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Hydrolysis kinetics and Electrophoresis Pattern of the Impact of the Kiwi Fruit Actinidine on Different Proteins of Rainbow Trout Meat

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ABSTRACT: Different methods are used to improve the quality of meat, one of which is to add proteolytic enzymes to the meat and tenderize it, thereby increasing the solubility of its proteins. Physical and chemical methods tenderize the meat by reducing or degrading myofibrillar proteins and connective tissues. In this study, the impact of kiwi protein on the physicochemical properties of Rainbow Trout meat was investigated. 2 cm cube pieces of fish meat were marinated in 20 ml of kiwi protein with the activity of 0.9 u/ml for a duration of 2, 4, 6, 8, 24, 48, 72, and 96 hours and then kept at 4 ± 1˚C. Afterwards, the actinidine enzyme was inactivated at 60°C for 10 minutes and the specimens were evaluated. The results showed that the hydrolysis of the muscle and the released amino acids significantly reduced the treatments pH in comparison to the control sample. The duration of enzyme activity significantly influenced the decomposition of fish meat proteins, increased the solubility and degree of hydrolysis, and reduced the peptide chain average length. The protein enzyme kinetic equation was suitable for modeling the enzymatic reaction of fish meat; the influence of kinetic parameters on enzyme and substrate variables was further investigated. Changing the concentration of the enzyme and substrate did not affect the parameter b but it positively influenced the parameter a. The results of SDS-PAGE electrophoresis showed that the kiwi enzyme enhanced the fish meat tenderness by affecting the sarcoplasmic and myofibrillar proteins.

Keywords: Electrophoresis, Fish, Hydrolysis Degree, Hydrolysis Kinetic, Peptide Chain Length, Kiwi.

Introduction

Fish meat is delicious and suitable for most people. Compared with other meat, fish have more water and less fat. The quantity of essential amino acids in this type of meat is equivalent to other types. With a digestion and protein intake of higher than 90%, fish meat is more easily digested compared to other meat. Fish and other marine organisms are abundant in minerals such as phosphorus, calcium, iodine, fluorine, and other micronutrients (Tavakoli *et al.*, 2005).

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Fish is a good Source for bioactive peptides and hydrolyzed proteins production, and hydrolysis of fish meat is a good economic strategy to achieve high biological value food from the Qualities aspects (Safdari *et al.*, 2014).

Furthermore, fish meat helps iron intake, prevents nutritional anemia, and plays a role in the treatment of many diseases such as rheumatism, pneumonia, migraine headaches, kidney disease, asthma, inflammation, autoimmune diseases, and certain cancers. The fatty acids that are unsaturated in fish are conducive to the development of the nervous system, the brain formation of the fetus and babies, and the treatment of depression (Tavakoli *et al.*, 2005). Fish hydrolyzed proteins are widely used and considered as suitable materials for cancer treatment owing to their bioactive peptides, chondroitin sulfate, and antioxidant properties. Moreover, due to the shortening of peptide chains, they have high digestibility and could, therefore, be applied as a protein supplement in humans, livestock, and aquatic animals (Alsmeyer *et al.*, 1974). The effective role of hydrolyzed proteins has been further proven as a good source of nitrogen in bacterial culture media (Ovissipour and Ghomi, 2008). Treatment with foreign proteases can be regarded as an appropriate method for tenderizing the meat. Proteases such as bromelain, papain, and ficin derived from plants, are commonly applied to tenderize meat (Koak *et al.*, 2011; Ha *et al.*, 2012). Extracted from kiwi fruit, actinidine is a protein with a molecular weight of 30,000 daltons; it belongs to a family of sulfhydryl proteases such as papain and similar to papain, it is a meat-tenderizer enzyme that is more effective (EL-GHARBAWI *et al.*, 2006 and Gilman *et al.*, 1946). When applied for meat tenderization, this enzyme prevents the formation of soft and sticky tissues on the meat surface, found in meat tenderized by other tenderizer enzymes such as papain (Lewis *et al.*, 1988; Kowlessur *et al.*, 1989). Actinidine is also applied as a digestive. In compounds that are commonly consumed to help digestion, particularly in people with digestive disorders such as inflammatory bowel disease, actinidine is utilized as a kind of protease. When used with papain as a protease, this protein is also able to remain active in the range of 2-45˚C, which is significantly less than the appropriate temperature for papain activity (70-60˚C) (Lewis *et al.*, 1988; CARNE *et al.*, 1978). Since 1991, researchers have examined the effects of a wide range of plant enzymes on different types of meat using the immersion method. They found that fruits such as kiwi, ginger, pineapple, figs, and papain increase the tenderness of the beef and improve the flavor of the product (Garg *et al.*, 2006; Han *et al.*, 2009; Naveenaa *et al.*, 2004; Toohey *et al.*, 2011; Wada *et al.*, 2002).The plant proteases found in such fruits can enhance the tenderness of meat tissues through influencing myofibrillar proteins and connective tissues and decomposing actomyosin, elastin, and collagen (owing to collagenase and elastase) (Lawrie *et al.*, 2006). The enzymatic hydrolysis of various parts of fish has been widely studied using commercial enzymes. Many of these studies have examined the effects of various enzymes, including papain enzymes with plant origin (HOYLE and Merritt, 1994), trypsin and chymotrypsin enzymes with animal origin (Shahidi *et al.*, 1995), and Alcalase, protamex, flavourzyme, Neutrase, and Promod with microbial origin (Aspmo *et al.*, 2005). Given the importance of actinidine in this study, we studied the influence of kiwi

protein on the physicochemical properties and electrophoretic pattern of Trout meat.

Materials and Methods

- *Extraction of kiwi protein*

Extraction was performed according to Paul *et al.* (1995) with some minor modifications. Employing a mortar, 10 g of peeled kiwi fruit was homogenized in liquid nitrogen. Next, to bring the final volume to 12 mL, an extraction buffer containing 150 µL of 100 mM phosphate buffer (pH = 6) from L (+) 10 mM ascorbic acid and 5 mM EDTA was added to the solution; afterwards, it was centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant was stored at -20 °C. Inorder to minimize proteinase activity, all processes related to extraction were carried out on ice (Afshar-Mohammadian *et al.*, 2011). The concentration of kiwi protein was measured by the Bradford method.

- *Determination of proteolytic activity*

The solutions and buffers used to determine the protease activity included 0.05 molar phosphate buffer (pH = 7.2), phosphate-cysteine-EDTA buffer solution with pH = 6 (7.1 g sodium phosphate dibasic anhydrous, 14 g sodium EDTA dihydrate, 1.6 g of cysteine hydrochloride monohydrate to prepare 1 liter of buffer), Trichloroacetic acid solution 30%, casein substrate solution (1% solution of casein in 0.05 M phosphate buffer), and papain stock (100 mg papain USP with 3 U/mg activity in 100 ml of phosphate-cysteine-EDTA buffer solution). Dilutions of 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 mg/100 ml of phosphate-cysteine-EDTA buffer solution were made from papain stock (Englund, *et al.*, 1968). 5 ml of a 1% casein solution was poured into every test tube (10 tubes related to blank and 10 tubes belonging to standards). The specimens were placed in a warm water bath at 40°C for 15 minutes; following the equilibration of the temperature, 2 ml of papain dilutions was added to standard tubes. First, 3 ml of trichloroacetic acid and then 2 ml of papain dilutions were added to the 10 control tubes (blank) which were incubated at 40°C for 60 minutes in a water bath. During this time, the tubes were shaken several times in the bathroom. The contents of the standard tubes became milkier and more opaque, indicating the enzymatic hydrolysis of casein by the papain enzyme. After 60 minutes, the reaction in standard tubes was stopped with the addition of 3 ml of the chloroacetic acids solution. The tubes were placed in a 40°C bath for another 30 minutes to ensure the coagulation of all the proteins. Protein clots were filtered through Whatman filter paper no. 42. The adsorption of standard tube solutions vs. the corresponding blank was read at 280 nm; the possible relationship existing between the absorption rate at 280 nm and the concentration and activity of the papain was further specified (Englund *et al.*, 1968). The kiwi activity (0.9 u/mg) was determined using the standard chart of the papain activity.

- *Preparation and Marinating*

Following slaughter, the fish was stored at 4°C for 24 hours. After separating the skin, meat was cut into cubes of 2 cm using a knife. Next, each piece of meat was packaged in a polyethylene bag (8×12 cm2). In order to calculate the best enzyme / fish meat ratio several pretreatments were done and the best ratio for this matter was 20 ml kiwi protein of Hayward cultivar with the activity of 0.9 u/mg (for each 8gr fish meat) was added to each piece; they were kept in a refrigerator at 4±1°C. After 2, 4, 6, 8, 24, 48, 72, and 96 hours, hydrolysis was stopped at 60°C

for 10 minutes. All experiments were done in 4 replications.

