Levan-type fructooligosaccharides synthesis by novel levansucrase-inulosucrase fusion enzyme

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Abstract

A novel strategy to enhance the yield of levan-type fructooligosaccharide (LFOS) was recently introduced, whereby levansucrase and inulosucrase reactions were coupled together in one pot. In order to simplify the process, in the present study we report the first example of a recombinant levansucrase-inulosucrase fusion protein and investigate its impact on LFOS production. Sequences for levansucrase from *Bacillus amyloliquefaciens* KK9 and inulosucrase from *Lactobacillus reuteri* 121 were fused genetically with a flexible eighteen residue glycine-serine peptide linker. SDS-PAGE analysis showed that the molecular weight of obtained fusion protein is approximately of 120 kDa, corresponding to the expected fusion protein molecular weight. Kinetic analysis revealed that after protein combination, the kinetic parameters of both enzymes are slightly changed. Biochemical characterization revealed that fusion did not affect the optimum pH and temperature for catalysis, but significantly change the stability of the enzyme. HPAEC-PAD analysis and enzymatic hydrolysis assays demonstrated that the fusion enzyme produced the desired higher yield of LFOS compared to those of individual levansucrases.

Keywords: fructan sucrase; fusion enzyme; fructooligosaccharide

1. Introduction

Fructooligosaccharides (FOS), also known as oligofructose, are a well-known prebiotic ingredient used in functional foods. They stimulate the growth of beneficial bacteria in the intestine, reduce levels of serum cholesterol, increase mineral absorption, and regulate the immune response [1, 2]. FOS could be classified into two groups according to the glycosidic linkages present: inulin-type fructooligosaccharide (IFOS) comprise linear chains of β-2,1-linked fructoses, while levan-type fructooligosaccharide (LFOS) are branched fructans with β-2,6-glycosidic bonds at the mainchain and β-2,1-glycosidic bonds connecting sidechains.

LFOS and IFOS can be synthesized from sucrose using levansucrase (E.C. 2.4.1.10) and inulosucrase (E.C. 2.4.1.9), respectively. These enzymes are classified as glycoside hydrolase family 68 (GH68) and exhibit high structure similarities [3, 4]. In addition to producing fructose-based oligosaccharidues, these enzymes also synthesize high molecular weight polysaccharide (levan or inulin), which have lower bioavailability than the oligosaccharide [5, 6]. Many studies have shown that the ratio of polymerization to oligosaccharide synthesis is dependent on both the reaction conditions and enzyme source. Levansucrase from Gram positive bacteria, such as *Bacillus subtilis* [7], *Bacillus licheniformis* [8, 9] and *Bacillus amyloliquefaciens* [6], mainly synthesize a high molecular weight levan without accumulating LFOS [10]. In addition, levansucrase from Gram negative such as *Erwinia amylovora* [11] and *Zymomonas mobilis* [12] mainly synthesizes short-chain LFOS with low polymer yield. Inulosucrases synthesize different ratio of IFOS and inulin depending on the reaction conditions [13, 14].

In recent years, it has been found that structurally different fructooligosaccharides and polysaccharides have distinct biological effects. LFOS, which comprise mainly β2-6 linkages, possess anticancer [15-17], antioxidant [18] and immunomodulatory activity [19, 20]. On the other hand, the β2-6 linked polymer fructan specifically activates Toll-like receptor (TLR) 3, 7 and 9, while inulin (β2-1) activates TLR 2 and TLR 4 [21]. TLRs are a group of transmembrane proteins that play a role in the immune response against pathogen [22]. Furthermore, LFOS have been selectively used as carbon sources for growth of beneficial intestinal microbiota [23, 24]. The biological activities of fructooligosaccharides are also dependent on the degree of polymerization (DP) [25-27]. FOSs with DP < 9 display a high prebiotic activity compared to long-chain FOS and fructan. Due to this fact, protein engineering and computational protein design have been employed on levansucrase to limit polymerization reactions and to increase the accumulation of LFOS [6, 9, 10, 28]. However, the mutant enzyme generally exhibit a high hydrolysis activity, resulting in a substantial amounts of free fructose in the reaction. Alternatively, the levansucrase reaction can be coupled with endolevanase, the enzyme that catalyze the hydrolysis of levan to a short- or medium-chain oligofructose [29, 30].

Additionally, a fusion protein between levansucrase and endolevanase was also recently reported reaction duration for this bi-enzyme system need to be strictly regulated to preventing excessive hydrolysis and to controlling the size of LFOS obtained [31].

Recently an approach for increasing LFOS production was reported using levansucrase/inulosucrase mixed enzyme reactions [32]. We found that some IFOS species obtained from inulosucrase could competitively bind to the levansucrase active site, resulting in a decrease in enzyme processivity. This approach enhanced the production of a broad spectrum of LFOS, ranging from short to medium chain length (DP4 - DP15). Further developing this concept, herein, a novel levansucrase-inulosucrase fusion protein was constructed and expressed in *Escherichia coli* for one-pot synthesis of LFOS. Specifically, levansucrase from *Bacillus amyloliquefaciens* KK9 and inulosucrase from *Lactobacillus reuteri* 121 were fused together with a flexible GS peptide linker. Biochemical properties and kinetic parameters of the fusion enzyme were analyzed and compared to those of the individual enzymes. The obtained levansucrase-inulosucrase fusion enzyme was used to synthesize LFOS from sucrose, the LFOS pattern and the ratio between oligo- and polysaccharide synthesis was determined.

2. Materials and methods

2.1 Construction of levansucrase-inulosucrase fusion enzyme gene

PCR reactions were performed using PrimeStar™ DNA polymerase (Takara Bio™). *Escherichia coli* Top10 strain was used as a host for plasmid construction. Enzyme chimeras were constructed from the plasmid vector pET-LSKK9 and pET-INU by PCR overlapping extension [33], using the primers described in Table 1. The PCR product was purified by 1% agarose gel electrophoresis, digested by *Nde*I and *Xho*I and ligated into pET-21b vector, resulting in the pET-LSKK9-INU expression vector that encoded Lev-$(GGS)_6$-Inu fusion protein.

After that, site-directed mutagenesis was performed in order to construct the genes that encoded levansucrase-inactive and inulosucrase-inactive versions of fusion proteins, named D86NLev-$(GGS)_6$-Inu and Lev-$(GGS)_6$-D272NInu, using pET-LSKK9-INU as a template. D86 and D272 are the essential catalytic nucleophiles of levansucrase and inulosucrase, respectively. D86NLev-$(GGS)_6$-Inu is a fusion protein which the residue corresponded to D86 of levansucrase was substituted by ASN, while the residue corresponded to D272 of inulosucrase was substituted by ASN for Lev-$(GGS)_6$-D272NInu. Mutations were introduced by PCR overlapping extension [33], using the primers described in Table 1. The obtained plasmids were verified by DNA sequencing.

Table 1 Nucleotide sequences of primers used in this study.

Primer	Sequence (5'-3')
F_LSKK9-NdeI	GCGTATCACCATATGAACATCAAAAAGATTGTAAAACGAGC
R_LSKK9-GS	GCTGCCACCGCTACCACCAGAACCACCGCTACCGCCTTGGTT GACTGCAGTTTGTCCTTG
F_INU-GS	GGTGGTAGCGGTGGCAGCGGTGGCAGCGGTGGCAGCGACA CAAATATTGAAAACAATGATTC
R_INU-XhoI	GGTGCTCGAGTTTTAATCCATAACCAATTAAG
F_LSKK9-D86N	CTCGATGTGTGGGACAGCTGGCCGCTC
R_LSKK9-D86N	GAGCGGCCAGCTGTCCCACACATCGAG
F_INU-D272N	CCTTTAGATGTATGGAATTCATGGCCAGTTC
R_INU-D272N	GAACTGGCCATGAATTCCATACATCTAAAGG

2.2 Expression and purification of levansucrase, inulosucrase and fusion enzymes

All proteins were expressed using *E. coli* BL21 (DE3) as expression host according to method described previously [34, 35]. The cells carrying recombinant plasmid were cultured in LB broth supplement with 100 μg/ml ampicillin, 5 g/l glucose and 10 mM $CaCl_2$ at 37°C, shaking at 250 rpm. Until OD600 reached 0.4–0.6, IPTG was added into the cultures to the final concentration of 0.1 mM. After that, the cells were further cultured at 30°C, 200 rpm for 18-20 h. The cells were harvested by centrifugation at 5,000 xg for 20 min, resuspended in 25 mM phosphate buffer (pH 7.4), and then lyzed by ultrasonication. The cell debris were separated from the crude enzyme extracts by centrifugation at 12,000 xg for 30 min. Recombinant proteins were further purified by Nickel affinity chromatography using Ni sepharose™ 6 fast flow bead (GE Healthcare). The column was equilibrated by 25 mM phosphate buffer (pH 7.4) before loading the crude proteins. The unbound proteins were washed with the same buffer containing 20 mM imidazole and 500 mM NaCl. Finally, the target protein was eluted from the column using 200 mM imidazole buffer (pH 7.4). The purity of the obtained proteins was checked by SDS-PAGE.

