DEVELOPMENT OF FUNCTIONAL PROTEIN POLYMERS PREPARED BY A LACCASE-CATALYZED CROSS-LINKING REACTION

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In the name of Allah, The Most Beneficent and The Most Merciful

This thesis is fully dedicated to my beloved parents and family

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CHAPTER I GENERAL INTRODUCTION

1.1 Introduction

Protein is a well-defined biopolymer showing specific biological functions. One of the most useful functionalities of proteins, especially enzymes, is its ability to become a probe molecule in diagnostic applications. The catalytic activity of enzymes for specific substrates is an important feature to develop molecular probes in diagnostic applications such as enzyme immunoassay (EIA) and enzyme-based biosensors. The use of enzymes as probes in EIA includes two common examples: enzyme-linked immunosorbent assay (ELISA) and enzyme-multiplied immunoassay test (EMIT). Alkaline phosphatases (AP), β-galactosidases, and peroxidases are the common enzymes applied as probes or reporter enzymes in ELISA [1]. Some other proteins are also applicable as probes in bioimaging applications. Nowadays, researchers are trying to improve the activity as well as functionality of proteinaceous probes by conjugating enzymes with a docking protein or fusing the enzymes to prepare a multimeric or polymeric enzyme. Conjugation of some units of protein into polymeric form is a potent strategy to improve the functionality and activity of protein probes because substrate conversion could be accelerated when different protein units are properly connected. Conjugation and polymerization of protein are thus a potent strategy to solve the functionality limitation of proteinaceous probes leading to the design of an artificial enzyme [2,3]. However, joining two or more proteins to create polymeric protein exhibiting intended functionality remains challenging for researchers [4,5]. Therefore, the preparation of polymeric proteins needs careful consideration in the design of protein units and the strategy of polymerization reaction.

Preparation of polymeric proteins is possible by engineering the proteins using some strategies such as genetic fusion or conjugation reaction of proteins [6-8]. Conjugation of proteins to form artificial protein conjugates has been developed to expand the functionalities of proteins or to create a unique and novel property of the conjugates. Genetic modification via tandem fusion of proteins is one of the widespread strategies to design artificial protein conjugates [4,9-11]. The genes encoding the protein of interests (POIs) can be expressed in one expression cassette and joined by adding some flexible linkers [10-14]. However, this strategy only works if the POIs folded appropriately. The number of protein units to be fused or conjugated is limited. Some unexpected results might occur in this strategy, such as deactivation, misfolding, and problems in protein expression and purification [4,15,16]. Moreover, the genetic fusion only works for preparing protein fusion and cannot be directly applied for preparing protein-polymer conjugates, protein-DNA hybrids, or protein-immobilized nanoparticles (NPs) [3,6,17,18]. Therefore, it is essential to choose an accurate strategy of protein conjugation reaction that can be applied for preparing different types of protein-based materials.

Post-translational modification (PTM) is another common strategy that can be applied to form protein polymers. In general, PTMs refers the covalent modification of proteins after they are biosynthesized. Proteins may undergo PTMs to become their mature forms after being synthesized by ribosomes. PTMs occur at its N- or C-terminus or on the internal side chains of amino acids [19] and modify the existing functional group of amino acids covalently. Inspired by nature, the researchers have developed some methods of PTMs that can be applied and performed through *in vitro* reaction. The PTMs can avoid the problems in protein expression of fusion proteins that commonly appeared in the preparation of tandem fusion. Compared to the tandem fusion which has a limit number of fused proteins, the PTMs enable a higher degree of conjugation such as polymerization of protein. This strategy provides some advantages such

as wide options of catalyst, the high yield of conjugates, and a simple reaction. The concept of natural PTMs also can be applied for the preparation of broad types of artificial protein conjugates, including protein-protein [20-22], protein-NPs [23,24], protein-polymer [25-27], protein-lipid [28,29] and protein-nucleotides conjugates [30] as well as specific protein immobilization on a specific solid surface (**Figure 1.1**) [31]. The PTMs have also been extended to broad methods of protein conjugates preparation such as chemical, enzymatic, chemo-enzymatic, metal and photo triggered cross-linking, and self-assembly. Therefore, the idea of PTMs are useful and effective for modification and conjugation of proteins.

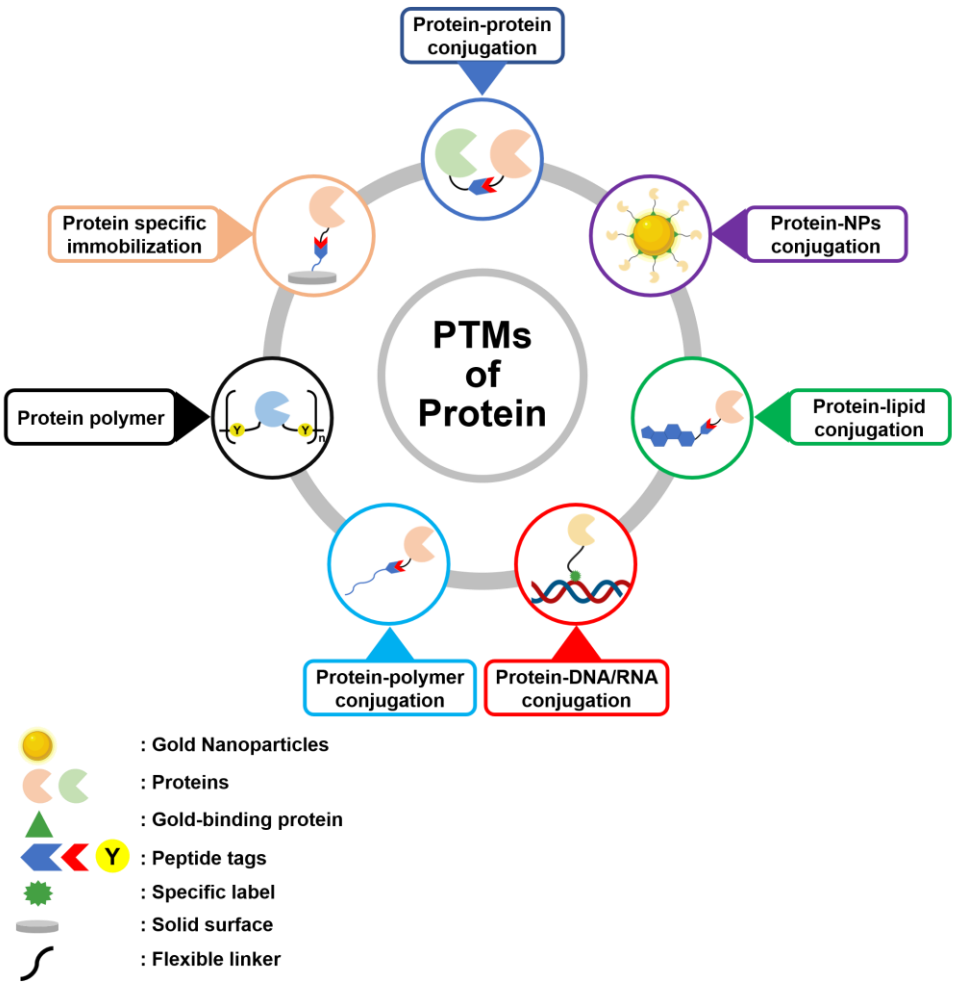


Figure 1.1 A variety of proteinaceous materials prepared through post-translational modifications (PTMs). Native and engineered proteins can be employed as protein targets or models in the PTMs.

Cross-linking reaction and self-assembly of proteins are two primary methods that commonly applied in the preparation of protein polymers. Both methods offer simplicity and specificity of the reaction. Importantly, compared to genetic modification, both methods are also applicable to avoid problems in protein expression. The cross-linking reaction of proteins is a powerful method for preparing many types of protein conjugates and protein polymers as well, which offers covalent bond formation to connect protein units of protein polymers. It needs a catalyst to perform the cross-linking reaction. While self-assembly of protein units to form protein polymers via noncovalent interactions offers some benefits such as spontaneous, controlled, designed, and self-organized reactions (**Figure 1.2**) [32]. It utilizes the noncovalent interactions to supervise the self-assembly of protein units, which offers the possibility to imitate the characteristics of native proteins. The non-covalent interactions are applied to control the assembly by facilitating the connection of protein units [33]. Compared to the covalent polymerization using chemicals or enzymes, self-assembly processes are reversible, dynamic, and directional [15,34,35]. Besides, the self-assembly method endows new non-covalent protein polymers with some attractive properties such as self-healing, stimuli-responsiveness, processability, recyclability etc. [36]. However, self-assembly needs an accurate design of the monomer unit. According to the simplicity, accessibility, and availability of catalyst of reaction provided by cross-linking reaction methods, makes it a better strategy in the preparation of protein conjugates and polymers. **Table 1.1** shows the summary of the comparison of both strategies in the preparation of protein polymer. The cross-linking reaction methods provide more advantages than genetic medication via tandem fusion methods.

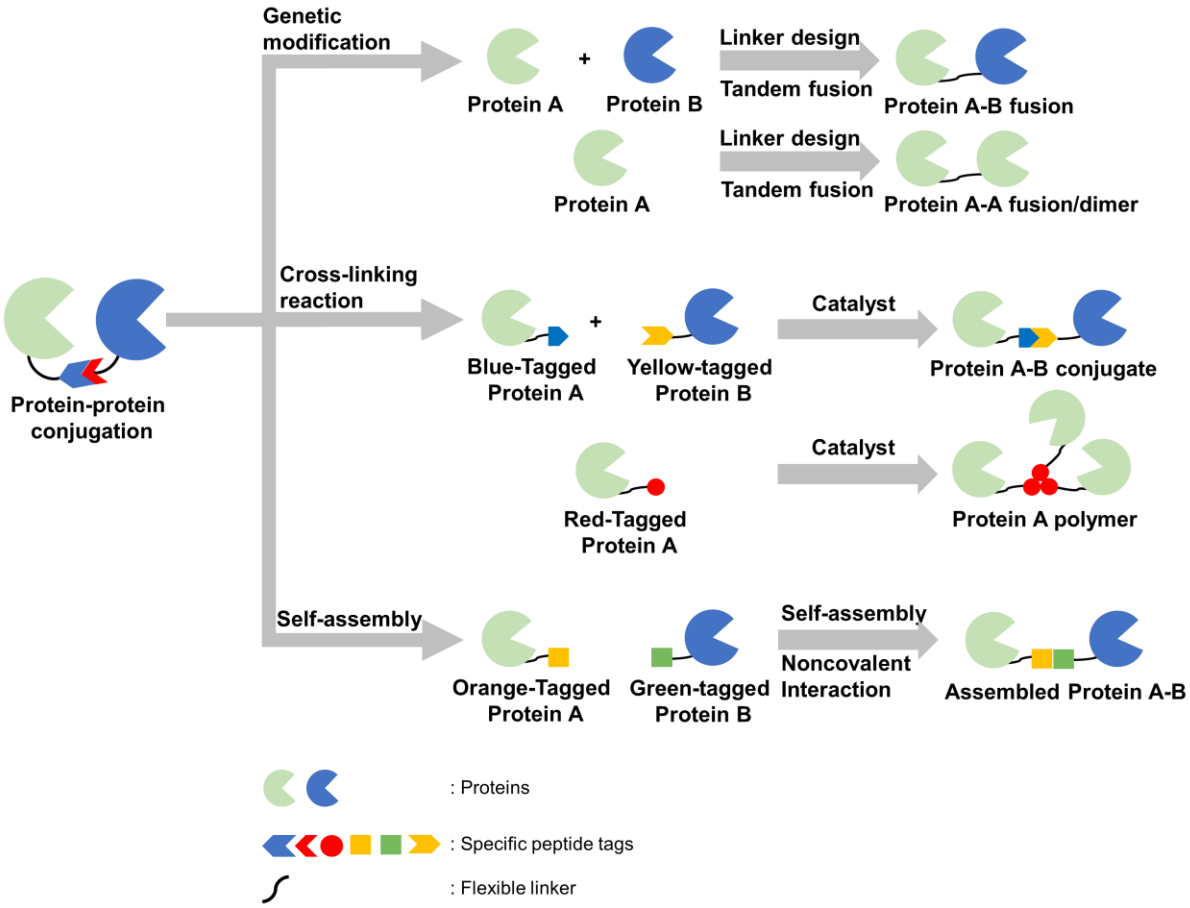


Figure 1.2 Three common strategies in the preparation of protein conjugates or protein polymers. Genetic modification through tandem fusion is a method commonly used for protein fusion preparation. Two or more genes encoding proteins were fused and engineered to one expression cassette. There are neither covalent nor noncovalent bond formation involved in this method. Another two common strategies are cross-linking and self-assembly of reaction of proteins. The protein cross-linking reaction is a method of joining protein that can be conducted through *in vivo* or *in vitro* reaction. Chemicals and enzymes commonly catalyze the formation of a covalent bond between protein units. The self-assembly methods are also applicable in the preparation of protein conjugates and polymers. It provides some options for noncovalent interactions to connect the protein units in the polymers. Both cross-linking and self-assembly reactions need genetically engineered proteins to perform the reaction. Proteins with specific peptide tags or sequences can assist the site-specific cross-linking or self-assembly reaction. The arrow in blue, red, and yellow; red circle; yellow and green rectangular indicating the peptide tags for cross-linking and self-assembly reaction.

Table 1.1 Comparison of two common strategies in the preparation of protein polymer

Genetic modification via tandem fusion	Cross-linking reaction	Self-assembly
Advantages: • Simple method, end-to-end genetic fusion [4,14,37]. • Protein conjugates or polymers expressed in the same expression cassette [11,14,38]. • The failure in protein conjugation can be avoided [12].	**Advantages:** • Wide options of protein cross-linker or catalyst [16] • Applicable for in vivo and in vitro reaction [16,38,40]. • Problems in protein expression and purification can be avoided [41]. • Able to cross-link native protein with a specific strategy and cross-linker [42-44]. • Specific sites and amino acid residues of proteins can be applied as a target of cross-linking [45-47]. • High site-specificity when enzymes used as catalyst [20,21,48].	**Advantages:** • Spontaneously [32]. • No catalysts needed [49]. • The polymerization reaction is well directed and controlled [15,34,45].
Disadvantages: • Protein misfolding is occurred in some cases [14] • The number of protein units to be fused is limited [12,13]. • The order of proteins in the polymer is essential [50]. • Problems in protein expression and purification are still challenging [12]. • The partners of fusion proteins should be physico-chemically compatible [5,8].	**Disadvantages:** • High temperature of reaction when chemicals used as catalyst [51]. • Cross-linking product cannot be controlled due to a rapid reaction [52].	**Disadvantages:** • Time-consuming process [49]. • Protein units connected through noncovalent interactions [32]. • The design of the monomer unit must be accurate for a successful assembly [32].

The advantages offered by the cross-linking reaction make them the most recommended and useful in the preparation of protein polymers. However, due to its wide option of catalysts, the strategy in the preparation of protein models or target as well as the catalyst selection needs carefully considerations. Thus, a proper strategy and catalyst are the two most important factors for a successful cross-linking reaction for protein polymers preparation.

1.2 Cross-Linking Reaction of Proteins

The cross-linking reaction of protein is described as the reaction of joining two or more proteins through covalent bonds. It can create newly assembled macromolecules that have functionalities and physicochemical characteristics that are originated from the individual protein units [16]. However, in some cases, the characteristics of cross-linked proteins are different from the single unit of protein depending on the structure of the polymeric products. Notably, the protein cross-linking reaction to form a large biomolecule has a substantial impact on the characteristics of proteins monomers due to the multivalency effect of the protein units in the resulting polymeric structure of proteins [2,15]. This effect may lead to the different functionality and activity of the protein polymer with its monomers.

The new covalent bonds between protein monomers comprising of a protein polymer can be formed by the catalyzation of chemicals [53-55], enzymes [22,45,56,57], chemoenzymatic [58,59], metals [2,60] and lights [61]. While fusion proteins prepared by genetic fusion did not involve the covalent bond formation to connect the protein units. The crucial step is designing a peptide linker to connect the protein units (**Figure 1.2**). The sequence of amino acids, flexibility, and properties of the linker can affect the preparation of protein fusion [4,11-13]. Moreover, compared to tandem fusion for protein conjugation, which only can be done through in vivo reaction, the cross-linking reaction can be conducted by *in vivo* and *in vitro* reaction. The cross-linking reaction also can target some natural amino acids (NAA) for modification sites. However, not all amino acids are accessible due to the location of amino acids on the surface of proteins and their abundance as well. Some natural amino acids: serine, glutamine, glutamic acid, glycine, lysine, and aspartic acid, were located on the surface of proteins (**Table 1.2**). They also have a high value of average surface accessibility (ASA), which means that they can be recognized and reacted with the catalyst in a cross-linking reaction. Another amino acid also can be targeted as the modification site; however, further modifications are needed

[62]. Some researchers also applied non-natural amino acids (NNAA) for cross-linking reaction or specific labeling of proteins. However, engineering a protein with NNAA needs careful consideration, since most enzymes cannot recognize it. The further chemical reaction is needed to modify or to cross-link NNAA of a protein. Thus protein cross-linking through existing NAA is preferable for most researchers.

Table 1.2 The amino acids used as a target in protein cross-linking reaction: amino acid, location, functionality, natural abundance, and their average surface accessibility.

Amino acid	Location[a]	Functionality[b]	Natural abundance	ASA[b]
Cysteine	C	Thiol	1.36	0.268
Isoleucine	C	Aliphatic	5.97	0.273
Tryptophan	C	Indole	1.08	0.279
Phenylalanine	C	Benzyl	3.86	0.290
Valine	C	Aliphatic	6.87	0.306
Tyrosine	C	Phenol	2.92	0.319
Leucine	C	Aliphatic	9.66	0.321
Methionine	C	Thioether	2.42	0.364
Alanine	C	Aliphatic	8.26	0.405
Histidine	M	Imidazole	2.27	0.425
Threonine	M	Hydroxy	5.34	0.480
Proline	M	Aliphatic	4.69	0.502
Arginine	M	Guanidine	5.53	0.539
Asparagine	M	Carboxamide	4.06	0.568
Serine	S	Hydroxy	6.55	0.568
Glutamine	S	Carboxamide	3.93	0.573
Glutamic Acid	S	Carboxylic acid	6.75	0.586
Glycine	S	-	7.08	0.588
Lysine	S	Primary amine	5.85	0.607
Aspartic Acid	S	Carboxylic acid	5.46	0.615

[a]Location of the amino acids in the core (C), intermediate (M) and surface (S)
[b]Average surface accessibility

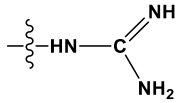
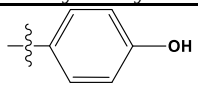
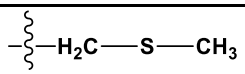
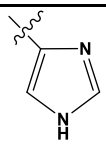
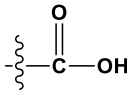
1.2.1 Chemical Cross-Linking Reaction of Proteins

The application of enzymes as a catalyst for synthetic organic chemistry has increased significantly for the past three decades. Most of the nonspecific approaches of the chemical cross-linking reaction of proteins are to observe the effect of cross-linking reaction on the protein stability, designing novel enzymes or proteins with high selectivity, activity, and stability. The cross-linking reaction can be accomplished by a massive variety of chemicals as cross-linkers with distinct properties and aim for different reactive groups on the proteins [56]. It is ranging from organic to inorganic chemical reagents. Some commercial products of protein cross-linkers are now also available and provide a high yield of protein conjugates. However, the use of chemicals as a catalyst in the protein cross-linking reaction is not site-specific, fairly high temperature of the reaction, and organic solvents that requires to solubilize chemicals can cause protein deactivation and denaturation in most cases. Therefore, the selection of strategy and chemical reagents is a crucial step in the preparation of chemical cross-linking of proteins. **Table 1.3** shows the reactive groups that commonly become the target in chemical cross-linking as well as protein modifications. The reactive groups that commonly used are —NH2, —COOH, —SH, —OH, and aryl—OH. Even though other amino acids have a lower value of ASA, it is still possible to become a target of a cross-linking reaction. Some reactive groups such as thioether, imidazole, and guanidino were rarely used and reported due to the lack option of chemicals. Small chemical molecules can avoid the steric hindrance of the proteins and react with the hidden amino acids.

Most of the chemical reactions that involve in protein cross-linking reaction are substitution, oxidation, and an addition reaction. In consequence, the substitution and addition reaction involve the leaving groups, which is varied based on the chemicals used. Moreover, activation of nucleophiles also needed and essential to initiate the reaction. Such activation requires some catalyst and specific solvent and temperature. It makes the chemical cross-

linking takes more time than enzymatic cross-linking that can proceed within minutes. In the next session, the reactive groups that commonly used in the chemical cross-linking of proteins are briefly explained.

Table 1.3 List of chemical agents that used as a catalyst in cross-linking reaction.

Reactive group	Amino acid	Chemical agents
 Primary amine	N-Terminus ε-amino group of Lysine	Triazolecarbaldehyde [63], Glutaraldehyde [51], Formaldehyde [64], Isothiocyanate [65], Sulfonyl chloride [65], Disuccinimidyl suberate [66], Succinimidyl acetate [67], N-hydroxy-sulfosuccinimide (Sulfo-NHS) [68]
 Guanidino group	Arginine	α-dicarbonyl compounds through Maillard reaction [69-71]
 Thiol	Cysteine	Maleimide, haloacetamides [53], vinyl sulfone [72]
 Hydroxyl	Serine Threonine	Hydrazide [73]
 Phenol	Tyrosine	Metalloporphyrins [55], Palladium porphyrins [61], thioesters of glutathione (GS-thioesters) [74], formaldehyde through Mannich-Type reaction [75]
 Thioether	Methionine	Iodoacetic acid [76]
 Imidazole	Histidine	4-hydroxynon-2-enal (HNE) [77]
 Carboxylic acid	C-Terminus Aspartic acid Glutamic acid	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) [68]

1.2.1.1 Primary amines

The conventional methodologies for the cross-linking reaction of protein are selected by taking advantage of the functional groups reactivity of the said chain of amino acids [78]. A typical

cross-linking reaction based on NAAs is commonly simple and easy to conduct. It does not require specialized techniques. The primary amines of proteins can provide reactivity by just becoming reactive nucleophiles. Lysine exists in most of proteins and is commonly on the protein surface. Lysine comes up with approximately 10% of the overall amino acid sequence of a protein [31]. It has good stability and very reactive against electrophilic agents without any activation steps. Therefore, lysines are the most used for the nonspecific covalent cross-linking reaction.

N-hydroxysuccinimide (NHS) esters are the most used chemicals to react with primary amine groups to form a stable peptide bond. This method has been reported by Wang Group, who immobilized a protein called ricin. Ricin is a protein that inhibits the synthesis of protein. They used NHS ester to cross-link ricin on a gold surface. Ricin has nine exposed lysine residues and can react with NHS ester [68]. The reaction of ricin with NHS ester also can be applied to analyze the side of the ricin structure. The AFM analysis observed that single ricin molecules have different structures because of the covalent coupling of the lysines. Imidoesters also applicable to cross-link the protein through primary amines or lysines (**Figure 1.3**A). The amine group of a protein could attack the carbonyl groups of imidoesters to form a stable intermediate. Another carbonyl can be attacked by another protein to form protein conjugates. Therefore, the amine–amine coupling is useful and not involving the prior modification of proteins.

1.2.1.2 Thiols

The thiol is a stronger nucleophilic than a primary amine (at pH below 9.0 lysines are protonated). Cysteine could become a promising tag for protein cross-linking reactions because multiple contacts can be avoided. If the proteins do not have free cysteines, it could be inserted by a site-directed mutagenesis method. A typical chemical cross-linker to cross-link or

coupling the protein via thiols groups is that of maleimides (**Figure 1.3**B) [53]. It reacts with cysteines stoichiometrically with high efficiency and specificity. The reduced protein is commonly used in maleimide-cysteine coupling reactions. Dithiothreitol (DTT) is commonly applied to prevent the disulfide bridges formation and inactivate the cysteines. The removal of those reducing agents is mandatory for avoiding the rivalry among the target thiol and thiol of the reducing agent. The cross-linking reaction must be conducted instantly right after DTT removed to prevent reoxidation of the thiols. The maleimide-modified proteins can react with other thiols groups and connect the proteins covalently. Ménard and co-workers reported a site-specific reaction of a cytochrome P450 enzyme from human through a maleimide-cysteine coupling [53]. The cytochrome P450 enzyme was cross-linked on agarose beads and silica microspheres that functionalized with maleimide. They mutated other cysteines, allowing the cytochrome P450 enzyme to cross-link on the solid supports site-specifically. Interestingly, cytochrome P450 enzyme was found to still be active after the cross-linking reaction.

1.2.1.3 Phenols

The existence of tyrosines on protein surfaces is moderately rare. It has a low value of ASA and commonly located in the core part of proteins. However, it is possible to introduce it to the protein surface genetically, without changing the redox sensitivity or overall charge state. Tyrosines are frequently overrepresented close to the protein active sites. Therefore, aiming tyrosine residues for cross-linking reaction requires thorough attention.

Francis and co-workers reported an interesting reaction of protein cross-linking using tyrosine. They applied the Mannich-type reaction for selective bioconjugation of protein through tyrosine residues (**Figure 1.3**C). Interestingly, they finished the reaction in mild conditions of reaction (pH 6.5 at RT to 37°C). However, reaction time still becomes a problem.

It took 18 hours to complete the reaction [75]. This reaction is going to substitute the common reaction, which requires significant heating and concentrated formaldehyde.

Kim and co-workers reported another approach in the protein cross-linking through tyrosine residues in 2011. They used a special chemical Pd(II)-5,10,15,20-tetra-(methylpyridinium)porphyrin (Pd(II)TMPyP). An electron acceptor was also added to the reaction to mediate the formation of free tyrosil radicals from tyrosine residues of proteins. The formation of radical porphyrin cation was led by the transfer of electron from the palladium porphyrins to ammonium persulfate (APS) as an electron acceptor. This reaction is swift; only 5 seconds required to connect the protein through cross-linking reaction. However, this reaction is the requirement of an electron acceptor, which may oxidize the protein target or some cellular components if we used membrane proteins as the target [61].

1.2.1.4 Carboxyl group

The protein cross-linking reaction utilizing the carboxylic acid reactive group is interesting and promising since the aspartic and glutamic acid found a main portion of the surface exposed amino acids. The carboxyl group can react with amines using the common coupling reagents, which also used for peptide synthesis. The EDC or *N*,*N*-dicyclohexyl carbodiimide (DCC) is commonly used to activate the carboxyl group and resulting in the peptide bond formation of proteins (**Figure 1.3**D) [68].

1.2.1.5 Other reactive groups

Tryptophan is another attractive target of amino acid for the modification of proteins. The use of rhodium carbenoid reagents has been reported to modify protein through the indole side-group and resulted in the formation of alkylated indoles. However, this reaction requires low pH conditions (pH 1.5−3.5), which could have unfavorable effects on the proteins [75].

