Food Hydrocolloids for Health 2 (2022) 100080

Contents lists available at ScienceDirect

Food Hydrocolloids for Health

journal homepage: www.elsevier.com/locate/fhfh

Enzymatic modification of Fish Gelatin and Beet Pectin using Horseradish peroxidase

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ARTICLE INFO

Keywords:
Structural characterization
Fish Gelatin
Beet Pectin
Enzymatic cross-linking
Intermolecular conjugation

ABSTRACT

The Fish Gelatin (FG), a good alternative for unhealthy and limited socio-cultural mammalian gelatin appears to possess endogenous structural limitations. The goal of this work was to use enzymatic crosslinking to modify cold-water Fish Gelatin (FG) with Beet Pectin. Reaction conditions were optimized by a single factorial experiment and covalent crosslinking was measured by ultraviolet (UV)-Vis spectroscopy at 340 nm to indicate Horseradish Peroxidase (HRP) catalyzes Beet Pectin (BP). At 50 °C for 4 h, the highest weight ratio of heterologous adducts between FG-BP was 1:3, with HRP and Hydrogen peroxide (H_2O_2) of 2 μg/mL and 0.067%, (v/v), respectively. Intermolecular cross-linking was found between treated samples using ATR-FTIR and Sodium Dodecyl Sulphur and Polyacrylamide Gel Electrophoresis (SDS-PAGE). The heterologous product, control FG, and BP as well as a mixture of untreated FG-BP had a β-sheet of 41.14%, 39.65%, 39.9%, and 40.0%, respectively. The maximum reduction in elution was obtained in heterogeneous FG-BP complex. Furthermore, a schematic mechanism for Cold-water Fish Gelatin and Beet Pectin was proposed. Overall, peroxidase crosslinked BP was able to modify cold-water Fish Gelatin. The use of Horseradish peroxidase on Fish Gelatin could provide a practical way of building the FG-BP complex as a basis for understanding the FG functionalities comprehensively.

1. Introduction

Mammalian gelatin accounts for the bulk of the world's gelatin manufacturing (Karim & Bhat, 2009). Notwithstanding, mammals can transmit bovine sponge from encephalopathy disease and also restraint from the religious sectors such as Judaism and Hinduism as well as vegetarians (da Trindade Alfaro et al., 2015; Gómez-Guillén et al., 2009) which have necessitated a search into alternative sources of gelatin (Huang et al., 2017).

Gelatin is an extracted protein obtained from the thermal denaturing of collagen and has several applications in both food and non-food industries (Stevenson et al., 2020). Fish Gelatin (FG) is increasingly gaining demand as consumers consider it a safe and healthy product. However, FG especially the cold-water species are facing huge industrial application limitations because of poor physicochemical properties (Karim & Bhat, 2009). These poor physicochemical characteristics were attributed to a low quantity of hydroxyproline, proline, and amino acids in the collagen (Fu et al., 2019). Many applications have been proposed to change the functional characteristics of FG to extend its industrial usage to overcome the limitations. Typically, a protein-polysaccharide combination is used to successfully modify structural and functional improvement (Warnakulasuriya & Nickerson, 2018). Protein and polysaccharide complexes could be obtained by various means using chemical, enzymatic, and Maillard heat reactions. Due to the low generation of potentially hazardous by-products, the enzymatic approach is chosen over alternative methods for forming covalent interactions between protein and polysaccharides (Liu et al., 2017). Transglutaminase, peroxidase, and laccase are the most commonly used enzymes to induce protein and polysaccharide complex (Littoz, McClements, 2008). Pectin and microbial transglutaminase modification could be utilized to create FG with improved rheological characteristics, gel strength, and melting temperature (da Trindade Alfaro et al., 2015; Huang et al., 2019).

Due to phenolic components like Ferulic acid, pectin polysaccharides are said to interact with protein enzymatically. A typical example of these polysaccharides is BP. BP is noted to possess feruloyl and acetyl moieties. These moieties are known to be catalyzed oxidative peroxidase enzymes such as laccase and HRP through free radical mechanisms Li et al., 2012; Zaidel et al., 2013). The mechanism of Horseradish

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https://doi.org/10.1016/j.fhfh.2022.100080
Received 1 October 2021; Received in revised form 20 March 2022; Accepted 8 July 2022

peroxidase catalyzed Ferulic acid to promote protein-polysaccharides has been studied extensively (Zaidel et al., 2013). This catalytic action involves a three-step cyclic reaction as shown in Equations (1) to ((3). The process begins with native Horseradish peroxidase enzyme (HRPN) which is first oxidized by H_2O_2 to HRP_I (oxidized Horseradish peroxidase) and then reduced to HRP_{II} (reduced Horseradish peroxidase) in two sequential one-electron transfers. Reducing substrates (R-H), typically a small molecule phenol derivative, produces one molecule of water (H_2O) and then returns the enzyme to its native state. Finally, the substrate is oxidized to free radicals (R•) to promote the reaction. The equation below indicates the reaction mechanism of HRP.

$$HRP_N + H_2O_2 \rightarrow HRP_I + H_2O \quad (1)$$

$$R \cdot HRP_I + R - H \rightarrow HRP_{II} + \quad (2)$$

$$HRP_{II} + R - H \rightarrow HRP_N + R^{\bullet} + H_2O \quad (3)$$

Herein, we elucidated the possible peroxidase-mediated conjugation of FG and Sugar Beet Pectin or Beet Pectin (SBP or BP). FG and SBP were used as protein and carbohydrate models, respectively, as well as HRP as an enzyme

2. Materials and methods

2.1. Materials

Cold-water Fish Gelatin (Mw. 100kDa and 99% purity) was purchased from Hubei Jusbeng Technology Co., Ltd (Wuhan, China), SBP or BP (degree of esterification of 53.7% and 99% purity) was purchased from Herbestreith and Fox Corporate Group (Neuenburg, Wutt, Germany), HRP with 1000 activity units (U) per mg of enzyme solid was also procured from Sigma-Aldrich (St. Louis, MO, USA). H_2O_2 was obtained from Beijing Chemical Works (China). All other reagents used were of analytical grade. Analytical methods were performed using Millipore Milli-Q water (Waters-Millipore Corporation, Milford, MA, USA).

