Biochemical characterizations and food applications of carbohydrate active enzymes secreted from microorganisms

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General introduction

Enzymes have gained recognition globally for their widespread uses in various sectors of industries, e.g., food, agriculture, chemicals, medicine, and energy[1]. Genetic changes using classical breeding technology and recombinant DNA technology can easily be done on microbial cells for elevated enzyme production and scientific development[2]. Therefore, microorganisms are favored as enzyme sources for industrial enzymes due to easy availability and fast growth rate.

The global enzymes market size was reached at USD 12.46 billion in 2022 and it is projected to surpass around USD 20.5 billion by 2030, growing at a compound annual growth rate (CAGR) of 6.42% during the forecast period 2022 to 2030[3]. Food and feed applications account for 55–60% of the global enzymes market. The global industrial enzyme market in these applications is driven by three leading categories: carbohydrases, proteases, and lipases[4]. (1) Carbohydrases segment accounts for almost 50% of the industrial enzymes market, up to 70% for the food and beverages application. (2) Proteases segment accounts for 25–30% of the global market. (3) Lipases segment account for less than 10% market share. Among them, carbohydrases such as amylase, cellulase, hemicellulase, pectinase, and glucose oxidase occupy the largest share in food applications. These commercial enzymes used for food industry are mostly secreted extracellularly.

In the Carbohydrate-Active enZYmes (CAZy) database, carbohydrases are classified according to sequence similarity into the following five enzyme classes[5-7]. (1) Glycoside Hydrolases (GHs): hydrolysis and/or rearrangement of glycosidic bonds. (2) GlycosylTransferases (GTs): formation of glycosidic bonds. (3) Polysaccharide Lyases (PLs): non-hydrolytic cleavage of glycosidic bonds. (4) Carbohydrate Esterases (CEs): hydrolysis of

carbohydrate esters. (5) Auxiliary Activities (AAs): redox enzymes that act in conjunction with other enzymes in CAZy database. These families continue to expand today, with 5 new families established in 2020, 4 new families in 2021, and 4 new families in 2022[8-20]. Namely, unknown proteins secreted from microorganisms have not been completely studied and have the possibility to establish novel family and EC number. Thus, unknown hypothetical proteins possessing academically and industrially important functions might exist among microbial extracellular proteins.

In Chapter 1, the author investigated biochemical characteristics of two GH134 β-1,4-mannanases, yellow mold AoMan134A and actinobacteria SsGH134, belonging to novel GH134 family. This GH134 was newly established by Shimizu et al. 2015[21]. This previous study reported β-mannan-induced hypothetical protein from *Aspergillus nidulans*. This protein shared no homology to reported β-mannanases but displayed hydrolytic activity toward β-mannan. Moreover, based on the reverse genetic analysis, this protein was found to be the predominant β-1,4-mannanase involved in β-mannan degradation in *A. nidulans*. However, the functionalities and usefulness of β-1,4-mannanase belonging to GH134 had not been studied in detail. Therefore, biochemical characterization of two GH134 β-1,4-mannanases were carried out to discuss usefulness for food applications and processing.

In Chapter 2, the author investigated the commercial enzyme applications for plant-based meat analogs. Currently, the widening gap between the current supply of meat and its future demand has increased the need to produce plant-based meat analogs. Despite ongoing technical developments, the texture, color, flavor, taste, and nutrition of plant-based meat analogs differ from those of traditional meat products. The use of various additives to generate a meat-like texture, juiciness, mouthfeel, and flavor raises questions about sensory

qualities, nutrition, clean labeling, and consumer confidence[22,23]. In addition, meat analogs should be as clean as possible and made with natural ingredients to fulfill consumer demands with respect to health benefits[24]. Thus, the author investigated the usefulness of laccase and cyclodextrin glucanotransferase for meat analogs to meet these demands of further functionalities and clean label trends.

Unknown hypothetical proteins possessing industrially important functions still exist among microbial extracellular proteins and even well-known commercial enzymes have the ability to solve the novel technical challenges corresponding to the needs of the times. In this paper, the author investigated the biochemical characterizations and food applications of carbohydrate active enzymes secreted from microorganisms.

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Chapter 1. Characterization of β-1,4-mannanase belonging to novel GH family 134

Section 1. Biochemical characterization of thermostable β-1,4-mannanase belonging to the glycoside hydrolase family 134 from *Aspergillus oryzae*

Introduction

Hemicellulose is the second most abundant polysaccharide found in nature, and it is usually associated with cellulose and lignin in plant cell walls[1,2]. The β-mannans (glucomannan, galactomannan, and galactoglucomannan) together with xylans are the major components of hemicelluloses[3,4]. Ivory nuts, konjac, coffee beans, softwoods, and red algae are especially abundant in β-mannans[5-10]. Glucomannan comprises a β-1,4-linked backbone containing mannose, or a combination of glucose and mannose residues, and it is acetylated at the O-2 and/or O-3 positions[11,12]. Galactomannan and galactoglucomannan have branched galactose side chains linked to the backbone mannoses by an α-1,6-bond[13]. Glucomannan from konjac and galactomannan from guar gum are storage polysaccharides that are useful in the food industry[14,15].

Mannanolytic enzymes are important reagents in industrial biorefinery processes such as the production of second-generation biofuels from plant biomass[16]. The complete degradation of complex β-mannans requires the synergistic action of a set of mannanolytic enzymes with various substrate specificities. Filamentous fungi produce several mannanolytic enzymes including β-1,4-mannanase (EC 3.2.1.78), α-galactosidase (EC 3.2.1.22), β-mannosidase (EC 3.2.1.25), acetylmannan esterase (EC 3.1.1.6), and βglucosidase (EC 3.2.1.21)[13,17,18]. Among them, β-1,4-mannanases that digest the internal β-

1,4-linkage of the mannan backbone are crucial for β-mannan degradation, and these enzymes are ubiquitous in viruses, bacteria, and eukaryotes[19].

Based on sequence similarity, the β-1,4-mannanases have been classified into glycoside hydrolase (GH) families 5 (GH 5), 26 (GH 26), and 113 (GH 113)[20]. Many filamentous fungi including *Trichoderma reesei*, *Aspergillus niger*, *Aspergillus nidulans*, and *Podospora anserina* produce β-1,4-mannanases belonging to the GH 5 and GH 26 families[21-25]. The authors recently discovered a novel β-1,4-mannanase called AnMan134A, which they isolated from *Aspergillus nidulans*, which has no homology with proteins of known function including extant β-mannanases belonging to the GH 5, 26, and 113 families. This novel β-1,4-mannanase has been placed in the GH 134 family in the CarbohydrateActive enZYmes (CAZy) database[26]. AnMan134A reacts with β-mannan and mannooligosaccharides with mannotriose recognition, and it has high catalytic efficiency (k_{cat}/K_m) toward mannohexaose (M_6) compared to the β-1,4-mannanase, AnMan5C, which belongs to the GH 5 family. The author also showed that AnMan134A can act synergistically with AnMan5C toward glucomannan and galactomannan[26].

Beta-1,4-mannanases are widely used in a broad range of industries including biofuel production, oil production, food and animal feed production, coffee production, textiles, and pulp bleaching[13,19]. Thermostable β-1,4-mannanases that are capable of hydrolyzing β-mannans during biofuel production, oil drilling, and coffee production[13,19,27-30] have been identified; these β-1,4-mannanases belong to the GH 5 and GH 26 families[22,31-34].

The filamentous fungi *Aspergillus oryzae* and *Aspergillus niger* are generally accepted as being safe in food by the USA Food and Drug Administration, and they have

been applied to produce fermented foods, organic acids, beneficial secondary metabolites, and various enzymes. These fungi have been used to produce fermented foods, organic acids, beneficial secondary metabolites, and various enzymes. In Japan, *Aspergillus oryzae* has been used for over 1000 years in the traditional food industry. Homologs of AnMan134A are also found in some proteobacteria, actinobacteria, zygomycota, basidiomycota, and ascomycota including *A. oryzae*, but interestingly, these homologs are not found in *A. niger*[26].

In this study, the author aimed to characterize the enzymatic properties, including heat, pH, solvent, detergent, and metal-ion stabilities of AoMan134A isolated from *A. oryzae*.

Results

Purification and characterization of recombinant AoMan134A expressed in P. pastoris

AoMan134A encoded by the AO090038000445 gene in *A. oryzae* is orthologous to AnMan134A having a 70% amino acid sequence identity[26]. Recombinant AoMan134A was expressed in the methylotrophic yeast *Pichia pastoris*, and Zeocin™-resistant transformants were evaluated for β-1,4-mannanase activity on plates containing Azo-carob galactomannan. Among the 30 transformants on the plates having β-1,4-mannanase activity, the colony with the largest clear halo was selected to produce recombinant AoMan134A. Examination of extracellular β-1,4-mannanase activity at different time points following induction of protein expression revealed that activity was maximal (460 U mL^{-1}) after a 96-h incubation at 28°C. SDS-PAGE analysis of the crude supernatant from 12 to 96 h following induction showed that AoMan134A had a molecular mass of 19 kDa (Fig. 1-1A). The recombinant AoMan134A derived from culture supernatants after 96 h of induction was purified by anion exchange chromatography and gel filtration. The purified protein appeared as a single band

on SDS-PAGE having a molecular mass of 19 kDa (Fig. 1-1B). Using MALDI-TOF-MS, the author then investigated any potential changes in the molecular mass of AoMan134A before, and after, endoglycosidase H de-glycosylation. The molecular mass of the purified protein before and after endoglycosidase H de-glycosylation did not change, indicating that AoMan134A produced in *P. pastoris* is not Nglycosylated. This is consistent with the fact that AoMan134A does not contain any potential N-glycosylation sites (N-X-T/S). Based on this, the author used recombinant AoMan134A without endoglycosidase H de-glycosylation for the enzyme characterization studies reported here.

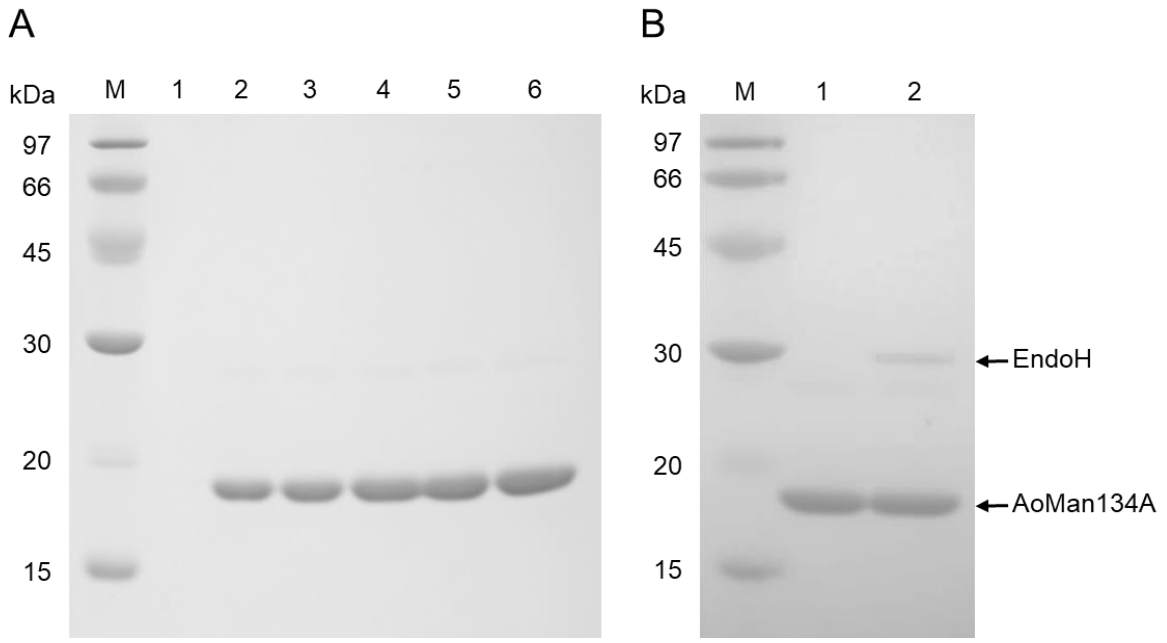

Figure 1-1. AoMan134A expressed in *P. pastoris* resolved by SDS-PAGE. (A) Culture supernatants (20 μL/lane) from *P. pastoris* expressing recombinant AoMan134A resolved by SDS-PAGE. Lanes 1 - 6, Culture supernatants after induction for 0, 12, 24, 48, 72, and 96 h; Lane M, protein molecular mass makers. (B) Purified AoMan134A (1 μg) resolved by SDS-PAGE stained with Coomassie brilliant blue. Lane 1, purified AoMan134A; 2, AoMan134A after de-glycosylation with endoglycosidase H (Endo H).

The optimal temperature and pH for the activity of recombinant AoMan134A were determined using glucomannan as a substrate (Figs. 1-2A, 1-2B). The optimal reaction temperature for AoMan134A was 30°C (Fig. 1-2A), whereas the optimal pH for AoMan134A activity was 6.0, with a preferred pH range between 5.0 and 6.5 (Fig. 1-2B). The author then determined the kinetic parameters of AoMan134A using galactomannan and glucomannan from different sources as substrates (Table 1-1). The catalytic efficiency (k_{cat}/K_m) of AoMan134Awas 6.8-fold higher toward locust bean gum galactomannan (k_{cat}/K_m = 527 mL $s^{-1}mg^{-1}$) than toward guar gum galactomannan (k_{cat}/K_m = 77 mL $s^{-1}mg^{-1}$), but similar toward locust bean gum galactomannan and konjac flour glucomannan (k_{cat}/K_m = 564 mL $s^{-1}mg^{-1}$) (Table 1-1). The specific activities of AoMan134A toward glucomannan and galactomannan from locust bean gum were 1,280 and 1,170 U mg^{-1}, respectively.

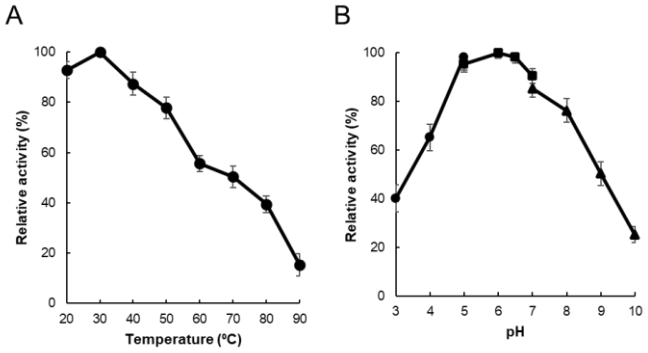

Figure 1-2. Temperature and pH optima for the enzymatic activity of AoMan134A.

(A) Optimal temperature for AoMan134A with glucomannan as substrate. Glucomannan (0.5%) in 475 μL of 50 mM sodium phosphate buffer (pH 6.0) was pre-incubated at temperatures ranging from 20°C to 90°C. (B) Optimal pH for AoMan134A. Enzyme reactions were conducted at 30°C for 60 min over a pH range of 3.0 – 10.0 in 50 mM sodium acetate (pH 3.0 – 5.0; ●), 50 mM sodium phosphate (pH 5.0 – 7.0; ■), or 50 mM Tris-HCl (pH 7.0 – 10.0; ▲). The activity of AoMan134A after incubation at 30°C for 60 min at pH 6.0 was set at 100%. Data are shown as means ± SE (error bars) of three independent experiments.

Table 1-1. Apparent kinetic parameters of AoMan134A towards β-mannans.

Substrate	K_m(mg/mL)	k_{cat}(/sec)	k_{cat}/K_m(mL/sec/mg)
Galactomannan (Galactose:Mannose)			
Locust Bean Gum (1:4)	1.1 ± 0.1	580 ± 20	527
Tara Gum (1:3)	2.1 ± 0.4	520 ± 50	248
Guar Gum (2:3)	3.8 ± 0.4	290 ± 40	77
Glucomannan			
Konjac flour	1.1 ± 0.3	620 ± 30	564

Data are presented as means ± standard deviation of three experiments. AoMan134A (1.0 μM) was incubated with β-mannans in 50 mM sodium phosphate buffer (pH 6.0) at 30°C.

Thermostability of AoMan134A toward glucomannan, β-mannan, and mannohexaose substrates

The thermostability of AoMan134A was determined using glucomannan as a substrate. Purified AoMan134A was incubated at 60, 70, 80, or 90°C for 120 min (Fig. 1-3A). After incubation at 70°C for 120 min, the β-1,4-mannanase activity decreased to 50% of its maximal activity at 30°C (Fig. 1-3A). After incubation at 90°C for 120 min, the β-1,4-mannanase activity of AoMan134A decreased to 28% (Fig. 1-3A), indicating that AoMan134A is stable at high temperatures.

Although *A. nidulans* AnMan134A generated mannobiose (M_2), mannotriose (M_3), and mannotetraose (M_4) from galactose-free β-mannan[26], AoMan134A produced M_2, M_3, M_4, and mannopentaose (M_5), with M_3 being the predominant reaction product under the

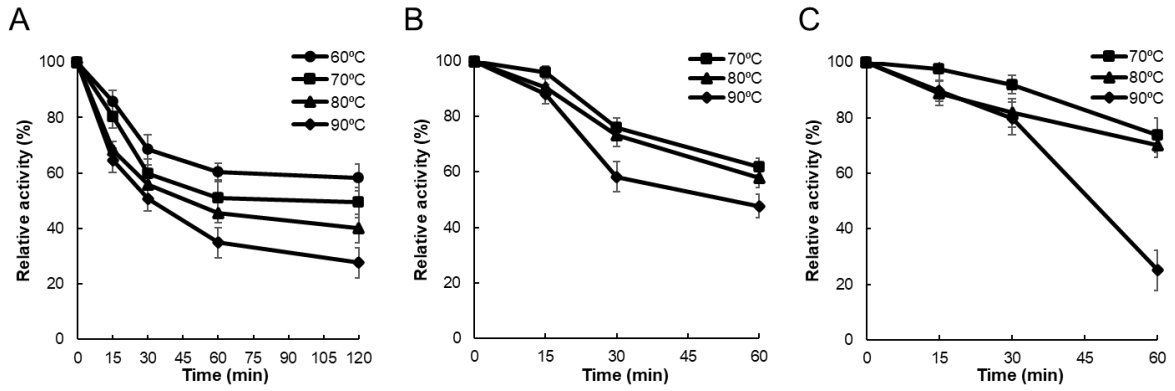

Figure 1-3. Thermostability of AoMan134A using glucomannan, β-mannan and mannohexose as substrates. (A-C) Purified AoMan134A (final concentration 1.0 μM) was incubated at a range of temperatures from 60°C to 90°C and then residual activity against 0.5% glucomannan (A), 0.5% galactose-free β-mannan (B), or 1.0 mM mannohexaose (C) was measured after further incubation at 30°C for 60 min at the optimal pH (pH 6.0). The activity of AoMan134A after incubation at 30°C for 60 min was set at 100%. Data are presented as means ± standard deviation (error bars) of independent four experiments.

same conditions (Fig. 1-4). The thermostability of AoMan134A was assayed by monitoring the reaction products generated when β-mannan was used as the substrate (Fig. 1-3B). After incubation at 70°C for 60 min, the β-1,4-mannanase activity of AoMan134A decreased to 62% of the activity seen at 30°C (Fig. 1-3B). Further increases in temperature to 80 and 90°C reduced the activity to 60 and 50% of the activity seen at 30°C, respectively (Fig. 1-3B). Similar results were obtained when glucomannan was used as the substrate (Fig. 1-3A).

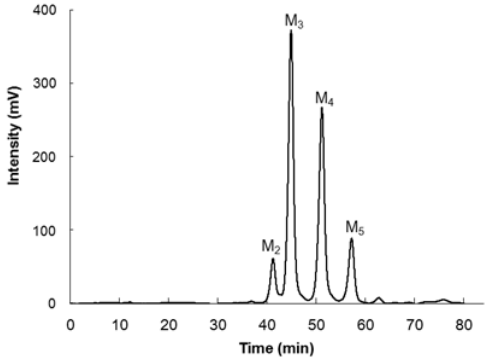

Figure 1-4. Hydrolysis of galactose-free β-mannan and mannohexaose by AoMan134A. Reaction products of 0.5% galactose-free β-mannan catalyzed after hydrolysis by AoMan134A were monitored using HPLC. M_2, mannobiose; M_3, mannotriose; M_4, mannotetraose; M_5, mannopentaose, M_6, mannohexaose.

Although AoMan134A did not hydrolyze M_2, M_3, and M_4, it produced M_2 and M_3 from M_5 and converted M_6 to M_2, M_3, and M_4 with M_3 being the predominant reaction product (data not shown). Based on this, the author assessed the thermostability of AoMan134A toward M_6 by measuring the reaction products obtained after incubation at 70, 80, and 90°C for 15, 30, and 60 min. (Fig. 1-3C). The activity of AoMan134A toward M6 decreased to 73% after incubation at 70°C for 60 min (Fig. 1-3C). Further increases of temperature to 80 and 90°C for 60 min resulted in 70 and 26% of the AoMan134A activity remaining, respectively (Fig. 1-3C). Taken together, these results suggested that AoMan134A is highly thermostable. The β-1,4-mannanase activity of AoMan134A was also seen to be thermostable when M_5 was used as the substrate (data not shown).

Stability of AoMan134A at various pH values assessed using glucomannan substrate

After incubation for 90 min at 30°C at pHs ranging from 5.0 to 10.0, AoMan134A retained > 75% of its maximal activity (Fig. 1-5). These findings indicated that AoMan134A

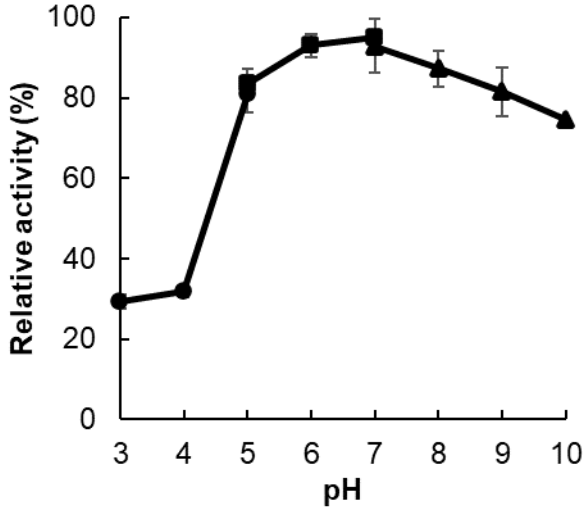

Figure 1-5. Stability of AoMan134A at various pHs.

Purified AoMan134A (final concentration 1.0 μM) was incubated in 50 mM sodium acetate (pH 3.0 - 5.0), 50 mM sodium phosphate (pH 5.0 - 7.0) and 50 mM Tris-HCl (pH 7.0 - 10.0) at 30°C for 90 min. The residual enzyme activity was then measured at the optimal pH (pH 6.0). The activity of AoMan134A incubated at pH 6.0 was set to 100%. Data are presented as means ± standard deviation (error bars) of three independent experiments.

remains very stable over a pH range of 5.0 to 10.0. This optimal pH range differs from those of other fungal β-1,4-mannanases that generally have an optimal pH in the acidic range[13].

Effect of organic solvents, detergents, and metal ions

AoMan134A activity was unaffected by up to 20% acetone, whereas 20% methanol, ethanol, and isopropanol similarly decreased the activity to about 60% (Fig. 1-6A). AoMan134A retained about 42%–56% of its maximal activity in the presence of 10% SDS, TritonX-100, or Tween-20 (Fig. 1-6B). The β-1,4-mannanase activity of AoMan134A was inhibited 38% by 20 mM Fe^{2+}, and 72% by 20 mM Cu^{2+}, whereas 20 mM Mn^{2+} and Zn^{2+}

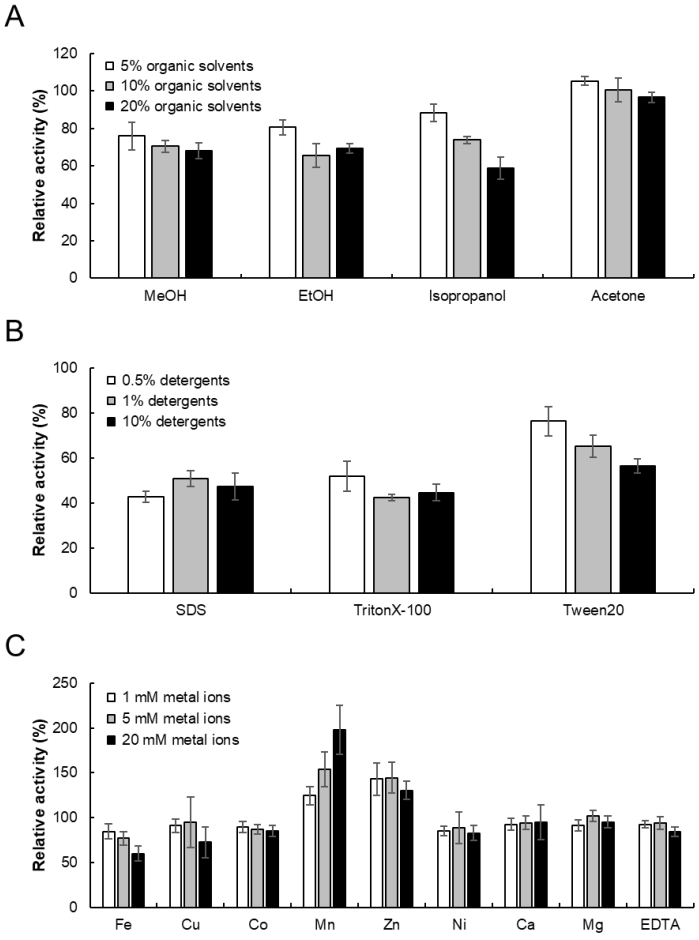

Figure 1-6. Effects of organic solvents, detergents and metal ions on AoMan134A. (A-C) Activity of AoMan134A (1.0 μM) in the presence of different levels of the indicated, organic solvents (A), detergents (B), or metal ions (C) was measured after incubation at 30°C for 60 min at pH 6.0. The activity of AoMan134A incubated at optimal pH (pH 6.0) in the absence of organic solvents, detergents, or metal ions was set at 100%. Data are presented as means ± standard deviation (error bars) of three independent experiments.

notably increased the enzyme activity of AoMan134A 2.0- and 1.4-fold (Fig. 1-6C). The effect of Mn^{2+} was dose-dependent over 1-20 mM, whereas the effect of Zn^{2+} was maximal at 1-5 mM. Co^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+} or EDTA did not affect the enzyme activity (Fig. 1-6C).

