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Research Article

Purification and enhancement of the thermal stability of pepsin enzyme from *Scomberomorus commerson* stomach by polyethylene glycol graphene oxide

Shobar S.R.[1], Rezaei M.[1*], Naghdi S.[1], Taghavi Moghadam A.[2]

1 Seafood Processing Department, Marine Sciences Faculty, Tarbiat Modares University, Noor, Iran
2 Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Ahvaz, Iran
*Correspondence: rezai_ma@modares.ac.ir

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Abstract

The viscera of narrow-barred Spanish mackerel (*Scomberomorus commerson*) fish contains abundant alkaline and acidic proteases that can be isolated using various methods. We purified pepsinogen from *S. commerson* viscera using ammonium sulfate precipitation, Sephadex G-50 gel filtration, and DEAE-cellulose ion-exchange chromatography. Two peaks corresponding to pepsinogen were observed during gel filtration and further purified using ion-exchange chromatography. Additionally, we investigated the effects of polyethylene glycol graphene oxide (GO-PEG) at a concentration of 3 mg/mL on enzyme activity and stability. Results demonstrated a significant enhancement in enzyme activity and increased stability at higher temperatures compared to the control group. These findings suggest that GO-PEG can improve enzyme production and stability. However, purified pepsin was found to be inactive in the presence of 0.1 M pepstatin A. In conclusion, *S. commerson* viscera offers a valuable source for pepsin purification. The purification process involving ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography successfully obtained purified pepsinogen. The use of GO-PEG exhibited promising potential in enhancing enzyme activity and stability, highlighting the role of nanosheets in improving enzyme thermal stability.

Introduction

As the aquaculture and fishing industries have grown significantly over the last decade, the marine processing industry has also experienced significant growth (De Melo Oliveira *et al.*, 2020; Bahram *et al.*, 2022). Therefore, this results in the production of a large number of raw remains, including 30 to 60 percent of the original weight of each fish, during the seafood processing of aquatic animals (Ribeiro Cardoso dos Santos *et al.*, 2020; Lamas and Massa, 2023; Shaviklo *et al.*, 2023). Fishmeal, fish oil, and fertilizer are currently produced from these wastes, whereas these products could be used to create more economically valuable materials (Mohanty, 2020; Naghdi *et al.*, 2023a). So, a suitable way to decrease production costs is to produce high-value products such as peptides, omega-3 fatty acids, sulfated polysaccharides, and enzymes (Taktak *et al.*, 2021; Naghdi *et al.*, 2023 b, c). Moreover, these products may be worth much more than their original products (Akanbi *et al.*, 2019; Ribeiro Cardoso dos Santos *et al.*, 2020). Due to the high concentration of digestive enzymes in marine animals' viscera, which make up a large part of their by-products, researchers and industry attention has been drawn to the extraction and purification of enzymes, especially aspartic proteases (pepsin) in the stomach and serine proteases (trypsin, chymotrypsin, collagenase, and elastase) in the viscera (Akanbi *et al.*, 2019; Ribeiro Cardoso dos Santos *et al.*, 2020; Naghdi *et al.*, 2023 b). Pepsin, which is created by changes in the pepsinogen structure when exposed to hydrochloric acid (HCl), is a marine enzyme that belongs to the class of aspartic proteases isolated from various sources and is used in a wide variety of industrial processes, such as collagen extraction, cheese production, fish silage production, fish processing, and medical research (Saranya and Jayapriya, 2018; Akanbi *et al*., 2019). Furthermore, marine enzymes have demonstrated excellent activity across different temperatures and pH ranges compared to enzymes derived from land animals (Zhao *et al.*, 2013). In this regard, various methods have been used to extract, purify, and identify enzymes, including ammonium sulfate precipitation, gel filtration, anion exchange chromatography, and polyacrylamide gel electrophoresis (Saranya and Jayapriya, 2018; Akanbi *et al*., 2019). It is worth noting that some methods, such as the use of nanoparticles and materials with unique features in enzyme extraction and purification, have received less attention, while they can be effective and appropriate methods. In this regard, few studies have been published on applying and using some materials and nanoparticles, such as graphene oxide nanosheets, to improve enzyme activity.

Today, graphene and graphene oxide have received much attention due to their properties, such as their large specific surface areas that can be ideal for the adsorption of many compounds (Naghdi *et al.*, 2022). Further, they are useful for absorbing and transporting biomolecules on surfaces (Yao *et al.*, 2015). However, it is important to note that graphene has defects, such as low solubility, which can be solved by modifying their surfaces (Chaudhary *et al.*, 2021). In light of this,

previous studies have found that increasing graphene solubility and dispersibility increases its biological safety (Chaudhary *et al.*, 2021; Naghdi *et al.*, 2022). A clear example of this problem can be found in graphene, which is very hydrophobic and lacks hydrophilic groups containing oxygen, which are essential to aqueous solution solubility. It is known that graphene oxide is soluble in water, but it accumulates in physiological buffers when present in salt (Mao *et al.*, 2021). Due to these reasons, polymer-graphene hybrids have been investigated to improve biocompatibility and decrease toxicity (Chaudhary *et al.*, 2021; Naghdi *et al.*, 2022). Polyethylene glycol has been widely used, as a biocompatible polymer to carry drugs and modify graphene and other nanomaterials (Catania *et al.*, 2021).

Narrow-barred Spanish mackerel (*Scomberomorus commerson*) is an epipelagic and oceanic species that lives in shoals in shallow coastal waters (10 to 70 meters deep) and is usually associated with coral reefs and shallow rocky areas. It is an important fish worldwide in commercial, traditional, and recreational fishing (Rengasamy *et al.*, 2016). According to the FAO report, the global catch of this species by 2020 was approximately 295 thousand tons.

Based on the above statements, this study aimed to purify and characterize the biochemical properties of pepsin obtained from the stomach of *Scomberomorus commerson* and investigate the effects of nanographene oxide on increasing pepsin recovery and thermal stability.

Material and methods

Collecting S. commerson viscera

Fresh *S. commerson* was prepared from a local fish market located in Bandar Abas (Iran). After washing and cleaning the fish, the guts and intestines were separated, and the fish stomach was washed well until its internal contents were empty. It was packed in plastic bags and placed in a 1:3 ice pack.

Preparation of the raw extract

The fish stomachs were transferred to an ultra-freezer at -80°C and put in a standard refrigerator for defrosting. The fish stomach was cut into smaller pieces and then ground. Using a homogenizer, it was ground and homogenized with cold acetone in a ratio of 1:3. Afterward, the homogenized mixture was filtered with filter paper overnight at room temperature and the next day in a 1:10 ratio with 20 mM Tris-HCL buffer with a pH equal to 7.5 for three hours. Then, it was centrifuged at 15,000 g for 20 min at 4°C. Next, the supernatant solution from the centrifuge was collected as a crude pepsinogen extract (CPS) and stored in a freezer at -20°C (Rengasamy *et al.*, 2016).

