EXTRACTION OF COLLAGENOLYTIC ENZYME FROM FISH VISCERA BY PHASE PARTITIONING (PEG/CITRATE) AND ITS POTENTIAL FOR INDUSTRIAL APPLICATION*

**ABSTRACT**

Internal viscera fish are potential sources of protein biomolecules of biopharmaceutical interest. However, this residue is frequently discarded inappropriately. The possibility to obtain byproducts of higher added value is a reality. Inside this view attention must be given to processes for the recovery and extraction of target molecules. However, the high cost of processing these residues is one of the obstacles to their reuse; techniques that facilitate their handling and make the process cheaper are desirable, such as extraction in a two-phase aqueous system. Thus, the aim of this study was to extract collagenolytic enzymes from common snook (Centropomus undecimalis) using a two-phase aqueous system (polyethylene glycol/citrate), according to the 2^4 factorial design, using as variables: molar mass of PEG (M_{PEG}), PEG concentration (C_{PEG}), citrate concentration (C_{CIT}), pH, still, considering purification factor (FP), partition coefficient (K), and yield (Y). The collagenolytic activity of the crude extract was 102.41 U mg^{-1}, after partitioning, was purified 3.91 times (M_{PEG}: 8000; C_{PEG}: 20.0%; C_{CIT}: 20.0% and pH 6.0). Inhibition (U mg^{-1}) was observed in benzamidine (22.51), TLCK (21.05), TPCK (21.29), and PMSF (23.05), signaling to be a serine-protease. The results showed the advantage of this semipurification technique as concerns to the low cost of extraction and purification, adding value to the fishing source material and allocating the residues from its processing to the industry.

**Keywords:** biotechnology; collagenase; by-products; residue.

---

Jessica Costa SILVA1
Luiz Henrique Svintiskas LINO1
Márcia Nieves CARNEIRO DA CUNHA1
Juanize Matias da Silva BATISTA1
Vagne Melo OLIVEIRA1
Ana Lúcia Figueiredo PORTO1

1 Universidade Federal Rural de Pernambuco - UFRPE, Departamento de Morfologia e Fisiologia Animal - DMFA, Laboratório de Tecnologia de Produtos Bioativos - LABTECBIO. Av. Dom Manoel de Medeiros, s/n, CEP: 52.171-900, Recife, PE, Brazil. vagne_melo@hotmail.com; analuporto@yahoo.com.br (*corresponding authors).

---

*This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 1 (Process nº 8888.119817/2016-01). This study is part of Jessica Costa Silva master’s dissertation, which is deposited in its entirety in UFPF’s digital repository.

Received: June 15, 2020
Approved: December 11, 2020

---

EXTRAÇÃO DE ENZIMA COLAGENOLÍTICA DAS VÍSCERAS DE PEIXES POR PARTIÇÃO DE FASES (PEG/CITRATO) E SEU POTENCIAL PARA APLICAÇÃO INDUSTRIAL

**RESUMO**

Visceras internas de peixes são fontes potenciais de biomoléculas proteicas de interesse biofarmacêutico. No entanto, esse resíduo é frequentemente descartado de forma inadequada. A possibilidade de obtenção de subprodutos de maior valor agregado é uma realidade. Dentro desta visão, atenção deve ser dada aos processos de recuperação e extração de moléculas-alvo. Como o custo do processamento desses resíduos é um dos obstáculos para sua reutilização, técnicas que facilitem seu manejo e barateiem o processo são desejáveis, como a extração em sistema bifásico. Assim, o objetivo deste estudo foi extrair enzimas colagenolíticas de robalo-flecha (Centropomus undecimalis) usando sistema bifásico (polietilenoglicol/citrato), de acordo com o planejamento fatorial 2^4, tendo como variáveis: massa molar do PEG (M_{PEG}), concentração de PEG (C_{PEG}), concentração de citrato (C_{CIT}), e pH; ainda, considerando: fator de purificação (FP), coeficiente de partição (K) e rendimento (Y) como respostas. A atividade colagenolítica do extrato bruto foi de 102,41 U mg^{-1}. Após o particionamento, a amostra foi purificada 3,91 vezes (M_{PEG}: 8000; C_{PEG}: 20,0%; C_{CIT}: 20,0% e pH 6,0), sendo inibida (U mg^{-1}) pela benzamidina (22,51), TLCK (21,05), TPCK (21,29), e PMSF (23,05), sinalizando tratar-se de uma serina-protease. Os resultados mostraram a vantagem desta técnica de semipurificação no que diz respeito ao baixo custo de extração e purificação, adicionando valor à matéria-prima pesqueira e desvencilhando os resíduos de seu processamento para a indústria.

