The Effect Protease Inhibitor on the Composition of Molecular Weight of Abalone (*Haliotis varia* Linnaeus) Fractions

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Abstract. Arginine is one of the important components of the latest technology in making the desensitizing materials in the field of dentistry. Abalone is one of the marine biotas containing a high level of arginine. The extraction process and fractionation of Abalone (*H. varia* Linnaeus) to obtain the gel of desensitizing agent require a certain procedure to obtain the molecular weight as expected. This research aims to observe the effects of the addition of the protease inhibitor on the composition of the molecular weight of the abalone fractions as a preliminary study for being used as dental material. Furthermore, a preparative chromatography procedure was conducted using the qualitative thin layer chromatography (TLC). The fractions results were dried and dissolved into the solution, centrifuged at stages, and then the supernatant was taken. Subsequently, the composition of molecular weight was observed with 12% SDS-PAGE electrophoresis gel. Then, it was analyzed using Image J software to measure the density of the band of electrophoresis gel and determine a molecular weight composition. The data were tested statistically using One-way ANOVA and continued with post hoc Tukey's HSD. The results of the analysis showed a significant difference in the composition of molecular weight ($p < 0.05$). Post hoc analysis showed a significant difference ($p < 0.05$) between fractions groups. According to the results of the present study, it can be concluded from this study that protease inhibitor has an effect on the molecular weight composition of heavy, medium and light levels.

INTRODUCTION

Prosthodontics procedures on vital teeth needed for the manufacture of fixed partial denture restoration may trigger discomfort after preparation procedures since it may lead to dentin hypersensitivity and pulp irritation [1]. The prevalence of dentin hypersensitivity is reported to be between 10% -35% depending on the study population [2].

Full crown preparation on vital teeth exposes 1 cm^2 of dentin containing more than 3 million tubules/cm^2 [3]. The Brannstrom Hydrodynamic Theory put forward in 1963 is a generally accepted theory to explain the occurrence of dentin hypersensitivity. The theory states that stimuli that cause pain, can increase the flow of intra-tubular dentinal fluid, or change the direction of fluid so that it stimulates nerves around odontoblasts, resulting in dentin hypersensitive [4].

Desensitizing agents such as fluorine, strontium salts, oxalates, glutaraldehydes, and bioactive glass are useful for treating dentin hypersensitivity. Despite the general assumption on its efficacy, these materials have the disadvantage of short durability and poor effectiveness leading to a short term efficacy of therapeutic effects due to the daily brushing process. Moreover, these materials dissolve in acidic drinks and have an imperfect closure of tubules [4]. To overcome such a problem, the current developments in anti-sensitivity technology are based on the consideration of the significance of saliva as a natural substance to reduce dentin hypersensitivity. An important component of this latest technology is arginine, which is a positively charged amino acid with a physiological pH of (6.5-7.5) and the molecular weight of L–arginine of 174.2 kDa [5]. Interaction of arginine and calcium carbonate in vivo triggers the deposition of phosphate, arginine, calcium, and carbonate on the surface of the dentin and in the dentinal tubules [6]. One marine biota that has a high content of arginine amino acids is abalone [7]. Some studies also mention that abalone meat has high mineral content [8]. Hence, the making of abalone as a desensitizing agent is expected to increase the durability of therapeutic effects since it is not soluble and more effective. However, the procedure of extraction and fractionation of abalone shells either with the addition or without the addition of protease inhibitor has never been done. Therefore, this study was conducted to understand the proper composition of molecular weights from abalone fractions.

MATERIAL AND METHODS

Characteristics test of abalone shellfish

This study used the Abalone shells *(Haliotis varia* Linnaeus) of natural FO biota derived from F3 cultivated by the Research Center for Marine Fisheries, Gondol, Buleleng, Bali. The identification of shellfish was carried out at the Animal Systematics Laboratory of the Faculty of Biology, Gadjah Mada University. This abalone shell is a species of *Haliotis varia* Linnaeus, 1758.

