Biocatalysis and Agricultural Biotechnology 24 (2020) 101509

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: http://www.elsevier.com/locate/bab

Separation and partial purification of collagenolytic protease from peacock bass (*Cichla ocellaris*) using different protocol: Precipitation and partitioning approaches

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ARTICLE INFO

Keywords:
ATPS
Partial purification
Partitioning
Separation
Waste

ABSTRACT

This comparative study provides a useful protocol for the separation and partial purification of collagenolytic proteases obtained from peacock bass, by using precipitation and partitioning. In the precipitation process, 30% acetone (*PF* = 1.76; *Y* = 198%) and ethanol (*PF* = 2.56; *Y* = 272%) were used, while 30–60% fraction of $(NH_4)_2SO_4$ was the most efficient in the separation process (*Y* = 201%; *PF*: 4.28).For the use of the three-phase partitioning (TPP), the sample was submitted to different proportions of *t*-butanol (1.0:0.5, 1.0:1.0, 1.0:1.5 and 1.0:2.0; v/v) and fixed proportion of $(NH_4)_2SO_4$ (30%). The assay with the best *Y* (232%) of the collagenolytic protease in the interphase was in the ratio of 1.0:0.5, which also resulted in higher *PF* (5.07) of this same phase. The formation of the two-phase aqueous partitioning (ATPS) was obtained by mixing polyethylene glycol(1500, 4000 and 8000), according to a 2^4-factorial design. The highest values of *PF* (8.24) and *Y* (116%) were obtained at pH 8.0, 12.5% (w/w) PEG 8000, and 10.0% (w/w) phosphate. PEG-collagenolytic was physicochemically characterized and exhibited optimal state at a temperature of 55 °C and pH 7.5, which was activated by Ca^{2+} and inhibited by Zn^{2+}; this activity was reduced when exposed to PMSF and TLCK. The PEG-collagenolytic showed three bands of protein, with molecular weights ranging from 10.0 to 60.3 kDa. The protease hydrolyzed native and fish skin collagen. Therefore, separation of the target molecule by precipitation and partitioningmay be a viable alternative. Furthermore, ATPS presented physicochemical characteristics compatible with the industrial interest.

1. Introduction

It is interesting for the fishing industry to use simple, fast, and low cost techniques for the separation and partial purification of protein biomolecules obtained from beneficiation residues of fish by-products (Khawli et al., 2019), such as precipitation, using organic solvents (acetone, ethanol, for example) or saline solution (ammonium sulfate $(NH_4)_2SO_4$, for example), or even successive stages of centrifugation and filtration, aiming at improving the degree of purity of the biomolecule (Crowell et al., 2013; Novák and Havlíček, 2016; Oliveira et al., 2019a;

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https://doi.org/10.1016/j.bcab.2020.101509
Received 13 July 2019; Received in revised form 8 December 2019; Accepted 21 January 2020
Available online 24 January 2020
1878-8181/

Table 1
Experimental design 2^4 for collagenolytic protease from peacock bass (*Cichla ocellaris*) partition using PEG/phosphate.

Variables	Levels		
	Low (−1)	Central (0)	High (+1)
[a]$M_{PEG\ (g/mol)}$	1500	4000	8000
[b]$C_{PEG(\%\ w/w)}$	12.5	15	17.5
pH	6.0	7.0	8.0
[c]$C_{PHO(\%\ w/w)}$	10	12.5	15

[a] PEG molar mass.
[b] PEG concentration.
[c] Phosphate concentration.

Table 2
Separation of collagenolytic proteases from peacock bass (*Cichla ocellaris*) with ammonium sulfate, ethanol, and acetone fractionation.

Fraction	*Total protein (mg)*	CA (U/mg)	Y (%)	PF
Crude extract	1.06	106.86	100	1
Acetone precipitation 30%	1.19	187.34	198.05	1.76
Acetone precipitation 60%	1.29	39.63	45.12	0.42
Acetone precipitation 90%	0.90	33.36	26.77	0.31
Ethanol precipitation 30%	0.84	367.79	272.56	2.56
Ethanol precipitation 60%	0.96	85.86	73.00	0.8
Ethanol precipitation 90%	0.74	59.73	39.30	0.56
Ammonium sulfate fractionation 0–30%	0.84	422.25	157.12	3.95
Ammonium sulfate fractionation 30–60%	0.83	457.59	201.85	4.28
Ammonium sulfate fractionation 60–90%	0.46	263.48	116.22	2.46

CA = Collagenolytic activity.
Y= Yield.
PF = purification factor.

Simas et al., 2019; Soto-sierra et al., 2018; Vidal et al., 2010; Yoshikawa et al., 2012).

In addition to the more traditional methods, the fishing industry has been encouraging the use of new techniques for obtaining protein molecules. Another feasible alternative for the recovery of biomolecules of alkaline proteases, derived from fish viscera (Ketnawa et al., 2014a) of Pacific white shrimp (*Litopenaeus vannamei*) hepatopancreas (Kuepethkaew et al., 2017), and biomolecules extracted from microalgae *Nannochloropsis* sp. (Qiu et al., 2019), is the use of three-phase partitioning (TPP), a non-chromatographic tool and emerging for macromolecule recovery (Gagaoua, 2018), in which different proportions of solvents (usually, *t*-butanol,1-butanol, 1-propanol and/or 2-propanol), and fixed concentrations of salt (usually, $(NH_4)_2SO_4$, dipotassium phosphate - K_2HPO_4, sodium citrate - $Na_3C_6H_5O_7$) are used, forming three distinct phases: top phase, interphase, and bottom phase (Ketnawa et al., 2014a). This technique is considered to be economical and efficient, since it optimizes a very fast large scale production of the protein, often potentiating the proteolytic activity, it has been increasingly applied in the recovery and purification of macromolecules, as a single step or combined with other types of methods (Gagaoua and Hafid, 2016; Gagaoua, 2018; Yan et al., 2017).

Another bioseparation technique, aqueous two-phase systems (ATPS) is used as a non-chromatographic method for extraction, recovery and purification of target molecules, especially enzymes such as proteases. Its efficiency is related to the study and analysis of its components in order to obtain a high purification factor (PF), yield (Y) and specific activity of the biomolecule of interest (Nadar et al., 2018; Wanderley et al., 2017). ATPS may be composed of a variety of aqueous solution components, among which two immiscible polymers (often polyethylene glycol-PEG and dextran), or a mixture of a polymer and a salt (phosphate, sulfate or citrate) (Baskaran et al., 2018; Shad et al., 2018; Silva et al., 2018; Vobecká et al., 2018) is present. Partitioning of biomolecules is influenced by weight, size and concentration of the polymer, concentration and type of the salt, temperature, pH (Baskaran et al., 2018; Poonsin et al., 2017; Shad et al., 2018), size, hydrophobic interactions, and conformation of the target biomolecule; with the last parameter being considered as the key factor (Grilo et al., 2016). In addition, ATPS extraction has some peculiar advantages, such as rapidity, high selectivity, optimization and high recovery rate (Shad et al., 2018; Vobecká et al., 2018), low cost (Wu et al., 2017), simplicity, effectiveness in the separation of biomolecules from contaminants (Phong et al., 2018) and eco-friendliness because of component recycling (Iqbal et al., 2016; Soares et al., 2015).

As seen so far, there are various separation and/or purification techniques for obtaining biomolecules from aquatic sources (Ketnawa et al., 2014a; Kuepethkaew et al., 2017). This feedstock is required by the global enzyme market for providing proteases of industrial interest such as trypsin, chymotrypsin and collagenase (Oliveira et al., 2019a). Fish by-products residues have high enzyme content (Khawli et al., 2019), especially collagenolytic proteases (Oliveira et al., 2019a), that are highly required in therapeutic procedures (Abood et al., 2018; Villegas et al., 2018), cultures of animal tissues, meat tenderization (Bhagwat and Dandge, 2018), collagen extraction (Ahmed et al., 2018), production of peptide fragments (Oliveira et al., 2019a; Wu et al., 2018), wool finishing in textile industry (Singh et al., 2016), and leather processing, with reduction in the amount of chemicals (Bhagwat and Dandge, 2018; Kanth et al., 2008).This work aimed to extract collagenolytic proteases from peacock bass (*Cichla ocellaris*), using precipitation and partitioning techniques, and biochemically characterizing separation with the highest purification factor.

2. Materials and methods

2.1. Obtaining the raw material

Intestinal viscera of peacock bass (*Cichla ocellaris*) (Brazilian Genetic Heritage n°A25441A) were obtained from fishermen of the Municipality of Petrolândia (8°59′25.2S 38°14′27.4W), Pernambuco, Brazil. The material was refrigerated and taken to the Laboratório de Tecnologia de Produtos Bioativos of the Federal Rural University of Pernambuco, Recife, Brazil, for processing and production of the crude extract.

