UNIVERSIDAD DE SONORA

DIVISIÓN DE CIENCIAS BIOLÓGICAS Y DE LA SALUD

DEPARTAMENTO DE INVESTIGACIÓN Y POSGRADO EN ALIMENTOS

Programa de Posgrado en Ciencias y Tecnología de Alimentos

Caracterización bioquímica, termodinámica y cinética de catepsina D del hepatopáncreas de la almeja japonesa (*Ruditapes philippinarum*), almeja reina (*Dosinia ponderosa*) y calamar gigante (*Dosidicus gigas)*

TESIS

Como requisito parcial para obtener el grado de:

DOCTOR EN CIENCIAS DE LOS ALIMENTOS

Presenta:

M.C. Francisco Cadena Cadena

Hermosillo, Sonora enero, 2020

APROBACIÓN

Caracterización bioquímica, termodinámica y cinética de catepsina D del hepatopáncreas de la almeja japonesa (*Ruditapes philippinarum*), almeja reina (*Dosinia ponderosa*) y calamar gigante (*Dosidicus gigas*)

Francisco Cadena Cadena

José Luis Cárdenas López
Director de tesis

Josafat Marina Ezquerra Brauer
Secretario

Hisila del Carmen Santacruz Ortega
Vocal 1

Francisco Javier Cinco Moroyoqui
Vocal 2

Alonso Alexis López Zavala
Vocal 3

Hermosillo Sonora enero de 2020

Hermosillo, Sonora a enero de 2020.

Asunto: Cesión de derechos

UNIVERSIDAD DE SONORA
P R E S E N T E.

Por este conducto hago constar que soy autor y titular de la obra denominada **Caracterización bioquímica, termodinámica y cinética de catepsina D del hepatopáncreas de la almeja japonesa (*Ruditapes philippinarum*), almeja reina (*Dosinia ponderosa*) y calamar gigante (*Dosidicus gigas*)**, en los sucesivo LA OBRA, realizada como trabajo terminal con el propósito de obtener el Grado de **Doctor en Ciencias de los Alimentos**, en virtud de lo cual autorizo a la Universidad de Sonora (UNISON) para que efectúe la divulgación, publicación, comunicación pública, distribución, distribución pública, distribución electrónica y reproducción, así como la digitalización de la misma, con fines académicos o propios de la institución y se integren a los repositorios de la universidad, estatales, regionales, nacionales e internacionales.

La UNISON se compromete a respetar en todo momento mi autoría y a otorgarme el crédito correspondiente en todas las actividades mencionadas anteriormente.

De la misma manera, manifiesto que el contenido académico, literario, la edición y en general cualquier parte de LA OBRA son de mi entera responsabilidad, por lo que deslindo a la UNISON por cualquier violación a los derechos de autor y/o propiedad intelectual y/o cualquier responsabilidad relacionada con la OBRA que cometa el suscrito frente a terceros.

A T E N T A M E N T E

LIC. GILBERTO LEÓN LEÓN
Abogado General
UNIVERSIDAD DE SONORA

Francisco Cadena Cadena

DEDICATORIA

A:

Dios, por darme la oportunidad de vivir y por estar conmigo en cada paso que doy, por fortalecer mi corazón e iluminar mi mente y por haber puesto en mi camino a aquellas personas que han sido mi soporte y compañía durante todo el periodo de estudio.

Mi madre María Jesús Cadena (QEPD) y mi padre Porfirio Cadena, por darme la vida, quererme mucho y creer en mí ya que siempre me apoyaron. Mamá y Papa gracias por apoyarme en mi carrera, todo esto se lo debo a ustedes

A mi hija Arya Ximena Cadena y mi esposa Erika Arely Núñez, quiénes me brindaron todo su apoyo y comprensión durante el curso del doctorado, así como en la elaboración del presente trabajo.

.

Mis hermanos, Ana, Jesús y Carlos por estar conmigo y apoyarme siempre.

Todos mis amigos Raquel, Ángel, Santiaguin, Solecito, Claudia, Ivan y Encinas a todos ustedes muchas gracias por haberme ayudado tanto y por compartir los buenos y malos momentos.

Mis suegros José Jesús Núñez y María Ofelia Portillo por el cariño y apoyo incondicional que siempre me han manifestado.

A mis sinodales Dra. Marina, Dr. Cinco, Dr. Alexis y Dra. Hisila que me apoyaron durante todo el curso del Doctorado y durante la realización de este trabajo. Muy especialmente quiero dedicarle este trabajo a mi director de tesis Dr. José Luis Cárdenas López usted ha sido mi mano derecha y quien me ha guiado en el complicado proceso. Es cierto, no ha sido nada fácil, sin embargo, gracias a su ayuda, esto ha parecido un tanto menos complicado. El resultado de mi tesis ha sido espectacular, mejor de lo que esperaba y una gran parte del desarrollo de ese

excelente trabajo se lo debo a usted. Que dios lo bendiga y solo resta decirle muchas gracias Dr. José Luis.

AGRADECIMIENTOS

El presente trabajo de tesis primeramente me gustaría agradecele a Dios por bendecirme para llegar hasta donde he llegado.

A la UNIVERSIDAD DE SONORA por darme la oportunidad de estudiar y ser un maestro en ciencias.

A mi director de tesis, Dr. José Luis Cárdenas López por su esfuerzo y dedicación, quien con sus conocimientos, su experiencia, por su visión crítica de muchos aspectos cotidianos de la vida, por su rectitud en su profesión como docente, por sus consejos, que me ayudaron a formarme como persona e investigador, por su paciencia conmigo y su motivación ha logrado que yo pueda terminar mis estudios con éxito.

Son muchas las personas que han formado parte de mi vida profesional a las que me encantaría agradecerles su amistad, consejos, apoyo, ánimo y compañía en los momentos más difíciles de mi vida. Algunas están aquí conmigo y otras en mis recuerdos y en mi corazón.

También me gustaría agradecer al DIPA y a CONACYT por la beca brindada durante mi estancia en el Doctorado y por el proyecto “Caracterización fisicoquímica de catepsinas L y D del hepatopáncreas de calamar gigante” Conacyt Ciencia Básica 182348 dirigido por el Dr. José Luis Cárdenas López.

CONTENIDO

INTRODUCCIÓN

Los moluscos son organismos ampliamente distribuídos a nivel mundial, sin embargo debido a fenomenos como la explotación excesiva de los recursos pesqueros en general, así como a los cambios climáticos, las estrategías de producción desde hace varias decadas se han enfocado a su producción a través de sistemas de cultivo. No obstante para poder etablecer las mejores condiciones de producción y entender el comportamiento que los organismos marinos presentan en los ambientes marinos se requieren estudios que permitan un mayor conocimiento de las mismas.

Los principales moluscos consumidos en el mundo son las almejas reina (*Doosinia ponderosa*) y la japonesa (*Ruditapes philippinarum*), situándose solo por debajo del ostión (Bagnara Vivanco, 2008) y el calamar gigante. La producción de éste último organismo se ha visto mermada en los últimos 5 años en las costas Mexicanas, atribuido entre otras a problemas climáticos como el fenómeno de la Niña y el Niño (Morales-Bojórquez *et al.,* (2017). Lo que ha propiciado la migración del mismo hacía otras zonas del continente americano como Costa Rica y Perú. Los fenómenos de migración se dan sobre todo por la búsqueda de alimentación.

Un nutriente importante dentro de la dieta de los organismos marinos son las proteínas. En el caso particular del calamar se ha reportado que en su principal órganos digestivo el hepatopáncreas, las principales actividad enzimática es proteolítica, como la tripsinica, quimotripsinica, aminopéptidica y carboxipéptidica.

Así mismo se ha detectado alta actividad enzimática a pH ácidos en el hepatopáncreas las principales enzimas que se han estudiado son las proteasas alcalinas (Ezquerra-Brauer *et al*. 2002; Osuna-Ruiz *et al*. 2010). Sin embargo, se sabe que existe una alta actividad enzimática a pH ácidos (Cárdenas-Lopez y Haard, 2005). Una de las principales enzimas lisosomales aislada del hepatopáncreas de muchos moluscos es la catepsina D (EC 3.4.23.5) (Tiscar *et al*., 2004; Venugopal y Kumar, 2014).

La catepsina D se encuentra en todos los organismos actuando en el recambio proteico (Balti *et al*., 2010; Davies, 1990). Esta enzima desempeña un papel importante en la degradación intracelular de hormonas polipeptídicas, factores de crecimiento, la insulina, el glucagón (Benes *et al*., 2008) y también participa en la activación *in vitro* de los precursores enzimáticos de catepsinas B y L aisladas del hígado y riñón de rata (Nishimura *et al*., 1988). Así mismo se ha reportado que su actividad se incrementa ante el desarrollo de varios tipos de carcinomas (Benes *et al*., 2008; Fusek *et al*., 2005). Se ha detectado en altas concentraciones en los pacientes con el mal de Parkinson (Crabtree *et al*., 2014), siendo también un blanco en el tratamiento de la enfermedad de Alzhéimer (Di Domenico *et al*., 2016), así como involucrada en la regulación de la apoptosis o muerte celular programada (Blanco-Labra *et al*., 1996).

En organismos marinos la catepsina D ha sido aislada de algunas especies de peces como la carpa (*Cyprinus carpio*) (Goldman-Levkovitz *et al*., 1995), el arenque (*Clupea harengus*) (Nielsen *et al*., 2001), del mejillón (*Mytilidae galloprovincialis*) y del hepatopáncreas de calamares (*Sepia*) (Balti *et al*., 2010;

Gildberg, 1987). En la langosta americana (*Homarus americanus*) se asocia con la digestión encontrándose en el jugo gástrico (Rojo *et al*., 2010), mientras que en la *Lampetra japonica* está relacionada con el sistema reproductor (Xiao *et al*., 2015). En otros invertebrados se encuentra presente en la digestión de proteínas del alimento (Padilha *et al*., 2009; Rojo *et al*., 2010; Williamson *et al*., 2002).

Se sabe que la catepsina D jueba un papel fundamental tanto en el sistema inmune como digestivo de los organismos vivos (Tiscar *et al*., 2004; Venugopal y Kumar, 2014). Existen muchas estrategias que pueden tomar los organismos para una adecuada proteólisis. Sin embargo, a nivel enzimático la principal es la síntesis de enzimas con diversas características, ya sea, enzimas que han incrementado su eficiencia catalítica a expensas de la estabilidad térmica o, enzimas que aumentan su estabilidad térmica a expensas de su eficiencia catalítica (Russell, 1998). La catepsina D posee una alta eficiencia catalítica, por lo que se ha detectado que se requieren concentraciones bajas de la enzima para que se lleve a cabo la reacción química, así mismo se ha reportado que pueden inactivarse a temperaturas moderadas, lo que permite detener los procesos enzimáticos sin comprometer la integridad de los productos (Feller, 2013; Siddiqui *et al*., 2006).

En cuanto a la caracterización estructural de la catepsina D en organismos marinos, a la fecha se tienen reportes principalmente en crustaceos (Martinez-Alarcon *et al*., 2018). Las cuáles al compararse con la presente en el bazo bovino, la proveniente de los crustáceos estudiados exhibe mayor eficiencia catalítica y ausencia de estructuras tipo lazo en la estructura de la enzima. Estas diferencias constituyen adaptaciones que le permiten a la catepsina D catalizar sus sustratos a

bajas temperaturas (Rojo *et al*., 2013). Estas caracteristicas estan asociadas a su estructura secundaria principalmente a motivos βαβαβ; es decir, las hélices α y las hojas β se van intercalando en la secuencia peptídica relacionados por un eje de rotación, y es aquí donde los dos motivos βαβαβ forman una estructura que tipo red (Bhattacharyya, 2016). En algunas proteínas existen residuos acoplados en algunas de las hojas β centrales, principalmente al final de las mismas. Esta presencia de acoplamiento les confiere estabilidad a las hélices α así como también al hecho de que se encuentran muchos residuos importantes en las asas de la proteína y otras regiones accesibles al solvente (Bottoms *et al.* 2002).

A pesar de los estudios antes mencionados, en moluscos la información que se tiene sobre la catepsina D en el tracto digestivo de los organismo es escasa, la mayoría de los trabajos se han centrado en la actividad que la misma posee en la región anatómica que mayormente se consume, su manto (Luna-Raya *et al*., 2014), y aún no se conoce la biogénesis lisosómica en estos organismos ni cuales enzimas participan en su digestión. Por lo tanto, debido a la gran diversidad biológica que presenta el grupo de moluscos y al papel fisiológico que la Catepsina D posee, se requieren realizar mayores estudios que permitan profundizar más en el conocimiento de esta enzima. En este trabajo se llevó a cabo la extracción y purificación de la catepsina D de tres moluscos de interés comercial la almeja japonesa, la almejra reina y el calamar gigante. La caracterización de la enzima purificada se realizó mediente el establecimiento de parámetros bioquímicos (masas moléculares), físicos (pH y temperatura), cinéticos y estructurales. El desarrollo de esta investigación coadyuvará al conocimiento de las características bioquímicas y termodinámicas de la catepsina D. y sienta las bases para próximas

investigaciones que permitan explorar cómo las características estructurales responsables de la actividad de la enzima impactan en el desarrollo de los organismos.

REFERENCIAS

Bagnara Vivanco, M. (2008). Descripción del sector mitilicultor en la región de Los Lagos, Chile: evolución y proyecciones. FAO Actas de Pesca y Acuicultura.

Balti, R., Hmidet, N., Jellouli, K., Nedjar-Arroume, N., Guillochon, D., & Nasri, M. (2010). Cathepsin D from the hepatopancreas of the cuttlefish (Sepia officinalis): purification and characterization. Journal of agricultural and food chemistry, 58(19), 10623-10630.

Benes, P., Vetvicka, V., & Fusek, M. (2008). Cathepsin D—many functions of one aspartic protease. Critical reviews in oncology/hematology, 68(1), 12-28.

Bhattacharyya, M., Bhat, C. R., & Vishveshwara, S. (2013). An automated approach to network features of protein structure ensembles. Protein Science, 22(10), 1399-1416.

Blanco-Labra, A., Martinez-Gallardo, N. A., Sandoval-Cardoso, L., & Delano-Frier, J. (1996). Purification and characterization of a digestive cathepsin D proteinase isolated from Tribolium castaneum larvae (Herbst). Insect Biochemistry and Molecular Biology, 26(1), 95-100.

Bottoms, C. A., Smith, P. E., & Tanner, J. J. (2002). A structurally conserved water molecule in Rossmann dinucleotide-binding domains. Protein Science, 11(9), 2125-2137.

Cardenas-Lopez, J. L., & Haard, N. F. (2005). Cysteine proteinase activity in jumbo squid (Dosidicus gigas) hepatopancreas extracts. Journal of food biochemistry, 29(2), 171-186.

Crabtree, D., Dodson, M., Ouyang, X., Boyer-Guittaut, M., Liang, Q., Ballestas, M. E. Zhang, J. (2014). Over-expression of an inactive mutant cathepsin D increases endogenous alpha-synuclein and cathepsin B activity in SH-SY5Y cells. Journal of neurochemistry, 128(6), 950-961.

Davies, D. R. (1990). The structure and function of the aspartic proteinases. Annual review of biophysics and biophysical chemistry, 19(1), 189-215.

Di Domenico, F., Tramutola, A., & Perluigi, M. (2016). Cathepsin D as a therapeutic target in Alzheimer's disease.

Ezquerra-Brauer, J., Haard, N. F., Ramírez-Olivas, R., Olivas-Burrola, H., & Velazquez-Sánchez, C. J. (2002). Influence of harvest season on the proteolytic activity of hepatopancreas and mantle tissues from jumbo squid (Doswicus gigas). Journal of food biochemistry, 26(5), 459-475.

Feller, G. (2013). Psychrophilic enzymes: from folding to function and biotechnology. Scientifica, 2013.

Fusek, M., & Vetvicka, V. (2005). Dual role of cathepsin D: ligand and protease. Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia, 149(1), 43-50.

Gildberg, A. (1987). Purification and characterisation of cathepsin D from the digestive gland of the pelagic squid Todarodes sagittatus. Journal of the Science of Food and Agriculture, 39(1), 85-94.

Goldman-Levkovitz, S., Rimon, A., & Rimon, S. (1995). Purification properties and specificity of cathepsin D from Cyprinus carpio. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 112(1), 147-151.

Luna-Raya, M. C., García, J. I. U., Zavala, C. A. S., Mata, M. Á. C., & Morales, L. F. B. (2014). Diagnóstico del consumo del calamar gigante en México y en Sonora. Economía Sociedad y Territorio.

Martínez-Alarcón, D., Saborowski, R., Rojo-Arreola, L., & García-Carreño, F. (2018). Is digestive cathepsin D the rule in decapod crustaceans? Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 215, 31-38. doi: https://doi.org/10.1016/j.cbpb.2017.09.006

Morales-Bojórquez, E., y Pacheco-Bedoya, J. L. (2017). A mantle length structured stock assessment model for the jumbo squid, Dosidicus gigas, fishery of the Ecuadorian Pacific: a limited data approach. *Marine Biology Research*, *13*(4), 417-428.

Nielsen, L. B., & Nielsen, H. H. (2001). Purification and characterization of cathepsin D from herring muscle (Clupea harengus). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 128(2), 351-363.

Nishimura, Y., Kawabata, T., & Kato, K. (1988). Identification of latent procathepsins B and L in microsomal lumen: characterization of enzymatic activation and proteolytic processing in vitro. Archives of biochemistry and biophysics, 261(1), 64-71.

Padilha, M. H. P., Pimentel, A. C., Ribeiro, A. F., & Terra, W. R. (2009). Sequence and function of lysosomal and digestive cathepsin D-like proteinases of Musca domestica midgut. Insect Biochemistry and Molecular Biology, 39(11), 782-791.

Rojo, L., & García-Carreño, F. (2013). Cold-adapted digestive aspartic protease of the clawed lobsters Homarus americanus and Homarus gammarus: biochemical characterization. Marine Biotechnology, 15(1), 87-96.

Rojo, L., Sotelo-Mundo, R., García-Carreño, F., & Gráf, L. (2010). Isolation, biochemical characterization, and molecular modeling of American lobster digestive cathepsin D1. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 157(4), 394-400.

Russell, N. J. (1998). Molecular adaptations in psychrophilic bacteria: potential for biotechnological applications Biotechnology of Extremophiles (pp. 1-21): Springer.

Siddiqui, K. S., & Cavicchioli, R. (2006). Cold-adapted enzymes. Annu. Rev. Biochem., 75, 403-433.

Tiscar, P. G., & Mosca, F. (2004). Defense mechanisms in farmed marine molluscs. Veterinary research communications, 28, 57-62.

Venugopal, A., & Kumar, N. S. (2014). Biochemical characterization of cathepsin D from the mussel Lamellidens corrianus. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 169, 25-30.

Williamson, A. L., Brindley, P. J., Abbenante, G., Prociv, P., Berry, C., Girdwood, K., . . . Dalton, J. P. (2002). Cleavage of hemoglobin by hookworm cathepsin D aspartic proteases and its potential contribution to host specificity. The FASEB Journal, 16(11), 1458-1460.

Xiao, R., Zhang, Z., Wang, H., Han, Y., Gou, M., Li, B., . . . Li, Q. (2015). Identification and characterization of a cathepsin D homologue from lampreys (Lampetra japonica). Developmental & Comparative Immunology, 49(1), 149-156.

DESARROLLO DEL TRABAJO DE INVESTIGACIÓN

Para probar la hipótesis planteada, el trabajo experimental se dividió en tres etapas, las cuales se describen en los siguientes tres capítulos

Capítulo 1: caracterización de la enzima catepsina D del hepatopancreas de calamar gigante (*Dosidicus gigas*)

Este capítulo consiste en un manuscrito titulado Effect of Temperature and pH on the Secondary Structure and Denaturation Process of Jumbo Squid Hepatopancreas Cathepsin D. El manuscrito contiene información acerca del efecto que puede tener el pH y la temperatura en la estructura secundaria de la proteina**.**

Capítulo 2: Caracterizacion de catepsina D del hepatopancreas de las almejas japonesa (*Ruditapes pHilippinarum*) y almeja reina (*Dosinia ponderosa*)

Este capítulo consiste en el manuscrito efecto de la temperatura y ph en la estructura secundaria y el proceso de desnaturalizacion del hepatopancreas de la almeja reina (*Dosinia ponderosa*)

Capítulo 3: Cambios en actividad, estructura secundaria y proceso de desnaturalización de catepsina D del hepatopáncreas de la almeja reina *Dosinia ponderosa* a diferentes condiciones de temperatura y pH.

Este capítulo contiene información sobre la caracterizacion y purificacion de catepsina D del hepatopancreas de la almeja almeja reina (*Dosinia ponderosa*) y como la temperatura y el pH afectan la estructura secundaria de la proteina.

Send Orders for Reprints to reprints@benthamscience.net

Protein & Peptide Letters, 2019, *26*, 532-541

RESEARCH ARTICLE

Effect of Temperature and pH on the Secondary Structure and Denaturation Process of Jumbo Squid Hepatopancreas Cathepsin D.

Cadena-Cadena Francisco[1], Cárdenas-López José Luis*[,1], Ezquerra-Brauer Josafat Marina[1], Cinco-Moroyoqui Francisco Javier[1], López-Zavala Alonso Alexis[2], Santacruz-Ortega Hisila del Carmen[3] and Rivero-Espejel Ignacio Alfredo[4]

[1]Departamento de Investigación y Posgrado de Alimentos, Universidad de Sonora, Hermosillo, Sonora, México; [2]Departamento de Ciencias Químico Biológicas, Universidad de Sonora, Hermosillo, Sonora, México; [3]Departamento de Investigación en Polímeros y Materiales, Universidad de Sonora, Hermosillo, Sonora, México; [4]Centro de Graduados e Investigación en Química, Instituto Tecnológico de Tijuana, Tijuana, Baja California, México

Abstract: ***Background*:** Cathepsin D is a lysosomal enzyme that is found in all organisms acting in protein turnover, in humans it is present in some types of carcinomas, and it has a high activity in Parkinson's disease and a low activity in Alzheimer disease. In marine organisms, most of the research has been limited to corroborate the presence of this enzyme. It is known that cathepsin D of some marine organisms has a low thermostability and that it has the ability to have activity at very acidic pH. Cathepsin D of the Jumbo squid (*Dosidicus gigas*) hepatopancreas was purified and partially characterized. The secondary structure of these enzymes is highly conserved so the role of temperature and pH in the secondary structure and in protein denaturation is of great importance in the study of enzymes. The secondary structure of cathepsin D from jumbo squid hepatopancreas was determined by means of circular dichroism spectroscopy.

***Objective*:** In this article, our purpose was to determine the secondary structure of the enzyme and how it is affected by subjecting it to different temperature and pH conditions.

***Methods*:** Circular dichroism technique was used to measure the modifications of the secondary structure of cathepsin D when subjected to different treatments. The methodology consisted in dissecting the hepatopancreas of squid and freeze drying it. Then a crude extract was prepared by mixing 1: 1 hepatopancreas with assay buffer, the purification was in two steps; the first step consisted of using an ultrafiltration membrane with a molecular cut of 50 kDa, and the second step, a pepstatin agarose resin was used to purification the enzyme. Once the enzyme was purified, the purity was corroborated with SDS PAGE electrophoresis, isoelectric point and zymogram. Circular dichroism is carried out by placing the sample with a concentration of 0.125 mg / mL in a 3 mL quartz cell. The results were obtained in mdeg (millidegrees) and transformed to mean ellipticity per residue, using 111 g/mol molecular weight/residue as average. Secondary-structure estimation from the far-UV CD spectra was calculated using K2D Dichroweb software.

***Results*:** It was found that α helix decreases at temperatures above 50 °C and above pH 4. Heating the enzyme above 70°C maintains a low percentage of α helix and increases β sheet. Far-UV CD measurements of cathepsin D showed irreversible thermal denaturation. The process was strongly dependent on the heating rate, accompanied by a process of oligomerization of the protein that appears when the sample is heated, and maintained a certain time at this temperature. An amount typically between 3 and 4% α helix of their secondary structure remains unchanged. It is consistent with an unfolding process kinetically controlled due to the presence of an irreversible reaction. The secondary structure depends on pH, and a pH above 4 causes α helix structures to be modified.

***Conclusion*:** In conclusion, cathepsin D from jumbo squid hepatopancreas showed retaining up to 4% α helix at 80°C. The thermal denaturation of cathepsin D at pH 3.5 is under kinetic control and follows an irreversible model.

Keywords: Jumbo squid, cathepsin D denaturation, circular dichroism, oligomerization, Far-UV CD, hepatopancreas.

ARTICLE HISTORY

Received: December 20, 2018
Revised: March 22, 2019
Accepted: March 23, 2019

DOI: 10.2174/0929866526666190405124353

*Address correspondence to this author at the Departamento de Investigación y Posgrado de Alimentos, Universidad de Sonora, Hermosillo, Sonora, México; Tel: 526621517311; Fax: 526622592207; E-mail: joseluis.cardenas@unison.mx

1875-5305/19 $58.00+.00

1. INTRODUCTION

Cathepsin D is a lysosomal enzyme found in most organisms acting on protein turnover [1]. The activity of this enzyme in humans is high in some types of carcinomas (breast cancer) [2], Parkinson's disease [3], and is a target in the treatment of Alzheimer's disease [4]. In marine species, cathepsin D is associated with the reproductive system of some organisms, in fish its activity is increased before spawning, functioning as a biomarker; hence, fish farmers can use the activity of this enzyme to take the necessary measures in production. In some species such as Japanese lamprey (*Lampetra japonica*) and American lobster (*Homarus americanus*), cathepsin D has been proposed as a digestive enzyme [5, 6].

The functionality of any protein is closely related to its structure [7, 8]. Circular Dichroism (CD) is a technique for detecting conformational changes that may be associated with the activity, regulation, structure of a protein, changes in DNA or in any molecule. CD spectroscopy measures the variation in absorption of left and right circularly polarized light as it passes through a chiral or optically active substance. Thus, conformational transitions can also monitored by far-UV CD [9]. Circular dichroism allows determining the secondary structure of any protein because at those wavelengths the chromophores are the peptide bonds [10], enabling the calculation of percentage of α helix, β sheet and random coils [11]. It is possible also to observe modifications in the structure of proteins by the interaction with other molecules such as drugs or antigens that can affect its functionality, changes in chirality are a good indication of the continuous changes of folding and, especially, of the modifications of the secondary and tertiary structure [12]. CD can also be used as a function of temperature to determine the thermodynamics of denaturation of the protein. When the deployment of a protein is reversible and there is only a single deployment transition, the thermodynamic parameters evaluated by CD are similar to those that can be estimated using differential scanning calorimetry [13].

There is very scarce information on the structure of cathepsin D, only the structure of human cathepsin D is known [14, 15]. In some invertebrates, cathepsin D is secreted in the hepatopancreas to digest food extracellularly [16, 17]. In Japanese common squid (*Todarodes pacificus*) [18] and pelagic squid (*Todarodes sagittatus*) [19] cathepsin D is secreted by the hepatopancreas and is involved in the digestion of food. In some clams such as *Lamelidus corianus* participates in the digestion of food [20]. In most marine organisms it is a thermolabile enzyme [5, 21].

The range of activity of catepsin D is in pH range of 3 to 5. However, most have an optimal pH of 3 and lose activity above its optimum. Optimal temperature is in the 40 to 50 °C range [22]. Cathepsin D is known to contain very little content of α helix [14, 23]. However, there are no reported three-dimensional structures of cathepsin D from marine organisms. This enzyme is a specific endopeptidase that hydrolyzes hydrophobic amino acids located in a microenvironment that is generated when there are more than two nearby hydrophobic amino acids. Therefore, secondary specificity becomes very important in this type of enzymes because they prefer a hydrophobic amino acid at the P2 site specifically for amino acids such as Glu, Phe and Leu, and in the P2' residue and basic residues in P3 [24-26].