Extraction procedure

Abalone (*Haliotis varia* Linnaeus) meats were taken and frozen at -26^0C. 50 g of frozen Abalone meat was washed, dried, cut into pieces, blended, and added with 150 ml of HCl 0.1 N. The extraction results were centrifuged in a 15 ml tube for 20 minutes at a speed of 5000 rpm. Tablet-shaped protease inhibitors were added to every tubes, 1 tablet for each tube. The supernatant was taken and 60 ml of solution was added with 0.5 M Tris (hydroxymethyl) aminomethane, 30 mM 2-Mercaptoethanol, and 2 mM EDTA disodium dihydrate [9]. The resulting mixture was centrifuged in a 15 ml tube for 20 minutes at a speed of 5000 rpm. The supernatant was taken and freeze-dried for 24 hours. The freeze-dried extraction was dissolved into 18 ml of 50 mM acetate buffer eluent with a pH of 5.0 and then filtered with filter paper.

Preparative chromatography procedures

The preparative chromatography procedure was performed with the stationary phase of Sephadex G-25 and the mobile acetate buffer phase of 50 mM and a pH of 5.0. The process used a glass column of 60 cm height and 2 cm in diameter. A 20 g of Sephadex G-25 matrix was packed in a column, 150 ml of acetate buffer was added and allowed to condense for 24 hours. The acetate buffer eluent was added until the glass column was full and allowed to drip until within 2 cm from the deposited Sephadex G-25. Abalone extract of 3 ml was dropped circularly along the column wall. Eight ml of fractions result was poured into a tube with a flow rate of 1 ml per minute.

Thin layer chromatography procedure

The qualitative thin layer chromatography (TLC) test was performed with the stationary phase of silica gel 60 F254 and the mobile phase of n-butanol 3.5 ml, acetone 3.5 ml, glacial acetic acid 1 ml, and distilled water of 1 ml. The test of active substances in each fraction was conducted by taking every 10 µl of liquid per fraction and then dropped on silica gel and poured into a chamber containing a mobile phase. The active substances were made visible

and grouped by ninhydrin painting. Once the substances were divided into fractions, each treatment was freeze-dried for 24 hours and heated in the oven with 40°C for 24 hours.

Analysis of protein levels

A total of 50 µl samples were dissolved in 1000 µl working reagents (pierce BCA protein assay kit, Thermo fisher Scientific),The solution was homogenized and put in an incubator at 37^0C for 30 minutes and cooled at room temperature. Measurements were made with a spectrophotometer at ʎ = 562 nm.

Determination of protein profiles with SDS-PAGE

The gel used for this procedure consists of a stacking gel with a concentration of 5% and a separating gel with a concentration of 12%. Before electrophoresis, 20 mg of dry sample was dissolved in 500 µl acetate buffer solvent and centrifuged in cold increments for 5 minutes at 7000 rcf, 9000 rcf, 11,000 rcf, 13,000 rcf, 15,000 rcf, 17,000 rcf, and 19,000 rcf. The supernatant was taken then denatured by heating for 2 minutes. Sampling was carried out at the same protein concentration for each fraction. Protein markers were used as a guide to determine the molecular weight of each fraction. Electrophoresis results in the form of bands are calculated by Retardation Factor (Rf) of each band with the formula [10].

RESULTS AND DISCUSSION

Percentage of molecular weight composition

Molecular weight profiles are shown on gel 1 and gel 2 electrophoresis (Fig.1 and Fig.2). Based on the results of the calculation of Rf and log molecular weight on the marker protein, it will be obtained a regression equation linear, i.e. y = -0.0031x + 2.0656. Protein bands from the SDS-PAGE results are calculated Rf values, then included in the linear regression equation. The fractionated protein bands were performed by the SDS-PAGE technique showed the protein band in a gel 1 with a molecular weight respectively 180,80,70,69,47,42,36,30,15 kDa and in a gel 2 respectively 180,88,38 kDa.

