PROTEIN INTERACTION STUDIES OF CROSS-LINKED ENDOLEVANASE AGGREGATES FROM *BACILLUS LEHENSIS* G1

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Graphical abstract
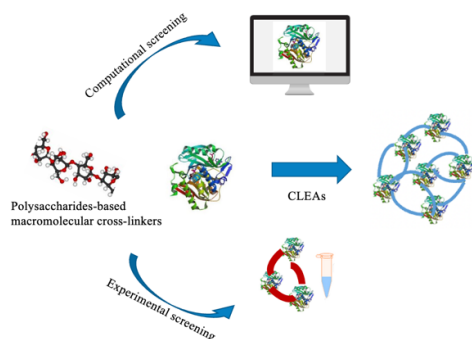
Abstract

The efficiency of cross-linked enzyme aggregates (CLEAs) is mainly affected by the strength and binding site of the formed linkages between the enzyme and cross-linker. Therefore, this study investigated the impact of different macromolecular cross-linkers on various functional groups, their binding energy, and intermolecular interaction in generating CLEAs of endolevanase from *Bacillus lehensis* G1 (rlevblg1), through the combination of computational and experimental analysis. Due to the distanced bonding of dextran from the active site, rlevblg1 cross-linked with dextran (rlevblg1-dex-CLEAs) exhibited the highest binding affinity (− 7.1 kcal/mol) and activity recovery compared to six other cross-linkers. Thus, the role of computational cross-linker screening is confirmed as a crucial step to predict strong attachment and construct efficient CLEAs.

Keywords: Endolevanase, enzyme immobilization, cross-linked enzyme aggregates, macromolecular cross-linkers, computational analysis

Abstrak

Kecekapan agregat enzim silang silang (CLEAs) dipengaruhi terutamanya oleh kekuatan dan tapak pengikatan hubungan yang terbentuk antara enzim dan penghubung silang. Oleh itu, kajian ini menyiasat kesan penghubung silang makromolekul yang berbeza pada pelbagai kumpulan berfungsi, tenaga pengikatnya, dan interaksi antara molekul dalam menghasilkan CLEA endolevanase daripada Bacillus lehensis G1 (rlevblg1), melalui gabungan analisis pengiraan dan eksperimen. Oleh kerana ikatan dextran yang berjauhan dari tapak aktif, rlevblg1 yang dipaut silang dengan dextran (rlevblg1-dex-CLEAs) mempamerkan pertalian pengikatan tertinggi (-7.1 kcal/mol) dan pemulihan aktiviti berbanding enam penyambung silang yang lain. Oleh itu, peranan penyaringan silang silang pengiraan disahkan sebagai langkah penting untuk meramalkan lampiran yang kukuh dan membina CLEA yang cekap.

Kata kunci: Endolevanase, imobilisasi enzim, enzim agregat terpaut silang. pemaut silang makro, analisis komputer

1.0 INTRODUCTION

The carrier-free immobilization technique, CLEAs (cross-linked enzyme aggregates) method has gained considerable attention in industrial applications [1, 2]. CLEAs provide several outstanding advantages including reusability, high stability against harsh operating environments, high catalytic activity, low production cost due to the exclusion of supports, ease of formation, the ability to combine two enzymes or more and the use of partially purified enzymes [1]. CLEAs formation consist of two principal processes which are enzyme precipitation by aggregating agents like non-ionic polymers, organic solvents or salts, afterwards cross-linking of the precipitated enzymes by bifunctional reagent [3].

Glutaraldehyde (GA) is the most utilized cross-linking agent to develop CLEAs of different enzymes due to its low price and availability in the commercial market [4]. However, the catalytic activity of certain enzymes, particularly enzymes with few lysine residues, were reduced after cross-linking with GA [5]. Therefore, macromolecular polysaccharides such as chitosan [6], pectin [7], and dialdehyde starch [8] were used as alternative cross-linkers in CLEAs formation [9]. Nevertheless, applying different macromolecular cross-linkers to immobilize the same enzyme has different impact on the activity recovery [10]. Formation of cyclodextrin glucanotransferase-CLEAs exhibited the highest activity recovery when chitosan was used as a cross-linker followed by dialdehyde-starch, pectin, benzoquinone, (succinic acid *N*-hydroxysuccinimide ester) (EG-NHS) and polyethylene glycol 8000 (PEG8000) [11]. Similarly, the highest activity recovery of maltogenic amylase-CLEAs was obtained using chitosan, l-lysine and ethylene glycol as cross-linkers, respectively [6]. In accordance with these results, it is essential to screen the enzyme with several cross-linkers as well as understand the intermolecular interaction in order to develop the most active CLEAs. However, experimental screening of multiple cross-linkers with the enzyme of interest is a laborious and time-consuming procedure [11].