- Physicochemical properties

- pH

The pH of the samples was measured directly using a pH meter (Testo230 tissue).

- Extraction of total protein

Total protein (sarcoplasmic + myofibrillar) extraction was carried out based on Joo *et al.* (1999). Accordingly, 40 ml of 0.1 M phosphate buffer (pH = 7.2) containing 1/1 M potassium iodide was added to 2 grams of minced meat; the sample was homogeneous and then centrifuged at 4°C for 20 minutes at 1500 g. The supernatant (total protein) was transferred to new micro-tubes and stored at -20°C. Protein concentrations were measured using a Biuret method (Joo *et al.*, 1999).

- Extraction of sarcoplasmic proteins

The sarcoplasmic proteins were extracted according to Toledra *et al.* (1992). Primarily, 20 g of minced meat samples were mixed with 200 ml phosphate buffer 0.02 M (ph=7.4) in a 1:10 ratio and then homogenized. This mixture was centrifuged for 20 minutes at 4°C at 15770 g. The supernatant (sarcoplasmic section) was sterilized through filtering with 0.22 µm filter and kept at -20°C until use. Protein concentrations of supernatant were measured using a Biuret method (Molina *et al.*, 1992).

- Extraction of myofibrillar protein

The extraction of myofibrirole protein was carried out according to Kelies *et al.* (1995). Using a homogenizer, 25 grams of minced meat was homogenized with 25 mL buffer solution (3°C, pH = 7.6) containing 0.25 M sucrose, 0.05 M Tris, and 1 mM EDTA at 16000 rpm for 30 seconds. The homogeneous mixture was centrifuged with 1000 g for 10 minutes. Following separation, the solid section was again mixed with 25 ml of buffer solution (3°C, pH = 7.6) containing 0.05 molar tris and 1 molar EDTA; after vortexing, it was centrifuged with 1000 g for 10 minutes, and the used supernatant was discarded. For the third-order, extraction was performed with 25 mL of 0.15 molar KCl (cold) solution. The resulting deposition, which was actually the isolated myofibrillar proteins, was stored in 10 ml of MFI buffer (100 mM potassium chloride, 20 mM potassium phosphate (pH=7), 1 mM EDTA, and 1 mM magnesium chloride, 1 mM sodium azide) (Claeys *et al.*, 1995). The concentration of the proteins was measured using the Biuret method.

- Determination of the hydrolysis degree

The hydrolysis degree was specified via the method developed by Nissen *et al.* (1986). The orthophetaldialdehyde indicator had to be daily provided. 7.620 g di-sodium tetraborate with 10 molecules of water and 200 ml sodium dodecyl sulfate (SDS) was allowed to be dissolved in 150 ml of water. The dissolution of 160 mg orthophetaldialdehyde (97%) was performed in 4 ml of ethanol and added to the previous solution; afterwards, 400 µl of beta-mercaptoethanol was also added and made to volume 200 ml using distilled water. Following the extraction of myofibrillar, sarcoplasmic, and total proteins from the control sample, the proteins were dried by freeze-drying, 0.1 g of proteins was dissolved in distilled water, and the concentration of proteins was measured using Biuret method. The solution was pasteurized at 85°C for 3 minutes and then cooled down to

hydrolysis temperature (50°C); prior to adding the enzyme, the solution pH was adjusted to 8 via sodium hydroxide 4 N and 2% concentration of meat proteins and kiwi protein were added to the solution. By heating at 60°C for 10 minutes, the reaction was stopped after 2, 4, 6, 8, 24, 48, 72, and 96 hours; 400 µl of protein hydrolyzed samples was mixed with 3 ml OPA reagent for 5 seconds, and after 2 minutes of storage at room temperature, the absorbance was read at 340 nm(standard used was serine). The hydrolysis degree (DH) was calculated by the following equations:

Degree of Hydrolysis= $h/h_{tot} \times 100\%$ Equation 1

where h is the number of hydrolyzed peptide bonds and h_{tot} is the total number of peptide bonds present (h_{tot} value is 8.8 meqv/g of protein).

$h = \text{serin-NH2} - \beta / \alpha$ Equation 2

where serine-NH2 is meqv serine-NH2/g protein and α and β values are 1.00 and 0.40, respectively.

- ***Determination of the average peptide chain length***

The average peptide chain length (PCL) of the hydrolyzed protein samples was calculated according to the following equation (Marambe *et al*., 2008):

Average peptide chain length= 100/DH Equation 3 (Chavira*et al*., 1984)

where DH is the hydrolysis degree.

- ***Electrophoresis***

Electrophoresis for proteins was performed by Laemmli method. The sample was mixed based on a 1:1 (v/v) ratio with a sample buffer (Tris-HCl 0.5 M with pH = 6.8, 4% SDS, 20% glycerol, and 10% beta-mercaptoethanol). 20 µl of the sample was electrophoresed in a polyacrylamide gel (5% stacking gel and 12% separator gel) with 30 mAh current. Following electrophoresis, the staining of the gel was performed by use of a staining solution (0.1% (w/v) Coomassie R-250, 45% methanol, and 10% acetic acid). The destaining solution contained water, acetic acid, and methanol in a ratio of 8:1:1 (v/v/v). Protein markers were utilized as an indicator with molecular weights of 10 to 250 amu (Sinaclon Co.).

- ***Enzymatic hydrolysis kinetics***

The speed of an enzymatic reaction can be determined by two methods: one is to measure the concentration of the substrate (S) in the environment, and another is to measure the amount of the product (p), the latter giving more accurate results. In systems composed of an enzyme and a substrate, the change occurring in the substrate over time is initially linear; however, later, with the reduction in the concentration of the substrate in the environment, the amount or rate of the substrate change decreases (Fatemi, 2016). An important criterion for measuring the ability of an enzyme is to determine its kinetic properties. Kinetics is a systematic approach to analyzing and quantitatively measuring the effect of factors such as enzyme concentration, substrate concentration, environmental conditions such as temperature, and pH on enzyme activity. The use of Michaelis–Menten equation is the most facile way to achieve kinetic enzyme. The relationship between the rate of product formation (catalyze rate) and the enzyme concentration and the substrate was determined (Mortazavi *et al*., 2007). To evaluate the impact of diverse concentrations of substrate and enzyme on enzymatic reaction, different

concentrations of substrate (18-45 mg/ml) with a fixed concentration of enzyme (0.7 g) and different concentrations of the enzyme (0.8-1.4 mg/ml) with a fixed concentration of substrate were prepared. Based on the method of Nielsen *et al.* (1986), over-time changes in the hydrolysis degree were measured using orthophetaldialdehyde indicator. All experiments were conducted under the same conditions at 50°C and six replications.

- *Statistical Analysis*

All experiments were performed with four replications. The data were analyzed in a completely randomized design at 95% confidence level using SPSS version 22 software. The mean of data was compared by the Duncan method at 95% confidence level. All charts were drawn by Excel.

Results and Discussion

- *pH changes*

According to Table 1, the pH of the control sample was higher than other treatments due to the high initial pH of the meat. The pH of the treatments significantly decreased in comparison to the control sample because of the lower initial pH value of the kiwi fruit protein. Muscle hydrolysis can result in the release of amino acids and reduce pH. According to Rawdkuen and Katnawa (2011), the use of bromelain extract reduced pH in treatment samples compared with the controls (Ketnawa *et al.*, 2011). The pH value is very important in meat products as it significantly influences such qualitative and physicochemical properties as water holding capacity, tenderness, and juiciness (Goli *et al.*, 2007). PH changes due to post-mortem metabolism and the use of substances added to meat during the process are technological (Gault, 1985).

- *Protein solubility*

Over time, the concentration of meat proteins increases. The increase in the enzyme activity or the contact time with the substrate leads to further hydrolysis and increased solubility. Generally, the increase in the activity of proteolytic enzymes augments the efficiency of the production of soluble nitrogen (soluble protein). If the substrate concentration is beyond a certain limit (> 8%), the hydrolysis rate is reduced (Kristinsson *et al.*, 2000). The difference lying in protein solubility can be due to the difference in meat composition (Rawdkuen *et al.*, 2013). Increased protein solubility by enzyme was reported by Buckley *et al.* (1974) and Kim *et al.* (1981). The increased solubility in treatment samples could be owing to the increased permeability of myofibrils, which can be easily decomposed (Figure1).