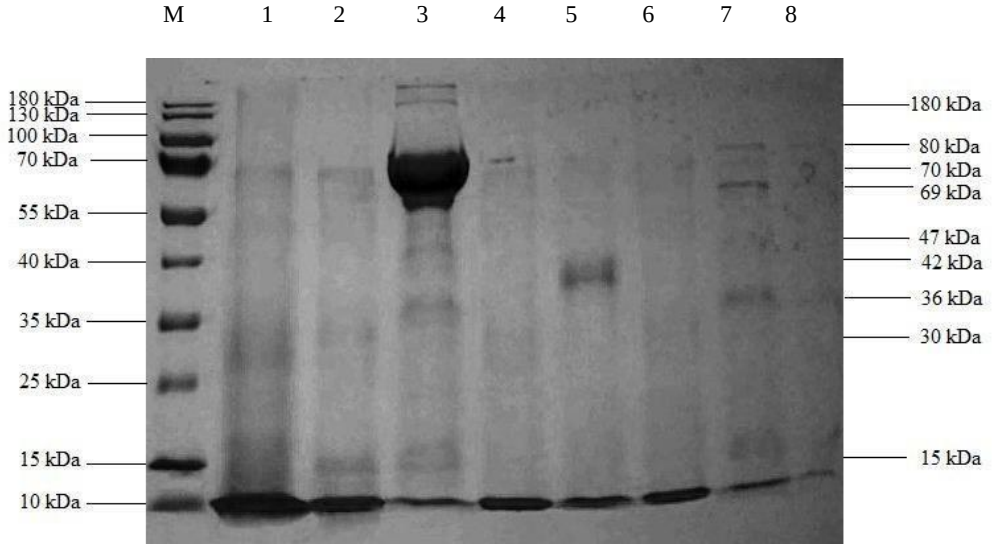

FIGURE 1. Molecular weight profile on gel 1 with molecular weight marker (M) and lane 1-8 were protein from fractions

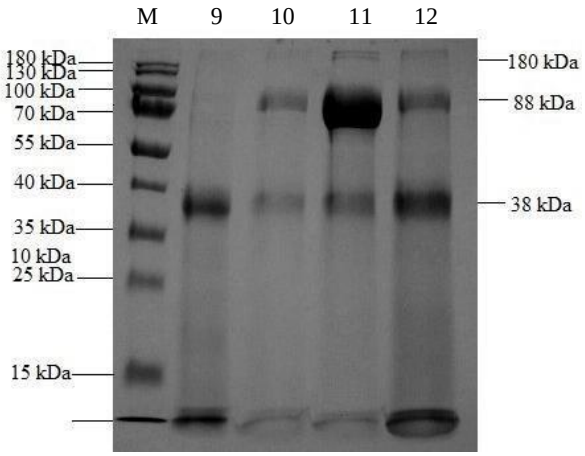

FIGURE 2. Molecular weight profile on gel 2 with molecular weight marker (M) and lane 9-12 were protein from fractions

Quantitative analysis of the density of a band electrophoresis gel with Image J software produces a composition of molecular weight percentage (kDa) in each gel as shown in table 1 and fig.3. The test indicated that there were significant differences in the composition of the molecular weight of heavy, medium and light categories ($p < 0.05$) for each fractions (table 2-4). Post hoc analysis showed a significant difference of molecular weight composition ($p < 0.05$) between each fractions (table 5).