Computational analysis is a practical method to predict the best equivalent binding mode between a macromolecular protein and a ligand [12], hence it saves time and material. Homology modeling and molecular docking provide insight for the fundamental intermolecular interaction between the protein and cross-linker [11], thus facilitating the selection of the suitable cross-linker as well as enhancing the cross-linking efficiency. Moreover, this technique would predict the binding site of the cross-linker on the enzyme surface which is crucial in order to avoid penetrating the cleft of the catalytic amino acids, thereby ensuring better substrate accessibility and improving the catalytic activity [13].

Therefore, computational estimation was expected to facilitate the finding of the suitable macromolecular cross-linker for endolevanase from

Bacillus lehensis G1 (rlevblg1). Previously, rlevblg1 was immobilized via CLEAs using GA [14] and dialdehyde-starch (DAS) [15] serving as cross-linking agents. Compared to GA, the larger size of DAS helped in decreasing the compactness of CLEAs, improving substrate accessibility and producing longer levan-type-fructooligosaccharides (L-FOS). Surprisingly, upon optimization, the activity recovery of CLEA-DAS was relatively low (67.6 %) compared to CLEA-GA (102.7%). Based on these findings, the concept of using macromolecular cross-linker would enhance the catalytic properties of the immobilized rlevblg1, yet there is a need to find the best possible macromolecular cross-linker for rlevblg1, investigate the intermolecular interactions and confirm the binding site in order to maximize the activity recovery after cross-linking.

This study focuses on determining the effect of several macromolecular cross-linkers on various functional groups, their binding energy, and the molecular interplay of surface amino acid residues of rlevblg1 in cross-linking. This study investigated the intermolecular interaction of seven cross-linkers, namely, cellulose, chitosan, dialdehyde-starch (DAS), dextran, pectin, polyethylene glycol 8000 (PEG8000) and sodium alginate with rlevblg1. The *in silico* results were confirmed by evaluating the retained activity recovery of the experimental screening.

2.0 METHODOLOGY

2.1 Material

All standard laboratory grade chemicals and reagents used in this study were purchased from Thermo Fisher scientific (USA), Sigma-Aldrich (USA) and Merck (Germany) unless stated otherwise.

2.2 Construction of rlevblg1 Model Structure and Molecular Docking Analysis

The three-dimensional (3D) structure of endolevanase from *Bacillus lehensis* G1 (rlevblg1) was constructed using the homology modeling carried out in the Modeller 9.13 software. The protein sequence of rlevblg1 is shown in the supplementary section (Figure S1). Fructofuranosidase from *Schwanniomyces occidentalis* (PDB ID: 3KF3) was used as the template because of its low expectation value (E-value) with sequence identity and similarity exceeding 30% would generate an adequate model [11]. Among of hundreds of rlevblg1 generated model, the model with the smallest energy value; based on the discrete optimize protein energy (DOPE) and molecular pdf; was further evaluated by the root mean square deviation (RMSD) and the Ramachandran plot [16].

Subsequently, docking analysis was performed via subjecting the 3D structure of rlevblg1 (receptor) to different cross-linkers (ligands). The .pdb file of each cross-linker (cellulose, chitosan, dialdehyde-starch

(DAS), dextran, pectin, polyethylene glycol 8000 (PEG8000) and sodium alginate) was acquired from the PubChem database [17]. Interactions between rlevblg1 and cross-linkers were simulated using the Autodock Vina (1.5.6, 2014) software [18]. The pdbqt files of receptors and ligands were generated from their conventional PDB files using Autodock Tools [19]. Hydrogen atoms were appended to these macromolecules, and their partial-atomic charges were computed. A matrix of 80 × 86 × 88 points, centered at coordinates X: 17.089, Y: 8.993, and Z: 24.869, was employed in the configuration file of AutoDock Vina to evaluate the entire rlevblg1 enzyme. Other docking variables were set as default.