2.2. Preparation of crude extract and total protein

The intestinal crude extract of *C. ocellaris* used in the enzyme extraction was obtained using a1:5 (w/v) ratio of tissue to extraction buffer (0.05 M Tris-HCl pH 7.5, containing 5 mM $CaC1_2$) (Sigma, St. Louis, MO, USA), according to methodology described by Oliveira et al. (2019a,b). After maceration, the suspension was homogenized for 5 min at 12 rpm and 4 °C, at time intervals of 3 min, using a homogenizer, model RW 20 S32 (IKA, Guangzhou, China). The homogenate was centrifuged for 30 min at 12,000 x *g* and 4 °C with a Sorvall Superspeed Centrifuge RC-6 (Thermo Fisher Scientific, Waltham, MA, USA). The extract produced was warmed (45 °C, 30min.), and then centrifuged again (10,000 x *g*, 8 min, 4 °C)(Oliveira et al., 2017a,b). The final material was defined as final crude extract and used for separation in the separation techniques. The protein content either of the crude extract or the purified enzyme was determined by the bicinchoninic acid (BCA) method (Smith et al., 1985).

2.3. Proteolytic and collagenolytic activity

The non-specific proteolytic activity was determined using the ELISA reader and microplate. To this end, 50 μl 1% (w/v) azocasein (Sigma, St. Louis, MO, USA) was incubated with 30 μl of the enzyme crude extract for a period of 60 min at 25 °C and protected from illumination. After incubation, 240 μl of 10% (w/v) trichloroacetic acid (TCA) was added,

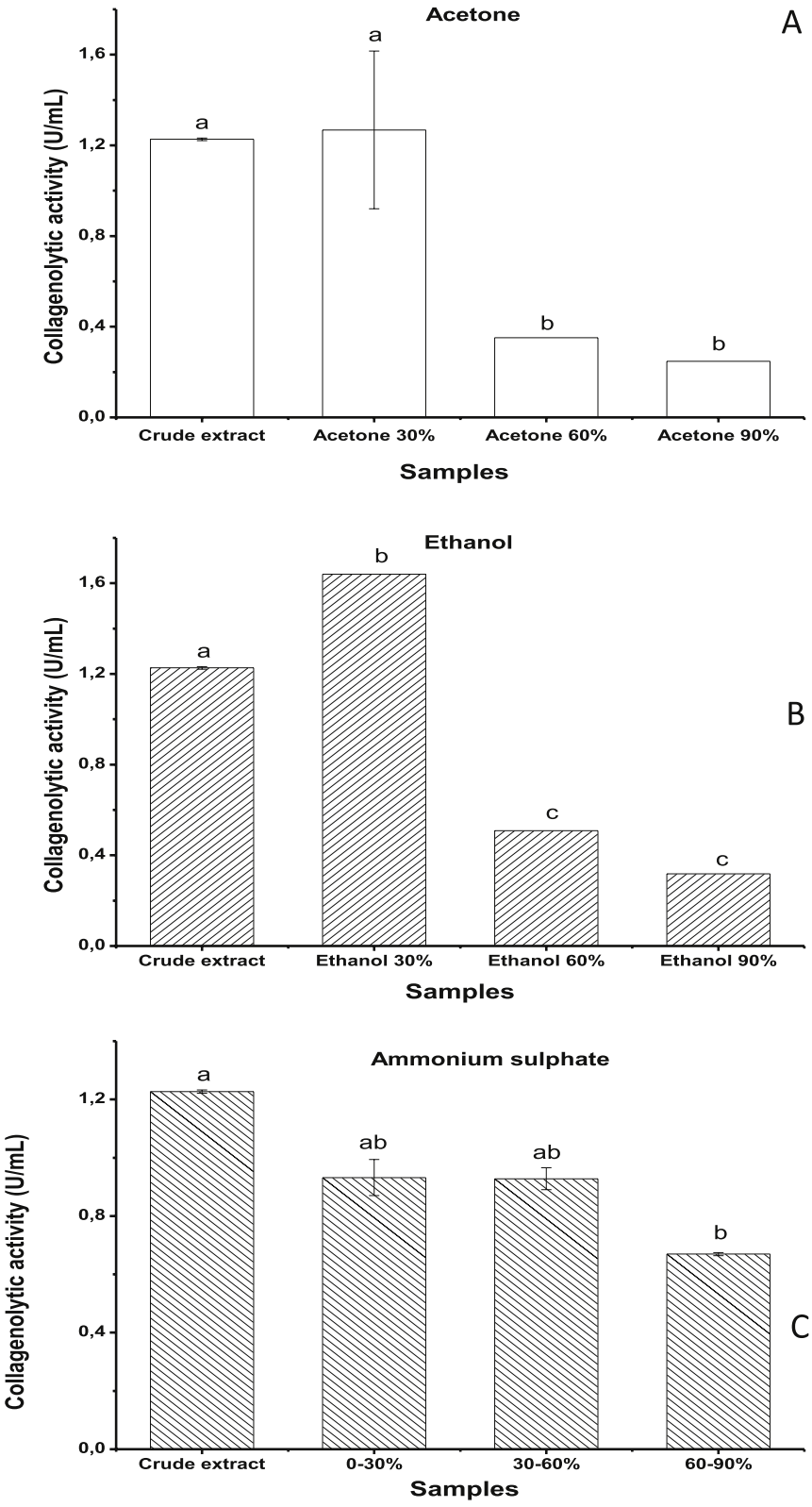
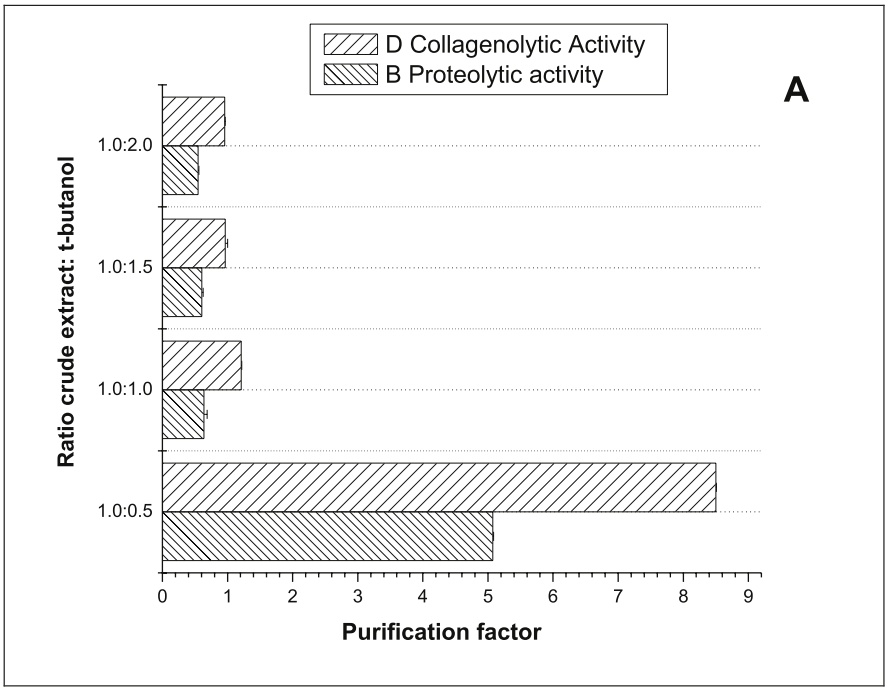


Fig. 1. Comparison between the average activities inside precipitation methods: (A) Acetone; (B) Ethanol; (C) Ammonium sulfate. Data are expressed as mean ± SD. Means with different letters differ significantly ($p < 0.05$) by one-way ANOVA followed by Tukey test and Bonferroni test. Assays were performed in triplicate. Data populations were tested for normality by the Shapiro-Wilk, Lilliefors and Kolmogorov-Smirnov tests and homogeneity of variances was verified by the Levene, Levene^2 and Brown-Forsythe tests. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

centrifuging after 15 min at 8000 x g for 5 min. At the end, the supernatant (70 μl) was added to 1 M NaOH (130 μl). The reading was observed at 450 nm (Alencar et al., 2003). The collagenolytic activity was determined using Azo dye-impregnated collagen (azocoll, Sigma, St. Louis, MO, USA) as the substrate after 5 successive cycles of washing with the extraction buffer and centrifugation (10,000 x g, 3 min, 4 °C) (Oliveira et al., 2017a,b).