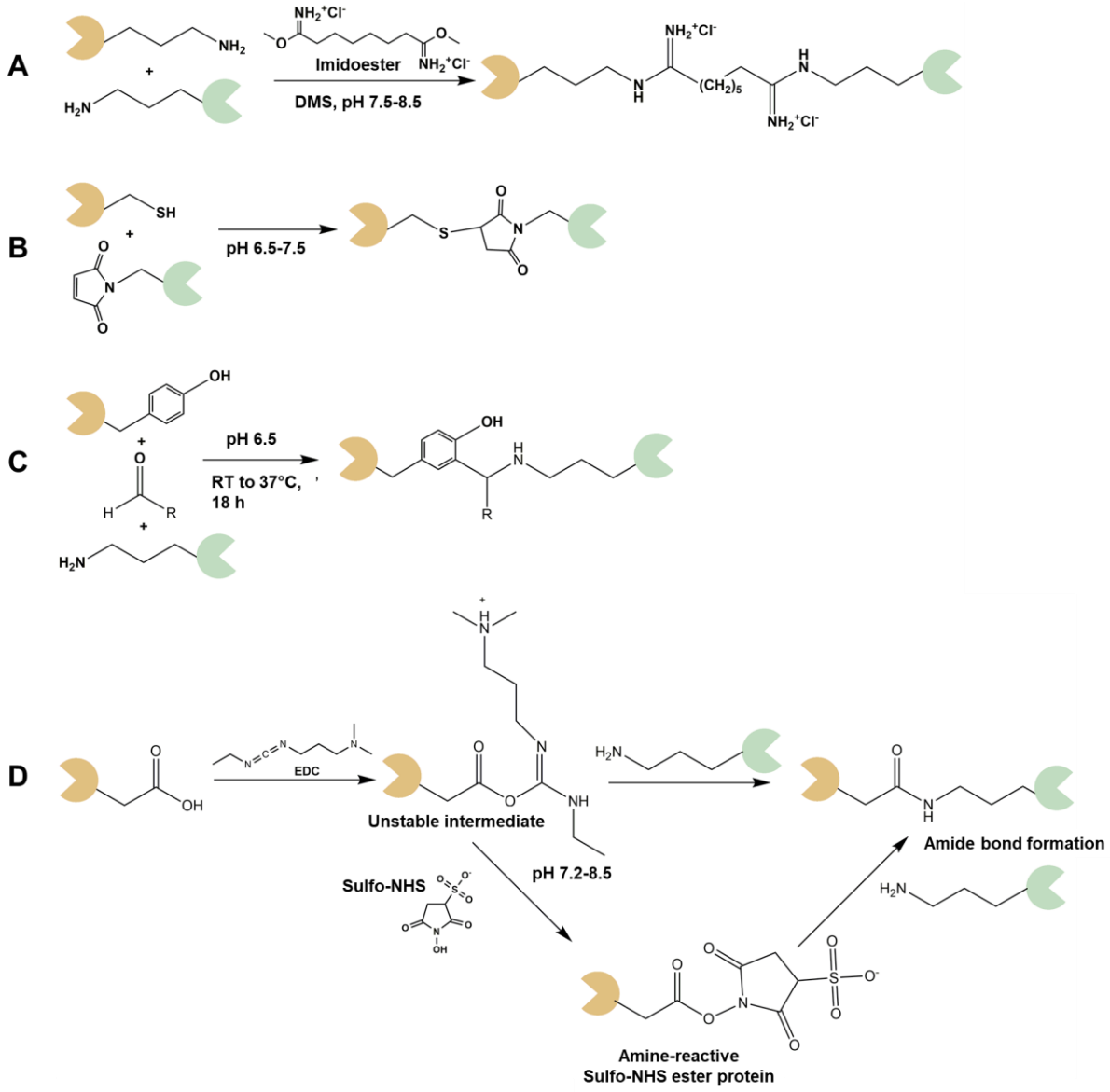


Figure 1.3 Schematic reaction of some common chemical cross-linking reactions targeting some reactive groups. (A) the reaction of imidoester with primary amine groups in DMS. (B) the reaction of the thiol group of protein with a maleimide-modified protein in a mild condition reaction. (C) the reaction of tyrosine and lysine in the presence of formaldehyde (Mannich reaction) proposed by Francis and co-worker. This reaction conducted in mild conditions of reaction and takes 18 hours. (D) reaction if the carboxyl group with EDC. It produces a reactive intermediate product which can react further with sulfo-NHS or with a primary amine group of lysine, resulting in a newly formed amide bond.

The site-specificity is still a significant concern in the chemical cross-linking reaction of proteins—lack of specificity resulting in the formation of heterogeneous products. The selective modification of a protein by using chemicals is possible and to be successful for reactions that used engineered proteins, for example, the engineered protein with site-directed

mutagenesis or specifically labeled-proteins. Chemical cross-linking reaction of genetically engineered proteins is a better strategy than the use of native proteins as a target of proteins. One of the common approaches is the alkylation reaction of tyrosine or cysteine-modified proteins due to the low abundance of cysteines and strong nucleophilicity of the thiolate anion. However, once again, the site-specificity and effectivity of the chemical cross-linking reaction of proteins will be determined by the catalyst. Thus, the selection of a catalyst is a crucial issue in the chemical cross-linking of proteins.

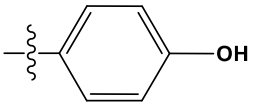
Enzymes are known as better options to substitute chemical agents in most of chemical reactions including the cross-linking reaction of peptides, proteins, and chemicals. The site-specific reaction is the most interesting feature of enzymes in the cross-linking reaction of proteins. The enzymes will only recognize and react with a specific residue or sequence of the proteins, which is very useful in the design of the protein targets. Although the enzyme options for the cross-linking reaction of proteins are not as much as chemical agents, however, it is not a limitation to applying it as a catalyst in the cross-linking reaction of proteins.

1.2.2 Enzymatic Cross-Linking Reaction of Proteins

Enzymatic cross-linking reaction for protein modifications has been attracting researchers due to its high site-specificity of reaction. The amino acid targets in the enzymatic cross-linking reaction of proteins are not as much as a chemical cross-linking reaction. It is reasonable since a large molecule and complex structure of enzymes cannot recognize and react with hidden amino acids. Enzymes would be a powerful catalyst for the cross-linking reaction of proteins if it reacted with genetically modified target proteins, which harboring specific peptide or sequence tags [7,16,18,21]. Reactive and specific peptides or sequence tags are essential for enzymatic cross-linking. For example, the protein cross-linking reaction catalyzed by horseradish peroxidase (HRP) [21,57,79-81], laccase [48,52,82-84], lysyl oxidase [85],

transglutaminase [86-88], lipoic acid ligase [47], biotin ligase [89], bacterial sortase [45], and phosphopantetheinyl transferase (PFTase) [58] require additional peptide or sequence tags for successful site-specific cross-linking reaction. However, the protein ubiquitination, which involves enzymatic cascade reaction, has not been considered for cross-linking applications due to its complexity of the enzymes and the ATP dependence reaction. The cross-linking reactions can be distinguished based on the reaction mechanism, i.e., (1) covalent bond formation through reactive intermediates or species catalyzed by oxidoreductases, and (2) direct covalent bonding formation catalyzed by transferases, hydrolases, and peptidase via proteinyl–enzyme–thioester intermediates.

Table 1.4 List of enzymes that used as catalyst in cross-linking reaction.

Recognition site or sequence	Amino acid	Enzymes
 Primary amine	N-Terminus ε-amino group of Lysine	Transglutaminase [86-88], Lysil oxidase [85]
 Thiol	Cysteine	Laccase [90], peroxidase [91], protein farnesyltransferase (PFTase) [92]
 Thiol	Tyrosine	Peroxidases [21,57,79-81], tyrosinases [43,93,94], laccases [48,52,82-84]
CXPXR	Cysteine	formylglycine-generating enzyme (FGE, EC 1.8.3) [95]
CAAX	Cysteine	Protein Farnesyltransferase (PFTase, EC 2.5.1.58) [92]
D**S**LEFIASKLA	Serine	phosphopantetheinyl transferase (PPTase, EC 2.7.8.7) [58]
T/SXXX**G**	N-terminal glycine	N-myristoyltransferase (NMT, EC 2.3.1.97) [96]
GFEID**K**VWYDLDA	Lysine	Lipoate−protein ligase A (LplA, EC 6.3.1.20) [47]
LPXT**G**	Glycine	Sortase A (SrtA, EC 3.4.22.70) [45]
GLNDIEAQ**K**IEWHE	Lysine	Biotin−[acetyl-CoA-carboxylase] ligase (BirA, EC 6.3.4.15) [89]

1.2.2.1 Transferases

Transferases catalyze a reversible transfer of chemical groups from a molecule to other molecules. Transpeptidation reaction is one of the examples which has been investigated to connect two different proteins through two peptide sequences. Fructosyltransferase and transglutaminase are some of the typical industrial transferases. They are commonly used in food processing industry: another transferase, carbohydrate transferases, also applicable to cross-link proteins with glycans.

Transglutaminases (TGs, EC 2.3.2.13) is an acyltransferase that catalyzes the transfer reaction of a γ-carboxyamine group of glutamines to the ε-amine group of lysines (**Figure 1.4**A). Some of TGs are calcium-dependent enzymes. TGs catalyzes the deamination of glutamine by water-assisted hydrolysis if there are no amine substrates in the mixture. Some transferases require a peptide sequence for their activity in PTMs. One of example is protein farnesyltransferase (PFTase) (EC 2.5.1.58), which catalyzes the transfer of farnesyl diphosphate analogs to protein substrates on a cysteine residue of CAAX recognition sequence from C-terminus (**Figure 1.4**B) [92]. Another example is phosphopantetheinyl transferase (PPTase) (EC 2.7.8.7), which catalyzes the transfer coenzyme A to the protein for the further installation of the 4′-phosphopantetheine to a serine residue on the POI. The transfer of 4′-phosphopantetheine is releasing ADP to the conserved DSLEFIASKLA motif (**Figure 1.4**C) [58].

N-myristoyl transferase (NMT) (EC 2.3.1.97) is an enzyme that can catalyze the addition of myristic acid analogs to proteins through an N-terminal glycine residue transformation of proteins through a T/SXXXG signaling motif (**Figure 1.4**D) [96]. Lipoate−protein ligase A (LplA) (EC 6.3.1.20) is an enzyme that catalyzes the acylation of lysine residues of the GFEIDKVWYDLDA tag. Interestingly, azide acid and coumarin analogs are also recognized by LplA, given a suitable length of the methylene linker (**Figure 1.4**E) [47].

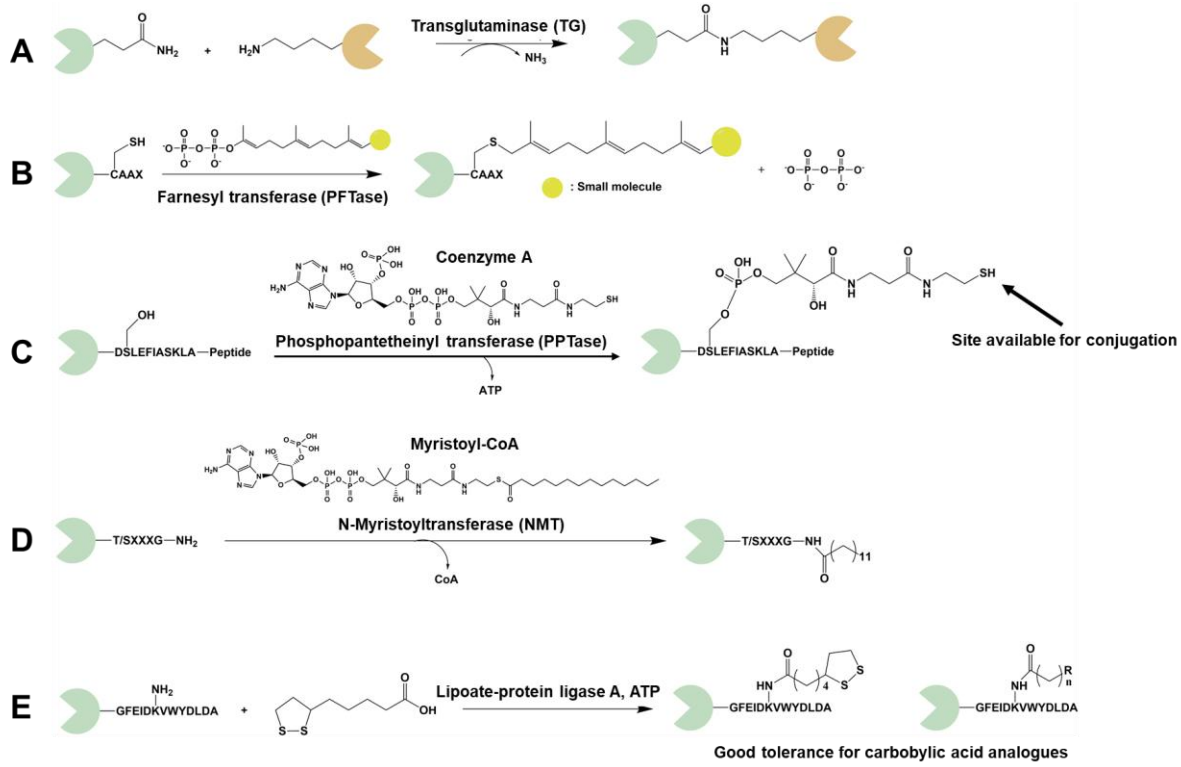


Figure 1.4 Schematic illustration of the enzymatic cross-linking reaction catalyzed by transferases. (A) transglutaminases (TGs) catalyze the transfer reaction of a γ-carboxyamine group of glutamines to the ε-amine group of lysines to form cross-linked proteins. (B) The PFTase catalyzes the transfer of farnesyl diphosphate analogs to protein substrates on a cysteine residue of the CAAX recognition sequence from C-terminus. (C) PPTases which catalyze the transfer coenzyme A to the protein for the further installation of the 4′-phosphopantetheine to a serine residue on the POI. The transfer of 4′-phosphopantetheine is releasing ADP to the conserved DSLEFIASKLA motif. (D) is an enzyme that can catalyze the addition of myristic acid analogues to proteins through a T/SXXXG signaling motif. (E) Lipoate−protein ligase A that catalyzes the acylation of lysine residues of the GFEIDKVWYDLDA tag.

1.2.2.2 Hydrolases

The hydrolases have been used in various industries, including fine chemical production, leather, food, textiles, and animal nutrition. Most of the hydrolases are produced on a large scale, delivering rapid access to the researchers. Hydrolases catalyze hydrolysis reaction of specific bonds, such as hydrolysis of C−N bonds of a peptide by the peptidases. Hydrolases cover approximately 75% of commercial hydrolytic enzyme sales. Hydrolases mostly applied for cutting existing peptide bonds rather than forming them.

A common hydrolase that applied for protein cross-linking experiments is sortase. Sortases catalyze the cross-linking reaction of proteins that signaling a specific sequence, LPXTG. It is a calcium-dependent enzyme. Sortase A (SrtA) (EC 3.4.22.70) is the best example of sortases which isolated from *Staphylococcus aureus* [45]. It has been used widely by researchers for modifying proteins. Although SrtA is more appropriately defined as a transpeptidase. The cysteine residues on the active site of SrtA cleaves the amide bond of Thr−Gly of LPXTG of the target protein, to form an intermediate called protein-enzyme-thioester. Next, the amine as a nucleophile attacks the intermediate of enzyme-acyl-protein to form a peptide bond. The amine group of the oligoglycine then catalyzes the discharge of the protein from SrtA. This mechanism involving polyglycine inspired the researchers to design accurate substrates for SrtA-catalyzed cross-linking reaction.

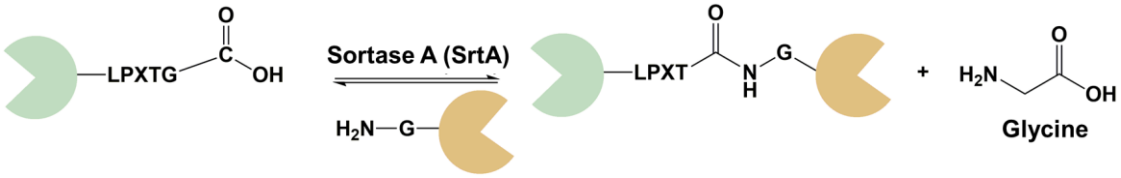


Figure 1.5 A common hydrolase that applied for protein cross-linking experiments is sortase. Sortases catalyze the cross-linking reaction of proteins that signaling a specific sequence, LPXTG

1.2.2.3 Ligases

Ligases are enzymes that catalyze the bond formation between two molecules. Because they require ATP or expensive cofactor rejuvenation for their enzymatic activity, it makes them rarely employed in industrial processes. The biotin−ligase (BirA) EC 6.3.4.15) from *Escherichia coli* is a ligase cofactor that playing an essential role in the biotin covalent attachment to a lysine within a long peptide sequence tag GLNDIEAQKIEWHE [89]. It is most

studied by researchers due to its functionality to recognize biotin analogs, which enables the biorthogonal fusion of chemical derivatives into the peptide backbone of the POI.

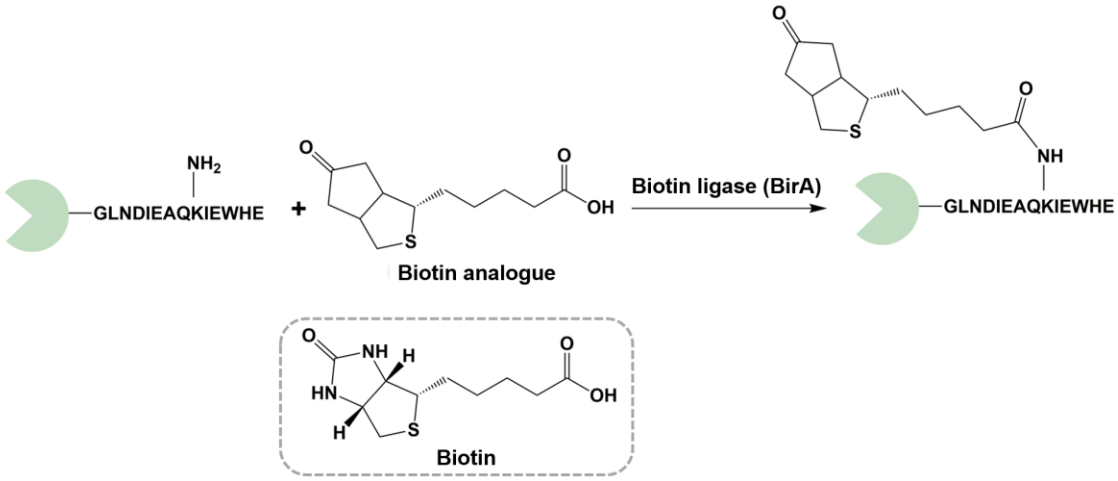


Figure 1.6 The biotin−ligase is a cofactor ligase that playing an essential role in the biotin covalent attachment to a lysine within a long peptide sequence tag GLNDIEAQKIEWHE.

The three major groups of enzymes above are beneficial in the preparation of protein conjugates and protein labeling. However, they cannot be employed in the polymerization reaction of proteins due to their limitation in the reaction. They cannot conduct oxidation reactions on the substrates to form a reactive free radical, which is required for non-enzymatic polymerization reaction of proteins. There are some enzymes in oxidoreductases family which can oxidize substrate and form free radicals, which initiates the cross-linking reaction between proteins. The free radicals coupling occurs non-enzymatically without the formation of tripartite complexes like other enzymes. Therefore, oxidoreductases could become good options to be employed as a catalyst in the polymerization reaction of proteins.

1.2.2.4 Oxidoreductases

Oxidoreductases (EC1) are well known as enzymes that catalyze reduction and oxidation (redox) reactions. The members are tyrosinases, laccases, peroxidases, catalases, and glucose oxidases. Most of them are produced on an industrial scale. There are also some commercially

available oxidative enzymes for protein functionalization, such as lysyl oxidase, sulfhydryl oxidase, and formylglycine-generating enzyme (FGE).

Tyrosinases (EC 1.14.18.1) catalyze the four-electron oxidation of tyrosine residues to o-quinones. It also oxygen-dependent enzymes. The formed quinones can easily cross-link with cysteinyl, lysyl, or tyrosyl residues (**Figure 1.5**A) [97]. Tyrosinase-catalyzed cross-linking reaction of proteins is an appealing transformation for the pharmaceutical or food company because they produce water as a sole byproduct.

Lysyl oxidases (EC 1.4.3.13) are amine oxidases. It also copper-dependent enzymes, as same as other oxidoreductases. It is found in the cross-linking reaction of elastin chains and collagen, followed by a simultaneous reduction of O_2 to H_2O_2. The lysine side chain oxidized by lysil oxidase to formaldehyde, which applicable to produce cross-linked products through two different pathways (**Figure 1.7**B). In the first pathway, the aldehyde (from oxidation reaction of amine) can react with other aldehyde groups of protein to form an aldol product. The second pathway, a Schiff base formation through the reaction with amine of the second lysine with the aldehyde [85].

Sulfhydryl oxidases (EC 1.8.3.2) are another oxidoreductase, which provides site-specific oxidation reaction [91]. It is a flavin-dependent oxidase. It catalyzes the oxidation of cysteine residues of proteins to form disulfide bonds by using molecular oxygen and produced H_2O_2 as a byproduct. Sulfhydryl oxidases are interesting biocatalysts due to its activity to catalyze intra- and intermolecular disulfide bond formation of proteins.

Peroxidases (EC 1.11.1.7) also form free radicals from proteins that can cross-link the proteins. It typically reacts not only with tyrosine but also with lysine and cysteine and resulting in heterogeneous products. However, tyrosine residues are preferable to react with peroxidases than other residues (**Figure 1.7**C). Further protein modifications are needed to proceed with the site-specific cross-linking of protein catalyzed by peroxidases. For example, introducing

tyrosine residues exposed to the surface of the proteins [22,79,98]. The significant problem of peroxidases in the H_2O_2 which is required in the reaction as an electron acceptor. The extra amount of H_2O_2 is harmful and unacceptable in food and pharmaceutical products.

Laccases (EC 1.10.3.2) are multi-copper enzymes that primarily employed textile and pulp industries as a catalyst for the degradation of aromatic polymers. It catalyzes the oxidation reaction of phenolic compounds using molecular oxygen results in the formation of free radicals and water (**Figure 1.7**C). The free radicals can undertake radical reactions, such as polymerization, hydration, disproportionation, and fragmentation. Laccase-catalyzed cross-linking reaction is commonly producing heterogeneous products, guiding to the dityrosine, isodityrosine formation, or a higher degree of tyrosine coupling, and disulfide intermolecular bonds of proteins. Laccases become an exciting option for the food industry because the sole by-product is water. Thus, recent reports on laccase-mediated modification of food proteins show a promising strategy and results which will expand the utility of laccases in PTMs.

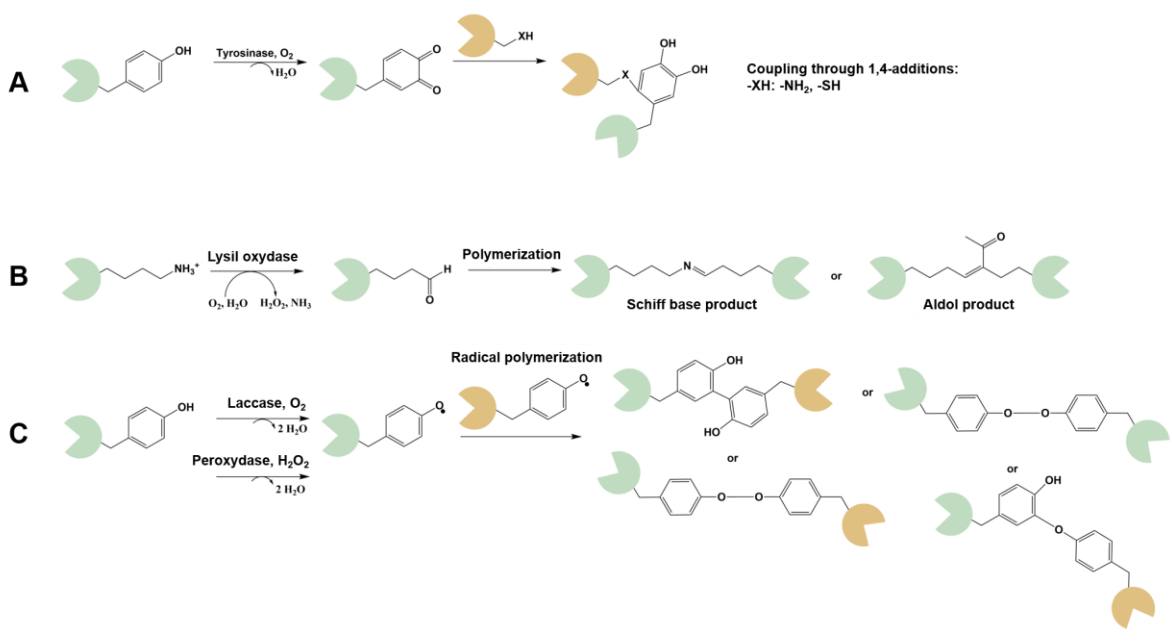


Figure 1.7 Schematic illustration of the enzymatic cross-linking reaction catalyzed by oxidoreductases. (A) The oxidation reaction of phenolic moieties of tyrosine by tyrosinase to form a quinone and further protein cross-linking with another protein. (B) Lysil oxidase oxidizes the protonated primary amine group to form an aldehyde group, which followed by the polymerization reaction of protein through two pathways. (C) laccases and peroxidases cross-link the protein through radical polymerization.

Peroxidases and laccases are a promising catalyst for the polymerization reaction of proteins due to their activity to catalyze the oxidation reaction of the substrate to form very reactive free radicals. They also commercially available, which makes them easier to be applied in the various polymerization reaction of proteins. Peroxidases have been applied in many polymerization reactions of proteins than laccases. Although they have the same mechanism of the reaction, however, one of the major differences is the source of oxygen for the oxidation reaction. Laccases use O_2 as its terminal oxidant, while peroxidases use H_2O_2. The presence and excessive concentration of H_2O_2 are harmful to most protein and unacceptable in food and pharmaceutical products. Therefore, laccases can become an alternative and replace the use of peroxidases in the polymerization reaction of proteins.

1.2.3 Effect of structure of model proteins on the reactivity of laccase

Laccases can oxidize some amino acid residues, including tyrosine, cysteine, and tryptophan. According to Table 1.1, those amino acids are in the core domain of most proteins. Additionally, their ASA value is extremely low, which is mean that it is hard to be recognized and reacted with a massive molecule like enzymes. Modification of protein is required to perform the cross-linking reaction catalyzed by laccase. Mattinen and co-workers investigated the effect of the structure of the protein model, coactosin, on the laccase-catalyzed cross-linking reaction. There are four tyrosines, two cysteines, and two tryptophan, on the structure of coactosin. However, only tyrosine residue at the position 137 of amino acid sequence or Y137 is exposed to the surface of coactosin (**Figure 1.8**A) [90]. Tyrosine is preferable to oxidize by laccase than other amino acids. It is reasonable since the oxygen consumed less oxygen than other amino acids, which makes the reaction faster and efficient (**Figure 1.8**B). However, coactosin also perform better in oxygen consumption experiment than cysteine and tryptophan due to the presence of Y137. The presence of Y137 was also responsible for the formation of dimer, trimer, and

tetramer of coactosin (**Figure 1.8**C). The *Trametes hirsuta* laccase (ThL) recognized and oxidized Y137 to form oligomers of coactosin. Interestingly, the absence of Y137 in truncated coactosin resulted in an unreacted monomer of coactosin (**Figure 1.8**D). It suggests that ThL reacted with coactosin site-specifically only with Y137. There are another three tyrosine residues on the coactosin; however, ThL did not recognize it as its substrate. The position of tyrosine residues and steric hindrance could become the reasons that caused this finding. Another problem is that the activity of ThL in the polymerization reaction of coactosin was prolonged. Regarding the oxygen consumption results, it takes more than 2 hours to oxidize the coactosin. **Figure 1.8**C shows that the dimers of coactosin were formed after 2 hours of reaction. While trimer and tetramer were formed after 3 hours of reaction. It suggests that the structure of coactosin also affected its reactivity against ThL. These findings were clearly showing the importance of the protein structure and available tyrosine for a successful laccase-catalyzed polymerization reaction of proteins. The proper structure of the protein model could improve the reactivity of protein against laccase, which may lead to a rapid polymerization reaction of proteins.