2.3 Activity assay and biochemical characterization

The activity of individual and fusion enzymes was determined by DNS methods as described previously [8, 21]. The purified enzymes were added into 0.5 ml of 10%(w/v) sucrose containing 50 mM phosphate buffer (pH 6.0). After incubation at 50°C for 10 min, the reactions were terminated by adding 0.5 ml DNS reagent [21] and boiled for 10 min. The color of the solution will be changed from yellow to orange or red that related to the concentration of reducing sugar in the reaction. The concentration of reducing sugar released by enzymes was determined by spectrophotometer at 540 nm using glucose as external standard. One unit (U) of enzyme was defined as the amount of enzyme required to release one μmol reducing sugar per min.

Optimum pH for catalysis of fusion enzymes was evaluated by assaying the enzyme activity at 50°C in Britton–Robinson universal buffer (a solution of phosphoric, acetic, and boric acids, each 0.04M; pH 3-12) [36]. The optimum temperature of enzymes was determined in phosphate buffer (pH 6.0) at temperature range of 20-60°C. Thermostability of enzymes was determined by incubating 0.2 mg/ml purified enzymes in 50 mM phosphate buffer pH 6.0 at 30-50°C from 0 to 8 h. Then, the remaining activity of enzyme was measured at 50°C, pH 6.0.

2.4 Determination of kinetic parameters

The purified enzymes were incubated with 0.5 ml of substrate solution containing 0 - 480 mM sucrose and 50 mM phosphate buffer (pH 6.0) at 37°C. The reactions were terminated by adding 0.5 ml DNS reagent and boiling for 10 min. The amount of reducing sugar released was determined by spectrophotometer at 540 nm using glucose as external standard. The activity *versus* sucrose concentration curves were plotted and fitted with Michaelis-Menten equation for obtaining K_m and k_{cat} values.

2.5 FOSs synthesis

FOSs were synthesized using 5 U/ml enzyme, 250 mM sucrose and 50 mM phosphate buffer (pH 6.0). After incubation at 30°C for 24 h, The reactions were then terminated by boiling for 10 min. The reaction mixtures were diluted to appropriate concentration before analyzing the LFOS pattern by HPAEC-PAD.

2.6 High-performance anion exchange chromatography analysis

HPAEC-PAD analysis was performed using a Dionex™ ICS 5000 system with CarboPac™ PA-100 column. Separation was achieved by a linear gradient of 0 – 0.5 M sodium acetate containing 150 mM NaOH solution at a flow rate of 1 ml/min for 35 min.

2.7 Determination of percentage of oligo- and polysaccharide synthesis

The total transglycosylation products (polysaccharide + oligosaccharide) were determined by measuring the amount of fructose, using DNS assay, released after hydrolysis by fructanase mixture (Megazyme™). Prior to treating with fructanase, the samples were treated by 1%(w/v) $NaBH_4$ to remove the free fructose and glucose background. After that, the reduction reaction was terminated using acetate buffer (pH 4.5). Polysaccharide was isolated by acetone precipitation (1:1 water to acetone) and totally hydrolyzed by fructanase mixture (Megazyme™). The amount of fructose

released was determined by DNS assay. The amount of oligosaccharide was calculated by subtracting the amount of polysaccharide from the total transglycosylation products.

2.8 Homology modeling and molecular dynamics (MD) simulation

The homology model of Lev-$(GGS)_6$-Inu was built by GalaxyWEB (http://galaxy.seoklab.org/) [37]. The H++ server [14], [23], [24] was used to determine the protonation state of all amino acids of the protein structure at pH 6.0. The AMBER20 package was used to perform molecular dynamics (MD) simulation to evaluate the dynamic behavior of Lev-$(GGS)_6$-Inu [38, 39]. The model was visualized using PyMOL.

3. Results and discussion

3.1 Construction, expression, and purification of levansucrase-inulosucrase fusion proteins

Protein fusion enables the construction of multi-functional enzymes [40]. This approach can integrate cascade reactions into a single enzyme molecule, which facilitates a one-step reaction and reduces enzyme production and purification costs. In this study, levansucrase and inulosucrase were linked by a flexible glycine–serine peptide linker, which has previously been used to construct multidomain proteins as it assists in maintenance of native enzyme conformation [41, 42].

As illustrated in Fig. 1, the levansucrase gene was linked to N-terminal of inulosucrase gene via $(GGS)_6$ linker. The levansucrase gene was amplified from pET-LSKK9 using F_LSKK9-NdeI and R_LSKK9-GS primers. The obtained PCR product, defined as fragment 1, consisted of the levansucrase gene at 5' end and the part of GS peptide linker at 3' end. Likewise, the inulosucrase gene was amplified from pET-INU using F_INU-GS and R_INU-XhoI primers. This PCR product, fragment 2, has another part of GS linker at 5' end and inulosucrase gene at 3' end. Note that the 3' end of fragment 1 and 5' end of fragment 2 are overlapped and complementary. These two fragments were used as template for a second PCR reaction using F_LSKK9-NdeI and R_INU-XhoI primers (Fig. 1). The obtained full length fusion gene was cloned into pET-21b expression vector to produce pET-LSKK9-INU.

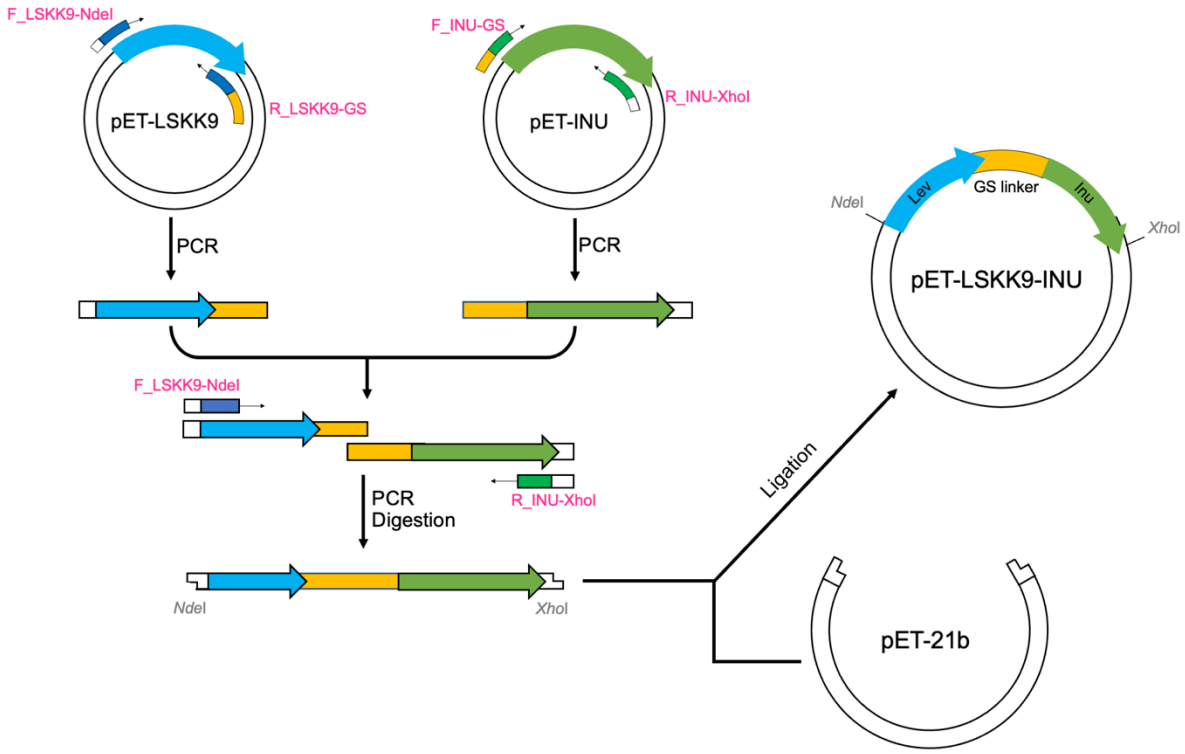

Fig. 1 Schematic overview of pET-LSKK9-INU construction.

Since levansucrase and, inulosucrase display similar catalytic activities, it is difficult to determine the kinetic and biochemical characteristics of the individual enzymes in a fusion protein. Hence, D86NLev-$(GGS)_6$-Inu and Lev-$(GGS)_6$-D272NInu, the levansucrase-inactive and inulosucrase-inactive versions of fusion proteins, were constructed and used as control. D86 and D272 are the essential catalytic nucleophiles of levansucrase and inulosucrase, respectively [6, 43]. Site-directed mutagenesis was employed to construct these catalytic-inactive fusion enzymes using pET-LSKK9-INU as a template. All fusion enzymes, shown in Fig. 2A, were expressed in *E. coli*, followed by His-tag purification. SDS-PAGE analysis showed that all fusion proteins can be expressed in *E. coli*, and successfully purified by Ni-column (Fig. 2B). These analyses also indicated molecular weights of the fusion proteins of ca 120 kDa, which corresponded well to the calculated molecular weights (ca 127 kDa).

