Table S1. qRT-PCR primers/probes used in this study

| | |
|---|---|
| Blon0248 R | AGCGACGGACGAAGTGC |
| Blon0248 F | ATTCCGCCGTGACGCTT |
| Blon0248 TM | 6FAM-CCACTTCCGAGGCCGGAACAT--BBQ |
| Blon0426 F | GATGTGGATTCCGCAACC |
| Blon0426 R | AAACGCACGACCTGAGG |
| Blon0426 TM | 6FAM-TGGCAACGTTGTCGTGGAGGC—BBQ |
| Blon2335 F | CCTGTTCAACCAGGATGAGTC |
| Blon2335 R | CCGTCCACGACGAAGTAG |
| Blon2335 TM | 6FAM-TCTGGCTTCCATCTGGCCGATCAT--BBQ |
| Blon2336 F | ATCACGCTCACCCTCCC |
| Blon2336 R | ACATCGTCGAAGCGGAGT |
| Blon2336 TM | 6FAM-AGCCCACGACGATCAACGCCA--BBQ |
| 0346F-q | ACCATCACGGCTACCTGTTC |
| 0346R-q | ACGGGCATCCGTTTATTACA |

# Table S2: Crystallographic statistics

| Data collection | |
|---|---|
| Space group | $P2_12_12_1$ |
| Unit Cell (Å, °) | $a$=82.61, $b$=106.1, $c$=122.4 |
| MW Da (residue) | 53036 (478) [1] |
| Mol (AU) | 2 |
| SeMet (AU) | 20 |
| Wavelength(Å) | 0.9793 (peak) |
| Resolution(Å) | 42.0-1.90 |
| Number of unique reflections | 84888 [2] |
| Redundancy | 4.9 (4.9) [3] |
| Completeness (%) | 99.8 (100.0) [3] |
| $R_{merge}$ (%) | 12.5 (81.4) [3] |
| I/σ(I) | 13.5 (1.5) [3] |
| Phasing | |
| $R_{Cullis}$ (anomalous) (%) | 85 |
| Figure of merit (before/after DM) (%) | 19/89 |
| Refinement | |
| Resolution | 42.0-1.90 |
| Reflections (work/test) | 80222/4023 |
| $R_{crystal}/R_{free}$ (%) | 16.2/20.5 |
| Rms deviation from ideal geometry<br>Bond length (Å)/angle (°) | 0.006/1.003 |
| No.of atoms (Protein/HETATM) | 7121/952 |
| Mean B-value (Å$^2$)<br>(mainchain/sidechain) | 24.3/30.0 |
| Ramachandran plot statistic (%) | |
| Residues in most favored regions, | 90.2 |
| in additional allowed regions, | 9.2 |
| in generously allowed regions, | 0.1 |
| in disallowed region | 0.5 [4] |

[1] Not including cloning artifact; [2] Including Bijvoet pairs; [3] (Last resolution bin, 1.93-1.90 Å); [4] The residue V206 is located in a sharp turn. The residue E30 is located at the end of a helix and forms a hydrogen bond to Y443 and salt bridges to R351 and R444, respectively. The two residues in each monomer are well-defined in electron density maps.

Table S3. α-L-fucosidase characteristics

| Enzyme | Optimum expression conditions | Optimum pH enzyme | Optimum Temperature |
|---|---|---|---|
| Blon_0248 | 28°C 0.5 mM IPTG 6h | 6.5 | 37°C |
| Blon_0426 | 28°C 0.5 mM IPTG 6h | 6 | 37°C |
| Blon_2335 | 37°C 0.5 mM IPTG 6h | 7.5 | 37°C |
| Blon_2336 | 37°C 0.5 mM IPTG 6h | 6.5 | 37°C |
| Blon_0346 | 28°C 0.5 mM IPTG 6h | 6 | 37°C |