Discussion

Thermophilic and thermostable β-1,4-mannanases have the advantages of reducing risk of contamination, increasing substrate solubility, and improving mass transfer rates[35]. The thermostability of GH 5 and GH 26 β-1,4-mannanases at temperatures ranging from 40°C–70°C, over time periods from 10 min to 24 h, has been summarized[19]. Furthermore, highly thermophilic and/or thermostable β-mannanases have been isolated from various fungi including *Aspergillus fumigatus* IMI 385708[36], *Aspergillus nidulans* XZ3[23], *Aspergillus niger* BK01[21], *Humicola insolens* Y1[31], *Penicillium oxalicum* GZ-2[37], *Rhizomucor miehei*[38], and *Talaromyces leycettanus* JCM12802[32]. The β-1,4-mannanase activity of AoMan134A towards glucomannan was 58%, 50%, 40%, and 28% of the maximal activity at 30°C after incubation at 60°C, 70°C, 80°C and 90°C for 120 min, respectively. These findings indicate that the thermostability of AoMan134A is at least equal to that of other highly thermostable β-1,4-mannanases. Compared with other thermostable β-1,4-mannanases, AoMan134A exhibits similar specific activities towards glucomannan and galactomannan from locust bean gum (1,280 and 1,170 U/mg, respectively)[35,39].

AoMan134A is stable over a neutral to alkaline pH range. Other fungal β-1,4-mannanases, for example, those from *Humicola insolens* Y1, *Chaetomium* sp. and *Rhizomucor miehei* are also tolerant of alkaline pH[31,38,40]. This neutral to alkaline pH tolerance of AoMan134A could provide an obvious advantage in the kraft pulp and detergent

manufacturing industries, which require the use of a high pH. Furthermore, AoMan134A being both thermostable and having a neutral to alkaline pH tolerance, could provide a benefit in the production of fermentable sugars from biomass, since increasing fermentation temperatures would accelerate process time, lower energy costs, lower the risk of microbial contamination, and improve fermentation yields[41,42].

AoMan134A retained 97%, 69%, 61%, and 68% of the original activity in the presence of up to 20% organic solvent (acetone, ethanol, isopropanol, and methanol, respectively), 47%, 45% and 56% of the original activity in the presence of up to 10% detergent (SDS, TritonX-100, and Tween-20, respectively) and 62% and 72% of the original activity in up to 20 mM Fe^{2+} and Cu^{2+} respectively. The β-1,4-mannanase activity of AoMan134A was notably increased in the presence of 20 mM Mn^{2+} and Zn^{2+} being increased 2.0- and 1.4-fold respectively. These latter findings are similar to those seen for the β-1,4-mannanases from *Xanthomonas campestris*[43] and *Bacillus sp*. JB-99[44], possibly because Mn^{2+} and Zn^{2+} might be associated with active alkaline phosphatase, which is essential for enzyme activity and the maintenance of its conformational stability[45]. Different metal ions at different concentrations have been shown to affect β-1,4-mannanases[43]. The effect of metal ions on β-1,4-mannanase activity might be related to the net overall electric charge on the surface of the enzyme[33,34]. Since saccharification can be combined with fermentation, an enzyme such as AoMan134A that is stable to metal ions, solvents, and high temperatures would be useful for the conversion of lignocellulose to biofuels[21,41,42].

Based on amino acid sequences, many filamentous fungi have been shown to possess β-1,4-mannanases that belong to the GH 5 and GH 26 families, which are both classified within the largest glycoside hydrolase clan, GH-A[20]. Enzymes in this clan share

the triose phosphate isomerase (TIM) $(\beta/\alpha)_8$-barrel fold. Recently, Jin *et al.* have reported the three-dimensional structure of SsGH134A, an enzyme with 56% identity to AoMan134A, that also belongs to the GH 134 family, and possesses a novel catalytic mechanism[46]. Based on this, family GH 134 β-mannanases adopt a lysozyme-like fold with a strong overall resemblance to family GH 19 chitinases, GH 22 C-type lysozymes, GH 23 G-type lysozymes, and GH 124 cellulases. Interestingly, two GH 22 C-type lysozymes, from bacteriophage T4 and chicken, and a GH 19 chitinase from *Hevea brasiliensis* have also been reported to be highly thermostable[47-49]. This information will be useful in designing site-directed mutagenesis studies designed to clarify the mechanism of AoMan134A's thermostability.

The major components of hemicelluloses are β-mannan polysaccharides, which are abundant in plant cell walls along with cellulose and lignin[1,2]. The diverse industrial applications of β-1,4-mannanases include de-inking of paper waste, clarifying fruit juices, and upgrading feed and fodder[19]. β-1,4-mannanases are also used in a variety of other industrial processes, such as the extraction of vegetable oil from leguminous seeds, reduction of viscosity in extracts during the manufacture of instant coffee, improvement in the consistency of beer, and wood biopulping[50]. One of the most important commercial applications of thermostable β-1,4-mannanase is to hydrolyze β-mannans to enhance the flow of oil and gas during drilling operations[51] in high-temperature wells[52]. The AoMan134A described herein should therefore be of interest to the energy, food, and pulp industries, and could be of relevance to studies of the lignocellulosic biomass conversion technology[53].

Methods

Chemicals and reagents

M_2, M_3, M_4, M_5, M_6, konjac glucomannan, locust bean gum galactomannan, guar gum galactomannan and galactose-free β-mannan (prepared from carob galactomannan with all α-galactosyl residues removed by α-galactosidase) were purchased from Megazyme International (Bray, Ireland). Galactomannan from Tara gum was obtained from Mitsubishi-Kagaku Foods Corporation (Tokyo, Japan).

Preparation of recombinant proteins

A. oryzae RIB40 was obtained from NITE Biological Resource Center (Tokyo, Japan). Single-strand cDNA was synthesized from total RNA extracted from disrupted fungal cells as described[54-56]. Complementary DNA fragments encoding the AoMan134A (AO090038000445) gene were prepared by PCR using the oligonucleotide primer set FwAoman134A (5'-CGGGGTACCGCTCCAACTCCCGATGCTTCC-3') and RvAoman134A (5'-ATAGTTTAGCGGCCGCTTAGATGGCACGAACATTGACCCAAA-3'). The PCR product was purified, digested by *Kpn*I and *Not*I and then ligated into pPICZα-A (Novagen, Darmstadt, Germany) that had been digested with the same restriction enzymes. The resulting plasmid was introduced into *Pichia pastoris* KM71H (Invitrogen, Carlsbad, CA) and the resulting strain was cultured to produce recombinant AoMan134A as described[26]. The culture supernatant was concentrated using an Amicon Ultra filter unit (Merck-Millipore, Billerica, MA, USA). The concentrated protein solution was fractionated on a HiTrap Q column (GE Healthcare) using a linear gradient of 0 - 0.5 M NaCl in 25 mM Tris-HCl (pH 8.0). The 0.4 M NaCl fraction was concentrated using an Amicon Ultra filter unit (Merck-Millipore). The purified β-1,4-mannanase was further applied to a Superose 6 10/300 GL column (GE Healthcare) and recombinant protein was eluted with 20 mM Tris-

HCl (pH 8.0) containing 150 mM NaCl and dialyzed against 20 mM Tris-HCl (pH 8.0). All protein purification steps proceeded at 4°C. Protein concentrations were assayed using Bradford Protein Assays (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as the standard. The purified AoMan134A was cleaved with Endoglycosidase H (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions to remove N-linked glycans.

Enzyme assays

The activity of β-1,4-mannanase was assayed in 0.5-mL reaction mixtures containing 50 mM sodium phosphate (pH 6.0), 0.2 - 5% (w/v) substrates and purified protein. Reactions were incubated at 30°C, and AoMan134A was removed from the reaction solution using a Nanosep® Centrifugal Device (Pall Corporation, Port Washington, NY, USA) as described in the instruction manual. Flow-through fractions were boiled at 100°C for 30 min, and then the reducing sugars produced by β-1,4-mannanase were measured using 3,5-dinitrosalicylic acid (DNS) as described[57]. Standard curves were prepared based on solutions containing various concentrations of mannose. One unit of β-1,4-mannanase activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugar (mannose equivalents) per minute.

Thermostability of purified AoMan134A

The thermostability of AoMan134A was determined using glucomannan, galactose-free β-mannan and manno-oligosaccharides as substrates. Purified β-1,4-mannanase was incubated at temperatures ranging from 60°C to 90°C for 15, 30, 60, and 120 min. Residual

β-1,4-mannanase activity was assayed in 0.5-mL reaction mixtures containing 50 mM sodium phosphate (pH 6.0), substrates and purified protein. The amount of reducing sugars produced by β-1,4-mannanase was measured using DNS method. Soluble products released from β-mannan and M_6 were determined by monitoring post-column derivatized reducing sugars that were separated using a Prominence reducing-sugar HPLC analytical system (Shimadzu, Kyoto, Japan) equipped with a fluorescence detector. The supernatant was separated on a Shim-pack 4.0 × 250-mm ISA-07/S2504 column (Shimadzu) with a linear gradient of 0.1 M potassium borate buffer (pH 8.0) and 0.4 M potassium borate buffer (pH 9.0) for 90 min at a flow rate of 0.6 mL min^{-1}. The activity of β-1,4-mannanase towards M_6 was determined by HPLC as a decrease in substrate.

pH stability of purified AoMan134A

The author evaluated the stability of β-1,4-mannanase at various pH values using glucomannan as a substrate. The enzyme was incubated in 50 mM sodium acetate (pH 3.0 - 5.0), 50 mM sodium phosphate (pH 5.0 - 7.0) and 50 mM Tris-HCl (pH 7.0 - 10.0) for 1 h and then residual β-1,4-mannanase activity against 0.5% glucomannan was measured at the optimal pH (pH 6.0).

Effects of organic solvents, detergents and metal ions on β-1,4-mannanase activity

The author determined the effects of the solvents, methanol, ethanol, isopropanol and acetone, the detergents SDS, Triton X-100 and Tween 20, the metal ions, Cu^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Ca^{2+}, Mg^{2+}, and EDTA on the enzymatic activity of β-1,4-mannanase by measuring residual activity against 0.5% glucomannan after a 60-min incubation at 30°C.

Summary

A β-1,4-mannanase, termed AoMan134A, that belongs to the GH 134 family was identified in the filamentous fungus *Aspergillus oryzae*. Recombinant AoMan134A was expressed in *Pichia pastoris*, and the purified enzyme produced mannobiose, mannotriose, mannotetraose, and mannopentaose from galactose-free β-mannan, with mannotriose being the predominant reaction product. The catalytic efficiency (k_{cat}/K_m) of AoMan134A was 6.8-fold higher towards galactomannan from locust bean gum, than towards galactomannan from guar gum, but similar towards galactomannan from locust bean gum and glucomannan from konjac flour. After incubation at 70°C for 120 min, the activity of AoMan134A towards glucomannan decreased to 50% of the maximal activity at 30°C. AoMan134A retained 50% of its β-1,4-mannanase activity after heating at 90°C for 30 min, indicating that AoMan134A is thermostable. Furthermore, AoMan134A was stable within a neutral-to-alkaline pH range, as well as exhibiting stability in the presence of a range of organic solvents, detergents, and metal ions. These findings suggest that AoMan134A could be useful in a diverse range of industries where conversion of β-mannans is of prime importance.

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Section 2. Characterization of pH-tolerant and thermostable GH 134 β-1,4-mannanase SsGH134 possessing carbohydrate binding module 10 from *Streptomyces* sp. NRRL B-24484

Introduction

The internal β-1,4-linkage of the mannan backbone is randomly hydrolyzed by β-1,4-mannanases that have been isolated from bacteria, fungi, plants and animals[1-4]. Based on sequence similarity, β-1,4-mannanases have been classified into the glycoside hydrolase (GH) families 5 (GH 5), GH 26, GH 113 and GH 134[1-5]. The GH 26 β-1,4-mannanases were found predominantly in bacteria such as *Bacillus subtilis*, *Cellvibrio japonicus*, *Clostridium cellulovorans*, *Clostridium thermocellum*, *Klebsiella oxytoca* KUB-CW2-3, *Pantoea agglomerans* and *Reticulitermes speratus*[6-11]. Some bacteria such as *Bacillus licheniformis*, *Cellulosimicrobium* sp. HY-13, *Streptomyces thermolilacinus*, *Thermotoga petrophila* and *Vibrio* sp. strain MA-138 reportedly produce GH5 β-1,4-mannanase[12-16]. The primary structure of GH 5, GH 26 and GH 113 β-1,4-mannanases considerably differ, but their spatial arrangements are similar and they have a $(\beta/\alpha)_8$-barrel protein fold; thus they are grouped into the GH-A clan[2,4]. The three-dimensional structure of the GH 134 β-1,4-mannanase SsGH134 from *Streptomyces* sp. NRRL B-24484 has recently been reported[17]. A representative GH family 134 β-1,4-mannanase displays a fold closely related to that of hen egg-white lysozyme but acts with an inversion of stereochemistry[17]. The structures of the Michaelis and product complexes suggest a Southern hemisphere $^1C_4 \rightarrow {}^3H_4^{\ddagger} \rightarrow {}^3S_1$ conformational itinerary along the reaction coordinate, with the product relaxing to a Michaelis-mimicking $^1C_4$ conformation (possibly via a $^3H_4$ conformation) after the reaction is

complete. *Streptomyces* sp. NRRL B-24484 has an operon encoding a series of proteins that are predicted to facilitate the deconstruction and metabolism of β-mannan, including a GH2 β-mannosidase, two GH5 β-mannanases, SsGH134, sugar ABC transporters, and a mannose-6-phosphate isomerase[17]. In addition, SsGH134 possesses a carbohydrate binding module (CBM) 10 that is attached via a short Gly-Ser linker to its catalytic domain.

Glycoside hydrolases often display modular architectures comprising CBM[4,18]. In general, CBM increase substrate concentrations around catalytic domains, which leads to increased catalytic efficiency[18]. Carbohydrate binding modules have been classified into 81 families according to amino acid sequences, binding specificity and structure. Among them, CBM10 is mainly fused to cellulase, β-xylanase and β-1,4-mannanase from bacteria[19-22]. CBM10 binds to insoluble microcrystalline cellulose and insoluble β-mannan[20,21].

The present study uncovered the enzymatic characteristics of SsGH134 and the role of the CBM10 domain at the N-terminal region. The enzymatic properties of SsGH134 differed from those of bacterial β-1,4-mannanases that belong to GH 5 and GH 26. The author also found that CBM10 disruption increased catalytic efficiency and decreased pH, solvent and detergent stabilities.

Results

Alignment of CBM10 from various glycoside hydrolases

The author aligned CBM10 amino acid sequences of GH 134 β-1,4-mannanase from *Streptomyces* sp. (SsGH134, WP_030268297.1) as described above. Ponyi et al. found using site-directed mutagenesis that aromatic amino acid residues in CBM10 sequences from PfXyn10A (GH 10 xylanase from *Pseudomonas fluorescens*) are highly conserved, and that

W497, Y498, W512 and W514 (previously represented as W7, Y8, W22 and W24) of CBM10 play roles in binding to cellulose and β-mannan[20]. Four of the CBM10 amino acid residues that interact with polysaccharides (Fig. 2-1) were highly conserved. In contrast, the essential residue W497 of CBM10 in PfXyn10A sequence was not conserved in SsGH134 (Fig. 2-1). SsGH134 homologs are distributed in some actinobacteria and proteobacteria[5]. Among them, SsGH134 from *Streptomyces* sp. NRRL B-24484, KIQ61562 (NCBI accession number) from *Kitasatospora griseola*, WP_062059199 (NCBI accession number) from *Cellvibrio* sp. OA-2007, AIF91528 (NCBI accession number) from *Alteromonadaceae bacterium* Bs12 and AIF91558 (NCBI accession number) from *A. bacterium* Bs02 were fused with CBM10. The first Trp of CBM10 was highly conserved in WP_062059199, AIF91528 and AIF91558 from a proteobacterium, while the substitution of the first Trp with Gly was presented in SsGH134 and KIQ61562 from an actinobacterium. These indicate that

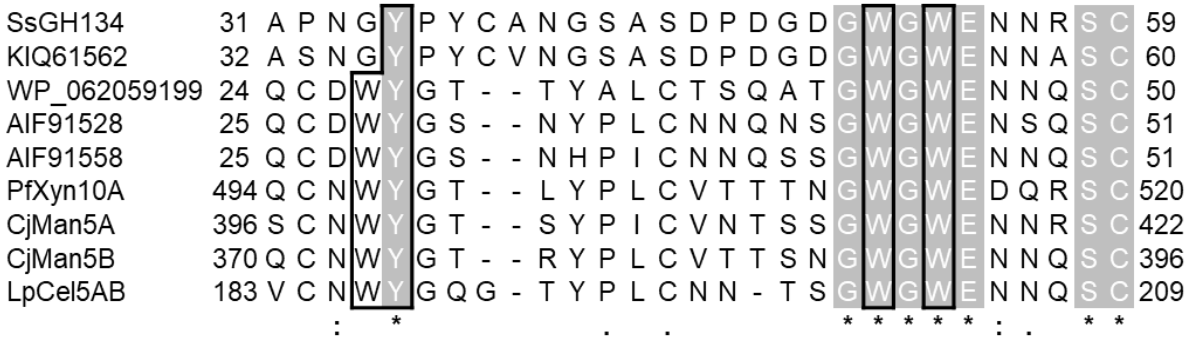

Figure 2-1. Amino acid alignment of CBM10. Amino acid sequences of CBM10 were aligned using ClustalW. Amino acids that might interact with cellulose are boxed, and identical amino acids are indicated in gray. Dots and colons indicate conserved amino acids with substitutions. CBM10 of SsGH134 (WP_030268297.1) from *Streptomyces* sp., CBM10 of KIQ61562 from *K. griseola*, CBM10 of WP_062059199 from *Cellvibrio* sp. OA-2007, CBM10 of AIF91528 from *A. bacterium* Bs12, CBM10 of AIF91558 from *A. bacterium* Bs02, CBM10 of CjMan5A (WP_012488914.1) from *C. japonicus*, CBM10 of CjMan5B (WP_012488072.1) from *C. japonicus*, CBM10 of PfXyn10A (WP_012488068.1) from *P. fluorescens*, CBM10 of Cel5AB (ABS72374.1) from *L. pedicellatus.*

replacing the first Trp with Gly might be conserved in actinobacteria (Fig. 2-1).

Purification of recombinant SsGH134 and SsGH134-ΔCBM10 expressed in E. coli

SsGH134 deposited in NCBI under accession number WP_030268297.1 is orthologous to AnMan134A (GH134 β-1,4-mannanase *Aspergillus nidulans*) and AoMan134A (GH134 β-1,4-mannanase from *A. oryzae*), having 61% and 56% amino acid sequence identity, respectively[5,23]. The author prepared recombinant SsGH134, SsGH134-ΔCBM10 (CBM10-linker-truncated SsGH134) and SsGH134-G34W (substitution of Gly34 to Trp) (Fig. 2-2A) as N-terminal His_6-tagged proteins in the *E. coli* expression system described in Materials and Methods. The recombinant SsGH134, SsGH134-ΔCBM10 and SsGH134-G34W migrated as a single band on SDS-PAGE after thrombin digestion of the His_6 tag and purification (Fig. 2-2B).

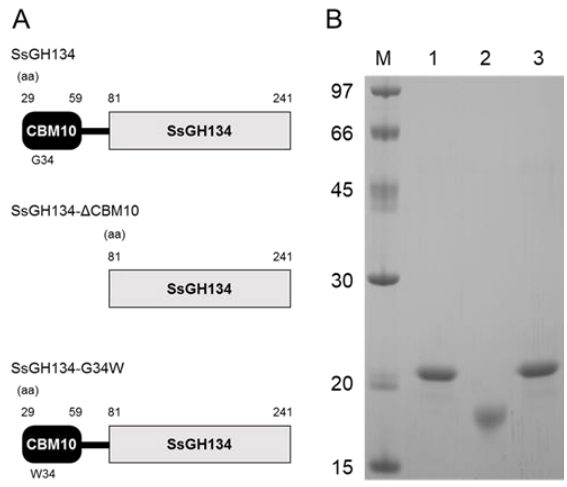

Figure 2-2. Schematic representation and SDS-PAGE analysis of purified recombinant SsGH134, SsGH134-ΔCBM10 and SsGH134-G34W. (A) Schematic representation of SsGH134, SsGH134-ΔCBM10 and SsGH134-G34W. (B) SDS-PAGE of recombinant SsGH134, SsGH134-ΔCBM10 and SsGH134-G34W. Purified enzymes resolved by SDS-PAGE were stained with Coomassie brilliant blue. Lane M, Protein standard; lane 1, SsGH134; lane 2, SsGH134-ΔCBM10; lane 3, SsGH134-G34W.

Binding assay of SsGH134, SsGH134-ΔCBM10 and SsGH134-G34W

The enzymes SsGH134, SsGH134-ΔCBM10 and SsGH134-G34W were mixed with MCC, β-mannan, chitin and xylan, and then the amounts of enzyme that bound or not to polysaccharides were evaluated by SDS-PAGE (Fig. 2-3A). The enzymes bound with high affinity to MCC (Fig. 2-3A). Binding of SsGH134, SsGH134-ΔCBM10 and SsGH134-G34W to cellulose, β-mannan, chitin and xylan was determined in a series of binding isotherm experiments (Fig. 2-3B). These enzymes bound to MCC, β-mannan and chitin, but not to xylan, indicating that SsGH134 binding was independent of CBM10.

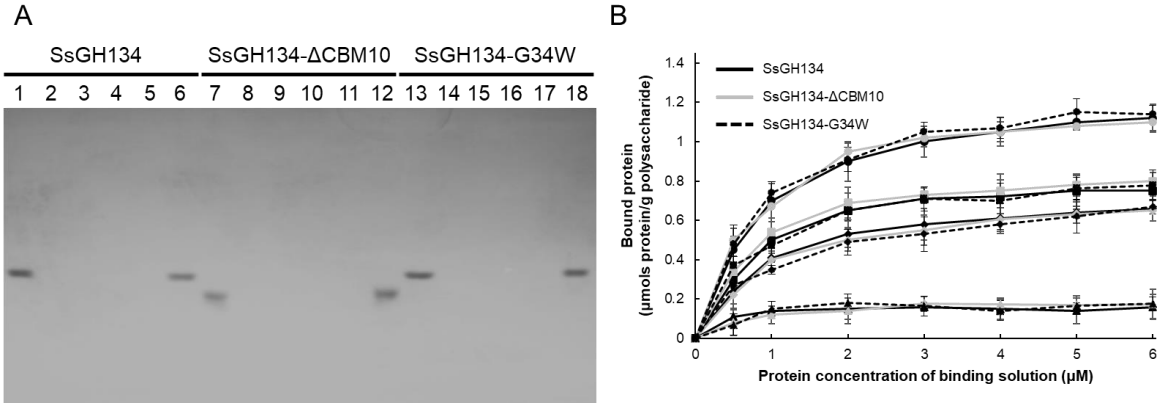

Figure 2-3. Binding assays of SsGH134, SsGH134-ΔCBM10 and SsGH134-G34W. (A) SsGH134, SsGH134-ΔCBM10 and SsGH134-G34W were incubated with MCC (15 mg) at 37°C for 30 min then input (lanes 1, 7 and 13), unbound (lanes 2, 8 and 14), wash (lanes 3-5, 9-11 and 15-17) and bound (lanes 6, 12 and 18) fractions were resolved by SDS-PAGE. (B) Binding was assayed in 100 μL-reaction mixtures containing 50 mM sodium phosphate (pH 5.0), 1.0 mg (w/v) substrates and 0.5 - 10.0 μM of purified enzymes. SsGH134, SsGH134-ΔCBM10 and SsGH134-G34W were incubated with MCC (circles), β-mannan (squares), chitin (diamonds) and xylan (triangles) at 37°C for 30 min, and then pelleted by centrifugation at 15,000 × g for 10 min. The amount of unbound proteins and bound proteins were measured at 280 nm. Error bars, means ± SE of three independent experiments.

Biochemical characterization of SsGH134 and SsGH134-ΔCBM10

The optimal pH and temperature for SsGH134 and SsGH134-ΔCBM10 activity were determined using glucomannan as a substrate. The optimal pH was 5.0, (preferred range, pH 4.0 - 7.0; Fig. 2-4A) and the optimal reaction temperature was 40°C (Fig. 2-4B) for both enzymes. The catalytic efficiency (k_{cat}/K_m) of SsGH134 and SsGH134-ΔCBM10 was 8.1- and 7.5-fold higher towards glucomannan than galactomannan (Table 2-1). The catalytic efficiency (k_{cat}/K_m) of SsGH134-ΔCBM10 towards glucomannan and galactomannan was 2.4- and 2.6-fold higher than that of SsGH134 (Table 2-1), suggesting that CBM10 on SsGH134 inhibits β-1,4-mannanase activity.