2.3 Preparation and Optimization of Cross-linked rlevblg1 Aggregates

Enzyme (rlevblg1) expression, purification and development of CLEAs was performed using the protocol of Abd Rahman, Jaafar [15] with some modifications. For enzyme precipitation, 60% of ammonium sulphate was added to 0.4 mg/mL of the enzyme with 200 rpm orbital stirring at 4 °C for 1 h. The aggregated enzyme was then cross-linked by the addition of the optimal concentration (0.8% (v/v)) of the macromolecular cross-linker [15] (cellulose, chitosan, dialdehyde-starch (DAS), dextran, pectin, polyethylene glycol 8000 (PEG8000) and sodium alginate) in total volume of 1 mL with constant agitation of 200 rpm at 4 °C for 1 h. The enzyme assay [20] and recovery activity of immobilized enzyme were calculated from equation 1 and 2, respectively:

(1)
$$\text{Enzyme activity} \left(\frac{U}{mL}\right)$$
$$= \frac{\text{Fructose released (μmol)} \times \text{Total volume of assay (mL)}}{\text{Volume of enzyme (mL)} \times \text{Time of assay (min)} \times \text{Volume in cuvette(mL)}}$$

(2)
$$\text{Recovery activity (\%)} = \frac{\text{Activity of CLEAs} \left(\frac{U}{mg}\right)}{\text{Activity of free enzyme} \left(\frac{U}{mg}\right)} \times 100\ \%$$

3.0 RESULTS AND DISCUSSION

3.1 Computational analysis

3.1.1 Homology Modeling and Structure Refinement

The rlevblg1 structural homology modeling was performed using comparative protein modeling [21]. The primary sequence of rlevblg1 was submitted to two different protein alignment servers (NCBI-BLAST and HHpred) to identify the homologous structures (Table 1). In this study, fructofuranosidase from *S. occidentalis* (PDB ID: 3KF3, 1.90 Å) was used as the template owing to its low E-value (6 × 10⁻⁶⁷) and adequate sequence identity (33%) [22] when compared with rlevblg1. To determine the structural and folding similarities between both proteins, the 3D

structure of rlevblg1 (Figure 1 A) was aligned against 3KF3 with PyMOL (Figure 1 B), and the RMSD value was 0.224 Å with the structure similarity of 84%. The model was validated using the Ramachandran plot analysis (Figure 2), with 93.4% of the residues occurring in highly preferred observations and 5.1% in preferred observations.

Table 1 The alignment search result against the PDB database

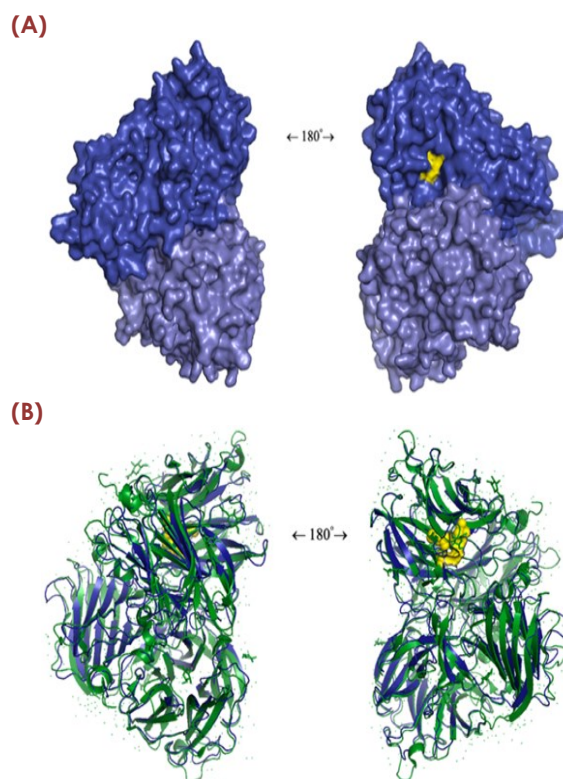| Server	Template (PDB code)	Identity	E-value
NCBI-BLAST	Fructofuranosidase (3KF3)	33%	6×10^{-67}
	Invertase (3KF5)	33%	8×10^{-67}
	E230A-fructofuranosidase (3U75)	33%	5×10^{-66}
	D50A-fructofuranosidase (3U14)	33%	1×10^{-65}
HHpred	Exoinulinase (6J0T)	31%	1×10^{-47}
	Invertase (4EQV)	30%	4.3×10^{-49}
	Endo-inulinase (3SC7)	29%	1.9×10^{-47}
	Levan fructotransferase (4FFH)	27%	1.9×10^{-49}

(A)

(B)

