CHAPTER 4 RESULTS AND DISCUSSION

4.1 Characterization of materials

4.1.1 Proximate composition of fish protein powder

Fish powder was prepared from fish mince by chopping and drying in hot air oven until weight constant. The proximate compositions of fish protein powder were determined as described by AOAC method [47]. Fat, protein moisture and ash content were analyzed as shown in Table 4.1

Table 4.1 Chemical composition of fish protein powder

Chemical composition	***Percent (%)***
Fat	4.28±0.07
Protein	84.93±0.45
Moisture	6.42±0.15
Ash	2.78±0.01

Values are mean ± SD, Protein conversion factor is 6.25 [57].

4.1.2 Thermogravimetric analysis (TGA)

Thermal decomposition of fish protein and Kraft lignin were studied. Table 4.2 shows T_d (thermal decomposition) of fish protein and Kraft lignin. The initial weight loss around 100 ºC is due to the evaporation of water [16]. This result was shown in appendix B.1. Thermal degradation temperatures were determined from T_d curves that samples start to degrade at around 190°C for fish protein and 150 °C for Kraft lignin. For Kraft lignin, it could not be degraded due to the presence of strong carbon-carbon linkages and other chemical groups such as aromatic groups, which are very resistant to treatment [58]. In addition, the initial stage in thermal degradation of lignin is concerned with hydroxyl groups [59]. Therefore, the temperature of 140 °C which is lower than degradation temperature was taken to study rheological properties of plasticized fish protein/Kraft lignin blend during the heating in order to avoid degradation.

Table 4.2 T_d (thermal decomposition) and T_g (glass transition temperature) of fish protein and Kraft lignin.

Sample	***T_d (°C)***	***T_g (°C)***
Fish protein	192	167.2±1.0
Kraft lignin	165	151.7±0.7

4.1.3 Differential Scanning Calorimetry (DSC)

Table 4.2 displays the DSC results which are the second DSC scan of fish proteins and Kraft lignin respectively. This sample shows the glass transition temperature. Fish protein has glass transition temperature at 167.2 ºC. For Kraft lignin, it melted has a glass transition temperature at 151.7 ºC. Glass transition temperature of protein and Kraft lignin are very close to those reported by Dean et al. [60]. In this work, the first DSC heating curve is often affected by desorption of moisture and by enthalpy relaxation effects. Thus, the results from the second heating curve were used for the various calculations.

4.2 Rheological properties of plasticized fish protein/ Kraft lignin blend

Rheological behavior represents the most important property of biomaterial during processing

4.2.1Torque rheology

Mixing of fish protein/Kraft lignin-plasticizer blends has an influence on the properties of thermo mechanically processed biomaterial. In processing plastics, the material is melted, transferred and then solidified. The transfer or flow process is critical to processing. An understanding of the flow properties of the material is important for manufacturing of biomaterial.

Figure 4.1 shows the equilibrium torque and equilibrium temperature of plasticized fish protein/ Kraft lignin blend at 0–70% Kraft lignin content during mixing at 80 °C 100 rpm for 15 min. Equilibrium temperature of polymer blend increased more than initial process temperature because shear induces heat during mixing [61], which is attributed to the thermal energy dissipation due to the high medium viscosity. In addition, increasing % of Kraft lignin content can decrease equilibrium torque and temperature. Therefore, addition of Kraft lignin decreased viscosity of polymer blend.

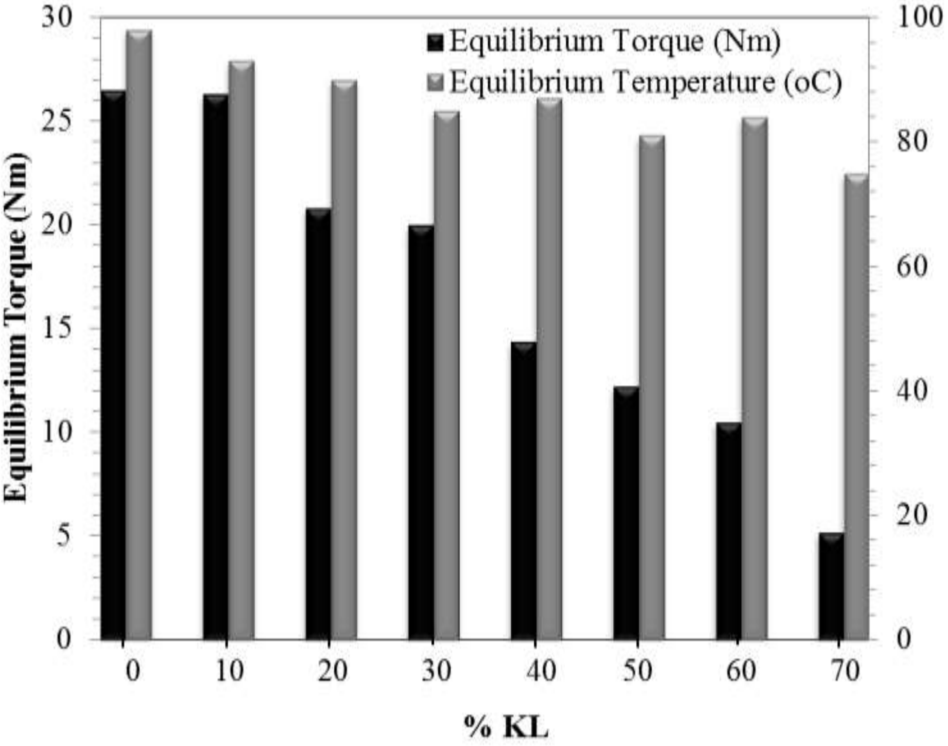


Figure 4.1 Equilibrium torque and equilibrium temperature of plasticized fish protein/ Kraft lignin blend with 0-70%Kraft lignin content at 80 °C 100 rpm for 15 min.