**Palavras-chave:** biotecnologia; colagenase; subprodutos; resíduo.
INTRODUCTION

The need for reuse of waste is a practice that has been increasingly encouraged, especially organic solids, such as those from the fishing industry. During the processing of fish there is a lot of organic matter that is produced, among them: head, tail, fins, skin, scales and internal viscera (stomach, intestine, pyloric caecum, mesentery, among others), most of these materials are discarded. Special attention must be given to proteic macromolecules of that have high biotechnological interest. Recently, fishing waste has been the subject of research on their applications in human and animal health (Ideaia et al., 2020; Oliveira et al., 2020).

From digestive viscera of aquatic organisms, it is possible to extract various proteases (pepsin, trypsin, chymotrypsin and collagenases and/or enzymes with similar catalytic functions) (Oliveira et al., 2017a; Gurumallesh et al., 2019; Silva et al., 2019). Enzymes with collagenolytic properties of aquatic organisms have already been recovered and partially purified from digestive residues of Neotropical fish species (Silva et al., 2019; Oliveira et al., 2017b; Liu et al., 2019). The collagenolytic property (collagen cleavage) is quite required in industry, mainly biomedical and pharmaceutical, being applied in surgical procedures (Zhao et al., 2019), intracerebral therapies (Chen-Roetling et al., 2019), wound healing (Abood et al., 2018), extraction of collagen and production of bioactive peptides (Bhagwat and Dandge, 2018), fibrosis treatment (Villegas et al., 2018), and treatment of Dupuytren (Zhang et al., 2019), thus making these enzymes fall into the category of high commercial value proteases (Oliveira et al., 2017c).

Various methods of separating protein biomolecules for biopharmaceutical use have been used to obtain collagenolytic enzymes with a higher degree of purity, the main ones being chromatographic (ion exchange, molecular exclusion) (Kim et al., 2002; Park et al., 2002). However, the high cost of obtaining it makes it necessary and desirable to constantly search for alternative sources and for extraction processes that are fast, efficient and low cost, such as liquid-liquid extraction, using the aqueous two-phase system (ATPS) (Oliveira et al., 2020). ATPS is a form of partitioning where the system is formed by two distinct phases employing a polymer (usually, polyethylene glycol, PEG) and a salt (citrate, sulfate, phosphate, for example). In aquaculture, the extraction by ATPS has been successful in the recovery of microalgae products (Phong et al., 2018; Suarez Garcia et al., 2018), mainly ribulose-1,5-biphosphate carboxylase/oxygenase (Suarez Ruiz et al., 2018), proteases from hepatopancreas of Pacific white shrimp (Senphan and Benjakul, 2014), and alkaline proteases from albacore tuna (Thunnus alalunga) liver (Sripokar et al., 2017) and spleen (Poonsin et al., 2017), and intestine from peacock bass (Cichla ocellaris) (Oliveira et al., 2020). Thus, this study aimed to partition a collagenolytic protein from digestive waste of common snook (Centropomus undecimalis), using two-phase aqueous system (PEG/citrate) for future biotechnological applications.

MATERIAL AND METHODS

Materials

Azocoll substrate, Tris, polyethylene glycol (PEG) of 400, 3350 and 8000 molar mass, dehydrate tri-sodium citrate and monohydrate citric acid salts used were obtained from Sigma-Aldrich®, Saint Louis, USA. HCl-Merck, Darmstadt, Germany. All other reagents were used of analytical grade.

Extraction of collagenolytic enzyme

Digestive viscera of common snook (Brazilian Genetic Heritage nºA25441A) were collected from fish sellers in the public market of São José, Recife, Pernambuco, Brazil. The viscera were transported under refrigeration in ice bags to the Bioactive Technology Laboratory, LABTECBIO, Federal Rural University of Pernambuco, UFRPE. The enzymatic extraction was done by the description by Oliveira et al. (2019). Primarily, 30 g of intestinal viscera were macerated in 50 mM Tris-HCl extraction buffer, homogenized (IKARW 20D S32, Guangzhou, China) for 5 min., at 10,000-12,000 rpm (4°C), and centrifuged at 12,000 x g (Sorvall® Superspeed RC-6 Centrifuge, Thermo Fisher Scientific, Waltham, MA, USA) for 30 min. at 4°C. The supernatant was preheated at 55°C for 30 min., was centrifuged again. The resulting supernatant was called crude enzyme extract, used in later analyzes, as shown in Figure 1. The common snook (Centropomus undecimalis) crude extract was subjected to ATPS PEG/citrate technique to extract and pre-purify of collagenolytic protease (Figure 1).

Protein determination

The protein concentrations of all samples were determined by the method of bicinchoninic acid (BCA) according to Smith et al. (1985).

