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Hydrophobic interaction chromatography as separation method of alkaline proteases from viscera of *Scomberomorus sierra*

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Abstract

This study focused on recovering alkaline proteases from the viscera of *Scomberomorus sierra* through hydrophobic interaction chromatography. Three alkaline proteases were partially separated using this chromatographic technique; two of them, with molecular weights of 19 and 31 kDa, were identified as trypsin-like enzymes according to inhibition assays. The 31 kDa alkaline protease, the only isolated enzyme, was purified under following chromatographic conditions: ammonium sulfate 13% (w/v) and ethylene glycol 27% (w/v); this enzyme showed maximum activity at pH 9 – 10 and 50 – 60 °C and was strongly inhibited by soybean trypsin inhibitor (SBTI) and porcine trypsin inhibitor (TPI). A third alkaline protease with molecular weight of 20 kDa was partially separated and inhibited by tosyl phenylalanyl chloromethyl ketone (TPCK), showing optimum activity at pH 9 – 11 and 60 °C. These results show that the viscera of *Scomberomorus sierra* may be useful as source of proteases.
Key Words: chymotrypsin, hydrophobic interaction chromatography, *Scomberomorus sierra*, trypsin.

Cromatografía de interacción hidrofóbica como método de separación de proteasas alcalinas de vísceras de *Scomberomorus sierra*

Resumen

Este estudio se enfocó en recuperar proteasas alcalinas de vísceras de *Scomberomorus sierra* mediante cromatografía de interacción hidrofóbica. Tres proteasas alcalinas se lograron separar parcialmente usando esta técnica cromatográfica; dos de ellas con pesos moleculares de 19 y 31 kDa fueron identificadas como enzimas tipo tripsina de acuerdo a ensayos de inhibición. La proteasa alcalina con peso molecular de 31 kDa, única enzima aislada, fue purificada bajo las siguientes condiciones cromatográficas: sulfato de amonio l3% (p/v) y etilenglicol al 27% (p/v); esta enzima mostró actividad máxima a pH 9 – 10 y 50 – 60 °C y fue fuertemente inhibida por el inhibidor de tripsina de soya (SBTI) como por el inhibidor de tripsina porcina (TPI). Una tercera proteasa alcalina con peso molecular de 20 kDa fue parcialmente separada e inhibida por tosil fenilalanil clorometil cetona (TPCK), la cual mostró actividad óptima a pH 9 – 11 y 60 °C. Estos resultados muestran que las vísceras de *Scomberomorus sierra* podrían ser de utilidad como fuente de proteasas.
Palabras Clave: quimiotripsina, cromatografía de interacción hidrofóbica, *Scomberomorus sierra*, tripsina.

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Introduction

Scomberomorus sierra, better known as tropical sierra, is one of the main fish species caught by fishermen in Sonora and it represents one of the ten fisheries with the highest production in Mexico; its consumption is based solely on muscle, while the rest (digestive tract, skin, head and bones) is discarded. Viscera represents about 10% (weight) of the whole sierra fish, and is a potential source of proteolytic enzymes such as alkaline proteases. Currently, this sort of enzymes have called attention of the food industry due to its physicochemical properties such as optimum temperature and pH, stability and thermal inactivation (Klomklao, Benjakul, Visessanguan, Simpson & Kishimura, 2005; Nalinanon, Benjakul, Visessanguan & Kishimura, 2009); therefore, it is important to investigate the adequate separation conditions to carry out the isolation from fish wastes. The development of techniques to separate and purify enzymes is a pre-requirement to study their structures, functions and potential applications in food industry (Brian & Lenhoff, 2008; Queiroz, Tomaz & Cabral, 2001); these methods focus on maintaining the concentration and enzimatic activity, factors that allow the enzymes to work in the best way once they are recovered from the native environment.

