Cross-linked enzyme aggregates (combi-CLEAs) derived from levansucrase and variant inulosucrase are highly efficient catalysts for the synthesis of levan-type fructooligosaccharides

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Abstract

Efficient and convenient access to short to medium chain length levan-type fructooligosaccharides (LFOS) is needed in order realise the nutritional potential of this class of bioactive oligosaccharides. While LFOS are synthesised by fructansucrase enzymes, these reactions are routinely associated with high molecular weight fructan polymer formation. Recent studies have shown that FOS production can be enhanced by the combination of levansucrase and inulosucrase in a one-pot reaction. In the present study, the novel mixed enzyme cross-linked enzyme aggregates (combi-CLEAs) based on levansucrase (Lev) and N543A variant inulosucrase (Inu) were prepared by ammonium sulfate precipitation, followed by glutaraldehyde cross-linking. The effect of Lev and Inu ratio on the activity of combi-CLEAs was explored. The results showed that >70% of total sucrase activity was recovered after immobilization and that the combi-CLEAs produced high amounts of LFOS (degree of polymerisation 3 to 21), while high molecular weight polysaccharide production was much reduced. Biochemical characterisation indicated that the optimum pH and temperature of combi-CLEAs (pH 5.5 and 50°C, respectively) were comparable to those of free enzyme; however, the stability of the enzyme was improved. In addition, these combi-CLEAs have operational stability for several reaction cycles, which makes them very attractive for biotechnology applications.

Keywords: Fructooligosaccharide; levansucrase; inulosucrase

Introduction

Prebiotic oligosaccharides have received increasing attention as components of functional foods and nutraceutical products. Of all prebiotics, fructooligosaccharides (FOS) are well-for their ability to selectively promote the growth of bacteria in human gastrointestinal (GI) tract, where they stimulate the enteric immune system [1-4]. FOS can be classified into two groups, based on the type of glycosidic linkage between fructose units. The first group is inulin-type fructooligosaccharides (IFOS), which are found in many plants and fruits [5, 6]. These molecules contain fructosyl units covalently linked by β2-1 linkages. Besides extraction from the plants, IFOS can also be synthesised from sucrose using the fungal β-fructofuranosidase [7, 8] or bacterial inulosucrase [9, 10]. On the other hand, levan-typed fructooligosaccharides (LFOS) mainly bear β2-6 linkages between fructose units. This kind of FOS has not been found in the plants but can also be synthesised by levansucrase [11-13].

Many studies have shown that biological activities of oligosaccharides, such as their prebiotic properties, are strongly dependent on their degree of polymerisation (DP) [2, 14, 15]. In the case of inulosucrase, the DP of its products can be modified by enzyme engineering [9, 16]. In contrast, the DP of LFOS is more challenging to control because the engineered levansucrases mainly produce short-chain LFOS. Moreover, mutants of the latter enzymes also became highly hydrolytic, resulting in a predominant production of monomeric fructose in the reaction [17, 18]. An alternative method to produce the LFOS is to use the hydrolysis activity of endolevanase or endoinulinase to control the size of LFOS. These enzymes can be used to partially hydrolyse the synthetic levan (two-step reaction) or directly added into the levansucrase reaction mixture [19, 20]. Nevertheless, the hydrolysis activity of endolevanase is difficult to control and to obtain the desired size of LFOS, the duration of bi-enzymatic reaction has to be strictly regulated [19].

Enzyme immobilisation is a technique that was been widely used to improve enzyme properties, such as stability and specificity. It can be divided into two main methods: carrier-bound and carrier-free immobilisation. For the latter case, the enzyme molecules are cross-linked together by bi-functional cross-linking agents, providing insoluble aggregates of biocatalysts, called “cross-linked enzyme aggregates (CLEAs)” [21-24]. This technique was used to prepare highly stable and reusable biocatalyst, such as β-galactosidase [25], amylase [26], β-mannanase [27], inulosucrase [28] and levansucrase [29]. In addition, multiple enzymes can be co-immobilized by this technique, generating “combinatorial cross-linked enzyme aggregates (combi-CLEAs)”, which are biocatalysts with multiple different enzyme activities. Various combi-CLEAs have been prepared for one-pot synthesis of high-value products, for example, the

combi-CLEAs of amylosucrase, maltooligosyltrehalose synthase, and maltooligosyltrehalose trehalohydrolase for one-pot synthesis of trehalose from sucrose [30]; combi-CLEAs of hydroxynitrile lyase and nitrilase for synthesis of enantiomerically pure (*S*)-mandelic acid [31]; and combi-CLEAs of amylase, glucoamylase, and pullulanase for one-pot hydrolysis of starch [32].

Previously, we demonstrated that the addition of inulosucrase to levansucrase reactions sharply increased the yield of levan-type fructooligosaccharides (LFOS) at the expense of high molecular weight fructan polymer [33]. 1-Kestose and nystose synthesized from inulosucrase modulated the size distribution of LFOS with concentration-dependent manner. In addition, Lev-Inu fusion enzyme was recently prepared [34]. As such LFOS are considered useful for many biotechnology applications. In this study, combi-CLEAs containing both levansucrase and inulosucrase activity were investigated. N543A variant inulosucrase, which majorly produce 1-kestose and nystose, was utilized for immobilized biocatalyst preparation. The effect of Lev-Inu ratio on activity and product chain length range was explored. The biochemical properties and stability of the immobilised enzyme were studied and compared to that of free enzyme. Finally, the operational stability of these combi-CLEAs were investigated, showing their potential for application of in fructooligosaccharide production.

