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AMBIENTAL Y BIOALIMENTARIA

**BIOPEPTIDES FROM FOOD BY-PRODUCTS: PRODUCTION,
FRACTIONATION AND BIOACTIVITY ASSESSMENT**

TESIS DOCTORAL

Fátima Arrutia Rodríguez

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

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2.- Autor	
Nombre: Fátima Arrutia Rodríguez	DNI/Pasaporte/NIE:
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RESUMEN (en español)

Es bien conocida la importante generación de residuos de la industria alimentaria y la importancia de llevar a cabo procesos y desarrollar técnicas y usos finales de dichos sub-productos con el fin de avanzar hacia una industria alimentaria sostenible.

En esta Tesis Doctoral se plantean procesos de reutilización de algunos de los sub-productos de esta industria para convertirlos en sofisticados compuestos finales con posible utilización en medicina y en la industria farmacéutica, como son los péptidos bioactivos.

Como materias primas se han utilizado la sangre procedente de mataderos y el suero lácteo. En concreto, se ha trabajado con la seroalbúmina bovina, presente tanto en el suero de quesería (minoritaria) como en la sangre (en mucha mayor concentración), y con un concentrado de proteínas de suero, obtenido tras la concentración por ultrafiltración del suero de queserías. Estos dos productos han sido suministrados por empresas y centros de investigación colaboradores con el trabajo que se presenta.

Para la obtención de los productos finales se ha llevado a cabo una primera etapa de hidrólisis enzimática utilizando tripsina y pepsina como enzimas. El objetivo de esta fase ha sido la de obtener un elevado grado de hidrólisis de los substratos sin buscar la modelización del proceso, sino para obtener los péptidos a partir de la proteína. Para ello, ha sido necesario en algunos casos (por ejemplo en el caso de la seroalbúmina) estudiar posibles procesos de pre-tratamientos previos a la hidrólisis, a fin de incrementar el grado de hidrólisis obtenido.

Tras esta etapa, se han identificado, mediante técnicas instrumentales, los péptidos obtenidos, y determinado con ayuda de simulación computacional las características más importantes de cada péptido o grupos de péptidos.

Posteriormente, se ha estudiado el fraccionamiento de dichos hidrolizados utilizando técnicas con membranas, con el objetivo de obtener permeados con un número más reducido de péptidos con propiedades biológicas de interés.



Los fraccionamientos de hidrolizados constituyen un tema muy novedoso que posiblemente experimentará un importante desarrollo en el futuro. En este apartado, los objetivos se han dirigido esencialmente a estudiar los efectos del pH del medio y el tamaño de poro de las membranas sobre la transmisión de los péptidos obtenidos, y no se ha realizado una optimización desde el punto de vista de la densidad de flujo de permeado de las membranas, por considerar ésta una etapa posterior para otros trabajos.

Por último, se ha realizado un esfuerzo importante para, en colaboración con otros centros y departamentos de investigación, estudiar los efectos *in vitro* e *in vivo* de algunas de las fracciones obtenidas. Los estudios se han centrado en las propiedades relacionadas con el síndrome metabólico, por constituir éste un problema de salud generalizado y con alta prevalencia en países del Primer Mundo. Los síntomas clave del síndrome metabólico son la hipertensión, la obesidad abdominal y la resistencia a la insulina entre otros, y además presentan la característica de estar muy relacionados entre sí, así como con procesos oxidativos.

Todos los ensayos realizados en este trabajo se ha desarrollado a escala de laboratorio, si bien se han seleccionado métodos que podrían tener un paso de escala relativamente sencillo técnicamente.

RESUMEN (en Inglés)

The food industry is characterized by the production of large volumes of effluent streams that also have a high polluting power. In this Thesis, several processes are proposed in order to upcycle some of these by-products, with the double aim to develop high-value bioactives that could be used in the medical and the pharmaceutical areas.

The by-product streams selected for utilization were cheese whey and cattle blood, and the focus was put on the protein component. In particular, two protein substrates were employed: bovine serum albumin, which is present in both blood and whey; and a whey protein concentrate, obtained from whey by ultrafiltration. All proteins were kindly supplied by academic and industrial collaborators. Precisely, the high-value bioactives under study are protein fragments with beneficial properties towards health called "Bioactive Peptides".

For the bioactive peptides production, a first stage of enzymatic hydrolysis was performed, using trypsin and pepsin as enzymes. The objective was to fragment the proteins in an array of peptides. For this, it was necessary, in some cases, to study as well protein pre-treatments that would enhance protein digestion. The modelling of the hydrolysis processes was not among the objectives of this Thesis.



In the second place, appropriate instrumental techniques were applied with the aim of identifying the obtained peptides, and modelling software was used to predict the peptides characteristics that could be relevant for the study.

In the third place, membrane fractionation of the produced hydrolysates was evaluated. The goal was to use a technology easy to scale-up, considering industrial applicability, to obtain a product enriched in the most promising peptides. The efforts were mainly directed to study the influence of the medium pH and the membrane pore size on the peptides transmission across the membrane. No optimization was intended, as it was considered as a topic for future studies based on these ones.

Finally, with some of the enriched fractions obtained, a collaborative study with other departments and universities was conducted about the biological activities of those peptide fractions. The activities studied were related with a group of metabolic conditions closely interrelated with each other whose prevalence has experienced a steep increase in recent years. The major symptoms (hypertension, glucose intolerance, high cholesterol and abdominal obesity) have been grouped under the term "metabolic syndrome".

Moreover, several of these pathologies are also related with oxidative stress, which frequently damages our body. All of these conditions are chronic, and thus generate increased medical costs because the patient has to be treated lifelong.

All of the work conducted for this Thesis was performed at laboratory scale. However, the methodologies used are all susceptible to be scaled-up.



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Apellidos: Arrutia Rodríguez	Nombre: Fátima
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Ángela Puente Salamanca
Francisco Amador Riera Rodríguez
Carlos Menéndez
Ulises A. González



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Coautor7 <input type="checkbox"/> Doctor <input type="checkbox"/> No doctor . Indique nombre y apellidos

Rebeca Fernández
Carlos Menéndez
Ulises A. González
Francisco Amador Riera Rodríguez

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Khaled Mohammed-Geba



Vicerrectoría d'Organización Académica
Vice-rectorate for Academic Organization

Coautor3	<input checked="" type="checkbox"/> Doctor	<input type="checkbox"/> No doctor .	Indique nombre y apellidos
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Coautor8	<input checked="" type="checkbox"/> Doctor	<input type="checkbox"/> No doctor .	Indique nombre y apellidos

Hoang Do-Huu
Yaisel J. Borrell
Asmaa Galal-Khallaf
Alba Ardura
Francisco Amador Riera Rodríguez
Eva García-Vázquez

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Abstract

The food industry is characterized by the production of large volumes of effluent streams that also have a high polluting power. In this Thesis, several processes are proposed in order to upcycle some of these by-products, with the double aim to develop high-value bioactives that could be used in the medical and the pharmaceutical areas.

The by-product streams selected for utilization were cheese whey and cattle blood, and the focus was put on the protein component. In particular, two protein substrates were employed: bovine serum albumin, which is present in both blood and whey; and a whey protein concentrate, obtained from whey by ultrafiltration. All proteins were kindly supplied by academic and industrial collaborators. Precisely, the high-value bioactives under study are protein fragments with beneficial properties towards health called "Bioactive Peptides".

For the bioactive peptides production, a first stage of enzymatic hydrolysis was performed, using trypsin and pepsin as enzymes. The objective was to fragment the proteins in an array of peptides. For this, it was necessary, in some cases, to study as well protein pre-treatments that would enhance protein digestion. The modelling of the hydrolysis processes was not among the objectives of this Thesis.

In the second place, appropriate instrumental techniques were applied with the aim of identifying the obtained peptides, and modelling software was used to predict the peptides characteristics that could be relevant for the study.

In the third place, membrane fractionation of the produced hydrolysates was evaluated. The goal was to use a technology easy to scale-up, considering industrial applicability, to obtain a product enriched in the most promising peptides. The efforts were mainly directed to study the influence of the medium pH and the membrane pore size on the peptides transmission across the membrane. No optimization was intended, as it was considered as a topic for future studies based on these ones.

Finally, with some of the enriched fractions obtained, a collaborative study with other departments and universities was conducted about the biological activities of those peptide fractions. The activities studied were related with a group of metabolic conditions closely interrelated with each other whose prevalence has experienced a steep increase in recent years. The major symptoms (hypertension, glucose intolerance, high cholesterol and abdominal obesity) have been grouped under the term "metabolic syndrome".

Moreover, several of these pathologies are also related with oxidative stress, which frequently damages our body. All of these conditions are chronic, and thus generate increased medical costs because the patient has to be treated lifelong.

All of the work conducted for this Thesis was performed at laboratory scale. However, the methodologies used are all susceptible to be scaled-up.

Resumen

Es bien conocida la importante generación de residuos de la industria alimentaria y la importancia de llevar a cabo procesos y desarrollar técnicas y usos finales de dichos sub-productos con el fin de avanzar hacia una industria alimentaria sostenible.

En esta Tesis Doctoral se plantean procesos de reutilización de algunos de los sub-productos de esta industria para convertirlos en sofisticados compuestos finales con posible utilización en medicina y en la industria farmacéutica, como son los péptidos bioactivos.

Como materias primas se han utilizado la sangre procedente de mataderos y el suero lácteo. En concreto, se ha trabajado con la seroalbúmina bovina, presente tanto en el suero de quesería (minoritaria) como en la sangre (en mucha mayor concentración), y con un concentrado de proteínas de suero, obtenido tras la concentración por ultrafiltración del suero de queserías. Estos dos productos han sido suministrados por empresas y centros de investigación colaboradores con el trabajo que se presenta.

Para la obtención de los productos finales se ha llevado a cabo una primera etapa de hidrólisis enzimática utilizando tripsina y pepsina como enzimas. El objetivo de esta fase ha sido la de obtener un elevado grado de hidrólisis de los substratos sin buscar la modelización del proceso, sino para obtener los péptidos a partir de la proteína. Para ello, ha sido necesario en algunos casos (por ejemplo en el caso de la seroalbúmina) estudiar posibles procesos de pre-tratamientos previos a la hidrólisis, a fin de incrementar el grado de hidrólisis obtenido.

Tras esta etapa, se han identificado, mediante técnicas instrumentales, los péptidos obtenidos, y determinado con ayuda de simulación computacional las características más importantes de cada péptido o grupos de péptidos.

Posteriormente, se ha estudiado el fraccionamiento de dichos hidrolizados utilizando técnicas con membranas, con el objetivo de obtener permeados con un número más reducido de péptidos con propiedades biológicas de interés. Los fraccionamientos de hidrolizados constituyen un tema muy novedoso que posiblemente experimentará un importante desarrollo en el futuro. En este apartado, los objetivos se han dirigido esencialmente a estudiar los efectos del pH del medio y el tamaño de poro de las membranas sobre la transmisión de los péptidos obtenidos, y no se ha realizado una optimización desde el punto de vista de la densidad de flujo de permeado de las membranas, por considerar ésta una etapa posterior para otros trabajos.

Por ultimo, se ha realizado un esfuerzo importante para, en colaboración con otros centros y departamentos de investigación, estudiar los efectos *in vitro e in vivo* de algunas de las fracciones obtenidas. Los estudios se han centrado en las propiedades relacionadas con el síndrome metabólico, por constituir éste un problema de salud generalizado y con alta prevalencia en países del Primer Mundo. Los síntomas clave del síndrome metabólico son la hipertensión, la obesidad abdominal y la resistencia a la insulina entre otros, y además presentan la característica de estar muy relacionados entre sí, así como con procesos oxidativos.

Todos los ensayos realizados en este trabajo se ha desarrollado a escala de laboratorio, si bien se han seleccionado métodos que podrían tener un paso de escala relativamente sencillo técnicamente.

INTRODUCTION

1. Introduction:

Estimates show that around 100 million tonnes of food are wasted annually in the European Union (EU). These resources, unfittingly termed “waste”, would be enough to feed all the hungry people in the world two times over. Since the term “waste” implies that the material is no longer of any use, the term “by-product”, or even sometimes “co-product”, are currently preferred. Directive 2008/98/EC of the European Parliament and the Council of the EU established a difference between “waste” and “by-product”. While the former is defined as “any substance or object which the holder discards or intends or is required to discard”, for the latter it is specified that it is “a substance or object, resulting from a production process, the primary aim of which is not the production of that item”. Therefore, “by-product” includes the concept that it could be used after the production process.

Hence, food by-products upcycling is a current global dire concern. Initiatives as the REFRESH (Resource Efficient Food and dRink for the Entire Supply cHain) project, by the EU, are trying to address it. The project runs from July 2015 to June 2019, and is focused on the reduction of avoidable waste and the improvement of the valorisation of food resources.

Due to the large volumes generated and the high pollutant load carried, both blood and whey are problematic effluents of the food industry. However, their high protein content suggests great potential for use. Whey proteins have been given a PDCAAS (Protein Digestibility-Corrected Amino Acid Score) value of a 100%, which is the highest possible (Dullius, Goettert, & de Souza, 2018). The PDCAAS is the FAO (Food and Agriculture Organization of the United Nations) / WHO (World Health Organization) preferred method for the measurement of the protein value in human nutrition. Likewise, meat proteins biological value is only overcome by that of whey and egg (Smithers, 2008).

Whey is the co-product of cheese and/or casein production, the watery fraction that remains after casein proteins coagulation, rich in lactose and protein. Whey composition may vary depending on the processing method (rennet or acid coagulation) and the milk source (cow, goat...). However, average values can be 93.12 - 93.42% water, 5.12 - 5.14% lactose, 0.76 - 0.85% protein, 0.09 - 0.36 fat, and 0.53 - 0.61 ash (Ward, 2008). The FAO expects an increase in developing countries dairy products consumption, which will, in turn, increase world milk production in a 23% by 2025, compared to the 2013 - 15 values (OECD/FAO, 2016).

Since for every kilogram of manufactured cheese, 9 L of whey are produced, to find large scale uses for this stream becomes urgent. Technological advances to concentrate and dry whey have allowed the production of a wide range of powders, being the most representative whey powders (WP), whey protein concentrates (WPC), whey protein isolates (WPI) and whey protein hydrolysates (WPH). WP are used in animal feed mainly; but also in infant formula, to increase protein content. They are also used as a milk partial replacement in chocolates or biscuits manufacture; in food processing, as bulking agent in the preparation of meat or soups; and in dietary supplements. WPC are used in infant formula and dietary supplements as well, but also in cheese processing and sports nutrition. Finally, WPI and WPH are utilized in sports nutrition, clinical nutrition, beverages and pharmaceuticals (Hoogwegt group, 2017). Whey is also used to produce lactose and, in minority, other value-added products such as organic acids, biopolymers or bacteriocins. Despite this, there is still a need to find new routes of whey utilization, since estimates report that only a 50% of the total whey production is processed (Mollea, Bosco, & Marmo, 2013).

On the other hand, livestock production in Europe contributes considerably to the economy, with a 45% of the total agricultural activity (Animal Task Force, 2017). Blood is an important by-product of the meat industry that accounts for a 4% of the live animal weight. Bovine blood average composition is 80.9% water, 17.3% proteins, 0.23% lipids, 0.07% carbohydrates, and 0.62% minerals (Ofori & Hsieh, 2014). The disposal of blood according to the environmental regulations is an expensive process, and selling prices cannot compensate it. Several cultures have traditionally used blood to cook ethnic dishes, such as in blood sausages and black pudding, and in the animal feed industry blood is a common ingredient. However, it should be considered that the use of blood is subjected to strict hygiene regulations (EC, 2004). Although after the detection of bovine spongiform encephalopathy (BSE) in 1982, the tissues that are most likely to contain the infectious agent have to be destroyed as risk materials (EC, 2001), blood is not among those tissues. In the food industry, blood is a versatile additive, used for fat replacement, colour enhancement, emulsification, and meat preservation. In pharmacological applications, blood is used in many culture media, as reactive in serology assays, and as precursor for porphyrin and thrombin production (Alao, Falowo, Chulayo, & Muchenje, 2017). Despite this, it is estimated that the food industry currently uses only about 30% of slaughterhouse blood (Ofori & Hsieh, 2014).

Therefore, being both blood and whey underutilized liquid effluents of the food industry, rich in good quality protein of animal origin; it seems logical to study similar technologies to process them. Moreover, serum albumin (SA), one of the proteins used in this work, is present in both streams.

Protein itself is a valuable component, which can reach very high market prices in both animal feed and human food markets. However, going one step further and turning protein into high-value bioactives can offer additional health improving benefits beyond basic nutritional value. The protein-derived high value bioactives produced in this study are named “Bioactive Peptides (BP)”, and are defined as fragments encrypted in the primary sequences of proteins that confer health improving functions upon administration (Li-Chan, 2015). Hence, BP are inactive within the sequence of the parental protein, and thus have to be released in a proteolysis process (Urista, Fernández, Rodriguez, Cuenca, & Jurado, 2011).

BP are becoming more and more popular regarding its use as intervention agents against chronic human diseases. As an example, the European Commission (EC) project INPACT (Innovative Peptides Against Cancer and pathogenic bacteria, with advances in science, biopharmaceutical drug development, product market targeting, training, and communication), framed within the Horizon 2020, aims to develop a platform technology to produce innovative peptide-based drugs, to later transfer the knowledge to the pharmaceutical industry. A global health problem with an increasing trend nowadays is the so-called “metabolic syndrome” (MS). The term comprises a series of metabolic abnormalities (abdominal obesity, high blood pressure, high blood sugar, high cholesterol levels) associated with cardiovascular disease and diabetes type 2 (T2D) (Uenishi, Kabuki, Seto, Serizawa, & Nakajima, 2012). MS is an epidemic, and higher medical care costs have been associated with each of MS components by several studies (Boudreau, Malone, Raebel, Fishman, Nichols, Feldstein, et al., 2009; Nichols & Moler, 2011). BP can prove useful against MS, as they have been recognized to possess antihypertensive, lipid and glucose regulation activities among others (Korhonen & Pihlanto, 2006). Precisely, diabetes and cardiovascular disease have been reported to be among the causes of 36 million deaths each year (Li-Chan, 2015). Likewise, many health problems are derived from body oxidation processes, since the free radicals released damage cells in a process called “oxidative stress”. Cancer, high blood pressure, artery hardening, inflammation, and infertility are related diseases (Y. Li & Yu, 2015).

BP have attracted both the lay audience attention and research efforts over the last years. They constitute “natural” food-derived molecules, very well suited for being used as therapeutic agents due to a high bioactivity and specificity, a wide spectrum of action (some peptides (multifunctional) can even present different activities), low toxicity and low levels of accumulation in body tissues, small size (what makes easier delivery and reduces the likelihood of triggering undesirable immune responses (Mason, 2010)), structural diversity and fewer side effects than other drugs.

However, the major drawback for BP commercialization is the severe lack of large-scale economical production methodologies (Agyei & Danquah, 2011). Another additional problem is the fact that the methodologies employed often affect the structure-activity relationship of the peptides (Agyei, Ongkudon, Wei, Chan, & Danquah, 2016). Consequently, the main improvement needed in BP production is the development of affordable methods, easy to scale-up and gentle with delicate molecules. In this work, membrane technologies were the method of choice to study the separation of BP. Membrane separations are highly energy efficient, easy to scale-up and/or down, and use mild operating conditions (Fernández, Zhu, FitzGerald, & Riera, 2014). Another strategy to avoid costs was the evaluation of *in silico* tools, as an alternative to costly and time-consuming *in vitro* assays.

However, apart from the isolation of peptides from natural food sources, there is another option: synthetic peptide synthesis. It was for long unaffordable at large scale, until the development of an improved technique by Trimeris and Roche (Bruckdorfer, Marder, & Albericio, 2004), an hybrid solid-phase/in solution procedure. Solid-phase peptide synthesis (SPPS) and derived techniques have the great advantage that can be carried out in an automated mode. However, they are highly polluting processes, because they involve the use of toxic chemicals, as resins and linking reagents, and unwanted toxic reactions can occur (Koutsopoulos, 2017). The scalability, rather than the underlying chemistry, is the fact that has changed the most over the last five years. Currently, peptide synthesis is a trendy and profitable business, and there are many companies worldwide interested in this area. For instance, giants as Roche (Basel, Switzerland) or Lonza (Basel, Switzerland) have peptide synthesis lines; and Pepsyntha (Torrance, CA, USA), or AmbioPharm (North Augusta, SC, USA) are companies fully dedicated to peptide synthesis.

On the other hand, another factor that severely limits the use of BP in humans, is the lack of robust clinical trials (Gallego, Mora, & Toldrá, 2018). These trials are time-consuming and involve high costs and technical complexity, apart from ethical concerns. However, bioactive peptides clinical trials trend is optimistic, and the number of assayed peptides per year has steadily increased from 1 in 1970 to 20 in 2014 (Uhlir, Kyprianou, Martinelli, Oppici, Heiligers, Hills, et al., 2014). Nevertheless, prior to clinical trials, there is the need of testing in animal models. Most used animal models are spontaneously hypertensive rats (SHR), when testing antihypertensive peptides. In this work, the use of zebra fish (*Danio rerio*) as a model can set a precedent in fore coming *in vivo* studies.

OBJECTIVES

2. Objectives

The primary objective of this thesis work was to explore industrially applicable methodologies to valorise whey and blood by-product streams, focusing in the protein component. The developed methodologies were aimed to produce functional protein-derived products with the ability to promote health condition, i.e. Bioactive Peptides (BP).

Thus, from the general objective, the derived objectives follow:

- Perform enzymatic hydrolyses to obtain peptides from the parental proteins, using gastrointestinal enzymes with high specificity (trypsin and pepsin).
- With the aim of improving the protein digestion by the enzymes, study the suitability of using pre-treatments prior to the hydrolysis processes.
- Develop methodologies to assess the protein enzymatic digestion, identify the obtained peptides (i.e. know their aa sequence) and study the characteristics relevant to the subsequent fractionation processes.
- Propose Membrane Filtration Processes to fractionate the hydrolysates so as to obtain products enriched in BP or even purified peptides. Study how the peptides characteristics, as well as the medium conditions, could affect the filtration outcomes with regard to the peptides transmission.
- Evaluate the bioactivity of the fractions and the peptides obtained by using *in silico*, *in vitro* and/or *in vivo* techniques, with regard to current global epidemics.

Overall, this thesis should be understood as a preliminary study of a whole process of production, fractionation and evaluation of peptide fractions and peptides. The work is not focused on optimising any process, but to explore routes and methodologies with potential applicability. This is, the study will always focus on peptides behaviour and properties rather than on the operating conditions.

THEORY AND METHODOLOGY

3. Theory and methodology:

In this section, brief comprehensive descriptions will be provided about:

- The substrates used.
- The enzymes employed.
- The hydrolysis, fractionation and identification processes.
- Related theoretical knowledge.

3.1. Proteins

Proteins are polymers formed by amino acids (aa). Aa are organic molecules that contain both an amino and an acid group. There are 20 different aa that can form part of proteins in nature. They link with each other through an amide bond called the peptide bond. Annex I shows the structure of the 20 natural aa (obtained from www.compoundchem.com).

The so-formed proteins have a structure organized in four levels (see Figure 1): the primary structure refers to the linear sequence of aa; the secondary structure is related to the spatial disposition of those aa among the chain. The tertiary structure shows how the linear chain folds and the three-dimensional shape it acquires. Finally, the quaternary structure attends to proteins composed by more than one amino acidic chain, and how these chains are arranged in the space.

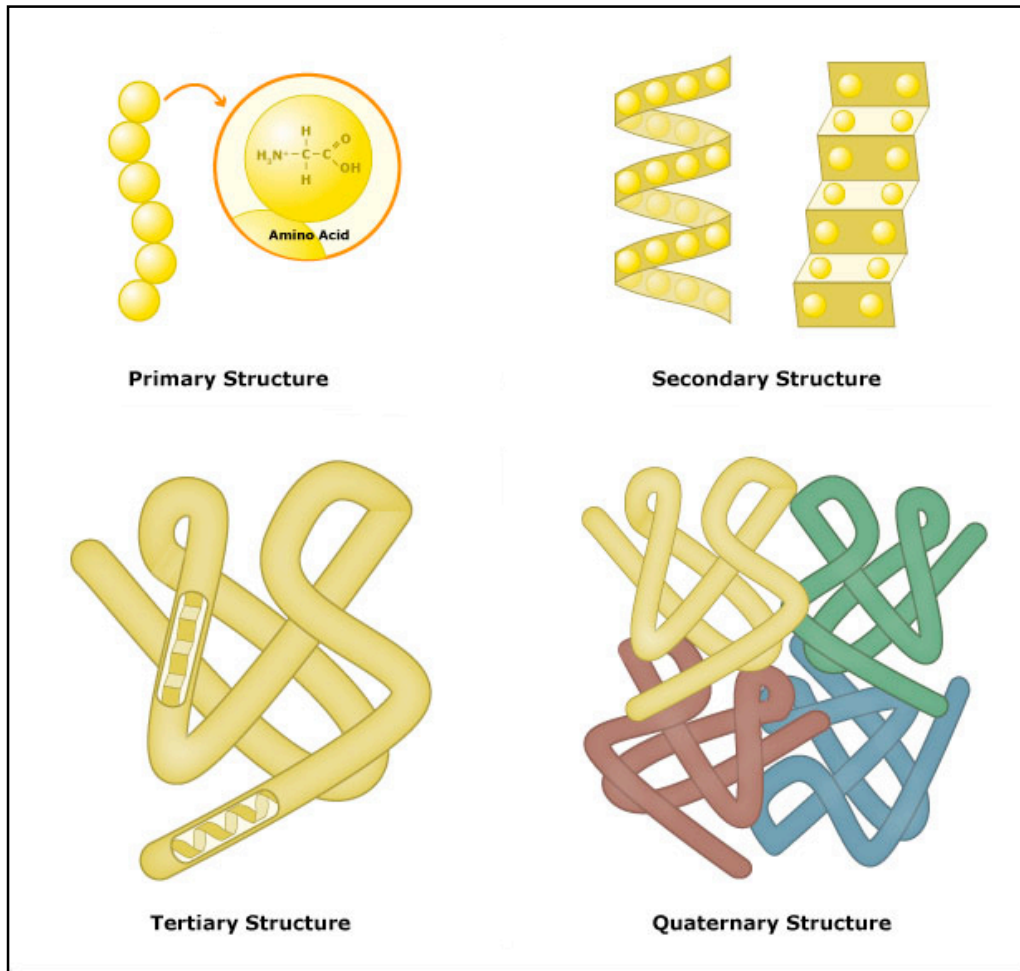


Figure 1: Protein structural levels. Image taken from the Eberly College of Science webpage (<http://science.psu.edu>).

3.2. Bovine Serum Albumin (BSA)

Bovine serum albumin (BSA) is a globular protein with a three-dimensional structure that resembles a heart (see Figure 2). It is divided into three helical domains; each of one can be sub-divided into two subdomains. Regarding the secondary structure, BSA has a high percentage of helical content, around 74%, and presents 17 disulphide bonds and a free thiol group. Serum albumins are relatively large proteins, with a molecular weight (MW) of around 66 kDa (583 aa), and their surface is negatively charged at neutral pH values, with an isoelectric point (pI) of around 4.8. Albumins constitute the main plasma protein in mammals.

As stated in the introduction, BSA is present in both whey and blood. BSA concentration in whey is approximately 0.03 - 0.06% (Blanca Hernández-Ledesma, Ramos, & Gómez-Ruiz, 2011), while in cattle blood is around 3.61% (Bah, Bekhit, Carne, & McConnell, 2013). Milk serum albumin is identical to the plasma one.

Albumins are multifunctional proteins, with a broad ligand-binding capacity. Among its functions, they contribute to the regulation of the blood pH, osmotic pressure, and the levels of Ca^{2+} and Mg^{2+} ; and serve as a reservoir for nitric oxide, a relevant signalling agent (Majorek, Porebski, Dayal, Zimmerman, Jablonska, Stewart, et al., 2012).

