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In-Depth Characterization of Debranching Type I Pullulanase from *Priestia koreensis* HL12 as Potential Biocatalyst for Starch Saccharification and Modification

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Abstract: Pullulanase is an effective starch debranching enzyme widely used in starch saccharification and modification. In this work, the biochemical characteristics and potential application of a new type I pullulanase from *Priestia koreensis* HL12 (HL12Pul) were evaluated and reported for the first time. Through in-depth evolutionary analysis, HL12Pul was classified as type I pullulanase belonging to glycoside hydrolase family 13, subfamily 14 (GH13_14). HL12Pul comprises multi-domains architecture, including two carbohydrate-binding domains, CBM68 and CBM48, at the N-terminus, the TIM barrel structure of glycoside hydrolase family 13 (GH13) and C-domain. Based on sequence analysis and experimental cleavage profile, HL12Pul specifically hydrolyzes only α-1,6 glycosidic linkage-rich substrates. The enzyme optimally works at 40 °C, pH 6.0, with the maximum specific activity of 181.14 ± 3.55 U/mg protein and catalytic efficiency (k_{cat}/K_m) of 49.39 mL/mg·s toward pullulan. In addition, HL12Pul worked in synergy with raw starch-degrading α-amylase, promoting raw cassava starch hydrolysis and increasing the sugar yield by 2.9-fold in comparison to the α-amylase alone in a short reaction time. Furthermore, HL12Pul effectively produces type III-resistant starch (RSIII) from cassava starch with a production yield of 70%. These indicate that HL12Pul has the potential as a biocatalyst for starch saccharification and modification.

Keywords: type I pullulanase; debranching enzyme; resistant starch; enzyme mixture

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Citation: Prongjit, D.; Lekakarn, H.; Bunterngsook, B.; Aiewviriyasakul, K.; Sritusnee, W.; Arunrattanamook, N.; Champreda, V. In-Depth Characterization of Debranching Type I Pullulanase from *Priestia koreensis* HL12 as Potential Biocatalyst for Starch Saccharification and Modification. *Catalysts* **2022**, *12*, 1014. https://doi.org/10.3390/catal12091014

Academic Editors: Paripok Phitsuwan and Ken-Lin Chang

Received: 19 August 2022
Accepted: 1 September 2022
Published: 7 September 2022

1. Introduction

Starch is one of the most abundant polymers in nature. The properties of native starch are seldom ideal for either food or industrial applications. In general, enzymes are used in large scale to convert native starch to starch-related products. These starch converting enzymes can be divided into four groups according to the mode of action on starch molecules consisting of (i) exo-acting enzyme, (ii) endo-acting enzyme, (iii) transferase, and (iv) debranching enzymes. Exo-acting enzymes, for example, glucoamylase (EC 3.2.1.3) and β-amylase (EC 3.2.1.2), processively hydrolyze α-1,4 glycosidic linkages in starch molecules from the external and release glucose or maltose as a final product. On the other hand, endo-acting enzymes, such as α-amylase (EC 3.2.1.1), randomly hydrolyze α-1,4 glycosidic linkages in starch molecules and liberate maltooligosaccharides as products. Transferases, such as amylomaltase (EC 2.4.1.25), hydrolyze α-1,4 glycosidic linkages of the donor molecule and transfer part of the donor to the glycosidic acceptor and generate new glycosidic linkages. Debranching enzymes, for example, pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68), can hydrolyze α-1,6 glycosidic linkages at the branching point of starch type polysaccharides and produce maltotriose, maltooligosaccharides, or amylose as a final product [1].

Generally, starch materials used in the starch-based industry consist of amylopectin branched molecule as a major component (approximately 80%), which contains 4–5% of branching point α-1,6 glycosidic linkages, and the rest comprise a linear molecule called amylose [1–3]. Therefore, starch-debranching enzymes (SDBEs) play a vital role in starch-rich materials in the bio-industry by eliminating the limit dextran component, a barrier branching point in starch molecules. This ability leads to an effective starch converting process. Principally, SDBEs take the part of the auxiliary enzyme and synergize with other enzymes; for example, the combination between pullulanase and glucoamylase was used to improve the quality of the final product [1,4,5]. In addition, the mixture of pullulanase and glucoamylase is commercially available; for instance, Attenuzyme® Pro from Novozyme (Bagsvaerd, Denmark) helps to accelerate fermentation in ultra-light beers production and increases brewhouse capacity. Furthermore, the wide application of SDBEs is not only used in starch saccharification but also in starch modification, such as pullulanase type I, which can be used in resistant starch type III (RS III) production instead of the chemical process or isolation from natural sources. Moreover, resistant starch has benefits as a functional food for people with diabetes [6,7].

Among starch-debranching enzymes (SDBEs), pullulan-hydrolyzing enzymes have been widely studied and used in many industries for decades. It can be categorized into two major groups according to specific glycosidic linkages, including pullulanase (type I pullulanase and type II pullulanase, also known as amylopullulanse) and pullulan hydrolase (type I, II, and III) [8–10]. Unlike the other types of pullulanase, type I pullulanase specifically hydrolyzes α-1,6 glycosidic linkages and releases only maltotriose as a final product when it acts on pullulan. In addition, pullulanase type I also acts on short chain branches (G1–G7) of starch-type polysaccharides, such as amylopectin, glycogen, and β-limit dextran [8]. In contrast, other types of pullulan-hydrolyzing enzymes exhibit bioactivity with both α-1,6 and α-1,4 glycosidic linkages yielding various final products.

Type I pullulanase (EC 3.2.1.41) is a member belonging to the glycoside hydrolase family 13 (GH13), which is classified into seven subfamilies (GH13_12, 13, 14, 20, 34, 35, and 39) according to the CAZy database (http://www.cazy.org/ accessed on 8 August 2022). Typically, type I pullulanase shows a multiple domain architecture containing carbohydrate-binding modules (CBM) located at N-terminal domain, catalytic domain, and C-terminal domain. CMBs play a vital role in polysaccharides binding and facilitate hydrolysis reaction. They are currently grouped with an affinity for starch, known as starch binding domains (SBDs), and have been reported and classified into 15 families, including CBM20, CBM21, CBM25, CBM26, CBM34, CBM41, CBM45, CBM48, CBM53, CBM58, CBM68, CBM69, CBM74, CBM82, and CBM83 [11]. Among them, CBM20, CBM21, CBM41, CBM48, CBM53, and CBM68 have been identified in pullulanase type I. Moreover, undefined X domains are also located at N-terminal pullulanase, for instance, X25 and X45 domains [8,12–14].

In this work, the type I pullulanase gene isolated from *Priestia koreensis* HL12 was recombinantly expressed in *Escherichia coli* BL21(DE3). This study aimed to investigate biochemical properties, starch-saccharification efficiency, and the starch-modification of the recombinant type I pullulanase. In addition, we also evaluated its potential application in an enzyme mixture and resistant starch production. Results suggested that the type I pullulanase from *P. koreensis* HL12 is a promising candidate enzyme for producing high-valued bio-based products from starch-based material.

2. Results

2.1. Pullulanase Gene Identification from P. koreensis HL12 and Sequence Analysis

The full-length *HL12PUL* pullulanase gene (2145 bp) was isolated from the genomic DNA of *P. koreensis* HL12 formerly classified as *Bacillus koreensis* [15,16]. The gene encodes a protein of 715 amino acid residues without a signal peptide. HL12Pul shows the highest amino acid sequence identity (99%) (100% similarity) to type I pullulanase from *Priestia koreensis* (WP_251605681.1), followed by *Anoxybacillus calidus* (WP_181537603.1) (57% identity and 74% similarity), *Metabacillus iocasae* (MBM7704871.1) (56% identity and 72% similarity),

Bacillus sp. FJAT-14578 (WP_028395221.1) (56% identity and 72% similarity) and *Priestia megaterium* (TYR82091.1) (55% identity and 71% similarity). Regarding the evolutionary analysis of HL12Pul and α-glucan debranching enzymes obtained from [3,9], HL12Pul was classified into type I pullulanase belonging to glycoside hydrolase family 13, subfamily 14 (GH13_14) (Figure 1).