Hydrophobic interaction chromatography (HIC), as a protein purification technique, has the advantage of using the protein hydrophobicity (a specific property of proteins) to promote their own separation as a result of hydrophobic interactions between immobilized hydrophobic ligands and non-polar regions on the protein surface. However, its disadvantage depends on the content, arrangement and distribution of the hydrophobic amino acids on the surface of the protein to obtain an optimal separation. It is well known that non-polar amino acids such as phenylalanine, tryptophan and valine are found on protein surface, as in lysozyme and albumin (Klotz, 1970; Hofstee, 1975), however this feature may be different according to the source and role that protein play in metabolism (Hofstee, 1975). In HIC, proteins adsorption increases when there is high concentration of salt on mobile phase, which promotes proteins to expose their hydrophobic aminoacids outside of the molecules by ions hydration (Tsumoto, Ejima, Senczuk, Kita & Arakawa, 2007), adquiring an ideal contact surface to interact with the stationary phase through van der Waals forces, which are principally involved in folding and structure stabilization of proteins. These forces do not have the enough energy to damage the structure, biological activity and stability of proteins. On the other hand, to achieve proteins elusion, salt concentration has to decrease, making proteins to adquire their native structure again as a result of the availability of water molecules by interacting with hydrophilic aminoacid, which come back simulating a native environment (Brian & Lenhoff, 2008; Queiroz, Tomaz & Cabral, 2001). It is important to emphasize that there are few reports about separation conditions for alkaline proteases by HIC (Osuna-Amarillas *et al*., 2012; Reyes-Guzmán *et al*., 2012) so this study was focused to recover alkaline proteases from viscera of *Scomberomorus sierra* through this chromatography technique, looking to reduce operation costs and to keep the biological activity of the enzymes.

Materials and methods

Materials

Azocasein, phenyl-methyl-sulfonyl-fluoride (PMSF), N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), soybean trypsin inhibitor (SBTI), porcine trypsin inhibitor (PTI), ethylene diamine tetra acetic acid (EDTA), brij-35, glycine, tris-buffer, acetic acid, pepstatin A, bestatin, cysteine proteases inhibitor (E-64) and molecular weight markers were purchased from Sigma Chemicals (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), acrylamide, ammonium persulfate, tetramethyl ethylene diamine and Coomassie blue G and R were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

Biological material

Scomberomorus sierra organisms with an average length and weight of 36 cm and 209 g, were captured on February at Kino bay, México. Immediately after their capture, the organisms were stored on ice beds and transported to Marine Laboratory at the Universidad de Sonora (Hermosillo, México). The time from fish capturing to evisceration was 24 hours. After that, visceral tissues from the 22 organisms were gathered and frozen at -80 °C to be later lyophilized, keeping the enzymatic activity.

Protein extracts

This step had as purpose to separate sarcoplasmic proteins from crude extract (CE) through solubility. Briefly, 5 g of lyophilized viscera were crushed and homogenized with Tris – HCl [50 mM] $CaCl_2$ [0.02 M] pH 7.8 in a ratio of 1:10 (w/v), using a tissue homogenizer (Wisd Laboratory Instruments, HG-15D). After that, the homogenized was centrifuged (Centrifuge, Thermo Scientific; Surval Biofuge Stratus; MA, USA), under next conditions: 14,000 x *g*, 1 hour and 4 °C. Recovered supernatant was mixed with Brij-35 (0.2 g of Brij-35/100 mL of supernatant), to separate and eliminate lipids from globular proteins; the mixture was shaken for 1 hour and left resting for 12 hours. Finally, the mixture was centrifuged again to recover the supernatant, which was labeled as crude extract (Olivas-Burrola, Ezquerra-Brauer, Rouzaud-Sández & Pacheco-Aguilar, 2001).

Alkaline proteases separation

HIC was used as separation stage according to the ability of alkaline proteases by interacting with hydrophobic groups available on stationary phase. Working conditions are

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presented next: phenyl sepharose CL-4B Sigma-Aldrich as stationary phase; column with 1.2 and 40 cm of diameter and length respectively; Tris – HCl [50 mM] $CaCl_2$[0.02 M] pH 8.0 as mobile phase; flow rate = 3 mL; elution gradient was a cold mixture composed by glycerol and buffer Tris – HCl [50 mM] $CaCl_2$ [0.02 M] pH 8.0. Two hundred fractions, 1.4 mL per fraction, were collected; each one of them were monitored spectrophotometrically at 280 nm to analyze the elution pattern of the proteins, being identified as picks of protein labeled with capital letters.

Protein determination

Protein estimation in chromatographic fractions was done by absorbance lectures at 280 nm using a UV-Vis spectrophotometer (Cary 50, Varian; CA, USA), to monitor the proteins separation pattern. On the other hand, total soluble proteins were determined in fractions by Bradford (1976), using bovine serum albumin as standard, information that was used later for the electrophoretic analysis.

Assay of proteolytic activity

The test consisted of the following: 300 µL of azocasein [2 mg/mL] were mixed with 100 µL of phosphate buffer [100 mM] pH 8 to be pre-incubated for 5 minutes at 37 °C before the catalytic reaction took place; after this, 100 µL (0.164 mg of protein) of CE was added in the mixture and it was incubated to begin the reaction for 1 hour. Reaction was stopped by adding 500 µL of trichloroacetic acid [25%, w/v] and cooling at 0 °C for 10 minutes. The solution was centrifuged at 5,000 x *g* for 5 minutes, after that, 800 µL of supernatant was recovered and mixed with 60 µL of NaOH [10M], to be read on a spectrophotometer at 428 nm. Proteolytic activity was calculated using the next equation: Abs_{428}/0.01 [=] activity units (An, Seymour, Juwen & Morrisey, 1994).