However, structural characteristics of the enzyme could help to establish a useful model for studying the evolution of enzymes as well as their relationship with folding and protein structure, it could also serve to understand the relationship between flexibility, activity, and stability, because cathepsin D may be considered the precursor to other aspartic proteases [27, 28]. In general, changes in secondary structure are to external parameters such as temperature, inhibitor concentration or pH [23]. In order to improve understanding changes in the secondary structure of cathepsin D in the hepatopancreas of jumbo squid, UV, fluorescence and circular dichroism analyses of a pure preparation of cathepsin D were carried out, determining the effect of temperature, pH and thermal denaturation, as well as its role in enzymatic stabilization of jumbo squid (*Dosidicus gigas*).

2. MATERIALS AND METHODS

Jumbo squid (*Dosidicus gigas*) was captured in waters of Santa Rosalía in the Gulf of California (28°40`107 N, 112°383 W] during spring 2016. Organisms were kept on ice and transported to Hermosillo to the laboratory of marine products at the University of Sonora. The hepatopancreas were dissected, freeze-dried, and kept at -80°C for all experiments.

2.1. Cathepsin D Extraction and Purification

The raw extract was prepared by mixing 1:1 w/v of freeze-dried hepatopancreas with sample buffer (100 mM sodium acetate pH 5.5) [17, 29]. The protein preparation was desalted by ultrafiltration (UF) using 50 kDa MWCO membranes (Amicon, Millipore, US). UF is a low pressure membrane separation process based in the size difference in of solutes. This technique separates the molecules according to a specific size in two fractions, one of proteins greater than 50 kDa in retentate fraction and proteins less than 50 kDa in filtrate fraction [30, 31]. Purification of the enzyme was performed by injecting the filtrate fraction to a 10 mL pepstatin agarose affinity chromatography column [20] with a washing stage with sample buffer. For elution, 20 mM Tris buffer pH 8.5 was used. SDS-PAGE Electrophoresis was carried out and performed according to Laemmli (1970). The gel was stained with 0.1% Coomassie blue for band visualization [32]. Protein in solution was monitored by following absorbance at 280 nm during chromatography and protein concentration was determined by Bradford assay using bovine serum albumin as standard for eluted fractions [33]. Purity of the enzyme was attained when there was a single band in SDS-PAGE and IEF, and maximum enzyme activity in both general and specific substrates.

2.2. Activity of Cathepsin D

The enzyme assay was performed following Barrett [34] methodology for a general protease assay and Yasuda [35] methodology with modifications for a specific cathepsin D

assay. The general assay consisted of mixing 600 µL haemoglobin with 600 µL 100 mM glycine buffer pH 3.5 with 50 µL of the sample, incubated for 10 min at 37°C, the reaction was stopped with 10% TCA (trichloroacetic acid) and centrifuged at 5000 x g for 5 min, after which the Abs was measured at 280 nm against a blank. All samples were done in triplicate [29, 34]. The specific assay consisted of using a specific fluorogenic substrate for cathepsin D consisting of N-Acetyl-Arg-Gly-Phe-Phe-Pro-7-amido-4-trifluoromethyl-coumarin (AcRGFFP-AFC). The assay was performed taking 10 µL of 200 mM AcRGFFP-AFC in 80 µL of 100 mM glycine buffer pH 3.5. The substrate and buffer were pre-incubated for 2 min at 45°C. The reaction started with the addition of 10 µL of the sample for 10 min, then adding 320 µL of 100 mM glycine pH 9.5 and 10 µL of 0.6 U/mL enzyme dipeptidyl-peptidase IV. The reaction was stopped by adding 2 mL of TCA at 5%. Cathepsin D cleaves the bond of substrate on Phe-Phe amino acids, leaving one fragment containing N-Acetyl-Arg-Gly-Phe (AcRGF) and the other fragment containing Phe–Pro–7–amido–4–trifluoromethyl coumarin (FP-AFC), the enzyme dipeptidyl peptidase IV was used to cleave the bond of the FP amino acids and release the molecule of AFC. The released AFC was measured in a fluorometer at 400 nm excitation and 505 nm emission. Control consisted of adding 1.6 mL of 0.5% trichloacetic acid TCA before the substrate was added [35].

2.3. Circular Dichroism (CD) Spectroscopy

Circular dichroism spectra were performed on a Jasco J-815 spectropolarimeter equipped with a Peltier temperature controller, using 1 cm path length quartz cell in the far-UV. The samples contained a final concentration of 0.125 mg/mL of purified cathepsin D [36]. Three analysis samples were collected and averaged to obtain the final spectra in all cases. The results were obtained in mdeg (millidegrees) and transformed to mean ellipticity per residue, using 111 g/mol molecular weight/residue as average. Secondary-structure estimation from the far-UV CD spectra was calculated using K_2D Dichroweb software [37, 38].

2.4. pH Effect on Secondary Structure

Effect of pH on the content of the secondary structure was obtained by placing the enzyme in different buffers at different pH values, ranging from 2 to 7 with 0.5 intervals. All buffers avoided the use of chlorine element and were chosen according to their appropriate pKa. In each case, the baseline was corrected with the buffer.

2.5. Thermal Denaturation

The thermal denaturation was followed by circular dichroism, using an enzyme concentration of 0.128 mg/mL in 50 mM sodium acetate buffer pH 3.5. The spectra were obtained using quartz cuvettes with 1 cm of optical path. The temperature was controlled with a Peltier. The thermal denaturation was followed by the change in ellipticity at 220 nm. The temperature change in the sample was 10-80°C and carried out at different heating rates (0.5°C/min, 2°C/min and 4°C/min with an accuracy of ± 0.02°C/min).

The reversibility of the transition was performed measuring the change in ellipticity at 220 nm. The increases in temperature were 1°C/min, from 5°C to 80°C, after which, solution was cooled rapidly at a rate of 15°C/min until reaching room temperature (25°C), the difference between the two ellipticities was expressed as the irreversibly denatured protein fraction.

3. RESULTS AND DISCUSSIONS

3.1. Purification and SDS Page

The chromatographic profile shows a single peak of protein eluted with Tris buffer at pH 8.5 (Figure **1**). The purification stages are shown in Table **1**. Approximately 3.3 mg/mL of the enzyme was obtained and the specific activity resulting from purification was 9680 ± 93 U/mg with a protein recovery yield of 21% of the original activity and 94 fold purification. The activity in the washing peak before elution was negligible. However, in the elution peak, a maximum activity of the enzyme was detected. In Figure **2** (**A-D**) the cathepsin D purification steps are shown. Figure **2 E** shows the isoelectric point of 5.2 and Figure **2F** shows a zymogram of the purified fractions with a single band. The purity of the enzyme was verified by SDS-PAGE, obtaining a single band of 38.5 kDa (Figure **2D**).

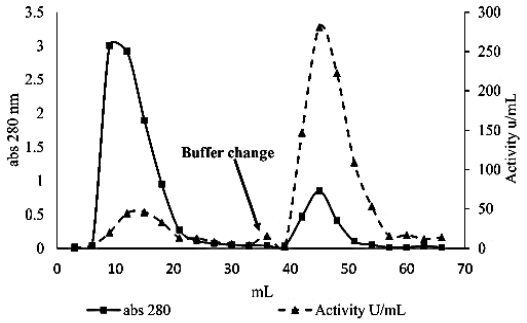


Figure 1. Affinity chromatography in pepstatin agarose of jumbo squid hepatopancreas extract, the column was equilibrated with 50 mM sodium acetate buffer in 20 mM NaCl at pH 5.0, followed by 50 mM Tris-HCl pH 8.8. The continuous line symbolizes absorbance at 280 nm, the hyphen line symbolizes the activity with the substrate AcRGFFP-AFC.

3.2. Structural Analysis by Circular Dichroism

3.2.1. Effects of Temperature

The increase in temperature can inflict an increase in the kinetic energy of the molecules, a disorganization of the aqueous envelope of the proteins and the destruction of weak interactions and disorganize the structure of the protein so that the hydrophobic interior interacts with the aqueous media and the aggregation and precipitation of the denatured protein occurs [39]. Figure **3** shows the spectrum of circular dichroism at different temperatures. The spectrum was characterized by having an intense and positive band at 189 ± 3 nm, an intense and negative band at 205-225 ± 5 nm. with a minimum of 119. These peaks are characteristic of

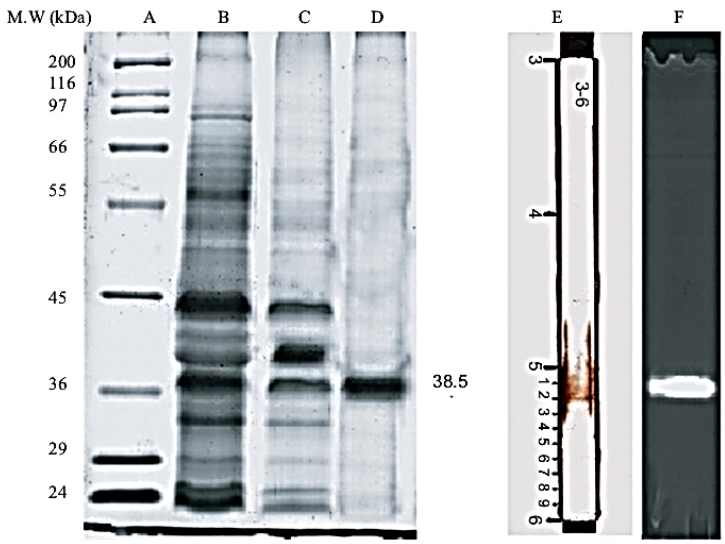


Figure 2. SDS-PAGE of purified cathepsin D from jumbo squid (*Dosidicus gigas*) hepatopancreas. (**A**) wide range molecular weight markers, (**B**) crude extract, (**C**) 50 kDa membrane (**D**) Cathepsin D in affinity chromatography using pepstatin agarose, (**E**) IEF of cathepsin D purified from jumbo squid hepatopancreas, (**F**) zymogram of cathepsin D purified from jumbo squid hepatopancreas.

Table 1. Purification table of cathepsin D from jumbo squid (*Dosidicus gigas*) hepatopancreas.

-	Total Volume (mL)	Total Activity (Units)	Total Protein (mg)	Specific Activity (U/mg protein)	Purification (X)	Yield (%)
Crude extract	100	34300	332.2	103 ±3	1.0	100
UF Membrane (50 kda)	80	23600	145	162±12	1.5	69
Affinity fractions	30	7260	0.75	9680±98	94	21

secondary β-sheet structures suggesting that this is the predominant structure in the enzyme [40]. However, at temperatures of 5 and 10°C and above 45°C the α-helix changes with a minimum at 210-220 ± 1 nm and 209 ± 2 nm with an intense band at 190 nm, which suggests the main structure continues to be β sheet but the α-helix undergoes modifications. The most affected secondary structure were the α helices [41].

Changes in α helix and β sheet are shown in Table **2**. It was observed that temperature affected mainly α helix, essentially in the loops formed from this type of secondary structure. Also the parameters of the secondary structure can be altered by the exposition of hydrophobic groups. The conformational transition that the protein undergoes is probably the result of exposure of the hydrophobic cavities and a disturbance of the microenvironments around the aromatic amino acid residues [42]. These changes are common in proteins such as ribonuclease A, which is an example of a protein that is mainly made up of β sheet [43].

The deformation of α helices at low temperatures most probably reflects transient α helix states that are stabilizing to disordered structures with increasing temperature [44]. In the case of wild boar (*Sus scrofa*) pepsinogen, it has a maximum of 193 nm and a minimum of 217 nm [45]. The spectra of these proteases are very similar and so the secondary structure content is very similar.

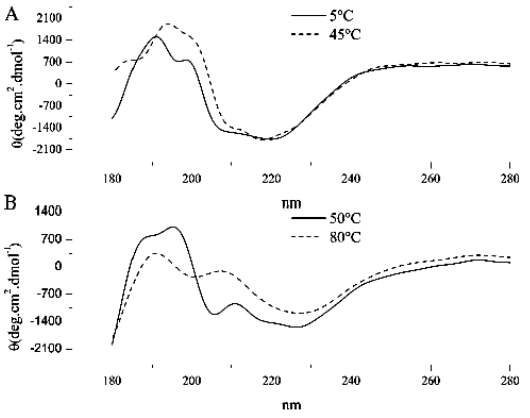


Figure 3. Circular dichroism spectrum of Cathepsin D from jumbo squid (*Dosidicus gigas*) hepatopancreas at different temperatures: **A)** 5, 40 and 45°C, and **B)** 50 and 80°C.

Table 2. Content of secondary structure of Cathepsin D from jumbo squid (*Dosidicus gigas*) hepatopancreas at different temperatures.

°C	Content of α Helix (%)	Content of β Sheet (%)
5	6.25^{c}±0.08	$46.88^{b,c,d}$ ±0.39
10	7.23^{d}±0.41	45.85^{b}±0.57
15	7.35^{d}±0.13	$45.90^{b,c}$±0.27
20	$7.88^{d,e}$±0.07	$47.14^{c,d,e}$±0.27
25	8.65^{f}±0.16	44.11^{a}±0.04
30	$8.35^{e,f}$±0.23	44.53^{a}±0.23
35	8.81^{f}±0.51	44.39^{a}±0.28
40	8.95^{f}±30	44.45^{a}±0.37
45	$8.25^{e,f}$±0.25	48.47^{f}±0.65
50	$5.20^{a,b}$±0.15	$48.40^{e,f}$ ±.04
55	$5.19^{a,b}$±0.22	48.79^{f}±0.047
60	$5.77^{b,c}$±0.26	48.52^{f}±0.40
65	5.42^{b}±0.08	$47.09^{b,c,d}$±0.59
70	$5.50^{b,c}$±0.13	$47.43^{d,e,f}$±0.50
75	$5.21^{a,b}$±0.11	$47.99^{d,e,f}$ ±0.19
80	4.56^{a}±0.406	48.00^{f} ±0.27

Averages with same superscript letter within a column are statistically equal in Tukey´s test at p≥0.05. Random coil not shown because the content remains constant.

The stability of the α helix depends on several factors and among the main are the amino acids that are present in the protein, the position of these amino acids within the helix and the interactions that may occur between the lateral chains of the amino acids that are forming this type of secondary structure are responsible for the modification of this structure [46]. The lateral chains located on the external part of the α helix avoid major steric impediment problems. Amino acids such as Ala, Glu, Leu, and Met are frequently found in helices [47]. Amino acids such as Pro are considered to be α-helix terminators because their α C does not have the freedom of rotation and because they are integrated into a ring, it interferes with the formation of hydrogen bridges [48]. Other amino acids such as Gly, Tyr, and Ser almost do not form part of these structures, due in part to the steric configuration of these amino acids, for example, amino acids with large R groups such as tyrosine and tryptophan or with small R-groups such as Gly destabilize α-helices in general the amino acids with polar groups are the worst helix formers due to freezing of the additional -CH2- bond that separates the main chain from the polar group [41, 47]. Another cause of instability of the α helix is the total dipole moment of the complete helix due to the individual dipoles of the C = O groups involved in the hydrogen bond. When an α helix is stable, it usually ends up with a charged amino acid which neutralizes the dipole moment [41, 47, 48].

Considering that only some amino acids are present in the α helix, the formation or deformation of α helix structures is a process that is driven by enthalpy and it depends on several factors like variations of pH, temperature changes and solvent composition alterations [8, 49]. In the case of temperature increase, the α helix begins to search for a more stable conformation [50]. In some cases, the deformation of the α helix adopts other types of conformations that give it more stability such as the β sheet [49]. In this way, the change of secondary structure at different temperatures can be explained. The rapid separation of the secondary structure at elevated temperatures usually indicates minimal hydrophobic interaction suggesting that hydrophobic interactions are not important for stabilizing α helix, but they are important in β sheet formation and stabilization [51-53]. The α helix structure is more easily formed than a β sheet. However, β sheet is more stable and is not affected by temperature and pH changes [51].

3.2.2. Effect of pH on the Secondary Structure

The stability of the secondary structure of cathepsin D is different at different pH conditions. This could be due to the effects of ionization that a side chain can acquire on the secondary structure of proteins [23]. Table **3** shows that the increase in pH modifies the content of the secondary structure and the α helix are possibly deformed and changed into new structures, mainly β sheet. Something similar was detected in human cathepsin D. At pH 7, the content of secondary structure changes and the pH acts as a regulator by modifying the secondary structure, basically deforming α helixes into β sheets [23]. Changes in this type of structures may be due to the type of residues present, for example, the presence of Gly and Pro could alter the structure due to their high ΔG° of formation [8].

Table 3. The content of secondary structure of Cathepsin D from jumbo squid (*Dosidicus gigas*) hepatopancreas at different pH.

-	Content of α Helix (%)	Content of β Sheet (%)	Content of Random Coil (%)
2.5	6.44^{c}±0.16	38.09^{a}±0.58	47^{a}±1.50
3.5	8.24^{d}±0.70	43.91^{b}±0.57	48^{a}±0.52
4	2.73^{a}±0.24	39.94^{a}±0.31	49^{a}±0.85
5	4.05^{b}±0.18	43.10^{b}±1.13	47^{a}±2.25
6	$3.48^{a,b}$±0.13	39.05^{a}±0.68	47^{a}±2.30
7	$3.53^{a,b}$±0.27	38.39^{a}±0.92	46^{a}±1.33

Averages with same superscript letter within a column are statistically equal in Tukey´s test at p≥0.05.

The K2D analysis on the Dicroweb page indicates that if pH is above optimum, the protein structure is predominantly

β sheet but the α helix is 8%. However, the increase in pH causes the reordering of amino acids, this suggests that pH is of great importance for enzymatic activity and promotes the appearance of random coils or β sheet [37]. For protein Z19 belonging to the α zein group from corn (*Zea mays L.*) it was found that pH increases the denaturation velocity [54].

The data suggests that the rate of denaturing depends on pH and grows with increasing alkalinity of the medium. In the case of the *Brugia malayi* parasite, the proteins were highly resistant to denaturing, the modification of the secondary structure is minimal in α helix and changes only occur at high pH, maintaining up to 50% of the secondary structure [55]. In contrast, the cathepsin D of jumbo squid easily loses α helix structure one pH unit above its optimal point, suggesting that it plays a very important role in secondary structure.

The denaturation of the protein due to pH can be explained due to the different ionization of the amino acids present in the protein. For example Glu and Asp, that at alkaline pH values are transformed to glutamate and aspartate due to the introduction of negative charges in the protein structure, thus promoting electrostatic interactions with the adjacent carboxyl group, structural instability is due to the reaction that can occur between the lateral chains of amino acids that are not forming α helix with those that are in a type of secondary structure [8, 51, 54, 56].

The activity of cathepsin D in pH and temperature experiments showed a non-linear relationship. As pH and temperature moved away from the optimal, the activity decreased. The optimum pH and temperature were 3.5 and 45°C respectively. The reduction of activity is attributed to the loss of stability of the enzyme due to modification of the secondary structure. It has been found that at basic pH, a β sheet blocks the active site of the enzyme, while at acidic pH (far from the optimal), the enzyme exhibits low activity probably due to autolysis [23]. As the temperature is increased, the thermodynamic stability of the protein approaches that of the working temperature. At the physiologically operational temperature conformational dynamics and specific activity increase and decrease outside the physiologically operating temperature [57].

3.2.3. Thermal Unfolding of the Cathepsin D

Thermal transition of protein denaturation depends on the heating rate because the unfolding process is under kinetic control and may be due to the presence of an irreversible reaction or to the change in the dynamic properties of the atoms inside the molecule, transitions generally appear at high temperatures, while at low temperatures simple harmonic vibrations predominate [58-61].

There is a direct relationship between the increase in temperature and electrostatic interactions, there is a greater amount of energy that strengthens the molecule, making the structure more rigid, rejecting conformational fluctuations [62]. Electrostatic interaction shows fortification with the increase of temperature, due to the dielectric property dependent on the temperature of the aqueous environment. This temperature increase indicates the presence of a positive entropic contribution because the electrostatic interaction itself is free energy. The positive entropy is related to the orientation of the water molecules with increasing temperature, this is manifested in the temperature dependence of the dielectric constant [63]. The electrostatic interactions are stabilized at 70°C, but at 10°C only half of them are active, which makes their structure unstable [64].

Supposing that the folding of the protein is a two-state process, the midpoint of denaturation would be that temperature (Tm) in which the folded and unfolded state are in equilibrium [65, 66]. Figure **4** shows thermal transition curves using circular dichroism at different heating rates and it is observed that the changes begin to appear around 50°C, which coincides with the change in α helices the enzyme shown in table 2. It is also observed that the Tm is modified according to the heating rate, a speed of slow heating of 0.5°C/min presents a Tm of 60°C while at higher speeds of 2°C and 4°C/min the Tm is modified passes to 65°C, this may be due to the fact that at lower heating speeds the protein can be stabilized by means of hydrogen bridges protect the hydrophobic microenvironment that is inside the molecule [67]. Samples heated to temperatures above 60°C showed no reversibility of the circular dichroism signal when cooled to 25°C. Three processes may be involved in the dissociation of proteins: the first is the rupture of the ionic bonds, the second is that secondary structure begins to dissociate hidden surfaces of exposure and the third is the loss of cations [68, 69].

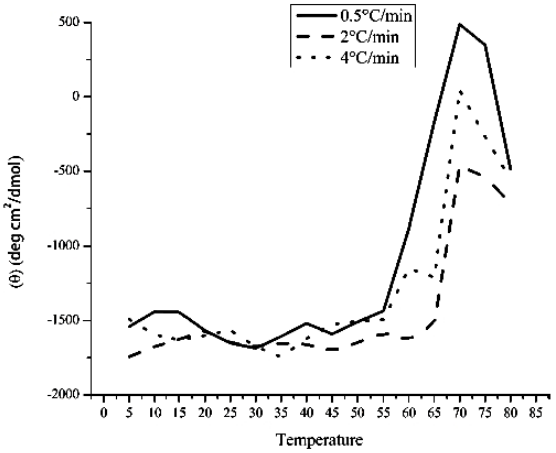


Figure 4. Cathepsin D from jumbo squid (*Dosidicus gigas*) hepatopancreas thermal transition curves as a function of the heating rate at pH 3.5. The process was followed by ellipticity monitoring at 220 nm. Heating rates: 0.5°C / min (dash point dash line), 2.0°C / min (continuous line), 4.0°C / min (dashed line).

In the case of cathepsin D, turbidity was observed in the solutions when heating above 70°C and more than 10 min. This suggests that irreversibility may be associated with unfolded protein aggregation. The turbidity changes are related to changes with the protein unfolding since the turbidity of the sample coincides at the temperature that begins the unfolding of the protein. In conclusion, the unfolding of cathepsin D is due to the unfolding of α-helix structure, this leads to intrinsic irreversibility that interferes

with thermodynamic analysis. A similar behavior was detected for apolipoprotein C1 and for asparaginase 2 in which the aggregation was detected from 63°C [58], also for *Aspergillus* acid proteases, in which a change and aggregation were detected from 70°C [70].

The native structure of cathepsin D was not completely lost, yet the β sheet structure increased and the random coils remained constant. Based on these results, the enzyme could have begun to suffer from changes in the disposition of α helix towards β sheets. These changes can be detected as an increase in the β sheet structure percentage. In the sorghum protein kafirin, the formation of aggregates start at 40°C and apparently prevent deformation of the α helix [71]. On the Z19 corn protein, it was found that the lack of changes in α helix may be due to the great role played by disulfide bridges that stabilize the protein since in this protein the disulfide bridges are present in great quantities [54, 71]. In the case of human cathepsin D, it has only 4 disulfide bridges, so a lower stability is expected. Therefore, it is reasonable that at this temperature the secondary structure will no longer recover [14-15, 23]. In the case of subtilisin, the temperature at which the secondary structure is not recovered is 61 to 71 °C depending on the rate of heating, the denaturation is a function of the heating rate because it could offer no time to adapt and so it is believed that there may be intermediate states of secondary structures such as in the case of enolase, which, as it is warming up, tends to dissociate its quaternary structure and aggregate, which prevents the α helix from being lost [68, 72]. When the sample was cooled rapidly, it retained 3-4% of the α helix.

Another important aspect is its melting temperature of 60°C, which is very similar to that reported for pepsinogen (62°C) [73]. It was observed that the process is markedly irreversible [60], for ribonuclease (63°C) and for chymotrypsinogen (57°C). However, it is higher than that reported for trypsin (54°C) and much lower than that reported for myoglobin (85°C), insulin (73°C) and lysozyme (66°C) [73].

The denaturation unfolding process was dependent on the heating rate, it is kinetically controlled due of an irreversible reaction, being the rapid speed of heating the condition that affects denaturation of the protein the least [60].

CONCLUSION

In conclusion, cathepsin D from jumbo squid hepatopancreas showed a tendency towards the conservation of secondary structure retaining up to 4% α helix at 80°C. These processes in the thermal denaturation are irreversible at low temperature.

The secondary structure of cathepsin D from jumbo squid hepatopancreas depends on the conditions of temperature and strongly depends on pH. The thermal denaturation of cathepsin D at pH 3.5 is under kinetic control and follows an irreversible model [23].

These findings are important for establishing the foundations for further studies aimed at the potential uses of the cathepsin D from jumbo squid (*Dosidicus gigas*) and also for understanding its role in protein hydrolysis.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study is ethically approved by CAP (Comisión Académica del Posgrado, or Graduate Academic Commission from the University of Sonora, Mexico in english)

HUMAN AND ANIMAL RIGHTS

No humans were used in this research. The animals used were according to the Guidelines of The national methodology of the official mexican standard NOM-062-ZOO-1999, titled "Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio" (Technical specifications for production, care and use of laboratory animals in english)

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

All the resources for the realization of this research were obtained from public funds through the project entitled "Physical and chemical characterization of cathepsins L and D of the giant squid hepatopancreas". Conacyt (Mexican Council for Science and Technology) Basic Science project # 182348 directed by Dr. José Luis Cárdenas-López.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

REFERENCES

[1] Press, E.M.; Porter, R.R.; Cebra, J. The isolation and properties of a proteolytic enzyme, cathepsin D, from bovine spleen. *Biochem. J.,* **1960**, *74*(3), 501-514. [http://dx.doi.org/10.1042/bj0740501] [PMID: 14434765]

[2] Fusek, M.; Vetvicka, V. Dual role of cathepsin D: Ligand and protease. *Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub.,* **2005**, *149*(1), 43-50. [http://dx.doi.org/10.5507/bp.2005.003] [PMID: 16170387]

[3] Crabtree, D.; Dodson, M.; Ouyang, X.; Boyer-Guittaut, M.; Liang, Q.; Ballestas, M.E.; Fineberg, N.; Zhang, J. Over-expression of an inactive mutant cathepsin D increases endogenous alpha-synuclein and cathepsin B activity in SH-SY5Y cells. *J. Neurochem.,* **2014**, *128*(6), 950-961. [http://dx.doi.org/10.1111/jnc.12497] [PMID: 24138030]

[4] Di Domenico, F.; Tramutola, A.; Perluigi, M. Cathepsin D as a therapeutic target in Alzheimer's disease. *Expert Opin. Ther. Targets,* **2016**, *20*(12), 1393-1395. [http://dx.doi.org/10.1080/14728222.2016.1252334] [PMID: 27805462]

[5] Rojo, L.; García-Carreño, F.; de Los Angeles Navarrete del Toro, M. Cold-adapted digestive aspartic protease of the clawed lobsters

Homarus americanus and *Homarus gammarus*: Biochemical characterization. *Mar. Biotechnol. (NY),* **2013**, *15*(1), 87-96. [http://dx.doi.org/10.1007/s10126-012-9461-4] [PMID: 22648335]

[6] Xiao, R.; Zhang, Z.; Wang, H.; Han, Y.; Gou, M.; Li, B.; Duan, D.; Wang, J.; Liu, X.; Li, Q. Identification and characterization of a cathepsin D homologue from lampreys (*Lampetra japonica*). *Dev. Comp. Immunol.,* **2015**, *49*(1), 149-156. [http://dx.doi.org/10.1016/j.dci.2014.10.014] [PMID: 25450905]

[7] Carter, C.W.; Sweet, R.M. Macromolecular Crystallography. Gulf Professional Publishing: *Houston*, Texas, **1997**.