Materials and methods

Production of recombinant inulosucrase (Inu)

Recombinant inulosucrases (wild-type and catalytic mutant N543A) from *L. reuteri* 121 were produced according to the methods described earlier [9]. In brief, the constructed inulosucrase-expressing plasmid was transformed into *Escherichia coli* BL21 (DE3). The recombinant *E. coli* was cultured in LB broth medium supplemented with 100 µg/mL ampicillin, 0.5 %(w/v) glucose and 10 mM $CaCl_2$. The culture was shaken at 250 rpm, 37 °C until an OD600 reached 0.4 – 0.6. Then, IPTG was added to a final concentration of 0.1 mM. The cells were further shaken at 200 rpm, at 37 °C for 18-20 h. The cells were harvested by centrifugation at 5000 x g for 20 min and lysed by ultra-sonication in buffer A (25 mM KH_2PO_4, 20 mM imidazole and 500 mM NaCl (pH 7.4)). The cell debris was separated from the crude extract enzyme by centrifugation at 15,000 x g for 20 min.

Production of a recombinant levansucrase (Lev)

Recombinant levansucrase from *B. licheniformis* RN01 [35] was produced according to the methods as described previously [33]. In brief, the constructed levansucrase plasmid was transformed into *E. coli* BL21 (DE3). The recombinant *E. coli* was cultured in LB broth medium supplemented with 15 µg/mL kanamycin and 10 mM $CaCl_2$, shaking at 250 rpm, 37 °C. Once an OD600 reached 0.6, the cells were further shaken at 250 rpm, at 16 °C for 18-20 h (without IPTG). The cells were harvested by centrifugation at 5000 x g for 20 min and lysed by ultra-sonication in buffer A. The cell debris was separated from the crude extract enzyme by centrifugation at 15,000 x g for 20 min.

Protein purification

Recombinant Inu and Lev were purified by nickel affinity purification. Crude extract enzymes were loaded into HisTrap™ HP column (5 mL, GE Healthcare) pre-equilibrated with buffer A. The column was washed with buffer A, and eluted with buffer B (25 mM KH_2PO_4, 500 mM imidazole and 500 mM NaCl (pH 7.4)). The fraction containing sucrase activity were collected and dialysed against 50 mM acetate buffer (pH 5.5).

Enzyme activity assay

Activity of Lev and Inu was determined in 50 mM acetate buffer (pH 5.5) containing 5% (w/v) sucrose and 1 mM $CaCl_2$ at 30 °C. The reaction was terminated by adding an equal volume of the DNS reagent [36] and boil for 10 min. The amount of reducing sugar was measured at 540 nm using 0–10 mM glucose as standard solutions. One unit of inulosucrase and levansucrase was defined as the amount of enzyme required to release 1 mmol of reducing sugar per min.

Preparation of combi-CLEAs from levansucrase and inulosucrase

For CLEAs preparation, 0.4 g $(NH_3)_2SO_4$ was added into 1 mL of mixed protein solution (containing 2U/mL of Lev, 0-4 U/mL of Inu and 0.2 mg/mL BSA). After keeping the mixture in shaking incubator at 4 °C, 50 rpm for 3 h, glutaraldehyde was added to a final concentration of 0.5% (v/v). The cross-linking reaction was performed at 4 °C for overnight and was then terminated by adding 100 µl of 1 M Tris-HCl (pH 7.0). The derived CLEAs were collected by centrifugation at 3,000 x g for 20 min and then washed 3 times with 10 mL ice-cold acetate buffer (pH 5.5). The percentage of recovered activity of the aggregated enzyme was determined by the observed sucrase activity of CLEAs/initial sucrase activity of free enzyme × 100%.

Scanning electron microscope (SEM) and FT-IR spectroscopy

A double-sided adhesive carbon disc (Agar Scientific Ltd, Stansted, England) was mounted on the surface of an aluminium pin stub and the freeze-dried samples were brushed onto this using a fine paintbrush. The stubs were then sputter-coated with approximately 5 nm platinum in a high-resolution sputter coater (Agar Scientific Ltd) and imaged using a Nova NanoSEM 450 field-emission scanning electron microscope (Thermo-Fisher, Eindhoven, The Netherlands). Imaging was done at 3kV and digital TIFF files were stored. The Fourier transform infrared (FT-IR) analysis was carried out using Spectrum™ BX FTIR spectrometer (PerkinElmer) in the range of 700 to 4000 cm^{-1}.

Biochemical characterisation of free and immobilised enzymes

The optimal pH for both CLEAs and free enzymes was determined by assaying enzymatic activity in the pH range of 3.6 – 8.0 using 50 mM acetate buffer (pH 3.6-6.0) and Bis-tris buffer (pH 6.0-8.0) at 30°C. For optimum temperature, the activity of both free and immobilised enzymes was assayed in 50 mM acetate buffer pH 5.5 at a temperature range of 20–70 °C. For enzyme stability, the activity of both free and immobilised enzymes was measured after incubating the enzymes in various pH buffer (acetate buffer pH 4.0-6.0 and Bis-tris buffer pH 7.0-8.0) at 30 °C and 50 °C for 2 h.

The operational stability of CLEAs was explored by assessing FOS synthesis in 8 experimental cycles. The reaction conditions were 2 U/ml of combi-CLEAs, 5% (w/v) sucrose, 50 mM acetate buffer (pH 5.5), at 30 °C for 30 min in an orbital shaker. After each cycle of operation, the reaction was terminated by removal of the biocatalyst by centrifugation at 3000 x g for 10 min. The pellets were washed 3 times with ice-cold acetate buffer (50 mM, pH 5.5). The remaining activity of CLEAs was assayed by the method as described above.