Figure S1

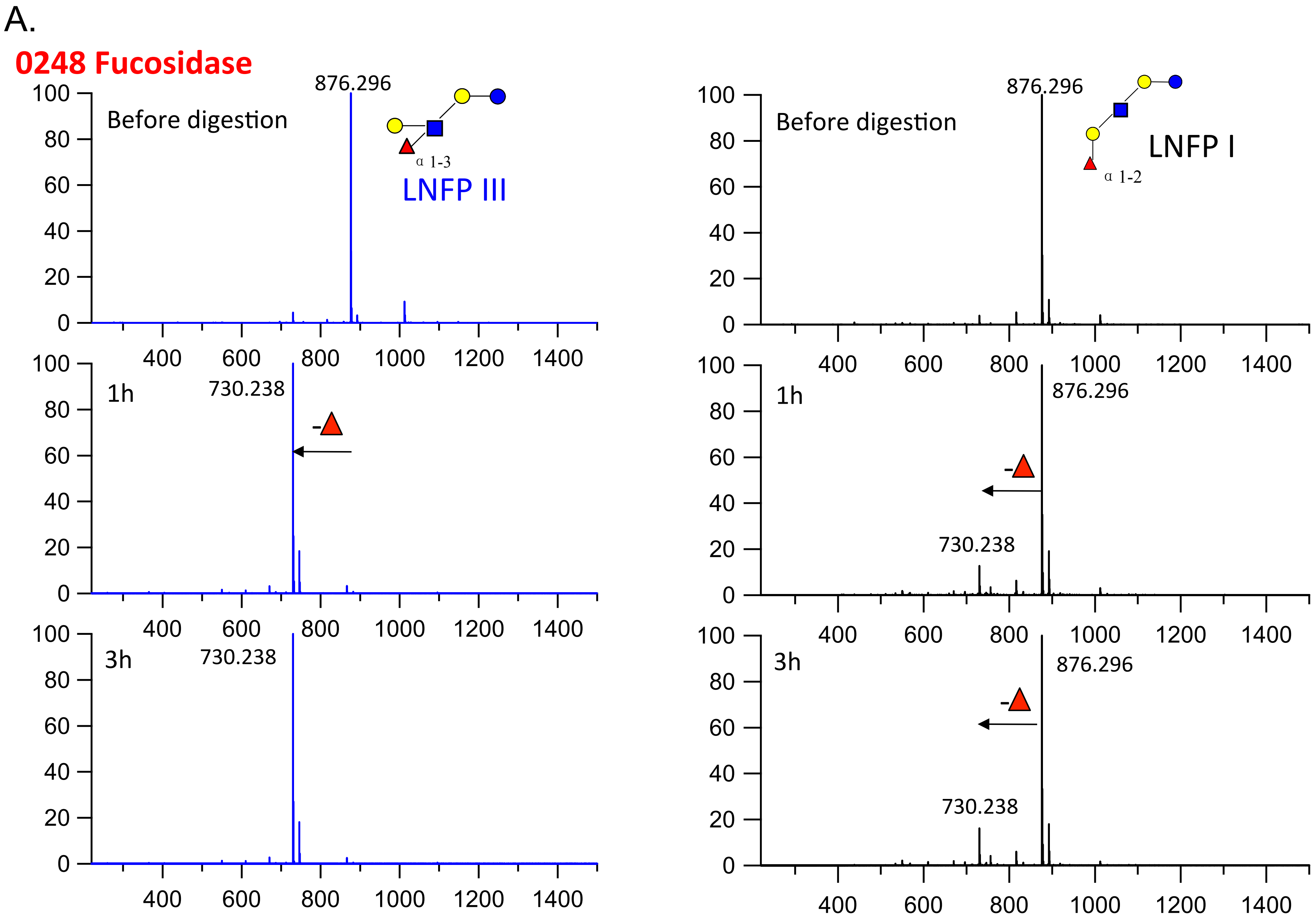

A.
0248 Fucosidase
Before digestion
876.296
α 1-3
LNFP III
1h
730.238
-▲
3h
730.238
Before digestion
876.296
LNFP I
α 1-2
1h
876.296
-▲
730.238
3h
876.296
-▲
730.238
0
20
40
60
80
100
400
600
800
1000
1200
1400

