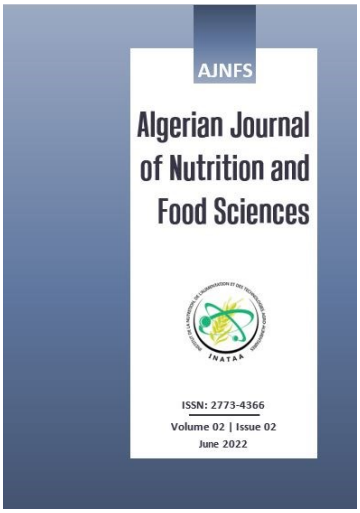

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Original Article

Thornback ray gelatin hydrolysate as an alternative preservative: Effect of hydrolysis degree

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Abstract *Thornback ray gelatin hydrolysates, at different hydrolysis degree (DH), are prepared by treatment with Bacillus subtilis A26 proteases. Their antioxidant potential was investigated by measuring the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and the reducing power, while their antibacterial activity was investigated by evaluating the inhibitory effect against 3 pathogenic strains (M. luteus, S. aureus and E. coli) using the agar diffusion technique. The two-antioxidant activities tested are dose dependent and negatively associated with DH. The DPPH scavenging activity reveals a median inhibitory concentration (IC50) of 1mg/ml and 1.36 mg/ml when the DH is 8% and 2.81%, respectively. Apart from the hydrolysate of which the DH is 11%, which has no effect on E coli and a markedly reduced effect against S. aureus, the various hydrolysates showed good anti-bacterial activity although it tends decreased as the DH increased. However, the observed difference remains small.*

Keywords *Thornback ray, Gelatin; Hydrolysate, Hydrolysis degree, Antioxidant, Antibacterial*

Résumé *Des hydrolysats de gélatine de raie bouclée, de différents degrés d'hydrolyse (DH), sont préparés par traitement avec des protéases de Bacillus subtilis A26. Leur potentiel antioxydant a été évalué en mesurant l'activité de piégeage des radicaux 2,2-diphényl 1-picrylhydrazyle (DPPH) et le pouvoir réducteur. L'activité antibactérienne a été évaluée contre 3 souches pathogènes (M. luteus, S. aureus et E. coli) par la technique de diffusion sur gélose. Les deux activités anti-oxydantes testées sont dose-dépendantes et montrent une tendance à diminuer lorsque le DH augmente. Cette tendance est plus claire pour le pouvoir réducteur que pour l'activité anti-radicalaire. Le test de piégeage du DPPH révèle une concentration inhibitrice médiane (IC50) de 1mg/ml et 1,36 mg/ml lorsque le DH est de 8% et 2,81% respectivement. Excepté l'hydrolysat dont le DH est de 11% qui n'a aucun effet sur E coli et un effet nettement réduit contre S. aureus, les différents hydrolysats montrent une bonne activité antibactérienne bien qu'elle diminue légèrement lorsque le DH augmente.*

Mots clés *Raie bouclée, Gélatine, Hydrolysat, Degré d'Hydrolyse, Antioxydant, Antibactérien*

Introduction

Foods are sensitive to oxidative modification and exposed to pathogenic/toxigenic microbes (*E. coli, Salmonella, Clostridium, Bacillus,* etc.) contamination. As a result, foods undergo a degradation of their rheological and nutritional characteristics, and can become dangerous for health due to the various deleterious products that appear there (hydroperoxides, aldehydes, mycotoxins). Because natural resources are limited and the world population is constantly growing, food preservation is necessary. One of the most valid approaches in this area is the addition of antioxidants and antimicrobials.

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Due to the side effects of traditional antioxidants (citric acid, butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT), etc.) and antimicrobials (sulphites, nitrites, benzoic acid, etc.), alternative products of natural origin are in high demand. Several compounds different by their origin (animal, vegetable, insects, etc.) and by their nature (essential oils, peptides, chitosan, etc.) are proposed (Aziz and Karboune, 2018). Protein derivatives of fishery and aquaculture co-products are particularly promising. Indeed, strong antioxidant and/or antimicrobial activities are expressed by many protein hydrolysates from different fish co-products or underutilized species (Barkia *et al.*, 2010; Jemil *et al.*, 2014; Pezechk *et al.*, 2019; Atef *et al.*, 2021; Agustin *et al.*, 2021).