2.4. Separation of collagenolytic protease using precipitation techniques

The enzymatic crude extract was subjected to precipitation in organic solvents (acetone and ethanol) at the solvent proportions of 30,



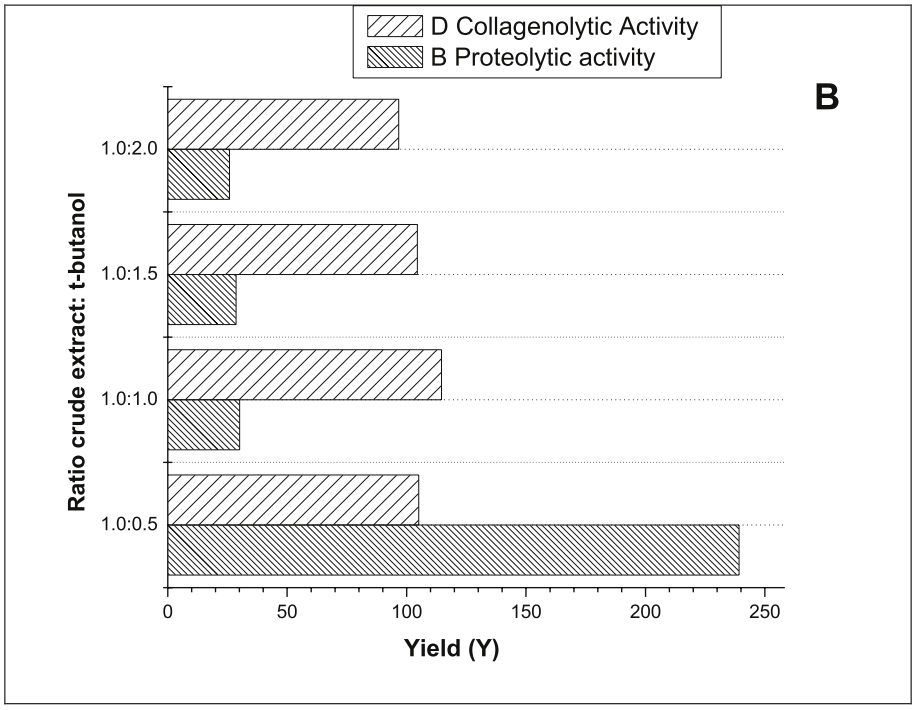


Fig. 2. Efficiency of partial purification of collagenolytic proteases from peacock bass (*Cichla ocellaris*) using TPP in the crude extract to *t*-butanol ratio at 1.0:0.5 (v/v).

60 and 90% of the total solution. Initially, the solvent mixture and extract were refrigerated at 4 °C for a period of 10 min, and then centrifuged at 8000 x g for 5 min. After that, the precipitated fraction was resuspended in the same enzymatic extraction buffer in the 2 ml volume. For the saline precipitation, $(NH_4)_2SO_4$ was slowly added to the enzyme solution at concentrations of 0–30, 30–60 and 60–90% saturation and maintained at 4 °C for a period of 4 h. The solution was centrifuged at 10,000 rpm at 4 °C for 30 min. The pellets were resuspended in a minimum amount of 2 ml of Tris-HCl buffer pH 7.5. The solution was then dialyzed. After the precipitation steps in solvents and saline, were determined total proteins, nonspecific proteolytic and collagenolytic activity of all fractions (Geethanjali and Subash, 2013).

2.5. *Two-phase partitioning (ATPS)*

2.5.1. *Experimental design and statistical analyses*

The biphasic system was prepared by mixing PEG and Na or K phosphate salts in different proportions according to a 2^4-factorial

Table 3
Matrix of the full factorial design (2^4) with conditions and results of the collagenolytic protease partition.

Run	M_{PEG}[a] (g/mol)	C_{PEG}[b] (%)	pH	C_{PHO}[c] (%)	K[d]	Y[e] (%)	P[f]
1[g]	1500	12.5	6.0	10	0	0	0
2	8000	12.5	6.0	10	0.69	40	3.47
3	1500	17.5	6.0	10	0.44	37	3.99
4	8000	17.5	6.0	10	1.53	142	2.20
5[g]	1500	12.5	8.0	10	0	0	0
6	8000	12.5	8.0	10	3.57	119	8.24
7	1500	17.5	8.0	10	0.80	103	3.43
8	8000	17.5	8.0	10	2.29	123	2.43
9	1500	12.5	6.0	15	1.64	121	2.97
10	8000	12.5	6.0	15	5.36	121	1.36
11	1500	17.5	6.0	15	2.59	100	1.50
12	8000	17.5	6.0	15	5.76	150	1.78
13	1500	12.5	8.0	15	1.80	122	1.48
14	8000	12.5	8.0	15	7.19	153	2.79
15	1500	17.5	8.0	15	7.12	169	1.91
16	8000	17.5	8.0	15	0.76	76	0.54
17	4000	15	7.0	12.5	0.76	124	3.67
18	4000	15	7.0	12.5	1.04	116	3.06
19	4000	15	7.0	12.5	1.46	117	3.15

[a] PEG molar mass.
[b] PEG concentration.
[c] Phosphate concentration.
[d] Partition coefficient.
[e] Yield.
[f] Purification factor.
[g] No phase formation after addition of extract.

design (Table 1) in which PEG molar mass (M_{PEG}, x_1), PEG concentration (C_{PEG}, x_2), phosphate salt concentration (C_{PHOS}, x_3) and pH (x_4) were selected as the independent variables, while purification factor (*PF*), partition coefficient (*K*) and activity yield (*Y*) as the responses. The tests were performed in graduated tubes with 15 mL conical tips. The system was loaded with 2 g of enzymatic crude extract, vortexed for 1 min and allowed to stand for 60 min at room temperature, for phase separation. Phase volumes were measured, and aliquots of each phase were taken separately and used for the determination of collagenolytic activity and protein concentration.

The experimental design was composed of 19 runs and 3 repetitions of the central point, which were required to calculate the pure error (Table 1). A linear regression model was employed to predict each response (*R*) according to the equation:where *R* is the predicted response, $b_0$is the interception coefficient and constant term, b_i and b_{ij} are the linear and interaction coefficients and x_i and x_{ij} are the coded values of the independent variables (M_{PEG}, x_1, C_{PEG}, x_2, pH, x_3 and C_{PHOS}, x_4). The relative significance of the main and interaction coefficients was assessed from the error estimate based on the third-order term at 95% confidence level. All statistical and graphical analyses were carried out using the Statistica 8.0 software (StatSoft Inc., Tulsa, OK, USA).

2.5.2. Partition and purification parameters

The distribution of collagenolytic activity between the two phases was expressed in terms of partition coefficient (*K*), defined as:

$$K = \frac{A_t}{A_b} \quad (2)$$

where A_t and A_b are the collagenolytic activities (U/mL) at the end of partition in the upper and lower phases, respectively.

The purification factor (*PF*) was calculated as follow:

$$PF = \frac{At/Ct}{Ai/Ci} \quad (3)$$

Where A_i is the initial collagenolytic activity in the crude extract and A_t is the collagenolytic activity in the upper phase (both in U/mL), while C_t and C_i are protein concentrations in the upper phase (mg/mL) and the crude extract, respectively.

The yield (Y %) was determined as the ratio of protease activity (A_t) (U/mL) in the top phase and in the crude extract (A_i) (U/mL) expressed as percentage (eq. (4)):

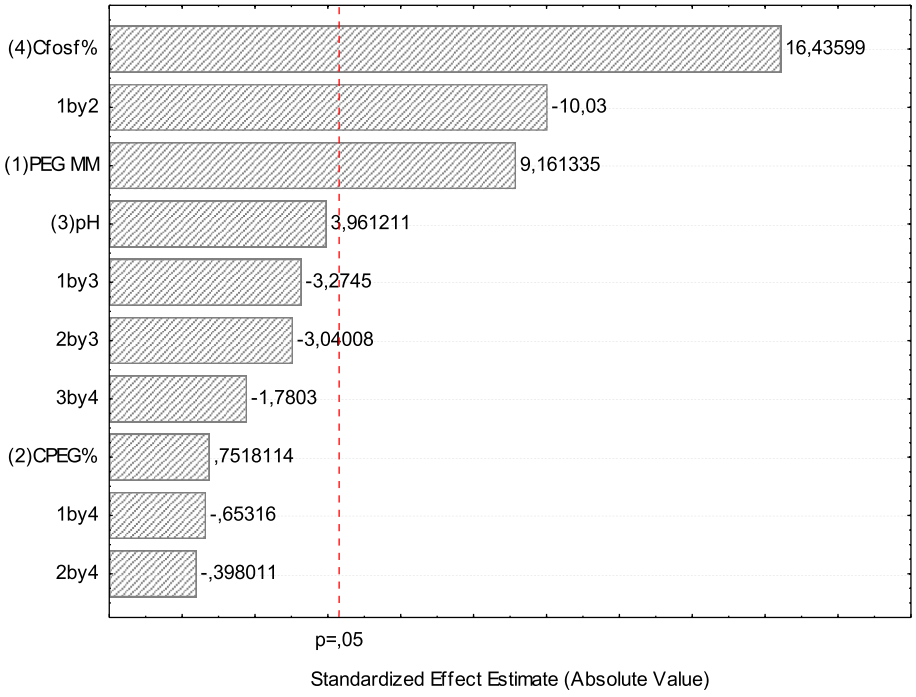


Fig. 3. Pareto chart of standardized effects of the factors. (1) PEG_{MM}: polyethylene glycol molar mass; (2) C_{PEG} %: polyethylene glycol concentration; (3) pH: pH factor; (4) C_{PHOS}%: phosphate concentration on the variable in 2^4-full factorial design1 by 2, 3, 4; 3 by 4; 2 by 3, 4 are the interaction effects between the factors. The validity of the model was verified by analysis of variance (ANOVA).