2.2. Experimental procedure

2.2.1. Preparation of stock solution

Stock phosphate buffer solution (pH 5.5, 0.1 M) was prepared from the mixture of sodium dihydrogen phosphate (NaH_2PO_4, 0.1 M) and disodium hydrogen phosphate (Na_2HPO_4, 100 mM). The solution was then adjusted by 1 M HCl or 1 M NaOH to obtain the desired pH (pH 5.5). FG (1% w/w) and BP (1% w/w) were prepared separately by dispersing dry powder in 0.1 M PBS (pH 5.5), stirred at room for complete hydration followed by storage at 4 °C overnight. HRP was prepared in Millipore Milli-Q water to 100 μg/mL and stored at 4 °C. Hydrogen peroxide was prepared to 1% (v/v). It was freshly prepared every day before the experiment.

2.2.2. FG-BP crosslinking using HRP/H_2O_2 system

A preliminary experiment was performed to estimate the right conditions for HRP/H_2O_2 system for successful reaction throughout the whole experiment using a modified version (Liu et al., 2015; Oudgenoeg et al., 2001). Different set of cross-linking reaction conditions; HRP (0.5-2 μg/mL), H_2O_2 (0.017, 0.033, 0.050 and 0.067%, v/v), time (30-240 min), pH (5-6.5), temperature (30-65 °C) were conjugated with the volume of BP (0.05-3 mL) and FG (1 mL). Water was added to the mixtures at 5 min intervals. The control experiment without HRP was prepared. The final optimized reaction system was as follows; weight ratio of FG-BP complex (1:3), temperature (50 °C), time (4 h), HRP (2 μg/mL), H_2O_2 (0.067% v/v), PBS (pH 5.5) and this was examined using UV-vis light absorption spectrometry (UV mini-1240, Shimadzu Corporation, Japan) analyzed at 340 nm. The reacted system was further incubated in a water bath at 50 °C for 4 h. After this, the reaction system was terminated by heating the sample for 10 min at 80 °C to inactivate the enzymes. The sample was concentrated at 50 °C for 24 h in a vacuum freeze dryer (LGJ-10, Beijing) to lyophilize the sample for further analysis. FG, BP and H_2O_2 without HRP were used as control groups. Plain FG and BP were also tested. FG-FG and BP-BP homogenous conjugation were carried out to better illustrate the hetero-conjugation FG and BP

2.4. Analytical procedure

2.4.1. Attenuated total reflection Fourier-transforms infrared (ATR-FTIR) spectroscopy

The sample was analyzed for the presence of different functional groups in a solid-state to avoid water absorption in the amide I region (Liu et al., 2015). The appropriate reaction product obtained in the preliminary experiment conducted in Section 2.2.2 was dried to solid powder using a vacuum freeze dryer analyzed (Thermo Electron Scientific Inc., Waltham, MA, USA). The lyophilized samples were scanned in mid-region of 4000-400 cm^{-1} at a resolution of 4 cm^{-1} in the ATR mode.

2.4.2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic mobility of concentrated reacted and plain samples under reduced conditions was obtained as performed by (Liu et al., 2017). Electrophoresis was carried out using polyacrylamide gel slabs of (10 × 8 cm (length × width) in a vertical slab electrophoresis apparatus (Model mighty small II, SE 250/SE 260, Hoefer Pharmacia Biotech Inc., Halliston, USA). A Bio-rad electrophoresis unit (Hercules, CA, USA) and already-made 15-20% Tris-glycine polyacrylamide gel (Solarbio Life Sciences, Beijing, China) were used. Samples (30 μL, 0.1% protein) were prepared in 0.01M Tris-glycine buffer (pH 8.8) containing 0.1% SDS, 20% glycerol, 0.02 mM of coomassie brilliant blue G-250) at 1:1 (v/v) ratio, 2- mercaptoethanol, 0.1% Bromophenol blue, and 50 mM/L EDTA. The mixtures were boiled at 100 °C for 1 min. The mixtures were centrifuged at 5000 rpm for 1 min. An aliquot of 5 μL was taken from the supernatant and loaded into the wells. The standard reference molecular-weight protein marker used was 11-245kDa (Sigma, St. Louis, MO, USA). Electrophoresis was carried out at a constant voltage of 100 V on a gel for 2 h. The gel sheet was stained for both protein (0.02 mM, 1.0 g Coomassie brilliant blue G-250) and polysaccharide (0.5% periodate-fuchsin solution for 30 min under running water to destain the gel (Liu et al., 2017).

2.4.3. Size exclusion chromatography (SEC)

The purity of the FG-BP complex was realized by SEC according to the modified procedure adopted from (Shim et al., 2009). The gel filtration column (0.1 × 100 cm) filled with Sephadex G-100 was placed upright on a stand. The column was equilibrated with washing buffer (Na_2HPO_4 and NaH_2PO_4, NaCl, pH 10 above fish gel IEP (Isoelectric point). Samples were dissolved in elution- containing tris base as reducing agent. Before SEC, centrifugation of sample at 5000 rpm for 45 s followed by filtration via 0.45 μm syringe-driven filter (Millex HV, PVDF, Millipore Corp., Billerica, MA) to remove any aggregate particles. The supernatant of 1.5 mL was injected into Sephadex G-100 with a column connected to the buffer reservoir and eluted at a flow rate of 0.25 mL/min. The eluents were fractionated with LKB Ultorac fraction collector fitted with 4 cm^{-3} capacity cups. The fractionated samples were determined at 280, 325, and 335 nm absorbance, respectively for protein, polysaccharides, and complex using UV-vis light spectrometry.

2.5. Statistical analysis

All the experiments were performed in triplicates independently. Origin (Origin Lab Co., Pro.8.0) and excel 2013 data processing and to create charts. Omnic 7.0 was used for IR peak analysis. Data samples were subjected to analysis of ANOVA using SPSS at probability levels of 0.05 ($p<0.05$).

