Research Article

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The role of the C domain in the thermostability of GH70 enzymes investigated by domain swapping

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Abstract: Sucrose-active enzymes belonging to the glycoside hydrolase (GH) family 70 are attractive tools for the synthesis of oligosaccharides, polysaccharides or glycoconjugates. However, their thermostability is an important issue for the development of robust and cost-effective enzyme-based processes. Indeed, GH70 enzymes are mesophilic and no thermophilic representatives have been described so far. Furthermore, structurally guided engineering is a challenge given the size of these proteins (120 to 250 kDa) and their organization in five domains. Herein, we have investigated the possible role of the domain C in the stability of GH70 enzymes. The alternansucrase (ASR) is the most stable enzyme of the GH70 family. Structural comparison of ASR to other GH70 enzymes highlighted the compactness of its domain C. We assumed that this atypical structure might be involved in the stability of this enzyme and decided to introduce this domain in another much less stable GH70 enzyme of known three-dimensional structure, the branching sucrase GBD-CD2. The chimeric GBD-CD2 exhibited a lower specific activity on sucrose substrate but its specificity was unchanged with the enzyme remaining specific for the branching of dextran *via* α -1,2 linkage formation. Interestingly, the chimera showed a higher melting temperature and residual activity than the wild-type enzyme after 10 min incubation at 30 °C showing that the domain C can affect GH70 enzyme stability and could be a potential target of both random or rational mutagenesis to further improve their stability.

Keywords: glucansucrase; branching sucrase; chimera; GH70 enzymes; domain C; enzyme stability.

Abbreviations

ASR, alternansucrase; CD, catalytic domain; DSF, differential scanning fluorimetry; GBD, glucan binding domain; GH, glycoside hydrolase; T_m , melting temperature.

1 Introduction

The alternansucrase (ASR) and the α -1,2 branching sucrase GBD-CD2 (GBD and CD stand for glucan binding domain and catalytic domain, respectively) are promising enzymes for the development of industrial applications including polysaccharide, oligosaccharide or glycoconjugate synthesis *via* the glucosylation of many different acceptors from sucrose substrate, an abundant and low-cost resource [1-5]. These efficient α -transglucosylases belong to the family 70 of the glycoside hydrolases (GH) according to the CAZy classification [6]. They are both mesophilic enzymes produced by lactic acid bacteria.

The ASR from *Leuconostoc citreum* NRRL B-1355 is one of the most stable enzymes of the GH70 family, with an optimum temperature of 45 °C [7], and a half-time life of 75 hours at 30 °C and 6 hours at 40 °C determined for the recombinant enzyme [8]. This old-known enzyme has attracted interest because of the peculiarity of the high molar mass polymer it produces from sucrose and which was named alternan because of the presence of alternative α -1,6 and α -1,3 linkages in the polymer main chain [9]. Thanks to its physico-chemical properties, the alternan polymer is a good candidate for replacing gum Arabic [10,11] and the oligoalternans have interesting prebiotic properties [12-14]. The three-dimensional structure of a truncated variant of this glucansucrase (ASRA2, comprising amino acids Ala29 to Gly1425; PDB ID: 6HVG) revealed that ASR adopts a U-shaped fold formed by five distinct domains A, B, C, IV and V like other GH70 enzymes [15-20]. Domains A, B, IV and V are made up by sequence fragments on either side of domain C [18]. Five

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other tertiary structures of ASR Δ 2 were solved in complex with different oligosaccharides (PDB IDs: 6SYQ, 6T1P, 6T16, 6SZI, 6T18). They enabled the identification of two sugar binding pockets (V-A and V-B) and revealed the presence of a unique oligosaccharide binding site at the surface of domain A [21].