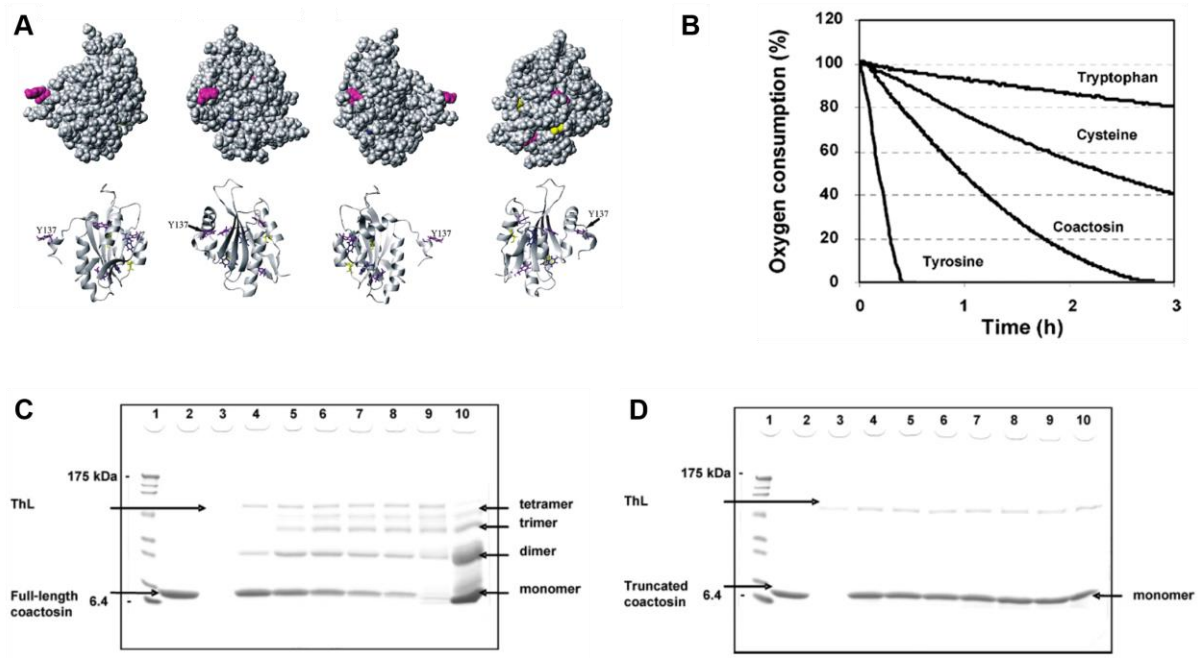


Figure 1.8 (A) the 3-D structures of coactosin (full-length); tyrosine residues (four) are shown in purple, cysteine residue (two) in yellow, and tryptophans (two) in blue. (B) time course experiment of oxygen consumption in *Trametes hirsuta* laccase (ThL)-catalyzed reactions with tyrosine, cysteine, and tryptophan and coactosin. (C) results of SDS-PAGE analysis of cross-linking reaction coactosin catalyzed by ThL. In lanes 4−9: incubation time (2, 3, 4, 5, 6, and 24 h); and lane 10: sample from the oxygen consumption measurement. (D) results of SDS-PAGE analysis of cross-linking reaction of truncated coactosin. In lanes 4−10: incubation time (1, 2, 3, 4, 5, 6, and 24 h). Reproduced from ref.90 Copyright with permission from the American Chemical Society (ACS).

1.2.4 Phenolic acids-assisted cross-linking reaction of proteins

Most of the reports on laccase-mediated protein cross-linking reaction employed some chemical mediators to assist reaction. The mediators are useful to cross-link of native or unmodified proteins. The mediators are commonly phenolic acids, such as ferulic acid [82,99-101], *p*-coumaric acid [43,101,102], caffeic acid [103], vanillic acid [42,101,104,105], and hydrolyzed oat spelt xylan (hOSX) [82]. Commonly it is added to the reaction mixture containing laccase and protein models. However, Hairan Ma and co-workers proposed a different approach in the use of a mediator. They employed a phenolic mediator, vanillic acid (VA), to modify the whey protein isolate (WPI) through a chemical reaction using NHS-ester,

to form a VA-modified WPI. The VA was attached to the lysine residues of WPI (**Figure 1.9**A). To confirm the attachment of VA to the MPI, they analyzed the MPI using a MALDI-TOF mass spectrum (**Figure 1.9**B). The changes in mass spectra confirmed the attachment of VA to MPI. The VA-MPI was then employed as a protein target in cross-linking reaction catalyzed by laccases. The reaction of unmodified MPI with laccase resulted in no oligomers and polymers formed. The MPI was remaining intact. It suggests that the laccase did not recognize MPI as a substrate (**Figure 1.9**C and F). While the additional free VA to the mixture also cannot cross-link the MPI. Although they increase the concentration of laccase from 0.15 to 0.60 U/mg of laccase, the MPI did not cross-link by laccase (**Figure 1.9**D and G). This result suggests that the additional mediators might somehow be not working to assist the laccase-catalyzed cross-linking reaction. Probably there are no tyrosine residues exposed to the surface that can react with free tyrosyl radicals of VA. The VA-MPI samples showed interesting results—cross-linked MPI with high molecular weight observed after incubated for 2 hours (**Figure 1.9**E). By increasing the incubation time, more polymeric MPI obtained, and almost all MPI monomer reacted with laccase. Additionally, the reaction time becomes shorter when the concentration of laccase increased four times from 0.15 to 0.60 U/mg of laccase. The polymeric MPI formed within 10 minutes (**Figure 1.9**H). These results suggest that modification of protein models is essential for the successful protein polymers formation. The modification also could minimize or even eliminate the use of mediators in laccase-catalyzed protein cross-linking reaction. Therefore, laccase, as well as enzymes-catalyzed cross-linking reaction is a combination of genetic modification of protein target and site-specific cross-linking reaction of enzymes. The genetic modification and strategy of the enzymatic reaction requires a careful consideration.

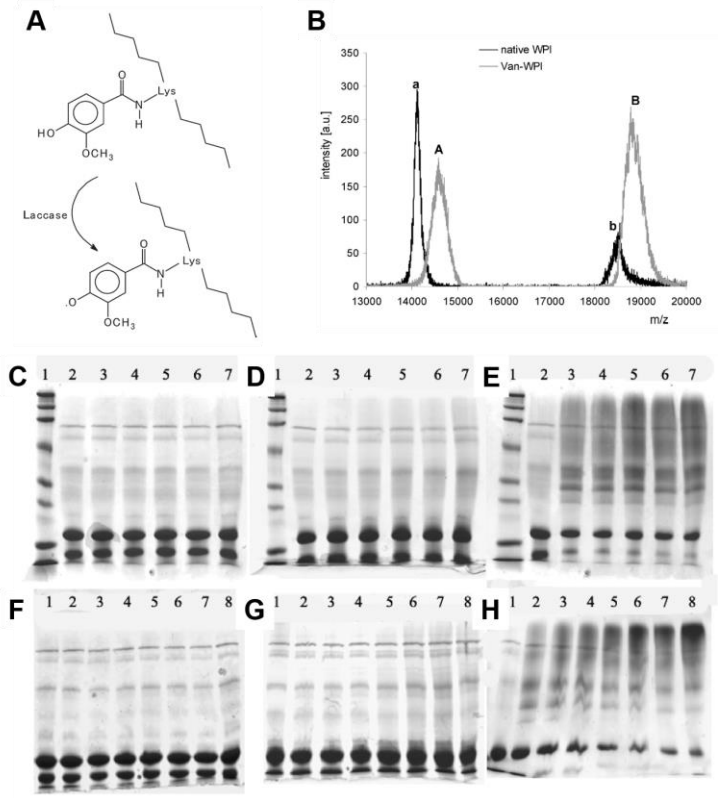


Figure 1.9 (A) Structure of vanillic acid, a mediator for a laccase in the cross-linking reaction of proteins. (B) Results of MALDI-TOF mass spectra analysis. Unmodified α-lactalbumin (a); unmodified β-lactoglobulin (b); VA modified α-lactalbumin (A); VA modified β-lactoglobulin (B). (C-E) results of SDS-PAGE analysis of cross-linking reaction of unmodified and modified WPI with laccase (0.15 U/mg). (C) WPI+laccase; (D) WPI+2 mM free VA+laccase; (E) VA-WPI+laccase. (F-H) results of SDS-PAGE analysis of cross-linking reaction of unmodified and modified WPI with laccase (0.60 U/mg). (F) WPI+laccase; (G) WPI+2 mM free VA+laccase; (H) VA-WPI+laccase. (C-E) Reaction time: lane 2-7, 0, 2, 4, 8, 6, 12, and 24 h. (F-H) Reaction time: lane 1-8, 0, 10, 20, 30 min, 1, 2, 4, 12 h. Reprinted from ref. 54. Copyright with permission from the American Chemical Society (ACS).

1.3 Laccase-Catalyzed Site-Selective Cross-Linking Reaction of Protein

Laccases were first isolated in 1883 from the lacquer tree, *Rhus vernicifera* [106]. It catalyzes the one-electron oxidation of substrates followed by four-electron reduction reaction of oxygen to form water. Laccase can also oxidize a broad range of inorganic and organic substrates, such as aromatic amines, phenol, non-phenols, and their derivatives. Laccase has been found in wide distribution in various fungi, some of higher plants, and bacteria [106]. White-rot fungi (WRF) are the most efficient laccase producers. WRF species were known can produce laccase, such

as *Pycnoporus cinnabarinus*, *Trichoderma atroviride*, *Polyporus sp*, *Cladosporium cladosporioides, P. sanguineus, Trametes trogii*, *T. versicolor*, *Cerrena sp*, and *Ganoderma lucidum* [107]. Peptides and proteins are known as weak substrates for laccases. It needs mediators, generally phenolic acids, to assist it in an enzymatic reaction against peptide or protein substrates. The good and GRAS grade of laccase has been developed by some companies, including Novozymes (Denmark) and Amano Enzyme Japan Co. Ltd. It is mostly employed in the baking industries.

1.3.1 Laccase-catalyzed site-selective cross-linking of tyrosine-tagged proteins

The use of enzymes can substitute the chemical agents that commonly and widely used in the cross-linking reaction of proteins. Enzymes offer a high site-specificity of the cross-linking reaction of proteins. It also offers mild conditions and rapid reaction. Laccases and peroxidases have the same mechanism of the reaction. They catalyze the oxidation of phenolic compounds to form free radicals, commonly free tyrosyl radicals, which will initiate the coupling reaction through a free radical mechanism. However, peroxidases require a chemical, hydrogen peroxide (H_2O_2), as an initiator to start the oxidation reaction. It became the most essential disadvantage of peroxidases.

Our research group has been employed a peroxidase, horseradish peroxidase (HRP), as catalyst for the cross-linking reaction of proteins [21,79,98] and preparation of redox-responsive hydrogels [108,109]. HRP provides a rapid protein cross-linking reaction. It also offers a site-specific cross-linking reaction of tyrosine-tagged (Y-tagged) proteins. Minamihata and co-workers in 2011 modified an *Escherichia coli* alkaline phosphatase (BAP) by adding a flexible linker with a tyrosine residue called Y-tag at the C-termini, which can be recognized by HRP. The Y-tagged BAPs was showing a significant reactivity against HRP and formed branched polymeric BAPs. However, they found that the enzymatic activity HRP-treated BAP

was slightly decreased after the reaction. The additional H_2O_2 could be responsible for the activity changes of BAPs. Another possibility is the large and branched polymeric structure of BAPs might affect its enzymatic activity.

Herein an alternative enzyme for the cross-linking reaction of proteins is explored. Laccases are good candidates that can become an alternative or even substitute HRP in the preparation of protein polymers. Laccase provides the same site-specificity as HRP. One of the advantages of laccases is it only uses oxygen as the oxidant for the oxidation reaction of phenolic compounds. According to the site-specificity of laccases against tyrosine, tyrosine-modified proteins will become a good substrate for laccases.

1.3.2 Controlling laccase-catalyzed cross-linking reaction

Controlling free radicals-mediated polymerization reaction is of proteins is still become a problem. The use of chemicals to control or stop the enzymatic polymerization reaction could become an interesting approach. However, the chemical could deactivate and denature the protein polymer and the enzyme as well. The use of molecular quenchers should become another alternative strategy to control the free radicals-mediated polymerization reaction of proteins. One of the options of molecular quenchers is cysteine. The thiol group of cysteine could capture the radicals and utilize it to form a disulfide bond. Another appropriate strategy is by modifying the protein target to mediate the controllable protein cross-linking, which required accurate design and strategy of the protein models and reaction.

1.4 Aim and outline of the thesis

This research aims to develop protein polymers prepared by a laccase-catalyzed site-specific cross-linking reaction. The protein polymers then applied as diagnostic probes in enzymatic immunoassay (EIA).

In the **Chapter 2**, the potential of a laccase, *Trametes sp.* laccase (TL), as catalyst for the site-specific cross-linking reaction of proteins was studied. The engineered bacterial (*Escherichia coli*) alkaline phosphatase (BAP) and chimeric antibody-binding protein (pG_2pAs) was applied as model proteins. Both proteins were engineered by adding a flexible linker containing tyrosine residue (Y-tags). The TL expected to recognize and oxidize the tyrosine residues of Y-tags to the formation of protein polymers. Unmodified BAP and pG_2pA are used to validate site-specificity of TL-catalyzed protein cross-linking reaction.

In the **Chapter 3**, the recombinant HRPs fused with single or double Y-tag were constructed and expressed in silkworm, *Bombyx mori*. TL was used as catalyst the formation of free tyrosyl radicals from Y-tagged HRPs, which initiated the random nonenzymatic radical polymerization of the HRP units through the tyrosine coupling of Y-tags. The covalent dityrosine bond also can be formed through an HRP-catalyzed self-cross-linking reaction in the presence of H_2O_2. The self-polymerization of Y-tagged HRPs by the addition of H_2O_2 is compared with TL-catalyzed polymerization of Y-tagged HRP to check the site-selectivity, mild reaction conditions and activity retention of the polymeric products.

In the **Chapter 4**, a new strategy for the formation of linear polymerization of BAP through the site-specific cross-linking reaction catalyzed by TL was demonstrated. A peptide loop containing a tyrosine residue (Y-Loop) was introduced to BAP to construct BAP-Loop-Y mutant. The BAP-Loop-Y is expected to form protein polymers with different morphology due to the rigid and short structure of Y-Loop. The performance of resultant polymers and copolymers is validated by an immunoassay due to its ability to bind the analytes with a better conformation.

Finally, in the **Chapter 5** the findings of this research are summarized and briefly discussed further direction related to this research.

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CHAPTER 2 LACCASE-CATALYZED BIOCONJUGATION OF TYROSINE-TAGGED FUNCTIONAL PROTEINS

2.1 Introduction

Protein cross-linking is defined as the process of combining proteins through covalent bonds. This cross-linking creates new assembled macromolecules that have physicochemical properties and functionalities that are derived from, but different to, the individual parent proteins [1]. In particular, the cross-linking of multiple protein molecules into one large macromolecule, i.e. protein polymerization, has a considerable effect on the properties and functionalities of the building block proteins, because of the multivalency of the protein units in the resulting protein polymer and its extremely large molecular structure [2,3]. The cross-linking of proteins to make protein polymers has been studied extensively in the field of food chemistry [4-7], but in these studies, the site specificity of the cross-linking sites on the proteins is not controlled and thus the structures of the protein polymers are random [8,9]. For functional proteins other than food proteins, controlled cross-linking is required to maintain their functions upon polymerization. Polymerization of proteins through a site-specific cross-linking reaction is a challenge for biochemists.

New covalent bonds between protein molecules can be formed by chemical [10-12], enzymatic [13-17], and chemoenzymatic [18-19] reactions. Chemical protein cross-linking can be achieved by an enormous diversity of cross-linking reagents with different characteristics and that target different functional groups on the proteins [14]. However, the use of chemical reagents for protein cross-linking is not site selective and can cause protein denaturation in some cases. Thus, chemical methods for protein cross-linking are not a good choice to produce protein polymers.

In contrast, enzyme-catalyzed, cross-linking methodologies for proteins have been receiving attention because of the mild reaction conditions and high substrate specificity using enzymes. Enzymes can be powerful tools to catalyze the site-specific cross-linking of proteins when combined with the genetic modification of target proteins to append peptide tags for post-translational modification (PTM) [1,20,21,22]. Reactive peptide tags for PTM play an important role in enzymatic recognition for cross-linking. For example, the enzymatic protein modifications catalyzed by biotin ligase [23], transglutaminase [17,24,25], lipoic acid ligase [26], bacterial sortase [16], horseradish peroxidase (HRP) [13,15,21], and phosphopantetheinyl transferase [18] use additional peptide tags to achieve site-specific protein cross-linking and modification. The amino acid tyrosine is highly effective in peptide tags and can be recognized by some enzymes. Tyrosine is hydrophobic and so the number of residues that can be placed on the surface of a protein is small, indicating that tyrosine can have high site specificity when used as the reactive residue in peptide tags [15]. The phenolic moiety of tyrosine is recognized by some enzymes, including HRP, tyrosinase, and laccase. In our previous reports, we used HRP to catalyze protein cross-linking via genetically introduced peptide tags containing tyrosine residues (Y-tags) [13,15,21,27]. The HRP-catalyzed protein cross-linking reaction must be initiated by the addition of hydrogen peroxide (H_2O_2), a strong oxidizing agent. HRP decomposes H_2O_2 rapidly, thus the effect of H_2O_2 on target proteins is minimal in most cases; however, when the concentration of the target proteins is high, the required amount of H_2O_2 for cross-linking becomes high as well, and the deactivation of target proteins by H_2O_2 is not negligible. Therefore, an alternative enzyme that can catalyze the oxidation of tyrosine residues without H_2O_2 is desirable.

Laccases can be a substitute for HRP to perform site-specific protein cross-linking with peptide tags. Laccases only require molecular oxygen to oxidize phenolic substrates and produce water as a side product [28]. It has been reported that laccases can catalyze the

oxidation of amino acids, including tyrosine, tryptophan, and cysteine [29]; food proteins [30]; and chemicals, such as aniline [31] and lignocatecol [32]. Laccases oxidize substrates through a one-electron oxidation reaction to form radicals and the generated radicals can react with each other to form polymerized products [33]. However, there has been no report to date, in which site-specific protein cross-linking or protein polymerization has been achieved using laccase. As laccases do not require H_2O_2 in their catalytic cycle, it may be beneficial to perform site-specific protein cross-linking and protein polymerization using laccases instead of HRP.

Therefore, we investigated site-specific protein cross-linking and polymerization using *Trametes sp.* laccase (TL). Recombinant *E. coli* alkaline phosphatases (BAPs) and antibody-binding chimeric proteins (pG_2pAs) possessing Y-tags were constructed as model proteins that are substrates for TL. Herein, we report the TL-catalyzed, site-specific cross-linking and co-cross-linking reactions of Y-tagged proteins (**Figure 2.1**). The BAP/pG_2pA polymer obtained was used as a highly sensitive detection probe for enzyme-linked immunosorbent assay (ELISA).

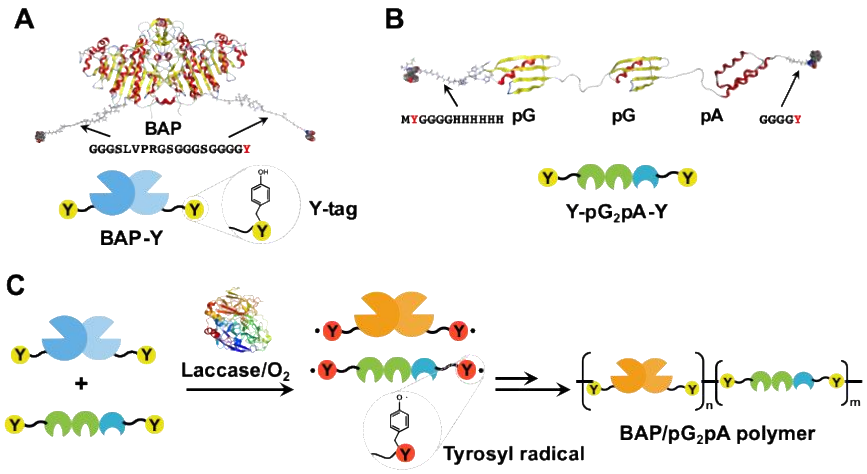

Figure 2.1 The schematic illustration of the laccase-mediated, site-specific, protein cross-linking reaction. (A) A schematic illustration of BAP-Y, possessing a Y-tag sequence at its *C*-terminus. (B) A schematic illustration of Y-pG_2pA-Y, composed of two pG domains and one pA domain, along with Y-tag sequences at both its *N*- and *C*-termini. The additional amino acid sequences are shown as a stick model in the molecular-model images and the Tyr residues of the Y-tags are highlighted as a Corey-Pauling-Koltun (CPK) model. (C) Scheme of the laccase-catalyzed co-cross-linking of BAP-Y and Y-pG_2pA-Y. Copyright 2018 reprinted with permission from Elsevier.

2.2 Materials and Methods

2.2.1 Material

TL was provided by Amano Enzyme Inc. (Nagoya, Japan). Para-nitrophenyl phosphate (*p*-NPP), potassium dihydrogen phosphate, and dipotassium hydrogen phosphate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Yeast extract and tryptone were purchased from Difco (Miami, USA). All reagents were used as received. The columns used: His-Trap FF Crude column 5 mL; HiPrep desalting column 60 mL; HiTrap Q HP anion exchange column 5 mL; and HiLoad Superdex 200 pg size exclusion chromatography column were purchased from GE Healthcare Life Sciences (Chicago, USA).

2.2.2 Construction of expression plasmids of BAPs and pG_2pAs

All of the model proteins used were expressed in *E. coli*. The amino acid sequence BAP was from *E. coli* BAP (PDB ID 1ALK). We modified and designed the expression vectors of the model proteins based on the plasmid vectors used in our previous reports [13,21], pET22b(+)-BAP-Y and pET22b(+)pG_2pA-Y. pG_2pA is a chimeric protein consisting of two protein G domains and one protein A domain in tandem [13]. The gene coding for YGGGG was added to the 5'-end of pG_2pA-Y in the pET22b(+)pG_2pA-Y vector to construct the expression vector for Y-pG_2pA-Y. The gene coding for pG_2pA was amplified by PCR and the obtained DNA fragment was cloned into the pET22b(+)-BAP-Y vector to construct the expression vector for BAP-pG_2pA-Y. The appropriate DNA domains were then deleted from pET22b(+) BAP-pG_2pA-Y to construct the expression vectors for BAP-pG and BAP-pG-Y. The full amino acid sequences of all the proteins used in this study are listed in the Table 2.1.

Table 2.1 The full amino acid sequences of all the proteins used in this study[a]

Protein ID	Amino acid sequence
BAP-Y	MKYLLPTAAAGLLLLAAQPAMA↓MDIGINSDPHHHHHHTPEMPVLENRAAQGDITAPG GARRLTGDQTAALRDSLSDKPAKNIILLIGDGMGDSEITAARNYAEGAGGFFKGIDAL PLTGQYTHYALNKKTGKPDYVTDSAASATAWSTGVKTYNGALGVDIHEKDHPTILEMA KAAGLATGNVSTAELQDATPAALVAHVTSRKCYGPSATSEKCPGNALEKGGKGSITEQ LLNARADVTLGGGAKTFAETATAGEWQGKTLREQAQARGYQLVSDAASLNSVTEANQQ KPLLGLFADGNMPVRWLGPKATYHGNIDKPAVTCTPNPQRNDSVPTLAQMTDKAIELL SKNEKGFFLQVEGASIDKQDHAANPCGQIGETVDLDEAVQRALEFAKKEGNTLVIVTA DHAHASQIVAPDTKAPGLTQALNTKDGAVMVMSYGNSEEDSQEHTGSQLRIAAYGPHA ANVVGLTDQTDLFYTMKAALGLKGGGSLVPRGSGGGSGGGGY
BAP-pG$_2$pA-Y	MKYLLPTAAAGLLLLAAQPAMA↓MDIGINSDPHHHHHHTPEMPVLENRAAQGDITAPG GARRLTGDQTAALRDSLSDKPAKNIILLIGDGMGDSEITAARNYAEGAGGFFKGIDAL PLTGQYTHYALNKKTGKPDYVTDSAASATAWSTGVKTYNGALGVDIHEKDHPTILEMA KAAGLATGNVSTAELQDATPAALVAHVTSRKCYGPSATSEKCPGNALEKGGKGSITEQ LLNARADVTLGGGAKTFAETATAGEWQGKTLREQAQARGYQLVSDAASLNSVTEANQQ KPLLGLFADGNMPVRWLGPKATYHGNIDKPAVTCTPNPQRNDSVPTLAQMTDKAIELL SKNEKGFFLQVEGASIDKQDHAANPCGQIGETVDLDEAVQRALEFAKKEGNTLVIVTA DHAHASQIVAPDTKAPGLTQALNTKDGAVMVMSYGNSEEDSQEHTGSQLRIAAYGPHA ANVVGLTDQTDLFYTMKAALGLKGGGSTYKLVINGKTLKGETTTEAVDAATAEKVFKQ YANDNGVDGEWTYDDATKTFTVTEKPEVIDASELTPAVTTYKLVINGKTLKGETTTKA VDAETAEKAFKQYANDNGVDGVWTYDDATKTFTVTEGGGGSDVDNKFNKEQQNAFWEI LHLPNLNEEQRNGFIQSLKDDPSQSANLLAEAKKLNDAQAPKLVPRGSGGGSGGGGY
BAP-pG	MKYLLPTAAAGLLLLAAQPAMA↓MDIGINSDPHHHHHHTPEMPVLENRAAQGDITAPG GARRLTGDQTAALRDSLSDKPAKNIILLIGDGMGDSEITAARNYAEGAGGFFKGIDAL PLTGQYTHYALNKKTGKPDYVTDSAASATAWSTGVKTYNGALGVDIHEKDHPTILEMA KAAGLATGNVSTAELQDATPAALVAHVTSRKCYGPSATSEKCPGNALEKGGKGSITEQ LLNARADVTLGGGAKTFAETATAGEWQGKTLREQAQARGYQLVSDAASLNSVTEANQQ KPLLGLFADGNMPVRWLGPKATYHGNIDKPAVTCTPNPQRNDSVPTLAQMTDKAIELL SKNEKGFFLQVEGASIDKQDHAANPCGQIGETVDLDEAVQRALEFAKKEGNTLVIVTA DHAHASQIVAPDTKAPGLTQALNTKDGAVMVMSYGNSEEDSQEHTGSQLRIAAYGPHA ANVVGLTDQTDLFYTMKAALGLKGGGSTYKLVINGKTLKGETTTEAVDAATAEKVFKQ YANDNGVDGEWTYDDATKTFTVTEKPEVIDASELTPAVT
BAP-pG-Y	MKYLLPTAAAGLLLLAAQPAMA↓MDIGINSDPHHHHHHTPEMPVLENRAAQGDITAPG GARRLTGDQTAALRDSLSDKPAKNIILLIGDGMGDSEITAARNYAEGAGGFFKGIDAL PLTGQYTHYALNKKTGKPDYVTDSAASATAWSTGVKTYNGALGVDIHEKDHPTILEMA KAAGLATGNVSTAELQDATPAALVAHVTSRKCYGPSATSEKCPGNALEKGGKGSITEQ LLNARADVTLGGGAKTFAETATAGEWQGKTLREQAQARGYQLVSDAASLNSVTEANQQ KPLLGLFADGNMPVRWLGPKATYHGNIDKPAVTCTPNPQRNDSVPTLAQMTDKAIELL SKNEKGFFLQVEGASIDKQDHAANPCGQIGETVDLDEAVQRALEFAKKEGNTLVIVTA DHAHASQIVAPDTKAPGLTQALNTKDGAVMVMSYGNSEEDSQEHTGSQLRIAAYGPHA ANVVGLTDQTDLFYTMKAALGLKGGGSTYKLVINGKTLKGETTTEAVDAATAEKVFKQ YANDNGVDGEWTYDDATKTFTVTEKPEVIDASELTPAVTLVPRGSGGGSGGGGY
Y-pG$_2$pA-Y	MYGGGGHHHHHHTYKLVINGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYDD ATKTFTVTEKPEVIDASELTPAVTTYKLVINGKTLKGETTTKAVDAETAEKAFKQYAN DNGVDGVWTYDDATKTFTVTEGGGGSDVDNKFNKEQQNAFWEILHLPNLNEEQRNGFI QSLKDDPSQSANLLAEAKKLNDAQAPKGGGGY

[a]Protein/peptide fragments are labeled with different colors: Red: Signal peptide; Orange: Hexahistidine tag; Purple: Alkaline phosphatase; Green: Flexible linker; Cyan: Protein G; Dark red: Protein A; Grey: thrombin recognition sequence; Blue: Y-tag.

