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Optimisation of *Dosidicus gigas* mantle proteolysis at industrial scale

Iria Barcia [a], M. Luz Sánchez-Purriños [a], Mónica Novo [a], Ana Novás [a], Julio F. Maroto [b], Ramiro Barcia [a,*]

[a] *Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Santiago de Compostela, Campus de Lugo, E-27002 Lugo, Spain*
[b] *Centro Tecnológico del Mar, Fundación CETMAR, E-36208 Vigo, Spain*

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Abstract

The use of enzymatic preparations in food processing is very old and has a number of advantages such as the high specificity of the enzymes, which avoids unwanted products, and their use at moderate temperatures, hence reducing alterations in the most labile components of food. The aim of this work is to supply information that would allow the use of *Dosidicus gigas,* as a source of raw material, in the preparation of diverse frozen products. With this purpose, we studied the effect of seven proteases: collagenase F, collagenase H, collagenase/dispase, papain, pronase, subtilisin and trypsin, and centered on the rheological and sensory evaluation of the changes which occurred in the texture of *D. gigas*, in order to establish the optimal conditions for the use of each enzyme.

Keywords: *Dosidicus*; Squid; Proteolysis; Sensory evaluation; Technological optimisation

1. Introduction

The squid *Illex argentinus* is the cephalopod most commonly used by frozen-seafood processing companies because it fulfills all the requirements: the tonnage available is usually abundant, even with certain annual fluctuations, and its size and organoleptic characteristics have confirmed it as the most suitable species. In fact, machinery and technology have developed in relation to the behaviour of this raw material, during handling.

However, the availability of this raw material might decrease anytime and, for this reason, finding alternative species that fit the technological requirements currently available in the processing rooms seems a sensible enterprise. *Dosidicus gigas*, the Humboldt, jumbo or giant squid is among these species. This is a Pacific species distributed from southern California to southern Chile (Klett, 1996).

Fresh squid has a sticky consistency and it is not easy to chew. The mantle contracts after cooking and assumes a round shape, owing especially to shrinkage of the skin. This happens because under the pigmented skin there is a white, gummy epithelium covering the mantle (Fig. 1) which cannot be easily eliminated, troubling industrial processing (Ramírez Olivas, Rouzaud Sández, Haard, Pacheco Aguilar, & Ezquerra Brauer, 2004). The frozen mantle presents the same feature.

There are different studies on squid tenderisation using protease solutions (Venugopal, Lakshmanan, Doke, & Bongirwar, 2000), obtained from squid hepatopancreas (Kolodziejska, Pacana, & Sikorski, 1992) or from other sources, such as papaya latex (Borresen, 1992) or from raw extract from the lysosomes of bovine spleen mixed with commercial bromelain (Melendo, Beltrán, & Roncales, 1997, 1998). Works and studies on the activity of the proteases present in different *Dosidicus* organs and tissues are also available (Cárdenas-López & Haard, 2005; Ezquerra-Brauer, Haard, Ramírez-Olivas, Olivas-Burrola, & Velázquez-Sánchez, 2002). However, these treatments

* Corresponding author. Tel.: +34 982 252303x22208; fax: +34 982 252195.
E-mail address: bnbarcia@lugo.usc.es (R. Barcia).

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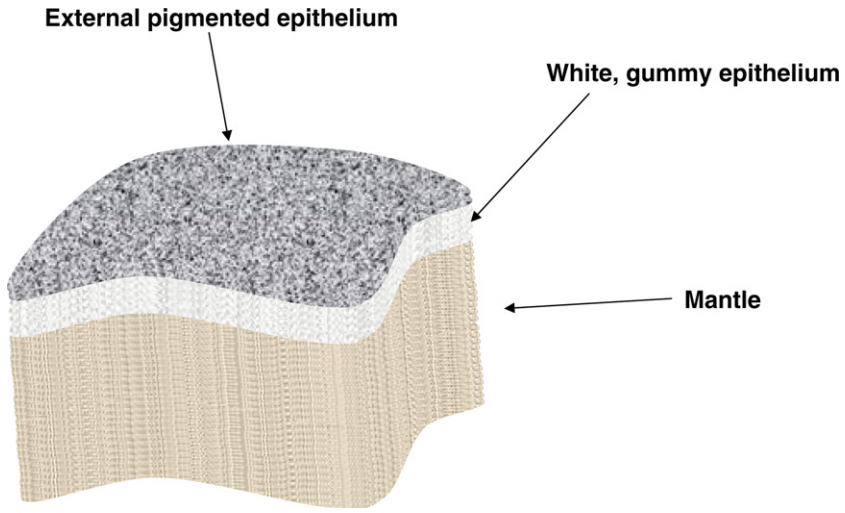