4.2.2 Capillary rheology

In all plastics conversion processes the flow characteristics are important in defining process parameters. For this reason, it is not surprising that the measurement of polymer melt flow has been considered as a critical factor in determining the processability of the polymer. Therefore, this study was to investigate the effect of Kraft lignin addition on flow properties of plasticized fish protein blend.

Viscosity data can also provide some information about changes in molecular structure [33]. Viscosity of fish protein blends with 0-70% Kraft lignin were determined by using capillary rheometer which is a technique whereby a sample undergoes extrusion through a die of defined dimensions. The test sample was pushed by a piston driven at temperature of 140 ^{o}C through a capillary die and measured its rheological properties like shear rate and shear viscosity. The data was obtained from steady shear measurement as shown in Figure 4.2.

The shear viscosity of plasticized fish protein/ Kraft lignin blend decreased when shear rate increased. The shear viscosity of the blends was recorded in the shear rate range of 10-1000 s^{-1}. However, the testing region for fish protein blend with 0% Kraft lignin could not exceed 500 s^{-1} with the same amount of sample. Thus, the melt of the blend exhibited more stability under high shear. The addition of Kraft lignin resulted in a decrease of shear viscosity of mixed blend.

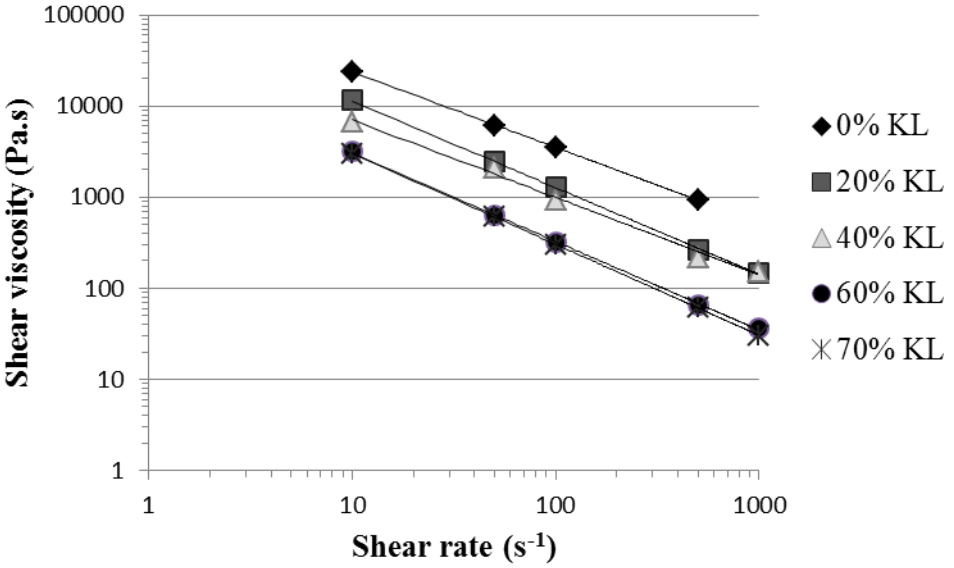


Figure 4.2 Viscosity of Fish protein blends with 0-70% Kraft lignin content determined by capillary rheometer at 140 °C die temperature

The relationship between the shear viscosity and shear rate can be described by power law equation according eq. (3.6). Power-law index and flow behavior consistency were calculated as shown in Table 4.3. K is often known as the flow behavior consistency. This describes the overall range of viscosities across the part of the flow curve that is being modeled. Also, if the Power law region includes $1s^{-1}$ shear rate then K is the viscosity or stress at that point. The exponent n is known as the Power Law Index (or sometimes the rate Index). For a shear thinning fluid: $0<n<1$, the more shear-thinning the product, the closer n is to zero. For experiment, the power-law index of fish protein-based material is 0.17. Values for the power index for protein ranging from 0.12 to 0.78 have been reported in the literatures [33, 49, 62]. All samples represented shear thinning behavior and their ability to flow. Power-law index and the consistency coefficient decreased when Kraft lignin content increased. This may be explained by a low molecular weight of Kraft lignin (MW= 2000-5000 Da) [11, 38, 42] compared to fish protein (MW= 8-600 kDa) [2, 63]. In addition, addition of Kraft lignin may increase chain-mobility at high temperature and high shear rate. Therefore, the presence of Kraft lignin in the mixture seemed to improve the flow properties substantially. The rheological properties of fish protein-based biomaterial strongly affected by the behavior of Kraft lignin and were expected to play an important role in any process developed to produce bioplastics from fish protein.

Table 4.3 Power-law model parameters of plasticized fish protein/ Kraft lignin blends

Sample	**Consistency (Pa.s^n)**	**n**
0% KL	145693^a±21442	0.17^a ±0.01
20% KL	105266^b± 4981	0.02^b ± 0.03
40% KL	47727^c± 3976	0.14^a ± 0.01
60% KL	28459^c± 428	0.03^b ± 0.00
70% KL	30235^c± 117	0.00^b ± 0.00

4.3 Thermomechanical properties

In order to investigate the effect of Kraft lignin as a filler on viscoelastic properties of fish protein material, fish protein materials with 0-70% Kraft lignin were determined by using Dynamic mechanical analysis (DMA).