Figure S1

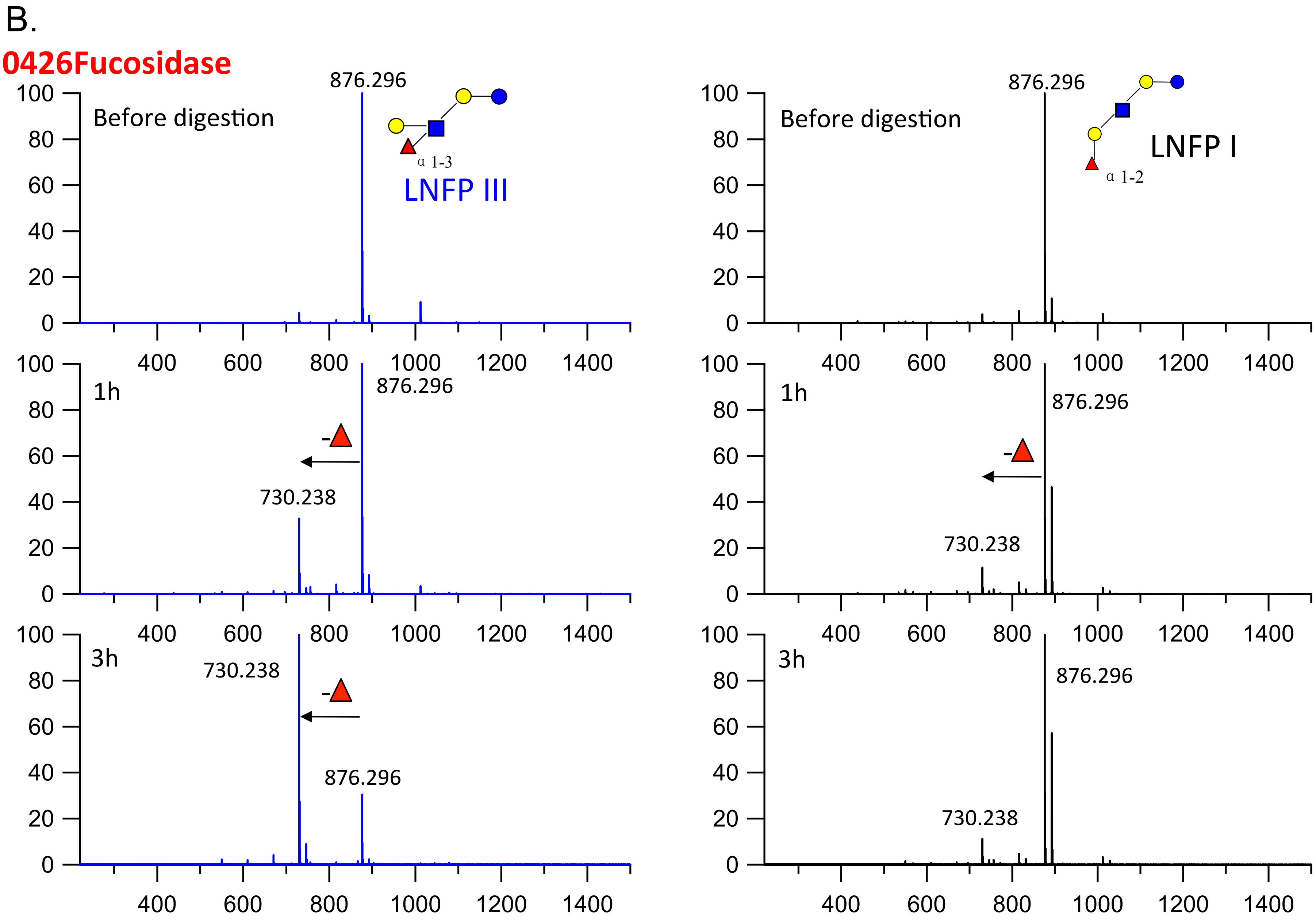