2.2.3 Expression and purification of BAPs and pG$_2$pAs

The competent cells of *E. coli* BL21(DE3) Star were transformed with the expression vectors with a heat-shock method. A single colony of transformants was selected from the agar plates

(containing 100 µg/mL of ampicillin sodium) and inoculated into 3.5 mL of LB media containing 100 µg/mL of ampicillin sodium. The media was cultured for 6-7 hours at 37°C. The pre-cultured media were then poured into 500 mL of TB media containing 100 µg/mL of ampicillin sodium and incubated until the OD_{600} reached ~0.5. The expressions of BAPs and pG_2pAs were induced by the addition of isopropyl-β-D-thiogalactoside (IPTG) (0.1 mM of final concentration) and followed by shaking for 18 h at 18°C. The cells were then collected by centrifugation at 5000 g for 20 min at 4 °C. The cell pellets were washed with TBS (25 mM Tris-HCl, 137 mM NaCl, 2.68 mM KCl, pH 7.4) 3 times. The suspensions of the cell were frozen and stored at -80°C.

The frozen cell suspensions were thawed and sonicated on ice for 15 min. The supernatant was separated from insoluble cell components by centrifugation at 5000 g for 20 min at 4°C. The supernatants were initially applied to an equilibrated HisTrap FF Crude column (equilibrated with the binding buffer: 20 mM Tris-HCl pH 7.4, 500 mM NaCl, 35 mM imidazole). The column was washed with a five column bed volumes of the binding buffer and BAPs and pG_2pAs were eluted with a gradient of the elution buffer (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 500 mM imidazole) from 0% to 100%. The fractions containing the proteins were collected and pooled. The pooled fractions were then desalted into 10 mM Tris-HCl (pH 8.0) using HiPrep Desalting column and applied to an anion exchange column, HiTrap Q HP (equilibrated with 10 mM Tris-HCl, pH 8.0). The BAPs and pG_2pAs were then eluted with a gradient of the elution buffer (10 mM Tris-HCl pH 8.0, 500 mM NaCl) from 0% to 100%. The fractions containing the proteins were pooled and then concentrated to a volume of ~5 mL using Amicon Ultrafiltration (MWCO 50 kDa). The concentrated solutions of BAPs and pG_2pAs were applied to the size exclusion chromatography (SEC) column (HiLoad Superdex 200 pg). The SEC mobile phase was 50 mM Tris-HCl, 150 mM NaCl, pH 7.5. The fractions were analyzed with SDS-PAGE analysis and the fraction containing the target proteins at high

purity was collected. The fractions were then concentrated using the Amicon Ultrafiltration (MWCO 50 kDa) and finally the concentration of the proteins were measured by BCA assay using BSA as the standard.

2.2.4 Polymerization of proteins

The polymerization of BAP-Y, BAP-pG_2pA-Y, and Y-pG_2pA-Y, and the co-polymerization of BAP-Y/Y-pG_2pA-Y were conducted in 10 mM Tris-HCl (pH 8.0) solution with a total volume of 50 µL. The pH of the reaction was optimized. The concentrations of BAP and pG_2pA were 2.5 µM. The reaction mixture was incubated at 37 °C for an appropriate time length. The samples were analyzed by SDS-PAGE. The percentage of efficiency of polymerization was determined by using ImageJ Software version 1.51s (provided by National Institute of Health, USA) by compared area of a control to the samples and multiple with a hundred.

2.2.5 Western blotting analysis on BAP/pG_2pA polymers

The SDS-PAGE gel was directly transferred to a midi size of Turbo Transfer Blot PVDF membrane (BioRad) by using Trans-Blot Turbo Transfer System. Ten milliliter of blocking buffer (1 % BSA in TBST) was added to the membrane in a clean container and incubated for 1 hour at RT. After the blocking buffer removed, 10 mL of anti-HisTag antibody (diluted 5000 times by the blocking buffer) (Proteintech, Cat No. 66005-1-Ig) was added and incubated for 1 hour at RT. Then, the membrane washed three times with 10 mL of TBST for 5 minutes. After washing, 10 mL of Goat anti-Mouse IgG (H+L) horseradish peroxidase (HRP) (diluted 5000 times by the blocking buffer) (Thermo Scientific, Cat No. 62-6520) was added and incubated for 1 hour at RT. Then, the membrane washed three times with 10 mL of TBST for 5 minutes. After washing, the membrane then placed on sample tray of BioRad Molecular Imager ChemiDoc XRS+. Then, a luminescence solution, ImmunoStar LD (Wako, Japan) was

added onto the membrane. Then, the chemiluminescence reaction of substrate and enzyme was captured by using the imager.

2.2.6 Activity assay of BAP

The enzymatic activity of the BAPs was measured by the dephosphorization of *p*-NPP. Samples of BAP solutions (10 µL) were pipetted into 490 µL of 1 mM *p*-NPP in 1 M Tris-HCl (pH 8.0) in a 1 mL quartz cell for UV-vis spectrophotometry. The absorbance at 410 nm was monitored for 3 min at 25 °C. The measurements were replicated three times for three individual samples for each BAP.

2.2.7 ELISA assay of BAP/pG$_2$pA polymers

The BAP/pG$_2$pA polymers were used as detection probes for ELISA. OVA (albumin from chicken eggs) was diluted in Tris-buffered saline (TBS) buffer (pH 7.4) to 10 µg/mL and incubated in a 96-well microplate with 100 µL/well at 4 °C overnight. The wells were washed three times with 200 µL of TBST (TBS buffer containing 0.1% Tween 80). One percent bovine serum albumin (BSA) solution in TBST was added (200 µL/well) to block the surface of the wells. After incubation for 1 h at RT, the wells were washed three times with 200 µL of TBST. The primary antibody, rabbit Anti-OVA-IgG (C6534-2 mL; Sigma–Aldrich), was diluted with TBST containing 1% BSA to a concentration of 1 µg/mL and the diluted solution was added to each well with a volume of 100 µL. After incubation for 2 h at RT, the wells were washed three times with 200 µL of TBST. Then, diluted BAP/pG$_2$pA polymers (100 µL/well) were added to each well and incubated for 1 h at RT. After washing the wells with 200 µL of TBST three times, the catalytic activities of the immobilized BAP/pG$_2$pA polymers were measured with *p*-NPP as the substrate at 25 °C. The absorbance at 410 nm was measured for 1 h at 25 °C using a 96-well microplate reader. In addition, the BAP/pG$_2$pA polymers were synthesized by

the HRP reaction. The concentration of HRP was 4 μM. The concentration of H_2O_2 added to the mixture of the Y-tagged proteins was 5 μM. The reaction was conducted for 0.5, 1, and 24 h. The TL and HRP-catalyzed BAP/pG_2pA polymers were then used in the ELISA in the same manner as described above.

2.2.8 Statistical analysis

ANOVA and Student's t-test functions in Microsoft Excel (Version 2016 for Macintosh, Microsoft Corp, Redmond, WA, USA) were used to perform statistical analysis. P values of < 0.05 were considered statistically significant.

2.3 Results

2.3.1 Laccase-catalyzed site-specific cross-linking of Y-tagged proteins

First, we first evaluated the ability of the TL to catalyze cross-linking reaction of Y-tagged proteins. **Figure 2.2** shows the results of SDS-PAGE analysis of Y-tagged proteins treated with TL. In the lanes for Y-tagged BAPs and pG_2pAs treated with TL, dimer and oligomers (>210 kDa) were generated, which corresponded to the cross-linked products of the Y-tagged proteins. We used a BAP without Y-tags, BAP-pG (BAP linked with a single protein G domain) and pG_2pA as controls. There were no cross-linked products observed with BAP-pG (**Figure 2.2**A) or pG_2pA (**Figure 2.2**B), indicating that laccase specifically recognized the tyrosine residues in the Y-tag. These results agree with the report from Mattinen et al., [29] in which coactosin was treated with laccase. This group reported that laccase can recognize tyrosine residues that are exposed on the surface of the protein. In our study, the Y-tag was tethered to the termini of proteins with flexible linkers, making the tyrosine residues in the Y-tag recognized readily by laccase.

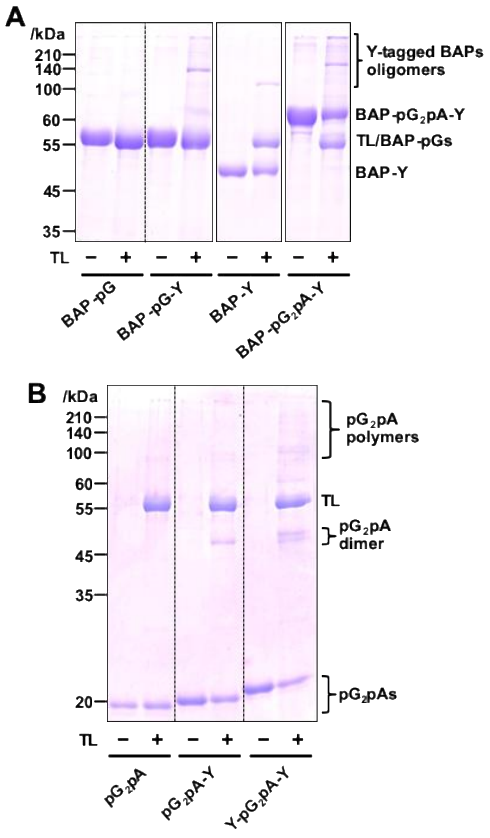

Figure 2.2 SDS-PAGE of TL-catalyzed, site-specific cross-linking of BAP and pG_2pA at 37 °C for 30 min. The concentration of TL was 3.6 μM in 10 mM Tris-HCl, pH 8.0. (A) The cross-linking of BAP catalyzed by TL; BAP-pG, BAP-pG-Y, BAP-Y, and BAP-pG_2pA-Y. (B) Cross-linking of pG_2pA, pG_2pA-Y, and Y-pG_2pA-Y catalyzed by TL. The concentrations of BAP and pG_2pA were 2.5 μM. Copyright 2018 reprinted with permission from Elsevier.

2.3.2 Effect of pH on the cross-linking of Y-tagged proteins catalyzed by TL

Next, we evaluated the effect of pH on the cross-linking of Y-tagged proteins. **Figure 2.3** shows the results of SDS-PAGE analysis of BAP-Y treated with TL under different pH conditions. It is clear that as the pH increased, the amount of cross-linked BAP-Y also increased. In addition, a higher concentration of TL resulted in approximately 70 % of cross-linked BAP-Y, indicating that the cross-linking of BAP-Y catalyzed by TL is slow and is not completed within the reaction time of 30 min. At lower values of pH, the tyrosyl radicals are protonated and thus the cross-linking of tyrosyl radicals will be inhibited [35]. The optimum reactivity of TL is reported to be under acidic conditions (pH 2.2–5.5) [36]; however, the cross-

linking of tyrosine residues occurs more efficiently under slightly basic conditions, probably because of differences in the substrate properties.

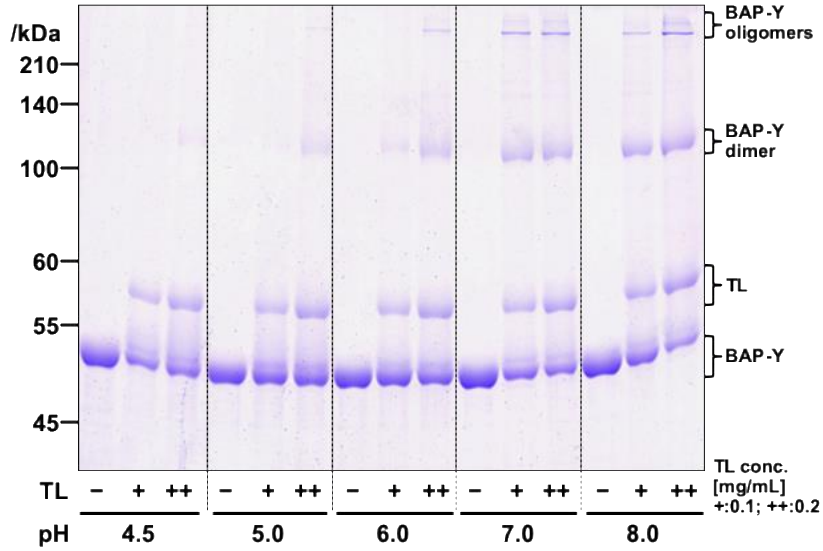

Figure 2.3 The effect of the pH conditions on the cross-linking efficiency of BAP-Y catalyzed by TL. The BAP-Y concentration was 2.5 μM. The concentrations of TL were 1.8 (+) and 3.6 (++) μM. The buffers used to adjust the pH were 10 mM of acetate buffer for pH 4.5, 5.0- and 10-mM phosphate buffer for pH 6.0 and 7.0, and 10 mM Tris-HCl for pH 8.0. The reaction was conducted for 30 min at 37 °C. Copyright 2018 reprinted with permission from Elsevier.

2.3.3 Time-dependence of TL-catalyzed cross-linking reaction of Y-tagged proteins

As suggested in Figure 2.3, the cross-linking of Y-tagged proteins by TL is slow and is time dependent; thus, we evaluated the time course of the cross-linking efficiency of the Y-tagged proteins. The results of the TL-catalyzed cross-linking of BAP-Y and Y-pG_2pA-Y at different incubation times are shown in **Figure 2.4**. The cross-linked products of each Y-tagged protein can be seen in the first 5 min of the TL reaction, and the amount of the cross-linked product increased as the reaction time increased. Approximately 74 % of the Y-pG_2pA-Y monomers had disappeared at 30 min, while the monomeric BAP-Y remained approximately 17 % even after reaction for 2 h. The differences in the reactivity of BAP-Y and Y- pG_2pA-Y is probably caused by the differences in their molecular structures. While Y-pG_2pA-Y is relatively small

(approx. 22 kDa) and its molecular shape is quite linear (Figure 2.1B), BAP-Y is a large dimeric protein (approx. 100 kDa in total, 50 kDa for each subunit). Therefore, as the cross-linking reaction proceeds, the reaction of cross-linked BAP-Y conjugates with TL is more sterically hindered than for the cross-linked Y-pG_2pA-Y conjugates. Y-pG_2pA-Y also possesses two Y-tags at its *N*- and *C*-termini, which will increase the cross-linking efficiency. As a result, recognition of the unreacted Y-tags in the BAP-Y conjugates by TL was inhibited, and such unreacted subunits of BAP-Y appeared as an intact BAP-Y band in the SDS-PAGE analysis, while Y-pG_2pA-Y was cross-linked almost completely. TL uses molecular oxygen solubilized in solution to oxidize substrates, thus the TL reaction continues as long as oxygen is being supplied from air and there is a substrate that is recognized by TL. Prolonging the reaction time will result in formation of protein polymers with a higher polymerization degree, but making such large polymers often results in unfavorable occurrences, such as precipitation. Thus, we chose 30 min as the standard reaction time for TL-catalyzed protein cross-linking, as Y-pG_2pA-Y was almost completely cross-linked after that time.

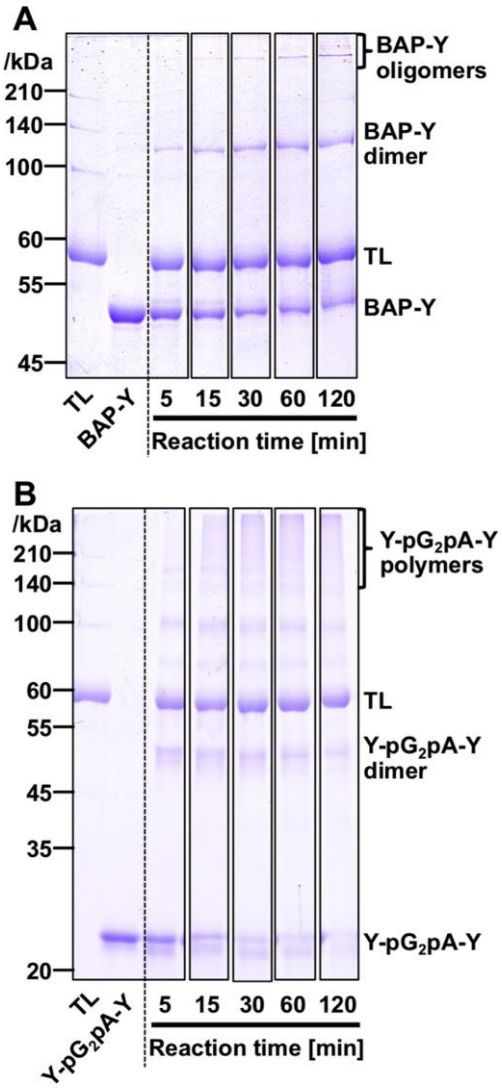

Figure 2.4 Time course analysis of the cross-linking of BAP-Y (A) and Y-pG$_2$pA-Y (B) catalyzed by TL. The BAP-Y and Y-pG$_2$pA-Y concentrations were 2.5 μM. The concentration of laccase was 3.6 μM. The reactions were conducted in 10 mM Tris-HCl, pH 8.0, and aliquots of solution were taken at each reaction time and heated with 4X SDS-PAGE sample buffer at 95 °C for 3 min. Copyright 2018 reprinted with permission from Elsevier.

2.3.4 TL-catalyzed co-cross-linking of BAP-Y and Y-pG$_2$pA-Y and ELISA using the BAP/pG$_2$pA copolymer

Next, we performed co-cross-linking of two Y-tagged proteins, BAP-Y and Y-pG$_2$pA-Y, to create a detection probe for ELISA. **Figure 2.5**A shows the results of SDS-PAGE analysis of the cross-linked products of BAP-Y and Y-pG$_2$pA-Y prepared by the TL reaction. As the ratio of BAP-Y to Y-pG$_2$pA-Y increased, more high molecular-weight bands were generated,

indicating the formation of highly cross-linked polymers. The Y-pG_2pA-Y band disappeared at all ratios of BAP-Y to Y-pG_2pA-Y, indicating that all the Y-pG_2pA-Y was incorporated in the highly cross-linked polymers. Then, using these BAP/pG_2pA polymers prepared with different molar ratios, we conducted an ELISA to evaluate the functionality of the BAP/pG_2pA polymers as detection probes (**Figure 2.5**B). The ELISA results clearly showed that as the ratio of BAP-Y to Y-pG_2pA-Y increased, the signals from the BAP/pG_2pA polymers were amplified. This result indicated that the ratio of BAP-Y and Y-pG_2pA-Y in the co-polymers varied according to the molar ratio of them in the reaction mixture. The co-polymer prepared with a ratio of 75:1 (BAP: pG_2pA) showed the highest signal, which was 22-fold higher than for the polymer prepared with a 1:1 ratio. The signal slightly decreased when the BAP/pG_2pA polymer was prepared at a ratio of 100:1 and this is probably because the large number of BAP-Y moieties in the BAP/pG_2pA polymer sterically inhibited the binding of the pG_2pA domains to IgG (**Figure 2.5**C).

As can be seen in Figure 2.5A, because of the high concentration of BAP-Y, a large amount of BAP-Y remained in the solutions. Hence, we increased the reaction time with TL to form BAP/pG_2pA polymers (at a ratio of 25:1) with a higher degree of cross-linking. In addition, we prepared BAP/pG_2pA polymers by reaction with HRP and their functions were compared with those prepared by reaction with TL. As the reaction time of the TL treatment was increased, the cross-linking degree of the BAP/pG_2pA polymer increased, which can be seen from the stronger band intensities in the stacking gel region (**Figure 2.6**A). In contrast, the cross-linking degree of the HRP-treated polymers was not dependent on the reaction time, because the HRP reaction proceeded much faster than the TL reaction and was complete within 30 min.

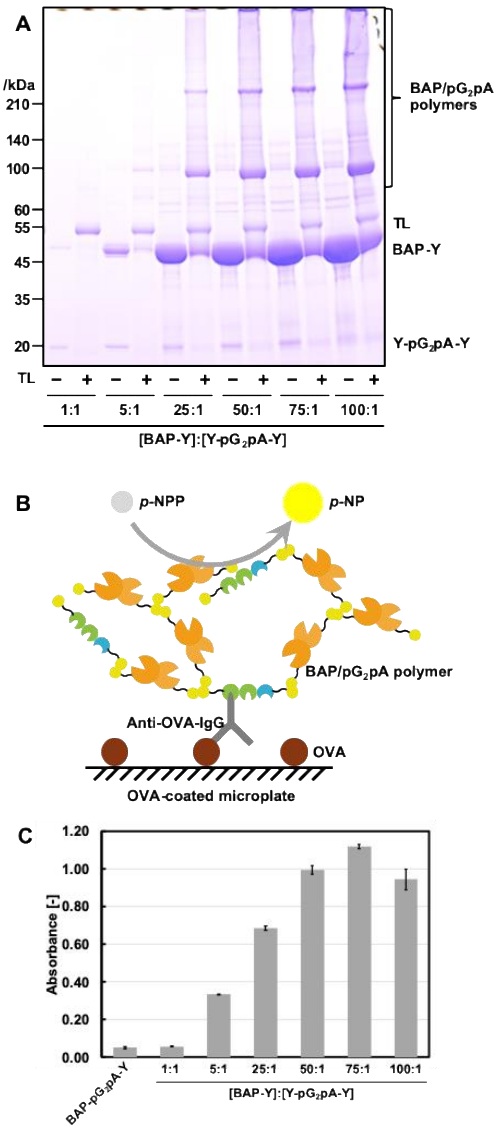

Figure 2.5 (A) SDS-PAGE analysis of laccase-catalyzed hetero cross-linking of BAP-Y and Y-pG2pA-Y. The molar ratios of BAP-Y to Y-pG2pA-Y were 1:1, 5:1, 25:1, 50:1, 75:1, and 100:1. The concentration of Y-pG2pA-Y was fixed at 0.25 μM. The cross-linking reaction was conducted in 10 mM Tris-HCl, pH 8.0, for 30 min at 37 °C. (B) A schematic illustration of indirect ELISA using BAP/pG2pA polymers. (C) Results of ELISA detecting OVA using BAP/pG2pA polymers prepared at different molar ratios of BAP-Y to Y-pG2pA-Y. A chimera protein of BAP–pG2pA-Y was used as the control. The concentration of the pG2pA unit was fixed at 0.25 nM for all the samples. The absorption of OVA onto the microplate was conducted at 10 μg/mL in TBS (pH 7.4). Copyright 2018 reprinted with permission from Elsevier.

Lastly, an ELISA was conducted using the BAP/pG2pA polymers (**Figure 2.6**). The signals decreased as the reaction time of the TL-treatment was extended, and the polymer

prepared after incubation for 24 h with TL had a drastically decreased signal, which was reduced by approximately 40% compared with the polymer prepared after incubation for 30 min. The BAP/pG_2pA polymers prepared using HRP with reaction times of 30 min and 2 h showed comparable signals, but the signals of the polymer prepared after incubation for 24 h were reduced to approximately 65%. In **Figure 2.6**A, for the samples treated with TL and HRP for 24 h, all of the band intensities were decreased compared with the other samples. This result suggested that some amounts of the BAP/pG_2pA polymers were lost during incubation for 24 h, probably because of absorption, and this explains the decreases in the signals of the samples prepared by incubation for 24 h. However, the decrease in the signals from the polymer formed after incubation for 24 h with TL was much larger than from the polymer prepared with HRP. This larger decrease is because of the formation of extremely large protein polymers by the TL reaction. The BAP/pG_2pA polymer prepared by the HRP reaction showed lower signals than that of the polymer prepared by TL and this also suggests that making a very large protein polymer diminishes the overall functionality of the protein polymer (**Figure 2.6**B). The protein cross-linking reaction catalyzed by TL is slow, but this means that the degree of cross-linking of the protein polymers can be easily controlled by varying the reaction time and the concentration of TL, indicating an advantage for using TL over HRP for protein cross-linking.