The branching sucrose GBD-CD2 from *Leuconostoc citreum* NRRL B-1299 is an α -1,2 branching sucrose that performs α -1,2 glucosylation of exogenous isomaltooligosaccharides or α -1,6 linked dextran using sucrose as glucosyl donor. Up to 40% of α -1,2 osidic linkages, a rare linkage in nature, can be introduced [4]. The resulting α -1,2 glucooligosaccharides are resistant to the hydrolysis by digestive enzymes from both humans and animals and show prebiotic activity [22-24]. GBD-CD2 is also an attractive tool for the glucosylation of other oligosaccharides or hydroxylated molecules. For instance, the wild-type enzyme and several mutants catalysed the glucosylation of tetrasaccharides entering in the composition of different oligosaccharidic motifs found in *Shigella flexneri* O-antigens (notably in the serotypes 3a, 2b, 3a, 4a or 4b) and stand as interesting tools for the chemo-enzymatic synthesis of carbohydrate-based vaccines [25,26]. This enzyme was also successfully engineered for flavonoid glucosylation and several variants were obtained, which increased naringenin conversion from 14% to 60% and morin from 20% to 67% compared to the wild-type [27]. The three-dimensional structure of a truncated version of GBD-CD2 (ΔN_{123} -GBD-CD2) was solved in 2012 (PDB ID: 3TTQ). Two sugar binding pockets (V-L and V-K) were structurally characterized in the domain V of the enzyme, confirming the designation “glucan binding domain” [28]. However, a limit to GBD-CD2 utilization is its relative poor stability, with a half-life time of 10 hours at 30 °C and only 15 minutes at 40 °C [4].

Structural comparisons of ASR and GBD-CD2 further revealed that although the C-domain of ASR contains longer loops, it also harbours a higher number of β -strands and a higher level of interactions than those found in GBD-CD2. The C-domain is the only domain in GH70 enzymes consisting of a contiguous segment, forming a Greek key motif at the bottom of the ‘U’ fold and acting as a pedestal. We therefore hypothesized that the greater compactness of the ASR C-domain might enhance the thermostability of this enzyme. By analogy, the introduction of the ASR C-domain into the structure of GBD-CD2 could thus increase the stability of the latter. It is this hypothesis that we wanted to study here by creating a chimeric protein comprising domains A, B, IV, V of GBD-CD2 grafted onto the C domain of ASR and by characterizing the biochemical properties of the chimera obtained.

2 Results and discussion

2.1 Structural comparison of domain C of GH70 enzymes

The domain C was extracted from the whole tertiary structure (Fig. 1A) for analysis. The structural comparison of the domain C of ASR with those of other GH70 sucrose active enzymes (i.e. GTF180, GTF-SI, GTFA, GBD-CD2 and DSR-M) highlighted significant differences (Fig. 1B). First, ASR presents three peptide insertions: ⁸⁸²SSGKDLKDGE⁸⁹⁰, ⁹¹³QDNS⁹¹⁶ and ⁹⁹⁶KQDGT¹⁰⁰⁰, insertion 1, 2 and 3, respectively in Figure 1. Notably, insertion 3 is part of a β -hairpin (from Thr991 to Glu1005) and is only found in the ASR structure.

In addition, four ionic interactions can be predicted in the domain C of ASR against two for DSR-M, GTF-SI and GTF180 and none for GTFA and GBD-CD2. Similarly, ASR domain C harbours seven π - π stacking interactions, whereas only two or three are predicted for the others. Sequence alignment of the domain C of ASR and GBD-CD2 shows that the two domains share 49.64% of identity, while their extremities are highly conserved (Fig. 2). In addition, the number of residues Ile, Val, Leu and Phe showing the highest hydrophobicity according to Kyte and Doolittle scale [29] is higher in ASR than in GBD-CD2 domain C. Many of them participate in a hydrophobic interface with domain A or into local hydrophobic cores of domain C (Figs 2 and 3).