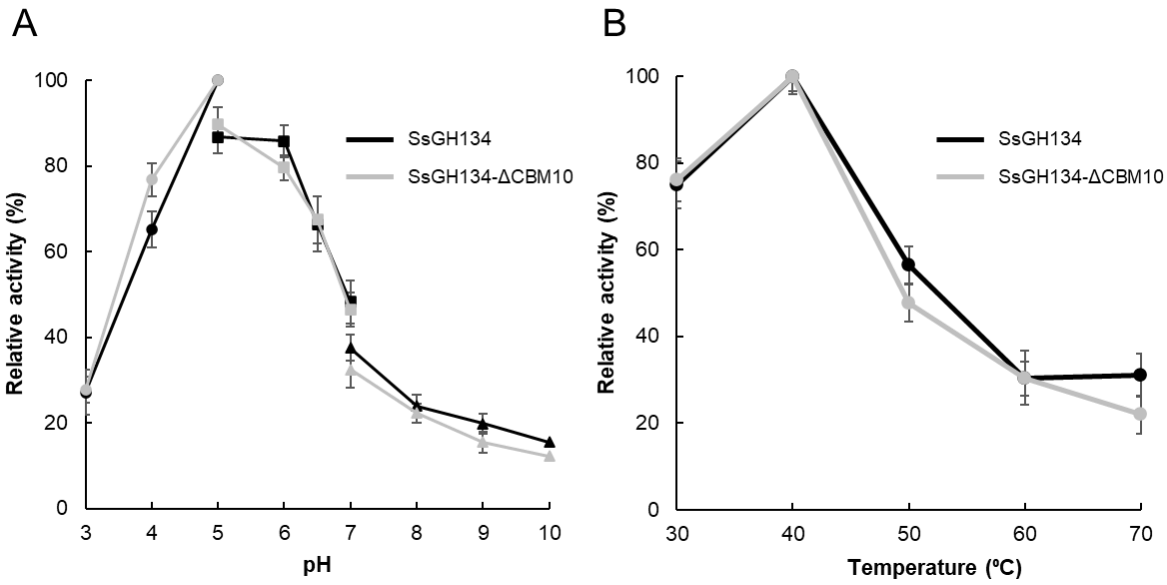

Figure 2-4. Optimal pH and temperature for enzymatic activity of SsGH134 and SsGH134-ΔCBM10. (A) Optimal pH of SsGH134 and SsGH134-ΔCBM10. Enzyme reactions proceeded at pH 3.0 – 10.0 in 50 mM sodium acetate, pH 3.0 – 5.0 (circles), 50 mM sodium phosphate, pH 5.0 – 7.0 (squares) and 50 mM Tris-HCl, pH 7.0 – 10.0 (triangles). (B) Optimal temperature of SsGH134 and SsGH134-ΔCBM10. Enzyme reactions proceeded at 30°C – 70°C for 60 min. Error bars, means ± SE of three independent experiments.

Table 2-1. Apparent kinetic parameters of recombinant SsGH134 and SsGH134-ΔCBM10 towards β-mannans.

Enzyme	Substrate	K_m (mg/mL)	k_{cat} (/sec)	k_{cat}/K_m (mL/sec/mg)
SsGH134	Glucomannan	2.8 ± 0.9	340 ± 40	121
	Galactomannan	7.8 ± 0.3	120 ± 20	15
GH134-ΔCBM10	Glucomannan	1.8 ± 0.4	530 ± 70	294
	Galactomannan	5.4 ± 0.5	210 ± 50	39

Stability of pH and thermostability of SsGH134 and SsGH134-ΔCBM10

Both SsGH134 and SsGH134-ΔCBM10 were stable at a pH range of 3.0 - 7.0 for

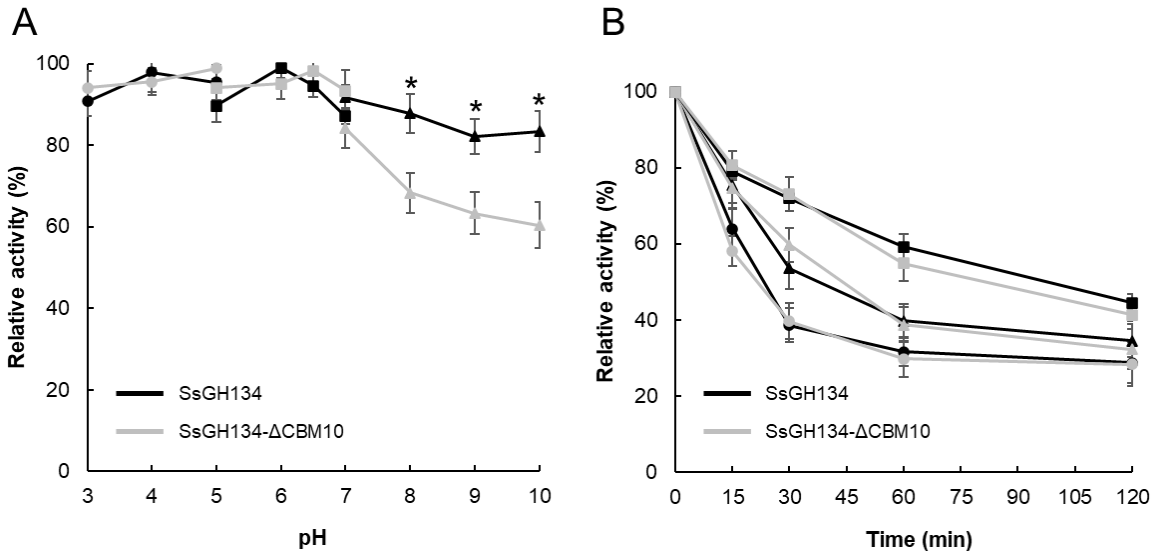

Figure 2-5. SsGH134 and SsGH134-ΔCBM10 pH stability and thermostability. (A) Purified AoMan134A incubated in 50 mM sodium acetate, pH 3.0 - 5.0 (circles), 50 mM sodium phosphate, pH 5.0 - 7.0 (squares) and 50 mM Tris-HCl, pH 7.0 - 10.0 (triangles) for 90 min. (B) Purified SsGH134 and SsGH134-ΔCBM10 incubated at 80°C (squares), 90°C (triangles) and 100°C (circles). Residual enzyme activity against 0.5% glucomannan was then measured at optimal pH (pH 5.0). Activity of SsGH134 and SsGH134-ΔCBM10 incubated at pH 6.0 was set to 100%. Data are presented as means ± standard deviation of three experiments. *$p < 0.005$, Student's *t* test.

120 min, but SsGH134 was more stable than SsGH134-ΔCBM10 at pH 7.0 - 10.0 (Fig. 2-5A). The thermostability of purified SsGH134 and SsGH134-ΔCBM10 was determined by incubation with glucomannan as a substrate at 80°C, 90°C and 100°C for 120 min (Fig. 2-5B). The activity of both β-1,4-mannanases decreased to ~ 60% and ~ 30% after incubation at 80°C for 60 min, and 100°C for 120 min, respectively, indicating that both are stable at high temperatures and have similar thermostability profiles (Fig. 2-5B).

Effect of organic solvents and detergents

The β-1,4-mannanase SsGH134 retained about 49% – 99% of its maximal activity in the presence of 20% organic solvents and 10% detergents, whereas the activity of SsGH134-ΔCBM10 was about 32%–52% lower than that of SsGH134 in the presence of 20% methanol,

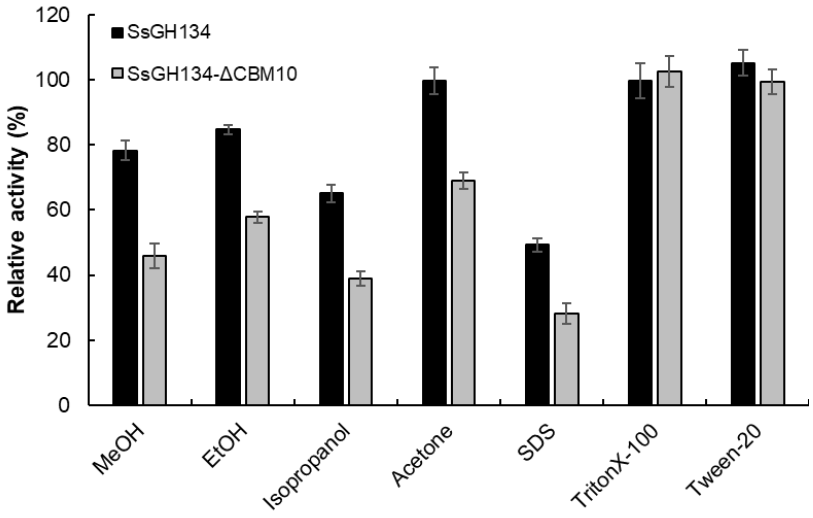

Figure 2-6. Effects of organic solvents and detergents on SsGH134 and SsGH134-ΔCBM10 activity. Activities of SsGH134 and SsGH134-ΔCBM10 (5.0 μM) in presence of 20% organic solvents and 10% detergents measured after incubation at 30°C for 60 min at pH 6.0. Activities of SsGH134 and SsGH134-ΔCBM10 incubated at optimal pH (pH 5.0) without organic solvents or detergents were set at 100%. Data are presented as means ± standard deviation (error bars) of three independent experiments.

ethanol, isopropanol, acetone and 10% SDS (Fig. 2-6). These findings indicated that the SsGH134-ΔCBM10 was less stable than SsGH134 against solvents and SDS. The SsGH134 and SsGH134-ΔCBM10 activities were unaffected by 10% TritonX-100 and Tween-20.

Discussion

Most β-mannanases isolated from bacteria belong to GH 26, and some GH5 β-mannanases have been described[6-16]. Bacterial GH 5 and GH 26 β-mannanases were maximally active at neutral to alkaline pH[1,2,4]. The pH stability of bacterial β-1,4-mannanases belonging to GH 5 and GH 26 has been summarized[4]. According to that review, most bacterial β-1,4-mannanases are significantly tolerant of a pH range of 6.0 – 9.0. Some bacterial β-1,4-mannanases are pH-stable under acidic conditions[24]. The SsGH134 isolated from *Streptomyces* sp. in the present study was maximally active between pH 4 – 6.5 (Fig. 2-4A) and retained > 80% of its maximal activity after incubation for 90 min at 30°C and pH 3.0 – 10.0 (Fig. 2-5A). These findings indicated that the biochemical properties of SsGH134 differed from those of bacterial GH 5 and GH 26, and fungal GH 134 β-1,4-mannanases.

SsGH134 β-1,4-mannanase retained 44%, 35% and 30% of its maximal activity at 30°C towards glucomannan after incubation at 80°C, 90°C and 100°C for 120 min. This indicated that SsGH134 is at least as thermostable as other highly thermostable β-1,4-mannanases[1-4]. The GH 5 and GH 26 β-1,4-mannanases are thermostable at a temperature range of 40°C – 70°C for 10 min to 24 h, which confers the advantages of reduced risk of contamination, increased substrate solubility, and improved mass transfer rates[25]. In fact, highly thermostable β-1,4-mannanases are used to hydrolyze galactomannan in oil drilling operations[1,4,26], to enhance the flow of oil and gas from wells at temperatures > 80°C.

CBM10 is found in bacterial cellulases, β-xylanases and β-1,4-mannanases and it binds to microcrystalline cellulose and insoluble β-mannan[20,21]. The first Trp residue involved in the binding to polysaccharides was not conserved in CBM10 of SsGH134 (Fig. 2-1). The binding of SsGH134 and SsGH134-ΔCBM10 to insoluble microcrystalline cellulose and insoluble β-mannan indicated that deleting CBM10 did not affect the ability of SsGH134 to bind to polysaccharides. SsGH134-G34W did not change the binding activity towards the polysaccharides. In contrast, deleting the CBM10 of GH 5 β-1,4-mannanases, Man5A and Man5B from *Cellvibrio japonicus*, abolished binding of the GH 5 β-1,4-mannanases to insoluble microcrystalline cellulose and insoluble β-mannan[21]. These findings indicate that the GH 134 domain binds with high affinity to polysaccharides.

CBM10 disruption affected the catalytic efficiency of SsGH134 (Table 2-1). The fusion and/or deletion of CBM changes the enzymatic properties of glycoside hydrolase[28-33]. Generally, CBM increases substrate concentrations around a catalytic domain, which increases catalytic efficiency[4,18]. The CBM10 of SlMan from *Streptomyces* sp. Increased catalytic efficiency towards galactomannan and glucomannan[27]. In contrast, some studies have found that β-mannanase activity increases after CBM deletion[28-30]. The catalytic efficiency (k_{cat}/K_m) of SsGH134-ΔCBM10 towards glucomannan and galactomannan was 2.4- and 2.6-fold higher than that of SsGH134 (Table 2-1), suggesting that the CBM10 of SsGH134 did not increase the substrate concentration, because the CBM10 did not function in binding to polysaccharides (Fig. 2-3). A similar phenomenon has been identified in other glycoside hydrolases with a deleted CBM domain[28-30].

SsGH134-ΔCBM10 and wild-type SsGH134 had similar pH, temperature optima and thermostability, and reduced stability at higher pH ranges (Figs. 2-4 and 2-5). These

findings are similar to those of glycoside hydrolase with CBM13 from *Streptomyces* sp. S27, with CBM9 and CBM22 from *Clostridium stercorarium* and with CBM22 from *Clostridium thermocellum*[31-33]. In addition, the stability of SsGH134-ΔCBM10 was lower than that of SsGH134 in the presence of 20% organic solvents and 10% SDS. Therefore, the CBM10 of SsGH134 might affect the structural integrity of the catalytic domain under a broad pH range. Here, the author presented the enzymatic characteristics of SsGH134 and described the role of the CBM 10 domain at its N-terminal region. The enzymatic properties differed between SsGH134 belonging to GH 134 and bacterial GH 5 and GH 26 β-1,4-mannanases. The author also showed that deleting CBM10 increased the catalytic efficiency and decreased the pH stability and the activity in the presence of organic solvents and detergents.

Methods

Chemicals

Low viscosity konjac glucomannan, carob galactomannan and insoluble β-mannan were purchased from Megazyme International (Bray, Ireland). Microcrystalline cellulose (MCC) and chitin were obtained from Funakoshi (Tokyo, Japan). Xylan from beech wood was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Preparation of recombinant proteins

The codon-optimized form of a DNA fragment encoding *SsGH134* (NCBI accession number WP_030268297.1) from *Streptomyces* sp. NRRL B-24484 was synthesized for expression in *Escherichia coli*. The *SsGH134* gene was inserted into pET28a (Novagen, Darmstadt, Germany). A CBM10-linker-truncated SsGH134 (SsGH134-ΔCBM10) was

generated using QuikChange site-directed mutagenesis kits (Stratagene, San Diego, CA, USA) and the primer set: forward SsGH134-ΔCBM10: 5'-ACCTGTGGTTCTTATACGGTTGGCG-3'; reverse, SsGH134-ΔCBM10: 5'-CGGGGCGGTTTCGAATTCGGATCCG-3'. The Gly34 of SsGH134 replaced with Trp (SsGH134-G34W) was also constructed using QuikChange site-directed mutagenesis kits (Stratagene, San Diego, CA, USA) and the primer set: forward SsGH134-G34W: 5'-TGGTATCCGTACTGCGCTAATGGTAGTG-3'; reverse SsGH134-G34W, 5'-GTTCGGGGCGGTTTCGAATTCGGAT-3'. The resulting plasmids were introduced into *E. coli* BL21 CodonPlus(DE3) (Novagen) and cultured in LB containing 50 μg/mL kanamycin sulfate for 16 h, and then 2-mL portions were agitated at 120 rpm in 200 mL of LB containing 50 μg/mL kanamycin sulfate at 37°C. When the optical density reached 1.0, isopropyl-thio-β-D-galactoside (0.2 mM) was added to the medium and the mixtures were further shaken at 120 rpm for 16 h at 25°C. The *E. coli* cells were harvested, suspended in 50 mL of 20 mM Tris-HCl buffer (pH 8.0) containing 150 mM NaCl and disrupted by sonication. Cell-free extracts were obtained from suspensions after centrifugation at 6,000 × g for 15 min, and then soluble fractions were further separated by centrifugation at 100,000 × g for 30 min. These fractions were passed through columns (φ5 × 20 mm) containing nickel-nitrilotriacetic acid agarose (QIAgen, Hilden, Germany) that were washed with 10 mL of 20 mM Tris-HCl buffer (pH 8.0) containing 50 mM imidazole. Proteins were eluted with the same buffer containing 300 mM imidazole. After dialysis with 20 mM Tris-HCl (pH 8.0), the protein solution was fractionated on 1-mL HiTrap Q columns (GE Healthcare, Waukesha, WI, USA) using a linear gradient of 0 – 0.5 M NaCl in 20 mM Tris-HCl (pH 8.0). The purified recombinant protein was finally dialyzed against 20 mM Tris-HCl (pH 8.0). All protein

purification steps proceeded at 4°C. The protein concentrations were measured at 280 nm.

Binding assay of SsGH134, SsGH134-ΔCBM10 and SsGH134-G34W

The reaction mixture containing 50 mM sodium acetate buffer (pH 5.0), SsGH134, SsGH134-ΔCBM10 or SsGH134-G34W, and 15 mg/mL of microcrystalline cellulose (MCC), insoluble β-mannan, chitin or xylan was incubated at 37°C for 30 min and then separated by centrifugation at 15,000 × g, for 10 min. The supernatant fraction was decanted and the precipitate was suspended in 50 mM sodium acetate buffer (pH 5.0). The wash and precipitate fractions were separated from the suspension by centrifugation and analyzed using SDS-PAGE.

Cellulose, insoluble β-mannan, chitin or xylan binding was quantified as described (20). Mixtures (100 μL) containing 0.5 – 10.0 μM of enzymes and 1.0 mg (w/v) MCC, insoluble β-mannan, chitin or xylan were incubated in 50 mM sodium acetate (pH 5.0), and then pelleted by centrifugation at 15,000 × g for 10 min. The concentrations of the enzymes in the supernatant fractions were measured at 280 nm.

Enzyme assays

The activity of β-1,4-mannanase was assayed in 0.5-mL reaction mixtures containing 0.2 – 5.0% (w/v) substrates and purified proteins that were incubated at 37°C. Enzymes were removed from reaction solutions using a Nanosep® Centrifugal Device (Pall Corporation, Port Washington, NY, USA) as described by the manufacturers, and flow-through fractions were boiled at 100°C for 30 min. Reducing sugars produced by β-1,4-mannanase were measured using the DNS method. Standard curves were prepared based on

solutions containing different concentrations of mannose.

Stability of pH and thermostability of purified SsGH134 and SsGH134-ΔCBM10

The pH stability of SsGH134 and SsGH134-ΔCBM10 was determined using glucomannan as a substrate. The β-1,4-mannanases were incubated in 50 mM sodium acetate (pH 3.0–5.0), 50 mM sodium phosphate (pH 5.0–7.0), and 50 mM Tris-HCl (pH 7.0–10.0) for 90 min, and then residual β-1,4-mannanase activity against 0.5% glucomannan was measured at the optimal pH of 5.0. The thermostability of SsGH134 and SsGH134-ΔCBM10 was determined using glucomannan as a substrate. Purified β-1,4-mannanases were incubated at temperatures ranging from 80°C to 100°C for 15, 30, 60, and 120 min. Residual β-1,4-mannanase activity was assayed at pH 5.0. The amount of reducing sugars produced by β-1,4-mannanase was measured using the DNS method.

Effects of organic solvents and detergents on β-1,4-mannanase activity

The author determined the effects of 20% solvents, methanol, ethanol, isopropanol and acetone, 10% detergents, SDS, Triton X-100 and Tween-20 on the enzymatic activity of β-1,4-mannanase by measuring residual activity against 0.5% glucomannan after a 60-min incubation at 30°C.

Sequence analysis

Amino acid sequences of CBM10 in GH134 β-1,4-mannanase from *Streptomyces* sp. NRRL B-24484 (SsGH134, WP_030268297.1) were aligned with the SsGH134 homologs, KIQ61562 from *Kitasatospora griseola*, WP_062059199 from *Cellvibrio* sp. OA-2007,

AIF91528 from *Alteromonadaceae bacterium* Bs12 and AIF91558 from *A. bacterium* Bs02, and GH 5 β-1,4-mannanases from *Cellvibrio japonicus* (CjMan5A, WP_012488914.1), GH 5 β-1,4-mannanase from *Cellvibrio japonicus* (CjMan5B, WP_012488072.1), GH 10 β-xylanase from *Pseudomonas fluorescens* (PfXyn10A, WP_012488068.1), and GH 5 cellulase from *Lyrodus pedicellatus* (Cel5AB, ABS72374.1) using ClustalW (http://www.clustal.org).

Summary

A GH 134 β-1,4-mannanase SsGH134 from *Streptomyces* sp. NRRL B-24484 possesses a carbohydrate binding module (CBM) 10 and a glycoside hydrolase 134 domain at the N- and C-terminal regions, respectively. Recombinant SsGH134 expressed in *E. coli*. SsGH134 was maximally active within a pH range of 4.0 – 6.5 and retained > 80% of this maximum after 90 min at 30°C within a pH range of 3.0 – 10.0. The β-1,4-mannanase activity of SsGH134 towards glucomannan was 30% of the maximal activity after an incubation at 100°C for 120 min, indicating that SsGH134 is pH-tolerant and thermostable β-1,4-mannanase. SsGH134, SsGH134-ΔCBM10 (CBM10-linker-truncated SsGH134) and SsGH134-G34W (substitution of Gly34 to Trp) bound to microcrystalline cellulose, β-mannan and chitin, regardless of the presence or absence of CBM10. These indicate that GH 134 domain strongly bind to the polysaccharides. Although deleting CBM10 increased the catalytic efficiency of the β-1,4-mannanase, its disruption decreased the pH, solvent and detergent stability of SsGH134. These findings indicate that CBM10 inhibits the β-1,4-mannanase activity of SsGH134, but it is involved in stabilizing its enzymatic activity within a neutral-to-alkaline pH range, and in the presence of various organic solvents and detergents. The author believe that SsGH134 could be useful to a diverse range of industries.

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Chapter 2. Enzyme application for plant-based meat analogs

Section 1. Improved functional properties of meat analogs by laccase catalyzed protein and pectin crosslinks

Introduction

In the last two decades, there has been a 58% growth in the global demand for meat due to an increase in the global population and rapid development of economy[1]. Moreover, the global population is expected to reach 9.7 billion by 2050, which will lead to a greater increase in protein demand[2]. However, limited land and water resources for livestock farming sustainability, rapid increase in animal welfare issues, undesirable effects on the environment, and change in climate have made it difficult to increase meat production to meet the future demand[3]. Thus, there is a gap between the present supply and future demand of meat; consequently, there is an increasing need to produce plant-based meat analogs as protein sources. Fresán and Sabaté reported that along with environmental benefits, human health benefits could also be achieved by changing the current dietary patterns to plant-based diets[4]. Moreover, to meet the demands of the expanding kosher and halal markets, it is necessary to develop plant-based meat analogs, instead of livestock-based or cell-based meat[5]. By 2025, more than 30% of the world's population may consume these types of food[5], making plant-based meat analogs one of the most popular topics within food and research communities.

Meat analogs are principally composed of texturized vegetable proteins (TVPs) that imitate the fibrillar structure of meat muscle[6]. Soy-based TVP is a plant-based protein

product that is cholesterol-free, with low concentrations of saturated fat and high concentrations of essential amino acids; it has several economical and functional benefits[7,8]. Soy-based TVP also presents characteristic functional properties, such as a high water holding capacity (WHC) and a good gelling behavior, fat absorption, and emulsification capacities[9]. It has been reported that when soy protein is used as a TVP constituent, the final product could mimic the texture, appearance, taste, smell, and functionality of red meat[5].

The presence of binders Is of importance as TVPs have no binding ability, considerably affecting the palatability of the final analog. Currently, methylcellulose (MC) is used in most commercial products[10] as it is a cheap binder and is considered safe for human consumption[11,12]. However, MC is chemically synthesized from cellulose and chloromethane in the presence of concentrated sodium hydroxide solution and is the main active ingredient in various laxatives. Therefore, concerns of MC not being a chemical-free binder and related risks necessitate the development of novel chemical-free binding systems for consumers and manufacturers. Moreover, the physical properties of existing plant-based meat analogs are still inferior to those of animal-based meat, especially when referring to texture, hardness, and juiciness[10,13]. These properties are crucial for the consumers' acceptance of food products and, hence, remain a critical obstacle[13]. Another challenge for plant-based meat analogs is the presence of anti-nutritional factors, such as protease inhibitors, tannins, and phytates. These factors decrease the digestibility and bioavailability of plant proteins compared with animal proteins[10,13]. Based on the above issue, there are many remaining challenges in making plant-based meat analogs available to a wide range of consumers.

Protein crosslinks can be introduced into food matrices using chemical, enzymatic,

and physical methods[14-16]. Among these approaches, transglutaminase (EC 2.3.2.13, TG) improves the physical properties of various protein-based foods[17,18]. This enzyme catalyzes the formation of an isopeptide bond between the glutamine residue side chains and lysine residue side chains[18]. Laccase (EC 1.10.3.2, LC) is also a protein-crosslink enzyme[19]. It oxidizes tyrosine phenols via a one-electron removal mechanism, producing phenoxy radicals. In proteins, the exposed tyrosyl side chains serve as substrates for oxidation by LC, resulting in the spontaneous production of subsequent protein crosslinks (dityrosine)[15,19-22]. However, the crosslinking reaction by LC oxidation cannot improve the physical properties of foods because proteins are poor substrates for LC[15,20,23,24]. Therefore, only TG has been commercially used as a crosslinking enzyme in the food industry.

Sugar beet pectin (SBP) consists of a linear α-1,4-D-galacturonic acid residue backbone, containing neutral sugars and ferulic acid, which are esters linked to arabinose and galactose side chains in the hairy region[25,26]. Approximately 50%–60% of ferulic acid groups are linked to the backbone of the arabinan side chains, whereas the rest are linked to galactose residues[27]. Traditionally, SBP has been used in the food industry as an emulsifying, thickening, and stabilizing agent[26,28]; it also forms stable gels by oxidative coupling and covalent crosslinks between beet pectin molecules via oxidative enzymes, such as LC and peroxidase[29,30]. The presence of covalent crosslinks in gels prevents post-gelation structural rearrangement and associated syneresis. As the crosslinks introduced by LC are heat-stable, firmness and elasticity can be retained[29]. This hydrogel is52hermoso-irreversible[31], which is a significant feature for food, biomedical, and biopharmaceutical applications[32]. So far, the use of an LC-catalyzed protein-SBP crosslink as a potential novel binding system for plant-based meat analogs has not been reported.