Fig. 1. Scheme of *Dosidicus gigas* mantle and skin.

are effective only when applied to the conventional species used in industry, such as *Loligo* and *Illex*, whereas they remain ineffective on *Dosidicus*. The effective treatment of *D. gigas* requires a very careful adjustment of treatment conditions because of the high endogenous proteolytic activity present in the mantle. If allowed to act, such activity may lead an excessively soft product. Therefore, if *Dosidicus* proteases are added to their own mantle and allowed to act in their optimal conditions, this will only favor a faster degradation of the tissue, thus making it more difficult to handle in industry.

In our case, as we used "pure" commercial enzymes, work conditions do not have to coincide with the optimal conditions of the proteases of the *Dosidicus* mantle, which slows down their activity and allows a more selective proteolysis, leading to an attractive product.

Collagen, in squid mantle, constitutes the main component of the connective tissue membranes and is responsible for its integrity. The content of collagen in squid mantle is about 2.6% (Sikorski, Scott, & Buisson, 1984).

The subepidermal connective tissue of the integument, of the cephalopods, consisted of a dense mass of collagen fibers with abundant fibroblasts, muscle fibers, chromatic cells, secretory cells and nervous structures (Bairati, Comazzi, & Gioria, 2003).

In cephalopods, there is a wide variety of types of collagen. Type V-like collagens are widely distributed in marine invertebrates, particularly crustaceans and mollusks (Sivakumar, Suguna, & Chandrakasan, 2000). Collagen type I-like and VI-like have also been detected in cephalopods (Bairati et al., 2003). On the other hand, collagen fibers of cephalopod dermis are closely related to proteoglycans (heparan sulfate and chondroitin sulfate) (Bairati et al., 2003), moreover, invertebrate collagens are exceptionally rich in sugars linked mainly *o*-glycosidically to hydroxylysine residues (Sikorski et al., 1984). During maturation, the proportion of collagen to total protein in the muscles increases while the extent of cross-linking does not change significantly (Sikorski et al., 1984).

The present work analyses the effectiveness of different enzymatic solutions in the treatment of *D. gigas* and suggests the optimal working conditions for their industrial use.

2. Materials and methods

2.1. Biological materials

The frozen-seafood processing company CONGALSA (Pobra do Caramiñal, Galicia, N.W. of Spain) supplied the mantles of *D. gigas*, frozen at −25 °C.

2.2. Proteases

All the proteases used in this work were supplied by the Sigma–Aldrich Co. (St. Louis, Missouri, USA), with the exception of pronase, supplied by Calbiochem (Darmstadtt, Germany).

2.3. Sample preparation for determination of protease activity

Dosidicus mantles were allowed to defreeze for 12 h, at 4 °C, prior to the experiments.

Next, the mantles were cut into pieces of 2 × 1 cm (approx. 2 g). Twenty-five samples were used in each assay.

In order to determine protease optimal operating conditions, we analysed the effect of the different proteases on the *Dosidicus* mantle protein extract by SDS-PAGE.

Mantle samples were weighed and 4 ml of Tris–HCl buffer 20 mM, pH 7.5, containing calcium acetate 12 mM, β-mercaptoethanol 10 mM and 10% Triton X-100 (homogenization buffer) were added per gram of mantle. The samples were then triturated and kept at 4 °C for 30 min, with steady shaking, in order to extract the proteins. Next, the

samples were centrifuged at 30,000*g* for 20 min at 4 °C and the supernatant was collected.

Then, protease solutions were prepared at the required concentrations. Ten microliters of each solution were added, respectively, to 100 µl samples of the supernatant obtained in the previous step and the mixture was allowed to stand at the temperature for the time that each enzyme required.

Then, the samples were mixed with Laemmli buffer (1970) at a rate 1:4 (vol/vol), heated at 95 °C for 5 min, and cooled immediately on ice. The electrophoresis was performed following Laemmli protocol (1970), loading 15 µg protein in each well. The amount of protein per sample was determined by the Bradford method (1976).

