



UNIVERSIDAD AUTÓNOMA DEL ESTADO DE HIDALGO

**INSTITUTO DE CIENCIAS DE LA SALUD
INSTITUTO DE CIENCIAS BÁSICAS E INGENIERÍA
INSTITUTO DE CIENCIAS AGROPECUARIAS**

**DOCTORADO EN CIENCIAS DE LOS ALIMENTOS Y SALUD
HUMANA**

TESIS DOCTORAL

**PROTEÍNAS DE TRUCHA ARCOÍRIS (*Oncorhynchus mykiss*)
COMO PRECURSORAS DE PÉPTIDOS ANTIOXIDANTES
CON CAPACIDAD ANTICANCERÍGENA**

Para obtener el grado de
Doctora en Ciencias de los Alimentos y Salud Humana

PRESENTA

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Instituto de Ciencias de la Salud

School of Medical Sciences

Área Académica de Nutrición

Número de control: ICSa-AAN/192/2026

Asunto: Autorización impresión de tesis

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Por medio de la presente se informa que el comité tutorial del trabajo de tesis titulado "Proteínas de trucha arcoíris (*Oncorhynchus mykiss*) como precursoras de péptidos antioxidantes con capacidad anticancerígena", realizado por la sustentante María Fernanda Escamilla Rosales con número de cuenta 244926, perteneciente al programa de Doctorado en Ciencias de los Alimentos y Salud Humana (DCASH), una vez que ha revisado, analizado y evaluado el documento recepcional de acuerdo a lo estipulado en el Artículo 110 del Reglamento de Estudios de Posgrado tiene a bien extender la presente **AUTORIZACIÓN DE IMPRESIÓN**. Por lo que la sustentante deberá cumplir los requisitos del Reglamento de Estudios de Posgrado y continuar con lo establecido en el proceso de grado vigente.

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Número de control: ICSa-AAN/190/2026

Asunto: Publicación de artículo

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Por este medio se informa que **María Fernanda Escamilla Rosales**, estudiante del **DOCTORADO EN CIENCIAS DE LOS ALIMENTOS Y SALUD HUMANA**, con número de cuenta 244926 ha publicado los artículos indizados, de los cuales se anexa a esta solicitud la primera hoja del artículo publicado:

Escamilla-Rosales, M. F., Olvera-Rosales, L., Jara-Gutiérrez, C. E., Jaimez-Ordaz, J., Santana-Sepúlveda, P. A., & González-Olivares, L. G. (2024). Proteins of Milk, Egg and Fish as a Source of Antioxidant Peptides: Production, Mechanism of Action and Health Benefits. *Food Reviews International*, 40(6), 1600–1620. <https://doi.org/10.1080/87559129.2023.2227974>. Factor de impacto: 6

Escamilla-Rosales, M. F., Pérez-Escalante, E., Jara-Gutiérrez, C. E., Santana-Sepúlveda, P. A., Álvarez-Álvarez, C. A., Castañeda-Ovando, A., & González-Olivares, L. G. (2024). Rainbow trout (*Oncorhynchus mykiss*) proteins as a source of antioxidant peptides with promising anticancer activity. *Future Foods*, 10(100509), 100509. <https://doi.org/10.1016/j.fufo.2024.100509>. Factor de impacto: 8.2

Escamilla-Rosales, M. F., Jara-Gutiérrez, C. E., Bautista-Ávila, M., Castañeda-Ovando, A., Álvarez-Álvarez, C., Santana-Sepúlveda, P. A., & González-Olivares, L. G. (2025). Antioxidant capacity of peptides derived from the enzymatic hydrolysis of rainbow trout (*Oncorhynchus mykiss*) skin: effect of pasteurization and ultrasonication pre-treatment. *Journal of the Chilean Chemical Society*, 69(4), 6234-6236.

Escamilla-Rosales, M. F., Jara-Gutiérrez, C. E., Villena-García, J., Bautista-Ávila, M., Castañeda-Ovando, A., Álvarez-Álvarez, C., Guzmán-Quimbayo, F., Santana-Sepúlveda, P. A., & González-Olivares, L. G. (2026). *In-silico* Identification and Experimental Validation of Rainbow Trout-Derived Anticancer Peptides. *Biointerface Research in Applied Chemistry*. 16(2), <https://doi.org/10.33263/BRIAC162.053>

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Debido a lo anterior, la estudiante cumple con los requerimientos de egreso establecidos por el programa de posgrado, al contar tanto con el trabajo de tesis como con un artículo (o en su caso tres artículos) publicados(s) en una revista(s) indizada(s). Por lo que solicitamos a usted tenga a bien permitir al doctorando dar continuidad al proceso necesario que conlleve a la obtención del grado de Doctor en Ciencias de los Alimentos y Salud Humana.

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«Lo que haces marca la diferencia, y tú debes decidir qué tipo de diferencia quieres marcar». — Jane Goodall

Dedicada a mi madre, mi esposo, mi familia y amigos; ustedes son quienes cada día me inspiran a alcanzar mis metas.

Agradecimientos

"Cada libro, cada tomo que ves, tiene alma. El alma de quien lo escribió, y el alma de quienes lo leyeron, vivieron y soñaron con él". — Carlos Ruiz Zafón

Quiero iniciar estos agradecimientos con mi familia, porque sin ellos esto no sería posible. **Silvia**, mamá, gracias por tu apoyo durante todas estas etapas, por ser siempre un refugio y un sostén cuando el cansancio me abrumaba. Gracias por acompañarme incluso en la distancia durante esos seis meses de estancia, por hacerme sentir capaz y, sobre todo, por ser mi mamá.

A mi esposo, **Gustavo**, amor, gracias por la familia que hemos formado, por tu apoyo y confianza incondicional, por apoyarme en este doctorado, incluso cuando implicó kilómetros de distancia. Gracias por tu amor, que siempre me dio fortaleza, y por acompañarme en cada etapa de este proyecto y motivarme a alcanzar mis metas.

A mi hermano **Marco**, gracias por tu apoyo y compañía a lo largo de estos años, por inspirarme a siempre ir por más. A mis primas **Ana** y **Bere**, gracias por los momentos compartidos, por las pláticas y risas que hicieron más ligeros estos días. A mis suegros, gracias por su apoyo.

Al doctor **Guillermo**, gracias por su asesoría y por esos momentos compartidos que siempre hicieron más ameno el trabajo, por enseñar y compartir desde su experiencia y conocimiento. Al doctor **Carlos**, gracias por recibirme en Chile, por su calidez, por su disposición para enseñarme y por compartir conmigo un poco de su país. A

la doctora **Paula**, gracias por recibirme con los brazos abiertos, por abrirme las puertas del laboratorio y de su familia, por su apoyo en esta investigación y por hacer que los días en Chile fueran tan alegres en su compañía. Al doctor **Claudio**, a **Teresa** y a **Karina**, gracias por recibirme en Coquimbo, por compartir su trabajo y por permitirme conocer un poco más de su país. Agradezco profundamente la estancia que realicé, la cual me permitió aprender sobre investigación y descubrir lo hermoso que es Chile. A las doctoras **Araceli**, **Mirandeli** y **Angélica**, gracias por su acompañamiento y asesoría durante este trabajo.

A **Laura**, **Emmanuel** y **Jovani**, gracias por su ayuda en el laboratorio y por hacer que las horas de trabajo fueran más ligeras. A **Mariel**, gracias por todos estos años de amistad, llenos de risas y, en ocasiones, de lágrimas, pero siempre acompañándonos y dándonos ánimo. A **Ibeth**, gracias por todas esas pláticas, risas y hasta regaños, pero también por siempre animarme. A ambas, gracias por una amistad que inició en el laboratorio pero que ha crecido con el tiempo.

Agradecimientos institucionales

Universidad Autónoma del Estado de Hidalgo



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Capítulo I

Descripción general del trabajo de investigación

Resumen

El cáncer es una de las principales causas de muerte a nivel mundial, con una incidencia en constante aumento. La eficacia de los tratamientos actuales se ve limitada por la presencia de efectos secundarios y resistencia adquirida por las células tumorales, lo que ha impulsado la búsqueda de nuevos compuestos bioactivos con potencial terapéutico. En este contexto, se han identificado péptidos bioactivos en subproductos de la trucha arcoíris (*Oncorhynchus mykiss*) con potenciales aplicaciones nutraceuticas y farmacológicas. El objetivo de este estudio fue obtener péptidos antioxidantes de proteínas de piel de trucha arcoíris (*Oncorhynchus mykiss*) a través de hidrólisis enzimática y síntesis orgánica para determinar su capacidad anticancerígena. Los resultados se presentan en seis capítulos, los cuales ofrecen una introducción al tema de investigación, la descripción de las metodologías empleadas y el análisis de los principales resultados. Al analizar los péptidos obtenidos *in silico* los resultados demostraron que no fueron citotóxicos en líneas tumorales a concentraciones ≤ 1 mg/mL, evidenciando la necesidad de refinar la metodología para la predicción de bioactividades. Posteriormente, al evaluar la obtención de péptidos bioactivos por hidrólisis enzimática comparando alcalasa y flavourzyme, se observó que una pasteurización previa a una hidrólisis de 6 horas, permitió obtener características relacionadas con la obtención de péptidos bioactivos, como mayor grado de hidrólisis y menor peso molecular, en comparación a la aplicación de ultrasonido como pretratamiento. Además, los hidrolizados con alcalasa requirieron menor cantidad de enzima y menor tiempo, en comparación con aquellos hidrolizados con flavourzyme, para ambos alcanzar una actividad antioxidante y perfil proteolítico similar. Por último, la optimización y purificación de los hidrolizados permitió obtener fracciones peptídicas no hemolíticas a concentraciones ≤ 1 mg/mL, que presentan mayor actividad antioxidante, capacidad de inhibir el crecimiento de bacterias Gram negativas y actividad citotóxica selectiva contra células de cáncer de mama y gástrico, relacionada con su capacidad para inducir la producción de ROS. En conjunto, esta investigación aporta evidencia de las propiedades bioactivas de hidrolizados de piel de trucha arcoíris, lo que abre el panorama a sus aplicaciones biomédicas y en el aprovechamiento integral de subproductos acuícolas.

Abstract

Cancer is one of the leading causes of death worldwide, with a steadily increasing incidence. The effectiveness of current treatments is limited by side effects and the development of resistance by tumor cells, which has driven the search for new bioactive compounds with therapeutic potential. In this context, bioactive peptides have been identified in rainbow trout (*Oncorhynchus mykiss*) byproducts, with potential nutraceutical and pharmacological applications. The aim of this study was to obtain antioxidant peptides from rainbow trout (*Oncorhynchus mykiss*) skin proteins through enzymatic hydrolysis and organic synthesis, and to determine their anticancer capacity. The results are presented in six chapters, which provide an introduction to the research topic, a description of the methodologies used, and an analysis of the main findings. Analysis of the peptides obtained *in silico* showed that they were not cytotoxic to tumor cell lines at concentrations ≤ 1 mg/mL, highlighting the need to refine methodologies for predicting bioactivities. Subsequently, during the evaluation of bioactive peptide production by enzymatic hydrolysis using alcalase and flavourzyme, it was observed that pasteurization prior to a 6-hour hydrolysis resulted in characteristics associated with bioactive peptide production, such as a higher degree of hydrolysis and lower molecular weight, compared to ultrasonication as a pretreatment. Furthermore, peptides hydrolyzed with alcalase required less enzyme and shorter hydrolysis time than those obtained with flavourzyme, while achieving similar antioxidant activity and proteolytic profiles. Finally, the optimization and purification of the hydrolysates yielded non-hemolytic peptide fractions at concentrations ≤ 1 mg/mL, which exhibited higher antioxidant activity, the ability to inhibit the growth of Gram-negative bacteria, and selective cytotoxic activity against breast and gastric cancer cells, likely related to their capacity to induce ROS production. Taken together, these findings provide evidence of the bioactive properties of rainbow trout skin hydrolysates, highlighting their potential biomedical applications and supporting the valorization of aquaculture byproducts.

1.1 Introducción

De acuerdo con la Agencia Internacional para la Investigación del Cáncer (IARC, 2024), el cáncer es una enfermedad que afecta a millones de personas y constituye una de las principales causas de muerte en el mundo. Además, prevé un aumento en los diagnósticos de cáncer, estimando que para el año 2050 la cifra alcance los 35 millones de casos. Las estrategias actuales se han centrado en la prevención, considerada la más favorable para disminuir la creciente incidencia; así como en la investigación y búsqueda de compuestos anticancerígenos con mayor especificidad, sensibilidad, precisión y menor toxicidad para las células no cancerígenas, con el propósito de desarrollar nuevos fármacos (Ghaly *et al.*, 2023; Nierengarten, 2024). En este sentido, los péptidos bioactivos anticancerígenos han cobrado relevancia (Ghaly *et al.*, 2023; Rafieezadeh, 2024; Zhang *et al.*, 2023).

Los péptidos son secuencias cortas de aminoácidos que pueden ser de origen natural o sintéticos. Se han descrito péptidos con diversas actividades biológicas, entre las que destacan antioxidante, antimicrobiana, antiinflamatoria y anticancerígena (Akbarian *et al.*, 2022). Investigaciones recientes se han centrado en los péptidos anticancerígenos, debido a su potencial aplicación en alimentos funcionales y fármacos, destacando su alta especificidad, capacidad de penetración tumoral y bajos efectos secundarios (Zhang *et al.*, 2023).

El entorno marino representa una fuente de gran diversidad biológica, donde se han identificado péptidos anticancerígenos en peces, mariscos, algas y algunos microorganismos (Rafieezadeh, 2024). También se han descrito péptidos antioxidantes de origen marino capaces de inducir apoptosis en células tumorales (Bielecka *et al.*, 2022; Yaghoubzadeh *et al.*, 2020).

Dentro de los péptidos bioactivos de fuentes marinas, se ha puesto especial atención en el aprovechamiento de los subproductos de la acuicultura, debido a que la creciente generación de desechos pesqueros a nivel mundial representa un problema ambiental (Nikoo *et al.*, 2023). El uso de estos subproductos para la obtención de compuestos bioactivos, no solo contribuye a la salud en la población, sino que también genera beneficios económicos en la industria (Jasrotia *et al.*, 2024).

En México hay estados cuya economía se sostiene por las actividades acuícolas. En este sentido, en el estado de Hidalgo, así como en otros, se cultiva la trucha arcoíris, cuya comercialización

genera subproductos que generalmente son desechados. Por lo que, en el presente proyecto se propone el aprovechamiento de la piel de trucha arcoíris, un subproducto de la acuicultura, como fuente para la obtención de péptidos bioactivos, que tengan un efecto en la prevención del cáncer o en el desarrollo de futuros fármacos.

1.4 Justificación

El cáncer es un grupo de enfermedades originadas por el crecimiento sin control de células anormales, con capacidad de invadir y diseminarse en tejidos y órganos. En México, esta enfermedad ocupa el tercer lugar en las principales causas de muerte. Los tipos de cáncer más frecuentes en el país incluyen el de mama, próstata, colorrectal, tiroides, cervicouterino, linfoma no Hodgkin, leucemia, estómago, riñón e hígado. La OMS y la IARC han estimado que en el país los casos de cáncer aumenten un 85% para el 2040. Actualmente, los tratamientos de cáncer consisten en quimioterapia, radiación y cirugía, sin embargo, debido a sus efectos secundarios se busca desarrollar nuevos fármacos que sean eficaces. Estudios han demostrado la presencia de agentes antitumorales como los péptidos bioactivos procedentes de especies marinas, que podrían ser considerados como posibles fármacos debido a que presentan menos efectos secundarios.

La trucha arcoíris es uno de los peces de mayor producción en el país y en el estado de Hidalgo. Presenta un alto valor nutricional y es comercializada entera o en filetes. Durante su procesamiento se obtienen subproductos como la cabeza, sangre, vísceras, aletas, esqueleto y piel, los cuales son desechados. La piel de trucha arcoíris es un subproducto compuesto principalmente por proteínas (41%). Estas proteínas contienen secuencias encriptadas de péptidos específicos que en algunos casos poseen características bioactivas. Existen diferentes métodos para hidrolizar las proteínas y liberar péptidos, uno de los cuales emplea enzimas, como la alcalasa y flavourzyme, que han mostrado ser eficaces en la obtención de secuencias peptídicas con posible actividad biológica que después deben ser aisladas e identificadas.

Estudios han reportado la presencia de péptidos bioactivos en la piel de trucha arcoíris y han revelado la importancia de estos péptidos centrados en su actividad antioxidante, la cual se ha documentado que ayuda a mantener un balance redox celular, por lo que ha sido relacionada a cierta acción anticancerígena en estudios *in vitro* sobre líneas celulares.

Por lo anterior, en este proyecto se busca tener un aprovechamiento de los subproductos de trucha arcoíris que presentan un alto valor nutricional, mediante la búsqueda de péptidos antioxidantes que presenten actividad anticancerígena.

1.5 Objetivos

1.5.1 General

Obtener péptidos antioxidantes de proteínas de piel de trucha arcoíris (*Oncorhynchus mykiss*) a través de hidrólisis enzimática y síntesis orgánica para determinar su capacidad anticancerígena.

1.5.2 Específicos

1. Obtención, caracterización y evaluación *in vitro* de péptidos sintéticos con potencial anticancerígeno a través de metodologías establecidas y análisis teóricos.
2. Establecer condiciones de diseño de experimentos mediante pruebas de reconocimiento.
3. Determinar las condiciones óptimas de hidrólisis enzimática de proteína de piel de trucha utilizando un diseño de experimentos factorial con puntos axiales con dos factores (tiempo de hidrólisis y relación proteína-enzima) en tres niveles, con dos enzimas comerciales alcalasa y flavourzyme, para la obtención de péptidos antioxidantes.
4. Caracterizar la hidrólisis enzimática óptima de proteína de piel de trucha a través del análisis de amino libres, del perfil proteico y de la separación cromatográfica, para determinar las características físicas de los péptidos obtenidos.
5. Identificar las secuencias peptídicas obtenidas a través de un análisis por MALDI-TOF.
6. Evaluar el efecto citotóxico de los péptidos obtenidos a través de pruebas sobre líneas tumorales y no tumorales humanas para determinar su potencial anticancerígeno.
7. Definir los efectos sobre el balance redox celular y vías de muerte celular regulada a través de citometría de flujo.

1.6 Metodología

La metodología planteada para el cumplimiento de los objetivos se puede observar en la Figura 1, desde la obtención de los péptidos *in silico* y de los hidrolizados de piel de trucha arcoíris, hasta la determinación de sus actividades biológicas.

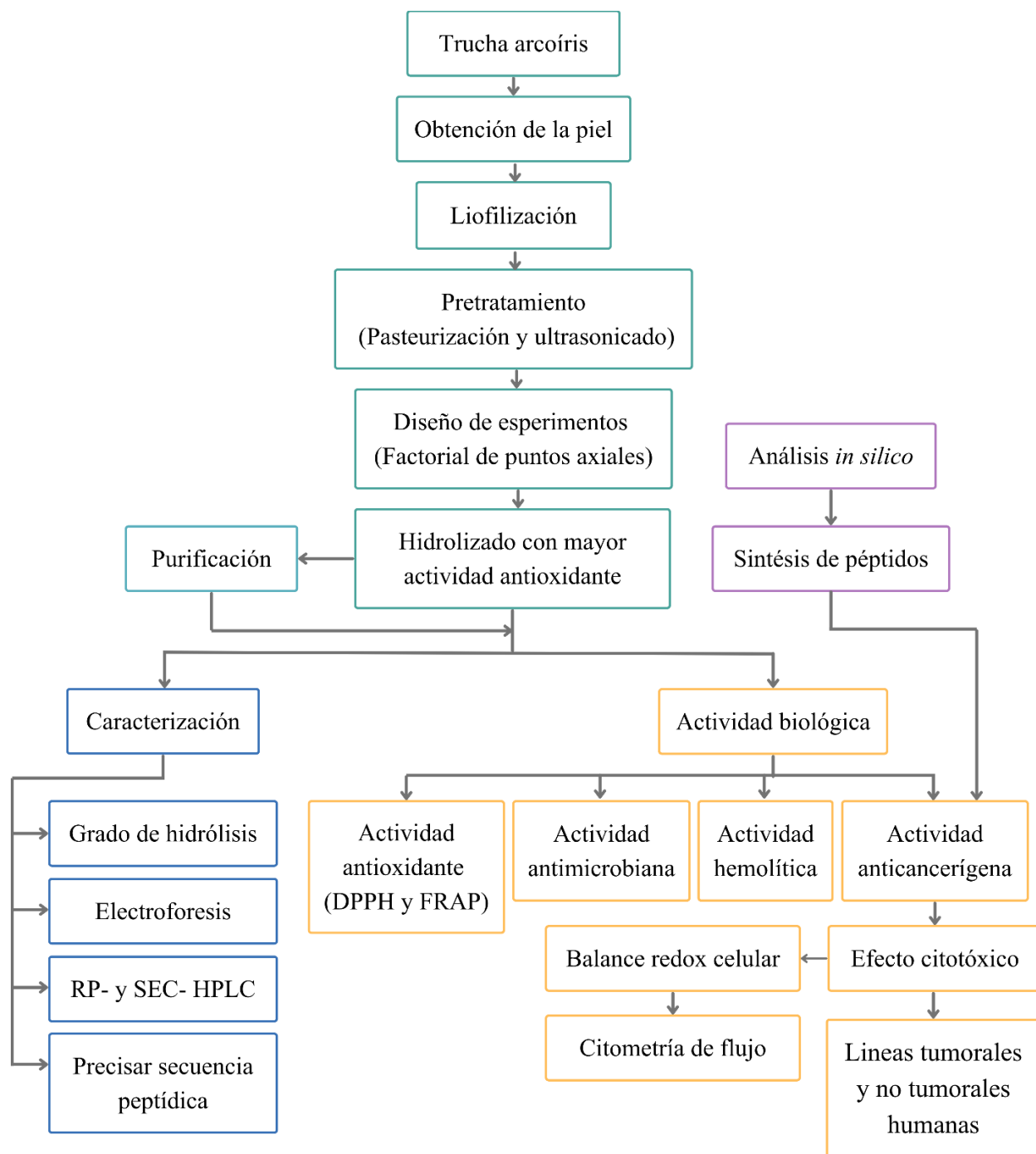


Figura 1. Esquema metodológico

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Capítulo II

Proteínas de leche, huevo y pescado como fuente de péptidos antioxidantes: obtención, mecanismo de acción y beneficios a la salud

Resumen

En este capítulo, se recopiló y analizó la información relevante sobre los antioxidantes presentes en las principales fuentes de proteínas de origen animal. A partir de este análisis, y resaltando la importancia de los péptidos antioxidantes, se elaboró una revisión, la cual se encuentra publicada en la revista *Foods Review International*. El contenido inicia con el estudio de las proteínas de la leche, describiendo los péptidos antioxidantes que se han secuenciado a partir de las caseínas y las proteínas del suero, además de mencionar el papel antioxidante de las vitaminas y el sistema enzimático presente en la leche. Posteriormente, se aborda la actividad antioxidante de compuestos del huevo, como las vitaminas E y A, el selenio, los fosfolípidos y los carotenoides. Se discute como estos componentes en conjunto con la ovoalbúmina y las secuencias peptídicas derivadas de esta y otras proteínas presentes en el huevo, contribuyen a la defensa frente al estrés oxidativo. También, hay un subtema dedicado a los componentes antioxidantes del pescado, en el cual se observa que la mayoría de las investigaciones se han enfocado en el uso de la piel, gelatina y colágeno como fuentes para la liberación de péptidos antioxidantes. En este apartado, se destaca la relevancia de las proteínas presentes en los subproductos de la industria acuícola, para la obtención de péptidos bioactivos. Para finalizar, la revisión describe los principales mecanismos de acción atribuidos a los péptidos para ejercer su actividad antioxidante. Estos mecanismos de acción incluyen la inhibición de la peroxidación lipídica, la activación de la vía de señalización Keap1/Nrf2, donde Keap1 es la proteína 1 asociada a ECH tipo Kelch y Nrf2 es el factor nuclear eritroide 2 relacionado con el factor 2, la disminución de la expresión de citoquinas proinflamatorias, la inactivación de especies reactivas de oxígeno y la quelación de metales. De manera general también se aborda la bioaccesibilidad de los péptidos, señalando que la acción de las enzimas digestivas puede modificar el tamaño de los péptidos y en consecuencia la actividad que expresan.

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Proteins of milk, egg and fish as a source of antioxidant peptides: production, mechanism of action and health benefits

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Abstract: Currently, there is a greater interest in natural antioxidant compounds, specifically, in those integrated to foods of animal origin, such as milk, eggs and fish. These foods present chemical compounds such as proteins, lipids, carotenoids, vitamins and minerals, whose antioxidant capacity has been studied. Peptides derived from proteins are compounds with the highest antioxidant activity. This review aimed at describing the antioxidant capacity of proteins and peptides derived from milk, egg and fish. Additionally, the mechanisms of antioxidant action, their bioavailability and the nutritional implications of each food will be discussed.

Keywords: antioxidant, bioactive peptide, protein, bioavailability, hydrolysis

Introduction

Oxidative stress is a condition given by the increased production of reactive oxygen species (ROS), mainly anionic radicals such as superoxide ($O_2^{\bullet-}$), singlet oxygen ($O_2^{\bullet}$), hydrogen dioxide ($HO_2^{\bullet}$), hydrogen (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$).^[1] These chemical species are generated by normal reactions in the body, acting as signaling molecules and activating various survival pathways sensitive to stress conditions.^[2] However, an increase in the concentration of these molecules leads to cell damage by lipid peroxidation, destruction of

protein structures, and DNA mutation^[3, 4], derived to onset or progression of neurodegenerative diseases, atherosclerosis, or cancer.^[5, 6]

Antioxidants help maintain oxidative balance and optimal health. These compounds are present in foods of animal origin and are related to minor components such as vitamins, minerals and carotenoids, but also fatty acids or proteins, which have been extensively studied. Also, various studies have shown that peptides with antioxidant properties could be released from protein hydrolysis in foods of animal origin.^[7, 8] These compounds, being of low molecular weight and simple structure, are more easily absorbed compared to native proteins.^[9] The use of this type of peptides has attracted special interest as they are considered natural and safe antioxidants.^[10] Therefore, this review aims at addressing the main antioxidant components in milk, eggs and fish with special emphasis on antioxidant peptides.

Antioxidant properties of milk

Dairy products are considered over 25 to 30% of an individual's normal diet. They are characterized as nutritionally complete foods due to their content of essential nutrients such as oleic acid, conjugated linoleic acid, omega-3 fatty acids, vitamins, minerals, and bioactive compounds such as antioxidants.^[11] The antioxidant capacity of milk is due to its proteins, mainly caseins (α -casein, β -casein and κ -casein) and to a lesser extent whey proteins (β -lactoglobulin, α -lactalbumin, lactoferrin, immunoglobulins, lactoperoxidase, lysozyme and serum albumin).^[12] General information on milk proteins are observed in Fig 1. Also, its sulfur amino acid content, mainly cysteine, vitamin A, E, carotenoids, enzyme systems such as lactoperoxidase, superoxide dismutase, catalase and glutathione peroxidase provide antioxidant activity.^[13]

Antioxidant properties of caseins and their derived peptides

Caseins are the proteins with the highest concentration in bovine and ovine milk and are found as macromolecular aggregates. Caseins are differentiated both by their structure and by their concentration of phosphate groups, which are 10, 5, and 1 mol per mol of α , β , and κ casein, respectively. These phosphate groups confer antioxidant capacity to the casein micelle.^[16] Phosphopeptides that possess antioxidant activity by preventing lipid oxidation by eliminating pro-oxidative metal catalysts are also derived from these proteins.^[17] It is

known that peptides with phosphoserine residues derived from α -casein and β -casein are involved in the inhibition of lipoxygenase activity due to the interaction that these residues have with divalent ions.^[18] Likewise, milk proteins have shown antioxidant capacity by trapping reactive oxygen species. That is why milk exhibits the ability to inhibit lipid auto-oxidation reactions.^[19]

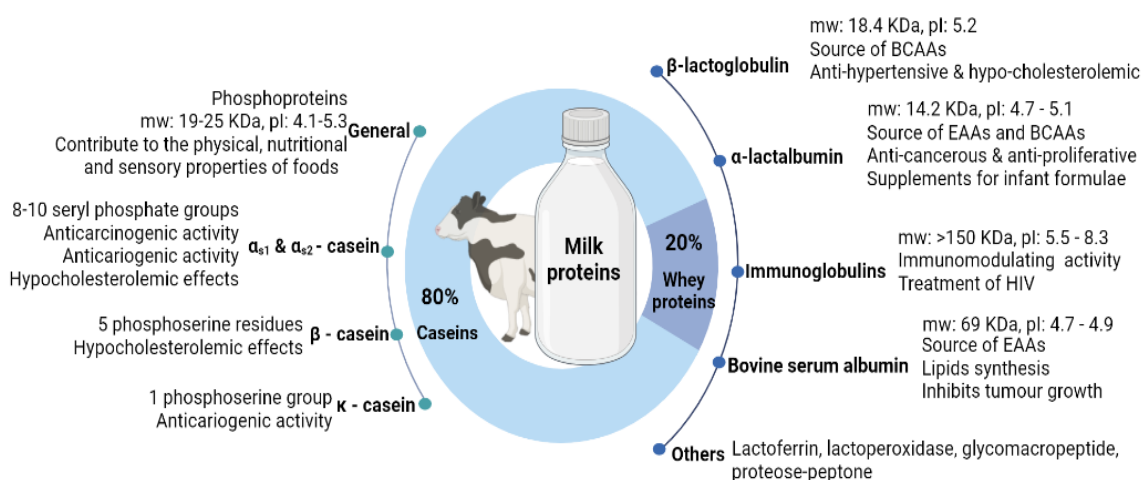


Figure 1. General characteristics and main bioactivities of milk proteins. Based on information from Davoodi *et al.*, & O’Kennedy.^[14, 15]

Many of these activities are determined by peptides that are derived from the breakdown of proteins through the gastrointestinal tract. This is how peptides with antioxidant capacity have been isolated from the different caseins. Due to their polar domains which contain serine residues, sequences such as -SerP-SerP-SerP-Glu-Glu, are chelating cations that complex with calcium, iron and zinc. In fact, phosphorylated caseins and/or their peptides in the aqueous phase could be a source of natural chelators to control lipid oxidation in food emulsions.^[20, 21]

Different chemical and physical characteristics of peptides are involved in antioxidant activity. Among them are the acid/base character of the peptide, its hydrophobicity, aromaticity, and ability to donate hydrogen. The presence of Val and Leu in the terminal amino group and Pro, His, Tyr, Trp in the peptide sequence, as well as the union of the Tyr-Trp and Glu-Leu amino acids, have also been related to a greater antioxidant capacity.^[22] In addition, the molecular weight is closely related to this activity, since smaller peptides exhibit greater antioxidant activity than those with longer structures. This has been revealed by

analyzing antioxidant sequences from different caseins (RHPHPHLSFM, ARHPPHLSF, HPHPHLSFM, VPYPQR, YVPR and VLPVPEK). Some amino acids have been removed from these sequences and it has been observed that the changes in antioxidant properties are more evident in peptides with shorter structures.^[23] These analyzed sequences were synthesized after a theoretical study was carried out.

However, it is known that the enzymatic hydrolysis of milk caseins with alcalase releases a peptide (RELEEL) with high antioxidant activity.^[24] In such a way that the antioxidant activity of these casein-derived peptides has been tested by enzymatic hydrolysis, verifying their ability to chelate metals. Such is the case of the LHSMK peptide, which was first reported by Lui *et al.*^[25] after carrying out casein hydrolysis with trypsin. These authors determined, in addition to the antioxidant activity commonly reported for other peptides, that this peptide regulates the activity of antioxidant enzymes in cellular oxidative damage processes. Likewise, casein hydrolysis with microbial proteases generates the antioxidant peptides YQLD, YFYP, FSDIPNPIGSEN and FSDIPNPIGSE that increase the expression of antioxidant enzymes by activating the Keap1-Nrf2 signaling pathway.^[22] Table 1 shows sequences of peptides derived from milk caseins and their mechanism of action.