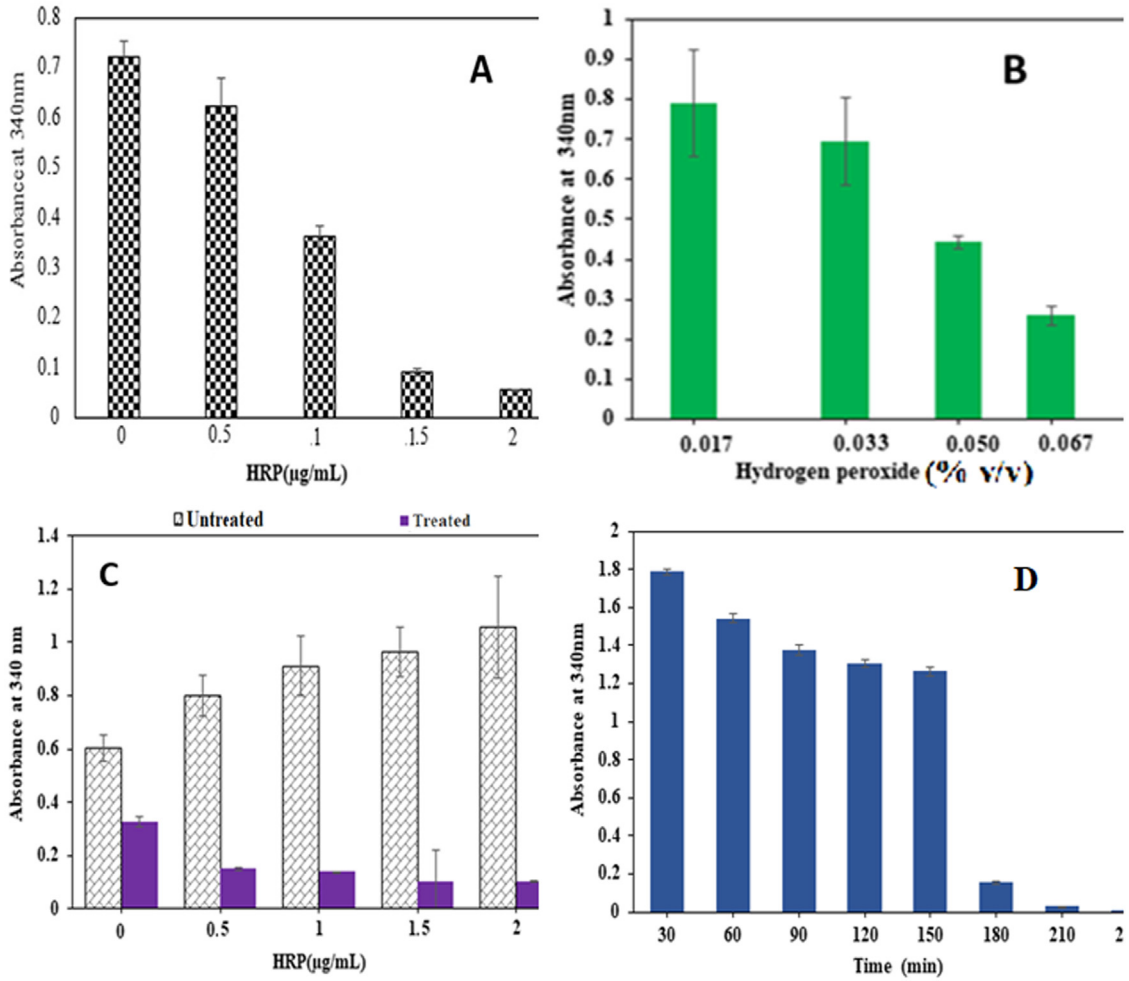


Fig. 1. Influence of different operation parameters on enzymatic reaction examined at wavelength of 340 nm using UV-vis spectroscopy–(A) HRP concentration (0-2 (µg/mL) (B) Hydrogen concentration (0.017, 0.033, 0.050, 0.067%, v/v) measured at FG-BP weight ratio of 1:3. (C) treated and untreated products measured at FG–BP weight ratios (1:0.05-3 mL) and (D) Effect of crosslinking duration (30-240) min.

3. Results and discussion

3.1. Optimization of reaction conditions for intermolecular conjugation of FG-BP

Complex

Fig. 1A shows the influence of enzyme activity on the reaction process within the peroxidase/hydrogen peroxide system. To select the optimum level of an enzyme (HRP), the enzyme concentration was varied over each group in the reaction mixture from 0-2 µg/mL. The absorption was measured at 340 nm using UV-vis light absorption spectroscopy. For FG and BP incubated with H_2O_2 without enzyme, high absorbance (0.72) was observed signifying the occurrence of more homo-conjugate linkage in the reaction chain. However, the addition of an enzyme resulted in a reduction of optical density (OD) or absorbance values. Increasing the concentration from 0.5 µg/mL to 2 µg/mL of peroxidase resulted in a drastic decline of absorption from 0.623 to 0.056 in the mixture. Similarly, absorbance decreased over time in the presence of 500 U/g laccase indicating oxidation of Ferulic acid in BP via laccases (Liu et al., 2015). The right concentration of enzyme for the final reaction was set at 2 µg/mL.

As depicted in Fig. 1B, the H_2O_2 concentration of 0.067% (v/v) significantly contributed to successful conjugation as compared to the samples with H_2O_2 concentration 0.017%, 0.033%,v/v, and 0.050%, v/v. A high concentration of H_2O_2 inactivated enzyme without any positive corresponding impact on UV absorbance intensities from the preliminary results. This was in line with (Liu et al., 2015) findings on hydrogen peroxide optimal analysis at various concentrations (0.006, 0.03, and 0.06%, v/v). The optimum value was chosen at 0.067%, v/v.

In Fig. 1C, the maximum UV absorption was obtained in the FG-BP weight ratio of 1:3. This weight ratio was enough to promote oxidation of Ferulic in BP to crosslink the Fish Gelatin during the reaction. There was a decrease in absorbance among the reacted samples. The absorbance in untreated products was high amidst increasing the concentration of BP. Contrary to this, samples with weight ratios of 0.25:1, 0.5:1, 1:1, 1.5:1, 2:1, and 3:1 mediated with enzyme caused some marginal differences in absorption intensities. There existed little significant differences in polymerization, 3:1 produced maximum FG-BP conjugation due to the high amount of Ferulic acid in the reaction. This agrees with the findings reported by (Liu et al., 2015) where reaction groups of 1:2, 1:4, and 1:10 were effective in activating reaction in CFG-BSA than a group of 0.25:1, 0.5:1, and 1:1.

