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Research Article

Production and Characterization of Protein Hydrolyzate from "Bibisan Fish" (Apogon Albimaculosus) as an Indigenous Flavor by Enzymatic Hydrolysis

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Abstract: This study aimed to product and characterize the physico-chemical and functional properties of protein hydrolysate bibisan fish by enzymatic hydrolysis using enzyme of biduri and papain. The results of physical analysis show yield of protein hydrolysates from bibisan fish ranged from 13.542% to 18.165%. Colors on protein hydrolyzate of bibisan fish, indicating decreasing enzyme of biduri, so hydrolysate colors is increasingly not bright. Chemical analysis show low moisture content (9-10%) and least lipid (0.03%) content. Protein content of around (63-75%). Level of rancidity fish protein hydrolysate ranged from 0.19 mmol/kg to 0.23 mmol/kg. Value of fish hydrolyzate Maillard products at various concentrations around 0.54-0.74. DH increased with increasing hydrolysis time around 6.77 to 11.98%. The results of WHC, in water absorption from 89.01 to 103.32%. While most high oil absorption capacity range around 68.06-80.09%. Amide I and II bands of protein hydrolysate appeared at the wavenumber of 1654.81 and 1548.73/cm. N-H stretching band appeared at 3292/20 cm.

Keywords: Bibisan fish, enzyme of bidury, functional properties, physico-chemical, papain enzyme

INTRODUCTION

One solution in reducing of dependence on imported food, especially food ingredient flavor is through the development of materials processing technology for the food industry based on local natural resources. Therefore, it needs to be explored further for the potential of indigenous flavor of local natural materials in Indonesia. One of the potential materials to be developed are inferior fish sourced from Indonesian seas, one of which is bibisan fish (Apogon albimaculosus). Especially at the time of harvest, the group inferior fish is very abundant and not be used yet widely as industrial raw materials.

Engineering technology for flavor enhancer's production can be developed through hydrolysis technique. By hydrolysis techniques, it will be produced amino acid L, a wide variety of peptides and nucleotides. The hydrolysis products can be a source of materials which cause "umami" (savory taste) and also as a source of flavor (Maga, 1998). Hydrolysis process can be carried out chemically or enzymatically. Chemical hydrolysis process can shorten, simplify and reduce the cost of manufacture, but the resulting flavor is not good for health and unguaranteed for safety (Anonym, 2000). Chemical hydrolysis techniques lately started to be avoided by most of the food ingredient industry in Indonesia. Enzymatic hydrolysis is the safest method and more profitable than chemically because hydrolysis enzymatically generate free amino acids and varying short chain peptides. The product has a wider range of uses in the food industry (Kunst, 2000).

Enzymatic proteolysis and solubilization of proteins from various sources studied extensively and has been described by several different authors over the last 60 years (Aspmo *et al*., 2005). Addition of proteolytic enzymes could make a hydrolytic process more controllable. Given the protease enzymes for the food industry has been mostly imported and are still relatively expensive, it is necessary to develop using protease enzymes sourced from local nature in Indonesia, one of which is a protease enzyme from Biduri plants (Calatropis gigantea). Results of previous studies indicate that extracts of the plant Biduri from the sap, stems and leaves great potential as a source of protease enzyme (Witono *et al*., 2007b). Results characterization of protease enzyme from plants Biduri, based strongly indicating specificity of the classic eksopeptidase which is very suitable for applications in the manufacture of a protein hydrolyzate (flavor enhancer) (Witono and Kang, 2010). Modification of the enzymatic hydrolysis process by added cysteine and synergism with the enzyme papain was found to improve the performance of Biduri proteases in inferior fish substrates hydrolyzing (Witono *et al*., 2011).