Antioxidant properties of whey proteins

The main proteins present in milk whey are β -lactoglobulin (β -LG), α -lactalbumin (α -LA), bovine serum albumin (BSA) and immunoglobulins (IG).^[36] To a lesser extent, lactoferrin (LF), lactoperoxidase (LP), proteosapeptone (PP) and glycomacropeptide (GMP) are also found. The latter is released by enzymatic casein hydrolysis in the cheese-making process, which is why it is only found in sweet whey.^[10, 37] These proteins have a globular structure with high solubility and are denatured by heat^[38], they form intramolecular bonds through disulfide bridges between sulfhydryl groups present in cysteine.^[39]

In addition to its nutritional value due to its high content of essential amino acids, whey proteins have various biological functions, which is why they represent a sector of interest in various food applications.^[40, 41] The antioxidant mechanisms that are reported for both whey proteins and their peptides obtained by enzymatic hydrolysis consist of the elimination of free radicals, hydrogen donation, metal chelation and the inhibition of lipid peroxidation.^[42]

Table 1. Antioxidant peptides derived from milk casein and action mechanisms*

Protein	Peptidic sequence	Action mechanism	Reference
α_{s1} caseína	EL, YPEL, PEL,	Removal of superoxide anions (SOSA)	[19]
	AYFYPEL	Oxygen radical absorbance capacity (ORAC)	[26]
	RYL, RYLG, RYLGY, RYLGYLE, YLG, YLGY, YLGYLE, LGY, AYFYPE, YFYPEL, FYPEL	ABTS radical scavenging activity	[27]
	PFPEVFGKE, KHQGLPQEVLENLL, APSFSDIPNPIGSENSE	ABTS and DPPH radical scavenging activity	[28]
α_{s2} caseína	PYVRYL, FALPQYLK, YQK, FPQY	Oxygen radical absorbance capacity (ORAC)	[26, 29]
β caseína	VKEAMAPK, KVLVPVPQK, AVPYYPQR	Inhibition of enzymatic and non-enzymatic lipid peroxidation	[30]
	VLPVPQK, LLY, VPYVQR, YQEPVLGPVR, YPFPGPI, YPFPGPIP, YPFPGPIN, YPFVE, YPFVEP, YGFL, YGFLP, YPVEPF	ABTS radical scavenging activity	[23, 27, 31–34]
κ caseína	AVRSPAQILQWQ, DKTEIPTINTIASGEPT, INNQFLPYPYYAKPA, KYIPIQYVLS, QYVLSRYPYGLN	ABTS and DPPH radical scavenging activity	[28]

*Adapted from Bielecka *et al.*^[35]

Various *in vitro* studies have indicated that whey protein hydrolysis, using enzymes such as alcalase, produces peptides that improve antioxidant activity in liposome models, reporting that whey protein isolate hydrolysates show greater radical scavenging capacity, Cu⁺ chelation and reducing power compared to non-hydrolyzed whey protein isolate.^[43, 44] In addition, in comparison with hydrolysates using other proteases, peptides generated with

alcalase have higher antioxidant activity.^[45, 46] Similarly, the hydrolysis of whey protein concentrate by trypsin has allowed the identification of strong antioxidant and cytoprotective peptides (ELKDLK and ALPMHIR), evidencing this effect in rat ileum cells (IEC-18) under stress conditions.^[47]

Besides the use of digestive enzymes for the generation of antioxidant peptides, lactic acid bacteria (LAB) have also been used. In this context, it has been reported that the fermentation of goat milk whey with *Lactobacillus plantarum* C4 allows the obtaining of these peptides.^[48] Similarly, the fermentation of cow's milk whey with various strains of *Lactobacillus plantarum* has allowed the obtention of peptides <1 kDa that exhibit better antioxidant activity compared to larger peptides.^[49]

The hydrolysis of β -LG has allowed the identification of antioxidant sequences such as WY, WYS and WYSLAM as well as peptides of >3 kDa (MHIRL, YVEEL, WYSLAMAASDI) obtained by hydrolysis with Corolase PP.^[50, 51] Similarly, sequences that participate in the chelation of specific metals have been identified, such as the TPEVDDEALEK peptide, which participates in the chelation of iron.^[52]

The hydrolysis of α -LA with DebitraseTM HYW20 has allowed the identification of a dipeptide (WC) with high antioxidant potential, which is also present in lactoferrin^[53], as well as the LDQW and INYW peptides derived from the hydrolysis with thermolysin, they inhibit totally the activity of the ABTS radical at 2.5 μ M.^[54]

The role of antioxidant peptides has also been explored in the context of intestinal microbiota, since it has been reported that this type of peptide can reverse the effects of dysbiosis on intestinal homeostasis by regulating ROS concentrations in the intestine. The imbalance between ROS and antioxidant systems causes intestinal microbiota dysbiosis that has also been associated with neurological disorders.^[55, 56] It has been observed that the variation in the concentrations of intestinal metabolites in individuals is associated with the levels of amino acids.^[57] Antioxidant peptides can alter microbial community structure by decreasing the abundance of pathobionts such as *Escherichia coli* and *Clostridium perfringens* that can generate large concentrations of ROS.^[58] The consumption of α -LA hydrolysates in a mouse model (C57BL/6), led to an increase in the ratio of Bacteroidetes to Firmicutes ^[59], while the

consumption of whey peptides in wistar rats and mice (C57BL/6) has led to a significant increase in the relative abundance of *Lactobacillus* spp. and *Bifidobacterium* spp.^[60, 61]

Peptides can also be released from the protein structure by the action of enzymes involved in digestion.^[62] Through simulated gastrointestinal digestion, it has also been possible to report the effect of antioxidant peptides, reporting a release of serum peptides from β -LG (ALMP, GDLE, TKIPA, VEELKPT), α -LA(VGIN) and BSA (AVEGPK), which are transported through the intestinal barrier in Caco-2 / HT-29 cells, and which inhibit the formation of free radicals in muscle and liver cells.^[63] The use of the bioactivity of the peptides obtained from the hydrolysis of milk proteins depends on their bioavailability, since the native structure is required to be preserved for their entry into the intestinal cell.^[64] Table 2 shows some antioxidant peptides derived from whey proteins.

Table 2. Antioxidant peptides derived from whey proteins.

Activity	Protein	Amino acid sequence	Reference
Antioxidant	β -LG	MHIRL, YVEEL, WYSLAMAASDI	[51]
	β -LG	ALMP, GDLE, TKIPA, VEELKPT	[63]
	β -LG	IDALNEK, HIRLS, KTKIPAVF, MAASDISLL, VEELKPT	[65]
	α -LA	WC	[53]
	α -LA	LDQW, INYW	[54]
	α -LA	VGINYWLAHK	[65]
	BSA	VGIN, AVEGPK	[63]

Antioxidant characteristics of other milk components

Several components of low concentrations in milk exhibit antioxidant properties. In the case of carotenoids, they usually accumulate in the membrane of the fat globule. This membrane is considered the most volatile site for auto-oxidation.^[66] However, β -carotene is recognized as a preventative antioxidant, which can inhibit the oxidizing capacity of singlet oxygen

molecules (250 to 1000 molecules).^[67] One of the mechanisms of action of these compounds is the inhibition of photo-oxidation, since β -carotenes absorb light, which causes a decrease in the formation and half-life of singlet oxygen.^[68]

Ascorbic acid, vitamin E and the minerals found in milk (calcium, magnesium, iron, selenium and zinc) have been shown to have antioxidant capacity. Ascorbic acid is one of the strongest and least toxic natural antioxidants. This is the most important hydrophilic soluble antioxidant in milk and its high free radical scavenging capacity is due to its low oxidation-reduction potential (330 mV).^[69] Ascorbic acid is capable of scavenging superoxide anion radicals, alkoxy radicals, and singlet oxygen.^[70] Furthermore, ascorbic acid significantly inhibits riboflavin degradation in the presence of light.^[69] α -tocopherol has 80 to 90% higher antioxidant power compared to β -, γ -, δ -tocopherols.^[71] α -tocopherol of E vitamin is found in the fat globule membrane. This vitamin inhibits singlet oxygen, eliminates free radicals, traps nitrogen oxide species, as well as inhibits proteolytic enzymes such as plasmin.^[72–74]

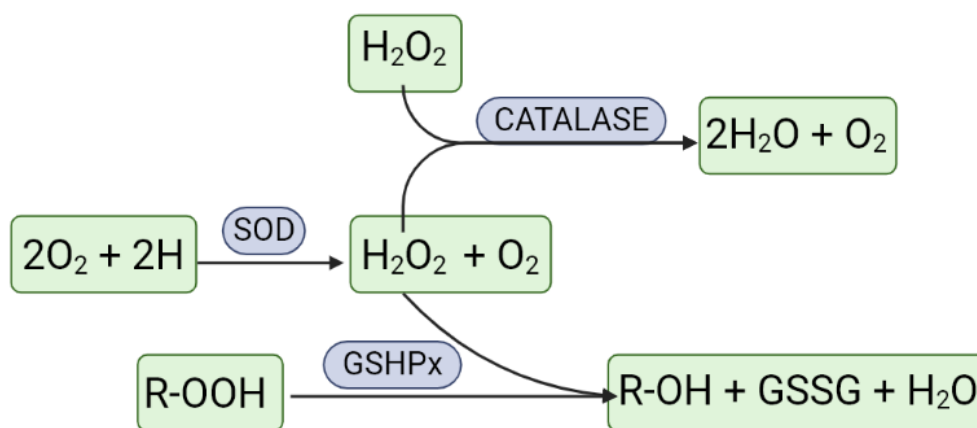


Figure 2. Mechanism of action of antioxidant enzymes present in milk. Adapted from Khan *et al.*^[13]

On the other hand, the enzymatic systems of milk play an important role in the antioxidant capacity of this food. Superoxide dismutase (SOD) catalyzes the removal of superoxide free radicals (O_2^-) and protects cells from damaging effects through the reaction in Figure 2. Another enzyme is glutathione peroxidase (GSHPx), which is a seleno-enzyme that provides protection against lipid peroxidation. It catalyzes the breakdown of H_2O_2 and organic hydroperoxides (R-OOH) by glutathione.^[75]

Finally, milk catalase is an enzyme that catalyzes the dismutation of H_2O_2 through a reaction in which another H_2O_2 molecule participates, in which one molecule is converted to O_2 and the remaining ones released are converted to two water molecules.^[76]

Antioxidant properties of eggs

The egg is not considered an antioxidant food, however, many of its components such as vitamins E and A, selenium, phospholipids and carotenoids exhibit antioxidant capacity.^[77] In addition to their high nutritional value, chicken egg proteins and related compounds (protein, peptide and amino acid hydrolysates) show many biological functions, including antioxidant activity. Hence, these have been used as functional ingredients in both the food and pharmaceutical industries.^[78]

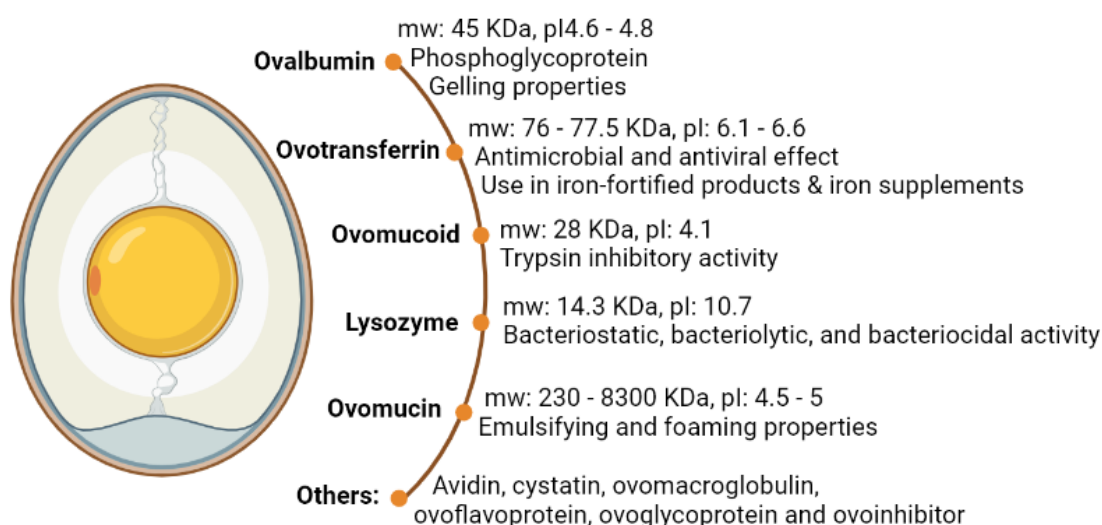


Figure 3. General characteristics and applications of egg proteins. Based on information from Guha *et al.*, & Abeyrathne *et al.*^[80, 81]

The approximate composition of egg white is 11% protein, with ovalbumin being the most abundant (54%) followed by ovotransferrin (12%), ovomucoid (11%), lysozyme (3.5%) and ovomucin (3.5%). However, other proteins have been identified in lower concentrations such as: avidin, cystatin, ovomacroglobulin, ovoflavoprotein, ovoglycoprotein and ovoinhibitor (Fig. 3).^[79] On the other hand, the main components of the egg yolk are lipids (31-35%) but it also includes 15-17% protein including lipovitellins (36%), livetins (38%), fosvitin (8%) and low-density lipoproteins (17%).^[80]

Antioxidant activity of egg proteins

Ovalbumin is the most abundant protein in egg white and its chemical composition contains a disulfide bond and four free sulfhydryl groups, which play a very important role in redox regulation, acting as chelators.^[82] Several studies have described an increase in the antioxidant capacity of this protein after a covalent union. Within these studies, some simple and complex saccharide unions have been carried out through emerging techniques such as ultrasound, favoring covalent unions.^[83–85]

Ovotransferrin is a protein with two globular subunits that contain a bound iron and interconnected by an alpha helix of nine amino acids, in addition to fifteen disulfide bonds that stabilize its structure.^[86] This protein is recognized for its antimicrobial activity. However, an increase in antioxidant activity has been detected due to the trapping of metal ions such as iron, magnesium, and copper, as well as conjugation with small molecules such as catechin.^[87, 88]

Other egg proteins that have been studied for their antioxidant activity are lysozyme, cystatin, and posvitin. Disulfide bridges and serine residues with phosphate groups embedded in their structures are the main characteristics of these proteins, which increase their capacity to trap metal ions and inhibit the formation of reactive oxygen species. Some of these proteins have been found to be capable of modulating the synthesis and release of nitric oxide, improving cellular oxidative stress.^[89] Another studied mechanism is the cystatin used in the protection of brain neurons against oxidative damage.^[90]

Egg-derived peptides with antioxidant properties

Peptides derived from egg proteins, which are fragments of between 2 to 20 amino acids, have been shown to be potentially more bioactive than the native proteins themselves.^[91] However, not only the size of the peptides is associated with antioxidant activity, but also the chemical composition has been shown to be important in this functional capacity. Sulphur-containing amino acids such as cysteine and methionine are prone to oxidation due to the sulfur content, which forms stable oxidation products by reactions with reactive species. Amino acids such as glutamine and asparagine, and hydrophobic amino acids such as proline,

alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, and tryptophan, also have a positive effect on antioxidant activity.^[92]

In addition to the fact that egg protein-derived peptides inhibit oxidative stress, they prevent oxidative damage through various mechanisms such as free radical scavenging, pro-oxidative metal ion chelation, reactive oxygen species inactivation, and hydroperoxide reduction.^[93–95]

The release of peptides has been studied through enzymatic hydrolysis. The use of pepsin generates peptides with molecular weights close to 3 kDa, increasing the antioxidant activity of egg proteins, which is attributed to the presence of aromatic amino acids, especially to the presence of tyrosine.^[96] However, the presence of methionine in the peptide structure may also be involved in this bioactivity, since methionine reduction is a very important antioxidant mechanism.^[97] For its part, the use of trypsin results in the release of fractions of raw egg proteins with molecular weights close to 1 kDa, which have exhibited great antioxidant power.^[98] Amino acid fractions such as VYLPR with antioxidant capacity measured through analysis by the DPPH technique, have been obtained by hydrolysis with alcalase.^[99] Other fractions with specific content of leucine, aspartic acid, glutamic acid and lysine have also been identified as potential antioxidants (HTKE, FFGFN, MPDAH and DHTKE).^[100]

Two peptides YLGAK and GGLEPINFN are obtained from the hydrolysis of egg white with papain. These peptides repress the formation of superoxide anion and hydroxyl radicals, preventing lipid peroxidation and showing reducing power.^[101] In addition to these enzymes, some commercial preparations such as Flavorzyme® have been used for the release of fractions with antioxidant capacity. However, the use of enzyme combinations, especially those involved in intestinal digestion (trypsin, pepsin and chymotrypsin) release peptides with aromatic amino acid residues which have been implicated in mechanisms of antioxidant activity. Within these sequences, there are three of great importance: WYGPD, KLSDW and KGLWE. The mechanism used by these fractions is through the inhibition of reactive peroxide and thiobarbituric acid molecules using free radical trapping and iron chelation pathways.^[102] Table 3 shows sequences of peptides derived from egg proteins obtained through enzymatic hydrolysis.

Table 3. Antioxidant peptides derived from egg proteins obtained by enzymatic hydrolysis*

Protein	Hydrolytic enzyme	Peptidic sequence	Reference
Ovotransferrin	Alcalasa	RVPSLM, TPSPR, DLQGK, AGLAPY, RVPSL	[103]
	Termolisin y pepsin	IRW, LKP	[104]
	Termolisin	WNIP, GWNI	[105]
Ovalbumin	Pepsin	YAEERYPIL, SALAM, YQIGL, YRGGLEPINF	[106]
	Alcalasa	DHPFLF, HAEIN, QIGLF	[77, 103]
	Protease P	AEERYP, AEERYP, DEDTQAMP	
Lisozime	Papain, trypsin	NTDGSTDYGILQINSR	[107]
	Pepsin, trypsin, quimotrypsin	RGY, WIR, VAW	[108]
	Pepsin	VAWRNRCKGTD, IRGCRL WIRGCRL, AWIRGCRL, WRNRCKGTD	[109]
White egg	Alcalasa	HANENIF, VKELY, TNGIIR, FFGFN, HTKE, DHTKE, VYLPR, EVYLPR, VEVYLPR, VVEVYLPR	[99, 100, 103]
Ovomucoid	Pepsin	WNWAD	[110]
Cystatin	Protease P	PVDENDEG	[77]

*Adapted from Benedé *et al.*^[111]

Antioxidant characteristics of other egg components

In addition to the proteins present in the egg, other minor components have antioxidant activity, such as selenium, which is one of the minerals that plays an antioxidant role as it forms part of selenocysteine, an amino acid that participates in the formation of selenoproteins, such as glutathione peroxidase.^[112] Also, there are carotenoids, which are

responsible for the coloration of the yolk, these have the ability to neutralize free radicals and prevent diseases caused by their accumulation. It has been observed that the supplementation of laying hen fed with foods that are a source of carotenoids increases the concentration of this antioxidant in the egg yolk.^[113]

Antioxidant properties of fish

Fish is a beneficial component for human health due to its high nutritional value and its antioxidant, anti-inflammatory, neuroprotective, cardioprotective, and hepatoprotective properties.^[114, 115] This food represents approximately 17% of the animal protein intake of the world population and 6.7% of all protein consumed in the world.^[114, 116, 117] Its nutritional composition presents variations between different species, and within the same species due to its diet, age, sex, time of year, temperature and environment.^[114, 116, 118] Thus, the average content of the different nutrients varies between 15-21% for proteins, 2-25% for lipids, 0.1-1% for carbohydrates and 0.5-2% for vitamins and minerals.^[118, 119]

Bioactive compounds with antioxidant properties can be obtained mainly in the edible part of fish meat as well as in the part of fish by-products including skin, scales, skeleton, bones, internal organs, head, and cooking juice.^[115, 120, 121] Thus, various bioactive compounds present in the fish and its by-products such as polyunsaturated fatty acids (PUFAs), collagen, gelatin, polysaccharides, minerals, and vitamins including zinc, iron, calcium, phosphorus, iodine, selenium, vitamins A, D, E, and various B vitamins (B3, B6, and B12), antioxidants, enzymes, and bioactive peptides are an attractive source of study for their valuable nutraceutical, pharmaceutical, and cosmetic potential.^[91, 97, 99]

Antioxidant activity of fish proteins

Collagen is a complex and an abundant structural protein present in the animal kingdom, which is characterized by its high content of glycine, proline and hydroxyproline.^[114, 117] About 28 different types of collagens have been found in higher metazoans, which are classified according to their primary structures and forms of supramolecular organization.^[117] In relation to the described literature, type I and II collagen have been extracted from fish

residues derived from the skin, skeleton, scales and cartilage in order to study their properties.^[114, 121, 122]

Fish collagen has shown up to 1.5 times more efficient absorption in the body and greater bioavailability compared to porcine and bovine collagen. However, it is important to consider that it is necessary to study a sustainable extraction process for its commercial exploitation.^[117] On the other hand, the antioxidant properties of collagen derived from the skin of different fish species (Table 4) have been demonstrated both *in vitro* and *in vivo*, observing its ability to sequester different free radicals such as DPPH, ABTS⁺, OH• and O²⁻, in addition to increasing the content of enzymes such as superoxide dismutase.^[117] Likewise, it has been observed that collagen derived from swim bladder and fish cartilage has similar *in vitro* antioxidant properties.^[117, 123]

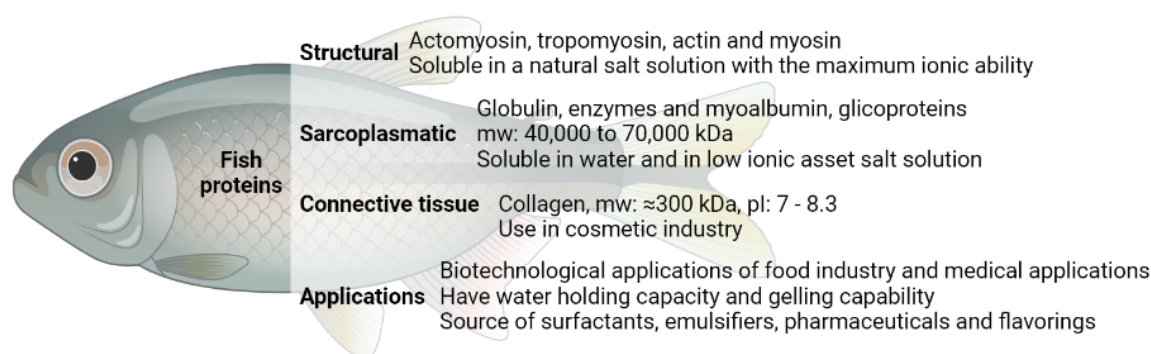


Figure 4. Characteristics and applications of fish proteins. Based on information from Alp-Erbay at al., & Khan *et al.*^[124, 125]

Partial hydrolysis and heat denaturation of collagen lead to obtaining gelatin.^[117, 121] The properties of the gelatin will depend on the characteristics of the collagen of origin and the extraction process. The composition of gelatin is closely related to the collagen of origin, which contains many essential amino acids such as glycine, which is the most predominant amino acid residue.^[117, 121] Other fish proteins are integrated into muscle, skin, and by-products with particular chemical and structural characteristics. For that reason, they could be integrated into many applications such as food industry, pharmaceuticals, cosmetics, and medical (Fig. 4).

Table 4. Proteins with antioxidant properties from residues derived from different species of fish*

Compound	By product	Specie	Activity / concentration	Reference
Collagen	Skin	<i>Lophius litulon</i>	1–8 mg/mL; 4 g/day	[128]
Collagen	Skin	<i>Oreochromis spp.</i>	2–7 mg/mL	[129]
Collagen / chitosan	Skin	Not specified	12–100 µg/mL	[130]
Collagen / gelatin	Skin	<i>Priacanthus hamrur</i>	DPPH y ABTS 1 mg/mL	[131]
Collagen	Swim bladder	<i>Nibea japonica</i>	12.5–50 µg/mL	[123]
Collagen/ gelatin	Skin	<i>Thunnus albacares</i>	DPPH 10–40 µg/mL ABTS 1 mg/mL	[132]
Collagen / gelatin	Cartilage	<i>Carcharhinus albimarginatus</i>	1–5 mg/mL	[133]
Collagen	Skin	<i>Scomber japonicus</i>	2.5–10 mg/mL	[134]
Collagen/ gelatin	Scale	<i>Katsuwonus pelamis</i>	0.1–5 mg/mL	[135]
Gelatin	Inner and outer layer	<i>Thunnus spp.</i> , <i>Hippoglossus spp.</i>	Ability to reduce iron 4 µmol FeSO ₄ *7H ₂ O /g	[126]
Gelatin	Skin	Salmon	Increase in SOD, GSH-Px, CAT and GSH activities.	[127]

*Adapted from Coppola *et al.*^[117]

A study of the antioxidant properties of gelatin (Table 4) obtained from the skin of two fish species, tuna (*Thunnus spp.*) and halibut (*Hippoglossus spp.*), has been described.^[126] In this study, the gelatins from these species showed certain antioxidant properties, measured by the ability to sequester free radicals and iron reduction; however, the mechanism is still unknown.^[126] Studies of the *in vivo* antioxidant capacity of gelatin obtained from salmon skin

were carried out in mice exposed to UV radiation. In this study it was shown that gelatin has a protective effect in mice by observing an increase in the activities of SOD, GSH-Px, catalase as well as the GSH level.^[127]

Peptides derived from fish with antioxidant properties

Fish processing generates a large number of by-products such as head, skin, viscera, backbone, scales, cartilage and blood, ranging from 25 to 70%.^[136] Decomposition of protein-rich fish by-products can be carried out through different methods, including chemical and enzymatic hydrolysis or microbial fermentation.^[136] In the case of enzymatic hydrolysis there are various proteolytic enzymes from animals, plants or microbes. Among these we have the enzymes pronase, bromelain, protease A and N, orientase, neutrase, protamex, validase, pancreatin and flavorzyme.^[136]

In addition to obtaining naturally active peptides, bioactive peptides can be recovered from this proteolytic processing, which contain between 2 and 20 amino acid residues (Fig. 5). These peptides have numerous activities, such as antihypertensive, antiproliferative, anticoagulant, immunomodulatory, antioxidant, among others.^[120, 136] The antioxidant activity of peptides may be the result of specific scavenging of ROS and radicals formed during peroxidation, protection against oxidative damage to DNA, scavenging of oxygen-containing compounds, or their ability to chelate metals.^{98]}

The radical scavenging activity can be influenced by the hydrophobicity/hydrophilicity, amino acid sequence, degree of hydrolysis, and molecular weight of the peptides.^[120, 137] It has been described that hydrophobic amino acids, including alanine, phenylalanine, isoleucine, leucine, valine, proline, methionine, among others, could act as proton or electron donors and/or as lipid radical scavengers.^[138, 139] In the same way, it has been shown that acidic amino acids, such as glutamic acid and aspartic acid, as well as basic amino acids such as arginine, lysine and histidine have antioxidant capacity as metal ion chelators due to carboxyl groups, and amino in the side chains.^[139] Several investigations have demonstrated the antioxidant activity of protein hydrolysates from fish by-products as shown in Table 5.

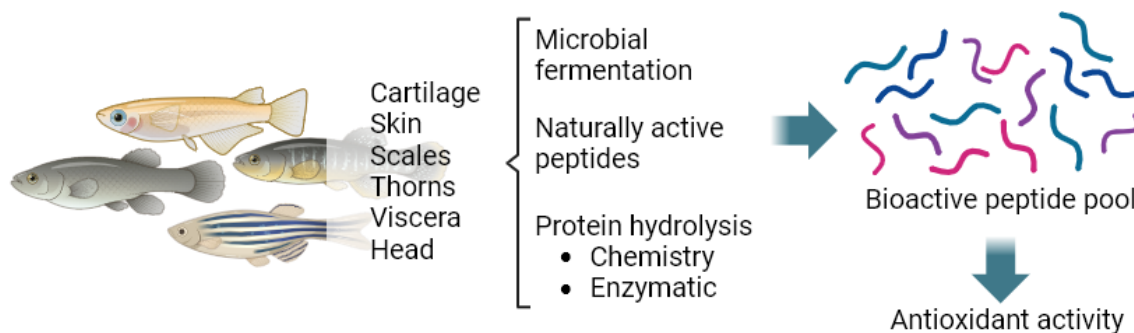


Figure 5. Obtaining and antioxidant mechanisms of bioactive peptides obtained from fish by-products of different species

Antioxidant characteristics of other fish components

Salmonids are an important source of carotenoids. These are pigments responsible for the wide spectrum of colors in fish and are characterized by their potential to reduce oxidative stress through the sequestration of ROS and free radicals, in addition to protecting low-density lipoproteins (LDL) against oxidation.^[121, 162] In fish the most common pigments are lutein, beta-carotene, alpha-, beta-doradexanthins, zeaxanthin, canthaxanthin and astaxanthin. The latter is widely distributed in both marine and freshwater fish, unlike lutein, which is only present in freshwater fish.^[114, 162]

On the other hand, hyaluronic acid derived from fish, or its by-products has a wide range of applications and is involved in many biochemical processes, such as cell signaling, tissue regeneration repair and morphogenesis, as well as providing redox balance or antioxidant. Its physicochemical properties, such as biodegradability, biocompatibility, zero toxicity, and immunogenicity, serve as excellent tools in biomedical applications.^[114]

Squalene is an organic molecule that participates as an intermediate for the biosynthesis of phytosterol or cholesterol in plants, animals and humans. High concentrations of this important natural biomolecule are found in the liver of certain fish species, especially sharks that live in the sea less than 400 meters deep.^[114, 163] Squalene is a highly unsaturated isoprenoid hydrocarbon, containing 6 double bonds. Due to this double bond structure, this isoprenoid acts as a strong antioxidant. It is also stable against the attack of peroxide radicals.^[114]

Table 5. Protein hydrolysates derived from fish by-products with antioxidant properties*

Fish	Scientific name	By-product	Peptidic sequence	Reference
Cod	<i>Gadus morhua</i>	Skeleton	ND	[140]
Japanese sole	<i>Paralichthys olivaceus</i>	-	VCSV, CAAP	[141]
Belanger's croaker	<i>Johnius belengerii</i>	Skeleton	GSTVPERTHPACPDFN	[142]
Alaskan collin	<i>Theragra chalcogramma</i>	Skeleton	LPHSGY	[143]
Sand fish	<i>Arctoscopus japonicus</i>	-	ATSHH	[144]
Dame	<i>Limanda aspera</i>	Skeleton	RPDFDLEPPY	[145]
Tilapia	<i>Oreochromis niloticus</i>	Skeleton	DCGYNYDEY	[146]
Conger	<i>Conger eel</i>	Muscle	LGLNGDDVN	[147]
Sardine	<i>Sardina pilchardus</i>	Muscle	LQPGQGQQ	[148]
Pacific hake	<i>Merluccius productos</i>	Muscle	PLFQDKLAHAK, AEAQKQLR	[149]
Salmon	<i>Salmo salar</i>	Seminal fluid	PR	[150]
Mackerel	<i>Magalaspis cordyla</i>	Skin	NHRYDR, GNRGFACRHA	[151]
Tiger croaker	<i>Otolithes ruber</i>			
Stripe	<i>Raja clavata</i>	Skin	AVGAT	[152]
Blue shark	<i>Prionace glauca</i>	Skin	EGR, GPR, GY, GF	[153]
Chinese carp	<i>Ctenopharyngodon idella</i>	Skin	PYSFK, GFGPEL, VGGRP	[154]
Pacific cod	<i>Gadus macrocephalus</i>	Skin	LLMLDNDLPP	[155]
Bluefin	<i>Navodon</i>	Skin	GSGGL, GPGGFI, FIGP.	[156, 157]
leatherjacket	<i>septrionialis</i>	Head	WEGPK, GPP, GVPLT	
Salmon	Not specified	Pectoral fin	FLNEFLHV	[158]
Black fish	<i>Parastromateus niger</i>	Viscera	AMTGLEA	[159]
		Eggs	DWMKGQ, MLVFAV,	
Tuna	<i>Katsuwana pelamis</i>		MCYPAST,FVSACSVAG, LADGVAAPA, YVNDAAATLLPR, DLDLRKDLYAN	[160]

* Adapted from Najafian *et al.*; Chalamaiah *et al.*; Atef *et al.*; Zamora-Sillero *et al.*^[120, 138, 139, 161]