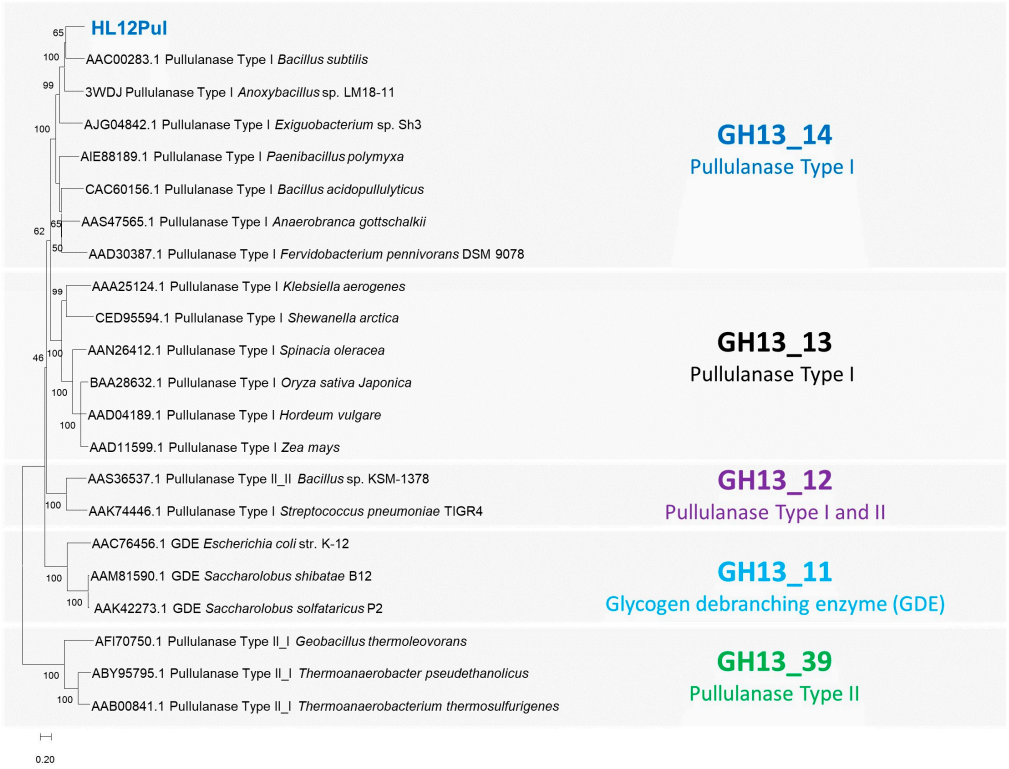

Figure 1. Evolutionary analysis based on full-length amino sequence alignment of HL12Pul and α-glucan debranching enzymes classified into various subfamilies of glycoside hydrolase family 13 (GH13) obtained from [3,9]. The neighbor-joining tree was constructed using the MEGA version 11 using the bootstrap method for estimation phylogenetic evolutionary via 1000 bootstrap replications.

Based on conserved domain annotation using three-dimensional structure prediction, HL12Pul comprises four domains, including (i) carbohydrate-binding domain family 68 (CBM68) (residues 1–87), (ii) carbohydrate-binding domain family 48 (CBM48) (residues 88–177), (iii) catalytic TIM barrel structure of glycoside hydrolase family 13 (GH13) (residues 187–621), and (iv) C-domain (residues 626–715) (Figure 2a). Based on the sequence logo prediction from the alignment of pullulanase type I classified as the GH13_14 subfamily, HL12Pul contained eight conserved sequence regions (CSRs) similar to the GH13_34 subfamily that has been previously reported [9] (Figure 2b). The proposed catalytic residues are the catalytic nucleophile (Asp407 in CSR-II), the proton donor (Glu436 in CSR-III), and the transition-state stabilizer (Asp527 in CSR-IV). In addition, the leucine next to the catalytic nucleophile involving with α-1,6 activity is found in CSR-II. Moreover, the signature motifs for type I pullulanase, IYETHIRDEFS and YNWGYNP, are also conserved in the CSR-VI and VII of HL12Pul, respectively (Figure 2b).

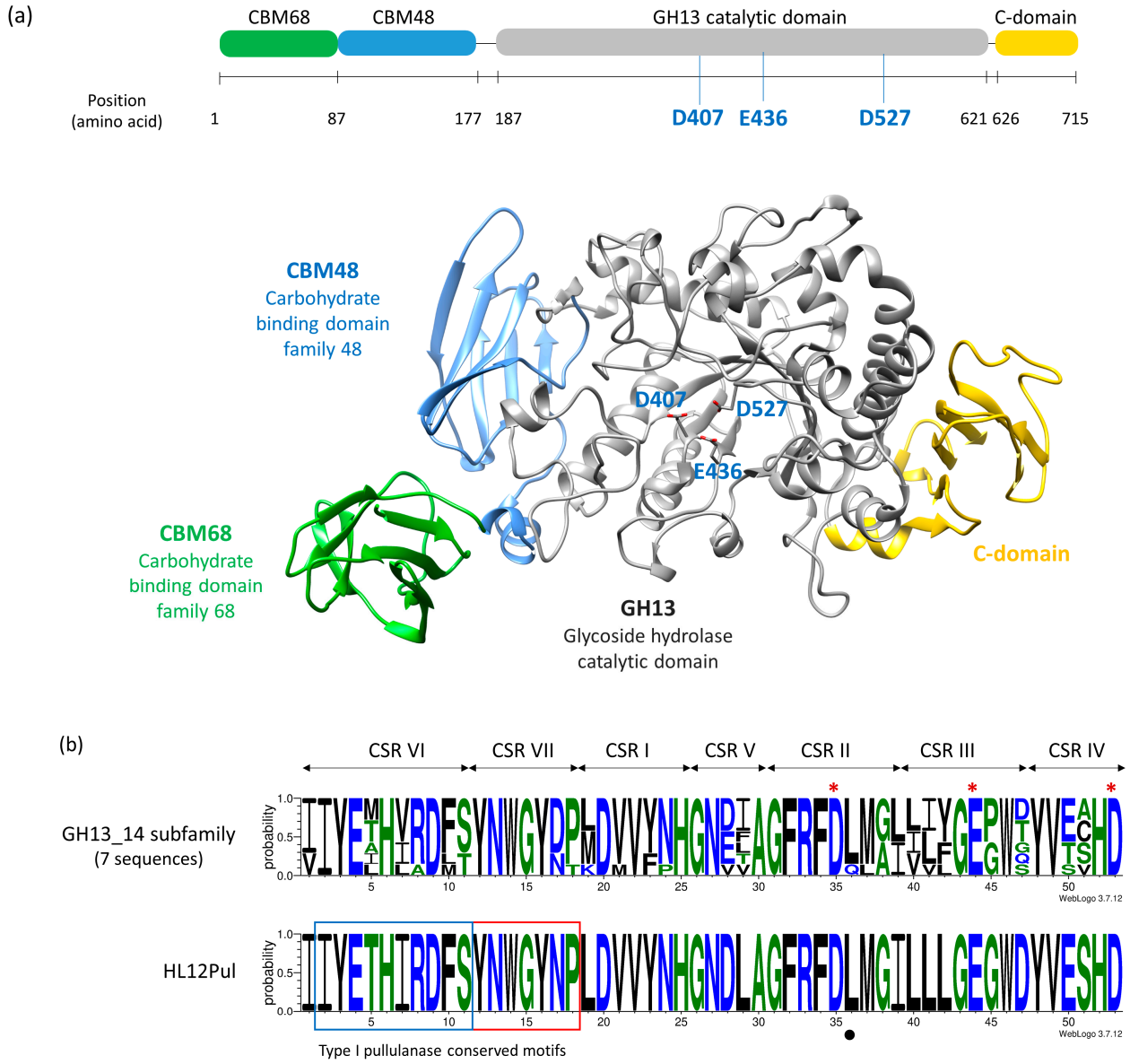

Figure 2. Schematic representation of domain organization in HL12Pul and the overall predicted three-dimensional structure of HL12Pul from *P. koreensis* HL12. (**a**) The structure was predicted based on type I pullulanase from *Anoxybacillus* sp. LM18-11 (PDB: 3wdh) using the SWISS-MODEL. The proposed catalytic residues were presented in the model (D407/E436/D527). (**b**) Sequence logos of eight conserved sequence regions (CSRs) found in HL12Pul and biochemically characterized pullulanase type I belonging to the GH13_14 subfamily. Seven type I pullulanases in sequence alignment are from *Anaerobranca gottschalkii* (AAS47565), *Bacillus acidopullulyticus* (CAC60156), *Bacillus subtilis* strain 168 (AAC00283), *Exiguobacterium* sp. SH3 (AJG04842), *Fervidobacterium pennavorans* Ven5 (AAD30387), *Paenibacillus polymyxa* Nws-pp2 (AIE88189), and *Anoxybacillus* sp. LM18-11 (3wdh). The proposed catalytic residues (D407/E436/D527) are indicated by red asterisks. The IYETHIRDFS and YNWGYNP conserved motifs of type I pullulanase are highlighted in the blue and red box, respectively. The unique residue important for α-1,6 glycosidic linkage hydrolyzing activity is indicated by the black circle.