Oligosaccharide synthesis using immobilised enzymes

2U/mL of differently prepared CLEAs was incubated with 5-20% (w/v) sucrose containing 50 mM acetate buffer pH 5.5 and 40 mM $CaCl_2$ since calcium is important for inulosucrase activity and stability [37]. After incubation at 30 °C for 24 h, the reaction was stopped by removing biocatalysts by centrifugation at 5000 x g for 10 min and boiling for 5 min. The reaction mixture was analyzed by HPAEC.

Product analysis

The amount of mono-, di-, oligo- and polysaccharide was determined by methods described previously with some modification [33]. In brief, polysaccharide was precipitated by addition of one volume (v/v) of acetone into the reaction medium and then centrifuged at 10,000 x g for 10 min. The

obtained precipitant was washed twice with 50% (v/v) acetone and totally hydrolyzed by boiling in 1M HCl for 30 min. The amount of polysaccharide was calculated from the amount of total monosaccharide released from hydrolysis reaction. The amount of glucose, fructose and sucrose was measured by HPLC using a Sugar-pak™ column (Water™) and an RI detector.

High-performance anion-exchange chromatography (HPAEC): HPAEC was performed by ICS 5000 system (Dionex) and a CarboPack PA100 column (Thermo Scientific) at a flow rate of 0.25 mL/min. The products were separated by using a linear gradient of 0 – 500 mM NaOAc in 150 mM NaOH for 35 min.

Results and discussion

Synthesis of LFOSs by a combination of levansucrase and inulosucrase

Recently, we established the effective synthesis of levan-type fructooligosaccharide (LFOS) by coupling the reaction of levansucrase (Lev) and inulosucrase (Inu) [33]. This method provided a very high yield of LFOS, at the expense of high molecular weight polymer. 1-Kestose, generated by both Lev and Inu, is a key intermediate or acceptor for levansucrase [33, 38]. The binding of 1-kestose to the active site of levansucrase may impact the expansion of the levan chain [33]. In the present study, novel combi-CLEAs of levansucrase and inulosucrase were prepared and used for the one-pot synthesis of LFOS. Since the addition of short-chain β-2,1 FOS, including 1-kestose and nystose, into levansucrase reaction could significantly increase the yield of LFOS obtained, the short oligosaccharide-producing mutant of Inu (N543A) [9] was selected for preparing combi-CLEAs in this study.

Before preparing the co-immobilised enzyme, the coupled reaction between soluble enzymes was examined. HPAEC showed that addition of Inu (either WT or N543A) into Lev reactions significantly increased the yield of oligosaccharide, while the yield of polymer was reduced (Fig 1), which is in line with results of previous studies [33]. Moreover, the HPAEC peak positions of products from mixed enzyme reactions corresponded to those from reactions with Lev alone, indicating that these reactions amplified only the signal of levan oligosaccharides but not inulin oligosaccharides. In comparison to the coupling reaction of WT_Inu and Lev, the coupling reaction of monomeric N543A_Inu and monomeric Lev produced higher amount of LFOS, so, a couple of Lev and N543A_Inu was selected for combi-CLEA

preparation.