Electrophoresis

Two 10% polyacrylamide gels in native conditions were prepared according to Laemmli (1970); one of them was formulated with azocasein [0.33%, w/v], and labeled as "azocasein gel". The azocasein gel was pre-incubated in phosphate buffer [100 mM] pH 8 for 30 minutes. In another gel, called "running gel", was injected 30 µL of each extract: *a* (4.4 µg of protein), *b* (2.6 µg of protein), *c* (1.5 µg of protein), *d* (2.3 µg of protein), *e* (3.0 µg of protein) and *f* (2.4 µg of protein); electrophoresis was performed in a Mini-PROTEAN 3, BIO-RAD®camera under next conditions: 200 Volts for 1 hour. Later, casein gel previously incubated was overlapped over the running gel to be incubated together at 37 °C for 2 hours. After that, gels were stained with amido black solution [0.1%, w/v] and acetic acid [7%, w/v] for 1 hour at 25 °C. Finally, acetic acid [7%, w/v] was used to distain gels (Heussen & Dowdle, 1980).

Inhibition assays

Extracts *a*, *b*, *c*, *d*, *e* and *f* were incubated at 25 °C for 30 minutes in tubes which contained different protease inhibitors such as SBTI[10 µM], PTI[10 µM], TPCK[10 µM], PMSF[5µM], cysteine proteases inhibitor (E-64)[10 µM], EDTA[1 µM], bestatine[50 µM] and pepstatin A[10 µM]. After the incubation time, residual activity was determined according to the methodology described by An, Seymour, Juwen & Morrisey (1994), using azocasein as substrate.

Optimum pH

pH effect on proteolytic activity of alkaline proteases was evaluated with a pH meter (Mettler Toledo, UK), under different pH conditions using next buffers: acetate [0.1 M] pH 4.0 – 6.0, Tris-HCl [0.1 M] pH 7.0 – 9.0, glycine – NaOH [0.1 M] pH 10.0 – 11.0. Residual activity was measured according to the methodology described by An, Seymour, Juwen & Morrisey (1994), using azocasein as substrate.

Optimum temperature

Temperature effect on proteolytic activity of alkaline proteases was evaluated at different temperature values: 24.6, 30, 37, 40, 50, 60 and 70 °C for 1 hour. Residual activity was measured according to the methodology described by An, Seymour, Juwen & Morrisey (1994), using azocasein as substrate.

Data analysis

Statistical design of this study was aimed to reduce the variation by replication. Reported data of alkaline proteases activity during separation and characterization assays are based on the average from three determinations. The variation among replicates was < 5%. Descriptive statistic, standard deviation, was used to analyze the data. Duncan's multiple-comparison test with significant 0.05 was used to determine differences among the effects of inhibitors on the enzymatic activity, where the kind of inhibitor represented to the factor with 8 levels and the inhibition percentage (by triplicate) the response variable.

Results and discussion

Extraction and isolation of alkaline proteases from viscera of *S. sierra*

The results about extraction and purification stages has been summarized in Table I. Previous purification process, CE showed 47.9 U/mg of specific activity. Once CE was passed by HIC, according to y axes that represent apparent protein concentration (Abs_{280nm}) and activity units (428 nm/0.01) in Figure 1, six activity peaks were found and each one of them could contain several proteins other than proteases, this is evident in Figure 2. Peaks with protease activity were labeled with capital letters (from A to F) to identify and analyze them. Fractions from peak A were collected, and the

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Extract	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Concentration (X-Times)
CE	81.9396	3925.8	47.9	100	1.0
a	2.0448	930.88	455.2	23.7	9.5
b	1.218	513.03	421.2	13.1	8.8
c	0.7196	374.22	520.0	9.5	10.9
d	1.0506	493.51	469.7	12.6	9.8
e	1.4184	516.6	364.2	13.2	7.6
f	1.1492	215.02	187.1	5.5	3.9
U represents to total activity units ($U = Abs_{428}/0.01$), CE represents to crude extract and letters from *a* to *f* are the extracts obtained by HIC					

Table I. Concentration (X-times) and activity of alkaline proteases in HIC-collected fractions from the protein crude extracts.