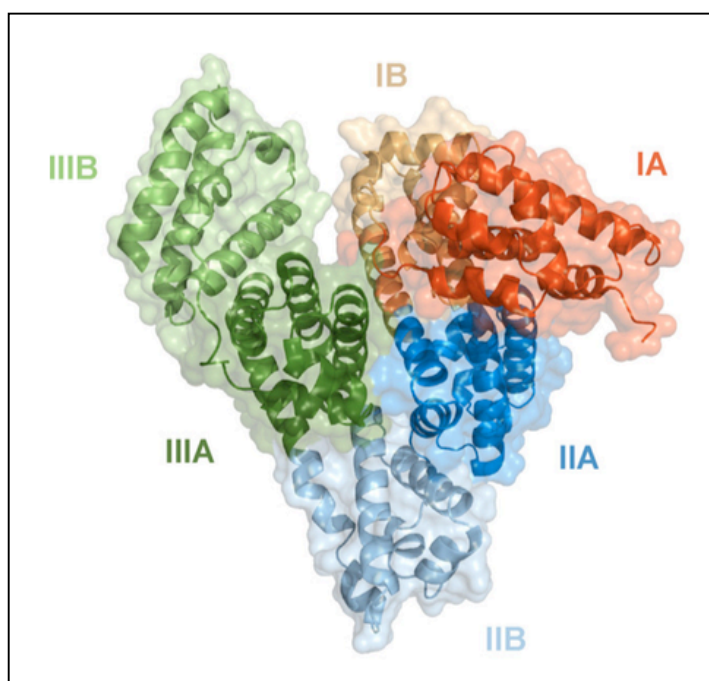


Figure 2: Bovine serum albumin (BSA) three-dimensional structure, showing subdomains. From Majorek, et al. (2012).

Serum albumins have the ability to undergo reversible conformational changes with variations of the medium pH values. Each one of the isomeric structures adopted are called “conformers”, and were first described by Foster (Foster, 1960). Table 1 summarises the conformers described until date.

Table 1: Bovine serum albumin (BSA) conformers with pH values. Adapted from Curvale (2009).

Conformer name	Transition pH value
E (Expanded)	2.7
F (Fast)	4.3
N (Normal)	8
B (Basic)	10
A (Aged)	10

The conformer N is the commonly described structure of BSA at neutral pH values. At pH 9, BSA acquires conformer B configuration, and then after 3 - 4 days conformer A.

BSA also undergoes structural changes with heat. However, studies are not exactly coincident about the transition temperatures, and in some cases differ greatly. This can be partially explained because denaturation not only depends on temperature, but also on other parameters such as pH, ionic strength or protein concentration. For instance, Su, Qi, He, Zhang, and Jin (2008) state that BSA structure remains mostly unchanged until 60 °C, and that above 65 °C aggregation starts. Aggregation happens because the molecule exposes its inner hydrophobic and thiol residues, which allows the formation of hydrophobic interactions, disulphide bridges and covalent bonds. Nevertheless, Murayama and Tomida (2004) established that the protein remained unchanged until 40 °C, and then underwent reversible changes until 52 °C. Above that temperature, the denaturation became irreversible, and above 60 °C aggregation started.

3.3. Whey Protein Concentrates (WPC)

Whey is the by-product of cheese making. The protein component can be concentrated by membrane filtration, commonly using hollow fibre or spiral wound membranes, to obtain whey protein concentrates (WPC), with around 35 - 80% protein content (w/w). The major whey proteins are β -lactoglobulin (β -lg) and α -lactalbumin (α -la).

Other minor components are serum albumin (SA), immunoglobulins (Ig), lactoferrin (LF), lactoperoxidase (LP), proteose-peptones (PP) and, in some cases, caseinomacropeptide (CMP). CMP consists in the 64 C-terminal amino acids of the κ -casein, which are released by pepsin during rennet coagulation. In case that coagulation is achieved with lactic acid, it is not present (Kinsella, 1990).

β -lg is a soluble globular protein, with a MW of 18.3 kDa and an aa chain of 162 residues long. It contains two disulphide bridges between C66 and C160, and C106 and C119; and a free thiol group at C121 (Boland, Singh, & Thompson, 2014). It is the major component of whey, representing on average around 50% of the total protein. There are at least 5 genetic variants of β -lg, although the most common ones are A and B, which only differ in two aa. Regarding the tertiary structure, β -lg is composed mainly of β -sheet strands (see Figure 3). β -lg can appear, depending on pH and temperature, as a single polypeptide chain, a dimer or an octamer mainly. For instance, the normal quaternary structure in milk (pH 6.5) is the dimer, and up to pH 8. From pH 6.5 to 3.7, it is assembled into octamers; and below pH 3.5 it is dissociated into monomers, which are the predominant form above pH 8 as well (Damodaran & Parkin, 2017; B Hernández-Ledesma, Recio, & Amigo, 2008).

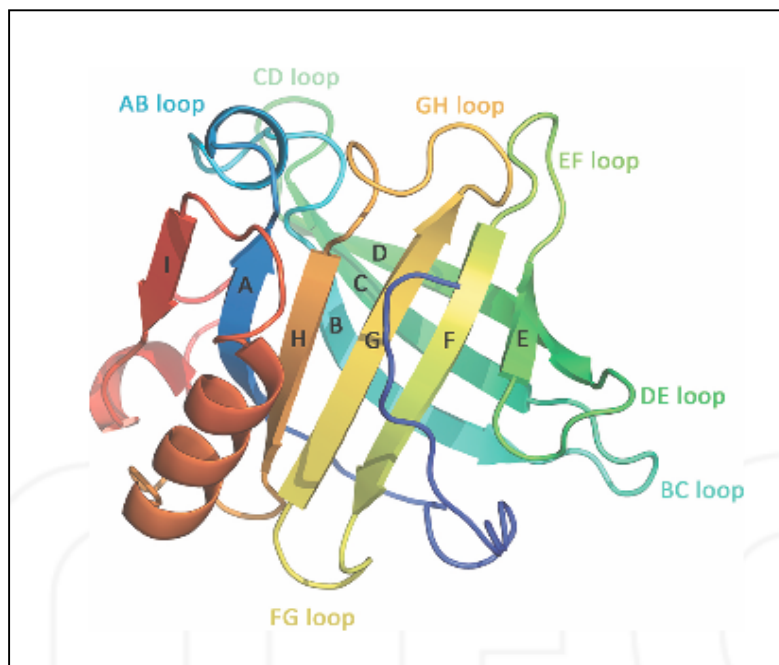


Figure 3: Crystal structure of a β -lactoglobulin (β -lg) monomer. Taken from (Crowther, Jameson, Hodgkinson, & Dobson, 2016).

α -la, the second main protein in whey, accounts for approximately 25% of whey proteins, and weights 14 kDa. The aa chain contains 123 residues and has four disulphide links and no sulfhydryl group. The three-dimensional structure (Figure 4) is composed of two domains, one big alpha helical, and one small β -sheet (Jovanović, Barać, & Maćej, 2005).

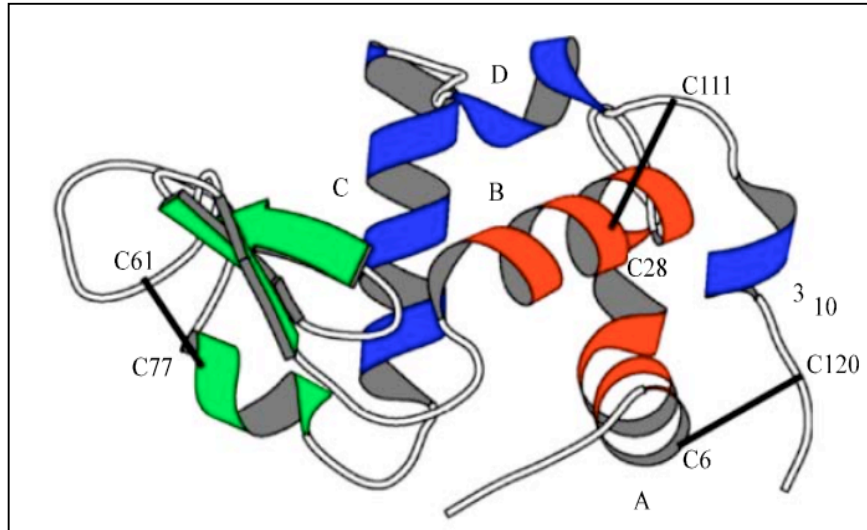


Figure 4: Crystal structure of α -lactalbumin (α -la). Obtained from Velusamy and Palaniappan (2011).

3.4. Trypsin

Trypsin (EC 3.4.21.4) is a protease that acts in the small intestine. It belongs to the class of serine proteases. They are called like that because they all have a very active S residue in their catalytic core. It is as well an endopeptidase, enzymes that hydrolyse peptide bonds located within the aa chain. They are opposed to exoproteases, which only hydrolyse terminal peptide bonds. The MW is 23.3 kDa, and in its active form, the enzyme possesses 224 aa and it has a globular three-dimensional form. Over 37 °C, trypsin starts to lose its activity. It breaks peptide bonds after R and K residues (see Appendix 1), this is, breaks the peptide bond in which the carbonyl group of these aa is involved. The specificity is due to an D residue in the catalytic core, that establishes saline bonds with the basic radicals of the positively charged aa (Müller-Esterl, 2008).

However, if a P residue follows, the cleavage will not occur, and if an acidic residue is on either side of the cleavage site, the rate of hydrolysis will be slower. Trypsin has a pH optimum between 7.5 and 8.5 (Worthington Enzyme Manual, 2017b).

3.5. Pepsin

Pepsin (E.C. 3.4.23.1), a member of the Peptidase A1 family, is the predominant digestive protease in the gastric juice of vertebrates. Pepsin has broad specificity with a preference for peptides containing linkages with aromatic or carboxylic L-amino acids. It preferentially cleaves C-terminal to F and L and to a lesser extent E linkages. The enzyme does not cleave at V, A, or G. Pepsin has a molecular weight of 34.5 kDa, and the optimum pH is between 1 and 4 (Worthington Enzyme Manual, 2017a).

3.6. Calculation of the degree of hydrolysis: pH-stat

The hydrolysis process is traditionally divided in three steps (Benítez, Ibarz, & Pagan, 2008) (Figure 5):

1. Formation of an enzyme-substrate complex (ES).
2. Cleavage of the peptide bond, resulting in one peptide (H-P') and the rest of the substrate (EP).
3. Separation of the peptide by the nucleophilic attack of a water molecule (H₂O).

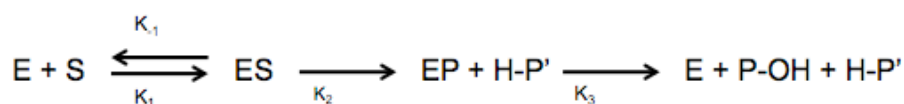


Figure 5: Traditional steps of the hydrolysis process.

If the hydrolysis process takes place in alkaline conditions, the medium pH becomes more acidic due to the breakage of the peptide bonds (Cheison, Schmitt, Leeb, Letzel, & Kulozik, 2010): when a peptide bond is broken, a free carboxyl and a free amino group are released (Figure 6).

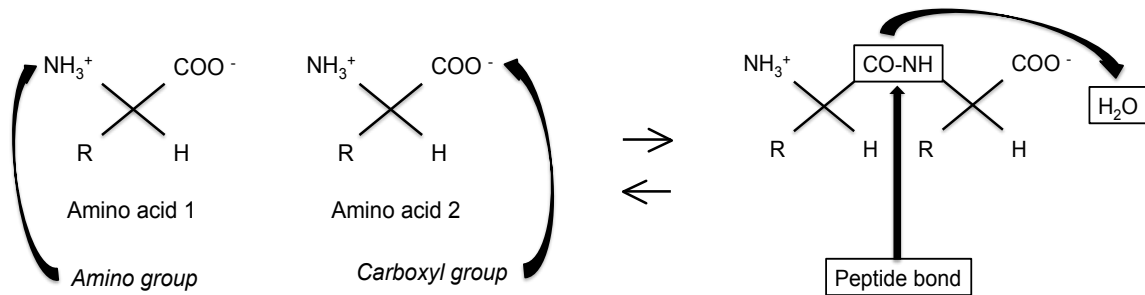


Figure 6: Breakage of a peptide bond and release of free amino and carboxyl groups.

Depending on the medium pH value and the group's pKa, they would either release or accept H^+ groups. The pKa is the pH value at which half of the species are in a de-protonated form (A^-) and half of them in a protonated form (AH). The relationship between the medium pH and the species pKa is defined by the Henderson-Hasselbalch equation (Equation 1) (Moore, 1985):

$$pKa = pH + \log HA/A^- \quad \text{Eq. [1]}$$

When the pKa is lower than the medium pH, the group's main form is the protonated one. Conversely, when the pKa is higher than the medium pH, the main form is the de-protonated one (Figure 7). The pKa value of an aa amino group is around 9 (it varies slightly depending on the specific aa), and that of the carboxyl group is around 2 (McGraw-Hill Education, 2017).

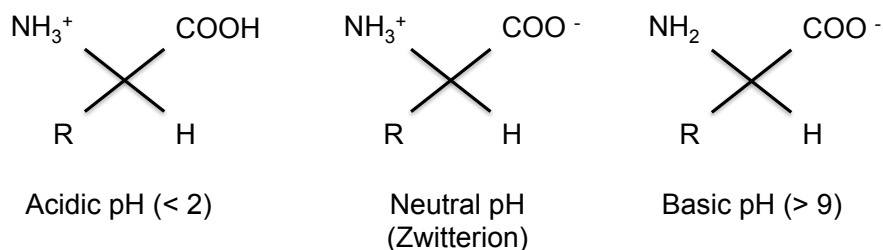


Figure 7: Protonated, zwitterionic and de-protonated amino acid (aa) forms.

Therefore, at pH values around 8 (trypsin optimum), the main aa form is the zwitterion. The amino group is mainly protonated but the carboxyl group is mainly de-protonated, and thus when the peptide bond is broken, the new generated carboxyl group releases H⁺ to the medium, lowering the pH value.

Thus, in order to maintain the pH value in the enzyme optimum, a base has to be added. This is the basis of the pH-stat method: a fixed pH value (normally the enzyme optimum) is maintained by adding base.

The degree of hydrolysis (DH) is a parameter of paramount importance to follow the hydrolysis extension, and is defined as the percentage of broken peptide bonds with regard to the intact protein. This is, the number of peptide bonds broken (h), compared to the total peptide bonds present in the substrate (h_{TOT}), represented by Equation 2 (Nielsen, Petersen, & Dambmann, 2001). They are both expressed in meq/g protein.

$$DH = h / h_{TOT} * 100 \quad \text{Eq. [2]}$$

In the pH-stat method, the DH is proportional to the amount of base consumed, and can be calculated with Equation 3 (Adler-Nissen, 1986):

$$DH = (V_B \times N_B / \alpha \times MP \times h_{TOT}) * 100 \quad \text{Eq. [3]}$$

Where h_{TOT} refers to the total peptide bonds present in the substrate. V_B is the mL of consumed base, N_B the normality of the base, α the average degree of dissociation of the amino groups, and MP the grams of substrate protein. α can be calculated using Equation 4 (Shi, He, & Qi, 2005).

$$\alpha = 10^{pH-pKa} / 1+10^{pH-pKa} \quad \text{Eq. [4]}$$

And the pKa can be calculated with Equation 5 (Camacho, González-Tello, Pérez-Dueñas, Guadix, & Guadix, 2001):

$$pK_a = 3,8 + 0,45 * pH$$

Eq. [5]

The DH depends upon both the hydrolysis conditions (substrate concentration, substrate to enzyme ratio, temperature, time, pH ...) and the enzyme used; and is affected as well by the substrate structure. In order to calculate h_{TOT} , the protein amino acid composition must be known. However, most common proteins h_{TOT} values have already been calculated and can be found in the literature.

The pH-stat method can also be used in acidic conditions. At pH2 (pepsin optimum), the amino acids carboxyl group is protonated as well. Thus, when a new group is released, it bonds H^+ and thus basifies the pH. Consequently, acid must be added in order to maintain the pH at a constant value. It is only necessary to change the parameter α by $(1 - \alpha)$, being α the dissociating factor of the carboxyl groups (Zhao, Sannier, & Piot, 1996).

However, as it is specified in the results and discussion section, when a protease with a determined specificity is used, the protein would never be digested completely, because only some peptide bonds will be susceptible of enzyme cleavage (Leeb, Götz, Letzel, Cheison, & Kulozik, 2015). Thus, with trypsin and pepsin, the DH would never be a 100%. Therefore, for these enzyme+protein systems, a maximum possible DH can be calculated: the $DH_{max\ the}$. Then, all the DH values are calculated taking the $DH_{max\ the}$ as the 100%.

Annex II shows how to calculate the $DH_{max\ the}$ for the different enzyme+protein systems used in this thesis.

3.7. Basis of ultra- and nanofiltration

Membrane filtration has been used extensively in the last four decades with the objectives of concentrate, purify, and/or fractionate solutions containing solids of different size, in real solutions, colloids or suspensions. Especially in the field of protein separation, there have been numerous membrane applications. Moreover, the WPC used in this work is a result of industrial application of membrane processes.

A filtration process is defined as a separation of two or more components from a fluid stream depending on size differences principally (Cheryan, 1998). The principle of membrane filtration is the selective sieve effect of a filter (the membrane) over molecules in suspension according to their size. The retention depends on both the particle size and the size of the filter pores.

Membrane filters are screen filters, this is, they perform the separation by retaining the particles on its surface, like a sieve. Depending on their internal structure, screen filters are classified in microporous or asymmetric (Cheryan, 1998), characterized by a thin skin over the surface of the membrane. The former are further classified in isotropic, when the pore size is uniform; and anisotropic, when the pore size is variable.

Mass transport through the membrane is pressure-driven process, in which the solution containing the species flows along the surface of the membrane due to a pressure difference. Those molecules small enough to pass through the membrane pores are collected from the permeate stream, and those too big go through the retentate stream.

Pressure-driven membrane separation processes are often classified based on the molecular size of the solutes and the pressure needed. Table 2 gives an overview of the classification.

Table 2: Membrane filtration classification according to the solute size and the pressure needed. Obtained from the National Energy Technology Laboratory webpage (<https://www.netl.doe.gov>).

Parameter	Microfiltration	Ultrafiltration	Nanofiltration	Reverse Osmosis
Pore Size	0.01 – 1.0 μm	0.001 – 0.01 μm	0.0001 – 0.001 μm	<0.0001 μm
Molecular Weight Cutoff	>100,000	1,000 – 300,000	300 – 1,000	100 - 300
Operating Pressure	<30 psi	20 – 100 psi	50 – 300 psi	225 – 1,000 psi
Membrane Materials	Ceramics, polypropylene, polysulfone, polyvinylidenedifluoride	Ceramics, polysulfone, polyvinylidenedifluoride, cellulose acetate, thin film composite	Cellulose acetate, thin film composite	Cellulose acetate, thin film composite, polysulfonated polysulfone
Membrane Configuration	Tubular, hollow fiber	Tubular, hollow fiber, spiral wound, plate and frame	Tubular, spiral wound, plate and frame	Tubular, spiral wound, plate and frame
Types of Materials Removed	Clay, bacteria, viruses, suspended solids	Proteins, starch, viruses, colloid silica, organics, dyes, fats, paint solids	Starch, sugar, pesticides, herbicides, divalent anions, organics, BOD, COD, detergents	Metal cations, acids, sugars, aqueous salts, amino acids, monovalent salts, BOD, COD

It should be considered that frontiers between different processes are diffuse and sometimes overlapping.

Therefore, membranes are also classified according to the size of the separated components. Nevertheless, in UF and NF, sizes are expressed as the “molecular weight cut-off (MWCO)”, instead of giving a size measure, and sometimes the prefix “nominal” is also used (NMWCO), as membrane manufacturers usually do.

Although in the first models filtration was perpendicular, in the case of this thesis the model of cross-flow filtration (see Figure 8) was used. Cross-flow filtration offers the advantage of reducing the amount of matter accumulated over the membrane surface.

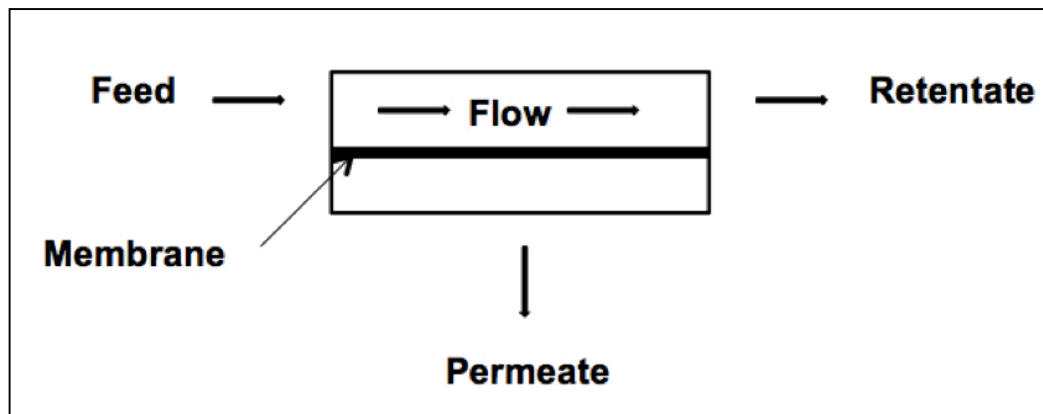


Figure 8: Schematic representation of a cross-flow filtration.

Smaller species are forced by the pressure gradient across the membrane to permeate the membrane pores, while larger species are rejected. This originates the initial feed stream to split in two different streams: the retentate, containing the rejected species; and the permeate, containing the transmitted species.

The basic parameters that define a membrane process are flux and selectivity. The flux (J) refers to the amount of solvent that flows through the membrane, and it is the result of applying the driving force (pressure). Nonetheless, it depends not only on the pressure applied, but also on the membrane intrinsic permeability and the osmotic pressure of the feed, result of the solutes nature and concentration. More detailed explanations can be found in (Cheryan, 1998; Schäfer, Fane, & Waite, 2005).

The flux is calculated in units of volume per units of area and time ($\text{m}^3 \times \text{m}^{-2} \times \text{s}^{-1}$). Selectivity (R) refers to the species rejected or permeated. For one particular solute, depends on its concentration in both the permeate stream (C_p) and the retentate stream (C_a), and is calculated by Equation 6:

$$R = 1 - C_p / C_a \quad \text{Eq. [6]}$$

The materials used to build membranes can be organic, inorganic or a combination of both. The membranes are arranged in modules, of which there are four categories: flat sheet, tubular, hollow fiber and spiral wound. Membrane modules are arranged depending on two basic membrane geometries: flat-sheet and tubular. From the former, flat-sheet and spiral wound modules can be fabricated; while for the latter, tubular and hollow fiber modules are derived.

The arrangement of a flat-sheet module consists in a basic unit: 2 membrane layers separated by a sieving layer which distributes the feed over the membranes. This basic unit can be repeated as necessary, depending the total filtration area needed. Figure 9 provides a schematic representation of the basic unit of a flat-sheet module.

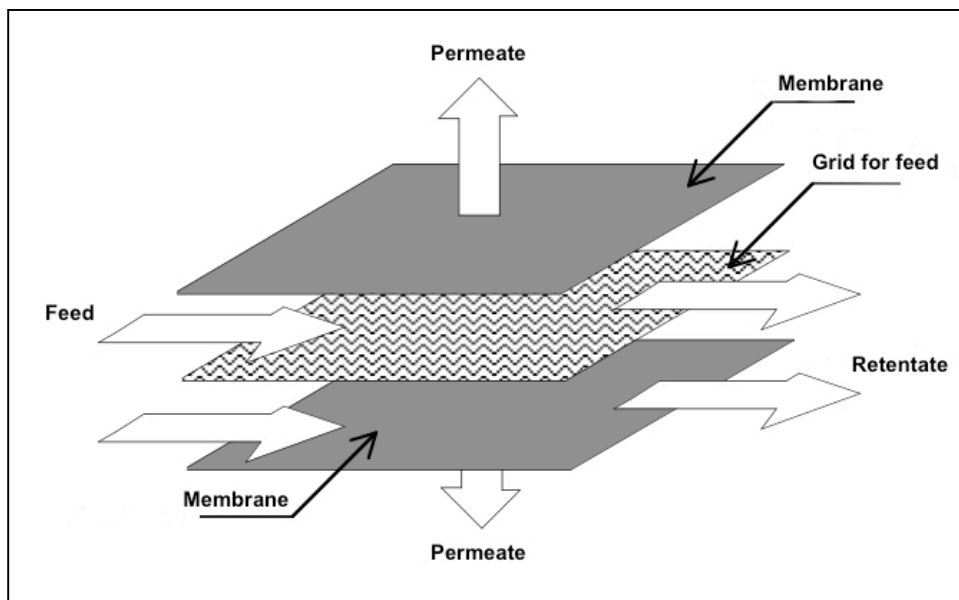


Figure 9: Flat-sheet membrane module schematic representation. Adapted from Christian (1999).

The process of membrane filtration has 2 major drawbacks: concentration polarization and fouling. These phenomena reduce the flux across the membrane and change the selectivity. The latter refers to the accumulation of solutes over the membrane surface. In most cases, cleaning has to be done at least once a day, in order to recover the permeability and selectivity of the membrane. However, cleaning procedures are aggressive, and reduce membranes lifespan (Corbatón-Báguena, Álvarez-Blanco, & Vincent-Vela, 2014). Concentration polarization is a consequence of cross-flow filtration: as a consequence of the pressure, there is an increase of the solute concentration over the membrane surface, which is responsible of the turnover of some species to the bulk solution, due to the chemical and also in some cases electrical gradient.

Ultra- and nanofiltration are pressure-driven membrane processes that separate solutes having a molecular weight between 10^3 and 10^6 Da and between 100 and 500 Da respectively (Atra, Vatai, Bekassy-Molnar, & Balint, 2005). In these cases, the selective permeation of species is not only the result of size differences, but also charge effects have an important role: electrostatic interactions between charged molecules and between those with the charged surface of the membrane.

3.8. *In silico* tools

The recent and steep improvement in computers and software has allowed introducing informatics tools on almost every aspect of science. In the field of BP, all the knowledge accumulated after two decades of identifying, isolating and testing peptides has been translated to mathematic algorithms for the development of *in silico* tools. Herein will be described some of the most relevant tools used in this thesis.

BIOPEP (<http://www.uwm.edu.pl/biochemia/index.php/en/biopep>) is an online database created and run by the Chair of Food Biochemistry at the Faculty of Food Science (the University of Warmia and Mazury, Olsztyn, Poland). It stores protein sequences, bioactive peptide sequences, and sensory peptides and aa; and it also offers prediction of allergenic proteins (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008). BP can be searched by their ID, name, type of bioactivity, the publication reference, their aa sequence, the number of aa, the mass or the InChIKey (International Chemical Identifier).

Apart from that, BIOPEP also offers information, when available, about the concentration of peptide necessary to inhibit the enzyme activity by half (IC₅₀, EC₅₀).

ExpASY is the Swiss Institute of Bioinformatics (SIB) Bioinformatics Resource Portal. It provides access to scientific databases and software tools in different areas of life sciences (Artimo, Jonnalagedda, Arnold, Baratin, Csardi, De Castro, et al., 2012). The resources used in this thesis fall under the proteomics category:

- **Compute pI/Mw** (https://web.expasy.org/compute_pi/): This tool calculates the theoretical pI and MW of a specified Swiss-Prot/TrEMBL entry (ID) or a user-entered aa sequence (in one letter code). The sequence pI is calculated using the pKa values of amino acids described in (Bjellqvist, Hughes, Pasquali, Paquet, Ravier, Sanchez, et al., 1993), which were defined by examining polypeptide migration between pH 4.5 to 7.3 in an immobilised pH gradient gel environment with 9.2M and 9.8M urea at 15°C or 25°C. The protein MW is calculated by the addition of average isotopic masses of amino acids in the protein and the average isotopic mass of one water molecule. Molecular weight values are given in Dalton (Da).

- **ProtParam** (<https://web.expasy.org/protparam/>): is a tool that computes various theoretical physico-chemical properties that can be deduced from a protein sequence, such as the MW, theoretical pI, aa composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY). The protein sequence also has to be entered as a specified Swiss-Prot/TrEMBL entry (ID) or a user-entered aa sequence (in one letter code).