Ammonium sulfate precipitation

Precipitation of pepsin enzyme and other proteins was performed with different percentages of ammonium sulfate ($(NH_4)_2SO_4$), then the precipitate was gathered by centrifugation for 15 min at 10,000g. Finally, the precipitates were suspended in extraction buffer (tris-buffer (10 mM Tris–HCl, pH 8.0) 1:10 (vol/vol), which was changed three times with the same buffer during this process (Wu *et al.*, 2009).

Gel filtration

Sephadex G 50, 100, and diethylaminoethyl-cellulose ion exchange gel filtration columns were used to purify the studied proteins. First, the Sephadex G column was washed with 20 mM Tris-HCl buffer with a pH of 7.5 to prepare the medium for separating the target protein. After that, the supernatant was filtered again with a 0.45-micron filter. The dialysis sample was centrifuged for 15 minutes at 4°C at 5000g to settle and separate the suspended particles in the sample. Then the filtered sample was slowly injected into the chromatography column and washed with 20 mM Tris-HCl buffer with a pH of 7.5. Fractions of 2.5 mL were collected from the column at a flow rate of 5 mL/hour, and then protein absorption of each fraction was performed with a spectrophotometer at a wavelength of 280 nm. Next, pepsin enzyme activity and its protein concentration were determined in each obtained peak. Afterward, the peak related to the pepsinogen enzyme was determined, and the fraction related to this peak was collected (Weng *et al.*, 2011).

Anion exchange chromatography

The pepsinogen enzyme fractions obtained from the purification step with gel filtration chromatography columns were injected into the diethylaminoethyl-cellulose ion exchange chromatography column for final purification. Before injection, the sample was concentrated in a freeze-dried system and then dissolved in 5 mL of 20 mM Tris-HCl buffer (pH 7.5), and dialysis was performed overnight at 4 °C. The chromatographic column was washed with 20 mM Tris-HCl buffer (pH 7.5) to reach equilibrium. The column was washed with a concentration gradient of buffer D (20 mM Tris-HCL, pH 7.5) containing 0.5 M NaCl. A fraction of 2.5 mL was collected at a 10 mL/h flow rate, and its absorption was determined at 280 nm. Also, dialysis was performed with 20 mM Tris-HCl buffer with pH equal to 7.5 with three changes at 4°C. The protein absorption of each fraction was recorded. Then obtained fractions were concentrated in a freeze dryer and stored in a freezer (Wu *et al.*, 2009).

Polyacrylamide gel electrophoresis

Using Laemmli (1970) SDS-PAGE method, the purity and protein bands related to the pepsin enzyme were verified. A protein marker was used to determine the molecular weight of the purified pepsin enzyme based on the movement of proteins along the gel or the Rf index. The gel is first placed on a clean, dry glass surface and faced towards the light source. Then, the gel length from the beginning of the separation gel to the blue bottom line of the gel (Dye front) and the distance of each band related to the protein marker from the beginning of the gel was determined with a ruler. Next, the value of Rf (the ratio of the distance of each band from the beginning of the gel to the total length) was calculated. The Rf graph was drawn with the logarithm of the molecular weight of each protein band on the X axis, and the Rf value of each one of the bands was written on the Y axis. Then, the Rf of the band corresponding to the purified enzyme sample was obtained and placed in

the Rf chart, and its molecular weight was determined (Rengasamy *et al.*, 2016).

Synthesize of graphene oxide nanosheet

Graphene oxide nanosheet was synthesized by the Hammer method. Raman spectroscopy and FTIR analysis were used to evaluate the GO nanosheets.

The development of NGO-PEG (polyethylene glycol graphene oxide nanosheet) nanosheets from graphene oxide nanosheets

Polyethylene glycol of nanographene oxide (NGO-PEG) was synthesized. Briefly, the graphene oxide (GO) was sonicated for 30 minutes and then centrifuged at 15,000 rpm to collect the lamellar crushed graphene oxide supernatant. This step was repeated until 500 mL of the supernatant was collected. The supernatant was sonicated for 30 minutes. Next, to prepare NGO-COOH, 1.2 g of NaOH and 1.0 g of $ClCH_2COONa$ were added to the nanographene oxide suspension and then subjected to ultrasound for 3 hours. The resulting solution was adjusted to pH 7 with 1 M hydrochloric acid. NGO-COOH was repeatedly washed with double-distilled water and kept overnight in a dry environment under a vacuum. To synthesize PEG-amine, GO-COOH was diluted with double distilled water. 10 mL of GO-COOH was placed in an ultrasonic bath with 100 mg of PEG-amine for 10 minutes. 19 mg of (N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride) EDC was stirred in the reaction mixture for another 30 minutes in an ultrasonic bath. Finally, 76 mg of EDC was added to the reaction mixture and stirred for 12 h. The reaction was stopped by adding mercaptoethanol and dialyzed in double-distilled water using a 300 kDa dialysis bag for three days to remove all reaction compounds. The final product (NGO-PEG) was collected in the supernatant after centrifugation at 15,000 rpm for three hrs. Then, the resulting solution was used to absorb the pepsin enzyme (Liu *et al.*, 2008).

Protein concentration

Protein concentration was measured by recording the absorbance at 280 nm of the sample solution or by Lowry *et al.* (1951) with bovine serum albumin as standard.

Pepsin activity assays

Hemoglobin substrate was used to determine pepsin enzyme activity because hemoglobin is broken down by an enzyme and converted into the amino acid tyrosine (Wald *et al.*, 2016). Its absorbance was determined at 280 nm wavelength. First, 1.25 mL of hemoglobin substrate solution was added to two test tubes. Then they were placed at 37°C for the same temperature. Then, 250 microliters of the sample diluted with 0.01 N hydrochloric acid was added to one of the tubes and placed at 37°C for 10 minutes. After this step, 2.5 ml of 5% trichloroacetic acid was added to the sample tube to stop the reaction. 2.5 mL of 5% trichloroacetic acid and then 250 microliters of the diluted sample with 0.01 N hydrochloric acids were added to the control tube. Then, the sample and control tubes were placed at 37°C for 5 minutes. Next, they were

centrifuged at 4,000 g for 20 minutes at 4°C, and the supernatant liquid, which had a clear appearance, was collected. Then, the absorbance of the control tube and the sample was recorded at a wavelength of 280 nm using a spectrophotometer. The unit of pepsin enzyme activity is calculated in terms of micromoles of tyrosine released in one minute per milligram of tissue weight and mg of protein from the following formula:

$$unit/\ mg\ protein = \frac{A280\ sample\ - A280\ blank\ \times 1000\ \times 4}{1250\ \times 10 \times mg/ml\ protein} \times \mathrm{DF}$$

Where, DF was the dilution factor, 1250 was the molecular constant coefficient for tyrosine, and 10 was the incubation period for the sample.