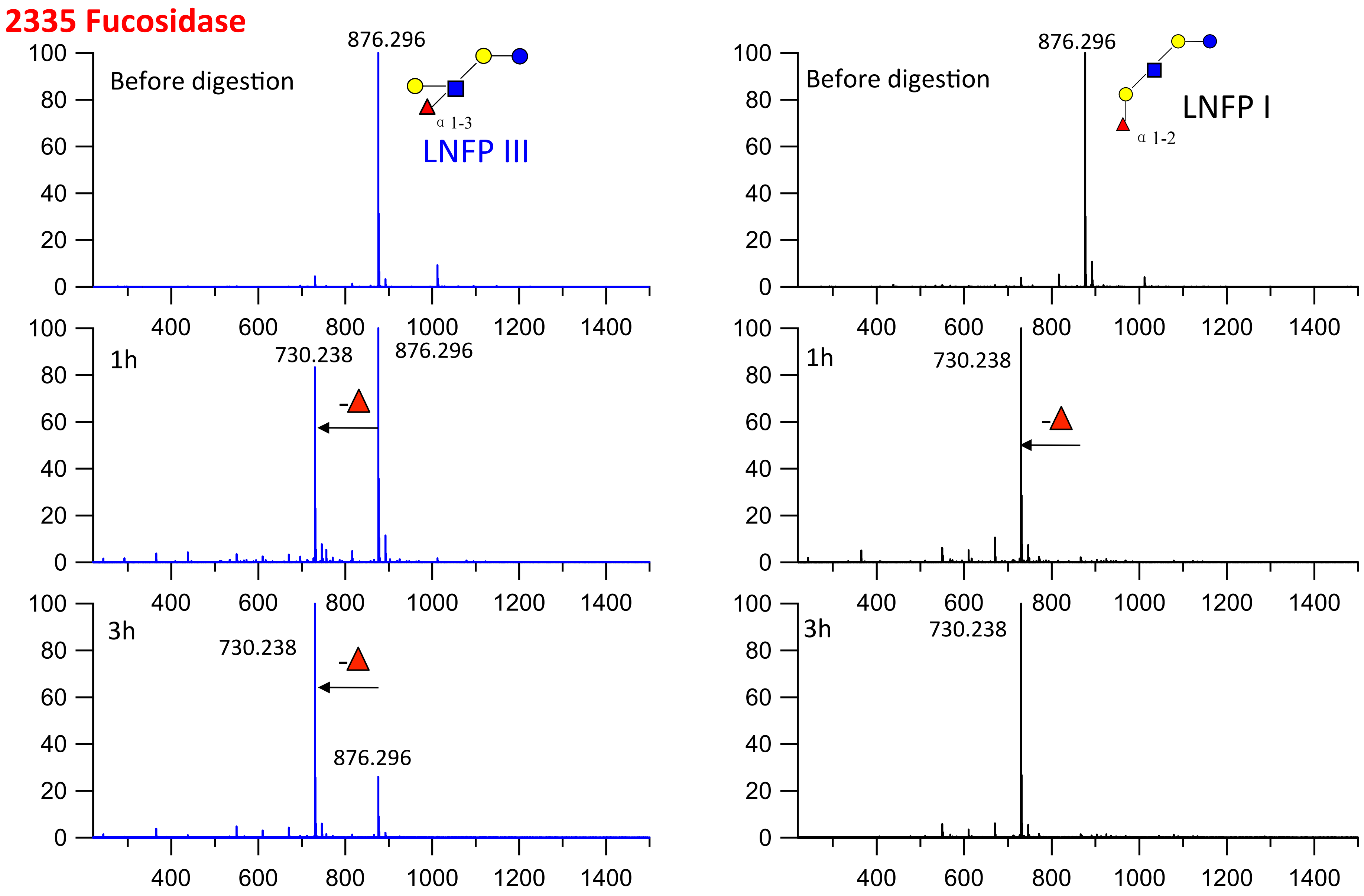
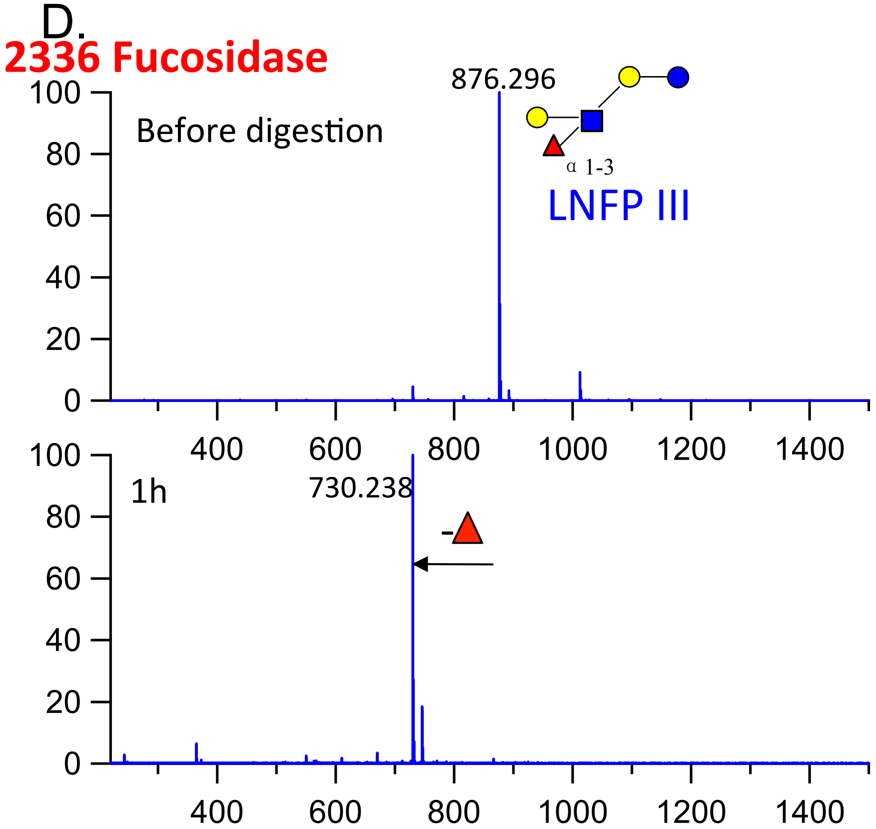