GRAVY values are calculated by adding the hydrophathy value for each residue (Kyte & Doolittle, 1982) and dividing by the length of the sequence. With this scale, each amino acid is given a hydrophobicity score between 4.6 and -4.6. A score of 4.6 is the most hydrophobic and a score of -4.6 is the most hydrophilic.

- **PeptideCutter** (http://web.expasy.org/peptide_cutter/): It is a tool that searches a protein sequence for protease cleavage sites. It predicts potential cleavage sites of proteases or chemicals in a given protein sequence. The proteases or chemicals have to be selected from a specified list. The theoretical cleavage prediction can be performed with only one or multiple proteases. The results can be displayed as a table of cleavage site positions or directly onto the entered sequence. The program specifies the number of cleavages performed, the probability of the cleavage to happen, the position of the cleavage in the aa sequence, and the resulting

peptide sequence. Annex III provides an example of the result obtained when digesting BSA with trypsin using the PeptideCutter tool.

- **FindPept** (<https://web.expasy.org/findpept/>): Is a tool to identify peptide sequences from their experimental masses. This is, from a mass spectrometry result, be able to infer to which aa sequences correspond that masses. It takes into account all artefactual chemical modifications, post-translational modifications (PTM) and protease autolytic cleavage. It also differentiates among specific protease cleavage, unspecific cleavage, protease autolysis or contaminants cleavage. Apart from the list of masses, the aa sequence of the parental protein has to be entered, as well as the enzyme used. Annex IV gives the results for a set of masses obtained from a mass spectrometry analysis (LC-MS) of a pepsin hydrolysate of BSA.

PeptideRanker (<http://bioware.ucd.ie/~compass/biowareweb/>) is a server that gives a peptide sequence a probability of being bioactive, based on a novel N-to-1 neural network algorithm. It should be noted that it is not a prediction of the degree of bioactivity, but the likelihood of the peptide to be bioactive. The information to be entered is the aa peptide sequences, in one letter code. The program will display the sequences list, each one given a bioactivity score.

3.9. Peptide bioactivities

Fosgerau and Hoffmann (2015) recently reported that more than 7000 naturally occurring peptides had been identified to the moment, and that 140 peptides were being clinically evaluated as therapeutics. Peptide therapeutics is gaining momentum because BP are recognised as being highly selective and efficacious and, at the same time, relatively safe and well tolerated.

In this thesis, the peptide bioactivities selected for study were among the most global and widespread at the moment. The metabolic syndrome (MS), a plethora of metabolic abnormalities closely interrelated, has recently been estimated as present in one of four adults. The prevalence is similar in men and women, but increases with age (Lau, Yan, & Dhillon, 2006). Key features are abdominal obesity, hypertension and hyperglycaemia, which frequently derives in type 2 diabetes (T2D). On the other hand, another process intimately related with diabetic and cardiovascular complications is the oxidative stress.

A brief summary of the interrelation of each health condition and the connected peptide bioactivity is given below.

Anti-hypertension (ACE inhibition): Hypertension occurs when the blood pressure is elevated in the arteries. Additionally, elevated blood pressure is a risk factor for other health conditions, such as cardiovascular disease (Asoodeh, Haghghi, Chamani, Ansari-Ogholbeyk, Mojallal-Tabatabaei, & Lagzian, 2014). Normal blood pressure is mainly regulated by the renin-angiotensin system (RAS) and the kallikrein-kinin system. The enzyme ACE (angiotensin converting enzyme; EC 3.4.15.1) plays a vital role in these systems, by degrading bradykinin and converting angiotensin I in angiotensin II. While angiotensin I is an inactive form, angiotensin II is a potent vasoconstrictor. Conversely, bradykinin is a vasodilator (Rawendra, Aisha, Chen, Chang, Shih, Huang, et al., 2014). Figure 10 provides a comprehensive scheme of ACE action.

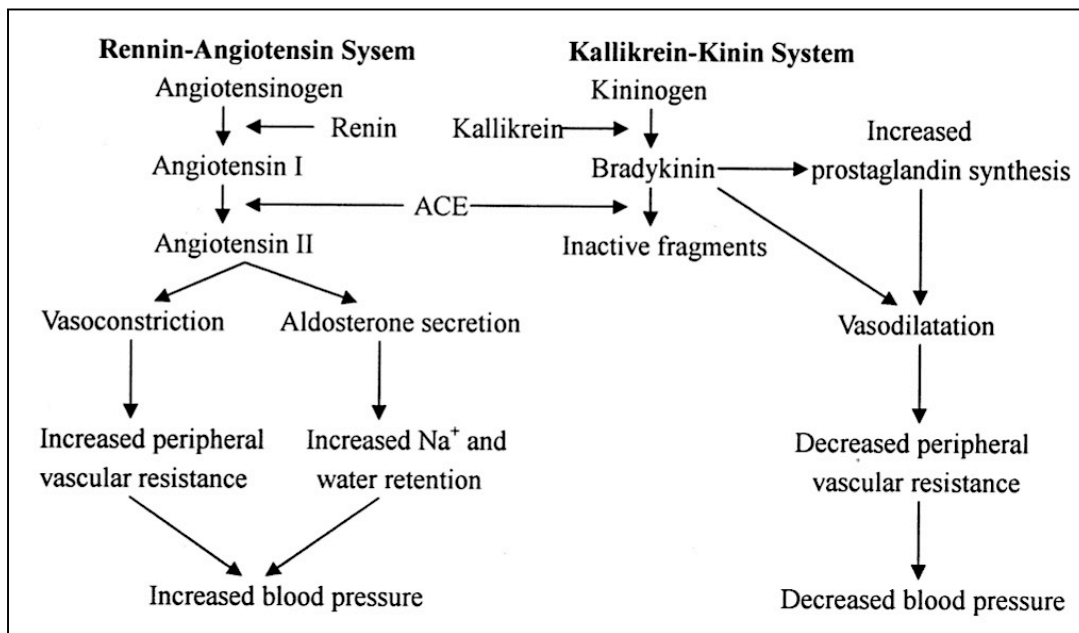


Figure 10: Angiotensin converting enzyme (ACE) mechanism of action regarding blood pressure homeostasis. Obtained from G.-H. Li, Le, Shi, and Shrestha (2004).

Glucose regulation (DPP-IV inhibition): Within the body, glucose levels regulation is critically influenced by the so-called “incretins”, hormones secreted by the gut after meal ingestion. The most important are glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Among its effects, the stimulation of pancreatic glucose-dependent insulin secretion, the inhibition of pancreatic glucagon release, the stimulation of

pancreatic β -cells proliferation and survival, the delay of gastric emptying, and the modulation of appetite are recognised (see Figure 11).

GIP and GLP-1 are both substrates for the enzyme DPP-IV (dipeptidyl peptidase-IV; EC 3.4.14.5) (I. M. E. Lacroix & Li-Chan, 2014). Thus, if inhibiting DPP-IV, the half-life of these hormones is prolonged.

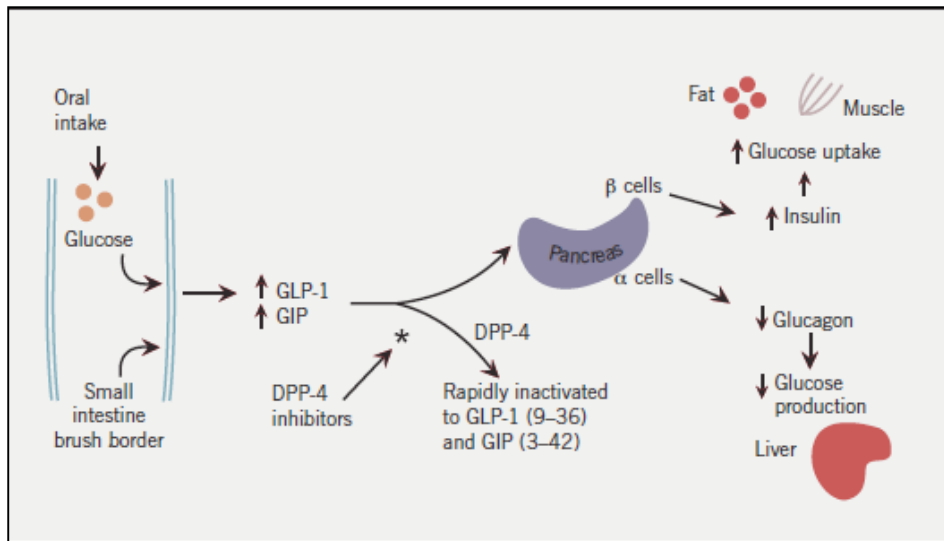


Figure 11: Dipeptidyl peptidase-IV (DPP-IV) inhibitors action on glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) activity. Obtained from McDougall, McKay, and Fisher (2011).

Anti-obesity (lipid metabolism regulation): Peptides are thought to exert several hypolipidemic mechanisms. One pathway is to disrupt the absorption of cholesterol, and also to elevate its catabolism. Another is to alter the enterohepatic bile acid circulation, since dietary cholesterol is incorporated into micelles with phospholipids and bile salts prior to intestinal uptake. Finally, peptides have also been shown to regulate lipogenic proteins and genes. Due to its role as nitric oxide precursor, R has been suggested as a contributor to hypolipidemic function (C. C. Udenigwe & Rouvinen-Watt, 2015). There is a differentiation among several forms of blood lipids: lipoproteins (HDL, low-density [LDL] and very low-density [VLDL]), triglycerides (TG), total cholesterol (TC), and free fatty acids (FFA). While HDL is catalogued as a “healthy” lipid, TG and LDL are undesirable. BP can revert abnormal ratios of these lipids, by reducing LDL, VLDL, TG and cholesterol levels, and increasing HDL levels.

One of the mechanisms of action is the modulation of the endogenous biosynthesis or metabolism of these lipids, through the alteration of the gene expression of involved enzymes, such as the fatty acid synthase (FAS), the hepatic phosphatidate phosphohydrolase (PAP), or the peroxisomal acyl-coenzyme A oxidase 1 (ACOX1). Also the elevation of the expression levels of the LDL receptor can regulate lipid homeostasis, since it is responsible for their assimilation. In addition, since dietary cholesterol is incorporated to the blood stream through micelle formation, and bile acids and phospholipids are critical for this process, lipid levels can also be regulated by bile acid peptide binding (Howard & Udenigwe, 2013).

Antioxidant capacity: When there is an excess of free-radicals formation, those can induce cellular apoptosis, by oxidising membrane lipids and proteins or the cell DNA. There is evidence that this oxidative stress negatively influences several age-related diseases (Collins, 2005; Halliwell, 2001). Antioxidant peptides are thought to scavenge free radicals through the oxidation of the aa. Other protective pathways are the active oxygen inactivation, the reduction of the formation of hydrogen peroxide or the chelation of metal ions. For instance, C and M can directly react with free radicals, and H can be used as a metal ion chelating agent, active oxygen quencher, and hydrogen free radical scavenger (Y. Li & Yu, 2015).

STATE OF THE ART

4. State of the art

Herein, a compilation of knowledge regarding the key aspects about BP that are considered in the thesis will be provided.

4.1. Bioactive peptides (BP) scope

During the last 2 decades, research about protein hydrolysis and BP production has experienced a steep increase (Li-Chan, 2015). However, research possibilities in the area of BP are still immense. Every step of the process offers a wide range of options, each of which will affect the final outcome. It is not only about choosing the protein source, the production methodology or the purification techniques; but also regarding the choice in how to study the bioactivity or how to administrate the bioactive formula. There is also the need to optimize existing technologies and develop new ones, especially in the area of BP identification and fractionation respectively. Figure 12 aims to represent the many ramifications of every step of BP production, which not necessarily follows the established order.

The first choice that affects the final type and number of BP released is the protein source, that can be plant (Orio, Boschin, Recca, Morelli, Ragona, Francescato, et al., 2017), animal (T. Lafarga, Rai, Oconnor, & Hayes, 2015) or algal-derived (Fan, Bai, Zhu, Yang, & Zhang, 2014). Each protein has a different composition in aa, what limits the BP that can be produced. Also, there is the possibility to use a purified protein, a mixture of proteins or even proteins in the whole substrate matrix of the source. An example would be to use only haemoglobin, a mixture of blood proteins or directly whole blood. Then, in order to release the peptide sequences, the protein has to be fragmented. Chemical hydrolysis has the disadvantage of lacking specificity, and also being a process that can destroy the L-form of the aa, or produce toxic or unwanted products (Aluko, 2012). Microbial fermentation has attracted the interest of the food industry, in particular the dairy industry, since starter cultures are already used in many production processes (yogurt, cheese...). However, microorganisms can generate unwanted metabolites as a consequence of their metabolism. Enzymatic hydrolysis is a highly specific methodology that offers a very wide range of possibilities, since each enzyme has a different specificity and optimal conditions. Even combinations of different enzymes have been tried.

Recombinant DNA techniques have been used for the production of peptides in microorganisms long ago (Y. K. Kim, Yoon, Yu, Lonnerdal, & Chung, 1999), and simulations of the gastrointestinal digestion process are also being carried out, to study the potential release of BP during normal food digestion (Sanchón, Fernández-Tomé, Miralles, Hernández-Ledesma, Tomé, Gaudichon, et al., 2018). There is also the possibility of adding other treatments to the production process: heat treatment, hydrostatic pressure or extrusion, for example, have been proven to in some cases improve the release of BP, while in others diminish it (Montoya-Rodríguez, de Mejía, Dia, Reyes-Moreno, & Milán-Carrillo, 2014; Quirós, Chichón, Recio, & López-Fandiño, 2007; C. Yang, Wei, Zhang, Zhang, Li, Hu, et al., 2005). The method used to purify the bulk of peptides obtained has an important impact as well, not only in the properties of the peptides, but also in the economic feasibility of the process. Separation and purification procedures in industrial biotechnology have been estimated to account for up to the 70% of the operating costs (Agyei & Danquah, 2011). Agyei, Ongkudon, Wei, Chan, and Danquah (2016) offers a comprehensive table highlighting the advantages and disadvantages of some of the most common fractionation methods. Predictive methods using computer software have the tremendous advantage of saving both time and costs. However, they are still used as exploratory approaches, and the outcome has to be confirmed by established methodologies such as mass spectrometry or Edman degradation, using a protein sequencer. As a traditional way to demonstrate bioactivity, *in vitro* tests are well spread. However, these tests are expensive and time consuming; and a lack of correspondence between them and *in vivo* assays has been many times reported (Foltz, Van Der Pijl, & Duchateau, 2010). Nevertheless, *in vivo* assays are also expensive and time consuming, and frequently there is no animal model of choice. This is why computer-aided methodologies are gaining momentum, although there is still the need to confirm their accuracy in replicating experimental procedures. Once the peptides have been validated, there is still the need to find ways to make them exert their effect in the human body. The administration route of choice has relevance, since for example, if using oral administration, the peptide would have to resist endogenous enzymes degradation and be able to reach the target organ. In relation with this, there is the problem of the bitter taste of the main part of the hydrolysates (S. Yang, Mao, Li, Zhang, Leng, Ren, et al., 2012).

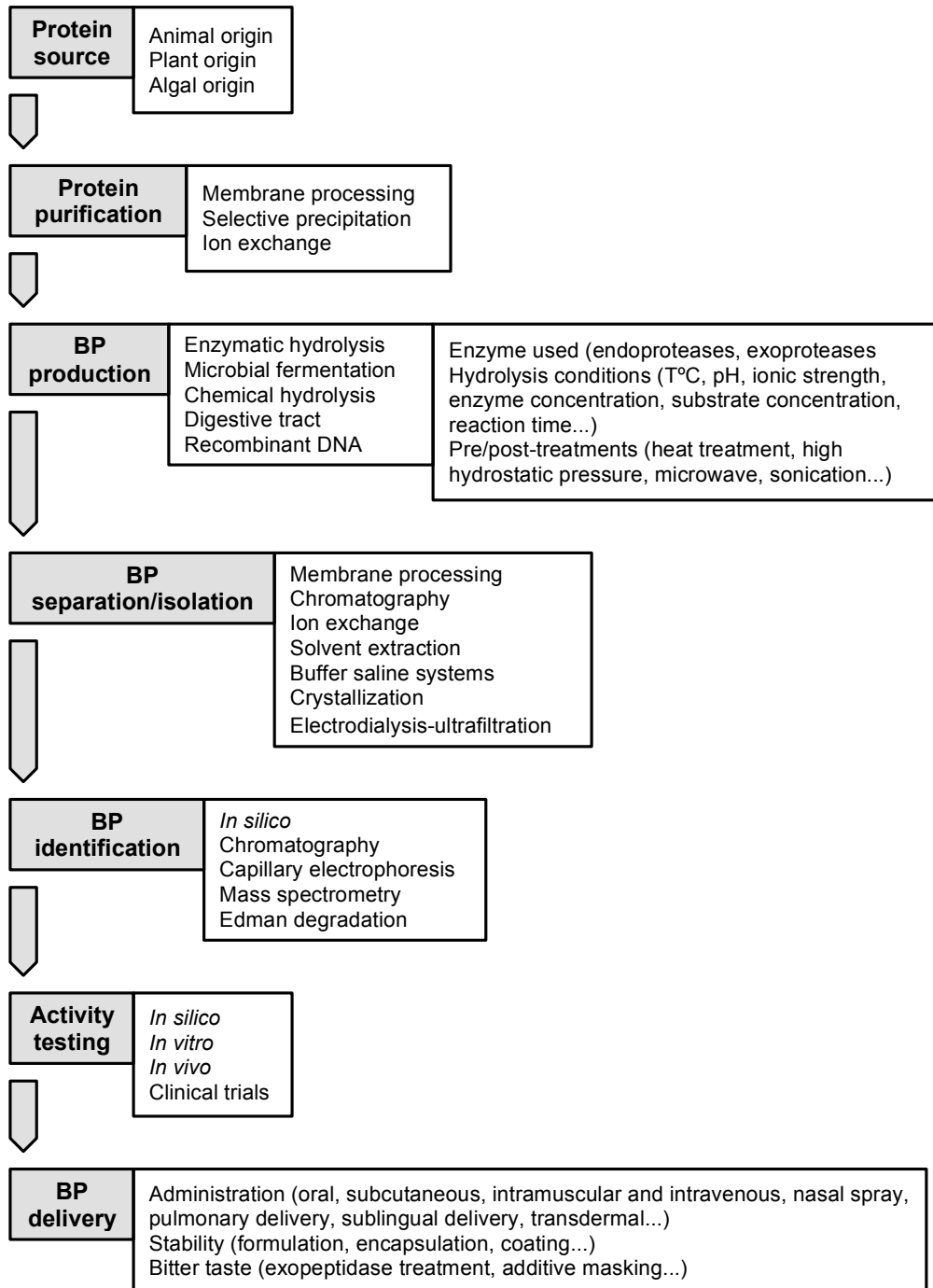


Figure 12: Bioactive peptides: possibilities regarding its production, purification, evaluation and administration.

4.2. Stages in the discovery of bioactive peptides (BP)

Until very recently, the classical approach to discover novel peptides had the subsequent broad stages:

Frist, the parental protein had to be fragmented in some way in order to release the peptide fragments. This was normally achieved by using either enzymes (*in vitro* digestions) or microorganisms (fermentations). Then, the peptides released had to be somewhat purified and identified. For that, chromatographic approaches were commonly the method of choice, to separate each peptide fragment, followed by mass spectrometry techniques to assign the aa sequence to the isolated fragment. Once the peptides released were correctly identified, the stage of bioactivity testing followed. Normally, some of the identified peptides were selected for synthesis, and the synthetic peptides were assayed using *in vitro* tests. There is no need to highlight that this approach is time-consuming and expensive; and often the finding of a promising peptide is done blindly by a trial and error approach (Connolly, O’Keeffe, Piggott, Nongonierma, & FitzGerald, 2015; Ferreira, Pinho, Mota, Tavares, Pereira, Goncalves, et al., 2007; I. M. E. Lacroix & Li-Chan, 2014; Otte, Shalaby, Zakora, & Nielsen, 2007; Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013). The next stage would involve *in vivo* trials to confirm the *in vitro* tests results, first using suitable animal models (Aydin, Aksoy, Aydin, Kalayci, Yilmaz, Kuloglu, et al., 2014; Tomas Lafarga, Aluko, Rai, O’Connor, & Hayes, 2016; Nelson & Reusch, 2014), and latter in humans performing clinical trials (H. Li, Prairie, Udenigwe, Adebisi, Tappia, Aukema, et al., 2011; Alice B Nongonierma & FitzGerald, 2015; Seppo, Jauhiainen, Poussa, & Korpela, 2003).

Nevertheless, since approximately 2014, several works have been published about the advantages of using *in silico* novel methodologies and discussing the suitability of *in vitro* trials to demonstrate peptide bioactivities (Agyei, Ongkudon, Wei, Chan, & Danquah, 2016; Li-Chan, 2015; Alice B. Nongonierma & FitzGerald, 2017).

4.3. Enzymatic hydrolysis of blood and whey proteins

As stated in the introduction section, by-product upcycling is a topic of great concern nowadays, and enzymatic hydrolysis to release BP is a currently promising methodology with industrial applicability in the agri-food sector. Several published works can be found in this area. Whey proteins, and specially WPC and WPI, have been hydrolysed by several groups with the same enzymes used in this thesis work. Bassan, Bezerra, Peixoto, da Cruz, Galán, Vaz, et al. (2016); Blayo, Vidcoq, Lazennec, and Dumay (2016); Galvao, Silva, Custodio, Monti, and Giordano (2001); Mota, Ferreira, Oliveira, Rocha, Teixeira, Torres, et al. (2006) used trypsin, while Kananen, Savolainen, Mäkinen, Perttilä, Myllykoski, and Pihlanto-Leppälä (2000); S. Kim, Ki, Khan, Lee, Lee, Ahn, et al. (2007); Pintado and Malcata (2000) also used pepsin. Going further and also considering bioactive peptide testing, the activities studied in this work have also been explored by some authors. With regard to glucose regulation, and specifically DPP-IV inhibition, I. M. Lacroix and Li-Chan (2012); I. M. E. Lacroix and Li-Chan (2014); Silveira, Martínez-Maqueda, Recio, and Hernández-Ledesma (2013); Tulipano, Sibilia, Caroli, and Cocchi (2011) are some of the few authors that study this bioactivity. Due to the publication dates it can be seen that is a recent topic of study. On the contrary, hypertension, represented by the inhibition of ACE, is clearly the most studied bioactivity to date among the considered (Ferreira, et al., 2007; Blanca Hernández-Ledesma, Recio, Ramos, & Amigo, 2002; Pan, Cao, Guo, & Zhao, 2012; A. Pihlanto-Leppälä, Koskinen, Phlola, Tupasela, & Korhonen, 2000). Antioxidant activity has also been studied extensively (Adjonu, Doran, Torley, & Agboola, 2013; Conway, Gauthier, & Pouliot, 2013; Blanca Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005; M. B. O'Keeffe, Conesa, & FitzGerald, 2017; Zhang, Wu, Ling, & Lu, 2013). Regarding blood proteins, haemoglobin is by far the most studied protein for BP production (Álvarez, Rendueles, & Díaz, 2013; Hu, Wu, Song, She, Zhao, Yang, et al., 2016), and that is precisely why SA was chosen instead in this work. Also, the majority of studies use the enzyme Alcalase (Barragán, Sánchez, & Montoya, 2016; Figueroa, Zapata, & Sánchez, 2016; Liu, Kong, Xiong, & Xia, 2010), while the use of trypsin or pepsin was only found on the works by Akemichi, Yeong-Man, Arakaki, and Takeda (1985); De Maria, Ferrari, and Maresca (2017); Özyiğit, Akten, and Pekcan (2016). Likewise, when considering bioactivity testing, only works about ACE inhibition and antioxidant capacity were found (De Noni & Floris, 2007; Hyun & Shin, 2000; Nakagomi, Fujimura, Ebisu, Sakai, Sadakane, Fujii, et al., 1998; Nakagomi, Yamada, Ebisu, Sadakane, Akizawa, & Tanimura, 2000; Wei & Chiang, 2009).

4.4. Membrane filtration of hydrolysates

Another process of relevance in the field of BP is the fractionation of the hydrolysates using membrane filtration procedures, one of the most promising methodologies regarding industrial scale-up. Membranes work without the addition of chemicals that could damage organic macromolecules, and operate under mild conditions. The processing costs are relatively low due to the small energy consumption, and scale-up is simple (Muro, Riera, & Fernández, 2013). Table 3 displays a collection of works about whey proteins hydrolysates fractionation using ultra and nanofiltration membranes. It can be seen that in several studies, the purpose was to analyse how the peptides permeated the membranes, as in this work. This study is relevant because there are no mathematical models to predict complex hydrolysates filtration. Only controlled mixtures of amino acids or peptides do comply with available models. In case of nanofiltration, the migration of species across the membrane is the result of two major parameters: peptide size and charge. Size exclusion is represented by the Ferry law (Ferry, 1936), corrected by Zeman and Wales (1981); while charge mechanisms are explained by the Donnan theory (Donnan, 1995). However, in many other cases, membrane filtration is just used as a way to increase hydrolysate bioactivity or purify peptides, without digging into the mechanisms responsible of the fractionation.

Table 3: Studies on filtration of whey or blood-derived hydrolysates.

Reference	Purpose
(Fernández, Zhu, FitzGerald, & Riera, 2014) (Fernández & Riera, 2013) (Fernández, Suárez, Zhu, FitzGerald, & Riera, 2013) (Fernández & Riera, 2012) (Ting, Gauthier, & Pouliot, 2007) (Butylina, Luque, & Nyström, 2006) (Lapointe, Gauthier, Pouliot, & Bouchard, 2005b) (Groleau, Lapointe, Gauthier, & Pouliot, 2004) (Yves Pouliot, Gauthier, & L'Heureux, 2000) (Y Pouliot, Wijers, Gauthier, & Nadeau, 1999)	Study peptide transmission and/or membrane selectivity
(M. B. O'Keeffe, Conesa, & FitzGerald, 2017) (Le Maux, Nongonierma, Murray, Kelly, & FitzGerald, 2015) (Martina B O'Keeffe & FitzGerald, 2014) (O'Loughlin, Murray, FitzGerald, Brodkorb, & Kelly, 2014) (O'Loughlin, Murray, Brodkorb, FitzGerald, & Kelly, 2014) (Power, Fernández, Norris, Riera, & FitzGerald, 2014) (Demers-Mathieu, Gauthier, Britten, Fliss, Robitaille, & Jean, 2013) (A. B. Nongonierma & FitzGerald, 2013) (A. B. Nongonierma & FitzGerald, 2013) (I. M. Lacroix & Li-Chan, 2012) (A. Pihlanto-Leppälä, Koskinen, Phlola, Tupasela, & Korhonen, 2000) (Hyun & Shin, 2000) (Mullally, Meisel, & FitzGerald, 1997)	Increase bioactivity of the fractions
(Cheison, Wang, & Xu, 2006) (Lapointe, Gauthier, Pouliot, & Bouchard, 2003) (Perea & Ugalde, 1996)	Study filtration parameters
(Prieto, Guadix, González-Tello, & Guadix, 2007) (Guadix, Camacho, & Guadix, 2006)	Reduce allergenicity
(Pan, Cao, Guo, & Zhao, 2012)	Purify a peptide
(Trusek-Holownia, Lech, & Noworyta, 2016)	Remove peptides
(Lapointe, Gauthier, Pouliot, & Bouchard, 2005a)	Study interactions
(Tavares, Amorim, Gomes, Pintado, Pereira, & Malcata, 2012)	Concentrate peptides

However, although the main purpose of the membrane filtration procedures in this thesis was to evaluate which peptides were preferentially transmitted under different conditions, it should be considered that filtration optimization was not included among the objectives. Filtration experiments should be understood as an exploratory approach to verify that membrane filtration was a technology suitable for the enrichment in BP from pepsin and trypsin whey and blood-derived hydrolysates.