In this study, the author aimed to develop a novel chemical-free binding system for meat analogs and to solve the physical and nutritional shortcomings of meat analogs. SBP was used as a mediator to improve the ability of LC to crosslink protein-based foods. First, the author investigated whether TVP-SBP crosslinks formed by LC could become a novel binding system for meat analogs. As a result, the presence of LC and SBP improved the moldability and binding ability of patties, regardless of the type, shape, and size of TVPs. Moreover, compared with MC, LC-catalyzed protein-SBP crosslinks reduced the cooking loss and improved the water/oil holding capacity and gastrointestinal digestibility of meat analog patties. These findings indicate that TVP-SBP crosslinks formed by LC improved physical and nutritional properties, potentially solving the current challenges faced by plant-based meat analogs.

Results and Discussion

Synergistic effect of LC on the formation of protein and SBP crosslinks

Soy protein solution, SBP solution, and soy protein + SBP solution were incubated with 100 units of LC for 20 min. As shown in lane 4, protein-SBP crosslinks were too large to enter the acrylamide gel (Fig. 3-1a). However, as shown in lanes 2 and 3, protein-protein crosslinks and SBP-SBP crosslinks were not induced by the LC reaction (Fig. 3-1a). The viscosity of the soy protein solution, SBP solution, and soy protein + SBP solution treated with LC was then measured. Although the viscosity of soy protein solution and SBP solution treated with LC did not change, the viscosity of soy + SBP solution treated with LC increased in a time-dependent manner (Fig. 3-1b). The soy + SBP solution treated with LC was finally gelled. These findings indicated that protein and SBP crosslinks were synergistically

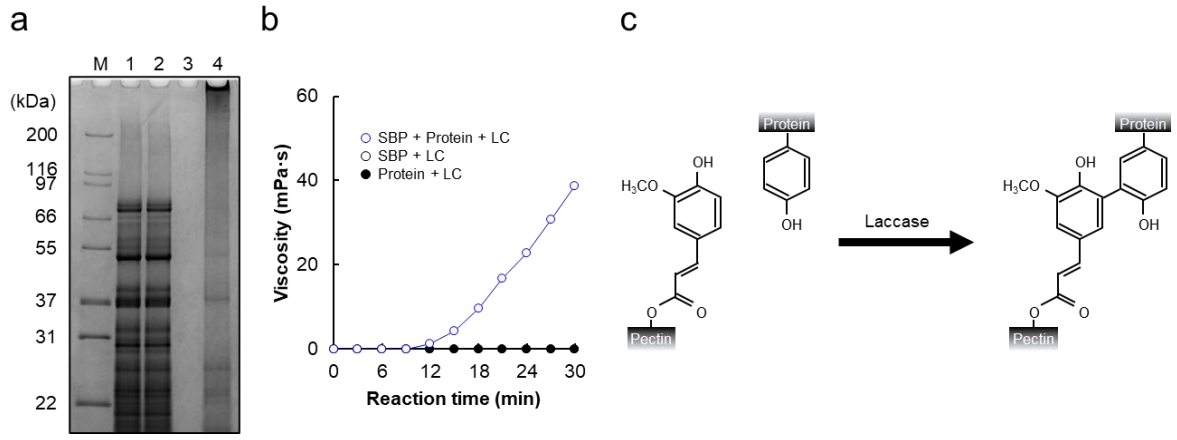

Figure 3-1. Synergistic effects of LC-catalyzed oxidative reaction on the formation of protein and SBP crosslinks. (a) SDS-PAGE analysis of soy protein and SBP treated with 100 U LC. Lane 1, non-treated soy protein and SBP; Lane 2, LC-treated soy protein; Lane 3, LC-treated SBP, Lane 4, LC-treated soy protein and SBP. (b) Apparent viscosity of soy protein and SBP solution treated with LC was measured using a viscometer. Open circle, LC-treated SBP; closed circle, LC-treated soy protein; blue circle, LC-treated soy protein and SBP. (c) Schematic of protein-SBP crosslink formation via LC-catalyzed oxidative reaction.

catalyzed by LC, leading to changes in their physical properties.

SBP gelation catalyzed by LC requires a longer incubation time and higher concentration of oxidation enzymes[29,33]. Previous studies have reported that proteins, such as fish gelatin, soy protein isolate, and whey protein isolate, can be crosslinked with SBP by oxidation enzymes[34-36]. As shown in Fig. 3-1c, LC can oxidize ferulic acid and tyrosine, leading to protein-SBP crosslinks[37-38]. This combination of SBP and protein provided additional properties to hydrogels and shortened the gelling time[29,33-39]. In this study, protein-SBP crosslinks were also formed by LC oxidation (Figs. 3-1a and 3-1b). Therefore, as a novel binding system for meat analogs, this protein-SBP gel could help develop food products with additional textural properties.

Improving physical properties of meat analogs by SBP-protein crosslinks

To investigate whether SBP-protein crosslinks formed by LC oxidation could be a novel binder for meat analogs, soy-based TVP and SBP were incubated with LC. MC was used as the control. Figure 3-2a shows the meat analog patties after incubation and cooking. Non-treated and LC-treated meat analog patties without a binder collapsed after incubation and grilling. Meat analog patties containing MC maintained their shapes with or without enzymes after grilling. Interestingly, LC-treated meat analog patties containing SBP maintained their shape after grilling. In contrast, non-treated meat analog patties containing SBP collapsed after incubation and cooking. Moreover, various plant-based TVPs were incubated with SBP and LC under the same conditions (Figs. 3-3 and 3-4). The results revealed that the presence of LC and SBP improved the moldability and binding ability of meat analog patties, regardless of the type, shape, and size of TVPs.

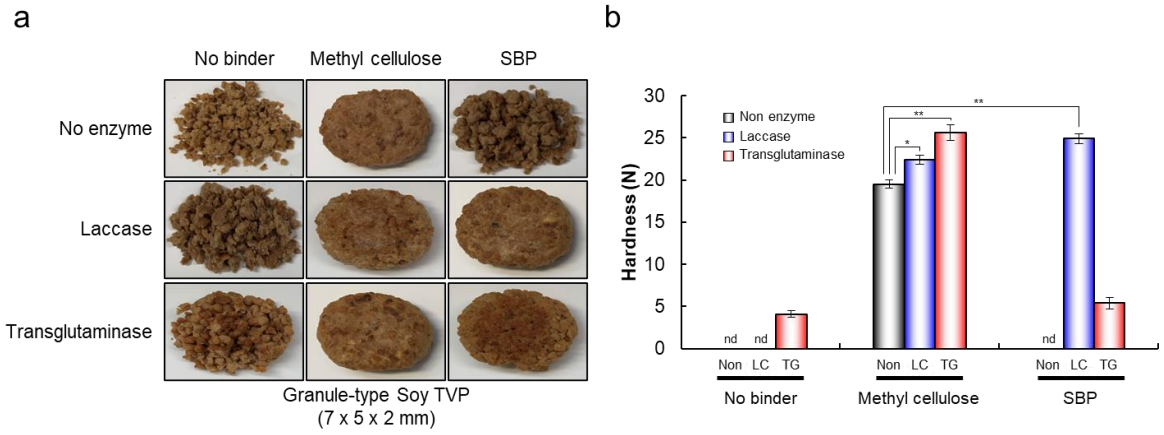

Figure 3-2. Appearance of grilled meat analogs treated with enzyme. (a) Meat analogs were treated with 20 U/g-TVP LC and 50 U/g-TVP TG, in the presence or absence of a binder (MC or SBP). (b) The hardness of meat analogs treated with each enzyme was measured using a rheometer. Data are presented as mean ± standard deviation of five experiments. *$p < 0.05$, **$p < 0.01$, Student's *t*-test. Non, non-treated meat analogs; LC, LC-treated meat analogs; TG, TG-treated meat analogs.

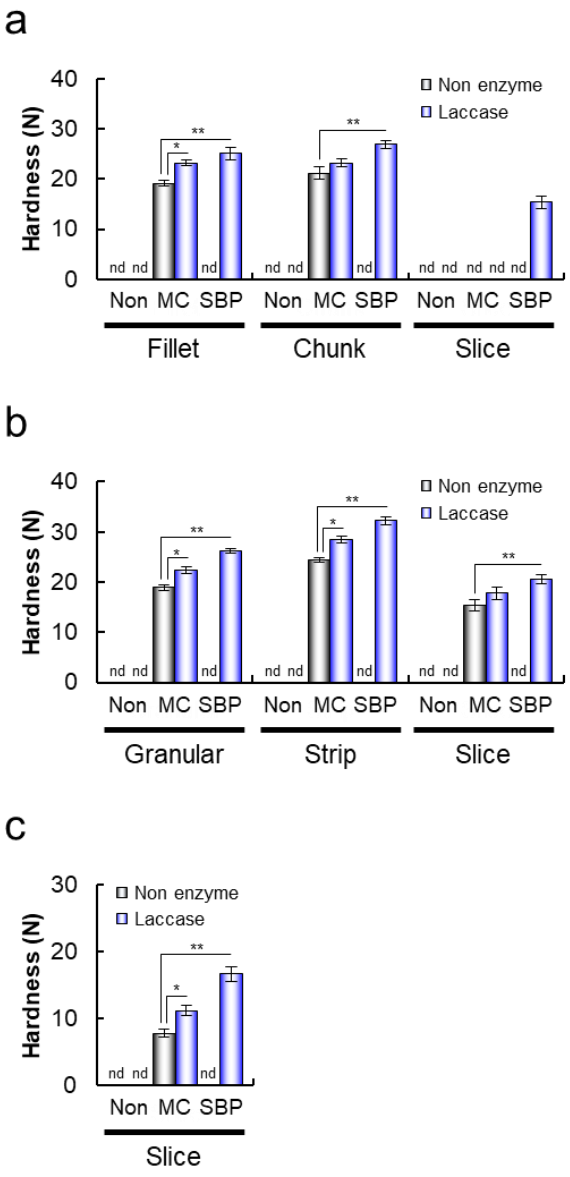

Figure 3-3. Hardness of meat analogs prepared from various TVPs. Various types of TVPs (plant origin, shape, and particle size) were investigated. Meat analogs were prepared from soy-based TVP (a), pea-based TVP (b), and gluten-based TVP (c). Non-meat analogs containing no binder; MC, meat analogs containing MC; SBP, meat analogs containing SBP. Black bar, non-treated meat analogs; blue bar, LC-treated meat analogs.

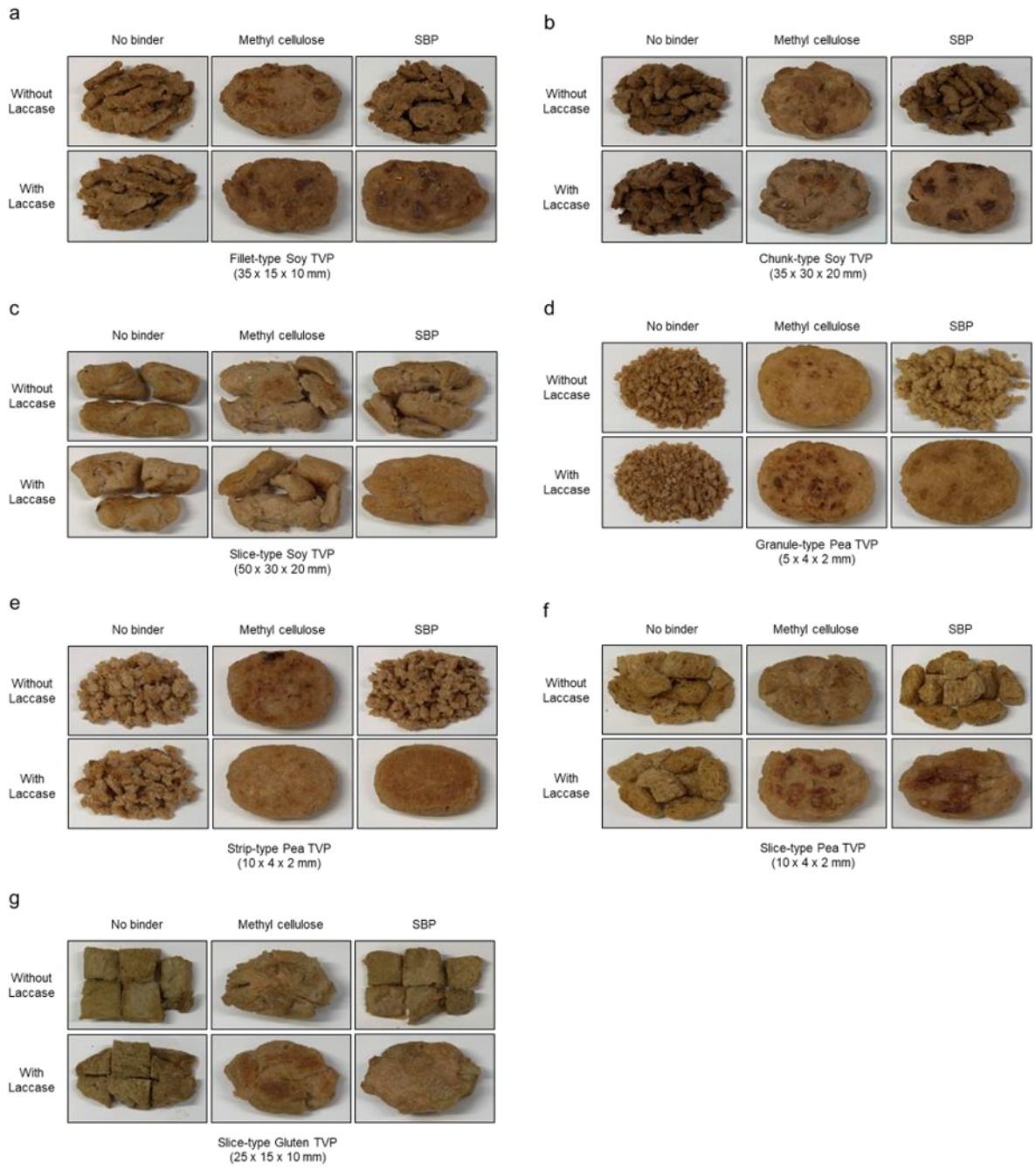

Figure 3-4. Appearance of grilled meat analogs treated with enzyme. Various types of TVPs (plant origin, shape, and particle size) were investigated. (a) fillet-type soy-based TVP (35 × 15 × 10 mm); (b) chunk-type soy-based TVP (35 × 30 × 20 mm); (c) slice-type soy-based TVP (50 × 30 × 20 mm); (d) granule type pea-based TVP (5 × 4 × 2 mm); (e) strip-type pea-based TVP (10 × 4 × 2 mm); (f) slice type pea-based TVP (10 × 4 × 2 mm); (g) slice type gluten-based TVP (25 × 15 × 10 mm).

The hardness of the meat analog patties after grilling was investigated using a rheometer (Fig. 3-2b). The hardness of patties containing MC was substantially higher in the order TG- > LC- > non-treated. Interestingly, the hardness of LC-treated patties containing SBP was substantially higher than that of non- and LC-treated patties containing MC. Moreover, as shown in Fig. 3-5, the hardness of meat analog patties was enhanced in an LC and SBP concentration- and time-dependent manner. The hardness of LC-treated patties with SBP plateaued at 32.2 N after 4 h of LC reaction, which was 1.7- and 7.9-fold higher than that of patties with MC and TG-treated patties. These findings indicate that TVP-SBP crosslinks offer great promise in changing the physical properties of plant-based meat analog products.

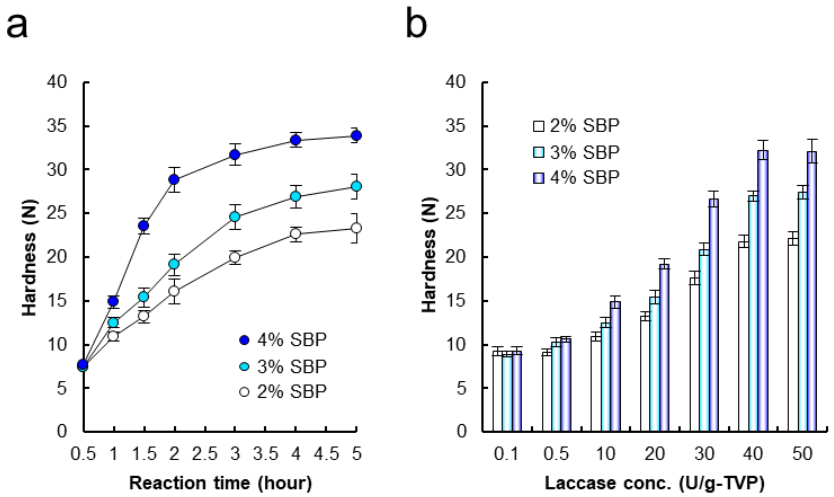

Figure 3-5. Hardness of grilled meat analog patties treated with LC. The hardness of the meat analogs treated with LC was measured using a rheometer. (a) Meat analogs containing 2%–4% SBP were treated with 20 U/g-TVP LC at 0.5–4.0 h. (b) Meat analogs containing 2%–4% SBP were treated with various concentrations of LC. Data are presented as mean ± standard deviation of five

Table 3-1 shows the TPA parameters (hardness, cohesiveness, springiness, and chewiness) of enzyme-treated meat analog patties containing binders. The chewiness of these patties was substantially higher in the order LC-treated patties with SBP > non-treated and LC-treated patties with MC > TG-treated patties. The hardness and chewiness of LC-treated patties increased in a SBP concentration-dependent manner, whereas the cohesiveness and springiness did not change. Our finding that the hardness and chewiness of LC-treated patties increased in an SBP concentration-dependent manner is similar to that of previous studies that incorporated other binders into patties. These previous studies reported that increasing concentration of binders such as MC and carrageenan proportionally increases the hardness and chewiness of products[7,40-42]. The hardness of LC-treated patties containing SBP was lower than that of animal-based patties containing MC[40]. Meat protein generally presents a higher degree of shrinkage. Therefore, the higher hardness of animal-based patties has been suggested to be due to muscle protein denaturation, which led to meat hardening[40,43]. Whereas, the hardness and chewiness of LC-treated patties containing SBP were similar to or higher than those of meat analogs containing other binders reported previously[7,40,41,44]. These findings suggest that TVP-SBP crosslinks formed by LC oxidation are a novel binder for meat analog patties. This is the first report of enhancing the moldability and binding ability of patties by TVP-SBP crosslinks by LC.

Table 3-1. Texture profile analysis of meat analogs.

Binder	Conc.	LC	TG	Hardness (N)	Cohesiveness	Springiness	Chewiness (N)
MC	2%	0 U/g-TVP	-	18.4 ± 0.6	0.70 ± 0.04	0.71 ± 0.05	9.1 ± 0.5
		20 U/g-TVP		21.5 ± 0.9	0.77 ± 0.03	0.81 ± 0.08	13.4 ± 0.7
SBP	2%	20 U/g-TVP	-	21.8 ± 0.3	0.78 ± 0.05	0.80 ± 0.05	13.6 ± 0.4
	3%			23.6 ± 0.5	0.77 ± 0.09	0.78 ± 0.07	14.2 ± 0.7
	4%			25.5 ± 0.7	0.79 ± 0.07	0.77 ± 0.06	15.6 ± 1.2
Non	-			7.1 ± 0.3	0.51 ± 0.06	0.57 ± 0.09	2.1 ± 0.3
MC	2%	-	50 U/g-TVP	23.1 ± 0.3	0.79 ± 0.08	0.77 ± 0.07	14.1 ± 0.7
SBP	2%			10.2 ± 0.4	0.57 ± 0.09	0.64 ± 0.08	3.7 ± 0.7

Meat analogs were treated with 20 U/g-TVP LC or 50 U/g-TVP TG in the presence of MC or sugar beet pectin. Data are presented as mean ± standard deviation of five experiments.

Improving physical properties of meat analogs by MC

In this study, MC was used as the control in all experiments. The mechanism by which gelation between MC and protein is achieved remains unclear. One standard theory is that the hydrophobic methyl groups of MC are surrounded by cage-like structures of water molecules[45]. However, with increasing temperature, the cage structure is disrupted, and the polymers gradually lose water[46,47]. At elevated temperatures, MC prefers hydrophobic association states, leading to the formation of strong gels[46,47]; the results of this study are consistent with those reported previously (Figs. 3-2b and 3-6). After grilling, the cooking loss and holding capacity of patties decreased, indicating the release of water. In contrast, the hardness of patties that had MC increased. Moreover, all TPA parameters of the LC- and TG-treated patties containing MC were higher than those of non-treated patties containing MC (Table 3-1). These findings suggest that TPA parameters of the patty are synergistically enhanced by crosslinking enzymes such as LC and TG.

Currently, MC is used in most of the commercial products[10]. However, only a few

studies have reported the effects of MC on the physical and functional characteristics of plant-based meat analog patties[48]. In this study, we investigated the properties of MC as a control binder, in detail. Selecting the right binder and its amount for meat analog products is crucial[10,48,49]. Therefore, the findings on patties containing MC expand our knowledge on their physical properties. This study will also contribute to a better understanding of the functional roles of MCs.

Cooking loss and water/oil holding capacity of meat analog patties

Cooking loss represents the degree of meat shrinkage during cooking, which is an important indicator for evaluating the juiciness and yield of the final product. As the amount of water and oil increased, a typical increase in the cooking loss of all patties was observed (Figs. 3-6a and 3-6b). Surprisingly, the cooking loss of LC-treated patties containing SBP was substantially lower than that of patties containing MC and TG-treated patties. When adding various amounts of water and oil, the cooking loss of LC-treated patties with SBP decreased by up to 8.9%-9.4% and 6.7%-15.6% compared with patties with MC and TG-treated patties, respectively. Water and oil holding capacity is an important factor, as it affects the quality and yield of fresh patties or their products[50]; the higher the holding capacity of patties, the more the juiciness. As the amount of water and oil increased, the water and oil holding capacity of all patties showed a typical decrease (Figs. 3-6c and 3-6d). The water and oil holding capacity of LC-treated patties containing SBP was substantially higher than that of patties containing MC and TG-treated patties. When adding various amounts of water and oil, the water and oil holding capacity of LC-treated patties with SBP increased by up to 5.8%-11.3% and 5.8%-12.4% compared with patties with MC and TG-treated patties,

respectively. These findings indicate that TVP-SBP crosslinks catalyzed by LC are superior to the high-network formed by MC, indicating that LC-treated patties containing SBP were juicier.

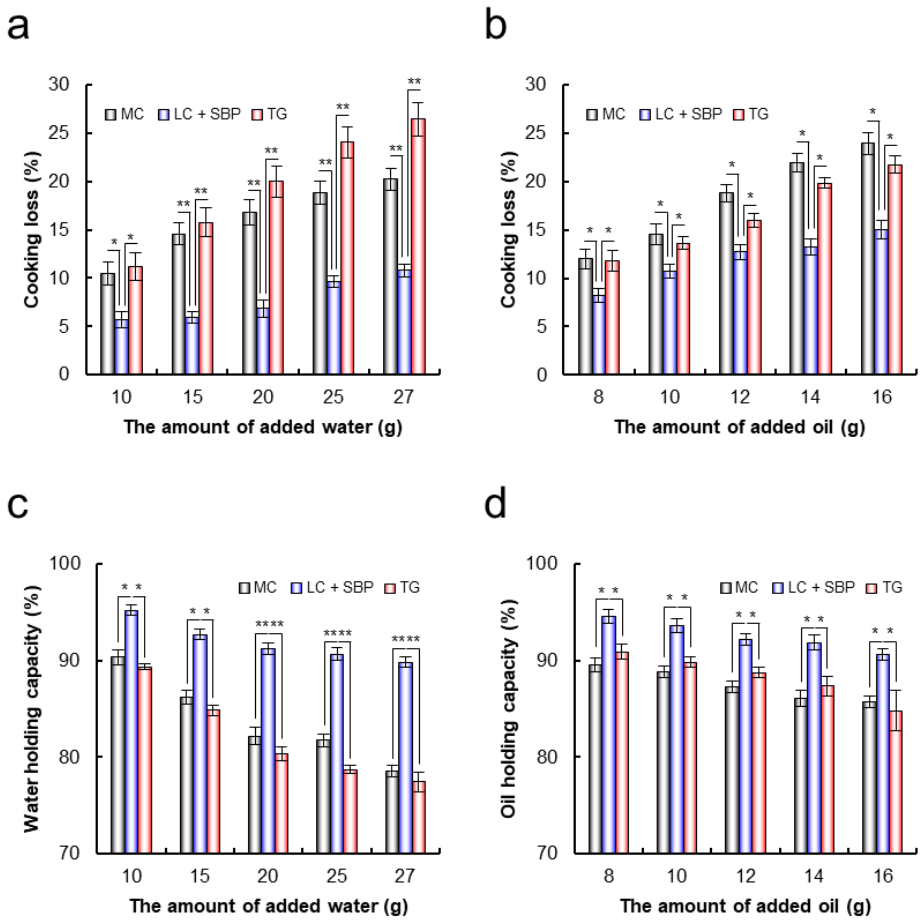

Figure 3-6. Cooking loss value and water/oil holding capacity of meat analogs. (a, b) Cooking loss was calculated as the percentage weight difference between the dough before cooking and after cooking. Meat analog containing different amounts of water (a) or oil (b) was treated with LC and cooked. (c, d) Holding capacity was calculated by comparing the weight of meat analogs before and after centrifugation. Meat analog patties containing different amounts of water (c) or oil (d) was treated with LC, cooked, and centrifuged. *$p < 0.05$, **$p < 0.01$, Student's *t*-test. MC, meat analog containing MC; LC + SBP, LC-treated meat analog containing SBP; TG, TG-treated meat analog.