Notably in Fig.1D, there was significant oxidation of Ferulic acid in the formation of BP-FG heteroadducts when the time was prolonged. As reaction duration was shortened at 30 min, polymerization was not sufficient which culminated from the short period of accessibility for bonding between the polymers. When the reaction was prolonged for 4 h, heterogeneous conjugates were highly synthesized linking to the fact that, enzyme activated by hydrogen peroxide was able to ignite more reaction with Ferulic acid. Same phenomenon between Horseradish

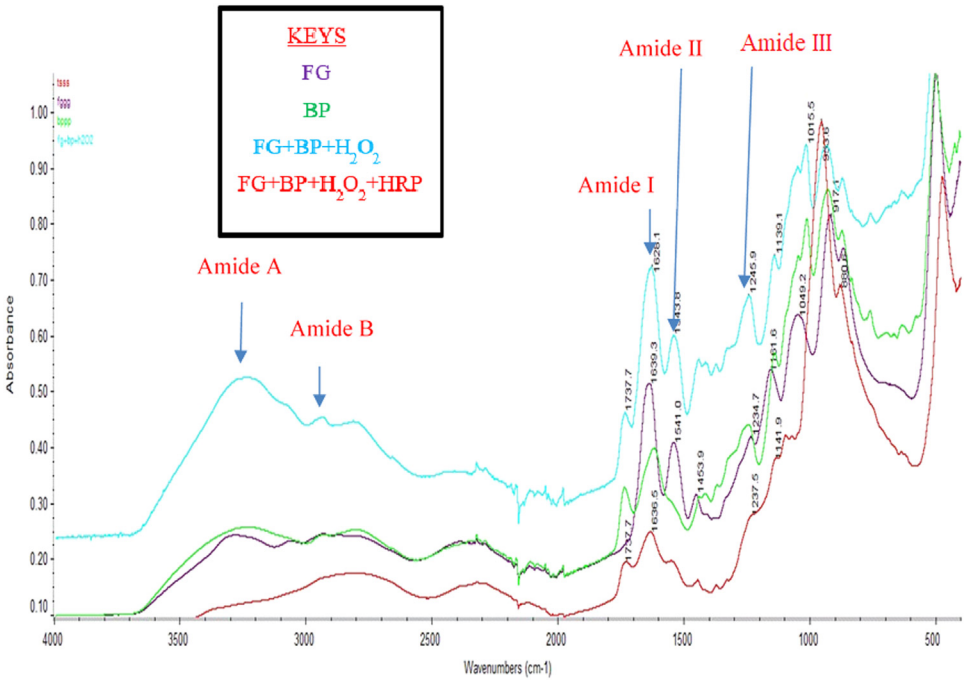


Fig. 2. ATR-FTIR absorption spectra of plain Fish Gelatin (black), Beet Pectin (green), Peroxide treated FG and BP (sea blue), Peroxide peroxidase FG cum BP (red). Gels were lyophilized before analysis (We suggested for better understanding using the online documents).

peroxidase-catalyzed cross-linking of feruloylated arabinoxylans with α-Casein (Boeriu et al., 2004). A maximum formation of β-casein-AX adducts was attained when the time was extended to 24 h in a combination with a low H_2O_2 concentration and molar ratio protein to enzyme between 10^2 and 10^4.

3.2. Characterization of complex (FG-BP)

3.2.1. Attenuated total reflection Fourier-transforms infrared (ATR-FTIR) spectroscopy

ATR-FTIR spectroscopy was employed to probe the conformational changes of protein functional groups and secondary structures like α-helix and β-sheet. From Fig. 2, the major bands of Fish Gelatin thus 1633, 1538, 1238, 3300, 2950, 1654, 1638 (cm^{-1}) correspond to C=O stretching vibrations in the amide I, C-N stretching and N-H bending vibrations in amide II and C-N bending in amide III (Staroszczyk et al., 2014), N-H stretching vibration of OH and NH groups in amide A (Jiridi et al., 2014), and vibration of C-H and NH_3^+ group in amide B (Hamzeh et al., 2018), α-helix (Barth, 2007) and β-sheet (Elavarasan et al., 2016).

The BP had the wavenumbers corresponding to the ester region (1740 cm^{-1}), carboxylate ion stretches (1600 and 1414 cm^{-1}), and amide peaks (1650 and 1550 cm^{-1}) (Marry et al., 2000). The amide peaks in BP are due to endogenous protein moieties, which serve as emulsifying properties (Lin, Guo, Ai, Zhang, & Yu, 2020). Meanwhile the slight shift of amide I band (1633 cm^{-1}) to high wavenumber, 1638.5 cm^{-1} indicates the presence of β secondary structures (Pavan, Chandra, Elavarasan, & Shamasundar, 2017). When BP was incorporated into FG in HRP/H_2O_2 mediated system a decrease of ordered structures as the result of complex formation was observed. The shift of absorption bands of the ester groups in the enzyme-treated sample to lower frequency (1737.7 cm^{-1}), compared to the band of BP (1740 cm^{-1}) shows the interaction of the ester group with the amide group in FG and this was analogous to other reports made (Sinthusamran et al., 2017). They ascertained that the sulphate group of FG-25 carrageenan (CG), FG-50CG, and FG-75CG band moved from lower wavenumbers 1241, 1242, and 1245 cm^{-1}, respectively as compared to 1250 cm^{-1} in pure CG gel.

Table 1
The secondary structure of plain fish gel, Beet Pectin, peroxide treated fish gel, and Beet Pectin is devoid of enzyme and peroxide peroxidase fish gel and Beet Pectin.

Treated Sample	Secondary Structure	
	α- helix (%)	β- sheet (%)
FG	13.56 ± 0.10^{ab}	39.65 ± 0.01^{a}
BP	14.44 ± 0.08^{a}	39.9 ± 0.02^{a}
FG+BP+H_2O_2	15.04 ± 0.06^{b}	40 ± 0.03^{a}
FG+BP+H_2O_2+HRP	14.1 ± 0.05^{a}	41.14 ± 0.04^{b}

Means of triplicate ± SD values in the same column with letters superscript are significantly different ($p<0.05$).