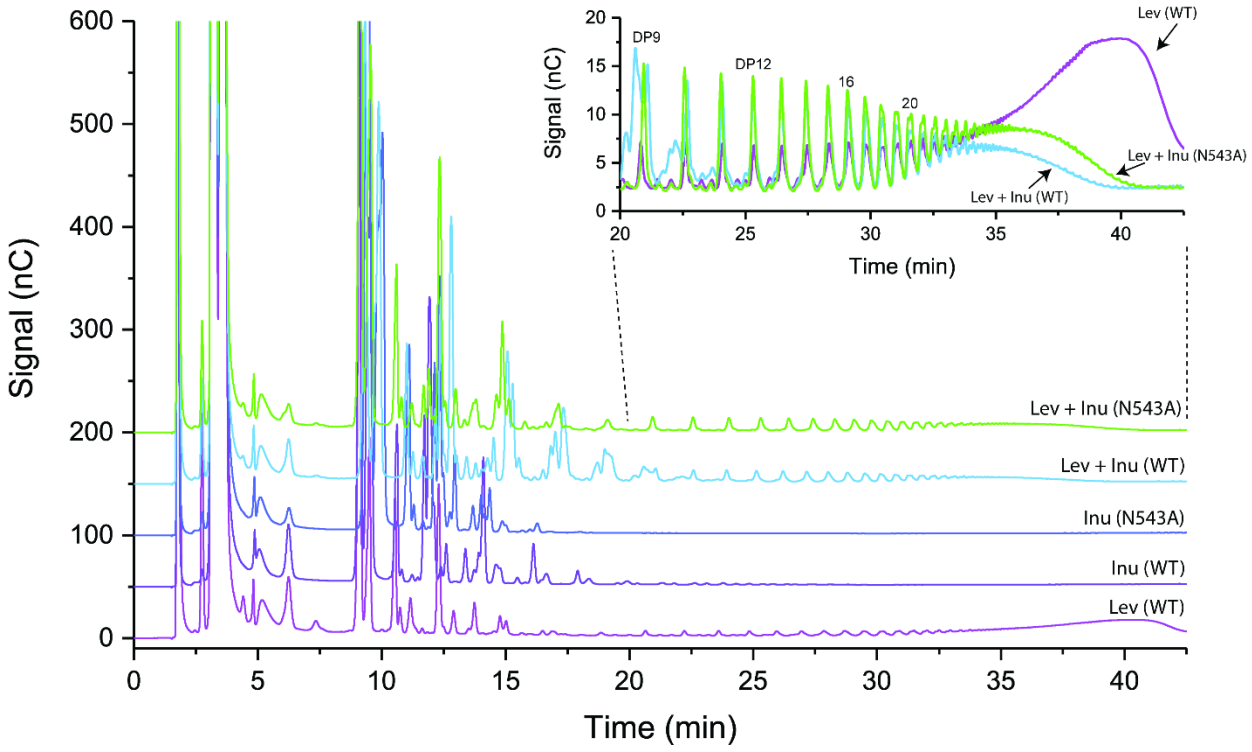


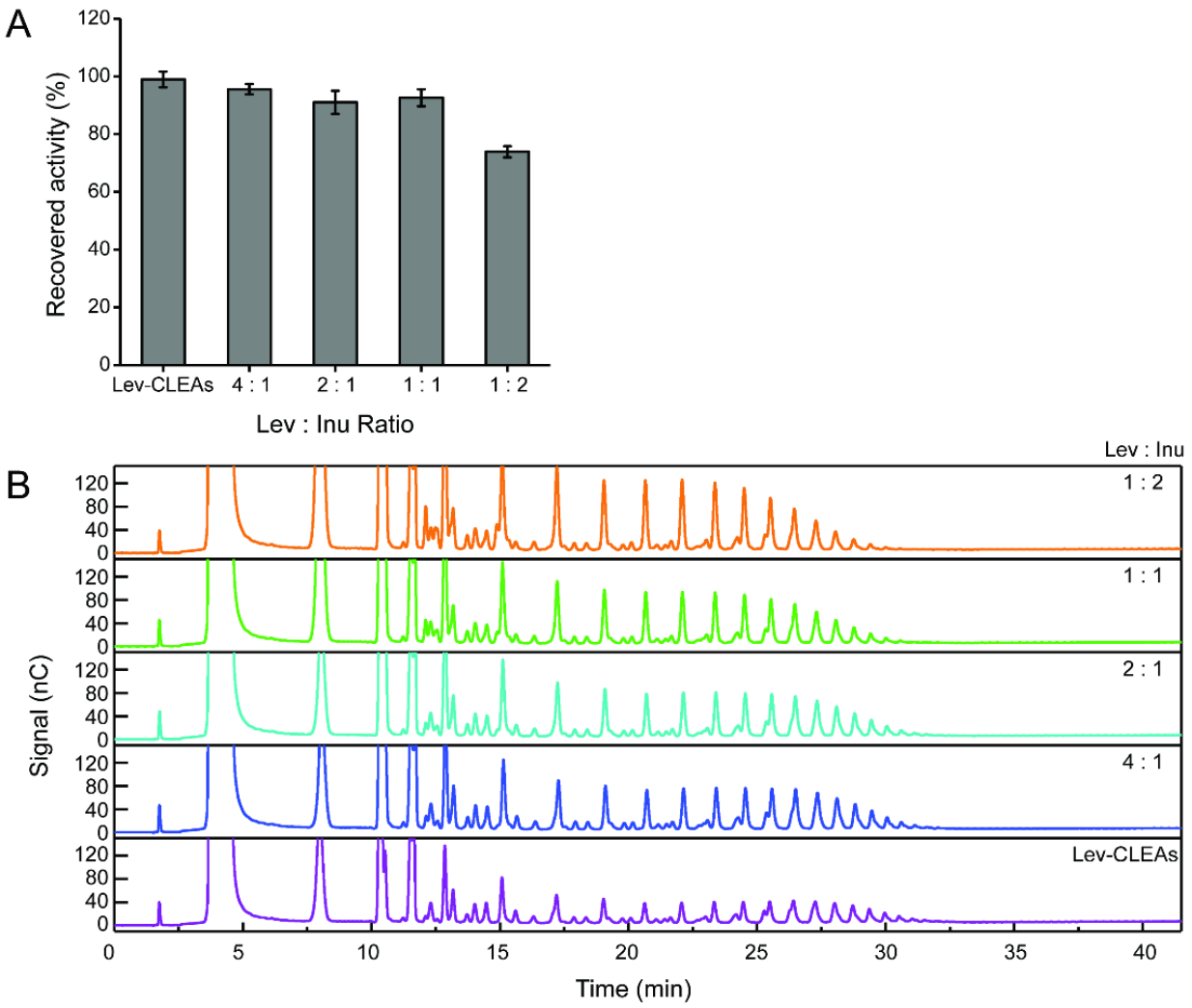
Figure 1 HPAEC chromatogram of fructooligosaccharide produced by Lev, Inu and mixed enzymes.