2.2. Heterologous Expression and Purification of Pullulanase

Type I pullulanase identified from *P. koreensis* HL12 was produced heterologously in *E. coli* BL21 (DE3) expression system as an intracellular soluble protein with a molecular weight of approximately 85 kDa after induction with 0.5 mM IPTG at 25 °C for 3 h. In

the purification step carried out using an immobilized metal ion affinity chromatography HisTrap^{TM} column (GE Healthcare Europe GmbH, Freiburg, Germany), HL12Pul was mostly eluted by 200 mM imidazole. After desalting and concentration, HL12Pul showed a single protein band (85 kDa) with more than 95% homogeneity (Figure S2a,b) and a production yield of 119 mg/L culture medium was obtained.

2.3. Biochemical Characterization

In order to explore the optimal working condition of HL12Pul, the effect of pH and temperature was tested against pullulan as a substrate. The effect of pH was measured under different pH with various buffers between pH 3.0–9.0. Under defined conditions, the HL12Pul optimally worked at pH 6.0 in both sodium citrate and sodium phosphate buffer and retained more than 50% of relative activity at pH 5.0–7.0 (Figure 3a). According to the effect of temperature examined for temperature ranging from 25–80 °C, HL12Pul exhibited a broad range of preferential working at low-temperature range with the highest relative activity at 40 °C and retained more than 55% relative activity under 40 °C. However, HL12Pul activity dramatically decreased under high temperature (50–80 °C) with residual activity less than 10% (Figure 3b). Therefore, HL12Pul provided optimal working conditions at pH 6.0, 40 °C with the highest specific activity of 191.09 ± 8.04 U/mg protein toward pullulan as substrate.

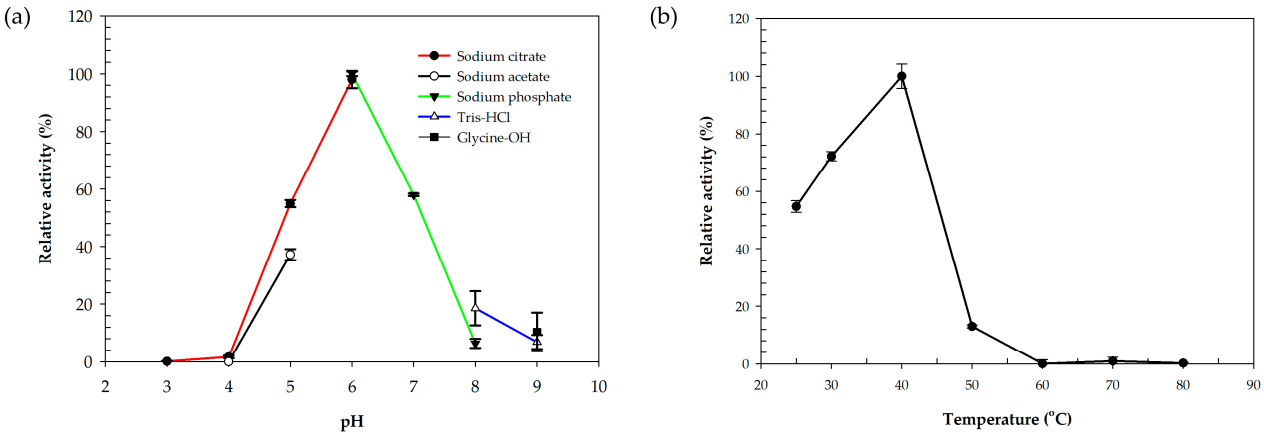

Figure 3. The effect of temperature and pH on pullulanase activity. (**a**) The influence of pH was measured in various buffers ranging from pH 3.0–9.0. (**b**) The influent of temperature on pullulanase activity was determined at temperatures ranging 25–80 °C.

The substrate specificity of HL12Pul was further evaluated against different substrates under the optimal working condition. HL12Pul capably hydrolyzed all substrates containing branching α-1,6 glycosidic linkage as component. The highest catalytic activity of HL12Pul was observed when pullulan was used as substrate, with a specific activity of 181.14 ± 3.55 U/mg protein, followed by starch from various botanical sources; cassava starch, potato starch, soluble starch, and rice starch, respectively. In contrast, no activity toward amylose, which contains solely α-1,4 glycosidic linkage, was detected (Table 1). These results imply that HL12Pul functions as type I pullulanase with a specificity towards only α-1,6 glycosidic linkage. In order to gain in-depth insight into debranching activity, the kinetic parameters of HL12Pul toward α-1,6 glycosidic linkage-rich pullulan was examined. HL12Pul presented V_{max} and k_{cat} values against pullulan with 143.58 ± 10.87 U/mg protein and 188.33 1/s, respectively. The K_m toward pullulan was 3.81 ± 0.68 (mg/mL).

Table 1. Substrate specificity of HL12Pul against various substrates.

Substrates	Relative Activity (%)	Specific Activity (U/mg Protein)
Pullulan	100.00 ± 1.96	181.14 ± 3.55
Amylopectin	9.78 ± 0.50	17.706 ± 0.92
Amylose	[a] ND	[a] ND
Soluble strach	29.57 ± 0.78	53.19 ± 2.02
Starch from cassava	33.99 ± 0.34	61.57 ± 0.61
Starch from rice	29.12 ± 0.25	52.75 ± 0.45
Starch from potato	31.51 ± 1.39	57.07 ± 2.52

[a] ND means not detectable.

2.4. Cleavage Pattern on Pullulan and Specific Linkages

To verify the type and mode of action of HL12Pul, the linkage-specific cleavage profiles toward α-1,6 glycosidic linkage rich pullulan and pure α-1,4 glycosidic linkage amylose were investigated. Based on the final product analysis obtained using TLC and HPLC, the hydrolysate of HL12Pul against pullulan yielded maltotriose (G3) as a final product. The effect of enzyme loading was observed toward pullulan hydrolyzing, in which completely hydrolyzed pullulan was observed under the reaction containing 10 and 20 U/g substrate enzyme loading (Figure 4a,b). When the enzyme loading of a 2 U/mg substrate was used, incomplete hydrolysis was observed from the presence of maltohexaose (G6) and larger oligomers. Importantly, no product was detected with pure α-1,4 glycosidic linkage amylose as a substrate. Regarding the cleavage pattern, HL12Pul specifically attacks only α-1,6 glycosidic linkage of branched substrate, and therefore, can be classified as type I pullulanase.

(a)