- *Determination of the hydrolysis degree*

Hydrolysis degree, which shows the breakdown of the peptide bands, is an important parameter considered in the evaluation of the properties of hydrolyzed proteins. It is highly important to control the amount of this degree because many of the hydrolyzed protein properties, including the amount of free amino acids, solubility, and molecular weight of the produced protein depend on the severity and degree of hydrolysis (Šližytė *et al.*, 2005). As shown in Table 2 and, with the increase in time, the degree of hydrolysis increased. On the other hand, the severe hydrolysis of the enzyme, which reduces the allergenic properties of proteins and makes them suitable for feeding children with allergies, can be estimated by examining the hydrolysis degree (Mahmoud, 1994). Klompong *et al.* (2007) investigated the functional properties and antioxidant activity of the hydrolyzed

protein Acanthopagrus latus. They observed more hydrolysis in the hydrolyzed proteins containing more enzymes. Batista *et al*. (2010) investigated the properties of hydrolyzed protein and the antioxidant properties of scabbardfish (Aphanopus) carbo. They reported that by increasing the amount of the protamex enzyme up to 2%, the hydrolysis degree was significantly enhanced. The results showed that peptide bands were more likely to break down in the presence of enzymes and nitrogen recovery increased. Taheri *et al*. (2011) reported a positive correlation between the degree of hydrolysis and nitrogen recovery, where the increase in the degree of hydrolysis augmented the recovered nitrogen. A similar result was obtained by Souissi *et al*. regarding the intestines of sardines (Souissi *et al*., 2007). Bagheri Kakash *et al*. (2019) showed that an increase in the hydrolysis time, increased the degree of hydrolyzing chicken meat using the enzyme kiwi fruit actinidin(Bagheri Kakash *et al*., 2019). . In line with the present study, many researchers, such as Ovissipour *et al*. (2010), Cao *et al*. (2008), and Kristinsson *et al*. (2000) also found that increased hydrolysis time increased the hydrolysis degree. Another property of the hydrolyzed proteins is the length of the hydrolysis peptides. The degree of hydrolysis has an inverse relationship with the peptide chain length, meaning that by increasing the degree of hydrolysis, the chain length of the average peptide is reduced.

Table 1. Comparison of the mean effects of kiwi protein at different times on the pH of meat fish samples $p < 0.05$

Treatment	control	2hr	4 hr	6 hr	8 hr	24 hr	48 hr	72 hr	96 hr
pH	6.19	5.55	5.32	5.32	5.11	4.91	4.88	4.83	4.79

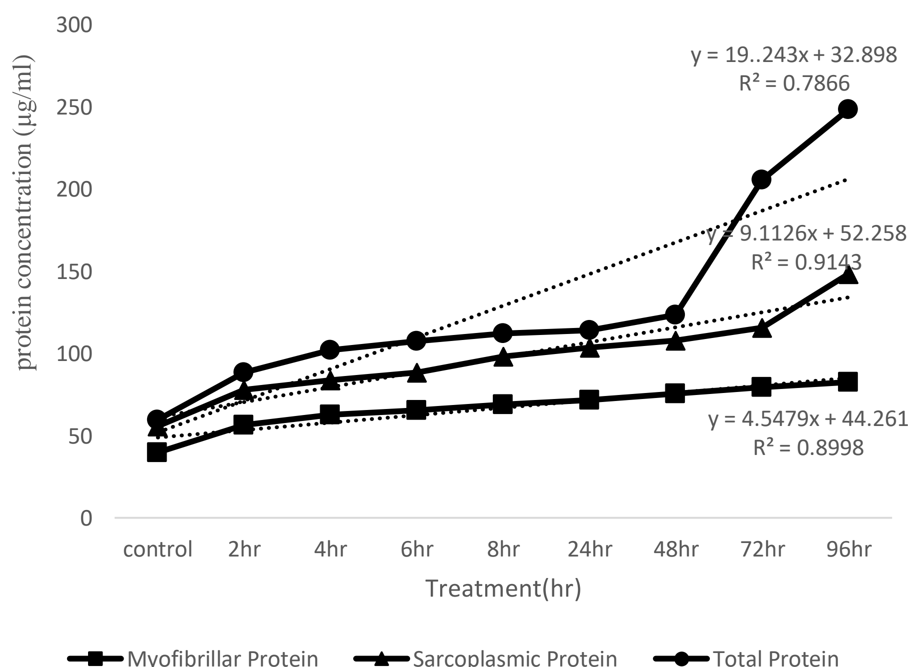

Fig. 1. The Effect of kiwi on changes in the concentration of fish protein.

Table 2. Results of the degree of hydrolysis and average peptide chain length of fish proteins

Protein type	Time	DH	PCL
Total proteins	2 hrs	0.24±0.006	413.24±4.13
	4 hrs	0.62±0.02	159.72±1.59
	6 hrs	0.71±0.009	140.82±1.4
	8 hrs	0.91±0.06	109.38±1.09
	24 hrs	1.06±0.009	93.60±0.93
	48 hrs	2.52±0.01	39.61±0.39
	72 hrs	2.89±0.02	34.56±0.34
	96 hrs	3.12±0.01	32.03±0.32
Myofibrillar proteins	2 hrs	1.39±0.02	71.82±0.63
	4 hrs	1.43±0.04	69.71±0.61
	6 hrs	1.97±0.01	50.59±0.46
	8 hrs	2.27±0.01	43.93±0.40
	24 hrs	2.82±0.01	35.44±0.33
	48 hrs	4.98±0.09	20.04±0.19
	72 hrs	5.19±0.10	19.24±0.18
	96 hrs	6.24±0.085	16.00±0.15
Sarcoplasmic proteins	2 hrs	1.44±0.01	69.10±0.69
	4 hrs	2.49±0.007	40.07±0.40
	6 hrs	2.51±0.03	39.81±0.39
	8 hrs	2.79±0.01	35.72±0.35
	24 hrs	3.08±0.01	32.37±0.32
	48 hrs	3.74±0.02	26.72±0.26
	72 hrs	5.04±0.11	19.82±0.20
	96 hrs	5.24±0.12	19.05±0.19

All values are the mean ± standard error; DH = Degree of hydrolysis; PCL = Average peptide chain length

- ***Effect of different concentrations of substrate and enzyme on enzymatic reaction***

As shown in Figure 2, by increasing the substrate concentration from 18 to 45 mg/ml, the hydrolysis degree decreased. Therefore, the substrate not only increased the reaction rate at low concentrations, but also inhibited enzyme activity at higher concentrations. In the process of enzymatic hydrolysis, selecting an optimal substrate concentration, maintaining a high reaction rate, and maintaining the activity of the enzyme can improve the enzyme efficiency and reduce the production cost.

- ***Effect of different enzyme concentrations on the enzyme reaction***

As shown in Figure3, the hydrolysis degree dramatically increased by increasing the enzyme concentration from 0.8 to 1.4 mg/ml.

In practical applications, selection of a low enzyme concentration in accordance with the conditions and controlling the degree of hydrolysis can also provide the catalyst cost (Qian *et al*., 2011). Similar results were obtained for sardines (Quaglia and Orban, 1987), shark (Kristinsson 1998), crab (Baek and Cadwallader, 1995), Menhaden (Hevia *et al*., 1976), calf bone (LINDER *et al*., 1995), whey protein (Mutilangi *et al*., 1995), casein (MOHAMED *et al*., 1992) and Chicken (Bagheri Kakash *et al*., 2019). As shown in Figures 3 and 4, the hydrolysis degree increased over time and then reached a constant value, having direct and inverse relationships with the enzyme concentration and the substrate concentration, respectively. The reaction speed decreases over time, especially in the middle and final stages of the reaction. This phenomenon could be owing to three reasons: 1) reduction in the concentration

of peptide bonds for hydrolysis, 2) inhibition of enzyme or product, 3) deactivation of the enzyme (Gonzàlez Tello *et al*., 1994).