The specific activity, purification fold, and percent protease recovery were calculated by the following formula:

Total pepsin enzyme activity = enzyme activity × total volume
Specific activity (U/mg) = Enzyme activity (U/mL) / Total protein (mg/mL)
Purification fold = Specific activity of a purified enzyme / The specific activity of a crude enzyme
% Protease recovery = Total activity × 100 Total activity of a crude extract

Biochemical properties

Pepsinogen activation

Pepsinogen was converted into pepsin by decreasing the initial solution pH to 2 with 0.1 M hydrochloric acid. Then, activated pepsin molecules activate other pepsinogens (Bougatef *et al.*, 2008).

Effect of temperature on enzymatic activity and thermostability

The impact of temperature on the activity of the enzyme in the sample was examined by conducting experiments at various temperatures, ranging from 5 to 70°C, with casein as the substrate. All treatments were conducted at a pH of 8.0 for a duration of 20 minutes. To assess thermal stability, the samples were incubated at different temperatures for 30 minutes in a water bath (model W350, Memmert, Germany), and the caseinolytic activity was determined using casein as the substrate under standard assay conditions. The sample that did not undergo any thermal pretreatment was used as the reference and considered to have 100% activity (Zhou *et al.*, 2008).

Effect of pH on enzymatic activity and stability

To determine the pH stability of the enzyme, firstly, enzyme extract with a volume equal to each of the following buffers with different pH (1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6. 6,5, 7) were combined and incubated for 15 minutes at 25ºC. Then, the incubated sample was mixed with the substrate-buffer solution and placed at 37ºC for 15 minutes. Then pepsin enzyme activity was measured. The relative activity of the pepsin enzyme was calculated through the ratio of enzyme

activity to the activity of the control sample (without incubation in a buffer with different pH) (Zhou *et al.*, 2008).

Effects of enzyme inhibitors on pepsin enzyme activity

The effect of different ratios of pepstatin A on pepsin enzyme activity was evaluated. For this purpose, the enzyme sample was incubated for 15 minutes at room temperature. The mixture was incubated with the substrate solution and was placed at 37ºC for 15 minutes. Then pepsin enzyme activity was measured. The test method of the control sample was the same as the enzyme sample, and only the control sample did not have an inhibitor. The percentage of the ability of the pepsin enzyme is calculated through the ratio of enzyme activity to sample activities (Wald *et al.*, 2016).

Effect of metallic ions

The enzyme sample was combined with different concentrations of bovine hemoglobin substrate, including 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28, 1.5, and 2 mM, as described by Wald *et al*. (2016). The mixtures were then incubated at 37 ºC for a duration of 15 minutes. Maximum enzyme activity (Vmax), Michaelis-Menten constant (Km), catalytic constant or conversion number (Kcat), and catalytic efficiency (Kcat/Km) were calculated by plotting the data on a Lineweaver–Burk double-reciprocal graph. $V_{max} = 1/a$ ، $K_m = b \times V_{max}$

The molar concentration of the purified enzyme was calculated based on the molecular weight determined from SDS-PAGE and the concentration of the purified enzyme from chromatography to assess the [E] value. The catalytic constant (Kcat) was calculated from the relation Vmax / [E].

Statistical analysis

One-way ANOVA and Duncan's test *(p<0.05)* were applied to determine the statistical differences between the data in different sections. All of the treatments were done in triplicate.

Results

Protein concentration and enzyme activity

The crude pepsinogen extract obtained from the stomach of *S. commerson* (CPS) was compared with crude pepsinogen enzyme extracts from bovine, sheep, and commercial pig pepsin enzymes in terms of several characteristics, including protein concentration of the crude extract and enzyme activity. These comparisons were conducted under the same conditions, and the results are presented in Table 1. The results indicate that CPS exhibited significantly higher protein concentration and enzyme activity compared to the crude extracts from cows and sheep but lower than the commercial pig enzyme *(p<0.05).* Furthermore, CPS was further analyzed using a G50 gel filtration chromatography column. Figure 1 illustrates that CPS exhibited eight distinct peaks, each representing a range of proteins with similar molecular weights. Complete purification of the crude extract would result in only one remaining peak. Additionally, during the measurement of protein concentration and enzyme activity of the peaks, it was discovered that peaks 2, 3, and 4 are associated with pepsinogen

and pepsin enzymes (protein concentration and enzyme activity data not shown).

Ammonium sulfate precipitation of pepsinogen from CPS

According to Figure 2, the CPS protein precipitation increased as the percentage of ammonium sulfate augmented. However, this upward trend continued until the ammonium sulfate solution became saturated. At concentrations higher than 67.5%, there were some insoluble parts of ammonium sulfate in the container. The highest rate of protein precipitation (59.92%) was observed with ammonium sulfate at the highest saturation percentage (67.5%). This fraction also exhibited the highest enzyme activity due to the presence of the most precipitated proteins (data not shown).

Table 1: Comparison of characteristics of obtained crude pepsin extracts from *S. commerson*, cow, and sheep with commercial pepsin enzyme.

The source of pepsin	RF	Protein %	Total protein (U.mg)	Total activity (U)	Specific activity (U.mg)
CPS	4.41	5.4	540	107.3	0.87318 [b]
Cow (Crude)	2.7	13.4	1340	75.37	0.1512 [d]
Sheep (Crude)	2.25	10.456	1045.6	136.57	0.2925 [c]
Pig (Commercial)	10	4.78	478	255.8	5.35 [a]

Different letters in the column indicate a statistically significant difference at the 5% level ($p<0.05$; n=3).

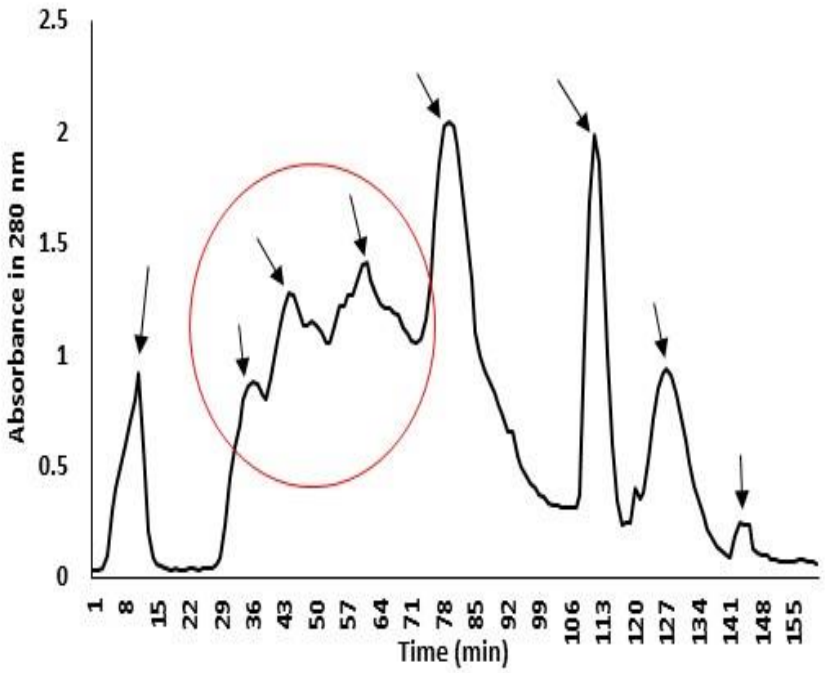

Figure 1: Chromatograms of the crude pepsinogen extract obtained from the stomach of *S. commerson* (CPS) on Sephadex G-50.