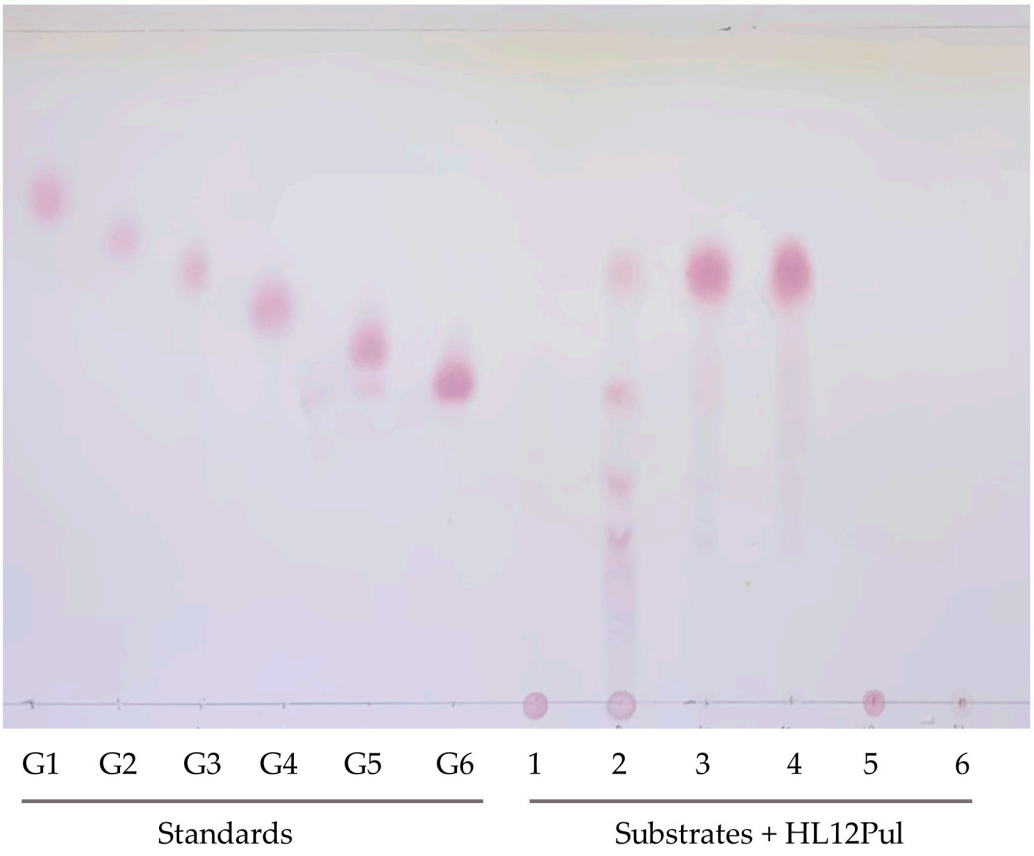

Figure 4. *Cont.*

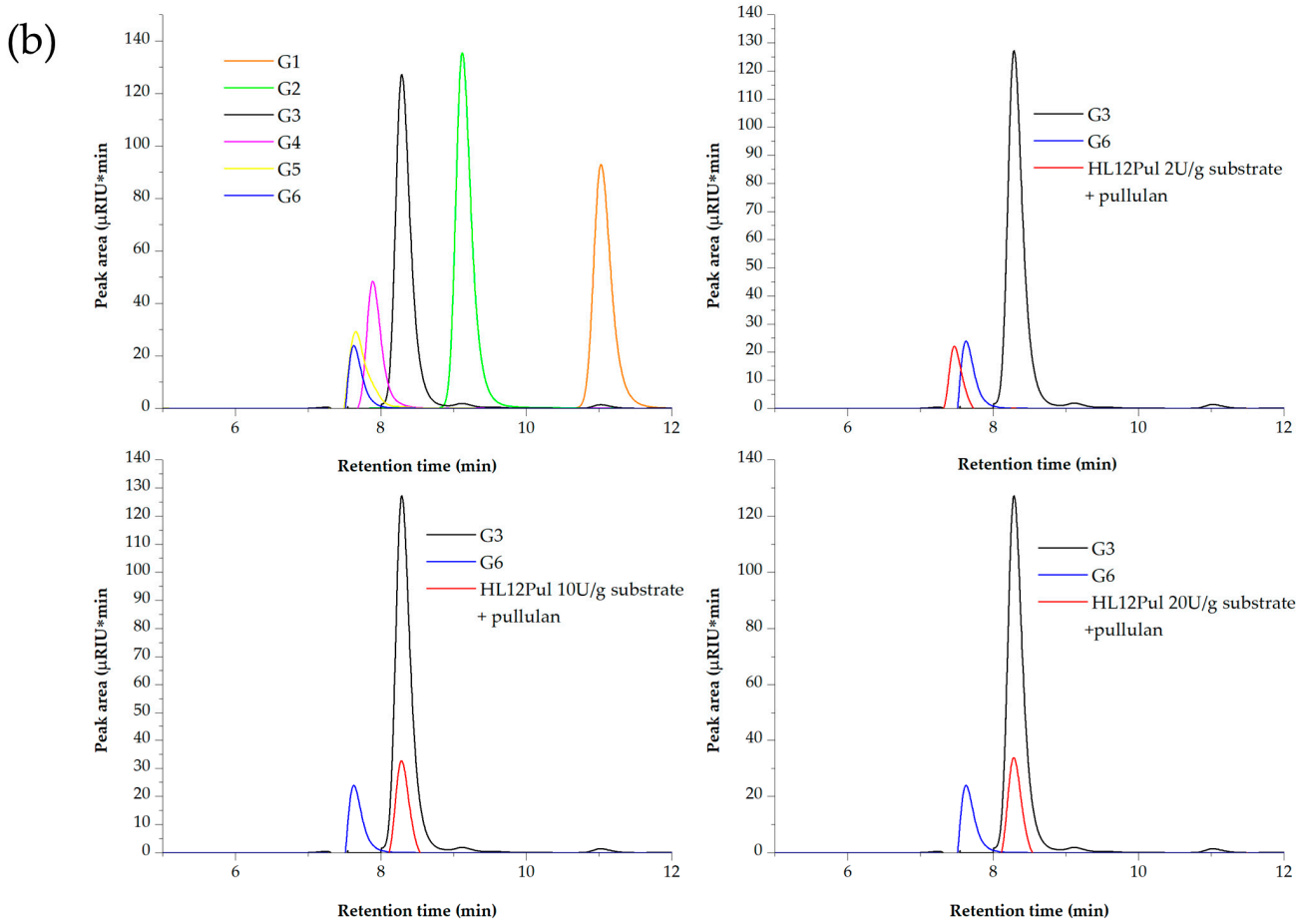

Figure 4. Verification of HL12Pul specific cleavage. (**a**) Specific cleavage of HL12Pul analyzed by the thin-layer chromatography technique. Lane 1: pullulan without enzyme; Lane 2–4: pullulan treated with 2, 10, and 20 U/g substrate of HL12Pul, respectively; Lane 5: amylose without enzyme; lane 6: amylose treated with 20 U/g substrate of HL12Pul. (**b**) High performance liquid chromatography. G1, G2, G3, G4, G5, and G6 represent glucose, maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose, respectively.

2.5. Potential of HL12Pul in Raw Starch Saccharification

The cooperative action between endo-acting α-amylases and debranching enzymes is important for efficient starch saccharification. Thus, the synergistic interaction of HL12Pul with raw starch degrading (RSD) α-amylase (HL11Amy) was then examined under a raw starch hydrolysis reaction at 40 °C for 30 and 60 min with varying HL12Pul dosage. The raw cassava starch treated with HL12Pul alone (1.6 μg protein) was not hydrolyzed (Figure 5a,b). Whereas the reducing sugar yield released from the reaction containing a mixture of type I pullulanase HL12Pul and RSD α-amylase HL11Amy (81.73 ± 3.52 mg/g substrate) was significantly increased compared to single HL11Amy (29.34 ± 1.53 mg/g substrate) (p-value < 0.05). This synergistic interaction provided an approximately 2.9-fold increasing yield compared to HL11Amy alone. In addition, the result demonstrated the synergistic potential of HL12Pul in the starch saccharification on the reaction carried out for 60 min, which gave 171.83 ± 6.51 mg/g substrate, equivalent to a 16.86% ± 0.48 conversion yield (Figure 5b).

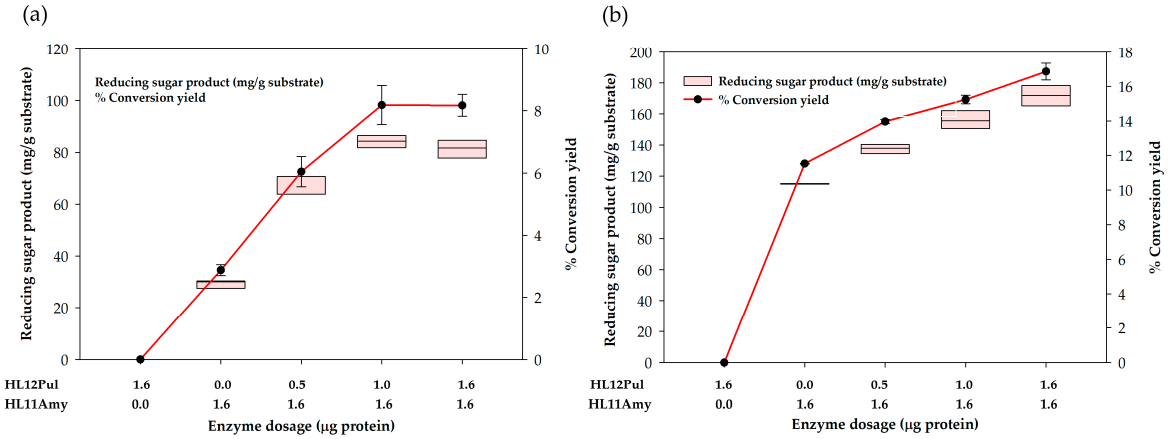

Figure 5. The evaluation of the synergistic interaction of type I pullulanase HL12Pul with RSD α-amylase HL11Amy. Varying enzyme loading of HL12Pul was added into a raw starch hydrolysis reaction. The reactions were incubated at 40 °C for (**a**) 30 min and (**b**) 60 min.

2.6. Enzymatic Process for Resistant Starch Production

HL12Pul was further applied for the production of retrograded resistant starch type III. The gelatinized cassava starch was treated with a varying dosage of HL12Pul (2, 4, 8, 16, and 32 U/g substrate) in pH 6.0 at 40 °C. After 24-h hydrolysis, the HL12Pul treated starch hydrolysate was clearer than that from the reaction without enzyme (Figure 6). Interestingly, the cloudy white precipitation formed after retrogradation at 4 °C. The resistant starch recovery increased depending on HL12Pul dosage, in which the highest recovery yield of 7.0 mg of treated starch (equivalent to 70% ± 6.25) was obtained from the 32 and 16 U/g substrate of HL12Pul.