Figure 1 (A) Homology model of endolevanase from *Bacillus lehensis* G1 (rlevblg1) using PyMOL. Domain A is shown in blue, domain B in purple and active site in yellow. (B) Alignment of the 3D structure of rlevblg1 and fructofuranosidase from *Schwanniomyces occidentalis* using PyMOL. Endolevanase is displayed in blue, fructofuranosidase in green and catalytic site in yellow
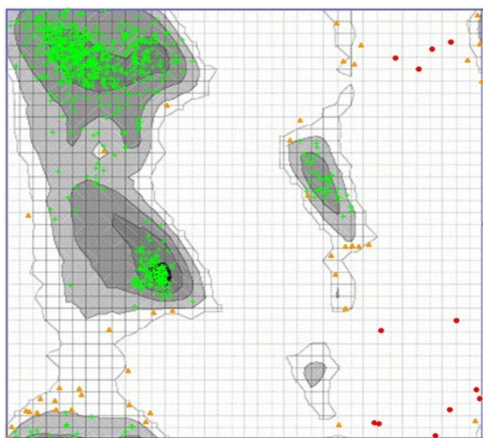
Figure 2 Ramachandran plot of the *in silico* structure of rlevblg1 using RAMPAGE server. Residues displayed in green are in highly preferred observations, orange are in preferred observations, and red are in questionable observations

3.1.2 Molecular Docking Analysis

The formation of CLEAs requires a robust protein cross-linking with the cross-linker to yield a durable CLEAs immobilization without a carrier [23]. Therefore, docking simulation was utilized to investigate the fundamental bindings between rlevblg1 and ligands (cross-linkers). The homologous 3D rlevblg1 based on modeling served as the receptor molecule, and 3D cross-linkers (cellulose, chitosan, dialdehyde-starch (DAS), dextran, pectin, polyethylene glycol 8000 (PEG8000) and sodium alginate) acquired from the PubChem database were used as ligands, generating nine conformations of ligand-rlevblg1 binding. The best configuration of each cross-linker interacting with rlevblg1 was determined using the smallest free binding energy value. A lower score of energy demonstrates better affinity or fitness for the protein-ligand binding [12].

Based on Table 2, among the compared macromolecular cross-linkers, dextran (-7.1 kcal/mol) showed the lowest binding energy, followed by chitosan (-7.0 kcal/mol), pectin (-6.7 kcal/mol), cellulose (-6.7 kcal/mol), sodium alginate (-6.6 kcal/mol), DAS (-4.7 kcal/mol), and PEG8000 (-4.0 kcal/mol). The strongest binding affinity of dextran was attributable to a high number of hydrogen bonds (12) interacting with six residues of the enzyme. For hydrogen bonds, seven were strong with less than 3.00 Å in length, and six residues were engaged in hydrophobic interactions (Supplementary Data, Table S1). In total, dextran interacted with eight amino acids, i.e., Asn, Asp, Glu, Phe, Thr, Trp, His, and Leu that are distanced from the active site (Asp20, Asp133 and Glu184) of rlevblg1, thus the interference of dextran with the catalytic reaction was confirmed to be avoided. However, other cross-linkers formed less hydrogen bonds and interacted with different amino acid residues of rlevblg1, according to the distinguished differences in their structure and

biochemical properties [11]. Cellulose, DAS and PEG8000 interacted near-with the vital amino acid residues of rlevblg1. Therefore, the cross-linkers were tested experimentally to confirm the reliability of the molecular docking analysis.

Table 2 Computational docking analysis of cross-linkers with rlevblg1

Cross-linkers (ligand)	Binding affinity (kcal/mol)	3-D schematic diagram interaction (catalytic site in yellow &cross-linker in red)	Hydrogen bound - residues involved	Hydrophobic interactions-residues involved
Cellulose	-6.7		Pro399, Glu398, Arg832, Asp846, Glu828, Asp394, Thr827, Arg395	Gly330, Phe400, Phe329
Chitosan	-7.0		Ser543, Lys607, Tyr377, Asn494, Asp378, Arg604, Asn683	Pro493, Tyr746, Glu684, Thr690,
DAS	-4.7		Gln8, Phe9, Thr341, Glu425, His472	His311, Trp312, Tyr343
Dextran	-7.1		Asn612, Thr614, Asn729, Glu731, Asn732, Asp733	Glu613, Phe661, Thr730, Trp737, His611, Leu663
Pectin	-6.7		Met759, Gly488, Thr487, Gly757	Pro476, Pro479, Gln480, Glu475, Trp755, Pro754, Met756, Pro486, Phe481, Ala758
PEG8000	-4.0		Gly70, Pro134	Pro21, Asn22, Ser69, Ser71, Asp133, Lys135, Gln253
Sodium alginate	-6.6		Thr445, Gly397, Gly403, Thr385, Ala387	Tyr444, His396, Ile443, Ans386