All together, these specific structural features (i.e. hydrophobic cores, hydrophobic interface, ionic or π - π stacking interactions) are likely to be important for protein folding and thermal stability, and let us assume that the ASR domain C could be involved in the higher stability of this enzyme. So, we hypothesized that it could enhance the stability of GBD-CD2, known to be less stable than ASR. To assess our hypothesis, we replaced the domain C of ΔN_{123} -GBD-CD2 by that of ASR. Importantly, the design and the construction of this chimeric enzyme were facilitated by the high level of conservation of the amino acid sequences found at the N- and C-terminal extremities of both domains C, limiting the risk of a complete destabilization of the overall fold due to domain C swapping (Fig. 2; orange dotted boxes).

2.2 Production of the chimeric enzyme

Using the protocol of production previously optimized for the wild-type enzyme ΔN_{123} -GBD-CD2 [31], the part of enzyme recovered in the insoluble fraction as aggregates or inclusion bodies was greater for the chimera (Fig. 4A). We

Figure 1: (A) View of the overall structure of ASR with the five domains: V (red), IV (yellow), B (green), A (blue) and C (purple). (B) Comparison of the domain C of available glucanase and branching sucrase structures.

Figure 2: Sequence alignment of the domain C of ASR and GBD-CD2 (ASR: 873-1030, GBD-CD2: 2424-2562). Arrows indicate the residues that differ in GBD-CD2 and that are thought to be involved in ASR domain C stability or folding. Blue triangle: hydrophobic interface with domain A; orange star: ionic interaction; pink circle: local hydrophobic core; green diamond: π - π stacking interaction. Orange dotted boxes: high similarity between the N-terminal and C-terminal parts of domains C. Alignment created with ENDscript 2 [30].

therefore tested the production of the wild-type enzymes and the chimera in the presence of a chaperone protein to facilitate protein folding. We used *Escherichia coli* BL21 star cells previously transformed with the commercial pTf16 plasmid harbouring the Tig chaperone. As shown in Figure 4, the production of enzymes in the soluble fraction increased and reached sufficient production levels to perform biochemical characterization (Fig. 4A). The ΔN_{123} -GBD-CD2-chimera was then purified and characterized to compare its stability, efficiency and specificity with those of the wild-type enzyme. To note, the Tig chaperone did not appear on the SDS-PAGE after purification (Fig. 4B).

2.3 Effect of domain C swapping on GBD-CD2 specificity and thermal stability

The specific activity of the ΔN_{123} -GBD-CD2-chimera was estimated at 11.6 U.mg⁻¹, versus 16.9 U.mg⁻¹ for the wild-type enzyme when tested on sucrose substrate only. With 69% of residual activity compared to the wild-type enzyme, the ΔN_{123} -GBD-CD2-chimera was impacted by the domain swapping but not to a major extent.

Furthermore, ¹H NMR spectra of the reaction products obtained from sucrose and 70 kDa dextran with ΔN_{123} -GBD-CD2 and ΔN_{123} -GBD-CD2-chimera are perfectly

Figure 3: Residues involved in domain C packing. (A) View of ASR domain C hydrophobic residues (Ile, Val, Leu, Phe, Cys, Met, Ala) involved in the interface between domain A and C (Met878, Leu895, Ile924, Val926, Val928, Leu958, Val959, Ile966, Val968, Leu1013, Ile1014, Leu1018, Val1020 and Val1022). (B) View of the residues involved into ionic interactions in ASR domain C (Asp881-Lys940, Lys935-Glu1005, Asp963-Lys1007 and Asp970-Lys974). (C) View of the residues involved into local hydrophobic cores in ASR domain C (Leu894, Phe900, Ile904, Ile927, Leu934, Leu936, Ile942, Leu944, Met946, Ala949, Ala957, Leu960, Leu978, Leu986, Phe988, Phe994 and Met1006). (D) View of the residues involved into π - π stacking interactions in ASR domain C (Tyr919-His950, His950-Tyr955, Phe988-Phe994, Phe988-Trp1021, Phe994-Tyr1004, Phe994-Trp1021 and Tyr1004-Tyr1017). (E) View of the residues involved into local hydrophobic cores in GBD-CD2 domain C (Leu2472, Leu2474, Leu2436, Leu2498, Ile2465, Ala2495, Leu2482, Leu2523, Met2484, Ala2515, Val2480, Ile2530). (F) View of the residues involved into π - π stacking interactions in GBD-CD2 domain C (Tyr2549-Trp2553, His2488-Tyr2493). The oxygen, nitrogen and sulphur atoms are coloured in red, blue and yellow, respectively.