2.4. Rheological studies

Twenty-five squid pieces of 2 × 1 cm were used in each assay. These were defreezed and subjected to incubation with the corresponding enzyme, at the concentration, temperature and for the time required. The pieces were always placed in the texture analyser Houmsfield H-10 KM in the same position, on their epithelium (Fig. 1). Shear force was measured on each sample using a triangular plunger with an angle of 30°.

The statistic program InStat™ GraphPad Software Inc., San Diego USA was used to analyse the results obtained and the comparisons between samples were performed with Student's *t*-test.

2.5. Sensory analysis

Early sample treatment was identical to that performed for the rheological studies. After the treatment with proteases, the samples were scalded with water, at 75 °C for 10 s, in order to inactivate the enzymes, rolled in breadcrumbs, pre-fried in sunflower oil at 180 °C for 15–20 s and frozen at −20 °C. Immediately before the tasting, the samples were cooked for 2–3 min in sunflower oil at 180 °C and kept in isothermal recipients, until presented to the tasters.

The analysis consisted of an assay of classification by ordering, according to the norm UNE87 023 AENOR (1997). With this purpose, a 12-member untrained sensory panel was asked to order four samples according to toughness.

As recommended by the norm UNE, the Friedman test (1937) was chosen to establish the statistical significance of the differences that the panelists might detect between samples.

When the Friedman test (1937) determined statistically significant differences between samples, the pairs of differing samples were identified using an analog of Fisher's least significant difference (1998).

3. Results

Table 1 shows the enzymes which were assayed to provoke tenderisation in the epithelium of *D. gigas*, together with the assay conditions. With the exception of collage-

Table 1
Assay conditions of the enzymes used in the treatment of *Dosidicus gigas*

Protease	Concentrations (µg/µl)	Time of incubation (min)	Temperature (°C)	pH
Collagenase H	0.75–3.75–15.2	From 1 to 480	25	7.5
Collagenase F	0.75–3.75–15.2	From 1 to 480	25	7.5
Collagenase/dispase	0.15–0.75	From 1 to 480	30	7.5
Papain	0.01–0.1–1.0	From 1 to 120	25	6.2
Pronase	0.004–0.007–0.015–0.075–0.757	From 1 to 60	40	7.5
Subtilisin	0.007–0.015–0.076–0.151–0.757	From 1 to 480	37	7.5
Trypsin	0.75–1.5–6.0–10.0	From 1 to 60	37	7.5

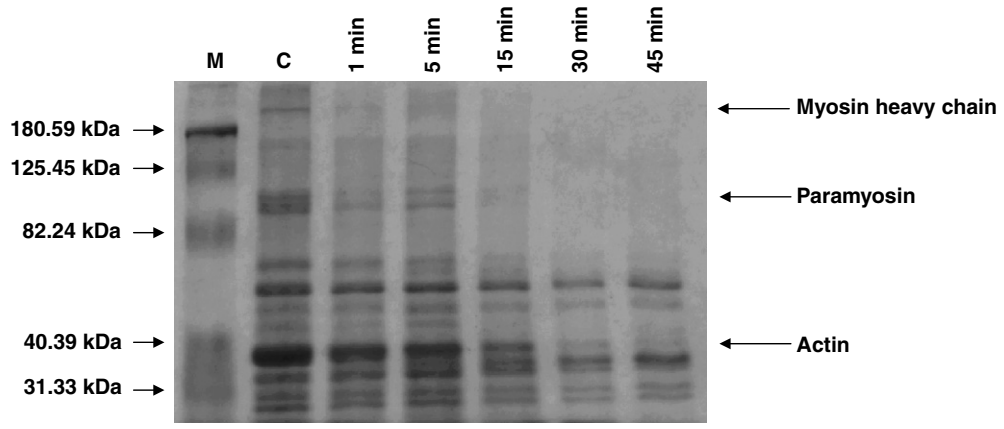

Fig. 2. Protein profile of mantle extracts from *Dosidicus gigas* treated at different times with 0.75 g L^{-1} of the enzymatic mixture collagenase/dispase at a temperature of 30 °C and a pH of 7.5. M: molecular weight markers; C: enzyme-untreated *Dosidicus* extract.