Preparation of combi-CLEAs from levansucrase and inulosucrase

Generally, CLEAs can be prepared by following two steps: precipitation of the enzyme molecules and cross-linking of the aggregated enzyme by a bifunctional cross-linker [22, 23, 28]. In this study, CLEAs of levansucrase (Lev-CLEAs) and combi-CLEAs of Lev and N543A_Inu were prepared using 40% (w/v) ammonium sulfate as a precipitant before cross-linking by glutaraldehyde. BSA was used as a protein feeder facilitating the protein precipitation in case where the protein concentration is low [39]. Since the Lev-Inu ratio is a key factor in determining the yield and chain length distribution of LFOS synthesised by mixed enzymes, the effect of Lev-Inu ratio on activity and LFOS yield of combi-CLEAs was investigated. The recovered activity of immobilised enzymes was determined from total sucrase activity because it is difficult to assay the activities of Lev and Inu separately. The result demonstrated that the recovered activity of combi-CLEAs decreased marginally when the inulosucrase concentration increased (Fig 2A). As free protein was not found in the soluble fraction, the reduction of enzyme activity might result from enzyme conformational changes, which generally occur upon cross-linking [40]. In comparison with our

previous study, cross-linked enzyme aggregates of inulosucrase (Inu-CLEAs) showed less recovered activity (around 40%) than that of Lev-CLEAs (around 99%) [41]. This may be the reason why the combi-CLEAs prepared by 1:2 Lev-Inu showed the lower recovered activity than other conditions.

The effect of the Lev-Inu ratio on LFOS chain length distribution was then explored by HPAEC-PAD (Fig 2B), which showed that the yield of LFOS tends to increase when the proportion of Inu in combi-CLEAs was increased. The higher amount of Inu generated a higher amount of 1-kestose and nystose, which presumably inhibited high molecular weight levan polymer formation and increased LFOS accumulation. Even though the combi-CLEAs prepared from 1:2 Lev-Inu showed the lowest recovered activity, they produced the highest yield of LFOS. Therefore, the 1:2 Lev-Inu combi-CLEA was selected for further evaluation. In comparison to fusion enzyme [34], co-immobilisation has more advantage since the stoichiometric ratio of the enzymes in biocatalyst could be adjustable. Also, Lev-CLEAs produced a higher amount of LFOS (Fig 3), suggesting that immobilisation may provide a microenvironment allowing only small acceptors to interact with the enzyme [28]. Nevertheless, this Lev-CLEAs still produced a lower amount of LFOS than that of combi-CLEAs, confirming cooperation between inulosucrase and levansucrase for LFOS production.



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207 **Figure 2** (A) Recovered activity of combi-CLEAs prepared by different Lev and Inu ratio. (B) HPAEC

208 chromatogram of LFOS produced by differently prepared combi-CLEAs.

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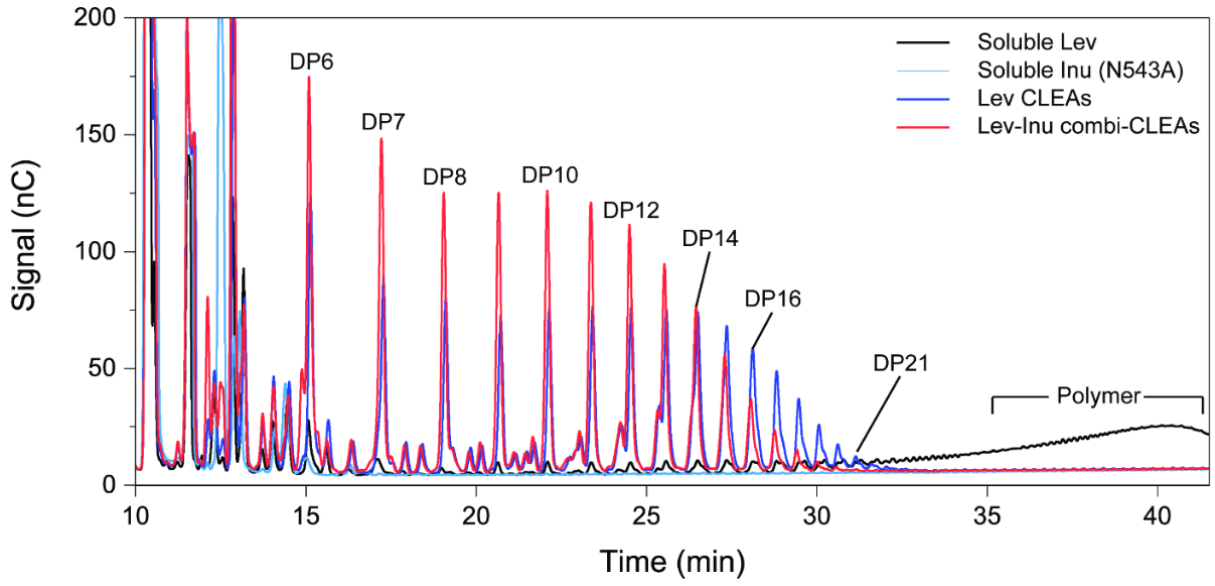

Figure 3 HPAEC chromatogram of FOS products synthesised from 5%(W/V) sucrose using free and immobilised enzymes.

Characterisation of combi-CLEAs from levansucrase and inulosucrase

Lev-CLEAs and combi-CLEAs were characterised by FT-IR spectroscopy and scanning electron microscope (SEM). Both appeared as yellow particle suspensions (Fig 4A). FTIR analysis showed the presence of the cross-linking reaction (Fig 4B): both free and immobilised enzymes showed a high absorption peak at ~3300 cm^{-1}, corresponding to the OH-stretching and −NH stretching vibrations, and high-intensity peaks at ~1700 cm^{-1}, corresponding to C=O stretching of amide I (1700–1600 cm^{-1}) of protein. For immobilized enzyme, the FT-IR spectra showed the intense peak at ~1600 cm^{-1} and ~1460 cm^{-1}. The peak at 1600 cm^{-1} was assigned to the C=N bond of Schiff's base linkages formed between glutaraldehyde and the amino group of the enzyme after cross-linking, while the peak at 1460 cm^{-1} corresponds to C-H bending frequencies of cross-linked glutaraldehyde [42, 43]. This result confirmed the presence of covalent linkages in the aggregated proteins.