Functional amino acids are those that participate in and regulate key metabolic pathways in the body, which is why they play an important role in the prevention and treatment of metabolic diseases.^[164] One of the essential amino acids in the body and that is obtained from

fish is taurine, which, in addition to other actions, has antioxidant properties.^[164] In addition, vitamin E is a potent antioxidant which prevents the oxidation of fatty acids in cell membranes.^[116] Marine tocopherols have first been isolated from salmon eggs and subsequently from tissues and organs of various fish, making them a considerable source of these beneficial molecules.^[121, 164]

Fish also contains a wide spectrum of minerals, where some of them have antioxidant properties such as Manganese (Mn), and Selenium (Se).^[116, 164] Regarding the latter, the incorporation of Se into the muscle of different fish species such as Atlantic salmon, rainbow trout, coho salmon and catfish has been described, for a greater bioavailability of this element.^[116]

Mechanism of action of antioxidant peptides

The antioxidant properties of peptides derived from animal proteins are related to their composition, structure, and hydrophobicity.^[111] It has been determined that aromatic amino acids such as Tyr, Trp, Met, Lys and His and amino acids containing sulfide groups such as Cys, have great antioxidant potential by donating protons to electron-deficient radicals.^[8, 54, 165]

The mechanism of action of antioxidant peptides has not been fully elucidated^[8] however, some investigators have suggested that the mechanism may involve reduced expression of metalloproteinases such as MMP-1, a collagenase that can generate damage to the connective tissue of the skin when there is an excess of reactive oxygen species^[166], the activation of the Keap1-Nrf2 pathways that allow the expression of antioxidant enzymes when the erythroid nuclear factor (Nrf2) binds in the cell nucleus with the ARE element (Antioxidant Response Element)^[28], decreases the expression of inflammatory cytokines through the inhibition of the transcription factor NF- κ B and microglia, which in turn decrease the expression of iNOS, TNF- α , IL-1 β and IL-6^[4], the inhibition of lipid peroxidation by stopping the chain reaction of fat oxidation, inactivation of reactive oxygen species (ROS) and capture of free radicals, chelation of metal ions by eliminating traces of metals such as iron or copper that facilitate oxidation, or a combination of these mechanisms (Fig. 6).^[8, 10, 167–169] Antioxidant peptides

have been reported to exert their effect via the intracellular conversion of cysteine to glutathione, a potent intracellular antioxidant that protects cells from ROS damage.^[168, 170]

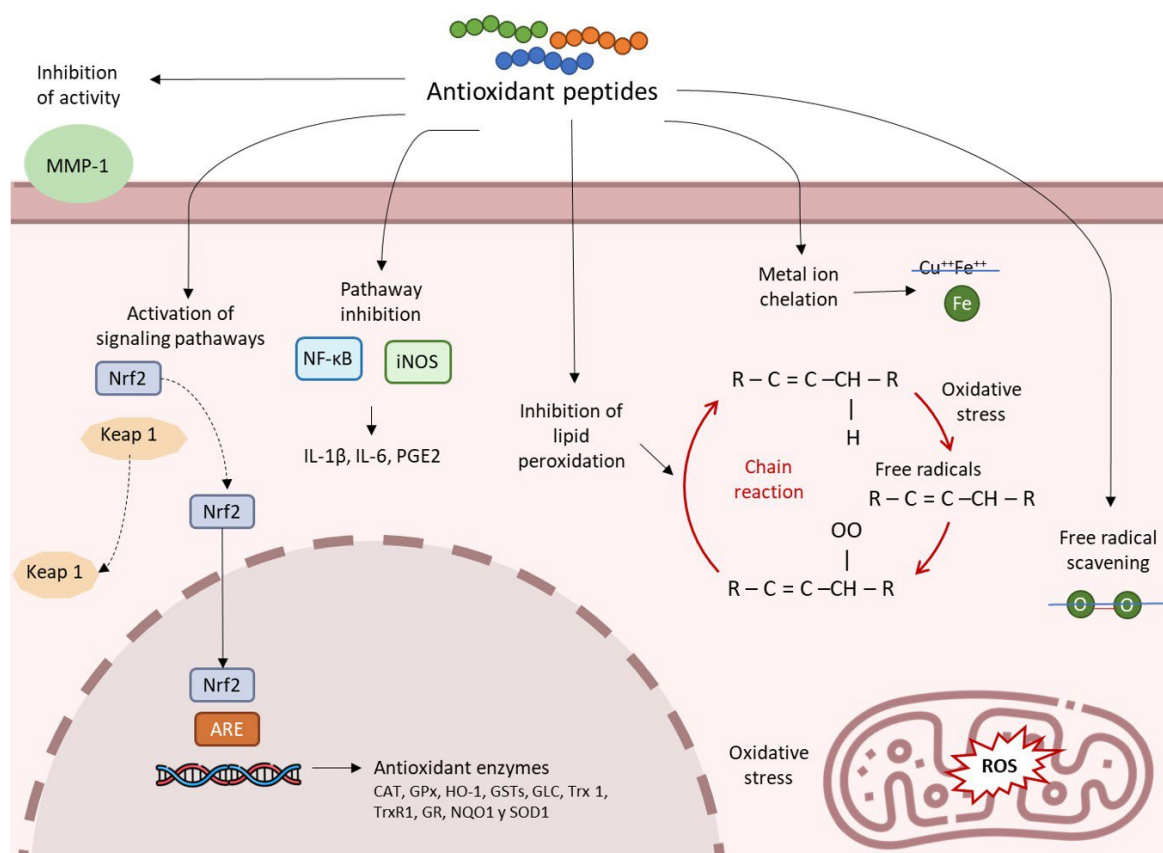


Figure 6. Mechanism of action of food-derived antioxidant peptides. Own elaboration based on information from Okagu *et al.*; Olvera-Rosales *et al.*^[168, 169]

Bioaccessibility of peptides

Antioxidant peptides can be incorporated into food or supplements for consumption; however, for reaching their site of action, they must maintain their structure and bioactivity after passing through the gastrointestinal tract. During digestion, peptides are susceptible to attack by proteases and peptidases found in the stomach and intestine^[171], these enzymes can cause that the peptide decreases or loses its activity, or breaks down into smaller peptides^[172, 173] (Fig. 7). The molecular weight and the sequence in the bioactive peptides are determinants for their bioavailability. Those that have proline or hydroxyproline in their sequence can resist the action of proteases.^[174] Both native peptides and those generated by the action of proteases can enter the intestinal cell through the action of the peptide transporter (PepT1) and the oligopeptide transporter (SOPT2).^[175]

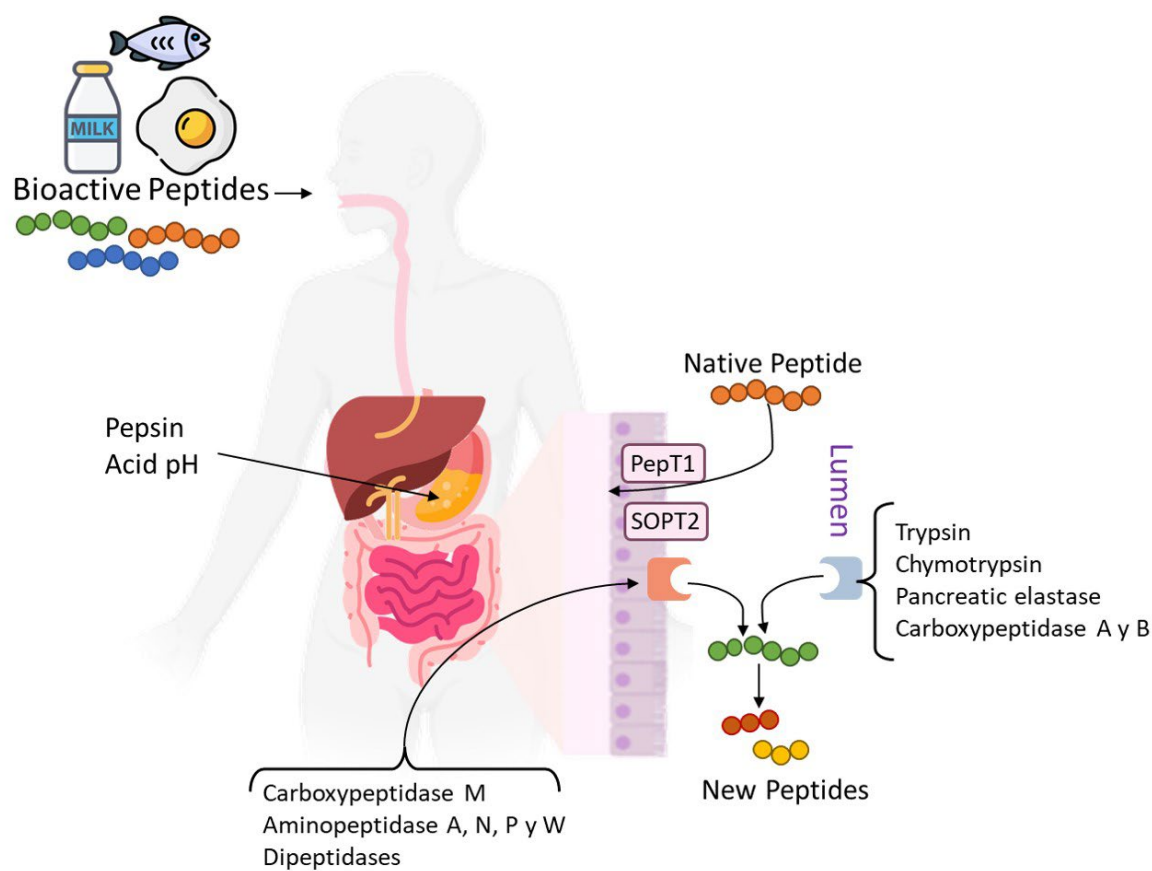


Figure 7. Bioavailability of food-derived bioactive peptides. Own elaboration based on information from Okagu *et al.*; Udenigwe *et al.*^[168, 174]

Conclusions

The food industry has developed through the modification of raw materials with a technological focus. However, new technologies are framed in the conservation of the functional properties of their macro and micro molecular components. Thus, the antioxidant properties of foods of animal origin are of great interest both in the field of food science and technology and in nutrition and human health. Therefore, in this revision it has been shown that there are components of high concentration and trace concentrations that have antioxidant activity in milk, eggs and fish. The main ones are those peptides fractions derived from the structural proteins of these foods, which exhibit varied mechanisms of action. In addition, the importance of the chemical composition of peptides and the synergism that exists between components to exert this bioactivity has been revealed. Knowing the antioxidant components of this type of food marks the challenges and opportunities that exist within the existing research on functional foods.

Disclosure statement

Authors reported no potential conflict of interest

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Capítulo III

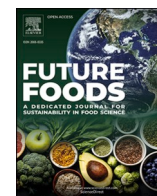
Proteínas de trucha arcoíris (Oncorhynchus mykiss) como fuente de péptidos antioxidantes con promisorio actividad anticancerígena

Resumen

El presente capítulo recopila y analiza la información científica reciente sobre el potencial de las proteínas de trucha arcoíris como precursoras de péptidos bioactivos, los cuales se han destacado por sus múltiples funciones biológicas incluyendo la actividad antioxidante y anticancerígena. Esta revisión, publicada en la revista *Future Foods*, presenta a los péptidos antioxidantes obtenidos específicamente de proteínas de trucha arcoíris, resaltando su potencial actividad anticancerígena y proporcionando una base para entender y aprovechar su potencial terapéutico y farmacológico.

En primer lugar, se describen los principales subproductos generados por la acuicultura de la trucha arcoíris, enfatizando su contenido proteico y su posible aprovechamiento en la obtención de compuestos bioactivos como los péptidos antioxidantes. Posteriormente, se mencionan los principales métodos empleados para su obtención, resaltando la hidrólisis enzimática, ampliamente reportada como una estrategia eficaz para generar péptidos con actividades biológicas.

Particularmente, se ha identificado que algunos péptidos derivados de la hidrólisis de proteínas de trucha arcoíris poseen actividad antioxidante, lo que les permite neutralizar especies reactivas de oxígeno y reducir el estrés oxidativo celular, un proceso estrechamente relacionado con la aparición y progresión de diversas enfermedades crónicas, incluido el cáncer. Más allá de esta función, ciertos péptidos han mostrado citotoxicidad selectiva frente a líneas celulares tumorales, especialmente en cáncer de mama y, en menor medida, en otros tipos como el cáncer gástrico. Estos hallazgos han motivado un análisis más profundo de sus mecanismos de acción y de la relación entre su estructura y función biológica, los cuales se describen en esta revisión. Finalmente, se incluye un análisis *in silico* de la búsqueda de péptidos derivados de proteínas de trucha con posible actividad anticancerígena, con el objetivo de enfatizar la capacidad de la trucha arcoíris como fuente de compuestos bioactivos de interés terapéutico.



Rainbow trout (*Oncorhynchus mykiss*) proteins as a source of antioxidant peptides with promising anticancer activity

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ARTICLE INFO

Keywords:

Antioxidant peptides
Oncorhynchus mykiss proteins
Anticancer bioactivity
Proteolysis
Peptide fractions

ABSTRACT

Fish production worldwide is aimed at muscle consumption, and this industry discards around 60 % of the fish in undervalued by-products. A widely consumed commercial species is the rainbow trout (*O. mykiss*), which has a consumption presence among commercial fish of 17 %. This makes it a significant species due to the amount of waste obtained after consumption. In addition, the protein content of muscle and by-products is relevant, as it is often a source of encrypted bioactive peptides, which can be released by hydrolytic mechanisms, such as enzymatic digestion, in vitro digestion, and microbial fermentation. Several studies have proved the bioactivity of peptides obtained from fish species that contain amino acid sequences with properties that interest human health. This article reviews the protein composition, the mechanism for obtaining peptides, action ways of antioxidant peptides, *in silico* sequences from the BIOPEP database, and their potential anticancer activity, contrasting with discoveries in other species and emphasizing the potential of *O. mykiss* proteins as a source for obtaining antioxidant peptides with anticancer capacity.

Introduction

Rainbow trout (*Oncorhynchus mykiss*), a salmonid species, shows rapid development, adaptability to different environments, and offers high nutritional value (Hardy, 2002). These traits drove its widespread cultivation globally. Over the past four decades, aquaculture production of rainbow trout reached 744 thousand tons in 2021 (FAO, 2022). Leading exporters include Norway, Chile, and Turkey (FAO, 2022). Mexico reported 2748 tons of trout production in 2023 (Comisión Nacional de Acuacultura y Pesca, 2023).

In both developed countries and emerging economies, such as Mexico, producers market gutted rainbow trout weighing <500 gs, with larger specimens (500 g to 2 kg) reserved for processed products like smoked fillets, hamburgers, and skewers (Joshi et al., 2008; Ortega and Valladares, 2017; Stanković et al., 2015). Trout farming supports economic growth in rural areas (D'Agaro et al., 2022) and offers a sustainable food option, given its lower ecological impact than livestock

like lamb and beef (Clune et al., 2017; Wilfart et al., 2023).

However, in the commercialization of rainbow trout, 60 % of the animal's weight is considered within the byproducts (skin, head, skeleton, viscera, blood) that are most often discarded (Stevens et al., 2018). Some authors consider that rainbow trout by-products only correspond 30 % (Nikoo et al., 2023). Although to obtain a greater yield of fish such as rainbow trout, strategies have been designed to take advantage of the byproducts. This is the case of the skin, which contains nutrients of interest for human health and industry, such as lipids (essential fatty acids) and, to a greater extent, proteins (collagen and essential amino acids) (Bruni et al., 2021; Rebolé et al., 2015; Xie and Liu, 2023). The collagen content in rainbow trout skin is 89.24 mg/g, and could be used to develop fish collagen, gelatin or collagen peptides (Xie and Liu, 2023).

In fact, from the proteins present in the skin of rainbow trout, sequences of amino acids considered bioactive peptides have been obtained through hydrolysis processes (Ucak et al., 2021). One of the

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<https://doi.org/10.1016/j.fufo.2024.100509>

Received 2 August 2024; Received in revised form 7 November 2024; Accepted 21 November 2024

Available online 22 November 2024

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methods for obtaining peptides is enzymatic hydrolysis using proteases (Sila and Bougatef, 2016). The next step is the purification process to separate small-sized peptides (Bartolomei et al., 2023). These peptides are considered to have bioactive potential due to the size and structure of their chain (Tonolo et al., 2019; Wang et al., 2024). Furthermore, peptides derived from rainbow trout could present different biological activities such as antioxidant, antihypertensive, antidiabetic, antithrombotic, and anticancer, among others (Bartolomei et al., 2023; Ketnawa et al., 2019; Naghdi et al., 2023; Yaghoubzadeh et al., 2020).

Regarding antioxidant peptides, they have been reported in trout skin (Ramezanzade et al., 2018; Vásquez et al., 2022). However, recently efforts have been made to identify the presence of anticancer activity in them (Yaghoubzadeh et al., 2020) since the mechanism of action of these peptides is associated with the REDOX balance. It is known that an imbalance in the levels of reactive oxygen species (ROS) induces carcinogenesis and progression of cancer because they are powerful oxidizing agents capable of damaging DNA (Shaik and Sarbon, 2022). Thus, antioxidant peptides would be an alternative to prevent these effects.

On the other hand, some characteristics that have been described in peptides with anticancer activity experimentally validated include a short length (5–50 residues), the presence of hydrophobic, with an imidazole or aromatic ring, positive charge, and the presence of leucine, histidine, and phenylalanine amino acids (Chai et al., 2021; Y. Zhang et al., 2023). These characteristics have also been identified in antioxidant peptides from marine sources.

Currently, treatments for this disease consist of chemotherapy and radiation, which attack both cancer cells and normal cells. In this sense, it has been reported that some anticancer peptides could kill cancer cells without affecting normal cells, which translates into faster recovery in patients (Y. Huang et al., 2011). Therefore, this review aims to present the potential of rainbow trout (*O. mykiss*) proteins for obtaining antioxidant peptides with anticancer capacity. Through it, the composition and importance of rainbow trout proteins, the enzymatic mechanism for obtaining antioxidant peptides, and the proposed mechanism to increase these peptides' antioxidant and anticancer capacity will be reaffirmed. Finally, some perspectives will be presented for the therapeutic use of antioxidant peptides from hydrolysis of *O. mykiss* proteins.

Methodology

A search was conducted in various scientific databases to obtain and analyze information on rainbow trout peptides' antioxidant, anticancer, and cytotoxic properties. The following electronic databases were used to find relevant articles: PubMed, Scopus, Dialnet, Latindex, Google Scholar. The keywords were rainbow trout (*Oncorhynchus mykiss*), rainbow trout proteins, antioxidant peptides, anticancer activity, fish proteins, and cytotoxicity. These words were essential to restrict and focus the search and were used individually and in combinations to maximize the relevance and specificity of the results obtained.

Articles published between 2013 and 2023 were considered, except for those articles that were relevant due to their results, articles in English or Spanish. Publications unrelated to the biological properties of rainbow trout peptides or other fish species were excluded. Finally, the articles were reviewed and updated, and relevant information on rainbow trout peptides was discussed, focusing on their antioxidant, anticancer, and cytotoxic properties and possible mechanisms of action.

Byproducts derived from rainbow trout

Rainbow trout farming spread in several countries due to its high nutritional value and rapid growth rate (Mustafa, 2016). The nutritional content of rainbow trout depends on factors such as age, size, the season of cultivation, environmental factors (S. Lee et al., 2020), water quality, diet, and feeding system (Fiordelmondo et al., 2020). However, only a portion of fish production is allocated for human consumption, while the

remainder is discarded. In the case of rainbow trout, 30 % of the animals weight corresponds to inedible parts or by-products, such as trimmings, skin, head, bones, guts, and blood (M. Nikoo, Regenstien, and Yasemi, 2023; Zapata et al., 2019). Fig. 1 shows the percentage that some byproducts represent concerning the weight of the fish.

Although the use of by-products for human consumption is limited, they are rich in nutrients, including minerals, vitamins, proteins, and lipids (Stevens et al., 2018; Zapata et al., 2019). Table 1 shows that protein predominates after humidity in the proximal composition of the rainbow trout by-products, with the bones and head having the highest ash content.

Due to their nutritional composition, attempts have been made to use these by-products for different purposes. Table 2 presents some nutrients in fish byproducts and some of their applications.

Some studies have demonstrated the importance of the chemical composition of the byproducts in obtaining compounds of scientific and commercial interest (Heydari et al., 2021; Javaherdoust et al., 2020; Li et al., 2019; Pando et al., 2018; Rothwell et al., 2005; Saito et al., 2009; Sharp et al., 2012). In this sense, proteins are predominant in studies to obtain bioactive compounds (Zapata et al., 2019). However, pro-oxidants must be considered to obtain bioactive peptides from by-products by enzymatic hydrolysis. It has been reported that hemoglobin, metal ions, and unsaturated lipids, combined with changes in temperature and pH during hydrolysis, can lead to hydrolysate oxidation (Nikoo et al., 2023). From this standpoint, the intensity of lipid oxidation varies according to the composition of the by-products, which affects the structure and antioxidant activity of the resulting peptides (Nikoo et al., 2023).

In addition, rainbow trout by-products offer a valuable opportunity for food utilization, particularly due to their potential nutritional benefits. Effective separation techniques can improve the quality of these by-products by isolating high-value, clean fractions while removing undesirable components (Hassoun et al., 2022). For example, methods such as enzymatic hydrolysis, membrane filtration, and centrifugation can be used to separate protein-rich fractions from fat, bones, and skin, resulting in cleaner products that are more suitable for human consumption (Hassoun et al., 2022; Raja et al., 2024).

The resulting fractions from these separation processes include protein hydrolysates and bioactive peptides, which may offer various health benefits, such as antioxidant and anti-inflammatory properties. Additionally, supercritical fluid extraction can further refine these by-products, effectively reducing contaminants (Majumder and Balange, 2023). This approach enhances the economic value of rainbow trout by-products and supports sustainable food production practices. By transforming waste into high-quality ingredients, we can promote the utilization of all fish parts, minimize waste, and contribute to improved food security.

Rainbow trout proteins

Proteins are part of rainbow trout's composition, as in most fish, and could be classified into structural, sarcoplasmic, and connective tissue proteins. In trout, structural proteins, such as actin, myosin, tropomyosin, and actomyosin, are essential components of the contractile apparatus responsible for muscle movement (Gokoglu et al., 2004).

In the case of actin is a myofibrillar protein that is present in trout and is part of the contractile structures of the muscle and the cytoskeleton (Krasnov et al., 2003; M. Wang et al., 2021). On the other hand, myosin, which is the main myofibrillar protein, is a 520 kDa hexamer with 6 polypeptide chains, 2 essential light chains, 2 regulatory light chains, and 2 heavy chains (Isik et al., 2020; Kristinsson and Hultin, 2004). Its light chains can regulate calcium binding (Donato et al., 2008). Its heavy chains are two 220 kDa polypeptides that give the protein water retention capacity, gel formation capacity, and emulsification (Kristinsson and Hultin, 2004). Finally, tropomyosin mediates the interaction between troponin and actin, which is required to regulate



Fig. 1. Rainbow trout by-products in percentage of body weight. Prepared with information from [Stevens et al. \(2018\)](#).

Table 1
Proximate chemical composition (%) of muscle and byproducts derived from rainbow trout.

	Moisture	Ashes	Fat	Protein	Reference
Muscle	67.27	1.58	16.41	21.22	(Xie and Liu, 2023)
Skin	72.11	1.74	17.75	21.92	(Xie and Liu, 2023)
Fish bone	59.60	–	17.70	15.90	(Fan et al., 2018)
Head	62.4	1.91	6.00	29.00	(Li et al., 2019)
Viscera	71.65	2.73	13.00	15.00	(Taheri et al., 2013)

muscle contraction ([Joyce, 2023](#)).

Other proteins found in fish are sarcoplasmic proteins. These proteins have the characteristic of being soluble in water, a solubility that decreases with an increase in ionic strength ([Chen and Jaczynski, 2007](#); [Lysenko et al., 2020](#)). Among the main sarcoplasmic proteins are myoalbumin, globulin, and some enzymes. Of the latter, it is known that their chemical and physical properties depend on their physiological location (skin, swim bladder, or muscles). Other sarcoplasmic proteins, such as α -enolase and creatinine, are related to the structural firmness of trout ([Kumar et al., 2020](#)).

Likewise, collagen is a connective tissue protein whose fish content depends on the species ([Heidari and Rezaei, 2022](#)). This protein is the main component of isinglass and has had applications in the beer industry as a fining agent. This is because collagen has a positive charge that attracts yeast cells with a negative charge. When they join, they precipitate and can be removed from the beer ([Walker et al., 2007](#)). Furthermore, due to their properties, they have been used in the cosmetics, pharmaceutical, food, and biomedical industries ([Rajabimashhadi et al., 2023](#)).

Furthermore, in the skin of rainbow trout, we can also find hydroxyproline (20.40 mg/g), which is a non-essential amino acid that stabilizes the collagen structure and intervenes in the thermal stability of the fish ([Johnston and Gillis, 2022](#)). The aging of the fish does not modify the content and composition of rainbow trout skin proteins. The primary amino acids in trout skin are lysine, phenylalanine, tyrosine, and leucine ([Kondratiuk and Otchenashko, 2021](#)). Many of these amino acids are bioavailable after the hydrolytic breakdown of the primary structure of proteins to obtain peptide fractions. Consequently, many of these peptides have bioactive characteristics.

Table 2
Applications of by-products derived from fish.

By-product	Component	Common Applications	Alternatives for use
Head	Proteins, peptides, lipids, collagen, gelatin, minerals (calcium), flavor (Stevens et al., 2018).	Fish meat, fish oil, food and animal grade hydrolysates, pet foods, nutraceuticals, and cosmetics (Stevens et al., 2018).	Used in the extraction of oil with a high content of unsaturated fatty acids (Li et al., 2019).
Skeleton (bones, meat, fins)	Proteins, peptides, lipids, collagen, gelatin, minerals (calcium), flavor (Stevens et al., 2018).		Use of rainbow trout bone micro-powder to improve texture properties and decrease degradation of surimi gel (Heydari et al., 2021). Used as a value-added component as it is a source of omega-3 long-chain polyunsaturated fatty acids (Pando et al., 2018).
Fish cuttings	Proteins, peptides, lipids (Stevens et al., 2018).		Protein hydrolysates from rainbow trout viscera increase the growth performance of fed fish (Javaherdoust et al., 2020). Oral administration of peptides derived from rainbow trout skin collagen can decrease plasma triglyceride levels (Saito et al., 2009).
Viscera	Proteins, peptides, lipids, enzymes (lipases) (Stevens et al., 2018).	Food and animal grade hydrolysates, fish meat, fish oil, fuel, and fertilizers (Stevens et al., 2018).	Fibrin from salmon is useful in the treatment of spinal cord injuries and in the production of bandages to control severe and uncontrolled bleeding (Rothwell et al., 2005 ; Sharp et al., 2012).
Skin	Collagen, gelatin, lipids, proteins, peptides, minerals, flavor (Stevens et al., 2018).	Fish meat, fish oil, cosmetics, leather or skin, fuel, and fertilizers (Stevens et al., 2018).	
Blood	Proteins, peptides, lipids, thrombin, and fibrin (Stevens et al., 2018).	Fuel, fertilizers and therapeutics.	

Enzymatic hydrolysis mechanisms for the fractionation of rainbow trout proteins

Obtaining bioactive peptides requires digesting a protein from an animal or plant source with an enzyme, evaluating the hydrolyzate's biological activity, and purifying and identifying the sequence with the highest activity (Chakrabarti et al., 2018). The known methods for producing bioactive peptides are fermentation using cultures of microorganisms, enzymatic hydrolysis using enzymes of plant origin or produced by microorganisms, and simulation of gastrointestinal digestion using digestive enzymes (Fig. 2). These methods can be used individually or in combination (Korhonen and Pihlanto, 2006).

Fermentation involves using microorganisms such as yeasts, fungi, or bacteria to hydrolyze the protein and obtain short peptides. The inoculate is first taken to the exponential growth phase before inoculating the sample (Rizzello et al., 2017). The fermentation time, the microbial strain used, and the type of food matrix from which the proteins determine the degree of hydrolysis (Chakrabarti et al., 2018). Combinations of different cultures can also be used to obtain shorter peptides (Ucak et al., 2021).

In the fermentation of rainbow trout by-products (heads, frames, and viscera) with lactic acid bacteria, smaller peptides were observed at 48 h. These bioactive peptides exhibited antioxidant properties and angiotensin-converting enzyme inhibition *in vitro*, and the most abundant peptide sequence was GQDGLSFPGP GPPG (Fernando, 2021).

On the other hand, bioactive peptides have been obtained through enzymatic hydrolysis to be used as functional ingredients in food manufacturing or as integral compounds in drugs (Shafiee et al., 2021). Combinations of enzymes can be used to generate smaller peptides, but it must be considered that each enzyme has its optimal pH and temperature conditions (Mora and Toldrá, 2023).

In the case of enzymatic hydrolysis, the optimum pH and temperature of the enzyme must be considered, in addition to optimizing factors such as the water-raw material ratio, the stirring speed, the enzymatic inactivation, and the reaction time. Oxidation must also be prevented by using nitrogen gas or adding antioxidants to improve the characteristics of the peptides obtained (Nikoo et al., 2023).

In the hydrolysis of rainbow trout heads, the effect of adding an antioxidant (butylated hydroxytoluene) in the retarding oxidative changes during the hydrolysis process with bromelain and papain was investigated. The antioxidant did not seem to affect the amino acid composition in the hydrolysates, but it improved the solubility

(Kvangarsnes et al., 2021).

Depending on the enzyme used, the peptides obtained can vary in chain length and amino acid sequence (Sila and Bougatef, 2016). Proteases generate large peptides and free amino acids, dipeptides, and tripeptides (Mora and Toldrá, 2023). Proteases are classified as aspartic peptidases, cysteine peptidases, metallopeptidases, or serine peptidases (Rawlings et al., 2014). In the serine-endopeptidases group is Alcalase, an enzyme that can hydrolyze protein and obtain peptides with bioactive (Borrajó et al., 2020). The most used proteases for protein hydrolysis in foods are the commercial enzymes Alcalase, Flavourzyme, and Protamex. However, pepsin, chymotrypsin, and trypsin have also been used for marine byproducts (Sila and Bougatef, 2016).

In rainbow trout viscera, peptides generated by enzymatic hydrolysis using Alcalase and Protamex have been compared with those produced by acid autolysis with endogenous enzymes from the viscera. Both commercial enzymes and acid autolysis, individually or in combination, effectively produce peptides smaller than 1 kDa, offering a more cost-effective alternative to the exclusive use of commercial enzymes (Domínguez et al., 2024).

After hydrolysis, it is advisable to purify the peptides because, during the hydrolysis process, peptides can also be generated that interfere with the activity of bioactive ones (Tacias-Pascacio et al., 2020). In this way, smaller peptides can be separated to which bioactive properties are attributed. One of the most important bioactive compounds of the fractions derived from the hydrolysis of proteins of marine species within the field of medicine is the group of antioxidant peptides (Chakniramol et al., 2022).

Antioxidant peptides from rainbow trout proteins

The antioxidant activity of peptides may result from the specific sequestration of ROS and radicals formed during peroxidation, protection against oxidative DNA damage, the sequestration of oxygen-containing compounds, their ability to chelate metals, the production of intracellular antioxidants, or the interaction with signaling pathways related to REDOX balance (Atef and Mahdi Ojagh, 2017; Lv et al., 2022; Mutalipassi et al., 2021). Fig. 3 shows a diagram of the different mechanisms of action of antioxidant peptides.