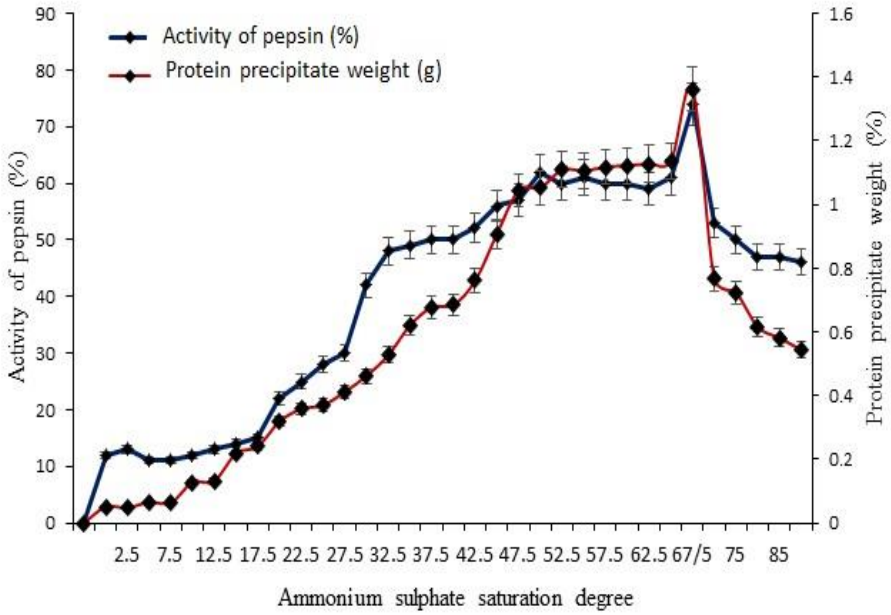

Figure 2: The amount of precipitated protein obtained from *S. commerson* stomach precipitated with different percentages of ammonium sulfate and their enzyme activity.

Purification of pepsin enzyme from S. commerson viscera

Table 2 presents the results of the purification of pepsin enzyme from *S. commerson* viscera in seven steps. Initially, the viscera extract was obtained using two buffers, one containing NaCl and $CaCl_2$ and the other without them. The results showed that increasing the concentrations of NaCl and $CaCl_2$ to 0.6 M and 30 mM, respectively, significantly increased the crude extract protein content and enzyme activity. Further purification was carried out using the crude extract from the buffer containing NaCl and $CaCl_2$. Next, it was acidified at pH 4 to remove some sensitive proteins, resulting in a 1.5-fold increase in purity compared to before this step. The extract was then precipitated using ammonium sulfate at 67.5% saturation, leading to a significant increase in purity. Subsequently, the use of 18% aluminum hydroxide gel further increased the purity by 2.25 times. To further purify the partially purified enzyme, a G50 gel filtration column was employed, resulting in the separation of two fractions (Fig. 3). Activity analysis determined that the second fraction was pepsin, which was further purified using DEAE-cellulose ion exchange in the final step (Fig. 4). Different letters in the column indicate a statistically significant difference at the 5% level (p<0.05; n=3).

Table 2: The results of the different purification steps.

Purification step	Parameter						
	Total volume (mL)	Total protein (mg)	Protein (mg.mL)	Activity (U)	Specific activity (U.mg)	Purity (fold)	Yield (%)
Step 1: Crude extract	100	1160	11.60	4060	3.5 [f]	1	100
Step 2: Acidification	44.39	515	5.15	2706	5.25 [e]	1.5	66.65
Step 3: Dried sulfate ammonium	6.57	76.26	0.76	1041	13.65 [d]	3.9	25.64
Step 4: Saturated ammonium sulfate	1.15	13.39	0.13	436	32.55 [c]	9.3	10.73
Step 5: Gel filtration Sephadex G-50	0.22	2.62	0.02	193	73.5 [b]	21	4.75
Step 6: Anion-exchange DEAE-cellulose	0.10	1.17	0.011	129	110.25 [a]	31.5	3.17

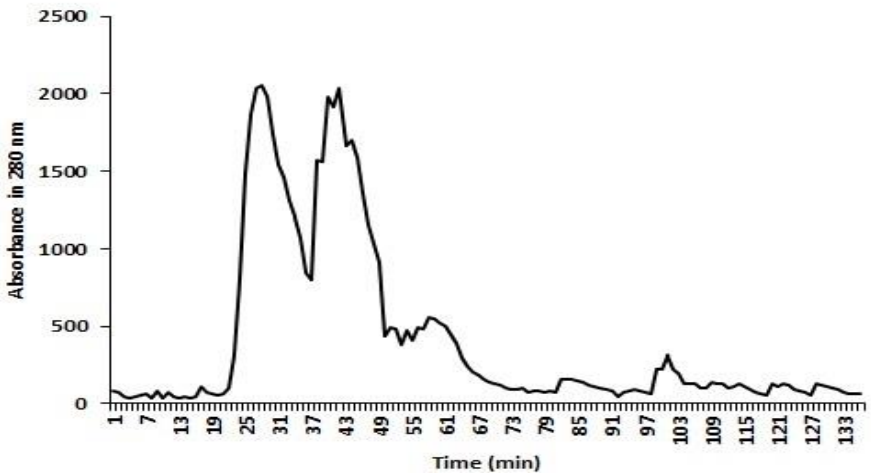

Figure 3: Gel filtration Sephadex G-50 of obtained pepsin from the step of enzyme purification by saturated ammonium sulfate.

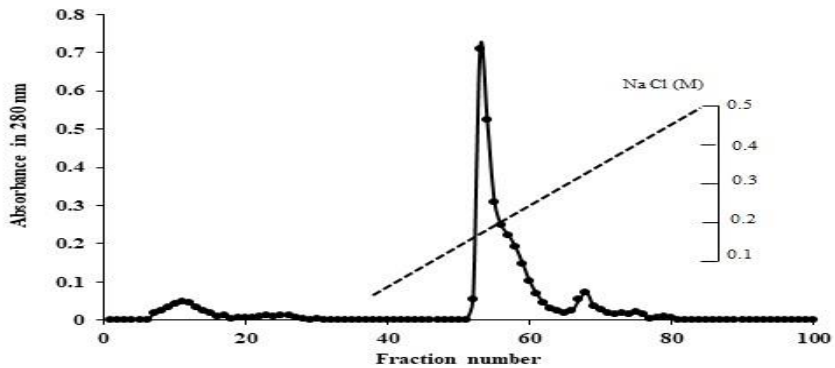

Figure 4: Anion-exchange DEAE-cellulose of obtained pepsin from step 7 of purification.