The structure of the Lev-$(GGS)_6$-Inu fusion enzyme was studied using homology modeling and molecular dynamics (MD) simulations (Fig. 2C-D). The RMSD plots show that the simulated systems are stable since 100 ns of simulations. Thus, the equilibrium structure of Lev-$(GGS)_6$-Inu fusion enzyme was extracted from the last 20 ns of MD trajectories (Fig. 2D). The obtained model suggests that the levansucrase moiety active site is well separated from that of the inulosucrase moiety, suggesting that each enzyme activity should not physically obstruct the activity of the other. Even though linker optimization may be needed to obtain the highest activity of the fusion enzyme [44, 45], the use of a

$(GGS)_6$ linker in the present study for levansucrase-inulosucrase fusion enzyme is in agreement with our further results in this study (see below).

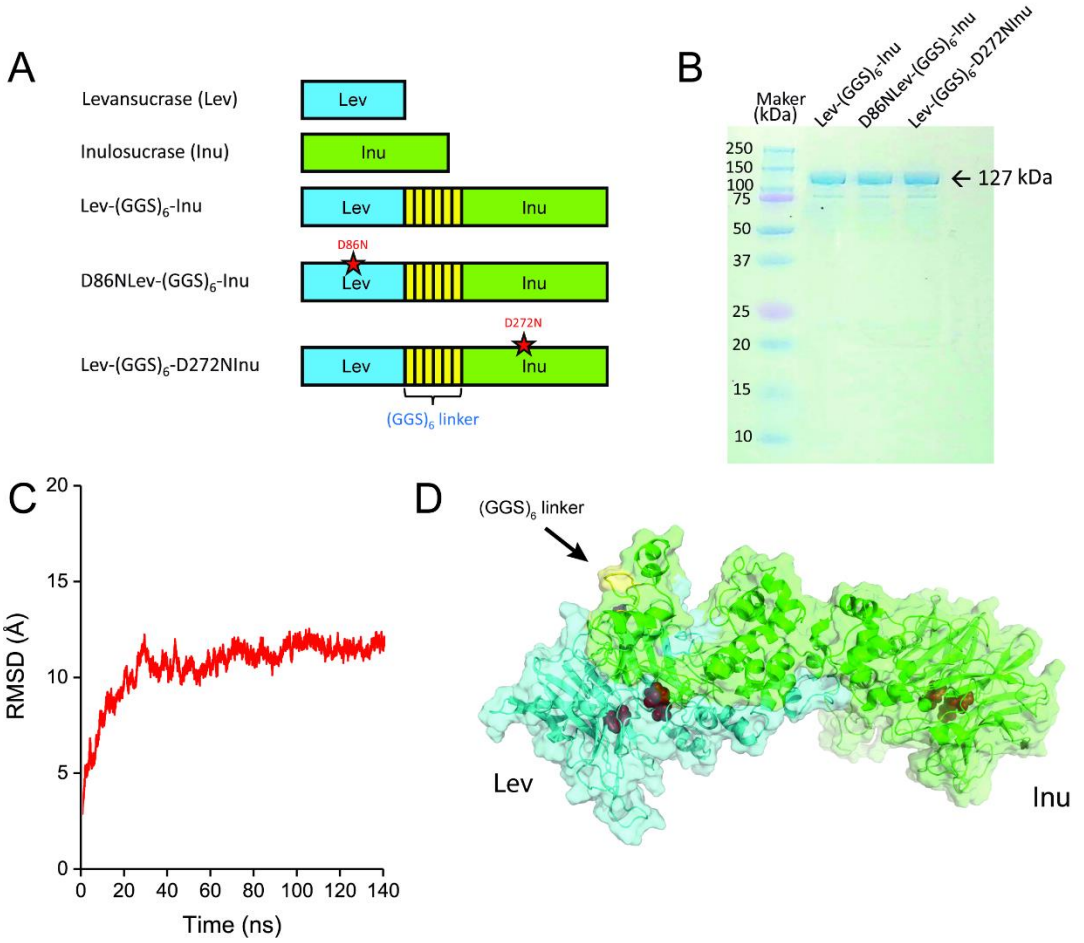

Fig. 2 (A) Schematic representation of enzymes in this study. (B) SDS-PAGE analysis of recombinant enzymes after His-tag purification. (C) All atom RMSD of Lev-$(GGS)_6$-Inu model during 120 ns of molecular dynamics simulations. (D) The equilibrium structure of Lev-$(GGS)_6$-Inu obtained by energy minimization and 120 ns molecular dynamics simulations. The catalytic residues were displayed as red spheres.

3.2 Kinetic analysis

After obtaining the fusion proteins, the effect of fusion on enzyme kinetic and biochemical characteristics was evaluated. Kinetic parameters of individual and fusion enzymes were determined based on sucrose concentration versus activity curves, fitted to the Michaelis–Menten equation (Fig. S1). As shown in Table 2, the k_{cat}/K_m value of levansucrase after fusion (equivalent to the k_{cat} of Lev-$(GGS)_6$-D272NInu) decreased by approximately of 10 % compared to the monomeric enzyme, while the k_{cat}/K_m value of inulosucrase and D86NLev-$(GGS)_6$-Inu were not significantly different. The reduction of Lev catalytic efficiency mainly resulted from the increase of K_m value of levansucrase

moiety [21.4 mM in monomeric Lev to 39.4 mM in the Lev-$(GGS)_6$-D272NInu]. The present of an inulosucrase moiety in fusion enzymes may competitively bind to sucrose, resulting in the lower affinity of levansucrase for their substrate. However, only minor changes of k_{cat} value for both enzymes upon fusion suggests that the $(GGS)_6$ linker has minimal impact on enzyme activity.

Table 2 Kinetic parameters of levansucrase (Lev), inulosucrase (Inu) and fusion enzymes. The experiment was performed in triplicate (mean ± standard error (SE)).

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
D86NLev-$(GGS)_6$-Inu	66 ± 7	615 ± 21	9.4
Lev-$(GGS)_6$-D272NInu	39 ± 5	92.0 ± 3.2	2.5
Lev-$(GGS)_6$-Inu	69 ± 10	729± 32	10.6
Lev	21 ± 2	102 ± 2	4.7
Inu	68 ± 9	631 ± 26	9.3

3.3 Biochemical characterization of fusion enzymes

Fusion of multiple enzymes may affect the structure, biochemical properties, and stability of individual enzyme components. Effect of pH and temperature on initial reaction rate of fusion enzymes were investigated in the pH and temperature range of 3.0-12.0 and 20-60°C, respectively. It was found that all fusion enzymes exhibited an optimum pH of 5.0-6.0 (Fig. 3A) and optimum temperature of 50°C (Fig. 3B), which was similar to the previous reports on levansucrase and inulosucrase [5, 9, 34]. The stability of Lev-$(GGS)_6$-Inu was then investigated at 30-50°C. As shown in Fig. 4, the activity of fusion enzyme dramatically decreased after the first hour of incubation at 40-50°C, indicating that it is not stable at these temperatures. Therefore, in comparison to the individual enzymes, the fusion enzyme exhibited lower stability. It probably is a result of higher molecular weight of fusion enzyme (> 120 kDa) and increased flexibility of the domain near peptide linker. This finding is similar to previous study of dextransucrase-dextranase fusion enzyme [46]. The decrease in thermostability of fusion enzyme could limit the application in FOS production. Based on the RMSF value obtained from MD simulation (Fig. S2), residue 150-155, and 435-438 located in Lev moiety were highly flexible compared to the neighbor ones. These residues might be the hotspot for protein denaturation. The stability of the fusion enzyme possibly improves by replacing these highly flexible residues by amino acid that could potentially form favorable interactions with their neighboring residues [8]. However, the fusion enzyme, free Lev and free Inu are stable for extended periods at 30°C (Fig. S4), where fusion enzyme's activity is ca 20% of that at the temperature optimum for the

enzyme. Nonetheless, a reduction in operating temperature is associated with a reduced costs for FOS synthesis.