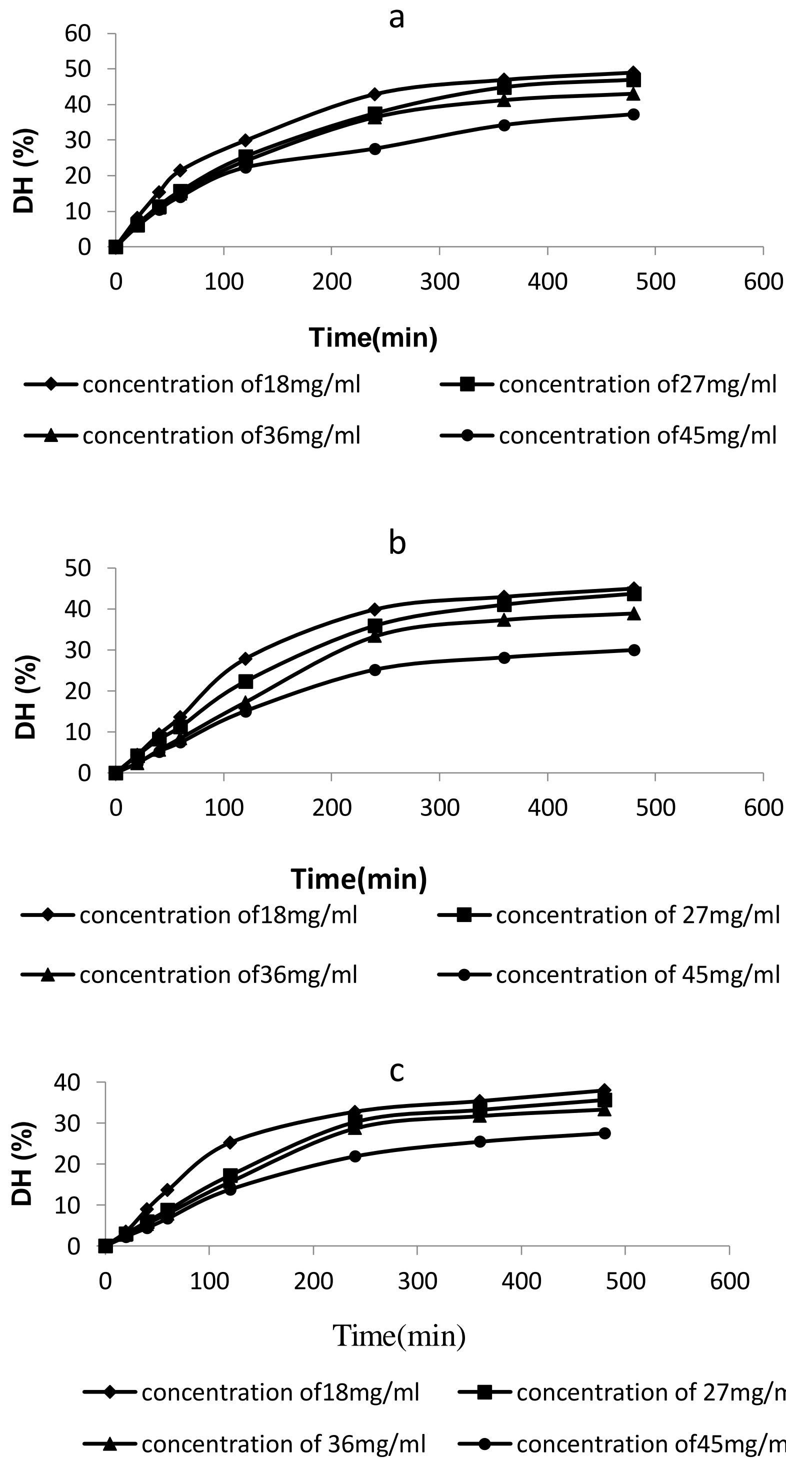

Fig. 2. Effect of different substrate concentrations on the degree of hydrolysis during the time, (a) total protein; (b) myofibrillar protein; (c) sarcoplasmic protein.

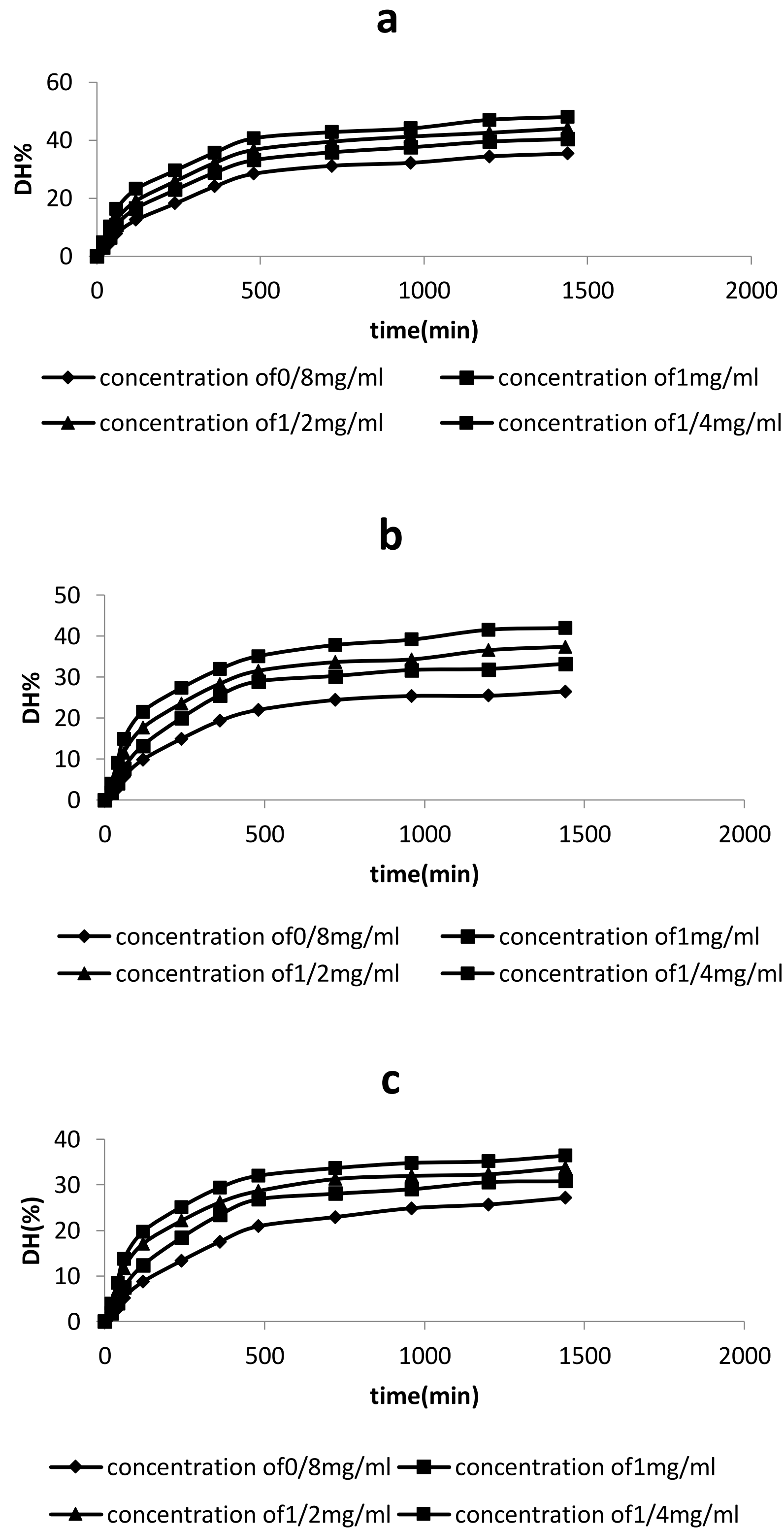

Fig. 3. Effect of different enzyme concentrations on the degree of hydrolysis during time a) total protein b) myofibrillar protein c) sarcoplasmic protein.

In order to determine the coefficients of the DH equation, DH changes were measured over time and the coefficients a and b parameters were determined using equation (4). Afterwards, by drawing a and b versus the enzyme to the substrate ratio, their equations were obtained and shown in Table 4. The results of Table 3 showed that the parameter b had a minor change, and the mean value of b for total fish meat was 0.073, the myofibrillar protein of fish with actinidine was 0.053, and for fish, sarcoplasmic protein with actinidin was 0.0716. The parameter decreased with the increase in the concentration of the substrate and augmented with the increase in the enzyme concentration; therefore, the relationship between a and E_0/S_0 is linear (Figure4) and their equations are:

$$DH = \frac{1}{b} ln(1 + abt) \quad \text{Equation (4)}$$

where a and b are constants and t is the hydrolysis time. In the model, the parameter a is expressed as ke_0 /s_0, where k is the reaction rate constant, e_0 is the initial enzyme concentration and s_0 is the initial substrate concentration. The parameter b is expressed as k_d /k, where k_d is the enzyme inactivation constant.

The following equations were used to determine the parameters of the Michaelis – Menten equation:

$$V = \frac{V_m[S]}{K+[S]} \quad \text{Equation (5)}$$

Where v is the initial reaction rate, [s] is the concentration of the substrate, vmax is the maximum rate, and KM is the Michaelis constant expressed in terms of concentration units (M/L). In other words, $K_M = \frac{1}{2}(vmax)$ is the substrate specific concentration at which half the maximum rate has been taken. The results of calculating Km, Vmax, and KS values are reported in Table 4. As shown, Vmax had the highest and lowest values for total protein and sarcoplasmic protein, respectively. KM shows the inclination of the enzyme for its substrate. By reducing the KM value, the inclination increased. According to Table 4, Vmax in total protein was higher than myofibrillar and sarcoplasmic protein, indicating the higher activity of the enzyme in total protein. The Km value was lower in the myofibrillar protein, indicating that the actinidine enzyme in the total protein had more affinity with the substrate and reached its maximum activity (Vmax) faster. A similar result was also obtained by Bagheri Kakash *et al*. (2019) about the effect of kiwi fruit actinidin on various proteins of chicken meat.