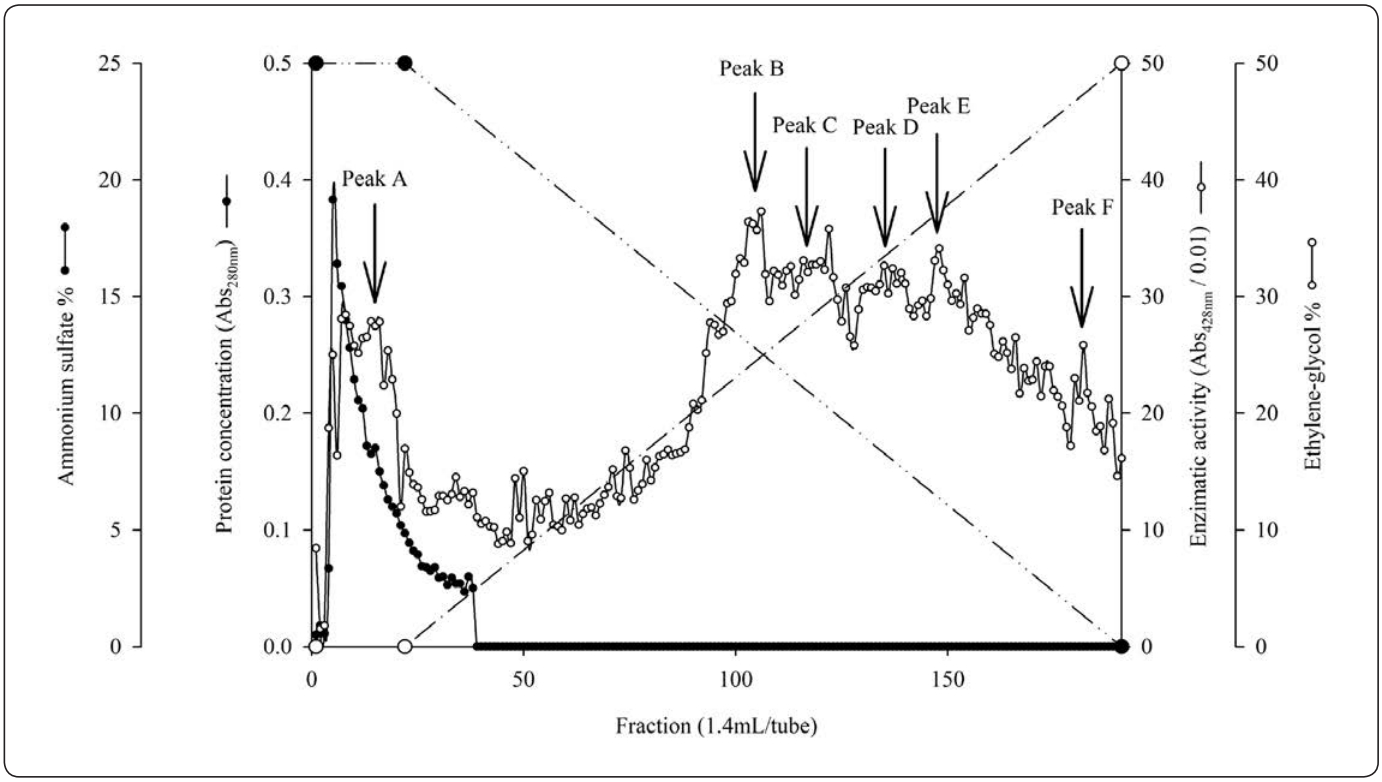

Figure 1. Hydrophobic interaction chromatography of crude extract from viscera of tropical *S. sierra.* Figure designed by the authors.

collected fraction was called "extract *a*". Same procedure was followed for the peaks B, C, D, E and F. Extract *c* showed the highest specific activity (520 U/mg) compared with extract *f*, which had the lowest specific activity (187.1 U/mg). Yield was apparently low due to the separation of protein (alkaline proteases) by HIC was distributed into different extracts. The extract *c* presented the highest specific activity, value that could be attributed to the high presence of alkaline proteases, such as trypsin and chymotrypsin, in the extract *c*.

Physicochemical properties and inhibitors effect on alkaline proteases from viscera of *S. sierra*

On the zymogram, Figure 2, was evident the presence of three alkaline proteases with molecular weights of 19, 20 and 31 kDa. At extracts *a* and *e*, there were not proteases separation, however at extract *b* was evident the isolation of an alkaline protease according to Figure 2 under next purification condition: ammonium sulfate [13%, w/v] and ethylene glycol [27 %, w/v]. This 30.9 kDa enzyme was inhibited