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The characteristics of hydrolysate directly affect the functional properties and the uses as food ingredients ((Kristinsson and Rasco, 2000). Fish protein hydrolysates have been shown to have potential for nutritional or pharmaceutical applications (Wergedahl *et al*., 2004). Functionality of food proteins has been defined as: any physicochemical property which affects the processing and behavior of protein in food systems as judged by the quality attributes of the final product (Kinsella, 1976). Fish protein hydrolysates have been well studied and reported in terms of their production, biochemical and functional properties (Kristinsson and Rasco, 2000). Functional properties of protein can be improved by enzymatic hydrolysis under controlled conditions (Quaglia and Orban, 1990). To improve the functional properties of proteins, enzymatic

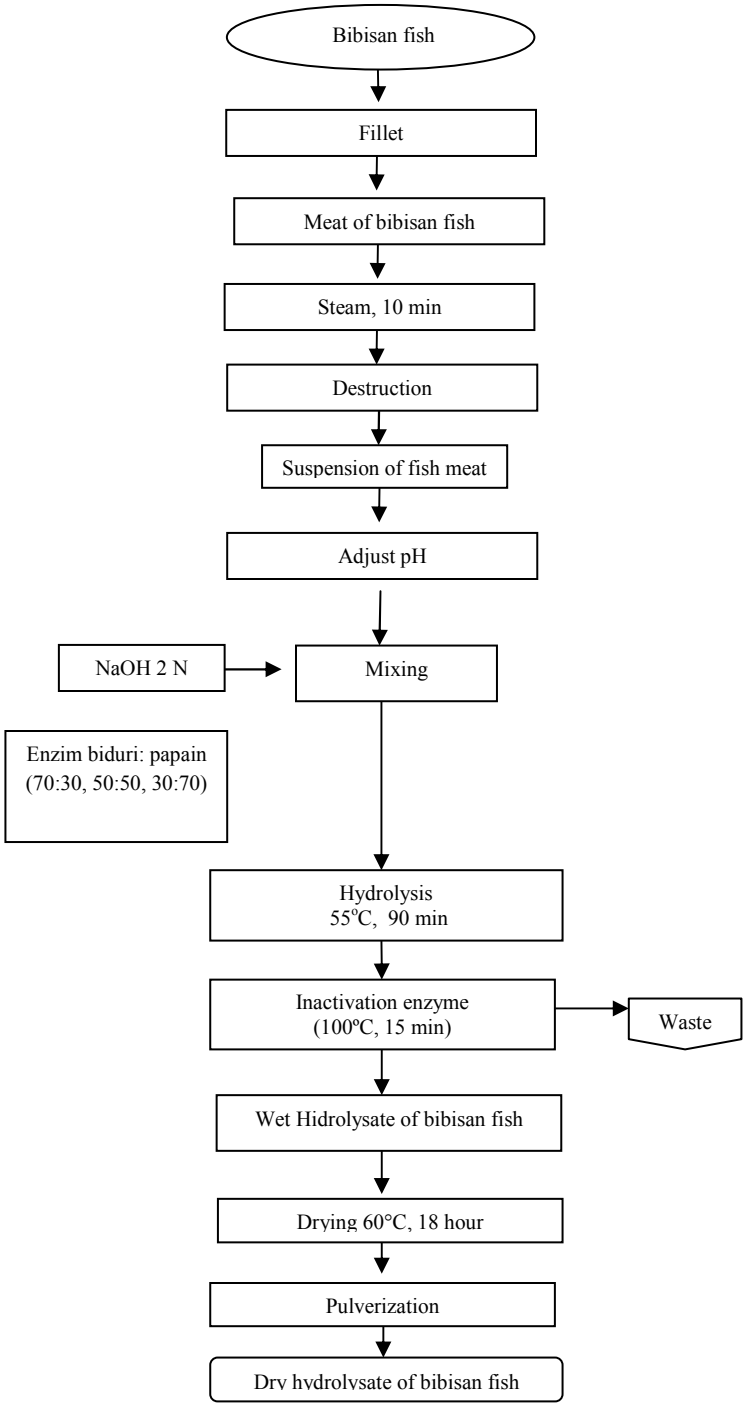

Fig. 1: Flowchart of protein hydrolyzate from bibisan fish

modification has been extensively employed. The objective of the present study was to evaluate the production and functional properties of protein hydrolysate from bibisan fish prepared by hydrolysis enzyme using biduri and papain.