Figure 4: (A) SDS-PAGE electrophoresis with and without tig chaperone for ΔN_{123} -GBD-CD2 and ΔN_{123} -GBD-CD2-chimera. 1: Soluble fraction ΔN_{123} -GBD-CD2; 2: soluble fraction ΔN_{123} -GBD-CD2-chimera; 3: soluble fraction ΔN_{123} -GBD-CD2 with tig chaperone; 4: soluble fraction ΔN_{123} -GBD-CD2-chimera with tig chaperone; 5: insoluble fraction ΔN_{123} -GBD-CD2; 6: insoluble fraction ΔN_{123} -GBD-CD2-chimera; 7: insoluble fraction ΔN_{123} -GBD-CD2 with Tig chaperone; 8: insoluble fraction ΔN_{123} -GBD-CD2-chimera with tig chaperone. (B) SDS-PAGE electrophoresis of the purified ΔN_{123} -GBD-CD2. 1: purified fraction A3; 2: purified fraction A6.

stackable (Fig. 5). The signals at 5.11 and 5.19 ppm are characteristic of the formation α -1,2 branching linkages in the linear dextran and their integration accounts for 36% α -1,2 linkage in both cases. These results indicate that domain C swapping did not affect the enzyme specificity, namely its ability to branch dextran molecules with glucosyl units linked through α -1,2 linkage.

The melting temperatures of ΔN_{123} -GBD-CD2 and ΔN_{123} -GBD-CD2-chimera were determined by differential scanning fluorimetry (DSF) (Fig. 6). Both DSF curves revealed the presence of two peaks (A and B) corresponding to two melting temperatures (T_m). The presence of different melting temperatures is often observed for multi-modular enzymes, such as the GH70 family enzymes. The first T_m (peak A) and the second one (peak B) correspond to T_m values of 37.7 °C and 44.5 °C for the chimera versus 37.1 °C and 41.6 °C for the wild-type enzyme. The variation of 0.6 °C and 2.9 °C for the two melting temperatures seems to indicate that the chimeric enzyme is more stable than the wild-type ΔN_{123} -GBD-CD2, even if the increase of T_m especially for the second peak must be taken with caution due the bad peak resolution.

Another way to compare enzyme stability is the determination of the residual activity after a period of incubation at a given temperature. After 10 minutes incubation at 30 °C, the percentage of residual activity was of 53% and 36% for the chimera and the wild-type ΔN_{123} -GBD-CD2, respectively. This difference is in agreement with the DSF results and shows that the chimeric enzyme is slightly more resistant to temperature than its wild-type homologue suggesting that the domain C swapping has slightly improved the stability of ΔN_{123} -GBD-CD2.

3 Conclusion

Studies focusing on stability improvement of GH70 family enzymes using protein engineering are scarce. One study reports the mutation of proline residues to serine or lysine to threonine in order to introduce stabilizing hydrogen bonds in the dextranase from *Leuconostoc mesenteroides* 0326 [32]. One mutant showed a 2-fold increase in catalytic efficiency, the same optimum temperature of 25 °C and a half-life time at 35 °C that increased from 6.6 minutes for the wild-type to 48.8

Figure 5: ^1H NMR spectra of ΔN_{123} -GBD-CD2 and its chimera. Reaction from 292 mM sucrose, 309 mM of 70 kDa dextran in 50 mM NaAc buffer pH 5.75 at 30 °C over a period of 24 hours.