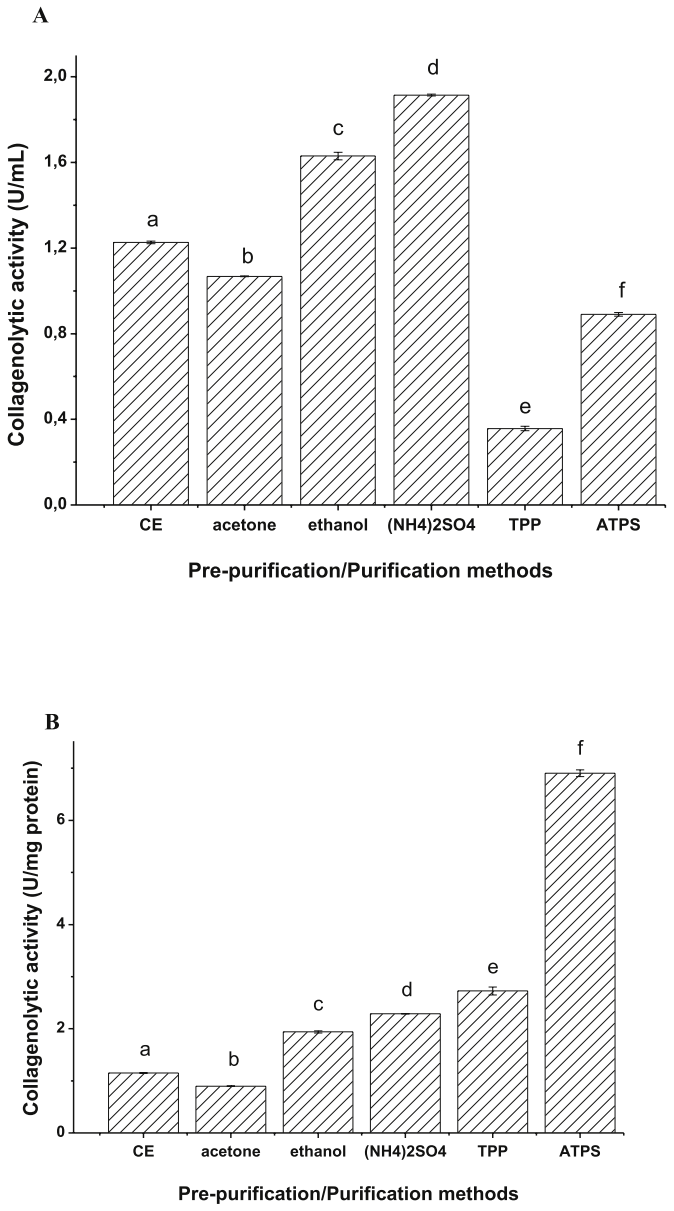


Fig. 4. Comparison between the average activities of the separation methods: (A) by total activity; (B) by specific activity. Data are expressed as mean ± SD. Means with different letters differ significantly ($p < 0.05$) by one-way ANOVA followed by Tukey test and Bonferroni test. Data populations were tested for normality by the Shapiro-Wilk, Lilliefors and Kolmogorov-Smirnov tests and the homogeneity of variances was verified by the Levene, Levene^2 and Brown-Forsythe tests. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

$$Y = \left(\frac{A_f}{A_i}\right) \cdot 100 \quad (4)$$

2.6. Three-phase partitioning (TPP)

2.6.1. Effect of the crude extract to t-butanol ratio on proteases partitioning

Separation by TPP was performed using t-butanol as solvent. *T*-butanol was added to crude enzyme extract in different ratios (1.0:0.5, 1.0:1.0, 1.0:1.5 and 1.0:2.0; v/v). The addition of $(NH_4)_2SO_4$ was constant at 30%. After the 60 min period, the mixture was centrifuged at 4500 x g for 12 min. The phases formed were separated. The lower phase and the interface were dialyzed for 24 h at 4 °C. After this period, the dosage of total proteins, nonspecific proteolytic and collagenolytic was determined (Rawdkuen et al., 2012).The purification factor was calculated according to equation (3) and the measurement with equation (4).

2.7. Physicochemical characterization

2.7.1. Effects of temperature and pH

The effect of temperature on the enzyme activity and stability was evaluated at temperatures ranging from 25 to 90 °C. For optimal temperatures, the assays were carried out by incubating the crude extract in a water bath. These assays were carried out in different pH ranges using the buffers: 0.5 M citrate–phosphate (pH 4.0–7.0), 0.1 M Tris–HCl (pH 7.5–8.5) and 0.1 M glycine-NaOH (pH 9.0–12.0), containing 5 mM $CaC1_2$. The highest enzymatic activity observed for the enzyme in different buffers was defined as 100%. The activity was calculated as the ratio between the enzymatic activity (Oliveira et al., 2019a).

2.7.2. Sensitivity to ions and inhibitors

The effect of metal ions on enzyme activity was investigated by adding the monovalent (K^+ and Na^+), divalent metal ions (Hg^{2+}, Pb^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Ba^{2+}, Mg^{2+} and Ca^{2+}) and trivalent metal ion (Al^{3+}) to the reaction mixture. The final concentration of each metal ion was 1 mM. Each ion was incubated for 30 min at a ratio of 1:1. The activity of the incubated samples was compared with that in absence of the corresponding metal ions. The activity was calculated as the ratio between the enzymatic activity, observed at the end of each incubation run, and that at the beginning, and expressed as percentage (%) (Oliveira et al., 2019a).

The sensitivity of enzymes under study to some inhibitors was tested: phenylmethylsulphonyl fluoride (PMSF), a serine-protease inhibitor; N-p-tosyl-L-lysin chloromethyl ketone (TLCK), a trypsin-specific inhibitor; benzamidine, a trypsin inhibitor; N-tosyl-L-phenylalaninechloromethyl ketone (TPCK), a chymotrypsin-specific inhibitor, all of them diluted in DMSO; ethylenediamine tetra-acetic acid (EDTA), a chelating compound; and β-mercaptoethanol, a reducing agent, diluted in deionized water. The final concentration of each inhibitor was 8 mM. Each ion was incubated for 30 min at a ratio of 1:1. The activity was compared to the reaction with absence of the corresponding inhibitors. The activity was determined as the percentage of the proteolytic activity in an inhibitor-free control sample (Oliveira et al., 2019a).

2.7.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a 12% polyacrylamide gel (Laemmli, 1970). The molecular mass was calibrated using a wide-range RN756E molecular mass marker (225, 76, 52, 38, 31, 24, 17, 12 kDa, GE Healthcare Life Sciences, São Paulo, SP, Brazil). Protein bands were detected by staining with silver.

2.7.4. Type I collagen test (native and skin)

The measure of the digestion of native collagen bovine Achilles tendon Type I (Sigma, St. Louis, MO, USA) and Crosslink-free collagen from *Cichla ocellaris* (Oliveira et al., 2019b).A reaction mixture, which contained 5 mg of collagen type I, 1 mL of 50 mM Tris-HCl (pH 7.5 with 5 mM $CaCl_2$) and 0.1 mL of the enzyme solution, was incubated at 37 °C for 12, 24, 36 and 48 h. The reaction was stopped by adding 0.2 mL of 50% trichloroacetic acid. After 10 min at room temperature, the solution was centrifuged at 1800 (×) g for 20 min. The supernatant (0.2 mL) was mixed with 1.0 mL of a ninhydrin solution, incubated at 100 °C for 20 min, and then cooled to room temperature. Subsequently, the mixture was diluted with 5 mL of 50% 1-propanol for absorption measurement at 570 nm. The concentration of hydrolyzate-amino acids was determined using a standard curve of L-leucine (Oliveira et al., 2019a; Park et al., 2002).