In addition to a strong antioxidant activity, a high antibacterial activity, in particular against gram+ strains (*Staphylococcus aureus* and *Bacillus subtilis*) was exhibited by protein hydrolysate from tilapia by-products

(*Oreochromis niloticus*) that was obtained using papain (Srikanya *et al.*, 2018). High antioxidant and antibacterial activities are also expressed by European eel by-product protein hydrolysate produced using savinase or protamex (Bougatef *et al.*, 2020). For its part, hydrolysates of *Scomber japonicas* fish fin waste protein concentrate that are prepared by Ediriweera *et al.* (2019) showed high antibacterial activity, but a low antioxidant activity.

In reality, a source of products is all the more useful, sought after, as it is more available, and allows a better financial advantage. Precisely, proteins from fish by-products are available in large quantities and can therefore allow the manufacture of products at low cost. In addition, the use of fishery by-products, in this sector, and in others, rids nature of a source of pollution and has a positive impact on the environment.

In addition to their sensitivity to the structure of substrate proteins and to protease specificity, the composition and properties of hydrolysate are influenced by hydrolysis conditions, particularly, the hydrolysis degree (DH) that expresses the percentage of cleaved peptide bonds relative to the total number of peptide bonds in the protein substrate. This parameter influences the size of produced peptides and, therefore, the functional and biological properties of hydrolysate. In particular, antioxidant activity increases with the increase in DH up to a value beyond which DH effect is reversed. The value of this limit DH varies according to the considered hydrolysate (Nasri *et al.*, 2014; Hmidet *et al.*, 2011). Furthermore, DH is negatively associated with antimicrobial activity (Thammasena *et al.*, 2020) and it affects organoleptic properties. Fish protein hydrolysates of 5-10% DH have good organoleptic properties (Chambers and Rasmussen, 1988).

The present study evaluates the potential of *Raja clavata* skin gelatin as a source of alternative antibacterial and antioxidant. To explore the relationship between peptide size and biological activities, different DHs are applied. The range of considered DH takes into account the relationship of this parameter with antioxidant, anti-bacterial and organoleptic properties. The identification of the DH leading a hydrolysate with significant antioxidant and antibacterial activities is sought.

Materials and methods

Reagents and Bacterial Strains

DPPH and glycine were purchased from Sigma Chemical Co. (St. Louis MO, USA). Pepsin was purchased from MP Biomedicals (Strasbourg, France). Other chemicals and reagents used were of analytical grade. All solutions were freshly prepared in distilled water.

Microbial strains

Antibacterial activities of MJTP extracts were tested against *Staphylococcus aureus* (ATCC 25923), *Micrococcus luteus* (ATCC 4698) and *Escherichia coli* (ATCC 25922).

Material

Fresh thornback ray by-products were purchased from the fish market of Sfax City, Tunisia. The samples were packed in poly-ethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w) and transported to the research laboratory within 30 min. Upon arrival, thornback ray skin was collected and scrapped to remove the adhering fat and foreign matter and cut into small pieces (0.5 cm x 0.5 cm). Prepared skin was then stored in sealed plastic bags at - 20 °C until used for gelatin extraction and analysis.

Proteolytic enzymes

Crude enzyme preparation from Bacillus subtilis A26 was prepared in our laboratory (Agrebi *et al.*, 2009). The method of Kembhavi *et al.* (1993) was used to measure the alkaline protease activity.

Preparation of Raja clavata gelatin hydrolysates

Thornback ray gelatin was extracted from the skin wastes following the method described by Lassoued *et al.* (2014). Glycine–HCl buffer (100 mmol/l, pH 2.0) in combination with commercial pepsin were used in the extraction process.

To elaborate the different thornback ray gelatin hydrolysates, the gelatin powder was dissolved in distilled water at 2.5% (w/v) and adjusted to the appropriate pH and temperature of *B. subtilis* A26 proteases (8.0, 40 °C). Enzymes were added to the mixture at an enzyme/substrate ratio (E/S = 50 U/mg of protein). During the reaction, the pH of each reaction mixture was maintained at the desired value by continuous addition of 1 M NaOH solution using a pH-stat after incubation for 5, 10, 15, 30, 60 and 240 min. The reaction was stopped by adding HCl solution (1N) until pH = 5.0 to inactivate enzymes. Gelatin hydrolysates were then centrifuged at 5000 g (MPW Med Instruments, Boremlowska, Poland) for 20 min to separate soluble and insoluble fractions. Finally, the soluble fractions referred to as protein hydrolysates, were freeze-dried using a freeze-dryer (Bioblock Scientific, Strasbourg, France) and stored at −20 °C for further use, less than one month.