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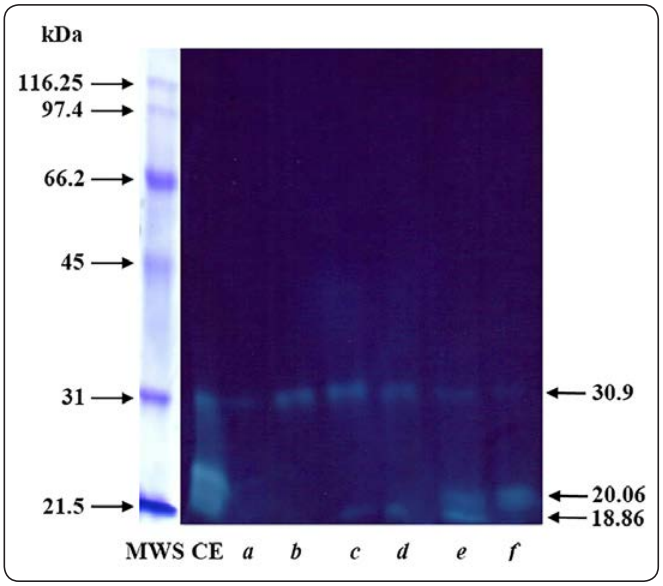

Figure 2. Page-native on alkaline conditions. MWS represents to molecular weight standards, CE represents to crude extract and the letters from a to *f* represent each extract from *S. sierra* viscera obtained by HIC. Figure designed by the authors.

92.6% and 86.1% by SBTI and PTI according to enzymatic assays (Figures 3 and 4). Valdez-Melchor, Ezquerra-Brauer, Cinco-Moroyoqui, Castillo-Yáñez & Cárdenas-López (2013) reported two trypsin isoforms from viscera of *S. sierra*, this evidence as well as the inhibition assays suggests that 31 kDa alkaline protease is a trypsin isoform; however, at least, the amino acids sequence have to be carried on as confirmation. Regarding fold-x and yield, this trypsin isoform was purified 8.8-fold and its purification process yield 13.1% according to Table I. Liu,Wang, Xu & Xu (2007) reported a similar molecular weight value (30.7 kDa) to trypsin isoform GT-A from carp intestine.

The 31 kDa trypsin isoform had an optimum pH of 9 – 10, and 50 – 60 °C as optimum temperature range according to the Figures 4 and 5. Kishimura, Hayashi, Miyashita & Nonami (2005) reported pH 8 and 60 °C as optimums of two trypsin isoforms (TR-I and TR-II), from viscera of Japanese anchovy (*Engraulis japonica*). Later Liu,Wang, Xu & Xu (2007) reported as optimums pH 8 and 38.5 °C for trypsin isoform GT-A, and pH 8.5 and 44 °C for GT-B from intestine of carp (*Ctenopharyn godonidellos*). Current studies have reported pH 9 and 55 °C as well as pH 10 and 50 °C as optimums for trypsins from viscera of Indian Mackerel (*Rastralliger kanagurta*) and golden gray mullet (*Liza aurata*) respectively (Bkhairia *et al*., 2016; Khandagale, Sarojini, Kumari, Suman Joshi & Nooralabettu, 2015).

On the other hand, in the extract *d* the 19 kDa alkaline protease was visible (Figure 2). Further, there was not inhibition by TPCK, but there were inhibitions by SBTI and PTI of 49.6% and 43.6% (Figure 3) respectively, indicating that 19 kDa alkaline protease may be another trypsin isoform, which agrees with previous studies (Valdez-Melchor, Ezquerra-Brauer, Cinco-Moroyoqui, Castillo-Yáñez & Cárdenas-López, 2013).

In the extract *f*, the 20 kDa alkaline protease was partially separated under next chromatography conditions, ammonium sulfate [2%, w/v] and ethylene glycol [48%, w/v], which may represent an option as previous stage for its possible isolation. This enzyme may correspond to chymotrypsin due to it is the only protein band to appear at extract *f* with that molecular weight, but it does not appear on extract *d* according to the zymogram (Figure 2), being this the last free of chymotrypsin extract according to inhibition assays (Figure 3); though, more studies have to be carried on to confirm our observations and assertions, even when the pattern –according to the electrophoresis, inhibition, pH and temperature studies– have shown a peculiar behavior. Further, extract *f* was 44.4% inhibited by TPCK, a specific inhibitor of chymotrypsin, according to Figures 3, putting its presence in evidence. Enzyme(s) at extract *f* present maximum activity under next condition: pH 9 – 11 and 60 °C. Kristjansson & Nielsen (1992) reported optimum activity at pH 9 and 55 °C for chymotripsin-like isoforms II and I from pyloric caeca of rainbow trout (*Oncorhynchus mykiss*), while Yang *et al*., (2009) reported pH 7.5 and 40 °C as well as pH 8 and 50 °C for chymotrypsin isoforms from hepatopancreas of crucian carp (*Carassius auratus*). Further, Castillo-Yáñez, Pacheco-Aguilar, García-Carreño, Navarrete-Del Toro & Félix-López (2006), reported pH 8 and 50 °C for chymotrypsin isolated from viscera of sardine Monterey (*Sardinopssagax caeruleus*) and Balti *et al*., (2012), reported as optimums pH 8.5 and 55 °C for chymotrypsin from hepatopancreas of cuttle fish (*Sepia officinalis*).