Biochemical characterization of pepsin purified S. commerson viscera

Optimal temperature and temperature stability

Figure 5a demonstrates that purified pepsin exhibits its highest activity at 37°C, with activity continuing up to 45°C. However, activity ceases at temperatures higher than 45°C.

Optimum pH and pH stability

Figure 5b illustrates the results of investigating pepsin enzyme activity at different pH levels. The highest activity of the pepsin enzyme was observed at pH 2, and it remained active up to pH 4.5.

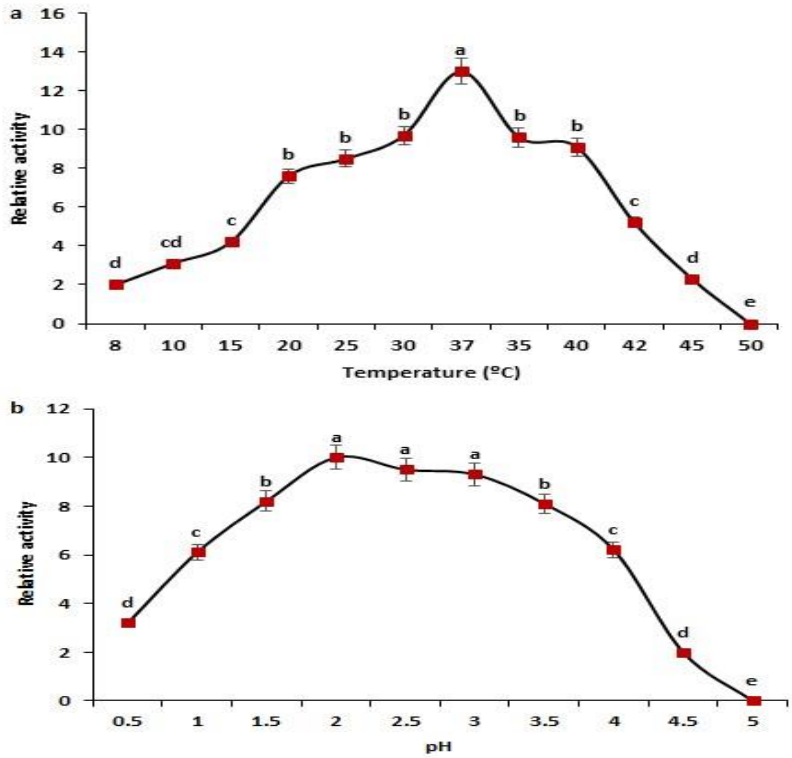

Figure 5: (a) Optimal temperature of purified pepsin. (b) Optimum pH of purified pepsin.

Effect of metal ions on the protease activity

Table 3: Effect of metal ions on the activity of purified pepsin from *S. commerson* viscera and commercial pig pepsin.

Metal ions	Concentration (mM . L)	Purified pepsin residual activity (%)	Pig pepsin residual activity (%)
Control	-	100 ± 0.00	100 ± 0.00
KCl	2	100 ± 0.90	99.1 ± 0.82
	5	100 ± 1.20	98.9 ± 0.91
NaCl	2	107 ± 1.12	105 ± 1.24
	5	112 ± 1.34	106 ± 1.43
$CaCl_2$	2	105 ± 0.96	102.7 ± 1.12
	5	108.2 ± 0.98	104.2 ± 0.97
$MgCl_2$	2	103.0 ± 1.20	103.8 ± 1.18
	5	107.2 ± 1.32	105.15 ± 1.23

Means ± Standard deviation; n=3.

Effect of protease inhibitor (pepstatin A)

The inhibitory effect of pepstatin on the activity of the pepsin enzyme purified from *S. commerson* viscera was observed to be complete at a concentration of 0.1 M.

Characterization of GO

Fourier transform infrared spectroscopy (FTIR) of GO

The result of FTIR of GO is shown in Figure 6 a. As seen in Figure 6 a, the visible peak in the 3407 cm^{1} is related to the stretching vibration of -OH in lactone and carboxylic acid groups (Naghdi *et al.*, 2022). A carboxylic acid group C=O stretching vibration can be attributed to the bond at 1731 cm^{-1}. Also, the sharp peaks at 1622 cm^{-1} and 1038 cm^{-1} are related to the bond of C=C and the alkoxy group C-O bond (Shin *et al.*, 2016). Besides, the peak around 1224 cm^{-1} is connected to the epoxy group C-O-C bond, indicating that functional groups contain abundant oxygen on the surface of GO nanosheets (Shin *et al.*, 2016; Naghdi *et al.*, 2022).

Atomic force microscopy (AFM) of GO

According to Figure 6 b, the results of the Atomic Force Microscopy (AFM) test indicate that the thickness of the synthesized graphene oxide sheets falls within the range of 0.4-0.6 nm, implying the presence of a monolayer and high quality of the synthesized graphene oxide. These findings align with those reported by Naghdi *et al.* (2022).

Raman spectroscopy of GO

The Raman spectroscopy analysis of graphene oxide revealed important information about its carbon structure. Figure 6 c displays the results of the Raman analysis. In the spectrum obtained, a vibrational band (Band D) belonging to SP3 carbon atoms was observed in the region of 1340 cm^{-1}. According to Naghdi *et al.* (2022), this range corresponds to carbon atoms that have oxygenated functional groups attached to them. Additionally, in the region of 1590 cm^{-1}, a vibrational band (Band G) belonging to SP2 carbon atoms was observed. This finding indicates the presence of intact carbon atoms within the graphite network. It is worth noting that the position of the D2 band for monolayer graphene is typically observed at 2679 cm^{-1}. However, for multilayer graphene, which contains 2-4 layers, the D2 band position shifts to higher wavenumbers (Wei *et al.*, 2016).

Investigating the effect of GO-PEG on the enzyme activity of purified S. commerson pepsin enzyme

In terms of solubility in water, the order of solubility for G, GO, and GO-PEG is as follows: GO-PEG > GO > G. Consequently, it was necessary to enhance the solubility of graphene oxide in water by incorporating polyethylene glycol (PEG) after synthesis. Subsequently, GO-PEG, which contains both oxide and hydroxide groups, was employed at different concentrations to adsorb pepsinogen from the stomach extract of *S. commerson*. The results (Fig. 7) demonstrated that 1 mg of GO-PEG to 3 mg of GO-PEG resulted in increased enzyme activity, followed by a relatively stable trend. Interestingly, the highest pepsin activity (67.67) was observed when using an aluminum hydroxide adsorbent at 18% concentration.