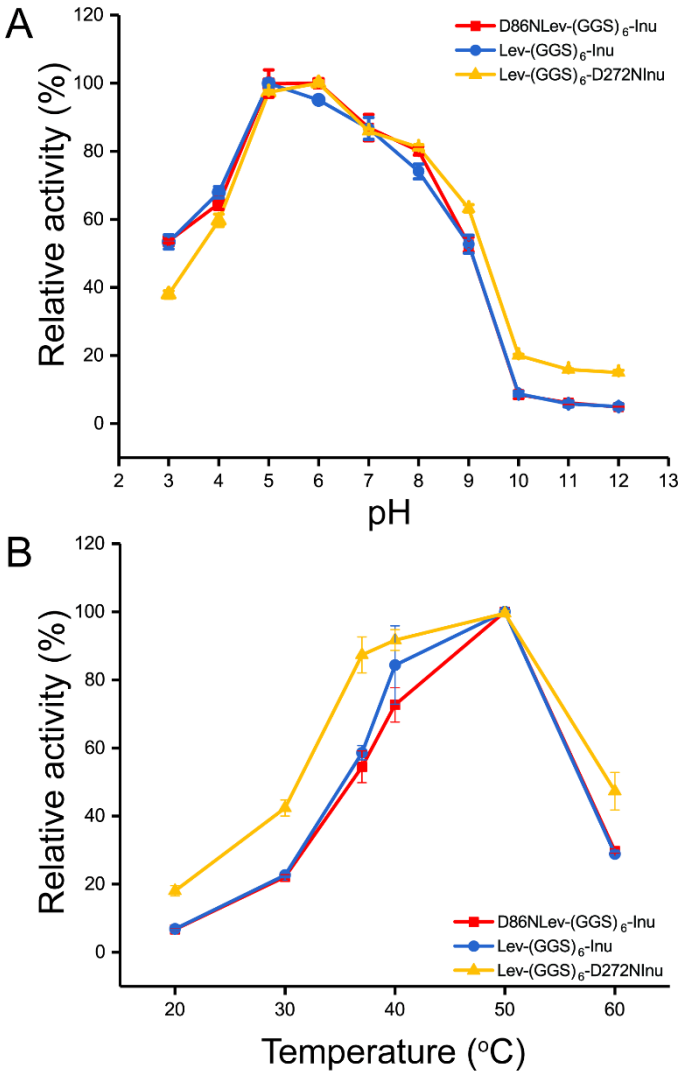

Fig. 3 Effect of pH and temperature on the initial reaction rate of fusion enzymes. (A) The effect of pH on the initial reaction rate was investigated at 50℃. (B) Effect of temperature on the initial reaction rate were performed at pH 6.0. The data represent means of three assays and error bars represent the standard error (SE) of three experiments.

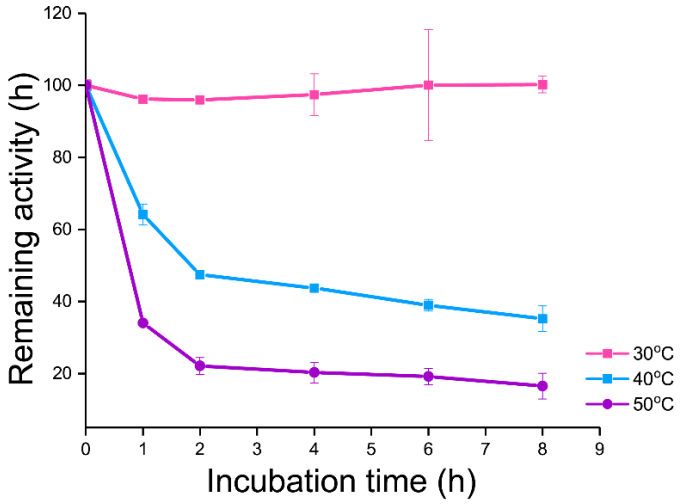

Fig. 4 stability of Lev-$(GGS)_6$-Inu fusion enzyme at 30, 40 and 50°C. The data represent means of three assays and error bars represent the standard error (SE) of three experiments.

3.4 Product analysis

FOS was synthesized using 5 U/ml enzymes and 250 mM sucrose at 30°C, since the fusion enzyme is stable at that temperature. HPAEC-PAD analysis showed that the pattern of FOS derived from D86NLev-$(GGS)_6$-Inu is similar to that of Inu, while Lev and Lev-$(GGS)_6$-D272NInu synthesized the same FOS pattern (Fig. 5). This result indicated that fusion by GGS linker did not affect the specificity of either levansucrase or inulosucrase. In the case of Lev-$(GGS)_6$-Inu, the product peaks observed in HPAEC-PAD chromatogram corresponded to the peak positions of Lev and Lev-$(GGS)_6$-D272NInu FOS. Furthermore, Lev-$(GGS)_6$-Inu produced the shorter chain FOS with higher peak intensity (Fig. 5). This indicated that fusion of levansucrase and inulosucrase into a single enzyme molecule could amplify the signal of levan-type fructooligosaccharide synthesized by levansucrase. In comparison to 1:1 stoichiometric mixture of free Lev and Inu, fusion enzymes synthesize slightly different FOS pattern, possibly due to the change in activity ratio of these enzymes (Fig. S3). This finding is on line with results from a previous study that showed β-2,1 FOS species obtained in the presence of inulosucrase significantly reduced the processivity of levansucrase in the same reaction mix and increased the synthesis of levan oligosaccharides at the expense of levan polymer, depended on the ratio of Lev:Inu [32].

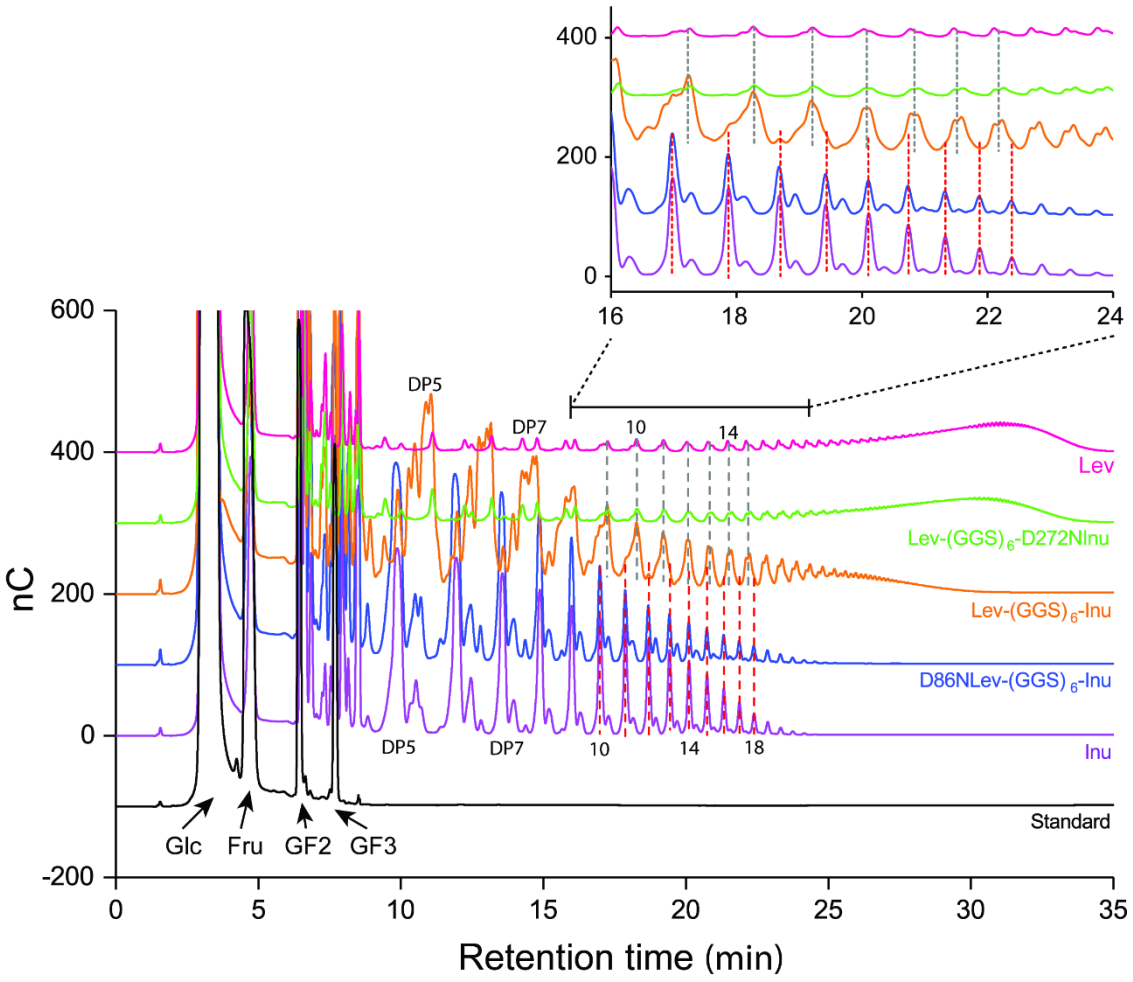

Fig. 5 HPAEC-PAD analysis of fructooligosaccharide synthesized by free and fusion enzyme.

Quantitative analysis of polysaccharide/oligosaccharide ratios (Fig. 6) indicated that the oligosaccharide/polysaccharide ratios between Inu and D86NLev-(GGS)6-Inu, and between Lev and Lev-(GGS)6-D272NInu were not significantly different. Oligosaccharides produced by Lev-$(GGS)_6$-Inu was ca 66% oligosaccharide, which was 2.3-times higher than that of Lev and Lev-$(GGS)_6$-D272NInu. Although Lev-$(GGS)_6$-Inu produced lower amount of glycan than Inu and D86NLev-$(GGS)_6$-Inu, HPAEC analysis clearly showed that Lev-(GGS)6-Inu's oligosaccharide is not inulin. Moreover, the size of LFOS synthesized by Lev-$(GGS)_6$-Inu was not much changed over time since the hydrolytic rate of levansucrase for levan is relatively low [47]. This is in contrast to the temporal control that is required to produce LFOS with a levansucrase-levanase fusion enzyme ($LevB_1SacB$), since levanase hydrolyzes levan to very short oligosaccharide [48].