4.5. Structure-activity relationship

From all the studies about BP, it was deduced that there was a relationship between several activities and several peptide primary structures:

ACE inhibition (anti-hypertension): ACE inhibitors have been reported to contain between 2 and 12 aa residues most frequently (Pan, Cao, Guo, & Zhao, 2012). ACE catalytic site comprises three hydrophobic aa (P, H and F). Therefore, the last three aa residues of the C-terminus have a great influence on the ACE inhibitory activity of the peptide (Li-Chan, 2015). Peptides having hydrophobic aa (see Annex I) at the three C-terminal positions have been described as potent inhibitors (Ferreira, et al., 2007), and also those having one at the C-terminus. W, Y, P and F have been described as the aa giving the highest potency when present at the C-terminus (Tomas Lafarga, O'Connor, & Hayes, 2014). In fact, there is a positive correlation between aa hydrophobicity and ACE inhibitory potency. It has also been suggested that positively charged aa in those positions also increase potency (Espejo-Carpio, De Gobba, Guadix, Guadix, & Otte, 2013), especially R. Regarding the N-terminal side, branched aliphatic aa, as well as aromatic or alkaline, have also been reported in potent ACE inhibitors (G.-H. Li, Le, Shi, & Shrestha, 2004). Another relationship with ACE inhibition is an internal P (Gupta, Tuohy, O'Donovan, & Lohani, 2015), however, it lowers the bioactivity when present in the N-terminus.

DPP-IV inhibition (anti-diabetic): Most of DPP-IV inhibitors comprise between 2 and 7 aa (Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013), and it is well established that they usually contain the structure X-P, P-X, or A-X in the N-terminus, being X any aa (Connolly, Piggott, & FitzGerald, 2014). It also has been reported that the primary structure has a greater influence than the aa composition for this bioactivity (Power, Fernández, Norris, Riera, & FitzGerald, 2014).

Lipid metabolism regulation (anti-obesity peptides): Some peptide sequences derived from bovine β -lg have been shown to possess lipid regulation activity, such as IIAEK (lactostatin), GLDIQK, ALPMH, VYVEELKPTPEGDLEILLQK, and HIRL (β -lactotensin) (Go & Mani, 2012; Nagaoka, Futamura, Miwa, Awano, Yamauchi, Kanamaru, et al., 2001; Yamauchi, Ohinata, & Yoshikawa, 2003). Besides, some structural models have been discovered, and efforts are being made in developing analogues, such as those of peptide YY (Nishizawa, Niida, Adachi, Masuda, Kumano, Yokoyama, et al., 2017) or GLP-1 (Blonde, Klein, Han, Zhang, Mac, Poon, et al., 2006).

Antioxidant capacity: Antioxidant peptides are typically composed of 5 - 11 aa. The most relevant characteristic is that they contain a high amount of hydrophobic aa (Martina B O'Keeffe & FitzGerald, 2014), and there is a positive correlation between the hydrophobicity and the potency.

WORKFLOW

5. Workflow

The following scheme summarises the work performed for this thesis. The scheme is organised in three columns:

- The left column represents the stage in the BP production sequence.
- The central column represents the protein source, enzyme and technologies used.
- The right column indicates the correspondence with published work.

The arrows indicate the process followed in each case.

Overall, we can differentiate 5 lines of work followed:

- BSA hydrolysis with trypsin.
- WPC hydrolysis with trypsin and membrane filtration of the hydrolysate.
- BSA hydrolysis with pepsin, membrane filtration of the hydrolysate, and *in silico* study of peptides bioactivity.
- BSA hydrolysis with trypsin, membrane filtration of the hydrolysate, and *in vitro* study of peptides bioactivity.
- WPC hydrolysis with trypsin, membrane filtration of the hydrolysate, liquid chromatography purification of the membrane permeate and *in vivo* testing of the bioactivity.

A more detailed explanation will be further provided in the results and discussion section.

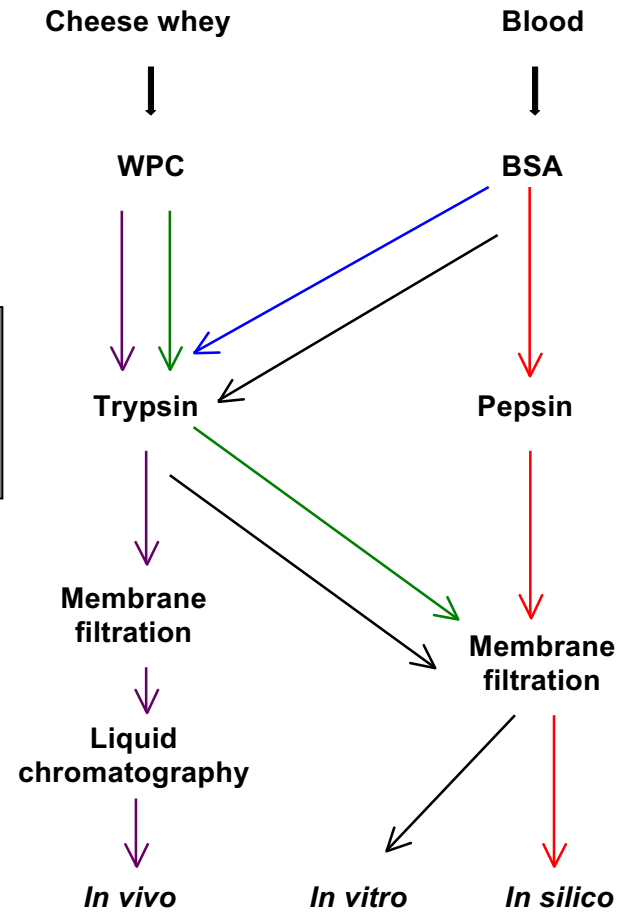
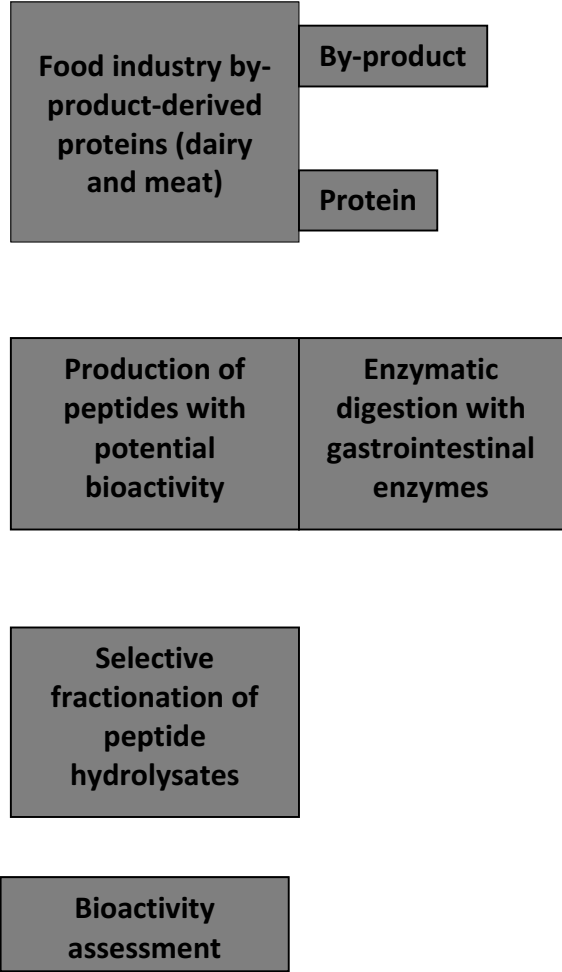
Arrutia F, Puente Á, Riera FA, Menéndez C, González UA. Influence of heat pre-treatment on BSA tryptic hydrolysis and peptide release. Food Chemistry. 2016;202:40-8. Impact factor: 4.529.

Arrutia F, Rubio R, Riera FA. Production and membrane fractionation of bioactive peptides from a whey protein concentrate. Journal of Food Engineering. 2016;184:1-9. Impact factor: 3.199.

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Mohammed-Geba K, Arrutia F, Do-Huu H, Borrell YJ, Galal-Khallaf A, Ardura A, et al. VY6, a β -lactoglobulin-derived peptide, altered metabolic lipid pathways in the zebra fish liver. Food & Function. 2016;7(4):1968-74. Impact factor: 2.791

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RESULTS AND DISCUSSION

6. Results and discussion

In this section, the work performed throughout the thesis and the results obtained will be presented and commented.

As specified in the manuscript title, the work is divided in three wide sections: production of BP, by means of enzymatic hydrolysis; fractionation of the hydrolysates and purification of peptides, by means of membrane filtration and/or liquid chromatography; and testing of fractions and isolated peptides, using *in vitro* and *in vivo* methodologies. In all the sections, *in silico* tools are also used to predict and model proteins digestion or peptides bioactivity and characteristics. Nevertheless, the sections should not be understood as separated blocks, but as interrelated and each of them necessary for the following stage.

All the work performed should be understood as an exploratory study, focused in testing the performance of the studied protein sources, enzymes and methodologies.

The BP were produced from proteins contained in food industry by-products. Two by-products were chosen (whey and blood), due to both their high production levels and their high polluting power.

As the blood-derived protein source, bovine serum albumin (BSA) was chosen. BSA is less studied than other major blood proteins (for instance haemoglobin) regarding BP production; and BSA is also present in whey. BSA was kindly supplied by Fedesa S.A. Laboratorios (San Luis, Argentina). It was obtained from bovine dehydrated plasma that was certified as free from bovine spongiform encephalopathy, by a process of thermal coagulation, selective precipitation using alcohols and membrane filtration. The resulting protein had a purity > 98%.

As the whey-derived protein source, a whey protein concentrate (WPC) was used. The WPC (Protarmor 80) was kindly donated by Armor Protéines (Saint Brice en Coglès, Brittany, France). Figure 13 shows the WPC chemical characterization per 100 g of powder.

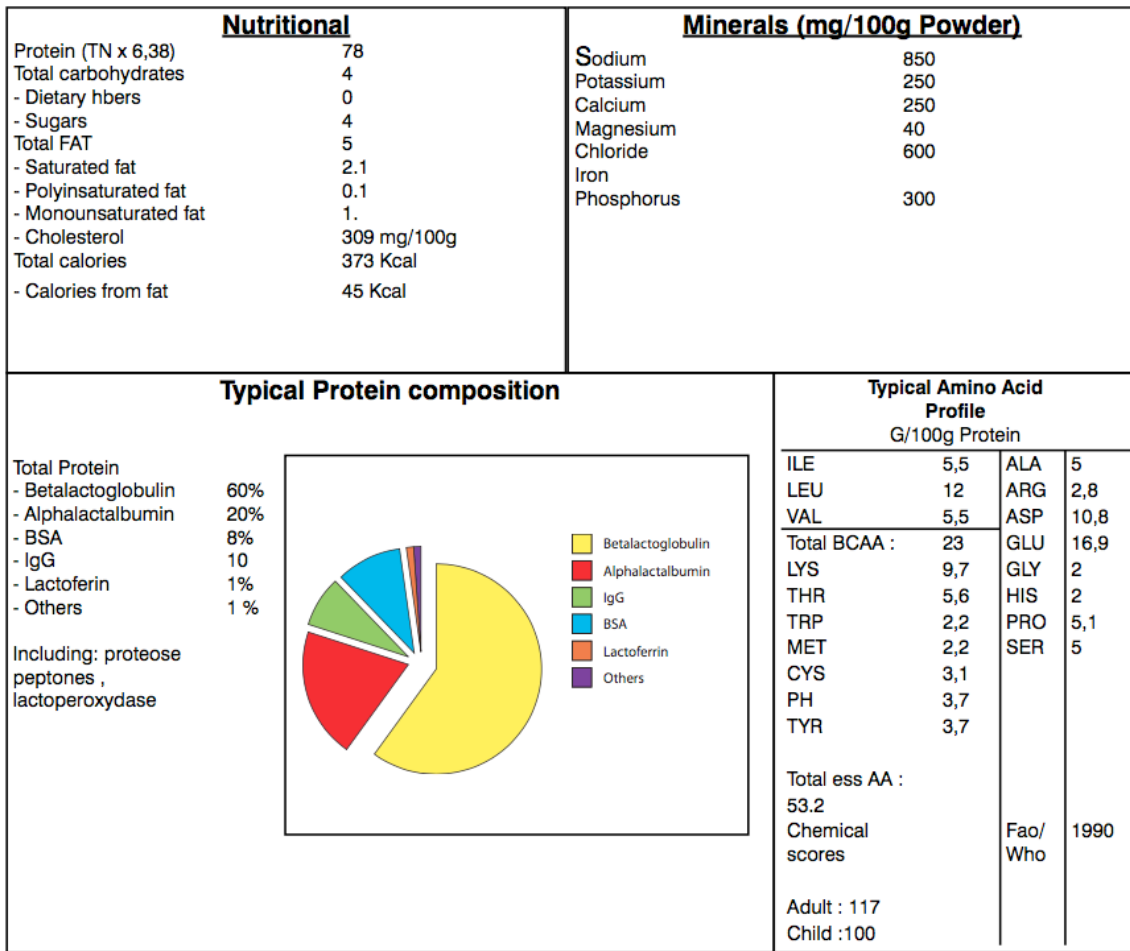


Figure 13: Protarmor 80 whey protein concentrate typical nutritional composition per 100 g of powder.

To digest the proteins and release the peptides, two gastrointestinal enzymes were used: pepsin and trypsin. Both enzymes are normally present within the human body and intervene in digestion. Also, they are easily available at food grade, and they are endopeptidases with a high degree of specificity, which makes easier to predict its action using bioinformatic software. They are also very interesting to compare, because trypsin acts at basic pH values (pH 8 is the optimum), while for pepsin the optimum is pH 2. As mentioned in section 3, proteins conformation depends on pH values, among other conditions, and protein conformation affects its digestion by the enzyme. Also, trypsin releases peptides with a terminal R and K. Anti-hypertension and lipid regulation, two of the bioactivities studied in this work, have been closely related with this aa (see section 4.5), while pepsin peptides have a wider variability.

As a summary of all the work performed, BSA was hydrolysed with trypsin and pepsin. The tryptic hydrolysis was less successful, and thus methodologies to enhance the digestion were assayed. For BSA pepsinolysis, the subsequent hydrolysate fractionation using filtration membranes was studied instead. The WPC was hydrolysed with trypsin and then fractionated using filtration membranes. BSA hydrolysate fractions were further tested for their bioactivity using *in vitro* methodologies. Finally, from the WPC, one peptide was selected for purification and testing using an animal model.

6.1. Bovine serum albumin tryptic hydrolysis to obtain bioactive peptides

BSA preliminary hydrolysis experiments using trypsin showed that the degree of hydrolysis (DH) obtained was always far from the maximum possible ($DH_{\max the}$), calculated as 14.09%. The average DH values obtained were around a 4% DH, which is approximately a 30% of the $DH_{\max the}$, independently of the quantity of enzyme used. It was seen in the bibliography (Adjonu, Doran, Torley, & Agboola, 2013; Nicoleta, Hintou, Stanciu, & Rapeanu, 2010) that to subject the protein to a thermal treatment prior to hydrolysis could enhance the digestion. However, some sources were contradictory. Therefore, in order to clarify if a thermal treatment would indeed enhance BSA tryptic hydrolysis, a study about it was conducted.

6.1.1. Influence of enzyme concentration and thermal pre-treatment on bovine serum albumin tryptic hydrolysis

The suitability of trypsin to hydrolyse BSA in order to release BP was studied. The influence of two parameters was evaluated, on both the DH and the number and type of peptides released: concentration of enzyme and a previous thermal treatment. The concentration of enzyme was studied because of economic concerns: the less enzyme used, the less costs. The thermal pre-treatment was applied with the aim of improving the DH values obtained.

First of all, there was the need to select the temperature and the duration of the thermal treatment, because each protein has a different denaturation behaviour depending on its structure at all four levels. For BSA, 4 different temperatures were selected (65, 75, 85 and 95 °C), considering the information available in the bibliography about BSA denaturation behaviour. There is a lot of controversy about the temperatures at which these processes take place, but BSA denaturation is thought to consist of a progressive loss of α -helix content, which is replaced by turns and β -sheet structures. At a certain point, protein aggregation also takes place, which means the exposure of hydrophobic and free sulfhydryl groups, previously buried inside the hydrophobic core of the native protein, that become available for intermolecular interactions. The thermal treatment duration was established as 1 hour, to make sure that there was enough time to allow the denaturation to happen, since normally in the bibliography treatments were no longer than 30 min.

While several published studies have evaluated the suitability of applying thermal pre-treatments in order to enhance proteolysis, this study went one step further, and also considered the type of peptides yielded, and the relative amounts of each of them. This double consideration constitutes a novelty among this type of works. Also, both the DH and the peptide profile were evaluated at frequent time intervals during all the treatment length, and not only the final result was considered. This allowed following the evolution of each individual peptide levels during all the treatment, giving more information on how the treatment affected each of them and the protein, and also information about the desired treatment length. In this case, peptides potential bioactivity was evaluated using the compiled knowledge about structure-activity relationships (see section 4.5).

Este capítulo (p. 67-75) se corresponde con el artículo:

Arrutia Rodríguez, Fátima et al. *Influence of heat pre-treatment on BSA tryptic hydrolysis and peptide release*. En **Food Chemistry**, 202, p. 40-48 (2016);
doi:10.1016/j.foodchem.2016.01.107

Debido a la política de autoarchivo de la publicación la versión de la editorial está disponible, únicamente para usuarios con suscripción de pago a la revista, en el siguiente enlace:

<http://dx.doi.org/10.1016/j.foodchem.2016.01.107>

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This work proved that for BSA trypsinolysis (0.3% trypsin concentration), the increase in the enzyme concentration had no statistically significant effect on the protein degree of digestion, at least in the range studied (1:50 to 1:200 E/S ratios). Moreover, it did not affect either the number of peptides released. Nevertheless, the amounts of peptides released did increase, although no quantitative measure was performed. Therefore, with regard to the economic aspects, small quantities of enzyme can be used to obtain BP from BSA using trypsin.

It also demonstrated that a previous thermal treatment enhanced both the DH achieved (from a 30% to a 50% of the maximum possible) and the number of peptides released (from 28 to 39, after 6 hours of thermal treatment). Furthermore, the main part of the peptides present in both hydrolysates was in similar or higher amounts in the thermally treated, since only three had decreased levels. However, the thermal treatment effects were different depending on the temperature chosen. While the 65°C treatment showed the same results as the lack of treatment, no significant differences were detected between 75, 85 and 95°C treatments. Thus, there is a threshold in the temperature used so that the treatment is effective somewhere between 65 and 75°C, which is also relevant economically, because heating treatments are expensive.

With regard to peptide amounts evolution over time, in both untreated and thermally treated hydrolysates the main part of the peptides increased their levels over time.

Peptide identification also showed that BSA-derived tryptic peptides had potential to be ACE inhibitors, due to its primary structure; and some of them also DPP-IV inhibitory and/or antioxidant (see section 4.5).

6.2. Hydrolysates fractionation after protein hydrolysis to enrich in bioactive peptides

When a protein enzymatic hydrolysis has been performed, the result is a protein hydrolysate in which there are peptides with potential bioactivity, but also peptides that may be undesirable (toxic, allergenic, bitter taste...), undigested protein fragments, enzymes...

Therefore, there is the need to use a technology that would allow separating the peptides with potential from the rest. To fractionate peptide hydrolysates, the separation technology employed has to be accurate enough to discriminate between the small differences in charge, size or hydrophobicity among peptides. It should also employ mild operation conditions, so as not to affect peptides structure, and be easy to scale up, thinking about industrial applicability.

In this thesis, membrane filtration suitability was mainly explored. Chromatography was also used in the isolation of a purified peptide, but for the rest of the work it was only used to study the peptides obtained, due to the fact that it is not affordable to scale it up to large volumes. Regarding membrane filtration, the focus is put on the influence of several parameters (hydrolysate nature, medium pH value and ionic strength, membrane NMWCO) on peptide transmission and membrane selectivity. Operation parameters are not studied, but constant ones are selected instead.

Two protein hydrolysates were subjected to membrane fractionation, a WPC tryptic hydrolysate and a thermally treated BSA tryptic hydrolysate. For the WPC tryptic hydrolysis, no pre-treatment was studied because the native protein hydrolysis already yielded DH values of around the half of the $DH_{\max \text{ the}}$, comparable to those obtained for BSA after the thermal treatment.

6.2.1. Membrane fractionation of a whey protein concentrate hydrolysate

The Protarmor 80 WPC was hydrolysed with trypsin, and the hydrolysate obtained was subjected to membrane filtration. No pre-treatment was applied this time because the native digestion already yielded DH values of almost half of the maximum possible. Since a WPC is composed of different proteins, trypsin selectivity for each of them was studied as well during the hydrolysis process.

For the membrane fractionation process, 3 different pH values were selected, so that the peptides and the membrane ionisable groups would be in different charge states. One pH value was basic (pH 8), one neutral (pH 6) and one acidic (pH4). The membranes selected were 2 flat-sheet polyethersulfone organic modules that varied in the NMWCO: one was of 5 kDa (PES5) while the other was of 1 kDa (PES1).

In this case, peptides bioactivity was evaluated by checking the sequences obtained with reported bioactive sequences found in the literature, and also using the database BIOPEP. Therefore, peptide bioactivity is not potential, but contrasted.

The tryptic hydrolysis of a the WPC at 5% concentration yielded a $44.22 \pm 1.62\%$ of the maximum possible DH after 6 hours, using an E/S ratio of 1:200. 42 peptide sequences were detected and identified in the hydrolysate, of which a 68.18% derived from β -lg, a 18.18% derived from α -la, and a 11.36% derived from BSA. β -lg was much more hydrolysed than α -la, as showed chromatographic analysis. A 16.7% of the peptides present in the hydrolysate had been previously reported as bioactive in the literature.

Both membranes retained undigested protein and fragments bigger than around 3.4 kDa. Despite the difference in the NMWCO, the peptide profile of both membrane permeates was very similar, with a variation of only 3 peptides. This, with the fact that the flux was reduced over time, suggests the formation of a polarized layer that performed an additional sieve effect over the membrane surface. Although the peptides transmitted (Tr) were almost the same, their respective Tr values were markedly lower for the lower NMWCO membrane, because lower were the fluxes (2.0 L/hm^2 for the PES1 membrane and 10.6 L/hm^2 for the PES5 membrane, on average). Consequently, membrane NMWCO had influence on the flux, and thus on Tr values, but not on the type of peptides transmitted.

Charge mechanisms, conversely, had a great influence on the type of peptides transmitted, since peptides Tr values varied greatly depending on the pH values. The variation followed the same trend in both membranes, suggesting that membrane composition has a marked influence. While the acidic peptides were nearly always rejected, for the basic and neutral peptides the highest Tr values were achieved at their isoelectric point, with few exceptions. Lowest Tr values were reached at acid pH values.

All the peptides with reported bioactivity were transmitted through the PES5 membrane, thus increasing the percentage of bioactive peptides to a 50%. However, two of them were not transmitted through the PES1 membrane, and some of the Tr only permeated at some pH values, indicating poorer performance for this last membrane.

Both membranes selectivity was similar, since best values were achieved at basic pH values and decreased with the acidification of the medium. Acidic peptides were easily separated from the other groups, although the efficacy decreased with the acidification of the medium, what also happened between basic and neutral peptides. Nevertheless, PES5 membrane always had better selectivity values.

6.2.2. Membrane fractionation of a bovine serum albumin peptic hydrolysate

Having trypsin suitability for digesting BSA already been tested, BSA was hydrolysed with a different gastrointestinal enzyme (pepsin). In this case, pepsin easily digested BSA, reaching DH values close to the $DH_{max\ the}$, and thus no pre-treatments were studied.

Next, the hydrolysate was fractionated using organic flat-sheet polyethersulfone membrane modules. The influence of the medium pH values was evaluated, with the purpose of finding the best conditions for enriching in peptides with potential bioactivity. Two membranes with different NMWCO were used (5 and 1 kDa), and two different pH values evaluated (2 and 10).

In order to assess the potential bioactivity of the peptides obtained, a bioinformatic server (PeptideRanker) was used. This server gives an overall bioactivity value, without considering specific bioactivities.

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Utilization of blood by-products: An *in silico* and experimental combined study for BSA usage

Fátima Arrutia¹, Rebeca Fernández¹, Carlos Menéndez², Ulises A. González² & Francisco A. Riera¹

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In order to exploit industrial discards, protein enzymatic hydrolysis is a currently popular methodology for obtaining bioactive peptides. However, once released, most promising peptides have to be selected from the mixture. In this work, the suitability of pepsin (EC 3.4.23.1) to hydrolyse serum albumin in order to obtain bioactive peptides was assessed. Then, a suitable process to obtain best separation of bioactive peptides was evaluated, using polyethersulfone membranes at different pH values. Serum albumin was easily hydrolysed by pepsin, reaching a DH value of the $65.64 \pm 1.57\%$ of the maximum possible. A 23.25% of the identified peptides possessed high bioactivity scores (greater than 0.5), and one of them had reported bioactivity (LLL). Charge mechanisms always predominated over the sieve effect, and best transmission was accomplished at pH values close to the peptides isoelectric points. Basic and neutral peptides with the highest scores were always the most transmitted. Membrane material had greater influence than NMWCO in determining peptide transmission. In order to obtain purified fractions rich in peptides with high bioactivity scores from serum albumin, polyethersulfone membranes (applicable to industrial scale) of 5 kDa MWCO should be used at basic pH values after pepsin digestion.

Bioactive peptides are defined as short amino acid fragments encrypted in the primary sequence of proteins that can confer health-related functions beyond basic nutritional benefits when orally administered¹. Bioactive peptides similarity with endogenous hormones and neurotransmitters is the reason why they can affect the major body systems when ingested. Many beneficial health effects have been described for these molecules, e.g., antimicrobial, antioxidative, antithrombotic, antihypertensive and immunomodulatory². One of the most common ways to obtain biopeptides from protein sources is enzymatic hydrolysis³. Enzymes offer the advantage of being highly specific in their mode of action, thus careful enzyme selection leads to different hydrolysates suitable for diverse applications⁴. Nevertheless, from all the peptides contained in a protein hydrolysate, not all of them will possess beneficial characteristics. Moreover, some might even be noxious or provide unwanted features⁵. Therefore, the need of a separation technology able to recognize small differences in charge, size, and hydrophobicity becomes evident.

Membrane systems can be used for separating the aforementioned complex mixtures. In fact, several authors have demonstrated the feasibility of high performance separations between proteins with very similar molecular size using membrane technologies^{6,7}. In ultrafiltration (UF) and nanofiltration (NF), both molecular sieving mechanisms and charge effects are known to play a role on selectivity. However, many aspects of the separation mechanisms are still not clear⁸. In fact, existing mathematical models are unable to predict the separation processes, especially when filtrating complex mixtures such as protein hydrolysates. Peptide-peptide, membrane-peptide or polarized layer-peptide and membrane interactions are complex. Moreover, other phenomena such as hydrophobic interactions, aggregation or fouling processes complicate the prediction of the separation⁹.

Peptide isolation or purification from complex hydrolysates has been widely achieved using chromatographic methods^{10,11}. However, the use of other technological approaches applicable at an industrial scale remains more scant¹². Membrane technologies are low-energy processes that use mild operation conditions and are thus gentle

¹Chemical Engineering and Environmental Technology Department, University of Oviedo, Julián Clavería, 8, 33006, Oviedo, Asturias, Spain. ²Research Institute in Chemical Technology (INTEQUI)-CONICET, Faculty of Chemistry, Biochemistry and Pharmacy, Universidad Nacional de San Luis (UNSL), Ejército de los Andes 950, 5700, San Luis, Argentina. Correspondence and requests for materials should be addressed to F.A. (email: farrutia@gmail.com)

with delicate molecules as proteins. They do not need the addition of chemicals and are very easy to scale-up¹³. There has been considerable interest in membrane filtration of protein hydrolysates during the last decade. However, the stress has been put in, for example, obtaining products with specific characteristics¹⁴ or filtration parameters optimization¹⁵; while little attention has been given to attempt to explain the mechanisms underlying the separation, except for^{16,17}.