MATERIALS AND METHODS

Material: Raw materials used in this study such as bibisan fish (Apogon albimaculosus) obtained from the Village Talang, Sumenep, Madura, Biduri and papain protease enzyme. The chemicals used are 2N NaOH, pH 8.2 phosphate buffer, Mix-Lowry (anhydrous Na2CO3, CuSO4, NaKtartat), Follin ciocalteau, BSA standard, tyrosine standard, 3% TCA, 15% TCA, TBA reagent (thiobarbituric acid), 37% HCl, isobutanol and 97% ethanol.

Tools used include: stainless steel blender, centrifuge Yenaco YC -1180 models and the tube, Roy Spectronic 21 D Melton and kuvetnya, Jen Way pH meter type 3320 (Germany), magnetic stirrer and stone stirernya Stuart Scientific, vortex Thermolyne type 16700, cabinets cooling, water bath GFL 1083, Ohaus analytical balance, an electric heater Gerhardt, spatula, vacuum ovens, vortex Max Type 16700 Maxi, 80 mesh sieve, distillates, the biuret, bottles films.

Methods:

Production of protein hydrolyzate bibisan fish: Bibisan was filled to separate the fish with the skin. Then the fish meat was steamed for 10 min and was destroyed so then resulting the fish meat suspension. After that pH adjustment was carried out using NaOH 2 N. Then added Biduri and Papain enzyme mixture with a ratio 70B:30P, 50B:50P, 30B:70P. Hydrolysis with temperature 55°C for 90 min. After that inactivation of the enzyme by temperature of 100°C for 10 minutes, so it resulting wet hydrolyzate. Then dried 60°C for 18 h and pulverized to produce dried fish meat hydrolyzate.

Analysis of chemical composition: The protein hydrolysate powder was analyzed in triplicates and then mean value was recorded. Protein, ash, fat and moisture content were determinated according to the method of AOAC (1995). Rancidity level is determined by TBA method (Sudarmadji *et al*., 1997). Maillard products determined by absorbance (Hofmann *et al*., 1999).

Degree of hydrolysis: Degree of hydrolysis was estimated according to Hoyle and Merritt (1994). To the supernatant, one volume of 20% trichloroacetic acid (TCA) was added to precipitate the intact protein, followed by centrifugation at 8000 g at 10°C for 20 min to collect the 10% TCA-soluble materials. The degree of hydrolysis (DH) was computed according to the following equation:

$$\%DH = 100\times(10\%\ \text{TCA-soluble N2 in the sample/total N2})$$

Analysis of physical charachteristic and functional properties: The color of the protein hydrolyzate powder is determined using the method of color reader (Fardiaz *et al*., 1992). Functional properties observed were including water holding capacity dan oil holding capacity by Subagio method (Subagio, 2006).

FTIR analysis: Infrared spectra between 4000 and 650/cm were recorded using a Perkin Elmer Spectrum 400 Infrared Spectrometer (Perkin Elmer Inc, Waltham, MA, USA). All experiments were performed at least in duplicate by Muyonga *et al*. (2004) (Fig. 1).

RESULTS AND DISCUSSION

Proximate analysis: Proximate analysis of protein hidrolysate of bibisan fish observed including moisture content, protein, fat and ash. The observation of the chemical composition of hydrolyzate from bibisan fish are presented in Table 1.

In the present study combination of biduri and papain enzyme was used to get optimal protein hydrolysate. The proximate analysis in terms of protein, lipid, moisture and ash contents of dried protein hydrolysate of bibisan fish were measured. The results were expressed as a mean of triplicate and represented in Table 1 respectively. Fish protein hydrolysate showed low moisture content (9-10%) and least lipid (0.03%) content. Protein content of around (34-41%) which was lower than earlier findings. Ovissipour *et al*. (2010) reported that protein hydrolysates of tuna ranges from 70-80%. The lyophilized protein hydrolysate from king fish showed protein content of around 85.57% (Abdulazeez *et al*., 2013). This may be due to differences in the type of enzyme used, incubation time and analytical methods used for estimation.