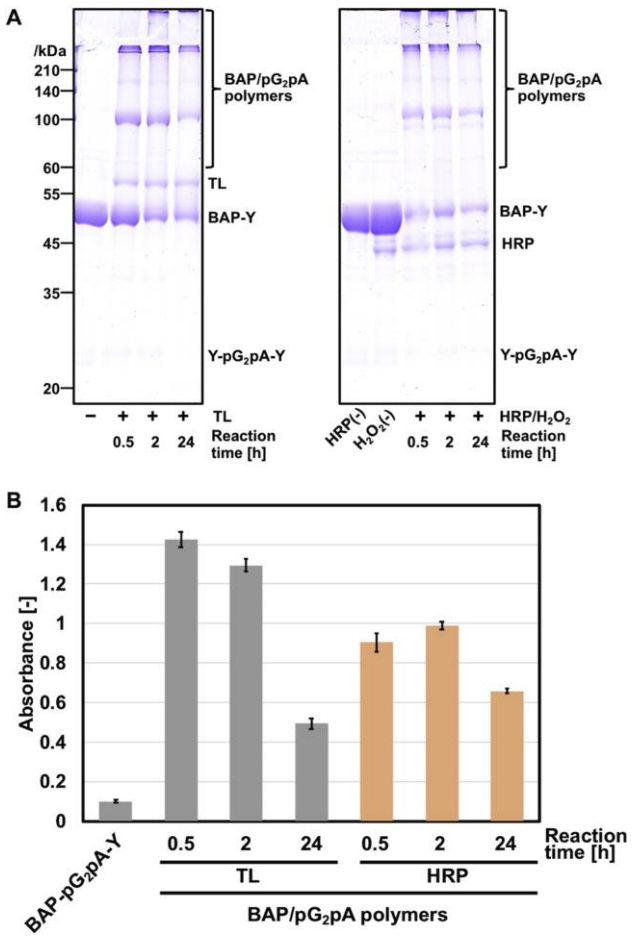

Figure 2.6 Comparison of the BAP/pG2pA polymers prepared by TL and HRP. (A) SDS-PAGE analysis of BAP/pG2pA polymers prepared using TL and HRP. The molar ratio of BAP-Y to Y-pG2pA-Y was 25:1. The concentrations of Y-pG2pA-Y, TL, and HRP were 0.25, 3.6 and 4 μM, respectively. The concentration of H_2O_2 was 5 μM. The reaction was carried out in 10 mM Tris-HCl, pH 8.0, for 0.5, 2, and 24 h at 37 °C. (B) The results of ELISA of BAP/pG2pA polymers prepared using either TL or HRP with different reaction times. The concentration of the pG2pA unit in the solution used for ELISA was fixed at 0.25 nM for all samples. The absorption of OVA onto the microplate was conducted at 10 μg/mL in TBS (pH 7.4). Copyright 2018 reprinted with permission from Elsevier.

2.4 Discussion

The tyrosine residues in flexible conditions can be recognized by TL, however most of the intrinsic tyrosine residues in proteins are located in the positions which are not directly accessible for TL. Some reports about laccase-catalyzed cross-linking of proteins used phenolic mediators to initiate the polymerization of proteins such as vanillin, vanillic acid, caffeic acid

[6], ferulic acid [4,5,29,37,38], *p*-coumaric acid [5]. The phenolic mediators are small therefore, they can be oxidized by TL efficiently to form radicals and then the phenolic radicals react with the intrinsic tyrosine residues of proteins to transfer their radicals. The tyrosyl radicals on the proteins subsequently react with each other to polymerize protein molecules. The oxidation of intrinsic tyrosine residues on proteins by the phenolic mediators occurs randomly, thus it is not favorable for maintaining the protein functions. In contrast, we added a flexible tyrosine residue at the termini of proteins as the Y-tags and the mediator-free and site-selective cross-linking of proteins was achieved by TL reaction, indicating that TL can be used as the enzyme for PTMs on funcitonal proteins.

Co-cross-linking of enzymes and antibody binding proteins can create detection probes. Alternatively, genetically fusing those proteins can provide a chimera protein, of which the conjugation ratio of component proteins is strictly controlled, however, it sometimes results in deminishing the protein functions due to incorrect folding of proteins. Since alkaline phosphatase (AP) is well known as one of reporter enzymes of immunohistochemistry (IHC), the APs or its AP-antibody binding protein conjugates will be applicable for applications such as ELISA and western blotting. Indeed, the enzymatic activity of BAP-pG_2pA-Y, which is the fusion protein of BAP and pG_2pA along with Y-tag, was lower than that of BAP-Y, suggesting that the tethering additional pG_2pA domains to the C-terminus of BAP affected the folding of BAP and decreased its catalytic activity. In addition, in order to create detection probes that exhibit high detection ability, the number of enzymes connected to the binding domain needs to be maximized, but the conjugation ratio of BAP and pG_2pA domains are always the same, 1:1 in such fusion protein, no matter how large protein polymer of BAP-pG_2pA is made. Thus, we believe that it is essential to make co-polymer of BAP and pG_2pA in the manner of PTMs to create high functional detection probes. In fact, we succeeded in making BAP-Y and Y-pG_2pA-Y co-polymers by using TL and and confirmed that they showed higher activity than

BAP-pG$_2$pA-Y in ELISA assay. The Western blot results of BAP:pG$_2$pA with various molar ratios also confirmed that the BAP/pG$_2$pA polymers were generated. The Mouse IgG anti-Histag monoclonal antibody that bound to the protein models was tracked the reaction of polymerization clearly. Compared to the signal obtained from BAP/pG$_2$pA polymer prepared at the molar ratio of 1:1, the signal obtained from the one prepared at 75:1 of molar ratio was 22-fold higher. However, the highest molar ratio, 100:1, was lower than 75:1. It might be caused by the size of the polymers was too large and the pG$_2$pA domains could not bind to the IgGs due to steric hinderance. Moreover, the activity of TL-catalyzed co-polymers of BAP-Y and Y-pG$_2$pA-Y in ELISA assay was higher than the HRP-catalyzed co-polymers. This is because of that the molecular size of the co-polymers prepared by the HRP reaction was larger than that of the ones formed by TL reaction (Figure 2.6A). These results obtained here clearly indicated the nessecity of controlling cross-linking degree of protein polymers to maximize the functionalities of them. TL has advantage over HRP in terms of controlling the cross-linking degree of proteins, due to its mild reaction condition (no need of H_2O_2) and slower reaction rate.

2.5 Conclusions

We demonstrated the TL-catalyzed cross-linking reaction of Y-tagged proteins to investigate the possibility of TL as an enzyme for PTMs targeting to Y-tags in this study. TL was able to catalyze cross-linking reaction of Y-tagged proteins specifically to yield protein polymers. Unlike small substrates, the TL-catalyzed protein cross-linking reaction occured efficiently at basic pH conditions but not in the acidic conditions. The BAP/pG$_2$pA polymers made by TL showed higher functionalities than the ones made by HRP. The results described here showed the potential of TL as an another enzyme for HRP to perform protein polymerization. Also, the need of controlling cross-linking degree of the protein polymers was revealed in this study and

TL possesses capabilities of achieving making protein polymers with different cross-linking degrees, which will open up the utilization of TL into biomedical and diagnosis fields of studies.

2.6 References

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CHAPTER 3 POLYMERIZATION OF HORSERADISH PEROXIDASE BY A LACCASE-CATALYZED TYROSINE COUPLING REACTION

3.1 Introduction

Polymerization of proteins or enzymes is a challenging research topic for a biochemist. In nature, some enzymes assemble to form dimers or oligomers, which is known as an essential biological event; for example, the assembly of pyrroline-5-carboxylate synthase (P5CS) and acetyl-CoA carboxylase (ACC) results in the improvement of the efficiency in cellular metabolisms [1-3]. Another example is a multienzyme complex bound on the cell wall surface of anaerobic bacteria, called cellulosome, which degrades complex cellulosic substrates [4]. Combining two or more proteins to form large and active polymers is one of the strategies to increase the activity, functionality and affinity of proteins for specific applications in several fields such as food chemistry [5,6], biotechnology and biomedicine [7]. The formation of new covalent bonds in protein polymerization is commonly assisted by chemical agents [8-13]. However, the lack of site-selectivity and protein deactivation or denaturation are problems that cannot be ignored. Enzymatic strategies [14-17] or chemoenzymatic techniques [18] are promising alternatives.

Horseradish peroxidase (HRP) is a well-known enzyme with broad applications. It is a reporter enzyme which is widely used as in immunohistochemistry, diagnostics, and biosensors [19-21]. Polymeric HRP may provide higher activity and performance than the monomeric HRP if it is utilized in enzymatic immunoassay (EIA) and organic synthesis catalysis. HRP and glucose oxidase (GO) are examples of glycoproteins, which are ideal and promising models for studying protein polymerization through the oxidation reaction using conventional methods such as chemical coupling with glutaraldehyde-mediated reductive amination [8, 22]. In an early report, Budnikova and Eryomin (2006) showed that the carbohydrate fragments of

HRP could be utilized for the polymerization reaction of HRP by using sodium periodate as an oxidizing agent [22]. However, recent reports on the polymerization of HRP utilizing the carbohydrate moiety followed chemical protocols, which required a one-day reaction and the use of inorganic solvents [8,19,22,23]. Another strategies of protein polymerization, including HRP, is the reversible addition-fragmentation chain transfer (RAFT) polymerization [24] and atom transfer radical polymerization (ATRP) [25]. RAFT polymerization is one of reversible deactivation radical polymerization (RDRP) methods that commonly used to synthesize protein-polymer conjugates that typically formed by grafting a polymer with protein using a chain transfer agent (CTA) or a small initiator molecules [24]. While, ATRP can be used in enzymatic-mediated radical polymerization of chemical monomers such as poly(ethylene glycol) methyl ether methacrylate (PEGMA). Some initiators are needed in ATRP such as ascorbic acid and alkyl halide (AH). This strategies are good for making chemical polymers, protein-polymer conjugates and some protein-protein conjugates with high molecular weight [26]. However, the use of chemicals, toxic solvents, initiator molecules, and high temperature of CTA attachment to the protein are some disadvantages of this strategy. In contrast, enzymatic polymerization of HRP provides an alternative strategy under mild conditions, high site-selectivity, retention of the activity of the HRP polymers and without targeting the carbohydrate moiety.

In our previous work, we modified the model proteins by adding specific and small peptide tags to target proteins to improve the enzyme-catalyzed cross-linking reaction of the proteins. Easily accessible and recognizable peptide tags can enhance the site-selectivity of reactions, which is a good strategy for enzymatic cross-linking of proteins. The phenolic moiety of the side chain of the tyrosine has unique chemical reactivity that has the potential to provide a quick method for the modifications of proteins including enzymatic or chemical reactions. Recently, we fused tyrosine-tags (Y-tags) with target proteins and polymerized them via

tyrosyl radicals to form a dityrosine-based protein polymer backbone [14,15,27]. The Y-tags can be recognized by some enzymes such as peroxidases [14,15,28], tyrosinase [16,17,29,30] and laccases [31–36]. Among those enzymes, laccase is a promising enzyme for post-translational modifications (PTMs) targeting Y-tagged proteins. It only requires molecular oxygen (O_2) to oxidize phenolic substrates and produce water as a side-product [37, 38]. In our previous report, we successfully applied a fungal laccase, *Trametes sp.* laccase (TL), to crosslink Y-tagged proteins in a site-selective manner and without additional phenolic mediators. TL performed the cross-linking reaction in a broad pH range. Therefore, it can be applied for the polymerization of a broad range of proteins with different stabilities depending on the solution pH [32].

Herein, we report the first enzymatic polymerization of HRP using TL as the catalyst. Our strategy did not utilize carbohydrate moieties of the HRP; the HRP was genetically modified with flexible peptide tags containing tyrosine (Y-tags) at its termini. TL site-selectively oxidized the phenolic moiety of the tyrosine residue of Y-tags in the recombinant HRPs to form free tyrosyl radicals and initiated the tyrosine coupling reaction to form active HRP polymers. Heteromeric cross-linking of different types of Y-tagged HRPs yielded active and large protein polymers, which can be applied for molecular diagnosis such as enzyme-linked immunosorbent assay (ELISA).

3.2 Materials and Methods

3.2.1 Preparation of Y-tagged HRPs

The Y-tagged HRPs were prepared based on our recent reports [39,40]. The gene-coding Y-tagged HRPs were ligated into the multicloning site of the pENTR11 vector with additional sequences (a L21 sequence of lobster and a *Bombyx mori* 30 K signal peptide gene) at its 5' end to construct the entry vectors of Y-HRP-Y, Y-HRP and Y-HRP-pG. The L21 sequence and 30 K signal peptide were used to improve the efficiency of the translation [41] and hemolymph secretion of HRPs [42], respectively. The baculovirus transfer vector was then constructed by joining the pENTR11 vectors carrying genes of HRPs and the pDEST8 vector by following the Gateway LR reaction protocols (Thermo Fisher Scientific, MA, USA). Then, the baculovirus that harboring the recombinant gene of Y-tagged HRPs were prepared according to the previous protocol [43]. The strain of silkworm that used is f38, which was provided by the Institute of Genetic Resources of Kyushu University. The larvae of silkworm were nourished at 24–27°C on mulberry leaves. The hemocoel of 3-day-old fifth instar silkworm larvae was injected with the recombinant baculovirus vector by using a Microliter™ syringe with a 30-gauge needle (Hamilton Co, USA). The larval legs were cut after four days of injection and the hemolymph was collected from each larva. The volume of hemolymph that obtained from one larva was approximately 0.4 mL. The Y-tagged HRPs were purified with a Ni-NTA column and anion exchange column from the hemolymph. The hemolymph samples containing HRPs were injected into the equilibrated Ni-NTA column (HisTrap Excel, 5 mL, GE Healthcare Life Sciences, Chicago, IL); equilibrated with a binding buffer (20 mM Tris-HCl pH 7.4, 35 mM imidazole, 500 mM NaCl). The column then was washed with the binding buffer (10 column volumes) and the bound protein was then eluted out with a gradient from 0 to 100% of an elution buffer (20 mM Tris-HCl pH 7.4, 500 mM imidazole, 500 mM NaCl). The fractions containing HRPs were collected and then applied to the HiPrep 26/10 Desalting

column (GE Healthcare Life Sciences) into 10 mM Tris-HCl (pH 8.0). The desalted protein solution was then injected to an anion exchange column (HiTrap Q HP, 5 mL, GE Healthcare Life Sciences) which was equilibrated with 10 mM Tris-HCl (pH 8.0). The HRP was eluted out by a gradient of 10 mM Tris-HCl (pH 8.0) from 0 to 100% that containing 500 mM NaCl. The fractions containing HRP were then collected and concentrated by using the 30 kDa MWCO of ultrafiltration membrane (Amicon Ultra-15 Centrifugal Filter Units, Merck Millipore Ltd., Billerica, MA, USA). The purified HRP concentration was determined by using bicinchoninic acid (BCA) assay with bovine serum albumin (BSA) was used as a standard (**Fig. S**1-**S**7 and **Table S**1 in supporting information).

Table 3.1 The full amino acid sequences of all the proteins used in this study[a]

Protein ID	Amino acid sequence
Y-HRP-Y	YGGAWSHPQFEKHHHHHHGGGENLYFQGGGSGGSQLTPTFYDNSCPNVSNIV RDTIVNELRSDPRIAASILRLHFHDCFVNGCDASILLDNTTSFRTEKDAFGN ANSARGFPVIDRMKAAVESACPRTVSCADLLTIAAQQSVTLAGGPSWRVPLG RRDSLQAFLDLANANLPAPFFTLPQLKDSFRNVGLNRSSDLVALSGGHTFGK NQCRFIMDRLYNFSNTGLPDPTLNTTYLQTLRGLCPLNGNLSALVDFDLRTP TIFDNKYYVNLEEQKGLIQSDQELFSSPNATDTIPLVRSFANSTQTFFNAFV EAMDRMGNITPLTGTQGQIRLNCRVVNSNSLVPRGSGGY
Y-HRP	YGGAWSHPQFEKHHHHHHGGGENLYFQGGGSGGSQLTPTFYDNSCPNVSNIV RDTIVNELRSDPRIAASILRLHFHDCFVNGCDASILLDNTTSFRTEKDAFGN ANSARGFPVIDRMKAAVESACPRTVSCADLLTIAAQQSVTLAGGPSWRVPLG RRDSLQAFLDLANANLPAPFFTLPQLKDSFRNVGLNRSSDLVALSGGHTFGK NQCRFIMDRLYNFSNTGLPDPTLNTTYLQTLRGLCPLNGNLSALVDFDLRTP TIFDNKYYVNLEEQKGLIQSDQELFSSPNATDTIPLVRSFANSTQTFFNAFV EAMDRMGNITPLTGTQGQIRLNCRVVNSNS
Y-HRP-pG	YGGAWSHPQFEKHHHHHHGGGENLYFQGGGSGGSQLTPTFYDNSCPNVSNIV RDTIVNELRSDPRIAASILRLHFHDCFVNGCDASILLDNTTSFRTEKDAFGN ANSARGFPVIDRMKAAVESACPRTVSCADLLTIAAQQSVTLAGGPSWRVPLG RRDSLQAFLDLANANLPAPFFTLPQLKDSFRNVGLNRSSDLVALSGGHTFGK NQCRFIMDRLYNFSNTGLPDPTLNTTYLQTLRGLCPLNGNLSALVDFDLRTP TIFDNKYYVNLEEQKGLIQSDQELFSSPNATDTIPLVRSFANSTQTFFNAFV EAMDRMGNITPLTGTQGQIRLNCRVVNSNSGGGSGGGSTYKLVINGKTLKGE TTTEAVDAATAEKVFKQYANDNGVDGEWTYDDATKTFTVTEKPEVIDASELT PAVT

[a]Protein/peptide fragments are labeled with different colors: Yellow: Y-tag; Red: Hexahistidine tag; Green: Flexible linker; Blue: Horseradish peroxidase; Brown: Protein G;

3.2.2 Activation of Y-tagged HRPs

The Y-tagged HRP apo-enzymes (10 μM) were mixed with hemin in three different molar concentration (10, 20 and 50 μM of hemin) (Sigma-Aldrich, MO, USA) in TBS (25 mM Tris-HCl pH 7.4, 150 mM NaCl) containing 1 mM $CaCl_2$ and 2% dimethylformaldehyde (DMF) and incubated overnight at 4°C. The 10 kDa MWCO of Slide-A-Lyzer^{TM} MINI Dialysis Device (Thermo Fisher Scientific) was used for buffer exchange in dialysis against TBS that containing 1 mM $CaCl_2$ for overnight at 4°C. Then, a 10 kDa MWCO of ultrafiltration membrane (Merck Millipore Ltd) was used to concentrate the dialyzed samples. The insertion of hemin into the structure of apo-HRPs was confirmed by measuring the UV-vis-absorption spectra on a Nanodrop 1000 (Thermo Fisher Scientific). The activated HRP concentrations were determined by using BCA assay with BSA as the standard.

3.2.3 Activity assay of HRP

The enzymatic activity of HRPs was measured by a 2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid ammonium salt (ABTS) assay. The substrate solution is consisting of 5 mg/mL of ABTS in 100 mM KH_2PO_4, pH 5.0 and 0.3% H_2O_2. The volume ratio of ABTS and H_2O_2 was 967 and 33 μL, respectively. This substrate solution was freshly prepared before the measurement. Sixteen microliters of the diluted HRPs solution (diluted by using 40 mM KH_2PO_4, pH 6.8 containing 0.25% BSA and 0.5% Triton-X100) was briefly mixed into 1 mL of the substrate solution, and the changes of absorbance at 405 nm was monitored for 3 min at 25°C on a on a UV-vis spectrophotometer (V-570, JASCO, Tokyo, Japan). The molar absorption coefficient of ABTS was used for the calculation (36800 M^{-1} cm^{-1} at 405 nm). One unit was defined as the enzymatic activity that oxidizes 1 μmol of ABTS in a minute at pH 5.0 at 25 °C.

3.2.4 Polymerization of HRPs

The polymerization reaction of Y-tagged HRPs and co-polymerization of Y-HRP-Y/Y-HRP-pG and Y-HRP/Y-HRP-pG were performed with a total volume of 50 μL in 10 mM Tris-HCl (pH 8.0) solution. The concentrations of the HRPs were 4 μM. The concentration of TL was optimized in this study. The mixture of reaction then was incubated for an appropriate time period at 37 ℃. The SDS-PAGE analysis (7.5-10% (w/v) of separating gel) was used to analyze the samples. Also, the self-cross-linking reaction of HRP was conducted by adding H_2O_2 solution of various concentrations into the HRPs and incubating at 37 °C.

3.2.5 ELISA assay of HRP polymers

The polymers of HRP were applied in ELISA assay as detection probes. The Tris-buffered saline (TBS) buffer (pH 7.4) was used to dilute the OVA (albumin from chicken eggs) to a final concentration 1 μg/mL and pipetted with a 100 μL/well into a 96-well microplate and incubated at 4 ℃ for overnight. The OVA-coated wells then were washed with 200 μL of TBST (TBS buffer containing 0.1% Tween 80) for 3 times. The blocking solution (TBST solution containing 1% BSA) was added (200 μL/well) and incubated for 1 h at RT. The wells then were washed with 200 μL of TBST for 3 times. The rabbit Anti-OVA-IgG (C6534-2 mL; Sigma–Aldrich) used as the primary antibody, which was diluted with TBST containing 1% BSA to a concentration of 1 μg/mL and then was added to each well with a volume of 100 μL and incubated for 2 h at RT. The wells then were washed with 200 μL of TBST for 3 times. The diluted HRP polymers then were added to each well (100 μL/well) and incubated for 1 h at RT. The catalytic activities of the immobilized HRP polymers were measured with ABTS as the substrate at 37 ℃ after washing the wells with 200 μL of TBST 3 times. The changes of absorbance at 405 nm was measured by using a 96-well microplate reader for 1 h at 37 °C.

3.2.6 Statistical analysis

ANOVA and Student's t-test functions in Microsoft Excel (Version 2016 for Macintosh, Microsoft Corp, Redmond, WA, USA) were used to perform statistical analysis. P values of < 0.05 were considered statistically significant.

3.3 Results and Discussion

3.3.1 The activity of silkworm-expressed Y-tagged HRPs

First, we confirmed the activity of the silkworm-expressed recombinant HRPs prepared in this study. We modified the HRPs by adding functional units, peptide tags for purification as well as a Y-tag and an antibody binding protein, which may have affected the activity of the HRPs. **Figure 3.1**A shows the construction of recombinant HRPs prepared in this study. The gene encoding HRPs were accompanied with gene encoding 30K signal peptide for secretion of HRP into the hemolymph of silkworms [41,42]. The spectrum of the purified recombinant HRPs before and after activation were shown in **Figure 3.1**B. The purified HRPs were in the form of apo-enzymes, showing no peak at 403 nm in the UV spectra, which corresponds to hemin-HRP complex; therefore, a further activation step was required to obtain the active form. After the activation process by incubating with hemin, Y-HRP, Y-HRP-Y and Y-HRP-pG showed a specific peak at 403 nm in the UV spectra, indicating the insertion of hemin into the HRP units (**Figure 3.1**B). Moreover, the activity of the HRPs depended on the presence of hemin (**Figure 3.1**C).

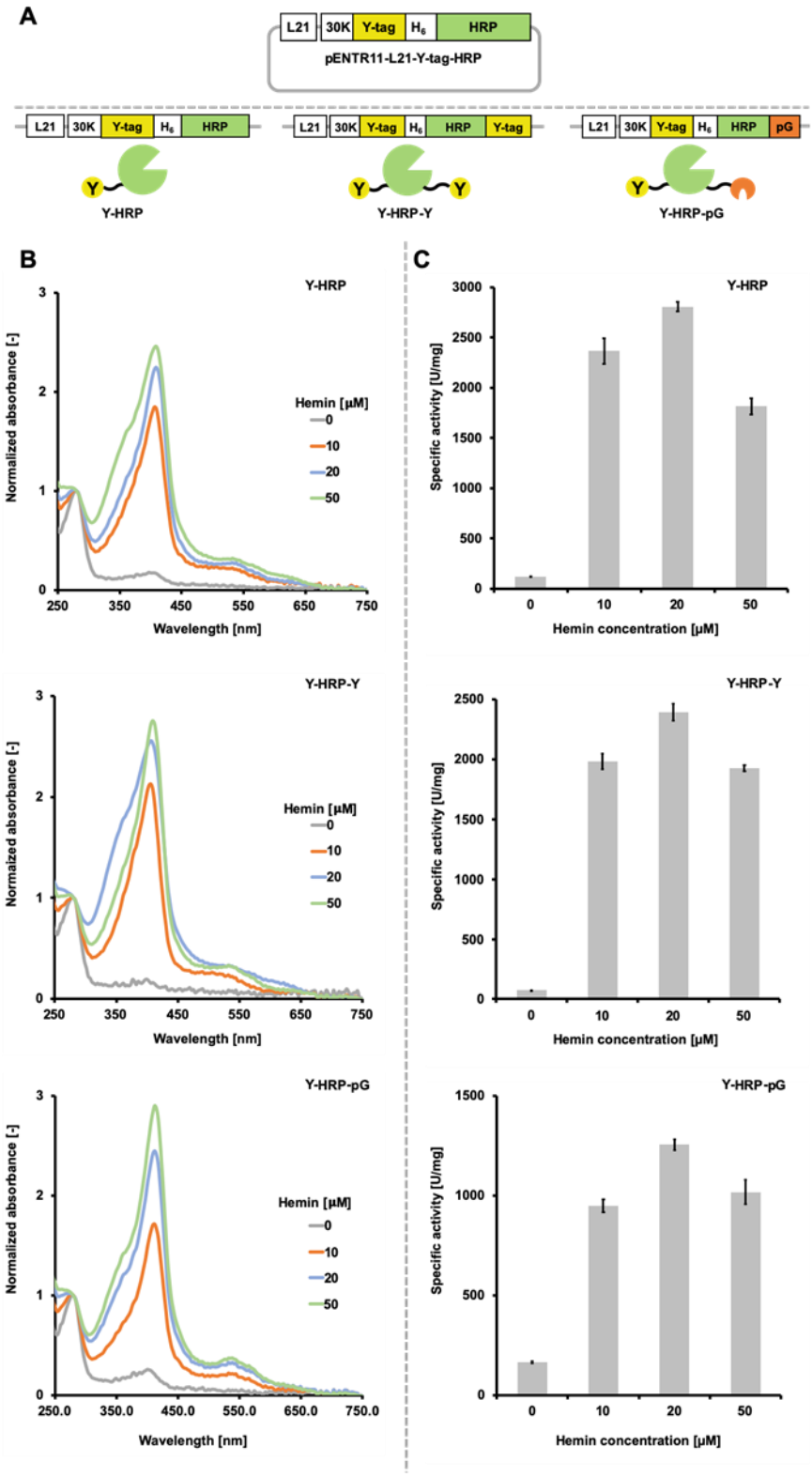

Figure 3.1 (A) A schematic illustration of the constructed expression plasmid of pENTR11 that was ligated with different gene encoded HRP with different modifications. (B) Spectrum of apo-form and activated Y-tagged HRPs with various concentration of hemin at 403 nm. (C) Enzymatic activity of Y-tagged HRPs by using ABTS as a substrate (5 mg/mL). Error bars represent the error of measurements from three individual samples (n = 3). Copyright 2019 reprinted with permission from Willey.

Two molar equivalents (2 eq.) or 20 μM of hemin to the HRP concentration was enough to fully activate the apo-enzyme of the HRPs; the activated HRPs showed the highest specific activity of all the Y-tagged HRPs. An excess concentration of hemin, 50 μM or 5 eq. relative to the HRPs, resulted in a lower activity of all the HRPs than in the case of 2 eq. of hemin. These results agreed with our previous study of HRP-pAG [40]. Compared with other Y-tagged HRPs, the Y-HRP showed the highest specific activity. The attachment of a flexible linker at the N- and C-termini of Y-HRP-Y and the fusion of a small protein at the C-termini of Y-HRP-pG could lower the catalytic activity of HRP. We observed the same trend when we genetically introduced a chimeric protein G and protein A (pG_2pA) to the *Escherichia coli* alkaline phosphatase (BAP) [32].

