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

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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, we investigated the synergistic effects of SBP and LC on BR decolorization of meat analog patties. We 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.
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, we added LC to pea-based patties containing BR and SBP (Fig. 1). Non-treated patties containing BR + methylcellulose (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 + SBP did not exude liquid (Fig. S1). The color tone of the LC-treated patties containing BR + MC (Fig. 2b,d). We also evaluated the color change of beef patties grilled under the same conditions. 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 (Fig. 2g,h). The waveform comparisons of non-treated patties containing BR + MC 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.

The currently established dye and pigment decolorization methods are classified into four main categories: physical, oxidative, enzymatic, and biological methods. Among them, the enzymatic method using laccase (LC; EC 1.10.3.2) has been applied in various industrial scenarios. LCs are oxidases that couple the reduction of molecular oxygen to the one-electron oxidation of a wide variety of phenols. 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 plant pigments, benzoxazoles, aromatic amines, and polymeric aromatic hydrocarbons. Therefore, they are widely used not only for the decolorization of synthetic dyes but also for bioremediation, detoxification, food processing, and biosensing.

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. In proteins, the exposed tyrosyl side chains serve as substrates for oxidation by LC, resulting in the spontaneous coupling of subsequent protein crosslinks (dityrosine). 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. LCs are oxidases that couple the reduction of molecular oxygen to the one-electron oxidation of a wide variety of phenols. 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 plant pigments, benzoxazoles, aromatic amines, and polymeric aromatic hydrocarbons. Therefore, they are widely used not only for the decolorization of synthetic dyes but also for bioremediation, detoxification, food processing, and biosensing.

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. In this study, we focused on developing a more effective and safe browning system for BR in plant-based meat analog patties using an enzymatic method. First, we investigated the synergistic effects of SBP and LC on BR decolorization of meat analog patties. Importantly, we 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.
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.

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. 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. Peroxidases and LCs oxidize phenols using a similar oxidation mechanism, but only oxygen molecules are required for the laccase reaction. 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 (Fig. 2d,f). These findings suggest that LC-catalyzed oxidation could degrade betanin (red) to betalamic acid.

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

Figure 2. 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.
hardness values of plant-based patties are lower than that of animal-based patties. In fact, the hardness values demonstrate a novel, effective, and safe browning process using LC and SBP for plant-based meat analogs containing BR pigment.

The higher hardness values of animal-based patties have been suggested to be due to muscle protein denaturation, which leads to meat hardening. The hardness and chewiness values of LC-treated patties containing SBP + BR were significantly higher than those of LC-treated patties containing SBP + MC (Fig. 3a,d). Moreover, the hardness and chewiness values of LC-treated patties containing SBP + BR were similar to those beef patties, the cohesiveness and springiness values were similar to those beef patties (Fig. 3a,d). These findings indicated that LC enhanced the physical properties of plant-based patties containing BR, especially in the presence of SBP.

Synergetic effect of LC and SBP on enhancing physical properties of patties. The physical properties of meat analog patties containing BR were investigated (Fig. 3). Similar to a previous study, non-treated patties containing SBP did not display 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 (Fig. 3a,d). Moreover, the hardness and chewiness values of LC-treated patties containing SBP + BR were significantly higher than those of LC-treated patties containing SBP + MC (Fig. 3a,d). 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 (Fig. 3a,d). These findings indicated that LC enhanced the physical properties of plant-based patties containing BR, especially in the presence of 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. It has been reported that the hardness values of plant-based patties are lower than that of animal-based patties. 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. 3a). 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. 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. This is because BR and SBP could function as mediators to enhance LC-induced protein crosslinking reactions (Figs. 3 and S2). 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. We measured the cooking loss of the different formulations of plant-based meat analog patties (Fig. 4). 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). 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

| 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 ± 1.1 | 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 ± 0.5  |
|                                  |    |     |    |    | 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 |

Table 1. Objective color measurements (L*a*b*) coordinates of plant-based and animal-based patties.
**Figure 3.** 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.

**Figure 4.** 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.
Putative enzymatic reactions that occurred in plant-based meat analog patties during grilling.

Figure S4 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 O₂, 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.

Conclusions

In this study, we developed an effective and safe browning system for BR in plant-based meat analog patties using LC + SBP. LC-treated patties containing BR and SBP showed remarkable browning after grilling and were comparable to beef patties. Additionally, LC-treatment improved the hardness of the patties containing BR. Thus SBP and LC enhanced browning as well as the 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 and would address the need for a more aesthetically appealing plant-based meat analog. Using our browning system, it would be possible to develop plant-based meat analogs that are more similar in appearance to meat patties and would also perform similarly to the meat patties during grilling. Such plant-based meat analogs would address the increasing demand for plant-based proteins.

Materials and 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 manu-
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 S1). 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.

Preparation of beef patties. Beef patties were prepared using ground beef, water, and MC. (Table S1). 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 Roskilly⁷. 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 (%) = (W1 − W2)/W1 × 100; W1 is the weight of the meat analog before grilling (g), and W2 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).

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Competing interests
The authors declare no competing interests.

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