Conclusions

Three alkaline proteases were detected in protein extracts from viscera of *Scomberomorus sierra* by zymogram. Two of them showed activity trypsin-like (19 and 31 kDa) and another one chymotrypsin-like (20 kDa) according to inhibition assays. 31 kDa enzyme was isolated from viscera of *Scomberomorus sierra* by HIC, showing maximal activity at pH 9 – 10 and 50 – 60 °C. Further, separation conditions such as ammonium sulfate [2%, w/v] and ethylene glycol [48%, w/v] were set to separate the 20 kDa alkaline protease from viscera of tropical sierra by HIC, presenting maximum activity at pH 9 – 11 and 60 °C. The results have opened the possibility to find the specific conditions to separate all the alkaline proteases from viscera of *S. sierra* in a few stages, keeping enzymatic activity and reducing costs and time, which could motive to the public and private initiative to scale the process to an industry level.

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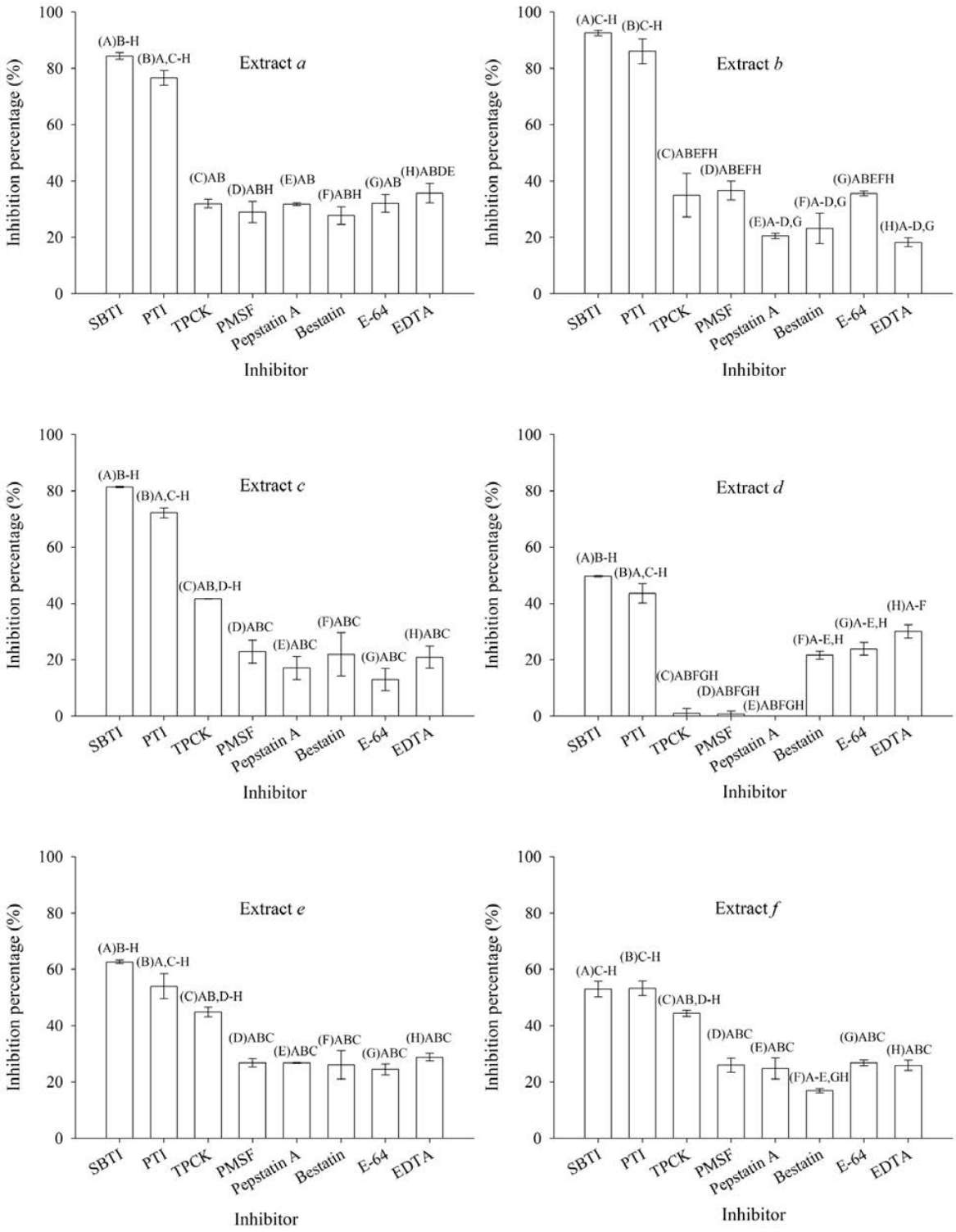