Figure 4.3 shows storage modulus (E') and tan δ of plasticized fish protein/Kraft lignin materials. As a function of temperature, storage modulus decreased down to minimum values, showing a rubbery-like plateau region about 120ºC. For tan δ, it was clearly found that two peaks appeared for all of the fish protein-based bioplastics. The lower temperature peak was attributed to the glass transition temperature (T_g) of the free glycerol [36]. The higher temperature peak corresponded to the T_g of the plasticized materials.

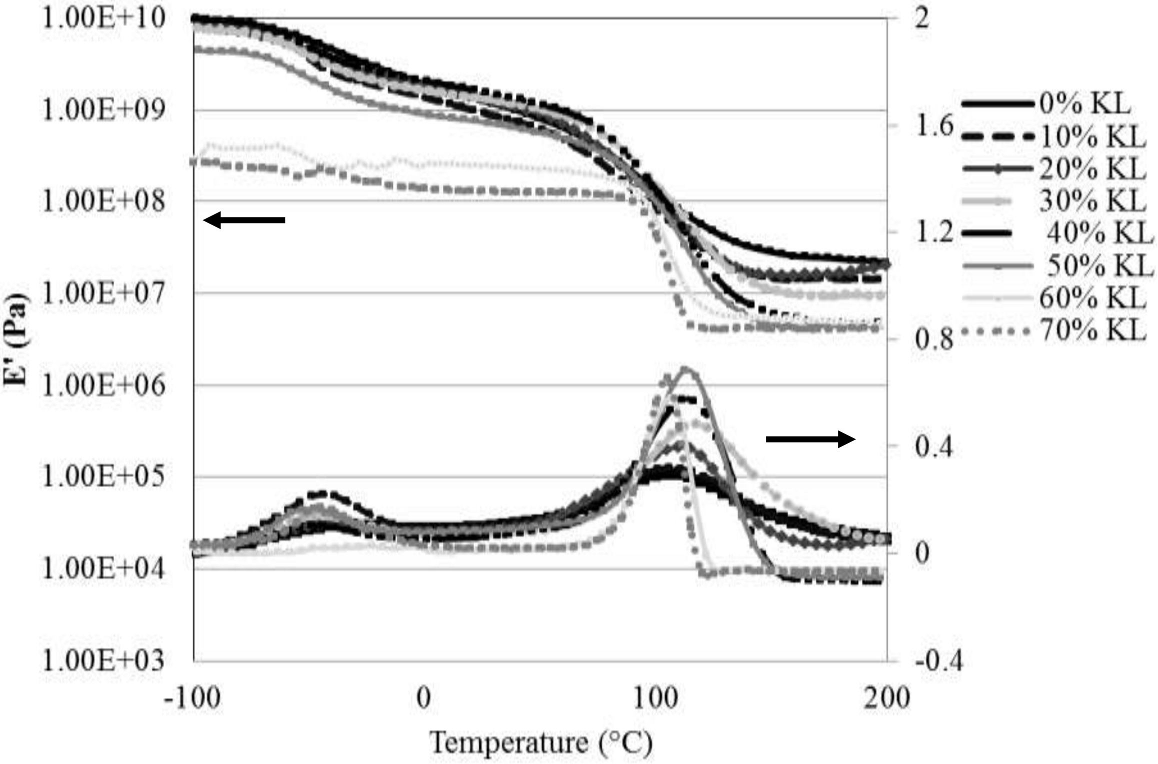


Figure 4.3 Storage modulus (E') and tan *δ* of plasticized fish protein/ Kraft lignin biomaterial

Table 4.4 shows the values of storage modulus E' in rubbery regions, tan *δ* peak height (E"/E') and the T_g which was determined by the temperature at maximum of tan *δ* peak height. Addition of Kraft lignin decreased storage modulus in rubbery state. Moreover, tan *δ* peak height increased with Kraft lignin content, suggesting an increase in liquidlike nature of materials. This may be associated with a low molecular weight of Kraft lignin and plasticization properties of Kraft lignin which has a number of hydroxyl groups [4]. The glycerol plasticized protein from liquidlike viscosity network structure. When the content of Kraft lignin increased, protein becomes more viscous and the resistance to small deformation decrease.

Processing of protein-based bioplastics on extrusion and injection molding is limited because protein blend has high viscosity due to protein aggregation during processing. Addition of Kraft lignin decrease viscosity of protein blend. Therefore, Kraft lignin is an alternative additive to enlarge the protein thermal processing window by decreasing the viscosity at high processing temperature.

As shown in Table 4.4, T_g of 0% Kraft lignin content is 105.5 ºC and T_g of 70% Kraft lignin content is 109.6 ºC. However, T_g of the material increased with Kraft lignin content increase and the maximum enhance was observed when Kraft lignin content reached to 30 wt%. Because, it may be associated with cross linking of plasticized fish protein/Kraft lignin biomaterial which might be due to the intra-molecular interactions of the hydroxyl and carboxyl groups in the fish protein and the methoxyl and carboxyl groups in Kraft lignin including hydrogen bonding and polar interactions [64, 65]. However, T_g of the materials are lower than pure fish protein and Kraft lignin because glycerol may have reduced the T_g by mechanisms other than those commonly associated with plasticization [66].