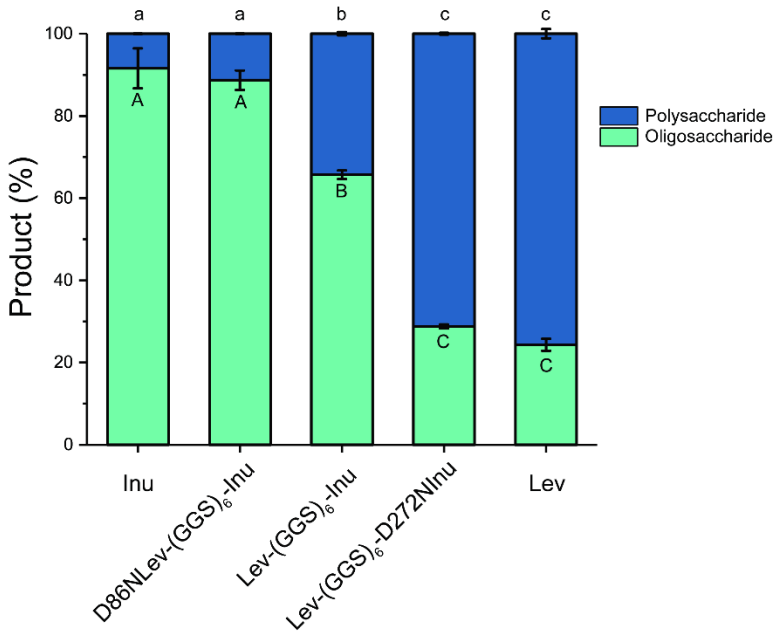

Fig. 6 Polysaccharide/oligosaccharide ratio of the product obtained from free and fusion enzyme. The data represent means of three assays and error bars represent the standard error (SE) of three experiments. Statistical differences were tested by one-way analysis of variance (ANOVA). Different letters indicate significant differences ($p < 0.05$) between samples.

5. Conclusion

In this study, a novel levansucrase-inulosucrase fusion enzyme (Lev-$(GGS)_6$-Inu) was constructed and expressed in *E. coli.* These two fructansucrases were joined through a highly flexible glycine-serine linker peptide. The obtained fusion enzymes clearly displayed a highly synergistic relationship between levansucrase and inulosucrase for LFOS synthesis. Biochemical characterization showed that fusion did not affect the optimum pH and temperature for catalysis, while kinetic parameters were only slightly changed. The fusion enzyme is stable for extended periods at operational temperature of 30°C. Taken together, our results suggest new application opportunities for levansucrase-inulosucrase fusion enzymes (Lev-$(GGS)_6$-Inu) in the production of bioactive levan-type fructooligosaccharides.

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7. References

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Supporting information

Levan-type fructooligosaccharides synthesis by novel levansucrase-inulosucrase fusion enzyme

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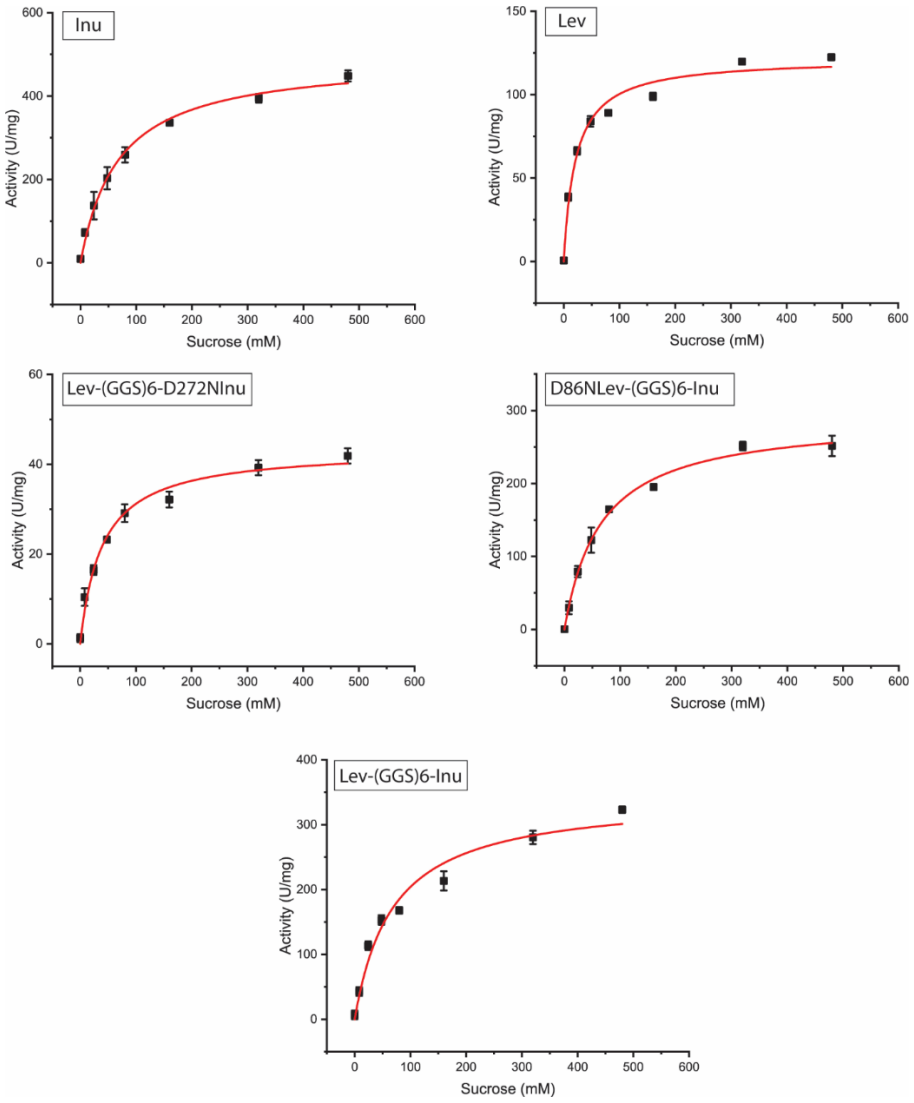

Figure S1 Graphs of relationship between initial velocity (V) and sucrose concentration (mM) for wild-type and mutant enzymes. Each data point is mean of three replicates ± standard error (SE).

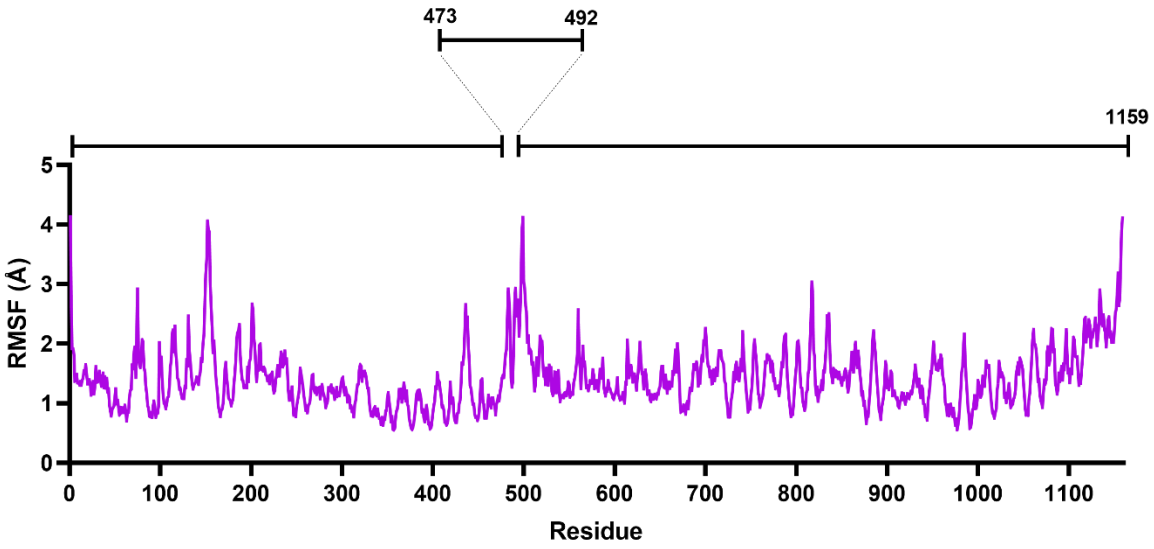

Figure S2 RMSF plot of Lev-$(GGS)_6$-Inu (Levansucrase domain, residue 1 – 473; GGS linker, residue 474 – 491; inulosucrase domain, residue 492 – 1159).