Table 2
Maximal shear force of the samples of *Dosidicus gigas* subjected to different treatments

Treatment of *Dosidicus gigas*	*Illex argentinus* control	*Dosidicus gigas* control	Collagenase H	Collagenase/dispase	Collagenase/dispase	Papain	Papain	Pronase	Pronase	Subtilisin	Trypsin	Trypsin	Trypsin
Incubation time (min)			60	60	60	60	60	60	60	60	60	30	30
Enzyme concentration (μg/μl)			15.2	0.15	0.75	0.1	0.01	0.075	0.757	0.076	1.5	1.5	6.0
Maximal shear force (N)	25.5 ± 3.4	43.9 ± 8.4	$28.3 \pm 3.4^{*}$	45.3 ± 10.1	46.2 ± 12.8	45.3 ± 12.1	44.7 ± 7.3	40.7 ± 3.6	42.3 ± 6.5	$22.1 \pm 3.7^{**}$	$17.7 \pm 9.7^{**}$	36.5 ± 9.5	$28.1 \pm 3.6^{*}$

$^{*}P < 0.01$ vs. *Dosidicus* control; $^{**}P < 0.0001$ vs. *Dosidicus* control; $n = 25$.

nase F, all the proteases assayed caused a significant degradation of the electrophoretic profile of the protein extracts. Fig. 2 shows the effect of incubation at different times with a concentration of 0.75 g L^{-1} of the mixture collagenase/dispase, as a cross-section sample of all the proteases assayed. After determination of the optimal operating conditions of the proteases, the rheological analysis was performed.

Table 2 shows the results obtained with the different proteases assayed. It is to be noted that only collagenase H, subtilisin A and trypsin offered positive results.

As observed in Fig. 3, the mantle of *D. gigas* shows a profile with two shear force peaks (Fig. 3b), whereas that of *I. argentinus* shows a single peak with a shear force of 25.5 ± 3.4 N, which is similar to the value of the first peak of the *Dosidicus* profile. This led us to conclude that the value 44.0 ± 8.4 N corresponding to the second peak is the shear force required to cut the tough epithelium.

Finally, the sensory analysis was performed using the three enzymes that tenderised the squid. However, the panelists detected an off-flavor in the samples treated with subtilisin A, which led us to remove this enzyme from the study. The definitive analysis was performed with untreated samples of *I. argentinus* and *D. gigas*, and samples of *Dosidicus* treated with either trypsin or collagenase H (Table 3).

As indicated in Section 2.4, the statistical analysis was performed with Friedman contrast, using the formula:

$$F = \frac{12}{JP(P+1)}\left(R_1^2 + R_2^2 + R_3^2 + R_4^2\right) - 3J(P+1)$$

where J is the number of panelists, $J = 12$; P is the number of samples analysed by each panelist, $P = 4$; R_1, R_2, R_3 and R_4 are the totals of the orderings attributed to the ensemble P of samples for the R panelists, $R_1 = 19$; $R_2 = 42.5$; $R_3 = 20$; $R_4 = 38.5$, therefore, $F = 22.475$.

Since three panelists assigned the same value to three samples, the critical value F' must be calculated

$$F' = \frac{F}{1 - \{E/[JP(P^2-1)]\}}$$

where $E = (n_1^3 - n_1) + (n_2^3 - n_2) + (n_3^3 - n_3)$; n_1, n_2 and n_3 are the number of samples with the same order number in the evaluation of the different panelists, $n_1 = 2$; $n_2 = 2$; $n_3 = 2$; $E = 18$, therefore, $F' = 23.05$.

For a signification level $\alpha = 0.01$; $J = 12$ panelists and $P = 4$ samples, the value obtained by means of approximations χ^2 is 11.34. Because F' is higher, the conclusion is that there are significant differences in toughness between the samples.