Extraction of aqueous two-phase systems (ATPS)

For enzyme extraction, the ATPS runs were formed in different concentrations according to the 2-factorial design, described in Table 1. The solution of sodium citrate (30% w w⁻¹) was prepared by mixing solutions of dihydrate tri-sodium citrate and monohydrate citric acid to obtain pH values (6.0, 7.0 and 8.0) and different volumes of these solutions were transferred to 15 mL-graduated tubes containing different PEG concentrations with different molar masses (400, 3350 or 8000 g mol⁻¹). This mixture was added the crude extract accounting for 20% (w w⁻¹) of total mass and after water addition up to a final mass of 5 g, the suspensions were then homogenized in a vortex for 1 min., and the two phases were separated by settling for 1 h. The influence of independents variables poly(ethylene glycol) molecular mass (MPEG), PEG concentration (CPEG), sodium citrate concentration (Ccitra), and pH, was verify under the response variables protease collagenolytic partition (K), yield (Y) and purification factor (PF) according to a 2-factorial complete design, composed of 16 runs; all independent variables were evaluated at two levels (+1 and -1) (Table 1), with three replicates at the central point (0)
Determining ATPS parameters

The protease collagenolytic partition coefficient ($K$) was determined as the ratio of collagenolytic activity (U mL$^{-1}$) in the PEG-rich top phase (CA$_T$) to that in the salt-rich bottom phase (CA$_B$): $K = \frac{CA_T}{CA_B}$.

The yield (Y) was determined as the ratio of the total collagenolytic activity in the top phase to that in the crude extract and expressed as a percentage: $Y = \left( \frac{CA_T \times V_T}{CA_{ce} \times V_{ce}} \right) \times 100$; where, $V_T$ and $V_{ce}$ are the volumes of the top phase and the crude extract, respectively.

The purification factor (PF) was defined as the ratio of the specific activity in the top phase to its initial value in the crude extract before partition: $PF = \frac{(CA_T/Pt)/(CA_{ce}/Pt_{ce})}$; where Pt (protein in phase rich PEG) and Pt$_{ce}$ (protein in crude extract) are the total protein concentrations (mg mL$^{-1}$) and CA$_T$ (collagenolytic in phase rich PEG) and CA$_{ce}$ (collagenolytic in crude extract) are collagenolytic activities (U mL$^{-1}$).

Collagenolytic assay

Collagenolytic activity was performed according to Oliveira et al. (2020). First, in microtube (2 mL), 5 mg of the azocoll, 500 μL of 50 mM Tris-HCl (pH 7.5) and 500 μL of enzymatic sample were added, incubated at 55°C for 30 min., under constant agitation. Then, the reaction was stopped (200 μL of 40% trichloroacetic acid, TCA) for 10 min., to then be centrifuged (10,000 x g for 10 min at 4°C). The reading was done at 520 nm. All tests were performed in triplicate.

Inhibitor sensitivity

The evaluation of sensitivity to inhibitors was carried out according to Oliveira et al. (2019). Briefly: in Eppendorf (2 mL), in the proportion of 1:1, the partitioned sample was subjected to Benzamidine, ethylenediaminetetraacetic acid (EDTA), Phenylmethylsulfonyl fluoride (PMSF), Tosyl-L-lysine chloromethyl ketone (TLCK), Tosyl phenylalanone chloromethylKylethyl ketone (TPCK), for a period of 30 min., to then determine the collagenolytic activity (U mg$^{-1}$). Data are expressed as mean ± SD. These data were statistically analyzed by ANOVA, followed by a post hoc (Tukey) test, when indicated. Differences between groups were accepted as significant at the 95% confidence level ($p <0.05$).
RESULTS

The two phases formation of intestine extracts was not observed in runs 1, 3, 9 and 11 after the addition of the crude extract in the system (Table 2). The results obtained of partition coefficient (K) indicating that the collagenolytic protease has higher affinity for the upper phase of the system (rich in PEG), since K > 1 values were obtained. It was observed that only in tests 5 and 7 the enzyme partitioned for lower phase of the system.

The effect of independent variables and their interactions evaluated of factorial design showed that K was positively influenced by the PEG molar mass (M_PEG) (Table 3).

In the runs formed by the polymer with molar mass 8.000 g mol⁻¹ higher values of partition coefficient were obtained (13.17 and 14.78), indicating once again the affinity of the collagenolytic protease for the upper phase of system (rich in PEG). The interaction between the M_PEG and pH (1x4) had a significant negative effect under the K for p < 0.05.

This interaction presented the antagonistic effect since a lower pH value combined with the higher polymer molar mass (8000 g mol⁻¹) favored the enzymatic partition for the polymeric phase. For the activity Yield (Y) values above 100% were observed in the upper phase of the system in the extraction of the collagenolytic protease from C. undecimalis (Table 2). As well as, in the statistical analysis of K, it was possible to observe a strong significative positive influence of M_PEG under Y in the upper phase (Y_{PEG}) and the negative influence of the interaction between M_{PEG} and pH (1x4) for p < 0.05 (Table 3).