3.3.2 Polymerization of Y-tagged HRPs

Next, we determined the reactivity of commercial HRP (comm HRP) and silkworm-expressed HRPs against enzymatic reaction of TL to form HRP polymers (**Figure 3.2**A). The band of comm HRP treated with TL remained intact, indicating that TL does not recognize HRP itself as its substrate (**Figure 3.2**B). In contrast, the Y-tagged HRPs prepared in this study resulted in polymerized products in the high molecular weight region after treatment by TL. HRP has some tyrosine residues in its structure, however, the solid and large structure of HRP with a molecular weight of 44 kDa, makes those tyrosine residues difficult to be recognized by TL. TL did not recognize any tyrosine residue on the surface of comm HRP, primarily because of the steric hindrance of HRP. However, the tyrosine residues in the flexible Y-tags at the termini of Y-HRP-Y, Y-HRP and Y-HRP-pG could be accessed, recognized, and oxidized efficiently by TL to form polymers (> 210 kDa) [32]. The Y-HRP-Y, which has two Y-tag at its N- and C-termini, showed the best cross-linking efficiency than the other single Y-tagged HRPs. The results indicated that TL recognized and oxidized Y-tagged proteins in a site-selective manner

and this result agreed with our previous findings of TL-catalyzed polymerization of alkaline phosphatases [32]. The Y-tagged HRPs formed polymers within minutes to hours by simply incubating with TL. The TL-catalyzed tyrosine coupling reaction of Y-tagged HRPs provides advantages over chemical cross-linking agents, such as site-selectivity, mild reaction condition and retaining activity of the resulted polymeric HRPs.

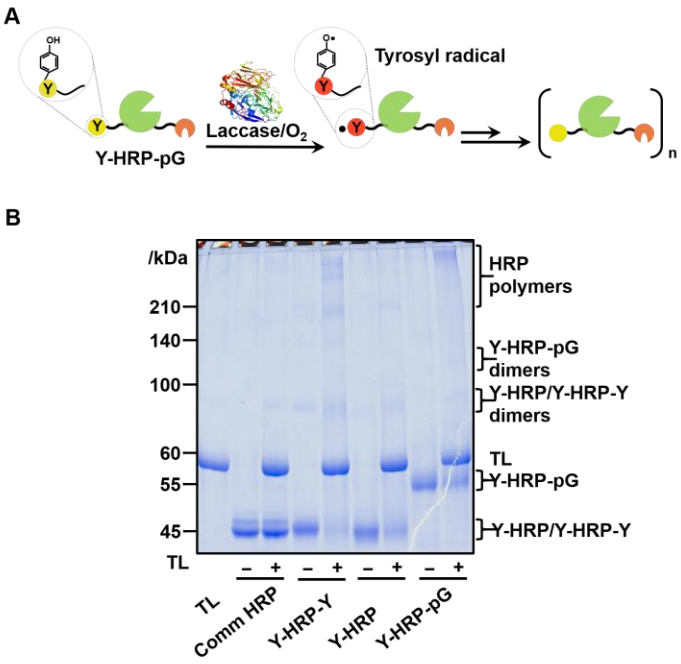

Figure 3.2 (A) A schematic illustration of the TL-catalyzed cross-linking reaction of a Y-tagged HRP used in this study, Y-HRP-pG, possessing a Y-tag sequence at its *N*-terminus. (B) SDS-PAGE analysis of TL-catalyzed cross-linking of comm HRP, Y-HRP-Y, Y-HRP and Y-HRP-pG. The concentrations of HRPs and TL were 4 and 3 μM, respectively. The reaction was conducted in 10 mM Tris-HCl, pH 8.0, at 37 °C for 2 h. Copyright 2019 reprinted with permission from Willey.

Herein, TL catalyzed the oxidative reaction of the phenolic moiety of tyrosine to form protein polymers without the use of a mediator. To the best of our knowledge, until now, researchers have used mediators to assist laccase in the oxidation reaction of phenolic compounds and to initiate the protein polymerization. Most of the mediators are phenolic acids and their derivatives including ferulic acid [17, 44, 45], *p*-coumaric acid [17, 33], caffeic acid [46, 47], vanillic acid [47] and hydrolyzed oat spelt xylan (hOSX) [17]. The mediators are small molecules and possess high reactivity against laccase to form radicals. The activated

mediators attack the phenolic moieties of the tyrosine residues of proteins to promote the polymerization of proteins; therefore, the cross-linking occurs in a random manner, not site-specifically. The tyrosine residues in the Y-tags in the recombinant HRPs could be recognized easily by TL and crosslinked to each other to form HRP polymers, substituting the function of mediators.

3.3.3 Comparison of self-cross-linking and TL-catalyzed cross-linking of Y-tagged HRPs

The addition of H_2O_2 to the Y-tagged HRPs can initiate their self-cross-linking, which is a simpler way to prepare HRP polymers than using TL. A comparison of self-cross-linking and TL-catalyzed cross-linking of Y-HRP-Y (**Figure 3.3**A), Y-HRP (**Figure 3.3**B), and Y-HRP-pG (**Figure 3.3**C) is shown in **Figure 3.3**. Indeed, the addition of H_2O_2 initiated self-cross-linking of Y-tagged HRPs. The efficiency of the self-cross-linking of the Y-tagged HRPs by H_2O_2 addition was comparable to that of the TL-catalyzed cross-linking reaction. However, the addition of H_2O_2 to the Y-tagged HRPs caused lowering of the activity of the resulting Y-HRP polymers, down to approximately 75% of the activity of the Y-tagged HRPs without the addition of H_2O_2 (**Figure 3.3**D). Even though only 2 μM H_2O_2 was added to 4 μM of the Y-tagged HRPs, the oxidation of hemin in the HRP unit occurred and caused loss of HRP activity [19, 20]. Interestingly, the TL treatment of the Y-tagged HRPs resulted in not only the polymerization of the HRPs, but it also enhanced the activity of the HRP polymers. The polymers of all the Y-tagged HRPs prepared by TL treatment showed higher activity than the controls (untreated HRPs). Even though TL can oxidize ABTS as its substrate, the activity of TL in the reaction mixture used to measure the HRP activity was much lower and was negligible. Also, the presence of H_2O_2 in the substrate solution for HRP activity measurement was harmful for TL and caused a decrease in the TL activity. Although it is not clear why the activity of the polymerized HRPs prepared by TL increased, the results indicated that the TL

reaction was more suitable for preparing the HRP polymers than using the self-cross-linking reaction of the Y-tagged HRPs. The active HRP polymers formed by TL are promising for further studies and applications.

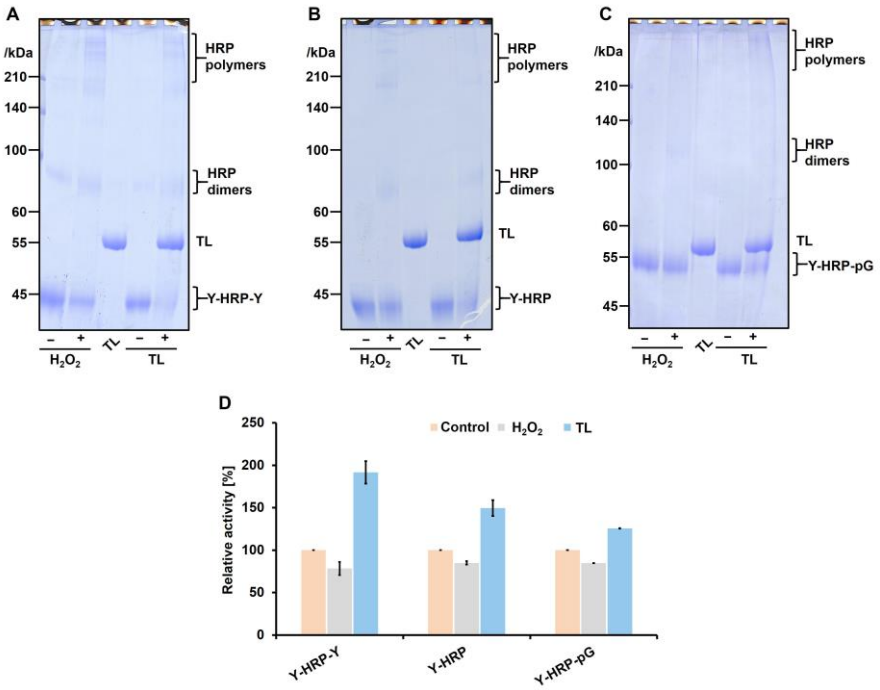

Figure 3.3 SDS-PAGE of hydrogen peroxide-mediated self-cross-linking and TL-catalyzed cross-linking of Y-tagged HRPs in 10 mM Tris-HCl, pH 8.0 at 37 °C for 2 h. The concentrations of Y-tagged HRPs, H_2O_2, and TL were 4, 2, and 3 μM, respectively. The self-cross-linking and cross-linking of (A) Y-HRP-Y, (B) Y-HRP and (C) Y-HRP-pG against H_2O_2 and TL concentration. (D) Relative activity of the Y-tagged HRPs after addition of H_2O_2 and TL treatment. The activity of Y-tagged HRPs without any treatment (Control) was calculated as 100%. Error bars represent the error of the measurements from three individual samples (*n* = 3). Copyright 2019 reprinted with permission from Willey.

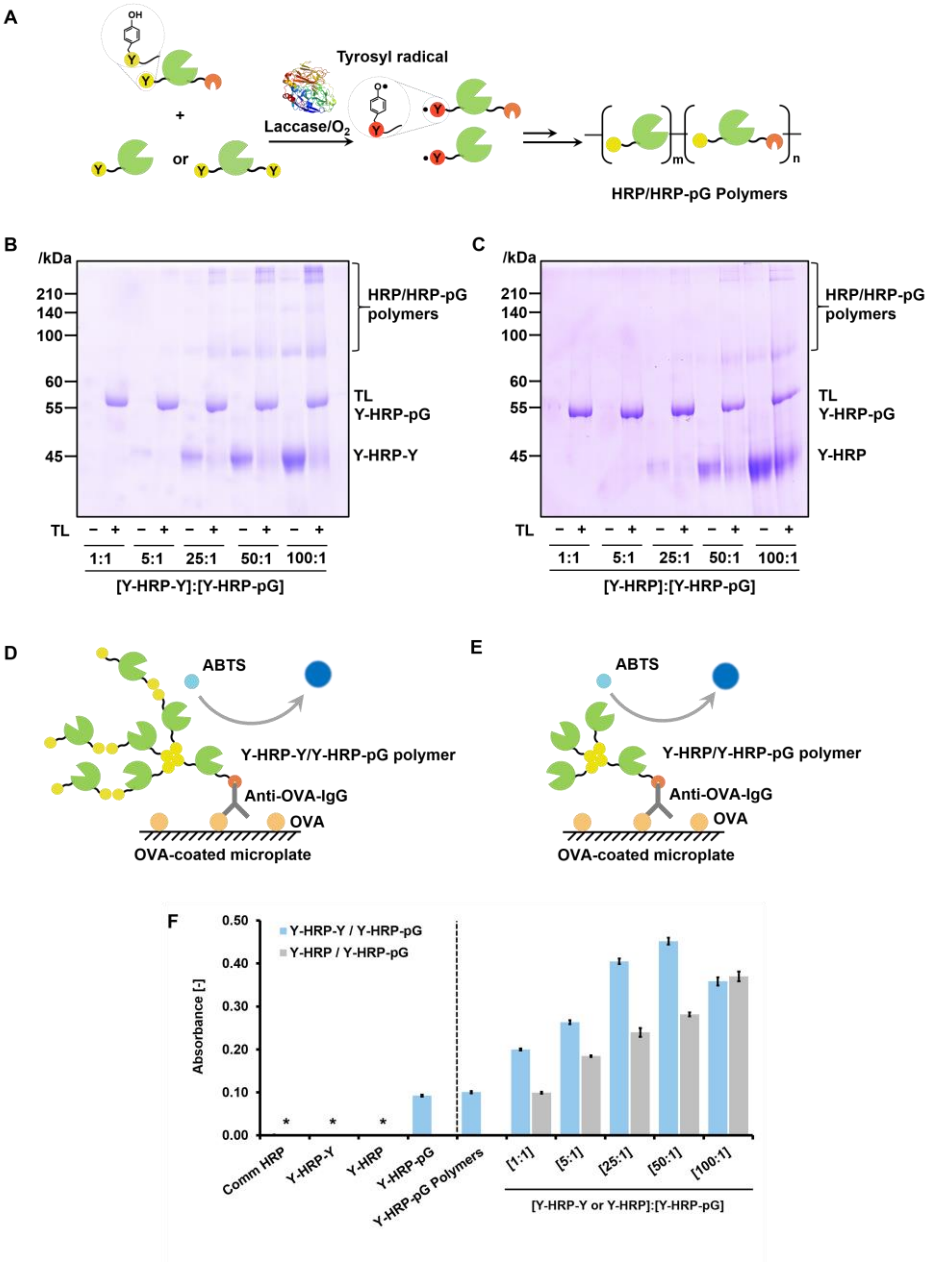

Figure 3.4 (A) Schematic illustration of the co-polymerization reaction of Y-HRP or Y-HRP-Y with Y-HRP-pG to form HRP/HRP-pG polymers. SDS-PAGE analysis of TL-catalyzed hetero-cross-linking of (B) Y-HRP/Y-HRP-pG and (C) Y-HRP-Y/Y-HRP-pG. The molar ratios of Y-HRP-Y or Y-HRP to Y-HRP-pG were 1:1, 5:1, 25:1, 50:1 and 100:1. The concentration of Y-HRP-pG was fixed at 0.25 µM. The cross-linking reaction was conducted in 10 mM Tris-HCl, pH 8.0, for 2 h at 37 °C. (D, E) A schematic illustration of HRP/HRP-pG polymers in ELISA. (F) Results of ELISA detecting OVA using Y-HRP-pG, Y-HRP-pG polymers and HRP/HRP-pG polymers prepared at different molar ratios of Y-HRP or Y-HRP-Y to Y-HRP-pG. Comm HRP, Y-HRP and Y-HRP-Y were used as controls. The absorption of OVA onto the microplate was conducted at 1 µg/mL in TBS (pH 7.4). Error bars represent the error of the measurements from five individual samples (n = 5). An asterisk indicates that there was no signal detected from the samples by the microplate reader. Copyright 2019 reprinted with permission from Willey.

3.3.4 HRP/HRP-pG polymers exhibit excellent functionality in ELISA

Lastly, we verified the ability of using HRP polymers as detection probes. To make a detection probe for ELISA, we conducted a co-polymerization reaction of Y-HRP-pG and Y-HRP-Y or Y-HRP. The schematic illustration of the TL-catalyzed co-polymerization of two Y-tagged HRPs is shown in **Figure 3.4**A. The results of the co-polymerization reaction of Y-HRP-pG and Y-HRP-Y or Y-HRP are shown in **Figure 3.4**B and C. The Y-HRP-pG concentration was fixed to 0.2 μM and the concentration of Y-HRP-Y and Y-HRP was varied from 0.2 μM to 20 μM. The co-polymerization of Y-HRP-Y and Y-HRP-pG (**Figure 3.4**B) yielded HRP/HRP-pG polymers (>210 kDa) with higher efficiency than that of Y-HRP and Y-HRP-pG (**Figure 3.4**C). **Figure 3.4**B suggesting that the two Y-tags in Y-HRP-Y efficiently crosslinked to form a polymeric HRP domain in the co-polymer with Y-HRP-pG. The pG unit in the HRP/HRP-pG polymer plays a role of anchoring domain and binds specifically to the immobilized Anti-OVA IgG along with the polymeric HRPs (**Figures 3.4**D and 3.**4**E). Finally, we compared the activity of the recombinant HRPs and their polymers in an ELISA assay. The comm HRP, Y-HRP- and Y-HRP, did not show any signal in ELISA assay, because those samples contained no pG domain (**Figure 3.4**F). However, the Y-HRP-pG and its polymers showed low absorbance with slight differences between each other, suggesting that the polymerization of Y-HRP-pG could not improve their functionality as detection probes. The result can be explained by the ratio of HRP and pG in the polymer of Y-HRP-pG, which is fixed at 1:1 regardless of the size of the Y-HRP-pG polymer formed. It is essential to increase the ratio of the enzyme against the binding domain to enhance the functionality of the detection probes. A 50:1 molar ratio of Y-HRP-Y/Y-HRP-pG showed the highest signal in ELISA, which was more than 4-fold higher than that of Y-HRP-pG and its polymers. However, the sample prepared with a Y-HRP-Y/Y-HRP-pG molar ratio of 100:1 resulted in a lower absorbance change than the one prepared with a ratio of 50:1. The steric hindrance of the large macromolecular

assembly of the HRP/HRP-pG polymer at the solid/liquid surface could be responsible for this result. The co-polymerization of Y-HRP/Y-HRP-pG was also performed under the same conditions. The sample prepared with a Y-HRP/Y-HRP-pG molar ratio of 100:1 showed the highest signal, which was more than 3-times higher than that of Y-HRP-pG and its polymers and the signal of Y-HRP-Y/Y-HRP-pG polymers prepared with the same molar ratio. These results suggest that the HRP/HRP-pG polymers prepared by the combination of Y-HRP-Y/Y-HRP-pG units resulted in a large macromolecular assembly with greater functionality. Overall, the HRP/HRP-pG polymers provide high functionality and activity for further development of diagnostic probes in biotechnology and biomedical applications.

3.4 Conclusions

In this study, we proposed a new strategy for the polymerization of HRP by creating Y-tagged HRPs and conducting polymerization of HRPs through the Y-tags using laccase. Compared with the self-cross-linking reaction of Y-tagged HRPs and chemical polymerization of HRP in the other reports, the TL-mediated cross-linking reaction of the Y-tagged HRPs provides site-selective polymerization at the Y-tags and mild reaction conditions, resulting in retaining of the activity of the resulting HRP polymers. The use of a Y-tag can substitute the use of mediators in laccase-catalyzed protein cross-linking reactions. The utilization of tyrosine coupling for new covalent bond formation resulted in active and highly functional HRP-based polymers, which could be further extended for the design of new biomaterials and bioconjugates applicable to biotechnology.

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CHAPTER 4 LINEAR POLYMERIZATION OF PROTEIN BY STERICALLY CONTROLLED ENZYMATIC CROSS-LINKING WITH A TYROSINE-CONTAINING PEPTIDE LOOP

4.1 Introduction

Conjugation of protein molecules into a large protein complex may expand the functionalities of proteins because the protein units in the complex may cooperatively work together. However, it is challenging to assemble a large number of proteins into a protein complex while retaining the functionality of each protein unit. Some protein units might get deactivated during conjugation if the conjugation occurs at a critical site, and some proteins may not be able to function in the complex because of steric hindrance. Therefore, it is essential to conjugate protein units in a site-specific manner, and the structure of the protein complex needs to be controlled appropriately, so that the protein units in the complex can work efficiently [1].

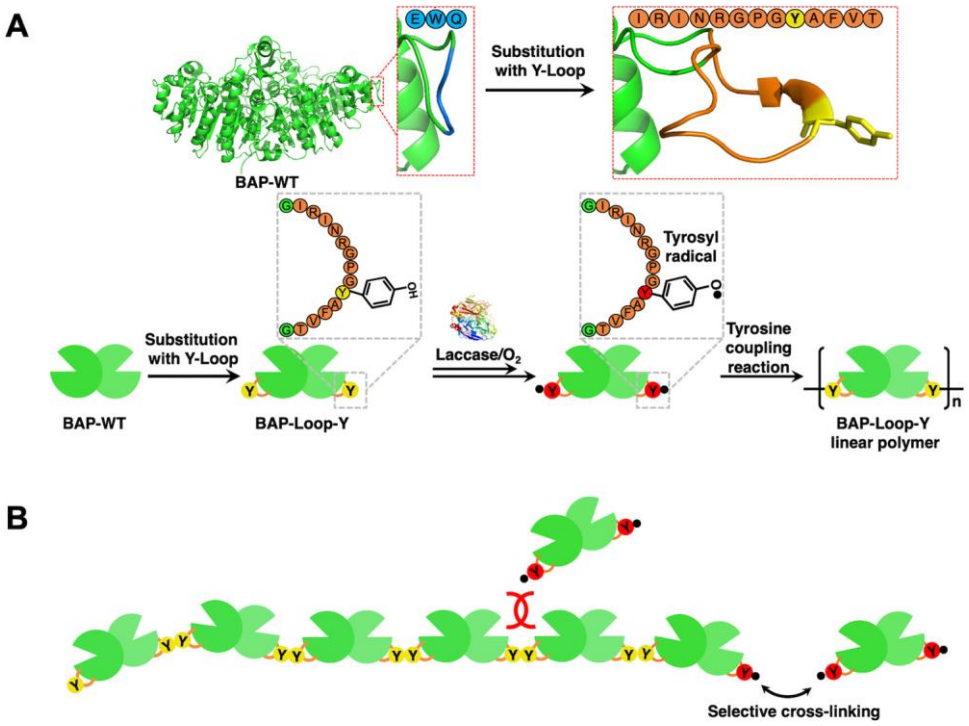
Genetic fusion of proteins is a common method to create artificial protein assemblies or protein polymers [2-7]. However, the possible number of protein units that can be conjugated is limited because of the difficulty in the expression of fusion proteins, and the tandem fusion strategy requires careful linker designs to organize the different proteins and control their characteristics [1,5,6]. Such strategies also decrease the degrees of freedom of the protein dynamics, which may lead to impaired activity and protein misfolding [1,2,8,9]. Another approach to assemble proteins into a protein complex is to use post-translational modifications (PTMs) [10-12]. PTMs enable a higher degree of polymerization than the genetic fusing method. Especially, PTMs using enzymatic reactions are ideal since enzymes can provide high site-selectivity with rapid and mild reaction conditions to retain the activity of protein units [13-16].

Horseradish peroxidase (HRP) and laccase have been utilized for making covalently cross-linked protein complexes with a high degree of conjugation, i.e., protein polymers. HRP and laccase are more suitable for making huge protein polymers than other PTM enzymes, such as transglutaminase [17,18], and sortase A [19,20]. This is because they conduct oxidation reactions on tyrosine residues to yield tyrosyl radicals, and the cross-linking between tyrosyl radicals occurs non-enzymatically without forming tripartite complexes, which are necessary for other enzymes [21-23]. In our previous studies, we demonstrated the polymerization of bacterial alkaline phosphatase (BAP), as well as HRP, by a *Trametes sp.* laccase (TL) reaction. Both BAP and HRP were recombinantly prepared and tethered with peptide tags containing tyrosine residues (Y-tag) [16,24]. The Y-tagged BAPs and HRPs were site-specifically cross-linked by TL to form polymers. Laccase has an advantage over HRP because it requires only molecular oxygen to catalyze the oxidation of tyrosine residues, while HRP requires hydrogen peroxide, which is potentially harmful to proteins [25].

The obtained dendritic protein polymers of BAPs and HRPs were utilized as detection probes in enzyme-linked immunosorbent assay (ELISA) by co-polymerizing with antibody binding proteins (ABPs), such as protein A (pA), and protein G (pG). Although the polymerization did not affect the activity of enzyme units in the protein polymers, a large number of enzyme units were required to co-polymerize with the ABPs to surpass the performance of the genetically-fused 1:1 conjugate. In previous work, the ELISA signal of the protein polymers became saturated at high molar ratios of enzyme to ABPs and even decreased at the highest molar ratio (100:1, enzyme units: ABPs) [16,24]. These results suggest that some of the enzyme and ABP units are buried inside the dendritic structure of the protein polymers and could not function appropriately. Also, the three-dimensionally dendritic protein polymers could not be packed efficiently onto the surface of an ELISA plate and they occupy a large surface area per protein polymer resulting in fewer enzyme units being immobilized [16,24].

One possible strategy to solve this problem is to create linear protein polymers [28]. A linear polymer is much simpler and less sterically hindered than 3D dendritic structures; thus, protein units in a linear protein polymer can work more efficiently. Also, a linear molecular structure is an ideal shape for maximizing the amount of protein polymer immobilized on the surface.

The conventional Y-tag sequences were highly flexible, so the cross-linked dityrosine could again be a substrate for TL, leading to the formation branched structures [23,26,27,29]. We anticipated that by introducing appropriate steric hindrance to the cross-linked dityrosine, the TL could not recognize it and thereby prevent the formation of branched structures and favoring linear polymers. Herein, we report the linear polymerization of engineered BAPs by using a tyrosine-containing loop peptide (Y-Loop). We genetically modified the BAP by substituting the loop domain of the BAP structure (219-221 aa) with a 13-mer Y-Loop (IRINRGPGYAFVT) to construct BAP-Loop-Y (**Scheme 4.1**A). We designed the Y-Loop by modifying the loop peptide sequence from the previous reports [30,32]. We anticipated that the Y-Loop could be recognized by TL to cross-link only once because the cross-linked Y-Loop will be too sterically encumbered to react again with TL (**Scheme 4.1**B). We conducted a scanning probe microscopy (SPM) analysis to confirm the structural differences between the BAP-Loop-Y polymer and the Y-tagged BAP (BAP-Y) polymer, which were prepared by TL treatment. Furthermore, copolymerization of BAP-Y or BAP-Loop-Y and a single Y-tagged ABP, pG_2pA-Y, was performed to form BAP-Y/pG_2pA-Y and BAP-Loop-Y/pG_2pA-Y copolymers. The ability of these copolymers to function as ELISA probes was also evaluated. Our proof-of-concept study on linear protein polymerization could contribute a new strategy to create highly functional one-dimensional protein complexes in biotechnological applications.



Scheme 4.1 The polymerization reaction of a Y-Looped bacterial (*E. coli*) alkaline phosphatase (BAP), BAP-Loop-Y, catalyzed by TL. (A) The BAP-Loop-Y were constructed by substituting the loop domain of the BAP structure (219-221 aa) with a 13-mer Y-Loop (IRINRGPGYAFVT) peptide. TL recognizes and oxidizes phenolic moieties of tyrosine residues on the Y-Loop to form reactive tyrosyl radicals, which then react with each other in a non-enzymatic way to form polymers. (B) A short and sterically hindered Y-Loop made the gap between the BAP-Loop-Y units very small and prevented another activated BAP-Loop-Y reacting at that position. Copyright 2020 reprinted with permission from the American Chemical Society (ACS).

4.2 Materials and Methods

4.2.1 Materials

TL was provided by Amano Enzyme Inc. (Nagoya, Japan). TL was dissolved in 100 mM sodium phosphate buffer, pH 6.0, and purified from impurities using an Amicon ultrafiltration device (50 kDa MWCO). Dipotassium hydrogen phosphate, sodium chloride, potassium dihydrogen phosphate, para-nitrophenyl phosphate (*p*-NPP), sodium dodecyl sulfate (SDS), hydrochloric acid, tetramethylethylenediamine (TEMED), and sodium ampicillin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Tryptone and yeast

extract were purchased from Difco (Miami, U.S.A). Trizma^{TM} base and imidazole were purchased from Sigma Aldrich (Tokyo, Japan). Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Takara Bio Inc. (Shiga, Japan). Acrylamide/bis solution 30% was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). All reagents were used as received.