TABLE 1. Percentage of molecular weight composition

Sample	Fractions	Molecular weight		
		Heavy (%)	Medium (%)	Light (%)
Gel 1 Sample 1	F1 PI O	57.60	3.65	38.75
Gel 1 Sample 2	F1 NPI O	19.01	13.16	67.83
Gel 1 Sample 3	F2 NPI O	81.42	11.90	6.68
Gel 1 Sample 4	F1 PI FD	23.81	40.05	36.15
Gel 1 Sample 5	F2 PI FD	1.20	96.41	2.39
Gel 1 Sample 6	F1 NPI FD	19.65	31.59	48.76
Gel 1 Sample 7	F2 NPI FD	10.70	51.22	38.08
Gel 1 Sample 8	F3 NPI FD	6.40	49.30	44.30
Gel 2 Sample 9	F2 PI PC	0.84	85.88	13.27
Gel 2 Sample 10	F3 PI FD	28.08	69.95	1.97
Gel 2 Sample 11	F3 PI O	75.87	23.76	0.37
Gel 2 Sample 12	F2 PI O	19.74	75.27	4.99

F : fraction 1, 2, 3
PI : protease inhibitor
NPI : non protease inhibitor
O : oven drying
FD : freeze drying

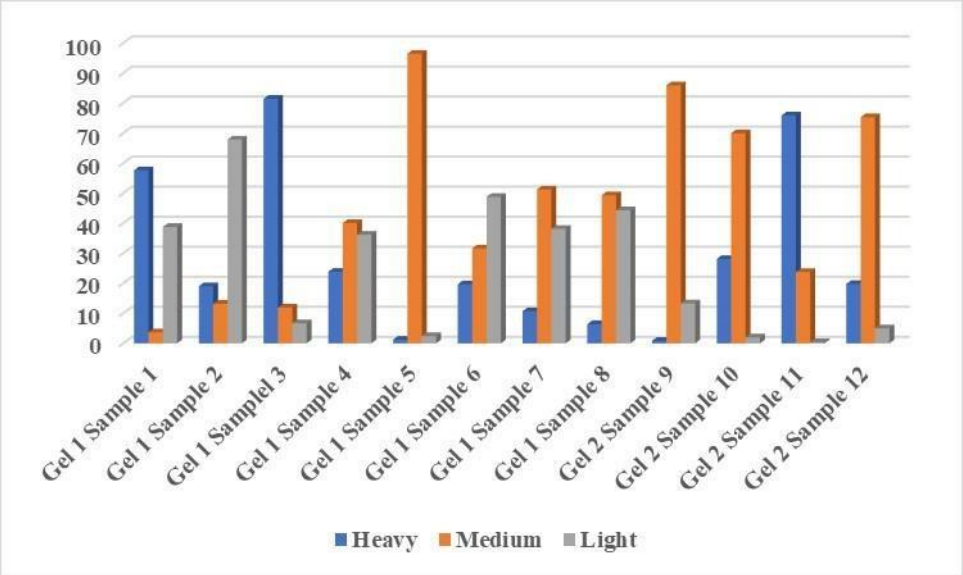

FIGURE 3. Molecular Weight Composition

TABLE 2. One-way ANOVA test results indicated the effect of the addition of protease inhibitors to the molecular weight composition in the heavy category (47.5-180 kDa) .

Source of variance	Square Number	Degree of Freedom	Quadratic Mean	F	Sig.
Between groups	25419.934	11	2310.903	51409.568	0.000
Within the group	1.079	24	0.045		
Total	25421.013	35			

TABLE 3. One-way ANOVA test results indicated the effect of the addition of protease inhibitor to the molecular weight composition in the medium category (30-47 kDa) .

Source of variance	Square Number	Degree of Freedom	Quadratic Mean	F	Sig.
Between groups	31115.478	11	2828.680	199932.563	0.000
Within the group	0.340	24	0.014		
Total	31115.817	35			

TABLE 4. One-way ANOVA test results indicated the effect of the addition of protease inhibitor to the molecular weight composition in the light category (10-29.5 kDa)

Source of variance	Square Number	Degree of Freedom	Quadratic Mean	F	Sig.
Between groups	17329.981	11	1575.453	65082.165	0.000
Within the group	0.581	24	0.024		
Total	17330.562	35			

TABLE 5. Tukey HSD result of the effect of protease inhibitors to the molecular weight of fractions