Table 4.4 Storage modulus (E') at 140^{o}C, glass transition temperature (T_g) and tan δ peak height of plasticized fish protein/ Kraft lignin biomaterial

KL (%)	**E' at 140ºC (MPa)**	**Tan δ peak height (E''/E')**	**T_g (°C)**
0%	$29.7^{a}\pm4.1$	$0.32^{e}\pm0.03$	$105.5^{c}\pm1.9$
10%	$16.9^{b}\pm0.03$	$0.34^{e}\pm0.03$	$107.8^{abc}\pm2.4$
20%	$15.2^{bc}\pm2.1$	$0.43^{de}\pm0.05$	$113.1^{ab}\pm2.5$
30%	$10.7^{cd}\pm4.2$	$0.49^{cd}\pm0.01$	$115.1^{a}\pm2.7$
40%	$8.1^{de}\pm0.2$	$0.59^{bc}\pm0.01$	$113.0^{ab}\pm0.5$
50%	$7.1^{de}\pm1.9$	$0.64^{ab}\pm0.07$	$111.3^{abc}\pm2.9$
60%	$5.9^{de}\pm0.6$	$0.59^{bc}\pm0.01$	$106.6^{bc}\pm0.7$
70%	$3.4^{e}\pm1.2$	$0.74^{a}\pm0.09$	$109.6^{abc}\pm5.0$

4.4 FTIR spectra

The cross linking of plasticized fish protein/ Kraft lignin biomaterial were supported by the FTIR spectrum. FTIR was used to investigate functional group and the interaction between fish protein and Kraft lignin because addition of Kraft lignin increases T_g of materials until the content of lignin reached to 30 wt%, so new chemical bond may occur.

Figure 4.4 shows the FTIR spectra of fish protein materials with 0-70% lignin content. The characteristic bands of fish protein at 3399 cm^{-1} represented O-H stretching vibration called Amide III. The bending vibration of N-H group and stretching vibration of C-N group called Amide II. That is attributed to an out-of-phase combination of CN stretch and in-plane NH deformation modes of the peptide group and also showed the peak at 1649 cm^{-1} representing carbonyl group called Amide I. The absorption in amide I region is the most useful for infrared spectroscopic analysis of the secondary structure of proteins [67]. For the FTIR spectra of material with 70% Kraft lignin showed various structural units that the products contain hydroxyl groups, indicated by the broad O–H stretching absorption 3390 cm^{-1}, C–H stretching bands of aromatic at 2936 cm^{-1}, aldehyde group indicated by H–CO stretching band at 2882 cm^{-1}, carbonyl group indicated by C=O stretching band at 1598 cm^{-1} and aromatic stretching band at 1462 cm^{-1} in agreement with literature [11, 68].

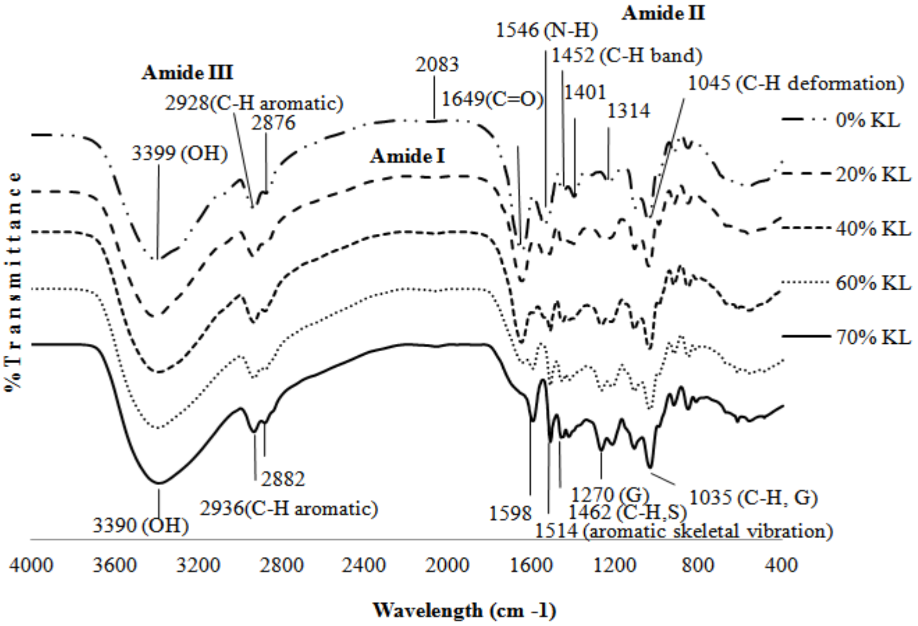


Figure 4.4 shows the FTIR spectra (top to bottom) of biomaterial with 0% Kraft lignin, 20% Kraft lignin, 40% Kraft lignin, 60% Kraft lignin and 70% Kraft lignin

However, cross linking of plasticized fish protein/Kraft lignin based biomaterial showed no new chemical bond between fish protein and Kraft lignin was observed. This suggests that these samples have the same main structure. In other words, this means that the biomaterial did not change the main structure of Kraft lignin and protein. This may be due to low cross-linking between fish protein and Kraft lignin [69].