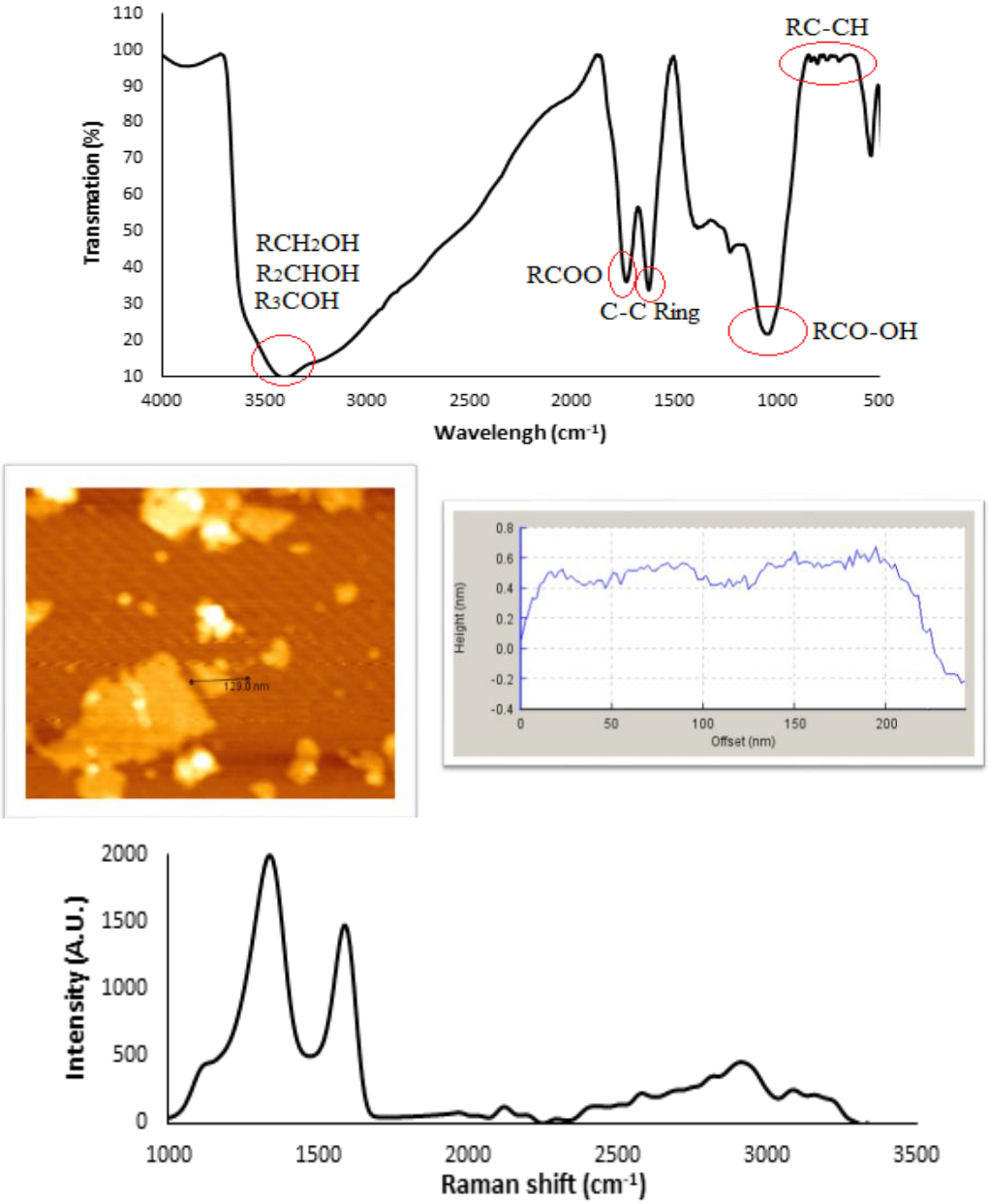

Figure 6: (a) FTIR spectrum, (b) AFM, and (c) Raman spectroscopy of GO nanosheet.

Whereas with GO-PEG, it was observed at a concentration of 3%. Notably, GO-PEG at a concentration of 3 mg/mL exhibited a pepsin enzyme activity nearly 1.5 times higher than that of enzymes adsorbed on an 18% concentration of aluminum hydroxide.

Investigation of the Effect of GO-PEG on the Thermal Stability of Purified Pepsin Enzyme from S. commerson

The results obtained in our study regarding the effect of GO-PEG on the thermal stability and activity of the purified pepsin enzyme from *S. commerson* provide (Fig. 8) valuable insights into the potential applications of graphene-based materials in biotechnology.

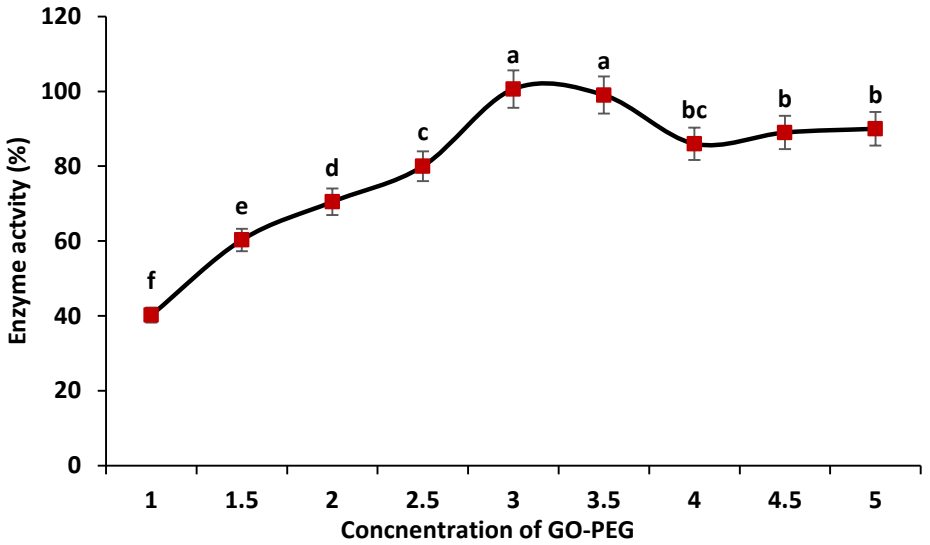

Figure 7: Investigating the effect of different concentration of GO-PEG on the pepsin enzyme activity purified from *S. commerson*.