Table 3. DH equation constant for fish meat

S		M		T		E_0/S_0	S_0	E_0
b	a	b	a	b	a			
0.065	0.395	0.048	0.395	0.058	0.568	0.039	18	0.7
0.048	0.222	0.042	0.292	0.057	0.406	0.026	27	0.7
0.048	0.201	0.037	0.213	0.054	0.349	0.019	36	0.7
0.062	0.168	0.058	0.192	0.072	0.303	0.016	45	0.7
0.081	0.129	0.069	0.167	0.089	0.265	0.022	36	0.8
0.081	0.229	0.062	0.247	0.086	0.308	0.027	36	1
0.091	0.417	0.053	0.379	0.083	0.383	0.033	36	1.2
0.097	0.543	0.052	0.523	0.084	0.543	0.038	36	1.4

E_0: Enzyme concentration, S_0: substrate concentration, E_0/S_0: Enzyme to Substrate ratio, a,b: parameter, T: total protein M: myofibrillar protein S: sarcoplasmic protein

Total protein : a= $10.761[E_0]/[S_0]+0.0921$

Myofibrillar protein : a= 12.763[E0]/[S0]-0.0531

Sarcoplasmic protein: a= 14.942[E0]/[S0]-0.126

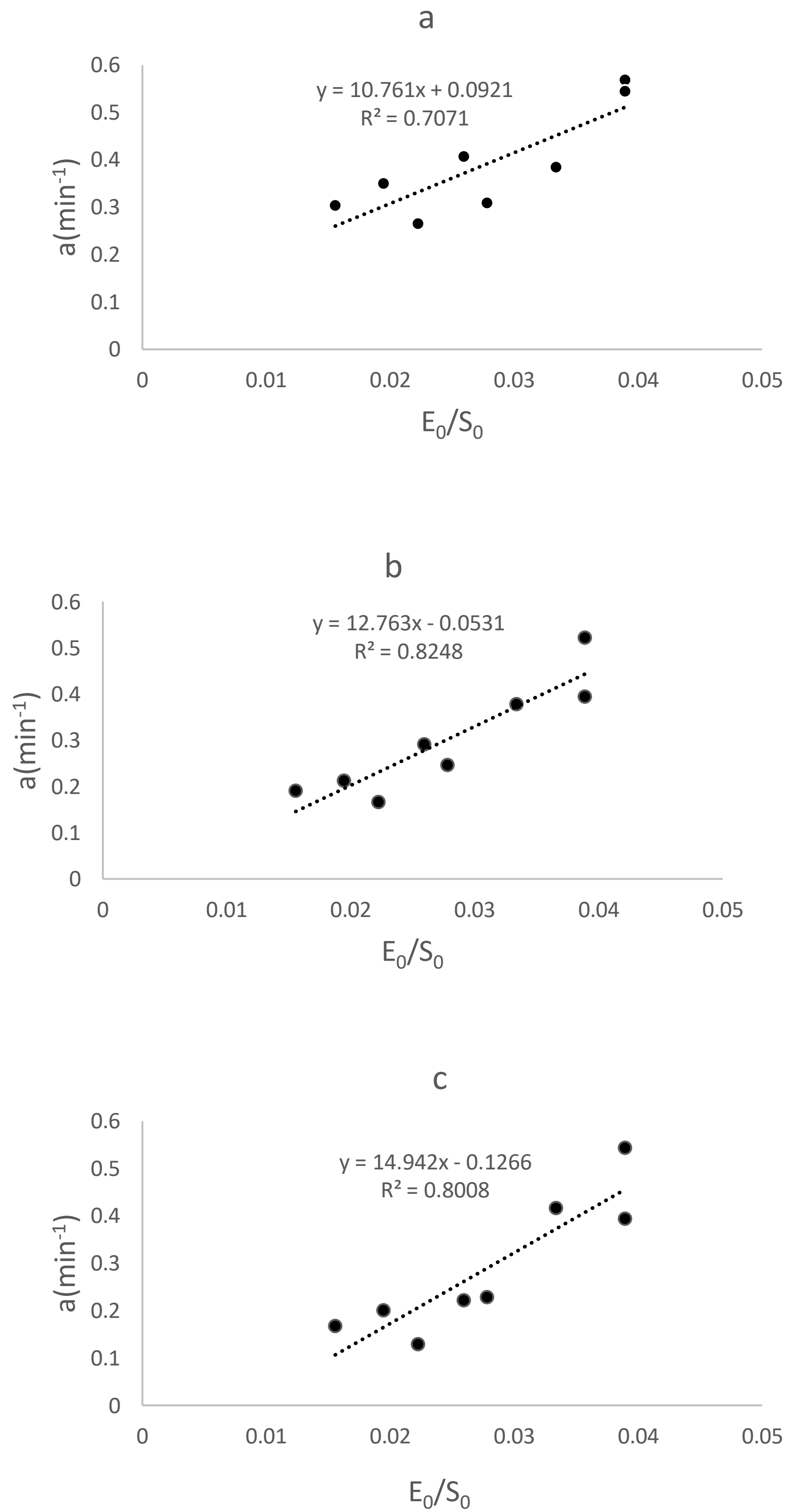

Fig. 4. Changes in a parameter against different E_0/S_0 a) total protein b) myofibrillar protein c) sarcoplasmic protein.

- *The effect of the enzyme on the electrophoretic pattern of meat proteins*

Figures 5, 6, and 7 show the electrophoretic pattern of proteins by SDS-PAGE for fish meats treated with actinidine enzyme at different times. The exact same protein patterns were observed for total, myofibrillar, and sarcoplasmic proteins in the control fish meat. Total protein bands are: Titin and Nebulin (600-3200 KDa), Filamin (280 kDa), Myosin heavy chain (223 kDa), myokinase (221 kDa), C protein (128 kDa), α-Actinin (103 kDa), Glycogen phosphorylase (97 kDa), pyruvate kinase (60 kDa), Phosphoglucose isomerase (51 kDa), desmine (53 kDa), creatine kinase (41 kDa), actin (42 kDa), aldolase (39 kDa), Glyceraldehyde Phosphate Dehydrogenase (36 kDa), lactate dehydrogenase (34 kDa) α- and β-tropomyosin (33 kDa), troponin T (30.5 kDa), phosphoglycerate mutase or kinase (30 kDa), thiosulfate isomerase (26 kDa), Myosin light chain (25 kDa), Troponin I (22 kDa), Troponin c (17.8 kDa), and Myoglobin (17 kDa). Proteolysis of muscle proteins in all treated enzymes increased over time with the reduction in the number and intensity of the protein band in the treated samples. As observed in the electrophoretic pattern of total fish proteins, all protein bands in the fish were completely eliminated from the first day, which is the result of the impact of Actinidine on the myofibrillar and sarcoplasmic proteins, causing meat tenderness.

Table 4. Enzyme kinetics parameters

S	M	T		
0.079	0.082	0.095	V_m	$V = \frac{V_m[S]}{K + [S]}$
36.33	28.7	33.25	K	
0.98	0.98	0.98	R^2	

V_m: maximum reaction velocity, K: Michaelis constant, T: total protein M: myofibrillar protein S: sarcoplasmic protein