The assignments of these intense peaks of lignin samples based on literature are summarized in Table 4.5

Table 4.5 IR peaks assignments for lignin [11, 59, 70]

Peak positions (cm^{-1})	**Peak assignments (Lignin)**
3420	O–H stretching
2960	C–H stretching
1590	C=C aromatic skeletal vibration
1510	C–C ring skeletal vibration
1460	C–H vibration
1420	C–O stretching
1393	Phenolic O-H- band
1271	aryl-O of aryl-OH and aryl-O-CH_3 ; guaiacyl ring (C=O group)
1182	C–O stretching
1102	Out of phase C-C-O stretch of phenol
1050	C–O stretching
969	C-CH and –HC=CH- deformation

Table 4.6 IR peaks assignments for protein [71, 72]

Type	***Peak positions (cm^{-1})***	***Peak assignments***
Amide I	1600-1614	Side-chain vibrations
	1616-1639	Intemolecular β-sheet
	1640-1650	Random coi1
	1651-1655	α-Helix (C=O stretching band C-N stretching and CCN deformation)
	1680-1688	Intermolecular β –sheet
Amide II	1550	NH bending and CN stretching C=O in-plane bending and NC stretching.
Amide III	1300	N-H bending and CN stretching CC stretching and CO bending

4.5 Protein aggregation of plasticized fish protein/ Kraft lignin biomaterial

4.5.1 Protein solubility

Solubility of protein biomaterial before and after processing is often regarded as a good indicator of cross-link formation during processing [4]. In order to study the effect of Kraft lignin on protein aggregation, changes in solubility of protein in SDS phosphate buffer were investigated. In this test, protein was dissolved in the SDS buffer. This protein fraction is called SDS-soluble protein. Then, DTE as a reducing agent was added to the remaining protein in order to cleavage disulfide bonds [66]. This SDS-insoluble protein was then dissolved in SDS-buffer. SDS-insoluble protein was a well established quantity, which reflects the covalent cross-linking degree of the protein network [31].

Protein content of SDS-soluble and SDS-insoluble fraction of Kraft lignin /fish protein molded at 100°C is shown in Table 4.7. After mixing and compression molding process, SDS soluble fraction of fish protein powder decreased dramatically from 64 to 18% compared to fish protein-based material with 0% Kraft lignin. This was attributed to heat treatment induced cross-linking during the molding process that led to an increase in covalent cross linking of protein by disulfide bonds, hydrogen bonds and hydrophobic interactions [4, 13, 73]. Total protein recovery was less than 100% in all samples. This may be explained by the original high protein of fish protein powder and the high processing temperature. The addition of Kraft lignin resulted in an increase of the SDS-soluble protein and total protein recovery compared to fish protein material without Kraft lignin. These results indicated a decrease of protein molecular weight of samples**,** suggesting the effect of Kraft lignin on protein aggregation.

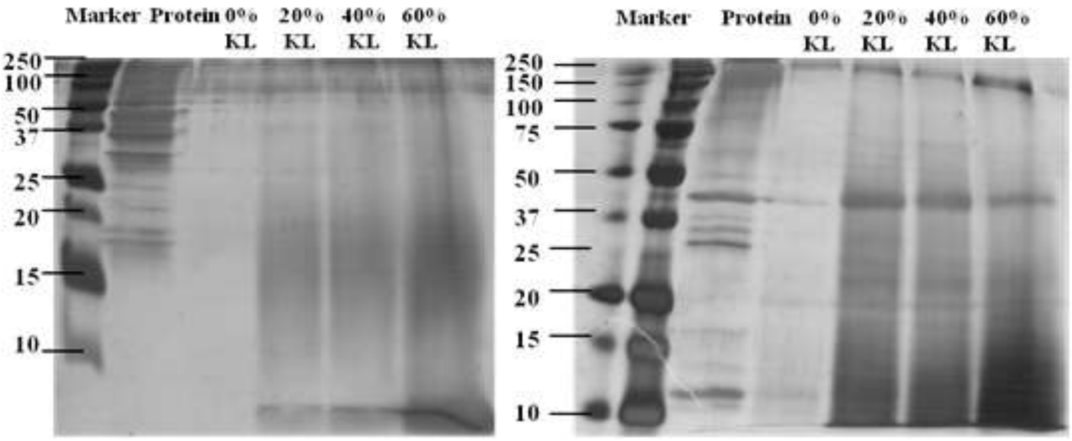
Table 4.7 Protein content (%) of SDS-soluble, SDS-insoluble fraction and total protein recovery of fish protein powder and plasticized fish protein/ Kraft lignin biomaterial molded at 100°C.

Sample	SDS-soluble protein fraction	SDS-insoluble protein fraction	Total protein recovery (%)
Fish protein	64^{a}±8	24^{a}±4	88
0% KL	18^{c}±2	8^{b}±3	26
20% KL	21^{bc}±1	11^{b}±2	33
40% KL	23^{bc}±4	12^{b}±3	35
60% KL	28^{b}±5	9^{b}±3	37

4.5.2 Protein molecular weight

As Kraft lignin is a free radical scavenger, it may interfere protein aggregation. The effect of Kraft lignin on protein aggregation was studied by measuring protein molecular weight of material using SDS-PAGE. 15% SDS-PAGE gel allows the separation of the majority of fish protein. The molecular weight of soluble fish protein and insoluble fish protein after molding with different Kraft lignin content were measured and showed in Figure 4.5.

Protein molecular weights of plasticized fish protein/ Kraft lignin molded at 100 °C were measured using 15% SDS-PAGE gel. Figure 4.5 (a) and (b) show respectively molecular weight of SDS-soluble and SDS-insoluble protein of plasticized fish protein/Kraft lignin materials. Molecular weight of fish protein powder ranges from 18 to 250 kDa in both SDS-soluble and insoluble. After compression molding, fish protein-based material presented only the low intensity band at 50-75 kDa for SDS-soluble and at 37-50 kDa for SDS-insoluble. Band of materials with 20-60% Kraft lignin contents presents the same trend as materials with 0% Kraft lignin. Therefore, no significant change of molecular weight of protein was observed in the studied molecular weight range (10-250 kDa) when Kraft lignin was added in the fish protein-based materials.