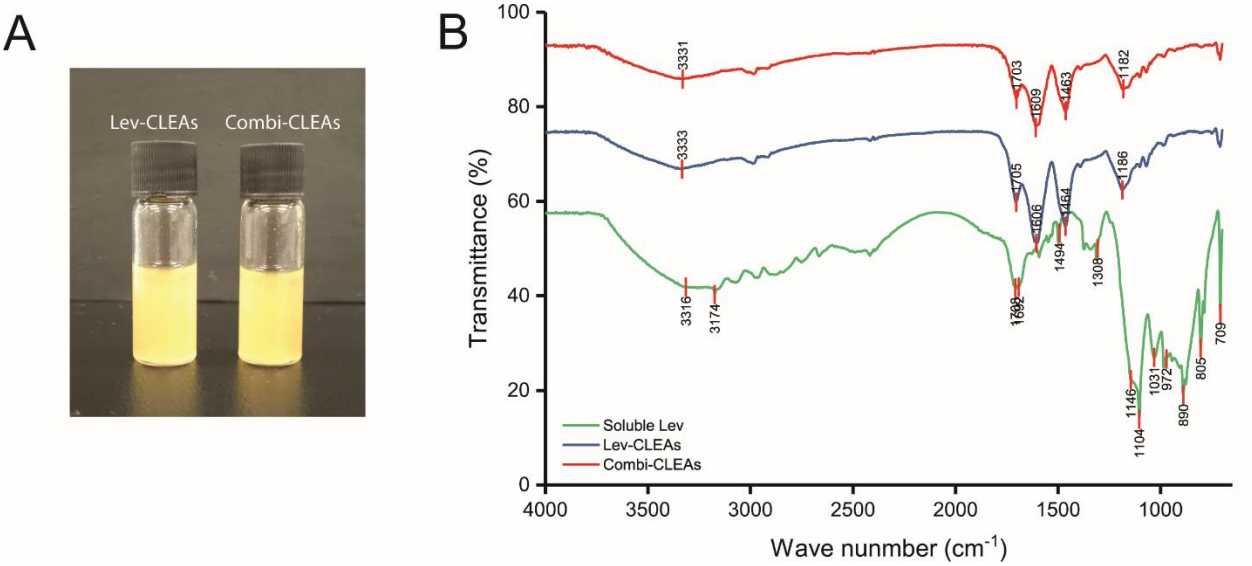

Figure 4 (A) Appearance of Lev-CLEAs and combi-CLEAs. (B) The FT-IR spectrum of free and immobilised levansucrase.

Scanning electron micrograph (SEM) demonstrated that the surface morphology of Lev-CLEAs and combi-CLEAs was uniform, with particles of an approximate diameter of 0.2 – 0.5 µm (Fig 5A-D). The structure of these CLEAs resembles the type 1 protein aggregates that were previously described by Schoevaart et al. [24]. However, both CLEAs have a porous structure, allowing substrate access to the active site of the enzyme. This might be the reason why the Lev-CLEAs and combi-CLEAs have very high activity after immobilisation.

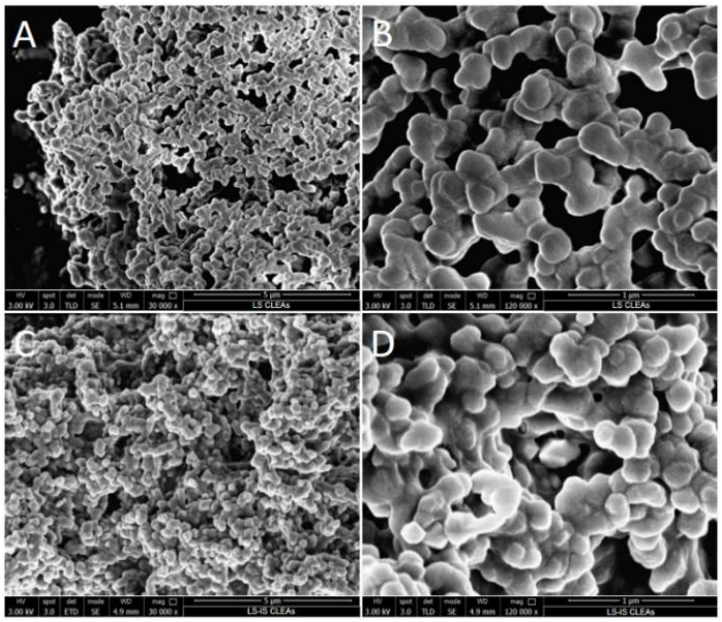

Figure 5 Scanning electron micrograph of (A-B) Lev-CLEAs and (C-D) Lev-Inu combi-CLEAs at magnification 30,000x (A and C) and 120,000x (B and D)

Effect of pH and temperature on activity and stability of free and immobilised enzymes

After immobilisation, the biochemical properties of the enzymes may change. So, their biochemical properties, including optimum pH and optimum temperature, need to be determined and compared to those of free enzymes. As shown in Fig 6A, the optimum pH of combi-CLEAs was comparable to that of the free Inu and Lev enzymes, while the combi-CLEAs were more active in the more acidic pH range were the free enzymes. It suggested that immobilisation by CLEAs can protect the protonation state of the active site of the enzymes. For the effect of temperature on enzyme activity, the combi-CLEAs exhibited similar optimum temperature to the free enzymes at 50°C (Fig 6B), while the combi-CLEAs were highly active at 60°C, a temperature at which the free Lev and Inu enzymes were inactive. This result suggests that immobilisation by CLEAs can retain the active conformation of the enzymes, resulting in increased enzyme stability.