Radical scavenging activity is influenced by the hydrophobicity/hydrophilicity, amino acid sequence, degree of hydrolysis, and molecular weight of the peptide (Atef and Mahdi Ojagh, 2017; Da Rocha et al., 2018). It has been described that hydrophobic amino acids, including

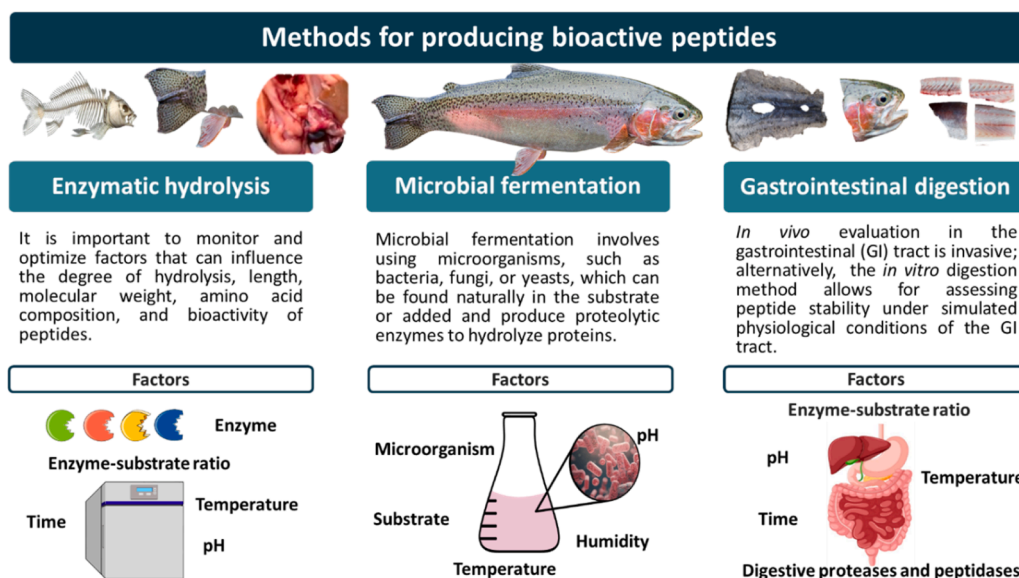


Fig. 2. Methods for producing bioactive peptides from rainbow trout by-products.

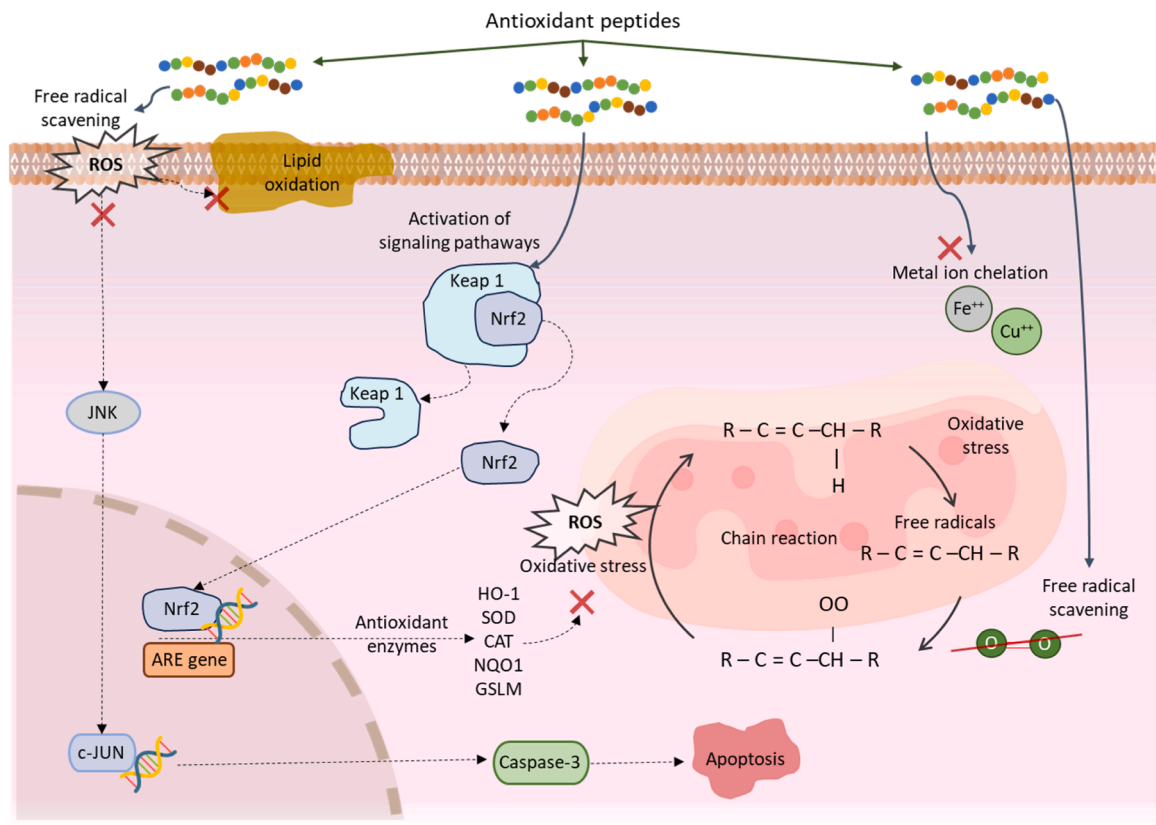


Fig. 3. Mechanism of action of antioxidant peptides. These can participate in the scavenging of free radicals, chelation of metal ions, signaling pathways and breaking the fat oxidation chain reaction. Adapted from [Olvera Rosales et al. \(2022\)](#).

alanine, phenylalanine, isoleucine, leucine, valine, proline, and methionine, among others, could act as proton or electron donors and/or as scavengers of lipid radicals ([Chalamaiah et al., 2012](#); [Zamora-Sillero et al., 2018](#)). In the same way, it has been shown that acidic amino acids, such as glutamic acid and aspartic acid, as well as amino acids, such as arginine, lysine, and histidine, have the antioxidant capacity as chelators of metal ions due to the carboxyl groups, and amino in the side chains ([Zamora-Sillero et al., 2018](#)).

Specifically, antioxidant peptides from marine proteins exert their activity by regulating the expression of cellular antioxidants ([Kong et al., 2023](#); [Mao et al., 2024](#)). Mediates activation of the Nrf2/Keap 1 signaling pathway ([Fig. 3](#)), which induces the expression of antioxidant enzymes when nuclear factor erythroid 2 (Nrf2) is released from the Kelch-type-ECH-associated protein (Keap 1) and binds to the response element antioxidant (ARE) ([Tonolo et al., 2020](#)). It has also been reported that they inhibit apoptosis by suppressing the activity of JNK and caspase 3 ([Xing et al., 2022](#)).

It has been shown that ROS induces DNA damage that can activate the JNK pathway. This pathway is associated with cellular stress, apoptosis, tumor onset, and development ([Lv et al., 2022](#)). [Fig. 3](#) illustrates that antioxidant peptides can inhibit this signaling pathway. Peptides exert cytoprotective effects that prevent ROS from phosphorylating JNK/c-JUN and trigger caspase expression and apoptosis, thus improving cell viability ([Deng et al., 2021](#); [Liu et al., 2019](#)).

In addition to the antioxidant activity presented by peptides, the amino acid glutamine, glycine and cysteine participate in synthesizing glutathione (GSH), an intracellular antioxidant ([Gould and Pazdro, 2019](#)). Therefore, it is considered that a peptide can exert antioxidant activity on its own but can also participate in synthesizing other intracellular antioxidants.

Several investigations have demonstrated that the peptides obtained from fish skin protein hydrolysates have antioxidant activity, as shown

Table 3
Sequences of antioxidant peptides derived from fish skin.

Species	Peptide sequence	Reference
<i>Jumbo skid</i>	HGPHLPL	(Mendis et al., 2005)
<i>Magalaspis cordyla</i>	AHATAA	(Sampath et al., 2011)
<i>Otolithes ruber</i>	GAAGPACAHA	(Sampath et al., 2011)
<i>Navodon septentrionalis</i>	GSGGL, GPGGPL, PIGP	(C. F. Chi et al., 2015a)
<i>Prionace glauca</i>	EGR, GPR, GY, GF	(Weng et al., 2014)
<i>Ctenopharyngodon idella</i>	PYSFK, GFGPEL, VGGRP	(Cai et al., 2015)
<i>Gadus macrocephalus</i>	LLMLDNDLPP	(Himaya et al., 2012)
<i>Raja clavata</i>	AVGAT	(Lassoued et al., 2015)
<i>Lates calcefer</i>	GLFGPR, GATGPQGGLGPR, VLGPF	(Sae et al., 2017)
<i>Scomberomorus niphonius</i>	GPY, GPTGE, PFGPD, GPTGAKG, PYGAKG, GATGPQG, GPFPGPM, YGPM	(J.-B. Zhang et al., 2019)
<i>Oreochomis niloticus</i>	GSGGL, GPGGFI, FIGP	(Ma et al., 2022 ; Y. Zhang et al., 2012)
<i>Cyprinus carpio</i>	EEL, YGDEY, GGYDEY, GGYDEYR, APDPGPGPM, GNAGPTGPAGPL, GAPGARGPNGY, SGPPVPGPIGPM	(Tkaczewska et al., 2018)
<i>Katsuwonus pelamis</i>	AY	(S.-Y. Zhang et al., 2022)
<i>Gadus macrocephalus</i>	GEGSE, GAGER, VPGGQG, VPEGD, MPAGE, GAGEEQ, LNPAG, GAPGETGD, GTGPA, LGSPN, GERGPAPNNQ, VFPGE, GAGGFGFAGG, LQGMF, LVGERGADGQ, GAPGPT, GPAGMFG, GPIGPTGPQ, PIGPFGPL, GPAGPHGPPGKDR, GPHGPPGKDR	(W. Wu et al., 2017)

in Table 3.

Bioactive peptides with antioxidant capacity have also been found in the skin of rainbow trout. The obtaining of peptides by enzymatic hydrolysis with Alcalase with a weight <30 kDa has been reported (Ramezanzade et al., 2018), also of antioxidant peptides with a weight <30 kDa using enzymatic hydrolysis with Alcalase and Flavourzyme (Yaghoubzadeh et al., 2020). However, the sequencing of these fractions has not been reported, although recently Bartolomei et al. (2023) reported sequences with antioxidant potential predicted by the BIOPEP database (Table 4).

Importance of the structure-activity relationship of antioxidant peptides

Different chemical and physical characteristics of peptides are involved in antioxidant activity. Among them are the molecular weight, hydrophobicity, amino acid sequence, acid/base character of the peptide, and aromaticity.

In rainbow trout hydrolysates, it has been shown that molecular weight is closely related to this activity (Bartolomei et al., 2023; Nami et al., 2023; Yaghoubzadeh et al., 2020), since smaller peptides with short sequences exhibit more significant antioxidant activity than those with longer sequences (Tonolo et al., 2019). Actually, low molecular weight is believed to facilitate the ability of peptides to cross the intestinal barrier and exert antioxidant effects (Pan et al., 2019).

The composition of amino acids in the amino-terminal and carboxyl-terminal regions is another essential factor (D. Wu et al., 2019). Fig. 4 shows a relationship between the characteristics and position of amino acids and the I in peptides. In general, a high presence of hydrophobic amino acids is observed, which, as some studies have shown, enhances the antioxidant capacity of the peptide (Zheng et al., 2016).

This high proportion of hydrophobic amino acids is also evident in Tables 3 and 4, which show antioxidant peptides from rainbow trout and other marine species. These hydrophobic amino acids enhance the peptides' affinity and reactivity with cell membranes, enabling them to cross the lipid bilayer and exert activities such as terminating lipid peroxidation within cells (Jin et al., 2018; Oliveira et al., 2017; Zhang et al., 2021).

Fig. 4 also illustrates that the presence of two phenylalanine or histidine residues in the sequence, or the inclusion of glycine, lysine, isoleucine, and valine in a peptide, enhances its antioxidant activity (Bougatef et al., 2010; Mendis et al., 2005). Amino acids with a ring in their side chain are also linked to the antioxidant activity of the peptide. For example, with its pyrrolidine ring, proline scavenges free radicals by interacting with singlet oxygen to exert its antioxidant effect (X. Pan et al., 2016). Histidine causes antioxidant activity due to its imidazole group, which has a chelating and trapping capacity for lipid radicals (Sila and Bougatef, 2016). The synergistic action of the indole, benzene, and phenol rings in the amino acids tryptophan, phenylalanine, and tyrosine is also associated with metal-chelating activity (Zou et al.,

Table 4
Sequences of potentially antioxidant peptides derived from rainbow trout (*O. mykiss*) proteins obtained from in-silico analysis.

Part of protein sequence	Bioactive peptide
IRLESDGSLDDVDEDDVEK	IR
TELHFNHFAENSAFGIVPQKSEDK	LH, EL, LHF AF, VP, GI, FG, TE, PQ, QP
DLKRTKVLADAQIMLDHMK	LK
TPVESGASSAENRAADSTMTTSKPK	KP RA, AA, GA, SG, KP, VE, TP, ST
RGGITCFLKVKCEEEMINDTMK	LK
QHIIDGKTHIINPTDQQRKDHEK	KD GE, DG, PT, EK
SEHEVQDAELRTLLQSSASRKTQK	EL DA, QK, EV, LQ, TQ, AEL, LR
IRCVEEKPVLSLCPVHPVAPSPNPK	PHV, IR, KP IR, VAP, AP, VP, KP, VE, PP, EK, PHLP
EVIRLEKDPPEMLK	KD, IR, LK
ALYTYQLQFKENEIPLKETEK	LY, LK
MSHSAVANGGGPGNHAYLTNPK	AY PG, GP, YL, AY, GP, GG, NG, PG, NK, HK, AV

2016).

Furthermore, various studies have determined that aromatic amino acids such as tyrosine and tryptophan, as well as lysine and histidine and sulfur amino acids such as methionine and cysteine, have great antioxidant potential by donating protons to electron-deficient radicals (Sadat et al., 2011; Wang and de Mejia, 2005).

In the case of sulfur amino acids such as cysteine and methionine, these are prone to oxidation due to the sulfur content, which forms stable oxidation products by reactions with reactive species (Udenigwe and Aluko, 2011).

Anticancer activity of antioxidant peptides

Cancer is a series of diseases caused by cell division of abnormal cells, which present mutations in DNA that affect cell proliferation and death, generating a tumor (Shaik and Sarbon, 2022). One of the causes related to the development of the disease is oxidative stress, a condition caused by the accumulation of ROS that can generate changes in the structure of biomolecules and cellular function (Xing et al., 2022).

Clearly, REDOX balance is essential to the physiological processes of normal cells. However, during cancer development, antioxidants and ROS affect cancer cells differently (Kumari et al., 2018). During the initiation and promotion phases, cancer cells need the production of ROS, which will promote carcinogenesis. In contrast, in the later stages of progression and metastasis, the cancer cell encourages the synthesis of antioxidant enzymes and molecules to protect itself from the attack of ROS (Xing et al., 2022). Therefore, it can be proposed that antioxidant peptides can suppress the production of ROS during the initiation and promotion stages, serve as an alternative therapy, and prevent cancer progression and metastasis stages.

In bioactive peptides, anticancer activity, also described in the literature as anticarcinogenic and antiproliferative activity, refers to the ability of a peptide to inhibit the development of cancer cells or counteract the effects of cancer (Shaik and Sarbon, 2022). These peptides have antiproliferative, antimutagenic, and antioxidant properties (Yaghoubzadeh et al., 2020). Antiproliferative peptides act against cancer cells through disruption of the cytoplasmic membrane, interaction of peptides with gangliosides, and induction of apoptosis (Ding et al., 2011). They are hydrophobic (key point), amphipathic, net charge, secondary structure in the membrane, and oligomerization capacity (Shaik and Sarbon, 2022). Fig. 5 shows the anticancer mechanism of antioxidant peptides.

Peptides of marine animal protein origin have shown the ability to generate apoptosis, antiproliferation, and cytotoxicity (Yaghoubzadeh et al., 2020). Table 5 shows peptide sequences from different species with antiproliferative effects tested in various cell lines. The peptide sequences must be purified and identified to establish the anticancer activity. Low-molecular-weight peptides have been related to greater molecular mobility and diffusivity than heavier ones, allowing for better interaction with cancer cells and enhancing their anticancer activity (Jumeri and Kim, 2011). Hydrophobicity modulation has been reported to be relevant against cancer cells (Huang et al., 2011).

Despite the few studies on the anticancer capacity of peptides derived from rainbow trout proteins, some have been carried out that demonstrate promising anticancer activity. Yaghoubzadeh et al.(2020) recently hydrolyzed rainbow trout skin proteins with Alcalase and obtained peptide fractions with anticancer activity. The authors found that fractions with the highest antioxidant capacity and <3 kDa had the best anticancer effects when tested in the HCT-116 colorectal cancer cell line. Furthermore, in Vázquez et al.(2022) study, after studying the antioxidant capacity of hydrolyzed fractions of *O. mykiss* Alcalase proteins, the cytotoxicity on the human colon adenocarcinoma cell line Caco - 2 / TC-7 of two hydrolytic derivatives with molecular weights between 7.14 and 0.55 kDa was tested. Cellular damage was found at concentrations higher than 0.5 mg/mL (Vázquez et al., 2022). This could be very important when these fractions are tested for their anticancer capacity.

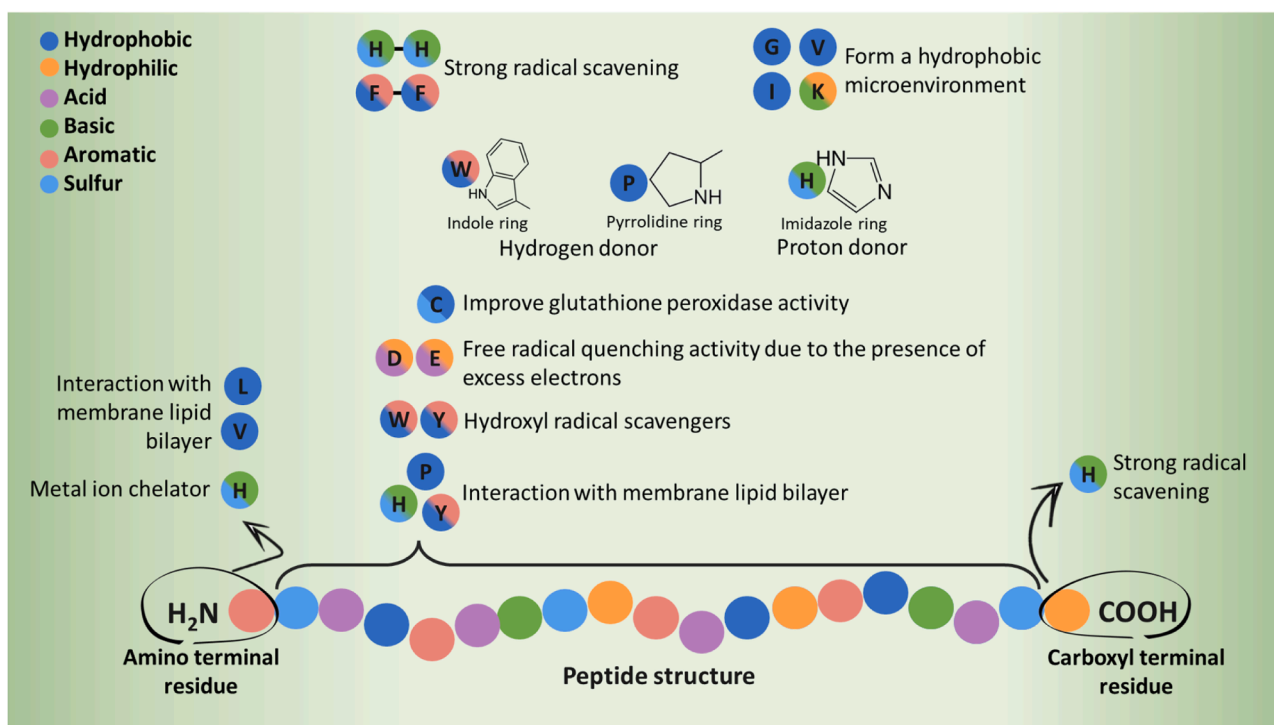


Fig. 4. Relationship between the chemical and physical characteristics of amino acids with the antioxidant activity of peptides. Prepared with information from Zou et al. (2016).

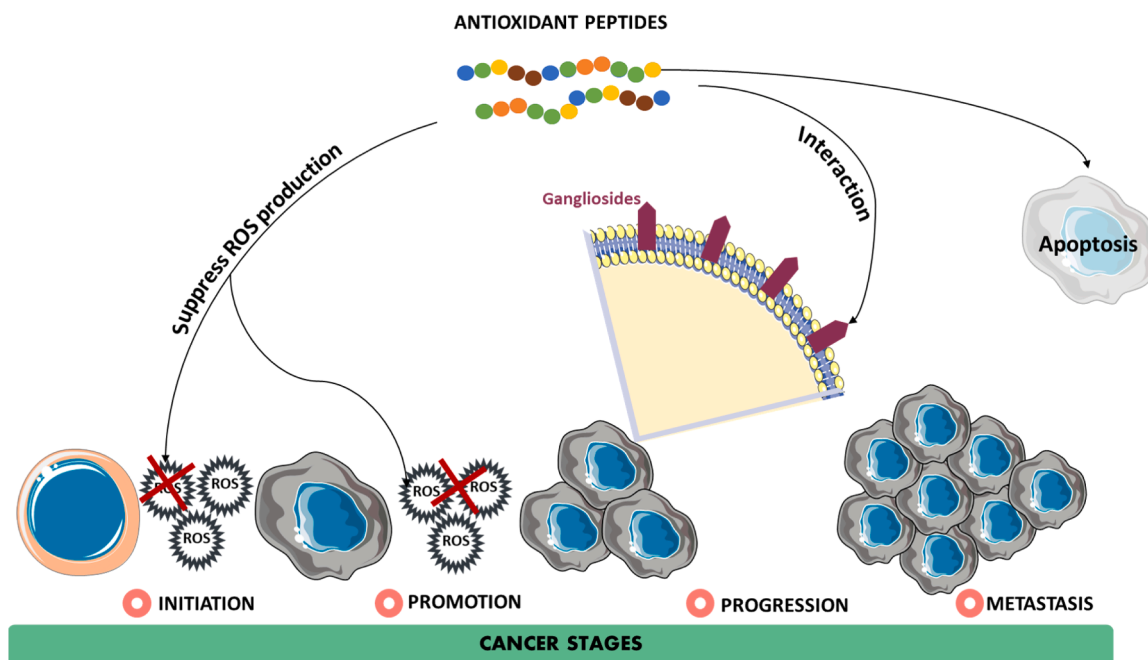


Fig. 5. Anticancer mechanism of antioxidant peptides. Antioxidant peptides suppress ROS production in the early stages of cancer. They interact with gangliosides, significantly enhancing the cytotoxicity and apoptotic activity of the peptides. They also generate cytoplasmic membrane disruption and induce apoptosis.

However, although the preceding studies have not identified peptide sequences with anticancer activity when searching through the BIOPEP database (Minkiewicz et al., 2008) for sequences of anticancer peptides derived from rainbow trout proteins, several can be located, listed in Table 6. This table presents the activity, sequence, and protein details categorized by rainbow trout protein. It includes the peptide names under which they are registered and indicates the locations of the amino acid fractions. Notably, the sequences exhibit repetition despite

originating from different proteins. The in silico data is significant, as these sequences have been recognized for their anti-cancer and antioxidant properties.

These results highlight the potential of proteins from this species for identifying sequences with anticancer properties. The review offers well-contrasted information alongside data from other marine species, emphasizing the significance of this species. It aims to explore utilizing both muscle and by-products of *O. mykiss* in obtaining peptide fractions

Table 5
Antiproliferative peptides in vitro identified in marine species.

Speies	Peptide sequence	Cell line	Reference
<i>Platycephalus fuscus</i>	MGPPGLAGAPGGAGA	HT-29 (Colon)	(Fu and Zhao, 2013)
<i>Setipinna taty</i>	YALPAH	PC-3 (Prostate)	(Song et al., 2014)
<i>Tegillarca granosa</i>	WPP	PC-3 (Prostate), DU-145 (Prostate), H-1299 (lung), HeLa (cervix)	(C.-F. Chi et al., 2015b)
<i>Bryopsis plumosa</i>	LAVISWKCEWNSLWKKRKRKT	NSCLC (lung)	(Kim et al., 2023)
<i>Arca subcrenata</i>	ISMEDVEESRKNGMHSIDVNHGDKHGRAYWADNTYLMKCMD-LPYDVLDTGGKDRSSDKNTDLVDLFELDMVPDRKNNECMNMIMDVIDTNTAARPYCYSLD-VNHGAGLSMEDVEEDK	HeLa (cervix), HepG2 (liver), HT-29 (Colon)	(L. Chen et al., 2013)
<i>Porphyra haitanensis</i>	VPGTPTKNLDSRP, MPAPSCALPRSVVPPR	MCF7 (Breast), HepG2 (liver)	(X. Fan et al., 2017)
<i>Acipenseridae</i>	VDSVLDVVRK	HeLa (cervix),	(Han et al., 2023)
<i>Thunnus tonggol</i>	LPHVLTPEAGAT y PTAEGVYMT	MCF7 (Breast),	(K.-C. Hsu et al., 2011)
<i>Platycephalus fuscus</i>	MGPPGLAGAPGEAGR	HT-29 (Colon)	(Nurdiani et al., 2022)
<i>Pleuronectes americanus</i>	GWGSFFKAAHVKGKHVGAALTHYL-NH ₂	A549 (lung)	(H.-C. Hsu et al., 2022)
<i>Thunnus tonggol</i>	LPHVLTGPAGAT	MCF7 (Breast)	(H. S. Lee et al., 2008)

Table 6
Anticancer peptide sequences of *O. mykiss* proteins obtained by in-silico analysis in the BIOPEP database.

Protein: Beta-2 adrenergic receptor, rainbow trout (<i>O. mykiss</i>)				
Peptide ID	Name of peptide	Activity	Sequence	Location
5472	Anticancer peptide	anticancer	RPK	[269–271]
8318	Dvl protein binding	anticancer	VVV	[155–157], [156–158], [302–304]
Protein: Glucocorticoid receptor, rainbow trout (<i>O. mykiss</i>)				
Peptide ID	Name of peptide	Activity	Sequence	Location
10,468	Antioxidative peptide	anticancer	CG	[404–405]
Protein: Gonadoliberolin III precursor, fragment, rainbow trout (<i>O.s mykiss</i>)				
Peptide ID	Name of peptide	Activity	Sequence	Location
5456	Anticancer peptide	anticancer	LKK	[67–69]
8318	Dvl protein binding	anticancer	VVV	[3–5], [45–47]
Protein: Desmin, fragment, rainbow trout (<i>O. mykiss</i>)				
Peptide ID	Name of peptide	Activity	Sequence	Location
4017	Anticancer peptide	anticancer	YK	[281–282]
5456	Anticancer peptide	anticancer	LKK	[224–226]
Protein: Na/K ATPase alpha subunit isoform 2 of rainbow trout (<i>O. mykiss</i>)				
Peptide ID	Name of peptide	Activity	Sequence	Location
5456	Anticancer peptide	anticancer	LKK	[25–27], [714–716], [761–763]
8318	Dvl protein binding	anticancer	VVV	[247–249], [916–918]
10,468	Antioxidative peptide	anticancer	CG	[454–455]
Protein: Triosephosphate isomerase of rainbow trout (<i>O. mykiss</i>)				
Peptide ID	Name of peptide	Activity	Sequence	Location
4017	Anticancer peptide	anticancer	YK	[52–53]
10,468	Antioxidative peptide	anticancer	CG	[26–27]

with anticancer potential.

Conclusions

Hydrolyzed proteins from *Oncorhynchus mykiss* (rainbow trout) are a promising source of peptides with anticancer potential, yet their application presents challenges and opportunities. A significant challenge is optimizing hydrolysis and fractionation processes to produce peptides with high biological activity and stability. Additionally, further research is needed to elucidate the specific structures of these peptides and their mechanisms of anticancer action, as the precise relationship between their sequences and their efficacy in pharmaceutical and cosmetic applications remains unclear. This gap offers an exciting opportunity for advances in biotechnology and protein chemistry to identify optimal peptide sequences and enhance their functional properties.

Another critical area is ensuring the safety and quality of trout by-products used as raw materials. These by-products must meet stringent safety standards to minimize risks in human applications. Safe handling practices are essential to reduce contaminants and ensure that the compounds derived are safe and effective, a priority for the pharmaceutical and cosmetics industries.

Moreover, the utilization of trout by-products supports waste reduction in the food industry and aligns with circular economy principles. Valorizing these by-products not only contributes to creating high-value products but also promotes environmental sustainability, underscoring the potential of rainbow trout as a source of safe and effective bioactive peptides for various industrial applications.

Ethical statement – studies in humans and animals

Authors declare that in the review derived from the article Rainbow trout (*Oncorhynchus mykiss*) proteins and composition: Source of antioxidant peptides with promising anticancer activity there are not experiments in animals or humans

CRedit authorship contribution statement

M.F. Escamilla-Rosales: Writing – original draft, Data curation. **E. Pérez-Escalante:** Investigation, Conceptualization. **C.E. Jara-Gutiérrez:** Writing – review & editing, Validation, Supervision, Investigation. **P.A. Santana-Sepúlveda:** Writing – review & editing, Resources,

Project administration, Investigation, Conceptualization. **C.A. Álvarez-Álvarez:** Resources, Project administration, Methodology. **A. Castañeda-Ovando:** Investigation, Data curation. **L.G. González-Olivares:** Writing – review & editing, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Capítulo IV

Identificación in silico, síntesis y validación in vitro de péptidos anticancerígenos de proteínas de trucha arcoíris

Resumen

El creciente interés en los compuestos anticancerígenos como agentes terapéuticos selectivos, junto con el uso de herramientas digitales para predecir secuencias con potencial bioactivo, ha fomentado la combinación de estrategias *in silico* e *in vitro* para potenciar la búsqueda de candidatos farmacológicos. En este capítulo se presentan los resultados de la validación *in vitro* de la actividad anticancerígena de los péptidos obtenidos *in silico* de las proteínas de trucha arcoíris (*Oncorhynchus mykiss*). Si bien, las investigaciones recientes han identificado numerosos péptidos *in silico* con una amplia diversidad de bioactividades, son relativamente pocos los estudios que han probado o validado experimentalmente su efecto biológico. Razón por la que el objetivo principal fue sintetizar y determinar la actividad anticancerígena de los péptidos identificados, datos que se encuentran publicados en la revista Biointerface Research in Applied Chemistry.

A partir de la identificación de cinco péptidos anticancerígenos provenientes de proteínas de trucha arcoíris mediante la base de datos BIOPEP, se sintetizaron empleando la metodología en fase sólida Fmoc-Rink amida. Posteriormente, se evaluó su efecto citotóxico sobre las líneas celulares PC-3 de cáncer de próstata humano, AGS de adenocarcinoma gástrico humano, MDA-MB-231 de cáncer de mama humano, MCF-7 cáncer de mama humano, HT-29 adenocarcinoma colorrectal humano, HeLa cáncer de cuello uterino y GES-1 de célula epitelial gástrica humana. Los resultados obtenidos indicaron que, bajo las condiciones evaluadas, los péptidos sintetizados no ejercieron una toxicidad significativa frente a las líneas celulares analizadas. Estos resultados resaltan la importancia de optimizar las estrategias y los criterios de selección utilizados al momento de realizar la búsqueda de compuestos bioactivos, así como acompañar el análisis *in silico* con una validación experimental. Lo anterior, con la finalidad de incrementar la posibilidad de identificar péptidos con potencial aplicación en el desarrollo de nuevas terapias contra el cáncer.

In-silico Identification and Experimental Validation of Rainbow Trout-Derived Anticancer Peptides

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Received: 14.08.2025; Accepted: 14.01.2026; Published: 15.04.2026

Abstract: Anticancer peptides have gained increasing attention as therapeutic agents, with marine species recognized as potential sources of bioactive peptides for pharmacological uses. This study aimed to evaluate the anticancer activity of *in silico*-predicted peptides derived from rainbow trout proteins. Peptides were synthesized using the solid-phase Rink amide/Fmoc method and tested for cytotoxicity via the sulforhodamine B assay across seven cancer cell lines. The results showed that, at concentrations from 0.01 µg/mL to 1 mg/mL, the five synthesized peptides generally did not exhibit significant toxicity against the cell lines tested, except for peptide CG, which resulted in 60.8% cell viability in the MCF-7 breast cancer line. These findings reveal the limitations of *in silico*-predicted peptides and emphasize the importance of refining predictive methods by combining multiple computational tools and physicochemical criteria to improve the chances of discovering anticancer peptides suitable for developing new cancer treatments.

Keywords: solid phase; cytotoxicity; cancer.

