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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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: SSQGKDLKDE906, KQDNS916 and KQDG1000C, 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 ΔN123-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 ΔN123-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
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\textsubscript{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).

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 glucansucrase 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].

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

The specific activity of the ΔN\textsubscript{123}-GBD-CD2-chimera was estimated at 11.6 U.mg\textsuperscript{-1}, versus 16.9 U.mg\textsuperscript{-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\textsubscript{123}-GBD-CD2-chimera was impacted by the domain swapping but not to a major extent.

Furthermore, 1\textsuperscript{H} NMR spectra of the reaction products obtained from sucrose and 70 kDa dextran with ΔN\textsubscript{123}-GBD-CD2 and ΔN\textsubscript{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, Leu938, Val958, Leu959, 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, Leu2498, Leu2465, 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.
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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\textsubscript{123}-GBD-CD2 and ΔN\textsubscript{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\textsubscript{m}). The presence of different melting temperatures is often observed for multi-modular enzymes, such as the GH70 family enzymes. The first T\textsubscript{m} (peak A) and the second one (peak B) correspond to T\textsubscript{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\textsubscript{123}-GBD-CD2, even if the increase of T\textsubscript{m} especially for the second peak must be taken with caution due to 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\textsubscript{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\textsubscript{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 dextranucrase 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
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.

Figure 5: 'H NMR spectra of ΔN123-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 ΔN123-GBD-CD2 and ΔN123-GBD-CD2-chimera.
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 sucrase ΔN\textsubscript{23}-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 ACGGCTCGTAAAAGCTATGTCTCTGTGGCCAA-CAATG and reverse primer TGTACGGGCATCTTGATTGTCAATG and reverse primer TGTACGGGCATCTTGATTGTCAATG-GACCATGCTCTTGCTGTGGCCAA-CAATG and reverse primer TGTACGGGCATCTTGATTGTCAATG-GACCATGCTCTTGCTGTGGCCAA-CAATG. The underlined sequence corresponds to the overlap with ΔN\textsubscript{23}-gbd-cd2 gene. Plasmid of pET53-ΔN\textsubscript{23}-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\textsubscript{23}-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 µg.mL\textsuperscript{-1} and chloramphenicol 20 µg.mL\textsuperscript{-1} was used to inoculate a culture at an OD\textsubscript{600nm} of 0.05 in ZYM-5052 auto-inducible medium [35] modified by supplementation with 100 µg.mL\textsuperscript{-1} ampicillin and 20 µg.mL\textsuperscript{-1} chloramphenicol, 4 mg.mL\textsuperscript{-1} arabinose, 0.75%(w/v) α-lactose, 1.5%(w/v) glycerol and 0.05% glucose for ΔN\textsubscript{23}-GBD-CD2 and ΔN\textsubscript{23}-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 OD\textsubscript{600nm} was 30. Cells were disrupted by sonication and debris was removed by a centrifugation step at 45,000 × 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\textsubscript{23}-GBD-CD2 and ΔN\textsubscript{23}-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 Superose12/60 (GE Healthcare) for the size exclusion step; and protein was eluted in MES buffer pH 5.5 at 30 mM with a cut-off of 50 kDa to 10-15 mg.mL\textsuperscript{-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 M\textsuperscript{-1}cm\textsuperscript{-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\textsuperscript{-1} of pure ΔN\textsubscript{23}-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\textsubscript{G}-GBD-CD2 and ΔN\textsubscript{G}-GBD-CD2 chimera branching reactions were performed using 1 U.mL\textsuperscript{-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 D.O. Deuterium oxide was used as the solvent, and sodium 2,2,3,3-tetradeterio-3-trimethylsilylpropanoate (TPSP) was selected as the internal standard (δ\textsubscript{H} = 0 ppm, δ\textsubscript{13C} = 0 ppm). \textsuperscript{1}H 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 \textsuperscript{1}H 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\textsuperscript{-1} of CaCl\textsubscript{2} 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; https://protein.bio.unipd.it/ring). It identifies and lists all the non-covalent interactions in the PDB loaded file (hydrogen bond, Van der Waals, π-π stacking, π-cation).

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