Rancidity level and product maillard: Biduri and papain concentration and hydrolysis time effect rancidity levels of fish protein hydrolysate that has been produced. Histogram of fish hydrolysate rancidity at various concentrations and long Biduri protease hydrolysis showed in Fig. 2. Level of fish hydrolysate

Table 1: Proximate composition of fish protein hydrolysate using Biduri and Papain enzyme

Ratio of Biduri: papain	Moisture content (%)	Fat content (%)	Ash content (%)	Protein content (%)
70B30P	9.763±0.001	0.033±0.00040	5.468±0.0006	75.908±0.0007
50B50P	10.826±0.003	0.0335±0.0007	2.715±0.0046	64.324±0.0052
30B70P	10.454±0.001	0.0371±0.0064	2.468±0.0054	63.615±0.0003

Data were presented as proximate analysis by AOAC method (AOAC, 1995), means ± SD (standard deviation), triplicate (n = 3)

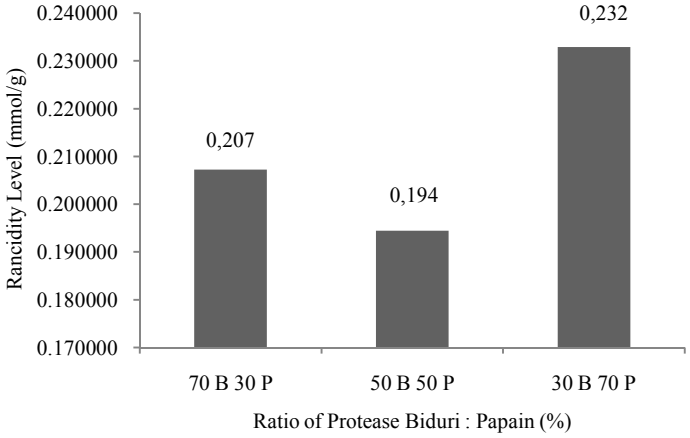

Fig. 2: Histogram of rancidity level protein hydrolisate from bibisan fish

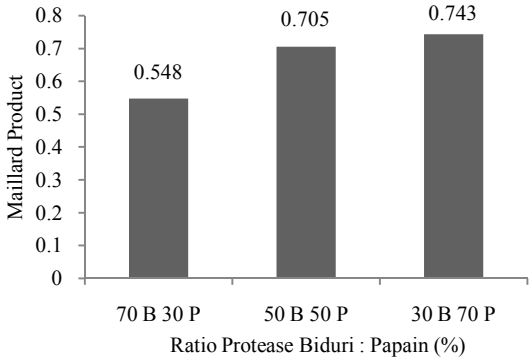

Fig. 3: Histogram of maillard product protein hydrolysate from bibisan fish

rancidity ranged from 0.19 mmol/kg to 0.23 mmol/kg. The greater concentration of Biduri protease enzyme, then rancidity level of (TBA value) fish protein hydrolyzate produced decreases. Increasing the proportion of Biduri enzyme causes increasing Maillard products then it could be expected to decrease the rate of oxidation because oxygen is difficult to penetrate.

Maillard product: Mailard reaction occurs between reducing sugars and primary amine groups. Amine group is derived from the breakdown of proteins that occur naturally in the ingredients. The value of fish hydrolysate Maillard products at various concentrations around 0.54-0.74. Maillard products are expressed in absorbance units. The higher absorbance so the increasing of Maillard products. It can be shown in Fig. 3. Figure 3 shows that the greater proportion of the papain enzyme in mixture of enzyme, the value of fish hydrolyzate Maillard products produced tends to be higher. This happens because the higher the activity of the papain that magnifies the short chain proteins thereby increasing the primary amine group. The more peptide bonds are hydrolyzed thus a primary amine group produced more and more. Maillard reaction is a reaction between the carbonyl group and the amine group primer (Witono *et al*., 2007). Thus the more concentration of enzymes, Maillard reaction that occurs is increased.