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1. Introduction

Cancer is a group of disorders characterized by the invasion and growth of abnormal cells [1]. Globally, it ranks among the leading causes of death, with 20 million new cases and 9.7 million deaths in 2022 [2]. Standard treatments such as surgery, radiation, and chemotherapy have significant drawbacks, including damage to normal cells and the development of tumor cell resistance [3]. Faced with this problem, more effective advanced therapeutic approaches have been suggested, such as gene therapy, stem cell use, ablation, and

targeted pharmacological treatments [4]. Likewise, bioactive peptides, both natural and synthetic, have been regarded as promising therapeutic options [2]. Specifically, marine peptides derived from proteins in edible products or by-products show potential for developing drugs with anticancer activity and treating conditions related to oxidative stress [3, 5]. These peptides are typically short, highly selective, exhibit effective tumor penetration, and are less immunogenic [6]. Additionally, because of their short length, their synthesis is simpler and less expensive.

In general, several mechanisms by which marine peptides induce apoptosis have been described, including microtubule or mitochondrial disruption, vascular inhibition, activation of death receptors, and direct cytotoxicity [7]. Some marine peptides approved by the US FDA include ziconotide, Adcetris®, eribulin, and trabectedin, which are derived from marine mollusks, sponges, and tunicates [8]. Several studies have characterized peptides obtained from collagen and from rainbow trout byproducts, revealing diverse bioactivities, including anticancer potential [9-11]. However, most of these studies do not report the peptide sequences.

A valuable approach to predicting peptide sequences with anticancer activity from proteins is *in silico* analysis [12]. In this context, the BIOPEP database enables the identification of potential bioactive sequences in food proteins using integrated tools [13]. This database is recognized and used in research for its broad coverage of real bioactive peptides. It also allows simulating hydrolysis conditions to predict peptides and their potential bioactivities with an accuracy of up to 54% [14]. The main limitation affecting the agreement between theoretical and experimental results is that not all laboratory conditions can be simulated.

These peptides can be produced through enzymatic hydrolysis, fermentation, or chemical synthesis. Chemical peptide synthesis relies on the condensation reaction between the carboxyl group of one amino acid and the amino group of another. In solid-phase synthesis, the peptide is attached to a resin, and amino acids protected by their amino groups and side chains are added sequentially, with their carboxyl groups coupling to the growing chain. Once the desired peptide is formed, it is separated from the resin and purified [5, 15]. This method enables the mass production of bioactive peptides for pharmaceutical applications [15].

Although the potential of *in silico* analysis and chemical synthesis for identifying and producing anticancer peptides has been recognized, evidence confirming their biological activity remains limited. Based on this gap, we hypothesized that peptides predicted *in silico* from rainbow trout proteins exhibit measurable anticancer activity upon chemical synthesis. Therefore, this study aimed to identify promising sequences through *in silico* screening, synthesize the selected peptides by solid-phase methods, and validate their biological potential by assessing *in vitro* cytotoxicity.

2. Materials and Methods

2.1. *In-silico* search for peptides in the BIOPEP database.

The protein sequences of *Oncorhynchus mykiss* were obtained from the UniProt database (<https://www.uniprot.org>), selecting only reviewed and complete proteins associated with this species (taxid: 8022). The sequences were downloaded in FASTA format and checked for redundancies or incomplete fragments. Then, each sequence was analyzed in the BIOPEP-UWM database (https://biochemia.uwm.edu.pl/biopep/start_biopep.php) using the Profile of Bioactive Peptides tool to identify peptides previously reported with anticancer activity. To do this, the sequences were entered individually into the search engine, and all subsequences

matching database entries that had the "anticancer" activity annotation or synonyms accepted by BIOPEP were recorded. In addition, database filtering criteria were applied, prioritizing peptides with higher frequency across multiple proteins, documented experimental evidence, and activity annotations supported by peer-reviewed literature. Only peptides with complete sequence information and confidence indicators reported in BIOPEP were retained for further analysis.

For each match, the peptide identifier in BIOPEP, the amino acid sequence, the protein of origin, and the associated bibliographic reference were documented. The position of the peptide sequence within the original protein and the type of experimental evidence reported by BIOPEP were also recorded. This process was repeated for all selected proteins, ensuring the traceability of the results by preserving the consultation dates, the database version used, and the search history. Based on these criteria, the final five peptides were selected as the most promising candidates for experimental validation. Finally, a summary table was created with the list of identified anticancer peptides, previously reported in *O. mykiss*.

2.2. Solid-phase chemical synthesis of peptides.

Peptide synthesis was performed in reactors with Rink amide resin 0.74 meq/g. The resin was washed and deprotected.

The amino acid was then added according to the peptide sequence, along with the corresponding coupling activator, the Oxime additive (Iris Biotech GmbH), and N, N-diisopropylethylamine (Iris Biotech GmbH). The activators used in the synthesis included: TBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) (Iris Biotech GmbH); HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (Iris Biotech GmbH); and HCTU (O-(1H-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (Merck).

Once all the amino acids in the sequence were added, the peptide was cleaved. For peptide CG, a solution of TFA, TIS, ultrapure water, and 2,2'-(ethylenedioxy) diethanol (DOTA) at 92.5:2.5:2.5:2.5 was used. After cleavage, the peptides were precipitated with cold ether and centrifuged for 10 minutes at 3500 rpm. Finally, the solvent was evaporated, and the peptides were lyophilized until further use. The mass of the synthesized peptide was confirmed by electrospray ionization mass spectrometry (ESI-MS), and its purity was analyzed through reversed-phase high-performance liquid chromatography (RP-HPLC).

The peptides were purified on Sephadex-G10 to be used in functional analysis.

2.3. Sulforhodamine B cytotoxicity assay.

The cytotoxic effect of synthetic peptides was tested on seven cell lines (PC-3, AGS, MBA-MB-231, MCF-7, HT29, HELA, and GES-1). Each cell line was plated in 96-well plates at a density of 5×10^3 cells per well in 100 μ L of medium and incubated for 24 hours before treatment. Treatments included a control with no intervention (C), a solvent control (CS) with added Milli-Q water, and the synthesized peptides at six different concentrations from 1 mg/mL to 0.01 μ g/mL, these values were chosen from published data that relate concentrations below 125 μ g/mL as extracts with high potential as therapeutic agents against cancer, while concentrations above 5 mg/mL have low potential [16]. The treatments were incubated for 72 hours. After incubation, cells were fixed with 50% w/v trichloroacetic acid for one hour at 4°C. They were washed with distilled water and stained with 50 μ L of 0.1% w/v sulforhodamine B

in 1% v/v acetic acid for 30 minutes. To remove excess dye, samples were washed with 150 μ L of 1% acetic acid, and the dye was solubilized with 100 μ L of 10 mM Tris Base. Absorbance was measured at 540 nm using a microplate reader. The percentage of viable cells was calculated using the viability formula:

$$\% \text{ viability} = \left(\frac{Abs_{\text{sample}}}{Abs_{\text{control}}} \right) \times 100 \quad (1)$$

Untreated cells were used as the reference control and considered as 100% viability. The results of six replicates were expressed as the mean $\pm$ standard deviation. Based on these data, the mean effective concentration (EC₅₀) for each extract and each cell line was determined using the SigmaPlot 11.0 software.

3. Results and Discussion

3.1. Peptide identification.

In silico analysis identified five sequences with potential anticancer activity derived from rainbow trout proteins. The features of these small peptides are shown in Table 1, including net charge, isoelectric point, and hydrophobicity analysis [17]. These peptides consist of combinations of two or three amino acids, including arginine, proline, lysine, valine, cysteine, glycine, leucine, and tyrosine. It has been reported that the presence of tyrosine, glycine, cysteine, and proline in ultrashort peptide sequences can significantly enhance their bioactivity [6]. Additionally, peptides containing basic residues such as arginine are common in tumor-homing peptides, which bind to cancer cells [18].

Table 1. *In silico* prediction of anticancer peptides from *O. mykiss* proteins using the BIOPEP database.

Sequence	Peptide ID	Name of peptide	Protein source (<i>O. mykiss</i>)	Net charge (pH 7) + Theoretical pI	Brief analysis
RPK	5472	Anticancer peptide	Beta-2 adrenergic receptor	+2; pI $\approx$ 11.8	Dominated by Arg and Lys, giving a strongly basic profile; high pI suggests strong cationic behavior typical of membrane-active anticancer peptides.
VVV	8318	Dvl protein binding	β 2-adrenergic receptor; Na/K ATPase α -2; Gonadoliberolin III precursor	0; pI $\approx$ 6.0	Neutral and hydrophobic, with a pI close to physiological pH; lacks ionizable side chains, contributing to a balanced but highly hydrophobic character.
CG	10468	Antioxidative peptide	Glucocorticoid receptor; Na/K ATPase α -2; Triosephosphate isomerase	0; pI $\approx$ 5.6	Contains Cys (pKa 8.3) but overall behaves as a neutral peptide at pH 7; slightly acidic pI due to terminal charges.
LKK	5456	Anticancer peptide	Na/K ATPase α -2; Gonadoliberolin III precursor; Desmin	+2; pI $\approx$ 11.1	Highly basic due to two Lys residues; elevated pI suggests strong positive charge density, favoring interaction with negatively charged membranes.
YK	4017	Anticancer peptide	Desmin; Triosephosphate isomerase	+1; pI $\approx$ 9.8	Lys contributes to a basic pI; Tyr adds moderate hydrophobicity and potential for π -interactions, balancing charge and aromaticity.

One of the identified peptides, made up entirely of three valine residues, can be considered hydrophobic. In this context, hydrophobicity has been shown to affect the anticancer and hemolytic activity of peptides, as well as their selectivity [19]. Peptide selectivity refers to the ability to target cancer cells without harming healthy tissues [20].

Additionally, ultrashort peptides are deemed suitable for biomedical uses and the development of new drugs.

The inclusion of additional predictive tools such as PeptideRanker, ToxinPred, and other complementary algorithmic platforms could further enhance the accuracy and robustness of peptide selection. While BIOPEP provides valuable information on previously reported bioactivities, tools like PeptideRanker offer probabilistic scoring based on machine-learning models that estimate the likelihood of a peptide exhibiting biological activity, enabling more quantitative prioritization of candidates. Likewise, ToxinPred enables the assessment of potential toxicity, an essential parameter for early filtering when selecting anticancer peptides, by helping differentiate active peptides from those with undesirable toxic profiles. Integrating multiple predictive approaches would therefore broaden the evaluation criteria, reduce false positives, and increase confidence in the final peptide shortlist by combining empirical evidence, activity likelihood estimates, and safety predictions into a unified selection strategy [21].

However, although several peptides with promising biological activities have been identified as potential drugs, their bioactivity is not always confirmed due to costs or difficulties in obtaining them [3]. In this context, the development of solid-phase peptide synthesis enables the large-scale chemical production of peptides [22]. This method was used to produce the five peptides listed in Table 1, which, after synthesis and cleavage, were analyzed by ESI-MS and RP-HPLC. Figure 1 displays the mass spectra together with their corresponding chromatograms, where each spectrum exhibits a dominant peak matching the expected molecular mass, and chromatographic profiles confirm the high purity of the synthesized peptides. Table 2 summarizes the analytical characterization of five synthesized peptides, including retention time (rt), purity percentage, and both theoretical and experimentally observed molecular masses. The retention times ranged from 1.31 to 1.42 minutes, indicating consistent chromatographic behavior typical of low-molecular-weight peptides. Purity levels exceeded 90% for most peptides, with YK achieving 100% purity, confirming the efficiency of both the synthesis and purification processes. The observed molecular masses, determined by ESI-MS, showed close agreement with the theoretical values. This strong correspondence validates the chemical identity of the synthesized peptides and supports the reliability of the synthetic procedure. Overall, the results confirm that the peptides obtained meet the analytical standards required for subsequent biological activity assays or functional studies.

Therefore, the sequences obtained are considered to have potential as anticancer agents. In the search for therapeutic alternatives for cancer, *in silico* prediction tools, combined with chemical synthesis and *in vitro* validation, offer a strategy to reduce time, costs, and effort.

Table 2. Analytical characterization of the synthesized peptides.

Peptide	rt*(min)	% purity	Molecular mass (Da)	
			Theoretical	Observed
RPK	1.38	90.2	398.4	399.3
VVV	1.38	90.7	314.4	315.3
YK	1.42	100	308.3	309.2
CG	1.31	91.7	177.2	352.5
LKK	1.38	96.5	386.5	387.4

* retention time

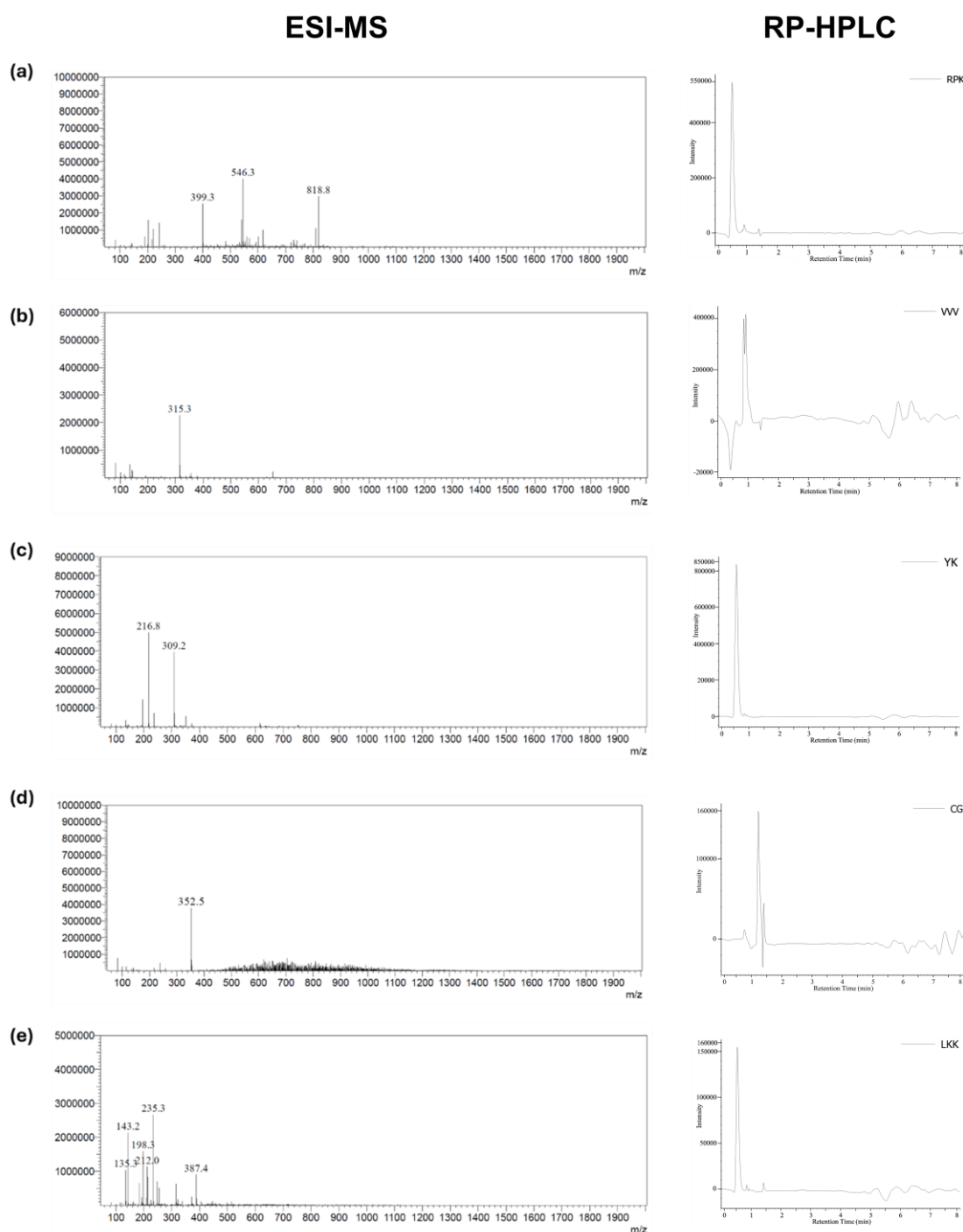


Figure 1. ESI-MS spectrum and chromatograms of the synthesized peptides obtained *in silico*. **(a)** RPK peptide; **(b)** VVV peptide; **(c)** YK peptide; **(d)** CG peptide; **(e)** LKK peptide. Improved resolution of chromatogram axes.

3.2. *In-vitro* cytotoxicity.

The cytotoxicity of six concentrations of the synthesized peptides, ranging from 0.01 $\mu\text{g/mL}$ to 1 mg/mL , was evaluated against seven tumor cell lines and one non-tumor cell line. Overall, the peptides did not show toxicity against the cells at these concentrations (data available in the supplementary material), as they presented viability percentages greater than 80%, with the exception of the CG peptide, which at 1 mg/mL showed a viability of $60.80 \pm 2.62\%$ in the MCF-7 breast cancer cell line, compared to $85.46 \pm 2.52\%$ in the non-tumorous line GES-1 (Figure 2). Due to its concentration and the effect observed in the GES-1 line, the peptide was considered to have mild cytotoxicity. The IC_{50} of all peptides is $>1 \text{ mg/mL}$.

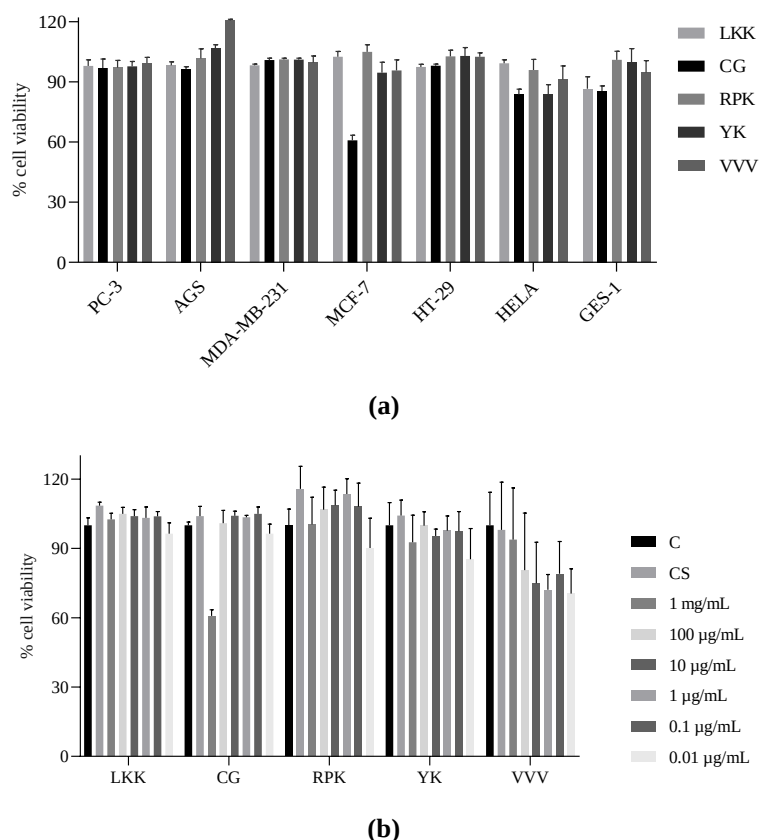


Figure 2. Percentage of cell viability in cell lines exposed to synthesized peptides. **(a)** cell lines exposed to 1 mg/mL of the synthesized peptides; **(b)** cell viability in breast cancer cell line MCF-7 exposed to different concentrations (0.01 µg/mL – 1 mg/mL) of the synthesized peptides. Data expressed as the mean of 6 replicates $\pm$ standard deviation. PC-3: Human prostate cancer, AGS: Human gastric adenocarcinoma, MDA-MB-231: Human breast cancer, MCF-7: Human breast cancer, HT-29: Human colorectal adenocarcinoma, HELA: Cervical cancer, and GES-1: Human gastric epithelial cell.

These results indicate that the *in silico* strategy for predicting peptides with potential anticancer activity was not sufficiently accurate, as significant activity was not confirmed in *in vitro* tests on the selected cell lines.

The results may be related to the characteristics of the peptides. The VVV peptide, being short and highly hydrophobic, is prone to forming insoluble aggregates due to the tendency of its side chains to enclose themselves within the structure, thereby decreasing their interaction with the cell membrane [23, 24]. Conversely, the LKK and RPK peptides are not very hydrophobic, which may hinder their interaction with membranes [24, 25]. In the specific case of peptide YK, its composition of an aromatic and a polar amino acid allows it to behave as a minimal amphipathic motif, a characteristic that may favor its interaction with membranes. However, its short length may limit its cytotoxic effect [24, 26]. With peptide CG, we observed a slight toxic effect, which could be related to cysteine's ability to form disulfide bonds and alter the peptide's structure, helping it to remain stable [27].

These results suggest the need for more selective and precise methodologies are needed for the identification of bioactive peptides. Specific refinements could further strengthen peptide selection. Computationally, molecular docking and short molecular-dynamics simulations would help assess binding affinity and structural stability beyond sequence-based predictions. QSAR models trained on anticancer peptide datasets could also improve discrimination of active motifs. Biochemically, testing simple stability-enhancing

modifications, such as N-terminal acetylation, C-terminal amidation, or limited D-amino acid substitutions, could increase peptide resistance to degradation while preserving activity [28].

Previous studies have identified *in silico* peptides with potential anticancer activity. For example, Wu *et al.* [13] used a bioinformatics strategy combining tools such as BIOPEP-UWM, PeptideRanker, ToxinPred, and ProtParam to analyze peptides derived from *Arca*. Although this strategy allowed them to identify 17 candidate peptides, only three showed anticancer activity after *in vitro* validation, while others even promoted cell proliferation.

Similarly, Law *et al.* [29] applied a strategy combining programs such as ADP3, AMPfun, ANTICP, ProtParam, ToxinPred2, and ADMETlab2.0 to design and predict anticancer peptides derived from the fish *Anabas testudineus*. Despite using multiple tools, only one peptide exhibited significant cytotoxicity against the MDA-MB-231 cell line.

In both cases, the combination of different tools enabled a more exhaustive selection of peptides with anticancer potential. Although this procedure does not guarantee bioactivity in *in vitro* tests, it does improve the selection of peptides with bioactive potential. Furthermore, to control peptide dynamics and aggregation *in silico*, structures could be designed in which hydrophobic and polar amino acids are strategically positioned [25].

4. Conclusions

Peptides identified *in silico* from rainbow trout proteins as potential anticancer agents did not exhibit the predicted activity after synthesis and *in vitro* evaluation. This finding reinforces the importance of complementing *in silico* searches with experimental assays that confirm or refute their bioactivity. It also highlights the value of simultaneously employing multiple digital tools to optimize peptide selection. Furthermore, the results underline the need to refine the predictive workflow by integrating complementary algorithms and structural evaluation techniques to reduce false-positive hits. While this approach does not guarantee the presence of biological activity, it does increase the likelihood of identifying bioactive peptides with pharmacological potential, key aspects in the search for new therapies against diseases.

A key limitation of this study is that the selected peptides were short, low-hydrophobicity peptides that lacked structural optimization, which likely reduced their stability and capacity to interact with cancer cells. These factors should be addressed in future selection and validation strategies, with a particular emphasis on strengthening the integration of *in silico* and *in vitro* tools to enhance the accuracy and efficiency of peptide-based drug discovery.

Author Contributions

Conceptualization, M.B., F.G., and L.O.; methodology, F.E., C.J., J.V., and P.S.; data curation, F.E., A.C., C.A., and F.G.; investigation, C.J., J.V., M.B., A.C., C.A., and L.O.; validation, C.J. and J.V.; writing—original draft preparation, F.E.; writing—review and editing, C.J., P.S., and L.O.; supervision, C.J., J.V., and L.O.; resources, P.S.; project administration, P.S. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

Funding

This study was funded by FONDECYT 1231088 and FOVI 230188 (Paula Santana) from ANID-Chile.

Acknowledgments

The authors appreciate the support of SEIHTI for the Ph.D. scholarship granted to María-Fernanda Escamilla-Rosales and the ANID-Chile for the project support.

Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary materials

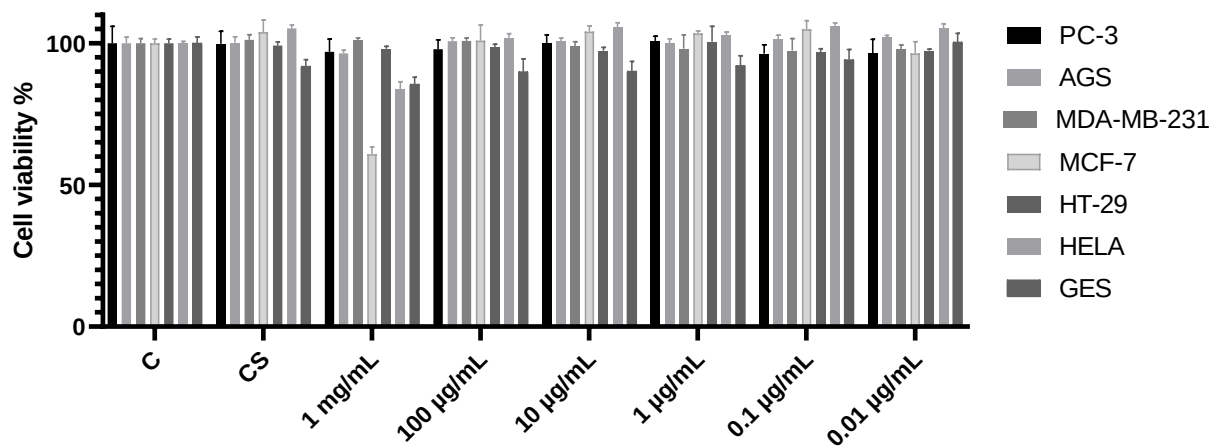


Figure S1. Percentage of viability of cell lines exposed to the CG peptide.

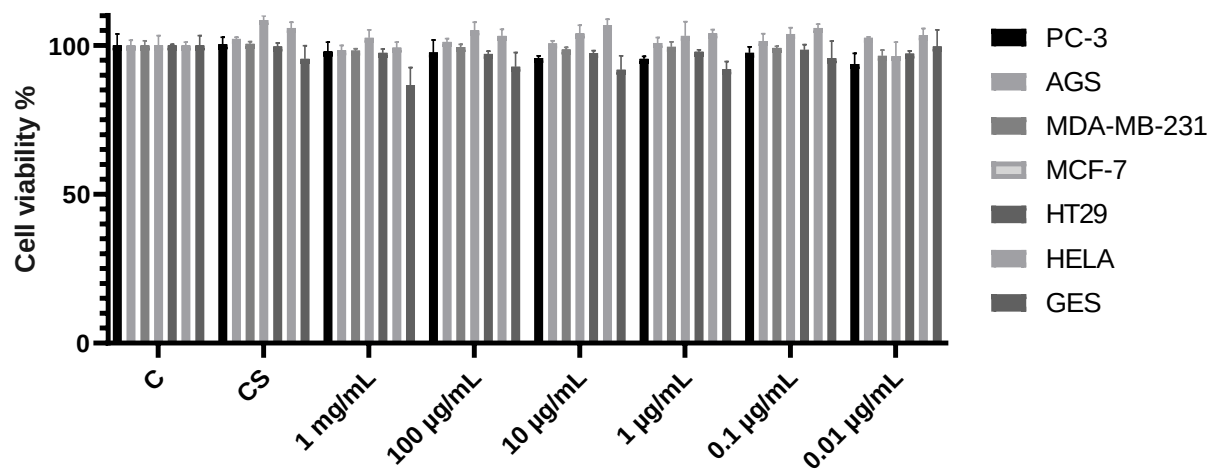


Figure S2. Percentage of viability of cell lines exposed to the LKK peptide.

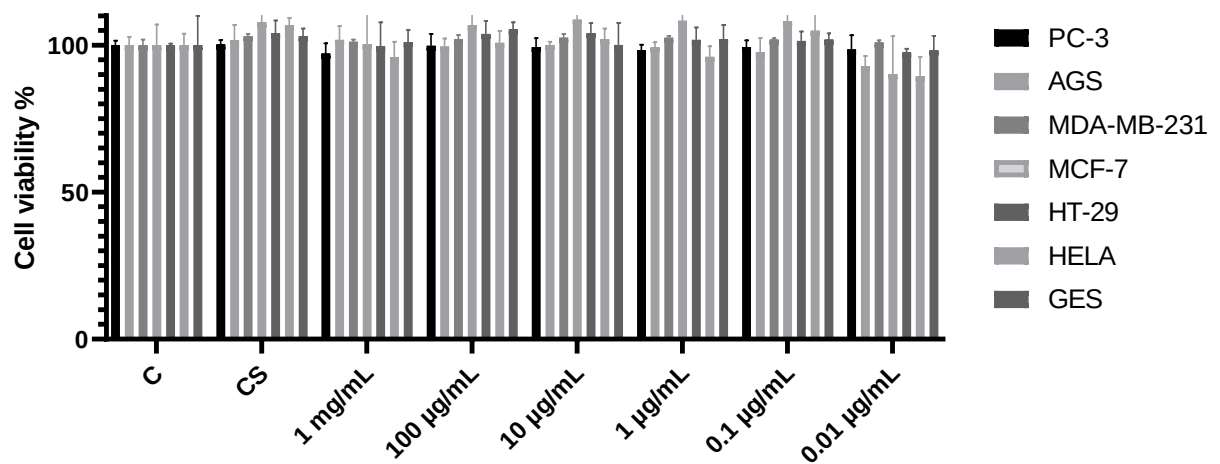


Figure S3. Percentage of viability of cell lines exposed to the RPK peptide.

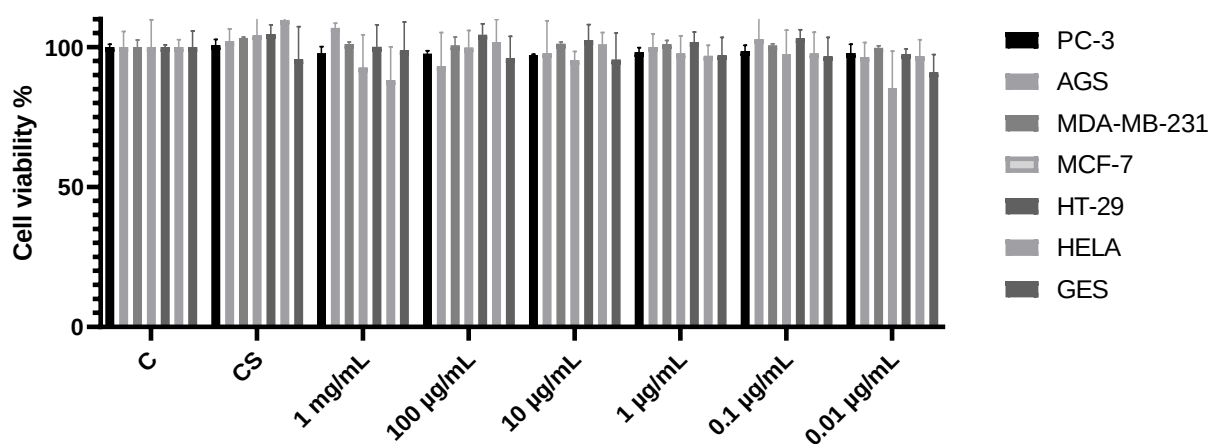


Figure S4. Percentage of viability of cell lines exposed to the YK peptide.

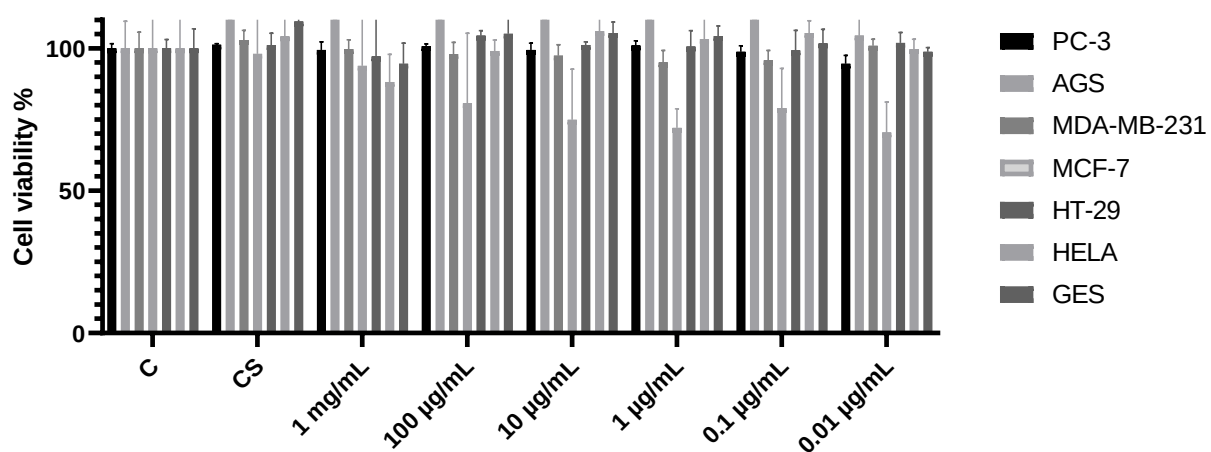


Figure S5. Percentage of viability of cell lines exposed to the VVV peptide.