Figure 3. Inhibitors effect on alkaline proteases activity from viscera of *S. sierra*. Every capital letter represents to different inhibitors to appreciate the statistic's analysis, for instance SBTI is represented by"A", capital letters located above the bar, TPI "B", TPCK"C", PMSF "D", pepstatin A"E", bestatin"F", E-64 "G" and EDTA "H". Above each bar the capital letter into parenthesis represents to the inhibitor that was compared *versus* the effect of the others inhibitors located outside of the parenthesis; it was done through Duncan's multiple-comparison test with significant 0.05. The capital letters no present above each bar mean that they did not show significant differences. Figure designed by the authors.

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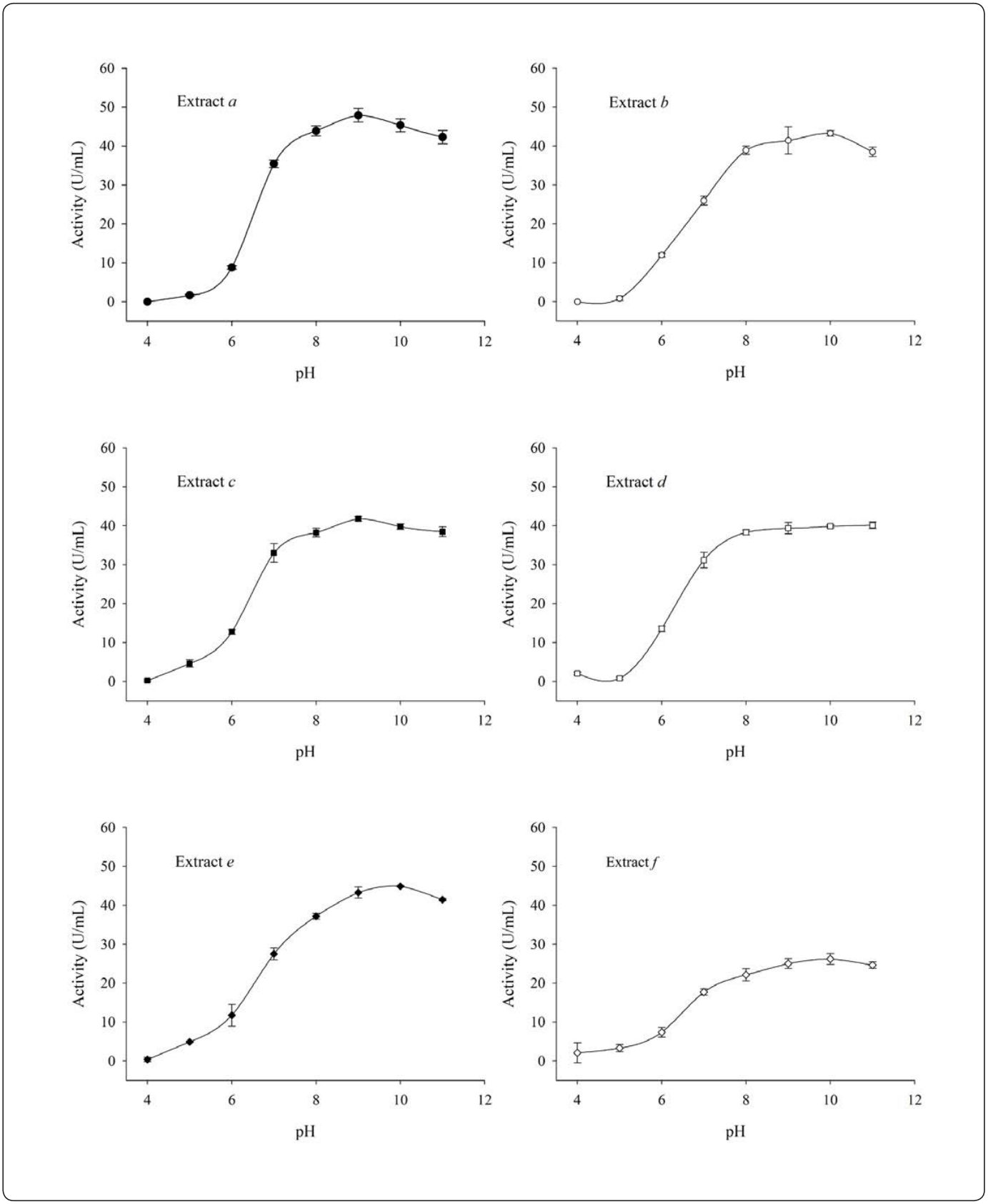

Figure 4. Optimum pH of alkaline proteases from viscera of *S. sierra*. Figure designed by the authors.