The highest purification factors (PF) 3.91 was obtained on the run 6, formed with PEG molar mass 8000 (g mol⁻¹) in 20.0% (w w⁻¹) concentration, 20% (w w⁻¹) of citrate concentration at pH 6.0 (Figure 2). Just like for K and Y activity, the independent variable M_{PEG} also had the strong significative positive effect on PF, already, the polymer concentration had a significative negative effect for this variable response. The interaction between variables the M_{PEG}, C_{PEG} and pH (1x2x4) was positively significant (p < 0.05) for obtaining for this variable (Table 3).

In the investigation of sensitivity to inhibitors, the common snook collagenolytic enzyme partitioned by the ATPS system showed reduced activity when exposed, in decreasing order (%): PMSF (23.08 ± 0.00⁰), benzamidine (22.51 ± 0.02⁰), TPCK (21.29 ± 0.02⁰), and TLCK (21.05 ± 0.01⁰), all tests being statistically significant.

Table 2. Matrix of the full factorial design (2⁴) with conditions and results of the collagenolytic protease partition.

| Ensaio | M_{PEG} (g mol⁻¹) | C_{PEG} (%) | C_{CIT} (%) | pH  | K   | Y_{PEG} (%) | PF |
|--------|------------------|-------------|-------------|-----|-----|-------------|----|
| 1      | 400              | 20          | 15          | 6   | *** | ***         | ***|
| 2      | 8000             | 20          | 15          | 6   | 9.39| 236.24      | 3.80|
| 3      | 400              | 24          | 15          | 6   | *** | ***         | ***|
| 4      | 8000             | 24          | 15          | 6   | 13.17| 220.07      | 0.30|
| 5      | 400              | 20          | 20          | 6   | 0.63| 20.04       | 0.01|
| 6      | 8000             | 20          | 20          | 6   | 14.78| 326.52      | 3.91|
| 7      | 400              | 24          | 20          | 6   | 0.81| 53.01       | 0.51|
| 8      | 8000             | 24          | 20          | 6   | 4.17| 120.98      | 1.15|
| 9      | 400              | 20          | 15          | 8   | *** | ***         | ***|
| 10     | 8000             | 20          | 15          | 8   | 2.43| 94.98       | 0.44|
| 11     | 400              | 24          | 15          | 8   | *** | ***         | ***|
| 12     | 8000             | 24          | 15          | 8   | 3.56| 110.75      | 1.02|
| 13     | 400              | 20          | 20          | 8   | 10.95| 207.51      | 2.32|
| 14     | 8000             | 20          | 20          | 8   | 3.30| 146.10      | 2.39|
| 15     | 400              | 24          | 20          | 8   | 1.16| 34.34       | 0.83|
| 16     | 8000             | 24          | 20          | 8   | 6.24| 123.52      | 1.24|
| 17 (C) | 3350             | 22          | 17.5        | 7   | 8.43| 164.26      | 1.23|
| 18 (C) | 3350             | 22          | 17.5        | 7   | 9.39| 200.07      | 1.18|
| 19 (C) | 3350             | 22          | 17.5        | 7   | 7.78| 179.65      | 1.20|

M_{PEG} = PEG molar mass; C_{PEG} = PEG concentration; C_{CIT} = Citrate concentration; K = Partition coefficient; Y = Activity yield; PF = Purification factor; ***No phase formation after addition of extract. (C) = Central point.

Table 3. Statistical analysis for the responses of collagenolytic protease by PEG-Citrate ATPS performed according to the 2⁴ experimental design.

| Variables | K   | Y_{PEG} | PF  |
|-----------|-----|---------|-----|
| (1) M_{PEG} | 13.41* | 14.80* | 91.51* |
| (2) C_{PEG} | -3.80 | -5.13* | -67.54* |
| (3) C_{CIT} | 4.16 | 5.14* | 59.01* |
| (4) pH | -4.72* | -3.61 | -12.40* |
| 1x2 | 2.11 | -1.22 | -50.49* |
| 1x3 | -4.19 | -3.61 | -4.75* |
| 1x4 | -11.29* | -8.31* | -57.76* |
| 2x3 | -6.84* | -5.11* | -17.09* |
| 2x4 | 0.29 | 0.12 | 31.95* |
| 3x4 | 5.49* | 3.36 | 33.22* |
| 1x2x3 | -0.91 | -1.21 | -0.04 |
| 1x2x4 | 6.42 | 5.85* | 66.41* |
| 1x3x4 | -1.08 | -1.33 | -12.14* |
| 2x3x4 | 1.92 | -0.76 | -38.59* |

K = Partition coefficient; Y = Activity yield; PF = Purification factor; M_{PEG} = PEG molar mass; C_{PEG} = PEG concentration; C_{CIT} = Citrate concentration; *Statistically significant p < 0.05.
DISCUSSION