Figure 6: (A) Evolution of the relative fluorescence and (B) derivative of the relative fluorescence units (RFU) during the denaturation of ΔN_{123} -GBD-CD2 and ΔN_{123} -GBD-CD2-chimera.

minutes for the mutant [32]. A second approach relied on disulphide bridge introduction in the dextranucrase and resulted in the identification of mutants revealing a shift of the optimum temperature from 25 °C to 35 °C but no improvement of thermostability [33]. Very recently, engineering was performed on the C-terminal part of dextranucrase dexYG domain V to improve the enzyme

stability. The domain V was artificially extended using ferredoxin as a suffix, and several truncations of this suffix were tested. The best improvement obtained was an increase of half-life time of 280% and 200% at 35 °C and 45 °C, respectively. These recent results highlight the potential of engineering domain V for stability [34], a strategy that could be coupled to domain C engineering.

In this study, we performed an exchange of domain C between two GH70 enzymes showing different thermal stability. Indeed, we noticed that the domain C, found at the basis of U-shape structure, is more compact in the ASR than in the GBD-CD2, our two model enzymes, and it was suspected to be involved in the highest stability of the ASR compared to that of the GBD-CD2. To test this hypothesis, we have successfully constructed and produced a chimeric enzyme, in which the domain C of GBD-CD2 was exchanged by that of ASR. Our results indicate that the branching sucrose ΔN_{123} -GBD-CD2 has slightly increased in thermal stability after acquiring the domain C of ASR, without modification of its specificity. Rigidification of domain C could thus be an approach that might deserve further investigations to increase the GH70 enzyme stability.

4 Experimental procedures

4.1 Chimera design and construction

Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) was used to align ASR and GBD-CD2 sequence and Genome Compiler (<http://www.genomecompiler.com/>) was used to construct and manipulate plasmid cards.

Domain C of ASR was amplified by PCR using *pET53-asr-Δ2* as template, Phusion® polymerase (NEB), forward primer ACGGCTCGTAAAGCTATGTCTCTGGTGGGCAAA-CAATG and reverse primer TGTACGGGCATCTTGATTGTCACTAGCTCCAAGTGGCACC. The underlined sequence corresponds to the overlap with ΔN_{123} -*gbd-cd2* gene. Plasmid of *pET53-ΔN₁₂₃-gbd-cd2* was amplified without the domain C with the primers GACAATCAAGATGCCCGTACAG and GACATAGCTTTTACGAGCCGTC. PCR products were purified with GenElute PCR Clean-up kit (Sigma) and DNA was quantified using a NanoDrop instrument. Assemblage of ΔN_{123} -*gbd-cd2* plasmid with *asr-Δ2* domain C was made using NEBuilder HiFi DNA Assembly kit with a molar ratio vector:insert of 1:2. Ligation product was transformed in home-made *E. coli* DH5α competent cells and sequences of the constructions were checked by sequencing (GATC).

4.2 Production and purification of chimera and wild-type enzymes

The *E. coli* BL21 DE3* strain transformed with a plasmid harbouring the Tf16 chaperone gene (Takara) was used for enzyme production. A pre-culture of transformed *E. coli* BL21 DE3* in LB medium supplemented with ampicillin