The FG-BP with H_2O_2 had peaks of 1628.1, 1543, and 1245.9 cm^{-1}. These new absorption peaks might have occurred due to electrostatic interaction between N-H and C=O in FG and carboxylic group in BP, though, the mixture was devoid of the enzyme. HRP mixture synergized with H_2O_2 encapsulating FG and BP recorded the highest β-sheet of 41.4% as shown in (Table 1) and this could be due to the intermolecular crosslinking of FG and BP to form C-O-C via amines and phenolic acids, respectively (Liu et al., 2015; Roberts et al., 2016).

Also, the β-sheet recorded a slight shift of amide I band (1633 cm^{-1}) to high wavenumber, 1638.5 cm^{-1} (Pavan, Chandra, Elavarasan, & Shamasundar, 2017). This could be attributed to interactions precipitated by the enzymes which include; hydrogen bond and electrostatic force (Hu et al., 2011). Ustunol (2014) distinguishes the importance of beta-sheet in the food protein who states that the β-sheet is more stable than the α-helix; therefore, proteins with large segments of β-sheet structures are likely to have higher denaturing temperatures. Additionally, a high amount of β-sheet may lead to an increment of an ordered structure of the protein's good stability (Gui et al., 2020). This modified gelatin could play a significant role in the pharmaceutical industry especially in capsules production with desired functional properties. It is reported that hard capsules which are usually obtained from cold- water Fish Gelatin had a low gelling temperature (12-13 °C) which is below the human body temperature (37 °C) because of the low content of proline and hydroxyproline. This problem could be solved if the gelatin is crosslinked to improve the imino acids content (Al-Nimry et al., 2021). These

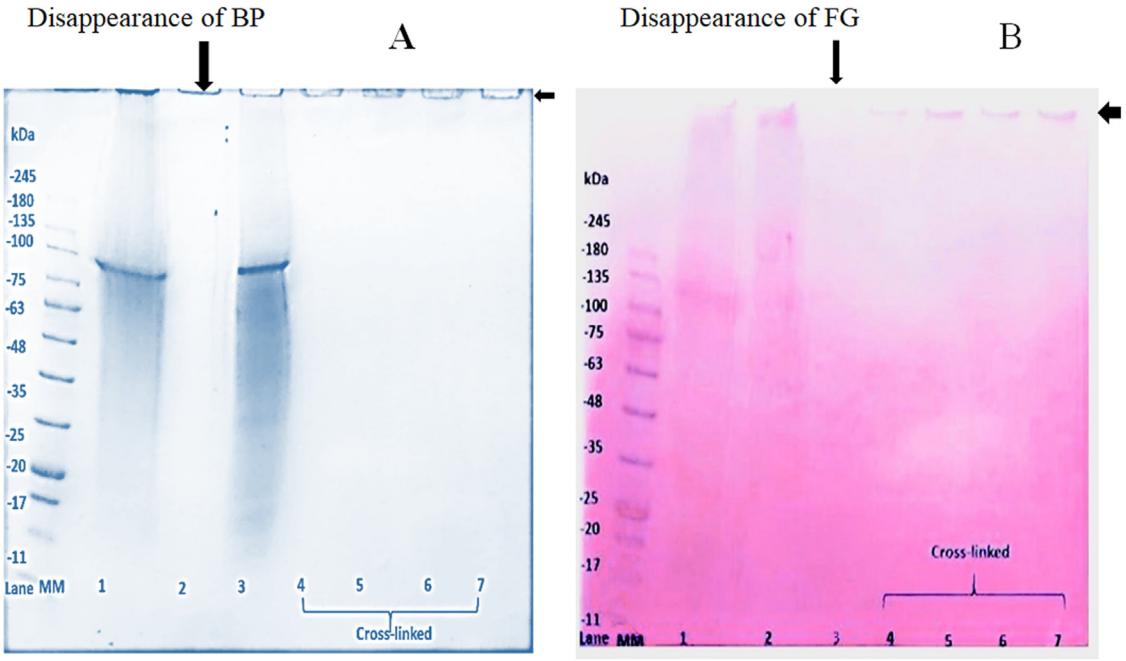


Fig. 3. A-B Analysis by SDS-PAGE of FG and BP conjugate (1:3) cross-linked by HRP/H_2O_2 system–(A) Protein stain (blue) and (B). Carbohydrate stain (pink). The labeled lanes are–MW–Molecular weight, 1. Native Fish Gelatin, 2. Pure Beet Pectin, 3. Fish Gelatin cum Beet Pectin treated with hydrogen peroxide without peroxidase, 4-7 Fish Gelatin and Beet Pectin mediated with peroxide/peroxidase (0.067%, v/v:0.5 μg/mL, 0.067%, v/v:1 μg/mL, 0.067%, v/v:1.5 μg/mL, and 0.067%, v/v:2 μg/mL), respectively. Horizontal arrows indicate the interface of stacking and resolving gels. Different gels were used. The protein (FG) component was injected in the 3rd lane in carbohydrate stain.

results indeed suggested that the interaction between amide groups of gelatin and ester and carboxylic groups of BP occurred, led to the formation of FG-BP conjugates in ATR-FTIR analysis.

According to scheme 1A-C, the enzyme crosslinked the Tyrosine moieties in FG and Ferulic acid in BP to produce heterologous products through a free radical mechanism using H_20_2 as a substrate in a combination of 3 mL of BP and 1 mL of FG. When the volume of BP increased, the enzyme was able to polymerize the Tyrosine residues in the FG leading to heteroconjugation of FG and BP. A similar observation occurred when two (2) molecules of FA and one (1) of glycine (GYG) molecule were covalently linked through peroxidase mediation. They noted HRP promoted GYG polymerization in the reaction when FA was added (Boriu, 2008). This phenomenon could induce a well -ordered amino acid constituents in the FG in the treated samples with strong and stabilized active ingredients which can be applied as active ingredients in the pharmaceutical industry (Karim & Bhat, 2009).