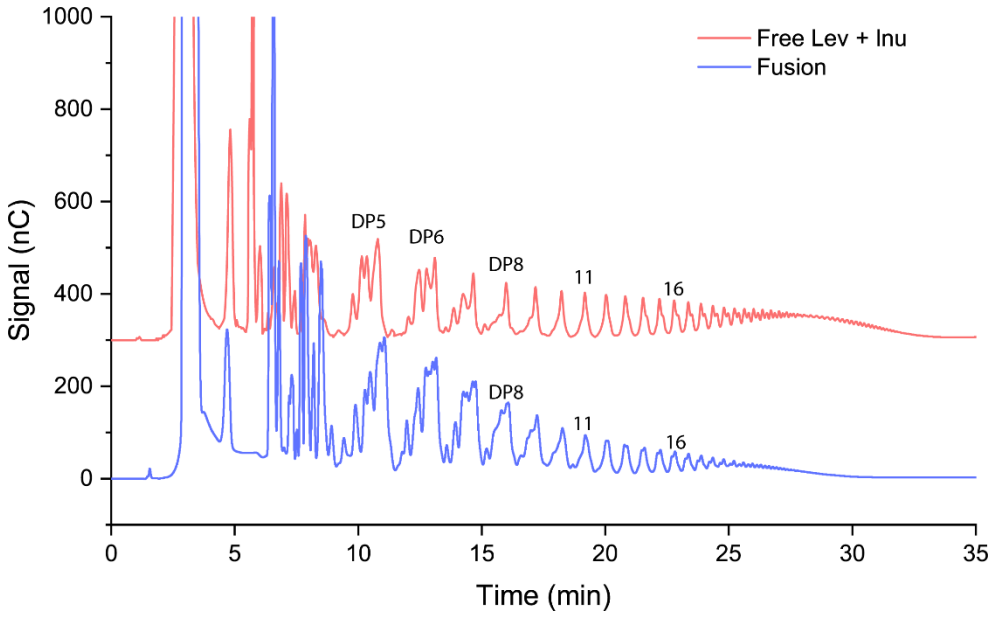

Figure S3 HPAEC-PAD chromatograms of reaction mixture obtained from Lev-(GGS)6-Inu and 1:1 mixture of levansucrase and inulosucrase.

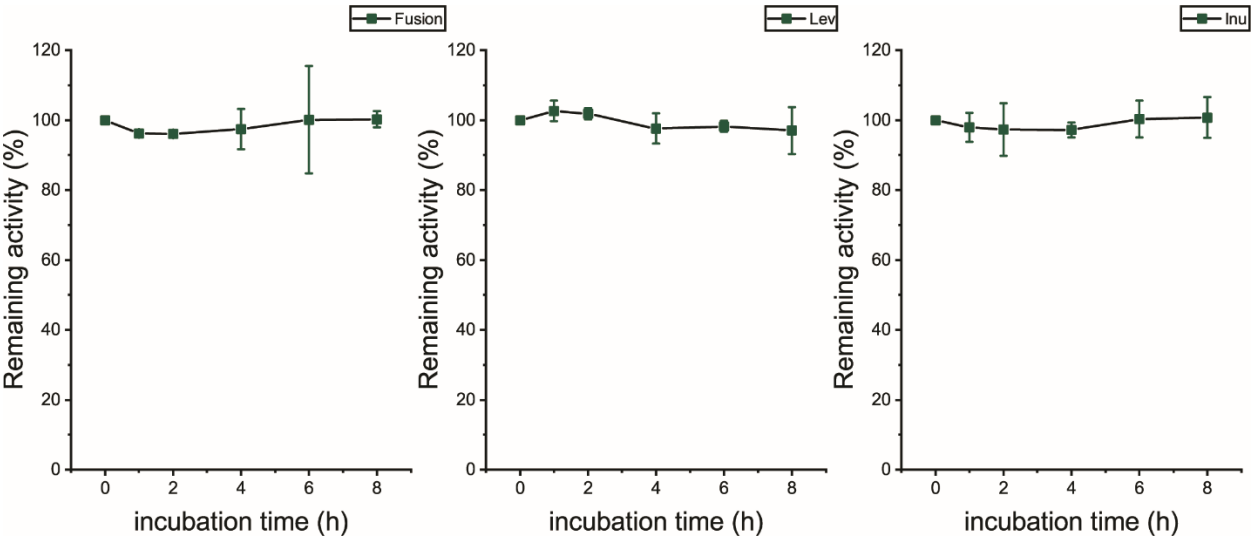

Figure S4 Stability of free Lev, free Inu and fusion enzyme at 30°C. The data represent means of three assays and error bars represent the standard error (SE) of three experiments.

fig1

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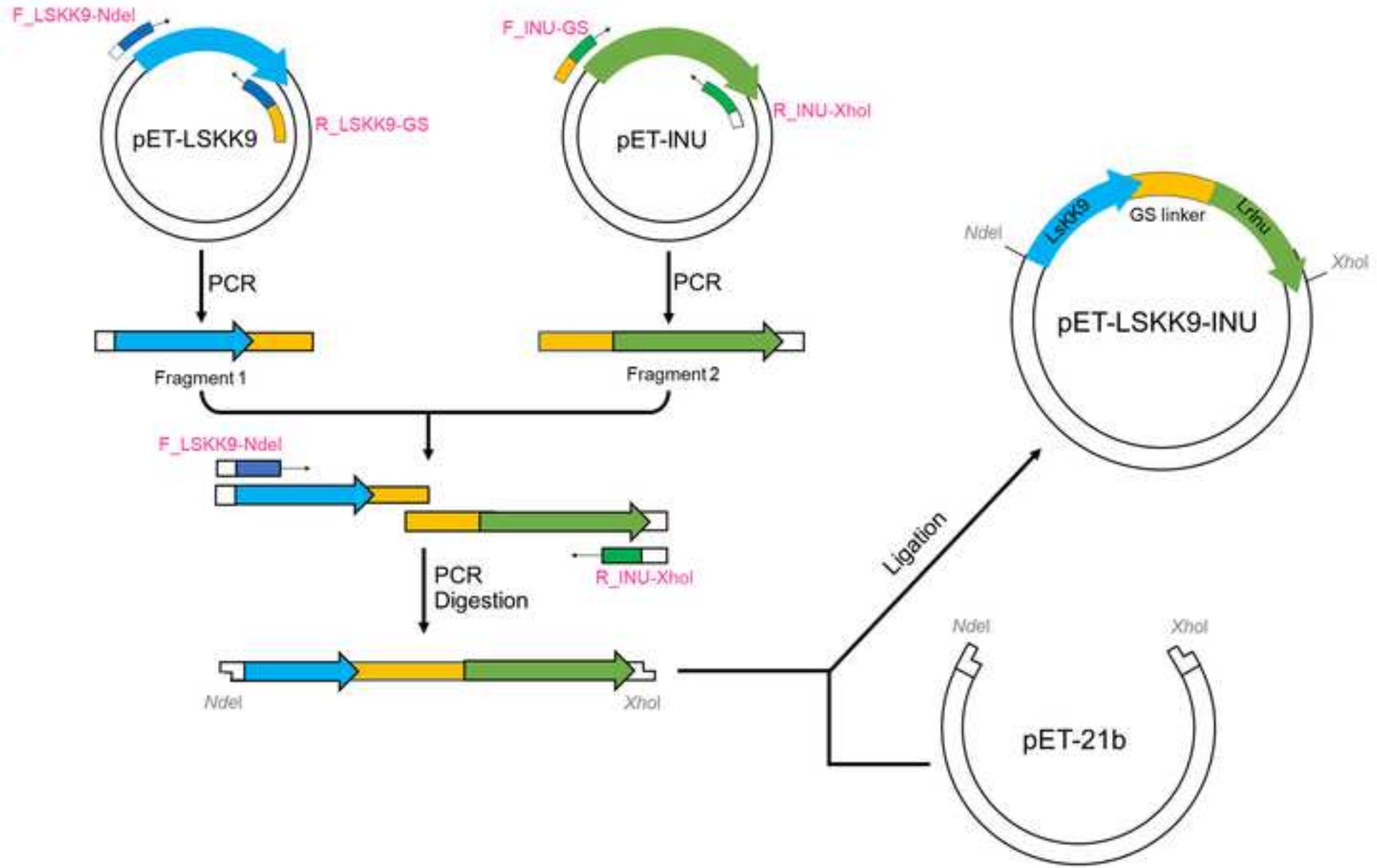