In order to identify the pairs of samples that differ significantly, the Fisher test (Fisher, 1998) was used. If i and j are two samples and R_i and R_j their sums of orderings, using a normal approximation, the two samples will be different if

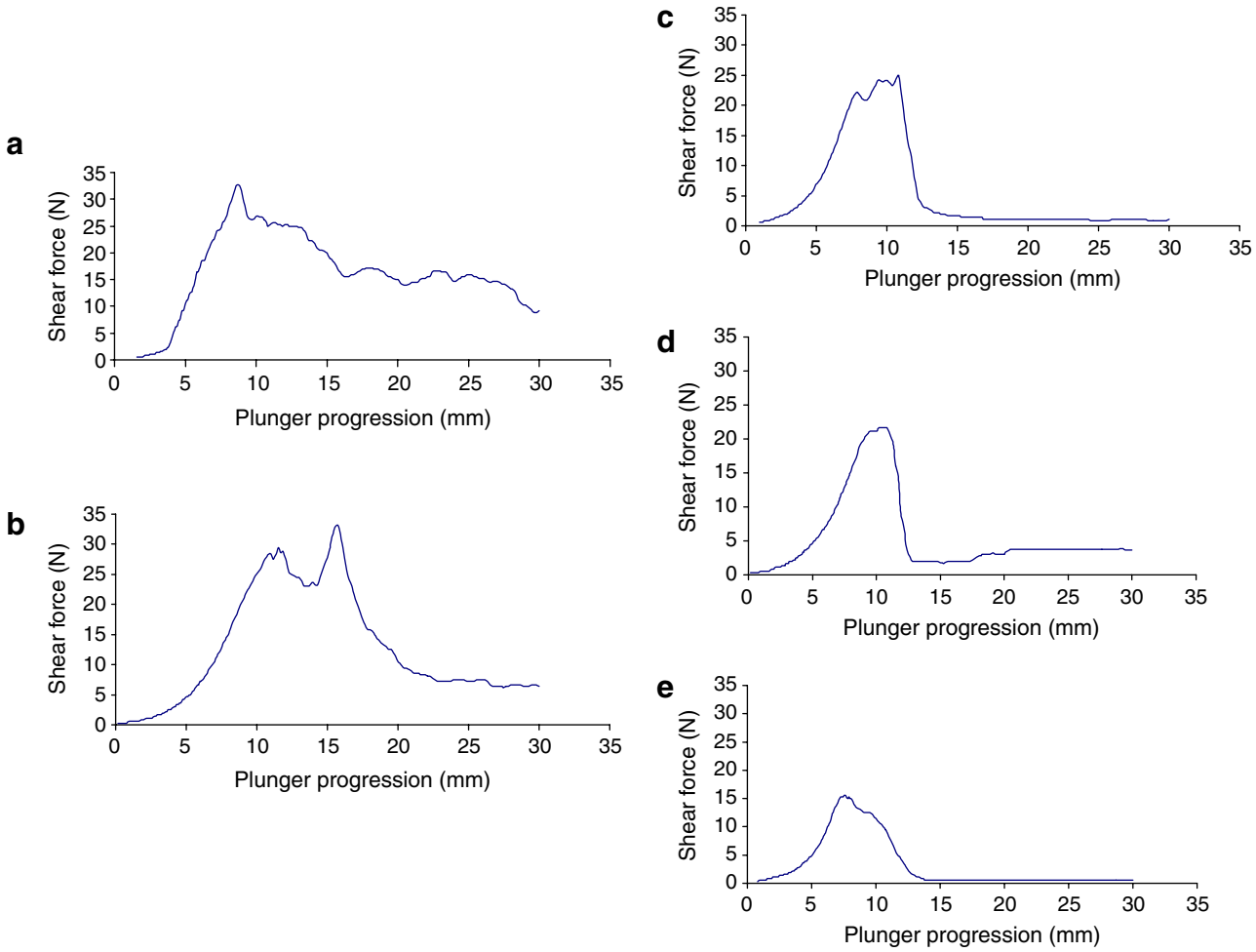

Fig. 3. Shear profiles of mantle of *Illex argentinus* (a) and enzyme-untreated mantle of *Dosidicus gigas* (b) and after treatment with collagenase H (c), subtilisin (d) or trypsin (e). The results presented correspond to the means of 25 samples.