2.7.5. Statistical analysis

Data are expressed as mean ± SD. Means with different letters differ

Table 4
Comparative study of molecule extraction by three-phase (TPP) and biphasic (ATPS) partitioning.

Source	Scientific name	Target molecule	Experimental conditions for extraction and purification of the target molecule	PF[a]	Y[b]	Reference
Three phase partitioning (TPP)						
Fish	*Cichla ocellaris*	Collagenolytic protease	*t*-butanol ratio of 1:0.5 (v/v) in the presence of 30% $(NH_4)_2SO_4$	5.07	239.20	*Present work*
Fish	*Pangasianodon gigas*	Alkaline protease	*t*-butanol ratio of 1:0.5 (w/v) in the presence of 50% sodium citrate	6.0	220.0	Ketnawa et al. (2014a)
Fish	*Pangasianodon gigas*	Alkaline protease	*t*-butanol ratio of 1:0.5 (w/v) in the presence of 50% $(NH_4)_2SO_4$	5.0	163.0	Rawdkuen et al. (2012)
Shrimp	*Litopenaeus vannamei*	Proteases	*t*-butanol ratio of 1:1 (w/v) in the presence of 30% $(NH_4)_2SO_4$	2.6	76.0	Senphan and Benjakul (2014)
Shrimp	*Litopenaeus vannamei*	Lipase	*t*-butanol ratio of 1:1 (w/v) in the presence of 50% $(NH_4)_2SO_4$	3.49	87.41	Kuepethkaew et al. (2017)
Plant	*Momordica charantia*	Invertase	*t*-butanol ratio of 1:1 (w/v) in the presence of 70% $(NH_4)_2SO_4$	10.48	20.28	Belligün and Demir (2019)
Plant	*Calotropis procera*	Proteases	*t*-butanol ratio of 1:1.05 (w/v) in the presence of 50% $(NH_4)_2SO_4$	6.92	182.0	Rawdkuen et al. (2010)
Plant	*Calotropis procera*	Proteases	*t*-butanol ratio of 1:1 (w/v) in the presence of 50% $(NH_4)_2SO_4$	14.91	215.0	Gagaoua et al. (2015)
Plant	*Amsonia orientalis*	Peroxidase	*t*-butanol ratio of 1:1 (w/v) in the presence of 20% $(NH_4)_2SO_4$	12.5	162.0	Karakus et al. (2018)
Bacteria	*Bacillus natto*	Nattokinase	*t*-butanol ratio of 1:1.5 (w/v) in the presence of 30% $(NH_4)_2SO_4$	5.6	199.5	Garg and Thorat (2014)
Fungi	*Ganoderma sp.*	Laccase	*t*-butanol ratio of 1:1.5 (w/v) in the presence of 20 and 90% $(NH_4)_2SO_4$	13.2	60.0	Rajeeva and Lele (2011)
Fungi	*Rhizopus arrhizus*	Lipase	*t*-butanol ratio of 1:0.5 (w/v) in the presence of 30% $(NH_4)_2SO_4$	19.1	71.0	Dobreva et al. (2019)
Fungi	*Aspergillus brasiliensis*	Naringinase	*t*-butanol ratio of 1:1 (w/v) in the presence of 30% $(NH_4)_2SO_4$	4.2	21.44	Shanmugaprakash et al. (2015)
Partitioning in aqueous two-phase system (ATPS)						
Fish	*Cichla ocellaris*	Collagenolytic protease	pH 8.0, 12.5% (w/w) PEG 8000, and 10.0% (w/w) phosphate	8.24	116.0	*Present work*
Shrimp	*Litopenaeus vannamei*	Proteases	pH 8.0, 15.0% (w/w) PEG 1000, and 250% (w/w) $MgSO_4$	8.6	65.5	Senphan and Benjakul (2014)
Shrimp	*Litopenaeus vannamei*	Lipase	pH 5.0, 25.0% (w/w) PEG 1000, and 15.0% (w/w) $MgSO_4$	5.19	78.46	Kuepethkaew et al. (2017).
Fungi	*Aspergillus sydowii*	Tannase	pH 6.0, 24.0% (w/w) PEG 6000, and 20.0% (w/w) sodium citrate	3.2	93.6	Albuquerque et al. (2020)
Fungi	*Rhizopus arrhizus*	Lipase	pH 7.0, 30.0% (w/w) PEG 4000, and 21.0% (w/w) $KNaC_4H_4O_6{\cdot}4H_2O$	6.1	217.71	Dobreva et al. (2019)
Fungi	*Penicillium sp.*	Collagenase	pH 7.0, 15.0% (w/w) PEG 3350, and 12.5% (w/w) phosphate	27.61	83.0	Wanderley et al. (2017)
Fungi	*Penicillium aurantiogriseum*	Collagenase	pH 6.0, 17.5% (w/w) PEG 1500, and 15.0% (w/w) phosphate	5.23	48.13	Lima et al. (2013)
Fungi	*Mucor subtilissimus*	Fibrinolytic protease	pH 7.0, 24.0% (w/w) PEG 6000, and 24.0% (w/w) sodium sulfate	4.5	116.0	Nascimento et al. (2016)
Fungi	*Aspergillus tamarii*	Proteases	pH 8.0, 24.0% (w/w) PEG 8000, and 20.0% (w/w) sodium citrate	3.95	55.8	Silva et al. (2017)
Fungi	*Aspergillus aculeatus*	Polygalacturonase	pH 8.0, 20.0% (w/w) PEG 8000, and 20.0% (w/w) sodium citrate	1.8	85.2	Silva et al. (2018)
Fish	*Thunnus alalunga*	Spleen trypsin	pH 7.0,25.0% (w/w) PEG 4000, and 15.0% (w/w) NaH_2PO_4	5.54	71.92	Poonsinet al. (2016)
Fish	*Thunnus alalunga*	Liver proteinase	pH 7.0, 25.0% (w/w) PEG 1000, and 20.0% (w/w) NaH_2PO_4	5.58	89.99	Sripokar et al. (2017)
Fish	*Pangasianodon gigas*	Alkaline protease	pH 8.0, 15.0% (w/w) PEG 2000, and 15.0% (w/w) sodium citrate	3.33	64.18	Ketnawa et al. (2013)
Fish	*Pangasianodon gigas*	Alkaline protease	40.0% EOPO3900–10.0% $MgSO_4$ with 17.0%NaCl	21.50	77.98	Ketnawa et al. (2014b)
Fish	*Thunnus alalunga*	Acid protease	25.0% (w/w) PEG 1000, and 20.0% (w/w) $MgSO_4$	7.2	85.7	Nalinanon et al. (2009)

[a] Purification Factor.
[b] Yield (%).

significantly ($p < 0.05$) by one-way ANOVA followed by Tukey test and Bonferroni test. Data populations were tested for normality by the Shapiro-Wilk, Lilliefors and Kolmogorov-Smirnov tests and the homogeneity of variances was verified by the Levene, Levene ^ 2 and Brown-Forsythe tests.

3. Results and discussion

3.1. Precipitation by solvents and saline

Precipitation alters the solvency potential of the solvent, decreasing the solubility of the solute (Novák and Havlíček, 2016). In the present experiment, the efficiency series on collagenolytic protease concentration, favoring the increase of purification factor, was in the order: $(NH_4)_2SO_4$> ethanol > acetone (Table 2).

The use of acetone as a precipitating agent concentrated the protein more than crude extract, reducing the amount of natural inhibiting agents and debris present in the tissue type (intestinal viscera), and enhancing collagenolytic activity (175.31%) when acetone-30% was added, as shown in Table 2. Acetone-60 and 90% significantly reduced ($p < 0.05$) enzymatic action (62.92 and 68.79%, respectively), as shown in Fig. 1. The use of ethanol-30% accentuated collagenolytic activity by 344.17%, in relation to the activity of the enzymatic crude extract. Ethanol-60 and 90% reduced 19.66 and 44.11%, respectively, collagenolytic protease activity. Increasing the concentration of both solvents, resulted in a drastic reduction in the recovery and purification factor of the collagenolytic material, majorly due to the destabilization of the enzyme in the face of unfavorable interactions between the exposed hydrophilic groups and the organic solvents tested at high concentrations (Crowell et al., 2013; Yoshikawa et al., 2012).