Table 2: Degree of hydrolysate fish protein hydrolysate

Ratio Biduri: Papain	Time (h)	DH (%)
70 B30 P	0.5	6.77±0.023
	1.0	7.43±0.058
	1.5	9.35±0.143
50 B 50 P	0.5	6.92±0.065
	1.0	7.69±0.175
	1.5	9.52±0.021
30 B 70 P	0.5	7.34±0.148
	1.0	9.15±0.105
	1.5	11.98±0.024

Data were presented as analysis of degree of hydrolysate using (Hoyle and Merritt, 1994), means ± Standard of Deviation (SD), triplicate (n = 3)

Degree of Hydolysis: Degree of Hydrolysis (DH), which is defined as the percentage of peptide bonds cleaved, is one of the basic parameters describing the properties of protein hydrolysates (Šližytė *et al*., 2005). DH is an important factor, which could influence molecular weight, the amount and composition of free amino acids, functional and nutritional properties of hydrolysates. Table 2 shows the progression of enzymatic hydrolysis of bibisan fish using combination enzymes (Biduri and Papain) during 1.5 h. DH increased (6.77- 11.98%) with increasing hydrolysis time. Biduri and papain combination with the addition of papain highest proportion is 70% further increase the degree of hydrolyzate. This is in par with earlier finding, Lee (2011) reported that the increasing addition of papain on fish protein hydrolyzate will increase the degree of hydrolyzate.

Physical analysis and functional properties: The physical characteristics analysis of protein hydrolyzate from bibisan fish including yield and color. From the

Table 3: Functional properties of protein hydrolysate bibisan fish

Ratio of Biduri: Papain (%)	Yield (%)	Color		
		L	A	B
70B30P	28.99	47.9	8.9	26.0
50B50P	21.03	45.7	9.4	27.2
30B70P	20.66	45.4	9.5	27.4

Data were presented as analysis of physical characteristic using (Fardiaz *et al*., 1992), means ± standard of deviation (SD), triplicate (n = 3)

Table 4: Physical characteristic of protein hydrolysate in bibisan fish

Ratio of Biduri: Papain (%)	OHC (%)	WHC (%)
70B30P	78.26±0.0983	90.89±0.01020
50B50P	68.06±0.0823	103.32±0.0147
30B70P	80.09±0.0658	89.01±0.05290

Data were presented as analysis of functional properties using Subagio method (Subagio, 2006), means ± Standard of Deviation (SD), triplicate (n = 3)

analysis results obtained, the more addition of the Biduri enzyme combination then the resulting yield is also growing. The yield of protein hydrolysates from fish bibisan ranged from 13.542 to 18.165%. It is associated with increased hydrolysis yield components such as dissolved solids, amino acids and other components. Colors on bibisan fish protein hydrolyzate indicate the decreasing concentration of Biduri enzyme addition and the increasing concentration of papain enzyme, so hydrolysate colors is increasingly not bright. The results can be seen in the value of L (Lightness) Table 3.

Functional properties that have been measured including water holding capacity and oil holding capacity. Water and oil absorption capacity is used to measure the magnitude of the ability to absorb water and oil then it is determined by centrifugation. The greatest water absorption capacity is on the same concentration of papain and Biduri addition on the sample. These results are presented in Table 4 water absorption capacity is affected by the protein content, where in the protein has hydrophilic and hydrophobic properties. But carbohydrates also been reported to affect the water absorption capacity (Appiah *et al*., 2011). Fish protein hydrolysates have an excellent water holding capacity (WHC) and can increase the cooking yield[8]. The increased concentration of polar groups such as COOH and NH_2 that is caused by enzymatic hydrolysis has a substantial effect on the amount of adsorbed water (Kristinsson and Rasco, 2000). In agreement with our results about of WHC, a similar trend was observed for fish protein hydrolysate in water absorption from 89.01 to 103.32% of sample in different combination enzyme (Table 4). While most high oil absorption capacity (68.06-80.09%) is also on the same sample with a comparison between the two enzymes. Large oil absorption capacity can increase the flavor and mouth feel of the food.

