron they did not diffract well. This meant we were unable to get a structure for the enzyme.

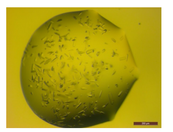


Figure 11: Crystals of BAD_1325

Conclusion

The preference of BAD_1325 GH32 for inulin suggests it may be the key enzyme that enables *B. adolescentis* to use long chain fructans.

Acknowledgements

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BBSRC bioscience for the future

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