(a) (b)

Figure 4.5 Protein molecular weight of SDS-soluble (a) SDS-insoluble (b) of fish protein powder and fish protein biomaterial with 0-70% Kraft lignin contents.

4.6 Free radicals

As Kraft lignin is known as free radical scavenger, it may interfere the protein aggregation by capturing free radicals during processing fish protein -based material. Therefore, electron spin resonance (ESR) signals of plasticized fish protein/ Kraft lignin biomaterial were investigated at 77 K.

ESR spectra corresponded to relatively stable free radicals of plasticized fish protein/ Kraft lignin biomaterial after mixing at 80 °C and compression molding at 100 °C are shown in Figure 4.6 (a) and (b), respectively. Plasticized fish protein/ Kraft lignin biomaterial after mixing presented ESR spectra clearly similar to that of after compression molding. The signal intensity of Fish protein-based biomaterial (0% Kraft lignin) indicated the nitrogen or nitroxyl radicals ($NO^{\cdot}$) at g = 2.00 [74, 75]. However, sulfur centered radicals such as thiyl and disulfide radicals were either absent or their signal was hidden in the nitrogen or nitroxyl signal. For Kraft lignin-based material (70% Kraft lignin), high intensity is observed at g = 2.00 corresponded aromatic radicals [76]. Therefore, ESR results indicated that no radical scavenging effect between Kraft lignin and fish protein during mixing and molding process was observed.

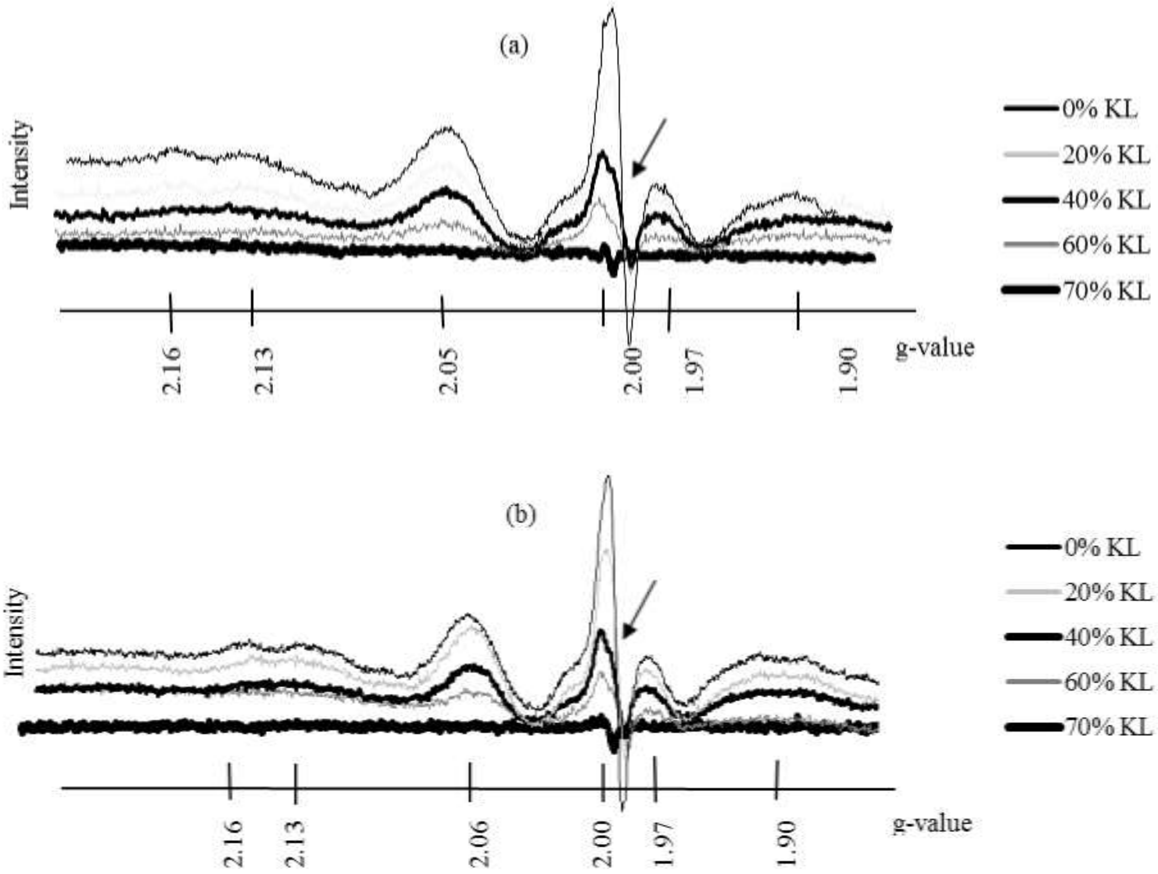


Figure 4.6 Free radicals of plasticized fish protein/ Kraft lignin biomaterial after mixing at 80°C (a) and molding at 100°C (b)

4.7 Mechanical properties of plasticized fish protein/Kraft lignin biomaterial

The functional properties of Kraft lignin-filled material are generally affected by the properties of their components. The mechanical properties of polymers blend was largely associated with distribution and concentration of inter- molecular interactions and intra-molecular interactions allowed by the sequence of the polypeptide chain [4]. Therefore, mechanical properties of Kraft lignin as a filler on fish protein material were investigated.