The degree of hydrolysis, defined as the per cent ratio of the number of peptide bonds broken to the total number of peptide bonds per unit weight (h_{tot}), in each case, was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis (Adler-Nissen, 1986).

Antioxidant activity

DPPH assay

The DPPH radical-scavenging activity of hysrolysates was determined by the method of Kirby and Schmidt (1997) with some modifications. A volume of 500 µl of each extract at different concentrations (1 to 5 mg/mL)

was added to 375 µl of 99% ethanol and 125 µl of DPPH solution (0.2 mM in ethanol) as free radical source. The mixtures were incubated for 60 min in the dark at room temperature. Scavenging capacity was measured spectrophotometrically (Labomed. Inc, USA) by monitoring the decrease in absorbance at 517 nm. In its radical form, DPPH has an absorption band at 517 nm which disappears upon reduction by an antiradical compound. Lower absorbance of the reaction mixture indicated higher free radical – scavenging activity (SA). DPPH radical – SA (%) was calculated as:

$$DPPH_{radical} - SA\ (\%) = \frac{A_{control} + A_{blank} - A_{sample}}{A_{control}} \times 100$$

Where $A_{control}$, A_{blank} and A_{sample} are, respectively, the absorbance of the control reaction (containing all reagents except the sample), the MJTP extract without DPPH solution and the MJTP extract with DPPH solution. The test was carried out in triplicate.

Ferric-reducing activity

The reducing power of hydrolysates was determined by the method of Yildirim *et al.* (2001). Sample solutions (0.5 ml) containing different concentrations of dried extract (1 to 5 mg/mL) were mixed with 1.25 ml of 0.2 M phosphate buffer (pH 6.6) and 1.25 ml of 10 g/l potassium ferricyanide solution. The mixtures were incubated for 30 min at 50 °C. After incubation, 1.25 ml of 100 g/l TCA was added and the reaction mixtures were centrifuged for 10 min at 3000 g. A 1.25 ml aliquot of the supernatant from each sample mixture was mixed with 1.25 ml of distilled water and 0.25 ml of 1.0 g/l ferric chloride solution in a test tube. After a 10 min reaction time, the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power. The control was conducted in the same manner, except that distilled water was used instead of sample. Values presented are the mean of triplicate analyses.

Agar diffusion method

Antimicrobial activities of the different extracts were tested according to the method described by Berghe and Vlietinck (1991). Hydrolysates were tested at a concentration of 100 mg/mL. Culture suspension (200 µl) of the tested microorganisms 10^6 colony-forming units (cfu)/mL of bacteria cells (estimated by absorbance at 600 nm). Then, bores (7 mm depth, 6 mm diameter) were made using a sterile borer and were loaded with 60 µl of sample at 100 mg/mL. The Petri dishes were kept, first for 4 h at 4 °C to allow extract diffusion in the agar, and then incubated for 24 h at 37 °C. Antimicrobial activity was evaluated by measuring the diameter of the growth inhibition zones in millimetres (including well diameter of 6 mm). The test was carried out in triplicate, and the values presented are the averages of three replicates.

Results and discussion

Enzymatic hydrolysis

One of the parameters influencing the properties of a hydrolysate is the degree of hydrolysis. The DHs corresponding to the different hydrolysis times are summarized in Table 1. They vary from 2.81 to 11%. This range of DHs is likely to include DHs which allow the production of hydrolysates which contain peptides of a size that are compatible with the antioxidant and the antibacterial activities, and which could, possibly, have a good organoleptic quality. Extension of the hydrolysis time resulted in more small size peptides (Li *et al.*, 2014).

Table 1: IC_{50} values of the different hydrolysates in radical scavenging activity

DH (%)	2.81	4.21	4.91	6.32	7.29	8
Time (min)	5	10	15	30	45	60
IC_{50} (mg/mL)	1.36	1.27	1.33	1.23	1.11	1

DH: hydrolysis degree, IC_{50}: median inhibitory concentration

Antioxidant activities

The evaluation of the antioxidant activity uses different tests because this activity involves several mechanisms. The effect of hydrolysis time on the radical scavenging and reducing power activities was investigated in order to determine the relationship of these activities to the DH. Both antioxidant activities tested are dose-dependent and positively associated with DH (Figure 1). The DPPH and reducing power activities both increased with the hydrolysis time for 60 min. Indeed, according to the results of the DPPH scavenging test, the IC_{50} varies from 1 mg/mL at DH 8% to 1.36 mg/mL at DH 2.81% (Table 1). This is in line with the observations of previous studies (Bougatef *et al.*, 2020, Agustin *et al.*, 2021, Zhang *et al.*, 2011, You *et al.*, 2009). However, a negative relationship between DH and antioxidant activity was observed in the enzymatic hydrolysate of yellowfin sole frame protein (Jun *et al.*, 2004). The antioxidant activity of a hydrolysate results from its composition in peptides (size, quantity, sequence) and in free amino acids (Wu *et al.*, 2003). Moreover, it is possible that the positive relationship of activity with DH would only exist if the size is greater than or equal to the activity size limit (Barkia *et al.*, 2010).