The use of the solvents in question may present some technical advantages like speed; however, it has the disadvantage of not being environmentally friendly. Geethanjali and Anitha Subash (2013), through separation of proteases extracted from *Labeo rohita* viscera, observed increased enzymes in 1.6 and 1.26, using acetone and ethanol, respectively. Vidal et al. (2010) have been able to reduce the amount of

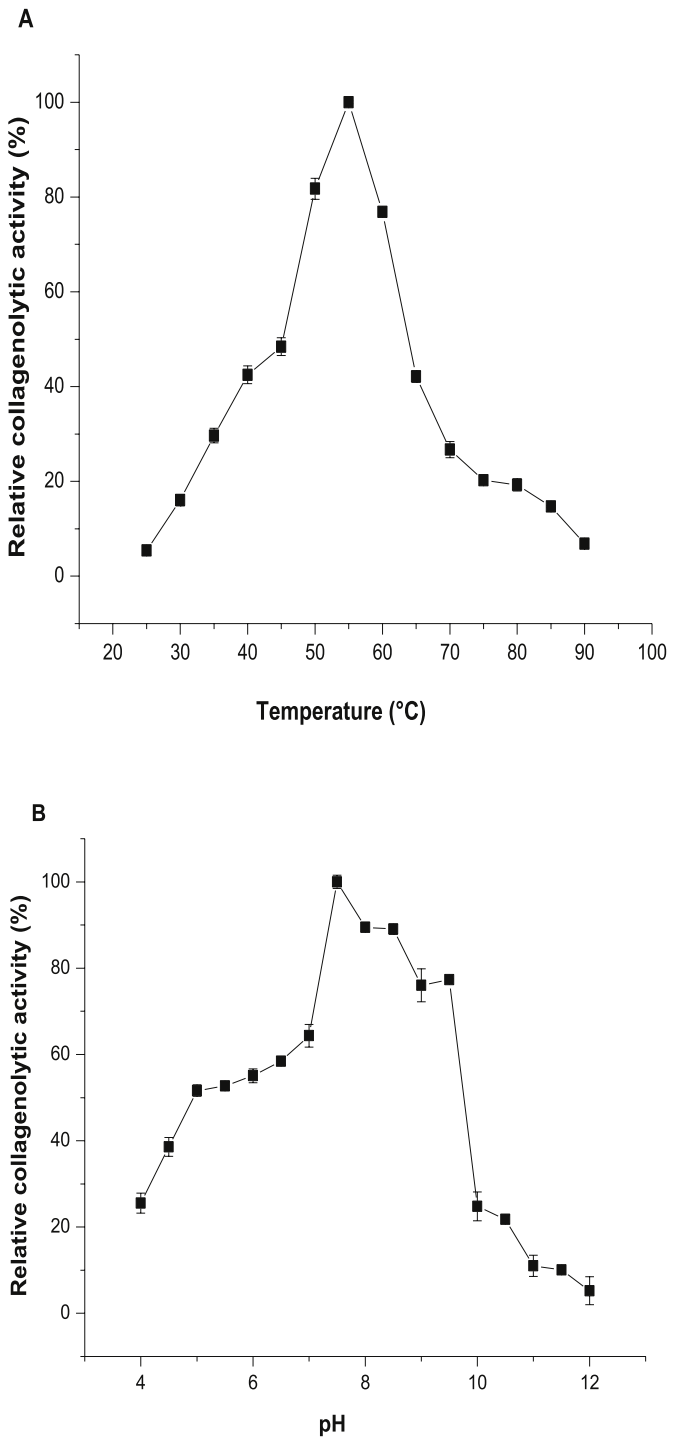


Fig. 5. Effects of temperature (A) and pH (B) on the relative activities of PEG-collagenolytic protease from peacock bass with 12.5% PEG 8000, 17.5% phosphate concentration and pH 8.0. The relative activities are expressed as percentages of the maximum obtained (A) in 0.05 M Tris–HCl buffer (pH 7.5) and (B) at 55 °C, respectively. The assays were performed in triplicate and data are expressed as mean ± SD.

contaminants by using acetone and ethanol by fractionating biomolecule extracted from brown algae *Spatoglossum schroederi*, corroborating the use of this type of solvent in the separation and partial purification of biomolecules from aquatic sources.

Collagenolytic protease activity was enhanced by precipitation with $(NH_4)_2SO_4$, with an increase of 395.14 (fraction: 0–30%), 428.21 (fraction: 30–60%) and 246.56% (fraction: 60–90%). The precipitation

Table 5
Effect of ions and inhibitors on the activity of PEG-collagenolytic protease from peacock bass (*Cichla ocellaris*).

Ions and Inhibitors	PEG-Collagenolytic activity (%)
Metal ions (1 mM)	
Control	100.0[a]
Cd^{2+}	54.29[b]
Cu^{2+}	41.98[b]
Zn^{2+}	74.26[b]
Al^{3+}	37.067[b]
Hg^{2+}	53.40[b]
Pb^{2+}	55.08[b]
Ca^{2+}	102.86[a]
Mg^{2+}	95.45[a]
Inhibitors (8 mM)	
Control	100.0[a]
PMSF	32.97[b]
TLCK	32.94[b]
TPCK	78.20[b]
Benzamidine	31.83[b]
EDTA	63.53[b]
β-Mercaptoethanol	40.63[b]

*Mean value ± standart deviation. Values followed by different superscript letters are significantly different at $P < 0.05$.

in the 30–60% fraction resulted in a maximum yield of 201.85%, and a purification factor of 4.28 (Table 2). Proteins have their solubility affected by the presence of ions and this has been advantageously and routinely used for their separation from aquatic sources. The presence of $(NH)_2SO_4$ displaces water from the protein's solvation layer, causing it to lose interaction with water, increasing protein-protein interaction and precipitating (Andevari et al., 2019; Novák and Havlíček, 2016). Similar results were described by Geethanjali and Subash (2013), for proteases purified with $(NH_4)_2SO_4$ (*PF* = 7.0, fraction = 0–20%) from *L. rohita* viscera. This type of protein precipitation partially purifies and maintains enzymatic activity (Novák and Havlíček, 2016), presenting the advantage of being low cost and efficient, viable for use in processes that do not require high degree of purification.

3.2. Three-phase partitioning (TPP)

In this study, TPP was utilized to separate collagenolytic proteases from intestinal viscera of *C. ocellaris*, and the ratio of *t*-butanol and crude enzymatic extract can be visualized in Fig. 2. The proportion with higher collagenolytic activity was 1.0:0.5 interphase. The *t*-butanol is miscible in water, but in saline it becomes immiscible, forming two liquid phases (alcohol floats to the top and saline water to the bottom). Potential protein inhibitors tend to be trapped in the upper phase. A third phase is formed (the intermediate) by the proteins present in the medium (Chew et al., 2018; Liu et al., 2019). Here, the increase in *t*-butanol volume led to the reduction of *Y* and *PF*, this effect was due to the protein denaturation process caused by high *t*-butanol concentrations (Belligün and Demir, 2019; Rawdkuen et al., 2012).

Similar interesting applications of TPP were also successfully used in the fish biotechnology industry. Biomolecules from aquatic organisms were recovered and purified, such as an alkaline protease from giant catfish (*P. gigas*) viscera, using a sodium citrate-*t*-butanol system (Ketnawa et al., 2014a). As far as other marine species are concerned, from the hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*), a two-stage *t*-butanol/$(NH_4)SO_4$ - PEG/$MgSO_4$ system allowed the recovery of a protease mixture (Senphan and Benjakul, 2014), this result is further corroborated by the extraction of an acid protease (Kuepethkaew et al., 2017), using the same extraction method, thus indicating the feasibility of using the technique in the fishing industry. Qiu et al. (2019) extracted biomolecules from the biomass microalgae of *Nannochloropsis* sp. by TPP, in the ratio of 1.0:2.0, with *t*-butanol as the solvent. Thus, the use of TPP by the fishing industry is already an efficient and alternative reality for the separation of proteases from different aquatic sources,