	G1S1	G1 S2	G1S3	G1 S4	G1 S5	G1 S6	G1 S7	G1 S8	G2 S9	G2 S10	G2 S11	G2 S12
G1 S1		39.23*	23.81*	33.79*	56.39*	37.95*	46.89*	51.21*	56.76*	29.52*	18.27*	37.86*
G1 S2			63.04*	5.43*	17.17*	1.27	7.671*	11.98*	17.53*	9.71*	57.49*	1.36
G1 S3				57.61*	80.21*	61.76*	70.71*	75.02*	80.57*	53.33*	5.54*	61.68*
G1 S4					22.60*	4.16*	13.10*	17.41*	22.96*	4.28*	52.06*	4.07*
G1 S5						18.45*	9.50*	5.19*	0.36*	26.88*	74.67*	18.53*
G1 S6							8.95*	13.25*	18.81*	8.43*	56.22*	0.09
G1 S7								4.31*	9.86*	17.38*	65.17*	9.03*
G1 S8									5.55*	21.69*	69.47*	13.34*
G2 S9										27.24*	75.03*	18.89*
G2 S10											47.78*	8.35*
G2 S11												56.13*

*Significance differences
Abbreviations: G, Gel and S,sampel

DISCUSSION

This study used meat samples from abalone shells (*Haliotis varia* Linnaeus) having arginine as the highest protein content. This is in accordance with the research which reported that abalone shellfish is one of the marine biotas of high content of amino acids arginine [7]. The results of this study showed that there were differences in the composition of heavy, light, and medium molecular weight in each fraction with and without the addition of protease inhibitors. The molecular weight range was categorized into three categories: light molecular weight (10-29.5 kDa), medium molecular weight (30-47 kDa), and heavy molecular weight (47.5-180 kDa).

The fraction with the addition of a protease inhibitor had a medium molecular weight composition, greater than the composition of the heavy, medium, and light molecular weight category as shown in table 1 or figure 3. This is in line with the function of protease inhibitors as a way to prevent proteolytic degradation during the protein extraction process. It is also consistent with the opinion which highlighting that protease inhibitors protect proteins from proteolytic degradation through inhibition of serine proteases, cysteine proteases, aspartic acid proteases, and aminopeptidases that are usually present in samples [11]. Protease catalyzes the breakdown of the hydrolytic protein into peptides or amino acids, resulting in the degradation of proteins deactivating or inhibiting proteases to buffer cell lysis or extract cells. In this study, the addition of protease inhibitors decreased the occurrence of lysis in the protein of the sample so that more molecules were in the medium molecular weight category.

The desensitizing agent is expected to close the dentinal tubules properly and have a longer therapeutic effect in the oral cavity without being dissolved by saliva. This is in accordance with the opinion that the opening of the dentinal tubules can increase the flow of intra-tubular dentinal fluid, or change the direction of the fluid that stimulates the nerves around odontoblasts, resulting in dentin hypersensitivity [4]. Arginine amino acid is the latest technology in desensitizing agent because it can bind with calcium carbonate to produce a complete dentinal tube closure. On this account, the research has been reported that arginine and calcium carbonate in vivo triggers the deposition of phosphate, arginine, calcium, and carbonate on the surface of the dentine and in the dentinal tubules [6]. In this study, the composition of medium molecular weight (30-47 kDa) is more visible in the fraction with the addition of protease inhibitors. Thus, the fraction with the addition of protease inhibitors is expected to meet the

desired desensitizing agent. In line with this, the research has been demonstrated that the dentinal tubule covering material has a molecular weight of 40 kDa [4].

CONCLUSION

Fractionation of abalone (*Haliotis varia* Linnaeus) with and without the addition of protease inhibitors results in different molecular weight compositions. The addition of protease inhibitors generates a medium molecular weight composition (30-47 kDa) with a greater percentage, while that without the addition of a protease inhibitor produces a lighter molecular weight composition (10-29.5 kDa).

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