[8] Nelson, D.; Cox, M. Lehninger Principles of Biochemistry. International Ed. Macmillan Learning: New York, NY, **2017**.

[9] Zolfagharzadeh, M.; Pirouzi, M.; Asoodeh, A.; Saberi, M.R.; Chamani, J. A comparison investigation of DNP-binding effects to HSA and HTF by spectroscopic and molecular modeling techniques. *J. Biomol. Struct. Dyn.,* **2014**, *32*(12), 1936-1952. [http://dx.doi.org/10.1080/07391102.2013.843062] [PMID: 24125112]

[10] Sreerama, N.; Woody, R.W. Estimation of protein secondary structure from circular dichroism spectra: Comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. *Anal. Biochem.,* **2000**, *287*(2), 252-260. [http://dx.doi.org/10.1006/abio.2000.4880] [PMID: 11112271]

[11] Woody, R.W. Circular dichroism. *Methods Enzymol.,* **1995**, *246*, 34-71. [http://dx.doi.org/10.1016/0076-6879(95)46006-3] [PMID: 7538625]

[12] Tousi, S.H.; Saberi, M.R.; Chamani, J. Comparing the interaction of cyclophosphamide monohydrate to human serum albumin as opposed to holo-transferrin by spectroscopic and molecular modeling methods: Evidence for allocating the binding site. *Protein Pept. Lett.,* **2010**, *17*(12), 1524-1535. [http://dx.doi.org/10.2174/092986611009011524] [PMID: 20937032]

[13] Greenfield, N.J. Using circular dichroism collected as a function of temperature to determine the thermodynamics of protein unfolding and binding interactions. *Nat. Protoc.,* **2006**, *1*(6), 2527-2535. [http://dx.doi.org/10.1038/nprot.2006.204] [PMID: 17406506]

[14] Baldwin; Bhat, T.N.; Gulnik, S.; Hosur, M.V.; Sowder, R.C.; Cachau, R.E.; Collins, J.; Silva, A.M.; Erickson, J.W. Crystal structures of native and inhibited forms of human cathepsin D: Implications for lysosomal targeting and drug design. *Proc. Natl. Acad .Sci. USA.,***1993**, *90*(14), 6796-6800. [https://dx.doi.org/10.1073%2Fpnas.90.14.6796] [PMID: 8393577]

[15] Grädler, U.; Czodrowski, P.; Tsaklakidis, C.; Klein, M.; Werkmann, D.; Lindemann, S.; Maskos, K.; Leuthner, B. Structure-based optimization of non-peptidic Cathepsin D inhibitors. *Bioorg. Med. Chem. Lett.,* **2014**, *24*(17), 4141-4150. [http://dx.doi.org/10.1016/j.bmcl.2014.07.054] [PMID: 25086681]

[16] Barker, P.L.; Gibson, R. Observations on the feeding mechanism, structure of the gut, and digestive physiology of the European lobster *Homarus gammarus* (L.) (Decapoda: Nephropidae). *J. Exp. Mar. Biol. Ecol.,* **1977**, *26*(3), 297-324. [http://dx.doi.org/10.1016/0022-0981(77)90089-2]

[17] Rojo, L.; Sotelo-Mundo, R.; García-Carreño, F.; Gráf, L. Isolation, biochemical characterization, and molecular modeling of American lobster digestive cathepsin D1. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.,* **2010**, *157*(4), 394-400. [http://dx.doi.org/10.1016/j.cbpb.2010.08.009] [PMID: 20817002]

[18] Komai, T.; Kawabata, C.; Amano, M.; Lee, B.R.; Ichishima, E. Todarepsin, a new cathepsin D from hepatopancreas of Japanese common squid (*Todarodes pacificus*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.,* **2004**, *137*(3), 373-382. [http://dx.doi.org/10.1016/j.cbpc.2004.01.006] [PMID: 15050524]

[19] Gildberg, A. Purification and characterisation of cathepsin D from the digestive gland of the pelagic squid *Todarodes sagittatus*. *J. Sci. Food Agric.,* **1987**, *39*(1), 85-94. [http://dx.doi.org/10.1002/jsfa.2740390110]

[20] Venugopal, A.; Siva Kumar, N. Biochemical characterization of cathepsin D from the mussel *Lamellidens corrianus*. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.,* **2014**, *169*, 25-30. [http://dx.doi.org/10.1016/j.cbpb.2013.12.003] [PMID: 24365170]

[21] Gudmundsdóttir, Á.; Fox, J.W.; Chlebowski, J.F.; Craik, C.S. Characteristics, protein engineering and applications of psychrophilic marine proteinases from Atlantic cod. *Studies in Organic Chemistry.,* **1993**, *47*, 205-214. [https://doi.org/10.1016/B978-0-444-89372-7.50027-0]

[22] Balti, R.; Hmidet, N.; Jellouli, K.; Nedjar-Arroume, N.; Guillochon, D.; Nasri, M. Cathepsin D from the hepatopancreas of the cuttlefish (*Sepia officinalis*): Purification and characterization. *J. Agric. Food Chem.,* **2010**, *58*(19), 10623-10630. [http://dx.doi.org/10.1021/jf102233d] [PMID: 20843039]

[23] Lee, A.Y.; Gulnik, S.V.; Erickson, J.W. Conformational switching in an aspartic proteinase. *Nat. Struct. Biol.,* **1998**, *5*(10), 866-871. [http://dx.doi.org/10.1038/2306] [PMID: 9783744]

[24] Scarborough, P.E.; Guruprasad, K.; Topham, C.; Richo, G.R.; Conner, G.E.; Blundell, T.L.; Dunn, B.M. Exploration of subsite binding specificity of human cathepsin D through kinetics and rule-based molecular modeling. *Protein Sci.,* **1993**, *2*(2), 264-276. [http://dx.doi.org/10.1002/pro.5560020215] [PMID: 8443603]

[25] Sun, H.; Lou, X.; Shan, Q.; Zhang, J.; Zhu, X.; Zhang, J.; Wang, Y.; Xie, Y.; Xu, N.; Liu, S. Proteolytic characteristics of cathepsin D related to the recognition and cleavage of its target proteins. *PLoS One,* **2013**, *8*(6), e65733. [http://dx.doi.org/10.1371/journal.pone.0065733] [PMID: 23840360]

[26] Keil, B. Specificity of proteolysis. Springer Science & Business Media: Berlin, **2012**; p. 336.

[27] Gildberg, A. Aspartic proteinases in fishes and aquatic invertebrates. *Comp. Biochem. Physiol. B,* **1988**, *91*(3), 425-435. [http://dx.doi.org/10.1016/0305-0491(88)90002-8] [PMID: 3148385]

[28] Vetri, V.; Militello, V. Thermal induced conformational changes involved in the aggregation pathways of beta-lactoglobulin. *Biophys. Chem.,* **2005**, *113*(1), 83-91. [http://dx.doi.org/10.1016/j.bpc.2004.07.042] [PMID: 15617813]

[29] Celis-Guerrero, L.E.; García-Carreño, F.L.; del Toro, M.A.N. Characterization of proteases in the digestive system of spiny lobster (*Panulirus interruptus*). *Mar. Biotechnol. (NY),* **2004**, *6*(3), 262-269. [http://dx.doi.org/10.1007/s10126-003-0032-6] [PMID: 15136918]

[30] Wang, T.; Wang, Y-Q.; Su, Y-L.; Jiang, Z-Y. Antifouling ultrafiltration membrane composed of polyethersulfone and sulfobetaine copolymer. *J. Membr. Sci.,* **2006**, *280*(1-2), 343-350. [http://dx.doi.org/10.1016/j.memsci.2006.01.038]

[31] Serna-Cock, L.; Velásquez, M.; Ayala, A.A. Efecto de la ultrafiltración sobre las propiedades reológicas de gelatina comestible de origen bovino. *Inf. Tecnol.,* **2010**, *21*(6), 91-102. [http://dx.doi.org/10.4067/S0718-07642010000600011]

[32] Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **1970**, *227*(5259), 680-685. [http://dx.doi.org/10.1038/227680a0] [PMID: 5432063]

[33] Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **1976**, *72*(1-2), 248-254. [http://dx.doi.org/10.1016/0003-2697(76)90527-3] [PMID: 942051]

[34] Barrett, A.J.; Cathepsin, D. Cathepsin D. Purification of isoenzymes from human and chicken liver. *Biochem. J.,* **1970**, *117*(3), 601-607. [http://dx.doi.org/10.1042/bj1170601] [PMID: 5419752]

[35] Yasuda, Y.; Kageyama, T.; Akamine, A.; Shibata, M.; Kominami, E.; Uchiyama, Y.; Yamamoto, K. Characterization of new fluorogenic substrates for the rapid and sensitive assay of cathepsin E and cathepsin D. *J. Biochem.,* **1999**, *125*(6), 1137-1143. [http://dx.doi.org/10.1093/oxfordjournals.jbchem.a022396] [PMID: 10348917]

[36] Kelly, S.M.; Jess, T.J.; Price, N.C. How to study proteins by circular dichroism. *Biochim. Biophys. Acta (BBA)-. Proteins and Proteomics,* **2005**, *1751*(2), 119-139. [http://dx.doi.org/10.1016/j.bbapap.2005.06.005]

[37] Whitmore, L.; Wallace, B.A. Protein secondary structure analyses from circular dichroism spectroscopy: methods and reference databases. *Biopolymers,* **2008**, *89*(5), 392-400.

[http://dx.doi.org/10.1002/bip.20853] [PMID: 17896349]

[38] Louis-Jeune, C.; Andrade-Navarro, M.A.; Perez-Iratxeta, C. Prediction of protein secondary structure from circular dichroism using theoretically derived spectra. *Proteins,* **2012**, *80*(2), 374-381. [http://dx.doi.org/10.1002/prot.23188] [PMID: 22095872]

[39] Raison, J.K. The influence of temperature-induced phase changes on the kinetics of respiratory and other membrane-associated enzyme systems. In: *Membrane structure and mechanisms of biological energy transduction.* Springer: Berlin, **1972**; pp. 559-583. [http://dx.doi.org/10.1007/978-1-4684-2016-6_30]

[40] Holzwarth, G.; Doty, P. The ultraviolet circular dichroism of polypeptides. *J. Am. Chem. Soc.,* **1965**, *87*(2), 218-228. [http://dx.doi.org/10.1021/ja01080a015] [PMID: 14228459]

[41] David, N.L.; Cox, M. Lehninger. Principios de bioquímica: España, **2015**.

[42] Sanei, H.; Asoodeh, A.; Hamedakbari-Tusi, S.; Chamani, J. Multispectroscopic investigations of aspirin and colchicine interactions with human hemoglobin: binary and ternary systems. *J. Solution Chem.,* **2011**, *40*(11), 1905-1931. [http://dx.doi.org/10.1007/s10953-011-9766-3]

[43] Tilton, R.F., Jr; Dewan, J.C.; Petsko, G.A. Effects of temperature on protein structure and dynamics: X-ray crystallographic studies of the protein ribonuclease-A at nine different temperatures from 98 to 320 K. *Biochemistry,* **1992**, *31*(9), 2469-2481. [http://dx.doi.org/10.1021/bi00124a006] [PMID: 1547232]

[44] Kjaergaard, M.; Nørholm, A.B.; Hendus-Altenburger, R.; Pedersen, S.F.; Poulsen, F.M.; Kragelund, B.B. Temperature-dependent structural changes in intrinsically disordered proteins: Formation of α-helices or loss of polyproline II? *Protein Sci.,* **2010**, *19*(8), 1555-1564. [http://dx.doi.org/10.1002/pro.435] [PMID: 20556825]

[45] Lees, J.G.; Miles, A.J.; Wien, F.; Wallace, B.A. A reference database for circular dichroism spectroscopy covering fold and secondary structure space. *Bioinformatics,* **2006**, *22*(16), 1955-1962. [http://dx.doi.org/10.1093/bioinformatics/btl327] [PMID: 16787970]

[46] Cantor, C.R.; Schimmel, P.R. Biophysical chemistry, part III: The behaviour of biological macromolecules. San Francisco, CA, **1980**.

[47] Pace, C.N.; Scholtz, J.M. A helix propensity scale based on experimental studies of peptides and proteins. *Biophys. J.,* **1998**, *75*(1), 422-427. [http://dx.doi.org/10.1016/S0006-3495(98)77529-0] [PMID: 9649402]

[48] Sengupta, D.; Behera, R.N.; Smith, J.C.; Ullmann, G.M. The α helix dipole: Screened out? *Structure,* **2005**, *13*(6), 849-855. [http://dx.doi.org/10.1016/j.str.2005.03.010] [PMID: 15939016]

[49] Qin, Z.; Buehler, M.J. Molecular dynamics simulation of the α-helix to β-sheet transition in coiled protein filaments: Evidence for a critical filament length scale. *Phys. Rev. Lett.,* **2010**, *104*(19): 198304 [http://dx.doi.org/10.1103/PhysRevLett.104.198304] [PMID: 20867006]

[50] Ding, F.; Borreguero, J.M.; Buldyrey, S.V.; Stanley, H.E.; Dokholyan, N.V. Mechanism for the α-helix to β-hairpin transition. *Proteins,* **2003**, *53*(2), 220-228. [http://dx.doi.org/10.1002/prot.10468] [PMID: 14517973]

[51] Barrow, C.J.; Yasuda, A.; Kenny, P.T.M.; Zagorski, M.G. Solution conformations and aggregational properties of synthetic amyloid β-peptides of Alzheimer's disease. Analysis of circular dichroism spectra. *J. Mol. Biol.,* **1992**, *225*(4), 1075-1093. [http://dx.doi.org/10.1016/0022-2836(92)90106-T] [PMID: 1613791]

[52] Bierzynski, A.; Kim, P.S.; Baldwin, R.L. A salt bridge stabilizes the helix formed by isolated C-peptide of RNase A. *Proc. Natl. Acad. Sci. USA,* **1982**, *79*(8), 2470-2474. [http://dx.doi.org/10.1073/pnas.79.8.2470] [PMID: 6283528]

[53] Macdonald, B.; McCarley, S.; Noeen, S.; van Giessen, A.E. Protein-protein interactions affect alpha helix stability in crowded environments. *J. Phys. Chem. B,* **2015**, *119*(7), 2956-2967. [http://dx.doi.org/10.1021/jp512630s] [PMID: 25591002]

[54] Cabra, V.; Arreguin, R.; Vazquez-Duhalt, R.; Farres, A. Effect of temperature and pH on the secondary structure and processes of oligomerization of 19 kDa alpha-zein. *Biochim. Biophys. Acta,* **2006**, *1764*(6), 1110-1118. [http://dx.doi.org/10.1016/j.bbapap.2006.04.002] [PMID: 16765112]

[55] Yadav, S.; Gupta, S.; Saxena, J.K. Monitoring thermal and chemical unfolding of *Brugia malayi* calreticulin using fluorescence and Circular Dichroism spectroscopy. *Int. J. Biol. Macromol.,* **2017**, *102*, 986-995. [http://dx.doi.org/10.1016/j.ijbiomac.2017.04.053] [PMID: 28416397]

[56] Nelson, R.J. An introduction to behavioral endocrinology. 5th edition. Sinauer Associates: Sunderland, MA, **2005**, pp. 722.

[57] Varley, P.G.; Pain, R.H. Relation between stability, dynamics and enzyme activity in 3-phosphoglycerate kinases from yeast and *Thermus thermophilus*. *J. Mol. Biol.,* **1991**, *220*(2), 531-538. [http://dx.doi.org/10.1016/0022-2836(91)90028-5] [PMID: 1856872]

[58] Tello-Solis, S.R.; Hernandez-Arana, A. Effect of irreversibility on the thermodynamic characterization of the thermal denaturation of *Aspergillus saitoi* acid proteinase. *Biochem. J.,* **1995**, *311*(Pt 3), 969-974. [http://dx.doi.org/10.1042/bj3110969] [PMID: 7487958]

[59] Arroyo-Reyna, A.; Hernández-Arana, A. The thermal denaturation of stem bromelain is consistent with an irreversible two-state model. *Biochim. Biophys. Acta,* **1995**, *1248*(2), 123-128. [http://dx.doi.org/10.1016/0167-4838(95)00014-L] [PMID: 7748893]

[60] Tello-Solís, S.R.; Romero-García, B. Thermal denaturation of porcine pepsin: A study by circular dichroism. *Int. J. Biol. Macromol.,* **2001**, *28*(2), 129-133. [http://dx.doi.org/10.1016/S0141-8130(00)00154-9] [PMID: 11164229]

[61] Bull, H.B.; Breese, K. Thermal transitions of proteins. *Arch. Biochem. Biophys.,* **1973**, *156*(2), 604-612. [http://dx.doi.org/10.1016/0003-9861(73)90311-1] [PMID: 4352419]

[62] Sinha, N.; Smith-Gill, S.J. Electrostatics in protein binding and function. *Curr. Protein Pept. Sci.,* **2002**, *3*(6), 601-614. [http://dx.doi.org/10.2174/1389203023380431] [PMID: 12470214]

[63] Okazaki, K.; Sato, T.; Takano, M. Temperature-enhanced association of proteins due to electrostatic interaction: a coarse-grained simulation of actin-myosin binding. *J. Am. Chem. Soc.,* **2012**, *134*(21), 8918-8925. [http://dx.doi.org/10.1021/ja301447j] [PMID: 22559201]

[64] Xia, Y-L.; Sun, J-H.; Ai, S-M.; Li, Y.; Du, X.; Sang, P.; Yang, L-Q.; Fu, Y-X.; Liu, S-Q. Insights into the role of electrostatics in temperature adaptation: a comparative study of psychrophilic, mesophilic, and thermophilic subtilisin-like serine proteases. *RSC Advances,* **2018**, *8*(52), 29698-29713. [http://dx.doi.org/10.1039/C8RA05845H]

[65] Muñoz, V.; Sanchez-Ruiz, J.M. Exploring protein-folding ensembles: A variable-barrier model for the analysis of equilibrium unfolding experiments. *Proc. Natl. Acad. Sci. USA,* **2004**, *101*(51), 17646-17651. [http://dx.doi.org/10.1073/pnas.0405829101] [PMID: 15591110]

[66] Bischof, J.C.; He, X. Thermal stability of proteins. *Ann. N. Y. Acad. Sci.,* **2005**, *1066*(1), 12-33. [http://dx.doi.org/10.1196/annals.1363.003] [PMID: 16533916]

[67] Back, J.F.; Oakenfull, D.; Smith, M.B. Increased thermal stability of proteins in the presence of sugars and polyols. *Biochemistry,* **1979**, *18*(23), 5191-5196. [http://dx.doi.org/10.1021/bi00590a025] [PMID: 497177]

[68] Kornblatt, M.J.; Lange, R.; Balny, C. Can monomers of yeast enolase have enzymatic activity? *Eur. J. Biochem.,* **1998**, *251*(3), 775-780. [http://dx.doi.org/10.1046/j.1432-1327.1998.2510775.x] [PMID: 9490051]

[69] Kornblatt, M.J.; Lange, R.; Balny, C. Use of hydrostatic pressure to produce 'native' monomers of yeast enolase. *Eur. J. Biochem.,* **2004**, *271*(19), 3897-3904.

[http://dx.doi.org/10.1111/j.1432-1033.2004.04326.x] [PMID: 15373835]

[70] Benjwal, S.; Verma, S.; Röhm, K.H.; Gursky, O. Monitoring protein aggregation during thermal unfolding in circular dichroism experiments. *Protein Sci.,* **2006**, *15*(3), 635-639. [http://dx.doi.org/10.1110/ps.051917406] [PMID: 16452626]

[71] Gao, C.; Taylor, J.; Wellner, N.; Byaruhanga, Y.B.; Parker, M.L.; Mills, E.N.; Belton, P.S. Effect of preparation conditions on protein secondary structure and biofilm formation of kafirin. *J. Agric. Food Chem.,* **2005**, *53*(2), 306-312. [http://dx.doi.org/10.1021/jf0492666] [PMID: 15656666]

[72] Tello-Solís, S.R.; Arroyo-Reyna, A. Estudio por dicroísmo circular de la desnaturalización térmica de la subtilisina BPN': Modelo irreversible de dos estados. *Rev. Soc. Quím. Méx.,* **2002**, *46*(2), 105-108.

[73] Bull, H.B.; Breese, K. Thermal stability of proteins. *Arch. Biochem. Biophys.,* **1973**, *158*(2), 681-686. [http://dx.doi.org/10.1016/0003-9861(73)90561-4] [PMID: 4782528]

CAPÍTULO 2

Effect of temperature and pH in secondary structure and denaturation process of Japanese clam (*Ruditapes philippinarum*) hepatopancreas cathepsin D

Francisco Cadena-Cadena[a], Josafat Marina Ezquerra-Brauer[a], Francisco Javier Cinco-Moroyoqui[a], Alonso Alexis López-Zavala[b], Hisila del Carmen Santacruz-Ortega[c], Ignacio Alfredo Rivero-Espejel[d], Ofelia Rouzand-Sández[a] and José Luis Cárdenas-López*[a]

[a]Departamento de Investigación y Posgrado de Alimentos, Universidad de Sonora, Hermosillo Sonora México
[b]Departamento de Ciencias Químico Biológicas, Universidad de Sonora, Hermosillo Sonora México
[c]Departamento de Investigación en Polímeros y Materiales, Universidad de Sonora, Hermosillo Sonora México
[d]Centro de Graduados e Investigación en Química, Instituto Tecnológico de Tijuana, Tijuana, Baja California, México

*Corresponding author:
Dr. Jose Luis Cardenas Lopez
e-mail: joseluis.cardenas@unison.mx
Tel: +52(662) 2-59-22-07

Abstract

Cathepsin D from the Japanese clam (*Ruditapes phillipinarum*) hepatopancreas was purified and partially characterized by affinity chromatography on pepstatin agarose. The purification fold and yield were 37 X and 19% respectively, with a molecular weight of 36.5 kDa estimated by SDS-PAGE. The isoelectric point was 6.6 determined by isoelectric focusing. The optimal activity of the enzyme was found from pH 3.0 to 3.5 and an optimum temperature at 45°C for hemoglobin substrate. The stability remained constant at a pH of 3 to 7, retaining more than 50% of its activity at 60 min and rapidly losing the activity at pH 2, 8 and 9. As for temperature, the activity it remained above 50% at 1 hour and 30 °C. However, it decreases rapidly from and above 40 °C. The protein structure is highly conserved, but

when temperature and pH change, there are corresponding changes in secondary structures α-helix and the β-sheet, as well as between the disordered structures and the β-turns, when one increases the other decreases to find a more stable conformation. Therefore, the role of pH and temperature in protein denaturation is of paramount importance. These changes are important for its relevance in the functionality of the protein. The process of denaturation of the enzyme is not dependent on the rate of heating and is not accompanied by oligomerization of the protein. A small percentage α-helix (1.5 and 4%) remains unchanged.

Keywords: Japanese clam, hepatopancreas, cathepsin D, secondary structure

1. Introduction

Clam production is one of the most important fisheries in the world [1, 2]. Japanese clam (*Ruditapes philippinarum*) is widespread in the world and represents 25% of total aquaculture and fisheries production, being the second most important economic species of clams [3, 4].

Cathepsin D is a lysosomal enzyme that is found in most organisms acting on protein turnover [5]. In humans this enzyme is very important since high activity is found in several types of carcinomas [6, 7]. In marine organisms, cathepsin D is associated with the reproductive system, and particularly in fish, this enzyme can function as a biomarker since its production and activity are linked to spawning [8]. In mollusks, cathepsin D is linked to the degradation of intracellular protein [9], antigen processing [10], hormonal maturation [11] and immune responses [12, 13]. In some species like the jumbo squid [14], neon flying squid [15] and two spot octopus [16], cathepsin D from the hepatopancreas has been found to be the main enzyme that digests food at acidic pH. In other organisms, such as American lobster [17] and

surf clam [18] cathepsin D presence has been detected in the gastric fluid, finding that this enzyme is adapted to low temperatures that fulfills digestive functions [19].

There is a relationship between the functionality and structure of a protein, the parameters of pH and temperature are the main factors that when modified can affect the activity of the protein and modify its structure, therefore, these changes must be taken into account to elucidate the functionality of the protein [20, 21]. Circular dichroism (CD) and differential scanning calorimetry (DSC) are excellent techniques for detecting conformational changes that may be associated with activity, regulation and structure of a protein, or in any type of molecule. CD spectroscopy is a well-known biophysical tool for the structural characterization of biopolymers such as proteins and nucleic acids. The physical basis of the technique is the differential absorption of polarized light to the right or left because the molecules contain or present a chiral center. [22]. Therefore, conformational transitions can also be monitored with distant UV CD. [23]. CD makes possible to determine the secondary structure of any protein because at wavelengths of 190 to 240 nm the chromophores are the peptide bonds [24], this allows the calculation of the percentage of α-helix, β-sheet, β turns and unordered structure [25, 26]. CD can also be used to determine the denaturation temperature of the protein. When the deployment of a protein is reversible and there is only a single deployment transition, the thermodynamic parameters evaluated by CD are similar to those that can be estimated using differential scanning calorimetry (DSC) [27, 28]. DSC measures the thermal capacity of a material as a function of temperature and is a powerful technique for studying protein folding and stability. The signal produced in a sample cell is compared to a blank cell without protein in the same environment. [29]. When the temperature of the cells is increased, the temperature differences between the target and the

sample cells are measured and calibrated continuously; therefore, the difference in heating energy can be used to calculate the calorific capacity of the protein. The heating capacity is recorded as a function of temperature. With the resulting thermogram analysis, enthalpy and thermal transition (Tm) can be obtained. [30-33].

Most of the described cathepsins D possess parameters that are out of the ordinary, for example, they contain very little α-helix content [34] or extreme isoelectric points [35]. Besides, there are few reports on the secondary and tertiary structure of marine organisms. Cathepsin D is an endopeptidase that hydrolyzes hydrophobic amino acids but it is not clear what the relationship is between protein folding structure and flexibility, activity and stability [36].

Due to the role played by cathepsin D in food degradation and the lack of information about it, it is important to deepen the understanding of the changes that occur in the secondary structure of cathepsin D when subjected to different pH and temperature conditions. Analyses of circular dichroism, differential scanning calorimetry, and UV of a pure preparation of cathepsin D were performed, determining the effect of temperature and pH on the secondary structure of cathepsin D as well as its thermal denaturation, this could be important to understand the role in the enzymatic stabilization of the Japanese clam (*Ruditapes philippinarum*).

2. Materials and Methods

2.1. Preparation of crude extract

The clams were collected in Kino Bay, Sonora, Mexico (geographical coordinates 28° 50' 0" North, 111° 56' 0" West) and transported to the laboratory. The hepatopancreas of ten clams was removed, minced, and extracted for 12 h at 4 °C under stirring with 50 mM sodium acetate buffer, pH 5.0, in a 1:2 ratio (w/v) following the procedures of Venugopal *et al.* [37]. The homogenate was centrifuged at 10, 000 x *g* for 1 h. The supernatant was filtered through an Amicon PM-50 membrane (Millipore Corporation, Bedford, MA), saved, and stored at 4 °C for further analysis.

2.2. Enzyme assay

The activity of cathepsin D was determined according to Celis-Guerrero *et al* [38]. The assay consisted of combining 100 µL of the enzyme, 600 µL of 0.1 M glycine-HCl buffer, pH 3.5, 3.5, and 600 µL of 0.5% hemoglobin (Hb). The reaction was performed at 40 °C for 10 min. At the end of the incubation period, 600 µL of 20% trichloroacetic acid (TCA) was added. The increment in TCA soluble peptides released due to the action of the enzyme was estimated at 280 nm. The enzyme activity was expressed as U/mL. One unit was defined as the change of 0.001 in absorbance at 280 nm [38].