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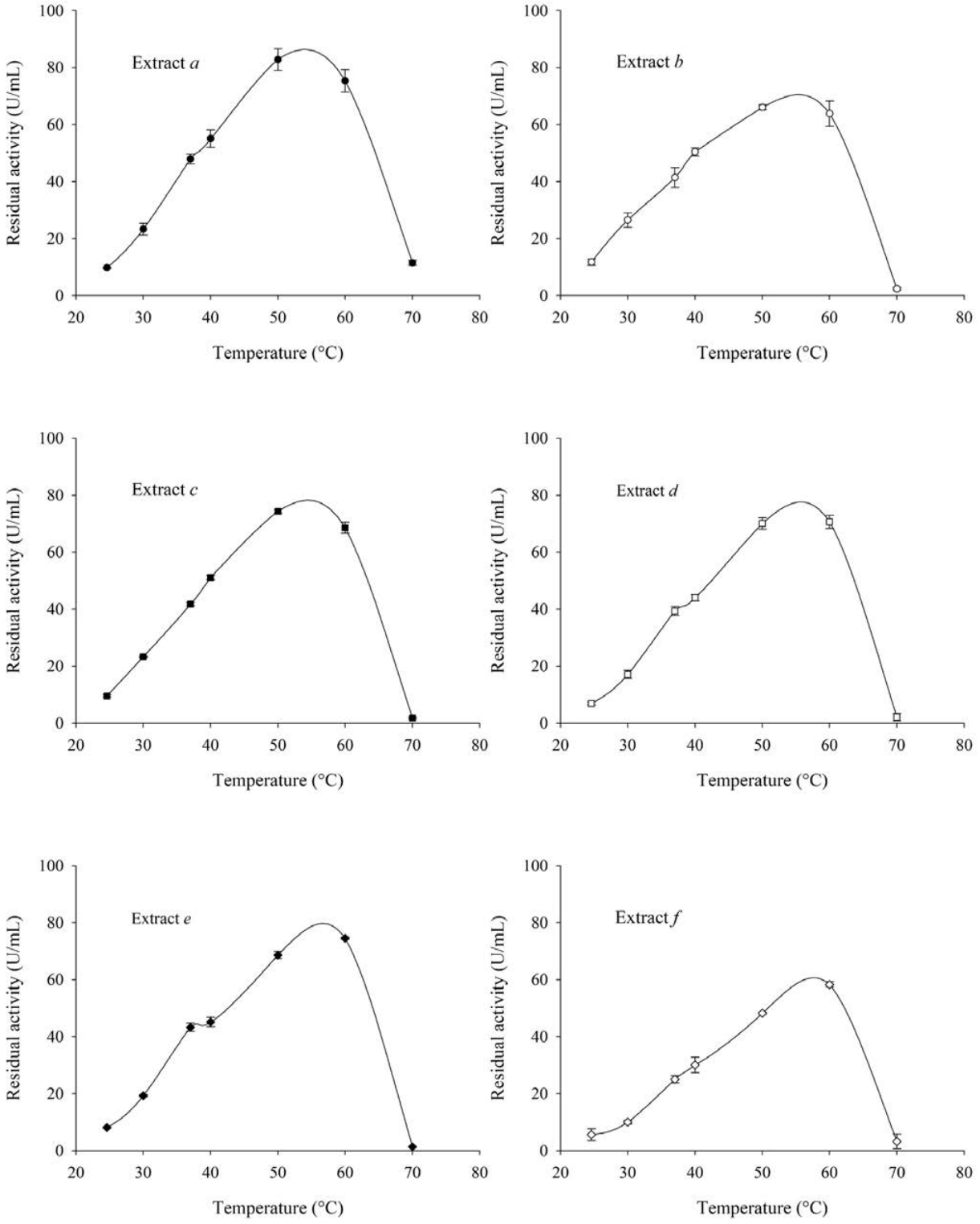

Figure 5. Optimum temperature of alkaline proteases from viscera of *S. sierra*. Figure designed by the authors.

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