MC is an effective amphiphilic cellulose derivative with the ability to absorb high amounts of water and oil[48,51,52]. In this study, the cooking loss and holding capacity of patties were more enhanced by LC-catalyzed protein-SBP crosslinks than by the high-network formed by MC (Fig. 3-6). SBP also has good amphiphilic properties, contributed by the protein moiety ferulate and acetyl groups, which impart hydrophobic properties, and the carbohydrate fraction, which imparts hydrophilic properties[53,54]. Oxidative enzymes, such as LC and peroxidase, can form a hydrated network, leading to hydrogel formation with a high water-holding capacity[30]. Here, the water holding capacity of patties was in the order of SBP + LC > MC (Figs. 3-6a and 3-6c), suggesting that TVP-SBP crosslinks formed a hydrogel-like network and improved the water retention capacity of plant-based meat analog patties.

LC treatment is an effective approach to prepare stable multilayered emulsions as it can crosslink proteins and SBP, and shrink the droplet size[55,56]. A previous study reported that an LC-treated multilayered protein-SBP stabilized emulsion improves emulsifiability compared with a non-treated protein and SBP emulsion[57]. Moreover, emulsions treated with LC and SBP exhibit droplets with a tight protein-polysaccharide membrane, higher emulsification stability during heating, oil retention, and freeze-thaw cycles, and at a wide pH range[55-57]. The results of this study are consistent with those reported previously; the oil holding capacity of patties was in the order SBP + LC > SBP + TG > MC + TG > MC > TG (Figs. 3-6 and 3-7). It is considered that LC formed a multilayered protein-SBP-stabilized emulsion and enhanced the emulsion stability and oil retention capacity of plant-based meat analog patties.

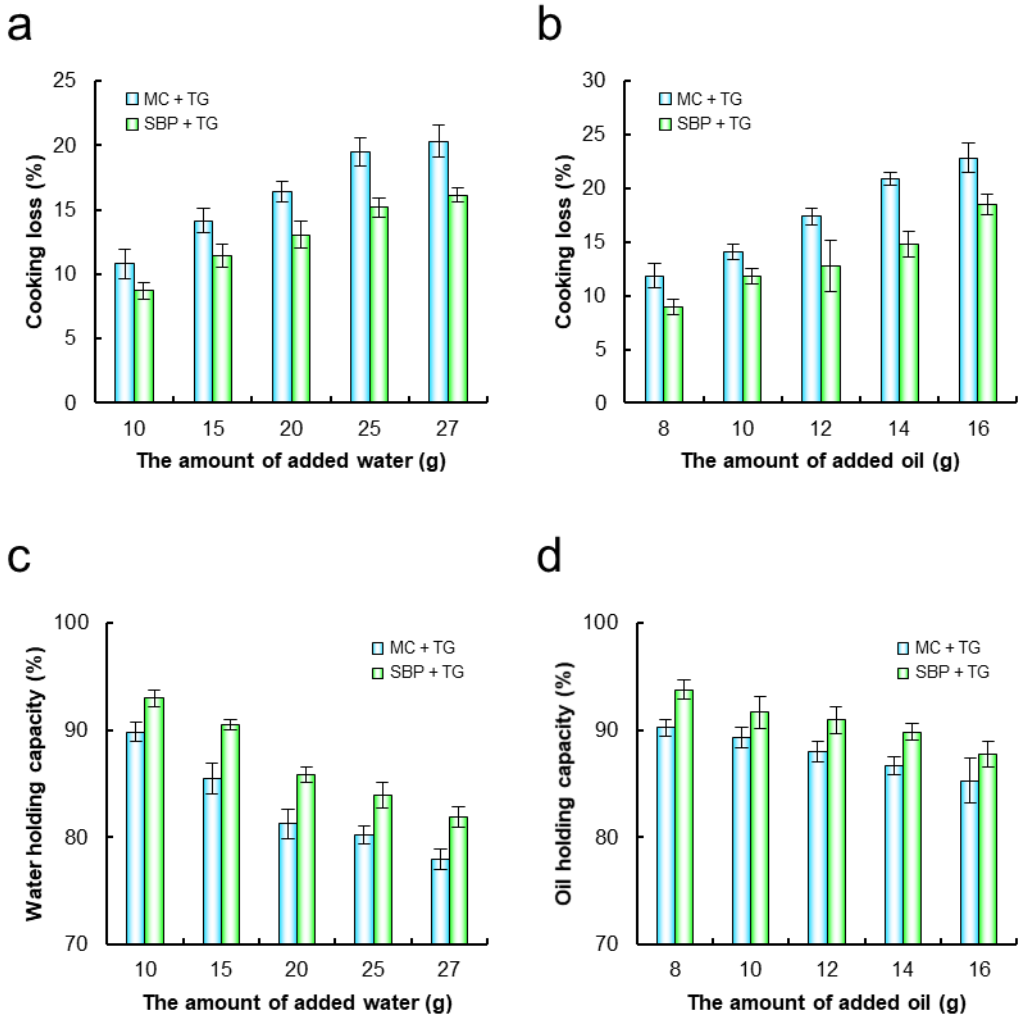

Figure 3-7. Cooking loss value and water/oil holding capacity of meat analogs treated with TG. (a, b) Cooking loss was calculated as the percentage weight difference between the dough before cooking and after cooking. Meat analog with different amounts of water (a) or oil (b) was treated with TG and cooked. (c, d) Holding capacity was calculated by comparing the weight of meat analogs before and after centrifugation. Meat analog with different amounts of water (c) or oil (d) was treated with TG, cooked, and centrifuged. *$p < 0.05$, Student's t-test. MC + TG, TG-treated meat analog including MC; SBP + TG, TG-treated meat analog including SBP.

In-vitro gastrointestinal digestibility of meat analogs

Figure 3-8 shows free amino nitrogen released from the plant-based meat analog patty and its dry residual weight after *in-vitro* gastrointestinal digestion by pepsin. The amount of free amino nitrogen released from LC-treated patties containing SBP was 2.3-fold higher than that from patties containing MC (Fig. 3-8a). The dry residual weight of LC-treated patties containing SBP was 2.2-fold lower than that of patties containing MC (Fig. 3-8b). These findings indicated that LC-treated patty containing SBP was easier to digest with pepsin and absorb nutrients from. This is considered to be because of higher water holding capacity, cohesiveness, and springiness (Table 3-1). Cohesiveness and springiness are the extent to which the gel withstands a second deformation relative to its resistance under the first deformation. Generally, the higher the water retention, cohesiveness, and springiness, the lower the density of food fragments after chewing. Therefore, the dispersibility of LC-treated patties containing SBP in the imitated gastric juice was considered to be high, leading to an enhanced pepsin activity toward the patties.

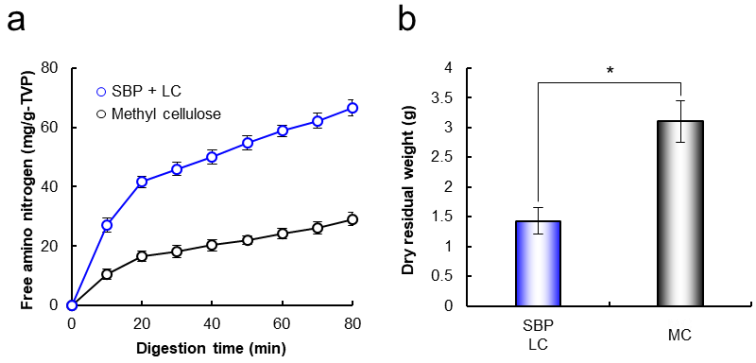

Figure 3-8. *In-vitro* gastrointestinal digestibility of meat analogs. The gastrointestinal digestion test was performed. Free amino nitrogen released from patties (a) was measured using the ninhydrin method, and then its dry residual weight (b) was measured. Data are presented as mean ± standard deviation of three experiments. *$p < 0.05$, Student's *t*-test

Meat as a protein source provides humans with the necessary nutrients and energy to function throughout the day[58]. Generally, meat analogs are considered to have slightly lower protein content than traditional meat products[10,13]. However, the digestibility of plant proteins is lower than that of animal-derived proteins because of different factors[59]. According to a previous study, all processing conditions, such as heat treatment, high pressure, pH change, protein fractionation, enzymatic reaction, milling, pressure, and fermentation, affect the nutritional availability of amino acids from proteins[60]. Here, replacing MC with TVP-SBP crosslinks catalyzed by LC as a binding system enhanced the digestibility of plant-based meat analog patties (Fig. 3-8a). Therefore, TVP-SBP crosslinks catalyzed by LC offer an option to overcome the poor nutritional availability of amino acids from plant-based meat analogs.

Based on recent scientific efforts, numerous studies have indicated the health benefits associated with the replacement of animal sources of protein with plant-based proteins, including reduced risks of type 2 diabetes, heart disease, and stroke[61,62]. Additionally, dietary fiber in meat analogs is considered to play an essential role in preventing large bowel disease, ischemic heart disease, and diabetes mellitus[63]. MC is a chemically modified polysaccharide, and this has raised concerns among the consumers and manufacturers as it is not a chemical-free binder. The SBP used in this study was a natural and chemical-free polysaccharide. Therefore, SBP offers great promises in not only enhancing physical and functional properties of patty but also improving nutritional availability and disease prevention.

Methods

Materials

Granule-type, fillet-type, chunk-type, and slice-type soy-based TVPs were purchased from Marukome Co., Ltd. (Nagano, Japan). Granule-type, strip-type, and slice-type pea-based TVPs were obtained from Puris LLC (Minneapolis, USA), SANSHO Co., Ltd. (Tokyo, Japan), and AJIGEN Co., Ltd. (Kagawa, Japan), respectively. Slice-type gluten-based TVP was purchased from Saniku Foods Co., Ltd. (Chiba, Japan).

SDS-PAGE analysis of crosslink

To investigate the synergistic effect of LC on the formation of SBP and protein crosslinks, the degree of crosslinking was measured by SDS-PAGE under reducing conditions with dithiothreitol (DTT). Assay of 10% (v/v) soy solution, 0.5% (v/v) SBP solution, and 10% (v/v) soy + 0.5% (v/v) SBP solutions was performed in reaction mixtures containing phosphate buffer (100 mM, pH 7.0) and LC (100 units). The reaction mixtures were incubated at 40°C for 20 min, and the reaction was terminated by boiling at 100°C for 5 min.

Rheological characterization

The viscosity of the soy solution, SBP solution, and soy + SBP solutions was measured using EMS-1000 (Kyoto Electronics Manufacturing Co., Ltd., Tokyo, Japan). Each solution was assayed in reaction mixtures containing phosphate buffer (100 mM, pH 7.0) and LC (500 units). The shear rate applied to the samples was 200 s^{-1}, and a preliminary measurement was conducted for 30 s to maintain the flow conditions. The temperature of the

samples was set to 40°C during the measurements. The variation in protein and SBP concentrations due to water vaporization was confirmed by gravimetric measurements.

Preparation for plant-based meat analogs

The base of the TVP matrix was prepared using TVPs and a binder (MC or SBP), followed by the addition of olive oil and enzyme solution additives (Table 3-2). First, dried TVP was immersed in water (five times in volume) for 2 h for hydration. After dehydrating the swollen TVP, it was mixed 2.0%–4.0% SBP or 2.0% MC at the final concentration. Unless otherwise noted, the TVP matrix was prepared in the absence and presence of 2.0% (final concentration) binder. Thereafter, different amounts of water or olive oil were added to each 25 g of TVP matrix. These were blended for 60 sec using a hand blender. Thereafter, LC (LC-Y120; Amano Enzyme Inc., Nagoya, Japan) was added to the TVP matrix and blended for 60 sec. LC used in this study was a commercially available food-grade product. According to the instruction manual of the manufacturer, the optimal reaction temperature for LC is 60°C, with a preferred temperature range between 25°C and 80°C (>65%). The TVP matrix was molded in a cylindrical mold (60 mm × 40 mm area and 25 mm height) and incubated at 25°C for 4 h. The matrix was then cooked in an oven at 200°C for 15 min and cooled to room temperature (20°C–25°C) before being used for further analysis.

Table 3-2. Mixing amounts of additives or enzymes in plant-based meat analog patties.

	Wet TVP (g)	Water[1] (g)	Oil[1] (g)	MC (%)	SBP (%)	LC (U/g-TVP)	TG (U/g-TVP)
Non-treated patties without binder	25	5	8	-	-	-	-
Non-treated patties with MC	25	5	8	2	-	-	-
Non-treated patties with SBP	25	5	8	-	2-4	-	-
LC-treated patties without binder	25	5	8	-	-	20	-
LC-treated patties with MC	25	5	8	2	-	20	-
LC-treated patties with SBP	25	5	8	-	2-4	20	-
TG-treated patties without binder	25	5	8	-	-	-	50
TG-treated patties with MC	25	5	8	2	-	-	50
TG-treated patties with SBP	25	5	8	-	2-4	-	50

[1] In the cooking loss and holding capacity test, the amounts of water and oil ware 10–20 g and 8–16 g, respectively.

Texture profile analysis

Texture profile analysis (TPA) was carried out using COMPAC-100II (Sun Scientific Co., Ltd., Tokyo, Japan) equipped with a cylindrical probe with an area of 31.4 mm. Meat analog patties were treated with 0.1–50 U/g-TVP LC and 50 U/g-TVP TG, respectively. Unless otherwise noted, it was performed with 20 U/g-TVP LC (5.0 mg). After grilling, meat analogs were prepared for the analysis and cut to a length of 15 mm to obtain homogeneous extrudates. The diameter of each extrudate was approximately 20 mm. A double compression cycle was performed at 1 mm/sec until a recorded deformation of 50% was achieved. The following parameters were evaluated: hardness, the maximum force recorded during the first compression; cohesiveness, the area of work during the second compression divided by the area of work during the first compression; springiness, the distance recorded during the second compression divided by the distance of the first compression; and chewiness, hardness × cohesiveness × springiness.

Cooking loss

The cooking method and conditions were determined based on the study of Pathare and Roskully[64]. The patties were cooked at 200°C for 15 min, depending on the temperature at the center of the meat analog, reaching 80°C. After cooking, the samples were cooled to room temperature (20°C–25°C). Cooking loss was calculated as the percentage weight difference between the patty before cooking and after cooking, using the following formula: cooking loss (%) = $((W_1 - W_2) / W_1) \times 100$; W_1: weight of the meat analog before grilling (g), W_2: weight of the meat analog after grilling (g).

Water/oil holding capacity

Water holding capacity and oil holding capacity were measured by modifying a previously described method[65,66]. Briefly, grilled meat analog (5 g) was placed in a 50-mL tube with gauze underneath. The tube was then centrifuged at 3,000 *g* for 10 min at 35°C. Holding capacity (HC) was calculated by comparing the weight of the meat analogs before and after centrifugation using the following formula: HC (%) = $(W_2/W_1) \times 100$; W_1: weight of the meat analog before centrifugation (g), W_2: weight of the meat analog after centrifugation (g).

In-vitro gastrointestinal digestibility of meat analogs

In-vitro gastrointestinal digestion tests were performed in 130-mL reaction mixtures containing NaCl (4.39 g), KCl (0.22 g), $CaCl_2$ (0.04 g), McIlvaine buffer (pH 5.0), meat analogs (25 g, finely cut), and 0.0065% pepsin from porcine gastric mucosa (Sigma-Aldrich,

St. Louis, MO, USA). The digestive reaction mixtures were incubated at 37°C and 60 rpm for 90 min, and 1 N HCl was added to the reaction mixtures every 5 min, resulting in pH 3.0 after 40 min. The solution was clarified using a Nanosep® Centrifugal Device (Pall Corporation, Port Washington, NY, USA) as described in the instruction manual. The free amino nitrogen was measured using the ninhydrin method[67].

Summary

The gap between the current supply and future demand of meat has increased the need to produce plant-based meat analogs. Methylcellulose (MC) is used in most commercial products. Consumers and manufacturers require the development of other novel binding systems, as MC is not chemical-free. The author aimed to develop a novel chemical-free binding system for meat analogs. First, the author found that laccase (LC) synergistically crosslinks proteins and sugar beet pectin (SBP). To investigate the ability of these SBP-protein crosslinks, texturized vegetable protein (TVP) was used. The presence of LC and SBP improved the moldability and binding ability of patties, regardless of the type, shape, and size of TVPs. The hardness of LC-treated patties with SBP reached 32.2 N, which was 1.7- and 7.9-fold higher than that of patties with MC and transglutaminase-treated patties. Additionally, the cooking loss and water/oil-holding capacity of LC-treated patties with SBP increased by up to 8.9%-9.4% and 5.8%-11.3%, compared with patties with MC. Moreover, after gastrointestinal digestion, free amino nitrogen released from LC-treated patties with SBP was 2.3-fold higher than that released from patties with MC. This is the first study to report protein-SBP crosslinks by LC as chemical-free novel binding systems for meat analogs.

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Section 2. Synergistic effects of laccase and pectin on the color changes and functional properties of meat analogs containing beet red pigment

Introduction

The widening gap between the current supply of meat and its future demand has increased the need to produce plant-based meat analogs as protein sources[1]. Developing better plant-based diets would address the current protein crisis and positively impact the environment and human health[1]. It has been estimated that progression from current omnivore diets to vegan and ovo-lacto vegetarian diets can achieve about 50% and 35% decrease of greenhouse gas emissions, respectively[2]. Also, numerous studies have reported the health benefits associated with the replacement of animal sources of protein with plant-based proteins, including reduced risks of type 2 diabetes, heart disease, and stroke[3,4]. Therefore, shifting the global food production system into a more sustainable one by developing plant-based analogs will not only address the future demand for protein sources but also support a healthy lifestyle and protect the environment.

Despite ongoing technical developments, the appearance, flavor, taste, and texture of plant-based meat analogs differ from those of traditional meat products. Among them, one of the unresolved challenges for plant-based meat analogs is their appearance, particularly their color[1,5]. Color is the first aspect of food products that consumers notice and a major contributor to consumer perception of taste and overall product acceptance[6]. Fresh, uncooked animal-based meats are bright red due to high oxymyoglobin content. When the meat is cooked, the metmyoglobin content increases, resulting in browning[7]. In contrast, most uncooked plant-based protein products are yellow or beige in color[1]. A major goal of

research in this field is to simulate the typical browning of cooked animal-based meat by mimicking the red-to-brown color change in meat analogs[8]. However, few studies have investigated color changes in plant-based meat analogs[1,5,9]. Therefore, plant-based meat analogs are presently unable to meet the consumer standard established by animal-based meat in terms of appearance and color.

In recent studies on novel, plant-based meat analogs, the red color of the raw product has been obtained by the addition of (1) beet red (BR) pigment or (2) soy leghemoglobin[9]. (1) BR pigment mainly consists of betanin/betanidin extracted from *Beta vulgaris* ssp., and is traditionally used as a natural red pigment in the global food industry because of its safety and low cost[10-12]. *B. vulgaris* is one of the top ten most potent antioxidant-rich vegetables[13]. Betalains (including betanin/betanidin) with high antioxidant activity were suggested to have a protective function on certain oxidative stress-related disorders, such as cardiovascular diseases, cancer, aging, and neurodegenerative disorders[14]. Therefore, BR pigment is an attractive food coloring agent. However, BR as a coloring agent for meat analogs has two limitations. First, meat analogs containing BR might remain red after grilling as betanin is thermo- and photostable. Therefore, it is difficult for consumers and companies to judge their degree of doneness. Second, overheating meat analogs containing BR may result in an unacceptable yellow color[1,5,8,9]. (2) Leghemoglobin is chemically and structurally similar to myoglobin and imparts cooked-color characteristics similar to those of animal-based meat to meat analogs[9,15]. However, soy leghemoglobin is a genetically modified protein that is overexpressed in the methylotrophic yeast *Pichia pastoris*. Some consumers and companies have concerns regarding the risk of using genetically modified proteins in meat analogs.

Further research is needed to simulate the color changing of raw to cooked meat safely and more effectively. Presently, browning of meat analogs is achieved by adding coloring ingredients or other precursor substances. Specifically, caramel colors, malt extracts, reducing sugars, and amino acids are added, masking the red color and imparting the final product with a brown appearance[1,5]. However, consumers and manufacturers have safety concerns over using these additives. In addition, reducing sugars and amino acids may react to produce mutagenic and carcinogenic compounds, including acrylamide and heterocyclic amine, via the Maillard reaction[16,17]. Moreover, the additives fail to mask the red color of the pigment completely. Therefore, consumer and manufacturer perceptions have necessitated the development of a safe and more effective browning system for meat analogs. The currently established dye and pigment decolorization methods are classified into four main categories: physical, oxidative, enzymatic, and biological methods[18]. Among them, the enzymatic method using laccase (LC; EC 1.10.3.2) has been applied in various industrial scenarios[19]. LCs are oxidases that couple the reduction of molecular oxygen to the one-electron oxidation of a wide variety of phenols[20-22]. The substrate-oxidizing activity of LCs can be enhanced using appropriate mediators. Consequently, LCs have been shown to catalyze the oxidation of a broad range of substrates such as phenol and its derivatives, benzenethiols, aromatic amines, and polycyclic aromatic hydrocarbons[23-25]. Therefore, they are widely used not only for the decolorization of synthetic dyes[26,27] but also for bioremediation, detoxification, food processing, and biosensing[28].

LC is used not only as a decolorizing enzyme but also as a crosslinking enzyme. Previous studies have reported that LC-catalyzed reactions formed pectin-chitosan films with hazardous gas removal abilities, arabinoxylan gels with higher gel abilities, and ferulate-

modified pullulan with swelling properties[29-31]. In proteins, the exposed tyrosyl side chains serve as substrates for oxidation by LC, resulting in the spontaneous coupling of subsequent protein crosslinks (dityrosine)[19,32-35]. However, proteins are poor substrates for LC because of the intra-protein location of most tyrosine residues. Therefore, it has been reported that the presence of a macromolecular mediator enhances the LC-catalyzed protein crosslinking reaction[33]. These mediator-protein crosslinks enhanced the heat resistance, chemical bond strength, viscosity, gel strength, emulsifiability, and foamability of films or foods[33,34,36]. In our previous study on plant-based meat analog patties, the presence of sugar beet pectin (SBP) as a mediator also enhanced the protein-crosslinking activity of LC, thereby improving the physical and nutritional properties[37].

In this study, the author focused on developing a more effective and safe browning system for BR in plant-based meat analog patties using an enzymatic method. First, the author investigated the synergistic effects of SBP and LC on BR decolorization of meat analog patties. Importantly, the author discovered that the red tones of the LC-treated patties containing BR and SBP were remarkably browned after grilling, compared with patties that did not contain SBP. Additionally, the hardness of LC-treated meat analog patties containing BR was higher than those that did not contain BR. Interestingly, the presence of SBP and LC enhanced the browning reaction and functional properties of meat analogs containing BR. To the best of our knowledge, this is the first report on a browning system for meat analogs containing BR using enzymatic methods.

Results and Discussion

Synergistic effect of LC and SBP on browning of meat analog patties containing BR.

To investigate whether SBP and LC have a synergistic effect on browning plant-based meat analogs, the author added LC to pea-based patties containing BR and SBP (Fig. 4-1). Non-treated patties containing BR + methylcelullose (MC) were used as controls. After grilling, a red liquid resembling fresh meat juices exuded from the control patties, whereas the LC-treated patties containing BR + MC + SBP did not exude liquid (Fig. 4-2). The red color tones of non-treated and LC-treated patties with BR + MC were not affected by the grilling process (Fig. 4-1). The color of non-treated patties containing SBP did not change either (data not shown). In contrast, LC-treated patties containing BR + SBP or BR + SBP + MC developed a brown color during grilling.

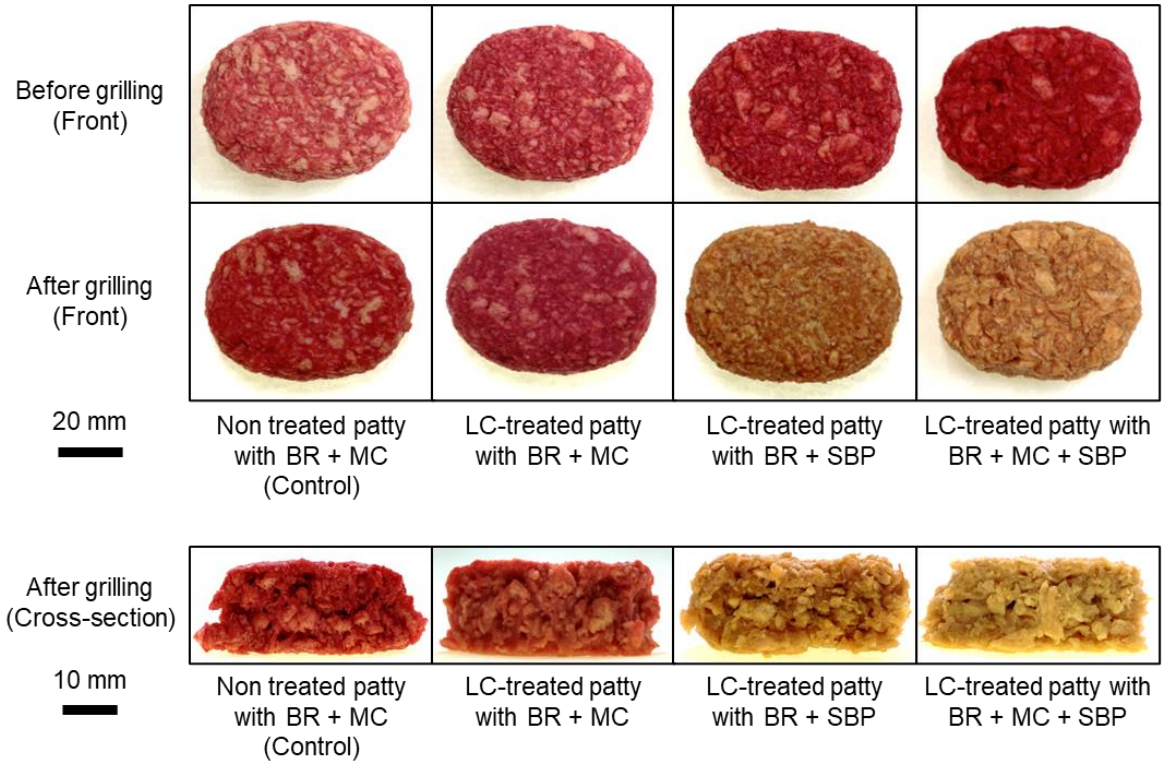

Figure 4-1. The appearance of the outside and inside of the meat analog patties before and after the grilling process.