3.2　Experimental screening

3.2.1　Expression and Purification of rlevblg1

Expression of rlevblg1 in *E. coli* BL21 (DE3) was conducted using an auto-induction medium at 25 °C under continuous shaking for 24 h [24]. After 24 hours, the cells were harvested and ruptured via sonication to collect crude rlevblg1 prior enzyme purification by AKTA Prime purification system (GE Healthcare). Table 3 summarizes the purification table of rlevblg1. The crude enzyme exhibited 489.3 U/mL and 5.5 mg/mL as enzyme activity and protein concentration, respectively. After purification, 66.5% of the enzyme activity was recovered with an increase in the protein concentration (6.9 mg/mL) and specific activity (235.6 U/mg). In addition, the single bond and expected size (~69.5 kDa) of purified rlevblg1 was successfully visualized using SDS-PAGE, as demonstrated in Figure 3.

Table 3　Purification table of rlevblg1

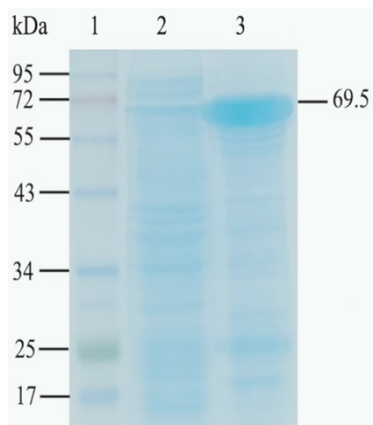| Sample	Enzyme activity (U/mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude	489.3	17125.5	192.5	88.9	100.0	1.0
Purified	1625.9	11381.3	48.3	235.6	66.5	2.7

Figure 3　SDS- PAGE analysis of crude and purified rlevblg1. 1: protein ladder, 2: crude rlevblg1 and 3: purified rlevblg1. The size of rlevblg1 is approximately 69.5 kDa

3.2.2　Effect of Cross-linker Types on the Activity Recovery of the Immobilized rlevblg1

To validate the computational screening of the suitable cross-linker, CLEAs was developed using several macromolecular cross-linkers including cellulose, chitosan, dialdehyde-starch (DAS), dextran, pectin, polyethylene glycol 8000 (PEG8000) and sodium alginate, (Figure 4). Compared to the other macromolecular cross-linkers, dextran retained the highest activity recovery (12.7%). Based on docking analysis, dextran interacted with the highest surface amino acid residues that were far from the catalytic sites of rlevblg1, therefore, substrate accessibility was facilitated and enzymatic activity was improved. Moreover, pectin, chitosan and sodium alginate were predicted to have a slightly weaker binding affinity than dextran and experimentally maintained 10.9%, 10.3% and 8.4% activity recovery, respectively. In addition, DAS had weak binding affinity (-4.7 kcal/mol) and exhibited low catalytic activity (5.9%). Interestingly, cellulose exhibited relatively strong binding affinity (-6.7 kcal/mol) toward rlevblg1 and retained low activity recovery (5.4%). This could be caused by the penetration of cellulose to the inner part of the enzyme residues that are near the active site (Table 2). In contrast, PEG8000 displayed possible penetration of the active site of rlevblg1 and the weakest binding affinity (-4.0 kcal/mol). However, rlevblg1 cross-linked with PEG8000 retained relatively high activity recovery (8.5%). Compared to cellulose (570,000 g/mol) [25], PEG8000 is a smaller molecule (8,000 g/mol) thus the active site of rlevblg1 was not completely blocked. In addition to the small size, PEG8000 is a nonionic surfactants [26] that can potentially alter the enzyme structure and disrupt the forces that hold the protein in its native conformation [27].
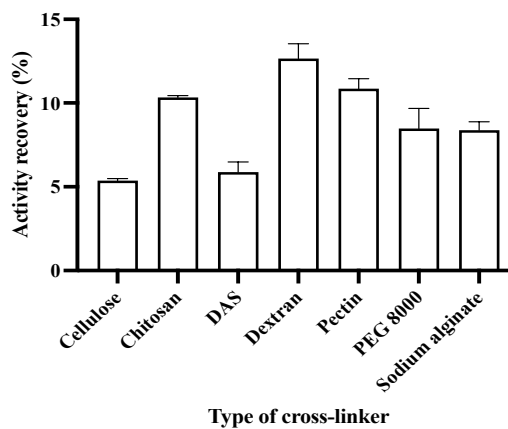