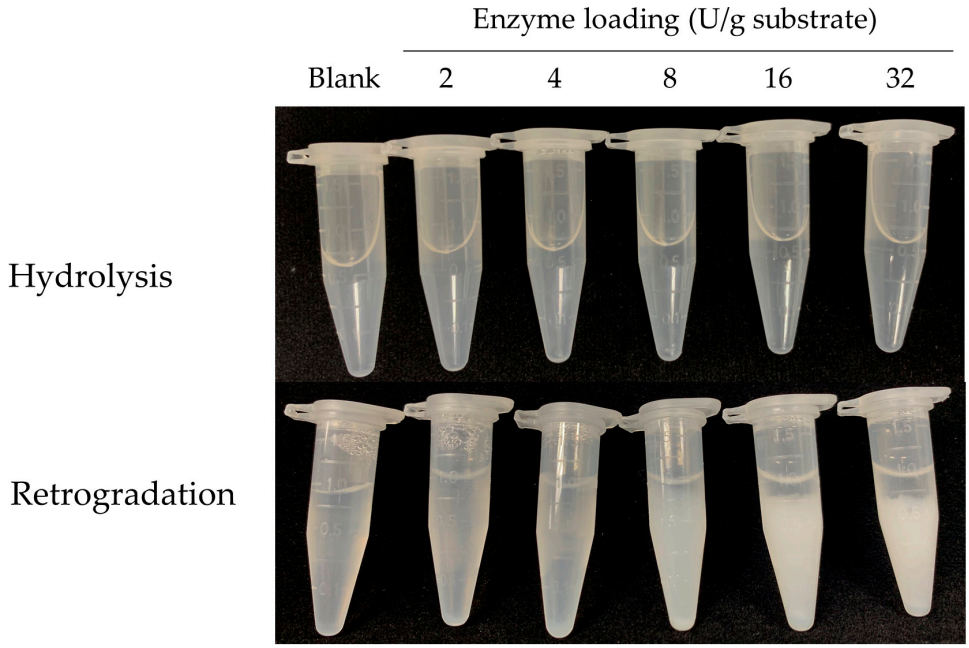

Figure 6. Resistant starch production via HL12Pul, the effect of varying enzyme dosage for resistant starch produced from cassava paste was evaluated. The varying HL12Pul loading contained 2, 4, 8, 16, and 32 U/g substrate.

Regarding the morphology of the HL12Pul-treated starch, as analyzed through a Scanning Electron Microscope (SEM), the overall structure of starch treated with HL12Pul significantly changed compared to untreated starch. The HL12Pul dosage demonstrated

an influence on the structure of the modified starch. Under 500× magnification, the modified starch treated with a high HL12Pul dosage (16 and 32 U/g substrate) formed a network polymer (Figure 7e,f), while the structure of modified starch treated with lower enzyme dosage (8 U/g substrate) was observed as a cross-linked polymer (Figure 7g,j). Importantly, the starch treated with 32 U/g substrate exhibited a more organized polymeric network structure than the starch treated with 16 U/g substrate HL12Pul loading (25,000× *g* magnification) (Figure 7h,i).

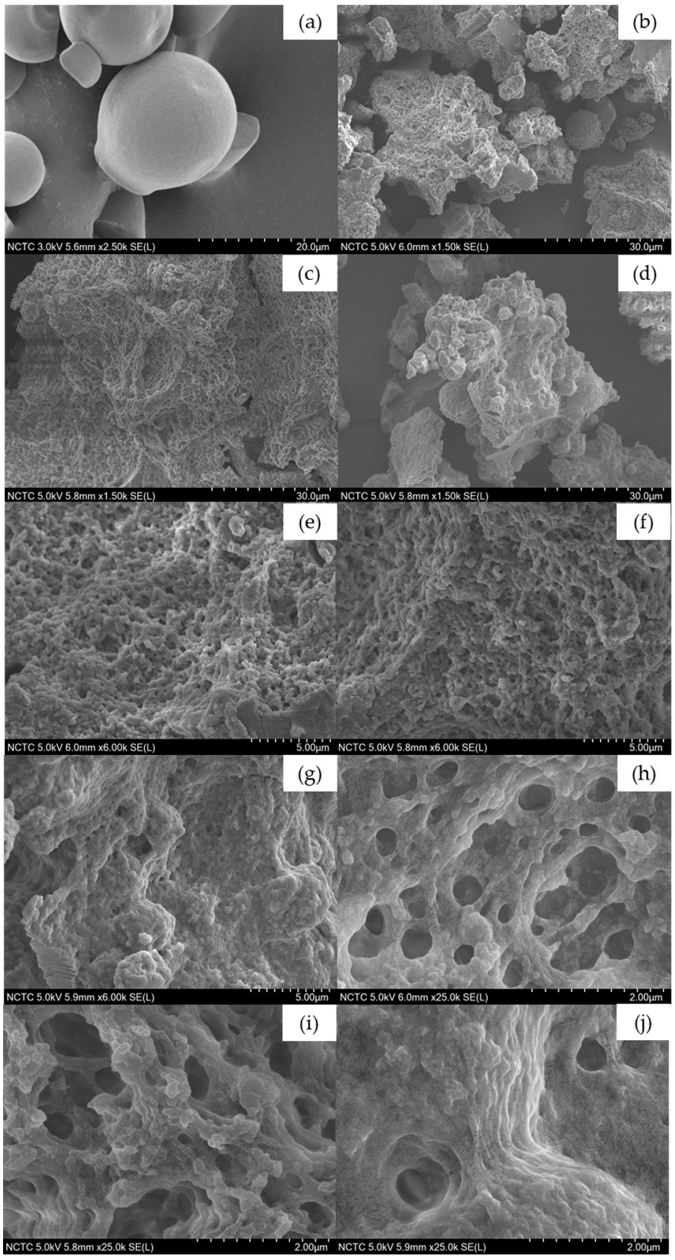

Figure 7. The morphology analysis of HL12Pul-treated modified starch using a Scanning Electron Microscope (SEM). The effect of enzyme loading against a starch structure was observed. (**a**) Untreated cassava starch; (**b**–**d**): Cassava starch treated with 32, 16, and 8 U/g substrate of HL12Pul, respectively, under 1500× magnification; (**e**–**g**): Cassava starch treated with 32, 16, and 8 U/g substrate of HL12Pul, respectively, under 6500× magnification; (**h**–**j**): cassava starch treated with 32, 16, and 8 U/g substrate of HL12Pul, respectively, under 25,000× magnification.

2.7. In Vitro Digestibility of Modified Starch

The resistance for the α-amylase digestibility of HL12Pul-treated modified cassava starch was investigated by in vitro digestion with α-amylase from porcine pancreas. Based on the product released from the in vitro digestion reaction, the native gelatinized starch and the retrograded gelatinized starch without HL12Pul treatment showed similar digestibility patterns, which were rapidly hydrolyzed at 20–120 min (Figure 8). On the other hand, after 120 min hydrolysis, the digested product from the HL12Pul-treated modified starch (12.0 ± 0.9 mg/g substrate) was significantly lower than the native starch (104.0 ± 7.4 mg/g substrate) and retrograded starch 83.9 ± 3.1 mg/g substrate). Hence, these results indicate that the HL12Pul-treated modified starch exhibits the function of type III resistant starch.

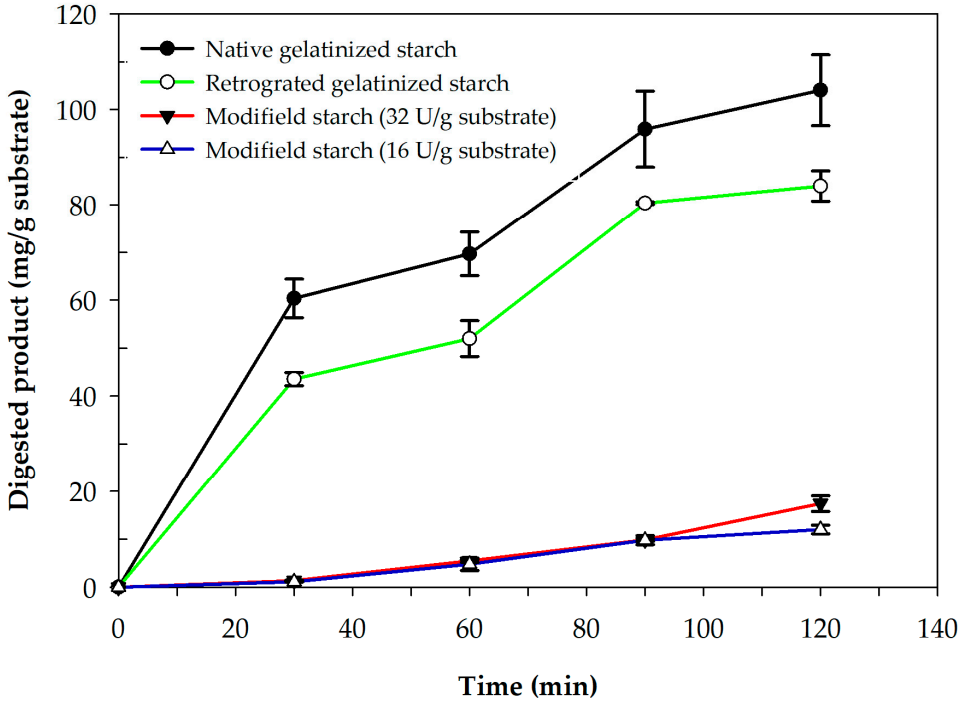

Figure 8. In vitro digestibility of HL12Pul-treated modified starch and native starch using α-amylase from porcine pancreas in 20 mM sodium phosphate buffer saline pH 7.0 at 37 °C for 30–120 min.