Besides, there is a dire need of finding industrial-applicable processes for obtaining valuable merchandises from food by-products, from both economical and environmental points of view¹⁸. Blood proteins are used mainly in food and feed industries as nutritional supplements, emulsifiers, stabilizers or clarifiers. Nevertheless, a big portion of blood is discarded as waste¹⁹. Bovine serum albumin (BSA), although a well known protein because of its ligand-binding capacity²⁰, has not extensively been used with the purpose of obtaining bioactive peptides, in contrast with other well studied blood proteins such as haemoglobin^{21,22}.

Therefore, this work had two main objectives: On the one hand, to present an exploratory procedure for obtaining a stream enriched in bioactive peptides from BSA, with future industrial applicability intended. Second, to assess the underlying mechanisms responsible for peptide transmission, with regards of membrane pore size and medium pH value.

Material and Methods

Materials. Pepsin (EC 3.4.23.1, from porcine gastric mucosa, activity ≥ 2500 U/mg protein) was from Sigma Aldrich (St. Louis, MO, USA). BSA isolated from cattle blood (purity $>98\%$, fat $<0.05\%$) was kindly supplied by Fedesa S.A. Laboratorios (San Luis, Argentina). Trifluoroacetic acid (TFA) was supplied by Merck (Darmstadt, Germany). Acetonitrile (ACN), formic acid and hydrochloric acid (HCl) were obtained from VWR (Barcelona, Spain). Sodium hydroxide was from Panreac (Barcelona, Spain).

Peptic hydrolysis of BSA. BSA (3 g) was dissolved in ultrapure water (1 L) (MilliQ system, Millipore, Billerica, MA, USA) under constant agitation, using a magnetically stirred hotplate (MR Hei-standard, Heidolph, Schwabach, Germany). Solutions were allowed to reach the working temperature (37°C) and the pH was adjusted to the enzyme optimum (2) using 2 M HCl. Hydrolysis reaction was started by the addition of pepsin at an enzyme to substrate (E/S) ratio of 4%. The pH values were maintained throughout digestion with an automatic pH regulator (pH burette 24 2S, Crison, Barcelona, Spain). Fresh aliquots were collected and every 15 min after enzyme addition. In each sample and in the final hydrolysates (3 h), the enzyme was inactivated by raising the pH until 7²³, using 0.1 N NaOH. Hydrolysates and samples were kept at -40°C . Hydrolysis experiments were performed in duplicate.

Calculation of the degree of hydrolysis (DH). The DH was estimated using the Equation (1)²⁴.

$$\text{DH}(\%) = (h/h_{\text{TOT}}) \times 100 = [A \times N_A / (1 - \alpha) \times h_{\text{TOT}} \times M_p] \times 100 \quad (1)$$

where h is the number of peptide bonds broken (meq/g protein), and h_{TOT} is the total number of peptide bonds in the protein substrate (meq/g protein). For this work, the previously calculated value of 8.8 was used²⁵. A is the acid consumption (mL), N_A is the normality of the acid (meq/mL), α is the average degree of dissociation of α -carboxylic groups and M_p is the mass of protein (g). In this study, α was calculated as in²⁶, and took the value of 0.325.

Since the DH is defined as the percentage of peptide bonds cleaved during an enzymatic reaction, a DH of 100% means the complete degradation of a protein to free amino acids. Nevertheless, DH values are always less than 100% when enzymes with specificity to individual peptide bonds are used. BSA contains 582 peptide bonds, of which 147 are theoretical possible cleavage sites for pepsin (see Supplementary information 1). The program PeptideCutter (http://web.expasy.org/peptide_cutter/) was used to calculate pepsin cleavage sites. According to this, during BSA pepsinolysis, a maximum possible DH (DH_{max}) of 25.26% can be achieved. Therefore, all the DH percentages were calculated relative to the theoretical DH_{max} , as in Leeb, *et al.*²⁷.

Membrane separation. *Membrane rig.* Two polyethersulfone (PES) membranes, each with a filtration area of 0.1 m^2 , were used. PES5 membrane (Millipore) had a nominal molecular weight cut-off (NMWCO) of 5 kDa, while PES1 (Sartorius, Goettingen, Germany) was of 1 kDa. Before first use, membranes were characterized with distilled water, and after every filtration run they were cleaned with 1 M NaOH and 2% H_3PO_4 . The cleaning protocol was a 10 min rinse followed by 30 min recirculation at 40°C at a constant transmembrane pressure (TMP) of 0.12 MPa for PES1 membrane and 0.20 MPa for PES5 membrane. The respective recirculation rates were 4 and $55 \text{ L/m}^2\text{h}$. The cleaning procedure efficiency was checked by measuring the pure water flux (J_w). J_w was always $\geq 95\%$ recovered. Membranes were stored in 20% ethanol (PES1) or 0.1 M NaOH (PES5) under refrigeration ($2-8^\circ\text{C}$). Both membranes were installed within a Pellicon 2 mini cassette holder (88 cm^2 & 0.11 m^2 , Millipore) connected to a 1 L jacketed glass tank reactor coupled to a thermostatic water bath for temperature control (Ulthatherm, P Selecta, Barcelona, Spain). Fluid was pumped through the system by a GJ series 120 pump (I-Drive, Micropump Inc., Vancouver, WA, USA).

Hydrolysate fractionation. All membrane experiments were performed at fixed conditions of TMP (0.2 MPa) and temperature (37°C), since membranes selectivity rather than parameter optimization was the interest of this work. Filtration was performed on full recirculation mode; at an average permeate flux (J_p) of $20.7 \pm 1.00 \text{ L/m}^2\text{h}$ for PES5 membrane and $7.8 \pm 0.21 \text{ L/m}^2\text{h}$ for PES1 membrane. So as to study the role of pH dependent changes on the selective permeation of hydrolysate peptides, two pH values were assayed for each membrane (2 and 10), selected to represent different charge states of both the peptide mixture and the membrane surface. The pH was

adjusted with 0.1 M HCl and 2 M NaOH. Before every filtration run the system was equilibrated for 15 min, and each pH value maintained for 1 h. All membrane experiments were performed in duplicate.

The observed transmissions (Tr_{obs}) of individual peptides through the membranes were calculated using Eq. (2):

$$Tr(\%) = \frac{A_{Pi}}{A_{Hi}} \times 100 \quad (2)$$

where A_{Pi} and A_{Hi} are the i peptide peak areas obtained from the UPLC chromatograms of each permeate and hydrolysate, respectively.

The theoretical transmissions (Tr_{theo}) were also calculated according to Eq. (3)²⁸:

$$Tr(\%) = [(1 - (\lambda \cdot (\lambda - 2)))^2] \cdot \exp(-0,7146\lambda^2) \times 100 \quad (3)$$

with

$$\lambda = \left(\frac{MW}{MWCO} \right)^{0,4}$$

being MW the molecular weight of the peptide (Da) and MWCO the molecular weight cut off of the membrane.

The separation factors (S) between two peptides or group of peptides ($S_{x/y}$) were calculated as the ratio of mean Tr values (%) with Eq. (4):

$$S_{x/y} = \frac{\sum_{i=1}^n Tr_i/n}{\sum_{j=1}^m Tr_j/m} \quad (4)$$

where n and m are the number of peptides comprised in groups x and y respectively. This value ($S_{x/y}$) represents the selectivity of the membrane to distinguish between specific peptides or groups of peptides.

Peptide identification. RP-UPLC coupled to tandem mass spectrometry (RP-UPLC-MS/MS) was performed on a Dionex Ultimate 3000 RS UHPLC (Thermo Fisher Scientific, Waltham, MA, USA) connected on-line to a Bruker Impact II Q-ToF mass spectrometer (Bruker, Billerica, MA, USA). The column used was a Bruker Intensity Trio C18 (50×2.1 mm, 3 μ m). Solvent A was composed of MilliQ water with 0.1% formic acid. Solvent B was composed of ACN with 0.1% formic acid. Gradient was 2% B for 1 min, from 2 to 35% B in 30 min, 35% B for 1 min, from 35 to 80% B in 4 min and 80% B for 1 min. The flux was 150 μ L/min and the injection volume 2 μ L. Separation was performed at 30 °C of temperature. Mass spectra were acquired in the positive ion mode using a 120 V fragmentation with a scan range of 50–3000 m/z . Nitrogen was used both as the drying gas at flow rate of 6.0 L/min and a temperature of 300 °C, and as the nebulizer gas at a pressure of 0.31 MPa. The capillary voltage was set at 4500 V. Only peaks with an intensity superior to the 10% of the most intense peak were considered for analysis, so as to avoid errors with sparse peptides. Identification analyses were all performed in duplicate. The ExPASy FindPept database (<http://ca.expasy.org/tools/findpept.html>) was used to identify the peptide masses obtained with the MS/MS analysis. FindPept performs a theoretical cleavage of a known protein by a known enzyme, displaying all possible peptide masses and their corresponding amino acid sequences resulting from both specific and non-specific cleavage. The program compares the experimental peptide masses to the theoretical ones, matching the similar and thus allowing identification. In this case, mass tolerance limit was set to 0.3 Da, and minimum peptide sequence length to 3 amino acids. The bibliography^{29–31} and BIOPEP database³² were searched to check if the identified peptide sequences had already been reported as bioactive. Peptide sequences GRAVY (average hydropathy score) and isoelectric points (pI) values were calculated with the ExPASy ProtParam tool (<http://www.expasy.ch/tools/protparam-doc.html>). The program Peptide Ranker (http://bioware.ucd.ie/~compass/biowareweb/Server_pages/peptideranker.php) was used to assess the potential bioactivity of the identified sequences³³. Peptide Ranker assigns each sequence a score depending on the calculated probability of being bioactive.

Hydrolysates, retentates and permeates protein content was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analyses. The results reported in this work are the average values of the experimental data, and the error bars indicate the relative experimental error. Analysis of variance (One-Way ANOVA) was carried out to assess whether the differences between groups or experiments were statistically significant, using the program StatPlus:Mac (AnalystSoft Inc., Walnut, CA, USA). The significance level was established for $P < 0.05$.

Results and Discussion

BSA hydrolysis with pepsin. BSA was hydrolysed with pepsin at the enzyme optimal conditions. The extent of protein hydrolysis was expressed as the DH, and monitored as a function of time (see Fig. 1). The DH values are relative to the DH_{max} . As expected, the DH increased with hydrolysis time. However, during the first 15 min., the slope of the curve was very sharp, while over the rest of the process the DH increased steadily, reaching a plateau from 120 min on. Similarly, Lacroix and Li-Chan³⁴ also found a slow down of pepsin activity after the first 30 min of hydrolysis. The maximum DH reached was a $65.54 \pm 1.57\%$ of the DH_{max} for the enzyme-substrate system. Although no bibliographical evidence was found of pure BSA pepsinolysis with the purpose of obtaining bioactive peptides in which the all the hydrolysate sequences were identified, several works about WPC and WPI hydrolysis with pepsin suggest BSA as a good substrate for the enzyme. For example, Kim, *et al.*³⁵ and Peña-Ramos and Xiong³⁶ found out that serum albumin was completely removed from SDS-PAGE gels after 30 min of

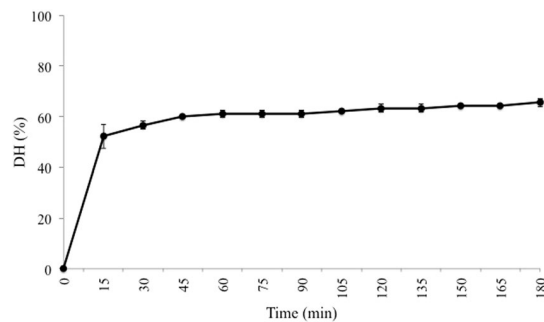


Figure 1. Hydrolysis curve for BSA (3 g/L) digestion with pepsin (4% E:S ratio) at 37°C and pH 2. Data are presented as mean \pm error.

pepsin hydrolysis of WPC solutions. Lacroix and Li-Chan³⁷, whilst not obtaining a complete degradation, proved that BSA was the most digested protein amongst the individual whey proteins assayed (Alpha-lactalbumin (α -la), β -lg, BSA and Lactoferrin (LF)).

For explaining the high DH obtained, it should also be taken into account the low pH at which pepsin digestion was conducted. As pH decreases from neutrality to acidity, BSA domains are separated and helical content is lost, replaced by unstructured regions. Below pH 3.13, BSA acquires a linear bead form called “E state” (expanded)³⁸. Pepsin preferentially cleaves peptide bonds with aromatic and hydrophobic amino acids³⁵, which are normally hidden in the interior of the globular proteins³⁹. With the unfolding, more of them become available for the enzyme^{40,41}, and thus hydrolysis is enhanced. Likewise, the rate of proteolysis has been demonstrated to depend on the conformation of proteins⁴². Although protein hydrolysis was not complete, final hydrolysate RP-UPLC chromatograms (chromatograms not shown) evidenced no BSA peak. However, large peptide fragments of around 5–6 kDa were detected in the hydrolysate (see section 3.2).

Peptide identification. For the sake of clarity, only peptides unequivocally identified and detected were considered. Peptide masses that could not be assigned to one single peptide sequence were 344.25, 360.21, 361.17, 375.22, 433.23, 462.27, 705.41 and 724.40, and thus were not considered. Peptides that were detected in any permeate but not detected in the hydrolysate were not considered either. A total of 19 peptide masses were excluded for this reason. Overall, 43 peptide masses were unambiguously assigned to peptide sequences. Of them, a 23.26% were acid, a 39.53% basic and a 37.21% neutral. Table 1 shows the identifications plus some relevant peptide characteristics. Only one of the identified peptides of Table 1 had previously been reported as bioactive, which is reasonable if we take into consideration the scarce works about peptide identification from BSA pepsinolysis. In fact, we have not found a complete report of the peptides yielded by pepsin digestion of BSA as the one we provide. Nevertheless, although IARRHPYF has not been reported as bioactive, a very close peptide sequence (IARRHPYFL) was reported as a neuropeptide³¹. The scores given by the program Peptide Ranker were used to assess peptides potential bioactivity (see Table 1). As it can be seen, a 23.25% of the peptides had bioactivity scores greater than 50%, indicator of probable bioactivity. The peptide with reported bioactivity is LLL, that, as expected, had a relatively good bioactivity score (0.57)⁴³.

Membrane fractionation. Peptide transmissions. Figures 2 and 3 display Tr values corresponding to filtration experiments. Solid bars represent Tr_{obs} , while dotted bars reflect Tr_{the} . Peptides were divided in three groups according to their pI values⁴⁴: acid ($pI < 5.0$), basic ($pI > 8.0$) and neutral ($5.0 < pI < 8.0$).

In general, it can be seen that Tr_{obs} were different from the calculated Tr_{the} for both PES1 and PES5 membranes, revealing the influence of other parameters apart from peptide size (see section 1). Tr values were generally higher than expected for PES1 membrane, while for PES5 membrane were both higher or lower. Additionally, Tr_{obs} changed greatly with pH, and the variation was markedly different for each peptide group. Similarly, when filtrating a tryptic β -lg hydrolysate with a 5 kDa polyethersulfone membrane, Fernández, *et al.*¹⁷ also realised that peptide Tr was mainly governed by charge mechanisms. Membrane NWCO had a relevant influence too, as Tr values were, in general, higher for PES5 membrane in the majority of the cases. At pH2, PES5 Tr_{obs} overall values were 2.23 times higher than PES1 Tr_{obs} values; and at pH10, the factor was 1.80. Despite the great difference in NMWCO, peptides transmitted across both membranes were the same, indicating that membrane material rather than pore size defines transmission.

In order to provide rough guidance about protein concentration in both membrane streams, a BCA assay was performed, indicating a protein content of 2.71 and 0.23 mg/mL in retentate and permeate streams respectively.

Acidic peptides. Speaking about general trends, acidic peptides were far more transmitted at acid pH value (30.96% on average) than basic pH value (5.85% on average), for both membranes. In fact, only two peptides were detected in the basic permeate streams (LYE and PELLY), and with very low Tr values, of less than 10% in all cases. This as well (see section 3.3.2) stresses the higher influence of charge state above other parameters, in this case ionic strength. In order to raise the pH from 2 to 10, base was added, and thus ionic strength increased. It is well known that an increase in salt concentration decreases electrostatic interactions⁴⁵. This usually leads to better Tr of the peptides with the same sign as the membrane. In this case, despite the smaller radius and the less intense repulsive forces, acidic peptides were not transmitted at basic pH values, except for LYE and PELLY. With

MW (Da)	Peptide sequence	Charge pH = 2 ^a	Charge pH = 10 ^b	Group ^c	GRAVY ^d	Bioactivity Score ^e
592.30	(F)/YAPEL/(L)	0.90	-2.50	A	-0.16	0.38
471.28	(A)/PELL/(Y)	0.90	-2.00	A	0.63	0.34
639.28	(E)/ACFAVE/(G)	0.90	-3.00	A	0.87	0.34
634.34	(A)/PELLY/(Y)	0.90	-2.50	A	0.24	0.32
596.29	(E)/YEATL/(E)	0.90	-2.50	A	0.02	0.13
424.21	(Y)/LYE/(I)	0.90	-2.50	A	-0.33	0.11
389.20	(E)/EQL/(K)	0.90	-2.00	A	-1.07	0.07
483.21	(E)/YEAT(L)	0.90	-2.50	A	-0.93	0.06
362.19	(E)/LTE/(F)	0.90	-2.00	A	-0.13	0.04
346.20	(F)/VEV(T)	0.90	-2.00	A	1.63	0.02
1059.58	(E)/IARRHPYF/(Y)	3.90	0.50	B	-0.75	0.73
731.42	(A)/WSVARL/(S)	1.90	0.00	B	0.60	0.68
517.28	(L)/HTLF/(G)	1.90	-1.00	B	0.68	0.59
401.29	(L)/LRL/(A)	1.90	0.00	B	1.03	0.56
1081.57	(L)/SQKFPKAEF/(V)	2.90	-1.00	B	-1.09	0.53
1075.52	(Y)/YANKYNGVF/(Q)	1.90	-1.50	B	-0.57	0.45
1122.65	(L)/PKLKPDPTL/(C)	2.90	-1.00	B	-1.27	0.43
1238.58	(L)/YANKYNGVF/(Q)	1.90	-2.00	B	-0.64	0.43
825.45	(E)/TYVPKAF/(D)	1.90	-1.00	B	0.19	0.30
1107.53	(E)/YSRRHPEY/(A)	3.90	-1.00	B	-2.59	0.28
550.33	(L)/IVRY/(T)	1.90	-0.50	B	0.73	0.20
893.48	(P)VSEKVTKC(C)	2.90	-2.00	B	-0.24	0.10
478.27	(L)/KTVM(E)	1.90	-0.50	B	0.38	0.09
801.52	(Q)/IKKQTAL/(V)	2.90	0.00	B	-0.27	0.09
1051.62	(L)/LKHKPKATE/(E)	4.90	-0.50	B	-1.68	0.08
838.48	(E)/HVKLVNE/(L)	2.90	-1.50	B	-0.27	0.05
1546.91	(L)/IVRYTRKVPQVST(P)	3.90	1.00	B	-0.34	0.05
414.20	(L)/FTF/(H)	0.90	-1.00	N	1.63	0.97
350.21	(L)/IAF/(S)	0.90	-1.00	N	3.03	0.82
514.23	(E)/YGFQ/(N)	0.90	-1.50	N	-0.60	0.79
358.27	(W)/LLL/(L)	0.90	-1.00	N	3.80	0.57
336.19	(F)/VAF/(V)	0.90	-1.00	N	2.90	0.56
423.22	(L)/GLAY/(P)	0.90	-1.50	N	0.98	0.46
330.20	(T)PTL/(V)	0.90	-1.00	N	0.50	0.35
713.42	(F)/AVEGPKL/(V)	1.90	-1.50	N	0.06	0.30
474.27	(D)RADL/(A)	1.90	-1.00	N	-0.60	0.26
382.20	(D)YLS(L)	0.90	-1.50	N	0.57	0.24
940.48	(E)/TYVPKAFD(E)	1.90	-2.00	N	-0.28	0.17
488.31	(Y)/AVSVL/(L)	0.90	-1.00	N	2.64	0.12
700.42	(L)/LPKIET(M)	1.90	-1.50	N	-0.23	0.10
417.27	(A)/VSVL/(L)	0.90	-1.00	N	2.85	0.08
688.43	(F)/VEVTKL/(V)	1.90	-1.50	N	0.68	0.05
533.29	(L)/VVSTQ/(T)	0.90	-1.00	N	0.68	0.03

Table 1. Peptides identified by LC-MS/MS in the BSA peptic hydrolysate (3 g/L) and membrane permeates, plus relevant physicochemical characteristics. Peptide sequences are arranged by group type. Each group is arranged in descending order by potential bioactivity. ^{a,b,d}Calculated with the ExPASy Molecular Biology Server (<http://www.expasy.org/>). ^cCalculated as in Lapointe, *et al.*⁴⁴. ^eCalculated with Peptide Ranker *in silico* tool (<http://bioware.ucd.ie/~compass/biowareweb/>).

regards to the charge effect, at pH values < than 4, the membrane loses its original negative charge⁴⁶, while acidic peptides are very near of their pI (see Table 1). Consequently, their charge is very small and thus they can be easily transmitted¹⁷, due to the lack of electrostatic repulsive forces. PELLY showed very low Tr values, and almost no difference between pH values. However, PELL, while only differing in one amino acid, showed much higher Tr values. At pH10, PELLY is even more charged than PELL. These results do not have to be contradictory, since Pouliot, *et al.*⁴⁷ showed that two peptides that only differed in one amino acid could be transmitted in a different way. Also, acidic peptides were all more transmitted through the membrane with higher MWCO, with no exceptions (Average Tr of 23.47% for PES1, while 35.45% for PES5). Regarding Tr_{the}, all acidic peptides were less transmitted than expected for PES5 membrane, but more transmitted than expected for PES1 membrane, highlighting

	pH2		pH10	
	PES1	PES5	PES1	PES5
A/N	135.92	102.52	1691.30	3765.36
A/B	1049.77	428.95	1962.46	4189.63
B/N	1426.88	418.43	116.03	111.27

Table 2. Separation factors for all the combinations membrane-pH assayed. A = acid peptides; B = basic peptides; N = neutral peptides.

interactions with PES membranes⁴⁸, so these interactions retained both hydrophobic peptides, lowering their Tr values.

Some basic peptides had very good bioactivity scores, such as IARRHPYF, WSVARL, HTLF or LRL. Interestingly, IARRHPYF (115.7%) was the most transmitted peptide, followed by HTLF (73.3%) and WSVARL (65.0%), across PES5 membrane at pH10. Therefore, filtration of BSA peptic hydrolysates at basic pH values through 5 kDa MWCO PES membranes is evidenced as a good strategy to enrich permeates in bioactive peptides.

Neutral peptides. Neutral peptides were in general more transmitted through the higher MWCO membrane. In this case, pH influence on transmission was very low, since almost all peptides were equally transmitted at both pH values. It can be seen in Table 1 that, for both pH values, the great part of the neutral peptides had similar absolute charge values (around 1), taking into account that charge sign was the opposite. Exceptions were PTL, which was much more transmitted at pH10; and YLS, that had very low Tr values. Some peptides were not transmitted at pH2 through one or both membranes, such as RADL, AVSVL, VVSTQ, LPKIET and AVEGPKL, with no apparent reason for this behavior.

Several neutral peptides also had very high bioactivity scores, and were also some of the best transmitted, such as FTF (best $Tr_{obs} = 57.8\%$), IAF (61.9%) and YGFQ (52.2%). Neutral peptides presented two advantages: they had overall Tr values very similar to the basic group and thus relatively high, and they had the same behavior at both pH values assayed. Further *in vitro* and even *in vivo* bioactivity assays would be interesting for neutral and basic peptides with high bioactivity scores that are transmitted in high percentages across PES membranes.

Separation factors. Table 2 summarizes the separation factors calculated for each peptide group at every set of conditions assayed. Overall, all the values were relatively high if compared with other works of hydrolysates filtration in which separation factors were similarly calculated. For instance, Fernández, *et al.*¹⁷ obtained separation factors that ranged from 0.9 to 291.8. In this work, the pH-membrane combination that presented the worst separation factors was pH2 and PES5 membrane; and the best pH10 and PES5 membrane. Fernández, *et al.*¹⁷ also obtained better separation factors at pH 10.

In order to achieve a good separation between acid and neutral peptides or acid and basic peptides, the latter combination would be the best choice as it had the highest value for both cases. However, when trying to separate basic and neutral peptides PES1 membrane at a pH value of 2 worked better. Interestingly, the best selectivity of the PES5 membrane at pH values of 10 is coincident with the enrichment in peptides with high bioactivity scores at those conditions.

In general, neutral and basic peptides were separated with more difficulty than each of them from the acid group. It should be taken into account that, although acid and neutral groups average molecular weight (MW) was very similar (493.85 and 504.04 kDa respectively), basic peptides were bigger in general (901.26 kDa), and thus sieving effect had an additional influence when separating this last group. This is why acid/basic separation was always higher than acid/neutral separation at all the conditions tested.

Conclusions

BSA at pH2 is a good substrate for pepsin, since a high DH value was achieved ($77.68 \pm 1.86\%$ of the DH_{max}). Besides, BSA pepsinolysis yields a 23.25% of peptides with high bioactivity scores (more than 0.50), and at least one peptide with confirmed bioactivity (LLL).

Regarding peptide Tr values, charge was the predominant mechanism responsible for transmission rather than size, since Tr_{obs} were different from Tr_{theor} and Tr_{obs} were different for each peptide group at each pH value assayed. Within each group, higher Tr values were achieved at the pH value that was nearest to the group pI. However, membrane MWCO also had an important influence, as peptide overall Tr values through PES5 membrane were approximately twice PES1 Tr values. Membrane material had greater influence than NMWCO in determining peptide transmission. Acidic peptides were more transmitted at pH2 and through PES5 membrane, but had low bioactivity scores. Consequently, filtration at pH2 is not recommended for obtaining permeates enriched in potentially bioactive peptides. Basic and neutral peptides, on the contrary, had better bioactivity scores, and were best transmitted at pH10 with PES5 membrane. Interestingly, most transmitted peptides were the ones that had highest bioactivity scores. Therefore, filtration of BSA peptic hydrolysates with a PES5 membrane at pH10 is evidenced as a good strategy, applicable to industrial scale, to obtain permeates enriched in neutral and basic peptides with very high bioactivity scores. In agreement with the latter, best separation factors were achieved at pH10 with PES5 membrane. In general, acid peptides were more easily separated from the other groups than neutral and basic between them. A new line of bioactivity testing research is suggested with peptides best transmitted and with high bioactivity scores.

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Author Contributions

F.A.R. has supervised the whole study. F.A. has written the manuscript and performed the experimental work. R.F. has helped with experimental work. C.M. and U.A.G. have provided the B.S.A. and assessed about the methodology employed in the study.

Additional Information

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BSA (at 0.3% concentration) peptic hydrolysis under the enzyme optimum conditions yielded a DH of a $65.54 \pm 1.57\%$ of the maximum possible, after 3 hours of digestion using an E/S ratio of the 4%. Therefore, the DH obtained with pepsin without any pre-treatment was higher than the one obtained with trypsin using a thermal pre-treatment. The fact that the E/S ratios were different does not seem to be relevant, since variation in trypsin concentrations did not significantly alter the DH obtained. As a result of the hydrolysis process, 43 peptide sequences were identified, from which a 23.25% were assigned probable bioactivity (bioactivity score $\geq 50\%$) by the program PeptideRanker. One of the identified peptides had also been reported in the literature as bioactive (LLL). There was a difference in the overall bioactivity of the peptides depending on their isoelectric group. Acidic peptides had an average of 0.18 on the bioactivity score, whereas basic peptides had a 0.33 and neutral peptides a 0.37.

Other parameters apart from peptide size influenced peptide transmission, since the observed transmission (Tr_{obs}) values were different from the theoretical (Tr_{the}) ones. Charge mechanisms were relevant because peptide Tr values changed with medium pH. Overall Tr values were higher for the highest NMWCO membrane (5 kDa), between 2.23 and 1.80 fold, depending on the pH values, compared to the 1 kDa membrane. Acidic and basic peptides were far more transmitted at pH values near their respective isoelectric points. However, the neutral group transmission values were very similar, regardless the pH. This behaviour was the same for both membranes. Selectivity values between acid and neutral or acid and basic peptides were always higher at basic pH value. However, between neutral and basic the selectivity was higher at acid pH value.