The specific test consisted of using a specific fluorogenic substrate for cathepsin D. The substrate was prepared by mixing 10 µL of 200 µM AcRGFFP-AFC (N-Acetyl-Arg-Gly-Phe-Phe-Pro-7-amido-4-trifluoromethylcoumarin) in 80 µL of 100 mM glycine buffer, pH 3.5, and warmed for 2 min at 45 °C. The reaction started by adding 10 µL of enzyme. After 10 min, 320 µL of 100 mM glycine, pH 9.5, and 10 µL of the enzyme dipeptidyl peptidase IV (0.6 U / mL) were added at the reaction mixture. Cathepsin D breaks the substrate on the

FF amino acids leaving two fragments, one being N-Acetyl-Arg-Gly-Phe (AcRGF) and the other one Phe-Pro-7-amido-4-trifluoromethylcoumarin (FP-AFC), whereas the enzyme dipeptidyl peptidase IV hydrolyzes the bond at Pro releasing the AFC. The AFC produced was monitored by measuring the fluorescence (excitation at 400 nm, emission at 505 nm). The control consisted of adding 0.5% TCA before the incorporation of the substrate [39] with 800 μL of buffer.

2.3. Protein quantitation

Protein content of the enzyme preparations were determined using the dye binding assay of Bradford [40] with bovine serum albumin as standard.

2.4. Cathepsin D extraction and purification

All purification steps were done at 4°C. Briefly, Five mL of pepstatin A agarose was packed in a 10 mL column, washed with 50 mM sodium acetate and 20 mM sodium chloride, pH 5.0 until 10 volumes were completed. One mL of desalted raw extract (equivalent to 2.0 mg of protein) was loaded onto the pepstatin A agarose affinity chromatography column [37]. After all the unbound protein was removed from the column with equilibration buffer, the enzyme activity was eluted with 50 mM Tris-HCl, pH 8.8. Protein elution profiles were estimated by monitoring their absorbance at 280 nm. The flow rate was 1 mL/min. Fractions of 3 mL were collected and frozen at -80°C for further analysis.

2.5. Electrophoresis and zymograms

The electrophoresis (Native and SDS PAGE) of purified enzymes was carried out according to Laemmli [41]. The protein separation was carried out at a constant voltage of 120 *V* for two hours in a 12 % acrylamide separation gel and a 4 % acrylamide stacking gel, both containing 1.35 % bis-acrylamide. Detection of bands of protein was visualized by staining with Coomassie blue or by a silver nitrate method [42-44]. Estimation of molecular weight of the enzyme was carried out using the following molecular weight standards: bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), bovine trypsinogen (24 kDa), trypsin inhibitor (20 kDa), lactalbumin (14.2 kDa), and aprotinin (6.5 kDa).

The zymogram technique was carried out to determine visually the presence of aspartic proteases. To obtain the zymograms, the sample was diluted in a 1:1 ratio with the sample buffer without heat treatment. The running buffer was 350 mM glycine and 140 mM acetic acid at a pH of 4.5. To perform the run, the position of the electrodes was inverted and the voltage was less than 100 V. At the end of electrophoresis, the gels were soaked in 100 mM glycine, pH 3.5 for 30 min and then in 0.5% Hb for 2 h. The gels were stained using 0.025% Coomassie Blue R250. The activity was visualized as clear bands indicating hydrolysis of the substrate [45].

2.6. Temperature and pH optima

The optimum temperature and pH of the purified enzyme were determined using Hb as a general substrate and AcRGFFP-AFC as a specific substrate. For pH optimum, the temperature was kept at 37°C. The general test consisted of mixing 500 μL of 2% Hb with 500 μL of glycine buffer, pH 3.5, and then adding 50 μL of sample. The mixture was incubated for 10 min at 37 °C and the reaction was stopped by the addition of 0.5% cold

TCA. The mixture was centrifuged at 5000 x *g* for 5 min and the protein content in the supernatant was estimated at 280 nm. All samples were made in triplicate [38, 46]. The specific test consisted in using a fluorogenic substrate for cathepsin D, as described previously. The control was prepared by adding 0.5% cold TCA before the substrate was added [39] with 800 µL of buffer, each at different pH.

2.7. Enzyme stability to pH and temperature

To determine pH stability, the purified enzyme (100µL) was incubated with 1000 µL of buffers of different pH (2-3.5 glycine, 4-6 acetate, 6.5-8 Tris base) at 37°C and sampling at 0, 30, 60, 90 and 120 min. The residual activity was then measured at optimal conditions after incubation time with Hb substrate as described above. The temperature stability of the enzyme was determined in 10 °C intervals at a range of 5 to 80°C, using 100 mM glycine buffer, pH 3.5. Appropriate aliquots of 100 µL were removed at 0, 30, 60, 90 and 120 min. The activity was expressed as % residual activity, and the comparison was made using the 50% residual activity reference [47].

2.8. Differential scanning calorimetry

DSC experiments were performed on a Nano-DSC II, Model 6100 (Calorimetry Sciences Corp.) instrument at scan rates of 0.05, 1.0 and 2 °C/min. The measurements were performed at temperature range 15–100 °C at the 3 bar excess pressure with a reference cell filled with 10 mM sodium acetate buffer pH 5. Volume of the sample cell was 0.5 mL containing 1 mg/mL of protein. The sample solution and reference solution were dialyzed with 10 kDa molecular weight cutoff dialysis tubing before the DSC experiment. Thermodynamic

parameters (ΔG, ΔH y ΔS) and transition temperature (Tm) were calculated with the data obtained in the CpCalc calorimeter software.

2.9. Kinetic parameters

The kinetic parameters maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) of the enzyme were determined. The reaction rate was measured at different concentrations of initial substrate Hb, in the range of 1-300 μM (1.5, 20, 50, 100, 150, 200, 250 and 300 μM). Estimation of K_m and V_{max} was obtained by fitting the observed values of v versus substrate concentration to the Michaelis–Menten equation using non-linear regression curve-fitting software (Graph- Pad Prism 5.0; San Diego, CA, USA) [39] .

2.10. Circular dichroism

Circular dichroism far spectra were obtained on a Jasco J800 spectropolarimeter equipped with a Peltier device. The methodology consisted of dissolving the sample in Tris base buffer, at different pH and temperatures, with a protein concentration of 20-200 μg, placing it in a quartz cell of 3 mL at different pH and different temperatures [28, 48]. The results were transformed into % secondary structure utilizing the K2D [49] and CONTIN software [50].

2.11. Statistics

For all the experiments descriptive statistics were used taking care of the randomness of the samples. The statistical design of the present study allowed to reduce the variation through replication. The respective cathepsin D activity data during purification and characterization were the average of three determinations.

3. Results and Discussion

3.1. Purification of cathepsin D

The chromatography with agarose pepstatin A was able to separate the activity for the cathepsin D of Japanese clam with Tris-HCl, pH 8.8 (Fig. 1), while the contaminants were removed at the first elution peak (Fig. 1). Table 1 shows the purification steps of the enzyme cathepsin D. Approximately 1.19 mg of the enzyme were recovered with a yield of 19% and purification of 37 fold. The specific activity of the purified enzyme was 5553 ± 220 U/mg of protein, obtaining results comparable to those reported for jumbo squid [14] , mussel [37] and sepia squid [51].

3.2. SDS PAGE, native electrophoresis, and isoelectric point

The profile of purified enzymes is shown in Fig. 2A, 3B and 3C. Cathepsin D of the Japanese clam has a molecular weight estimated at of 36.5 kDa (Fig. 2A), which is similar to those of cathepsins D detected in Japanese squid (*Todarodes pacificus*)[52] (36.5 kDa), jumbo squid (*Dosidicus gigas*) [14]**,** herring (*Clupea harengus*) (38 kDa) [53] and mussel (43 kDa)[37]. However, there are reports of this enzyme with lower molecular weights (10 and 28 kDa), like that from the European squid (*Todarodes sagittatus*))[35]. The isoelectric point was 6.6, which is slightly higher than those of enzymes of terrestrial organisms, but lower than that of the Japanese squid [51]. The zymogram reveals the presence of a unique band of hemoglobin hydrolysis, indicating the homogeneity of the purified cathepsin D (Fig. 2 B). Two main points were noticed in the zymogram. One is that at acidic pH cathepsin D was the only enzyme hydrolyzing hemoglobin (Fig. 2 E), which discards the presence of other acid proteases in the crude extract. Second, the intensity of the band increases when purified

(Fig. 2, F, G), due to the increasing specific activity in each purification step. That is consistent with Cadena-Cadena *et.al* [14] for jumbo squid (*Dosidicus gigas*), who reported that cathepsin D was the only enzyme with hydrolytic ability at very acid pH.

3.3. Temperature and pH optima

The optimal temperature and pH of cathepsin D are shown in Fig. 3. Cathepsin D showed a relative activity of more than 50% in a range of 30 °C to 60°C with an optimum of 45 °C to 50°C for the specific substrate (Fig. 3 A). Other species of mollusks, such as squids, have optimal temperatures of 40 and 45 °C [35, 52], Atlantic clam 45 °C [18], and mussels 60 °C [37]. Cathepsin D exhibited more than 50% of the activity with the substrate AcRGFFP-AFC at a pH range of 2-4, with an optimum in the range 3-3.5 (Fig. 3B). Above pH 4, the activity of cathepsin D rapidly decreased losing nearly 40% of activity, while at pH 5 only 10% of the activity remained. This pH value is very similar to those reported for other species of mollusks such as squid [35, 52], which showed a pH of 3.5, and for mussels (*Lamellidens corrianus*) and Atlantic cod the optimum pH was 3 [54].

The differences found between optimum pH for cathepsin D from Japanese clam with other mollusks are associated with secondary structure and to the pKa of the carboxyl groups. Protein conformation between different thermodynamic states as temperature or pH conditions are altered so that changes in the relative stabilities of the various amino acid groups play a fundamental role in the active site of the enzyme as well as in the electronic cloud formed around the active site, pH also influences the folding and detachment of certain regions of the protein [55, 56].

3.4. Temperature and pH stability

The stability of cathepsin D at different temperatures is shown in Fig. 4A. The enzyme was stable up to 50 °C and retained more than 50% of residual activity at 30 min. At 60 min it retains only 35% of residual activity. However, at 60 °C, the enzyme lost all its activity. Cathepsin D activity losses are minimal at 5 °C and reached 48% at 40 °C and 120 min incubation, at a higher temperature and longer exposure time the loss of enzyme activity is greater since proteins are easily denatured by higher temperatures. Although the thermal stability of proteins varies widely, many of them are slowly denatured above 25°C [57, 58]. When the temperature increases above a certain point, the kinetic energy of the molecules is increased and consequently the aqueous envelope of the protein is disorganized. In the same way, when the temperature rises, weak interactions are destroyed, such as the hydrogen bridges that are located outside the tertiary structure of the protein is disorganized, so that the hydrophobic interior is exposed to the aqueous medium. This produces the aggregation of the protein that at the moment is denatured [59, 60]. The loss of activity between 50 °C and 60 °C can also be attributed mainly to the loss of affinity of the enzyme with the substrate [61, 62]. Other purified enzymes such as cathepsin D from pig and rat, as well as porcine pepsin [63], have very similar thermal stability. However, the thermostability was much higher than that reported for tilapia [55, 64]. Cathepsin D from marine organisms has been shown to be different from other sources with respect to heat stability [46, 65]. However, cathepsin D of clam was rapidly inactivated at 60°C, just like chicken cathepsin D, which was reported as inactivated above 55°C[66, 67].

The pH stability is shown in Fig 4B. The higher stability of the enzyme was detected at a pH range of 3 to 6 retaining more than 50% of the activity at a time of 60 min. The cathepsin D

of the Japanese clam is less stable than that of sepia squid (*Sepia officinalis*), since it remained fully active at pH 3.0 after 60 min [51]. Other cathepsins of terrestrial organisms such as cathepsin D of the uterine coil have stability between 5 and 7, however, loses all its activity at a pH of 6.3 [68]. Proteins have a large number of ionizable groups that exhibit a variety of pKa values. Therefore the H^+ and OH^- ions affect not only the protein aqueous envelope but also directly affect the electrical charge of the acidic and basic chains groups. When the pH increases or decreases from the optimum of the enzyme, the lateral amino acid residues can undergo reversible ionization of the amino and carboxyl groups, making that the enzyme loses its activity. Alterations of this type modify the surface charge of the proteins and consequently eliminate the electrostatic interactions that stabilize the tertiary structure so that hydrophobic groups that interact with each other are exposed and often cause their precipitation. At this point the enzyme is already denatured and even when the optimum pH is returned, it will not recover activity [57, 58, 69]. This behavior is similar to that reported for enzymes that are not adapted to cold, which are expected to have an optimum temperature higher than cold adapted enzymes.

3.5. Kinetic parameters

The kinetic parameters provide information about the catalytic reaction mechanism of the enzyme as well as the specificity of the enzyme. The maximum velocity (Vmax) was 958 nM/min (Fig. 5), whereas the Km was 245 nM. It has been shown that cathepsin D efficiency of marine animals is greater than their terrestrial counterparts. Chicken cathepsin D presented a Km of 22.7 μM, while its Vmax was 15.12 μM/min [66]. For human cathepsin D [37] a Km of 1070 μM has been reported. That indicates that the affinity is higher for Japanese clam

cathepsin D than for terrestrial organisms. This could be due to the need for more efficient enzymes due to feeding habits of clams, since clams are filtering animals.

3.6. Thermodynamic parameters

The thermal transition (Tm) of Japanese clam cathepsin D determined by DSC was 72, 74.5 and 76 °C depending on the scanning speed as shown in Fig. 6A. This variation may be since deployment process is an irreversible reaction of two states. At low temperatures, the harmonic vibrations of the atoms predominate, while at higher temperatures the kinetic energy that is introduced into the molecule modifies the dynamic properties of the atoms with loss of hydrogen bonds and therefore, makes it impossible to maintain the secondary structure in the molecule. The point where the entire secondary structure is lost is the equilibrium temperature between the unfolded and folded states [29, 70, 71]. The differences in the signals at the different heating rates may be due to the breakdown of a small number of hydrogen bonds before it reaches denaturation, which decreases the amount of thermal energy that must be absorbed to break the native configuration and pass at a denatured configuration [72], the same can be seen in Fig. 8, where the decrease in α-helix and the increase in the β-sheet is observed. Once the temperature limit has been exceeded, the specific structure of the protein is loosened and the peptide chain is deployed or rearranged to another in other more stable structures. To be reversible, a sufficient amount of specific configuration must be retained to allow the return to the native configuration. However, this is not observed in cathepsin D of the Japanese clam, the modifications are so extreme that it is impossible to recover its secondary structure. This denaturation is a two-state process, one native and the other deployed, it can be seen in Fig. 6 that there is a dependence on the heating

rate with respect to the transition curves, which implies that the process is under kinetic control [72-74] .

However, in CD the temperatures for Tm were 73.5, 77.2 and 78.3 °C at the same heating speeds. The difference in Tm with DSC may be due to the difference in technique principle because the calorimeter measures the heat absorbed or released for the protein [29], while CD only measures light deflection to the left or right. However, being in the process of denaturation the chain of amino acids tends to seek a more stable conformation, which can result in the formation of different structures that can be detected by CD as a change in secondary structure making the temperature of denaturation rise. These structures are the result of exposure of hydrophobic amino acids by the rupture of the aqueous envelope, and therefore the breakdown of hydrogen bonds that keep the secondary structure of the molecule [75-77]. The stability of the protein can be defined as the difference in free energies between native states and denatured in a dynamic equilibrium between native and denatured conformations [78]. In CD, the measurement is at 222 nm, in this region, the chromophore is the peptide bond. Therefore this measures the changes that occur in the electronic densities of that peptide bond at that wavelength [28, 33]. The thermodynamic parameters are shown in Table 2. The denaturation temperatures are very similar to those reported for g globulin (72.9 °C), β-lactalbumin (72.8 °C), lysosimide (70.5 °C) and lactoferrin (69.0 °C). However, it is different from the temperatures reported for chymotrypsin (55.7 °C), xanthine oxidase (61.4 °C) and seroalbumin (62.2 °C) [79].

It is known that the thermal denaturation of a protein presents a very large change in its structure, especially in proteins that have few disulfide bonds, as in the case of human cathepsin D, which only has 4 disulfide bonds, so it would be expected that the denaturation

temperature to be lower [34, 80, 81]. However, cathepsin D from Japanese clam had an unusually high Tm compared to proteins of terrestrial origin, which is related to the difference in ΔG (160 KJ), mainly due to its amino acid composition and low amount of α-helix which makes the protein more stable [14, 82]. This high denaturing temperature in the cathepsin D of the Japanese clam may be related to the amino acids present in the primary structure of the protein, there is a greater quantity of amino acids that can form disulfide bridges, which confer a certain stability to the protein. It may also be due to the ability of the molecule to form secondary structures that presumably are more stable, this can be seen in the variation of the secondary structure and how it changes from α-helix to β-sheet or from β-turns to un-ordered as the temperature increases.

The thermal denaturation of cathepsin D can occur because heating induces a denatured state in which residual secondary structures are due to some peptides avoiding exposure to the solvent [82]. This denaturation process is a spontaneous process in the direction of native state to denatured state at low temperatures, related to the loss of activity at a very short time (Fig. 4) and is also related to the loss of α-helix around 70°C (Fig. 8). This may be due to the fact that the denaturation of Japanese clam cathepsin D is an irreversible process. In proteins such as corn zeins the denaturation temperature is higher due to the high concentration of disulfide bridges give stability to the protein [83], while subtilisin and enolase have a high dependence on the rate of heating due to the formation of intermediates of secondary structure[73, 84]. The same is observed in cathepsin D of the Japanese clam that at temperatures of 80 °C retains a reduced number of α-helices. It has been found that the free energy difference between folded and non-folded conformations is only ~ 45 KJ/mol; this suggests a very small balance between the native state and the denatured state. The pH of the

buffer in which the protein is found plays a very important role since it must exert a stabilizing effect on the protein that would help maintain folding, storage, stabilization and prevention of aggregation [85, 86].

3.7. Structural analysis by circular dichroism

3.7.1. Effect of pH

The effect of pH on the secondary structure of cathepsin D was evaluated by CD and results are shown in Fig. 7. A loss of approximately 82% of α-helix structure at pH 7 and a loss of 78% at pH 2 was observed. The β-she*et al*so loses 50% structure, the β turns and the disordered structure remained constant in a 16 to 20% content. It was observed a relationship between the gain and loss of secondary structures because when there was a loss of α-helix is observed a gain of β-sheets and vice versa. The same pattern was observed in cathepsin D from jumbo squid, reaching up to 4% of α-helix [14].

The increase and loss of β-sheets and α-helix could be a process of unfolding and re-prolonging with which they achieve greater flexibility in the polypeptide chain [87], which may be due to the exposure of some non-polar groups that are embedded within the polypeptide chain were exposed to the molecular surface with which has some interaction with the polar solvent after the pH change, mainly hydrogen bridges, which contributes to the molecule moving towards red in the electromagnetic spectrum (increase in wavelength), which makes the non-polar groups move to a more hydrophilic location [88, 89]. This is observed in other proteins such as jumbo squid (*Dosidicus gigas*)[14] or protein isolates WPI (β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA), and immunoglobulins (IgG). [90, 91].

The activity of the cathepsin D of the Japanese clam is associated with the alpha structure as that, in order to have an optimal activity there must be an approximate 8% of α-helix. This same observation was made by Cadena-Cadena *et.al* [14] for jumbo squid cathepsin D. Lee *et.al* [34] found that the interconnection between active and inactive forms of cathepsin D is reversible and can be regulated by pH. At a pH of 7.5 they reported the appearance of a new β-sheet blocking the active site.

For Japanese clam, cathepsin D an increase was observed in the percentage of β-sheet at a pH of 5, ranging from 29% to 33%. That is related to the self-regulation of the enzyme by joining the carboxylate side chains near the active site and thus regulating in that way the pH-dependent enzyme activity.

3.7.2. Effect of Temperature

Temperature induces changes in the secondary structure of proteins in aqueous solution, causing disorganization of their aqueous envelopes and breakdown of internal bonds (mainly hydrogen bonds). This causes non-polar groups to be exposed causing aggregation and denaturation of the protein [75, 92]. Figure 8 shows the secondary structure content of the Japanese clam (*Ruditapes phillipinarum*) at different temperatures.

At temperatures of 10 to 30 ° C the percentage of α-helix and β-sheet was 10% and 29%, respectively, while from 40 ° C and up to 50 ° C the α-helix increased to 16 % of and the β-sheet decreased to 26%. At 60 ° C the percentage of α-helix was reduced 74% and the percentage of β-sheets increased first to 34% and then to 82% at 70 ° C. These changes are related to the loss of activity and the irreversible denaturation of the protein. It was also observed that the losses of one type of secondary structure meant the gain of the other. For instance, at temperatures of 50 and 60 °C the α-helix showed a decrease of almost 50% but,

in contrast, the β-sheet and the disordered structure showed increases of 34 and 21, respectively. Another important change was that β-turns showed a decrease of 50% at 30 °C.

These changes in α-helix to β-sheet and β-turns in the unordered structure were reported in sol and gel proteins. It was found that the protein that had a greater deployment in its secondary structure was an isolate of the actomyosin-paramyosin complex, this could be due to the decomposition of hydrogen bonds and electrostatic interactions [93]. A greater denaturation of the protein translates as a greater unfolding, which will allow for a greater rearrangement of the protein with the exposure of the hydrophobic regions which seek to stabilize and therefore seek the formation of new structures [93-95]. These movements are also observed in polyproline II in which, when the temperature rises, an increase in β-sheet and a decrease in other structures is observed, mainly due to the exit of water from the aqueous envelope due to the kinetic energy that it is induced by increasing the temperature, which suggests that dehydration of these regions of the protein favors the formation of β-sheets [96], in case of the BSA, it was found that by incubating the protein at 37°C, its α-helix content (65%) is reduced (30% to 300 min) while the β-sheet content (4%) increased (17% at 300 min), this indicates that the most stable conformation is that of the β-sheet [97]. Cadena-Cadena *et al* [14], observed that, when the temperature is increased, cathepsin D enzyme of the jumbo squid (*Dosidicus gigas*) hepatopancreas does not completely lose its secondary structure and retaining only 4%. However, in cathepsin D of the Japanese clam only conserves about 2%, which indicates possibly a higher percentage of non-polar amino acids that are agglomerating and in their α-helix form. It is known that the process of formation of an α-helix is controlled by enthalpy [98, 99]. However, the loss of side-chain entropy is a major determinant of the helix-forming, a process naturally occurring in amino

acid residues [100]. The change from α-helix to β-sheet occurs primarily because β-sheet structures (mainly antiparallel) have a greater number of conformational degrees of freedom, which can assume a greater diversity of spatial configurations than those produced in α-helix. This flexibility of configuration is due to hydrogen bonding between β-sheets. Therefore, its spatial configuration reflects the effects that arise from hydrogen and electrostatic interactions in the internal packing of β-sheets with other secondary structures of the protein [101].

4. Conclusions

The present work provides new evidence on the biochemistry and protein structure of cathepsin D of the Japanese clam (*Ruditapes phillipinarum*) hepatopancreas. The secondary structure of cathepsin D from Japanese clam hepatopancreas is dependent on temperature and even more on pH. The most affected secondary structure was α-helix, that at extremely high temperature, retains up to 4 %, while at extreme pH conserves only 1.5%. Therefore, pH is of the most importance for secondary structure in this enzyme.

Ethics approval and consent to participate

This study is ethically approved by CAP (Comisión Académica del Posgrado, or Graduate Academic Commission) from the University of Sonora, Mexico.

Human and animals right

No humans were used in this research. The animals used were according to the Guidelines of The National Methodology of the Official Mexican Standard NOM-062- ZOO-1999, titled

"Especificaciones técnicas para la producción, cuidado y uso de los animales de laboratorio" ("Technical specifications for production, care and use of laboratory animals").

Funding

All the resources for the execution of this research were obtained from public funds through the project entitled "Physical and chemical characterization of cathepsins L and D from jumbo squid hepatopancreas". Conacyt (Mexican Council for Science and Technology) Basic Science project # 182348 directed by Dr. José Luis Cárdenas-López.

Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

References

[1] FAO, © FAO 2006-2018. Software para la pesca y la acuicultura. FishStat Plus - Programa informático universal para series cronológicas de estadísticas pesqueras. In: Departamento de Pesca y Acuicultura de la FAO [en línea]. Roma. Actualizado 14 September 2017. . (2018).
[2] SIAP, Servicio de Información Agroalimentaria y Pesquera (SIAP), in: CONAPESCA (Ed.), Mexico, 2018.
[3] FAO, El estado mundial de la pesca y la acuicultura. Cumplir los objetivos de desarrollo sostenible. Roma. Licencia: CC BY-NC-SA 3.0 IGO., 2018.
[4] T.T.T. Ngo, H.-S. Yang, K.-S. Choi, Temporal variation in the reproductive effort and tissue biochemical composition in manila clam, *Ruditapes philippinarum* from a sand flat on the East Coast of Jeju Island Korea. Ocean and Polar Research 40 (2018) 15-22.
[5] E.M. Press, R.R. Porter, J. Cebra, The isolation and properties of a proteolytic enzyme, cathepsin D, from bovine spleen. Biochemical Journal 74 (1960) 501.
[6] P. Benes, V. Vetvicka, M. Fusek, Cathepsin D—many functions of one aspartic protease. Critical Reviews in Oncology/Hematology 68 (2008) 12-28.
[7] S. Basu, S. Cheriyamundath, N. Gavert, T. Brabletz, G. Haase, A. Ben-Ze'ev, Increased expression of cathepsin D is required for L1-mediated colon cancer progression. Oncotarget 10 (2019) 5217.
[8] M. Yamashita, S. Konagaya, High activities of cathepsins B, D, H, and L in the white muscle of chum salmon in spawning migration. Comparative biochemistry and physiology B, Comparative biochemistry 95 (1990) 149-152.