Capítulo V

Capacidad antioxidante de péptidos derivados de la hidrólisis enzimática de la piel de trucha arcoíris (Oncorhynchus mykiss): Efecto del pretratamiento de pasteurización y ultrasonificación

Resumen

En este capítulo se presentan y discuten los resultados obtenidos sobre el efecto de la pasteurización y la ultrasonificación como pretratamientos aplicados antes de la hidrólisis enzimática con alcalasa o flavourzyme de la piel de trucha arcoíris. El objetivo fue evaluar como estos pretratamientos influyen en la actividad antioxidante de los hidrolizados. Para cumplir el objetivo, se caracterizaron los hidrolizados obtenidos y se determinó su actividad antioxidante mediante los ensayos de DPPH y FRAP, con el fin de identificar cuál pretratamiento mejoraba la respuesta antioxidante. Los resultados de este estudio se encuentran publicados en la revista *Journal of Chilean Chemical Society*.

Para poder comparar el efecto de los pretratamientos, las muestras se homogeneizaron y, a partir de búsqueda bibliográfica se establecieron las condiciones de pasteurización, ultrasonificación e hidrólisis (relación enzima: proteína, temperatura, tiempo y pH) que previamente se habían reportado como óptimas para maximizar el grado de hidrólisis y actividad antioxidante. A los hidrolizados obtenidos se les determinó el grado de hidrólisis mediante la liberación de grupos amino libres; además, se cuantificó el contenido de proteína y se separaron los péptidos de bajo peso molecular mediante electroforesis en gel de poliacrilamida Tris-Tricina. Posteriormente, se midió la actividad antioxidante utilizando dos métodos: DPPH, que permite identificar la actividad antioxidante por compuestos hidrofóbicos, y FRAP para evaluar la capacidad del compuesto de reducir el ion férrico Fe^{3+} a ferroso Fe^{2+} . En general, la pasteurización es el pretratamiento que mayor efecto tiene sobre el perfil proteolítico y actividad antioxidante de los hidrolizados. Finalmente, la información contenida en este capítulo ayudó a establecer los parámetros necesarios para el diseño de experimentos enfocados en optimizar la hidrólisis enzimática de las proteínas de piel de trucha arcoíris.

ANTIOXIDANT CAPACITY OF PEPTIDES DERIVED FROM THE ENZYMATIC HYDROLYSIS OF RAINBOW TROUT (*Oncorhynchus mykiss*) SKIN: EFFECT OF PASTEURIZATION AND ULTRASONICATION PRE-TREATMENT

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ABSTRACT

Rainbow trout skin and viscera are considered non-commercial by-products with high protein content. This research aimed to determine the incidence of pasteurization and ultrasonication during the obtaining of antioxidant peptides by the hydrolysis of trout skin proteins with Alcalase and Flavourzyme. Solutions of freeze-dried skin were prepared (20 % w/v), and two pretreatments were performed: pasteurization (90 °C for 10 min) and a combination of pasteurization and ultrasonication (40 kHz x 15 min). Hydrolysis was for 8 hours with Alcalase (pH 9, 55°C) and Flavourzyme (pH: 7, 50°C) sampling every 2 hours. The degree of hydrolysis was determined by TNBS and SDS-PAGE. Antioxidant activity was determined by DPPH and FRAP. The ultrasonication does not affect the release of peptides with antioxidant capacity. In addition, greater antioxidant capacity was determined in hydrolysates with Flavourzyme. Results would allow establishing the conditions for the optimized obtaining of this type of peptide fractions.

Keywords: Enzymatic hydrolysis; antioxidant activity; skin trout proteins; bioactive peptides, pre-treatment.

INTRODUCTION

Rainbow trout (*Oncorhynchus mykiss*) is characterized by its ability to adapt to different habitats by modifying its nutritional content [1]. However, the increase in the production and commercialization of this species has generated a greater amount of waste such as heads, bones, skin, and viscera [2]. These wastes have a high protein content, so they are used in the generation of bioactive peptides, which are of interest to the food, pharmaceutical, or cosmetic industries [3]. One method to obtain these peptides is through enzymatic hydrolysis [4]. Currently, in search of increasing the yield and biological activity of these peptides, the use of enzymes has been combined with the application of new technologies [5]. One of these technologies is ultrasonication which, through cavitation, shear, shock waves, and heat, causes physical and chemical changes that affect the protein, increasing the degree of hydrolysis and improving the antioxidant capacity of the peptides [6]. In the case of pasteurization, this heat treatment causes a breakdown of the protein, facilitating the enzymatic reaction and resulting in a greater number of peptides with antioxidant activity [7], [8]. That is why the objective was to know the effect of pasteurization and ultrasonication as pretreatments in enzymatic hydrolysis on the antioxidant activity of rainbow trout skin peptides.

EXPERIMENTAL SECTION

On a farm in the state of Hidalgo, Mexico, 15 fresh rainbow trout were acquired. The skin was separated, cleaned, and cut into 2 cm pieces to be frozen and freeze-dried. With freeze-dried rainbow trout skin, a 1:4 (w/v) suspension was prepared with deionized water, and it was used to prepare a 20% solution with 0.1 M Tris-HCl buffer. Subsequently, the solutions were divided into two groups: pasteurization (90°C x 10 min) as pretreatment and pasteurization plus ultrasonication (40 kHz x 15 min). The suspensions were hydrolyzed with Alcalase (pH 9, 55°C) and Flavourzyme (pH: 7, 50°C) for 0, 2, 4, 6 and 8 hours.

Free amino groups were measured using the trinitrobenzene sulfonic acid (TNBS) method [9]. 250 µL of sample, 2 ml of phosphate buffer, and 2 mL of 0.1% TNBS solution were mixed. It was incubated for 1 h at 50°C, and the reaction was stopped with 4 ml of 0.1 N HCl. The absorbance was measured at 340 nm. To determine the content, a standard curve was performed with glycine. The results were expressed as g/L of free amino groups (NH²).

The separation of the peptides was carried out by Tris-Tricine polyacrylamide gel electrophoresis following the methodology of Schägger and von Jagow [10], with modifications by González-Olivares et al., [11]. Separation and concentration gels were made with 15% and 4% acrylamide respectively, from a 40% Bio-Rad Acrylamine solution (19:1 ratio of acrylamide: bisacrylamide with 5% crosslinker) in Tris-HCl buffer. In an electrophoresis chamber, the gels were placed in Tris-Tricine-SDS-HCl buffer (pH: 8.25), and the samples were injected together with the standard. The electrophoretic run was run at 95V for 6 hours. Gels were stained with Coomassie blue G-250 (Bio-Rad) and analyzed by Gel-Doc software.

The antioxidant activity of the hydrolysates was measured by the DPPH and FRAP methods following the methodology proposed by Ramírez-Godínez et al., [12]. For the determination by DPPH, 100 µL of hydrolysate, with 2.9 mL of DPPH reagent (0.1 mM 2,2-diphenyl-1-picrylhydrazyl in methanol) was placed in tubes. After 50 min the absorbance was measured at 515 nm. A standard curve was performed with Trolox. The results were expressed as IC₅₀. For the FRAP methodology, 250 µL of the hydrolysates together with 1 ml of FRAP reagent (300 acetate buffer pH 3.6, 10mM TPTZ solution, and 20 FeCl₃ 6H₂O solution) were added to 10 mL with deionized water. They were incubated at 37°C for 15 min and the absorbance was measured at 593 nm. A curve was performed with FeSO₄ · 7H₂O in 40 mM HCl. The results were expressed as mg equivalents of Fe²⁺.

The protein concentration in the supernatants of the rainbow trout skin suspensions had a significant difference before pasteurization and after pasteurization (Table 1). In the case of the suspension at pH 7, it was 1.84 to 1.99 g/L and in the suspension at pH 9, it was 1.67 to 1.83 g/L, before and after pasteurization respectively. The higher concentration of proteins in pasteurized suspensions is because exerted by heating and a pH higher than 6 [7], [13]. Due to these results, pasteurization pretreatment was applied to all samples. Likewise, the enzymatic hydrolyses were applied according to the optimal pH of each of the enzymes used (Alcalase pH 9 and Flavourzyme pH 7). Four different suspensions were obtained to hydrolyze according to the pretreatments and enzymes used (PA, pasteurized sample hydrolyzed with Alcalase; PUA, pasteurized and ultrasonicated sample hydrolyzed with Alcalase; PF, pasteurized sample hydrolyzed with Flavourzyme and PUF to the pasteurized and ultrasonicated hydrolyzed sample with Flavourzyme).

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Table 1. Effect of pasteurization on the protein content of rainbow trout skin suspension after centrifuge*

Treatment	pH	Protein	
		Supernatant mg/L	Sediment %
No treatment	7	1848.69 ± 25.96	17.34
	9	1673.99 ± 10.57	16.39
Pasteurization	7	1990.26 ± 13.91	17.00 ± 0.28
	9	1832.63 ± 38.37	14.47 ± 0.40

*20% (w/v) suspension of rainbow trout paste with 0.1M Tris-HCl buffer

The protein content of the supernatant and sediment were determined with the Bradford and Kjeldahl methods, respectively.

According to the results obtained in the analysis by the TNBS technique, differences were observed in the concentration of free amino groups (Table 2). The Alcalase enzyme caused a higher degree of hydrolysis even immediately after adding the enzyme (PA: 5.97 g/L NH₂⁻, PUA: 5.00 g/L NH₂⁻). Subsequently, a decrease in the concentration of free amino groups was observed. In contrast, those hydrolyzed with Flavourzyme presented lower concentrations of these groups at the initial time (PF: 1.40 g/L NH₂⁻, PUF: 0.85 g/L NH₂⁻). However, the release of free amino groups increased after 2 hours of hydrolysis (PF: >7.51 g/L NH₂⁻ and PUF: >6.16 g/L NH₂⁻). In general, a greater release of amino groups was observed in the hydrolysates at 6 h regardless of the enzyme used. This effect on the release of amino groups is related to the specificity of the enzyme used. It is known that during the first hours of hydrolysis, Alcalase generates a greater number of cleavages to the right of the peptide bonds with glutamic acid. This amino acid is mainly present in the α1 subunit of trout skin collagen [14], [15].

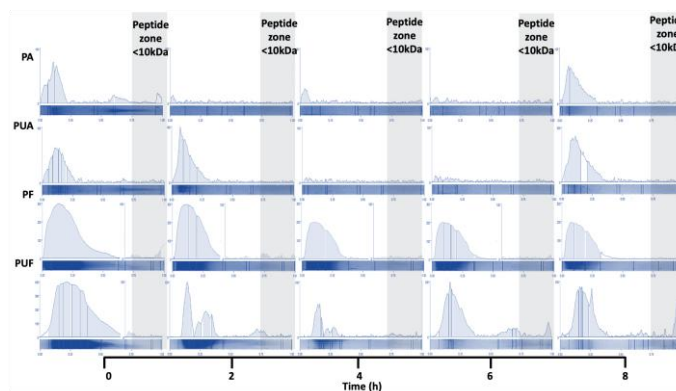
Table 2. Effect of sonication and enzyme on the degree of hydrolysis of pasteurized rainbow trout skin.

Hydrolysis time (h)	Hydrolysis degree Free amino groups [NH ₂ ⁻] (g/L)			
	PA	PF	PUA	PUF
0	5.97 ± 0.02 ^{Ae*}	1.40 ± 0.02 ^{Cc}	5.00 ± 0.01 ^{Bc}	0.85 ± 0.02 ^{Dc}
2	6.47 ± 0.01 ^{Cd}	7.51 ± 0.03 ^{Ad}	7.25 ± 0.07 ^{Bc*}	6.16 ± 0.07 ^{Dd}
4	8.51 ± 0.02 ^{Dc}	10.14 ± 0.02 ^{Ab}	9.34 ± 0.07 ^{Bb}	8.69 ± 0.03 ^{Cc}
6	9.59 ± 0.01 ^{Ca}	10.65 ± 0.01 ^{Aa}	10.64 ± 0.01 ^{Aa}	10.17 ± 0.01 ^{Ba}
8	8.76 ± 0.01 ^{Bb}	7.79 ± 0.03 ^{Cc}	6.25 ± 0.01 ^{Dd}	8.99 ± 0.07 ^{Ab}

Results expressed as mean of three repetitions ± standard deviation. ^{a-c} In columns indicate significant difference between the hydrolysis time. ^{A-D} Between rows, indicate significant difference between treatments.

In the case of Flavourzyme, being endo- and exopeptidase, it generates a higher degree of hydrolysis compared to other enzymes such as Alcalase, papain, pepsin, or trypsin, as has been reported in hydrolysis of sturgeon skin [16], [17]. Regarding the effect of ultrasonication as pretreatment, an increase in the concentration of free amino groups was observed only in those hydrolyzed with Alcalase. In the other treatments, the change in concentration was without any effect. It has been reported that the degradation of collagen during ultrasonication could cause the aggregation of compact proteins resistant to the hydrolysis of some enzymes, which results in a new conformation of the structures [18], [19].

After peptide separation by SDS-PAGE, the gels were analyzed and the molecular weights of the fractions obtained were determined (Figure 1). In the electropherograms, high molecular weight fractions were observed at time 0 which correspond to unhydrolyzed trout skin proteins (collagen). This high molecular weight protein represents 60% of the rainbow trout skin composition, mainly type I collagen, which has two chains α1 and α2 [20], [21]. Using SDS-PAGE electrophoresis in native collagen, the α chains that make it up have been identified with weights between 111 to 117 kDa; cross-linked β and γ components with weights similar to 200 kDa can also be identified with less intensity [20], [22]. It has also been identified that in partial hydrolysis of collagen peptides with weights of 50 to 90 kDa are found, while more extensive hydrolysis generates peptides with weights less than 40 kDa [23].

**Figure 1.** Electropherograms of peptide separation in hydrolyzed rainbow trout skin. PA, pasteurized sample hydrolyzed with Alcalase; PUA, pasteurized and ultrasonicated sample hydrolyzed with Alcalase; PF, pasteurized sample hydrolyzed with Flavourzyme and PUF to the pasteurized and ultrasonicated hydrolyzed sample with Flavourzyme.

However, in the hydrolysates with Alcalase (PA and PUA) peptide fractions with molecular weight greater than 25 kD were present. In contrast, in those hydrolyzed with Flavourzyme (PF and PUF), the highest concentration of peptides was those with molecular weights less than 10 kDa. It is known that the ability to produce lower molecular weight peptides by Flavourzyme corresponds to its endo- and exo-peptidase capacity, which could be involved in a higher degree of hydrolysis and, consequently, greater production of lower molecular weight peptides [24].

The antioxidant activity of the hydrolysates by the DPPH method was expressed as IC₅₀ (Table 3). It was observed that the antioxidant capacity of the PF hydrolysates (8.20–1.43 µg/mL) was greater than that of the PA hydrolysates (21.33–1.79 µg/mL), and it was at 6 h when the highest antioxidant activity was found (PA: 2.47 and PF: 1.43) (Table 3). In contrast, the FRAP results (Table 3) demonstrated greater antioxidant activity at 8 hours in PA and PF samples, while in PUF it was at 6 hours. Furthermore, no relationship was observed between the degree of hydrolysis or enzyme.

Table 3. Antioxidant activity of hydrolysates with Alcalase and Flavourzyme from pasteurized and ultrasonicated pasteurized rainbow trout skin.

Hydrolysis time (h)	PA	PF	PUA	PUF
DPPH (IC ₅₀ µg/mL)				
0	21.33 ± 0.46 ^{Ba}	8.20 ± 0.06 ^{Da}	19.73 ± 0.40 ^{Ca}	36.12 ± 1.00 ^{Aa}
2	4.81 ± 0.04 ^{Cc}	2.69 ± 0.03 ^{Db}	9.00 ± 0.06 ^{Bb}	10.68 ± 0.19 ^{Ab}
4	5.61 ± 0.06 ^{Cb}	2.14 ± 0.02 ^{Dc}	8.40 ± 0.10 ^{Ac}	7.67 ± 0.12 ^{Bc}
6	2.47 ± 0.04 ^{Cd}	1.43 ± 0.01 ^{De}	7.07 ± 0.04 ^{Ad}	4.05 ± 0.07 ^{Bd}
8	1.79 ± 0.02 ^{Ce}	1.74 ± 0.00 ^{Cd}	8.05 ± 0.08 ^{Bc}	9.66 ± 0.13 ^{Ab}
FRAP (mg eq. Fe ²⁺ /100 ml)				
0	1.20 ± 0.09 ^{Ba}	1.20 ± 0.03 ^{Ba}	1.22 ± 0.02 ^{Aa}	1.20 ± 0.03 ^{Da}
2	1.19 ± 0.13 ^{Bb}	1.60 ± 0.01 ^{Aa}	0.77 ± 0.03 ^{Cc}	0.83 ± 0.03 ^{Ec}
4	1.10 ± 0.01 ^{Bb}	1.13 ± 0.01 ^{Bb}	0.92 ± 0.02 ^{Bc}	1.72 ± 0.02 ^{Ba}
6	1.58 ± 0.03 ^{Ab}	1.06 ± 0.16 ^{Bc}	0.81 ± 0.03 ^{Cd}	2.03 ± 0.04 ^{Aa}
8	1.73 ± 0.02 ^{Aa}	1.75 ± 0.01 ^{Aa}	0.99 ± 0.01 ^{Bc}	1.59 ± 0.06 ^{Cb}

Results expressed as mean of three repetitions ± standard deviation. ^{a-c} In columns indicate a significant difference between the hydrolysis time. ^{A-D} Between rows indicate significant differences between treatments.

Ultrasonication did not favor the antioxidant activity in the hydrolysates with Alcalase or Flavourzyme. Previously Yaghoobzadeh et al.[25], reported a similar behavior of the antioxidant activity in rainbow trout skin hydrolysates, in which the hydrolysates with Flavourzyme presented greater inhibitory power by the DPPH method than those obtained with Alcalase. Also, when comparing the values obtained in this work, trout skin hydrolysates achieve greater antioxidant activity by DPPH, compared to what was reported for stingray skin gelatin hydrolysates, which presented an IC₅₀ value of 1.98. mg/mL [26].

The antioxidant activity in peptides has been related to the presence of amino acids with nucleophilic side chains with sulfur (taurine, cysteine, and methionine) or with aromatic side chains (tryptophan, tyrosine, and phenylalanine), amino acids that are present in skin proteins of fish [27].

For the enzymatic hydrolysis of rainbow trout skin, the factors that allowed obtaining better characteristics such as higher soluble protein content, a higher degree of hydrolysis, low molecular weight peptides (<10 kDa), and higher antioxidant activity were pasteurization, hydrolysis by 6 hours and the use of flavourzyme. While ultrasonication as pretreatment did not improve the characteristics of the hydrolysates. These results could be the beginning of the optimization of processes for obtaining peptides with antioxidant activity by enzymatic hydrolysis of trout skin and waste byproducts.

FUNDING

This study was funded by FOVI 230188 (Paula Santana) from ANID-Chile

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Capítulo VI

Nuevos péptidos de proteínas de piel de trucha arcoíris (Oncorhynchus mykiss): Optimización y caracterización de hidrólisis enzimática

Resumen

La creciente demanda por ingredientes funcionales de origen natural ha impulsado la búsqueda de fuentes alternativas y sostenibles para el desarrollo de productos bioactivos. En este contexto, los residuos de la industria pesquera, como la piel de pescado, han cobrado relevancia como materia prima valiosa para la obtención de péptidos bioactivos. Estos compuestos, obtenidos mediante hidrólisis enzimática de proteínas, han demostrado poseer propiedades beneficiosas como actividad antioxidante, antihipertensiva, antimicrobiana y antiinflamatoria, entre otras. En particular, la piel de la trucha arcoíris (*Oncorhynchus mykiss*), un subproducto comúnmente desechado, contiene colágeno y otras proteínas de alto valor biológico que pueden ser transformadas en péptidos multi-bioactivos mediante procesos biotecnológicos controlados. La hidrólisis enzimática se presenta como una estrategia eficiente y selectiva para liberar secuencias peptídicas con potencial funcional. Sin embargo, la obtención óptima de estos péptidos depende de diversos factores como el tipo de enzima, las condiciones de reacción y el diseño experimental adecuado.

Este capítulo presenta detalladamente los resultados experimentales obtenidos de la optimización y caracterización de hidrólisis enzimática, en el cual se describen a lo largo de este trabajo de investigación. En el estudio se desarrolló un proceso optimizado de hidrólisis enzimática empleando un diseño factorial con puntos de estrella, seguido de una caracterización de los hidrolizados en términos de su perfil proteolítico y actividades biológicas, con la finalidad de informar los hallazgos más relevantes de la investigación, así como evidenciar el potencial biotecnológico del aprovechamiento de subproductos de la acuicultura para el desarrollo de ingredientes funcionales con aplicaciones en las industrias alimentaria, farmacéutica y cosmética.

Introducción

Actualmente, existe un creciente interés de parte de la población por compuestos bioactivos con efectos benéficos en la salud humana. Algunos de estos compuestos incluyen los polifenoles, antocianinas, carotenoides, ácidos grasos y péptidos, que pueden obtenerse de los alimentos (Jakubczyk et al., 2020). Los péptidos, son secuencias de aminoácidos que provienen de las proteínas y que pueden ser capaces de regular funciones fisiológicas (Karaś, 2019). Investigaciones recientes se han enfocado en la obtención de péptidos a partir de fuentes proteicas no convencionales, como los desechos acuícolas, que representan el 70% del pescado procesado en la industria (FAO, 2022). Estos desechos incluyen cabezas, vísceras, piel, huesos y escamas, que tienen un impacto ambiental desfavorable al desecharlos, lo que motiva su aprovechamiento como materia prima (Pratama et al., 2022).

Durante la digestión gastrointestinal de las proteínas se liberan péptidos de manera natural (Jakubczyk et al., 2020; Karaś, 2019). Sin embargo, también puede generarse de forma controlada mediante hidrólisis enzimática, utilizando enzimas de origen vegetal (papaína o bromelina), animal (pepsina, tripsina, quimiotripsina) o bacteriano (neutrasa, alcalasa o flavourzyme) (Mazorra-Manzano et al., 2018). Las enzimas alcalasa y flavourzyme son de las más utilizadas en investigación debido a su amplia selectividad y especificidad de sustrato, liberación de péptidos de bajo peso molecular, estabilidad a valores de pH alcalinos, alto grado de hidrólisis y liberación de péptidos bioactivos (Merz et al., 2015; Tacias-Pascacio et al., 2020). Una hidrólisis enzimática óptima requiere la regulación de factores como la relación proteína-enzima, el tiempo de hidrólisis, el pH y temperatura de la reacción, debido a que estas condiciones influyen en las propiedades de los péptidos generados (Li-Chan, 2015). En este contexto, el diseño de experimentos (DoE) constituye una herramienta estadística que permite evaluar de manera simultánea la interacción de estas variables, optimizar el proceso y predecir las condiciones que maximizan la respuesta con un número reducido de ensayos experimentales. Entre las metodologías DoE, el diseño central compuesto se ha aplicado para optimizar los procesos de hidrólisis enzimática, ya que permite evaluar los efectos de interacción y construir modelos matemáticos estadísticamente válidos (Abdi et al., 2018). Además de estos parámetros, también se ha informado que la aplicación de un pretratamiento al sustrato, como la desnaturalización térmica, mejora la eficiencia de la hidrólisis

al aumentar la accesibilidad de la enzima, siempre que se preserve la integridad de los aminoácidos (Borad et al., 2017).

Péptidos bioactivos con capacidad antioxidante, son capaces de contrarrestar estrés oxidativo (Bartolomei et al., 2023; Nami et al., 2023; Sae et al., 2017; Tkaczewska et al., 2018; Zhang et al., 2012). El estrés oxidativo surge de un desbalance entre la producción y eliminación de especies reactivas de oxígeno en células y tejidos, y está vinculado a enfermedades crónicas como obesidad, diabetes, enfermedades del corazón y cáncer (Pizzino et al., 2017). Por lo tanto, se ha propuesto que los péptidos antioxidantes son agentes prometedores para la prevención y el tratamiento del cáncer, especialmente en las primeras etapas de la enfermedad. Dada la complejidad del cáncer y su resistencia a las terapias convencionales, los esfuerzos se han centrado cada vez más en identificar nuevos compuestos anticancerígenos eficaces, de origen natural y con menos efectos adversos (Alemán et al., 2011).

En este contexto, se ha informado que varios péptidos antioxidantes ejercen efectos antiproliferativos sobre las células tumorales al modular las vías celulares implicadas en la proliferación y apoptosis (Bielecka et al., 2022; Chen et al., 2013; Yaghoubzadeh et al., 2020). Es importante destacar que un solo péptido puede presentar múltiples bioactividades, lo que convierte a estos compuestos en candidatos para aplicaciones en la industria alimentaria, farmacéutica y cosmética (Zaky et al., 2022). Por lo tanto, el objetivo de este estudio fue optimizar el proceso de hidrólisis enzimática de piel de trucha arcoíris (*Oncorhynchus mykiss*) para la obtención de péptidos con actividad antioxidante, así como también determinar su potencial anticancerígeno.

Metodología

Tratamiento de muestras

Se adquirieron 15 ejemplares de trucha arcoíris (*Oncorhynchus mykiss*) frescas de 500 g en un centro piscícola del estado de Hidalgo, México. Las truchas se evisceraron y la piel se separó y se limpió para retirar restos de músculo. Después la piel se cortó en cuadrados de 2 cm, se congeló y se liofilizó. La piel liofilizada se suspendió 1:4 (p/v) en agua desionizada. A partir de la suspensión, se realizaron dos soluciones al 20% de piel de trucha con buffer Tris-HCl 0.1 M, una con pH 7 y otra con pH 9. Estas soluciones se pasteurizaron a 90°C por 10 min y se almacenaron a -20°C para

su hidrólisis con las enzimas comerciales Alcalase® enzyme, *Bacillus licheniformis* y Flavourzyme, proteasa de *Aspergillus oryzae*.

Diseño de experimentos para optimización de hidrólisis enzimática

Para conocer el efecto de los factores de tiempo y la relación enzima:proteína (E:P) expresada como relación masa/masa (p/p), durante la hidrólisis de piel de trucha arcoíris sobre la variable respuesta de actividad antioxidante por DPPH y FRAP, se empleó un diseño de experimentos factorial con puntos axiales (Tabla 1).

Tabla 1. Factores y niveles del diseño factorial con puntos axiales

Factores	Niveles				
	- α	-1	0	+1	+ α
Relación E:P (p/p)	5:100	6:100	10:100	14:100	15:100
Tiempo (horas)	4	4.59	6	7.41	8

Tabla 2. Condiciones de los hidrolizados del diseño factorial con puntos axiales

Experimento	Relación E:P	Tiempo (H)
1	10:100	4.00
2	15:100	6.00
3	10:100	6.00
4	10:100	8.00
5	10:100	6.00
6	5:100	6.00
7	10:100	6.00
8	6:100	4.59
9	10:100	6.00
10	6:100	7.41
11	14:100	4.59
12	10:100	6.00
13	10:100	6.00
14	14:100	7.41

Con los valores establecidos se obtuvo un diseño con 14 combinaciones de condiciones para hidrolizar la piel de trucha (Tabla 2). El análisis de varianza, el ajuste del modelo del diseño y la optimización de la respuesta se realizaron con el software Minitab 18.

Las soluciones pasteurizadas se hidrolizaron con flavourzyme (50°C y pH 7) y con alcalasa (55°C y pH 9) siguiendo las condiciones de relación E:P y tiempo descritas en la Tabla 2. Para inactivar la reacción se colocaron en agua en ebullición por 10 min. Los hidrolizados se centrifugaron y el sobrenadante se almacenó a -20°C para los análisis posteriores. Se cuantificó proteínas por el método Bradford.

Caracterización del perfil proteolítico y actividad antioxidante de hidrolizados del diseño de experimentos

Grado de hidrólisis por TNBS

La liberación de grupos amino libres se analizó por el método del ácido trinitrobenzenosulfónico (TNBS) (Adler-Nissen 1979). Para ello fueron mezclados 250 µL de hidrolizado, 2 mL de buffer de fosfatos 0.21 M pH 8.2 (KH₂PO₄ y K₂HPO₄) y 2 mL de solución de ácido picrilsulfónico al 0.1%. Esta solución se incubó por 1 h a 50°C, y la reacción se detuvo con 4 mL de HCl 0.1 N. Se midió la absorbancia a 340 nm en espectrofotómetro. Para determinar el contenido, se realizó una curva estándar con glicina (0 - 0.25 mg/mL). Los resultados se expresaron como g/L de grupos amino libres (NH₂-).

Electroforesis en Gel de poliacrilamida Tris-Tricina SDS-PAGE

Se realizó electroforesis en gel de poliacrilamida Tris-Tricina para la separación de los péptidos presentes en los hidrolizados del diseño de experimentos siguiendo la metodología de González-Olivares et al. (2011) con modificaciones. Se prepararon geles de acrilamida (relación 19:1 de acrilamida:bisacrilamida con 5% de crosslinker) 12.5% de T. Los geles se colocaron en una cámara con buffer Tris-Tricina-SDS-HCl (pH 8.25), se inyectaron las muestras y el estándar. La electroforesis se llevó a 95V durante 4 horas. La tinción de los geles se realizó con plata (Silver Stain Plus, Bio-Rad) y se analizaron mediante Gel-Doc con el software Image J.

Capacidad antioxidante y poder reductor de hierro

Para el método de DPPH se utilizaron 100 μ L de hidrolizado y 2.9 mL de reactivo DPPH (2,2-diphenyl-1-picrylhydrazyl 0.1 mM en metanol). Se midió la absorbancia en espectrofotómetro a 515 nm pasados 50 minutos y se utilizó una curva estándar con trolox (0 – 35 μ M). Los resultados fueron expresados como porcentaje de DPPH remanente.

Para el método de FRAP se colocó 250 μ L de hidrolizado, 1 mL de reactivo FRAP (buffer acetatos 300 mM pH 3.6, solución TPTZ (2,4,6-tripiridil-s-triazina) 10mM y solución $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 20mM) y 8.75 mL de agua desionizada. La reacción se mantuvo a 37°C por 15 min y se midió la absorbancia a 593 nm. Se realizó una curva con $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ en HCl 40 mM. Los resultados se expresaron como mg equivalentes de Fe^{2+} (Ramírez-Godínez et al., 2017).

Experimentos confirmatorios de la optimización de la hidrólisis enzimática

Para confirmar la validez del modelo matemático obtenido del análisis del diseño de experimentos, se realizó un experimento adicional por triplicado empleando las condiciones óptimas predichas para maximizar la actividad antioxidante por DPPH y FRAP en la hidrólisis de piel de trucha arcoíris con alcalasa y flavourzyme. Posteriormente, las muestras hidrolizadas se liofilizaron y se codificaron como HA (piel de trucha arcoíris hidrolizada con alcalasa) y HF (piel de trucha arcoíris hidrolizada con flavourzyme).

Caracterización de los hidrolizados optimizados

La caracterización de los hidrolizados HA y HF se realizó con los procedimientos de TNBS, electroforesis y actividad antioxidante anteriormente descritos. Además, se realizó la separación de péptidos por RP-HPLC y SEC-HPLC.

La separación por polaridad de los péptidos de los hidrolizados HA y HF se realizó en un sistema HPLC (Perkin Elmer) equipado con un detector UV. Se empleó una columna RP-C18 (4.6 x 150; Zorbax, Agilent). Para la elución de los péptidos se siguió un gradiente lineal del 100 a 0 en 85 min. Se emplearon dos fases móviles, fase A: agua:acetonitrilo (ACN) 98:2 con 0.10% de ácido trifluoroacético (TFA) y fase B: agua:ACN 35:65 con 0.10% de TFA. Se utilizó un flujo de 1 mL/min y las detecciones se realizaron a 220 nm y 280 nm.