6.3. Hydrolysates, membrane fractions and peptides bioactivity assessment

The assessment of the bioactivity is performed throughout all the work using different methodologies and strategies: performing bibliographical searches and using BIOPEP database, using the accumulated knowledge on structure-activity relationship, using *in silico* predictive software, or performing *in vitro* and *in vivo* trials. Precisely *in vitro* and *in vivo* works on bioactivity testing will herein be presented.

Although traditional *in vitro* methodologies are currently under criticism, due to the length, high costs and poor predictability; they are still the method of choice to prove peptides bioactivity in a first stage, and should therefore be known by anyone exploring the field of BP.

For the *in vitro* tests, BSA was thermally treated and then hydrolysed with trypsin to release peptide fragments. The resulting hydrolysate was subjected to membrane filtration, using two different membrane materials and three different pH values. All the different permeate fractions were then assayed *in vitro* for anti-hypertension and antioxidant bioactivities, using the ACE inhibition and the DPPH radical scavenging *in vitro* tests.

For the *in vivo* testing, a purified peptide was used instead of a membrane fraction. Membrane methodologies were shown to be able to obtain peptide fractions containing a few peptides, but not fractions containing only one peptide. With the aim to study a technology able to achieve this, liquid chromatography (RP-HPLC) was studied at a preparative scale. Liquid chromatography is a more accurate methodology than membrane filtration. However, it involves the use of organic solvents, and the scale up becomes quickly unaffordable. Still, it is widely used in the pharmaceutical industry. In this case, the protein source was β -lg, the main protein in the WPC. β -lg was hydrolysed with trypsin and then subjected to membrane filtration. The permeate fraction was further subjected to preparative RP-HPLC, and a fraction containing only one peptide sequence was selected. This fraction was injected on a suitable animal model to study its effect over lipid metabolism.

6.3.1. Antioxidant and anti-hypertensive *in vitro* activities of bovine serum albumin hydrolysate fractions

In this work, the possibility of obtaining bioactive peptide fractions from BSA by means of a simple procedure of only 2 steps (hydrolysis and membrane filtration) was evaluated, as an exploratory study for possible industrial application. In this case, the focus was put on bioactivity testing, rather than on membrane filtration. Two bioactivities were considered: antioxidant capacity, through the DPPH radical scavenging assay, and anti-hypertension, through the ACE inhibition assay. Therefore, all permeates were subjected to this two assays.

The methodology used was, firstly, a tryptic hydrolysis of BSA at a concentration of 3 g/L, in order to release the peptides from the protein. The protein was previously subjected to a thermal treatment in order to enhance the enzyme cleavage and thus the peptide yield. The thermal treatment lasted 1 hour and was conducted at 75°C. The hydrolysis lasted 6 hours, and was performed using the pH-stat methodology, at trypsin optimum conditions (pH 8 and 37°C) and using an E/S ratio of 1:50. Then, the obtained hydrolysate was filtered through a nanofiltration/ultrafiltration flat rig at three different pH values (4, 6 and 8) with two different membrane modules (polyethersulfone and cellulosic) of 0.1 m² of filtration area, so as to compare membrane performance. The operation parameters were constant throughout the filtration process: for the polyethersulfone flat-sheet module of 5 KDa NMWCO, the settings were a transmembrane pressure (TMP) of 1.5 bar, a temperature of 37 °C and a recirculation rate of between 19 and 38 L/m²h. For the cellulosic flat-sheet module of NMWCO 5 kDa the settings were the same, except the recirculation rate, which was constant at around 45 L/m²h. The obtained permeates were analysed by liquid chromatography-tandem mass spectrometry technology (LC-MS) in order to obtain the peptide sequences present in each permeate, as in all the works published. Finally, different concentrations of every permeate were evaluated for their *in vitro* antihypertensive and antioxidant activities through ACE-inhibition and DPPH radical scavenging tests. The ACE inhibition test was performed as in Chibuike C Udenigwe, Lin, Hou, and Aluko (2009), measuring the percentage of inhibition as:

$$\% \text{ ACE inhibition} = \frac{[(\text{absorbance of the blank} - \text{absorbance of the sample}) * 100]}{\text{absorbance of the blank}}$$

The DPPH radical scavenging assay was performed using as reagents 0.1 M sodium phosphate buffer at pH 7 with Triton X-100 at 1% concentration (v/v). The DPPH was dissolved in methanol to a final concentration of 0.1 mM, while the samples were dissolved in the prepared buffer. The blank (double distilled water), the control (GSH), and the samples so prepared were placed in 96-well microplates, incubated for 30 min at room temperature in the dark, and the absorbance measured at 517 nm. The percentage of DPPH radical scavenging activity was determined as:

$$\% \text{ DPPH radical scavenging activity} = [(\text{absorbance of the blank} - \text{absorbance of the sample}) * 100] / \text{absorbance of the blank}$$

The hydrolysis process with the previous thermal treatment allowed achieving a DH of the $49.66 \% \pm 2.20$ of the maximum possible. This result is in agreement with all the previous heat-treated BSA tryptic hydrolysis performed using the same parameters, which always yielded around a 50% DH in reference to the $DH_{\max_{the}}$. Figure 14 shows the average trend of all the hydrolyses performed (6 in total).

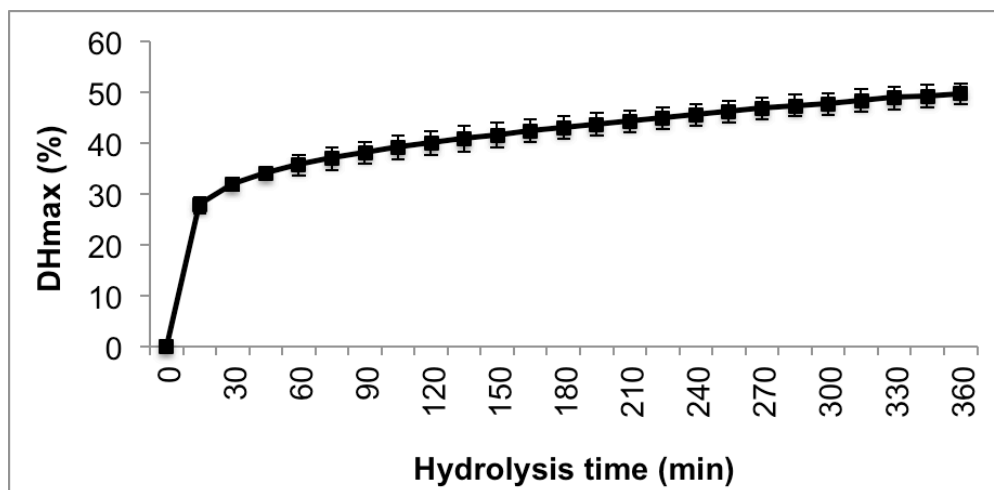


Figure 14: Average degree of hydrolysis (DH) values obtained in bovine serum albumin (BSA) tryptic hydrolysis at 0.3% concentration and 1:50 E/S ratio, after a 1 h thermal pre-treatment at 75°C.

Regarding the filtration process, both membranes were compared (see Figure 15) according to the only variable parameter, which was the flux rate. While for the cellulosic membrane the average flux was constant with pH, the polyethersulfone membrane had significant higher fluxes at pH 8, compared to pH 6 and 4. Moreover, polyethersulfone membrane fluxes were always lower than those of the cellulosic membrane.

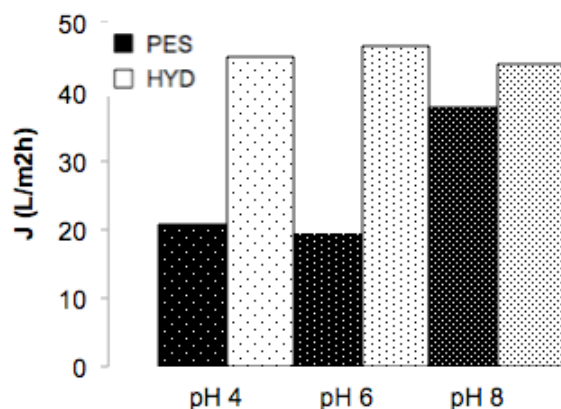


Figure 15: Average flux rates during bovine serum albumin trypsinolysis hydrolysates filtration through a 5 kDa NMWCO polyethersulfone flat-sheet membrane (PES) and a 5 kDa NMWCO cellulosic flat-sheet membrane (HYD).

The behaviour of the PES membrane was consistent with other works in which polyethersulfone flat-sheet membranes were used (Fernández, Zhu, FitzGerald, & Riera, 2014). At acidic pH values the peptides tend to aggregate and those high MW aggregates increase the fouling of the membrane surface. Conversely, the utilized cellulosic membranes were specifically designed to avoid fouling issues, such as flux decline.

For the antioxidant assays, three different hydrolysate concentrations were assayed (10, 1 and 0.1 mg/mL). Table 4 summarises the different samples assayed and the nomenclature, for both types of bioactivity *in vitro* assays.

Table 4: Samples assayed for antioxidant and/or anti-hypertensive *in vitro* activity.

Sample	Sample code
5 kDa polyethersulfone permeate pH 8	PP8
5 kDa polyethersulfone permeate pH 6	PP6
5 kDa polyethersulfone permeate pH 4	PP4
5 kDa cellulosic permeate pH 8	PC8
5 kDa cellulosic permeate pH 6	PC6
5 kDa cellulosic permeate pH 4	PC4
BSA tryptic hydrolysate	H
Bioactivity positive control	C

Figure 16 summarises the results for antioxidant activity. Results are expressed in relation with the control (GSH), which always has 100% inhibition potency.

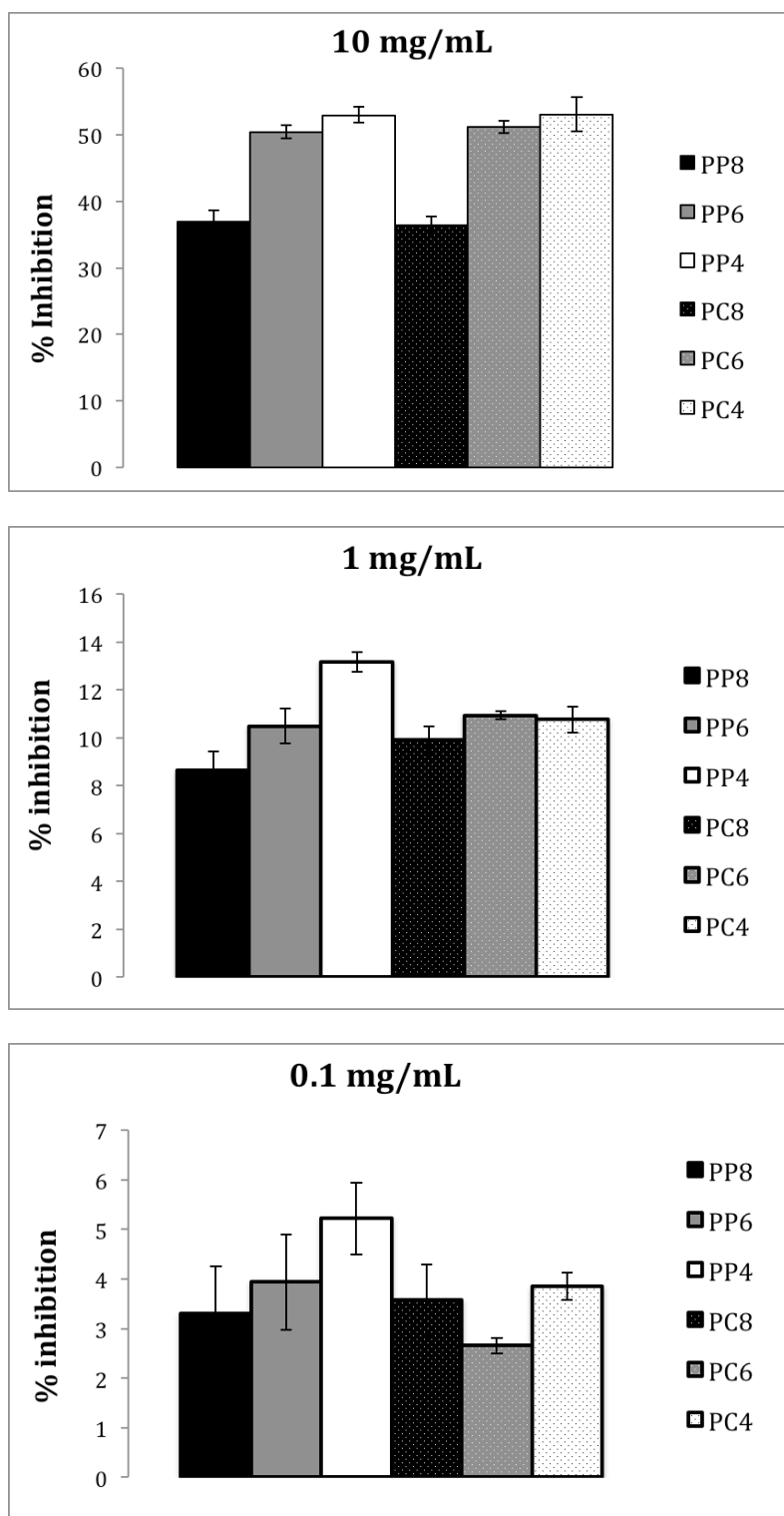
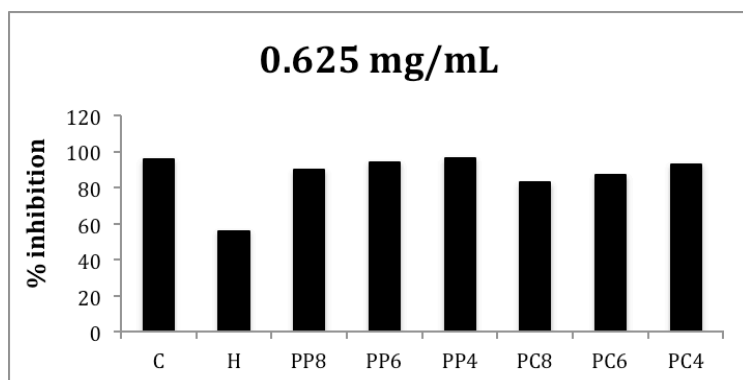
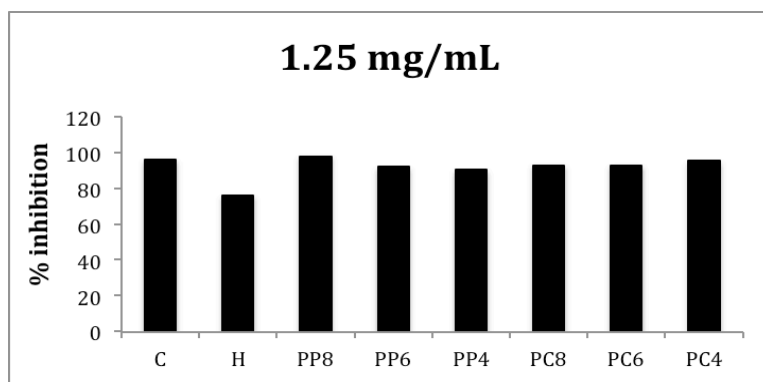
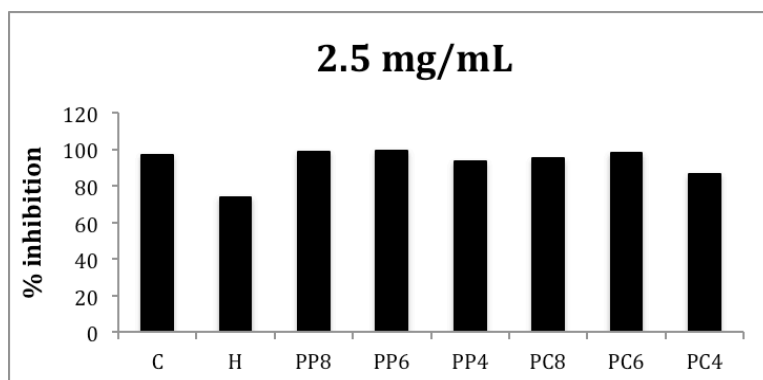
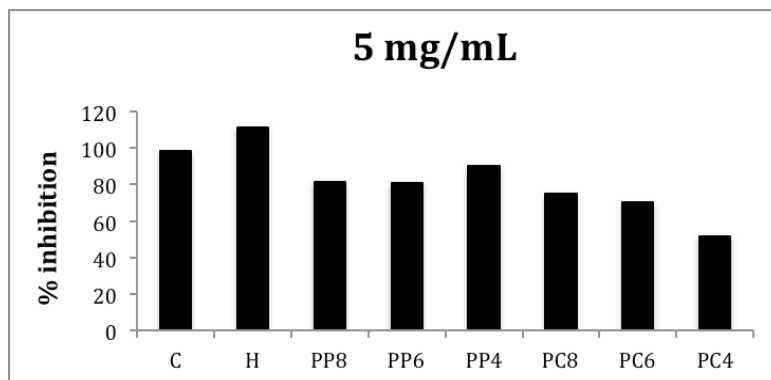


Figure 16: Antioxidant capacity of bovine serum albumin (BSA) trypsinolysis hydrolysates permeates. PP refers to the polyethersulfone membrane, and PC to the cellulosic membrane. The numbers make reference to the pH value of the filtration medium.

The antioxidant activity of all permeates was only comparable to that of the control only for the highest dose (10 mg/mL). There is a markedly decrease in all permeates bioactivity with the decrease in the sample concentration. The highest dose (10 mg/mL) showed a similar trend, with lower activity in both pH 8 membrane permeates and almost the same for pH 6 and 4. For the lowest doses (1 and 0.1 mg/mL), that relationship is broken.

Considering the 10 mg/mL results, it is surprising to see how the permeates corresponding to two different membrane materials (polyethersulfone and cellulose) show the same values in bioactivity and the same variation with pH. The expected result would be a different bioactivity, since different membrane materials would interact differently with the peptides in solution, and thus allow different species to be transmitted. Nevertheless, in the light of the results obtained, we could conclude that the hypothesis of the formation of a polarized layer affecting membrane permeation (section 7.2.1) is supported as well for these results.

For the anti-hypertensive assays, 5 different hydrolysate concentrations were assayed (5, 2.5, 1.25, 0.625 and 0.3125 mg/mL). Figure 17 provides the obtained results.



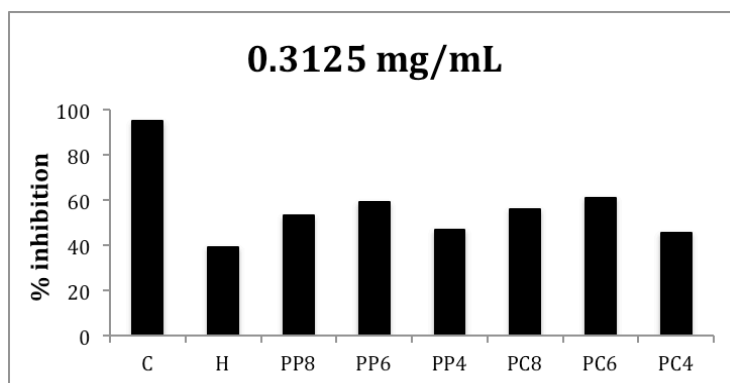


Figure 17: Anti-hypertensive capacity of bovine serum albumin (BSA) trypsinolysis hydrolysates permeates. PP refers to the polyethersulfone membrane, and PC to the cellulosic membrane. The numbers make reference to the pH value of the filtration medium.

All the permeates had very high ACE inhibition, around 100%, at all the concentrations tested except the lowest one (0.3125 mg/mL), with which average % inhibition was around 60%. The hydrolysate also showed ACE inhibition, but in this case the activity more clearly decreased with the decrease in concentration. In fact, the permeates always had more bioactivity than the hydrolysate, except for the highest dose (5 mg/mg). Therefore, although differences between them, all permeates were promising regarding antihypertensive and antioxidant properties. In this case, it is also surprising that all the permeates had very similar inhibition potency.

Therefore, it can be concluded that both polyethersulfone and cellulosic membranes yielded permeates with very similar degree of bioactivity, regarding antioxidant and anti-hypertensive activities. Highest permeate concentrations always had higher bioactivity. Best results were around 60% inhibition for antioxidation and around 100% inhibition for anti-hypertension.

Membrane Technologies for Obtaining Bioactive Fractions from Blood Main Protein: An Exploratory Study for Industrial Application

F. Arrutia, F.A. Riera

Abstract— The meat industry generates large volumes of blood as a result of meat processing. Several industrial procedures have been implemented in order to treat this by-product, but are focused on the production of low-value products, and in many cases blood is simply discarded as waste. Besides, in addition to economic interests, there is an environmental concern due to bloodborne pathogens and other chemical contaminants found in blood. Consequently, there is a dire need to find extensive uses for blood that can be both applicable to industrial scale and able to yield high value-added products. Blood has been recognized as an important source of protein. The main blood serum protein in mammals is serum albumin.

One of the top trends in food market are functional foods. Among them, bioactive peptides can be obtained from protein sources by microbiological fermentation or enzymatic and chemical hydrolysis. Bioactive peptides are short amino acid sequences that can have an positive impact on health when administered.

The main drawback for bioactive peptide production is the high cost of the isolation, purification and characterization techniques (such as chromatography and mass spectrometry) that make unaffordable the scale-up. On the other hand, membrane technologies are very suitable to apply to the industry because they offer a very easy scale-up and are low-cost technologies, compared to other traditional separation methods.

In this work, the possibility of obtaining bioactive peptide fractions from serum albumin by means of a simple procedure of only 2 steps (hydrolysis and membrane filtration) was evaluated, as an exploratory study for possible industrial application.

The methodology used in this work was, firstly, a tryptic hydrolysis of serum albumin in order to release the peptides from the protein. The protein was previously subjected to a thermal treatment in order to enhance the enzyme cleavage and thus the peptide yield. Then, the obtained hydrolysate was filtered through a nanofiltration/ultrafiltration flat rig at three different pH values with two different membrane materials, so as to compare membrane performance. The corresponding permeates were analyzed by liquid chromatography-tandem mass spectrometry technology in order to obtain the peptide sequences present in each permeate. Finally, different concentrations of every permeate were evaluated for their *in vitro* antihypertensive and antioxidant activities though ACE-inhibition and DPPH radical scavenging tests.

The hydrolysis process with the previous thermal treatment allowed achieving a degree of hydrolysis of the 49.66% of the maximum possible. It was found that peptides were best transmitted to the permeate stream at pH values that corresponded to their isoelectric points. Best selectivity between peptide groups was achieved at basic pH values. Differences in peptide content were

found between membranes and also between pH values for the same membrane. The antioxidant activity of all permeates was high compared with the control only for the highest dose. However, antihypertensive activity was best for intermediate concentrations, rather than higher or lower doses. Therefore, although differences between them, all permeates were promising regarding antihypertensive and antioxidant properties.

Keywords—Hydrolysis, membrane filtration, bioactive peptides, bovine serum albumin.

F. Arrutia and F. A. Riera Authors are with the Chemical Engineering and Environmental Technology Department, University of Oviedo, Asturias, 33006 Spain (e-mail: farrutia@gmail.com).

6.3.2. Isolation of a β -lactoglobulin-derived peptide and effect on zebra fish lipid metabolism

In this work, the bioactivity of a WPC-derived peptide was assayed using an animal model, with regard to its influence in the lipid metabolism. While the traditional approach is to test peptides bioactivity through *in vitro* assays, there have been many critics to this approach. Peptides fate within the body can be altered by endogenous proteases digestion or the impossibility to permeate some biological barriers. Therefore, the use of an animal model eliminates these drawbacks, although the process is lengthier. Other common approach when assaying BP, is to first identify the peptides present in the hydrolysate or fraction, and then *de novo* synthesize them in order to test them. However, in this case the purified peptide fraction was assayed, what demonstrates the accuracy of the process developed, which can compete with synthetic synthesis. The use of zebra fish is appropriate for the measurement of the lipid metabolism, since the metabolic routes that trigger obesity are very similar to those happening in humans.

In vivo bioactivity testing can confirm *in silico* or *in vitro* predictions. Only clinical trials would remain as a later phase, and those stood out of the scope of this research.

The peptide sequence isolated from β -lg was VAGTWY (VY6). VY6 was obtained from a commercial bovine β -lg, kindly supplied by Davisco Foods International (Le Sueur, MN, USA). First, a tryptic hydrolysis was performed over trypsin, in order to release the peptide sequences. Former WPC hydrolyses had confirmed that β -lg was an appropriate substrate for trypsin, since the protein was almost completely digested. The hydrolysate was ultrafiltered with a flat-sheet polyethersulfone membrane module of 5 kDa NMWCO at pH 8, and the permeate fraction obtained was further purified by preparative RP-HPLC separation using a C18 column and acetonitrile as organic solvent. The purified fraction, which only contained the peptide sequence VY6, as tested by analytic chromatography, was then subjected to the *in vivo* assays.

The effects of VY6 were analysed at two levels: on hepatic triglycerides and both hepatic and free cholesterol levels, and on gene expression, through the measurement of the mRNA levels of 2 obesity-related genes. Also, two different peptide doses were assayed, labelled as “low” or “high”.

Este capítulo (p. 111-117) se corresponde con el artículo:

Mohammed-Geba, Khaled; Arrutia Rodríguez, Fátima et al. *VY6, a β -lactoglobulin-derived peptide, altered metabolic lipid pathways in the zebra fish liver* . En **Food and Function**, 7(4), p. 1968-1974 (2016); doi:10.1039/c6fo00003g

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<http://dx.doi.org/10.1039/c6fo00003g>

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Title: VY6, a β -lactoglobulin-derived peptide, altered metabolic lipid pathways in the zebra fish liver.

K. Mohammed-Geba^{a,b}, F. Arrutia^c, H. Do Huu^d, Y. J. Borrell^a, A. Galal-Khallaf^{a,b}, A. Ardura^a, Francisco A. Riera^c, Eva Garcia-Vazquez^{a,†}.

Abstract Today enormous research efforts are focused on alleviating the massive, adverse effects of obesity. Short peptides are key targets for research as they can be generated from natural proteins, like milk. Here we conducted Trypsinogen digestion of Beta-lactoglobulin (β -Lg), the major mammalian milk protein, to release the hexamer VY6. It was assayed *in vivo* for its activities on lipid metabolism using zebra fish as a vertebrate model. Zebra fish juveniles were injected with two different doses of the peptide: 100 and 800 μ g g⁻¹ fish and left for 5-days before sacrificing. Lipids measurements showed significant reduction in liver triglycerides and free cholesterol, as well as increased liver HDL cholesterol. Dose-dependent increases of mRNA levels of the genes coding for the enzymes acyl coenzyme A oxidase 1 (*acox1*) and Lipoprotein lipase (*lpl*) were also found. The whole results suggest significant anti-obesity activity of the β -Lg-derived VY6 peptide. Its use as a nutraceutical is discussed.

A. Introduction

More than 40 million children under 5 years old and more than 1.4 billion adults above 20 years old are suffering obesity^{1,2}. The dimension of the problem is enormous and researchers and health agents are looking together for remedies to prevent and control the pandemics. The search of food components, preferably derived from natural products, able to decrease metabolic lipid contents and controlling cholesterol levels is therefore a priority.

Genetics has indeed a role in obesity. Among several enzymes responsible for lipids catabolism, acyl-coenzyme A oxidase 1 (*Acox1*) and lipoprotein lipase (*Lpl*) are of special importance. The product of the transcript *acox1* catabolizes both fatty acids and reactive oxygen species (ROS) in the peroxisomal β -oxidation process. In liver and adipose tissues, it is the first enzyme in such metabolic oxidation route^{3,4}. Up-regulation of *acox1* gene expression is correlated with obesity-inhibitory treatments in the livers of different experimental models and at different stages of life, starting very early in the development^{5,6}. Likewise, the enzyme produced by *lpl* transcript is a potent controller of lipids, chiefly acting as rate-limiting enzyme for the hydrolysis of triglycerides and being a potent anti-hypertriglyceridemia molecule⁷. Therefore, any food product able to activate these two genes is expected to contribute to control obesity.