[9] J. Krause, S.C. Tshidino, T. Ogawa, Y. Watanabe, V. Oosthuizen, B. Somai, K. Muramoto, R.J. Naudé, Purification and partial characterization of ostrich skeletal muscle cathepsin D and its activity during meat maturation. Meat Science 87 (2011) 196-201.
[10] L.C. Hsing, A.Y. Rudensky, The lysosomal cysteine proteases in MHC class II antigen presentation. Immunological Reviews 207 (2005) 229-241.
[11] S. Yasothornsrikul, D. Greenbaum, K.F. Medzihradszky, T. Toneff, R. Bundey, R. Miller, B. Schilling, I. Petermann, J. Dehnert, A. Logvinova, Cathepsin L in secretory vesicles functions as a prohormone-processing enzyme for production of the enkephalin peptide neurotransmitter. Proceedings of the National Academy of Sciences 100 (2003) 9590-9595.
[12] A.K. Dixit, P. Dixit, R.L. Sharma, Immunodiagnostic/protective role of cathepsin L cysteine proteinases secreted by Fasciola species. Veterinary parasitology 154 (2008) 177-184.
[13] U. Menike, K. Ariyasiri, J.-y. Choi, Y. Lee, W.D.N. Wickramaarachchi, H.K.A. Premachandra, J. Lee, M. De Zoysa, Manila clam, *Ruditapes philippinarum* Cathepsin D. The Korean Journal of Malacology 29 (2013) 155-161.
[14] F. Cadena-Cadena, J.L. Cárdenas-López, J.M. Ezquerra-Brauer, F.J. Cinco-Moroyoqui, A.A. López-Zavala, H.d.C. Santacruz-Ortega, I.A. Rivero-Espejel, Effect of temperature and pH on the secondary structure and denaturation process of jumbo squid hepatopancreas cathepsin D. Protein and Peptide Letters 26 (2019) 532 - 541.
[15] K. Zhang, R. Wei, R. Song, Extraction of Cathepsin D-Like Protease from Neon Flying Squid (Ommastrephes bartramii) Viscera and Application in Antioxidant Hydrolysate Production. Biomolecules 9 (2019) 228.
[16] L.E. Ibarra-García, D. Tovar-Ramírez, C. Rosas, Á.I. Campa-Córdova, J.M. Mazón-Suástegui, Digestive enzymes of the Californian two-spot octopus, Octopus bimaculoides (Pickford and McConnaughey, 1949). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 215 (2018) 10-18.
[17] L. Rojo, R. Sotelo-Mundo, F. García-Carreño, L. Gráf, Isolation, biochemical characterization, and molecular modeling of American lobster digestive cathepsin D1. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 157 (2010) 394-400.
[18] H.C. Chen, R.R. Zall, Partial Purification and Characterization of Cathepsin D-Like and B-Like Acid Proteases from Surf Clam Viscera. Journal of Food Science 51 (1986) 71-75.
[19] P.B. Szecsi, Pepsin B, Handbook of Proteolytic Enzymes (Third Edition), Elsevier, 2013, pp. 36-37.
[20] S. Nakai, E. Li-chan, Structure Modification and Functionality of Whey Proteins: Quantitative Structure-Activity Relationship Approach. Journal of Dairy Science 68 (1985) 2763-2772.
[21] F. Muneer, E. Johansson, M. Hedenqvist, T. Plivelic, R. Kuktaite, Impact of pH Modification on Protein Polymerization and Structure–Function Relationships in Potato Protein and Wheat Gluten Composites. International journal of molecular sciences 20 (2019) 58.
[22] A.J. Miles, B.A. Wallace, Circular dichroism spectroscopy of membrane proteins. Chemical society reviews 45 (2016) 4859-4872.

[23] M. Zolfagharzadeh, M. Pirouzi, A. Asoodeh, M.R. Saberi, J. Chamani, A comparison investigation of DNP-binding effects to HSA and HTF by spectroscopic and molecular modeling techniques. Journal of Biomolecular Structure and Dynamics 32 (2014) 1936-1952.
[24] N. Sreerama, R.W. Woody, Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. Analytical biochemistry 287 (2000) 252-260.
[25] R. Woody, Circular dichroism. Methods Enzymol 246 (1995) 34–71.
[26] S. Hamed-Akbari Tousi, M. Reza Saberi, J. Chamani, Comparing the interaction of cyclophosphamide monohydrate to human serum albumin as opposed to holo-transferrin by spectroscopic and molecular modeling methods: Evidence for allocating the binding site. Protein and peptide Letters 17 (2010) 1524-1535.
[27] N.J. Greenfield, Using circular dichroism collected as a function of temperature to determine the thermodynamics of protein unfolding and binding interactions. Nature protocols 1 (2006) 2527.
[28] S.M. Kelly, T.J. Jess, N.C. Price, How to study proteins by circular dichroism. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics 1751 (2005) 119-139.
[29] B. Ibarra-Molero, A.N. Naganathan, J.M. Sanchez-Ruiz, V. Muñoz, Chapter twelve - modern analysis of protein folding by differential scanning calorimetry, in: A.L. Feig (Ed.) Methods in Enzymology, Academic Press, 2016, pp. 281-318.
[30] P.L. Privalov, Stability of proteins small globular proteins, Advances in protein chemistry, Elsevier, 1979, pp. 167-241.
[31] P.L. Privalov, Stability of proteins: proteins which do not present a single cooperative system, Advances in protein chemistry, Elsevier, 1982, pp. 1-104.
[32] P.L. Privalov, P.L. Mateo, N.N. Khechinashvili, V.M. Stepanov, L.P. Revina, Comparative thermodynamic study of pepsinogen and pepsin structure. Journal of Molecular Biology 152 (1981) 445-464.
[33] J. Wen, K. Arthur, L. Chemmalil, S. Muzammil, J. Gabrielson, Y. Jiang, Applications of differential scanning calorimetry for thermal stability analysis of proteins: qualification of DSC. Journal of pharmaceutical sciences 101 (2012) 955-964.
[34] A.Y. Lee, S.V. Gulnik, J.W. Erickson, Conformational switching in an aspartic proteinase. Nature Structural & Molecular Biology 5 (1998) 866-871.
[35] A. Gildberg, Purification and characterisation of cathepsin D from the digestive gland of the pelagic squid *Todarodes sagittatus*. Journal of the Science of Food and Agriculture 39 (1987) 85-94.
[36] S. Bjelland, A. Gildberg, G. Volden, Degradation of human epidermal keratin by fish pepsin. Archives of dermatological research 280 (1988) 119-123.
[37] A. Venugopal, N.S. Kumar, Biochemical characterization of cathepsin D from the mussel *Lamellidens corrianus*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 169 (2014) 25-30.
[38] L.E. Celis-Guerrero, F.L. García-Carreño, M.A.N. del Toro, Characterization of proteases in the digestive system of spiny lobster (*Panulirus interruptus*). Marine Biotechnology 6 (2004) 262-269.
[39] Y. Yasuda, T. Kageyama, A. Akamine, M. Shibata, E. Kominami, Y. Uchiyama, K. Yamamoto, Characterization of new fluorogenic substrates for the rapid and sensitive assay of cathepsin E and cathepsin D. Journal of Biochemistry 125 (1999) 1137-1143.

[40] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72 (1976) 248-254.
[41] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (1970) 680-685.
[42] H. Blum, H. Beier, H.J. Gross, Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8 (1987) 93-99.
[43] H.M.G. Pérez, Electroforesis en geles de poliacrilamida: fundamentos, actualidad e importancia. Universo Diagnóstico 1 (2000) 31-34.
[44] C.-M. Tsai, C.E. Frasch, A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Analytical Biochemistry 119 (1982) 115-119.
[45] M. Dıaz-López, F.J. Moyano-López, F.J. Alarcón-López, F.L. Garcıa-Carreno, M.A.N. del Toro, Characterization of fish acid proteases by substrate–gel electrophoresis. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 121 (1998) 369-377.
[46] A.J. Barrett, Cathepsin D. Purification of isoenzymes from human and chicken liver. Biochemical Journal 117 (1970) 601-607.
[47] R.G. Valdez-Melchor, J.M. Ezquerra-Brauer, F.J. Cinco-Moroyoqui, F.J. Castillo-Yáñez, J.L. Cardenas-Lopez, Purification and partial characterization of trypsin from the viscera of tropical sierra (Scomberomorus sierra) from the Gulf of California. Journal of Food Biochemistry 37 (2013) 694-701.
[48] S.M. Kelly, N.C. Price, The application of circular dichroism to studies of protein folding and unfolding. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology 1338 (1997) 161-185.
[49] M.A. Andrade, P. Chacon, J.J. Merelo, F. Moran, Evaluation of secondary structure of proteins from UV circular dichroism spectra using an unsupervised learning neural network. Protein Engineering, Design and Selection 6 (1993) 383-390.
[50] S.W. Provencher, J. Gloeckner, Estimation of globular protein secondary structure from circular dichroism. Biochemistry 20 (1981) 33-37.
[51] R. Balti, N. Hmidet, K. Jellouli, N. Nedjar-Arroume, D. Guillochon, M. Nasri, Cathepsin D from the hepatopancreas of the cuttlefish (*Sepia officinalis*): purification and characterization. Journal of Agricultural and Food Chemistry 58 (2010) 10623-10630.
[52] T. Komai, C. Kawabata, M. Amano, B.R. Lee, E. Ichishima, Todarepsin, a new cathepsin D from hepatopancreas of Japanese common squid (*Todarodes pacificus*). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 137 (2004) 373-382.
[53] S. Goldman-Levkovitz, A. Rimon, S. Rimon, Purification properties and specificity of cathepsin D from *Cyprinus carpio*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 112 (1995) 147-151.
[54] P.A. Wang, J. Stenvik, R. Larsen, H. Mæhre, R.L. Olsen, Cathepsin D from Atlantic cod (*Gadus morhua L.*) liver. Isolation and comparative studies. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 147 (2007) 504-511.
[55] S.T. Jiang, F.P. Nei, H.C. Chen, J.H. Wang, Comparative study on the cathepsin D from banded shrimp (*Penaeus japonicus*) and grass shrimp (*Penaeus monodon*). Journal of Agricultural and Food Chemistry 40 (1992) 961-966.

[56] E.P. O'Brien, B.R. Brooks, D. Thirumalai, Effects of pH on proteins: predictions for ensemble and single-molecule pulling experiments. Journal of the American Chemical Society 134 (2011) 979-987.
[57] D. Voet, J.G. Voet, Biochemistry, 4th Edition, John Wiley & Sons, 2004.
[58] N.L. David, M. Cox, Lehninger. Principios de bioquímica, España, 2015.
[59] Y.R. Espinosa Silva, Estudio de la desnaturalización en frío de proteínas mediante simulaciones computacionales. (2016).
[60] A.R. López-Flores, C. Luna-Urban, J.J. Buenrostro-Figueroa, R. Hernández-Martínez, S. Huerta-Ochoa, H. Escalona-Buendía, C.N. Aguilar-González, L.A. Prado-Barragán, Efecto del pH, temperatura y fuente de proteína y carbohidratos en la producción de proteasas por *Yarrowia lipolytica* encultivo sólido. Revista mexicana de ingeniería química 15 (2016) 57-67.
[61] C. Capasso, W.E. Lees, A. Capasso, R. Scudiero, V. Carginale, P. Kille, J. Kay, E. Parisi, Cathepsin D from the liver of the antarctic icefish (*Chionodraco hamatus*) exhibits unusual activity and stability at high temperatures1. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology 1431 (1999) 64-73.
[62] A.M. Draper, M.G. Zeece, Thermal stability of cathepsin D. Journal of Food Science 54 (1989) 1651-1652.
[63] A.F. Sinkovits, B.C. Bryksa, T. Tanaka, R.Y. Yada, Understanding the structure–function role of specific catalytic residues in a model food related enzyme: Pepsin. Enzyme and Microbial Technology 40 (2007) 1175-1180.
[64] L. Zhao, S.M. Budge, A.E. Ghaly, M.S. Brooks, D. Dave, Extraction, purification and characterization of fish pepsin: a critical review. Journal of Food Processing & Technology 2011 (2012).
[65] A.A. Iodice, V. Leong, I.M. Weinstock, Separation of cathepsins A and D of skeletal muscle. Archives of Biochemistry and Biophysics 117 (1966) 477-486.
[66] S.N. Jamdar, P. Harikumar, Purification, identification and characterization of aspartic proteases of chicken intestine. Journal of Food Biochemistry 40 (2016) 451-462.
[67] M.G. Zeece, K. Katoh, Cathepsin D and its effects on myofibrillar proteins: A review. Journal of Food Biochemistry 13 (1989) 157-178.
[68] J.F. Woessner, R.J. Shamberger, Purification and properties of cathepsin D from bovine uterus. Journal of Biological Chemistry 246 (1971) 1951-1960.
[69] D.R. Davies, The structure and function of the aspartic proteinases. Annual review of biophysics and biophysical chemistry 19 (1990) 189-215.
[70] H.B. Bull, K. Breese, Thermal transitions of proteins. Archives of Biochemistry and Biophysics 156 (1973) 604-612.
[71] S.R. Tello-Solís, A. Arroyo-Reyna, Estudio por dicroísmo circular de la desnaturalización térmica de la subtilisina BPN': Modelo irreversible de dos estados. Revista de la Sociedad Química de México 46 (2002) 105-108.
[72] M. Levy, A.E. Benaglia, The influence of temperature and pH upon the rate of denaturation of ricin. Journal of Biological Chemistry 186 (1950) 829-847.
[73] S.R. Tello-Solís, B. Romero-García, Thermal denaturation of porcine pepsin: a study by circular dichroism. International Journal of Biological Macromolecules 28 (2001) 129-133.
[74] S.R. Tello-Solís, S. Schiavon-Nieto, G.-L.K. Karina, P.-B.M. Cristina, Desplegamiento térmico de colágeno bovino tipo I. RevistaTendencias en Docencia e Investigación en Química 1 (2015) 12-19.

[75] J.K. Raison, The influence of temperature-induced phase changes on the kinetics of respiratory and other membrane-associated enzyme systems, Membrane Structure and Mechanisms of Biological Energy Transduction, Springer, 1972, pp. 559-583.
[76] A.J. Barrett, J.F. Woessner, N.D. Rawlings, Handbook of proteolytic enzymes, Elsevier, 2012.
[77] M. Ahmad, S. Benjakul, Extraction and characterisation of pepsin-solubilised collagen from the skin of unicorn leatherjacket (*Aluterus monocerous*). Food Chemistry 120 (2010) 817-824.
[78] K.A. Dill, D. Shortle, Denatured states of proteins. Annual review of biochemistry 60 (1991) 795-825.
[79] M. Rüegg, U. Moor, B. Blanc, A calorimetric study of the thermal denaturation of whey proteins in simulated milk ultrafiltrate. Journal of Dairy Research 44 (1977) 509-520.
[80] Baldwin, T.N. Bhat, S. Gulnik, M.V. Hosur, R.C. Sowder, R.E. Cachau, J. Collins, A.M. Silva, J.W. Erickson, Crystal structures of native and inhibited forms of human cathepsin D: implications for lysosomal targeting and drug design. Proceedings of the National Academy of Sciences 90 (1993) 6796-6800.
[81] F. Franks, Protein Destabilization at Low Temperatures, in: C.B. Anfinsen, F.M. Richards, J.T. Edsall, D.S. Eisenberg (Eds.) Advances in Protein Chemistry, Academic Press, 1995, pp. 105-139.
[82] S. Rahman, A. Islam, M.I. Hassan, J. Kim, F. Ahmad, Unfoldness of the denatured state of proteins determines urea: Methylamine counteraction in terms of Gibbs free energy of stabilization. International Journal of Biological Macromolecules 132 (2019) 666-676.
[83] V. Cabra, R. Arreguin, R. Vazquez-Duhalt, A. Farres, Effect of temperature and pH on the secondary structure and processes of oligomerization of 19 kDa alpha-zein. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics 1764 (2006) 1110-1118.
[84] M.J. Kornblatt, R. Lange, C. Balny, Can monomers of yeast enolase have enzymatic activity? The FEBS Journal 251 (1998) 775-780.
[85] P.L. Privalov, Thermodynamics of protein folding. The Journal of Chemical Thermodynamics 29 (1997) 447-474.
[86] B. Anumalla, N.P. Prabhu, Counteracting Effect of Charged Amino Acids Against the Destabilization of Proteins by Arginine. Applied Biochemistry and Biotechnology (2019) 1-15.
[87] J. Jiang, J. Chen, Y.L. Xiong, Structural and emulsifying properties of soy protein isolate subjected to acid and alkaline pH-shifting processes. Journal of Agricultural and Food Chemistry 57 (2009) 7576-7583.
[88] S. Ghobadi, M.R. Ashrafi-Kooshk, H. Mahdiuni, R. Khodarahmi, Enhancement of intrinsic fluorescence of human carbonic anhydrase II upon topiramate binding: Some evidence for drug-induced molecular contraction of the protein. International journal of biological macromolecules 108 (2018) 240-249.
[89] M. Miriani, S. Iametti, F. Bonomi, M. Corredig, Structural changes of soy proteins at the oil–water interface studied by fluorescence spectroscopy. Colloids and Surfaces B: Biointerfaces 93 (2012) 41-48.
[90] M.A.B. Siddique, P. Maresca, G. Pataro, G. Ferrari, Effect of pulsed light treatment on structural and functional properties of whey protein isolate. Food Research International 87 (2016) 189-196.

[91] W. Chen, W. Wang, X. Ma, R. Lv, R.B. Watharkar, T. Ding, X. Ye, D. Liu, Effect of pH-shifting treatment on structural and functional properties of whey protein isolate and its interaction with (−)-epigallocatechin-3-gallate. Food Chemistry 274 (2019) 234-241.
[92] H. Fabian, C. Schultz, D. Naumann, O. Landt, U. Hahn, W. Saenger, Secondary Structure and Temperature-induced Unfolding and Refolding of Ribonuclease T1 in Aqueous Solution: A Fourier Transform Infrared Spectroscopic Study. Journal of Molecular Biology 232 (1993) 967-981.
[93] I.J. Tolano-Villaverde, H. Santacruz-Ortega, I.A. Rivero-Espejel, W. Torres-Arreola, G.M. Suárez-Jiménez, E. Márquez-Ríos, Effect of temperature on the actomyosin-paramyosin structure from giant squid mantle (*Dosidicus gigas*). Journal of the Science of Food and Agriculture (2019).
[94] H. Sanei, A. Asoodeh, S. Hamedakbari-Tusi, J. Chamani, Multi-spectroscopic investigations of aspirin and colchicine interactions with human hemoglobin: binary and ternary systems. Journal of Solution Chemistry 40 (2011) 1905-1931.
[95] M. Kjaergaard, A.B. Nørholm, R. Hendus–Altenburger, S.F. Pedersen, F.M. Poulsen, B.B. Kragelund, Temperature-dependent structural changes in intrinsically disordered proteins: Formation of α–helices or loss of polyproline II? Protein Science 19 (2010) 1555-1564.
[96] A.J.M. D'Souza, D.S. Hart, C.R. Middaugh, S.H. Gehrke, Characterization of the Changes in Secondary Structure and Architecture of Elastin−Mimetic Triblock Polypeptides during Thermal Gelation. Macromolecules 39 (2006) 7084-7091.
[97] D. Chakravarty, J. Janin, C.H. Robert, P. Chakrabarti, Changes in protein structure at the interface accompanying complex formation. IUCrJ 2 (2015) 643-652.
[98] F. Ding, J.M. Borreguero, S.V. Buldyrey, H.E. Stanley, N.V. Dokholyan, Mechanism for the α-helix to β-hairpin transition. Proteins: Structure, Function, and Bioinformatics 53 (2003) 220-228.
[99] Z. Qin, M.J. Buehler, Molecular dynamics simulation of the α-helix to β-sheet transition in coiled protein filaments: Evidence for a critical filament length scale. Physical Review Letters 104 (2010) 198304.
[100] T.P. Creamer, G.D. Rose, Side-chain entropy opposes alpha-helix formation but rationalizes experimentally determined helix-forming propensities. Proceedings of the National Academy of Sciences 89 (1992) 5937-5941.
[101] F.R. Salemme, D.W. Weatherford, Conformational and geometrical properties of β-sheets in proteins: II. Antiparallel and mixed β-sheets. Journal of Molecular Biology 146 (1981) 119-141.

Figure captions

Fig. 1. Affinity chromatography in agarose pepstatin of Japanese clam (*Ruditapes philippinarum*) hepatopancreas. The column was equilibrated with 50 mM sodium acetate buffer in 20 mM NaCl pH 5.0 followed by elution with 50 mM Tris-HCl, pH 8.5. The continuous line symbolizes absorbance at 280 nm, the dashed line symbolizes the activity with the specific substrate AcRGFFP-AFC, the arrow indicates change of buffer.

Fig. 2. SDS-PAGE of purified cathepsin D from Japanese clam (*Ruditapes philippinarum*) hepatopancreas. (A) wide range molecular weight markers, (B) crude extract, (C) 50 kDa membrane, (D) purified cathepsin D in pepstatin agarose affinity chromatography fractions, (E) zymogram of crude extract, (F) zymogram of 50 kDa membrane fractions, (G) zymogram of purified cathepsin D in pepstatin agarose affinity chromatography fractions, and (H) IEF of purified cathepsin D.

Fig. 3. Cathepsin D from Japanese clam (*Ruditapes phillipinarum*) hepatopancreas optimum pH (A) and optimum temperature (B). The result is the average of three determinations.

Fig. 4. Stability of purified cathepsin D of the Japanese clam (*Ruditapes phillipinarum)* hepatopancreas (A) temperature (B) pH in a time span of 120 min. The dotted line represents the threshold of 50% of the activity. The result is the average of three determinations

Fig. 5. Japanese clam (*Ruditapes philippinarum*) hepatopancreas cathepsin D Michaelis-Menten graph for hemoglobin substrate (using Prism ver 5.0 software). The error bar represents three determinations. The values are the average of three determinations.

Fig. 6. Cathepsin D from Japanese clam (*Ruditapes phillipinarum*) hepatopancreas thermal transition curves as a function of the heating rate at pH 3.5. The process was followed by heat capacity and ellipticity. Heating rates: 0.5 °C/min (continuous line), 1.0 °C/min (dashed line) and 2.0 °C/min (point line). Experiments were monitored in A) Circular dichroism ellipticity at 220 nm, adjusted by Boltzmann equation with an r^2 above 0.95. B) Differential scanning calorimetry.

Fig. 7. Estimation of changes of secondary structure by circular dichroism at different pH of cathepsin D from Japanese clam (*Ruditapes phillipinarum*).

Fig. 8. Estimation of changes of secondary structure at different temperature of Japanese clam (*Ruditapes phillipinarum*) hepatopancreas cathepsin D.

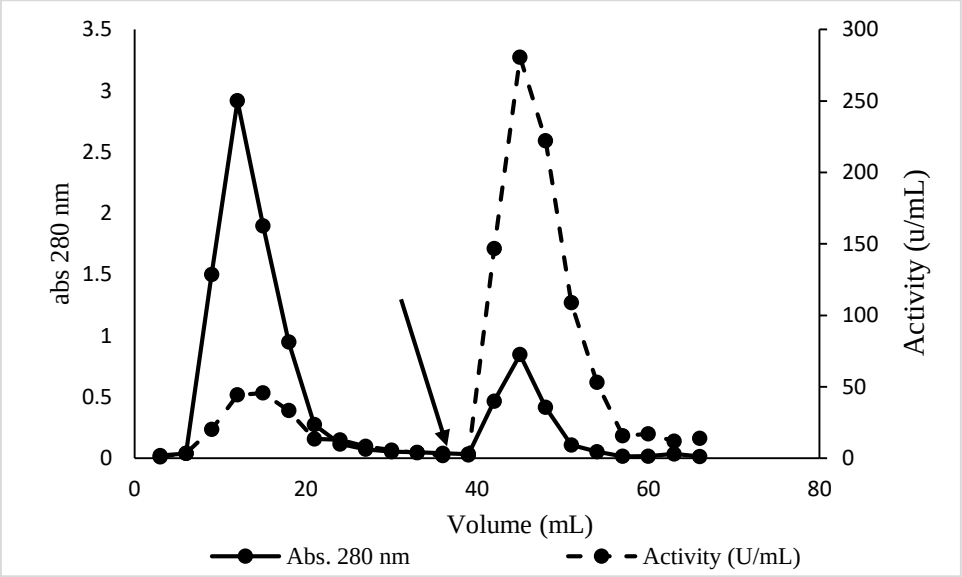

3.5
3
2.5
2
1.5
1
0.5
0
abs 280 nm
300
250
200
150
100
50
0
Activity (u/mL)
0
20
40
60
80
Volume (mL)
Abs. 280 nm
Activity (U/mL)

Fig. 1.

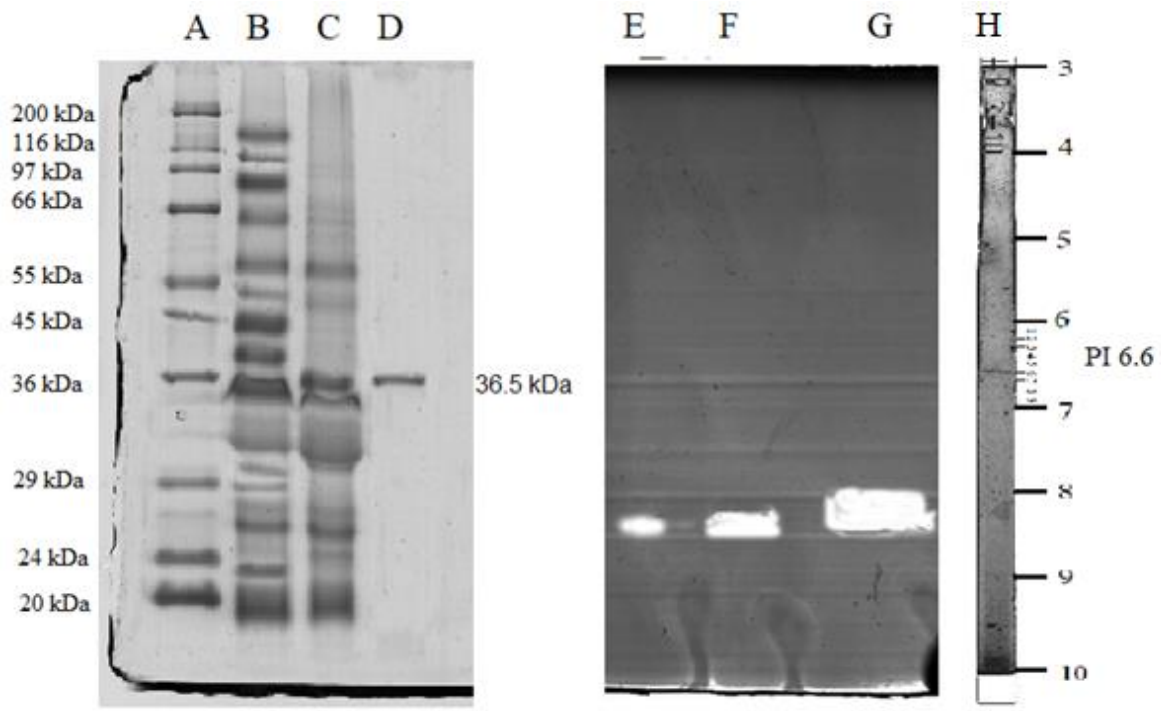

A
B
C
D
E
F
G
H
200 kDa
116 kDa
97 kDa
66 kDa
55 kDa
45 kDa
36 kDa
29 kDa
24 kDa
20 kDa
36.5 kDa
3
4
5
6
7
8
9
10
PI 6.6

Fig 2.