100 $\mu\text{g.mL}^{-1}$ and chloramphenicol 20 $\mu\text{g.mL}^{-1}$ was used to inoculate a culture at an $\text{OD}_{600\text{nm}}$ of 0.05 in ZYM-5052 auto-inducible medium [35] modified by supplementation with 100 $\mu\text{g.mL}^{-1}$ ampicillin and 20 $\mu\text{g.mL}^{-1}$ chloramphenicol, 4 mg.mL^{-1} arabinose, 0.75%(w/v) α -lactose, 1.5%(w/v) glycerol and 0.05% glucose for ΔN_{123} -GBD-CD2 and ΔN_{123} -GBD-CD2 chimera production (conditions optimized previously [31]). After 24 hours at 23 °C, cells were harvested by centrifugation and re-suspended in binding buffer containing 20 mM phosphate buffer, 20 mM imidazole (Merck Millipore), 500 mM NaCl and 50 mM sodium acetate buffer pH 5.75. Final $\text{OD}_{600\text{nm}}$ was 30. Cells were disrupted by sonication and debris was removed by a centrifugation step at 45,000 $\times g$ for 30 minutes at 8 °C. Purification was performed with ÄKTA Xpress system (GE Healthcare). Twenty mM imidazole and 500 mM NaCl were added to the soluble fraction prior to the purification of ΔN_{123} -GBD-CD2 and ΔN_{123} -GBD-CD2 chimera. Two-step purification was performed in a cold chamber at 12 °C using: (i) a HisTrap HP 1 mL column (GE Healthcare) for the affinity step; and (ii) a Superose12 16/60 (GE Healthcare) for the size exclusion step; and protein was eluted in MES buffer pH 6.5 at 30 mM with 100 mM NaCl and 0.05 g.L^{-1} CaCl_2 . Purified fractions were pooled together and concentrated using AmiconUltra-15 with a cut-off of 50 kDa to 10-15 mg.mL^{-1} . Purification was checked by SDS-PAGE electrophoresis using NuPAGE 3-8% Tris-Acetate protein gels (Invitrogen), and protein concentration was assessed by spectroscopy at 280 nm using a NanoDrop instrument. The theoretical molecular weight and molar extinction coefficient of the enzymes were calculated using the ExPASy ProtParam tool (<https://web.expasy.org/protparam/>). The molecular weight is of 127.56 kDa and 157.26 kDa for the GBD-CD2-chimera and ASR-chimera, respectively. Extinction coefficient is of 196310 and 213730 $\text{M}^{-1}.\text{cm}^{-1}$ for the GBD-CD2-chimera and ASR-chimera, respectively.

4.3 Activity measurement

Activity was determined in triplicate at 30 °C in a Thermomixer (Eppendorf) using the 3,5-dinitrosalicylic acid method [36]. Fifty mM sodium acetate buffer pH 5.75, 292 mM sucrose and 0.002 mg.mL^{-1} of pure ΔN_{123} -GBD-CD2 enzyme were used. One unit of activity is defined as the amount of enzyme that hydrolyses 1 μmol of sucrose per minute.

4.4 Enzymatic reactions and product characterization

ΔN_{123} -GBD-CD2 and ΔN_{123} -GBD-CD2 chimera branching reactions were performed using 1 U.mL⁻¹ of pure enzyme with 292 mM sucrose and 309 mM of 70 kDa dextran in 50 mM NaAc buffer pH 5.75 at 30 °C over a period of 24 hours. These polymers were purified by dialysis using 14 kDa cut-off cellulose dialysis tubing (Sigma-Aldrich) in water and analysed by NMR. NMR samples were prepared by dissolving 10 mg of purified product in 0.5 mL D₂O. Deuterium oxide was used as the solvent, and sodium 2,2,3,3-tetradeuterio-3-trimethylsilylpropanoate (TSPD₄) was selected as the internal standard ($\delta^1\text{H} = 0$ ppm, $\delta^{13}\text{C} = 0$ ppm). ¹H spectra were recorded on a Bruker Avance 500-MHz spectrometer operating at 500.13 MHz. The data were processed using TopSpin 3.0 software. One-dimensional ¹H NMR spectra were acquired by using a zgpr pulse sequence (with water suppression). Spectra were performed at 298 K.

4.5 Enzyme melting temperature determination

DSF experiments were performed in triplicates with 7 μM of pure enzyme in 50 mM sodium acetate buffer pH 5.75 supplemented with 0.5 g.L⁻¹ of CaCl₂ and 10 X of SYPRO orange (Life Technologies). A ramp from 20 °C to 80 °C was applied with 0.3 °C increments on a C1000 Thermal Cycler apparatus from Bio-Rad.

4.6 Structural analysis of the enzymes

To analyse the network of interactions in the structure, the RING [37] web server was used (Residue Interaction Network Generator; <http://protein.bio.unipd.it/ring>). It identifies and lists all the non-covalent interactions in the PDB loaded file (hydrogen bond, Van der Waals, ionic, π-π stacking, π-cation).

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