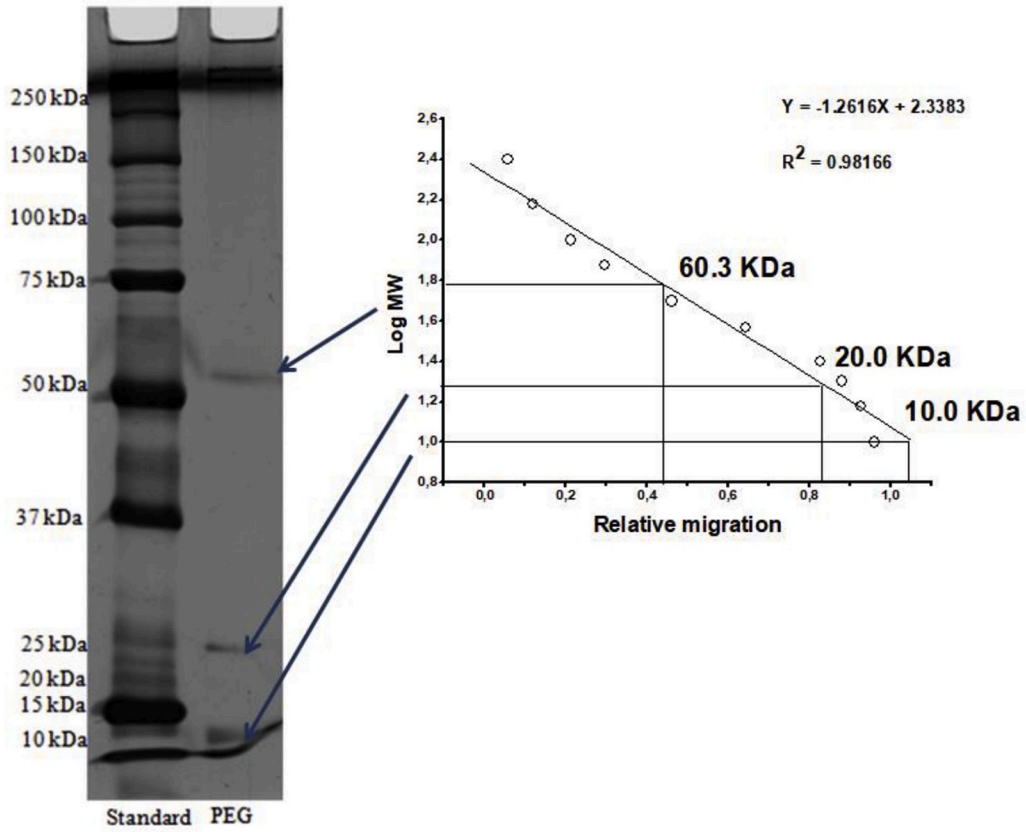


Fig. 6. Molecular mass of PEG-collagenolytic protease from peacock bass (*Cichla ocellaris*) with 12.5% PEG 8000, 17.5% phosphate concentration and pH 8.0.

such as the collagenolytic described in this paper, with the sole aim of targeting the best and most economical proportion of employing solvent in the separation steps.

3.3. Aqueous two-phase system (ATPS)

The results of experimental design for partition coefficient (K)are presented in Table 3. According to the binodal curve obtained with PEG 1500 g/mol (Ferreira et al., 2009), no phase separation was observed for runs 1 and 5, as salt and PEG concentrations were below the curve. The influence of the independent variables, PEG molar mass (MPEG), PEG concentration (CPEG), pH, and phosphate salt concentration (CPHO), on the collagenolytic protease partition coefficient, is described by equation (5) (where*x1, x2, x3*,and *x4* are the coded values for these independent variables):

$$K = 2.36 + \mathbf{0.79}x_1 + 0.06x_2 + 0.35x_3 + \mathbf{1.43}x_4 - \mathbf{0.87} \times 1 \times 2 - 0.28 \times 1 \times 3 - 0.06 \times 1 \times 4 - 0.26 \times 2 \times 3 - 0.03 \times 2 \times 4 - 0.15 \times 3 \times 4 \quad (5)$$

The interactions of the independent variables M_{PEG}, CPHO, and M_{PEG}/C_{PEG} (bold numbers), were statistically significant at 95% confidence level. Despite this, all other terms were maintained in the model to minimize the error. Thus, in conformity with the results presented in Table 3, in most runs, collagenolytic proteases partition occurred preferentially on the top phase rich, which is in PEG.

According to equation (2), the interaction of M_{PEG} and C_{PEG} is negative effect, indicating that an increase in C_{PEG} and a decrease on M_{PEG}, or vice versa causes an improvement in partition coefficient of collagenolytic protease in the top PEG rich phase. In this work, the results obtained indicate that, the polymer size does not create a repulsive effect on collagenolytic protease partition, and the interaction of both variables is more significant in the coefficient partition (runs 6 and 15). The lower molecular weight PEGs may interact strongly with proteins, while higher molecular weight PEGs have the ability to form intramolecular force. Systems with PEGs of higher molecular mass give the highest resolution to facility partitioning, due to hydrophobicity and exclusion of volume effect (Tubio et al., 2004).

The transfer of the protein into one of the phases requires the breaking of the components' interacting phases, in order to create a cavity in which the protein is added. Therefore, the energy balance can be positive or negative, depending on whether the protein/polymer interactions are attractive or repulsive, and this depends on the PEG molecular weight (Picó et al., 2007; Raja et al., 2011). The higher the molecular weight of the polymer, the lower the concentration of polymer required for phase separation. The concentration of PEG provides a similar effect, since a high polymer concentration provides a greater number of polymer units involved in the separation of the protein, and thus a larger interaction of the target molecule partitions into the PEG phase, which is as a result of the increased number of hydrophobic interaction that can be formed between the protein and the polymer molecules (Raja et al., 2011; Saravanan et al., 2008).

PEG-Collagenolytic showed different yield values (from 37 to 169%) (Table 3). Yields above and near 100% are frequently reported for enzyme extraction, using ATPS. These results are probably explained by the elimination of inhibitors during the recovery process, and by the composition of the systems, in which PEG modifies the structure of the enzyme active site, consequently favoring the enzymatic activity (Mayerhoff et al., 2004; Pancera et al., 2002).

$$Y = 102.18 + 17.02x_1 + 13.98x_2 + \mathbf{9.76}x_3 + 27.97x_4 - 6.82 \times 1 \times 2 - 7.37 \times 1 \times 3 - \mathbf{18.60} \times 1 \times 4 - 4.33 \times 2 \times 3 - 16.91 \times 2 \times 4 - 6.06 \times 3 \times 4 \quad (6)$$

The mean variables that influenced the yield response, were PEG molar mass and phosphate salts concentration (Fig. 3). The effect of the M_{PEG} and C_{PHO} interaction (x_1x_4) was negative, implying that high concentration of phosphate salt causes migration of collagenolytic

protease to the PEG rich phase. This can be explained by a salting out effect, where the biomolecule is directed to the other phase, because of the great amount of salt in the bottom phase (Yang et al., 2008).

However, the addition of salts in an aqueous solution of PEG leads to an orderly arrangement of water molecules (Nalinanon et al., 2009) around the PEG molecules due to the ability of the salts to destabilize the structure of water. The formation of a layer of water around cations results in a more compact structure, with a smaller volume occupied by the PEG molecule. The volume occupied by the polymer increases with chain length (or molecular weight) and the polymer concentration, which results in reduced space for biomolecules in the top phase. Consequently, the biomolecules tend to partition to the bottom phase, which is a phenomenon termed as volume exclusion effect (Porto et al., 2008; Rosso et al., 2012). Phosphate ions can influence the protein partition, by means of electrostatic interactions between biomolecules and the components of ATPS system. With an increase in C_{PHO}, negatively charged proteins prefer the PEG-rich phase, because of the repulsion force caused by salt anions (Silva et al., 2009).The behavior observed in the statistical analysis for purification factor (*P*) obtained in this study, is also in agreement with the yield response, i.e. the negative effect of the M_{PEG} and C_{PHO} interaction had negative influence in the *P*.

$$P = 2.53 + 0.47x_1 - 0.16x_2 + \mathbf{0.22}x_3 - 0.59x_4 - 0.96 \times 1 \times_2 + 0.43 \times 1 \times_3 - \mathbf{0.65} \times 1 \times_4 - 0.37 \times 2 \times_3 - 0.20 \times 2 \times_4 - 0.33 \times 3 \times_4 \quad (7)$$

According to the statistical analysis, increase in pH (x3) has a positive effect on the Y and P responses, i.e., at alkaline pH, protein partition is preferentially aligned to the top PEG rich phase. The pH of the system influences the ionizable groups of a protein, alters the protein surface charges, and also the isoelectric point. At high pH values, the protein is more negatively charged than at low pH, and therefore, the partition coefficient of the protein increases with increasing pH (Saravanan et al., 2008),which may also be due to the electrostatic interactions between the protein and the PEG units (Vats and Banerjee, 2006).