Figure 4　The influence of different macromolecular cross-linkers on the activity recovery of the immobilized rlevblg1. The experiments were conducted in triplicate and error bars represent standard deviations

4.0　CONCLUSION

Studying the interactions and screening of cross-linkers to develop an efficient cross-linked enzyme aggregates (CLEAs) using the combination of computational analysis and experimental data was proven to be a useful strategy. Dextran displayed the strongest binding affinity (-7.1 kcal/mol) toward rlevblg1 as well as highest activity recovery (12.7%) among six other cross-linkers. These findings confirm the promising potential of computational analysis in

developing qualified CLEAs for many industrial applications.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.
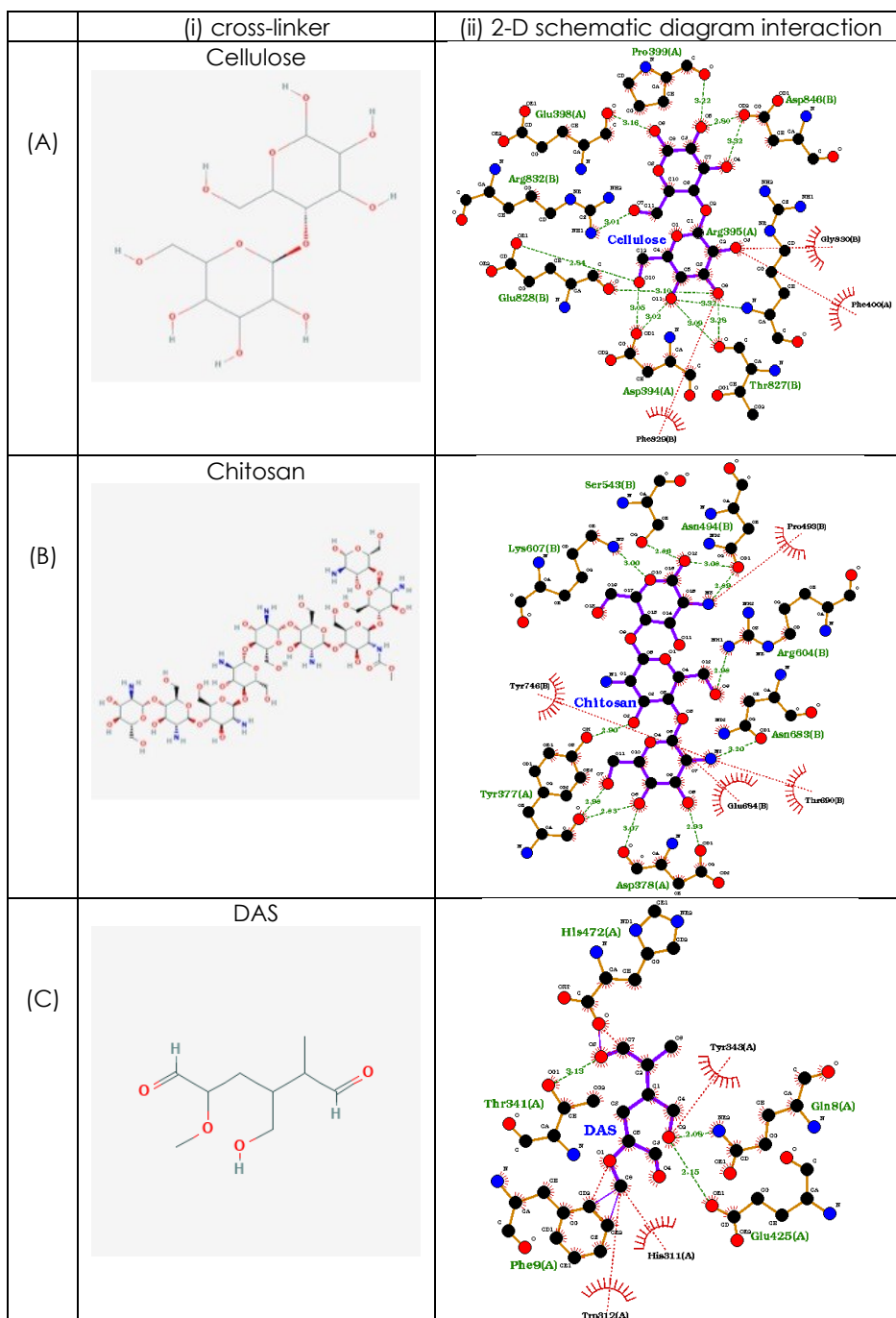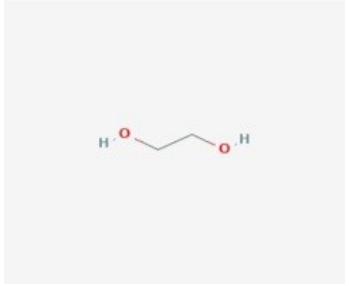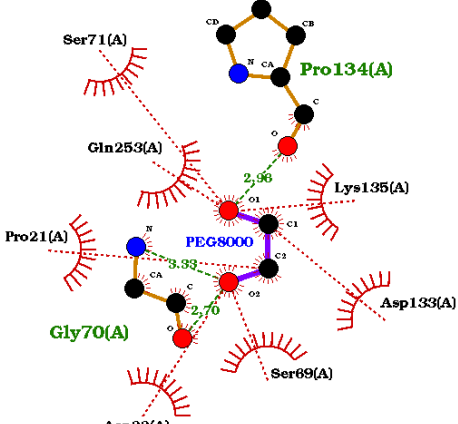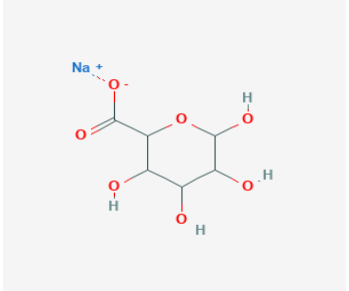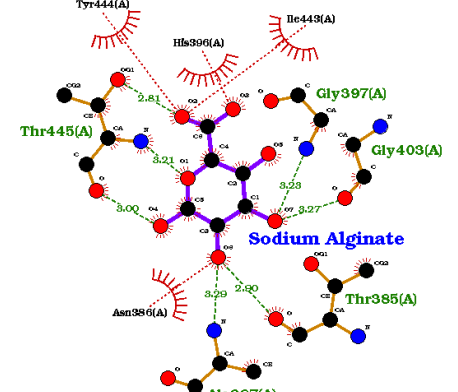
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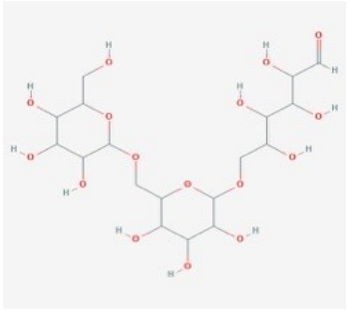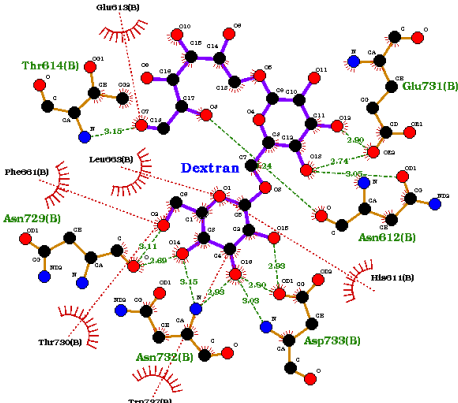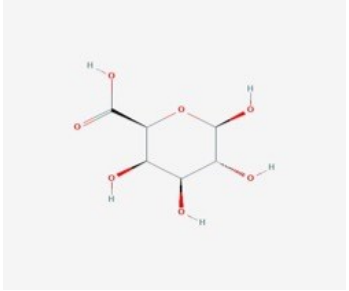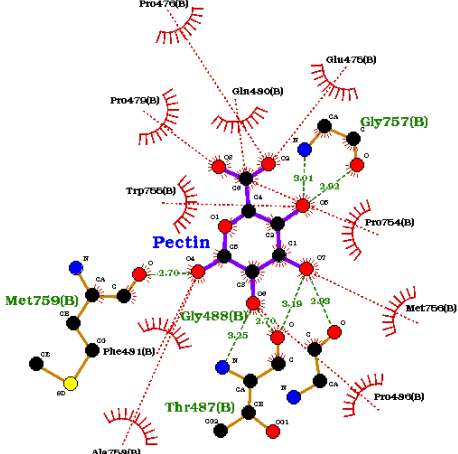
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Supplementary Data

Table S1 Docking analysis between rlevblg1 and cross-linkers. (A) cellulose, (B) Chitosan, (C) DAS, (D) dextran, (E) pectin, (F) PEG 8000, and (G) sodium alginate. (i) 2-D structure of cross-linker obtained from PubChem and (ii) 2-D schematic diagram interaction where the purple line represents the ligand, green dashed line represents hydrogen bond, and red dashed line represents hydrophobic interaction.