Structural characteristics of bibisan fish protein hydrolysates: Fourier Transform Infrared (FTIR) spectroscopy is an important and well established technique to study secondary structure of proteins and polypeptides. Nine characteristic FTIR absorption band, namely A,B and I-VII can be observed from a typical IR spectra, of which amide I band (1700-1600/cm) is the most sensitive and widely used in studies of protein secondary structure. Amide I band is mainly due to C = O stretching vibration (about 80%) of the amide group coupled with in-plane NH bending (less than 20%) (Kong and Yu, 2007). Amide II (1575-1480/cm) derives mainly from in-plane NH bending and CN stretching vibration and shows less protein conformational sensitivity compared with amide I, while other amide vibrational bands have less practical use in protein conformational studies (Kong and Yu, 2007). Amide I and II bands of protein hydrolysate appeared at the wavenumber of 1654.81 and 1548.73/cm (Fig. 4). Amide I bands of protein hydrolysate was found at around 1630/cm (Benjakul *et al*., 2009).

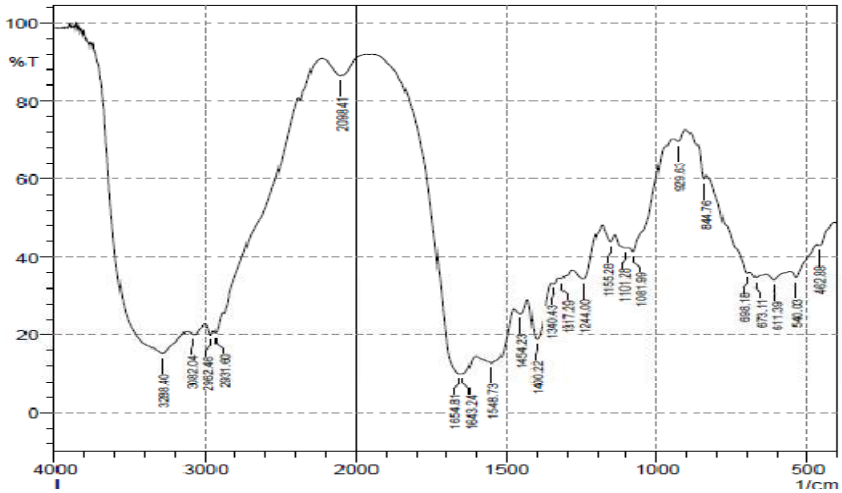

Fig. 4: Structure of "bibisan fish hydrolysate with ratio biduri: Papain (70B:50P)

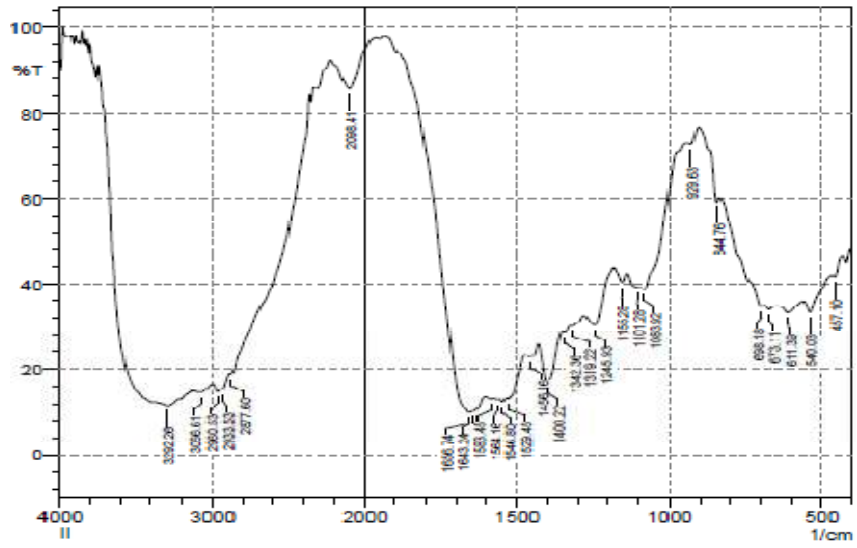

Fig. 5: Structure of "bibisan fish hydrolysate with ratio biduri: Papain (50B:50P)