Table 3
Decodification and calculation of the sums of ordinations of the sensory analysis

Panelist	Samples				Sum of ordinations
	Illex (control)	*Dosidicus* (control)	Trypsin (*Dosidicus*)	Collagenase H (*Dosidicus*)	
1	4	2.5	1	2.5	10
2	2	3	1	4	10
3	1	3	2	4	10
4	2	4	1	3	10
5	1	3	2	4	10
6	1	4	2.5	2.5	10
7	1	4	2	3	10
8	1	4	2	3	10
9	1	4	2	3	10
10	1	4	2.5	2.5	10
11	2	3	1	4	10
12	2	4	1	3	10
Sum of ordinations per sample (R_i)	19	42.5	20	38.5	120

$|R_i - R_j| \geqslant 16.29$; being $16.29 = 2.576\sqrt{\{JP(P+1)\}/6}$. For a signification level $\alpha = 0.01$; being $2.576 = t_{0.005,\infty}$.

In view of Table 4, the samples treated with trypsin have a degree of toughness similar to those of *I. argentinus* because $|R_i - R_j| < 16.29$.

4. Discussion

The modification of raw materials for obtaining quality food has been one of the earliest fields in human research. This led from the early agricultural techniques to genetic

Table 4
Differences between pairs of samples according to the Fisher test

Pairs of samples	$\lvert R_i - R_j \rvert$	$2.576\sqrt{\{JP(P+1)\}/6}$ for $\alpha = 0.01$ and $2.576 = t_{0.005,\infty}$	Conclusion
Illex–Dosidicus	$\lvert 19 - 42.5 \rvert = 23.5$	16.29	Different
Illex–Collagenase H	$\lvert 19 - 38.5 \rvert = 19.5$	16.29	Different
Illex–Trypsin	$\lvert 19 - 20 \rvert = 1$	16.29	Equal
Dosidicus–Collagenase H	$\lvert 42.5 - 38.5 \rvert = 4$	16.29	Equal
Dosidicus–Trypsin	$\lvert 42.5 - 20 \rvert = 22.5$	16.29	Different
Collagenase H–Trypsin	$\lvert 20 - 38.5 \rvert = 18.5$	16.29	Different

selection, which allowed, in many cases, the maintenance and improvement in the production of foods demanded by society.

The use of proteases in the food industry includes a great variety of processes, and referring to seafood processing, we could list the obtaining of hydrolysed fish protein, protein recovery from filleting waste or from crustacean waste or prawn peeling.

In general myosin is the main protein hydrolysed during storage and is responsible of the loss of quality and functionality of giant squid mantle muscle (Dublán-García, Cruz-Camarillo, Guerrero-Legarreta, & Ponce-Alquicira, 2006; Ramírez Olivas et al., 2004). In exchange, the actin is the component of the mantle muscle least susceptible to breakdown, although increasing the duration of frozen storage produced more extensive degradation (Stanley & Hultin, 1984).

In view of Fig. 2, the first protein to be degraded is that with a molecular weight above 180 kDa, which may correspond to myosin heavy chain, whereas one of the latest is actin.

Databases offer abundant information on different techniques for the modification of certain characteristics of food. For instance, there are specific enzymatic products for squid skinning in the market but they remove only the external pigmented epithelium, not affecting the inner one.

In 1985 Raa, Hjelmeland, and Gilbert eliminated the skin of diverse squids (*Loligo*, *Illex*, etc.) with a pre-treatment using a saline solution, at 5% and 45 °C, for 10 min to soften the proteoglycan layer, leading to the activation of endogenous enzymes that provoke skin tenderisation.

Borresen (1992) used an enzymatic preparation from papaya at 3 °C and 5% of salt. Melendo et al. (1997) used bromelain and a lysosomic raw extract from bovine spleen, at pH 7 and 37 °C, for 30 min.

However, these treatments are effective only in *Loligo* and *Illex* but they do not affect *Dosidicus* epithelium.

In this case, the aim is to degrade the white, gummy epithelium covering the *Dosidicus* mantle, without tenderising the mantle. The main component of the connective tissue membranes in squid mantle is collagen (Sikorski et al., 1984). For this reason, we thought that collagenases would not damage the mantle because of their high specificity. However, our results show that they are ineffective at the concentrations used, as only collagenase H provoked significant tenderisation, but the panelists did not notice it. On the other hand, the use of a higher collagenase concentration would make the commercial transformation of the cephalopod economically unviable.

As for the rest of the proteases used, only subtilisin and trypsin offered positive results in *Dosidicus* tenderisation, but subtilysin A causes the appearance of a noticeable and unpleasant spicy flavor that makes it unacceptable for the market.

Therefore, out of the enzymes assayed, only trypsin provided adequate results for an industrial application.

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