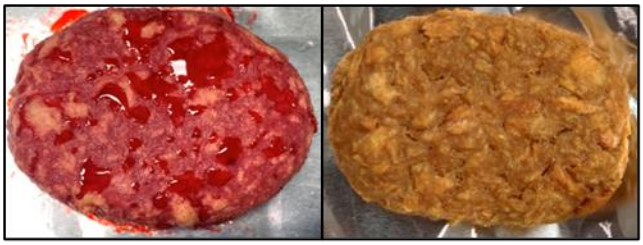

Figure 4-2. The appearance of meat analog patties immediately after grilling. Non-treated patty with BR + MC (Control). LC-treated patty with BR +MC + SBP.

To investigate the color change in greater detail, the objective color attributes of the patties after grilling were measured using a colorimeter (Fig. 4-3). The color tone of the LC-treated patties containing BR + MC was slightly different from that of the control (Figs. 4-3a and 4-3b). Compared with the control, the reflectance of LC-treated patties containing BR + SBP or BR + SBP + MC increased in the 500–600 nm range and decreased in the 600–700 nm range (Figs. 4-3b and 4-3d). This indicates that the color of the patties changed from red to brown. Interestingly, the reflectance difference of LC-treated patties containing BR + SBP + MC was higher than that of LC-treated patties containing BR + MC (Figs. 4-3b and 4-3d). The author also evaluated the color change of beef patties grilled. Similar to that of the LC-treated patties, the reflectance of beef patties also increased in the 500–600 nm range and decreased in the 600–700 nm range (Figs. 4-3g and 4-3h). The waveform comparisons of non-treated patties containing MC (control) with meat before grilling and LC-treated patties containing BR + SBP or BR + SBP + MC with meat after grilling were similar (Fig. 4-4). Next, the color of the meat analog patties was characterized using the $L^*a^*b^*$ coordinates (Table 4-1). It is indicated that a^* (redness) decreased and b^* (yellowness) increased in LC-treated patties containing BR + SBP or BR + SBP + MC after grilling.

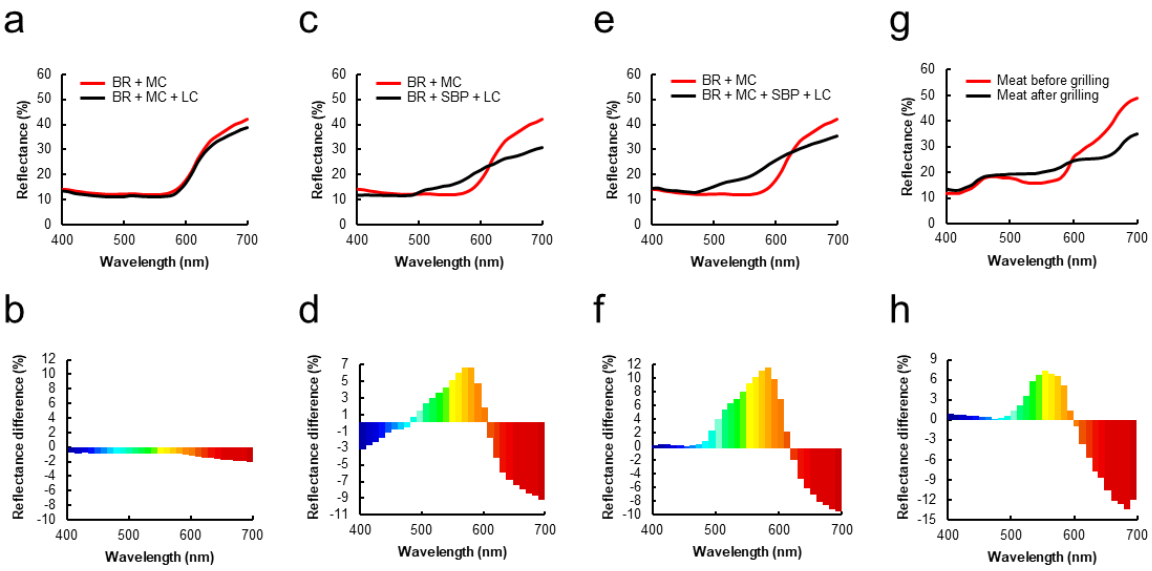

Figure 4-3. Reflectance of meat analog patties and beef patties. The reflectance values of (a, b) LC-treated patties containing BR + MC, (c, d) LC-treated patties containing BR + SBP, and (e, f) LC-treated patties containing BR + MC + SBP were measured using a colorimeter. Non-treated patties with BR + MC were used as controls. (g, h) Beef patties before and after grilling.

Similarly, *a** decreased and *b** increased under the same conditions in beef patties. The *L** value (lightness) of LC-treated patties containing SBP was higher than that of patties without SBP. At the same time, the decrease rate in the *L** value of animal-based patties was higher than plant-based patties. These findings of reflectance and *L*a*b** values indicated that the browning system for BR by LC + SBP in plant-based patties could effectively imitate the color changes of animal-based patties.

Table 4-1. Objective color measurements (*L*a*b) coordinates of plant-based and animal-based patties.**

Formulation	MC	SBP	BR	LC	Grilling process	*L**	*a**	*b**
Non-treated patty with BR + MC (Control)	+	-	+	-	Before	48.7 ± 1.7	48.5 ± 0.9	3.9 ± 1.1
					After	45.1 ± 2.1	47.9 ± 1.0	3.9 ± 0.5
LC-treated patty with BR + MC	+	-	+	+	Before	47.9 ± 3.5	48.4 ± 0.6	3.6 ± 1.0
					After	44.6 ± 1.7	47.0 ± 2.8	3.5 ± 0.5
LC-treated patty with BR + SBP	-	+	+	+	Before	53.7 ± 2.4	47.9 ± 2.4	3.8 ± 1.4
					After	52.7 ± 1.1	9.3 ± 1.1	43.5 ± 2.2
LC-treated patty with BR + MC + SBP	+	+	+	+	Before	55.6 ± 0.8	48.3 ± 3.2	3.7 ± 2.1
					After	54.1 ± 1.5	9.4 ± 1.6	46.6 ± 1.8
Beef patty	+	-	-	-	Before	54.8 ± 0.6	55.1 ± 1.7	3.5 ± 0.9
					After	45.2 ± 1.5	4.0 ± 1.3	39.7 ± 1.3

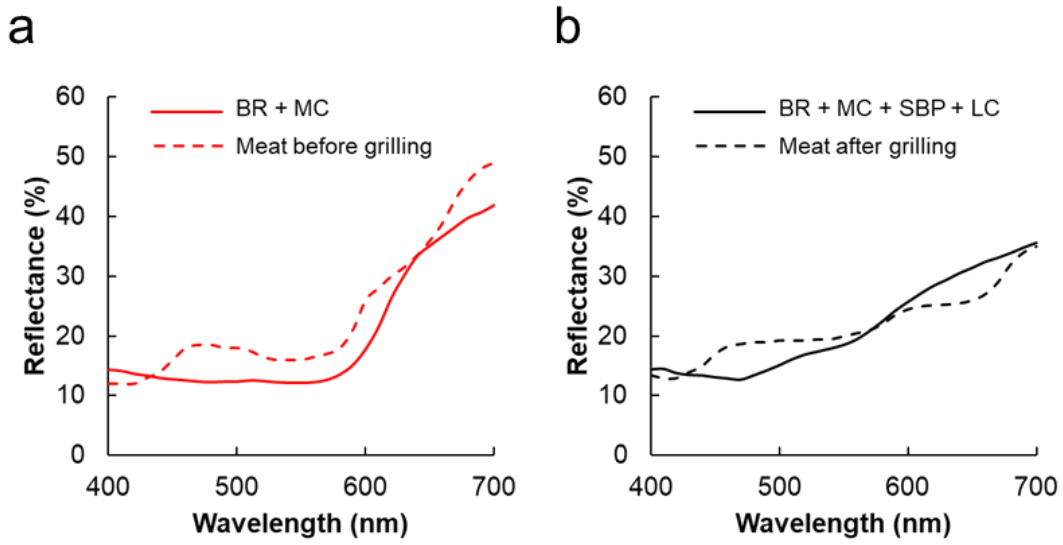

Figure. 4-4. Reflectance of meat analog patties and beef patties. (a) The comparison between non-treated patty containing BR and beef patty before grilling. (b) The comparison between LC-treated patties containing BR + MC + SBP and beef patty before grilling.

It is known that red betanin is oxidized by endogenous peroxidases in living red beets, resulting in color changes due to the formation of yellow betalamic acid and colorless cyclodopa[38-40]. Peroxidase is a hydrogen peroxide-requiring enzyme. Therefore, peroxidase decolorization of betanin has limited applicability in the food industry because hydrogen peroxide produces unacceptable off-flavors due to lipid oxidation, resulting in quality deterioration[41]. Peroxidases and LCs oxidize phenols using a similar oxidation mechanism, but only oxygen molecules are required for the laccase reaction[19]. The reflectance of LC-treated patties containing BR + SBP or BR + SBP + MC increased in the 500–600 nm (yellow) range and decreased in the 600–700 nm (red) range (Figs. 4-3d and 4-3f). These findings suggest that LC-catalyzed oxidation could degrade betanin (red) to betalamic acid (yellow) and cyclodopa (colorless), similar to the peroxidase-catalyzed reaction. The LC used in this study is a commercially available food-grade product that requires oxygen molecules alone to catalyze the reaction[19]. Betalamic acid has also been identified as a natural yellow pigment in plants[42]. Cyclodopa, a major metabolite derived from tyrosine, is a precursor of melanin in animals[43] and betalain pigment in plants[44]. Humans have ingested foods containing these compounds for many years. Moreover, betalamic acid has been found beneficial in controlling diabetes mellitus by suppressing pancreatic amylase activities[45,46]. Therefore, these findings demonstrate a novel, effective, and safe browning process using LC and SBP for plant-based meat analogs containing BR pigment.

Synergistic effect of LC and SBP on enhancing physical properties of patties.

The physical properties of meat analog patties containing BR were investigated (Fig. 4-5). Similar to a previous study[37], non-treated patties containing SBP did not display

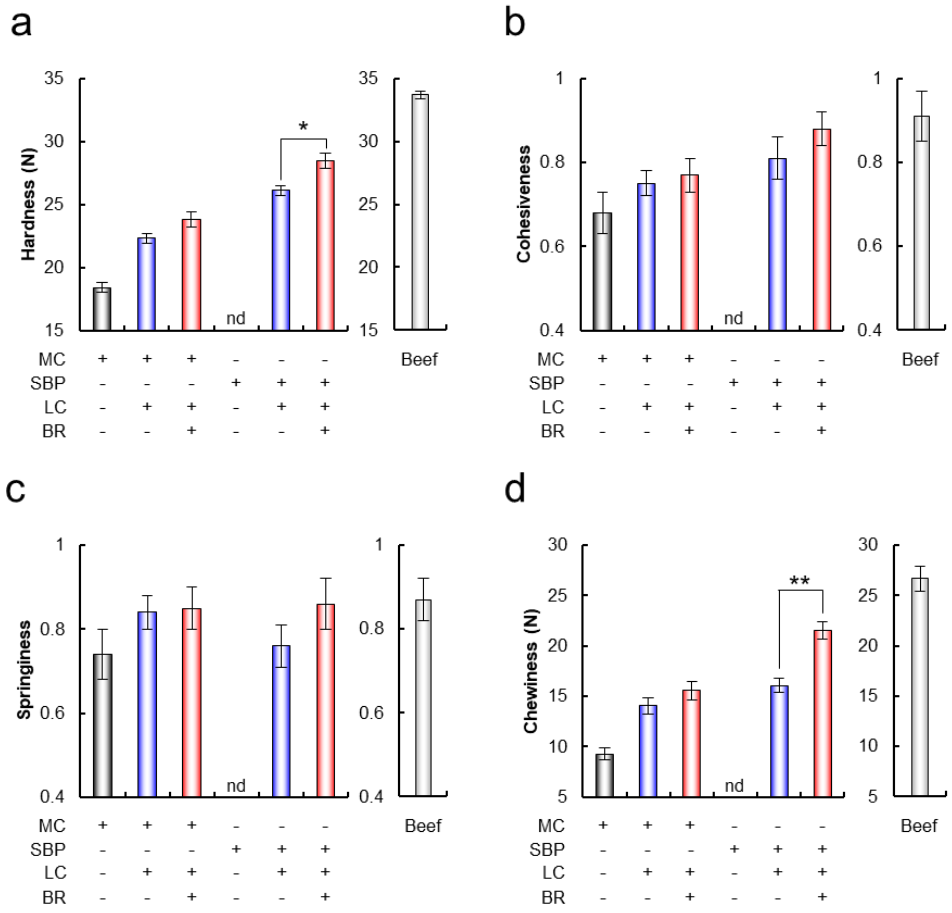

Figure 4-5. Texture profile analysis of meat analog patties containing BR and beef patties. (a) Hardness, (b) Cohesiveness, (c) springiness, (d) chewiness. Data are presented as mean ± standard deviation of five experiments. *$p < 0.05$, **$p < 0.01$, Student's *t*-test.

moldability or binding ability. Interestingly, the hardness and chewiness values of LC-treated patties containing MC + BR were slightly higher than those of LC-treated patties containing MC (Figs. 4-5a and 4-5d). Moreover, the hardness and chewiness values of LC-treated patties containing SBP + BR were significantly higher than those of LC-treated patties containing SBP (Figs. 4-5a and 4-5d). While the hardness and chewiness values of these patties were lower than those of the beef patties, the cohesiveness and springiness values were similar to those beef patties (Figs. 4-5a and 4-5d). These findings indicated that LC enhanced the

physical properties of plant-based patties containing BR, especially in the presence of SBP.

LC is also known as a protein-crosslinking enzyme. Generally, proteins are poor substrates for LC because of the intra-protein location of most tyrosine residues. It has been reported that the presence of a low-molecular phenolic mediator enhances the LC-catalyzed protein crosslinking reaction[47,48]. The mediator radicalized by LC can oxidize the intra-protein tyrosine residues where LC cannot directly attack, resulting in protein crosslinking[32]. Therefore, phenolic mediators such as vanillin, ferulic acid, and catechin could enhance the LC-catalyzed crosslinking reaction[49-51]. Interestingly, in this study, the presence of BR enhanced the hardness and chewiness attributes of the meat analog patties (Fig. 4-5). BR pigment consists mainly of betanin, which is a phenolic compound[10-12]. The presence of BR pigments could enhance LC-catalyzed crosslink reaction. Therefore, the author investigated the effects of BR on crosslinks formed by LC, in detail. As shown in Figure 4-6, the degree of LC-induced crosslinks increased in the following order: protein + SBP + BR > protein + SBP > protein. These findings suggest that BR could function as an LC mediator and enhance the degree of SBP-protein and protein-protein crosslinking induced by LC. Furthermore, LC in the presence of SBP caused a synergistic browning reaction in plant-based meat analog patties containing BR (Figs. 4-3 and 4-5). These findings suggest that SBP acts as a mediator of LC-induced browning in meat analogs containing BR.

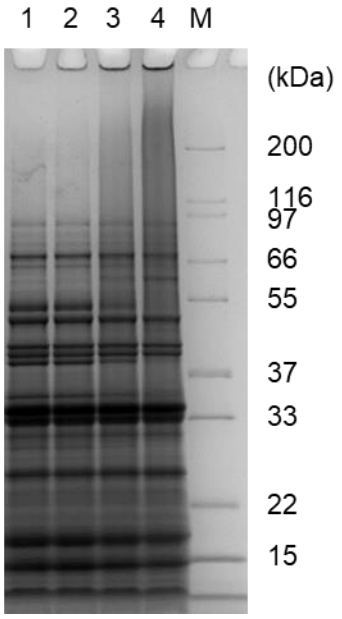

Figure 4-6. Synergistic effect of LC combined with BR on the formation of protein crosslinks in meat analog patties. SDS-PAGE analysis was performed on pea protein and BR treated with 250 U of LC. Lane 1: non-treated pea protein; Lane 2: LC-treated pea protein; Lane 3: LC-treated pea protein and SBP; Lane 4: LC-treated pea protein, BR, and SBP.

Despite ongoing technical developments, the physical properties of existing plant-based meat analogs are still inferior to those of animal-based meat, especially in terms of texture and hardness[5,9]. It has been reported that the hardness values of plant-based patties are lower than that of animal-based patties[52]. In fact, the hardness values of LC-treated patties containing SBP + BR and LC-treated patties containing MC + BR was lower than that of beef patties grilled under the same conditions (Fig. 4-5a). Meat proteins generally exhibit a higher degree of shrinkage. The higher hardness values of animal-based patties have been suggested to be due to muscle protein denaturation, which leads to meat hardening[52,53]. The hardness and chewiness values of LC-treated patties containing SBP + BR and LC-treated patties containing SBP + MC + BR were similar to or higher than those of previously described plant-based meat analog patties[52,54-56]. This is because BR and SBP could function as mediators to enhance LC-induced protein crosslinking reactions (Figs. 4-5 and 4-6). These findings offer a novel strategy for improving the physical properties of plant-based meat

analog patties and getting closer to the meat patty textures.

Cooking loss of meat analog patties and beef patties.

The author measured the cooking loss of the different formulations of plant-based meat analog patties (Fig. 4-7). Cooking loss, which reflects the degree of meat shrinkage during cooking, is an important indicator of the juiciness and yield of the final product. The cooking loss of patties containing SBP was lower than that of patties without SBP (Fig. 4-7). The presence of SBP in the formulation reduced the cooking loss by 4.4%–5.2%, compared to that in the absence of SBP. Our previous study also reported a similar phenomenon in which LC-induced SBP-protein crosslinks reduced the cooking loss and improved the water/oil holding capacity of meat analog patties[37]. The use of MC as a binder is associated with the problem of low water/oil holding capacity[37,52,57]. In fact, a red liquid that looked like fresh meat juices exuded from non-treated patties containing BR + MC after the grilling process (Fig. 4-2). This is an unacceptable phenomenon for consumers and manufacturers. However, the LC-treated patties containing BR + MC + SBP exuded no such liquid (Fig. 4-2). This effect also occurred with LC-treated patties containing BR + SBP (data not shown). These findings indicate that enhancing the water holding capacity by adding SBP and LC to the formulation could overcome these unacceptable phenomena and enhance the juiciness of plant-based meat analog patties.

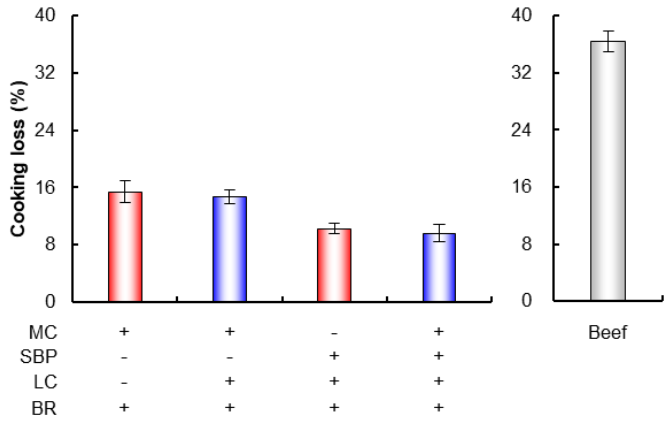

Figure 4-7. Cooking loss values of meat analog patties and beef patties. Cooking loss was calculated as the percentage weight difference between the dough before and after grilling. *$p < 0.05$, Student's *t*-test.

The author also measured the cooking loss of beef patties (Fig. 4-7). The cooking loss value was 36.0%. This is in line with a previous study that reported a 40% cooking loss for commercial beef[58]. Moreover, Marchi et al. reported that cooking and volume loss values of animal-based burgers were higher than those of plant-based burgers[59]. This superiority of plant-based burgers could be explained by the greater dietary fiber content, as incorporating dietary fibers in meat products reduced their cooking loss[60]. Similarly, in this study, the cooking loss value of meat patties was significantly higher than that of plant-based meat analog patties containing dietary fibers such as MC or SBP (Fig. 4-7). Cooking loss value greatly affects food intake yield for consumers; therefore, plant-based meat analogs could be a major food source in the future. Moreover, dietary fiber in meat analogs is considered to play an essential role in preventing large bowel diseases, ischemic heart diseases, and diabetes mellitus[61]. Therefore, plant-based meat analog patties offer great

promise in compensating the lack of the future protein source supply and contributing to disease prevention.

Furthermore, after the grilling process, the *L** value of LC-treated patties with SBP + BR was higher than that of patties containing MC + BR (Table 4-1). Previous studies reported that *L** values increased with increasing water/oil content of plant-based meat analog patties[62-64]. This is because small globules, such as water or oil, cause more light reflection[62,63]. Thus, it is suggested that LC-induced SBP-protein crosslinks decreased cooking loss, resulting in the increased *L** value of LC-treated patties containing SBP. In contrast to the LC-treated patties with SBP + BR, the *L** value of beef patties decreased after grilling (Table 4-1) as the cooking loss of beef patties was significantly higher than that of plant-based meat analog patties (Fig. 4-7). Furthermore, in this study, the pre-grilling *L** value of LC-treated patties containing SBP + BR was higher than that of patties containing MC + BR (Table 4-1). This is because SBP itself has good amphiphilic properties, contributed by the protein moiety ferulate and acetyl groups, which impart hydrophobic properties, and the carbohydrate fraction, which imparts hydrophilic properties[65,66]. This could enhance the redness saturation of the patty before the grilling process. Color is the first and most crucial element that consumers notice in food products[6]. Therefore, the addition of SBP and LC could enhance the value of plant-based meat analog patties by enhancing their appearance before and after the grilling process.

Putative enzymatic reactions that occurred in plant-based meat analog patties during grilling.

Figure 4-8 shows the putative browning and SBP-protein crosslinking reactions involved in plant-based meat analog patties treated with LC. As shown in Reaction *1*,

ferulates in SBP are oxidized by LC and O_2, producing feruloyl radicals. This radical indirectly oxidized betanin by acting as an LC mediator (Reaction *2*). Also, betanin would be a substrate for LC because it is a typical phenolic compound (Reaction *3*). Betanin (red) is degraded to betalamic acid (yellow) and cyclodopa (colorless) via feruloyl radical-catalyzed indirect oxidation (Reaction *2*) and LC-catalyzed direct oxidation (Reaction *3*). Therefore, it is suggested that LC-catalyzed oxidation in the presence of SBP synergistically caused a red-to-brown color change in plant-based meat analogs containing BR. In the next step, cyclodopa is formed via Reactions *2* and *3*, which is further oxidized to form radicalized cyclodopa (Reaction *4*). This radical indirectly oxidizes the ferulates in SBP and the tyrosine residues in protein, producing feruloyl radicals and tyrosyl radicals (Reaction *5*). LC-catalyzed oxidation also produces these radicals (Reaction *6*). Finally, feruloyl radicals and tyrosyl radicals spontaneously crosslinked, subsequently producing SBP-protein crosslinks (Reaction *7*). Therefore, it is suggested that cyclodopa radical-catalyzed indirect oxidation (Reaction *5*) and the LC-catalyzed direct oxidation (Reaction *6*) act synergistically to form SBP-protein crosslinks, resulting in enhanced physical properties of plant-based meat analog patties. LC-catalyzed oxidation reactions in the presence of SBP effectively facilitated BR browning reactions and SBP-protein crosslinking reaction through the above-mentioned reactions.

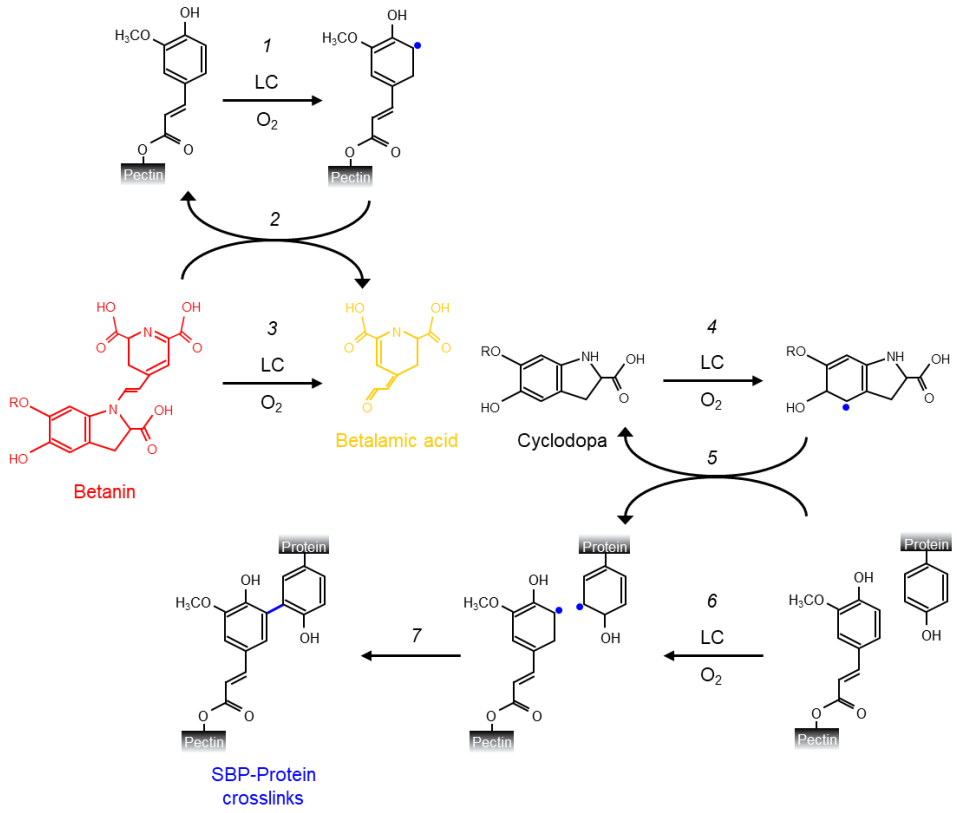

Figure 4-8. Putative reactions involved in browning and crosslinking reactions in plant-based meat analog patties. Reaction *1*: Oxidation reaction catalyzed by LC; Reaction *2*: Indirect oxidation reaction induced by radicalized ferulic acids in SBP; Reaction *3*: BR browning reaction by LC; Reaction *4*: Oxidation reaction by LC; Reaction *5*: Indirect oxidation reaction by radicalized cyclodopa; Reaction *6*: oxidation reaction by LC; Reaction *7*: SBP-protein crosslinking reaction by phenoxy

Methods

Materials.