The effect of Kraft lignin on mechanical properties was investigated and shown in Figure 4.7. This showed that the elongation at break of the materials decreases with an increasing Kraft lignin content. Young's modulus and tensile strength of the materials increase when Kraft lignin content increased. The maximum enhancement is observed when the content of lignin reached to 30 wt% because it has mainly protein component in material. Because, protein is consists of polar and non-polar side chains. There are strong intra- molecular interactions and inter-molecular interactions. Therefore, the strong polar interactions between side chains of protein molecules restrict segment rotation and molecular mobility, which lead to increase in modulus, and tensile strength. However, tensile strength of materials decreased at more than 30 % Kraft lignin may be due to the effect of natural properties of Kraft lignin which is brittleness [38]. The effect of Kraft lignin on mechanical properties can shown data in Table 4.8. The results revealed that blend ratio of 40:30:30 (fish protein: Kraft lignin: glycerol) showed maximum value in Young's modulus and tensile strength. It was also concluded that polymer blends of this ratio had a high stress resistance and elasticity.

Mechanical properties of plasticized fish protein/ Kraft lignin biomaterial were low when compared to other protein in literatures. Sun et al., 2008 reported that Thermo-molded at 105ºC of wheat gluten plastics plasticized with glycerol presented the tensile strength of 3.03 MPa, elongation at break of 288.5 % and Young's modulus of 20.0 MPa. In addition, Zhang et al., 2000 studied mechanical properties of extruded soy protein sheets plasticized with glycerol that showed, tensile strength of 15.6 MPa, and 133% elongation at break of 112% and Young's modulus of 374 MPa.

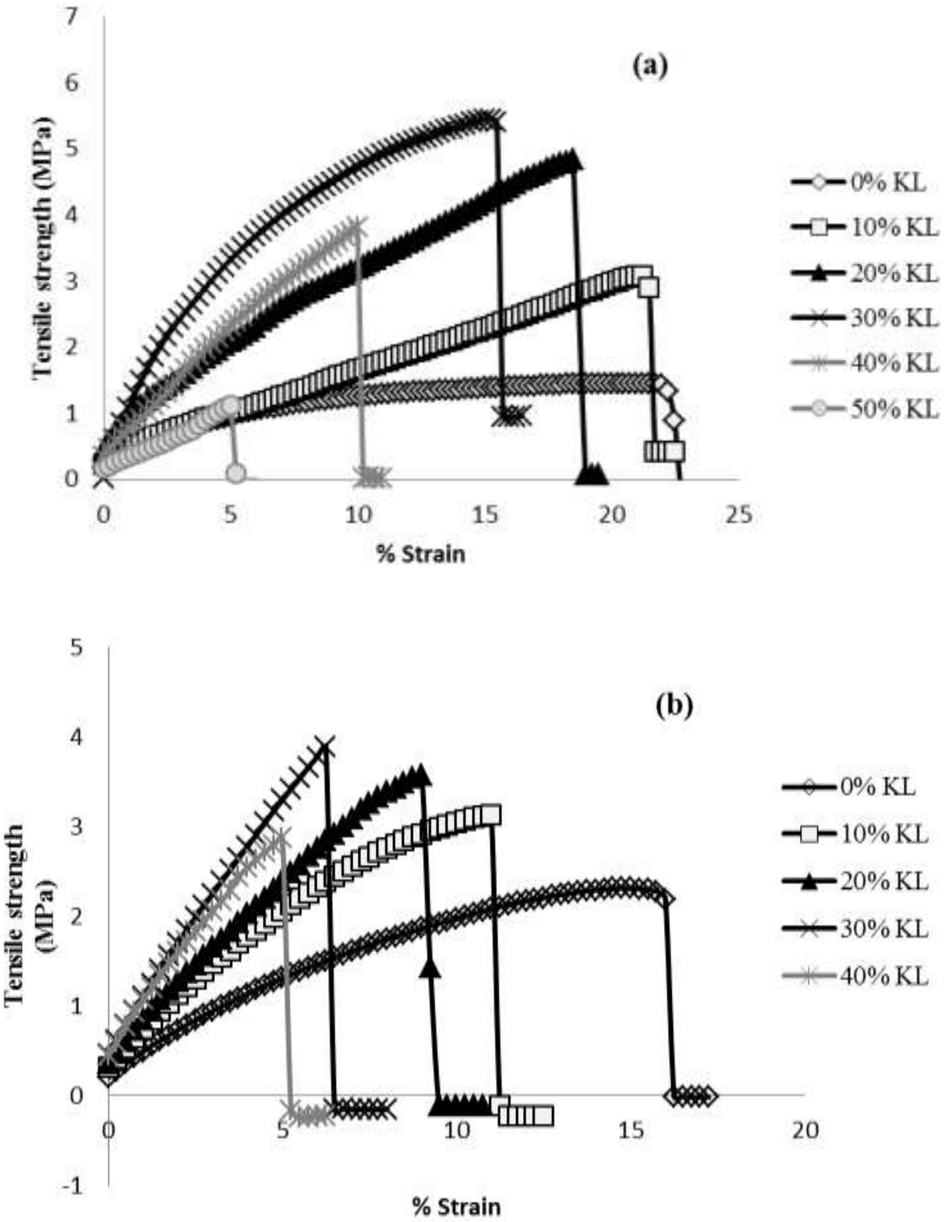


Figure 4.7 Mechanical properties of plasticized fish protein/ Kraft lignin biomaterial molded at 100 ºC (a) and 130ºC (b)

The effect of molding temperature on mechanical properties of plasticized fish protein/Kraft lignin biomaterial at molded 100 ºC and 130ºC was investigated and shown in Table 4.8. The tensile strength and Young's modulus of material increased as molding temperature increased because at high temperature induced cross-linking between protein and Kraft lignin result in protein aggregation as high strength.