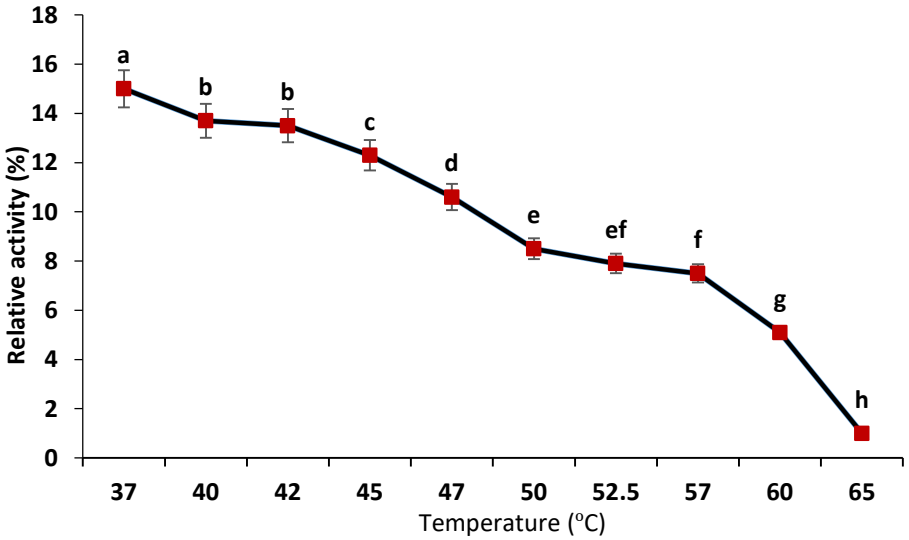

Figure 8: Investigating the effect of GO-PEG on the thermal stability of pepsin enzyme purified from *S. commerson*.

Discussion

The obtained difference in protein concentration and enzyme activity between CPS and the crude extracts from cows and sheep can be attributed to the fact that CPS comes from a fish with a carnivorous diet, whereas cows and sheep are grass-fed. Moreover, the commercial pig enzyme was completely pure, while the three prepared crude extracts were not purified (Bastos *et al.*, 2020). The protein concentration of CPS can vary due to several factors, including the source of extraction, catching season, and the animal diet (Wald *et al.*, 2016). It has been proven that animals with a carnivorous diet have more active stomachs and produce higher levels of pepsin enzymes (Zhao *et al.*, 2011). As a result, more pepsinogen and pepsin can be extracted and purified from their mucus and stomach walls (Zhao *et al.*, 2011). When only gastric mucus is

used for extraction, the protein concentration tends to be lower, but the enzyme activity is higher (Ribeiro Cardoso dos Santos *et al.*, 2020). On the other hand, if the raw extract is derived from stomach tissue, the protein concentration will be higher, but the enzyme activity may be lower due to the presence of non-specific proteins in the stomach walls and tissue (Nayak *et al.*, 2021). Additionally, the extraction method employed also influences the protein concentration and, consequently, the enzyme activity (Mohanty and Majumdar, 2020).

It has been reported that the initial step in purifying proteolytic enzymes involves an ammonium sulfate solution use. Adding ammonium sulfate to enzyme extracts increases the surface tension of water molecules, leading to enhanced hydrophobic interactions between proteins and water. Consequently, the proteins reduce their surface area to minimize contact with water, resulting in precipitation. In a study by Bougatef *et al.* (2008), ammonium sulfate saturated to 20% to 70% was used firstly in purifying pepsin from *Mustelus mustelus*. Subsequently, further purification was performed using gel filtration Sephadex G-100 and anion-exchange DEAE-cellulose. Similarly, Klomklao *et al.* (2006) utilized 30-70 percent saturated ammonium sulfate to purify the crude enzyme extract from *Coryphaenoides pectoralis*. Wald *et al.* (2016) purified both pepsinogen and pepsin from rainbow trout (*Oncorhynchus mykiss*) stomachs using ammonium sulfate saturation ranging from 30% to 50%. They also employed Sephadex G-100 and ion-exchange DEAE-cellulose for additional purification of the enzymes. Sholeh *et al.* (2020) examined the purification of pepsin and pepsinogen from *Hemigaleus balfouri* stomach using varying degrees of ammonium sulfate saturation, ranging from 0-20% to 20-40%, 60-40%, and 80-60%. They discovered that the highest enzyme activity was observed at the 20-40% degree of saturation.

The final step in purification resulted in a specific activity of 110.25 (U/mg), which was 1.5 times higher than the previous step. Precipitation with ammonium sulfate is commonly employed as one of the initial steps in protein purification to separate unwanted proteins. It is an effective method to reduce the volume of the tested mixture. High concentrations of ammonium sulfate can decrease the solubility of proteins by removing water molecules, leading to their precipitation. Each protein has a specific range of ammonium sulfate concentrations in which it precipitates, depending on its characteristics. Maintaining a constant pH and temperature during the experiments is critical to ensure repeatability. Based on the results of the present study, 70% saturated ammonium sulfate was utilized for protein precipitation. However, protein concentration, specific enzyme activity, purity, and yield for obtained pepsin by the seven purification steps, including Step 1: Crude extract 1, Step 2: Crude extract 2, Step 3: Acidification, Step 4: Dried sulfate ammonium, Step 5: Saturated ammonium sulfate, Step 6: Gel filtration Sephadex G-50, Step 7: Anion-exchangege DEAE-cellulose, were (8.36, 2.69, -,-), (11.60, 3.5, 1, 100), (5.15, 5.25, 1.5, 66.65), (0.76, 13.65, 3.9, 25.64), (0.15, 30.73, 7.78,

11.37), (2.62, 0.02, 73.5, 4.75), and (0.011, 110.25, 31.5, 3.17), respectively. Yoko Miura *et al.* (2015) conducted a study in which they purified the pepsinogen enzyme using three different chromatography methods, including DEAE-Sephacel, Sephadex G100, and MonoQ FPLC. Their results indicated that the protein concentration, enzyme activity, and yield for these three methods were (14/ 0, 18, 16), (14, 38, 0.05), and (0.017, 51, 5.8), respectively (Miura *et al.*, 2015). The results of Weng *et al.* (2011) indicated that the protein concentration, enzyme activity, efficiency, and purity of pepsin enzyme purified from *Monopterus albus Zuiew* fish using saturated ammonium sulfate, DEAE-Sephacel, and Sephacryl S-200 were (3.35, 2, 68.2, 1.1), (0.09, 6.3, 6.4, 3.5) and (0.017, 15.1, 2.7, 8.4), respectively. In a similar study, Nalinanon *et al.* (2010) investigated the purification of the pepsin enzyme from *Thunnus alalunga*. Their findings showed that the crude extract had a protein concentration of 99.89 and an enzyme activity of 0.124 U. For further purification, five steps were used, including Sephacryl S-200, Sephadex G-50, DEAE-cellulose, Sephadex G-50, and DEAE-cellulose. As a result of purification steps including steps one, two, three, four, and five, the concentration, enzyme activity, efficiency, and purity of pepsin were found to be (18.50, 0.275, 39, 2), (0.87, 3.53, 25, 168), (19 0, 10.8, 17, 513), (0.10, 12.8, 10, 608), and (0.04, 13.8, 0.5 and 658), respectively. Our results in the current study were similar to those in the above investigation.