From the study, including the statistical analysis for the three variables responses (*K*, *Y* and *P*), the best results was obtained in run 6 (pH 8.0, M_{PEG} 8000 g/mol, C_{PEG} 12.5% w/w and C_{PHO} 10% w/w); suggesting that hydrogenionic/hydroxylic forces favors the recovery in the top phase where most of the recovery occurred in alkaline pH. The validity of the model was verified by analysis of variance (ANOVA), and all determination coefficient-R^2 were around 0.80; a value close to 1 indicated agreement between the experimental data and the predicted model. The estimated effects and the corresponding *p*-values indicate that, the independent variables have a significant effect on the studied response ($p < 0.05$).

ATPS for fish protease extraction have been successfully reported, such as those isolated from the stomach of Albacore tuna (*Thunnus alalunga*), using the PEG/$MgSO_4$ system (Nalinanon et al., 2009), from the liver (Sripokar et al., 2017) and spleen (Poonsin et al., 2017) using PEG/NaH_2PO_4 system, besides being applied in the extraction of alkaline proteases from viscera of farmed giant catfish (*Pangasianodon gigas*) by PEG/sodium citrate system (Ketnawa et al., 2013). Thus, as described in this work, the application of ATPS system for recovery of biomolecules from water sources is highly feasible, especially from species of tropical fish and Neotropical, to obtain alkaline proteases collagenolytic potential.

3.4. Statistical analysis of separation methods

Here, the best results of each type of separation were statistically compared (precipitation with acetone, ethanol, ammonium sulfate-$(NH_4)_2SO_4$, TPP and ATPS partitioning). According to Fig. 4A, separation by ammonium sulfate and ethanol were statistically the best methods according to total collagenolytic activity due to their highest protein accumulation. However, this protein accumulation did not result proportionally in accumulation of collagenolytic enzymes as evidenced (also statistically significant) by the specific activities in Fig. 4B where TPP and mainly ATPS yielded, respectively 119.1 and 301.8% in relation to the classical method of ammonium sulfate precipitation.

Table 4 shows a comparison between the purification factors (PF) and yields (%) of several studies using TPP and ATPS. In this table there are enzymes from several sources, however for the best of our knowledge the present study is the first report on purification of collagenolytic enzymes from fish using TPP and ATPS. In relation to PF, the values in most of cases are in the same order of magnitude, excepting the collagenases from the fungus *Penicillium sp*. described by Wanderley et al. (2017) and alkaline proteases from the fish *Pangasianodon gigas* by Ketnawa et al. (2014a) for ATPS whereas for TPP the invertase from the plant *Momordica charantia* (Belligün and Demir, 2019), proteases from the plant *Calotropis procera* (Gagaoua et al., 2015), peroxidase from plant *Amsonia orientalis* (Karakus et al., 2018) as well as lipase and laccase from the fungi *Rhizopus arrhizus* and *Ganoderma sp.*, respectively (Dobreva et al., 2019; Rajeeva and Lele, 2011). On the other hand, in relation to the yield, the present results with *C. ocellaris* showed the highest value for TPP and the second highest value for ATPS. Besides this performance of extraction in both systems, fish collagenolytic enzymes are economically more viable since they may come from fisheries and aquaculture wastes, in contrast to microbial collagenases which require time-consuming steps using expensive culture mediums. Thus, the partitioned enzyme was designated PEG-collagenolytic and used for partial characterization and hydrolysis testing with collagenous proteins.

3.5. Partial characterization and collagen hydrolysis

Here, PEG-collagenolytic had its physicochemical characteristics determined (effect of temperature and pH, sensitivity to metal ions and inhibitors, electrophoretic profile) and tested for its efficiency in cleavage of native and commercial collagen.

The effect of temperature and pH, on the relative PEG-collagenolytic activity, was investigated, and the results are presented in Fig. 5. The maximum activity was observed at a temperature of 55 °C (Fig. 5A); according to the described purified collagenolytic enzymes extracted from fish species that have undergone recovery and purification. The enzyme reduced more than 50% of its activity after reaching a temperature of 65 °C. The effect of pH was investigated in the range of 4–12, with the results shown in Fig. 5B. Optimal activity was observed at pH 7.5, maintaining more than 60% relative activity in the range of 6.5–9.5. Parameters, such as temperature and pH, are limiting factors for a good performance of enzymes that have collagenolytic properties of producing peptides (Daboor et al., 2012), which can be exploited in the food, cosmetic, and pharmacologic industry.

Tests with metal ions, and natural and synthetic inhibitors, are described in Table 5. Only Ca^{2+} and Mg^{2+} showed no significant difference ($p < 0.05$) compared with the control group. There was inhibition, in descending order, by the following ions: Al^{3+}, Cu^{2+}, Hg^{2+}, Cd^{2+}, Pb^{2+}, Zn^{2+} and Mg^{2+}. Regarding the inhibitors tested, there was a significant difference between all treatments, compared to the control group. The highest degree of inhibition was specific for trypsin inhibitors (Benzamidine and TLCK); however, a high degree of inhibition (67.03%) was also detected for the serine protease inhibitor (PMSF). The test with metalloprotease inhibitors (EDTA) demonstrated reduced activity, but in a lower degree in relation to that for serine protease inhibitors. Similar results for temperature, pH, sensitivity to ions and inhibitors have been described for collagenolytic serine proteases partially purified from the waste (viscera) of smooth weakfish *Cynoscion leiarchus* (Oliveira et al., 2017a,b).

Enzymes belonging to the class of metalloprotease generally require Zn^{2+} to maintain optimum activity and stability, while having their activities significantly reduced after exposure to EDTA, a chelating agent for the Ca^{2+}. Thus, the increase in activity induced by Ca^{+}, and the inhibition brought about by classical inhibitors of serine proteases suggest that the enzyme in question belongs to the group of serine collagenolytic

proteases, corroborating the reported for purified collagenases of Mackerel *Scomber japonicus* (Park et al., 2002), Filefish *Novoden modestrus* (Kim et al., 2002) and smooth weakfish *C. leiarchus* (Oliveira et al., 2017a,b).

The PEG-collagenolytic showed three bands of protein, with molecular weights ranging from 10.0 to 60.3 kDa (Fig. 6).This variation in molecular mass has already been reported for collagenases from aquatic sources, as this type of enzyme does not have a single source, as reported for collagenolytic enzymes extracted and purified by different methods from fish waste (a mixture of haddock, herring, ground fish and flounder) (Daboor et al., 2012), which has been mainly described as in the serine protease class (Kim et al., 2002; Park et al., 2002).

In the collagenous protein hydrolysis test, PEG-collagenolytic was able to cleave both types of substrates tested, varying only according to the cleavage time. In the assay with bovine Achilles tendon collagen type I and skin collagen (Oliveira et al., 2019b), cleavage was detected at 24 h (38.82 and 6.58 U/mg), 36 h (149.29 and 134.55 U/mg) and 48 h (268.77and 454.21 U/mg), respectively. Specificity tests with collagen have been reported for marine collagenases described by Park et al. (2002), Daboor et al. (2012), Oliveira et al. (2017a,b) and Abood et al. (2018). The production of peptides from type I collagen is highly desirable for the industrial segments, especially the biomedical sector, since these types of peptides are potential sources of antibacterial, anti-inflammatory, antioxidant and/or immunomodulator activity (Oliveira et al., 2017a,b), indicating the feasibility of using the PEG-collagenolytic to hydrolyze collagen of different sources aiming the production of peptides of industrial interest.

4. Conclusions

This article provides a useful protocol for separation and partial purification of collagenolytic proteases, as a way of exploiting waste discarded by the fishing industry. All the techniques employed were efficient in increasing the collagenolytic protease purification factor, suggesting its use when the necessity of a collagenase without a high degree of purification, of obtaining fast (textile industry, tannery industry, for example), and providing a suitable destination for neglected aquaculture waste, with the advantage of almost full reuse of all waste employed, arises. Moreover, PEG-collagenolytic presented similar biochemical parameters with enzymes purified by more complex and expensive techniques currently being used, which makes their practical application feasible.

CRediT authorship contribution statement

Vagne de Melo Oliveira: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing. **Márcia Nieves Carneiro da Cunha:** Conceptualization, Methodology, Writing - original draft. **Caio Rodrigo Dias de Assis:** Conceptualization, Validation, Formal analysis, Writing - original draft. **Juanize Matias da Silva Batista:** Investigation. **Thiago Pajeú Nascimento:** Investigation. **Juliana Ferreira dos Santos:** Investigation. **Carolina de Albuquerque Lima:** Writing - original draft, Writing - review & editing. **Daniela de Araújo Viana Marques:** Writing - original draft, Writing - review & editing. **Ranilson de Souza Bezerra:** Supervision, Project administration, Funding acquisition. **Ana Lúcia Figueiredo Porto:** Supervision, Project administration, Funding acquisition.

Acknowledgements

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 8888.119817/2016-01.

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