Table 4.8 Mechanical properties of plasticized fish protein/ Kraft lignin biomaterial molded at 100ºC and 130ºC

Kraft lignin (%)	**Molded at 100ºC**			**Molded at 130ºC**		
	Young's modulus (MPa)	**Tensile strength (MPa)**	**Elongation at break (%)**	**Young's modulus (MPa)**	**Tensile strength (MPa)**	**Elongation at break (%)**
0%	$26.5^{cd}\pm8.0$	$1.6^{c}\pm0.6$	$22.5^{a}\pm9.3$	$27.9^{c}\pm2.6$	$1.8^{d}\pm0.5$	$10.9^{a}\pm3.1$
10%	$29.3^{c}\pm4.5$	$3.0^{b}\pm0.4$	$21.3^{ab}\pm5.6$	$40.2^{b}\pm2.6$	$3.3^{c}\pm0.3$	$10.9^{a}\pm0.5$
20%	$39.1^{ab}\pm7.8$	$4.4^{a}\pm0.6$	$20.9^{ab}\pm4.2$	$66.1^{a}\pm14.8$	$4.4^{b}\pm0.8$	$8.6^{ab}\pm2.0$
30%	$42.2^{a}\pm4.4$	$4.9^{a}\pm1.0$	$19.5^{ab}\pm4.1$	$67.1^{a}\pm5.8$	$5.3^{a}\pm0.3$	$8.5^{ab}\pm2.0$
40%	$30.2^{bc}\pm6.0$	$3.6^{b}\pm0.2$	$12.2^{bc}\pm2.0$	$64.6^{a}\pm3.7$	$3.7^{bc}\pm0.7$	$6.5^{b}\pm1.7$
50%	$18.3^{d}\pm0.5$	$1.2^{c}\pm0.1$	$6.1^{c}\pm1.3$	$5.8^{d}\pm8.5$	$0.2^{e}\pm0.1$	$0.9^{c}\pm0.8$

4.8 Water absorption of plasticized fish protein/Kraft lignin biomaterial

Water absorption of plasticized fish protein/ Kraft lignin biomaterial after immersion in water containing 0.05% NaN_3 to avoid the microbial growth for 1 week was determined.

Figure 4.8 shows water absorption of fish protein-based bioplastics with 0-70% Kraft lignin content. Addition of Kraft lignin content decreased water absorption of the plasticized fish protein/ Kraft lignin biomaterial because Kraft lignin is relatively hydrophobic and aromatic in nature [11, 43, 77]. Therefore, the high lignin content limits water absorption of biomaterial. Meanwhile, studying the effect of alkaline lignin on the water absorption of the soy protein plastics showed as same trend by Huang et al. [15]. Tests of water absorption proved that the introduction of hydrophobic alkaline lignin effectively reduced water absorption with an increase of alkaline lignin content from 240 to 85%.

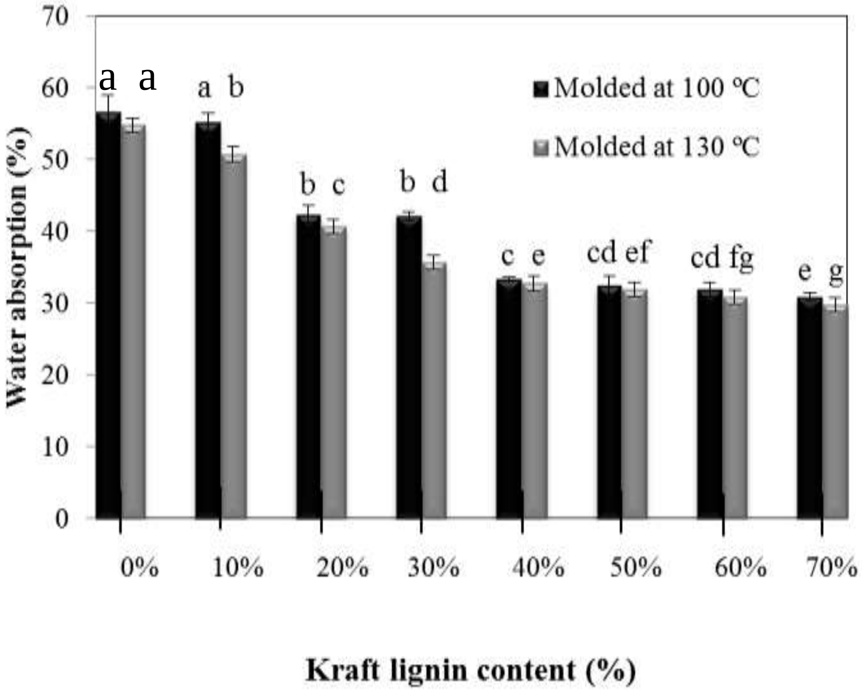


Figure 4.8 Water absorption of fish protein-based biomaterial with 0-70% Kraft lignin content at mold 100ºC and 130 ºC

The effect of molding temperature on water absorption of plasticized fish protein/ Kraft lignin biomaterial with difference Kraft lignin content at molded 100ºC and 130 ºC was investigated and shown in Figure 4.8. Water absorption decreased as molding temperature increasing. Since, when exposed to higher temperatures, more protein molecules unfold resulting in more entanglement (aggregation) and packing tighter during the molding process, which could inhibit water absorption. Water absorption results suggest that the cross-link of the polymer is increased by the presence of the molding temperature.