Antibacterial activity

According to the tests carried out, the hydrolysates of different DH are able to inhibit the growth of used bacterial strains, as evidenced by the presence of clear zones around the deposit wells (Figure 2 and Table 2). Such property is observed with hydrolysates of several other proteins (Wald *et al.*, 2016; Pezechk *et al.*, 2019; Jemil *et al.*, 2014; Atef *et al.*, 2021). For example, Jemil *et al.* (2014) reported that hydrolysates of meat protein from several fish (*Sardinella aurita, Salaria basilisca, Zosterizessor ophiocephalus, Dasyatis pastinaca*) have an antibacterial effect that is more effective against Gram+ than against Gram–. Da Rochaa *et al.* (2018)

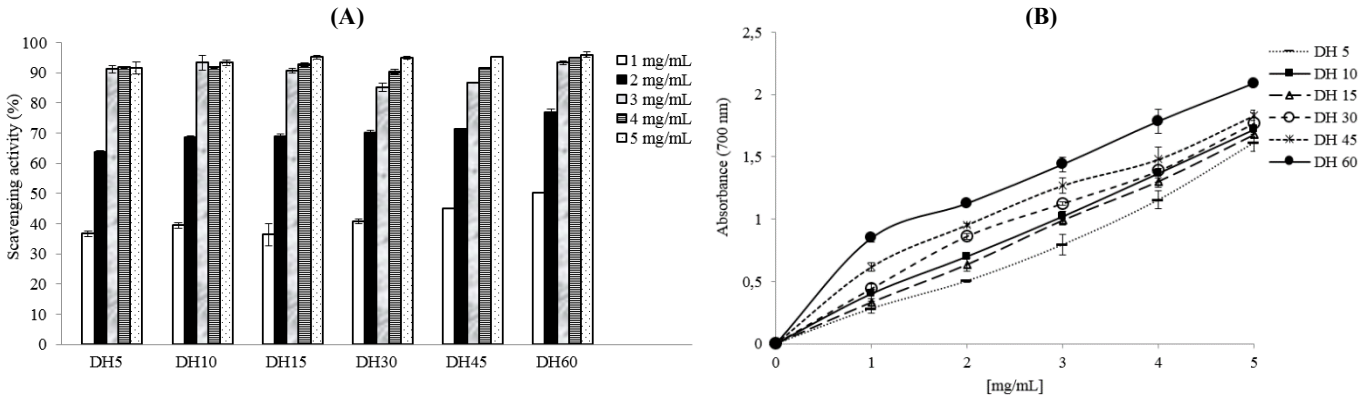

Figure 1. Antioxidant effect of different concentration of the hydrolysates (A) DPPH radical scavenging and (B) reducing power activities.