The common snook has been identified as a promising species for supplying enzymes, such as trypsin, chymotrypsin (Concha-Frias et al., 2016), leucine aminopeptidase, carboxypeptidase A, lipase, α-amylase, acid phosphatases, alkaline phosphatase (Jimenez-Martinez et al., 2012), and collagenases (Silva et al., 2019). The richness in proteases of the sea bass intestinal viscera has aroused interest for investigations that may lead to the reuse of its residues and at the same time provide alternative sources of biomolecules. Silva et al. (2019) extracted protease from common snook intestinal viscera using only maceration and centrifugation techniques. Here, the aqueous two-phase system (ATPS) was chosen due to the efficiency in the extraction of collagenolytic proteases described for waste from fishery resources, mainly in the efficient extraction of proteases (Kuepethkaew et al., 2017; Phong et al., 2018; Poonsin et al., 2017; Sripokar et al., 2017; Suarez Garcia et al., 2018; Suarez Ruiz et al., 2018; Oliveira et al., 2020).

ATPS was a fast method for extracting proteases of digestive viscera from common snook, corroborating with what was described by Porto et al. (2007) and Amaral et al. (2020) when they submitted proteases to the same partitioning conditions, observing the non-formation of phases when displacement of salt and polymer concentrations were below the binodal curve for PEG 400 g mol\(^{-1}\). The PEG phase potentialized the enzymatic activity, meeting that described by Oliveira et al. (2020) in which the elimination of inhibitors from the enzymatic extract of peacock bass is reported, favoring the increase in collagenolytic activity (pH 8.0; 12.5% PEG 8000 g mol\(^{-1}\); 10.0% phosphate).

Kuepethkaew et al. (2017) reported partitioning using ATPS (25% PEG 1000 g mol\(^{-1}\); 15% MgSO\(_4\); pH 5.0) as an efficient recovery technique with good yield and high degree of purification; while Ketnawa et al. (2013) reported an increase in yield (365.53%) when performing a second ATPS cycle (15% PEG 2000 g mol\(^{-1}\); 15% sodium citrate; pH 8.5) in digestive viscera extracts of giant catfish (Pangasianodon gigas) for partitioning alkaline proteases, indicating that ATPS can be expanded to industrial production.

The statistical analysis of results showed that the independent variable \(M_{\text{PEG}}\) had a strong influence for all the variables evaluated in the present study (K, \(Y_{\text{PEG}}\), and PF). \(M_{\text{PEG}}\) had a positive influence indicating that the higher molar mass of the polymer (8000 g mol\(^{-1}\)) favored the enzymatic partition for the upper phase of the ATPS, improved \(Y\) activity, and increased the purification factor.

Generally, with the increase in molar mass and concentration of polymer, occurs the K values decreasing. This phenomenon is known as the volume exclusion effect, since, the available free volume is reduced for the protein in the PEG-rich phase, leading

---

**Figure 2.** Scatter plot graphic of the simultaneous effects of PEG molar mass (\(M_{\text{PEG}}\)) and PEG concentration (\(C_{\text{PEG}}\)) on the Purification Factor (PF) in the top phase to extraction of collagenolytic protease from *C. undecimalis* using factorial design 2\(^4\) by aqueous two-phase systems.
to the partition of the biomolecules to the lower phase of the system (Iqbal et al., 2016; Nadar et al., 2017).

However, as increase in the polymer molar mass occurs simultaneous increases in your hydrophobicity, due to increase in hydrophobic area to hydrophilic group ratio (Leong et al., 2015) which results in an increase in the number of hydrophobic interactions formed between the protein and the polymer molecules. Collagenolytic proteases have hydrophobic regions that favor their interaction with PEG and their partitioning to the upper phase (PEG-phase) of the system. Others authors report this behavior when studying the partitioning of these enzymes in ATPS formed by PEG-salt (Rosso et al., 2012; Oliveira et al., 2020).

After the partitioning step, the samples were submitted to sensitivity to inhibitors. The extracts of the common snook (C. undecimalis) showed a marked reduction in activity when subjected to classical inhibitors of serine proteases, mainly trypsin and chymotrypsin, presenting a significant difference in all treatments ($p < 0.05$). Similar inhibition results by Benzamidine, TLCK, and PMSF were found for the pre-purified collagenolytic enzyme from smooth weakfish (Cynoscion leiarchus) (Oliveira et al., 2017a). Enzymes belonging to the class of metalloproteases activities are significantly reduced after exposure to EDTA. In a parallel, the correlation of the effects of the inhibitors on the enzymatic action can indicate the class of the target enzyme.

According to the results described herein, it is likely that the purified enzyme in question is of the serine protease class, corroborating with those described by Teruel and Simpson (1995), Roy et al. (1996), Kim et al. (2002), Park et al. (2002), Wu et al. (2010), Souchet et al. (2012), Oliveira et al. (2017a), Silva et al. (2019), and Oliveira et al. (2020) for collagenolytic serine proteases extracted from fish viscera and other sources, such as winter flounder (Pseudopleuronectes americanus), shore Crab (Carcinus maenas), Filefish (Novodden modestus), Mackerel (Scomber japonicus), red sea bream (Pagrus major), Snow Crab (Chionoecetes opilio), fresh water prawn (Macrobrachium rosenbergii), Smooth weakfish (C. leiarchus), Bluefish (Pomatomus saltatrix), and peacock bass (Cichla ocellaris), respectively.