3. Discussions

Pullulanases, α-glucan debranching enzymes, are important enzymes involved in the starch hydrolysis pathway in living organisms. In this work, the full-length *HL12PUL* gene was isolated from *P. koreensis* HL12 (formerly known as *Bacillus koreensis* [15]), an amylolytic enzyme producing bacteria isolated from soil associated with sago stem. The biochemical characteristics of type I pullulanase from *P. koreensis* were described for the first time in this work. HL12Pul exhibited the highest identity to type I pullulanase from *Priestia koreensis* (99%) and was classified into subfamily 14 of glycoside hydrolase family 13 (GH13_14), which consists of type I pullulanases from a range of bacteria, such as Gram-positive bacteria *Anaerobranca gottshalskii*, *Exiguobacterium acetylicum*, *Paenibacillus polymyxa*, and *Geobacillus thermopakistaniensis* [9], while type I pullulanases belonging to GH13_12 and GH13_13 are generally identified from firmicutes, especially streptococci and plants, respectively [9,12]. The modular structure of HL12Pul consists of four domains, including (i) CBM68, (ii) CBM48, (iii) GH13 catalytic domain, and (iv) C-domain according to type I pullulanase from *Anoxybacillus* sp. LM18-11 [3]. Based on amino acid sequence alignment, HL12Pul contains CSR-I, CSR-II, CSR-III, and CSR-IV referring to the conserved motifs for all GH13 families and CSR-V, CSR-VI, and CSR-VII; additional specificity motifs positioned near the C-terminus of domain B corresponding to nomenclature were

established for the entire alpha-amylase family GH13 [17,18]. The catalytic domain of HL12Pul is composed of a typical $(\alpha/\beta)_8$ TIM barrel, which is the signature structural fold of GH13 families, with the proposed catalytic residues: Asp407 (catalytic nucleophile) Glu436 (proton donor), and Asp527 (transition-state stabilizer) conserved in member of GH13_14 subfamily type I pullulanase [3]. Amino acid residue next to the catalytic nucleophile in CSR-II plays important roles in the recognition of α-1,6-glucosidic linkage. The replacement of valine with alanine, a smaller amino acid, changed the linkage preference from α-1,6 to α-1,4 glycosidic linkages of GH13_31 1,6-α-glucosidase from *Streptococcus mutans* [19] and GH13_20 from *Thermoactinomyces vulgaris* [20]. The type I pullulanases hydrolyzing only α-1,6-glycosidic linkage, such as GH13_12 from *Streptococcus agalactiae*, GH13_13 from *Hordeum vulgare* and GH13_14 from *Anoxybacillus* sp. LM18-11 contain large amino acids, methionine, or leucine at this residue in CSR-II [9,21] as same as Leu408 found in HL12Pul. The signature motif of type I pullulanases, IYELHIRDEFS, being a part of $(\alpha/\beta)_8$ TIM barrel was also conserved in CSR-VI of HL12Pul [9,22]. In addition, the consensus sequence YNWGYDP in CSR-VII, a highly conserved motif for all type I pullulanase enzymes, can be found in HL12Pul [9,12,23,24]. These therefore confirmed that HL12Pul was classified as type I pullulanase.

Generally, CBM48 is closely related to the starch binding domain CBM20, but CBM48 has only one starch binding site (SBS) corresponding to CBM20 SBS1, whereas CBM20 generally has two SBSs. CBM48, involved in substrate recognition, is mostly observed in debranching enzymes belonging in GH13 subfamilies. According to sequence comparison, CBM48 in HL12Pul contains only one starch-binding site (SBS) with two conserved tryptophans (Trp119 and Trp151) and a functionally important arginine (Arg145) (Figure S1) corresponding to SBS1 in CBM20 and CBM48 three-dimensional structures solved as complex with an alpha-glucan [9,11,25,26]. In the case of CBM68, based on several studies, the CBM68 domain at the N-terminus has been reported to play a beneficial role for catalytic efficiency and thermostability, in addition to assisting the substrate binding. The CBM68-truncated mutant of thermostable pullulanase (PulA) from *Anoxybacillus* sp. LM18-11 showed remarkable lower in the optimum temperature and specific activity comparing to the wild type [3,27]. Moreover, typically pullulanase belonging in subfamily GH13_14 also presented the domain arrangement with others composition, especially possessing the CBM41 [28]. On the other hand, HL12Pul presented the notable CBM arrangement in this group, which exhibits CBM68 together with the SBD of family CBM48. Regarding the functional analysis of CBM68 of PulA through domain rearrangement, the CBM68 located on the N-terminus of PulA significantly enhanced substrate specificity and enzyme catalytic efficiency by increasing the k_{cat}/K_m value (737.78 mL/mg·s) comparing to recombinant PulB with CBM41 (190.44 mL/mg·s) [29]. The alanine substitution of four key substrate binding residues (Tyr14, Val91, Gly92, and Arg96) in PulA confirmed the crucial role of Val91 and Gly92 on substrate binding affinity and catalytic efficiency [27]. Regarding structural superimposition with PulA from *Anoxybacillus* sp. LM18-11 (PDB: 3wdh), CBM68 of HL12Pul contained two key amino acid residues for pullulan binding (Ile85 and Gly86).

Regarding substrate specificity against different substrates, HL12Pul displayed remarkably specificity on α-1,6 glycosidic linkage-rich pullulan with the specific activity of 181.14 ± 3.55 U/mg protein, followed by starch from different botanical sources containing the mixture of amylose and amylopectin [1]. However, amylose representing pure α-1,4 glycosidic linkage molecule could not be hydrolyzed by HL12Pul. The result corresponded with the cleavage pattern analysis, in which HL12Pul specifically cleaved only α-1,6 glycosidic linkage in pullulan by releasing maltotriose (G3) as a final product. These results indicated that HL12Pul exhibit type I pullulanase characteristics, which are in accordance with evolutionary and conserved region analysis, in which HL12Pul shares high similarity with type I pullulanase in the GH13_14 subfamily and contains a unique α-1,6 glycosidic linkage hydrolysis-related motifs; hence, HL12Pul was classified as type I pullulanase. In several studies, maltotriose (G3) was detected as a product obtained from the complete

hydrolysis of pullulan by the type I pullulanase of *Paenibacillus polymyxa* Nws-pp2, *Anoxybacillus* sp. SK3-4, *Bacillus megaterium*, *Exiguobacterium* sp. SH3 [24,30–32]. Based on the enzyme kinetic analysis, HL12Pul showed greater kinetic parameters toward pullulan compared to previous reports with the K_m, V_{max}, and k_{cat} values of 3.81 ± 0.68 mg/mL, 143.58 ± 10.87 U/mg protein and 188.33 1/s, respectively [24,33,34].

Considering the temperature profile, most of type I pullulanases have been previously reported as thermophilic enzymes with optimal temperature ranging 50–80 °C [30,32,35–39]. There are few reports of cold-adapted type I pullulanases, for example from *Paenibacillus polymyxa* Nws-pp2 (35 °C), *Bacillus cereus* Nws-bc5 (35 °C), and *Exiguobacterium* sp. SH3 (45 °C) [24,31,40]. In this work, HL12Pul exhibited the maximum catalytic activity of HL12Pul at 40 °C pH 6.0, while it was able to retain more than 50% residual relative activity under low temperature range (25–40 °C). This suggests that HL12Pul can be applied for starch modification and hydrolysis reaction under ambient temperature, leading to the reduction of energy input required for these processes.

Remarkably, HL12Pul cooperated with RSD α-amylase HL11Amy by promoting raw cassava starch saccharification, in which the reducing sugar yield was increased by approximately 2.9-fold compared to the use of HL11Amy alone. The hydrolysis of α-1,6 glycosidic linkage at the branching point by HL12Pul increased the accessibility of α-amylase to its linear α-1,4 chain substrate and, thus, resulted in the increased hydrolysis activity of HL11Amy. Generally, pullulanase type I was utilized in synergism with other enzymes, such as glucoamylase and α-amylase [1,4,5,41]. This demonstrated that HL12Pul is a promising auxiliary enzyme for various industrial applications, such as starch saccharification.