The results of optimal temperature and temperature stability of pepsin purified *S. commerson* viscera are consistent with the findings of Wald *et al.* (2016) in rainbow trout (*Oncorhynchus mykiss*) and Miura *et al.* (2015) in largemouth bass (*Micropterus salmoides*). The optimal temperature range and temperature stability are two crucial indicators of pepsin activity (Miura *et al*., 2015; Wald *et al*., 2016). Deviating from the optimal temperature, either lower or higher, decreases the activity of fish pepsin. The optimal temperature for pepsin varies among different fish species (Morellon-Sterling *et al.*, 2022; Patil *et al.*, 2022). Cold-water fish generally have lower optimal temperatures compared to warm-water fish. Different types of fish pepsin have distinct optimal temperatures, and pepsin derived from cold-water fish tends to have lower optimal temperatures than that from warm-water fish (Salelles *et al.*, 2021; Stanforth *et al.*, 2022). This discrepancy can be attributed to the lower activation energy for Arenos formation in pepsin enzymes obtained from cold-water fish, which explains their lower optimum temperature and higher temperature stability compared to those from warm-water fish (Morellon-Sterling *et al.*, 2022; Stanforth *et al.*, 2022). Nalinanon *et al.* (2010) reported that pepsin obtained from albacore tuna exhibited temperature stability within the range of 20°C to 50°C, with a significant decrease in stability observed at temperatures above 50°C. A decrease in enzyme activity at higher temperatures is often attributed to denaturation and structural disruption of pepsin (Murthy *et al.*, 2018; Patil *et al.*, 2022).

Similar results to those obtained in optimum pH and pH stability evaluation

were reported by Wald *et al.* (2016) for purified pepsin from rainbow trout (*Oncorhynchus mykiss*) and by Miura *et al.* (2015) for pepsin from largemouth bass (*Micropterus salmoides*). The optimal pH (the pH at which enzyme activity is maximum) and pH stability (the pH range at which the enzyme is active) significantly affect the pepsin enzyme activity. Enzyme activity decreases when the pH deviates from its optimal value (Morellon-Sterling *et al.,* 2022). Pepsin is an acidic protease, and its activity is maintained at low pH values. Its activity has been attributed to protein denaturation at pH above 6 (Wald *et al.,* 2016; Morellon-Sterling *et al.,* 2022; Stanforth *et al.,* 2022). Generally, if a studied fish species has multiple types of pepsin, their optimal pH values are usually similar. However, this may not be the case for all species (Stanforth *et al.,* 2022).

The study found that the activity of both pepsin enzymes was increased by metal ions such as NaCl, $CaCl_2$, and $MgCl_2$ at concentrations of 2 and 5 mM, compared to the control samples. However, the KCl ion at both concentrations showed no significant difference in activity compared to the control samples. The effect of calcium and magnesium salts on pepsin enzyme activity extracted from fish in the present study is consistent with the findings of El-Beltagy *et al.* (2004) and Klomklao *et al.* (2006). However, it is interesting to note that $MgCl_2$ and $CaCl_2$ had no effect on the activity of pepsin obtained from *Thunnus alalunga* (Nalinanon *et al.,* 2010), *Thunnus tonggol*(Nalinanon *et al.,* 2008), *Scophthalmus maximus* (Wang *et al.,* 2006), and *Coryphaenoides pectoralis* (Klomklao *et al.,* 2007). In contrast, NaCl had an increasing effect on pepsin activity in some fish species and a decrease in others. It should be mentioned that metal ions can act as cofactors in increasing pepsin enzyme activity or, in some cases, decrease it (Bougatef, 2013; Stanforth *et al.,* 2022). It has also been observed that divalent cations have a more pronounced effect on pepsin enzyme activity compared to monovalent cations (Bougatef, 2013).

Pepstatin is a specific inhibitor that acts on aspartic proteases, including fish pepsin. It binds to fish pepsin and prevents binding the enzyme to its substrate, resulting in complete inhibition of its activity (Wald *et al.,* 2016; Stanforth *et al.,* 2022). Wald *et al.* (2016) reported that purified pepsin from the stomach of rainbow trout (*Oncorhynchus mykiss*) was completely blocked by an equimolar ratio of pepstatin A, which is in contrast to isolated pepsin. This result suggests that the sensitivity of pepsin to pepstatin may vary depending on its source or purification method. In a study by Zhou *et al.* (2008), it was observed that a 1:1 ratio of pepstatin inhibitor to pepsin enzyme caused complete inhibition of pepsin III and IV from mandarin fish, while a ratio of 1:10 was required to inhibit pepsin I and II. This finding indicates that different types of pepsin may have varying sensitivities to pepstatin inhibition.

Our findings align with previous studies conducted by Yao *et al.* (2015) and Jin *et al.* (2012), which investigated the influence of GO and GO-PEG on the activity and thermal resistance of trypsin and other serine proteases. Yao *et al.*

(2015) reported that GO-PEGs could enhance trypsin activity and improve its thermal stability. They demonstrated that GO and GO-PEG could protect trypsin from thermal denaturation at high temperatures, suggesting their potential as efficient modulators for this enzyme. Our study extends these findings by showing that GO-PEG can enhance pepsin thermal stability and activity, a different serine protease. Similarly, Jin *et al.* (2012) observed that PEGylated GO nanosheets selectively enhanced trypsin activity and thermostability while they did not meaningfully affect the chymotrypsin or proteinase activity. These findings are consistent with our results, where GO-PEG improved the thermal stability of pepsin. The selective enhancement of enzyme activity by GO-PEG suggests that the interactions between graphene-based materials and specific proteins are highly dependent on their molecular structures and properties. The molecular dynamics (MD) simulations conducted by Yao *et al.* (2015) shed light on the underlying mechanisms of the interactions between enzymes and GO. They revealed that trypsin was adsorbed onto the GO surface through interactions with cationic and hydrophilic amino acids, leading to the stabilization and protection of its active site. These findings provide a mechanistic understanding of the enhanced thermal stability observed in our study and highlight the potential of graphene-based materials as carriers or supports for enzymes in various biotechnological applications. Moreover, Srivastava *et al.* (2018) demonstrated the thermal stability enhancement of crude cellulase treated with GO. Their results showed that GO acted as a carrier or support, enabling the enzymes to maintain the activity at higher temperatures. This finding aligns with ours, where GO-PEG improved the thermal stability of pepsin, enabling its activity to maintain at elevated temperatures. The obtained results demonstrate that GO-PEG can enhance pepsin thermal stability and activity, which is in line with previous studies on trypsin and cellulase. The mechanistic insights provided by MD simulations further support the potential of graphene-based materials as selective and efficient modulators for specific enzymes. These findings open up new possibilities for the nanobiological systems development and pave the way for further investigations into the interactions between graphene-based materials and various enzymes.

S. commerson viscera proves to be a valuable source for pepsin purification. The purification process involving ammonium sulfate fractional precipitation, gel filtration, and ion-exchange chromatography obtained purified pepsinogen effectively. Additionally, GO-PEG use showed promising results in enhancing enzyme activity and thermostability. These findings highlight that GO-PEG has the potential to serve as a modulator for enhancing enzyme production and stability.

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Conflicts of interest

The authors declare no conflicts of interest.

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