CONCLUSIONS

In this study, collagenolytic protease was successfully extracted and semi purified from internal viscera from common snook using the biphasic partition method. Because it is a fast and simple technique when compared to the most improved chromatographic methods, it was possible to prove its feasibility for large-scale application in fish farming residues. All intestinal waste was reused, suggesting that this practice can be included in management plans to reduce environmental impact. The salt used (citrate) is not considered a polluting element and the polymer used can also be reused, which would reduce processing costs. The partitioned enzyme was recovered with a high degree of purification similar to the collagenolytic serine-proteases extracted using chromatographic techniques. Thus, the use of fish residues as a promising raw material in the supply of collagenolytic proteases for biopharmaceutical applications is suggestive, mainly to compose formulations of healing ointments.

ACKNOWLEDGEMENTS

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 1 (Process nº 8888.119817/2016-01).

REFERENCES

Abood, A.; Salman, A.M.M.; Abdelfattah, A.M.; El-Hakim, A.E.; Abdel-Aty, A.M.; Hashem, A.M. 2018. Purification and characterization of a new thermophilic collagenase from Nocardiosis dassonvillei NRC2aza and its application in wound healing. International Journal of Biological Macromolecules, 116: 801-810. http://dx.doi.org/10.1016/j.ijbiomac.2018.05.030.

Amaral, Y.M.S.; Silva, O.S.; Oliveira, R.L.; Porto, T.S. 2020. Production, extraction, and thermodynamics protease partitioning from Aspergillus tamarii Kita UCP1279 using PEG/sodium citrate aqueous two-phase systems. Preparative Biochemistry & Biotechnology, 50(6): 619-626. http://dx.doi.org/10.1080/10826068.2020.1721535.

Barros Neto, B.; Scarminio, I.S.; Bruns, R.E. 2003. Como fazer experimentos: pesquisa e desenvolvimento na ciência e na indústria. 2ª. ed. Campinas: Unicamp. 401p.

Bhagwat, P.; Danjge, P. 2018. Collagen and collagenolytic proteases: A review. Biocatalysis and Agricultural Biotechnology, 15: 43-55. http://dx.doi.org/10.1016/j.bcab.2018.05.005.

Chen-Roetling, J.; Cao, Y.; Peng, D.; Regan, R.F. 2019. Rapid loss of perithematonal cell viability in the collagenase intracerebral hemorrhage model. Brain Research, 1711: 91-96. http://dx.doi.org/10.1016/j.brainres.2019.01.014.

Concha-Frias, B.B.; Alvarez-González, C.A.; Gaxiola-Cortes, M.G.; Silva-Aramibia, A.E.; Toledo-Aguero, P.H.; Martinez-Garcia, R.; Camarillo-Coop, S.; Jimenez-Martinez, L.D.; Arias-Moscoso, J.L. 2016. Partial characterization of digestive proteases in the Common snook Centropomus undecimalis. International Journal of Biology, 8(4): 1-11. http://dx.doi.org/10.5539/ijb.v8n4p1.

Gurumallesh, P.; Alagu, K.; Ramakrishnan, B.; Muthusamy, S. 2019. A systematic reconsideration on proteases. International Journal of Biological Macromolecules, 128: 254-256. http://dx.doi.org/10.1016/j.ijbiomac.2019.01.081.

Ideia, P.; Pinto, J.; Ferreira, R.; Figueiredo, L.; Spinola, V.; Castilho, P.C. 2020. Fish processing industry residues: a review of valuable products extraction and characterization methods. Waste and Biomass Valorization, 11: 3223-3246. http://dx.doi.org/10.1007/s11707-019-00739-1.

Iqbal, M.; Tao, Y.; Xie, S.; Zhu, Y.; Chen, D.; Wang, X.; Huang, L.; Peng, D.; Sattar, A.; Shabbir, M.A.B.; Hussain, H.I.; Ahmed, S.; Yuan, Z. 2016. Aqueous two-phase system (ATPS): An overview and advances in its applications. Biological Procedures Online, 18: 1-18. http://dx.doi.org/10.1186/s12575-016-0048-8.
Jimenez-Martinez, L.D.; Alvarez-González, C.A.; Tovar-Ramírez, D.; Gaxiola, G.; Sanchez-Zamora, A.; Moyano, F.J.; Alarcón, F.J.; Marquez-Couturier, G.; Gisbert, E.; Contreras-Sánchez, W.M.; Perales-Garcia, N.; Arias-Rodriguez, L.; Indy, J.R.; Páramo-Delgado, S.; Palomino-Albarran, I.G. 2012. Digestive enzyme activities during early ontogeny in Common snook (Centropomus undecimalis). Fish Physiology and Biochemistry, 38(2): 441-454. http://dx.doi.org/10.1007/s10695-011-9525-9.