	(i) cross-linker	(ii) 2-D schematic diagram interaction
(A)	Cellulose 	
(B)	Chitosan 	
(C)	DAS 	

(D)	Dextran	
(E)	Pectin	
(F)	PEG8000	
(G)	Sodium alginate	

ATGATTCATCCATTAAGTGATGACAAAACATATTATACAGAGCCTTACCGTCCACAGTTCC
ATTTCTCGTCTCCAACTGGAAATTTAGCTGACCCCAACGGACTCGTTTATTATGAAGGAGA
ATATCATTTGTTCCATCAAAAGAATGGGACGTGGGCACATGCAATAAGTAGCGATTTACT
TCATTGGAACCATATGCCAATTGCATTAGAGCACGATGAACTTGGTCAAGCGCTTTCCGG
AAGTGTCGTTGTTGATGAAGAAGATTCTTCTGGCTTGTTTGAAGGAAAAGCCGGACTTGTT
GCTCTATATACGAATACAGAGGGCGGAGAAGCTCAGTCTATTGCGTACAGTCAAGATAAT
GGTCGAACGTGGGAGCGCTATGGTGAAAATCCGGTCATTCAAAACCCAGGTATAAAAGAT
TTTCGAGACCCGAAAGTGTTCTGGCACAACGAAACGTCACAATGGGTGATGGTCGTGTCT
ACAGATAAGTCCGTTACGTTTTATCAATCACAAAATTTAATTTATTGGAAGTACGCCAGTC
GCTTTGGTGATGGTGAAGGCTCCCATGTAGCTGTTTGGGAGTGCCCGGATCTTTTTCGCTT
ACCTGTAGATGGAAATACCGAAAATGAGAAATGGGTACTTCATGTGAGTGTCGGTGATAA
CGAAGAAACAAATGGTTCAACAGCTCAGTATTTTATTGGTGAATTTGATGGCACTACATTT
ACAAACGATCATGATCCAGAAGAGGTGCTGATTACAGACTATGGCCAAGATTTTTATGCG
GCACAAACATTTTCAAATACAGAGAACGATAAAGTGATTTGGCTTGGATGGATGGCAAAT
TGGCGCTATCCGTATCAATCACCTACTAATCCTTGGATGGGTGCCATGTCCATTCCAAGAG
AATTAAGTTTAGTTACAAATGATCAAGGGGAGGTTCGCCTTTTTCAACAGCCGATTCATTG
GCTAGATGATCTATCAGCTAAACAGGAACCGCTTCAAACATTCCATGTGAGTGGGGAGAA
AGCTTTACCATTAAAGGTCTCAGGTACAACCTATCGGTATGAAGCAACCGTTAGATGGGA
TGAATTGACTGAATTTGGCGTGCGACTAAGACATGGAGATGGAATGGAGTCACTTATCGG
TTTTGATGCAGCGTACGATAAACTATTTCTTGATCGAACAAATGCTGGATTAGAAACGATT
GTTGATCGTCATGGTGAGCCGTTTCCATTTGGTAGACGTTATGAAACGGATTATAGAGCG
AGCAGAGGAGAATTGAAACTCACCGCATTAGTTGATGAATCCTCTATTGAGTTATTCATA
AATGATGGTGAACTTGTTTTTACATCTCTTATCTACACAGATCCTACGAATGATGGGATTG
AGTGGTATGCTGATGGGGGTACCATCCATGTATCTGATTCTACTTTTACGTATTTTCATAA
TGTCTGGAGACAACCGGTAGAAGAAGGAGCATTTGAGCGACTTGTGACCAACGTGAATCA
AGCTCGTTTGAAAGTAGGAGAGTATTTGCCCCTCTCTGCTGCTCACAAGCCTGATTACGAA
GAGGCTAGCGGTGAAACCTTAAGCTGGAAAAGCTTAGATAGTAACATCGTCGACATTCGT
AATGAAACCGATGAAGGGGTAGTCATTAAAGCTCTAAACGAAGGCGTTACAGAGATTGTT
GTAACAAACGAATCACAAGGACTAGAAAAAACAATCCGACTTTTTATTACCAAATAG

Figure S1 Nucleotide sequence of endolevanase from *Bacillus lehensis* G1