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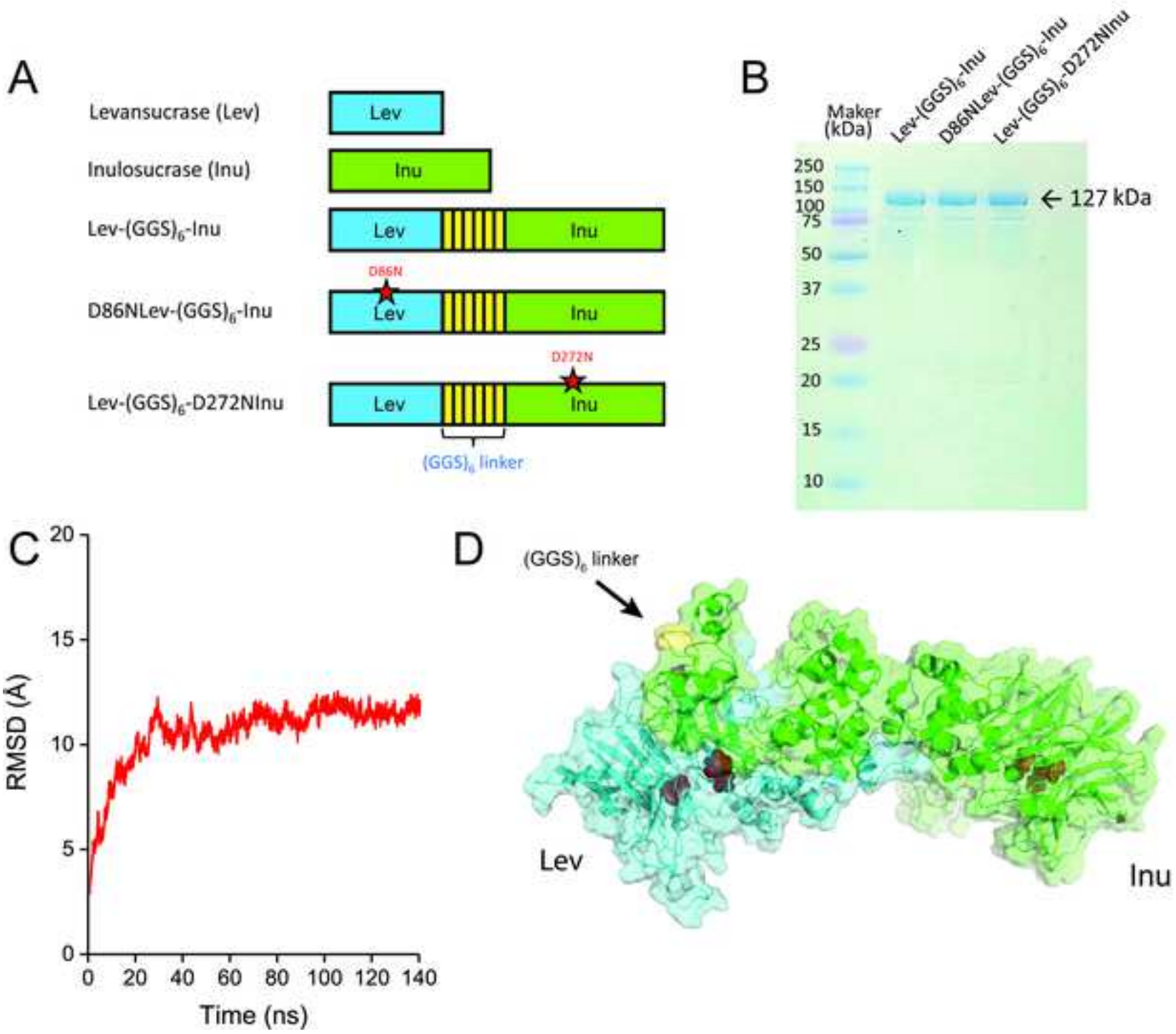

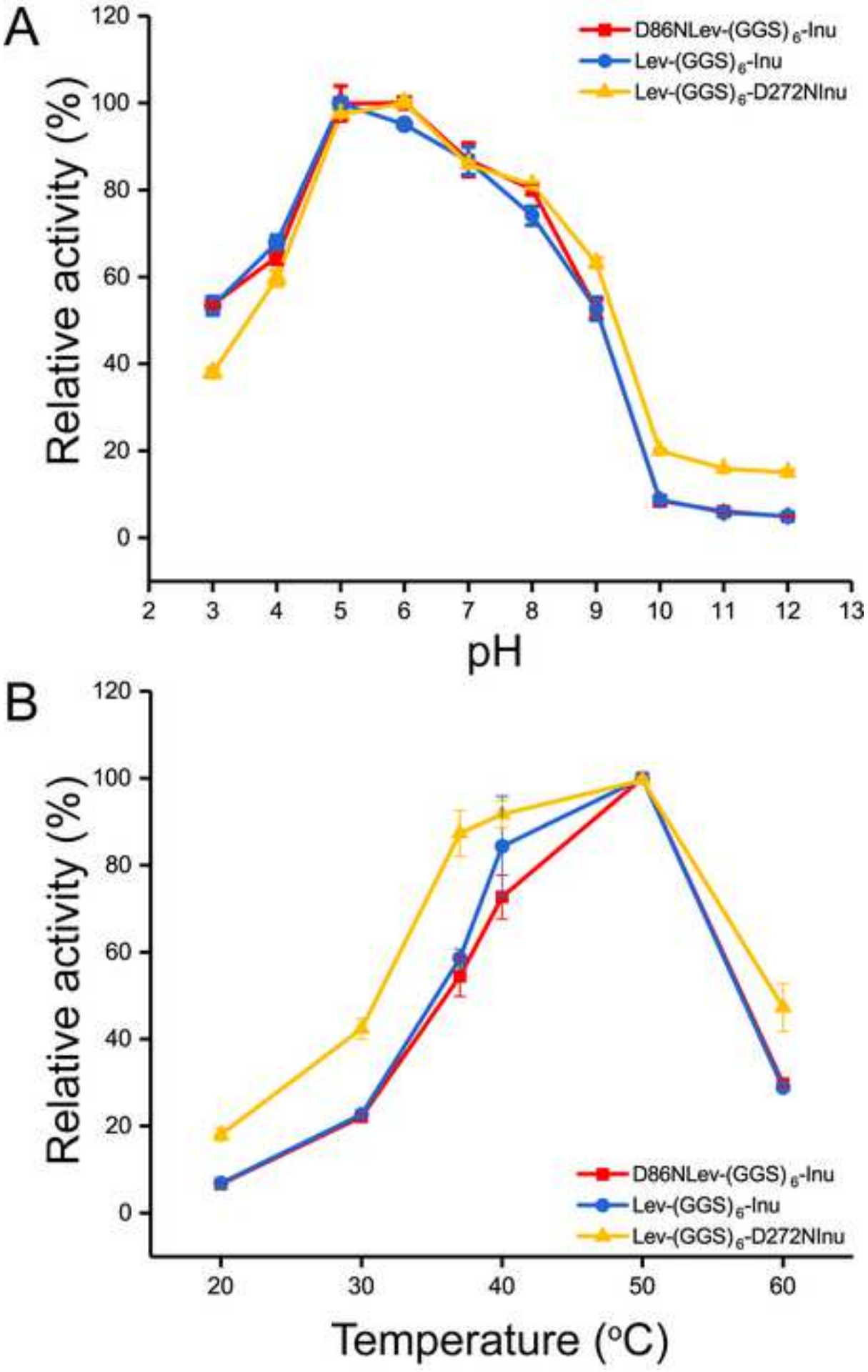

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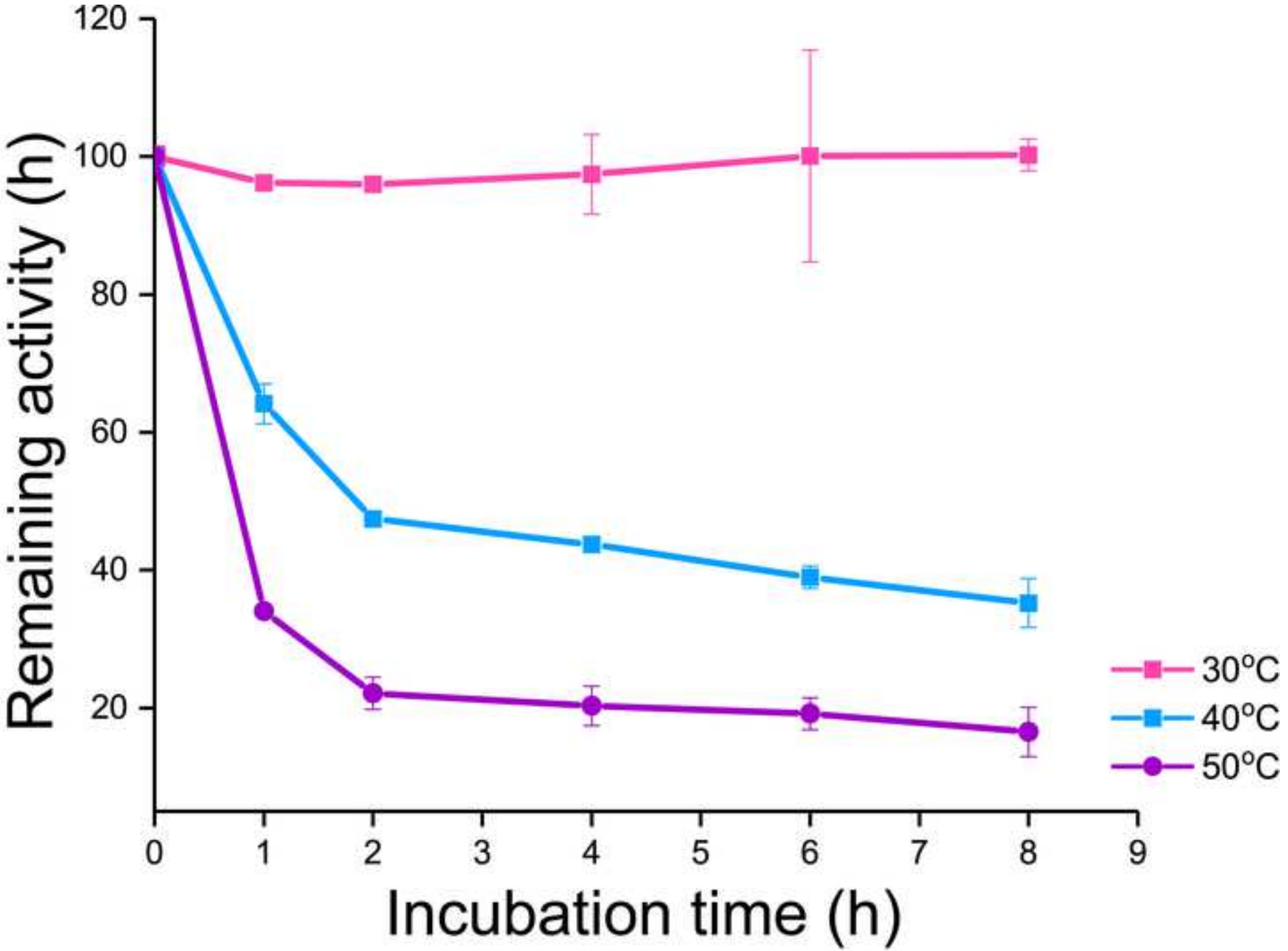