Ketnawa, S.; Benjakul, S.; Ling, T.C.; Martinez-Alvarez, O.; Rawdkuen, S. 2013. Enhanced recovery of alkaline protease from fish viscera by phase partitioning and its application. Chemistry Central Journal, 7: 1-9. http://dx.doi.org/10.1186/1752-153X-7-79.

Kuepethkaew, S.; Sangkharaik, K.; Benjakul, S.; Klomkla, S. 2017. Use of TPP and ATPS for partitioning and recovery of lipase from Pacific white shrimp (Litopenaeus vannamei) hepatopancreas. Journal of Food Science and Technology, 54: 3880-3891. http://dx.doi.org/10.1007/s13197-017-2844-9.

Kim, S.K.; Park, P.J.; Kim, J.B.; Shahidi, F. 2002. Purification and characterization of a collagenolytic protease from the filefish, Novodiden modestus. Journal of Biochemistry and Molecular Biology, 35(2): 165-171. http://dx.doi.org/10.5483/bmbrep.2002.35.2.165.

Leong, Y.K.; Lan, J.C.-W.; Loh, H.-S.; Ling, T.C.; Ooi, C.W.; Show, P.L. 2015. Thermoseparating aqueous two-phase systems: Recent trends and mechanisms. Journal of Separation Science, 39(4): 640-647. http://dx.doi.org/10.1002/jssc.201500667.

Liu, Y.; Liu, Z.; Song, L.; Ma, Q.; Zhou, D.; Zhu, B.; Shahidi, F. 2019. Effects of collagenase type I on the structural features of collagen fibres from sea cucumber (Stichopus japonicus) body wall. Food Chemistry, 301: 1-7. http://dx.doi.org/10.1016/j.foodchem.2019.125302.

Nadar, S.S.; Pawar, R.G.; Rathod, V.K. 2017. Recent advances in enzyme extraction strategies: A comprehensive review. International Journal of Biological Macromolecules, 101: 931-957. http://dx.doi.org/10.1016/j.ijbiomac.2017.03.055.

Oliveira, V.M.; Assis, C.R.D.; Hercuvano, P.N.; Cavalcanti, M.T.H.; Bezerra, R.S.; Porto, A.L.F. 2017a. Collagenase from Pacific white shrimp (Litopenaeus vannamei) hepatopancreas. Journal of Food Science and Technology, 100(5): 503-510. http://dx.doi.org/10.1007/s12091-017-1786-4.

Oliveira, V.M.; Assis, C.R.D.; Hercuvano, P.N.; Cavalcanti, M.T.H.; Porto, A.L.F. 2017b. Purification and characterization of a collagenolytic protease from the fish hepatopancreas. Journal of Biochemistry and Molecular Biology, 35(6): 87-95. http://dx.doi.org/10.1016/j.jbiosc.2018.03.005.

Oliva, M.; Cunha, M.N.C.; Assis, C.R.D.; Silva, J.M.; Nascimento, T.; Santos, J.F.; Duarte, C.A.L.; Marques, D.A.V.; Bezerra, R.S.; Porto, A.L.F. 2020. Separation and partial purification of collagenolytic protease from peacock bass (Cichla ocellaris) using different protocol: Precipitation and partitioning approaches. Biocatalysis and Agricultural Biotechnology, 24: 1-13. http://dx.doi.org/10.1016/j.bcab.2020.101509.

Phong, W.N.; Show, P.L.; Chow, Y.H.; Ling, T.C. 2018. Recovery of biotechnological products using aqueous two-phase systems. Journal of Bioscience and Bioengineering, 126(3): 273-281. http://dx.doi.org/10.1016/j.jbiosc.2018.03.005.

Santos, P.N.; Assis, T.P.; Porto, A.L.F.; Pessoa, A. 2007. Removal of proteases from Clostridium perfringens fermented broth by aqueous two-phase systems (PEG/citrate). Journal of Industrial Microbiology & Biotechnology, 34(8): 547-552. http://dx.doi.org/10.1007/s10295-007-0230-8.

Santos, P.K.; Sriskopar, P.; Benjakul, S.; Simpson, B.K.; Visessanguan, W.; Klomkla, S. 2017. Major trypsin like-serine proteinases from albacore tuna (Thunnus alalunga) spleen: biochemical characterization and the effect of extraction media. Journal of Food Biochemistry, 41(2): 1-9. http://dx.doi.org/10.1111/jfbc.12323.