Granule-type pea-based textured vegetable protein (TVP) and sugar beet pectin (GENU pectin type BETA BI-J) were obtained from SANSHO Co., Ltd. (Tokyo, Japan). BR pigments and pea protein isolate (PPI) were purchased from KUMAMOTO BEET RED Co., Ltd. (Kumamoto, Japan) and Roquette Frères (Lestrem, France), respectively. Ground beef

was obtained from a local supermarket in Nagoya (Japan). LC (LC-Y120; Amano Enzyme Inc., Nagoya, Japan) is a commercially available food-grade product. According to the manufacturer's instructions, the optimal reaction temperature for LC is 60°C, with a preferred temperature range of 40–70°C (> 80% activity).

Preparation of plant-based meat analogs.

The base of the TVP matrix was prepared using TVPs and a binder (MC or SBP), followed by the addition of olive oil, BR, and PPI (Table 4-2). First, dried TVP was immersed in water (1:5 mass-to-volume ratio) for 2 h for hydration. After dehydrating the swollen TVP, it was mixed with 2.0% SBP or 2.0% MC at the final concentration. Thereafter, 5 g water, 8 g olive oil, 2.5 g PPI, and 0.5 g BR were added to each 25 g of TVP matrix. The samples were blended for 60 s using a hand blender. Thereafter, LC was added to the TVP matrix and blended for 60 s. The TVP matrix was molded (60 mm × 40 mm area and 25 mm height). The matrix was then cooked at 150°C for 15 min and cooled to room temperature (20–25°C) before being used for further analysis.

Table 4-2. Mixing amounts of additives or enzymes in plant-based meat analog patties.

Formulation	Wet TVP (g)	Ground beef (g)	Water (g)	Oil (g)	PPI (g)	BR (g)	MC (%)	SBP (%)	LC (U/g TVP)
Non treated patty with BR + MC (Control)	25	-	5	8	2.5	0.5	2	-	-
LC-treated patty with BR + MC	25	-	5	8	2.5	0.5	2	-	50
LC-treated patty with BR + SBP	25	-	5	8	2.5	0.5	-	2	50
LC-treated patty with BR + MC + SBP	25	-	5	8	2.5	0.5	2	2	50
Beef patty	-	35	5	-	-	-	2	-	-

Preparation of beef patties.

Beef patties were prepared using ground beef, water, and MC. (Table 4-2). First, 2.0% MC at the final concentration and 5 g water were added to 35 g ground beef. The samples were blended for 60 s using a hand blender. Thereafter, the meat patties were molded. The matrix was then cooked at 150°C for 15 min and cooled to room temperature (20–25°C) before being used for further analysis.

Color analysis.

The color of the cooked meat analogs was measured using a colorimeter (Chroma Meter CM-700d/600d; Konica Minolta, Tokyo, Japan). The color analysis results were expressed according to the Commission International de l'Eclairage (CIE) system and reported as Hunter *L** (lightness), *a** (redness), and *b** (yellowness).

Cooking loss.

The cooking method and conditions were determined based on the study by Pathare and Roskilly[67]. The patties were cooked at 150°C for approximately 15 min, depending on the time taken for the temperature at the center of the meat analog to reach 80°C. After cooking, the samples were cooled to room temperature (20–25°C). Cooking loss was calculated as the percentage weight difference between the patty before cooking and after cooking, using the following formula: cooking loss (%) = $[(W_1 - W_2)/W_1] \times 100$; W_1 is the weight of the meat analog before grilling (g), and W_2 is the weight of the meat analog after grilling (g). The cooking loss of beef patties was estimated following a similar method.

Texture profile analysis.

Texture profile analysis was carried out using a COMPAC-100II (Sun Scientific Co., Ltd., Tokyo, Japan) equipped with a cylindrical probe of 31.4 mm area. After grilling, meat analogs and beef patties were prepared for the analysis and cut to a length of 15 mm in order to obtain homogeneous pieces of extrudates. The diameter of each sample was approximately 20 mm. A double compression cycle was performed at 1 mm/s until a recorded deformation of 50% was achieved. Five replicates were used for each sample. The following parameters were evaluated: hardness, the maximum force recorded during the first compression; cohesiveness, the area of work during the second compression divided by the area of work during the first compression; springiness, the distance recorded during the second compression divided by the distance of the first compression; and chewiness, hardness × cohesiveness × springiness.

SDS-PAGE analysis of crosslinking.

To investigate the synergistic effects of LC on the formation of crosslinks between proteins, betanidin, and SBP, the degree of crosslinking was measured by SDS-PAGE. 10% (v/v) pea + 1.0% RB pigment solution, 0.5% (v/v) SBP + 1.0% RB pigment solution, and 10% (v/v) pea + 0.5% (v/v) SBP + 1.0% RB pigment solution were assayed in 10.0-mL reaction mixtures containing 100 mM phosphate buffer (pH 7.0) and 250 units of LC. The reaction mixtures were incubated at 40°C and then stopped by boiling at 100°C for 5 min. Samples were prepared with an SDS-PAGE buffer (62.5 mM Tris-HCl pH6.8, 10% glycerol, 2% SDS, 5% DTT, and 0.002% BPB) under reducing conditions, and resolved on a 10-20% separating gel using an electrophoresis buffer (25 mM Tris, 19 mM glycine, and 0.1% SDS).

Summary

The widening gap between current supply of meat and its future demand has increased the need to produce plant-based meat analogs. Despite ongoing technical developments, one of the unresolved challenges of plant-based meat analogs is to safely and effectively imitate the appearance of raw and cooked animal-based meat, especially the color. This study aimed to develop a more effective and safe browning system for beet red (BR) in plant-based meat analog patties using laccase (LC) and sugar beet pectin (SBP). First, the author investigated the synergistic effects of SBP and LC on BR decolorization of meat analog patties. The author discovered that the red tones of LC-treated patties containing BR and SBP were remarkably browned after grilling, compared to patties that did not contain SBP. Notably, this color change by LC + SBP was similar to that of beef patties. Additionally, the hardness of LC-treated meat analog patties containing BR was higher than those that did not contain BR. Interestingly, the presence of SBP and LC enhanced the browning reaction and functional properties of meat analogs containing BR. This is the first report on a browning system for meat analogs containing BR using enzymatic methods to the best of our knowledge.

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Section 3. Cyclodextrins produced by cyclodextrin glucanotransferase mask beany off-flavors in plant-based meat analogs

Introduction

The widening gap between the supply and demand for meat has increased the need to produce plant-based meat analogs as protein sources[1]. It has been estimated that the progression from current omnivorous diets to vegan and ovo-lacto vegetarian diets can decrease greenhouse gas emissions by approximately 50% and 35%, respectively[2]. In addition, numerous studies have reported health benefits associated with replacing animal sources of protein with plant-based proteins, including reduced risks of type 2 diabetes, heart disease, and stroke[3,4]. Therefore, developing better plant-based diets would address the current protein crisis and positively impact the environment and human health[1].

Meat analogs are principally composed of textured vegetable proteins (TVPs) that imitate the fibrillar structure of meat muscle[5]. Soybean proteins are widely used for the production of TVPs. Soy-based TVP is a plant-based protein product that is cholesterol-free, with low concentrations of saturated fat and high concentrations of essential amino acids [6,7]. Despite ongoing technical developments, the appearance, flavor, taste, and texture of plant-based meat analogs differ from those of traditional meat products[1,8,9]. One of the unresolved challenges for plant-based meat analogs is their flavor, particularly the off-flavors from soy[1,8,9]. As soybean protein has an unpleasant beany and grassy odor, it affects the overall flavor of meat analogs, limiting consumer acceptability and restricting the development of meat analogs as food products[10].

The beany flavor is a combination of more than 20 volatile compounds produced

during soybean growth and processing of soybean[11,12]. These compounds can be mainly divided into fatty aldehydes, fatty alcohols, fatty ketones, furans, furan derivatives, and aromatic compounds[13]. Among these, hexanal, 1-octen-3-ol, and benzaldehyde are considered the compounds that primarily contribute to the off-flavor[12]. Currently, there are three main approaches for the removal of soybean beany flavor: genetic engineering, physical, and chemical methods[12]. However, these approaches cannot solve the technical challenges associated with beany off-flavors.

Cyclodextrins (CDs) are cyclic oligosaccharides composed of six or more α-1,4-linked glucose units that are also industrially produced from starch using the enzyme cyclodextrin glucanotransferase (CGT, EC 2.4.1.19). CDs with 6, 7, and 8 α-glucose residues are called α-CD, β-CD, and γ-CD, respectively. CDs can form inclusion complexes with hydrophobic chemicals as they possess a hydrophilic exterior and a hydrophobic interior[14]. Therefore, CDs are used to remove cholesterol from dairy and egg products, decaffeinate coffee, and for flavor encapsulation[14]. Moreover, CD-mediated masking or reduction of undesirable flavors has shown considerable promise in food applications[12,14]. In a previous study, the addition of CDs to soymilk decreased the concentration of volatile compounds associated with beany flavors[15]. Zhu and Damodaran (2018) reported the volatile compound removal properties of CDs on soy protein isolates[16]. However, masking the beany flavors of soy-based TVP and meat analogs using CDs has not been extensively studied.

CDs were assigned a “generally recognized as safe” (GRAS) status by the U.S. Food and Drug Administration (US-FDA) and an E number by the European Food Safety Authority (EFSA) and are considered as safe food additives[17,18]. Therefore, CD-mediated masking or reduction of beany flavors is an effective and safe approach. However, the

increasing consumer demand for clean-label food demands the reduction of additives, and the scientific community and food industry are developing more acceptable strategies for decreasing the beany flavor of plant-based meat analogs[19]. Among these, using food-grade enzyme catalysis is an attractive tool as enzymes do not need to be listed as an additive, provided that they are inactive in the finished product (e.g., denatured, inactivated enzymes). Therefore, the masking effect of CGT-produced CDs could be an attractive tool for treating clean-label foods. However, the masking efficiency of CDs produced by the CGT reaction within foods has not been extensively studied.

This study investigated the masking effects of CDs produced using CGT on meat analogs composed of soy-based TVP. CGT treatment effectively reduced the volatilization amounts of the known beany off-flavor-producing compounds compared to the non-treated patties. Moreover, CGT treatment improved the texture of the patties and increased their water- and oil-holding capacity. These findings indicated that CDs produced by the CGT reaction could effectively mask off-flavors of meat analogs and improve their physical properties while meeting clean-label requirements.

Results and Discussion

Biochemical characterization of food-grade CGT

In this study, the author used the cyclodextrin glucanotransferase “Amano” as the commercially available food-grade CGT. The author first investigated the optimal temperature and pH of this CGT for the production of CD from starch. The optimal temperature and pH for CGT production were determined using potato starch. The optimal temperature of CGT was 60°C, with a preferred temperature range (>80%) between 40°C and

60°C (Fig. 5-1A). The optimal pH was 6.0, with a preferred pH range (>80%) of 6.0 and 7.0 (Fig. 5-1B). Next, under optimum conditions (60°C, pH 6.5), the CGT-catalyzed conversion rate of each CD was measured by HPLC (Fig. 5-2). The total CD conversion yield by the 2 h CGT-catalyzed reaction was 17.4 g/L (Fig. 5-1C). The amounts of α-CD, β-CD, and γ-CD produced increased in a time-dependent manner, reaching 4.0 g/h (46.0%), 3.2 g/h (36.8%), and 1.5 g/h (17.2%), respectively.

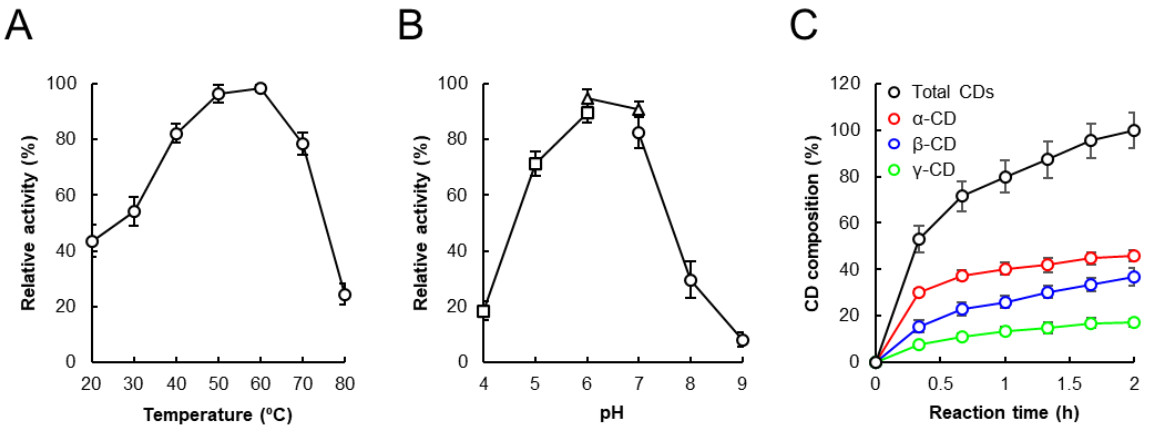

Figure 5-1. Biochemical characterization of food-grade cyclodextrin glucanotransferase (CGT). (A) Optimal temperature for CGT activity. Enzyme reactions proceeded at temperatures ranging from 20–80°C. (B) Optimal pH for CGT activity. Enzyme reactions proceeded over a pH range of 4.0–9.0 in 50 mM sodium acetate (pH 4.0–6.0, square), 50 mM sodium phosphate (pH 6.0–7.0, triangle), and 50 mM Tris–HCl (pH 7.0–9.0, circle). (C) Composition of cyclodextrin produced from potato starch through CGT reaction. Data are presented as the mean ± standard deviation of three experiments.

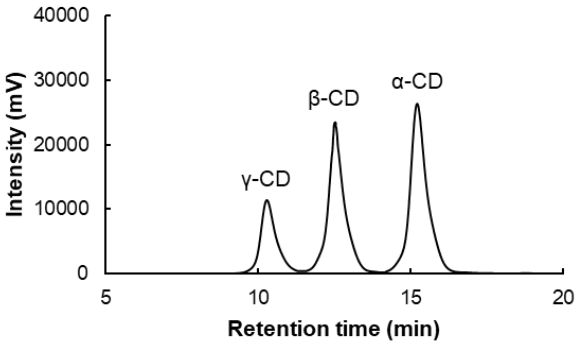

Figure 5-2. HPLC chromatography of α-, β-, and γ-cyclodextrins produced by cyclodextrin glucanotransferase.

The production ratios of α-CD, β-CD, and γ-CD were strongly affected by the origin of the CGT. The food-grade CGT used in this study is secreted by *Paenibacillus macerans*. Previous reviews have summarized the production ratio of CDs by CGTs obtained from various microorganisms: 96.5% α-CD obtained using CGT from *Klebsiella oxytoca*, 95% α-CD obtained using CGT from *Thermoanaerobacter*, 51% α-CD, and 32% β-CD obtained using CGT from *Clostridium* spp., 30% α-CD and 51% β-CD obtained using CGT from *Bacillus circulans*, and 36% β-CD, and 61% γ-CD obtained using CGT from *Brevibacillus spp*[20,21]. Compared to these studies, the CGT used in this study produced α-CD and β-CD more evenly (Fig. 5-1).

Effects of CDs produced by CGT on beany off-flavors of the meat analog patties

Next, the author investigated whether CGT could produce sufficient CDs in plant-based meat analog patties. All the ingredients used in this study were of food-grade. After treating the patties containing starch with CGT, CDs generated by the enzymatic reaction of CGT were extracted from the patties and quantified. The plant-based patty pH level was 6.48 ± 0.02. As a result, the production of α-CD, β-CD, and γ-CD reached 8.5, 5.8, and 2.8 g/L, respectively. The total yield of CD was 17.1 g/L. These findings showed that CGT could produce sufficient CD yield in the plant-based meat analog patty matrixes.

Next, the author investigated whether the CDs produced by CGT could mask the off-flavors released from the plant-based meat analog patties. After molding and grilling, the volatile components released from each meat analog patty were measured using HS-HPME-GC/MS in the SIM mode. Figure 5-3 shows the chromatographic analysis of n-hexanal, 1-

octen-3-ol, and benzaldehyde, the major off-flavor-producing compounds. The major fragment ions of these compounds were at 56 (Fig. 5-3A), 57 (Fig. 5-3B), and 77 *m/z* (Fig. 5-3C). Interestingly, volatilization amounts of the three compounds released from CGT-treated patties containing starch were lower than those released from non-treated patties (Fig. 5-3). Other volatile components, generally known as beany off-flavors, were analyzed according to Wang et al. (2021)[12]. Table 5-1 shows the volatilization amounts of 12 beany off-flavor-producing compounds released from each plant-based meat analog patty. Volatilization amounts of hexanal, heptanal, nonanal, benzaldehyde, octanal, hexanol, 1-octen-3-ol, 2-pentylfuran, and furfural released from CGT-treated patties containing starch were lower compared to that released from the other three patties. These findings indicate that CDs produced by CGT can mask the beany off-flavors released from plant-based meat analog patties.

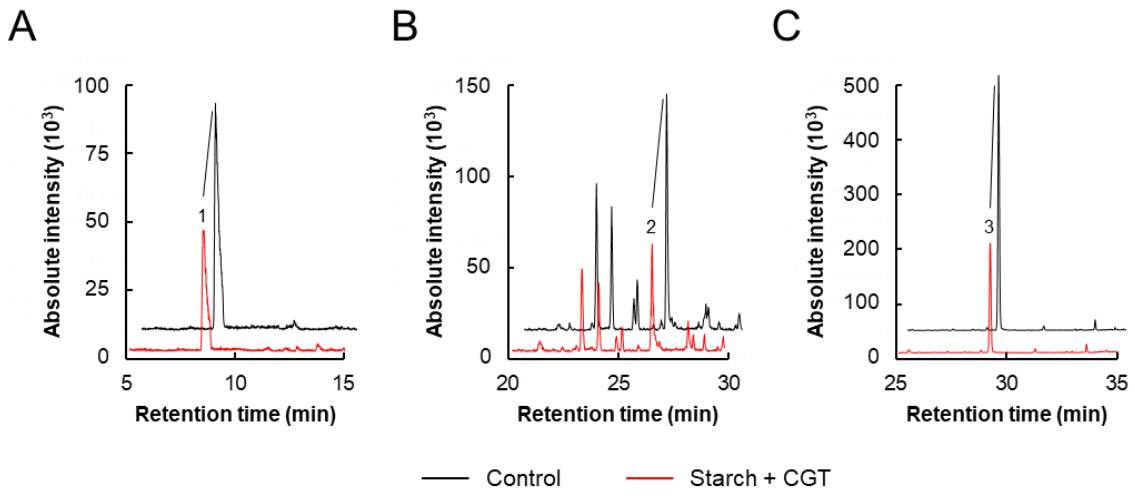

Figure 5-3. Detection of beany off-flavors in the meat analog patties using headspace-gas chromatography/mass spectrometry (HS-HPME-GC/MS). *n*-Hexanal (A), 1-octen-3-ol (B), and benzaldehyde (C) were detected by HS-GC/MS in the SIM mode. The major fragment ions of these compounds were 56 (A), 57 (B), and 77 (C) *m/z*.

The binding affinity of CDs for nonpolar compounds is related to their cavity size[22]. It has been reported that α-CD forms complexes with aliphatic chains, β-CD forms complexes with aromatic and heterocyclic compounds, and γ-CD binds larger molecules, such as steroids[22,23]. The CGT used in this study mainly produced α-CD and β-CD (Fig 5-1C), and the volatilization amounts of various fatty alcohols, fatty aldehydes, and aromatic compounds decreased (Table 5-1). The ability of the different CDs to mask beany flavors is not well-studied. Therefore, the author investigated the effect of α-CD, β-CD, and γ-CD on the efficiency of masking beany off-flavors. As shown in Table 5-1, CGT produced 1.7% CDs in soy-based patties. Based on this, each CD was added to the soy-based patties at a final concentration of 2%, and all patties were grilled and analyzed under the same conditions (Table 5-2). The ability to mask fatty aldehydes (hexanal, heptanal, octanal, and nonanal) and fatty alcohols (1-hexanol and 1-octen-3-ol) was in the order α-CD > β-CD > γ-CD. The ability to mask benzaldehyde, 2-pentylfuran, and furfural decreased in the order β-CD > α-CD > γ-CD. Interestingly, none of the CDs decreased the volatilization amounts of unsaturated aldehydes such as 2-octenal and 2-nonenal. These findings indicated that the binding affinity to off-flavors was different among α-CD, β-CD, and γ-CD, and that α-CD and β-CD could effectively mask off-flavors volatilized from soy-based patties.

Table 5-1. Volatile components released from meat analog patties with and without CGT and starch. Data are presented as the mean ± standard deviation of three experiments.

Volatile components	RT (min)	Starch	CGT	Starch + CGT
Aldehyde				
Hexanal	8.43	1.09 ± 0.09^{a}	1.12 ± 0.07^{a}	0.52 ± 0.14^{b}
Heptanal	12.11	0.98 ± 0.04^{a}	1.06 ± 0.09^{a}	0.58 ± 0.18^{b}
Octanal	31.11	1.17 ± 0.09^{a}	0.93 ± 0.08^{a}	0.58 ± 0.13^{b}
Nonanal	17.15	1.12 ± 0.03^{a}	1.07 ± 0.11^{a}	0.63 ± 0.09^{b}
2-Octenal	17.37	1.13 ± 0.12^{a}	1.06 ± 0.09^{a}	1.02 ± 0.06^{a}
2-Nonenal	29.89	1.06 ± 0.07^{a}	0.94 ± 0.04^{a}	1.12 ± 0.22^{a}
Benzaldehyde	29.17	1.12 ± 0.05^{a}	1.21 ± 0.14^{a}	0.67 ± 0.10^{b}
Alcohol				
1-Hexanol	21.47	0.98 ± 0.10^{a}	1.14 ± 0.15^{a}	0.61 ± 0.21^{b}
1-Octen-3-ol	26.52	1.07 ± 0.09^{a}	1.15 ± 0.13^{a}	0.55 ± 0.07^{b}
Heterocyclic compound				
Pyrazine	19.16	1.04 ± 0.03^{a}	0.97 ± 0.14^{a}	1.12 ± 0.08^{a}
Acid				
Acetic acid	26.23	0.98 ± 0.21^{a}	1.15 ± 0.16^{a}	0.95 ± 0.24^{a}
Furan				
2-Pentylfuran	13.96	1.15 ± 0.05^{a}	1.11 ± 1.06^{a}	0.48 ± 0.19^{b}
Furfural	26.74	1.21 ± 0.24^{a}	1.09 ± 0.14^{a}	0.63 ± 0.12^{b}

Relative values of volatile components from each analog patty compared with those from the control patty are shown. Data are presented as mean values ± standard deviation of three independent experiments. Different letters in the same row mean significant differences at *$p<0.05$. CGT, cyclodextrin glucanotransferase; RT, retention time of each compound

In this study, α-CD effectively removed fatty alcohols/aldehydes, and β-CD removed

benzaldehyde (Table 5-2). This difference in the affinity of CDs for nonpolar compounds is related to the size of the cavity. The hydrophobic inner cavity of the α-CD interacts with the acyl chain of the alcohol/aldehyde, resulting in the tight-fitting of an acyl chain with a 4.0 Å diameter (cross-section) within the 4.7–5.3 Å cavity of the α-CD. In contrast, the β-CD cavity interacts with the aromatic ring of benzaldehyde, leading to the tight-fitting of the ring (5.8–6.3 Å) within the 6.0–6.5 Å cavity of β-CD[24]. These interactions could enhance van der Waals forces, further enhancing the interaction energy of the complex[16]. In contrast, the CDs could not eliminate unsaturated aldehydes with shorter hydrophobic chains (Table 5-1). This is because the binding affinity of CDs to acyl chains is generally affected by the chain length of the fatty alcohol/aldehyde[25]. Moreover, γ-CD was unable to interact with alcohol/aldehyde, benzaldehyde, and other volatile compounds in soy-based patties (Table 5-2). This is because its cavity size (7.5–8.3 Å) is larger than these volatile compounds, resulting in poor van der Waals and hydrophobic interaction[26]. These results indicated that the presence of α-CD and β-CD, but not γ-CD, was sufficient to mask any off-flavors. Therefore, the CGT used in this study was suggested to be suitable for masking beany off-flavors from soy-based foods, as it mainly produces α-CD and β-CD.

Table 5-2. Volatile components released from meat analog patties with and without each CD. Data are presented as the mean ± standard deviation of three experiments.