β -lactoglobulin (β -lg) is one of the chief proteins that remain soluble within non-skimmed or skimmed milk⁸. β -lg-derived peptide VAGTWY (VY6) has already been assayed *in vitro* for different biological activities, and exhibits anti-diabetic⁹, anti-bacterial^{10,11}, and ACE-inhibitory and opioid-like properties^{12,13}. However, to date no assays have been reported for anti-obesity properties, neither in vertebrate models. VY6 showed some glucose regulatory capability in mice¹⁴. However, they administered whole β -lg hydrolysates, not the purified peptide. In fact, the utilization of zebra fish as an animal model

to assay the aforementioned peptide bioactivities constitutes a novelty, since rats are normally the animal of choice^{15,16,17,18}.

Zebra fish is an animal model of choice for studies of human obesity because the metabolic routes that control the processes conducting to obesity in zebra fish are quite similar to the human's^{19,20}. In this study, VY6 was obtained from β -lg by tryptic hydrolysis, and then isolated using a two-step process: ultrafiltration of the hydrolysate and semi-preparative RP-HPLC fractionation of the permeate. It is important to highlight that bioactivity assessments are usually performed using synthetic peptides, rather than directly purified peptides from the source of origin, as in the present case, as synthesized peptides lack functional inhibitors or activity-modifiers deliberately added from the original protein during the course of its purification for isolation of targeted peptides^{21,22,23}. VY6 was subsequently assayed for its anti-obesity properties. *In vivo* assays were performed using zebra fish (*Danio rerio*) as an animal model, focusing on lipid contents and in the modification of the *acox1* and *lpl* gene expression by reverse transcription-polymerase chain reaction (RT-PCR) technique that is used in several studies dealing with the effect of different nutraceuticals on physiological performance in human and experimental animals^{24,25,26}.

B. Materials and Methods

B.1. Production of the peptide VY6

The commercial substrate of Bovine β -lg (supplied by Davisco Foods International Inc., Le Sueur, MN, USA) contained 97.9 % protein (w/w) of which 91.5 % were β -lg, according to the manufacturer. Trypsin (T1426 from bovine pancreas TPCK treated, activity of ≥ 10000 units/mg protein), acetonitrile grade HPLC and TFA were purchased from Sigma-Aldrich (St. Louis, MO, USA), VWR (Barcelona, Spain) and Panreac (Barcelona, Spain), respectively. All other chemicals were of analytical grade.

Bovine β -lg solutions were prepared by solubilizing the protein powder in 3 L of distilled water until a concentration of 30 g L⁻¹ was reached. The pH and temperature values were set to 8 and 37 °C respectively. Hydrolysis was carried out for 23h by adding trypsin at an enzyme:substrate ratio of 1:450 (w/w). The pH was kept constant with a pH-stat device model 842 Titrando (Metrohm Ion Analysis, Herisau, Switzerland), that added 1M NaOH when necessary. Reaction was stopped by lowering the pH with 0.1 M HCl, when the degree of hydrolysis (DH) reached the target value of 6.8 %. The calculation of the DH was based in the pH-stat method²⁷, using Eq. (1).

$$(1) \quad DH(\%) = \frac{B \cdot N_B}{\alpha \cdot MP \cdot h_{TOT}} \times 100$$

Where B (mL) is the volume of consumed base, N_B (mol L⁻¹) the normality of the base, M (g) the mass of protein, h_{TOT} the total number of peptide bonds in the substrate protein. This value was previously calculated by Cheison et al. (2010)²⁸ as 7.2 meqv g⁻¹ protein for β -lg. α is the average degree of dissociation of α -amino groups in the hydrolysis system β -lg + Trypsin. In the present study, α was calculated according to Camacho et al. (2001)²⁹, and it took the value of 0.979 at pH 8. For the membrane permeation experiments, the hydrolysates were first diluted in distilled water to a concentration of 15 g L⁻¹. The membrane used for the fractionation process, made of polyethersulfone (PES) with 5 kDa molecular weight cut off (MWCO) and 0.1 m² filtration area (Millipore, Billerica, MA, USA), was installed within a Pellicon 2 mini holder (Millipore, Billerica, MA, USA). The filtration conditions were pH 8, temperature 37 °C and transmembrane pressure (TMP) 7.5×10⁵ Pa. All membrane experiments were performed in duplicate.

Peptides present in the permeate stream were further separated by a Varian ProStar semi-preparative RP-HPLC (Varian, Palo Alto, CA, USA), equipped with a fraction collector. Permeate samples were injected into a Dynamax Microsorb 300-8 C18 (250×21.4 mm, Varian, CA, USA) column, preceded by a guard Dynamax Microsorb C18 column (Varian, CA, USA). The system was run at ambient temperature and a flow rate of 10 mL min⁻¹, and light detection was set to 214 nm. Mobile phase A was composed of 0.1 % TFA (v/v) in MilliQ water (Millipore, Billerica, MA, USA) and mobile phase B was 0.1 % TFA (v/v) in acetonitrile. The gradient was: 5% B (0.1% TFA (v/v) in acetonitrile) for 8 min, 5 to 45 % B in 142 min, 45 to 80 % B in 20 min, 80 to 95 % B in 5 min and returning to the starting conditions in 1 min.

The collected fractions were analysed with a Voyager-DE STR matrix assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF/MS) (Applied Biosystems, Waltham, MS, USA). Sample preparation was performed with Zip-Tip pipette tips containing C18 reverse-phase media (Millipore, Billerica, MA, USA). A α -cyano-4-hydroxy-cinnamic acid (10mg mL⁻¹ in 50 % Acetonitrile-0.1 % TFA) (Sigma, St. Louis, MO, USA) matrix was used. The MW range for the MS analysis was from 500 to 3500 Da, and for external calibration a MSCAL2 calibration kit (Sigma, USA) was used. Mass spectrometry acquisition was performed in reflectron mode and positive

polarity, with an acceleration voltage of 25 kV. The spectra were processed with software Data Explorer (Applied Biosystems, Waltham, MS, USA). Peptide identification was carried out by MW, using the FindPept tool on the ExPASy server (<http://web.expasy.org/findpept/>).

The fractions were further analysed by direct-infusion electrospray mass spectrometry (ESI-MS/MS), so as to confirm the correct identification of peptide, using a quadrupole time-of-flight mass spectrometer equipped with an electrospray source (Qstar XL, Applied Biosystems, Waltham, MS, USA). The obtained MS/MS spectra were processed using Analyst QS 1.1 (Applied Biosystems, Waltham, MS, USA).

The peptide of interest (VY6) was stored until zebra fish tests using a Cryodos laboratory freeze-dryer (Telstar Industrial, S.L., Terrassa, Barcelona, Spain).

B.2. *In vivo* trial: zebra fish treatment, sampling and measurements

The experiments with living zebra fish followed the EU standards for animal experiments and were approved by the Committee of Ethics of the Asturias Principality as it is in the Directive of the University of Oviedo of 13 of February of 2014 (BOPA 26/02/2014).

Juvenile zebra fish *Danio rerio* were purchased from ZF Biolabs (Madrid, Spain), where they were raised under the minimum levels of consanguinity. Fifteen fish were acclimated for 14 days to normal, dechlorinated freshwater at ambient temperature (22 °C) and normal summer light regime. They were fed with standard fishmeal for tropical aquarium fishes (Dajana Tropica Basic), containing 7 % crude oils and fat materials, one dose a day as recommended. Despite being the knowledge about the real requirements of nutrients lacking in zebra fish and many other ornamental fishes, the need for lipids should be adjusted since the low energy demands of the fish make them prone to fatty deposition³⁰. The experimental fish were divided at random in three groups of five fish of similar weights, ranging between 0.8796 ±0.096 and 1.145±0.19 g. Two groups were treated with two different doses of VY6, and the control group was treated with water.

Fish were anaesthetized using sublethal 50 mg L⁻¹ of Ethyl 3-aminobenzoate methanesulfonate (Tricaine, Santa Cruz Biotechnology, Cat. no. 10743661). Each fish was intraperitoneally-injected with 10 μ L of one of the following doses: low (20 μ g μ L⁻¹: final *in vivo* injected dose=200 μ g g⁻¹ fish) or high (80 μ g μ L⁻¹: final *in vivo* injected dose=800 μ g g⁻¹ fish) of the peptide. In the control group the Zebra fish were injected with sterile water. For the injection 10 μ L Hamilton syringes were used (Hamilton® GASTIGHT® syringe, cemented needle volume 10 μ L, needle size 26s Ga-cone tip, Cat. no. 80039). After treatment the fish were kept in 20 L aquaria, under normal environmental and feeding conditions, in a closed-recirculation water system with mechanical filters for 7 days, renewing daily 20 % of tank waters.

For sampling, fish were anaesthetized by a lethal dose of Tricaine (100 mg L⁻¹). Each fish was dissected and the liver removed. Twenty mg of the liver tissue were placed in RNase-

free 2 mL Eppendorf tube and immediately flash frozen in liquid nitrogen (N₂, 196 °C) for later total RNA extraction, and the rest were kept in sterile 2 mL Eppendorf tube that was also flash frozen in liquid N₂ for measurement of lipids. Both aliquots were maintained at -80 °C until further analyses.

Total lipids were extracted from liver using the method of Folch et al. (1957)³¹ with slight modifications. In brief, ultrafrozen livers (- 80 °C) were individually homogenized in liquid N₂, then resuspended with strong vortexing in 500 µL of 2:1 chloroform:methanol mixture. The reconstituted samples were centrifuged at 10,000 g for 10 min. The supernatants were transferred to new 1.5 mL Eppendorf tubes, the process of adding 500 µL of 2:1 chloroform:methanol was repeated twice. Later on, 100 µL of 0.9 % NaCl were added to the supernatant and the tubes were thoroughly vortexed for strong mixing. The tubes were then centrifuged at 4.000 g for 30 min. The lower layer containing lipids was collected to a new 1.5 mL eppendorf tube that was incubated at 37 °C for 2 days for Chloroform:Methanol mixture evaporation. The resulting lipids were reconstituted with 250 µL of 10 mM KH₂PO₄ a pH7, with strong vortex until complete lipids dissolution. In these samples, triglycerides, free cholesterol, and HDL cholesterol were measured using commercial kits from Spinreact (Cat. no. 1001310, 41035, and 10010196-respectively). KH₂PO₄ (10 mM, pH7) was used as negative control. External standard curve method was used for measurement of concentration in relation to the fluorescences emitted by lipids standards prepared as five, half-serial dilutions from each major standard provided with the lipids estimation kits. Extracted lipids samples and controls were applied in duplicates in a 96-well microplate for each treatment for measurement. Intra-specific errors were calculated as standard error of means of measurements between the duplicates of the same sample.

B.3. RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Total liver RNA was extracted from each specimen using GeneMATRIX Universal RNA Purification Kit (Eurx, Cat. no. E3598) according to manufacturers' instructions, reconstituting the tissue lysing buffer with 2-mercaptoethanol (Sigma-Aldrich, M3148-250ML). Extreme caution was taken to avoid introduction of RNase into the working materials or samples. All glass materials used for preparation of reagents for RNA extraction were previously sterilized at 180°C for 16 h. Working station, pipettes, centrifuges, and all other working materials were thoroughly cleaned with absolute ethyl alcohol. Diethylpyrocarbonate (DEPC, Merck, Cat. no. 8.41751.0005) was used for preparation of necessary dilutions of any material. After RNA extraction, the quality of RNA was checked using 2 %, RNase-free agarose gel (UltraPure™ Agarose, LifeTechnologies, Cat. no. 16500-100), prepared with 1X TBE (Eurx, Cat. no. E0230-01). 1 µL of RNA was loaded into the agarose gel using RNase-free gel-loading buffer (LifeTechnologies, Cat. no. AM8556.1). Success of extraction was confirmed by the presence of 2 bands, of the 28S rRNA and the 18S rRNA, with neither a smear or lower bands of

degradation, nor high molecular weight band of genomic DNA. RNA quantity was determined through the spectrophotometric measurement of absorbance at A260/280 by BioPhotometer Plus (Eppendorf). Samples with A260/280 = 1.8-2 were considered adequate for cDNA synthesis. 500 ng of RNA from each liver samples were used for synthesis of cDNA, using EurxdART RT-PCR kit (E0802-01).

Semi-quantitative RT-PCR approach was used for assessing the effects of the injected hexamer on two enzymes' genes related to lipid degradation and oxidation in liver, that are Peroxisomal acyl-coenzyme A oxidase 1 (*acox1*) and Lipoprotein lipase (*lpl*). The primers for *acox1* were: *acox1*Fw: 5'-ACAGCAGAGCAAGAGTAACG-3' and *acox1*Rv: 5'-TGAAGGGCATAAAGCAGAGC-3'. The primers for *lpl* were: *lpl*Fw: 5'-CGCAGGAGCAGCAAGATG-3' and *lpl*Rv: 5'-GTTCAAAGTAGGCATAATGTAGGG -3'. Beta actin (*actb*) was used as reference gene as its expression does not change under different chemical treatments to the zebra fish³². The primers used for *actb* amplification in liver cDNA were β -*act*Fw: 5'-CGAGCTGTCTTCCATCCA-3' and β -*act*Rv: 5'-TCACCAACGTAGCTGTCTTTCTG-3'^{33,34,35}. All PCRs were carried out using thermal cycler Applied Biosystems Model 2720-2. The PCR mix contained in the previously mentioned RT-PCR kit, consisted of 1x PCR buffer, 0.2 mM dNTPs, 0.4 mM of each primer, and 2.5 units of *Opti-Taq*DNA polymerase. The PCR program consisted of an initial denaturation step at 95 °C for 10 min, then 30 cycles of 95°C for 30 s, 50 °C for 30 s, and 30 s at 72 °C for the extension. The annealing temperature (50°C) was chosen according to preliminary optimization trials. Finally, PCR products were visualized in 2 % agarose gel stained by SimplySafe (Eurx, Cat. no. E4600-01) nucleic acids intercalating dye. The intensity of the resulting bands were measured using the program ImageJ³⁶. The values of each band in the gel were normalized dividing the band intensity by the intensity of the band of the reference gene (*actb*) of the same sample.

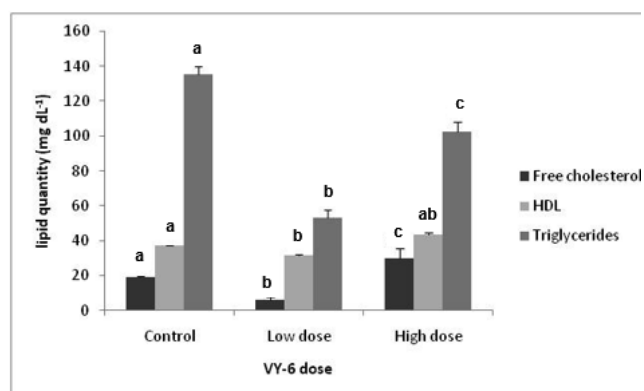
B.4 Statistical analyses

For all measurements, the results were analyzed using one-way ANOVA in the program Statgraphics Centurion IX. Tukey

HSD was used as post-hoc test to estimate statistical differences between

n groups of fish in pairwise tests. Differences were considered significant at P<0.05.

C. Results



C.1. VY6 production

As stated in section B.1, the identity of the peptide was confirmed by MALDI-TOF/MS and MS/MS. Additionally, it was characterized by RP-HPLC so as to assess the purity of the fraction. No additional peaks were found in the chromatogram, as it can be seen in Figure 1. The peptide was characterized by a retention time (RT) of 14.83 min. The National Center for Biotechnology Information (NCBI) database was searched for the bovine β -lg sequence, so as to confirm that VY6 sequence was within the protein sequence. VY6 corresponded to the 15th-20th amino acid residues.

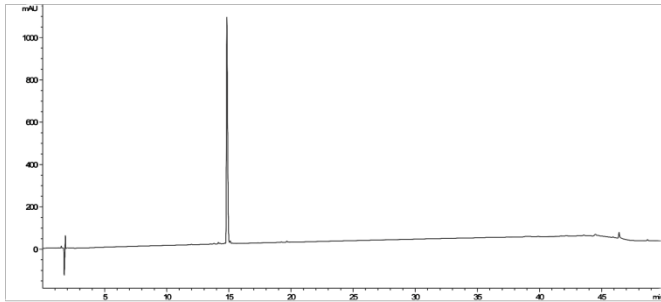


Fig. 1: RP-HPLC chromatographic profile of the semipreparative RP-HPLC isolated fraction identified as VY6 by MALDI-TOF/MS and MS/MS.

C.2. Effect of VY6 on zebra fish hepatic lipids

For all measurements, the ranges of linearity of standard curves covered adequately the concentrations used. r^2 in all cases ranged between 0.999-1. Moreover, very low intraspecific error were found between duplicates of each sample measure ($1-3 \times 10^{-3}$ for triglycerides and free cholesterol, and $4-5 \times 10^{-3}$ for HDL) (see Table 1). Peptide injection induced significant reduction in the levels of triglycerides in liver (one-way ANOVA with $F= 75.35$, $P<0.001$; pairwise values in the Tukey's test are all significant, see Table 2) The decrease was more intense in response to the lower dose of the peptide injected (about one half of the control level; Figure 2), than with the high dose for which the reduction of triglycerides was significant (comparison control-high dose with a p-value of 0.007; Table 2). For the free cholesterol the F-value in a one-way ANOVA was 11.36 ($P=0.009$); however, the significant pairwise comparison was between the groups treated with the high and the low dose (Table 2), but not with the control that was intermediate between the two treated groups. A similar pattern was found for HDL, with the low-dose treatment exhibiting the lowest concentration and the control being intermediate (Figure 2). In this case all the pairwise comparisons were significant (Table 2 below) due to very low within-treatment variance.

Fig 2. Levels (mg dL^{-1}) of free cholesterol ($\times 100$), HDL/LDL cholesterol and triglycerides in zebra fish liver after treatment with VY6. Results are represented as average \pm standard error of means. Different letters (a,b,c) above bars of each lipid category refer to significant

differences among this lipid's measured concentrations in response to injection with nil, low, and high doses of VY-6 ($P<0.01$).

Table 1. Values of regression coefficients (R^2), ranges tested for serial standards used for metabolites calculations, and intraspecific errors among duplicates of samples.

Lipids	Treatment groups	Intraspecific error	R^2	Linearity range
Triglycerides	Control	0.001	0.999	250-0.4 mg dL^{-1}
	Low dose	0.002		
	High dose	0.003		
Free cholesterol	Control	0.001	0.999	250-0.4 mg dL^{-1}
	Low dose	0.002		
	High dose	0.003		
HDL	Control	0.004	0.999	42-0.34 mg dL^{-1}
	Low dose	0.005		
	High dose	0.004		

Table 2. Pairwise Tukey's test values for the differences between experimental groups in average of lipid concentrations, below diagonals. P-values: above diagonals.

Lipids	Treatment groups	Control	Low dose	High dose
Triglycerides	Control	-	0.0002	0.007
	Low dose	17.22	-	0.001
	High dose	6.721	10.5	-
Free cholesterol	Control	-	0.078	0.185
	Low dose	3.839	-	0.008
	High dose	2.879	6.718	-
HDL	Control	-	0.012	0.012
	Low dose	6.078	-	0.0005
	High dose	6.078	12.16	-

C.3. RT-PCR of *acox1* and *lpl* mRNA

The intraperitoneal injection of zebra fish with the hexamer peptide induced a dose-dependent increase of the expression of *acox1* and *lpl* genes, responsible for the degradation of fatty acids and triglycerides, respectively (Figure 3).

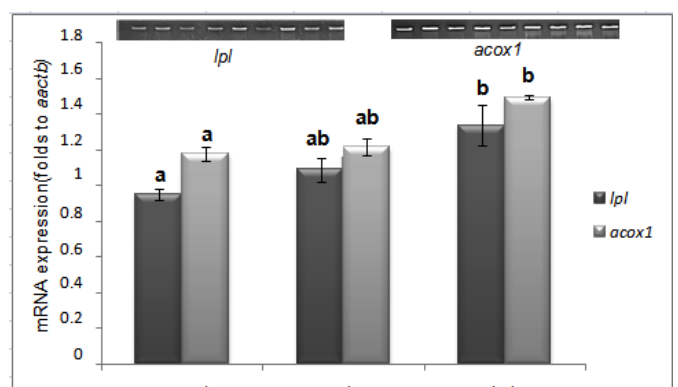


Fig 3. mRNA expression measured as mRNA levels of *lpl* and *acox1* transcripts in relation to *actb* as reference gene. Above: the RT-PCR patterns for *lpl* (left) and *acox1* (right) 3 control, 3 low dose, and 3 high dose samples. Results are represented as average \pm standard error (bars). Different letters (a,b) above bars of each gene refer to significant differences among this gene's measured expression in liver in response to injection with nil, low, and high doses of VY-6 ($P < 0.01$).

The effect of the treatment was statistically significant for *acox1* (one-way ANOVA with $F = 13.11$, $P = 0.002$), and marginally significant for *lpl* (one-way ANOVA with $F = 4.065$ and $P = 0.09$). The dose-dependent effect is reflected in the second case in a significant difference between the control and the group treated with the higher dose (Table 3).

Table 3. Pairwise Tukey's test values for the differences between experimental group means of liver mRNA level of *lpl* (above diagonal) and *acox1* (below diagonal) genes. Significant values are marked as ** ($p < 0.01$) and *** ($p < 0.001$).

	Control	Low dose	High dose
Control	-	2.173	4.159 **
Low dose	0.642	-	2.016
High dose	5.943***	4.258**	-

D. Discussion

Zebra fish is considered a good model for metabolic assays of different human foods, drinks, drugs and nutraceuticals in general. For instance, it was used to test the efficiency of human nutraceuticals-delivering emulsions³⁷, algal polyphenols against stress³⁸, developmental anomalies resulting from inhibition of some essential mitochondrial energy production cycles enzymes by human drugs³⁹, green tea as obesity inhibitor⁴⁰, plant-derived flavonoids as Taxifolin against oxidative DNA damage⁴¹, probiotics and their effects on the immune systems and also in reproduction^{42,43}, as well as many other, mostly human-related, nutraceuticals and drugs.

In the current trial, VY6 peptide enhanced the levels of HDL and reduced triglycerides efficiently. Enhancement also of mRNA levels of *lpl* and *acox1* enzymes in the zebra fish, our vertebrate experimental model, can refer to a positive role that VY6 plays as a nutraceutical, since these enzymes are active participants in fatty acids degradation. It is the first time to elucidate these physiological capabilities for the peptide VY6, what makes them indeed interesting and novel results. They strongly support the activity of VY6 peptide on lipid metabolism, and suggest this peptide may have an effect in preventing or combating obesity in vertebrates.

More in detail, *acox1* mediates both fatty acids and reactive oxygen species (ROS) metabolism in the peroxisomal β -oxidation process in liver and adipose tissues as the first

enzyme in this metabolic oxidation route^{3,4}. Up-regulation of *acox1* gene expression correlates with obesity-inhibitory treatments in different experimental models. In general, its enhancement is always concomitant to anti-lipid deposition effects in almost all levels of vertebrates, yet we were the first to demonstrate it in zebra fish in response to β -lg-derived peptide VY6. For examples, *acox1* enhanced upon feeding mice on high fat diet mixed with concentrate of n-3 polyunsaturated fatty acids from fish oil, what eventually led to obesity reduction⁴⁴. Moreover, *acox1* enhanced together with body fat reduction in obese zebra fish given green tea extract^{5,6}. Broiler chickens given basal diet mixed with dehydroepiandrosterone (DHEA) shown enhance *acox1* levels⁴⁵. Likewise, *lpl* is a potent controller for lipids, chiefly acting as rate-limiting enzyme for the hydrolysis of triglycerides and potent anti-hypertriglyceridemia⁷. Our results clearly demonstrated this anti-hypertriglyceridemic effect, with both concentrations tested of VY6 showing significant reduction in triglycerides. However, the more significant enhancement of *lpl* by the high dose than by the low dose may refer to VY6 capability to stimulate more *lpl* synthesis at the high dose. Adult liver in zebra fish is the organ where maximum *lpl* expression can be found, in comparison to other organs in the body⁴⁶. Its expression in livers further enhances upon feeding animals on high fat diets^{47,48}.

Alongside with the increase of expression of the lipid-degrading enzyme genes, the anti-obesity effect of the assayed peptide is clearly revealed by significantly lower levels of hepatic "bad" cholesterol and significantly higher levels of the "good cholesterol" HDL. However, it seems that the low dose of the VY6 showed better overall results for lipids levels than the higher dose. For the insignificance of both *acox1* and *lpl* increase in response to the low dose of VY6, it should be taken into account that enzymes' mRNA synthesis is often dependent upon the available quantity of the enzymes' proteins. This means that if there is a high level of enzyme protein quantity and activity, the need for mRNA transcription for production of more enzyme units diminishes. We believe that this is the explanation of our case since "bad" lipids levels were much lower in response to VY-6 low dose than the high dose. Both *lpl* and *acox1* activities are governed via posttranslational mechanisms and the discrepancy for their mRNAs/proteins levels is known^{49,50,51}.

The VY6 peptide could be used as a nutraceutical if its effects on lipid metabolism are confirmed with further experiments with some mammalian models and even possibly with volunteers. Now it is known that animal and plant proteins are active source of nutraceuticals, which is a role extending far beyond their traditional role in nutrition. Food-derived peptides exhibit antimicrobial properties, blood pressure-lowering effects, cholesterol-lowering ability, antithrombotic and antioxidant activities, enhancement of immunomodulatory effects, inhibit cancer cell growth, enhance mineral uptake, diabetes, obesity, obesity-induced inflammation and exhibit potent opioid activities^{52,53,54}. They can be effectively used then to reduce risk of disease or to enhance certain physiological functions.

Conclusions

The peptide VY6 derived from β -lg exhibited positive impacts on lipid deposition in the zebra fish liver. This, besides other previously demonstrated functions of that peptide, recommends VY6 as a promising, milk-derived nutraceutical that may exhibit positive effects against obesity and DPP-IV production.

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Overall, the work demonstrated that VY6, obtained by a hydrolysis and fractionation process, had enough purity to show bioactivity. It also proved that VY6 is effective against obesity, so a novel function is described herein for this peptide, already described as anti-hypertensive, anti-diabetic, antimicrobial and immunomodulatory (Madureira, Tavares, Gomes, Pintado, & Malcata, 2010; Pellegrini, Thomas, Bramaz, Hunziker, & von Fellenberg, 1999; Anne Pihlanto-Leppälä, Rokka, & Korhonen, 1998; Silveira, Martínez-Maqueda, Recio, & Hernández-Ledesma, 2013).

Concerning the lipid levels, triglycerides were significantly reduced by both doses compared to the control. However, for free cholesterol, only the low dose showed significant reduction. Conversely, for HDL only the high dose showed an increase. In addition, VY6 also increased the expression of the two genes involved in the degradation of fatty acids and triglycerides, although in this case the high dose was more effective.

Therefore, VY6 purified fraction was able to exert a positive effect on lipid metabolism at 2 levels (gene expression and lipid levels).

CONCLUSIONS

7. Conclusions:

- A thermal pre-treatment of 6 hours between 65 and 75°C increases the DH obtained in BSA trypsinolysis (0.3% BSA concentration, 1:50 E/S ratio) from roughly a 30% to a 50% DH, and the number of peptides released from 28 to 39. Besides, no peptides found in the untreated hydrolysate were lost in the thermally treated, and the relative amounts of the main part of them did increase. Higher temperatures do not significantly improve digestion, while lower temperatures show no difference with untreated digestions.

- Trypsin hydrolysis of BSA, and moreover with a thermal pre-treatment, releases peptides with potential to be ACE inhibitors, DPP-IV inhibitors and/or antioxidants, as it can be deduced from their primary sequence.

- When hydrolysing a WPC at 5% concentration with trypsin, a $44.22 \pm 1.62\%$ of the maximum possible DH was obtained, after 6 hours of digestion and using an E/S ratio of 1:200. The hydrolysis yielded a 16.7% of peptides with previously reported bioactivity in the literature.