Santos, P.K.; Sriskopar, P.; Benjakul, S.; Simpson, B.K.; Visessanguan, W.; Klomkla, S. 2017. Major trypsin like-serine proteinases from albacore tuna (Thunnus alalunga) spleen: biochemical characterization and the effect of extraction media. Journal of Food Biochemistry, 41(2): 1-9. http://dx.doi.org/10.1111/jfbc.12323.

Santos, P.K.; Sriskopar, P.; Benjakul, S.; Simpson, B.K.; Visessanguan, W.; Klomkla, S. 2017. Major trypsin like-serine proteinases from albacore tuna (Thunnus alalunga) spleen: biochemical characterization and the effect of extraction media. Journal of Food Biochemistry, 41(2): 1-9. http://dx.doi.org/10.1111/jfbc.12323.

Santos, P.K.; Sriskopar, P.; Benjakul, S.; Simpson, B.K.; Visessanguan, W.; Klomkla, S. 2017. Major trypsin like-serine proteinases from albacore tuna (Thunnus alalunga) spleen: biochemical characterization and the effect of extraction media. Journal of Food Biochemistry, 41(2): 1-9. http://dx.doi.org/10.1111/jfbc.12323.

Santos, P.K.; Sriskopar, P.; Benjakul, S.; Simpson, B.K.; Visessanguan, W.; Klomkla, S. 2017. Major trypsin like-serine proteinases from albacore tuna (Thunnus alalunga) spleen: biochemical characterization and the effect of extraction media. Journal of Food Biochemistry, 41(2): 1-9. http://dx.doi.org/10.1111/jfbc.12323.
Suarez Garcia, E.; Suarez Ruiz, C.A.; Tilaye, T.; Eppink, M.H.M.; Wijffels, R.H.; Van Den Berg, C. 2018. Fractionation of proteins and carbohydrates from crude microalgae extracts using an ionic liquid based-aqueous two-phase system. Separation and Purification Technology, 204: 56-65. http://dx.doi.org/10.1016/j.seppur.2018.04.043.

Suarez Ruiz, C.A.; Emmery, D.P.; Wijffels, R.H.; Eppink, M.H.; Van Den Berg, C. 2018. Selective and mild fractionation of microalgal proteins and pigments using aqueous two-phase systems. Journal of Chemical Technology and Biotechnology (Oxford, Oxfordshire), 93(9): 2774-2783. http://dx.doi.org/10.1002/jctb.5711.

Sripokar, P.; Chaijan, M.; Benjakul, S.; Yoshida, A.; Klomklao, S. 2017. Aqueous two-phase partitioning of liver proteinase from albacore tuna (Thunnus alalunga): application to starry triggerfish (Abalistes stellaris) muscle hydrolysis. International Journal of Food Properties, 20(S2): 1600-1612. http://dx.doi.org/10.1080/10942912.2017.1350705.

Sriket, C.; Benjakul, S.; Visessanguan, W.; Kishimura, H. 2011. Collagenolytic serine protease in freshwater prawn (Macrobrachium rosenbergii): characteristics and its impact on muscle during iced storage. Food Chemistry, 124(1): 29-35. http://dx.doi.org/10.1016/j.foodchem.2010.05.098.

Statsoft Inc. 2008. Statistica, version 8.0. Tulsa: Statsoft. Inc.

Teruel, S.R.L.; Simpson, B.K. 1995. Characterization of the collagenolytic enzyme fraction from winter flounder (Pseudopleuronectes americanus). Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology, 112(1): 131-136. http://dx.doi.org/10.1016/0305-0491(95)00044-9.

Villegas, M.R.; Baeza, A.; Usategui, A.; Ortiz-Romero, P.L.; Pablos, J.L.; Vallet-Regí, M. 2018. Collagenase Nanocapsules: An Approach to Fibrosis Treatment. Acta Biomaterialia, 1(74): 430-438. http://dx.doi.org/10.1016/j.actbio.2018.05.007.

Wu, G.P.; Chen, S.H.; Liu, G.M.; Yoshida, A.; Zhang, L.J.; Su, W.J.; Cao, M.J. 2010. Purification and characterization of a collagenolytic serine proteinase from the skeletal muscle of red sea bream (Pagrus major). Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology, 155(3): 281-287. http://dx.doi.org/10.1016/j.cbpb.2009.11.014.

Zhang, D.; Earp, B.E.; Blazar, P. 2019. Risk factors for skin tearing in collagenase treatment of Dupuytren contractures. The Journal of Hand Surgery, 44(12): 1021-1025. http://dx.doi.org/10.1016/j.jhsa.2019.06.010.

Zhao, J.Z.; Blazar, P.E.; Mora, A.; Rozental, T.D.; Earp, B.E. 2019. Clinical outcomes of collagenase injections during a surgeon’s initial learning phase. Journal of Hand Surgery Global Online, 1(3): 161-165. http://dx.doi.org/10.1016/j.jhsg.2019.04.001.