The resistant starch can be divided, according to the production process, into five types [42]. The resistant starch produced by the enzymatic process was categorized as type III resistant starch (RSIII), which amylose molecule retrograded to form a double helices structure. For decades, type I pullulanases have been widely studied and utilized in the enzymatic RSIII production process. The advantages of this process include the high specificity of type I pullulanase acting only on a branching point α-1,6 glycosidic linkage and high amylose content forming double helices structures [6,43,44]. In this work, HL12Pul exhibited high efficiency in RSIII production (70% recovery) from gelatinized cassava starch. Typically, cassava starch contains 17–20% amylose (a linear chain molecule) and 80–83% amylopectin (a branch polymer of glucose) with high molecular weights [1,45,46]. The different enzyme dosages in starch treatment significantly differed modified starch architecture. According to SEM analysis, the lower HL12Pul dosage created cross-linkage polymeric structure. Nevertheless, the higher dosage reconstructed modified starch into network polymer. Regarding physiochemical properties of network polymer, RSIII exhibited distinctive mechanical and thermal properties [47], preventing HL12Pul-treated starch (16 and 32 U/g substrate) from dissolving at pasting temperature. Furthermore, the HL12Pul-treated starch was more resistant to α-amylase from porcine pancreas digestion compared to the native starch. The similar appearances of slow digestion of enzyme-treated resistant starch were observed in previous reports [10,43]. Based on its slow and hard digestibility, HL12Pul-treated starch can be applied as the functional ingredient for people with diabetes. Therefore, the results verify the significant potential of HL12Pul as a debranching enzyme for high effective RSIII production. In summary, regarding its enzymatic properties and significant synergistic interaction with RSD α-amylase, HL12Pul could be recognized as a potential debranching enzyme for an efficient starch saccharification and modification processes.

4. Materials and Methods

4.1. Chemicals, Bacterial Strains and Plasmids

For the production of recombinant proteins, the pJET1.2/blunt vector (Thermo Fisher Scientific, Waltham, MA, USA) and the pET28a vector (Novagen, Darmstadt, Germany) were used as cloning and expression plasmids, respectively. *E. coli* DH5α was used as a

plasmid propagation host, and *E. coli* BL21(DE3) (Novagen, Darmstadt, Germany) was used for recombinant protein expression. Recombinant raw starch degrading α-amylase was produced in our laboratory [46]. All chemicals and reagents used were analytical grade and derived from major chemical suppliers (Sigma, Merck, and Fluka). The following ingredients were bought from Megazyme (Wicklow, Ireland): amylose, pullulan, and maltooligosaccharides (M2–M6). Soluble starch was bought from Carlo-Erba (Cornaredo, Italy). Purchased from Sigma-Aldrich (St. Louis, MO, USA) were amylopectin, rice starch, potato starch, and α-amylase from porcine pancreas. Cassava starch was purchased from Thai Wah Food Products Public Co., Ltd. (Bangkok, Thailand).

4.2. Identification of HL12PUL Type I Pullulanase Gene from P. koeensis HL12

The full-length *HL12PUL* gene was isolated from the genomic DNA of *P. koreensis* HL12 extracted using GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) using BKHL12/Pul/F (5′-ATGAACGTCACCAAACGTGAGTAC-3′) and BKHL12/Pul/R (5′-TTCTCTACCTAAAATCAAGACGCT-3′) primers with Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The primer set was designed based on the pullulanase gene from *P. koreensis* genome sequence (accession number KOO46795.1). The temperature profile consisted of pre-denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 2 min 30 s, and a final extension step at 72 °C for 10 min. According to the manufacturer's protocol, the PCR product was purified by a GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) and cloned into pJET1.2/blunt vector (Thermo Fisher Scientific, Waltham, MA, USA) resulting in recombinant plasmid pJET1.2-*HL12PUL*, which was then transformed into *E. coli* DH5α. The inserted gene sequence was confirmed by Sanger sequencing (Macrogen, Seoul, South Korea). The amino acid sequence of HL12Pul was compared to amino acid sequences in the Protein database available in NCBI using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 4 August 2022). The signal peptide was predicted using SignalP 5.0 (https://services.healthtech.dtu.dk/service.php?SignalP-5.0, accessed on 4 August 2022). The conserved domain annotation was performed against Conserved Domains Database (CDD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml, accessed on 4 August 2022) [48]. Sequence alignment was performed using ClustalW [49]. The phylogenetic tree was constructed using the neighbor–joining method (bootstrap test of 1000 replicates) using MEGA 11 software [50]. The sequence logos of HL12Pul and 7 sequences of previously reported type I pullulanase classified in GH13_14 from [9] were created based on 8 conserved regions using the WebLogo 3.5.0 server [51]. The three-dimensional structure of HL12Pul was predicted through homology modeling using SWISS-MODEL [52,53] based on a crystal structure of type I pullulanase from *Anoxybacillus* sp. LM18-11 (PDB: 3wdh) [3] and visualized using Chimera software (UCSF, San Francisco, CA, USA) [54].

4.3. Heterologous Expression and Purification of Pullulanase

The recombinant plasmid was constructed using pET28a as an expression vector, the *HL12PUL* gene was amplified from pJET1.2-*HL12PUL* using specific primers hl12pul/F (5′-CCATGGACGTCACCAAACGTGAGTTT-3′) and hl12pul-R (5′-CTCGAGTTCTCTACCTAAAATCAAGACGCT-3′) with NcoI and XhoI restriction sites, which are underlined. After digestion, the gene fragments were inserted into the pET28a plasmid between NcoI and XhoI restriction sites to generate recombinant plasmids pET28a-*HL12PUL*, which were then transformed into *E. coli* DH5α by heat shock transformation [55]. The recombinant clones were plated on LB agar supplemented with 50 μg/mL kanamycin as a selection medium and incubated at 37 °C for 18 h. The transformants grown on the selection medium were verified according to colony PCR using both hl12pul/F, hl12pul/R specific primers and internal primers for DNA sequencing. Using the heat-shock approach, the plasmid harboring the *HL12PUL* gene was introduced into the *E. coli* BL21(DE3) expression host [55]. The

transformants were selected on selection medium cultivated at 37 °C for 18 h. The selected clones were validated by colony PCR using hl12pul/F- and hl12pul/R-specific primers.

For recombinant HL12Pul production, the validation clone was seeded on LB broth supplement with 50 µg/mL kanamycin on an orbital shaker at 200 rpm shaking speed for 18 h. The 1% (*v*/*v*) inoculum was inoculated into 800 mL LB broth supplemented with 50 µg/mL kanamycin. The culture was incubated at 37 °C with 200 rpm for 3 h; subsequently, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture to induce HL12Pul production. The culture was further incubated at 25 °C with 200 rpm for 3 h. Cells were collected by centrifugation at 8000× *g* at 4 °C for 10 min. After discarding the supernatant, the cell pellet was resuspended with cold 50 mM sodium phosphate buffer, pH 7.4, and lysed via sonification using 60% amplitude for 15 min with 10 s pulse (Sonics Ultrasonic Vibra Cell). The cell debris and inclusion bodies were removed by centrifugation at 12,000× *g* at 4 °C for 30 min. The soluble protein in clarified supernatant was collected. The proteins expressed were analyzed on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 15 mA for 65 min and stained with Coomassie Blue staining. Subsequently, Western blot analysis was performed for verified recombinant HL12Pul using a monoclonal anti-His tag antibody.

4.4. Purification of Recombinant HL12Pul

HL12Pul purification was performed using affinity chromatography. The soluble fraction was loaded into HisTrap™ HP column (GE Healthcare, Uppsala, Sweden) after the column was equilibrated with 10 column volumes (CVs) of binding buffer (20 mM sodium phosphate buffer pH 7.4, 0.5 M NaCl, and 20 mM imidazole). After that, the unbound protein was washed with 10 CVs of binding and washing buffer (20 mM and 50 mM imidazole), respectively. The elution step was carried out according to gradient elution using 5 CVs of elution buffer (20 mM sodium phosphate buffer, pH 7.4, 0.5 M NaCl) consisting of 100, 200, 300, and 400 mM imidazole, respectively. Fractions with HL12Pul were pooled into 10 kDa Macrosep® Centrifugal Filters (Pall, MI, USA) for desalting and concentration against 50 mM sodium phosphate buffer, pH 7.4. Protein concentration was determined according to the Bradford assay protocol (Bio-Rad, Hercules, CA, USA). The purified HL12Pul was evaluated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 15 mA for 65 min and visualized with Coomassie Blue staining along with Western blot analysis.

4.5. Enzyme Activity Assay

Pullulanase activity was determined based on liberated reducing sugar according to DNS method [56]. The reaction composition was 1% (*w*/*v*) pullulan dissolved in 50 mM sodium acetate buffer, pH 5.0, pre-incubated at 50 °C for 10 min, and an appropriate concentration of HL12Pul. The reaction mixture was incubated at 50 °C for 10 min. The amount of reducing sugar was calculated from the measured absorbance at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µmole of product per 1 min.