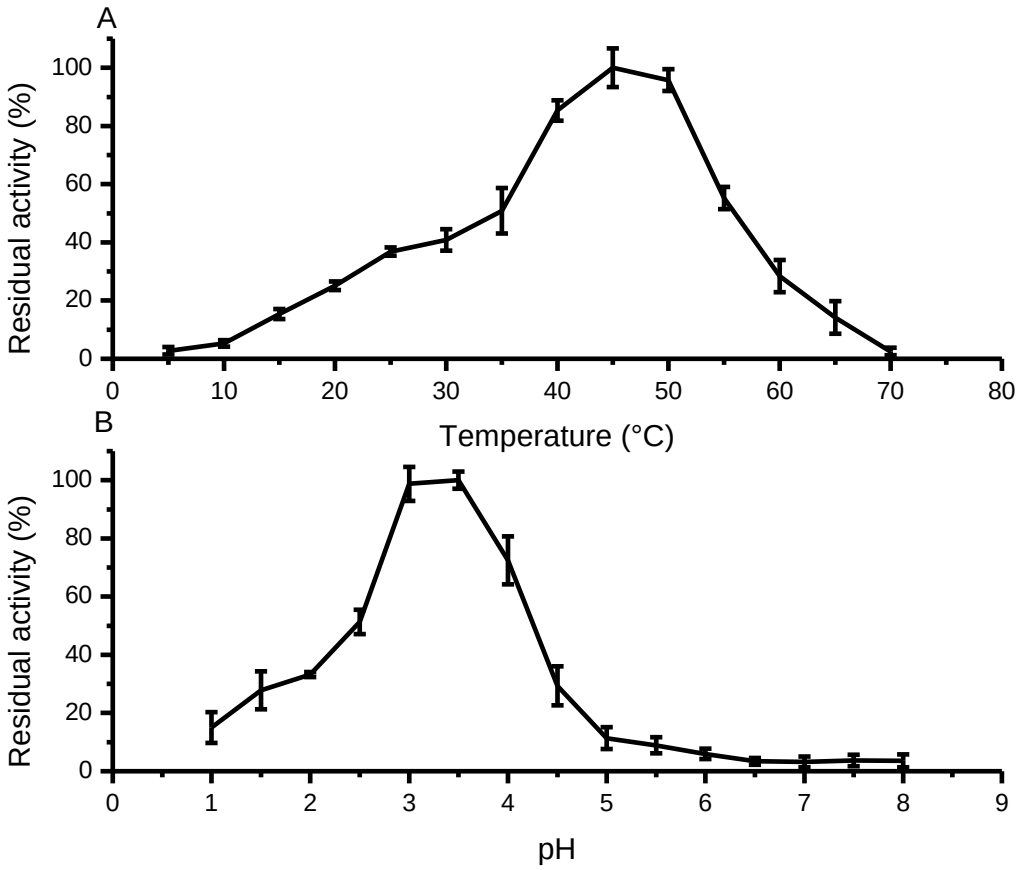
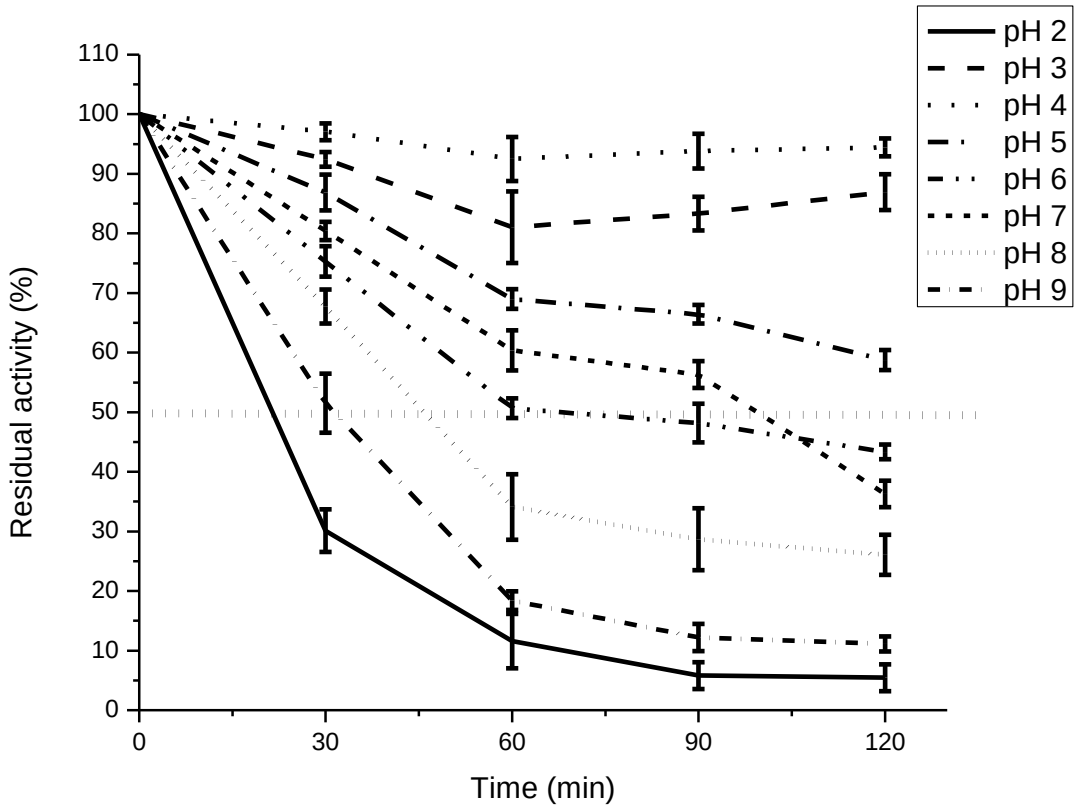

A
Residual activity (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
Temperature (°C)
B
Residual activity (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
pH

Fig. 3.


pH 2
pH 3
pH 4
pH 5
pH 6
pH 7
pH 8
pH 9
Residual activity (%)
Time (min)
0
10
20
30
40
50
60
70
80
90
100
110
0
30
60
90
120

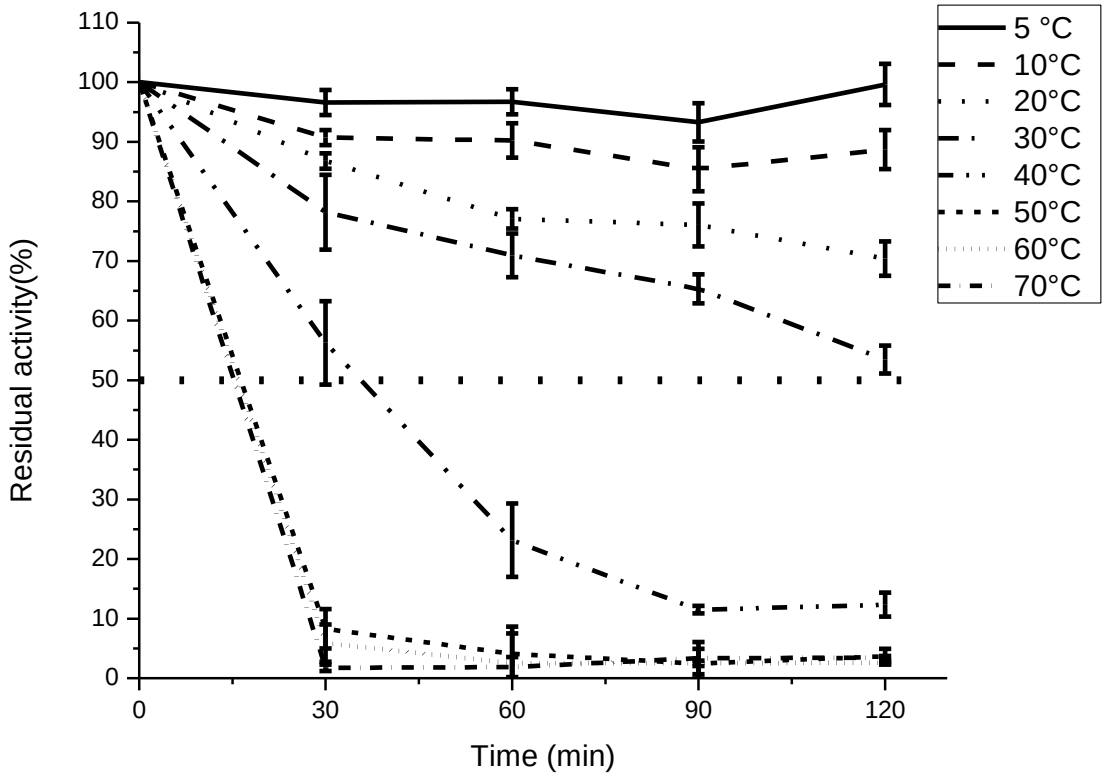

Residual activity(%)
Time (min)
0
10
20
30
40
50
60
70
80
90
100
110
0
30
60
90
120
5 °C
10°C
20°C
30°C
40°C
50°C
60°C
70°C

Fig. 4.

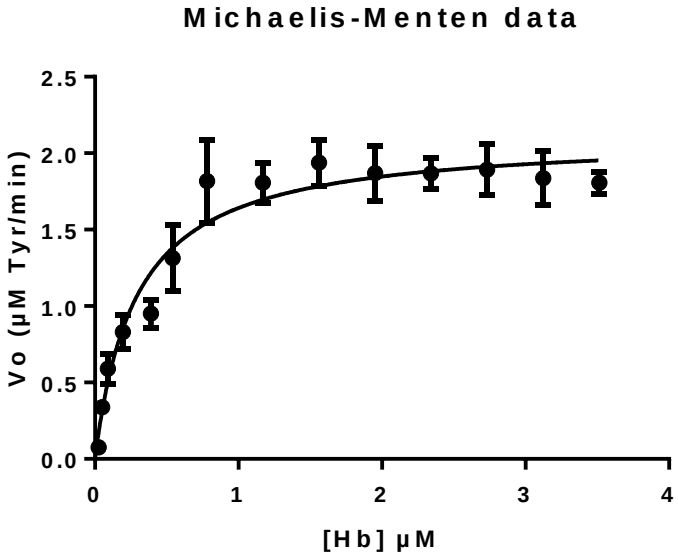

Michaelis-Menten data
Vo (µM Tyr/min)
2.5
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
[Hb] µM

Fig. 5.

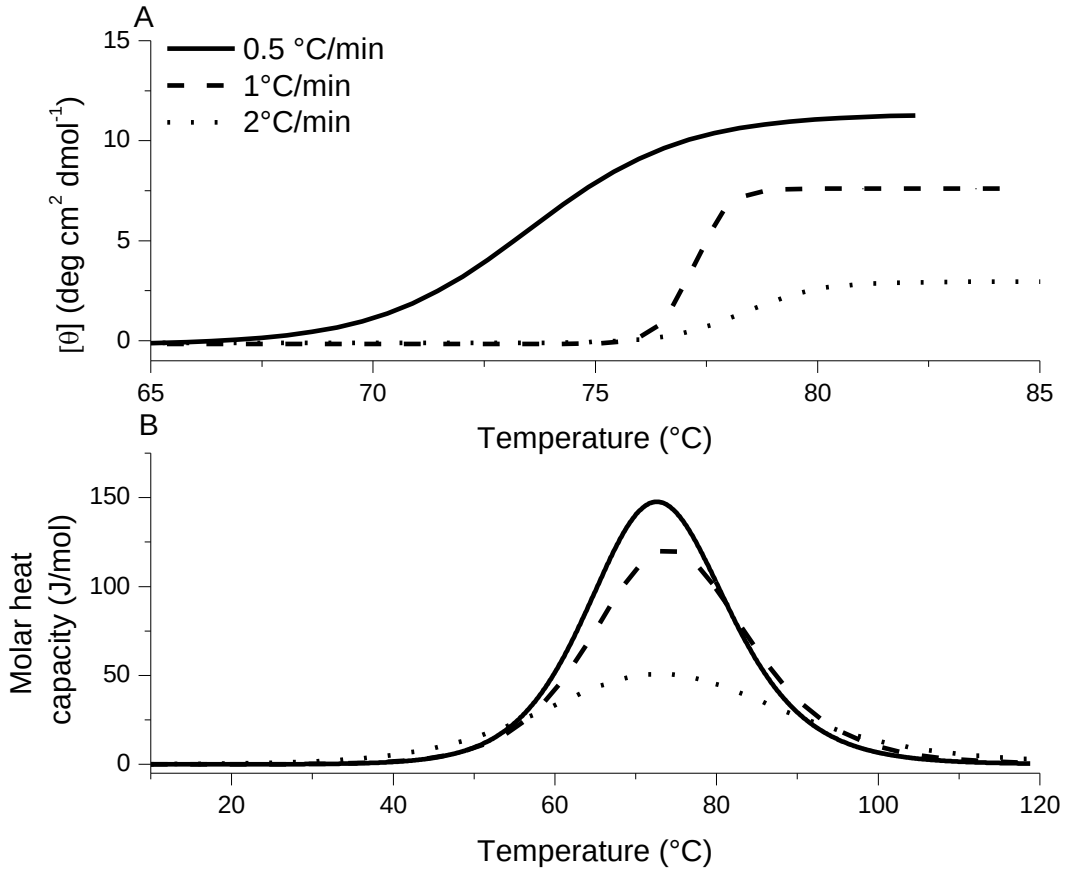

A
0.5 °C/min
1°C/min
2°C/min
[θ] (deg cm^2 dmol^{-1})
15
10
5
0
65
70
75
80
85
Temperature (°C)
B
Molar heat capacity (J/mol)
150
100
50
0
20
40
60
80
100
120
Temperature (°C)

Fig. 6.

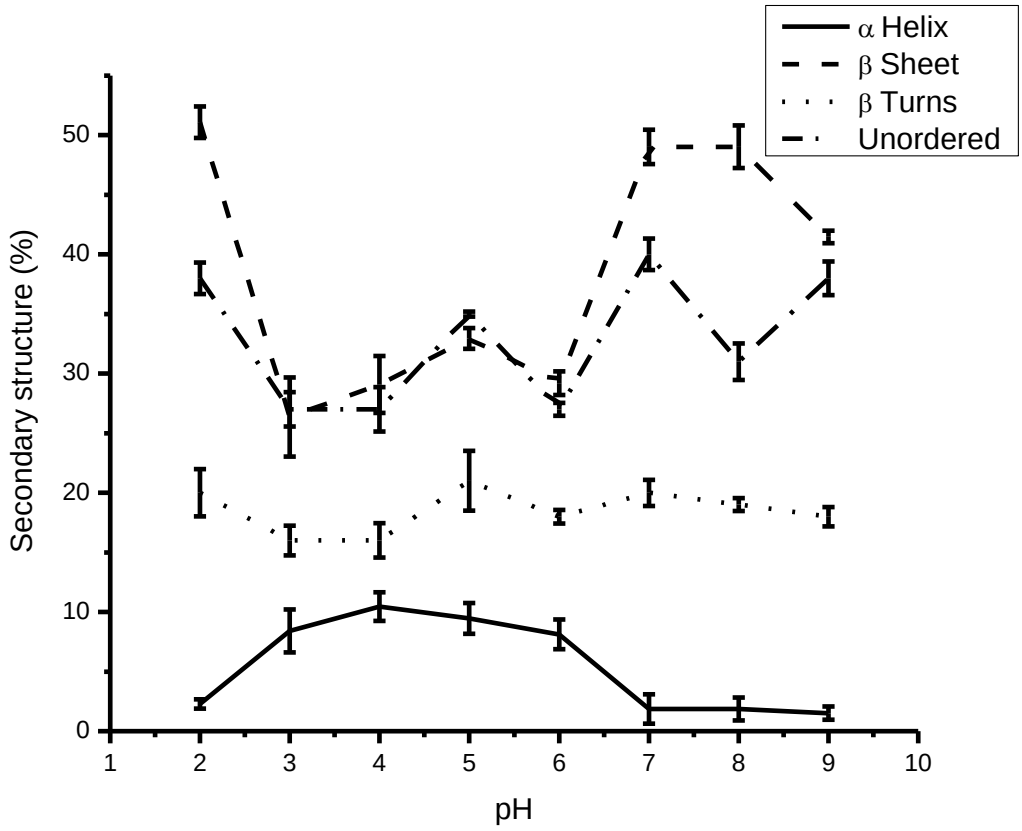

α Helix
β Sheet
β Turns
Unordered
Secondary structure (%)
0
10
20
30
40
50
1
2
3
4
5
6
7
8
9
10
pH

Fig. 7

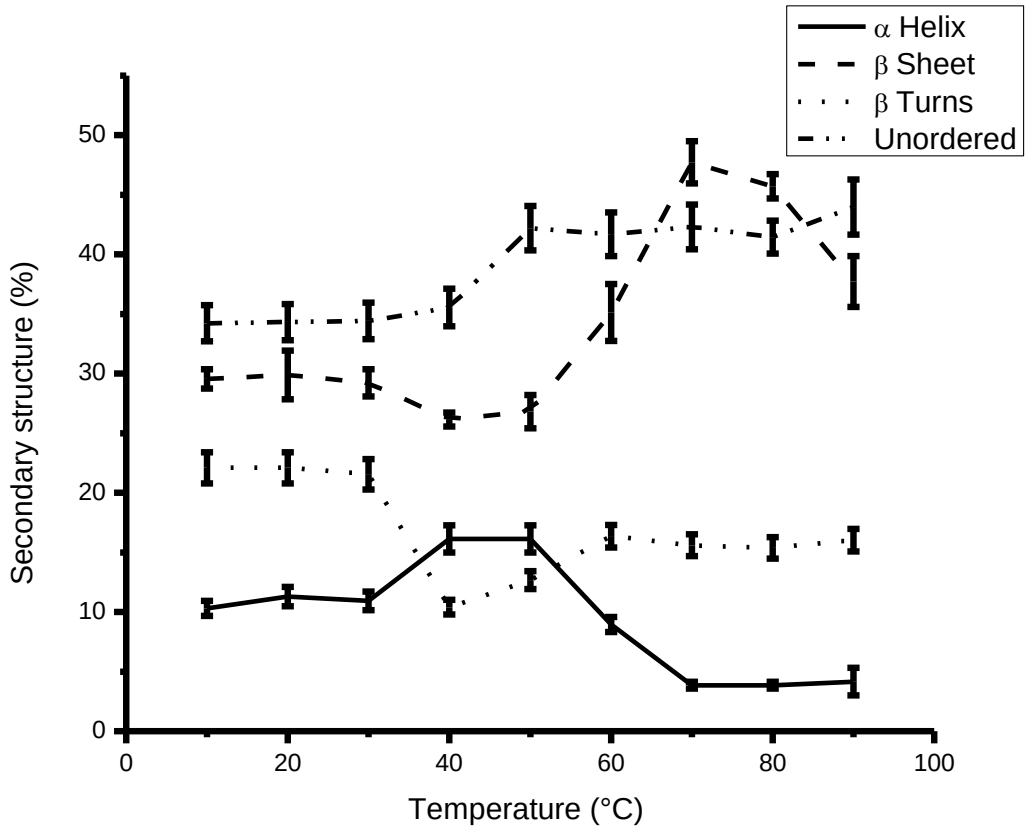

α Helix
β Sheet
β Turns
Unordered
Secondary structure (%)
Temperature (°C)
0
10
20
30
40
50
0
20
40
60
80
100

Fig. 8.

Table 1. Purification table of cathepsin D from Japanese clam (*Ruditapes philippinarum*) hepatopancreas.

	Total activity (U)	Total Protein (mg)	Specific activity (U/mg protein)	Purification (X)	Yield (%)
Crude extract	33,440	225	148	1	100
UF Membrane (50 kDa)	21,105	141	148	1	62
Affinity fractions	6,622	1.2	5,553	37	19

*The data is average of three determinations

Table 2. Thermodynamic parameters of cathepsin D from Japanese clam (*Ruditapes phillipinarum*) hepatopancreas.

Heat speed (°C/min)	Tm CD (°C)	Tm DSC (°C)	ΔG (KJ/mol)	ΔH (KJ/mol K)	ΔS (KJ)
0.5	73.5	72.0	-128.9	-422.5	-0.8
1	76.2	74.5	-85.7	-374.0	-0.8
2	78.3	76.0	-64.4	-239.0	-0.5

Tm= thermal transition, CD=circular dichroism, DSC=Differential scanning calorimetry
*Thermodynamic parameters cathepsin D was estimating using program CpCalc ver.2.1

CAPÍTULO 3

Cambios en actividad, estructura secundaria y proceso de desnaturalización de catepsina D del hepatopáncreas de la almeja reina *Dosinia ponderosa* a diferentes condiciones de temperatura y pH.

Cadena-Cadena Francisco[a], Cárdenas-López José Luis*[a], Ezquerra-Brauer Josafat Marina[a] Cinco-Moroyoqui Francisco Javier[a], López-Zavala, Alonso Alexis[b], Santacruz-Ortega Hisila del Carmen, Rivero-Espejel Ignacio Alfredo[e] and Ofelia Rouzand Sandez[a]

[a]*Departamento de Investigación y Posgrado de Alimentos, Universidad de Sonora, Hermosillo Sonora México*
[b]*Departamento de Ciencias Químico Biológicas, Universidad de Sonora, Hermosillo Sonora México*
[c]*Departamento de Investigación en Polímeros y Materiales, Universidad de Sonora, Hermosillo Sonora México*
[e]*Centro de Graduados e Investigación en Química, Instituto Tecnológico de Tijuana, Tijuana, Baja California, México*
**Corresponding autor, joseluis.cardenas@unison.mx*

Introducción

Los factores que afectan las estructuras secundarias y terciarias de las proteínas, son poco conocidos (White, 2013; White *et al.*, 1998). La catepsina D es una enzima lisosómica que se encuentra en todos los organismos actuando en el recambio proteico y recientemente se ha descubierto su participación en la degradación de proteínas del alimento (Jamdar *et al.*, 2016; Martínez-Alarcón *et al.*, 2018; Rojo *et al.*, 2013; Rojo *et al.*, 2010), en humanos, esta enzima está bien caracterizada debido a su presencia en varios tipos de carcinomas (Fusek *et al.*, 2005), es un blanco en el tratamiento de mal de Parkinson (Pal *et al.*, 2019) y actúa en la regulación de la activación de la las células inflamatorias durante la pancreatitis (Aghdassi *et al.*, 2018), en organismos marinos se sabe que la catepsina D tiene una intensa actividad durante el desove (Balti *et al.*, 2010; Sales *et al.*, 2019).

Se han realizado varios esfuerzos analíticos para dilucidar la relación que existe entre la estructura de la proteína y la actividad. Los estudios de caracterización fisicoquímica y

estructural de proteínas sugieren que estas proteínas tienen más bajo contenido de hélice α que las proteínas de origen terrestre (Balti *et al.*, 2010; Cadena-Cadena *et al.*, 2019) por lo que su funcionalidad está estrechamente relacionada con su estructura (Carter *et al.*, 1997; Nelson *et al.*, 2017).

El proceso de modificación estructural es complejo y no se ha aclarado por completo, porque depende de una gran cantidad de parámetros físicos y químicos, en especial de la temperatura y del pH (Militello *et al.*, 2004; Vaiana *et al.*, 2001). En general, la actividad de proteínas parece estar asociada principalmente con cambios conformacionales y estructurales de la molécula en los cuales la actividad es modulada por los movimientos que sufren las distintas estructuras secundarias de la proteína (Lee *et al.*, 1998), estas modificaciones pueden ser tan grandes que exponen algunos de los residuos no polares, los cuales al interaccionar con el solvente provocan la agregación de la proteína o son tan pequeñas que sólo hay formación o deformación de enlaces de hidrógeno (Nawrocka *et al.*, 2016). La necesidad de un estudio mas detallado de catepsina D en almejas resulta mas que evidente debido a sus implicaciones en la degradación de proteínas y a su participación en el sistema inmune (Asbjörn Gildberg, 1987). En estudios en calamar gigante se ha encontrado en el hepatopáncreas dos picos de actividad autolítica, resultando en catepsina L para el pico de pH 5 y catepsina D para el pico de pH 3 (Cardenas-Lopez *et al.*, 2005). En general, cambios en estructura secundaria son parámetros externos que pueden afectar la actividad de la enzima (Lee *et al.*, 1998). A fin de comprender los cambios en la estructura secundaria de catepsina D en la hepatopáncreas de la almeja reina calamar gigante se determio el efecto de la temperatura, pH y desnaturalización térmica, así como la relación entre la actividad y los cambios que tiene su estructura secundaria.

Materiales y métodos

Las almejas fueron recolectadas en Bahia de Kino, Sonora, México (localización geográfica 28° 50' 0" Norte, 111° 56' 0" Oeste) y transportado al laboratorio. El hepatopáncreas fue removido e inmediatamente se preparó el extracto crudo, durante 12 h a 4 ° C bajo agitación con buffer de acetato de sodio 50 mM, pH 5.0, en una relación 1: 2 (p/v) siguiendo los procedimientos de Venugopal *et al (*2014). El homogeneizado se centrifugó a 10,000 x g durante 1 h, y el sobrenadante se filtró a través de una membrana Amicon PM-50 (Millipore Corporation, Bedford, MA), y se guardó a 4 °C para su posterior análisis

Extracción y purificación de la catepsina D

Las almejas fueron recolectadas en la Bahía de Kino, Sonora, México (coordenadas geográficas 28 ° 50 '0 "Norte, 111 ° 56' 0" Oeste) y transportadas al Laboratorio. El extracto crudo se preparó mezclando 1:1 v/v del hepatopáncreas recién extraído de las almejas con buffer de muestra (acetato de sodio 100 mM pH 5,5) (Celis-Guerrero *et al.*, 2004). Se utilizó una membrana de ultrafiltración (UF. MWCO de 50 kDa (Amicon, Millipore, EE. UU.)) para desalar la preparación de proteínas. La purificación de la enzima consistió en la inyección de 10 ml del filtrado en la columna de cromatografía de afinidad utilizando un soporte de agarosa pepstatina (inhibidor de las proteasas asparticas) (Knight *et al.*, 1976). La etapa de lavado se realizó con buffer muestra. Para la elución, se usó buffer Tris 20 mM, pH 8,5. La electroforesis SDS-PAGE se realizó de acuerdo a Laemmli (1970), El zimograma en condiciones ácidas se llevó a cabo utilizando la metodología propuesta por Diaz-lopez *et.al.*

(1998) y el punto isoeléctrico se efectuó en tiras de 7 cm en pH 3-10 con una medida de 7 cm. Los geles se tiñeron con azul de Coomassie al 0,025% para la visualización de la banda (Laemmli, 1970). Durante la cromatografía, el monitoreo se realizó midiendo la absorbancia a 280 nm. La concentración de proteína se determinó mediante el ensayo de Bradford utilizando albúmina de suero bovino como estándar para las fracciones(Bradford, 1976), además se utilizó la actividad máxima de la enzima en sustratos generales y específicos (Yasuda *et al.*, 1999).

Actividad de catepsina D

Ensayo general

La actividad de proteasas aspárticas se obtuvo mediante el método Anson (1937) modificado por Celis-Guerrero (2004). Se utilizó como substrato hemoglobina de suero bovino 0.5% (p/v) disuelta en buffer de glicina-HCl 100 mM, pH 3.5. Para dar inicio a la reacción se añadió 500 µL de buffer glicina a 500 µL de solución de sustrato y 100 µL del extracto enzimático. La reacción es detenida después de 10 min a 45 °C con 500 µL de ácido tricloroacetico al 10% (TCA) frio, la centrifugación se realizó por 5 min a 9000 x g. La absorbancia del sobrenadante se mide a 280 nm.

Ensayo específico

El ensayo enzimático con el sustrato específico para catepsina D consta del péptido N-Acetil-Arg-Gly-Phe-Phe-Pro-7-amido-4-trifluorometilcoumarina (AcRGFFP-AFC) y se realizó siguiendo una modificación de la metodología reportada Yasuda (1999). La metodología consiste en mezclar 80 µL de buffer glicina 100 mM con 10 µL de AcRGFFP-AFC 200 µM a pH 3.5. El sustrato en el buffer se pre-incubó por 2 min a 45 °C. La reacción inició al añadir

los 10 µL de enzima. Después de 10 min se añadieron 320 µL de glicina 100 mM pH 9.5 y 10 µL de enzima dipeptidilpeptidasa IV a una concentración de 0.6 U/mL. La catepsina D rompe el sustrato sobre los aminoácidos F-F quedando dos fragmentos uno N-Acetil-Arg-Gly-Phe (AcRGF) y el otro Phe-Pro-7-amido-4-trifluorometilcoumarina (FP-AFC), se usa la enzima dieptidil peptidasa IV para romper el enlace de los aminoácidos unidos al AFC. El AFC liberado se midió en un fluorómetro a 400 nm de excitación 400 nm y 505 nm de emisión 505nm. El blanco o control consistió en agregar TCA antes de ser agregado el sustrato (Yasuda *et al.*, 1999).

Espectroscopía de dicroísmo circular (CD)

Los espectros de dicroísmo circular se realizaron en un espectropolarímetro Jasco J-815 equipado con un controlador de temperatura Peltier, utilizando una celda de cuarzo de 1 cm de longitud de trayectoria en el UV lejano. Las muestras contenían una concentración final de 0.02 mg/mL de catepsina D purificada. Se recogieron tres muestras de análisis y se promediaron para obtener los espectros finales en todos los casos.

Los resultados se obtienen en mdeg (milisegundos o milésimas de un grado) y se transformaron en elipticidad media por residuo, utilizando 110 g/mol de peso molecular promedio por residuo. La estimación de la estructura secundaria a partir de los espectros de CD de UV lejano se calculó utilizando el software K2D y CONTIN Dichroweb (Van Stokkum *et al.*, 1990).