La separación molecular de los péptidos presentes en los hidrolizados HA y HF se realizó con un sistema HPLC (Perkin Elmer) equipado con un detector UV. Se empleó una columna SRT SEC-150, 7.8 X 3000 mm. La elución de los péptidos se realizó con un flujo isocrático de 60 min a 0.5 mL/min. Se empleó como fase móvil una solución amortiguadora de fosfatos (0.1 M, pH 6.8), y la detección se realizó a 280 nm. Se utilizaron seroalbúmina B, ovoalbúmina, lactoglobulina y bacitracin A como marcadores de peso molecular.

Purificación de hidrolizados optimizados

Los hidrolizados HA y HF se purificaron mediante precipitación con acetona. Para la purificación se suspendieron 10 mg de las muestras liofilizadas en 100 µL de buffer de lisis (Tris-HCl 0.05 M, NaCl 0.1 M, EDTA 0.1 M, Tritón X-100 1% pH 7.5). Se agregó un volumen de acetona 50% a -20°C y se incubó a 0°C en agitación constante durante 4 horas. Posteriormente, se centrifugó a 12000xg 30 minutos a 4°C. Se recolectó el sobrenadante y se le agregaron seis volúmenes de acetona al 75% a -20°C y se incubó por 18 horas a 4°C en agitación constante. Después se centrifugó a 14000xg por 45 min a 4°C, y tanto el precipitado como el sobrenadante final se recuperaron, liofilizaron y almacenaron por separado. Se determinó el contenido proteico mediante el ensayo del ácido bicinonínico (BCA) para confirmar la distribución de los péptidos.

Para los análisis posteriores, solo se utilizó el sobrenadante final, estas muestras se codificaron como HA-P (para péptidos extraídos de la muestra hidrolizada con alcalasa) y HF-P (para péptidos extraídos de la muestra hidrolizada con flavourzyme).

Caracterización estructural y fisicoquímica de hidrolizados purificados

Cuantificación de proteínas

La cuantificación de proteínas en las muestras HA, HF, HA-P y HF-P se realizó con ensayo BCA con el BCA Protein Assay kit (Pierce, Thermo Scientific). Para la cuantificación 25 µL de cada muestra y 200 µL de reactivo de trabajo se colocaron en una microplaca de 96 pocillos, junto con la curva estándar de BSA (0.125-2 µg/µl). Se incubó por 30 minutos a 37°C y luego se registró la absorbancia a 562 nm. El análisis se realizó por duplicado.

Electroforesis en gel Tris-Tricina

La separación de péptidos presentes en las muestras HA, HF, HA-P y HF-P se realizó mediante electroforesis Tris-Tricina con la preparación de un gel compuesto por tres diferentes porcentajes de acrilamida (separador 15%, espaciador de 7,6% y concentrador 3.3%). Las muestras se prepararon con buffer de carga y se incubaron 5 min a 100°C. Los geles se colocaron en buffer de carga cátodo (Tris 0.1M, Tricina 0.1M y 0.1% de SDS, pH 8.25) y ánodo (Tris 0.2 M, pH 8.9). Se realizó una pre-corrida sin muestras por 15 min a 85V. Para la corrida electroforética las muestras junto a un estándar de peso molecular (SeeBlue) se corrieron a 100 V por 90 min. La tinción se realizó con SYPRO Rubí y se analizaron los geles con el software ImageJ.

Actividad biológica de hidrolizados optimizados y purificados

Actividad antioxidante

La actividad antioxidante de las muestras HA, HF, HA-P y HF-P liofilizadas se evaluó por el método DPPH. La técnica consistió en mezclar 100 µL del hidrolizado y 2.9 mL de reactivo DPPH 50 µM en etanol, después de 15 min, se realizaron las medidas de absorbancia a 517 nm. Este procedimiento se realizó para cada hidrolizado en tres concentraciones 1, 5 y 10 mg/mL. Los datos se reportaron como IC₅₀, a partir del porcentaje de DPPH remanente y la concentración de los hidrolizados. Los valores de IC₅₀ se compararon considerando BHT como antioxidante de referencia.

Actividad antimicrobiana

Se determinó la actividad antimicrobiana de las muestras HA, HF, HA-P y HF-P liofilizadas a concentraciones de 5 y 1 mg/mL frente a dos bacterias Gram negativas *Escherichia coli* (ML-35), y *Pseudomona aeruginosa* (ATCC27853) y una bacteria Gram positiva, *Staphylococcus aureus* (ATCC 25923). Para realizar el ensayo, cada cepa de la bacteria se cultivó en caldo de soya tripticasa (TSB) a 37°C. Este cultivo se diluyó 100 veces en TSB y se incubó a 200 rpm hasta obtener una densidad óptica (OD, 600nm) en fase logarítmica media. Luego, las bacterias se lavaron y centrifugaron 2 minutos a 6000 rpm. El pellet obtenido se resuspendió en TSB al 1% en solución salina fosfato (PBS) 10 mM y se ajustó a 1×10^6 UFC mL⁻¹. Se agregaron las muestras a las concentraciones establecidas y se incubó por 1 hora a la temperatura de crecimiento óptimo (37°C). Pasado el tiempo, las muestras incubadas y un control negativo se sembraron en placas de

agar de triptona y soja (TSA) por triplicado y se incubó entre 12-16 horas. Finalmente, se contaron las colonias formadas y se calculó la supervivencia de las bacterias (UFC/mL), con la Ecuación 1.

$$\text{UFC/mL} = \frac{\text{número de colonias} \times \text{Factor de dilución}}{\text{Volumen de muestra sembrada}} \quad (1)$$

Actividad hemolítica

Para la determinación de la actividad hemolítica de las muestras liofilizadas, se recolectó sangre de ratón en tubos EDTA. La sangre con anticoagulante se centrifugó a 2000xg 10 min a 4°C. El pellet obtenido fue resuspendido en PBS 1X y se centrifugó nuevamente, este ciclo se repitió 3 veces. Al precipitado de eritrocitos obtenidos se le añadieron 10 veces el volumen inicial con PBS 1X.

Las muestras liofilizadas se diluyeron en PBS 1X. En microtubos se agregó 1:1 (v/v) suspensión de hematocritos: dilución de la muestra y se incubó 1 hora a 37°C con agitación constante. Después se centrifugó a 3000xg por 5 min. En una microplaca de 96 pocillos se colocaron 80 µL del sobrenadante de la muestra incubada y 80 µL de agua Milli-Q. Luego se midió la absorbancia en espectrofotómetro a 540 nm. Como control positivo se usó solución al 0.5% de tritón X-100(v/v) y como control negativo se usó PBS 1X. Los resultados se expresaron como % de actividad hemolítica, calculados a partir de la Ecuación 2.

$$\% \text{ Hemolisis} = \left(\frac{\text{Abs}_{\text{muestra}} - \text{Abs}_{\text{PBS 1X}}}{\text{Abs}_{\text{Tritón X-100}} - \text{Abs}_{\text{PBS 1X}}} \right) \times 100 \quad (2)$$

Ensayo de citotoxicidad de sulforodamina B

Para este ensayo, 7 líneas celulares PC-3 (ATCC® CRL-1435TM), AGS (ATCC® CRL-1739TM), MDA-MB-231 (ATCC® CRM-HTB-26TM), MCF-7 (ATCC® CRL-3435TM), HT-29 (ATCC® HTB-38TM), HeLa (ATCC® CCL-2TM) y GES (Cytion® 305428), fueron sembradas en placas de 96 pocillos a una densidad de 5×10^3 células*pocillo⁻¹ en un volumen de 100 µL de medio DMEM/Ham's F-12 1:1 (v/v), suplementado con 10% de suero fetal bovino, 100 U/mL de penicilina, 100 µg/mL de estreptomycin y 1 mM de glutamina, e incubadas durante 24 horas, antes de cada tratamiento. Los tratamientos consistieron en un control sin intervención (C), un control solvente (CS) al que se le agregó agua Milli-Q y las muestras HA, HF, HA-P y HF-P a 6 concentraciones (1 mg/mL, 100 µg/mL, 10 µg/mL, 1 µg/mL, 0.1 µg/mL y 0.01 µg/mL) fueron incubados por 72 h. Después, las células fueron fijadas con 25 µL de ácido tricloroacético (TCA)

al 50% p/v durante una hora a 4°C y lavadas 3 veces por inmersión en agua destilada, tras lo cual se realizó la tinción con 50 µL de sulforodamina B al 0,1% p/v en ácido acético al 1% v/v, durante 30 minutos. Pasado el tiempo, las células fueron lavadas 3 veces con 150 µL de ácido acético al 1% y el colorante fue solubilizado con 100 µL de Tris Base 10 mM. Se midió absorbancia a 540 nm en lector de microplacas. El porcentaje de células viables se determinó con la Ecuación 3:

$$Viabilidad\ celular(\%) = \frac{Abs_{Células\ tratadas}}{Abs_{Control\ solvente}} \times 100 \quad (3)$$

También, se determinó el valor de la concentración efectiva media (EC₅₀) para los hidrolizados y purificados de todas las líneas celulares con el programa estadístico Sigma Plot 11.0. Con los valores de EC₅₀ se calculó el índice de selectividad (IS), aplicando la Ecuación 4.

$$IS = \frac{EC_{50}\ Células\ normales}{EC_{50}\ Células\ cancerigenas} \quad (4)$$

Determinación de ROS por citometría de flujo

Las células AGS y MCF-7 fueron incubadas con las muestras HA-P y HF-P a concentraciones de 50 y 100 µg/mL durante 48 h. Posteriormente, se realizó la tinción con la sonda DCFH₂-DA (acetato de 2,7-dicloro-dihidro-fluoresceína) 1 mM para la detección de especies reactivas de oxígeno (ROS) intracelulares. Las células se incubaron a oscuridad 1 h a 37 °C. Finalizando este tiempo, se retiró el medio y las células se lavaron dos veces con PBS 1X. Luego, se tripsinizaron y se recolectaron por centrifugación a 1500 rpm por 10 min. El pellet celular resuspendido en PBS 1X fue analizado por citometría de flujo utilizando el filtro FL1 (Fluorecence 1). Los resultados se expresan como el porcentaje de células teñidas con DCFH₂-DA.

Secuenciación de péptidos y predicción de su bioactividad in silico

Se realizó la secuenciación de péptidos de los hidrolizados optimizados HA y HF mediante nanocromatografía líquida acoplada a espectrometría de masas en tándem (nLC-MSMS) en la Universidad de Santiago de Compostela.

Las secuencias peptídicas obtenidas se analizaron *in silico* para predecir su potencial bioactivo mediante herramientas bioinformáticas, incluyendo UniDL4BioPep (<https://nepc2pvmzy.us-east-1.awsapprunner.com/>), BIOPEP-UWM: Analysis (https://biochemia.uwm.edu.pl/biopep/rec_pro1.php?x=104&y=8), PeptideRanker

(<http://distilldeep.ucd.ie/PeptideRanker/>), mACPred 2.0 (<https://balalab-skku.org/mACPpred2/>), HLPpred-FUSE (<http://thegleelab.org/HLPpred-Fuse/>), AnOxPePred - 1.0 (<https://services.healthtech.dtu.dk/services/AnOxPePred-1.0/>) y PeptideAtlas (<https://peptideatlas.org/>). El análisis se enfocó principalmente en la predicción de la actividad anticancerígena.

Análisis estadístico

Todos los experimentos se realizaron al menos por triplicado y los resultados se expresaron como media $\pm$ desviación estándar. El análisis estadístico se realizó con el software Minitab® 22.1. El diseño experimental para la optimización de la hidrólisis se analizó mediante la metodología de superficie de respuesta y el análisis de los efectos de las variables independientes y sus interacciones. La validación del modelo se realizó comparando los valores predichos y experimentales de DPPH y FRAP mediante prueba t de Student de una muestra.

Para los hidrolizados optimizados y purificados, se analizó la actividad antioxidante y antimicrobiana mediante ANOVA de una vía y la prueba post hoc de Tukey. Para las comparaciones de los porcentajes de actividad hemolítica se utilizó Kruskal-Wallis y se reportaron como mediana y rango intercuartílico. En el ensayo de citotoxicidad los valores de EC₅₀ se calcularon mediante regresión no lineal con el software Sigma Plot 11.0. Las diferencias estadísticas significativas se consideraron con un valor de $p < 0.05$.

Resultados y discusión

Optimización de la hidrólisis enzimática y respuesta antioxidante

Del análisis del diseño de experimentos se generaron las gráficas de superficie de respuesta que muestran el efecto de las interacciones que ejercen las variables de tiempo de hidrólisis y la relación E:P sobre la actividad antioxidante medida por los métodos DPPH Y FRAP de los hidrolizados de piel de trucha arcoíris (Figura 1).

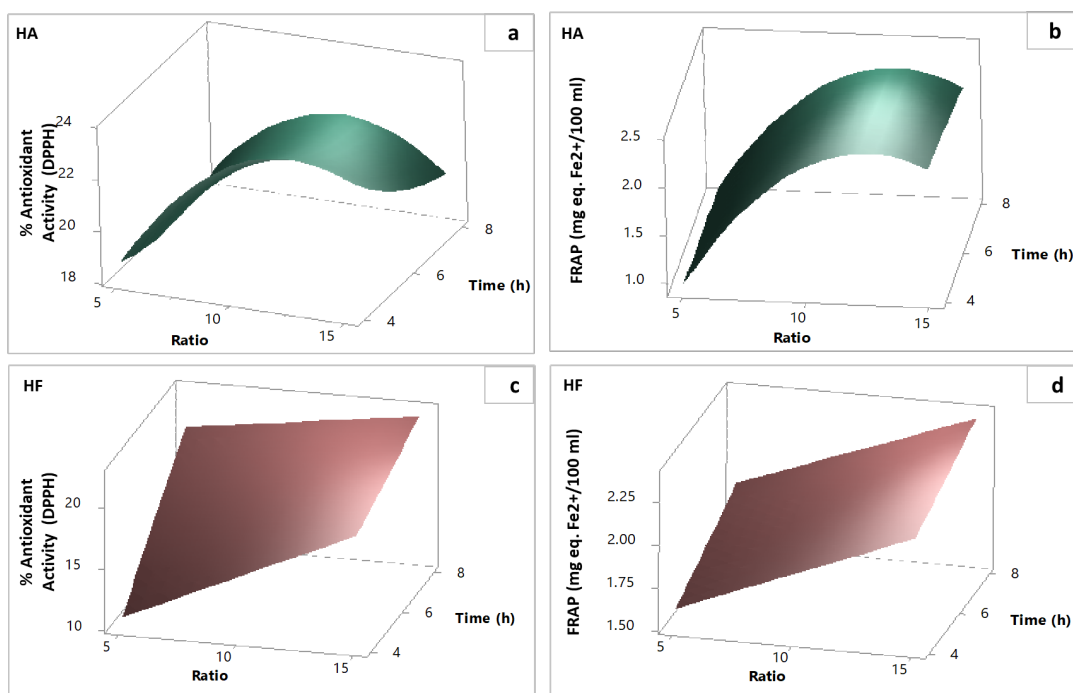


Figura 1. Gráficas de superficie de respuesta del efecto del tiempo y relación E:P para las variables de actividad antioxidante por DPPH y FRAP. HA: Hidrolizados de piel de trucha arcoíris con alcalasa y HF: Hidrolizados de piel de trucha arcoíris con flavourzyme.

En la Tabla 3 se encuentran los valores de R^2 que fueron mayores al 80% para los 4 modelos, este porcentaje indica un buen ajuste del modelo. Con la información del diseño se predijeron las condiciones óptimas de tiempo y de relación E:P que serían necesarias para maximizar la respuesta de actividad antioxidante en la hidrólisis de piel de trucha. Con el uso de la enzima alcalasa las condiciones fueron tiempo de 4 h y relación E:P 12.5:100, mientras que con flavourzyme 8 h y 15:100 de E:P. El valor $p > 0.05$ indica que la H_0 se acepta y no hay diferencias entre la respuesta predicha y la experimental, demostrando la validez del modelo.

Con la optimización de la hidrólisis enzimática, los hidrolizados de piel de trucha arcoíris con alcalasa y flavourzyme presentaron similitud en la respuesta de actividad antioxidante (Tabla 3). En hidrolizados de proteínas de peces la actividad antioxidante se atribuye a la composición, secuencia e hidrofobicidad de los aminoácidos alanina, valina, leucina, isoleucina, prolina, fenilalanina, triptófano y metionina (Pezeshk et al., 2019).

Tabla 3. Predicción y ajuste de la actividad antioxidante por diseño factorial con puntos axiales para hidrolizados de piel de trucha arcoíris

Enzima de hidrólisis	Variable de respuesta	Ajuste del modelo (R ²)	Respuesta predicha (máx.)	Respuesta experimental	Valor <i>p</i>	Ecuación del modelo
Alcalasa	DPPH*	90.67%	23.68	24.18	0.26	12.48 + 2.51A - 1.16B - 0.08A ² + 0.13B ² - 0.08AB
	FRAP**	83.13%	1.33	1.34	0.86	-0.96 + 0.359 A + 0.024 B - 0.01386 A ² - 0.0011 B ² - 0.0024 AB
Flavourzyme	DPPH*	81.44%	22.42	24.48	0.32	-5.13 + 1.40A + 2.95B - 0.14AB
	FRAP**	86.93%	1.33	1.32	0.842	0.592 + 0.0319A + 0.0394B - 0.00048AB

Donde A representa relación proteína-enzima y B el tiempo de hidrólisis. * Resultados expresados en % actividad antioxidante, ** Resultados expresados en (mg eq de Fe²⁺ en 100 mL). Sin diferencias estadísticas (*p*>0.05) entre la respuesta predicha y la experimental con T de student.

El ensayo de DPPH, utilizado en este estudio para evaluar la actividad antioxidante, se aplica comúnmente para evaluar péptidos y matrices alimentarias derivadas de proteínas. Yaghoubzadeh et al. (2020) reportaron una asociación positiva entre la actividad antioxidante y la hidrólisis de proteínas de piel de trucha arcoíris, consistente con las respuestas antioxidantes observadas en las condiciones optimizadas. Además, la actividad antioxidante depende de la composición general del hidrolizado, ya que puede surgir de los efectos combinados de una mezcla de péptidos de diferentes tamaños y residuos que interactúan entre sí (Sarmadi & Ismail, 2010). Este fenómeno se ha reportado para hidrolizados de proteína de piel de carpa donde la actividad antioxidante por FRAP del hidrolizado completo (23.76 %) superó la de sus 8 fracciones individuales (6 a 13.97 %) (Tkaczewska et al., 2018), lo que respalda las respuestas observadas en el presente estudio.

Caracterización de hidrolizados optimizados (HA y HF)

El perfil electroforético de los hidrolizados de piel de trucha arcoíris evidenció la presencia de bandas <10 kDa (Figura 2), así como algunas bandas de mayor peso molecular. Las diferentes bandas de pesos moleculares son resultado de las diferencias en las condiciones de hidrólisis, tipo de enzima y de la composición de la piel de trucha. También se puede atribuir a la formación de agregaciones de proteínas efecto de la pasteurización de la muestra (Sakuyama et al., 2024). Se ha descrito principalmente la presencia de colágeno de tipo I, compuesto por hetero trímeros $\alpha 1$ (I), $\alpha 2$ (I) y $\alpha 3$ (I), junto con sus componentes reticulados β (I) y γ (I) (Saito et al., 2001), con pesos moleculares cerca a los 100 kDa para las cadenas α y mayores a 200 kDa para los componentes β (Lee et al., 2016).

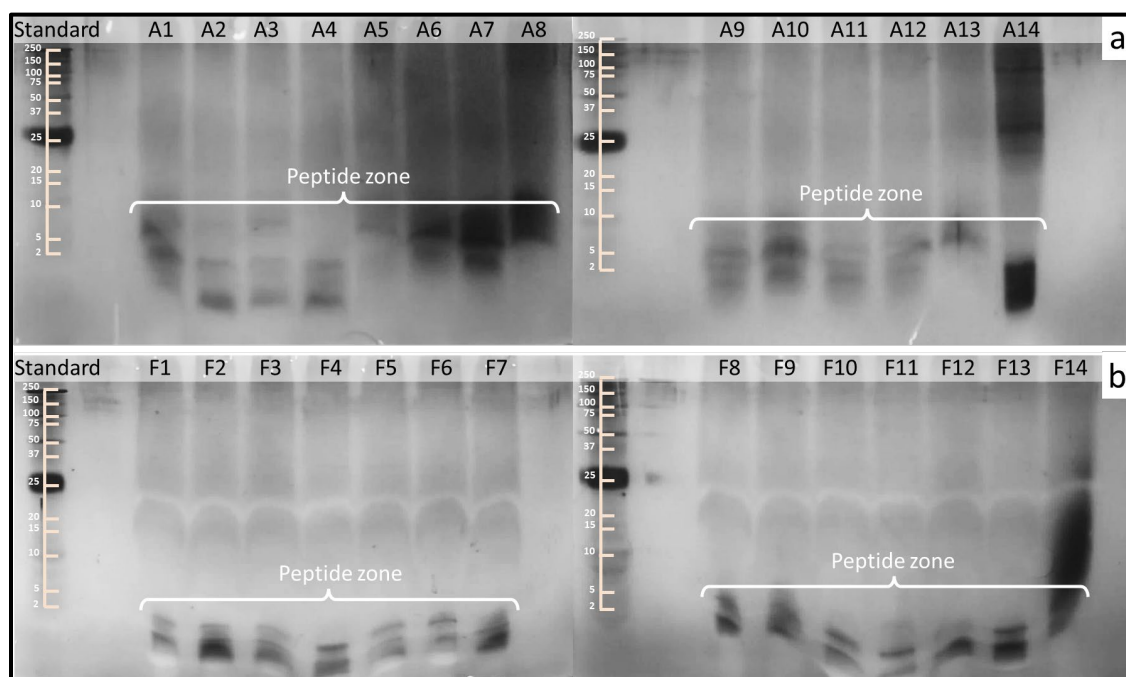


Figura 2. Electroforesis en gel poliacrilamida Tris-Tricina SDS-PAGE de hidrolizados de piel de trucha arcoíris correspondientes al diseño de experimentos. Tinción Silver Stain Plus (Bio-Rad). La imagen a, corresponde al diseño de experimentos de la hidrólisis de piel de trucha arcoíris con alcalasa, muestras de la A1 a la A14. La imagen b, corresponde al diseño de experimentos de la hidrólisis de piel de trucha arcoíris con flavourzyme, muestras de la F1 a la F14.

Estas diferencias en la distribución del tamaño de los péptidos pueden explicarse, en parte, por la especificidad de las enzimas utilizadas durante la hidrólisis. Flavourzyme es una mezcla de exo- y endopeptidasa que puede escindir enlaces tanto dentro de las proteínas como en sus extremos terminales específicamente en los residuos de aminoácidos N-terminales, lo que aumenta los sitios de escisión generando péptidos de menor tamaño (Alahmad et al., 2023; Hunsakul et al., 2022). En cambio, alcalasa es una endopeptidasa que rompe los enlaces peptídicos en los residuos de aminoácidos C-terminales, lo que puede resultar en una distribución del tamaño de los péptidos diferentes a la de Flavourzyme (Hunsakul et al., 2022).

En este sentido, Hou & Chen (2023) indicaron que, en hidrolizados de colágeno de piel de esturión, la enzima flavourzyme, produce péptidos de menor peso molecular en comparación con las enzimas pepsina, papaína, alcalasa y tripsina. Además, con la enzima alcalasa observaron bandas con pesos de 5, 10, 15 y 25 kDa y de 5 y 10 kDa, con flavourzyme. Se observó un patrón electroforético comparable en los electroferogramas mostrados en la Figura 3, que corresponden al análisis de la separación de péptidos mediante electroforesis de las muestras HA y HF (resultantes de la optimización del diseño). En ambos hidrolizados se obtuvieron bandas por debajo de los 10 kDa, indicando que las condiciones de hidrólisis fueron suficientes para activar la exopeptidasa y la endopeptidasa, y producir péptidos de bajo peso molecular.

La Figura 3 también muestra los cromatogramas que ilustran la distribución del peso molecular de los hidrolizados optimizados. En ambas muestras (HA y HF), se observaron péptidos de bajo peso molecular, con tamaño aproximado de 3 kDa. No obstante, el análisis mediante electroferograma revela la presencia de fracciones de mayor peso molecular, cercana a los 30 kDa. Un patrón similar de pesos moleculares se ha reportado en fracciones provenientes de hidrolizados de colágeno de piel de caballa española (*Spanish Mackerel*), con un rango de pesos de 5.04 a 47.82 kDa (Chi et al., 2014).

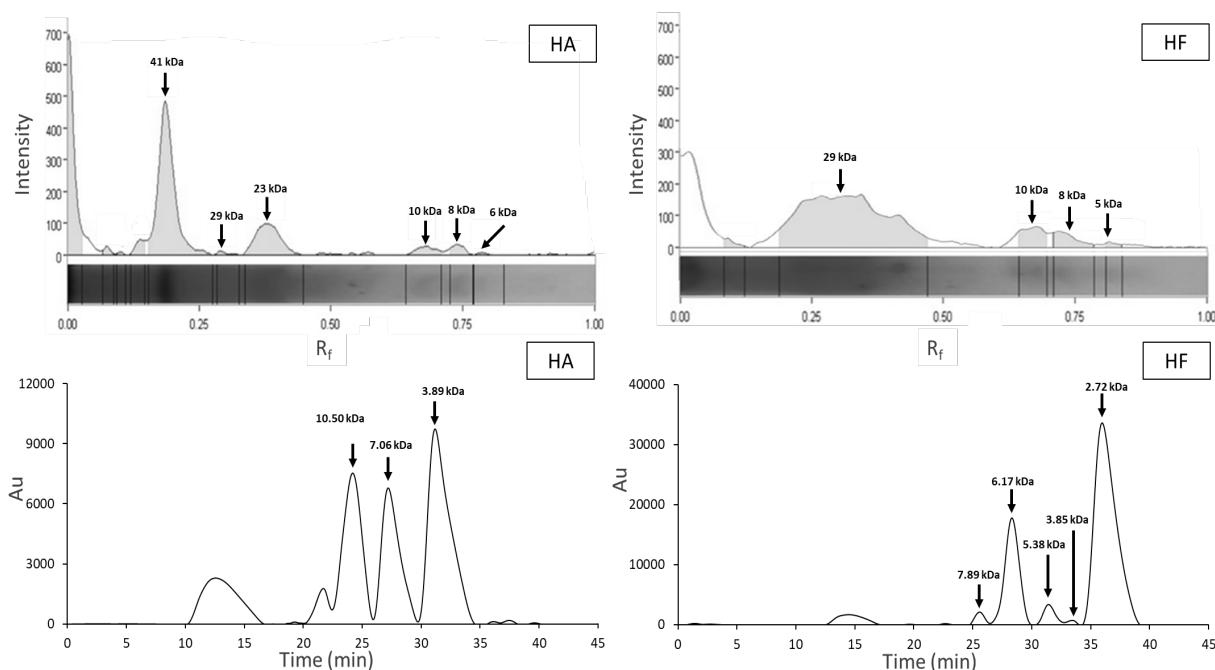


Figura 3. Separación de péptidos de hidrolizados de piel de trucha arcoíris bajo las condiciones óptimas de tiempo y relación enzima/proteína. En la parte superior electroferogramas y en la parte inferior cromatogramas de exclusión de tamaño molecular. HA: Hidrolizado de piel de trucha arcoíris con alcalasa. HF: Hidrolizado de piel de trucha arcoíris con flavourzyme.

La actividad proteolítica de cada enzima fue distinta debido a la especificidad de cada una, en comparación con flavourzyme que es endo- y exopeptidasa, alcalasa es una endopeptidasa que realiza cortes en los enlaces de los aminoácidos c-terminales (Hunsakul et al., 2022). Como se muestra en la Figura 4, los cromatogramas obtenidos por RP-HPLC presentan diferencias entre los hidrolizados de piel de trucha tratados con cada enzima. En la lectura a 220 nm, se detectaron enlaces peptídicos en ambos hidrolizados, con la elución de compuestos en los primeros 30 minutos. Estos péptidos pueden considerarse polares, debido a que el RP-HPLC separa los compuestos en función de su polaridad. Un perfil cromatográfico similar ha sido reportado en hidrolizados con actividad antioxidante obtenidos de escamas y esqueletos de tilapia roja (*Oreochromis spp.*); los que presentan 9 fracciones peptídicas polares que eluyen antes de los 25 min (Sierra, 2021).

Por otra parte, en los cromatogramas a 280, se identificaron 5 fracciones peptídicas con tiempos de retención menores a 30 min para las muestras HA y HF, sugiriendo que la actividad antioxidante que presentan estos hidrolizados se atribuye a la interacción de estas 5 fracciones.

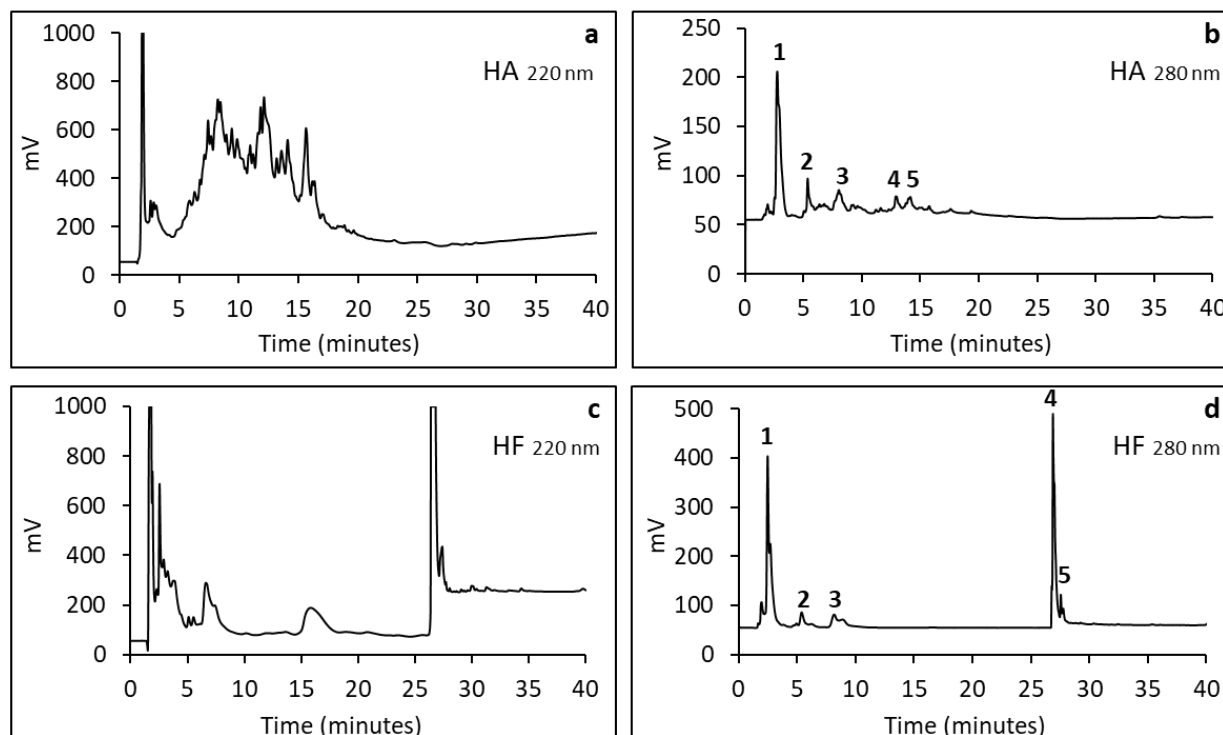


Figura 4. Cromatogramas de fracciones peptídicas en hidrolizados óptimos de piel de trucha arcoíris. En los cromatogramas a 280 nm se evidencia la elución de 5 fracciones peptídicas señaladas con los números del 1 al 5.