B.
0426Fucosidase
Before digestion
876.296
α 1-3
LNFP III
1h
876.296
-▲
730.238
3h
730.238
-▲
876.296
Before digestion
876.296
LNFP I
α 1-2
1h
876.296
-▲
730.238
3h
876.296
730.238
100
80
60
40
20
0
400
600
800
1000
1200
1400

Figure S1

C.


2335 Fucosidase
Before digestion
876.296
α 1-3
LNFP III
1h
730.238
876.296
-
3h
730.238
876.296
-
Before digestion
876.296
LNFP I
α 1-2
1h
730.238
-
3h
730.238
100
80
60
40
20
0
400
600
800
1000
1200
1400

# Figure S1



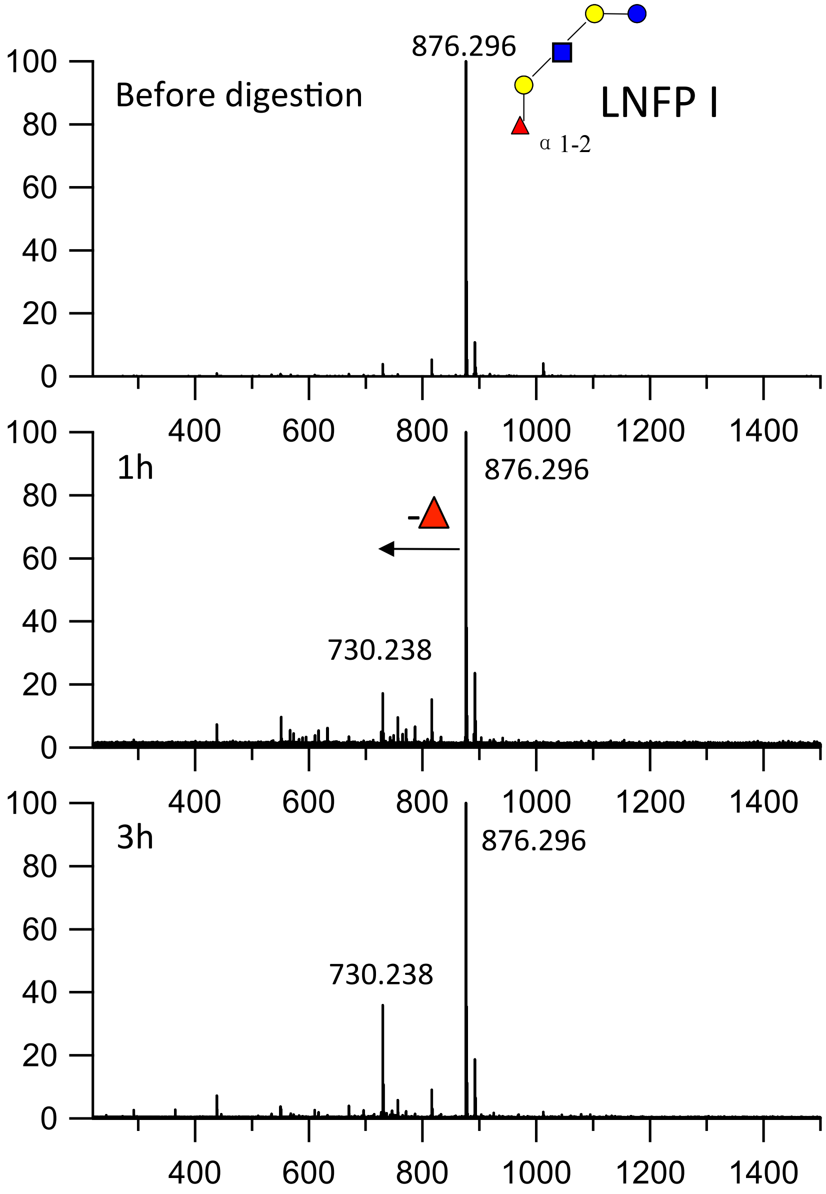


Figure S1. Digestion of fucosylated HMO with recombinant fucosidases. HMO standards were obtained from a commercial source. Incubation with Blon_0248 (A); Blon_0426 (B); Blon_2335 (C); Blon_2336 (D).

Table S4. Substrate preferences of HMO cluster fucosidases

| **Blon_2335** | **Relative activity** |
|---|---|
| 2’ Fucosyl lactose | 91.90% |
| 3’ Fucosyl lactose | 53.90% |
| H-disaccharide | 100% |
| Lewis a | 48.90% |
| Lewis x | 26.80% |
| | |
| **Blon_2336** | |
| 3’ Fucosyl lactose | 100% |
| Lewis a | 47.20% |
| Lewis x | 70.60% |