Volatile components	RT (min)	α-CD	β-CD	γ-CD
Aldehyde				
Hexanal	8.43	0.48 ± 0.09^{c}	0.78 ± 0.12^{b}	1.12 ± 0.05^{a}
Heptanal	12.11	0.55 ± 0.21^{c}	0.79 ± 0.15^{b}	1.09 ± 0.05^{a}
Octanal	31.11	0.61 ± 0.06^{b}	0.84 ± 0.14^{a}	1.05 ± 0.06^{a}
Nonanal	17.15	0.59 ± 0.05^{b}	0.83 ± 0.05^{a}	0.98 ± 0.12^{a}
2-Octenal	17.37	1.09 ± 0.07^{a}	0.96 ± 0.11^{a}	1.12 ± 0.20^{a}
2-Nonenal	29.89	1.16 ± 0.12^{a}	1.13 ± 0.07^{a}	1.10 ± 0.11^{a}
Benzaldehyde	29.17	0.84 ± 0.12^{ab}	0.63 ± 0.08^{b}	1.17 ± 0.13^{a}
Alcohol				
1-Hexanol	21.47	0.54 ± 0.13^{b}	0.86 ± 0.06^{a}	0.98 ± 0.22^{a}
1-Octen-3-ol	26.52	0.62 ± 0.09^{b}	0.83 ± 0.12^{a}	1.07 ± 0.11^{a}
Heterocyclic compound				
Pyrazine	19.16	1.07 ± 0.12^{a}	1.10 ± 0.02^{a}	0.97 ± 0.05^{a}
Acid				
Acetic acid	26.23	1.06 ± 0.25^{a}	0.94 ± 0.20^{a}	0.98 ± 0.14^{a}
Furan				
2-Pentylfuran	13.96	0.72 ± 0.16^{ab}	0.53 ± 0.22^{b}	1.07 ± 0.17^{a}
Furfural	26.74	0.90 ± 0.05^{a}	0.67 ± 0.04^{b}	1.02 ± 0.07^{a}

Relative values of volatile components from each meat analog patty compared with those from the control patty are shown. Data are presented as mean values ± standard deviation of three independent experiments. Different letters in the same row mean significant differences at *$p<0.05$. CD, cyclodextrin; RT, retention time of each compound

Beany flavors are volatilized not only from soy but also from pea, faba, mung,

chickpea, and lentil bean[27–31]. Recently, bean proteins have been developed as plant-based meat analogs. However, various off-flavor-producing compounds, including hexanal, heptanal, 2-octenal, 1-octen-3-ol, and benzaldehyde, were detected and linked to an unpleasant beany and grassy odor in the proteins isolated from these beans. It is known that most bean proteins used for producing plant-based meat analogs release the same off-flavors[28,32]. These compounds affect the overall flavor of the final product[30,32]. This phenomenon limits the consumer acceptability of the products[1,8,9]. CDs are directly added to bean-based foods to mask the off-flavors[15,16]. In this study, the CDs produced by the enzymatic activity of CGT on the starch added to the patties were effective in decreasing the compounds responsible for the beany off-flavors (Table 5-1). Additionally, as the CGT added to the patties would be rendered inactive during the cooking process, it would not be considered an additive. Therefore, CDs generated by CGT in food could be an attractive strategy to mask the beany off-flavors of plant-based meat analogs and meet the increasing consumer demand for clean-label food[19].

Sensory evaluation

The author evaluated the changes in the sensory characteristics (smell and taste) of the patties with CGT treatment. First, the author investigated the effect of CGT treatment on the smell of plant-based patties (Table 5-3). The plant-based patties were scored based on their odor (an unpleasant smell) and fragrance (a pleasant smell). Interestingly, CGT treatment significantly reduced the odor values of plant-based patties. In contrast, this treatment enhanced the fragrance values, albeit there were no significant differences. This suggested that CGT could reduce the unpleasant beany odor released from plant-based patties.

Table 5-3. Smell test for plant-based patties.

	Odor	Fragrance
Control	3.83 ± 1.23^{a}	2.65 ± 0.90^{a}
Starch + CGT	1.22 ± 0.54^{b}	4.27 ± 0.63^{a}

Smell of plant-based patties was evaluated by a panel of five experts. The samples were evaluated for two attributes (odor, and fragrance) and were scored on a scale from 1 (weak) to 5 (strong). Data shows the mean ± standard deviation of the 5 expert scores. Different letters in the same row mean significant differences at $*p<0.05$.

Table 5-4. Taste test for plant-based patties.

Taste	Sweetness	Sourness	Saltiness	Bitterness	Umami
Control	0.23 ± 0.05^{a}	1.90 ± 0.81^{a}	2.32 ± 0.96^{a}	1.82 ± 0.75^{a}	3.02 ± 1.12^{a}
Starch + CGT	0.33 ± 0.25^{a}	1.11 ± 0.58^{a}	2.88 ± 1.13^{a}	3.07 ± 1.29^{a}	2.65 ± 0.80^{a}

Taste of plant-based patties was evaluated by a panel of five experts. The samples were evaluated for five attributes (sweetness, sourness, saltiness, bitterness, and umami) and were scored on a scale from 1 (weak) to 5 (strong). Data shows the mean ± standard deviation of the 5 expert scores. Different letters in the same row mean significant differences at $*p<0.05$, as determined.

Next, the effects of CGT treatments on the taste of plant-based patties were evaluated (Table 5-4). The plant-based patties were evaluated based on five attributes: sweetness, sourness, saltiness, bitterness, and umami. The CGT treatment did not affect the sweetness, sourness, saltiness, bitterness, or umami of the plant-based patties ($p>0.05$), suggesting that the CGT treatment did not significantly affect the taste of the patties. These

results demonstrate that CGT treatment could be an effective method for masking the beany off-flavor of plant-based patties without affecting the taste.

In some previous studies, the addition of lower CD concentrations to soy-based foods did not affect their sensory attributes[33, 34]. It is indicated that these foods contained a quantity of volatile beany flavor compounds, well above their threshold values, compared with the addition amounts of CDs. According to Suratmen 2004, 0.5% CD-added soymilk contained hexanal, 1-octen-3-ol, and benzaldehyde concentrations that were 278-, 787-, and 8,150-fold more than the threshold value (0.05, 0.01, and 0.0004 μg/mL)[35, 36]. In the present study, CGT treatment produced about 2% CDs in plant-based patties, which was enough to reduce the beany odor by the sensory evaluation. In fact, the volatilization amounts of hexanal, 1-octen-3-ol, and benzaldehyde released from CGT-treated patties containing starch were only 0.55, 0.84, and 0.023 μg/g, that were 11-, 84-, and 57-fold more than the threshold value. Considering the individual differences in the sensitivity to the threshold and lower volatile amount than in the previous study, it is considered that the CGT treatment in this study improved the sensory characteristics of the plant-based patties.

The CGT-catalyzed reaction generally produces CDs, maltooligosaccharides, and dextrin. Maltooligosaccharides, and dextrin are known to be hydrolyzed by human salivary α-amylase, producing glucose and maltose[37, 39]. However, CGT treatment did not affect the sweetness of the plant-based patties (Table 5-4). It is possible that the hydrolysis by α-amylase did not provide significant sweetness as the in-mouth contact time used by the panel was extremely short (< 30 sec).

Physical properties of meat analog patties

Generally, most meat analog products contain starch[8]. Functionally, the addition of starch improves the interaction between the protein, lipid, and water components in meat analog patties and improves the texture and consistency of the patties[8]. Moreover, starch is often used as a bulking agent in meat analog products because of its effect on textural properties[39]. Thus, the author also investigated the adverse effects of partially converting starch to CDs using CGT on the physical properties of meat analog patties. Table 5-5 shows the TPA parameters (hardness, cohesiveness, springiness, and chewiness) of the meat analog patties. The hardness and chewiness of the patties containing starch were slightly higher than those of the control patties as the increase in the viscosity and shear of gelatinized starch by cooling enhances the hardness of the patties[39]. The hardness and chewiness of CGT-treated patties containing starch were equivalent to those of untreated patties containing starch (Table 5-5). These findings indicate that CGT treatment for masking beany off-flavors did not decrease the textural properties of the plant-based meat analog patties.

Table 5-5. Texture profile analysis of meat analogs. Data are presented as the mean ± standard deviation of three experiments.

	Hardness (N)	Cohesiveness	Springiness	Chewiness (N)
Non-treated patty (control)	20.6 ± 1.2^{a}	0.62 ± 0.03^{a}	0.65 ± 0.04^{a}	8.3 ± 0.3^{b}
Non-treated patty containing starch	22.5 ± 1.1^{a}	0.67 ± 0.08^{a}	0.70 ± 0.10^{a}	10.6 ± 0.8^{a}
CGT-treated patty	20.4 ± 1.3^{a}	0.61 ± 0.05^{a}	0.66 ± 0.06^{a}	8.2 ± 0.4^{b}
CGT-treated patty containing starch	21.5 ± 1.8^{a}	0.65 ± 0.09^{a}	0.71 ± 0.12^{a}	10.0 ± 0.6^{a}

Data are presented as mean values ± standard deviation of three independent experiments. Different letters in the same row mean significant differences at *$p<0.05$. CGT, cyclodextrin glucanotransferase

Cooking loss of meat analog patties

Next, the author investigated the adverse effects of cooking loss on meat analog patties. Cooking loss represents the degree of meat shrinkage during cooking and is an important indicator for evaluating the juiciness and yield of the final product. As the amount of water and oil increased, a typical increase in cooking loss of all patties was observed (Fig. 5-4). Interestingly, the cooking loss of CGT-treated patties containing starch was substantially lower than that of non-treated patties containing starch under the condition of higher water content (Fig. 5-4A). The cooking loss of CGT-treated patties containing starch was approximately 1.3-fold higher than that of non-treated patties containing starch. However, under conditions of higher oil content, the cooking loss of CGT-treated patties containing

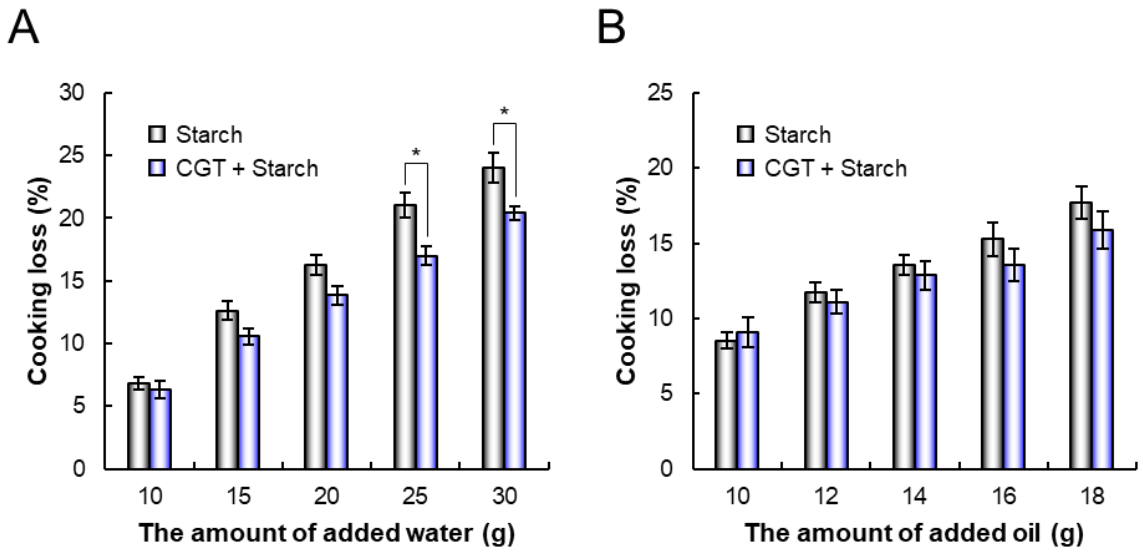

Figure 5-4. Cooking loss value of meat analog patties. (A, B) Cooking loss value was calculated as the percentage weight difference between the dough before cooking and after cooking. Meat analog patties containing different amounts of water (A) or oil (B) were treated with cyclodextrin glucanotransferase and starch and cooked. Data are presented as the mean ± standard deviation of three

starch was also slightly lower (Fig. 5-4B). These findings indicate that CGT treatment for masking beany off-flavors improves the cooking loss and water/oil-holding capacity of plant-based meat analog patties.

The use of MC lowers the water/oil-holding capacity[40,41]. Previous studies have reported that the cooking loss of plant-based patties containing MC is higher than that of patties containing other binders[40,41]. This is an unacceptable phenomenon for both consumers and manufacturers. In this study, the addition of starch to MC did not affect the water-holding capacity of plant-based patties, whereas CGT treatment enhanced the water-holding capacity of patties containing starch (Fig. 5-4A). Generally, starch gelatinization retains water, but starch retrogradation expels water as the temperature decreases, decreasing the water-holding capacity[42]. To prevent retrogradation, α-amylase treatment is used to decrease the amylose-chain length. Importantly, a previous study reported that CGT is a better antistaling enzyme than α-amylase because of its starch hydrolyzing and cyclizing activities[43]. Thus, it is possible that the partially decomposed starch and CDs formed by CGT enhanced the water-holding capacity of plant-based patties.

The CGT treatment also enhanced the oil-holding capacity of patties containing starch (Fig. 5-4B). In a previous study, the addition of β-CD and starch to a lipid solution formed an amylose–CD–lipid complex owing to the interaction between amylose and β-CD[44]. This complex has been reported to retain lipids and retard retrogradation[42,44]. In addition, it has been reported that the addition of α-CD helps retain fat globules in the microstructure and improves yields for animal-based products (chicken frankfurters)[45]. Thus, the author investigated the synergistic effects of CD and starch on the oil-holding capacity of patties under the condition of higher oil content (Fig. 5-5). Adding both CDs and starch

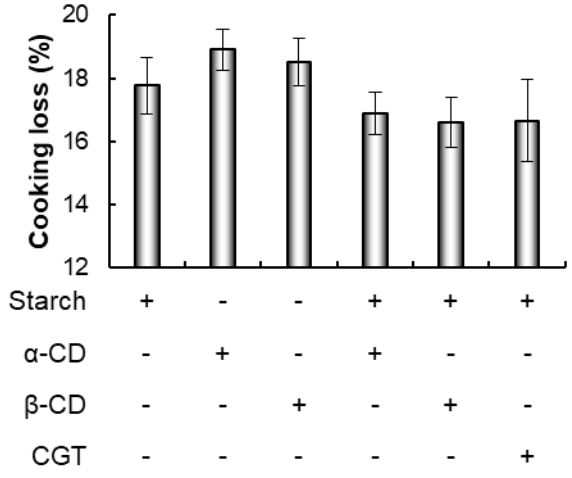

Figure 5-5. Cooking loss value of meat analog patties containing various saccharides. Cooking loss was calculated as the percentage weight difference before and after cooking. Meat analog patties were mixed with 10 g of water, 8 g of oil, 2% methylcellulose, and 2% of each saccharide (starch, α-CD, β-CD, and γ-D). Data are presented as the mean ± standard deviation of three experiments.

slightly decreased the cooking loss compared to the addition of CDs alone. These findings suggest that amylose–CD–lipid complexes formed by CGT can enhance the oil-holding capacity of plant-based patties.

Methods

Materials

Granule-type soy-based TVP was purchased from Marukome Co. Ltd. (Nagano, Japan). Potato starch was purchased from Nippon Star Chemical Co. Ltd. (Osaka, Japan).

Hexanal, heptanal, octanal, nonanal, 2-octenal, 2-nonenal, benzaldehyde, 1-hexanol, 1-octen-3-ol, pyrazine, acetic acid, 2-pentylfuran, and furfural were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). α-CD, β-CD, and γ-CD were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

CGT (Amano Enzyme Inc., Nagoya, Japan) is a commercially available food-grade product.

Enzyme assays

The activity of CGT was assayed in 1.0-mL reaction mixtures containing 100 mM sodium phosphate (pH 6.0) and 1.0% (w/v) potato starch at 20–80℃ for 2 h. The reaction was stopped by incubating at 100℃ for 5 min. After stopping the reaction, the reaction mixture was mixed with 0.005% iodine in 0.05% potassium iodide, and the absorbance was measured at 660 nm.

Preparation for plant-based meat analogs

The base of the TVP matrix was prepared using TVPs and a binder (methylcellulose (MC)), followed by the addition of water, olive oil, potato starch, and CGT (Table 5-6). First, 10 g of dried TVP was immersed in 15 mL water for 30 min for hydration. After hydration, the swollen TVP was mixed with 2.0% MC to obtain the final concentration. Thereafter, 10 g water, 3 g olive oil, 2.5 g soy protein isolates, and 1.0 g potato starch were added to 25 g TVP matrix. The samples were blended for 60 s using a hand blender. Subsequently, 100 U/g-starch CGT was added to the TVP matrix and blended for 60 s. The CGT used in this study was a commercially available food-grade product. The TVP matrix was molded (60 mm × 40 mm area × 25 mm height) and incubated at 60℃ for 2 h. The matrix was then cooked at 150℃ for 15 min and cooled to room temperature (20–25℃) before being used for further analysis.

Table 5-6. Amounts of additives or enzymes added to the plant-based meat analog patties.

Sample	Wet TVP (g)	Water (g)	Oil (g)	MC (%)	Starch (%)	CDs (%)	CGT (U/g-starch)
Non-treated patty (Control)	25	10	3	2	0	0	0
Non-treated patty with starch	25	10	3	2	2	0	0
CGT-treated patty	25	10	3	2	0	0	100
CGT-treated patty with starch	25	10	3	2	2	0	100
Non-treated patty with α-CD	25	10	3	2	0	5	0
Non-treated patty with β-CD	25	10	3	2	0	5	0
Non-treated patty with γ-CD	25	10	3	2	0	5	0

TVP, textured vegetable proteins; MC, methylcellulose; CDs, cyclodextrins; CGT, Cyclodextrin glucanotransferase

High-performance liquid chromatography (HPLC) analysis for CDs

The CDs in the reaction solution and patty matrix were analyzed by HPLC (Shimadzu, Kyoto, Japan). To extract CDs, the patty matrix was crushed using a mixer. The solutions containing various maltooligosaccharides and dextrin were added to 80% methanol and centrifugated at 15,000 × *g* for 10 min to precipitate saccharides with a molecular weight greater than oligosaccharides. Then, these solutions were incubated with 2.0 mg/mL glucoamylase (Sigma-Aldrich, St. Louis, USA) at 60°C for 3 h to hydrolyze all oligosaccharides, except CDs. After filtering through a membrane filter (pore size 0.45 μm), the supernatant containing CDs was injected into the HPLC. The supernatant was separated

on an MCI GEL CK04S (300 × 8.0 mm I.D.; Mitsubishi Chemical Corporation, Tokyo, Japan) connected to an evaporative light scattering detector system, with MilliQ water for 20 min at a flow rate of 0.4 mL/min at 80°C. Standard curves were prepared using solutions containing different concentrations of CD.

Headspace solid-phase microextraction-gas chromatography/mass spectrometry (HS-SPME-GC/MS)

Finely cut mold matrix (3 g) was added to 20 mL screw glass vials, and HS-SPME-GC/MS was performed using an autosampler (Shimadzu, Kyoto, Japan). 1,2-Dichlorobenzene (1.0 μL/g-sample) was added as an internal standard to check the retention time of different samples. Polydimethylsiloxane (PDMS; Shimadzu, Kyoto, Japan) and divinylbenzene (DVB)/PDMS (Shimadzu, Kyoto, Japan) were used as SPME fibers for extraction. The fiber was conditioned at 250°C for 30 min before each use. The vials were incubated for 10 min at 80°C in an autosampler with agitation (270 rpm). Volatile compounds were extracted by placing each fiber in a vial and exposing it to the headspace for 30 min at 80°C with agitation (270 rpm). The fiber was removed and inserted into the GC injection port. Desorption time for the SPME fiber injection was 3 min at 250°C. A gas chromatography system (GC-2030, Shimadzu, Kyoto, Japan) attached to a triple quadrupole MS (Shimadzu) was used. The injection port used was in split mode with a flow rate of 60 mL/min and a surge pressure of 100 kPa. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. An InterCap Pure-WAX column (30 m × 0.25 mm, film thickness 0.25 μm, GL Sciences Inc., Tokyo, Japan) was used for volatile compound separation. The oven temperature was programmed according to the manufacturer’s application datasheet for this

column, with slight modifications. The initial oven temperature was maintained at 40°C for 0.5 min. The temperature was then raised at 2°C/min to 120°C and finally at 10°C/min to 230°C, where it was kept constant for 5 min. The ion source temperature was 200°C, and the MS range was set to 29 to 450 *m/z*. The volatile compounds were identified by comparing the results with the mass spectra and retention indices from the spiked internal standard and a database developed using the National Institute of Standards and Technology library guidelines. To quantify volatile compounds, standard curves were generated from plant-based patties spiked to contain 0.1-, 1.0-, 5.0-, and 10-μg standard/g-sample for each volatile compound. Under the same conditions, these spiked samples were analyzed by HS-SPME-GC/MS.

Sensory evaluation

The smell and taste of plant-based patties were evaluated by five employees of Amano Enzyme Inc. trained in sensory evaluation. For taste tests, the samples were evaluated for five attributes (sweetness, sourness, saltiness, bitterness, and umami). For smell tests, the samples were evaluated for two attributes (odor and fragrance). The samples were scored on a scale from 1 (weak) to 5 (strong).

Measurements for texture profile analysis (TPA) of patties

TPA was performed using COMPAC-100II (Sun Scientific Co., Ltd., Tokyo, Japan) equipped with a cylindrical probe with an area of 31.4 mm[46]. After grilling, the meat analogs were prepared for analysis and cut to a length of 15 mm to obtain homogeneous extrudates. The diameter of each extrudate is approximately 20 mm. A double-compression

cycle was performed at 1 mm/s until a recorded deformation of 50% was achieved. The following parameters were evaluated: hardness, maximum force recorded during the first compression; cohesiveness, area of work during the second compression divided by the area of work during the first compression; springiness, the distance recorded during the second compression divided by the distance of the first compression; and chewiness, hardness × cohesiveness × springiness.

Cooking loss

The cooking method and conditions were determined based on a previous study[47]. The patties were cooked at 150°C for 15 min, or until the temperature at the center of the patty reached 80°C. After cooking, samples were cooled to room temperature (20–25°C). Cooking loss was calculated as the percentage weight difference between the patty before cooking and after cooking, using the following formula: cooking loss (%) = $[(W_1 - W_2)/W_1] \times 100$, where W_1 is the weight of the patty before grilling (g), and W_2 is the weight of the patty after grilling (g).

Summary

The widening gap between the supply and demand for meat products has increased the need to produce plant-based meat analogs as protein sources. Meat analogs are principally composed of soy-based textured vegetable proteins. Despite ongoing technical developments, one of the unresolved challenges for plant-based meat analogs is the off-flavor from soy, which limits their consumer acceptability. Among the various methods developed for overcoming this challenge, masking the beany flavors with cyclodextrins (CDs) is an

attractive, cost-effective, and safe strategy. However, the current established CD treatment method does not meet the requirement for a clean-label. This study aimed to develop more acceptable off-flavor-masking technologies for plant-based patties for modern clean-label preferences using enzymatic methods. The author used the cyclodextrin glucanotransferase (CGT), "Amano," as a commercially available food-grade CGT. The CGT-catalyzed reaction in plant-based patties yielded 17.1 g/L CD. As CGT could yield sufficient CD in the patties, the author investigated whether CDs produced by CGT could mask the off-flavors released from the plant-based patties. The CGT-treated patties had significantly lower volatilization amounts of the known beany off-flavor-generating compounds compared to the non-treated patties. Moreover, CGT treatment improved the texture of the patties and increased their water- and oil-holding capacity. As CGT is rendered inactive after cooking, it would not be considered an additive. These findings indicated that CDs produced by the CGT reaction could effectively mask off-flavors of meat analogs and improve their physical properties while meeting clean-label requirements.

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Conclusion

Commercial enzymes used for food industry are mostly secreted extracellularly. However, the functionalities and usefulness of extracellular enzymes have not been completely studied. Therefore, in this paper, the author focused on characterization of unknown enzymes secreted by microorganisms and usefulness of commercial enzymes for food processing.

In Chapter 1, the author described biochemical characterization of two GH134 β-1,4-mannanases, yellow mold AoMan134A and actinobacteria SsGH134, belonging to novel GH134 family. GH5 and GH26 β-1,4-mannanases mainly produced M1 and M2 from β-mannan, whereas GH134 β-1,4-mannanases produced M2, M3, M4, and M5 from β-mannan, with M3 being the predominant reaction product. Owing to their health-promoting effects, mannooligosaccharides have become a recent spurt of interest to nutraceutical and functional food sectors. Thus, GH134 β-1,4-mannanases might be suitable for producing prebiotic mannooligosaccharides. Moreover, AoMan134A retained 50% of its β-1,4-mannanase activity after heating at 90°C for 30 min, indicating that AoMan134A is thermostable. These findings indicated that the thermostability of AoMan134A is at least equal to that of other highly thermostable β-1,4-mannanases. AoMan134A could be of interest to the food, energy, and pulp industries.

In Chapter 2, the author described the commercial applications for plant-based meat analogs. The widening gap between the current supply of meat and its future demand has increased the need to produce plant-based meat analogs. Despite ongoing technical developments, functional properties of analog products differ from those of traditional meat. These limit consumer acceptability. The author tried to dissolve these outstanding challenges

of plant-based meat analogs. Section 1 described a novel binding system for meat analogs. Laccase formed pectin-protein crosslinks, improving binding ability of meat analogs. This crosslinks by laccase were the first reports about chemical-free novel binding systems for meat analogs. This enzymatic method could meet consumer's chemical-free trends. Moreover, Section 2 described novel color changing system of beet red used for plant-based patties. Red tones of laccase-treated patties containing beet red and pectin were browned after cooking. This could provide a safe and effective browning system for plant-based meat analogs products to simulate color changes in meat. Finally, Section 3 described novel off-flavor masking system. Cyclodextrin produced by glucanotransferase decreased volatile amounts of beany off-flavor compounds from analog products. This enzymatic method could meet consumer's clean-label trends because inactive enzymes do not need to be listed as an additive. Therefore, further enhancement of functionality and the achievement of label-free plant-based meat products by the enzymatic method are very effective and may attract increasing attention in the future.

In conclusion, unknown hypothetical proteins possessing industrially important functions still exist among microbial extracellular protein and even well-known commercial enzymes have the ability to solve the novel technical challenges corresponding to the needs of the times.

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List of publications

1. **Sakai, K.**, Mochizuki M., Yamada, M., Shinzawa, Y., Minezawa, M., Kimoto, S. et al. Biochemical characterization of thermostable β-1,4-mannanase belonging to the glycoside hydrolase family 134 from *Aspergillus oryzae*. *Applied Microbiology and Biotechnology* **101**, 3237–3245 (2017).
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1. Shimizu, M., Kaneko, Y., Ishihara, S., Mochizuki, M., **Sakai, K.**, Yamada, M. et al. Novel β-1,4-mannanase belonging to a new glycoside hydrolase family in *Aspergillus nidulans*. *Journal of Biological Chemistry* **290**, 27914-27 (2015).
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