The stability of combi-CLEAs was explored in the pH 4 – 8 at 30°C and 50°C (Fig 6C-D). The stability of combi-CLEAs and free Lev was compared because the aim of this research is to synthesise the levan oligosaccharides. At 30°C, the combi-CLEAs exhibit roughly the same stability to free Lev, while pre-incubation at a 50°C resulted in 100% of initial activity while free Lev retained only ~8%. Therefore, we

can conclude that the preparation of co-immobilized Lev and Inu can substantially promote enzyme stability, which is in line with previous studies in which CLEAs can increase the stability of levansucrase [29] and inulosucrase [41].

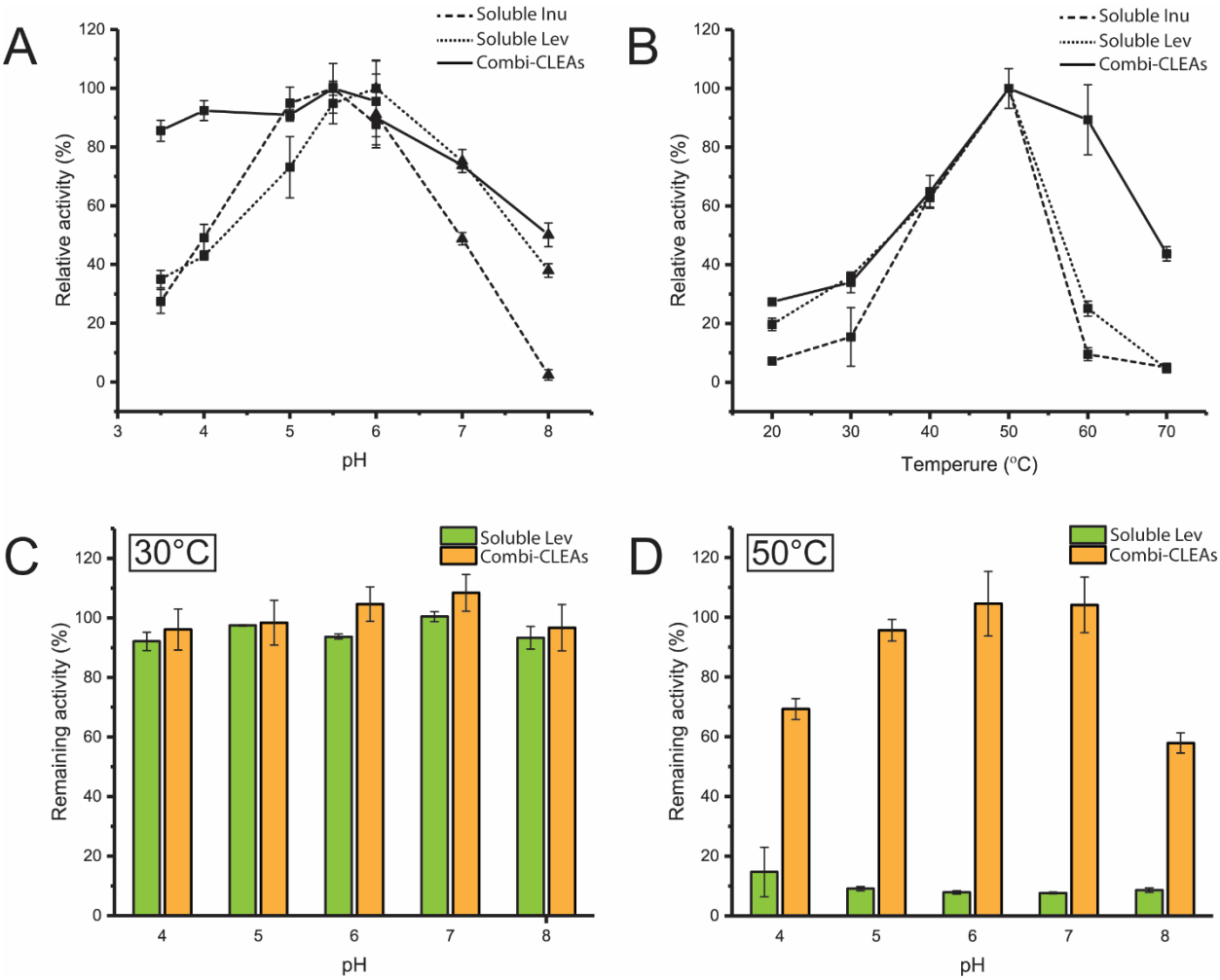

Figure 6 Effect of (A) pH and (B) temperature on the activity of the soluble and immobilised enzyme. (C) and (D) indicate the stability of soluble Lev and combi-CLEAs in the buffer pH 4 – 8 at 30°C and 50°C, respectively.

Synthesis of LFOS by combi-CLEAs

Although the optimum temperature of both levansucrase and inulosucrase are 50°C, the enzymes is not stable at this temperature. Therefore, the synthesis of LFOS was conducted at temperatures below

the optimum range (30°C) since this temperature affords the enzyme with greater transglycosylation activity and stability. Quantitative analysis by HPLC indicated that the combi-CLEAs produced lower amount of polysaccharide than those of free and immobilised Lev, while it produced highest amount of fructan oligosaccharide (Fig 7A-C). In comparison to other system, this combi-CLEAs provided access to a broad range of LFOS, which has not been reported before. Therefore, the combi-CLEAs of levansucrase and inulosucrase (N543A mutant) may prove useful for the synthesis of LFOS and future biotechnological applications.