4.2.2 Preparation of Y-tagged proteins

We constructed *E. coli* expression vectors of the proteins used in this study by using the plasmid vectors of pET22b(+)-BAP and pET22b(+)-pG_2pA as templates [14,24,47]. The pG_2pA is a chimeric protein composed of two protein G (pG) domains and a protein A (pA) domain fused in tandem [47]. To construct the expression vector of the BAP-Loop-Y, the DNA encoding the EWQ at the position of the loop domain of BAP was replaced with the DNA encoding the Y-Loop sequence (IRINRGPGYFVT). We designed the sequence of Y-Loop based on the loop peptide sequence from the previous reports [30,31]. The amino acid sequences of the proteins used in this study are listed in the Table 4.1. All the proteins were expressed and purified according to a previously reported protocol [14,24].

4.2.3 Enzymatic assay of BAPs

p-NPP was used as a substrate to measure the activity of the BAPs. The BAPs were diluted to an appropriate concentration using 10 mM Tris-HCl (pH 8.0). We pipetted 10 µL of BAP solutions into 990 µL of 1 mM *p*-NPP in 1 M Tris-HCl (pH 8.0) in a 1 mL quartz cell for UV-vis spectrophotometry. We monitored the absorbance at 410 nm for 3 min at 25 °C. The measurement was replicated three times for three individual samples of each BAP.

Table 4.1 The full amino acid sequences of all the proteins used in this study[a]

Protein ID	Amino Acid Sequence
BAP-WT	MKYLLPTAAAGLLLLAAQPAMA↓MDIGINSDPHHHHHHTPEMPVLENRAAQG DITAPGGARRLTGDQTAALRDSLSDKPAKNIILLIGDGMGDSEITAARNYAE GAGGFFKGIDALPLTGQYTHYALNKKTGKPDYVTDSAASATAWSTGVKTYNG ALGVDIHEKDHPTILEMAKAAGLATGNVSTAELQDATPAALVAHVTSRKCYG PSATSEKCPGNALEKGGKGSITEQLLNARADVTLGGGAKTFAETATAGEWQG KTLREQAQARGYQLVSDAASLNSVTEANQQKPLLGLFADGNMPVRWLGPKAT YHGNIDKPAVTCTPNPQRNDSVPTLAQMTDKAIELLSKNEKGFFLQVEGASI DKQDHAANPCGQIGETVDLDEAVQRALEFAKKEGNTLVIVTADHAHASQIVA PDTKAPGLTQALNTKDGAVMVMSYGNSEEDSQEHTGSQLRIAAYGPHAANVV GLTDQTDLFYTMKAALGLK
BAP-pG$_2$pA-Y	MKYLLPTAAAGLLLLAAQPAMA↓MDIGINSDPHHHHHHTPEMPVLENRAAQG DITAPGGARRLTGDQTAALRDSLSDKPAKNIILLIGDGMGDSEITAARNYAE GAGGFFKGIDALPLTGQYTHYALNKKTGKPDYVTDSAASATAWSTGVKTYNG ALGVDIHEKDHPTILEMAKAAGLATGNVSTAELQDATPAALVAHVTSRKCYG PSATSEKCPGNALEKGGKGSITEQLLNARADVTLGGGAKTFAETATAGEWQG KTLREQAQARGYQLVSDAASLNSVTEANQQKPLLGLFADGNMPVRWLGPKAT YHGNIDKPAVTCTPNPQRNDSVPTLAQMTDKAIELLSKNEKGFFLQVEGASI DKQDHAANPCGQIGETVDLDEAVQRALEFAKKEGNTLVIVTADHAHASQIVA PDTKAPGLTQALNTKDGAVMVMSYGNSEEDSQEHTGSQLRIAAYGPHAANVV GLTDQTDLFYTMKAALGLKGGGSTYKLVINGKTLKGETTTEAVDAATAEKVF KQYANDNGVDGEWTYDDATKTFTVTEKPEVIDASELTPAVTTYKLVINGKTL KGETTTKAVDAETAEKAFKQYANDNGVDGVWTYDDATKTFTVTEGGGGSDVD NKFNKEQQNAFWEILHLPNLNEEQRNGFIQSLKDDPSQSANLLAEAKKLNDA QAPKLVPRGSGGGSGGGGY
BAP-Y	MKYLLPTAAAGLLLLAAQPAMA↓MDIGINSDPHHHHHHTPEMPVLENRAAQG DITAPGGARRLTGDQTAALRDSLSDKPAKNIILLIGDGMGDSEITAARNYAE GAGGFFKGIDALPLTGQYTHYALNKKTGKPDYVTDSAASATAWSTGVKTYNG ALGVDIHEKDHPTILEMAKAAGLATGNVSTAELQDATPAALVAHVTSRKCYG PSATSEKCPGNALEKGGKGSITEQLLNARADVTLGGGAKTFAETATAGEWQG KTLREQAQARGYQLVSDAASLNSVTEANQQKPLLGLFADGNMPVRWLGPKAT YHGNIDKPAVTCTPNPQRNDSVPTLAQMTDKAIELLSKNEKGFFLQVEGASI DKQDHAANPCGQIGETVDLDEAVQRALEFAKKEGNTLVIVTADHAHASQIVA PDTKAPGLTQALNTKDGAVMVMSYGNSEEDSQEHTGSQLRIAAYGPHAANVV GLTDQTDLFYTMKAALGLKGGGSLVPRGSGGGSGGGGY
BAP-Loop-Y	MKYLLPTAAAGLLLLAAQPAMA↓MDIGINSDPHHHHHHTPEMPVLENRAAQG DITAPGGARRLTGDQTAALRDSLSDKPAKNIILLIGDGMGDSEITAARNYAE GAGGFFKGIDALPLTGQYTHYALNKKTGKPDYVTDSAASATAWSTGVKTYNG ALGVDIHEKDHPTILEMAKAAGLATGNVSTAELQDATPAALVAHVTSRKCYG PSATSEKCPGNALEKGGKGSITEQLLNARADVTLGGGAKTFAETATAG**IRIN** **RGPGYAFVT**GKTLREQAQARGYQLVSDAASLNSVTEANQQKPLLGLFADGNM PVRWLGPKATYHGNIDKPAVTCTPNPQRNDSVPTLAQMTDKAIELLSKNEKG FFLQVEGASIDKQDHAANPCGQIGETVDLDEAVQRALEFAKKEGNTLVIVTA DHAHASQIVAPDTKAPGLTQALNTKDGAVMVMSYGNSEEDSQEHTGSQLRIA AYGPHAANVVGLTDQTDLFYTMKAALGLK
pG$_2$pA-Y	MHHHHHHTYKLVINGKTLKGETTTEAVDAATAEKVFKQYANDNGVDGEWTYD DATKTFTVTEKPEVIDASELTPAVTTYKLVINGKTLKGETTTKAVDAETAEK AFKQYANDNGVDGVWTYDDATKTFTVTEGGGGSDVDNKFNKEQQNAFWEILH LPNLNEEQRNGFIQSLKDDPSQSANLLAEAKKLNDAQAPKGGGGY

[a]Protein/peptide fragments are labeled with different colors:
Red: Signal peptide; Orange: Hexahistidine tag; Purple: Alkaline phosphatase; Green: Flexible linker; Cyan: Protein G; Dark red: Protein A; Grey: thrombin recognition sequence; Blue: Y-tag.
Yellow Shaded: Y-Loop

4.2.4 TL-catalyzed polymerization of proteins

We conducted the polymerization of the BAPs and the copolymerization of BAP-Loop-Y/pG_2pA-Y and BAP-Y/pG_2pA-Y by mixing with TL in 10 mM Tris-HCl (pH 8.0) in a total volume of 50 µL. The final concentration of the BAP was 10 µM, and we optimized the concentration of TL and reaction time. The polymerization reaction was performed by incubating a mixture of the Y-tagged proteins and TL at 37 °C for an optimized length of time. The samples were analyzed by SDS-PAGE and native-PAGE.

4.2.5 Scanning probe microscopy (SPM) imaging of the BAP polymers

Samples of BAP monomers and polymers were diluted 10 to 50 times using 10 mM Tris-HCl (pH 8.0). Four microliters of the sample solution were then placed onto mica, which was freshly cleaved (Nilaco Corp., Tokyo, Japan), and incubated for 5-10 minutes at room temperature. The mica was then rinsed once using ultrapure water and dried under ambient conditions. The SPM images were taken using a Nanocute (Hitachi High-Tech Science Corp., Japan) using an SI-DF3P2 microcantilever (Hitachi) [48] in the dynamic force mode.

4.2.6 ELISA using BAP polymers

The Y-tagged BAP polymers were used as detection probes in an ELISA. The OVA (albumin from chicken eggs) was diluted to concentrations ranging from 10 µg/mL to 0.1 ng/mL using Tris-buffered saline (TBS), pH 7.4, and pipetted into a flat bottom microplate (96-well, 100 µL/well) and the microplate was incubated overnight at 4 °C. The OVA-coated wells were then washed with 200 µL of TBST (TBS containing 0.1% Tween 20), 3 times. The blocking solution (TBST solution containing 1% BSA) was added (200 µL/well) and incubated for 1 h at RT. The wells were then washed with 200 µL of TBST, 3 times. The rabbit anti-OVA-IgG (C6534-2 mL; Sigma–Aldrich), used as the primary antibody, was diluted with TBST containing 1%

BSA to a concentration of 1 μg/mL and then 100 μL was added to each well, followed by 2 h incubation at RT. The wells were washed with 200 μL of TBST, 3 times. The diluted BAPs, BAP-Loop-Y/pG_2pA, and BAP/pG_2pA polymers were then added to each well (100 μL/well) and followed by 1 h incubation at RT. After washing the wells with 200 μL of TBST, 3 times, 100 μL of *p*-NPP solution (1 mM in 1 M Tris-HCl (pH 8.0)) was added to each well. A 96-well microplate reader (BioTek, Synergy HTX) was used to monitor the changes of the absorbance at 410 nm for 1 h at 25 °C.

4.2.7 Size exclusion chromatography (SEC) of BAP copolymers

The SEC samples of BAP copolymers were prepared according to the same procedures for TL-catalyzed polymerization of proteins which mentioned above. The molar ratio of BAPs to pG_2pA-Y was set at 100:1 (50 μM to 0.5 μM) with a total volume of 1 mL in 10 mM Tris-HCl, (pH 8.0). The concentration of TL was adjusted to 5 μM to facilitate high copolymerization efficiency between the BAPs and pG_2pA-Y in a large volume. The polymerization reaction was conducted at 37 °C for 18 hours. The copolymer solution was then injected into the SEC column (HiLoad Superdex 200 pg, GE Healthcare). The SEC mobile phase was 10 mM Tris-HCl (pH 8.0). The fractions containing polymers were pooled and concentrated by using an ultrafiltration membrane (30 kDa MWCO) and analyzed with SDS-PAGE and native-PAGE. The enzymatic activity of the polymers was measured, and they were functionally evaluated as probes in an ELISA.

4.2.8 Statistical analysis

ANOVA and Student's t-test functions in Microsoft Excel (Version 2016 for Macintosh, Microsoft Corp, Redmond, WA, USA) were used to perform statistical analysis. P values of < 0.05 were considered statistically significant.

4.3 Results and Discussion

4.3.1 The enzymatic activity of recombinant BAPs

We employed *E. coli*-expressed BAPs with different structures as model proteins for the protein polymerization reaction. **Figure 4.1**A shows the constructs of the BAPs used in this study. We used the wild type BAP (BAP-WT) as the control. BAP-Y and BAP-pG_2pA-Y were tagged with a flexible peptide sequence containing a tyrosine residue (Y-tag) at the C-termini. All the BAPs were expressed in *E. coli* with high expression levels. Although the expression level of BAP-Loop-Y was as high as the other BAPs, we observed cleavage of BAP-Loop-Y during expression and purification, probably somewhere around the Y-Loop. The cleaved BAP-Loop-Y eluted at the same elution volume in SEC purification, indicating that the cleaved BAP-Loop-Y retained its tertiary and quaternary structure. Different constructs of BAP showed different specific activities, and the modification of the BAPs with Y-tags, especially BAP-Y, exhibited approximately 34% higher activity than that of the wild type (**Figure 4.1**B). However, BAP-pG_2pA-Y showed lower activity than the other BAPs; it showed approximately 55% of the specific activity of BAP-WT. This is consistent with the data obtained in our previous report [24], suggesting that genetically fusing pG_2pA to BAP is responsible for decreasing the BAP activity. BAP-Loop-Y showed comparable activity to that of BAP-WT. This result also agreed with previous reports [30,31]. The Y-Loop peptide was inserted into the BAP structure at a position away from the active site of BAP; therefore, there was no detrimental effect on the ability of the BAPs to recognize their substrates.

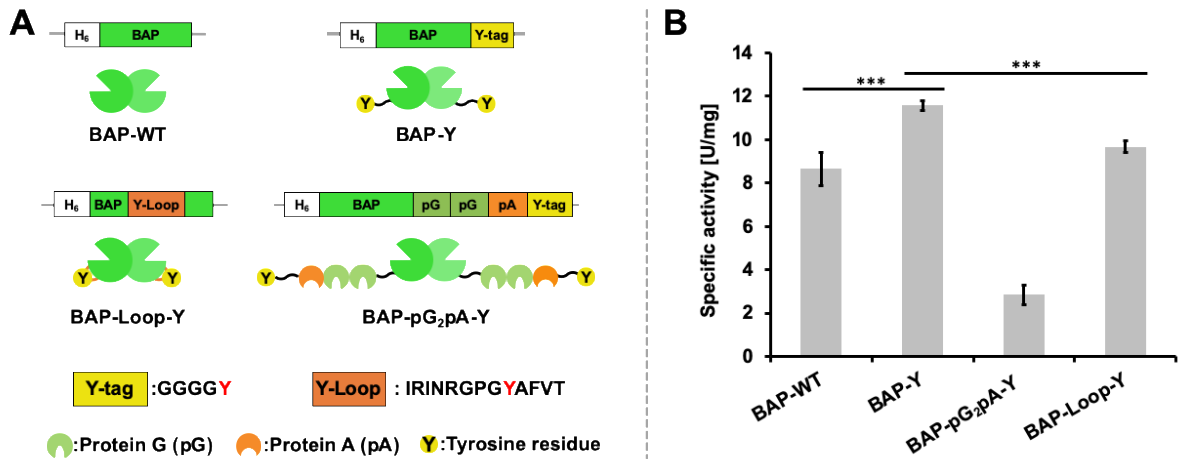


Figure 4.1 The recombinant bacterial (*E. coli*) alkaline phosphatases (BAPs) used in this study. (A) The constructs and structural illustration of the recombinant BAPs prepared in this study; BAP-WT, wild-type (unmodified) BAP; BAP-Y, BAP with a a peptide tag containing tyrosine residue (Y-tag) at C-termini; BAP-pG$_2$pA-Y, BAP with a Y-tagged chimeric antibody-binding protein, pG$_2$pA-Y, at C-termini; BAP-Loop-Y, BAP inserted Y-Loop sequence at 221-223 aa. Three of them were tagged with Y-tags that can be recognized by TL. (B) Specific activities of *E. coli*-expressed BAPs using *p*-NPP as the substrate. Error bars denote the standard error of the measurements from three individual samples (***$p < 0.001$). Copyright 2020 reprinted with permission from the American Chemical Society (ACS).

4.3.2 TL-catalyzed polymerization of BAPs

Next, we evaluated the TL-catalyzed cross-linking reaction of the BAPs. We optimized the concentration of TL and the reaction time to perform polymerization reaction with high efficiency. Then we fixed the concentration of TL and reaction time to 2 μM and 2 h, respectively. **Figure 4.2**A presents the native-PAGE analysis of the TL-treated BAPs. The BAP-Y, BAP-pG$_2$pA-Y, and BAP-Loop-Y formed polymers with high molecular weights that exceeded the upper limit of the separating gel and therefore were retained in the stacking gel. A wild-type BAP (BAP-WT) was employed as the control reaction. After the TL treatment, no cross-linked product was observed in the case of BAP-WT. Although there are some tyrosine residues in the structure of BAP-WT, TL did not recognize them as substrates. From these results, TL recognized the tyrosine residues in the Y-tags or Y-Loop, site-selectively. These results agreed with our previous reports [16,24]. The presence of Y-tags and Y-Loops, which

can be recognized and activated by TL, eliminates the use of phenolic mediators, such as vanillic acid [33,34], ferulic acid [35,36], *p*-coumaric acid [35,37], and caffeic acid [38-40], are typically used in a laccase-catalyzed cross-linking of proteins.

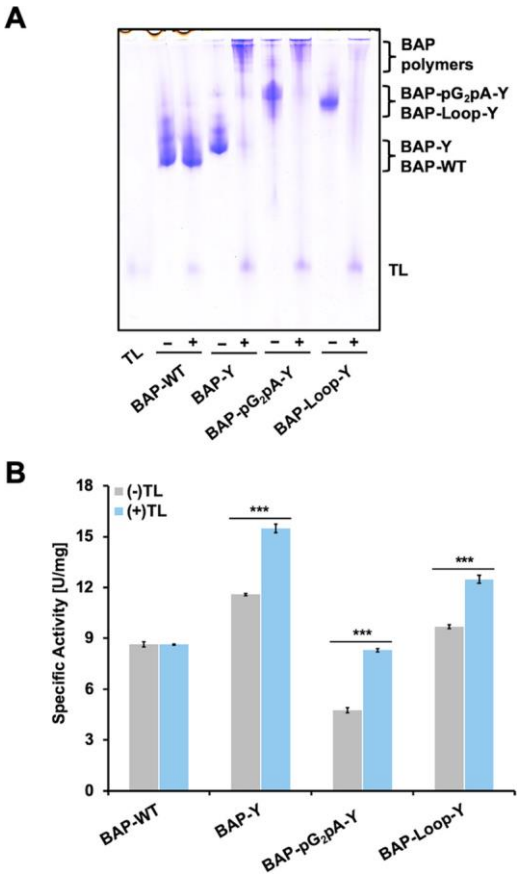


Figure 4.2 Native-PAGE analysis and relative activity of BAPs before and after TL treatment. (A) Results of the native-PAGE analysis of BAPs before and after TL treatment. The concentration of the BAPs and TL were 10 and 2 μM, respectively. The cross-linking reaction of the BAPs was conducted in 10 mM Tris HCl pH 8.0 at 37 °C for 2 h. (B) Enzymatic activity of the BAPs before and after TL treatment using *p*-NPP as a substrate. Error bars denote the standard error of the measurements from three individual samples (***$p < 0.001$). Copyright 2020 reprinted with permission from the American Chemical Society (ACS).

To confirm the activity changes of the BAPs after TL treatment, we measured the enzymatic activity of the BAP monomers and polymers using *p*-nitrophenyl phosphate (*p*-NPP) as a substrate (**Figure 4.2**B). Interestingly, the relative activity of the BAP polymers was higher than that of their monomers. We found similar results when we polymerized HRP by TL in our

previous work [16]. Although it is not clear why the activity increased after the polymerization of enzymes, we expect that the small substrate molecules require less time to be recognized by the catalytic sites of the protein as there are many catalytic sites in the polymer, resulting in faster reactions. TL is a more useful enzyme for the preparation of protein polymers than peroxidases, such as HRPs, which need additional hydrogen peroxide (H_2O_2) to initiate the polymerization reaction. Additional H_2O_2 may cause changes in enzymatic activity and deactivation of the model proteins.

The expected schematic polymerization reaction of a BAP by TL is shown in **Scheme 4.1**. TL oxidizes the phenolic moieties of the Y-tags or Y-Loop and generates free tyrosyl radicals, which react with each other to form dityrosine, trityrosine, or an even higher degree of cross-linked tyrosine derivatives [41,42]. Although the reactivity of Y-Loop should be lower than that of the Y-tags attached to the C-terminus of the BAPs (because of the steric hindrance), all of the BAP-Loop-Y units were cross-linked by TL treatment, indicating that the reactivity of Y-Loop is sufficient to make polymers. In the SDS-PAGE analysis of the TL-treated BAPs, the cross-linked BAP-Loop-Y showed clearer bands in a lower molecular weight region than BAP-Y and BAP-pG_2pA-Y. The sharper bands of the cross-linked BAP-Loop-Y suggest that the BAP-Loop-Y monomers were cross-linked with each other in a more uniform way than the other Y-tagged BAPs. Since the cross-linked Y-Loops are more sterically encumbered than the cross-linked Y-tags at the C-terminus, further cross-linking to Y-Loops to yield oligomeric BAPs was inhibited; this should lead to the formation of linear BAP-Loop-Y polymers (**Scheme 4.1**B).

4.3.3 Scanning probe microscopy (SPM) of BAP polymers

To validate the structural differences of BAP polymers, we analyzed the shape of BAP monomers and polymers by using an SPM in dynamic force mode. **Figure 4.3** shows the results of the SPM analysis of BAP monomers and polymers. Thanks to the high molecular weight of

BAPs (97.6, 99.6, 142.0, and 100.5 kDa for BAP-WT, BAP-Y, BAP-pG_2pA-Y, and BAP-Loop-Y, respectively), the monomers of the BAPs were successfully visualized as globular forms with heights ranging from 3.5 to 4.0 nm. However, some aggregates of BAP monomers were also observed. These may be caused by the drying process before the SPM analysis. There were no shape differences between BAP-WT monomer and TL-treated BAP-WT, indicating that BAP-WT was intact after TL-treatment. In contrast, BAP-Y formed a huge globular shape, suggesting that the BAP-Y monomers were connected to each other after the TL treatment. As the Y-tag at the C-terminus can cross-link multiple times, the BAP-Y polymer can form highly branched structures, which would be visualized as a huge globular shape in the SPM analysis. The BAP-pG_2pA-Y formed large irregular structures after TL treatment, but the shapes of the TL-treated BAP-pG_2pA-Y were different from those of the BAP-Y polymers. This is probably because of the presence of the chimeric protein, pG_2pA, the structure of which is linear, so the molecular structure of BAP-pG_2pA-Y monomer and also its polymers are not globular.

Interestingly, compared to other BAPs, the BAP-Loop-Y polymers had mostly linear shapes. The longest length of a BAP-Loop-Y polymer observed was approximately 350 nm and the average of length of BAP-Loop-Y polymers was 205±17 nm. This result strongly suggests that the BAP-Loop-Y units were cross-linked through the designed Y-Loop to yield linear polymers. A short and rigid Y-Loop consisting of 13 amino acid residues could eliminate the formation of branched structures because of the steric hindrance caused by the BAP-Loop-Y structure. Although the polymer length could not be controlled in this system yet, this is the first demonstration of the use of steric hindrance to control the structures of protein polymers, which were made via tyrosyl radical coupling reactions. Because of the better molecular packing on solid interfaces, linear protein polymers have the potential to exhibit better performance than hyperbranched globular protein polymers, especially in applications such as ELISA or western blotting where the reactions and interactions occur on a solid surface.

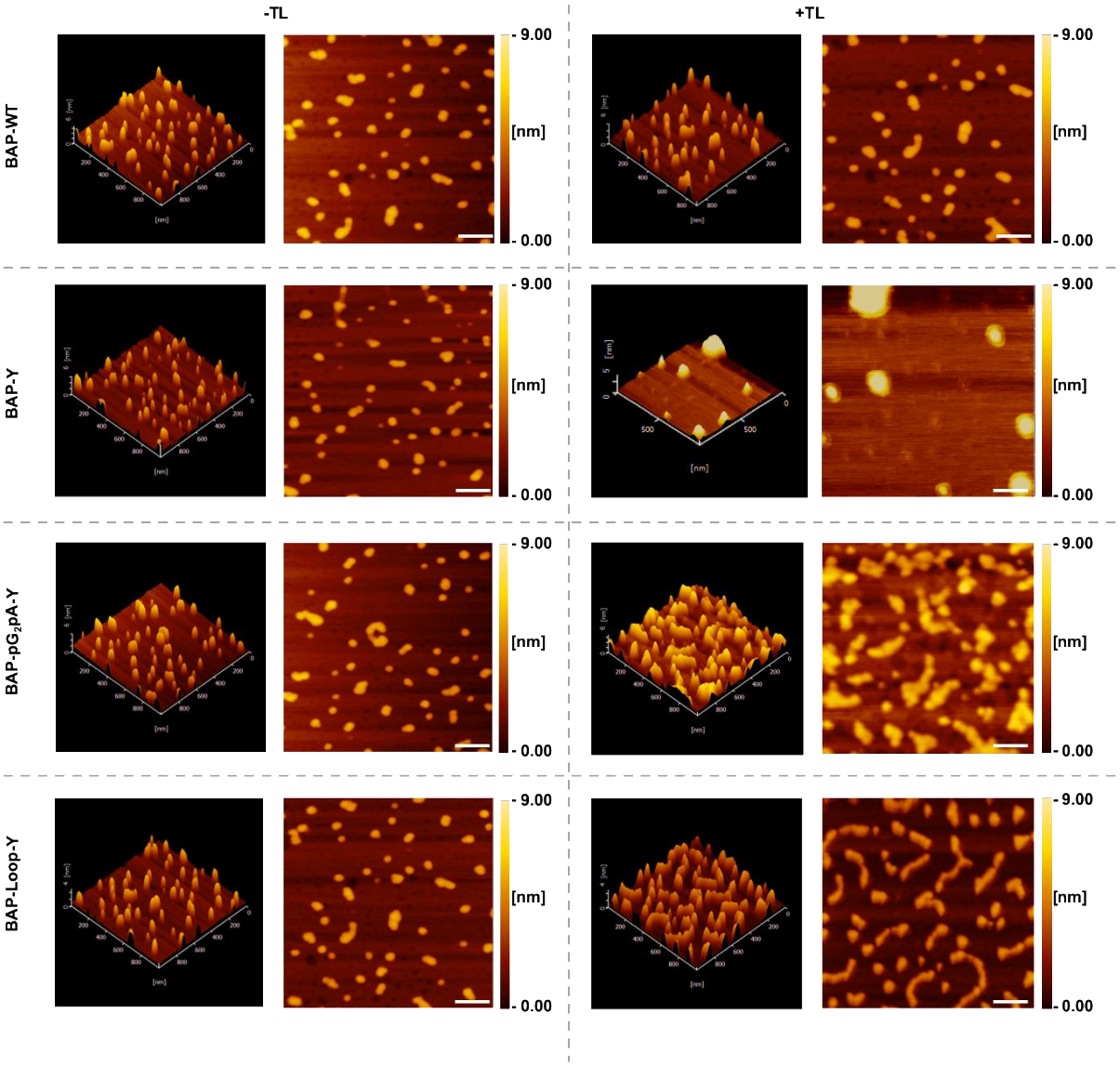


Figure 4.3 Results of the SPM analysis of the BAPs before and after TL treatment. The BAPs were diluted to a final concentration of 0.2 µM or ~0.02 mg/mL in 10 mM TrisHCl pH 8.0. Scale bars: 100 nm. Copyright 2020 reprinted with permission from the American Chemical Society (ACS).