3.2.2. Confirmation of intermolecular conjugation by SDS-PAGE

To confirm the covalent coupling of FG to BP profile during enzymatic cross-linking, SDS-polyacrylamide gel electrophoresis was employed (Selinheimo et al., 2008; Akhtar and Dickinson, 2007; Liu et al., 2017; Perrechil et al., 2014). Carbohydrates usually react with proteins via Maillard heat reaction to form high molecular-weight products which are normally identified by glycoprotein stained SDS-PAGE gels (Liu et al., 2017). The conjugated products show retained bands in stacking gel and rough bands in resolving gels. In this study, we proved the viability of using this assay to affirm the enzymatically-protein and carbohydrates conjugates. Fig. 3A shows the patterns of native BP and FG and enzyme-mediated mixture of FG and BP (FG + BP + HRP) in the presence of H_2O_2 at different combination of enzyme/peroxide (0.5 μg/mL: 0.067%, v/v, 1 μg/mL: 0.067%, v/v, 1.5 μg/mL: 0.067%, v/v, and 2 μg/mL: 0.067%, v/v).

Upon visualizing the gels, changes in characteristics bands of protein were noticed. As the conjugation reaction proceeds, there was drastic disappearance of lane 2 which indicates the carbohydrates portion, BP in the blue stained gel. Lane 1 appears to have a deeply stained band in pure Fish Gelatin signifying unreacted protein in the native state. However, lane 3 which is a mixture of untreated FG and BP H_2O_2 appears to experience the same phenomenon in lane 1. The enzyme-treated mixture in the weight ratio of 1:3 (FG: BP) from lanes 4-7 seems to be associated with a marked fainted band in the conjugated sample. The reaction between FG and BP activated by an enzyme causes more FG to polymerize the amount of native FG in the reaction. Elsewhere, a similar observation was ascertained by (Cura et al., 2009) on the treatment of sodium caseinate with laccase under a condition of 45 °C results in the formation of polymerization products. This was attributed to the disappearance of fragmented bands due to the fast rate of reaction activated by 45 °C· In Fig. 3B, the gel traces for carbohydrate component was shown by PAS (periodic acid Schiff) pink stained gel. When the sample was treated with enzyme from (0.017-0.067%, v/v):1 μg/mL) of H_2O_2 and HRP, respectively, the bands became faint. This is synonymous with results reported by (Fan et al., 2016) on Bovine Serum Albumin (BSA) and SBP conjugation. The bands in BSA were wide and dark as compared to fainted and narrow bands in the BSA-SBP complex and conjugation. Consequently, (Liu et al., 2017) reported similar work on BSA and Corn Fiber Gum (CFG) enzymatically cross-linked. They observed that enzymatic-treated CFG and BSA produce high-molecular-weight which settles at the interface of resolving and stacking gel. An indistinguishable scenario was observed in our work. When the concentration of enzyme was increased, more feruloly moieties were exposed for reaction. This leads to purple staining conjugate bands at the interface between stacking/resolving gel as clearly shown in lanes 4-7 among the cross-linked samples. The results gave more retained bands in FG-BP conjugates at the weight ratio of 1:3 which was induced by intermolecular crosslinking of FG with BP in the stacking gel with high molecular-weight. Further, to prove the feasibility of enzymatically-treated FG-BP conjugates by electrophoresis, the enzymatically-treated FG-BP protein stain band completely disappeared in lane 3. Hence, the concerted disappearance of the protein band in Fig. 3A and carbohydrate in Fig. 3B logically portrays another evidence of covalent bonding between the polysaccharide and protein.