Efecto de la purificación sobre la actividad antioxidante y propiedades estructurales

Mediante una precipitación con acetona, se recuperaron péptidos de bajo peso molecular a partir de los hidrolizados de piel de trucha arcoíris con alcalasa y flavourzyme. Este método ha sido empleado anteriormente por Fukutomi et al. (2005) para precipitar péptidos <10 kDa con acetona fría (-40°C), al igual que Kawashima et al. (2010) quienes utilizando acetona recuperaron péptidos de 2.5 kDa provenientes de suero de leche. Se ha demostrado que la recuperación de péptidos de bajo peso molecular mejora conforme aumenta la proporción de acetona (>60%), sumado a la incorporación de sales, como el NaCl con acetona al 80% que permite una recuperación de hasta el 99% de los péptidos de ~10 kDa (Baghalabadi & Doucette, 2020).

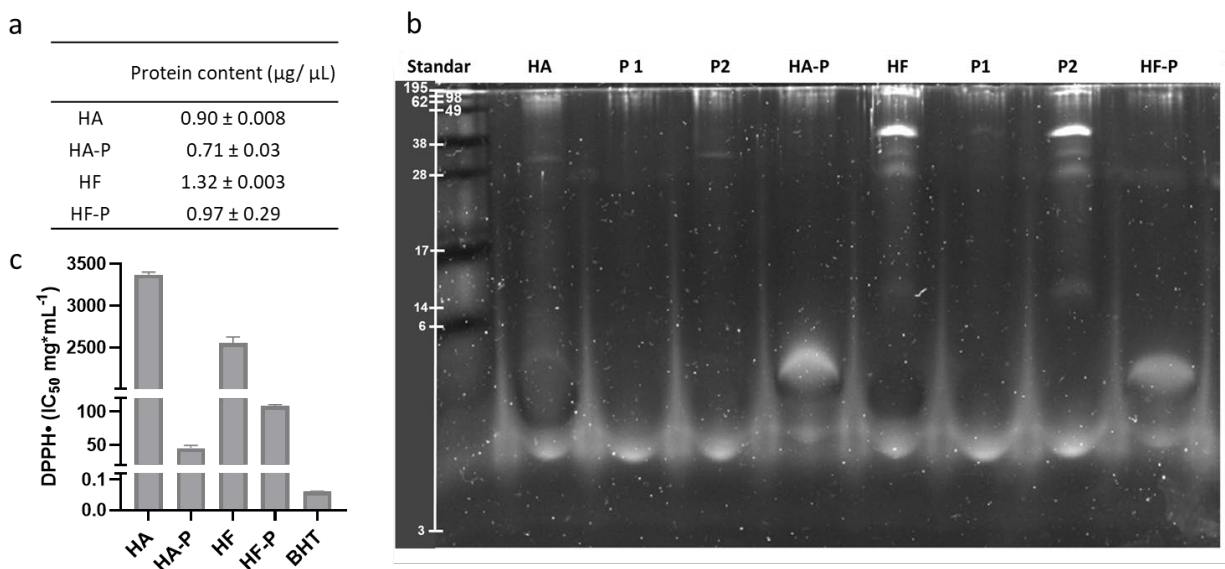


Figura 5. Efecto de la purificación sobre el perfil proteolítico y actividad antioxidante de los hidrolizados optimizados de piel de trucha arcoíris. a: Contenido de proteína por BCA kit; b: electroforesis Tris-tricina con tinción Sypro-Ruby. HA: hidrolizado con alcalasa, HF: hidrolizado con flavourzyme, P1 y P2: pellet 1 y 2 de la extracción con acetona, HA-P: muestra HA purificada, HF-P: muestra HF purificada; c: Actividad antioxidante por método DPPH.

En los hidrolizados HA y HF se observan cambios en el contenido de proteínas y en las bandas electroforéticas antes y después de la purificación usando buffer de lisis y acetona al 80% (Figura 5a-b), confirmando que con la purificación se recuperaron péptidos con pesos moleculares de <6 kDa. Al comparar el efecto de la purificación en la actividad antioxidante medida por el método de DPPH (Figura 5c), se puede identificar que hay un aumento en la capacidad antioxidante de los hidrolizados después de la purificación. En las muestras HA y HF el valor de IC_{50} es mayor que en sus respectivas muestras purificadas, demostrando que la purificación con acetona favoreció la recuperación de péptidos con actividad antioxidante.

La purificación con acetona permite concentrar péptidos aumentando la bioactividad de los hidrolizados debido a que los hidrolizados de pescado son una mezcla compleja de péptidos de diferentes tamaños con propiedades fisicoquímicas diversas. El proceso de purificación favorece la concentración de péptidos con bajo peso molecular, eliminando proteínas de alto peso y compuestos interferentes (Atef et al., 2021; Baraiya et al., 2024; Ennaas et al., 2015). Estos péptidos de bajo peso molecular son de interés por que se han relacionado con una mayor

biodisponibilidad, tasa de difusión en el cuerpo, funciones inmunitarias, antioxidantes, antimicrobianas, así como eficiencia terapéutica (Bazhenov et al., 2022).

Efecto de la purificación sobre las actividades biológicas de hidrolizados optimizados

Actividad antimicrobiana

Los péptidos provenientes de proteínas de peces también han demostrado actividad antimicrobiana contra bacterias Gram-positiva y Gram-negativas (Beaulieu et al., 2009; Robert et al., 2015; Sila et al., 2014). Su mecanismo de acción consiste en la formación de poros o el bloqueo de los gradientes iónicos de la membrana bacteriana, lo que provoca apoptosis (Wald et al., 2016). En el presente estudio, las proteínas hidrolizadas de piel de trucha (muestras HA y HF) no presentaron actividad antimicrobiana frente a *E. coli*, *P. aeruginosa* y *S. aureus* (datos no presentados). Sin embargo, después de la purificación con acetona, los hidrolizados (HA-P y HF-P) exhibieron actividad inhibitoria del crecimiento contra dos bacterias Gram negativas, aunque esta actividad no fue dependiente de la concentración a 5 y 1 mg/mL (Figura 6).

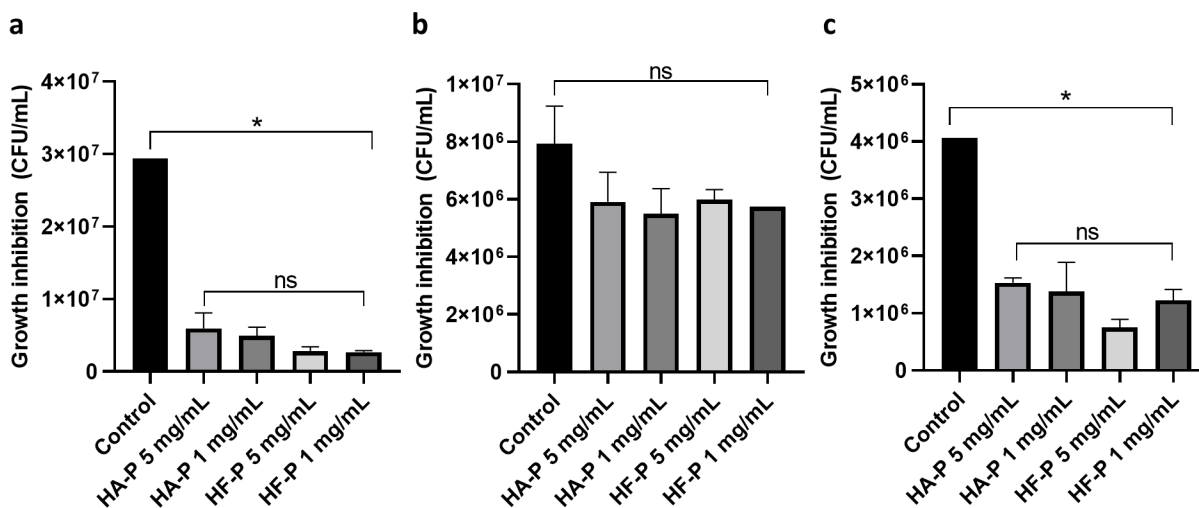


Figura 6. Inhibición del crecimiento de las bacterias *E. coli*, *S. aureus* y *P. aeruginosa* expuestas a hidrolizados purificados de piel de trucha arcoíris. Los datos se expresan como media ± DE, experimentos realizados por triplicado. Los cultivos se diluyeron a 1x10⁶ UFC mL⁻¹. a: *E. coli*, b: *S. aureus* y c: *P. aeruginosa*. Control: Control negativo, medio de cultivo sin inóculo bacteriano.

**p*>0.05, ns: sin diferencia estadística

Se ha demostrado que la hidrólisis de subproductos de peces libera péptidos que tienen acción contra bacterias, por ejemplo, hidrolizados de vísceras de trucha arcoíris o hidrolizados de subproductos de *Scomber scombrus* reportaron fracciones de péptidos que inhibían el crecimiento de bacterias grampositivas y gramnegativas (Ennaas et al., 2015; Wald et al., 2016).

No obstante, al igual que en este estudio, los hidrolizados de colágeno de piel de esturión (*Huso huso*) y subproductos de caballa (*Scomber scombrus*) no mostraron actividad antibacteriana y fue necesaria una purificación con acetona para concentrar los péptidos activos y obtener mejor actividad antibacteriana, atribuible a la hidrofobicidad de los péptidos purificados (Atef et al., 2021; Ennaas et al., 2015).

Además, se ha encontrado que péptidos antimicrobianos derivados de peces también pueden presentar actividad anticancerígena, debido a sus propiedades como carga positiva e hidrofobicidad que les permiten unirse a las membranas con carga negativa de las bacterias y a la fosfatidilserina de la membrana de la célula cancerígena (Felício et al., 2017).

Citotoxicidad

Se ha clasificado la toxicidad de los péptidos en tres grupos, péptidos citotóxicos, cuando la toxicidad es general; hemolíticos cuando son tóxicos sobre los glóbulos rojos, e inmunotóxicos, cuando modulan la respuesta inmunitaria de forma indeseable (Chaudhary et al., 2016). En este sentido, para que los péptidos bioactivos puedan tener una aplicación como posibles fármacos se debe de garantizar su efectividad y no toxicidad. Por lo que en los estudios de péptidos bioactivos es necesaria la realización de ensayos *in vitro* para evaluar la actividad citotóxica. Los principales métodos incluyen ensayo MTT (bromuro de 3-[4,5-dimetiltiazol-2-il]-2,5-difeniltetrazolio), LDH (Lactato deshidrogenasa), basados en ATP (Adenosín trifosfato) y lisis de eritrocitos (Greco et al., 2020; Oddo & Hansen, 2017).

Actividad hemolítica

El ensayo de hemólisis determina la destrucción de los glóbulos rojos ante un agente específico mediante cuantificación espectrofotométrica de la hemoglobina liberada (Ishiguro et al., 2020). Los eritrocitos son las células más abundantes en el sistema circulatorio, son de fácil cultivo y disponibilidad. Además, su falta de estructura de membrana interna facilita la estandarización e

interpretación de los resultados, por lo que se consideran eficaces para evaluar la toxicidad (Almeida et al., 2021; de Oliveira & Saldanha, 2010).

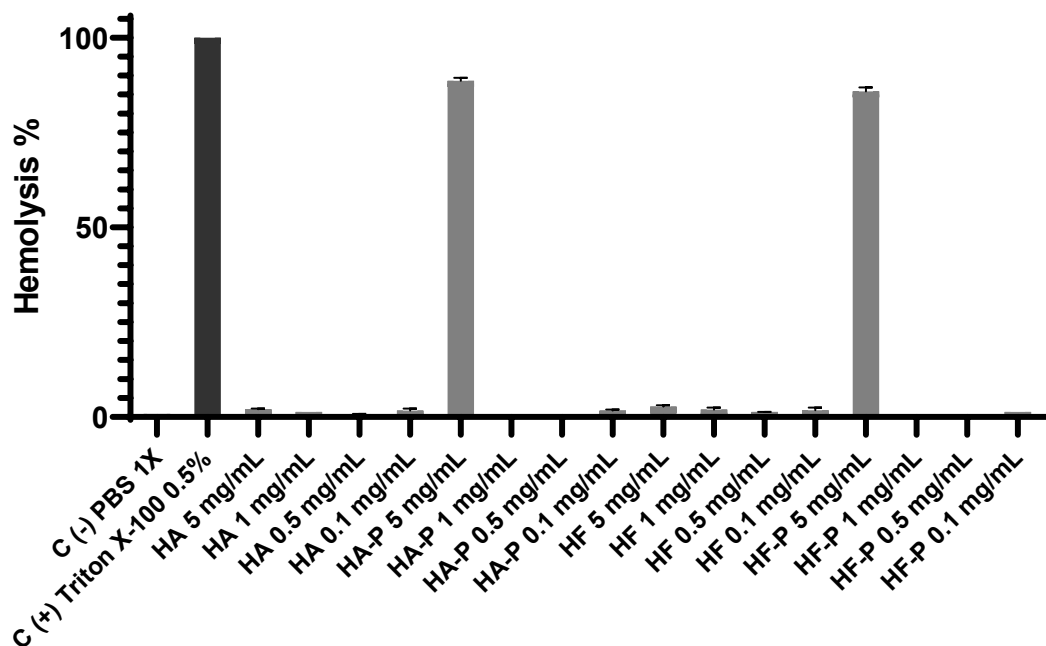


Figura 7. Actividad hemolítica de los hidrolizados de piel de trucha arcoíris. HA: hidrolizado con alcalasa, HF: hidrolizado con flavourzyme, HA-P: muestra HA purificada, HF-P: muestra HF purificada. Los datos se presentan como mediana y rango intercuartílico (n=3). Se observaron diferencias significativas entre los grupos por Kruskal-Wallis con ajuste por empates ($p < 0.05$).

Se ha establecido que un porcentaje de hemólisis inferior a 5% indica que un compuesto no es citotóxico o que provoca una mínima ruptura de glóbulos rojos (HK et al., 2023). Considerando este criterio, los resultados mostrados en la Figura 7 indican que los hidrolizados HA y HF no presentaron actividad hemolítica a concentraciones ≤ 5 mg/mL, mientras que en los hidrolizados purificados si hay daño a los glóbulos rojos a concentración de 5 mg/mL, con porcentajes de 88.5% para HA-P y 85.7% para HF-P.

Los hidrolizados de otras especies de peces también han mostrado baja o nula actividad hemolítica, como hidrolizados proteicos de *Liza aurata*, péptidos antimicrobianos de *Apostichopus japonicas* o colágeno tipo I de piel de Tilapia (Bkhairia et al., 2016; Zhang et al., 2022; Zhao et al., 2024).

Sin embargo, el aumento en la actividad hemolítica en las muestras purificadas podría deberse a una mayor concentración de péptidos hidrofóbicos.

Chen et al. (2007), indican que hay una correlación entre la hidrofobicidad de los péptidos con una mayor hemólisis, debido a que péptidos con mayor hidrofobicidad penetrarán más profundamente la membrana del eritrocito causando mayor hemólisis por la formación de poros o canales. Sin embargo, esto no depende solo de la hidrofobicidad general, sino de la distribución de aminoácidos hidrofóbicos dentro de la estructura del péptido, así como de las diferencias en la composición de las membranas blanco, particularmente entre células bacterianas o eucariotas (Fong-Coronado et al., 2024; Krishnamoorthy et al., 2023).

Actividad anticancerígena

La determinación de la actividad citotóxica contra células cancerígenas se realiza agregando la muestra a un medio con células que crecieron durante 24 horas previamente. Los hidrolizados de piel de trucha arcoíris con alcalasa y flavourzyme no presentaron actividad citotóxica contra las 7 líneas celulares probadas (Datos no presentados). Contrario a las muestras de los hidrolizados purificados HA-P y HF-P que sí resultaron citotóxicas.

En la Tabla 4, se identifican los valores de IC_{50} de las muestras HA-P y HF-P para las 7 líneas tumorales. Manosroi et al. (2006) sugieren que para considerar un agente como candidato terapéutico contra el cáncer se consideren valores de $EC_{50} < 125 \mu g \cdot mL^{-1}$, valor que establecieron al comparar con fármacos de referencia como vincristina y 5-FU. Tomando en cuenta esto, se pueden considerar a los hidrolizados HA-P y HF-P como potenciales candidatos terapéuticos contra la línea MCF-7, que corresponde a una línea celular de carcinoma de mama humano y HF-P contra la línea AGS, una línea celular de cáncer gástrico.

Aleman et al., (2011) describieron hidrolizados de gelatina de calamar usando alcalasa, los cuales presentaron actividad citotóxica contra la línea celular MCF-7 (IC_{50} : 0.81mg/mL) y células de glioma U87 (IC_{50} : 0.85 mg/mL), atribuyeron el efecto al peso molecular de sus péptidos (<2 KDa) y principalmente a la presencia de secuencias con capacidad de interactuar con la membrana de células tumorales, inducir la activación de caspasas y provocar lisis celular. Asimismo, se ha observado una relación entre la enzima utilizada en la hidrólisis y la actividad citotóxica del hidrolizado (Alemán et al., 2011; Mirzapour-Kouhdasht et al., 2021; Tekle et al., 2022), como

también se evidencia en el presente estudio. Sin embargo, un aspecto importante a considerar, además del IC₅₀, es el índice de selectividad de estos hidrolizados.

Tabla 4. Concentración inhibitoria media e índice de selectividad de hidrolizados purificados de piel de trucha arcoíris en líneas celulares tumorales y no tumorales humanas.

Cell lines	HA-P		HF-P	
	IC ₅₀ µg/mL	IS	IC ₅₀ µg/mL	IS
GES-1	342.8 ± 27.2	1.0	305.1 ± 16.9	1.0
MDA-MB-231	245.8 ± 7.8	1.4	272.8 ± 17.9	1.1
AGS	165.0 ± 24.4	2.1	28.9 ± 3.9	1.4
HT-29	332.1 ± 33.3	1.0	391.8 ± 28.7	0.8
HeLa	181.2 ± 23.9	1.9	197.3 ± 20.1	1.5
MCF-7	84.0 ± 4.0	4.1	85.1 ± 9.6	3.6
PC-3	283.6 ± 19.2	1.2	213.5 ± 17.4	1.4

PC-3: Cáncer de próstata humano, AGS: Adenocarcinoma gástrico humano, MDA-MB-231: Cáncer de mama humano, MCF-7: Cáncer de mama humano, HT-29: Adenocarcinoma colorrectal humano, HeLa: Cáncer de cuello uterino y GES-1: Célula epitelial gástrica humana. SI: Índice de selectividad.

Para calcular el índice de selectividad de la citotoxicidad que causa un compuesto (Koch et al., 2005), se utilizó la línea GES-1, que corresponde a una línea celular epitelial gástrica humana, como un control no tumoral. Este índice de selectividad es determinante para establecer el potencial terapéutico de un compuesto contra el cáncer, identificando la presencia o ausencia de efectos citotóxicos en células normales (Badisa et al., 2009). Los valores de IS ≥ 2 indican selectividad, es decir, que el compuesto afecta el doble o más células tumorales que las no tumorales (Koch et al., 2005; Badisa et al., 2009). Las muestras HA-P y HF-P tienen un potencial terapéutico contra el cáncer de mama al tener IS de 4.1 y 3.6 respectivamente, contra la línea MCF-7. Resalta que la muestra HA-P también presentó selectividad contra la línea AGS, con potencial terapéutico contra el cáncer gástrico. Este cáncer se considera uno de los más comunes a nivel mundial, con un mal pronóstico y alta tasa de mortalidad (Pan et al., 2015).

Como se observó en este estudio, se ha evidenciado que los péptidos pueden presentar efectos específicos dependiendo de la línea celular. Por ejemplo, el péptido VECYGPNRPF presentó actividad citotóxica en células AGS mediante su capacidad anti proliferativa de inducir arresto del ciclo celular, sin efecto significativo en líneas de cáncer de colon, hígado o cuello uterino (C2BBel, HepG2, HeLa) (Yaghoubzadeh et al., 2025). También, hay diferencias entre la actividad del hidrolizado crudo y los péptidos purificados, como se observó en hidrolizados de tunicados solitarios en donde dos fracciones con actividad antioxidante (DPPH y ABTS) inhibieron crecimiento de células AGS, DLD-1 y HeLa de manera dosis-dependiente relacionada con su hidrofobicidad, la presencia de residuos de tirosina y el bajo peso molecular de los péptidos, mientras que la actividad de los hidrolizados crudos no fue destacable (Jumeri, 2011).

Péptidos de peces también han demostrado actividad contra la línea MCF-7, como los péptidos de subproductos de atún, bacalao, platija, bacaladilla y salmón (Hsu et al., 2011; Picot et al., 2006), también hidrolizados de huevas de *A. tarichi* presentaron actividad anticancerígena dependiente de la dosis, con mayor actividad contra las células MCF-7 que las MDA-MB-231, debido a que estas últimas son más resistentes al tratamiento al derivar de un estado metastásico, la actividad de los péptidos se atribuyó a una acción multifactorial por activación de caspasas y sobreexpresión de p53 (Berköz et al., 2020), mecanismos relacionados con el aumento de ROS intracelulares, potenciando el daño celular y la inducción de apoptosis en células tumorales (Trachootham et al., 2009).

Como parte de la prevención y tratamiento de los tumores, las investigaciones se han enfocado en el desarrollo de estrategias que tengan un efecto en las distintas fases del proceso tumoral. En este sentido, las ROS, por el papel que desempeñan en las diferentes etapas del cáncer, son el foco de investigaciones con fines terapéuticos (Jia et al., 2020). Aunque las ROS promueven la metástasis, angiogénesis y tumorigénesis, una producción excesiva de ROS causa apoptosis (Sullivan & Chandel, 2014) y se ha demostrado que las células tumorales, debido a su alta tasa metabólica y disfunción mitocondrial, presentan niveles basales elevados de ROS y son más susceptibles a desequilibrios redox que las células normales (Redza-Dutordoir & Averill-Bates, 2016).

Es así que compuestos antioxidantes como péptidos o flavonoides pueden comportarse como moduladores del sistema redox y promover un aumento de ROS, actividad que ha sido descrita para la quercetina, un antioxidante capaz de incrementar los niveles de ROS intracelulares e inducir

apoptosis de células tumorales (Biswas et al., 2022). Algunos compuestos anticancerígenos aumentan la producción de ROS a niveles que pueden dañar el ADN de las células cancerosas, desencadenando señales de apoptosis (Arrieche et al., 2024).

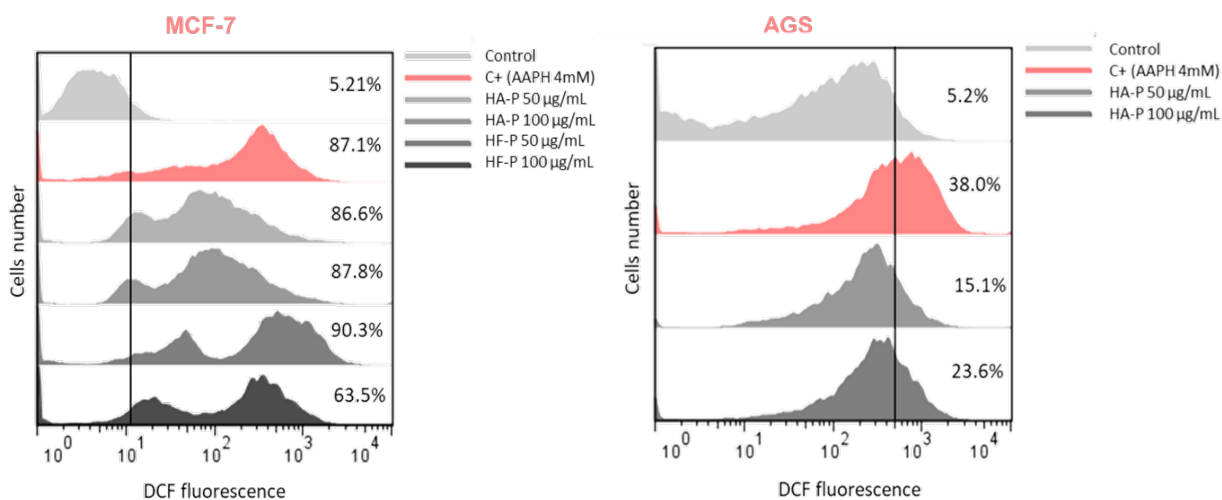


Figura 8. Análisis de citometría de flujo de especies reactivas de oxígeno (ROS) inducidos por los hidrolizados HA-P y HF-P en las líneas celulares MCF-7 y AGS.

En la Figura 8, se muestra como el tratamiento con HA-P a concentraciones de 50 y 100 µg/mL en la línea MCF-7 generó niveles de ROS comparables al control positivo AAPH 4 mM (2,2'-Azobis(2-amidinopropane) dihydrochloride). En cambio, la exposición de HF-P a 50 µg/mL produjo niveles de ROS superiores al control positivo, aunque con un aumento en la concentración del compuesto estos niveles disminuyeron, pero continuaron siendo superiores respecto al control y cercanos al control positivo. En las células AGS, la producción de ROS inducida por HA-P fue dependiente de la concentración, sin embargo, incluso a 100 µg/mL no se alcanzaron los niveles del control positivo, pero si por sobre los niveles del control por lo que se puede considerar generador de ROS.

Identificación de péptidos y predicción in silico de actividad anticancerígena en hidrolizados optimizados

Se identificaron 9 secuencias en los hidrolizados de piel de trucha arcoíris, de las cuales 7 corresponden a nuevos péptidos (Tabla 5). Las predicciones sugieren actividad anticancerígena de estas secuencias, con puntajes cercanos a 1 para las herramientas consultadas.

En las 9 secuencias identificadas encontramos aminoácidos hidrofóbicos, pero en mayor cantidad en GLPGPIGPPGPR, CPDPVIVCPDL PCK y GLPGPLGPPGP, relacionado con la predicción de su actividad en la Tabla 5. Se ha discutido sobre la relevancia de la secuencia de aminoácidos sobre la actividad antioxidante o anticancerígena que el péptido pueda expresar. La actividad antioxidante está estrechamente relacionada con la presencia de aminoácidos hidrofóbicos alanina, valina, leucina, isoleucina, prolina, fenilalanina, triptófano y metionina (Pezeshk et al., 2019).

Las secuencias identificadas están conformadas por 10 a 15 residuos de aminoácidos. Se ha descrito que en los péptidos un tamaño entre 3 y 25 residuos de aminoácidos y la hidrofobicidad les confiere una mejor interacción con las células por su mayor difusividad y movilidad molecular (Zapata & Gómez-Sampedro, 2024).

También se ha relacionado la presencia de los aminoácidos glicina, valina, isoleucina y lisina en el intermedio de la secuencia con la capacidad antioxidante del péptido, debido a que estos aminoácidos favorecen la formación de un ambiente hidrofóbico. La presencia de prolina se relaciona con la capacidad del péptido de donar hidrógenos, pero también con su capacidad de interactuar con la membrana de bicapa lipídica. Mientras que la presencia de cisteína mejora la actividad del glutatión peroxidasa (Escamilla-Rosales et al., 2024).

Tyagi et al., (2013) refieren que la presencia de los aminoácidos glicina, lisina, cisteína, fenilalanina, isoleucina y triptófano es común en péptidos anticancerígenos (Tyagi et al., 2013). En las secuencias obtenidas en este trabajo se encuentran estos aminoácidos, en especial la glicina, que puede relacionarse con los puntajes de predicción cercanos a 1 obtenidos para la actividad anticancerígena por 4 diferentes predictores.

En general, observamos que las predicciones *in silico* indicaron que los péptidos tienen alta probabilidad de ser antioxidantes y anticancerígenos. Estas herramientas predictivas integran

parámetros fisicoquímicos como el peso molecular, la carga neta, la hidrofobicidad y la composición de aminoácidos, características que son determinantes en las interacciones con células y en sus mecanismos de acción antioxidante (Singh et al., 2023).

El péptido CPDPVIVCPDLCK fue el único que se encuentra en ambos hidrolizados y que podría resultar citotóxico. Esto resalta la importancia de validar la actividad de los péptidos con ensayos experimentales que permitan comparar la actividad biológica predicha con la real. En general los resultados obtenidos mediante el análisis *in silico* resaltan el valor de las herramientas informáticas como un complemento en la caracterización funcional de los péptidos derivados de alimentos.

Tabla 5. Secuencias peptídicas identificadas en los hidrolizados de piel de trucha arcoíris y predicción de la actividad biológica

Sample	Secuencia	Mass	m/z	Peptide Ranker	AO ¹	FRS ²	Anti-Ca ³	Anti-Ca ⁴	Anti-Ca ⁵	Anti-Ca ⁶	HA ⁷	Tox ⁸
HA	GLPGPIGPPGPR *	1114	557.82	0.82	0.98	0.55	0.92	0.99	0.99	0.71	0.34	0.12
HA	GFAGPPGGDGQPGAK	1312	656.81	0.78	0.93	0.51	0.96	1.0	0.95	0.19	0.01	0.11
HA	PGPIGPPGPR*	943.5	472.77	0.91	0.97	0.55	0.95	0.99	0.99	0.89	0.05	0.12
HA	GPAGPPGSGGGNGPK	1207	604.29	0.89	0.98	0.49	0.87	0.97	0.98	0.48	0.05	0.12
HA y HF	CPDPVIVCPDLPCCK	1669	835.38	0.88	0.98	0.42	1.0	0.89	0.91	0.32	0.11	0.86
HA	GLPGPIGPEGPR	1146	573.81	0.73	0.95	0.52	0.89	0.98	0.98	0.48	0.02	0.11
HF	GLPGPLGPPGP	957.5	479.77	0.82	0.99	0.56	0.91	0.99	0.99	0.84	0.31	0.12
HF	GPPGPPGYCDSSQC	1460	730.78	0.84	0.99	0.53	0.99	0.83	0.97	0.33	0.05	0.12
HF	GIPGPLGPKGDN	1121	561.30	0.70	0.91	0.48	0.80	0.54	0.97	0.30	0.04	0.12

*Sequence(s) found in BIOPEP-UWM bioactive peptides database and peptide atlas

Valores cercanos a 1 indican mayor posibilidad de actividad

¹ Antioxidant activity predicted by UniDL4BioPep

² Free radical scavenger (FRS) activity predicted by AnOxPePred

³ Anticancer activity (alternative model) predicted by UniDL4BioPep

⁴ Anticancer activity (main model) predicted by UniDL4BioPep

⁵ Anticancer activity predicted by mACpred 2.0 (CBBL-SKKU)

⁶ Anticancer activity predicted by MLACP 2.0 (CBBL-SKKU)

⁷ Hemolytic activity predicted by HLPpred-Fuse

⁸ Toxicity predicted by HyPepTox-Fuse (CBBL-SKKU)

Conclusiones

El perfil proteolítico y la actividad antioxidante de los hidrolizados enzimáticos de piel de trucha arcoíris se favorece a las 6 horas de hidrólisis cuando se emplea la pasteurización como pretratamiento. El uso de alcalasa permitió alcanzar las condiciones óptimas con una menor cantidad de enzima y menor tiempo, lo que representa una alternativa eficiente para la obtención de hidrolizados bioactivos de piel de trucha arcoíris. Por su parte, la optimización y purificación en los hidrolizados de piel de trucha arcoíris permite obtener fracciones peptídicas polares de bajo peso molecular con actividades biológicas. Estos hidrolizados presentaron péptidos no hemolíticos con actividad citotóxica selectiva contra células de cáncer de mama y gástrico, relacionada con la capacidad de los hidrolizados para aumentar la producción de ROS.

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Conclusiones generales

Los péptidos *in silico* identificados de proteínas de trucha arcoíris no presentaron actividad anticancerígena significativa.

El perfil proteolítico y actividad antioxidante de hidrolizados enzimáticos de piel de trucha arcoíris se favorece a las 6 horas y con una pasteurización como pre-tratamiento.

La hidrólisis con alcalasa requiere menor cantidad de enzima y menor tiempo para optimizar su actividad antioxidante en hidrolizados enzimáticos de piel de trucha arcoíris.

La optimización y purificación en los hidrolizados de piel de trucha arcoíris permite obtener fracciones peptídicas polares de bajo peso molecular con actividades biológicas.

Los hidrolizados enzimáticos de piel de trucha arcoíris optimizados y purificados presentan péptidos no hemolíticos con actividad citotóxica selectiva contra células de cáncer de mama y gástrico, relacionada con la capacidad de los hidrolizados para aumentar la producción de ROS.

Se identificaron siete nuevos péptidos en los hidrolizados de piel de trucha arcoíris mediante secuenciación; mientras que, el análisis *in silico* permitió identificar su potencial actividad anticancerígena.