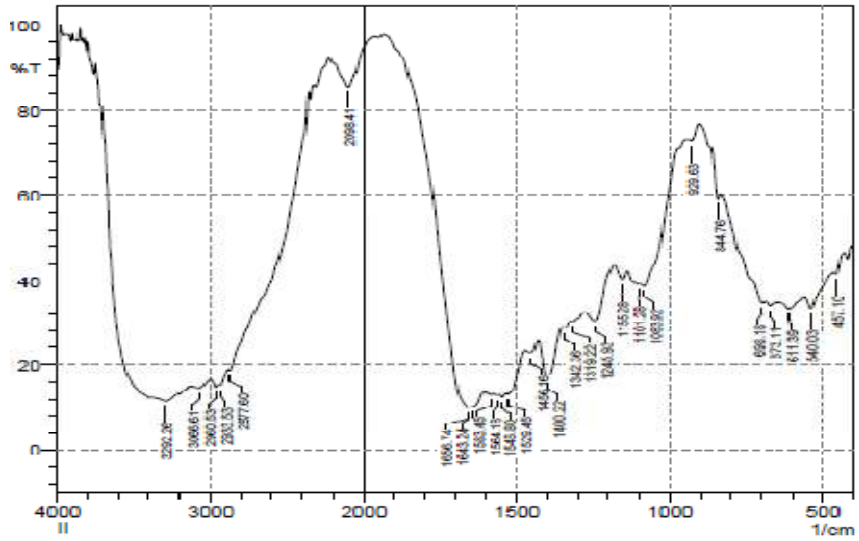

Fig. 6: Structure of "bibisan fish hydrolysate with ratio biduri: Papain (30B:70P)

Higher frequencies of amide I bands is attributed to greater loss of molecular order of triple helix due to uncoupling of intermolecular cross-links and disruption of intra molecular bonding when protein hydrolysate was extracted at higher temperature or longer time (Kittiphattanabawon *et al*., 2010; Ahmad and Benjakul, 2011). In addition, amide III band of protein hydrolysate was detected at 1235/cm which was associated with loss of triple-helix state of the molecules and transformation of α-helical to random coil structure due to denaturation of protein (Muyonga *et al*., 2004). Amide A band derives from the stretching vibration of N-H group (Kong and Yu, 2007). In this study, N-H stretching band appeared at 3292.20/cm (Fig. 5). N-H stretching vibration of amide A occurs normally at wave number of 3440-3400/cm (Muyonga *et al*., 2004). When N-H group of shorter peptides are involved in hydrogen bonding, the position of the band in amide A region shifts to lower frequencies. Amide A band of protein hydrolysate extracted for 5 h, shifted to lower wave number, compared with extracted for 1.5 h, indicating the involvement of N-H group of shorter peptide fragments in hydrogen bonding (Ahmad and Benjakul, 2011) (Fig. 6).

CONCLUSION

Bibisan fish can be developed into a food flavor, such as fish protein hydrolysate to reduce dependency on imported food ingredient in Indonesia. Production of fish protein hydrolysate using protease enzymes sourced from local nature in Indonesia, one of which is

a protease enzyme from Biduri plants (Calatropis gigantea). Modification of the enzymatic hydrolysis process by added cysteine and synergism with the enzyme papain.

The physical charachteristic of protein hydrolysates from bibisan fish, yield ranged from 13.542 to 18.165%. Colors on bibisan fish protein hydrolyzate indicate the decreasing concentration of Biduri enzyme addition and the increasing concentration of papain enzyme, so hydrolysate colors is increasingly not bright. Chemical analysis show low moisture content (9-10%) and least lipid (0.03%) content, Protein content of around (63-75%). Level of rancidity fish protein hydrolysate ranged from 0.19 mmol/kg to 0.23 mmol/kg. Value of fish hydrolyzate Maillard products at various concentrations around 0.54-0.74. DH increased with increasing hydrolysis time around 6.77 to 11.98%. Water absorption from 89.01 to 103.32%. While most high oil absorption capacity range around 68.06-80.09%. Amide I and II bands of protein hydrolysate appeared at the wave number of 1654.81 and 1548.73/cm. N-H stretching band appeared at 3292.20/cm.

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