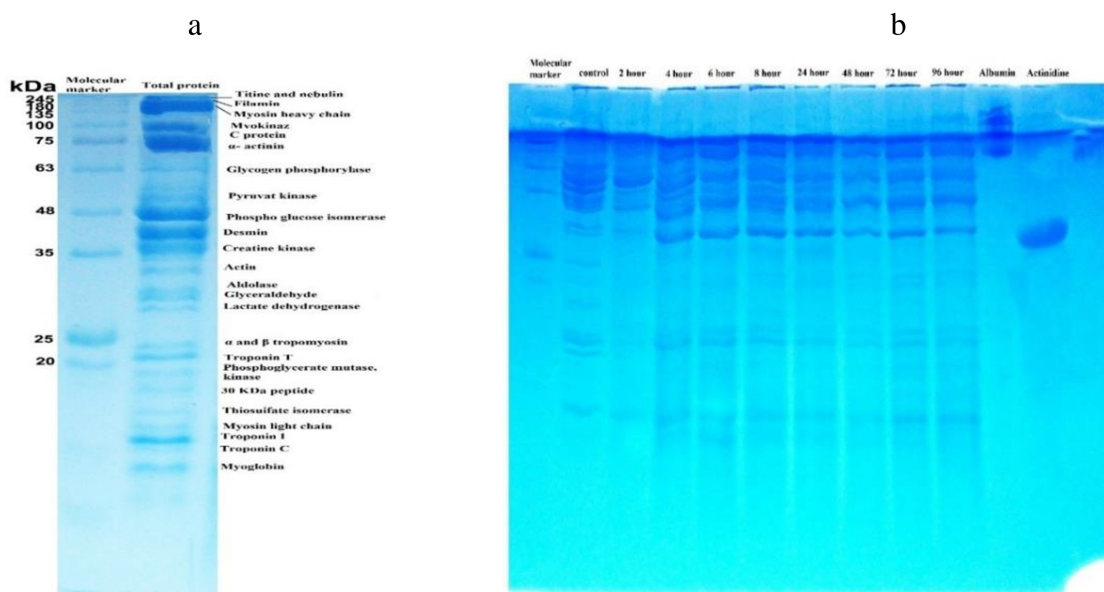

Fig. 5. Meat protein of the kiwifruit analysed with 1D-SDS–PAGE. (a) Fish total protein control (b) Fish total protein hydrolys during time.

Myofibrillar protein bands include: myosin heavy chain (223 kDa), C protein (128 kDa), α- actinin (103 kDa), actin (42 kDa), α- and β- Tropomyosin (33 kDa), troponin T (30.5 kDa), myosin light chain (25 kDa), troponin I (22 kDa), troponin c (17.8 kDa), Titin and Nebulin (600-3200 kDa), Filamin (280 kDa), and desmine (53 kDa) [11,24].Actinidine heydrolized a wide range of myofibrillar proteins, including actomyosin (actin and myosin) (42 and 223 kDa), Nebulin and Titin (650-3200 kDa), Filamin (280 kDa), actinin (100 kDa), and desmine (53 kDa), which is similar to the results of Christensen *et al*. (2009), Han *et al*.(2009), and Kaur *et al*. (2010).

The main bands of sarcoplasmic protein are: myokinase (221 kDa), glycogen phosphorylase (97 kDa), double band of pyruvate kinase and phosphoglucose isomerase (60 kDa), double band of creatine kinase (41 kDa) and aldolase (39 kDa), glyceraldehyde phosphate dehydrogenase (36 kDa), lactate dehydrogenase (34 kDa), thiosulfate isomerase (26 kDa), phosphoglycerate mutase or phosphoglycerate kinase (30 kDa), myoglobin (17 kDa) (Marcos *et al*., 2010; Christine *et al*., 2003). Fig. 7 shows the results pertaining to the SDS-PAGE of sarcoplasmic proteins of fish meat treated with the enzyme at different times of enzyme hydrolysis. Many of the protein bands disappeared due to the hydrolysis of sarcoplasmic proteins.

Investigating the changes in protein units over the hydrolysis period suggested that at the beginning of the hydrolysis, the increase in the mobility of these units was very slight; over time, their variation increased because with the progression of hydrolysis, the depth regions of the protein, which are more diverse in producer amino acids subjected to hydrolysis and action sites of enzymes, increased significantly, thereby increasing the intensity and speed of hydrolysis. The results of this study showed that Actinidine affected myofibrillar and sarcoplasmic proteins, causing meat tenderness.

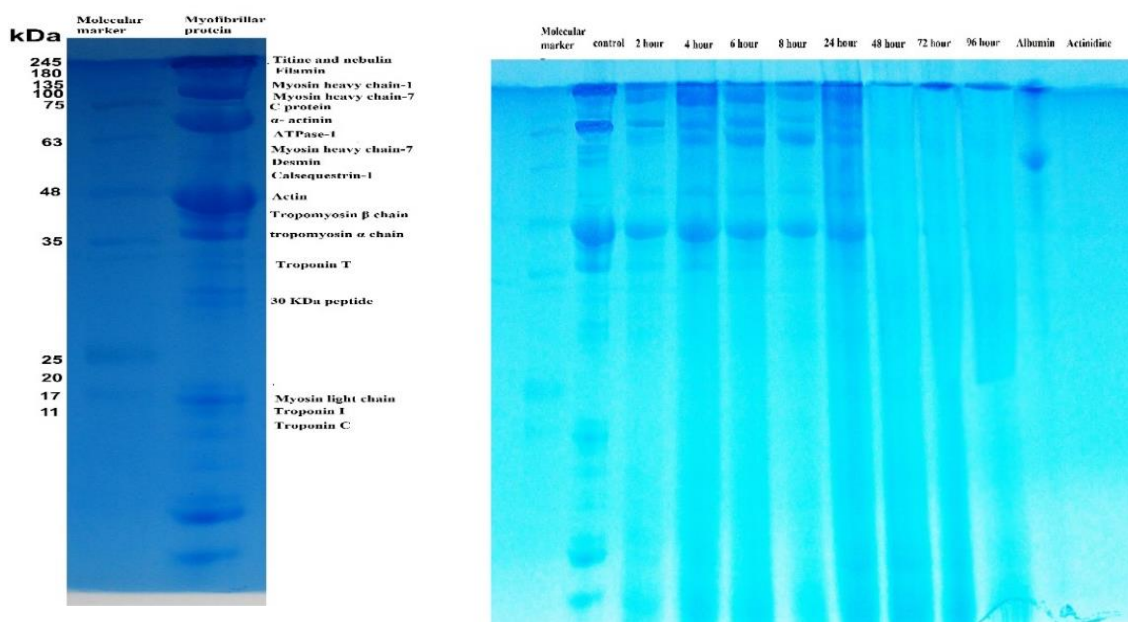

Fig. 6. Meat protein of the kiwifruit analysed with 1D-SDS–PAGE. (a) Fish myofibrillar protein control (b) Fish myofibrillar protein hydrolys during time.

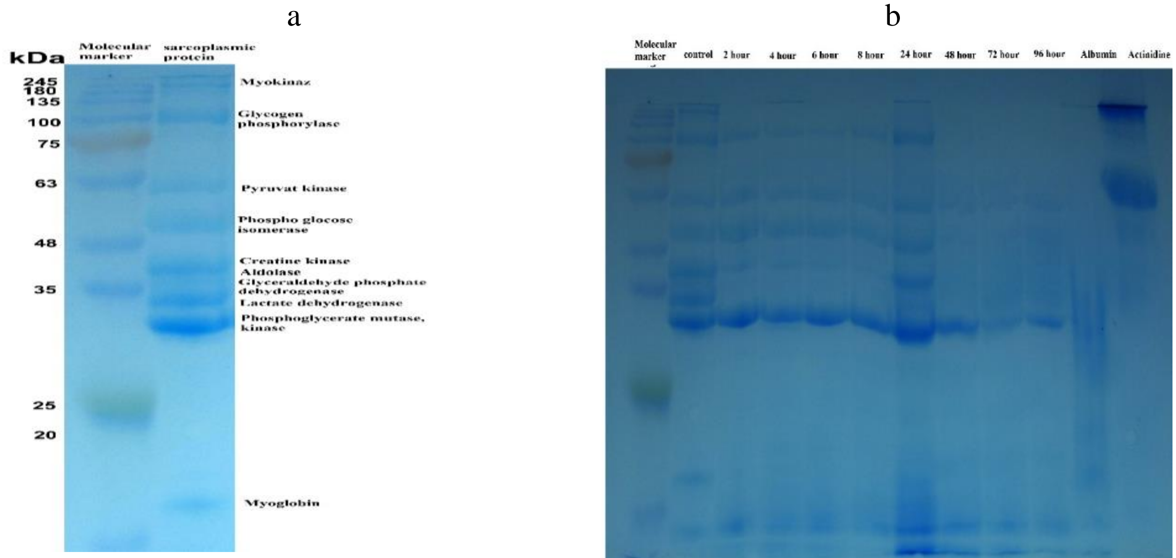

Fig. 7. Meat protein of the kiwifruit analysed with 1D-SDS–PAGE. (a) Fish sarcoplasmic protein control (b) Fish sarcoplasmic protein hydrolys during time.

Conclusion

Given the objective of the present study, tenderizing the fish meat, the results showed that the increase in hydrolysis time increased the degree of hydrolysis but reduced the peptide chain length. The electrophoretic pattern of fish proteins revealed that kiwi fruit tenderized the meat by affecting the myofibrillar and sarcoplasmic proteins. In conclusion, kiwi fruit tenderizes the fish and improves its characteristics.