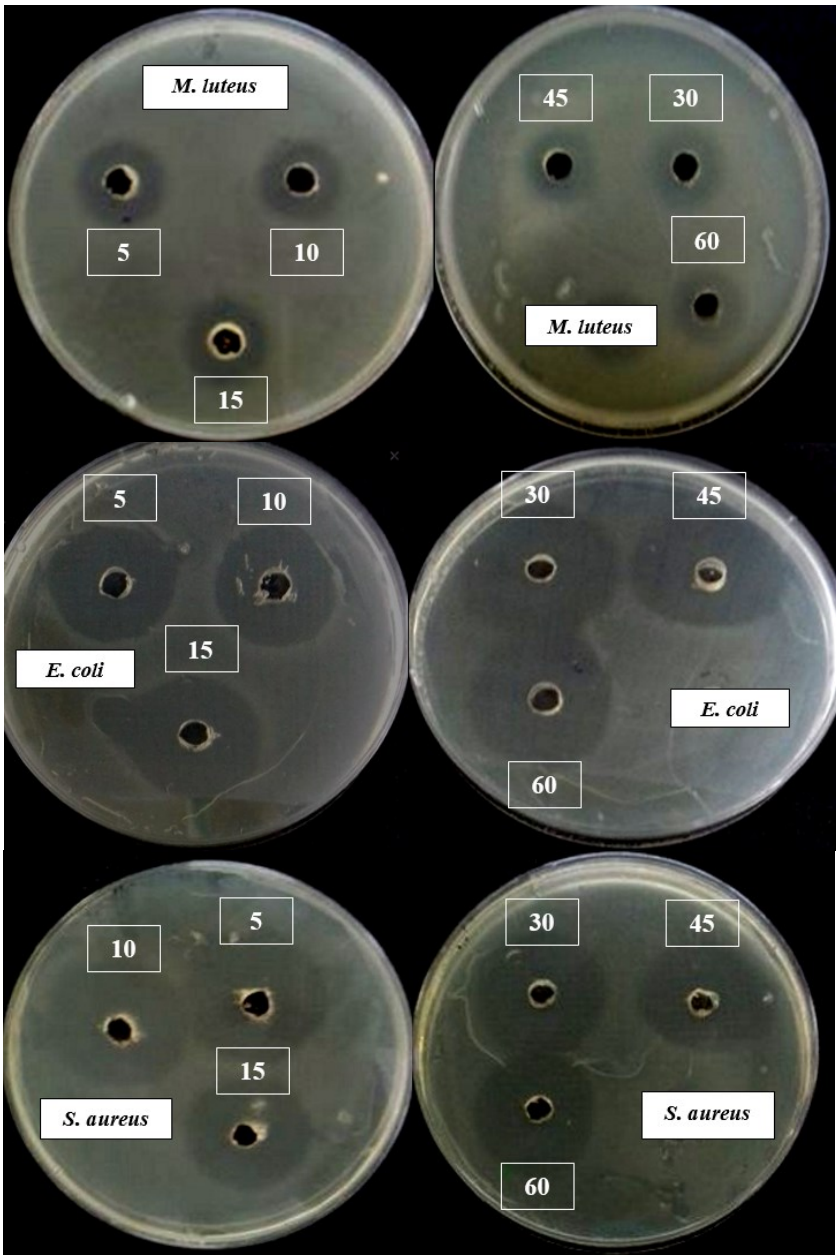

Figure 2. Antibacterial activity of different hydrolysates by agar diffusion method against *M. luteus, S. aureus and E. coli* (5: DH 2.81%; 10: DH 4.21%; 15: DH 4.92%; 30: DH 6.32%; 45: DH 7.29%; 60: DH 8%)

Table 2. Antibacterial activity of the different gelatin hydrolysates.

Bacterial strain	Hdrolysis time (min)	DH (%)	Inhibition diameter (mm)
M. Luteus **(Gram +)**	5	2.81	21
	10	4.21	20
	15	4.91	19
	30	6.32	18
	45	7.29	18
	60	8	18
S. aureus **(Gram +)**	5	2.81	30
	10	4.21	27
	15	4.91	25
	30	6.32	25
	45	7.29	25
	60	8	23
E. coli **(Gram -)**	5	2.81	28
	10	4.21	25
	15	4.91	25
	30	6.32	25
	45	7.29	25
	60	8	25

report a same observation with Argentine croaker (*Umbrina canosai*) protein hydrolysate. Present hydrolysates show significant activity against both Gram– and Gram+ bacteria with inhibition diameters ranging from 15 to 30 mm (Table 2).

For their part, Wald *et al.* (2016) reported that the antibacterial effect of rainbow trout by-product hydrolysates increased significantly with increasing DH. These results corroborate with the results of Pezeshk *et al.* (2019) who demonstrated that the size fraction < 3 kDa of the protein hydrolysate of yellowfin tuna (*Thunnus albacores*) viscera inhibits bacterial growth more effectively than the larger size fractions (3-10, 10-30 and 30 kDa <). On the contrary, Atef *et al.* (2021) did not observe an effect of the degree of hydrolysis on the antibacterial activity of Sturgeon Fish Skin Collagen hydrolysate (*Huso huso*).

In present study, activity tended to decrease as DH increased. In fact, the greatest inhibition halos are observed for the lowest DH (2.81%) and this against the three strains tested (Table 2). Further hydrolysis could cause inactivation of peptides by hydrolyzing them and/or producing new peptides that would be inactive.

Conclusion

Hydrolysis applied to *Raja clavata* gelatin released peptides with antioxidant activity and antibacterial capacity against the bacteria used. However, it appears that the DH must be less or equal to 8% in order to guarantee good antioxidant activity and powerful antimicrobial activity against all used bacterial strains. Thornback ray gelatin hydrolysate is an alternative as natural compounds that can help, in particular, in the preservation and safety of food.

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