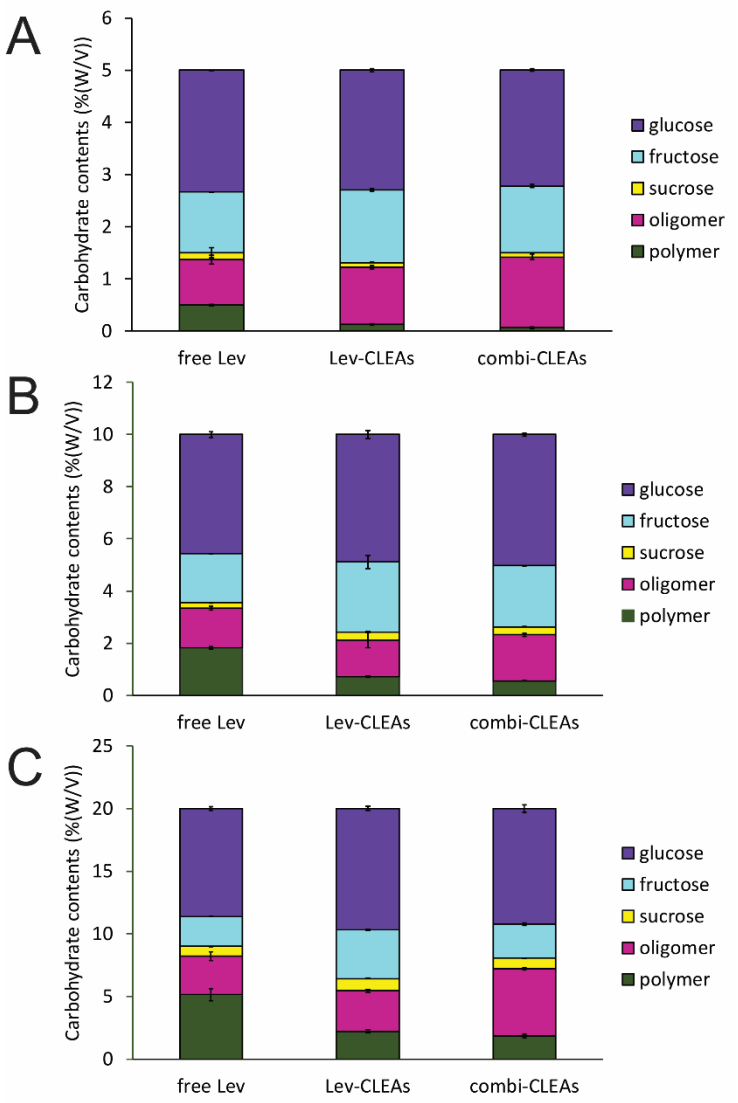

Figure 7 The carbohydrate composition after the conversion of sucrose by free Lev, Lev-CLEAs and combi-CLEAs. The reaction was performed using (A) 5%, (B) 10%, and (C) 20%(W/V) sucrose at 30°C for 24h.

Operational stability

One of the advantages of the immobilised enzymes is its reusability. The operational stability of combi-CLEAs was evaluated in a series of batch reactions, where the remaining activity was assessed after each cycle. As showed in Fig 8, the activity of combi-CLEAs decreased from 100% to 60% after three cycles of reuse. After that, the activity gradually decreased with increasing number of cycles d. The initial decrease of CLEAs activity in the early cycles might be a result of the denaturation of unstable enzyme molecules or desorption of non-covalently bound enzymes. This finding corresponds to many studies on immobilisation of enzymes, including levansucrase [44] and inulosucrase [28, 45].

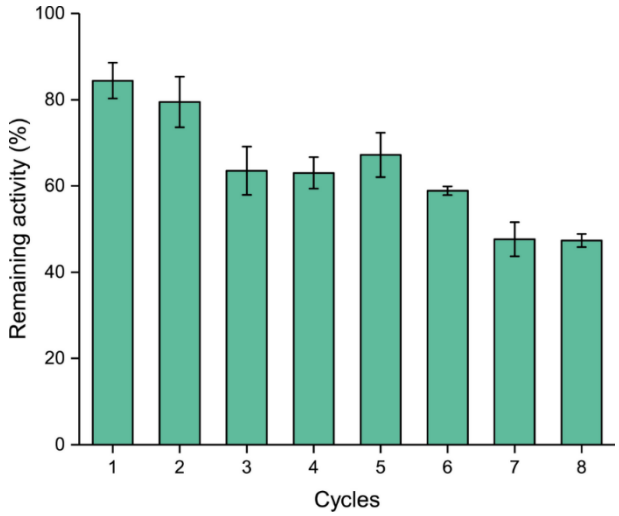

Figure 8 Operational stability of combi-CLEAs.

Conclusions

Herein we report the first preparation and characterization of combi-CLEAs from levansucrase and inulosucrase (N543A). In comparison with free levansucrase and Lev-CLEAs, the combi-CLEAs produced a higher amount of LFOS. Moreover, the immobilization also increased the stability of the enzyme. Product characterization by HPAEC-PAD indicated that this combi-CLEAs could produce a broader range of LFOS, which have not previously been accessible through other enzymatic synthesis procedures. These properties make combi-CLEAs of this type highly attractive as biocatalyst for the synthesis of bioactive LFOSs.

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Graphical Abstract