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References

Adler-Nissen, J. (1986). *Enzymic hydrolysis of food proteins*. London: Elsevier Applied Science. ISBN: 0-85334-386-1.

Afshar-Mohammadian, M., Rahimi-Koldeh, J. & Sajedi, R. (2011). The comparison of protease activity and total protein in three cultivars of kiwifruit of Northern Iran during fruit development. *Acta Physiologiae Plantarum*, 33(2), 343-348. http://dx.doi.org/10.1007/s11738-010-0553-3.

Alsmeyer, R. H., Cunningham, A.E. & Happich, M.L. (1974). Equations predict PER from amino acid analysis. *Food Technology*, 7 (28), 34–40. http://agris.fao.org/agris-search/search.do?recordID=US201303166075.

Aspmo, S. I., Horn, S. J. & Vincent, G.H. (2005). Enzymatic hydrolysis of Atlantic cod (Gadus morhua L.) *viscera. Process Biochemistry*, 5(40), 1957-1966. https://doi.org/10.1016/j.procbio.2004.07.011

Baek, H. & Cadwallader, K. (1995). Enzymatic hydrolysis of crayfish processing by-products. *Journal of Food Science*, 60(5), 929-935. http://dx.doi.org/10.1111/j.1365-2621.1995.tb06264.x.

Bagherikakash, S., Hojjatoleslamy, M., Babaei, G. & Molavi, H. (2019). Kinetic study of the effect of kiwi fruit actinidin on various proteins of chicken meat. *Food*

Science and Technology, *39*(4), 980-992. DOI:Dhttps://doi.org/10.1590/fst.14118

Batista, I., Ramos, C., Coutinho, J., Bandarra, N. & Nunes, M. (2010). Characterization of protein hydrolysates and lipids obtained from black scabbardfish (Aphanopus carbo) by-products and antioxidative activity of the hydrolysates produced. *Process Biochemistry, 45*(1), 18-24. https://doi.org/10.1016/j.procbio.2009.07.019

Cao, W., Zhang, C., Hong, J. & Ji, H. (2008). Response surface methodology for autolysis parameters optimization of shrimp head and amino acids released during autolysis. *Food Chemistry*, 109(1), 176-183. http://dx.doi.org/10.1016/j.foodchem.2007.11.080. PMid:26054279.

Carne, A. & Moore, C. H. (1978). The amino acid sequence of the tryptic peptides from actinidin, a proteolytic enzyme from the fruit of *Actinidia chinensis*. *The Biochemical Journal*, 173(1),73-83. http://dx.doi.org/10.1042/bj1730073. PMid:687380.

Chavira, R. J., Burnett, T. J. & Hageman, J. H. (1984). Assaying proteinases with azocoll. *Analytical biochemistry*, 136(2), 446-450. https://doi.org/10.1016/0003-2697(84)90242-2

Christensen, M., Tørngren, M., Gunvig, A., Rozlosnik, N., Lametsch, R., Karlsson, A. & Ertbjerg, P. (2009). Injection of marinade with actinidin increases tenderness of porcine M. biceps femoris and affects myofibrils and connective tissue. *Journal of the Science of Food and Agriculture*, *89*(9), 1607-1614. https://doi.org/10.1002/jsfa.3633

Claeys, E., Uytterhaegen, L., Buts, B. & Demeyer, D. (1995). Quantification of beef myofibrillar proteins. Meat Science, 39(2), 177-193. http://dx.doi.org/10.1016/0309-1740(94)P1819-H. PMid:22059824.

El-Gharbawi, M. & Whitaker, J. (2006). Factors affecting enzymatic solubilization of beef proteins. Journal of Food Science, 28(2), 168-172. http://dx.doi.org/10.1111/j.1365-2621.1963.tb00177.x.

Englund, P., King, T., Craig, L. & Walti, A. (1968). Ficin. I. Its isolation and characterization. *Biochemistry*, 7(1), 163-175. http://dx.doi.org/10.1021/bi00841a021. PMid:5758541.hin

Fatemi, H. (2016). *Food chemistry*. Tehran: Sahami Enteshatr.

Garg, V. & Mendiratta, S. (2006). Studies on tenderization and preparation of enrobed pork chunks in microwave oven. *Meat Science*, 74(4), 718-726. http://dx.doi.org/10.1016/j.meatsci.2006.06.003. PMid:22063229.

Gault, N. (1985). The relationship between water-holding capacity and cooked meat tenderness in some beef muscles as influenced by acidic conditions below the ultimate pH. *Meat Science*, 15(1), 15-30. http://dx.doi.org/10.1016/0309-1740(85)90071-3. PMid:22056073.

Gilman, A., Philips, F. S., Koelle, E. S., Allen, R. P. & St. John, E. (1946). The metabolic reduction and nephrotoxic action of tetrathionate in relation to a possible interaction with sulfhydryl compounds. *The American Journal of Physiology*, 147(1), 115-126. http://dx.doi.org/10.1152/ajplegacy.1946.147.1.115. PMid: 21000729.

Goli, T., Abi Nakhoul, P., Zakhia-Rozis, N., Trystram, G. & Bohuon, P. (2007). Chemical equilibrium of minced turkey meat in organic acid solutions. *Meat Science*, 75(2), 308-314.

http://dx.doi.org/10.1016/j.meatsci.2006.07.016. PMid: 22063663.

Gonzàlez-Tello, P., Camacho, F., Jurado, E., Páez, M. P. & Guadix, E. M. (1994). Enzymatic hydrolysis of whey proteins:I. kinetic models. *Biotechnology and Bioengineering*, 44(4), 523-528. http://dx.doi.org/10.1002/bit.260440415. PMid:18618786.

Ha, M., Bekhit, A. D., Carne, A. & Hopkins, D. (2012). Characterisation of commercial papain, bromelain, actinidin and zingibain protease preparations and their activities toward meat proteins. *Food Chemistry*, 134(1), 95-105. http://dx.doi.org/10.1016/j.foodchem.2012.02.071.

Han, J., Morton, J., Bekhit, A. & Sedcole, J. (2009). Pre-rigor infusion with kiwifruit juice improves lamb tenderness. *Meat Science*, 82(3), 324-330. http://dx.doi.org/10.1016/j.meatsci.2009.02.003. PMid:20416722.

Hevia, P., Whitaker, J. & Olcott, H. (1976). Solubilization of a fish protein concentrate with proteolytic enzymes. *Journal of Agricultural and Food Chemistry*, 24(2), 383-385. http://dx.doi.org/10.1021/jf60204a048. PMid:1254819.

Hoyle, N. T. & Merritt, J. H. (1994). Quality of Fish Protein Hydrolysates from Herring (Clupea harengus). *Journal of Food Science*, 1(59), 76-79. https://doi.org/10.1111/j.1365-2621.1994.tb06901.x.

Huff-Lonergan, E., Parrish, F.C. & Robson, R. M. (1995). Effects of postmortem aging time, animal age, and sex on degradation of titin and nebulin in bovine longissimus muscle. 4 (73), 1064-1073. https://doi.org/10.2527/1995.7341064x.

Joo, S., Kauffman, R. G., Kim, B. C. & Park, G. B. (1999). The relationship of sarcoplasmic and myofibrillar protein solubility to colour and water-holding capacity in porcine longissimus muscle. *Meat Science*, 52(3), 291-297. http://dx.doi.org/10.1016/S0309-1740(99)00005-4. PMid:22062578.

Kaur, L., Rutherfurd, S., Moughan, P., Drummond, L. & Boland, M. (2010). Actinidin enhances gastric protein digestion as assessed using an in vitro gastric digestion model. *Journal of Agricultural and Food Chemistry, 58*(8), 5068-5073. https://pubs.acs.org/doi/10.1021/jf903332a

Ketnawa, S. & Rawdkuen, S. (2011). Application of bromelain extract for muscle foods tenderization. *Food and Nutrition Sciences*, 2(5), 1-9. http://dx.doi.org/10.4236/fns.2011.25055.

Kim, K.S., Lee, S.Y. & Kee, K.W. (1981). Studies on tenderization of meat by use of proteolytic enzymes: study on the properties of proteolytic enzymes. *Research reports of the office of rural development. Livestock and Veterinary (Korea R.)*, 23,75-83. http://agris.fao.org/agris-search/search.do?recordID=XB8235093.

Klompong, V., Benjakul, S., Kantachote, D. & Shahidi, F. (2007). Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (Selaroides leptolepis) as influenced by the degree of hydrolysis and enzyme type. *Food Chemistry, 102*(4), 1317-1327. https://doi.org/10.1016/j.foodchem.2006.07.016.