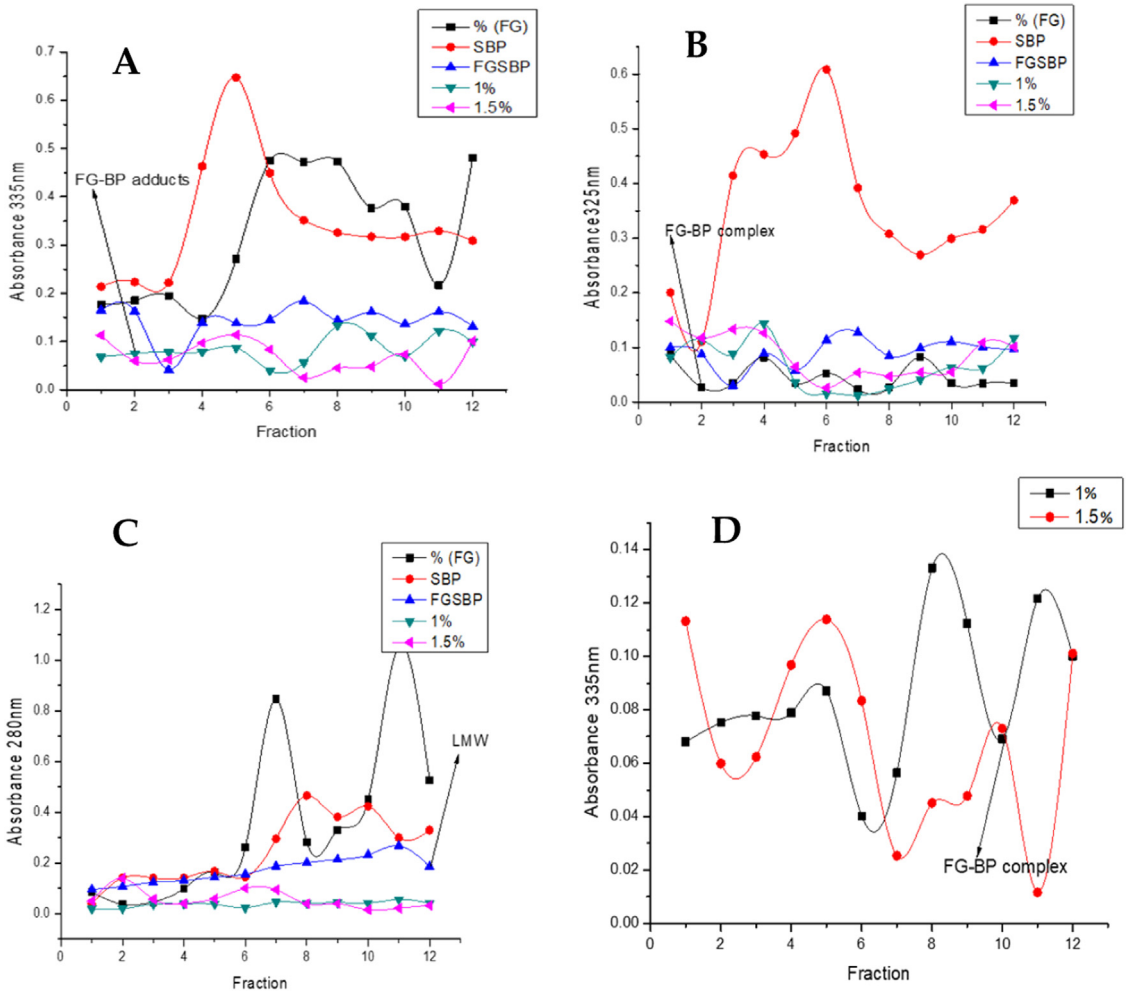


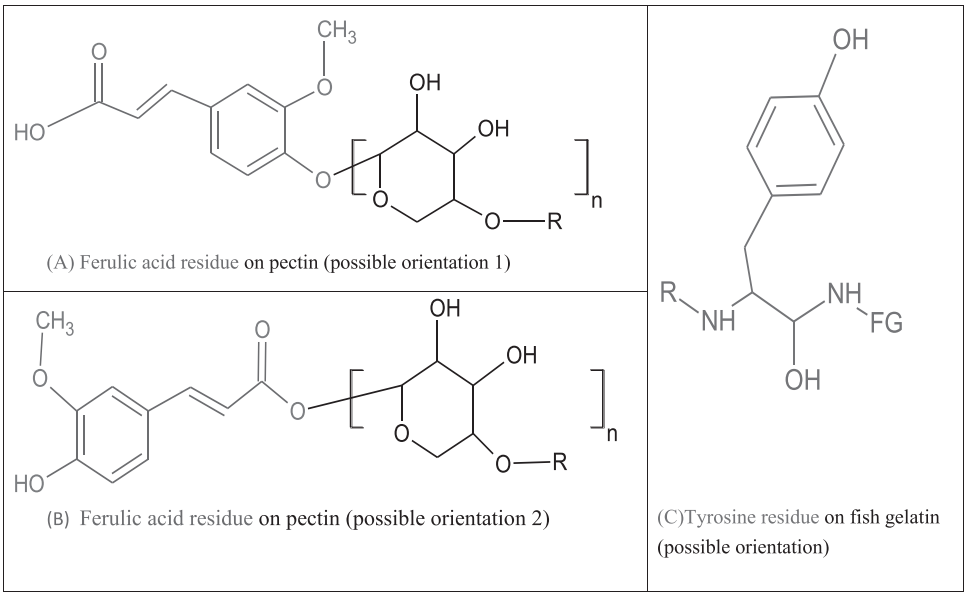
Fig. 4. A-D SEC-Sephadex G-100 Chromatograms of FG and purified FG-SBP enzymatic conjugates. The Figs. A, B and C (FG, SBP, untreated FGSBP and purified FG-SBP enzymatic conjugates varied at 0.033% and 0.050% (v/v) of hydrogen peroxide concentration) and D (comparative analysis of eluted peak between 0.033%, v/v and 0.050%, v/v conjugated FG-SBP purified mixture), respectively.

The conjugated polysaccharide-protein products are touted as a great determinant of gelatin quality because this product appears to have a high molecular weight as compared to the pure or untreated product. The higher the molecular weight, the better and stable the gelatin quality and functionality (Chen et al., 2018). Additionally, modified gelatin obtained in our work could act as a stabilized emulsifying agent according to the electrophoretic results in Fig. 3A-B, which indicated the intermolecular conjugation, which possibly showed that our products could have high molecular weight and as such exhibit good emulsifying properties [Surh et al., 2006]. Lastly, marine products are usually applied as drug deliverers. They are mostly preferred to synthetic polymers because they are biocompatible. They are modified through cross-linking to modify it properties to enhance its stability and circulation time (Jahanshahi et al., 2008). These are synonymous to our focus in the study to modify the existing cold-water Fish Gelatin in order to improve and expand its utilization in the pharmaceutical industry.

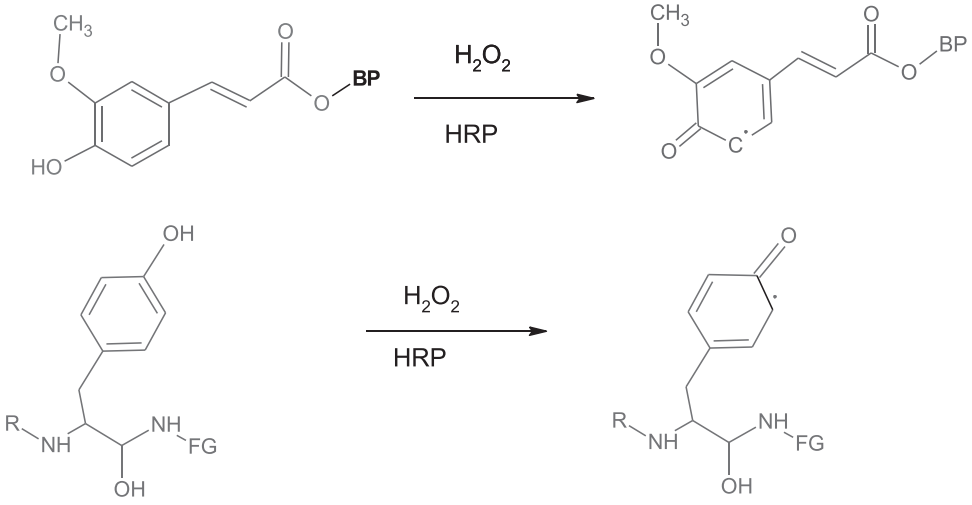
3.2.3. Size exclusion chromatography (SEC)

Conjugated FG-BP peroxidase/peroxide was separated from homopolymers by SEC using the Sephadex G-100 column. A preliminary experiment was conducted to determine the appropriate wavelength for protein, polysaccharide and conjugate (data not shown). Results shown by UV-spectroscopic measurements were 280 nm, 325 nm, and 335 nm for protein (FG), polysaccharide (BP) and FG-BP conjugate, respectively. Plain FG, BP, BP-FG with H_2O_2, and HRP treated FG-BP conjugates were estimated on all the wavelengths. Fig. 4A-D shows the analytical SE chromatograms of incubation of FG- BP/H_2O_2 system with or without HRP on the Sephadex G-100 column.