Temperatura y pH óptimos

El efecto de la temperatura sobre la actividad proteolítica de la catepsina D se evaluó en un rango de temperatura de 10 a 80 °C. La enzima en buffer glicina 100 mM, pH 3.5, se incubó a las diferentes temperaturas por 10 min, posteriormente se midió la actividad como se mencionó anteriormente. En el caso del pH óptimo el efecto sobre la actividad proteolítica de la catepsina D se evaluó a diferentes valores de pH: 2 - 3.5 (buffer de glicina 100 mM), 4 - 8 (buffer de acetato de sodio 100 mM). La actividad se midió incubando la enzima junto al sustrato a 37 °C, posteriormente se realizó el ensayo enzimático como se mencionó anteriormente (Balti *et al.*, 2010; Venugopal *et al.*, 2014).

Estabilidad a la temperatura y pH

Este experimento, fue diseñado para medir el efecto de la temperatura de almacenamiento sobre la actividad de catepsina D a pH constante, se llevó a cabo mediante el almacenamiento de la enzima a diferentes temperaturas (5, 10, 20, 30, 40, 50, 60, 70 y 80°C), manejando el mismo sistema de buffer que para la determinación de la temperatura óptima (Chen *et al.*, 1986; Valdez-Melchor *et al.*, 2013). De la misma forma, la determinación del pH de estabilidad consistió en el almacenamiento de la enzima a diferentes pH (2,3,4,5,6,7 y 8) en 120 min de incubación obteniendo alícuotas cada 30 min, y llevando a cabo el ensayo antes descrito (Chen *et al.*, 1986; Valdez-Melchor *et al.*, 2013).

2.8. Calorimetría diferencial de barrido

Los experimentos de calorimetría diferencial de barrido (DSC) se realizaron en un instrumento Nano-DSC II, Modelo 6100 (Calorimetry Sciences Corp.) a velocidades de exploración de 0.05, 1.0 y 2.0 °C/min. Las mediciones se realizaron en un rango de

temperatura de 15 a 100 °C con un exceso de presión de 3 bares con una celda de referencia llena con buffer de acetato de sodio 10 mM pH 5. El volumen de la celda de muestra fue de 0.5 mL que contenía 1 mg /mL de proteína. La solución de muestra y la solución de referencia se dializaron con tubos de diálisis de corte de peso molecular de 10 kDa antes del experimento DSC. Los parámetros termodinámicos (ΔG, ΔH y ΔS) y la temperatura de transición Tm se calcularon con los datos obtenidos en el software de calorímetro CpCalc.

Efecto de la temperatura and pH en la estructura secundaria

El efecto del pH y la temperatura sobre el contenido de la estructura secundaria se obtuvo colocando la enzima en diferentes buffers a diferentes valores de temperatura y pH, que van de 10 a 80 °C y de 2 a 8 respectivamente. Todos los buffers evitaron el uso de elementos de cloro y fueron elegidos de acuerdo con su pKa apropiado. En cada caso, la línea de base se corrigió con el buffer.

2.5. Desnaturalización térmica

La desnaturalización térmica fue seguida por dicroísmo circular, usando una concentración de enzima de 0.128 mg / ml en tampón de acetato de sodio 50 mM, pH 3,5. Los espectros se obtuvieron utilizando celdas de cuarzo con 1 cm de trayectoria óptica. La temperatura se controló con un Peltier. La desnaturalización térmica fue seguida por el cambio en la elipticidad a 220 nm. El cambio de temperatura en la muestra fue de 10-80 ° C y se realizó a diferentes velocidades de calentamiento (0.5 °C/min, 2 ° C/min y 4 °C/min con una precisión de ± 0.02 °C/min). La reversibilidad de la transición se realizó midiendo el cambio en la elipticidad a 220 nm. Los aumentos de temperatura fueron de 1 °C/min, de 5 °C a 80 °C, después de lo cual, la solución se enfrió rápidamente a una velocidad de 15 °C/min hasta

alcanzar la temperatura ambiente (25 °C), la diferencia entre dos elipticidades se expresaron como la fracción de proteína desnaturalizada irreversiblemente.

Resultados y discusión

Catepsina D extracción y purificación

El perfil cromatógrafico se muestra en la Fig. 1. Se observa un solo pico de actividad el cual aparece después de hacer el cambio de buffer de acetato de sodio pH 5.5 a tris base pH 8.8. La mayor parte de la proteína contaminante fue removida en el primer pico de elución. Un resumen de las etapas de purificación se muestra en la Tabla 1. Aproximadamente 4659.57 U/mg de proteína fueron obtenidas lo cual representa una purificación de 22 veces y un rendimiento de 36% de la actividad original. En el primer pico se detectó una cantidad muy pequeña de actividad por lo cual la podemos ignorar por su magnitud. Siendo en el segundo pico donde se detectó el máximo de absorción relacionado con la actividad de la enzima. En cuanto al extracto crudo obtenido del hepatopáncreas de la almeja reina la actividad específica (130.86 U/mg de proteina) es mayor a la reportada para el calamar gigante (103 U/mg de proteína) (Cadena-Cadena *et al.*, 2019), calamar japonés (*Todarodes pacificus*) (62.89 U/mg de proteína) (Komai *et al.*, 2004) y para calamar europeo (*Todarodes sagittatus*) (7 U/mg de proteína (Asbjörn Gildberg, 1987). Estas diferencias en el contenido de proteínas del extracto crudo pueden deberse al método de extracción, al manejo y época de captura o al hábitat de las especies, ya que los calamares tienden a habitar a profundidades muy grandes, por lo cual las enzimas tienen que tener características especiales (Ezquerra-Brauer *et al.*, 2002; Rongel *et al.*, 2011), mientras que las almejas viven en ambientes muy diversos, lo que influye en la expresión de las enzimas presentes.

En el análisis electroforético se detectó una sola banda en las fracciones de cromatografía (Fig. 2 D),), una sola banda mediante zimograma (Fig. 2 G) y una sola banda mediante IEF (Fig. 2 H). El peso molecular estimado es de 36 kDa, mientras que su punto isoeléctrico se mostró en 7.1. Estos datos están asociados a catepsina D de calamar japonés (*Todarodes pacificus*) (36. 5 kDa) (Komai *et al.*, 2004), Calamar gigante (*Dosidicus gigas)* (38.5) (Cadena-Cadena *et al.*, 2019) arenque (*Clupea harengus*) (38 kDa), mejillón (*Lamelidus corianus*) (43 kDa) (Venugopal *et al.*, 2014), incluso hay reportes de esta enzima con pesos moleculares menores como en el calamar europeo (*Todarodes sagittatus*) (10 y 28 kDa) (Asbjörn Gildberg, 1987; Asbjørn Gildberg, 1988), esto nos habla de la heterogeneidad de la enzima y cómo aún tratándose de la misma enzima esta puede tener distintas propiedades de acuerdo a los requerimientos de la especie (Haard *et al.*, 2000), el punto isoeléctrico es variable y se han detectado para humano en 5.2 (Maguchi *et al.*, 1988), para cerdo en 5.7 (Bird *et al.*, 1978), para calamar europeo en 8.5 (Asbjörn Gildberg, 1987) y para arenque en 8.29 (Nielsen *et al.*, 2001). El zimograma del extracto crudo revela la presencia de al menos cuatro bandas activas (Fig 2, carril E y F). Sin embargo, después de la purificación con columna de afinidad una sola banda es detectada (Fig 2 carril G).

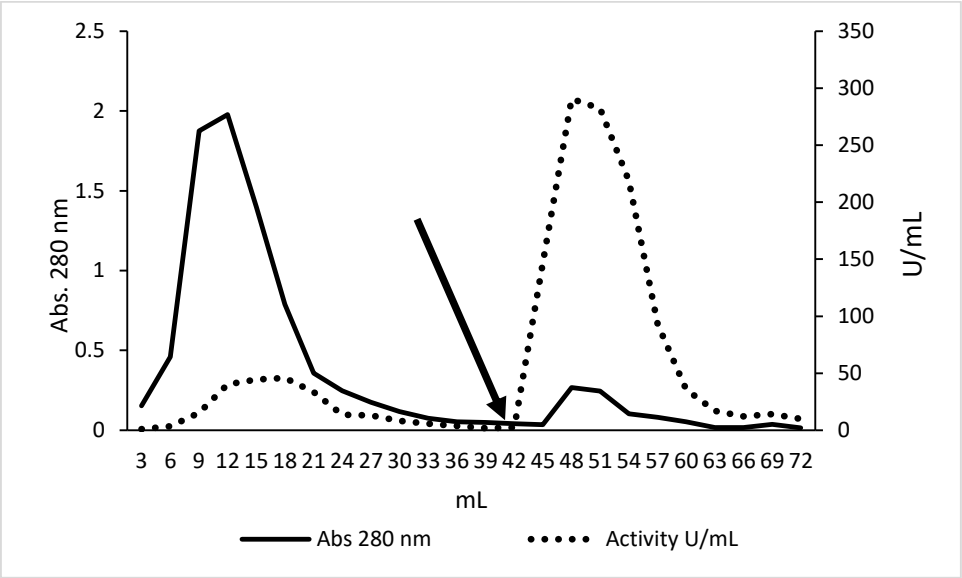


Fig. 1. Cromatografía de afinidad en pepstatina agarosa de la almeja del extracto de hepatopáncreas de almeja reina, la columna se equilibró con buffer de acetato de sodio 100 mM en NaCl 20 mM a pH 5,5, seguido de Tris-HCl 50 mM pH 8,8. La línea continua simboliza la absorbancia a 280 nm, la línea punteada simboliza la actividad con el sustrato AcRGFFP-AFC. La flecha inca el cambio de buffer.

Tabla 1. Tabla de purificación de la catepsina D de la almeja reina (*Dosinia ponderosa*)

	Actividad Total	Proteína neta (mg)	Actividad específica (U/mg proteína)	Rendimiento (%)	Purificación (X)
ECH Almeja Reina	28660	219	130.86	100	1
Filtrado 50 kDa	21248	20	1062.41	62	8
Afinidad	6132	1.316	4659.57	22	36

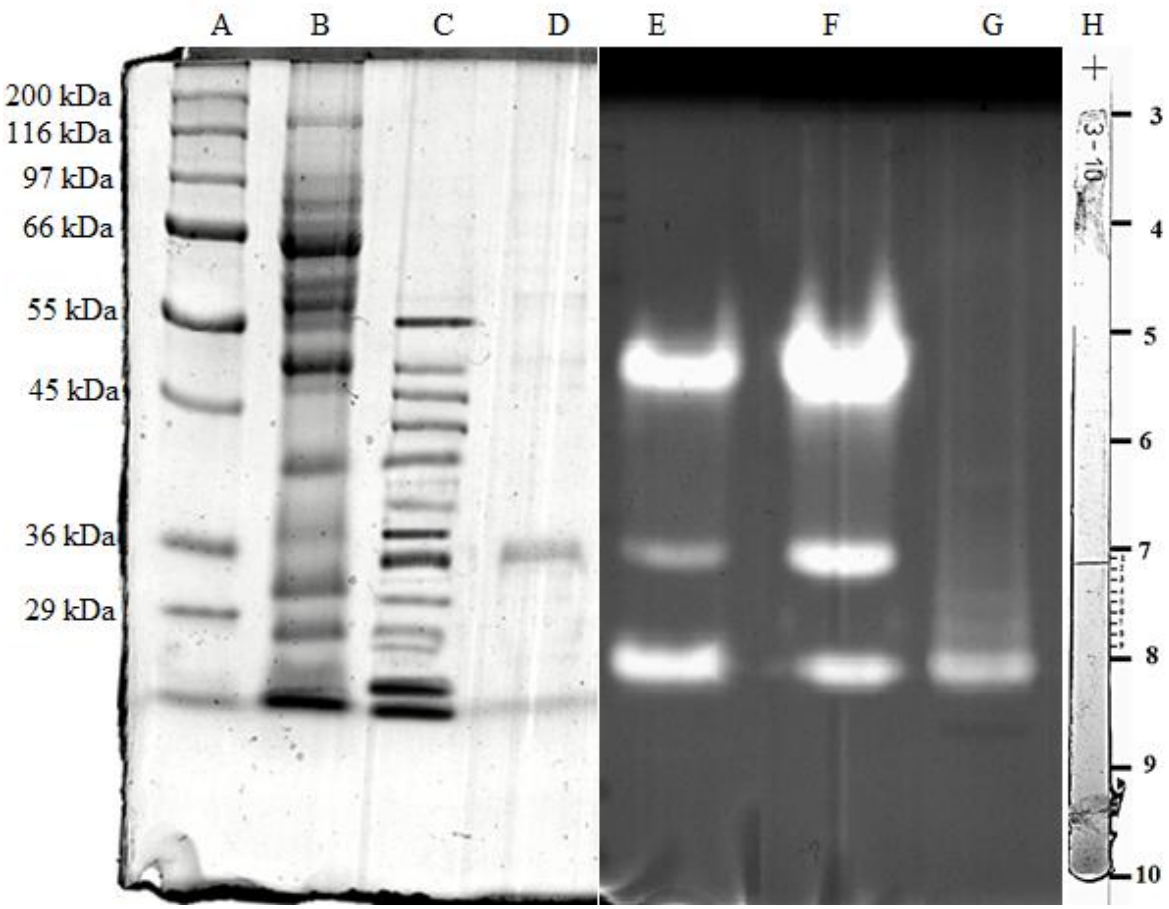


Fig 2. SDS-PAGE de catepsina D purificada de hepatopáncreas de almeja japonesa (*Ruditapes philippinarum*). (A) marcadores de peso molecular de amplio rango, (B) extracto crudo, (C) membrana de 50 kDa, (D) catepsina D purificada en fracciones de cromatografía de afinidad con agarosa de pepstatina, (E) zimograma de extracto crudo, (F) zimograma de 50 kDa fracciones de membrana, (G) zimograma de catepsina D purificada en fracciones de cromatografía de afinidad con agarosa de pepstatina, y (H) IEF de catepsina D purificada

Análisis bioquímico de la catepsina D

Temperatura óptima y pH óptimo

La enzima purificada es activa entre pH 2.5 y 4.5 presentando un pH óptimo de 3 a 3.5 para la almeja reina (Fig 3). Sin embargo, la actividad de la enzima catepsina D decrece un 58%por encima de pH optimo y un 40% debajo de su pH optimo, a pH de 5 ya se ha perdido prácticamente toda la actividad, esto nos demuestra la gran dependencia que tiene la enzima de la almeja al pH, este pH es muy similar al reportado para otras especies de moluscos como los calamares (Balti *et al.*, 2010; Cadena-Cadena *et al.*, 2019; Asbjörn Gildberg, 1987; Komai *et al.*, 2004) que presentaron un pH de 3.5, para el mejillón (*Lamellidens corrianus*) y para el bacalao del Atlántico (Wang *et al.*, 2007) el pH óptimo es de 3.0. La temperatura óptima de la catepsina D fue de 45 y 50°C. Sin embargo, se mantuvo activa desde los 30°C hasta los 60°C aumentando más de 70% su actividad al llegar a su óptimo. A temperaturas superiores a la óptima, la actividad disminuyo rápidamente (Fig 3 B) entre un 45 y un 54% de pérdida de actividad, en las temperaturas inferiores podemos observar la gran dependencia que tiene a la temperatura, ya que a abajo de los 30°C casi no muestra actividad. Otros moluscos, como los calamares presentan temperaturas óptimas entre 40 y 45 °C (Balti *et al.*, 2010; Cadena-Cadena *et al.*, 2019; Asbjörn Gildberg, 1987; Komai *et al.*, 2004), para almeja del atlántico fue de 45°C (Chen *et al.*, 1986), para el mejillón fue de 60°C (Venugopal *et al.*, 2014) y para el calamar neón fue de 60°C(Zhang *et al.*, 2019). Estas diferencias se pueden asociar a cambios en su estructura secundaria y al pKa de los grupos carboxilos de los extremos finales. Las conformaciones de proteínas cambian entre distintos estados termodinámicos a medida que las condiciones de temperatura o pH son alteradas por lo que los cambios en las estabilidades relativas de los diversos grupos de aminoácidos juegan un papel fundamental

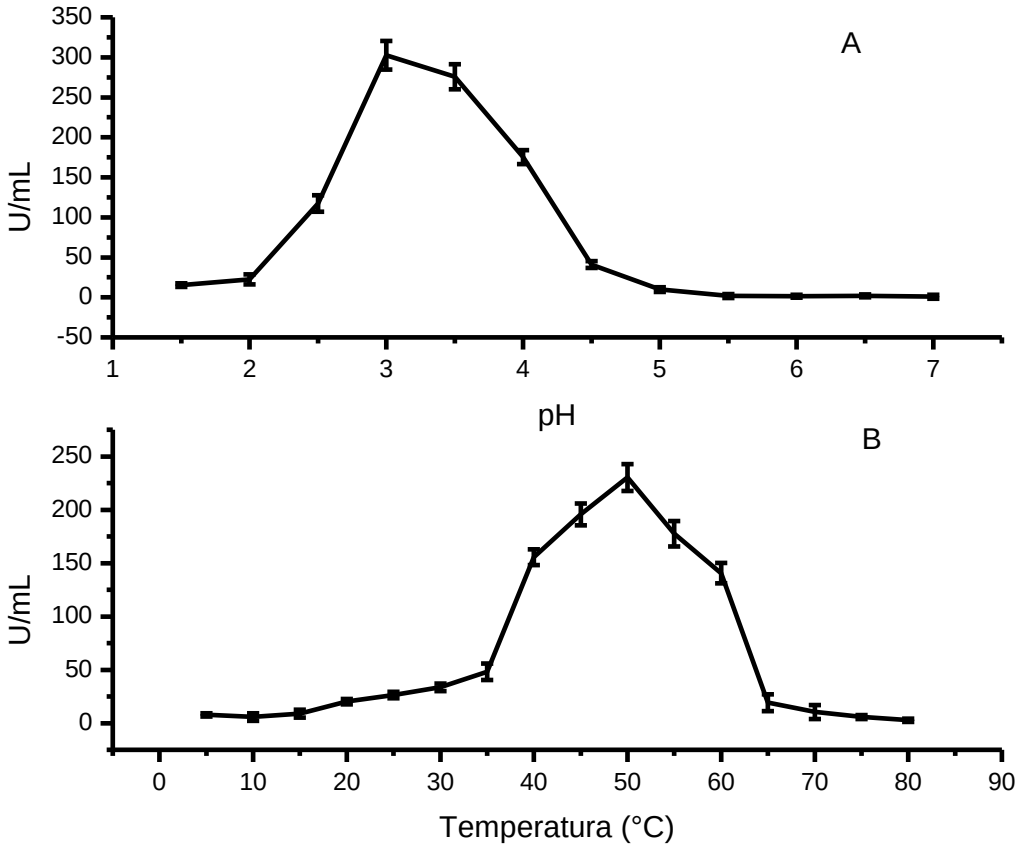


Fig 3. Efecto del pH y la temperatura en la actividad de catepsina D purificada de la almeja reina (A) pH y (B) temperatura. Los resultados son la media de experimentos realizados por triplicado.

en el sitio activo de la enzima así como en la nube electrónica formada alrededor del sitio activo, también el pH influye en el plegamiento y despegamiento de ciertas regiones de la proteína (S.-T. Jiang *et al.,* 1993; Lee *et al.*, 1998; O'Brien *et al.*, 2011). El hecho de que la temperatura óptima de organismos marinos sea menor que la temperatura óptima de sus homólogas terrestres está relacionado con la evolución de las enzimas a nivel estructural que le confiere un alto nivel de flexibilidad en su arreglo en comparación con las enzimas de los organismos terrestres (Benes *et al.*, 2008; Lee *et al.*, 1998).

Estabilidad a la temperatura y al pH

La estabilidad de la enzima al pH se muestra en la Fig 4 A. La mayor estabilidad de la enzima se detectó a pH 3,4,5 ya que conserva más del 80% de la actividad residual en un tiempo de 120 min. Sin embargo, aun en un pH de 6 y un tiempo de 120 min conserva más del 50% de la actividad residual, esta enzima es menos estable que la enzima de calamar sepia (*Sepia officinalis*), ya que presenta una retención del 100% de su actividad original a pH 3.0 después de 60 minutos de incubación (Balti *et al.*, 2010), otras catepsinas de organismos terrestres como la catepsina D del útero bovino tiene una estabilidad entre 5 y 7. Sin embargo, pierde toda su actividad a 60°C y un pH de 6.3 (Woessner *et al.*, 1971). Por otra parte, la estabilidad térmica de la enzima fue de los 10 a los 40°C ya que conserva mas del 50% de actividad en un tiempo de 120 minutos. Las proteínas poseen una gran cantidad de grupos ionizables que exhiben una variedad de valores de pKa por lo tanto los iones H+ y OH- afectan no solo la envoltura acuosa de las proteínas sino que también afectan directamente la carga eléctrica de los grupos ácidos y básicos de las cadenas laterales de los aminoácidos, por consiguiente cuando el pH aumenta o disminuye del óptimo de la enzima, se induce una ionización

reversible de los grupos amino y carboxilos presentes en los aspartatos y la enzima pierde su actividad. Las alteraciones de este tipo modifican la carga superficial de las proteínas y por consecuencia eliminan las interacciones electrostáticas que estabilizan la estructura terciaria por lo que se exponen grupos hidrofóbicos que interaccionan entre si y a menudo provoca su precipitación en este punto la enzima ya está desnaturalizada y aun cuando se regrese al pH óptimo de la misma esta no recuperara la actividad (David *et al.*, 2015; Piper *et al.*, 1965; Voet *et al.*, 2004).`

A mayor temperatura y mayor tiempo es mayor la pérdida de actividad de la enzima ya que las proteínas se destruyen con facilidad por las temperaturas elevadas. Aunque las estabilidades térmicas de las proteínas varían ampliamente, muchas de ellas se desnaturalizan lentamente por encima de 25 ºC (Voet *et al.*, 2004). Cuando la temperatura se eleva arriba de cierto punto la energía cinética de las moléculas se ve aumentada y por consecuencia se desorganiza la envoltura acuosa de la proteína. Del mismo modo, cuando aumenta la temperatura se destruyen las interacciones débiles como los puentes de hidrogeno que se encuentran en el exterior se desorganiza la estructura de terciaria de la proteína, de forma que el interior hidrofóbico se ve expuesto al medio acuoso por lo que se produce la agregación de la proteína que en estos momentos se encuentra desnaturalizada (Espinosa Silva, 2016; López-Flores *et al.*, 2016). La pérdida de actividad también se puede atribuir sobre todo entre los 50°C y 60°C a la pérdida de afinidad de la enzima con el sustrato (Capasso *et al.*, 1999; Draper *et al.*, 1989; Narinx *et al.*, 1997). Otras enzimas purificadas como la catepsina D purificada de puerco y rata tienen una estabilidad térmica muy similar. Sin embargo, tiene una termoestabilidad mucho mayor a la reportada para tilapia (S. T. Jiang *et al.*, 1992).

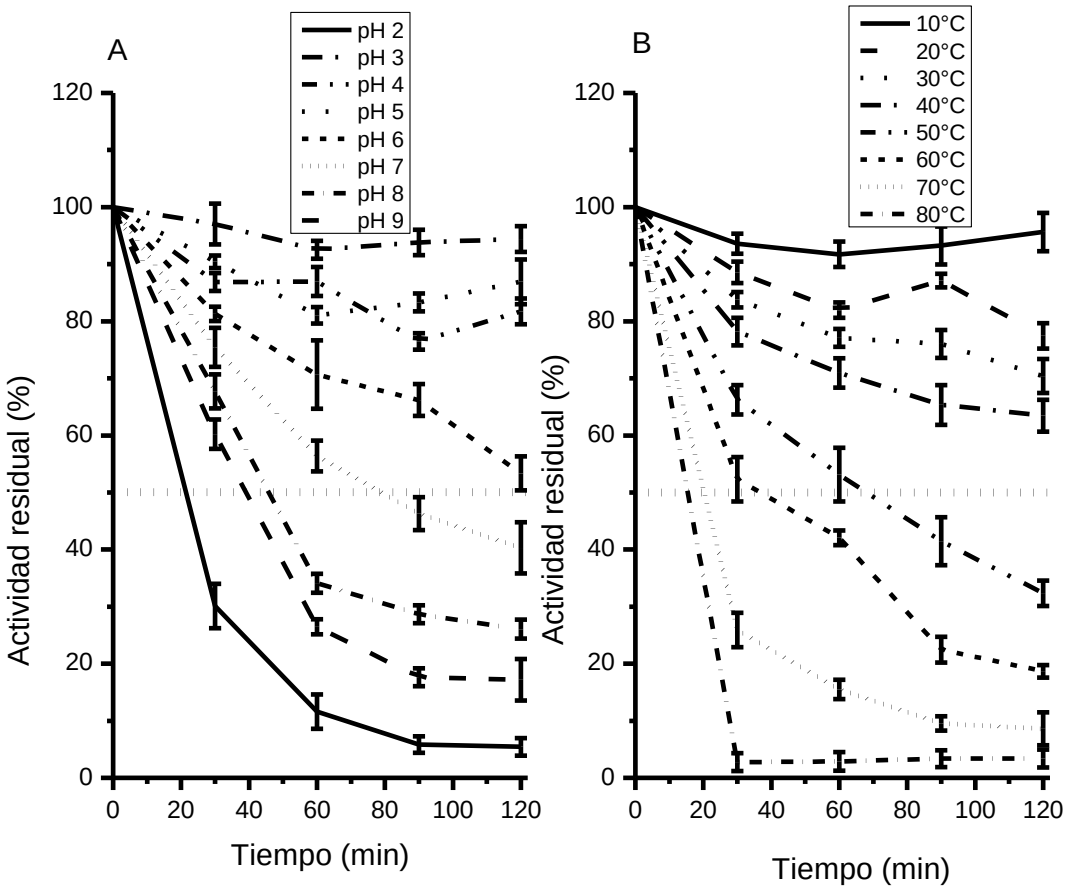


Fig 4. Estabilidad al pH (A) y a la temperatura (B) de la catepsina D purificada de la almeja reina (B) en un tiempo de 120 minutos, mostrando con la línea punteada el 50% de la actividad.

Análisis estructural de la catepsina D

Efectos de la temperatura

La forma en que las enzimas responden a la temperatura es fundamental en muchas áreas de la biología. En décadas pasadas el efecto de la temperatura sobre la actividad se entendía en términos de aumento o disminución de actividad enzimática (Copeland, 2000; Daniel *et al.*, 2009). Sin embargo, ahora está claro que estos efectos son insuficientes para explicar el efecto de la temperatura. Una vez que se ha separado una proteína de su entorno natural, se encuentra expuesta a muchos agentes que pueden dañarla irreversiblemente y uno de ellos es la temperatura que al incrementarse puede aumentar la energía cinética de toda la proteína. Con lo cual se van a debilitar interacciones iónicas principalmente interacciones no covalentes como los puentes de hidrogeno en el interior de proteína, con lo cual se empieza a exponer el interior hidrófobo de la proteína y por consecuencia se desnaturaliza la proteína (Raison, 1972; Voet *et al.*, 2004).