4.6. Determination of Biochemical Properties of Recombinant HL12Pul

To understand the working profile of HL12Pul, the biochemical characteristics were evaluated by measuring pullulanase activity acted on 1% (*w*/*v*) pullulan as substrate. To study the effect of pH, 1% (*w*/*v*) pullulan was dissolved in 50 mM different buffers ranging from 3.0 to 10.0, including sodium citrate buffer (pH 3.0–6.0), sodium acetate buffer (pH 4.0–5.0), sodium phosphate buffer (pH 6.0–8.0), tris-hydrochloride (pH 8.0–9.0), and glycine-sodium hydroxide (pH 9.0). The reaction mixtures were incubated at 50 °C for 10 min. Additionally, the effect of temperature was carried out by measuring pullulanase activity after 10 min incubation at varied temperatures ranging between 25 to 80 °C. The enzyme activities were measured from the amount of reducing sugar liberated by the enzyme activity, following the DNS method [56].

The substrate specificity of HL12Pul was investigated in terms of various substrates, including pullulan, amylopectin, amylose, soluble starch, starch from rice, starch from potato, and starch from cassava. The reaction mixture contained 1% (*w*/*v*) of substrate dissolved in 50 mM sodium phosphate buffer pH 6.0, with an appropriate concentration of HL12Pul, which was then incubated at 40 °C for 30 min. The amount of reducing sugar liberated during the enzymatic activity was determined according to the DNS method [56].

4.7. Determination of Kinetic Parameters on Pullulan

For an in-depth understanding of debranching activity of HL12Pul, kinetic parameters (K_m, V_{max}, and k_{cat}) toward α-1,6 glycosidic linkage rich pullulan were determined. The reaction mixture contained substrate concentration between 1.25–10.0 mg/L dissolved in 50 mM sodium phosphate buffer pH 6.0 with an appropriate concentration of HL12Pul. The mixture was incubated at 40 °C with an incubation time of 5–60 min. Michaelis-Menten's equation was used to calculate the kinetic values (K_m and V_{max}) for each substrate. Triplicate experiments were used to determine the standard deviation.

4.8. Verification of HL12Pul Specific Cleavage of Glycosidic Linkages

Due to a variety of pullulan-hydrolyzing, the specific glycosidic linkages cleavage of HL12Pul was examined in the reaction mixtures containing 1% (*w*/*v*) of substrates (pullulan and amylose) dissolved in 50 mM sodium phosphate buffer, pH 6.0. The maximum enzyme dosage (20 U/mg protein) was used in the amylose hydrolysis reaction, while the varying dosages at 2, 10, and 20 U/mg protein were used in pullulan hydrolysis reactions. The reaction mixtures were incubated at 40 °C for 24 h; afterward, hydrolysates were inactivated at 100 °C for 10 min and filtered via 0.2 μm filter membranes. The final products from hydrolysates were analyzed using the thin-layer chromatography (TLC) technique and high-performance liquid chromatography (HPLC).

The filtrate was applied to an Aminex HPX-87H ion exclusion column equipped with a refractive index (RI) detector, with 5 mM sulfuric acid as mobile phase and a flow rate of 0.5 mL/min at 65 °C. The sugar profile released from the hydrolysate was analyzed using TLC Silica gel 60 F_{254} (Merck KGaA, Darmstadt, Germany) as a stationary phase, and the mixture of isopropanol (propan-2-ol), acetic acid, and deionized water with ratio 4:1:1 was used as a mobile phase. The TLC plate was spotted with 1 μL of end products from the reaction and thoroughly sprayed with a fresh visualization reagent comprising 0.1% (*w*/*v*) orcinol (Sigma-Aldrich, St. Louis, MO, USA) dissolved in a mixture of 95% (*v*/*v*) absolute ethanol and 5% (*v*/*v*) sulfuric acid. The TLC plate was air-dried and heated on a hot plate until spots appeared.

4.9. Synergistic Effects of HL12Pul in Starch Saccharification

To develop an efficient starch hydrolyzing process, the synergistic effects of HL12Pul in starch saccharification were investigated by mixing with recombinant raw starch degrading α-amylase from *Roseateles terrae* HL11 (HL11Amy) [46], In the mixture design, the HL11Amy was fixed enzyme loading as 1.6 μg protein, while HL12Pul was varied as 0.5, 1.0, and 1.6 μg protein for raw cassava starch hydrolysis. The hydrolysis reaction contained 1% (*w*/*v*) raw cassava starch in 50 mM sodium phosphate buffer, pH 6.0, with the enzyme cocktail. The reaction mixture was incubated at the optimal condition of HL12Pul (pH 6.0, 40 °C) for 30 and 60 min in ThermoMixer® C (Eppendorf, Hamburg, Germany) at 1000 rpm shaking speed; subsequently, reactions were inactivated by boiling at 100 °C for 10 min. The supernatant containing the product was collected for total reducing sugar determination. The amount of reducing sugar was measured from the absorbance measurement at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmole of product per 1 min.

4.10. Enzymatic Process for Resistant Starch Production

Resistant starch was prepared according to 3 steps. Firstly, the enzymatic hydrolysis of high amylose content starch, 1% (*w*/*v*) of gelatinized cassava starch dissolved in 50 mM sodium phosphate buffer pH 6.0 was mixed with the varying enzyme dosage, including 2, 4, 8, 16, and 32 U/g substrate. The mixture was incubated at 40 °C for 24 h in the ThermoMixer® C (Eppendorf, Hamburg, Germany) at 1000 rpm shaking speed. The hydrolysis reaction was inactivated by boiling at 100 °C for 10 min. Secondly, retrogradation, the hydrolysate was incubated at 4 °C for 48 h and was centrifuged at 12,000× *g* for 5 min to separate the pellet. The pellet was rinsed with deionized water 3 times. Finally, pellet dehydration, the pellet was stored in a desiccator for 24 h. The recovery yield was calculated from the weight of the final product after dehydration (final weight of modified starch) compared with the initiation weight of substrate.

$$\% \text{ Recovery} = \frac{\text{Final weight of modified starch (mg / mL)}}{\text{Initiation weight of substrate (mg / mL)}} \times 100$$

4.11. Physiochemical and Biochemical Properties Evaluation of Modified Starch

The morphology of modified starch produced by HL12Pul was observed compared with native cassava starch carried out using a scanning electron microscope (SEM) (SU5000, HITACHI, Tokyo, Japan). The samples were dried and coated with gold. An electron beam energy of 10 kV was used for analysis.

Moreover, the digestibility of modified starch was evaluated. The in vitro digestibility was performed by comparing native gelatinized cassava starch, retrograded gelatinized cassava starch, and HL12Pul-treated modified cassava starch at 16 and 32 U/mg protein loading. Substrates were digested with α-amylase from porcine pancreas (Sigma-Aldrich, St. Louis, MO, USA) in 20 mM sodium phosphate buffer saline, pH 7.0 at 37 °C for 30–120 min. The reaction mixture was incubated in ThermoMixer® C (Eppendorf, Hamburg, Germany) at 700 rpm shaking speed. The digested product was determined according to the DNS method [56].

5. Conclusions

In this work, the biochemical characteristic of type I pullulanase belonging to GH13_14 subfamily from *P. koreensis* HL12 was firstly evaluated. The enzyme specifically cleaved only α-1,6 glycosidic linkage in the substrate. Regarding its synergistic interaction with raw starch degrading α-amylase and resistant starch production efficiency, the enzyme represents a promising debranching enzyme that could be applied to develop efficient starch saccharification and modification processes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/catal12091014/s1, Figure S1: Sequence alignment of CBM48 in HL12Pul and α-glucan debranching enzymes in GH13_11–14 subfamilies; Figure S2: Investigation of recombinant intracellular HL12Pul produced by *E. coli* BL21(DE3).

Author Contributions: Conceptualization, D.P., H.L., B.B., K.A., W.S. and V.C.; funding acquisition, D.P. and H.L.; investigation, D.P., H.L., B.B., K.A., W.S. and V.C.; methodology, D.P., H.L. and B.B.; writing–original draft, D.P., H.L., B.B., K.A., W.S., N.A. and V.C.; writing–review & editing, D.P., H.L., B.B., K.A., W.S., N.A. and V.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financial supported by Thailand Graduate Institute of Science and Technology (TGIST), National Science and Technology Development Agency (Grant number: SCA-CO-2563-12209-TH), Thammasat University Research Fund (Contract No. TUFT 042/2563), and Agricultural Research Development Agency (Public Organization) (Grant number: HRD65050090).

Data Availability Statement: Not applicable.

Acknowledgments: The authors acknowledge the support provided by Enzyme Technology Research Team, National Center for Genetic Engineering and Biotechnology, and the Department of Biotechnology, Faculty of Science and Technology, Thammasat University.

Conflicts of Interest: The authors declare no conflict of interest.

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