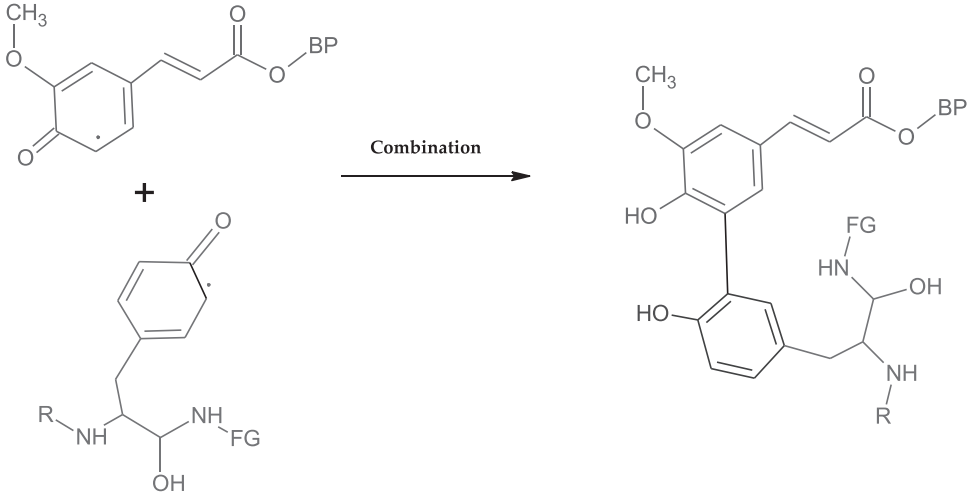
In Fig. 4A, UV signal for untreated BP had the highest peak followed by pure FG and H_2O_2 modified FG-BP. The treated sample had a reduced peak which was fairly lower than unpurified FG-SBP. As the concentration increased from (0.033 (1%)-0.050 (1.5%), v/v) of H_2O_2, peak height declined markedly. A possible reason for the peak reduction could be that majority of the Tyrosine was conjugated to SBP/BP (sugar Beet Pectin or Beet Pectin). Conversely, FG and SBP seem to have recorded high peaks implying most of the proteins and carbohydrates moieties were not reacted (Figueroa-Espinoza & Rouau, 1998). Fig. 4B shows chromatograms of various samples at 325 nm. This wavelength is noted for carbohydrates determination regarding the maximum detection of Ferulic acid at this wavelength band (325 nm) (Jung & Wicker, 2012). The polysaccharides appear to be of high eluting peak (shaded red) containing a large amount of Ferulic acid as a sign of the relative abundance of BP unreacted in the total mixture. Burying of feruloyl moieties in BP as well as amino residues of FG hinders the formation of C-C covalent aromatic bonds. Our work appeared to agree with the publication done by (Xiao et al., 2018) on dry-state Maillard reaction heat-induced Whey Protein Isolate (WPI)-SBP conjugates, in which they reported disruption of environment for heterogeneous aromatic side-chains formation culmination from loss of contribution between Tyrosines in WPI and Ferulic acid in SBP. Interestingly, protein components demonstrated



Scheme 1A. Ferulic acid residue in Beet Pectin and Tyrosine residue in Fish Gelatin.



Scheme 1B. HRP/H_2O_2 catalyzed radical formation of Ferulic acid (FA) residues and Tyrosine (Tyr) residues of Beet Pectin and Fish Gelatin, respectively.



Scheme 1C. Possible outcome for the enzyme catalyzed hetero-conjugation of FG and BP via Tyr-FA radical reaction.

some peak on carbohydrates wavelength though it was reported protein moieties are majorly detected on 280 nm (Xiao et al., 2018). According to Fig. 4C, a similar phenomenon in Fig. 4B also occurred at 280 nm, a wavelength normally suitable for amino acid residues detection. A high population of cross-linked protein conjugates eluted as depicted in the major peak (black colour). In this case, there is an appearance of BP (red colour) on 280 nm due to the proteinaceous materials it possessed (Fan et al., 2016). Enzymatic-treated FG-BP at different concentrations (0.033% and 0.050%, v/v) produced peaks with low absorbance intensities. The product with 0.050%wt and 0.050%,v/v of H_2O_2 mediated with 1 μg/mL of the enzyme had reduced and high peak profile, respectively. This is in agreement with a study reported by (Boriu, 2008) when they prepared HRP/H_2O_2 (100 μL/100μL) to cross-linked α-lactoalbumin and arabinoxylan. Finally, Fig. 4D depicts treated samples eluted on 335 nm, complex wavelength determined in a preliminary experiment (results not shown). This was conducted to estimate the treated products responsible for a higher molecular weight. As seen in Fig. 4C, with the right combination of peroxidase/hydrogen peroxide (1 μg/mL: 0.033%, v/v), respectively for conjugate reaction, absorption value reduced signifying the occurrence of crosslinking reaction for large molecular weight. This is reflected in absorption value of 0.033%, v/v (0.0699) and 0.050%, v/v (0.1199).

4. Conclusions

Protein-polysaccharide complexation has been noted for multiple commercial applications in both the food and non-food industries. This study was carried out to cross-link FG-BP in HRP/H_2O_2 system to modify the chemical and molecular structure of Fish Gelatin. The crosslinking between FG-BP was successful through an optimized experiment between the various cross-linking reaction parameters using UV-Vis Spectroscopy at 340 nm as confirmation of Horseradish catalyzes BP. The optimal weight ratio of heterologous adducts between FG-BP was 1:3, in a combination with HRP to H_2O_2 of 2 μg/mL and 0.067%, respectively at 4h under 50°C. Generally, the incorporation of peroxidase to FG-BP via hydrogen peroxide was found to be favorable for the modification of cold water Fish Gelatin with Beet Pectin. This work provides a suitable base for building the FG-BP complex to understand the functionalities of FG Scheme 1A, 1B, 1C.

Declaration of Competing Interest

The authors declare no conflict of interest or personal relationship that could have appeared to influence the work reported in this paper.

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