La Fig. 5 muestra el espectro del dicroísmo circular a diferentes temperaturas y diferentes pH. Se puede observar una banda una banda intensa negativa de 222 ± 4 nm a 228 nm con un mínimo de 222 nm. Estos picos son característicos de las estructuras secundarias de la hoja β, lo que sugiere que esta es la estructura predominante en la enzima. A medida que aumenta la temperatura el espectro de dicroísmo circular se reduce, esta reducción del espectro es consistente con un cambio en las estructuras secundarias debido a la pérdida de quiralidad, a medida que la elipticidad media del residuo se acerca a cero (Holzwarth *et al.*, 1965). Se puede observar una relación entre hélices α y hojas β a temperaturas de 70°C en adelante, las hojas β permanecen sin cambios hasta 70°C mientras que las hélices α permanecen constantes hasta 70°C, las vueltas β y la estructura desordenada están

relacionadas, ésta presenta una pérdida del 54% y después una ganancia del 60% de estructura secundaria, estos reacomodos estructurales son esenciales para la actividad de la enzima y está relacionado un incremento de actividad lo que nos muestra una dependencia a la temperatura de 40 a 60°C (Fig. 3). Los cambios de estructura secundaria a partir de 70°C están relacionados con relacionados con la pérdida de actividad y la desnaturalización irreversible de la proteína (Fig. 7), también podemos observar que las pérdidas de un tipo de estructura secundaria significan la ganancia de otro tipo. Las principales estructuras tridimensionales son las hélices α y las hojas β (Senthil *et al.*, 2019). Las transiciones entre hélices α y hojas β se observa universalmente para una amplia gama estructuras proteicas tanto para pH como para temperatura (Ding *et al.*, 2003; Koga *et al.*, 2003).

La estabilidad de la estructura secundaria (principalmente de las α hélices) depende de muchos factores, sin embargo, los factores determinantes son el acomodo de los aminoácidos, y el tipo de aminoácidos que se encuentran formando parte de la hélice α, ya que aminoácidos como GLY, LYS, ARG o los ácidos ASP y GLU se les considera como terminadores de α hélices debido a que causan impedimento estérico con el resto de las cadenas laterales de aminoácidos, en el caso de GLY es un aminoácido que tiene mucha flexibilidad por lo que puede desestabilizar la α hélice y en el caso de PRO es demasiado rígido con lo cual no puede formar puente de hidrogeno debido a su anillo y se le considera como un terminador de α hélices. Una α hélice siempre va a ser más estable en el centro que en las orillas por lo cual es de suma importancia que aminoácidos capaces de formar puentes de hidrógeno se encuentren al final de la α hélice para darle una mayor estabilidad (Nelson *et al.*, 2017). A pesar del incremento de temperatura, la estructura secundaria no se perdió por completo, si disminuyó el contenido de α hélice, pero se conserva un 3% de esta estructura secundaria

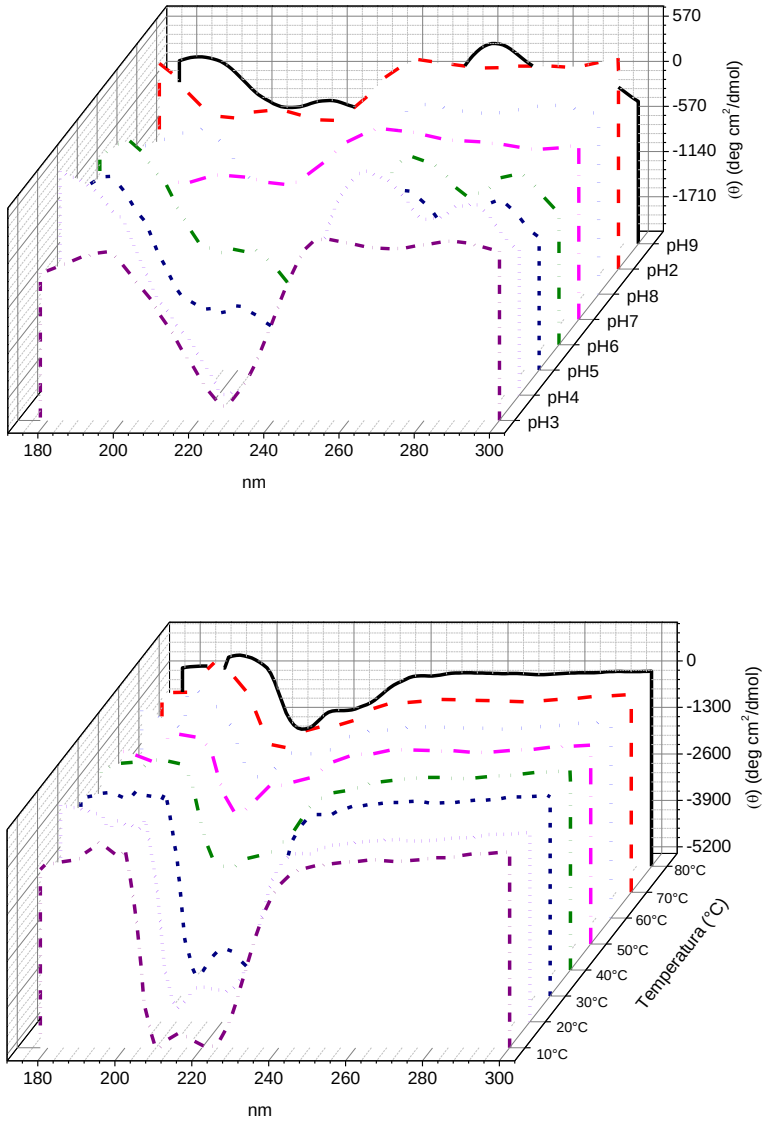


Fig 5. Espectros de dicroísmo circular de catepsina D de hepatopáncreas de la almeja japonesa A) espectro de pH y B) espectros de temperatura.

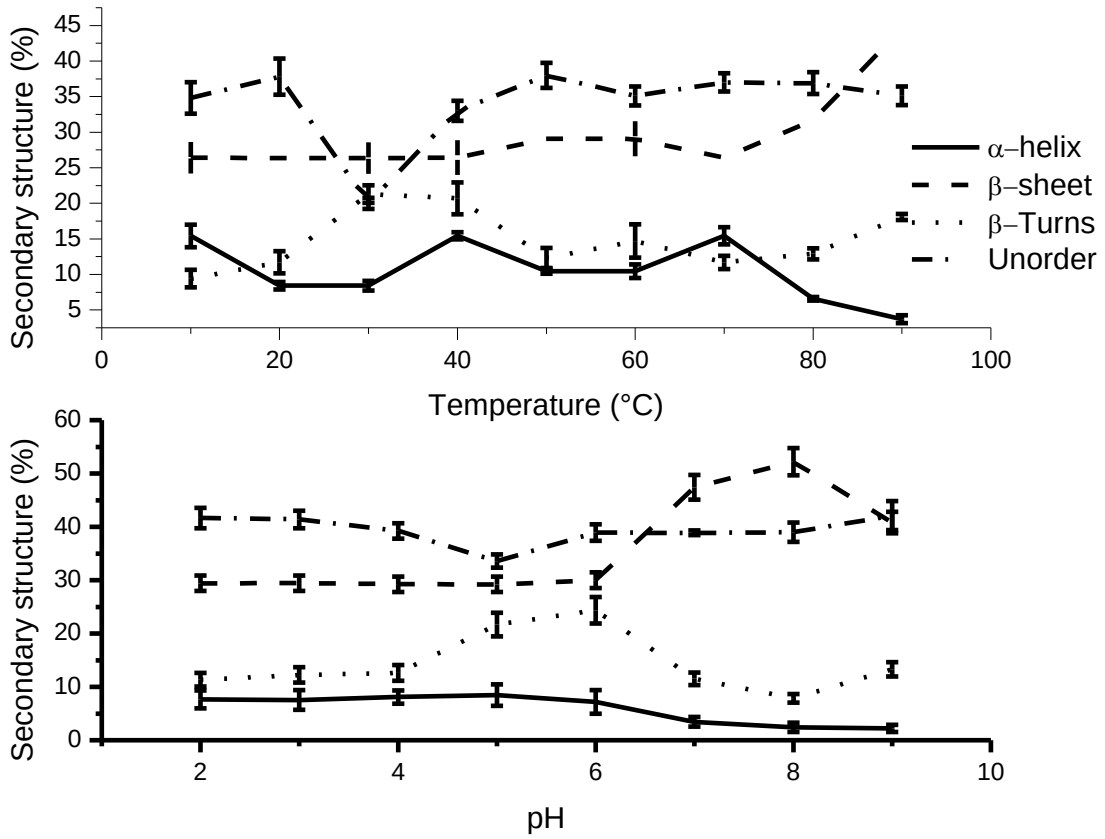


Figure 6. Estimacion de la estructura secundaria de la catepsina D del hepatopancreas a diferentes temperaturas de la almeja Reina (*Dosinia ponderosa)*.

mientras que las hojas β, las vueltas β y la estructura desordenada aumentaron, esto puede ser debido a la formación de otras estructuras secundarias o agregados proteicos. Este hecho fue ilustrado en zeinas de maíz (Cabra *et al.*, 2006), en prolaminas de sorgo (Gao *et al.*, 2005) y en catepsina D de calamar gigante (Cadena-Cadena *et al.*, 2019) donde a temperaturas elevadas se forman agregados de estructuras β intermoleculares, siempre tendiendo a la conservación de un pequeño porcentaje de α hélice. En proteínas de gluten se ha encontrado que el calentamiento afecta principalmente a las hojas β, sin embargo estos cambios no son permanentes ni en prolaminas ni en proteínas de gluten (Singh *et al.*, 2016).

Efecto del pH

Las proteínas son extremadamente sensibles al pH en un rango muy estrecho y afecta drásticamente su estabilidad. El cambio de pH produce cambios en las interacciones electrostáticas entre las cargas de los radicales de los aminoácidos y puede romper puentes de hidrógeno o facilitar la formación de otros puentes (Voet *et al.*, 2004).

La Fig. 5 muestra el espectro del dicroísmo circular a diferentes temperaturas y pH. Se puede observar una banda una banda intensa negativa que abarca todo el espectro (200 a 240 nm) con un mínimo de en 225 nm que corresponden a las características principales de las hojas β, se puede observar la pérdida de estructura secundaria a medida que aumenta el pH 3 a 9. Sin embargo, la mayor pérdida de estructura secundaria se observa a un pH de 2 y 9 mientras que a pH de 3 y 4 el espectro es prácticamente el mismo. En la Fig 6 se observa los cambios en la estructura secundaria y se observa que las α hélices permanecen constantes hasta un pH de 6 que es cuando presentan una reducción y un aumento de las hojas β, a un pH mayor a 4 la actividad comienza a disminuir (Fig 3), esta reducción puede estar relacionada con el

aumento de vuelta β y la disminución de la estructura desordenada, estos cambios en el pH se han observado en catepsina D humana, al incrementar el pH aparece una nueva hoja β que bloquea el sitio activo de la enzima (Lee *et al.*, 1998). El efecto que tiene el valor del pH sobre la estructura secundaria de las proteínas está relacionado con los grupos ionizables. El cambio en el valor de pH induce un cambio en la carga general de la proteína debido a que los grupos o sitios en los cuales se puede titular una proteína están ionizados, lo que finalmente afecta las interacciones electrostáticas que son indispensables para la estabilidad de la proteína, con estas modificaciones en la carga debido al pH esta no se distribuyen uniformente alrededor de la proteína (Hameed *et al.*, 2007; Linderstrom-Lang *et al.*, 1924). Sin embargo, la carga de las cadenas laterales no se modifica hasta que se rompe la envoltura acuosa de la proteína y como tal afectara la conformación de la proteína modificando sus parámetros de ΔG, ΔH y ΔS, en proteínas como lizosima mutante se ha encontrado que la sustitución de cargas debida al pH modifica la interacción electrostática, para los residuos cargados que están muy separados en la superficie de la proteína, la interacción electrostática entre ellos se atenúa por la distancia, debido a la alta constante dieléctrica del solvente y por el efecto de detección de los contraiones (Nick Pace *et al.*, 2000).

Conclusión

En conclusión, la estructura secundaria de la catepsina D de la almeja reina (*Dosinia ponderosa*) es más afectada por las cambios de pH que por cambios de temperatura. La desnaturalización térmica de la catepsina D a pH 3.5 es inusualmente alta bajo un modelo irreversible. La estructura secundaria más afectada fue la α hélice, que a temperaturas extremadamente altas retiene hasta un 4%, mientras que a pH extremo conserva solo un 2%. Por lo tanto, el pH es de la mayor importancia para la estructura secundaria en esta enzima.

Estos hallazgos son importantes para establecer las bases para futuros estudios dirigidos a los posibles usos de la catepsina D del de la almeja reina (*Dosinia* ponderosa) y también para comprender su papel en la hidrólisis de proteínas del alimento.

Referencias

Aghdassi, A. A., John, D. S., Sendler, M., Weiss, F. U., Reinheckel, T., Mayerle, J., & Lerch, M. M. (2018). Cathepsin D regulates cathepsin B activation and disease severity predominantly in inflammatory cells during experimental pancreatitis. *Journal of Biological Chemistry, 293*(3), 1018-1029.

Balti, R., Hmidet, N., Jellouli, K., Nedjar-Arroume, N., Guillochon, D., & Nasri, M. (2010). Cathepsin D from the hepatopancreas of the cuttlefish (*Sepia officinalis*): purification and characterization. *Journal of agricultural and food chemistry, 58*(19), 10623-10630.

Benes, P., Vetvicka, V., & Fusek, M. (2008). Cathepsin D—many functions of one aspartic protease. *Critical reviews in oncology/hematology, 68*(1), 12-28.

Bird, J. W. C., Spanier, A. M., & Schwartz, W. N. (1978). Cathepsins B and D: proteolytic activity and ultrastructural localization in skeletal muscle *Protein turnover and lysosome function* (pp. 589-604): Elsevier.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry, 72*(1-2), 248-254.

Cabra, V., Arreguin, R., Vazquez-Duhalt, R., & Farres, A. (2006). Effect of temperature and pH on the secondary structure and processes of oligomerization of 19 kDa alpha-zein. *Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics, 1764*(6), 1110-1118.

Cadena-Cadena, F., Cárdenas-López, J. L., Ezquerra-Brauer, J. M., Cinco-Moroyoqui, F. J., López-Zavala, A. A., Santacruz-Ortega, H. d. C., & Rivero-Espejel, I. A. (2019). Effect of temperature and pH on the secondary structure and denaturation process of jumbo squid hepatopancreas cathepsin D. *Protein and peptide Letters, 26*(7), 532 - 541.

Capasso, C., Lees, W. E., Capasso, A., Scudiero, R., Carginale, V., Kille, P., . . . Parisi, E. (1999). Cathepsin D from the liver of the Antarctic icefish *Chionodraco hamatus* exhibits unusual activity and stability at high temperatures1. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 1431*(1), 64-73. doi: 10.1016/S0167-4838(99)00039-4

Cardenas-Lopez, J. L., & Haard, N. F. (2005). Cysteine proteinase activity in jumbo squid (Dosidicus gigas) hepatopancreas extracts. *Journal of food biochemistry, 29*(2), 171-186.

Carter, C. W., & Sweet, R. M. (1997). *Macromolecular Crystallography Part A* (Vol. 276): Gulf Professional Publishing.

Celis-Guerrero, L. E., García-Carreño, F. L., & del Toro, M. A. N. (2004). Characterization of proteases in the digestive system of spiny lobster (*Panulirus interruptus*). *Marine Biotechnology, 6*(3), 262-269.

Copeland, R. A. (2000). *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis, 2nd Edition*.

Chen, H. C., & Zall, R. R. (1986). Partial Purification and Characterization of Cathepsin D-Like and B-Like Acid Proteases from Surf Clam Viscera. *Journal of Food Science, 51*(1), 71-75.

Daniel, R. M., Peterson, M. E., Danson, M. J., Price, N. C., Kelly, S. M., Monk, C. R., . . . Lee, C. K. (2009). The molecular basis of the effect of temperature on enzyme activity. *Biochemical Journal, 425*(2), 353-360.

David, N. L., & Cox, M. (2015). *Lehninger. Principios de bioquímica* (Omega Ed.). España.

Ding, F., Borreguero, J. M., Buldyrey, S. V., Stanley, H. E., & Dokholyan, N. V. (2003). Mechanism for the α-helix to β-hairpin transition. *Proteins: Structure, Function, and Bioinformatics, 53*(2), 220-228.

Draper, A. M., & Zeece, M. G. (1989). Thermal stability of cathepsin D. *Journal of Food Science, 54*(6), 1651-1652.

Espinosa Silva, Y. R. (2016). Estudio de la desnaturalización en frío de proteínas mediante simulaciones computacionales.

Ezquerra-Brauer, J., Haard, N. F., Ramírez-Olivas, R., Olivas-Burrola, H., & Velazquez-Sánchez, C. J. (2002). Influence of harvest season on the proteolytic activity of hepatopancreas and mantle tissues from jumbo squid (Doswicus gigas). *Journal of food biochemistry, 26*(5), 459-475.

Fusek, M., & Vetvicka, V. (2005). Dual role of cathepsin D: ligand and protease. *Biomedical papers of the Medical Faculty of the University Palacky, Olomouc, Czechoslovakia, 149*(1), 43-50.

Gao, C., Taylor, J., Wellner, N., Byaruhanga, Y. B., Parker, M. L., Mills, E. N. C., & Belton, P. S. (2005). Effect of preparation conditions on protein secondary structure and biofilm formation of kafirin. *Journal of agricultural and food chemistry, 53*(2), 306-312.

Gildberg, A. (1987). Purification and characterisation of cathepsin D from the digestive gland of the pelagic squid *Todarodes sagittatus*. *Journal of the Science of Food and Agriculture, 39*(1), 85-94.

Gildberg, A. (1988). Aspartic proteinases in fishes and aquatic invertebrates. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry, 91*(3), 425-435.

Haard, N. F., & Simpson, B. K. (2000). *Seafood enzymes: utilization and influence on postharvest seafood quality*: CRC Press.

Hameed, M., Ahmad, B., Fazili, K. M., Andrabi, K., & Khan, R. H. (2007). Different molten globule-like folding intermediates of hen egg white lysozyme induced by high pH and tertiary butanol. *Journal of biochemistry, 141*(4), 573-583.

Holzwarth, G., & Doty, P. (1965). The ultraviolet circular dichroism of polypeptides. *Journal of the American Chemical Society, 87*(2), 218-228.

Jamdar, S. N., & Harikumar, P. (2016). Purification, Identification and Characterization of Aspartic Proteases of Chicken Intestine. *Journal of food biochemistry, 40*(4), 451-462.

Jiang, S.-T., Her, Y.-H., Lee, J.-J., & Wang, J.-H. (1993). Comparison of the Cathepsin D from Mackerel (*Scomber australasicus*) and Milkfish (*Chanos chanos*) Muscle. *Bioscience, Biotechnology, and Biochemistry, 57*(4), 571-577. doi: 10.1271/bbb.57.571

Jiang, S. T., Nei, F. P., Chen, H. C., & Wang, J. H. (1992). Comparative study on the cathepsin D from banded shrimp (*Penaeus japonicus*) and grass shrimp (*Penaeus monodon*). *Journal of agricultural and food chemistry, 40*(6), 961-966.

Knight, C. G., & Barrett, A. J. (1976). Interaction of human cathepsin D with the inhibitor pepstatin. *Biochemical Journal, 155*(1), 117-125.

Koga, T., Taguchi, K., Kobuke, Y., Kinoshita, T., & Higuchi, M. (2003). Structural Regulation of a Peptide-Conjugated Graft Copolymer: A Simple Model for Amyloid Formation. *Chemistry–A European Journal, 9*(5), 1146-1156.

Komai, T., Kawabata, C., Amano, M., Lee, B. R., & Ichishima, E. (2004). Todarepsin, a new cathepsin D from hepatopancreas of Japanese common squid (*Todarodes pacificus*). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 137*(3), 373-382.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680-685.

Lee, A. Y., Gulnik, S. V., & Erickson, J. W. (1998). Conformational switching in an aspartic proteinase. *Nature Structural & Molecular Biology, 5*(10), 866-871.

Linderstrom-Lang, K., & Holter, H. (1924). CR Trav. Lab. Carlsberg 15.

López-Flores, A. R., Luna-Urban, C., Buenrostro-Figueroa, J. J., Hernández-Martínez, R., Huerta-Ochoa, S., Escalona-Buendía, H., . . . Prado-Barragán, L. A. (2016). Efecto del pH, temperatura y fuente de proteína y carbohidratos en la producción de proteasas por *Yarrowia lipolytica* encultivo sólido. *Revista mexicana de ingeniería química, 15*(1), 57-67.

Maguchi, S., Taniguchi, N., & Makita, A. (1988). Elevated activity and increased mannose-6-phosphate in the carbohydrate moiety of cathepsin D from human hepatoma. *Cancer research, 48*(2), 362-367.

Martínez-Alarcón, D., Saborowski, R., Rojo-Arreola, L., & García-Carreño, F. (2018). Is digestive cathepsin D the rule in decapod crustaceans? *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 215*, 31-38.

Militello, V., Casarino, C., Emanuele, A., Giostra, A., Pullara, F., & Leone, M. (2004). Aggregation kinetics of bovine serum albumin studied by FTIR spectroscopy and light scattering. *Biophysical chemistry, 107*(2), 175-187.

Narinx, E., Baise, E., & Gerday, C. (1997). Subtilisin from psychrophilic antarctic bacteria: characterization and site-directed mutagenesis of residues possibly involved in the adaptation to cold. *Protein Engineering, 10*(11), 1271-1279.

Nawrocka, A., Szymańska-Chargot, M., Miś, A., Kowalski, R., & Gruszecki, W. I. (2016). Raman studies of gluten proteins aggregation induced by dietary fibres. *Food chemistry, 194*, 86-94.

Nelson, D., & Cox, M. (2017). *Lehninger Principles of Biochemistry: International Edition*: Macmillan Learning.

Nick Pace, C., Alston, R. W., & Shaw, K. L. (2000). Charge–charge interactions influence the denatured state ensemble and contribute to protein stability. *Protein Science, 9*(7), 1395-1398.

Nielsen, L. B., & Nielsen, H. H. (2001). Purification and characterization of cathepsin D from herring muscle (Clupea harengus). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 128*(2), 351-363.

O'Brien, E. P., Brooks, B. R., & Thirumalai, D. (2011). Effects of pH on proteins: predictions for ensemble and single-molecule pulling experiments. *Journal of the American Chemical Society, 134*(2), 979-987.

Pal, P., Sadhukhan, T., Chakraborty, S., Sadhukhan, S., Biswas, A., Das, S. K., . . . Ray, J. (2019). Role of Apolipoprotein E, Cathepsin D, and Brain-Derived Neurotrophic Factor in Parkinson's Disease: A Study from Eastern India. *Neuromolecular medicine*, 1-8.

Piper, D. W., & Fenton, B. H. (1965). pH stability and activity curves of pepsin with special reference to their clinical importance. *Gut, 6*(5), 506.

Raison, J. K. (1972). The influence of temperature-induced phase changes on the kinetics of respiratory and other membrane-associated enzyme systems *Membrane Structure and Mechanisms of Biological Energy Transduction* (pp. 559-583): Springer.

Rojo, L., & García-Carreño, F. (2013). Cold-adapted digestive aspartic protease of the clawed lobsters *Homarus americanus* and *Homarus gammarus*: biochemical characterization. *Marine Biotechnology, 15*(1), 87-96.

Rojo, L., Sotelo-Mundo, R., García-Carreño, F., & Gráf, L. (2010). Isolation, biochemical characterization, and molecular modeling of American lobster digestive cathepsin D1. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 157*(4), 394-400.

Rongel, Á. G., Higuera, V. M. O., Aguilar, R. P., Yánez, F. J. C., Sánchez, M. E. L., Hurtado, S. V., & Ríos, E. M. (2011). Efecto del almacenamiento sobre la estabilidad de proteasas de vísceras de pez diablo (*Hypostomus plecostomus*). *Interciencia: Revista de ciencia y tecnología de América, 36*(8), 625-629.

Sales, C. F., Melo, R. M. C., Pinheiro, A. P. B., Luz, R. K., Bazzoli, N., & Rizzo, E. (2019). Autophagy and Cathepsin D mediated apoptosis contributing to ovarian follicular atresia in the Nile tilapia. *Molecular reproduction and development*.

Senthil, R., Usha, S., & Saravanan, K. (2019). Importance of Fluctuating Amino Acid Residues in Folding and Binding of Proteins. *Avicenna Journal of Medical Biotechnology, 11*(4), 339-344.

Singh, A., Lahlali, R., Vanga, S. K., Karunakaran, C., Orsat, V., & Raghavan, V. (2016). Effect of high electric field on secondary structure of wheat gluten. *International journal of food properties, 19*(6), 1217-1226.

Vaiana, S. M., Manno, M., Emanuele, A., Palma-Vittorelli, M. B., & Palma, M. U. (2001). The role of solvent in protein folding and in aggregation. *Journal of biological physics, 27*(2-3), 133-145.

Valdez-Melchor, R. G., Ezquerra-Brauer, J. M., Cinco-Moroyoqui, F. J., Castillo-Yáñez, F. J., & Cardenas-Lopez, J. L. (2013). Purification and partial characterization of trypsin from the viscera of tropical sierra (Scomberomorus sierra) from the Gulf of California. *Journal of food biochemistry, 37*(6), 694-701.

Van Stokkum, I. H. M., Spoelder, H. J. W., Bloemendal, M., Van Grondelle, R., & Groen, F. C. A. (1990). Estimation of protein secondary structure and error analysis from circular dichroism spectra. *Analytical biochemistry, 191*(1), 110-118.

Venugopal, A., & Kumar, N. S. (2014). Biochemical characterization of cathepsin D from the mussel *Lamellidens corrianus*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 169*, 25-30.

Voet, D., & Voet, J. G. (2004). *Biochemistry, 4th Edition*: John Wiley & Sons.

Wang, P. A., Stenvik, J., Larsen, R., Mæhre, H., & Olsen, R. L. (2007). Cathepsin D from Atlantic cod (*Gadus morhua L*) liver. Isolation and comparative studies. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 147*(3), 504-511.

White, S. H. (2013). *Membrane protein structure: experimental approaches*: Springer.

White, S. H., Wimley, W. C., & Ladokhin, A. S. (1998). Energetics of Peptide–Bilayer Interactions. *Methods in Enzymology, 295*, 62-87.

Woessner, J. F., & Shamberger, R. J. (1971). Purification and properties of cathepsin D from bovine uterus. *Journal of Biological Chemistry, 246*(7), 1951-1960.

Yasuda, Y., Kageyama, T., Akamine, A., Shibata, M., Kominami, E., Uchiyama, Y., & Yamamoto, K. (1999). Characterization of new fluorogenic substrates for the rapid and sensitive assay of cathepsin E and cathepsin D. *Journal of biochemistry, 125*(6), 1137-1143.

Zhang, K., Wei, R., & Song, R. (2019). Extraction of Cathepsin D-Like Protease from Neon Flying Squid (Ommastrephes bartramii) Viscera and Application in Antioxidant Hydrolysate Production. *Biomolecules, 9*(6), 228.

CONCLUSIONES

- Se logró establecer la presencia de catepsina D en el hepatopáncreas de la almeja japonesa, almeja reina y calamar gigante.
- La estabilidad al pH y a la temperatura están aumentados posiblemente por la poca presencia de α hélices.
- Su temperatura de transición (Tm) es inusualmente alta comparada con la que se reporta para organismos marinos, en el caso del calamar gigante su temperatura de desnaturalización es muy similar a la reportada para otros calamares.
- Su estabilidad térmica y estabilidad al pH coincide con los cambios en su estructura secundaria.
- Se sugiere que la catepsina D es la única enzima que actúa a un pH muy ácido en la almeja japonesa, almeja reina y calamar gigante.

RECOMENDACIONES

- Modelar la estructura proteica de las catepsinas D de calamar gigante, almeja reina y almeja japonesa.
- Identificar la secuencia N-terminal de aminoácidos de la catepsina D de calamar gigante, almeja reina y almeja japonesa.
- Conocer los parámetros termodinámicos de la unión entre el inhibidor y la catepsina D de calamar gigante, almeja reina y almeja japonesa mediante la calorimetría de titulación isotérmica (ITC).
- Conocer la especificidad de la catepsina D hacia diferentes sustratos.