Koak, J. H., Kim, H. S., Choi, Y., Baik, M. Y. & Kim, B. Y. (2011). Characterization of a protease from over-matured fruits and development of a tenderizer using an optimization technique. *Food Science and Biotechnology*, 20(2), 485-490. http://dx.doi.org/10.1007/s10068-011-0067-9.

Kowlessur, D., O'Driscoll, M., Topham, C., Templeton, W., Thomas, E. & Brocklehurst, K. (1989). The interplay of electrostatic fields and binding interactions determining catalytic-site reactivity in actinidin: a possible origin of differences in the behaviour of actinidin and papain. *The Biochemical Journal*, 259(2), 443-452. http://dx.doi.org/10.1042/bj2590443. PMid: 2719659

Kristinsson, H. (1998). *Reaction kinetics, biochemical and functional properties of salmon muscle proteins hydrolyzed by different alkaline proteases*. Washington: University of Washington.

Kristinsson, H. & Rasco, B. (2000). Fish protein hydrolysates: production, biochemical, and functional properties. *Critical Reviews in Food Science and Nutrition*, 40(1), 43-81. http://dx.doi.org/10.1080/10408690091189266. PMid:10674201.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature*, 227(5259), 680-685. DOI:10.1038/227680a0

Lawrie, R. & Ledward, D. (2006). *Lawrie's meat science* (7th ed.). Cambridge: Woodhead Publishing. http://dx.doi.org/10.1533/9781845691615.

Lewis, D. & Luh, B. S. (1988). Application of actinidin from kiwifruit to meat tenderization and characterization of beef muscle protein hydrolysis. *Journal of Food Biochemistry*, 12(3), 147-158. http://dx.doi.org/10.1111/j.1745-4514.1988.tb00368.x.

Liaset, B., Lied, E. & Espe, M. (2000). Enzymatic hydrolysis of by-products from the fish-filleting industry: chemical characterisation and nutritional evaluation. *Journal of the Science of Food and Agriculture*, 80(5), 581-589. http://dx.doi.org/10.1002/(SICI)1097-0010(200004)80:5<581::AID-JSFA578>3.0.CO;2-I.

Linder, M., Fanni, J., Parmentier, M., Sergent, M. & Phan-Tan-Luu, R. (1995). Protein recovery from veal bones by enzymatic hydrolysis. *Food Science*, 60(5), 949-952. http://dx.doi.org/10.1111/j.1365-2621.1995.tb06268.x.

Mahmoud, M., Malone, W. T. & Cordle, C. T. (1992). Enzymatic hydrolysis of casein. effect of degree of hydrolysis on antigenicity and physical properties. *Journal of Food Science*, 57(5), 1223-1229. http://dx.doi.org/10.1111/j.1365-2621.1992.tb11304.x.

Marambe, P., Shand, P. & Wanasundara, J. (2008). An in-vitro investigation of selected biological activities of hydrolysed flaxseed (*Linum usitatissimum* L.) proteins. *Journal of the American Oil Chemists' Society*, 85(12), 1155-1164. http://dx.doi.org/10.1007/s11746-008-1293-z.

Marcos, B., Kerry, J.P. & Mullen, A.M. (2010). High pressure induced changes on sarcoplasmic protein fraction and quality indicators. *Meat Science*, 1(85), 115-20. https://doi.org/10.1016/j.meatsci.2009.12.014

Mahmoud, M.I. (1994). Physicochemical and functional properties of protein hydrolysates in nutritional products. *Food Technology*, 59,89– 94. https://ci.nii.ac.jp/naid/10025368874/.

Molina, I. & Toldrá, F. (1992). Detection of proteolytic activity in microorganisms isolated from dry cured ham. *Journal of Food Science*, 57(6), 1308-1310. http://dx.doi.org/10.1111/j.1365-2621.1992.tb06843.x.

Mortazavi, A., Salary, R. & Zia ul Haq, H. (2007). *Modeling of food processes*.

Mashhad:Mashhad Ferdowsi University. https://doi.org/10.1016/C2013-0-16519-1.

Mutilangi, W. A. M., Panyam, D. & Kilara, A. (1995). Hydrolysates from proteolysis of heat-denatured whey proteins. *Food Science*, 60(5), 1104-1109. http://dx.doi.org/10.1111/j.1365-2621.1995.tb06302.x.

Naveena, B. M., Mendiratta, S. K. & Anjaneyulu, A. S. (2004). Tenderization of buffalo meat using plant proteases from *Cucumis trigonus Roxb* (Kachri) and *Zingiber officinale roscoe* (Ginger rhizome). *Meat Science*, 68(3), 363-369. http://dx.doi.org/10.1016/j.meatsci.2004.04.004. PMid:22062404.

Ovissipour, M., Benjakul, S., Safari, R. & Motamedzadegan, A. (2010). Fish protein hydrolysates production from yellowfin tuna *Thunnus albacares* head using Alcalase and Protamex. *International Aquatic Research.*, 2(2), 87-95. https://www.sid.ir/fa/journal/ViewPaper.aspx?id=118537

Paul, W., Amiss, J., Try, R., Praekelt, U., Scott, R. & Smith, H. (1995). Correct processing of the kiwifruit protease actinidin in transgenic tobacco requires the presence of the C-terminal propeptide. *Plant Physiology*, 108(1), 261-268. http://dx.doi.org/10.1104/pp.108.1.261. PMid:7784505.

Qian, J., Zhang, H. & Liao, Q. (2011). The properties and kinetics of enzymatic reaction in the process of the enzymatic extraction of fish oil. *Journal of Food Science and Technology*, 48(3), 280-284. http://dx.doi.org/10.1007/s13197-010-0128-8. PMid:23572747.

Quaglia, G. & Orban, E. (1987). Influence of the degree of hydrolysis on the solubility of the protein hydrolysates from sardine (*Sardina pilchardus*). *Journal of the Science of Food and Agriculture*, 38(3), 271-276. http://dx.doi.org/10.1002/jsfa.2740380311.

Rawdkuen, S., Jaimakreu, M. & Benjakul, S. (2013). Physicochemical properties and tenderness of meat samples using proteolytic extract from Calotropis procera latex. *Food Chemistry*, 136(2), 909-916. http://dx.doi.org/10.1016/j.foodchem.2012.08.077. PMid:23122144.

Safdari, Y., Saeedi Asl, M. R., Safari, R. & Jahed, J. (2014). Evaluation of enzymatic hydrolysis of rodent entrails and utilization of hydrolyzate as a source of peptone for Lactobacillus plantarum growth. *Journal of Innovation in Food Science and Technology,* 6(3), 17-26.

Shahidi, F., Han, X. & Synowiecki, J. (1995). Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). *Food Chemistry*, 53(3), 285-293. http://dx.doi.org/10.1016/0308-8146(95)93934-J.

Shargel, L., Wu-Pong, S. & Yu, A. B. C. (2005). *Applied biopharmaceutics and pharmacokinetics* (5th ed.). New York:McGraw-Hill. ISBN: 0-07-137550-3.

Šližytė, R., Daukšas, E., Falch, E., Storrø, I. & Rustad, T. (2005). Characteristics of protein fractions generated from hydrolysed cod (Gadus morhua) by-products. Process *Biochemistry, 40(6),* 2021-2033. https://doi.org/10.1016/j.procbio.2004.07.016

Souissi, N., Bougatef, A., Triki-Ellouz, Y. & Nasr, M. (2007). Biochemical and functional properties of sardinella (*Sardinella aurita*) by-product hydrolysates. *Food Technology and Biotechnology*, 45(2), 187-194. https://hrcak.srce.hr/27772.

Taylor, W. J. & Diers-Caviness, M. H. (2003). *A textbook of the clinical*

application of therapeutic drug monitoring. Irving: Diagnostic Division, Abbott Laboratories. ISBN: 0961490306, 9780961490300.

Tavakoli, H., Aghazadeh Mashghi, M., Dabbagh Moghaddam, A. & Sadeghzadeh Araqi, A. (2005). *TextBook Of Meat Hygiene and Inspection.* Marze danesh.

Toohey, E. S., Kerr, M. J., Van de Ven, R. & Hopkins, D. L. (2011). The effect of a kiwi fruit based solution on meat traits in beef m. *semimembranosus* (topside). *Meat Science*, 88(3), 468-471. http:// dx.doi.org/10.1016/j.meatsci.2011.01.028. PMid:21345602.

Wada, M., Suzuki, T., Yaguti, Y. & Hasegawa, T. (2002). The effects of pressure treatments with kiwi fruit protease on adult cattle semitendinosus muscle. *FoodChemistry*,78(2), 167-171. http:// dx.doi.org/10.1016/S0308-8146(01)00395-8.