4.3.4 TL-catalyzed copolymerization of Y-tagged proteins

We performed the copolymerization of BAP-Loop-Y or BAP-Y, with a single Y-tagged chimeric antibody-binding protein, pG_2pA-Y to create a bifunctional protein polymer that could be applied as a detection probe in an immunosorbent assay. The copolymer of BAP-Y and pG_2pA-Y should form highly branched protein polymers since both proteins possess

flexible Y-tags at their *C*-termini. On the contrary, we expected the BAP-Loop-Y/pG_2pA-Y copolymer to form linear structures incorporating pG_2pA-Y, mainly at the termini of the polymer. There are also some possibilities for pG_2pA-Y to attach to BAP-Loop-Y polymers as side branches. **Figure 4.4**A shows the native-PAGE analysis of the copolymers that were prepared by the copolymerization reaction of BAP-Y or BAP-Loop-Y with pG_2pA-Y catalyzed by TL. After TL treatment, both bands of pG_2pA-Y and BAP-Y or BAP-Loop-Y disappeared, and new bands in a high molecular-weight region were produced, indicating the formation of cross-linked copolymers. The co-cross-linking of pG_2pA-Y did not affect the activity of the BAPs since no decrease in activity was detected after TL treatment.

Figure 4.4B shows the results of the SPM analysis of the BAP-Y/pG_2pA-Y copolymer and BAP-Loop-Y/pG_2pA-Y copolymer. The shape of the BAP-Y/pG_2pA-Y copolymer was different from that of the BAP-Y polymers (Figure 4.3). Incorporation of pG_2pA-Y changed the globular structure of the BAP-Y polymers to form smaller globular protein polymers since pG_2pA-Y possesses only one Y-tag in a molecule thus attachment of pG_2pA-Y to the BAP-Y polymers terminates the polymerization reaction. In contrast, the shape of BAP-Loop-Y/pG_2pA-Y copolymer was as linear as the BAP-Loop-Y polymers. The longest and average length of a BAP-Loop-Y/pG_2pA-Y copolymer observed was approximately 500 nm and 255±35 nm, respectively. The monomer of BAP-Loop-Y appeared as 47±1 nm of globular shape in SPM analysis (Figure 4.3). It suggests that the degree of polymerization of BAP-Loop-Y was in the range of 5 to 10. This suggests that the pG_2pA-Y units were incorporated at the termini of BAP-Loop-Y polymers. Although some pG_2pA-Y might have been incorporated at the cross-linking site of the BAP-Loop-Y units in the polymers and bound as small branches, they are probably not visible by SPM. There is also a possibility that the pG_2pA-Y mostly cross-linked with itself and not with BAP-Loop-Y, which would result in pG_2pA-Y oligomers. The presence of the pG_2pA unit is essential for the BAP-Loop-Y polymer to bind to IgG.

Therefore, the function of the BAP-Loop-Y/pG$_2$pA-Y copolymer as a detection probe in an immunoassay was evaluated next.

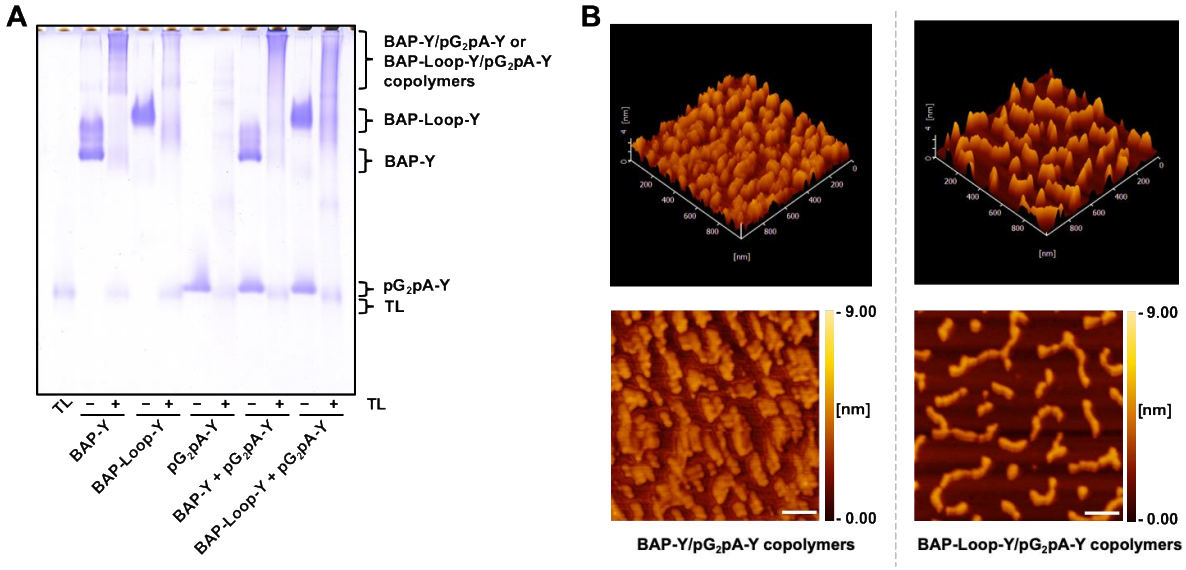


Figure 4.4 Copolymerization reaction of BAPs and pG$_2$pA-Y catalyzed by TL. (A) Results of the native-PAGE analysis of BAPs/pG$_2$pA copolymers after TL treatment. The concentration of both proteins was 10 µM. TL concentration was 2 µM. The reaction was conducted for 2 h at 37 °C. (B) SPM analysis of BAP-Y/pG$_2$pA-Y and BAP-Loop-Y/pG$_2$pA-Y copolymers prepared at a 1:1 molar ratio of BAPs to pG$_2$pA-Y. The copolymers were adsorbed onto mica after a 50 times dilution with 10 mM Tris-HCl pH 8.0. Scale bars: 100 nm. Copyright 2020 reprinted with permission from the American Chemical Society (ACS).

4.3.5 Performance of the BAP-Loop-Y/pG$_2$pA-Y copolymers as protein probes in an ELISA

We evaluated the functionality of the BAP-Y/pG$_2$pA-Y copolymer and BAP-Loop-Y/pG$_2$pA-Y copolymer in an OVA-detecting ELISA (**Figure 4.5**). A polymer of genetically fused BAP-pG$_2$pA-Y was used as a control, which corresponds to a copolymer of BAP and pG$_2$pA cross-linked at a 1:1 molar ratio. Both copolymers showed absorbance changes in the ELISA, indicating that the pG$_2$pA-Y and BAP-Y or BAP-Loop-Y were cross-linked to each other by TL treatment. The BAP-Loop-Y/pG$_2$pA-Y copolymer exhibited 2-fold higher absorbance in the ELISA than the BAP-Y/pG$_2$pA-Y copolymer (**Figure 4.5**A), despite the specific activity of BAP-Loop-Y being approximately 20% lower than that of BAP-Y (Figure 4.1).

These results shed light on the importance of controlling the molecular structure of protein polymers to maximize their functionality. The detection probes in ELISA are on a surface, so it is important to control the structure of the protein polymer structure so that they work efficiently [43,44]. A surface can be considered as a set of lines, so the ideal structure of protein polymer to maximize the molecular packing on a surface is linear. The copolymerization reaction of BAP-Y and pG_2pA-Y resulted in the formation of large highly-branched structures [24], while the BAP-Loop-Y/pG_2pA-Y copolymer formed linear structures (**Figure 4.5**B). The BAP-Loop-Y/pG_2pA-Y copolymer showed higher signals than the BAP-Y/pG_2pA-Y copolymers at the conditions of low OVA coating concentrations, where the molecular packing on a surface has less effects. This suggests that the pG_2pA-Y units in the linear copolymer work more efficiently than the units in the branched copolymers. The pG_2pA-Y units in the BAP-Y/pG_2pA-Y copolymers are embedded within the branched structures. On the other hand, the pG_2pA-Y units in the BAP-Loop-Y/pG_2pA-Y copolymer are most likely to be conjugated at the termini of linear structure, which is suitable for binding to IgGs. Also, the linear structures of BAP-Loop-Y/pG_2pA-Y copolymers provide better molecular packing on the surface of ELISA plate than the 3-dimensional highly-branched BAP-Y/pG_2pA-Y copolymer, resulting in a higher signal in the ELISA. The molecular packing on the surface was particularly significant when the OVA coating concentration was high and there were many antibodies on the ELISA plate surface [45]. The BAP-Loop-Y/pG_2pA-Y copolymer showed remarkably higher absorbance than the BAP-Y/pG_2pA-Y copolymers when there was a high OVA coating concentration.

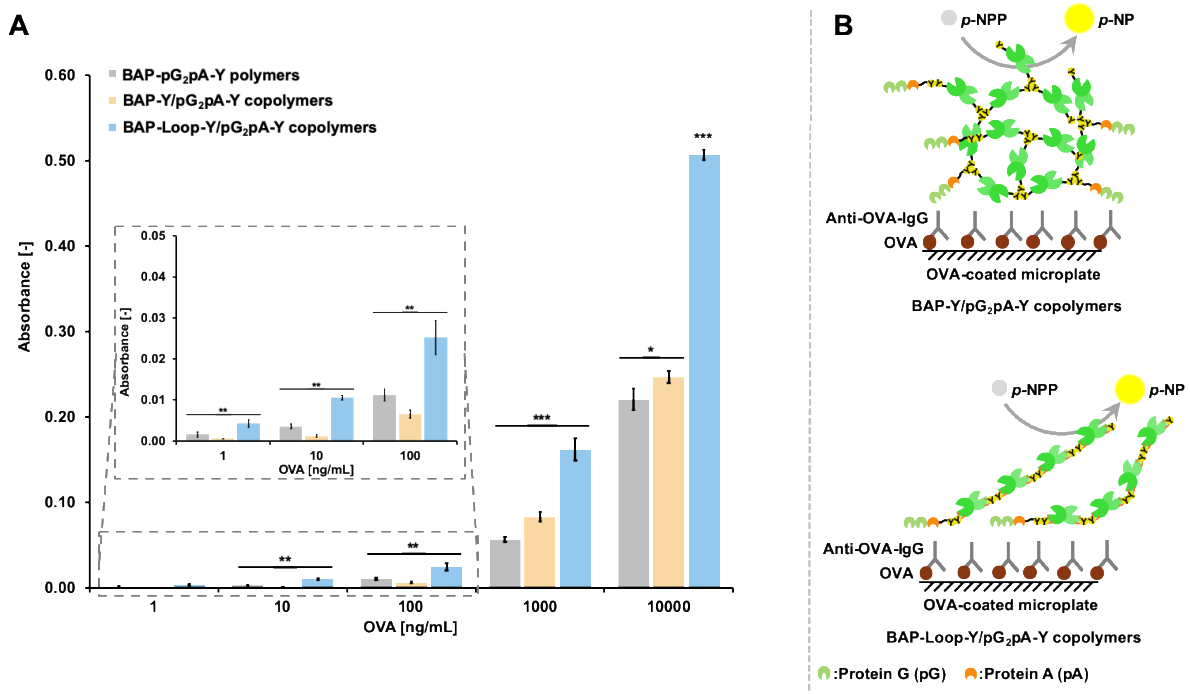


Figure 4.5 Results of the OVA-detecting ELISA using BAP-Y/pG2pA-Y and BAP-Loop-Y/pG2pA-Y copolymers. (A) Results of the OVA-detecting ELISA using BAP-Y/pG2pA-Y and BAP-Loop-Y/pG2pA-Y copolymers prepared at a 1:1 molar ratio of BAPs to pG2pA-Y. The polymer of genetically-fused BAP-pG2pA-Y was used as a control. The concentration of the BAP polymers and copolymers were fixed to 0.5 U/mL. The absorption of OVA onto the microplate was conducted at concentrations of 1 ng/mL to10 μg/mL in TBS (pH 7.4). (B) The expected structure of the BAP-Y/pG2pA-Y copolymer and BAP-Loop-Y/pG2pA-Y copolymer in the ELISA. The protein G (pG) (green) or protein A (pA) (orange) can bind to the IgG specifically. Error bars denote the standard error of the measurements from three individual samples (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Copyright 2020 reprinted with permission from the American Chemical Society (ACS).

4.3.6 BAP-Loop-Y/pG2pA-Y copolymers prepared with various molar ratios

In order to enhance the activity and functionality of the BAP-Y/pG2pA-Y and BAP-Loop-Y/pG2pA-Y copolymers as detection probes, it is crucial to increase the molar ratio of BAP-Y or BAP-Loop-Y against pG2pA-Y. The copolymers of BAP-Y or BAP-Loop-Y and pG2pA-Y were prepared at various molar ratios. **Figure 4.6** shows the results of OVA-detecting ELISA using the copolymers of BAPs and pG2pA-Y that were prepared at different molar ratios. As the ratio of the BAP against pG2pA-Y increased, the absorbance increased in the ELISA for the BAP-containing copolymers, indicating that a greater number of BAP units were

incorporated into the copolymers with pG_2pA-Y. Intriguingly, the BAP-Loop-Y/pG_2pA-Y copolymer showed an almost linear increase in absorbance in proportion to the ratio of BAP-Loop-Y, while the BAP-Y/pG_2pA-Y copolymer reached its peak at the ratio of 75:1; the absorbance decreased at the ratio of 100:1. At the ratio of 100:1, the BAP-Loop-Y/pG_2pA-Y copolymer exhibited 26-fold and 20-fold higher absorbance than BAP-pG_2pA-Y and its polymer, respectively. To fully understand the functionality of BAP and pG_2pA copolymers, we purified them from the remaining monomers using size exclusion chromatography (SEC). The copolymers with high molecular weights were completely separated from the monomers by SEC (**Figure 4.7**A-B). To confirm the functionality and activity of the purified BAP/pG_2pA copolymers, we applied them again in an OVA-detecting ELISA (**Figure 4.7**C). The purified copolymers performed better as detection probes than those that were not purified. The BAP-Loop-Y/pG_2pA-Y copolymer showed higher signals in ELISA than the BAP-Y/pG_2pA-Y copolymers. This result revealed that the morphology of the copolymers was the most critical feature to improve the functionality of the protein polymers as detection probes, not only for immunoassays but also for other diagnostic applications.

The use of a large number of enzymes in the copolymerization reaction with binding proteins results in the formation of highly branched structures in which the binding proteins tend to be buried inside of the structures and are not able to function correctly. Therefore, in previous studies of protein polymers using Y-tagged proteins, there is often an optimum ratio of enzyme to binding proteins to extract the best functionality as detection probes [16, 24, 46]. Since the molecular structure of the BAP-Loop-Y/pG_2pA-Y copolymer is linear and the pG_2pA-Y units are mostly conjugated at the terminus of the polymers, the pG_2pA-Y could efficiently bind to the antibody on the ELISA plate, while carrying a large number of enzymes. The results here demonstrate the advantages of linear protein polymers over highly-branched protein polymers.

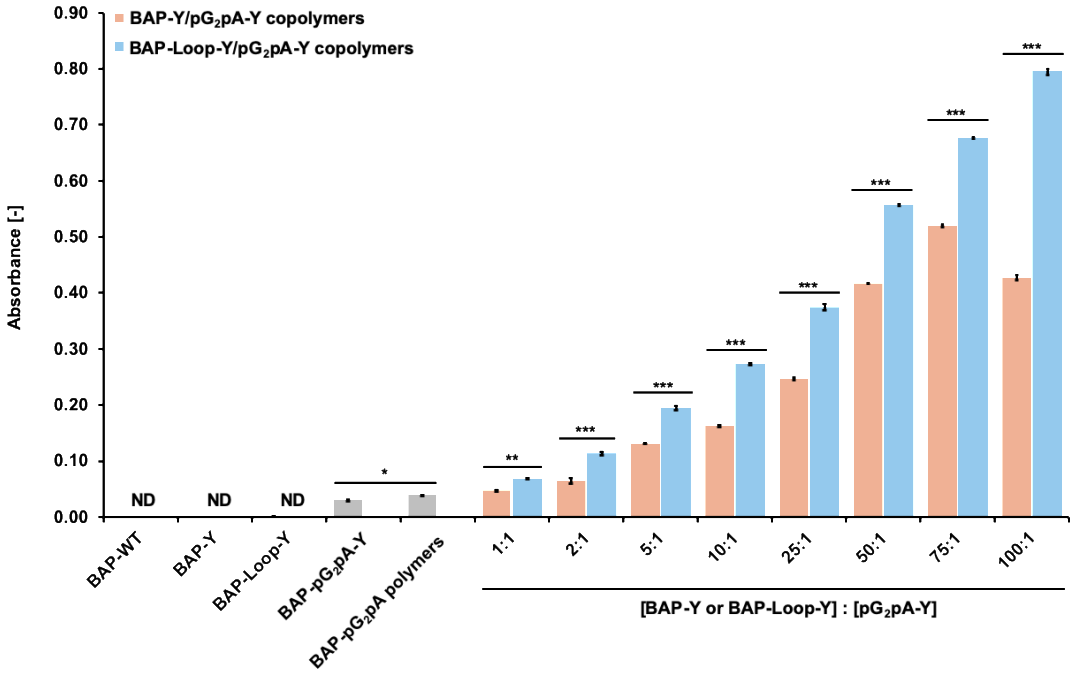


Figure 4.6 Results of the OVA-detecting ELISA using BAP and pG2pA-Y copolymers prepared at various molar ratios. The concentration of pG2pA-Y was fixed to 0.5 μM. While the concentrations of BAP-Y and BAP-Loop-Y were varied from 0.5–50 μM. The concentration of TL was 2 μM. The reaction was conducted in 10 mM Tris HCl, pH 8.0, at 37 °C, for 2 hours. All protein polymer solutions were diluted to fix the concentration of pG2pA-Y as binding domain to 1 nM. The concentration of OVA that coated the wells was 1 μg/mL in TBS (pH 7.4). ND: not detected. Error bars denote the standard error of the measurements from three individual samples (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Copyright 2020 reprinted with permission from the American Chemical Society (ACS).

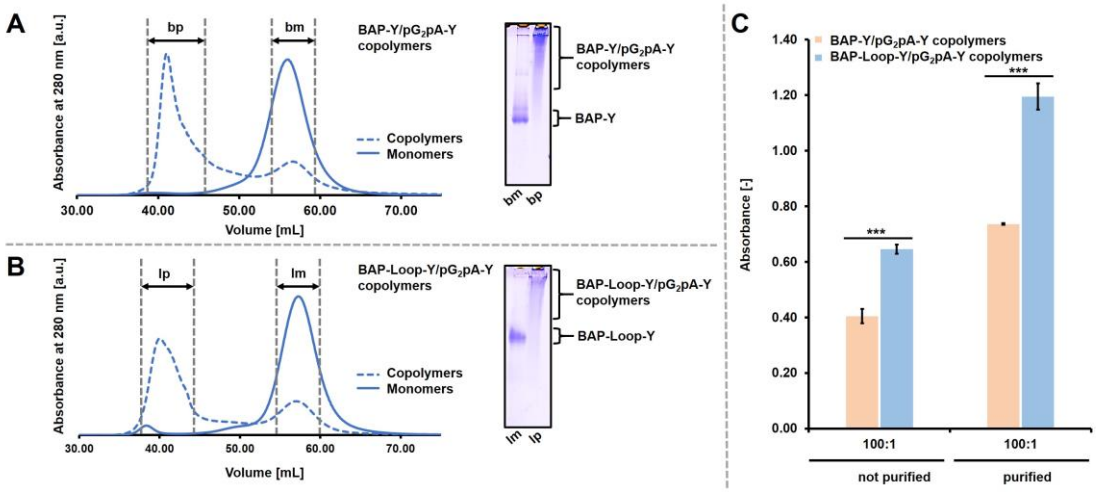


Figure 4.7 SEC, native-PAGE, and OVA-detecting ELISA results of the BAP/pG2pA copolymers that were prepared at a 100:1 molar ratio of BAP-Y or BAP-Loop-Y to pG2pA-Y. (A) BAP-Y/pG2pA-Y copolymers. (B) BAP-Loop-Y/pG2pA-Y copolymers. The SEC results of copolymers are shown as a dashed line. The mixed solution containing the BAPs and pG2pA-

Y without TL treatment was employed as the control (solid line). Native-PAGE analysis results of BAP-Y/pG$_2$pA-Y and BAP-Loop-Y/pG$_2$pA-Y copolymers stained using Coomassie blue. The separating gel was 10 % (%wt). (C) Results of the OVA-detecting ELISA of BAP-Y/pG$_2$pA-Y and BAP-Loop-Y/pG$_2$pA-Y copolymers from the pooled and concentrated fraction of polymers from SEC. The concentration of OVA that coated the well was 1 μg/mL in TBS (pH 7.4), while the concentration of the BAP/pG$_2$pA copolymers was fixed to 0.5 U/mL. Error bars denote the standard error of the measurements from three individual samples (***p < 0.001). Copyright 2020 reprinted with permission from the American Chemical Society (ACS).

4.4 Conclusions

We demonstrated a new strategy for controlling the structure of protein polymers by utilizing tyrosine-containing loop peptide, Y-Loop. The Y-Looped BAP, BAP-Loop-Y, formed linear polymers, whereas BAPs fused with a *C*-terminal Y-tag showed irregular shapes in SPM analysis. The sterically confined structure of the Y-Loop and a closely cross-linked BAP-Loop-Y caused steric hindrance around the cross-linked tyrosine residues at Y-Loops and prevented further cross-linking reactions and branch formations. Furthermore, linear BAP-Loop-Y/pG$_2$pA-Y copolymers that were prepared from the copolymerization reaction of BAP-Loop-Y and pG$_2$pA-Y showed excellent functionality and activity as protein probes in an OVA-detecting ELISA. These results suggested that the linear structure of the BAP-Loop-Y/pG$_2$pA-Y copolymers allowed them to pack better on the anti-OVA IgG surface than the globular BAP-Y/pG$_2$pA-Y copolymers. Interestingly, the absorbance of the BAP-Loop-Y/pG$_2$pA-Y copolymers that were prepared with higher molar ratios of reporter enzyme to IgG-binding protein (BAP-Loop-Y to pG$_2$pA-Y) increased across the whole range tested. Further study on the SEC fractions of the purified BAP-Loop-Y/pG$_2$pA-Y confirmed its excellent functionality and activity as an ELISA probe, which showed higher signal than the BAP-Y/pG$_2$pA-Y copolymers. It can be concluded that the size of polymers containing the reporter enzyme is not the only factor to improve their performance as a protein probe in an immunoassay. The shape of the protein polymers could be the most important factor in improving the functionality

and activity of protein polymers. This newly proposed linear polymerization of BAP strategy can contribute to the further development of functional protein polymers for specific applications in biotechnology, biomedical sciences, bioimaging, and diagnostics.

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CHAPTER 5 SUMMARY AND FUTURE PROSPECTIVES

Proteins nowadays are used in more wider fields, ranging from catalysis to nanoprobes in diagnostic applications. The limitation on the structure as well as the function of proteins could be improved by engineering them to create artificial enzymes or to form polymeric enzymes. Preparation of polymeric proteins as well as enzymes can be done by engineering the proteins using some strategies such as genetic modification via tandem fusion or site-specific cross-linking reaction of proteins. Polymerization of proteins to form protein polymers has been developed to expand the functionalities of proteins or to create a unique and novel property of the conjugates.

In Chapter 2, the TL-catalyzed cross-linking reaction of Y-tagged proteins was studied to investigate the possibility of TL as an enzyme for polymerization reaction of proteins. TL was able to catalyze cross-linking reaction of Y-tagged proteins specifically to yield protein polymers. Unlike small substrates, the TL-catalyzed protein cross-linking reaction occured efficiently at basic pH conditions but not in the acidic conditions. The BAP/pG_2pA polymers made by TL showed higher functionalities than those made by HRP. The results described here showed the potential of TL as an another enzyme for HRP to perform protein polymerization. Also, the need of controlling cross-linking degree of the protein polymers was revealed in this study and TL possesses capabilities of achieving functional protein polymers with different cross-linking degrees, which will open up the utilization of TL into biomedical and diagnosis fields of studies.

In Chapter 3, a new strategy for the polymerization of HRP was demonstrated by creating Y-tagged HRPs and conducting polymerization of HRPs through the Y-tags using laccase. Compared with the self-cross-linking reaction of Y-tagged HRPs and chemical polymerization of HRP in the other reports, the TL-mediated cross-linking reaction of the Y-tagged HRPs

provides site-selective polymerization at the Y-tags and mild reaction conditions, resulting in retaining the catalytic activity of the resulting HRP polymers. The use of a Y-tag can substitute the use of mediators in laccase-catalyzed protein cross-linking reactions. The utilization of tyrosine coupling for new covalent bond formation resulted in active and highly functional HRP-based polymers, which could be further extended to the design of new biomaterials and bioconjugates applicable to biotechnology.

In Chapter 4, a novel strategy for controlling the structure of protein polymers by utilizing tyrosine-containing loop peptide, Y-Loop, was explored. The Y-Looped BAP, BAP-Loop-Y, formed linear polymers, whereas BAPs fused with a *C*-terminal Y-tag showed irregular shapes in the SPM analysis. The sterically confined structure of the Y-Loop and a closely cross-linked BAP-Loop-Y caused steric hindrance around the cross-linked tyrosine residues at Y-Loops and prevented further cross-linking reactions and branch formations. Furthermore, linear BAP-Loop-Y/pG_2pA-Y copolymers that were prepared from the copolymerization reaction of BAP-Loop-Y and pG_2pA-Y showed excellent functionality and activity as protein probes in an OVA-detecting ELISA. These results suggested that the linear structure of the BAP-Loop-Y/pG_2pA-Y copolymers allowed them to pack better on the anti-OVA IgG-immobilized surface than the globular BAP-Y/pG_2pA-Y copolymers. Interestingly, the absorbance of the BAP-Loop-Y/pG_2pA-Y copolymers that were prepared with higher molar ratios of reporter enzyme to IgG-binding protein (BAP-Loop-Y to pG_2pA-Y) increased across the whole range tested. The shape of the protein polymers could be the most important factor in improving the functionality and activity of protein polymers. This newly proposed linear polymerization of BAP strategy can contribute to the further development of functional protein polymers for specific applications in biotechnology, biomedical sciences, bioimaging, and diagnostics.

The development of protein polymers will contribute in a range of applications that apply proteins or their processes. The protein polymerization reaction is one of interesting and

promising approaches to improve the functionality and activity of protein in bioprocesses. The researchers can design the desired structure and characteristics of the protein polymers for their designated reactions. The protein polymerization reaction also will open further development of artificial proteins, for example, polymeric bifunctional proteins for specific applications. Protein polymers also applicable not only as protein probes in diagnostic applications but also in bioimaging and biosensors. Protein polymerization can be an interesting strategy for preparing an effective antigen for vaccine development. Although there are no sufficient reports regarding the response of immune system against polymeric antigens, this strategy is worth pursuing. The advances in the research and development of antibody-drug conjugates (ADCs) needs some enzymes to connect the antibody and drugs. Polymeric ADCs can become a novel approach in the development of new ADCs. Other enzymes that used in protein polymerization reaction are also good candidates for preparing ADCs. Therefore, the study of protein polymerization reaction by using a laccase reported in this dissertation will contribute to the further development of functional protein polymers in the future.

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