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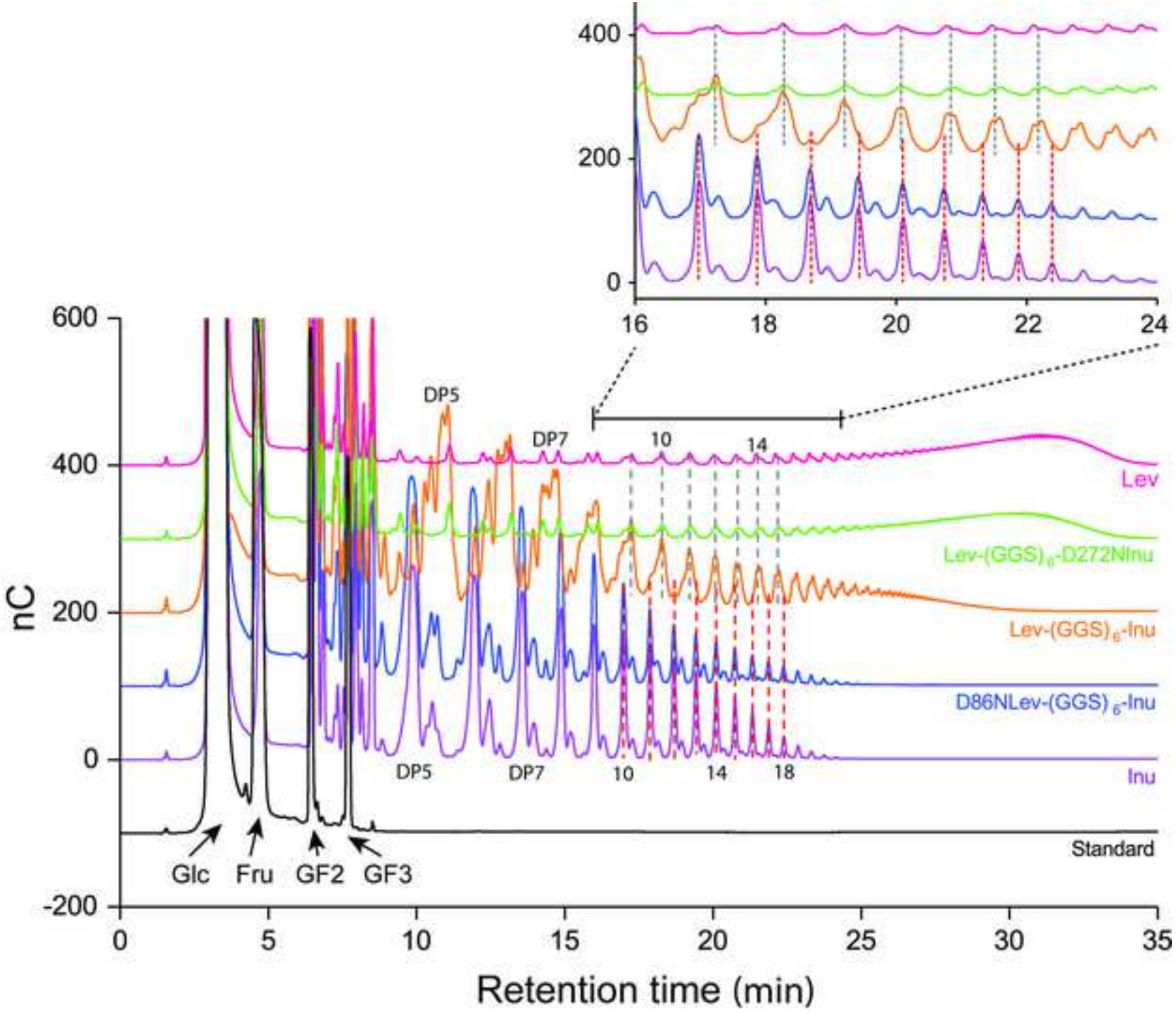

fig6

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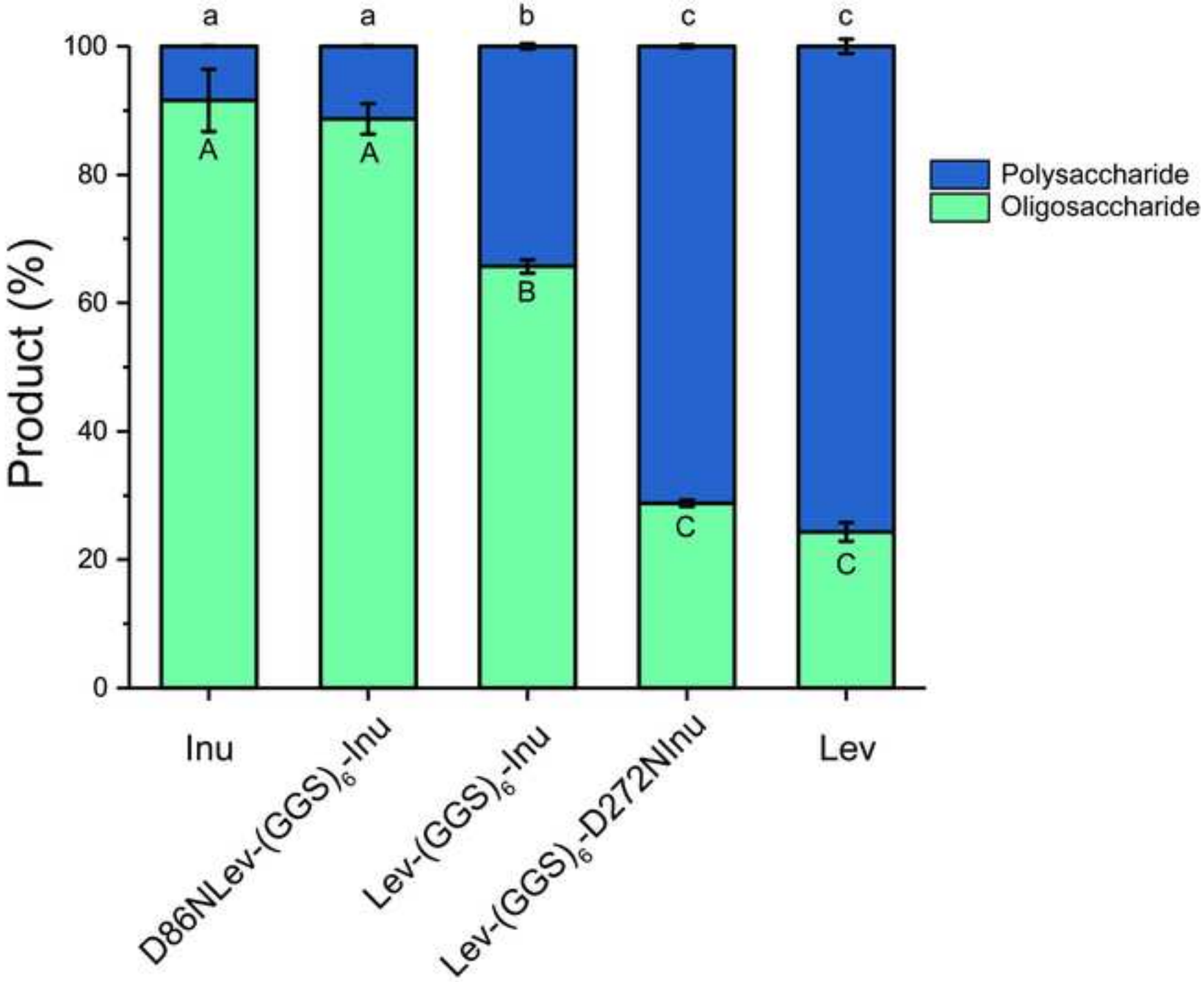

CRediT authorship contribution statement

TC: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration

KW: Investigation, Data Curation

MK: Investigation, Data Curation

KK: Resources

RF: Resources, Writing - Original Draft, Supervision

RP: Conceptualization, Resources, Supervision