- β -lg was much more hydrolysed than α -la when jointly digested in the WPC, since a 68.18% of the hydrolysate peptides derived from β -lg, and the chromatographic β -lg peak disappeared; while α -la chromatographic peak only experimented a 14% area reduction at the end of the hydrolysis and a 18.18% of the hydrolysate peptides derived from α -la.

- Filtration of the WPC tryptic hydrolysate with a 5 kDa NMWCO polyethersulfone membrane at pH 8 increased the percentage of peptides with reported bioactivity from a 16.7% in the WPH to a 50% in the membrane permeate. At pH8 membrane selectivity among peptide groups took the best values, and also peptides with reported bioactivity has greater Tr_{obs} values.

- BSA (at 0.3% concentration) pepsinolysis under the enzyme optimum conditions yielded a $65.54 \pm 1.57\%$ of the maximum possible DH after 3 hours of hydrolysis using an E/S ratio of the 4%, without any previous treatment; and a 23.25% of the obtained peptides were assigned probable bioactivity (bioactivity score $\geq 50\%$). Basic and neutral peptides had greater scores than acid peptides. One of the identified peptides was also reported in the literature as bioactive (LLL).

- Zebra fish was proven as a suitable novel animal model for studying the effects of bioactive peptides over lipid metabolism.

- VY6 [β -lg f (15-20)] significantly reduced in zebra fish triglycerides and cholesterol levels, and increased HDL levels. It also significantly increased the expression of the two genes involved in the degradation of fatty acids and triglycerides, although results were dose-dependent. Therefore, VY6 is reported as a novel anti-obesity peptide.

- Membrane filtration of BSA tryptic hydrolysates using both polyethersulfone and cellulosic membranes yields fractions with relevant *in vitro* bioactivity regarding antioxidation and anti-hypertension.

8. Conclusiones

- Un pre-tratamiento térmico de 6 horas a una temperatura en el rango de 65 a 75 °C aumenta el grado de hidrólisis obtenido de alrededor del 30% a un 50% en la digestión de BSA con tripsina, a una concentración de sustrato del 0.3% y usando un ratio E/S de 1:50. También aumenta el número de péptidos obtenidos de 38 a 39, y la cantidad relativa de la mayoría. Mientras que temperaturas por debajo de 65 °C no muestran diferencias con la ausencia de pre-tratamiento, temperaturas por encima de 75 °C tampoco mejoran el resultado obtenido.

- Tanto la hidrólisis nativa de BSA con tripsina, como añadiendo un pre-tratamiento térmico, libera péptidos con potencial de actividad anti-hipertensiva, anti-diabética y antioxidante, como puede ser deducido por su secuencia amino-acídica.

- La hidrólisis con tripsina de un WPC 80 al 5% de concentración libera un hidrolizado con un contenido en péptidos bioactivos del 16.7%. El grado de hidrólisis alcanzado es un $44.22 \pm 1.62\%$ del máximo posible tras 6 horas de digestión usando un ratio E/S de 1:200.

- Cuando la β -lg y la α -la están juntas en el WPC, la tripsina hidroliza preferentemente a la primera, ya que el pico cromatográfico de la β -lg era prácticamente inexistente al final de la hidrólisis, mientras que el de la α -la sólo había experimentado una reducción del 14%.

- La filtración con membranas planas de polietersulfona y 5 kDa de tamaño de poro de un hidrolizado de WPC con tripsina a pH 8 aumentó el porcentaje de péptidos bioactivos, de un 16.7% a un 50%.

- La digestión con pepsina de BSA a una concentración del 0.3% y en las condiciones óptimas de la enzima alcanzó un grado de hidrólisis del $65.54 \pm 1.57\%$ respecto del máximo posible, tras 3 horas de digestión. Un 23.25% de los péptidos del hidrolizado poseían probabilidad de ser bioactivos (puntuación > 50%) según el software PeptideRanker, y uno de los péptidos poseía actividad confirmada (LLL). Los péptidos básicos y neutros presentaron mejores puntuaciones que los ácidos.

- El pez cebra se ha revelado como un modelo animal adecuado para estudiar el efecto de péptidos bioactivos sobre el metabolismo lipídico.

- El péptido VY6 [β -lg f (15-20)] redujo los niveles de triglicéridos y colesterol, a la vez que aumentó los niveles de HDL en el pez cebra. También aumentó la expresión de 2 genes implicados en la degradación de ácidos grasos y triglicéridos. Éste efecto de éste péptido es descrito por primera vez en éste trabajo.

- La filtración con membranas de polietersulfona y celulosa de 5 kDa de tamaño de poro permite obtener, a partir de hidrolizados de BSA con tripsina, fracciones con actividad antioxidante y anti-hipertensiva *in vitro*.

PERSPECTIVES

9. Perspectives

Based on all the information reviewed and the work developed, the areas that would experience advances in the near future are predicted to be mainly three:

- **Bioinformatics:** there is a dire need to validate the performance of the current software programs that model BP production or test bioactivity. How the algorithms could be more precise? What will be the deviation accepted from the experimental results? Will the reliability be so that the experimental confirmation would become unnecessary? One possible route could be to profit from the works already stored in databases, as BIOPEP. A derived database could be created in which all the *in vitro* or *in vivo* works or clinical trials would be *in silico* replicated, and then the degree of correlation evaluated.

- **Applicability:** Published work about laboratory processes scale-up is still very scarce. There is the need to confirm that laboratory trials with intended industrial applicability are indeed feasible at large scale. There is also the need to report data such as yields, and perform cost-benefit analyses. An increase in the collaboration between the university and the industry, for example pharmaceutical industry or food industry, could be a way to profit from both the knowledge on the companies knowledge on production processes, and the universities proficiency in research.

- **Clinical trials:** There is the need to pass to that last phase. *In vitro* reported peptides bioactivity has been demonstrated to not always correspond to *in vivo* behaviour. Also, animal models have its limitations in replicating human responses. Biological, physio-chemical and engineering research groups that produce and study BP could establish more collaboration with medical research groups, hospitals or pharmaceutical companies, which could perform those clinical trials.

10. Bibliography

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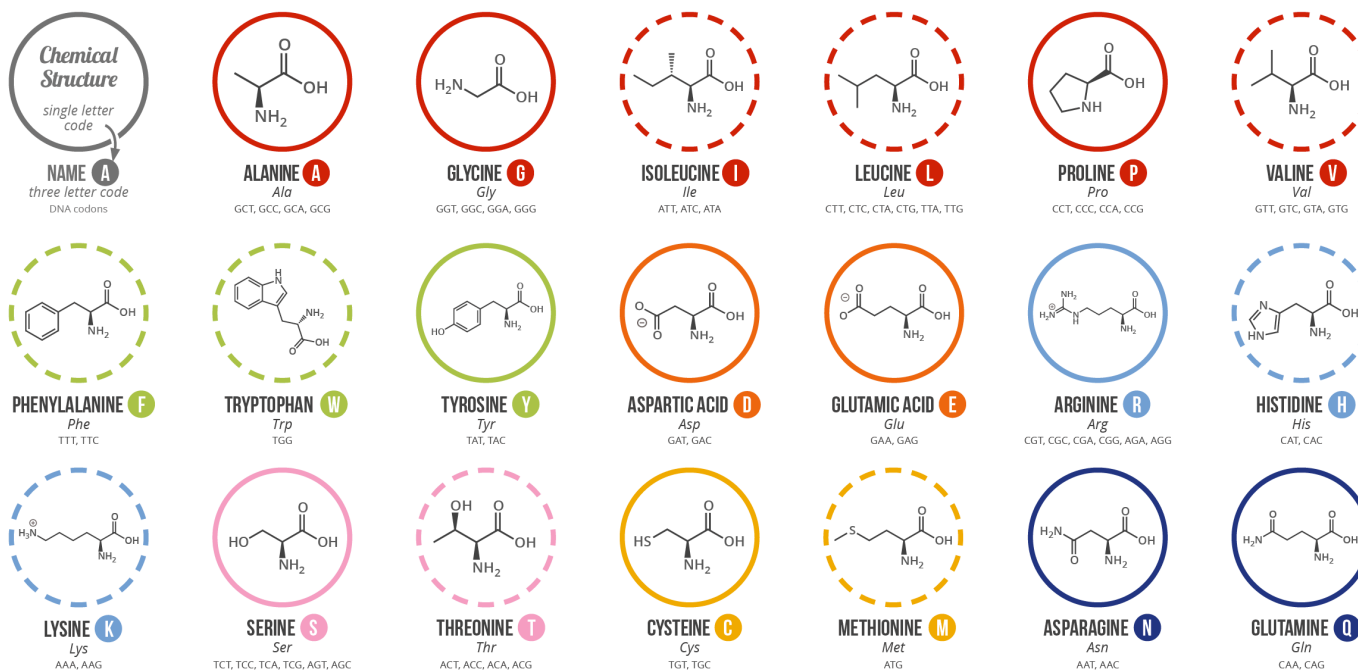
11. Annexes

11.1. Annex I: Structure and characteristics of the 20 natural amino acids (aa).

A GUIDE TO THE TWENTY COMMON AMINO ACIDS

AMINO ACIDS ARE THE BUILDING BLOCKS OF PROTEINS IN LIVING ORGANISMS. THERE ARE OVER 500 AMINO ACIDS FOUND IN NATURE - HOWEVER, THE HUMAN GENETIC CODE ONLY DIRECTLY ENCODES 20. 'ESSENTIAL' AMINO ACIDS MUST BE OBTAINED FROM THE DIET, WHILST NON-ESSENTIAL AMINO ACIDS CAN BE SYNTHESISED IN THE BODY.

Chart Key: ● ALIPHATIC ● AROMATIC ● ACIDIC ● BASIC ● HYDROXYLIC ● SULFUR-CONTAINING ● AMIDIC ○ NON-ESSENTIAL ○ ESSENTIAL



Note: This chart only shows those amino acids for which the human genetic code directly codes for. Selenocysteine is often referred to as the 21st amino acid, but is encoded in a special manner. In some cases, distinguishing between asparagine/aspartic acid and glutamine/glutamic acid is difficult. In these cases, the codes asx (B) and glx (Z) are respectively used.

11.2. Annex II: Guidelines to calculate maximum possible degree of hydrolysis ($DH_{\max the}$).

- System BSA+trypsin

BSA characteristics:

- Number of aa: 583
- MW: 66463 Da
- Number of peptide bonds: 582
- Number of peptide bonds susceptible of cleavage by trypsin: 82*

* Calculated with PeptideCutter

$$DH_{\max the} = (82/582) * 100 = 14.09\%$$

- System WPC+trypsin

Since WPC is composed of several proteins, each of which are present in a different percentage, it is necessary to first calculate the number of peptide bonds cleaved by trypsin for each of the proteins, and then calculate an average considering the percentage in which they are present in the WPC:

Proteins percentages in the WPC:

β-Ig: 60%

α-la: 20%

IgG: 10%

BSA: 8%

LF: 1%

Number of aa:

β -lg: 161

α -la: 141

IgG: 469

BSA: 582

LF: 707

Number of peptide bonds susceptible of cleavage by trypsin*:

β -lg: 18

α -la: 13

IgG: 37 (IgG heavy chain precursor)

BSA: 82

LF: 92

* Calculated with PeptideCutter

$$DH_{\max \text{ the}} = \left[\frac{(18/161) \cdot 0,6 + (13/141) \cdot 0,2 + (37/469) \cdot 0,1 + (82/582) \cdot 0,08 + (92/707) \cdot 0,01}{(0,6+0,2+0,1+0,08+0,01)} \right] \cdot 100 = 10.70\%$$

- System BSA+pepsin

BSA characteristics:

➤ Number of peptide bonds susceptible of cleavage by pepsin: 147*

* Calculated with PeptideCutter

$$DH_{\max \text{ the}} = (147 / 582) \cdot 100 = 25.26\%$$

11.3. Annex III: PeptideCutter theoretical prediction of tryptic bovine serum albumin digestion.

PeptideCutter

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PeptideCutter

The sequence to investigate:

```

10      20      30      40      50      60
DTHKSEIAHR FKDLGEEHFK GLVLIAFSQY LQQCPFDEHV KLVNELTEFA KTCVADESHA

70      80      90      100     110     120
GCEKSLHTLF GDELCKVASL RETYGMADC CEKQEPERNE CFLSHKDDSP DLPKLPDPN

130     140     150     160     170     180
TLCDEFKADE KFWGKLYE IARRHPYFYA PELLYYANKY NGVFQECQA EDKGACLLPK

190     200     210     220     230     240
IETMREKVL TSSARQLRCA SIQKFGERAL KAWSVARLSQ KFPKAEFVEV TKLVTDLTKV

250     260     270     280     290     300
HKECCHGDL ECADDRADLA KYICDNQDTI SSKLKECCDK PLEKSHCIA EVEKDAIPEN

310     320     330     340     350     360
LPPLTADFAE DKDVCKNYQE AKDAFLGSFL YEYSRRHPEY AVSVLLRLAK EYEATLEECC

370     380     390     400     410     420
AKDDPHACYS TVFDKCLKLV DEPQNLIKQN CDQFEKLG EY GFQNALIVRY TRKVPQVSTP

430     440     450     460     470     480
TLVEVSRSLG KVGTRCCTKP ESERPCTED YLSLILNRLC VLHEKTPVSE KVTKCCTESL

490     500     510     520     530     540
VNRRCFSAL TPDETYVPKA FDEKLFTFHA DICTLPDTEK QIKKQTALVE LLKHKPKATE

550     560     570     580
EQLKTMENF VAFVDKCAA DDKEACFAVE GPKLVVSTQT ALA

```

The sequence is 583 amino acids long.

Available enzymes

The enzyme(s) that you have chosen:

- Trypsin

You have chosen to display all possible cleaving enzymes.

At these positions the following enzymes cleave:

- Please note that the size of the peptides are calculated as if **all chosen enzymes were present** during digestion. If you want to obtain the size of the peptides resulting from the cleavage of only one enzyme, please, deselect the others.
- Please be aware of the fact that the present version of the PeptideCutter program does not take into consideration any kind of **modification** neither of the protein sequence nor of modifications evoked by the cleavage. Mass computations are based on [average masses](#) of the occurring amino acid residues, and giving peptide masses as [M]. If you want to select different parameters, we recommend to use [PeptideMass](#).

Position of cleavage site	Name of cleaving enzyme(s)	Resulting peptide sequence (see explanations)	Peptide length [aa]	Peptide mass [Da]	Cleavage probability
4	Trypsin	DTHK	4	499.524	100 %
10	Trypsin	SEIAHR	6	711.776	100 %
12	Trypsin	FK	2	293.366	87.9 %
20	Trypsin	DLGEEHFK	8	974.038	100 %

41	Trypsin	GLVLIAFSQYLQQCFDEHVK	21	2435.823	100 %
51	Trypsin	LVNELTEFAK	10	1163.336	93.1 %
64	Trypsin	TCVADESHAGCEK	13	1349.453	100 %
76	Trypsin	SLHTLFGDELCK	12	1362.563	100 %
81	Trypsin	VASLR	5	544.652	100 %
93	Trypsin	ETYGDMADCCEK	12	1364.480	100 %
98	Trypsin	QEPER	5	657.681	100 %
106	Trypsin	NECFLSHK	8	977.103	100 %
114	Trypsin	DDSPDLPK	8	885.926	100 %
116	Trypsin	LK	2	259.349	49.7 %
127	Trypsin	PDPNTLCDEFK	11	1278.399	86.4 %
131	Trypsin	ADEK	4	461.472	87.3 %
132	Trypsin	K	1	146.189	100 %
136	Trypsin	FWGK	4	536.631	100 %
143	Trypsin	YLYEIAR	7	927.068	90.4 %
144	Trypsin	R	1	174.203	10.5 %
159	Trypsin	HPYFYAPPELLYYANK	15	1889.140	100 %
173	Trypsin	YNGVVFQECQAEDK	14	1633.768	89.7 %
180	Trypsin	GACLLPK	7	700.894	100 %
185	Trypsin	IETMR	5	648.775	100 %
187	Trypsin	EK	2	275.305	95.7 %
194	Trypsin	VLTS SAR	7	732.835	100 %
196	Trypsin	QR	2	302.333	100 %
198	Trypsin	LR	2	287.362	100 %
204	Trypsin	CASIQK	6	648.775	100 %
208	Trypsin	FGER	4	507.547	90.7 %
211	Trypsin	ALK	3	330.428	100 %
217	Trypsin	AWSVAR	6	688.784	100 %
221	Trypsin	LSQK	4	474.558	100 %
224	Trypsin	FPK	3	390.483	94.1 %
232	Trypsin	AEFVEVTK	8	922.046	100 %
239	Trypsin	LVTDLTK	7	788.940	100 %
242	Trypsin	VHK	3	382.463	63.9 %
256	Trypsin	ECCHGDLLECADDR	14	1578.707	100 %
261	Trypsin	ADLAK	5	516.595	100 %
273	Trypsin	YICDNQDTISSK	12	1386.496	100 %
275	Trypsin	LK	2	259.349	92.8 %
280	Trypsin	ECCDK	5	596.671	29.3 %
285	Trypsin	PLLEK	5	598.740	100 %
294	Trypsin	SHCIAEVEK	9	1015.149	83.2 %
312	Trypsin	DAIPENLPPLTADFAEDK	18	1956.136	37.7 %
316	Trypsin	DVCK	4	463.549	100 %
322	Trypsin	NYQEAK	6	751.794	90.6 %
335	Trypsin	DAFLGSFLYEYSR	13	1567.718	85.5 %
336	Trypsin	R	1	174.203	10.5 %
347	Trypsin	HPEYAVSVLLR	11	1283.493	100 %

350	Trypsin	LAK	3	330.428	82 %
362	Trypsin	EYEATLEECCA	12	1388.527	90.6 %
375	Trypsin	DDPHACYSTVFDK	13	1497.599	76.8 %
377	Trypsin	LK	2	259.349	100 %
388	Trypsin	HLVDEPQNLIK	11	1305.497	100 %
396	Trypsin	QNCDQFEK	8	1011.074	94.7 %
409	Trypsin	LGEYGFQNALIVR	13	1479.699	100 %
412	Trypsin	YTR	3	438.484	82.3 %
413	Trypsin	K	1	146.189	84.5 %
427	Trypsin	VPQVSTPTLVEVSR	14	1511.739	100 %
431	Trypsin	SLGK	4	403.479	94.2 %
435	Trypsin	VGTR	4	431.492	100 %
439	Trypsin	CCTK	4	453.572	19.4 %
444	Trypsin	PESER	5	616.629	100 %
458	Trypsin	MPCTEDYLSLILNR	14	1667.956	100 %
465	Trypsin	LCVLHEK	7	841.036	100 %
471	Trypsin	TPVSEK	6	659.737	95.7 %
474	Trypsin	VTK	3	346.427	100 %
483	Trypsin	CCTESLVNR	9	1024.175	79.2 %
484	Trypsin	R	1	174.203	30.4 %
499	Trypsin	PCFSALTPDETYVPK	15	1667.894	94.1 %
504	Trypsin	AFDEK	5	608.649	94.7 %
520	Trypsin	LFTFHADICTLPDTEK	16	1851.104	100 %
523	Trypsin	QIK	3	387.479	85.5 %
524	Trypsin	K	1	146.189	75.5 %
533	Trypsin	QTALVELLK	9	1014.230	100 %
535	Trypsin	HK	2	283.330	52.4 %
537	Trypsin	PK	2	243.306	94.1 %
544	Trypsin	ATEEQLK	7	817.894	100 %
556	Trypsin	TVMENFVAFVDK	12	1399.625	85.9 %
563	Trypsin	CCAADDK	7	724.802	83.8 %
573	Trypsin	EACFAVEGPK	10	1050.195	100 %
583	end of sequence	LVVSTQTALA	10	1002.176	-

These are the cleavage sites of the chosen enzymes and chemicals mapped onto the entered protein sequence:

- You have chosen a block size of **60** for the map.
- Please note that the cleavage occurs at the **right side** (C-terminal direction) of the marked amino acid.
- You have the possibility to display the results of a single enzyme by **mouseclicking** on the respective enzyme name in the map.

```

                Tryps_(100%)
      Tryps_(87.9%) |
    Tryps_(100%) | |
Tryps_(100%) | | |
                Tryps_(93.1%)
    Tryps_(100%) |

```


11.4. Annex IV: FindPept identification of a set of peptide masses obtained by mass spectrometry analysis of a pepsin bovine serum albumin hydrolysate.

Interactive FindPept analysis

2/12/15 11:05

FindPept

[Home](#) | [Contact](#)

FindPept tool

The entered sequence is:

```

10      20      30      40      50      60
DTHKSEIAHR FKDLGEEHFK GLVLIAPFSQY LQQCPFDEHV KLVNELTEFA KTCVADESHA

70      80      90      100     110     120
GCEKSLHTLF GDELCKVASL RETYGMADC CEKQEPERNE CFLSHKDDSP DLPKLPDPN

130     140     150     160     170     180
TLCDEFKADE KKFVWGKLYE IARRHPYFYA PELLYYANKY NGVFQECQQA EDKGACLLPK

190     200     210     220     230     240
IETMREKVLTT SSARQLRCA SIQKFGERAL KAWSVARLSQ KFPKAEFVEV TKLVTDLTKV

250     260     270     280     290     300
HKECCHGDLLE CADDRADLA KYICDNQDTI SSKLKECCDK PLEKSHCIA EVEKDAIPEN

310     320     330     340     350     360
LPPLTADFAE DKDVCKNYQE AKDAFLGSFL YEYSRRHPEY AVSVLLRLAK EYEATLEBCC

370     380     390     400     410     420
AKDDPHACYS TVFDKLLHLV DEPQNLKQN CDQFEKLG EY GFQNALIVRY TRKVPQVSTP

430     440     450     460     470     480
TLVEVSRSLG KVGTRCCTKP ESERPCTED YLSLILNRLC VLHEKTPVSE KVTKCTESL

490     500     510     520     530     540
VNRPPCFSALE TPDETYVPKA FDEKLFTHA DICTLPDTEK QIKKQATLVE LKHKPKATE

550     560     570     580
EQLKTVMENF VAFVDKCAA DDKEACFAVE GPKLVVSTQT ALA

```

583 Amino Acids.

Entered peptide masses: 533.200 483.200 397.200 362.200 360.300 317.200 705.600 847.700 504.500 1015.900 1057.900 1107.700 802.200 496.600 697.400 1127.100 884.700 1547.500 514.200 564.400 1894.600 1122.900 713.600 1325.700 596.300 2012.900 2465.000 940.800 2984.500 1400.500 1082.000 1549.500 1060.100 1081.900 825.800 2727.900 2481.900 1076.700 2231.800 414.200 731.600 3623.400

Tolerance: ± 0.5 daltons

Using **monoisotopic** masses of the occurring amino acid residues and interpreting your peptide masses as **[M+H]⁺**.

Enzyme: **Pepsin (pH > 2)** ([P00791](#)).

Cysteine in reduced form.

[New FindPept Search](#) [PeptideMass](#) [FindMod](#) [GlycoMod](#)



[FindPept documentation](#)
[Mass values and considered PTMs](#)

Peptides resulting from the cleavage of contaminants:

User mass	DB mass	Δ mass (daltons)	type	contaminant	peptide	position	modifications	missed cleavages
317.200	317.182	-0.018	Keratin	P04264 Keratin, human (KRT1)	(E)/NAL/(K)	435-437		1
317.200	317.218	0.018	Keratin	P04264 Keratin, human (KRT1)	(E)/VKA/(Q)	354-356		0
				P04264				

360.300	360.213	-0.087	Keratin	Keratin, human (KRT1)	(A)/LDL/(E)	475-477	1
				P04264			
397.200	397.208	0.008	Keratin	Keratin, human (KRT1)	(Q)/SKY/(E)	375-377	0
				P04264			
504.500	504.266	-0.233	Keratin	Keratin, human (KRT1)	(A)/KEDL/(A)	455-458	1
				P04264			
596.300	596.293	-0.007	Keratin	Keratin, human (KRT1)	(L)/EIATY/(R)	478-482	2
				P04264			
1015.900	1015.542	-0.358	Keratin	Keratin, human (KRT1)	(A)/LQQAKEDLA/(R)	451-459	6
				P04264			
1015.900	1015.578	-0.321	Keratin	Keratin, human (KRT1)	(E)/VTINQSLQ/(P)	155-163	3
				P04264			
1057.900	1057.564	-0.336	Keratin	Keratin, human (KRT1)	(L)/QAKLDNLQ/(E)	302-310	5
				P04264			
1060.100	1060.538	0.438	Keratin	Keratin, human (KRT1)	(E)/DEINKRTNA/(E)	272-280	1
				P04264			
1060.100	1060.563	0.463	Keratin	Keratin, human (KRT1)	(E)/DIAQKSKAEA/(E)	360-369	4
				P04264			
1081.900	1081.552	-0.347	Keratin	Keratin, human (KRT1)	(A)/TYRTLLEGE/(E)	481-489	4
				P04264			
1082.000	1081.552	-0.447	Keratin	Keratin, human (KRT1)	(A)/TYRTLLEGE/(E)	481-489	4
				P04264			
1107.700	1107.517	-0.182	Keratin	Keratin, human (KRT1)	(E)/LKNMQDMVE/(D)	256-264	2
				P04264			
1400.500	1400.676	0.176	Keratin	Keratin, human (KRT1)	(Q)/QVDTSTRTHNLE/(P)	217-228	2
				P04264			
1400.500	1400.738	0.237	Keratin	Keratin, human (KRT1)	(A)/KNKLNLEDALQ/(Q)	441-452	5
				P04264			
1400.500	1400.772	0.271	Keratin	Keratin, human (KRT1)	(L)/RRRVDQLKSDQ/(S)	239-249	2

1400.500	1400.774	0.274	Keratin	P04264 Keratin, human (KRT1)	(A)/LKDAKNKLNLDLE/(D)	437-448	4
1400.500	1400.797	0.296	Keratin	P04264 Keratin, human (KRT1)	(E)/IQKVKSREREQ/(I)	173-183	3
1549.500	1549.847	0.347	Keratin	P04264 Keratin, human (KRT1)	(A)/LDLEIATYRTLLE/(G)	475-487	7
2012.900	2013.040	0.139	Keratin	P04264 Keratin, human (KRT1)	(L)/EQQNQVLQTKWELLQQ/(V)	202-217	11
2012.900	2013.061	0.161	Keratin	P04264 Keratin, human (KRT1)	(E)/NALKDAKNKLNLDLEDALQ/(Q)	435-452	8
3623.400	3622.962	-0.437	Keratin	P04264 Keratin, human (KRT1)	(E)/LNRVIQRLRSEIDNVKKQIS NLQQSISDAEQ/(R)	401-431	10
317.200	317.218	0.018	Keratin	P35908 Keratin, human (KRT2A)	(E)/VKA/(Q)	352-354	0
362.200	362.156	-0.044	Keratin	P35908 Keratin, human (KRT2A)	(L)/DVE/(I)	474-476	0
397.200	397.219	0.019	Keratin	P35908 Keratin, human (KRT2A)	(Q)/IHQ/(S)	324-326	0
504.500	504.266	-0.233	Keratin	P35908 Keratin, human (KRT2A)	(A)/KEDL/(A)	453-456	1
504.500	504.282	-0.218	Keratin	P35908 Keratin, human (KRT2A)	(E)/PIFQ/(G)	227-230	1
697.400	697.330	-0.069	Keratin	P35908 Keratin, human (KRT2A)	(L)/YHSKY/(E)	371-375	1
697.400	697.338	-0.062	Keratin	P35908 Keratin, human (KRT2A)	(E)/QRGEHA/(L)	429-434	2
802.200	802.467	0.266	Keratin	P35908 Keratin, human (KRT2A)	(E)/LQSKVDL/(L)	299-305	2
802.200	802.467	0.266	Keratin	P35908 Keratin, human (KRT2A)	(L)/QSKVDLL/(N)	300-306	2

825.800	825.362	-0.437	Keratin	P35908 Keratin, human (KRT2A)	(L)/YDAEISQ/(I)	317-323	3
847.700	847.398	-0.302	Keratin	P35908 Keratin, human (KRT2A)	(E)/LNNMQDL/(V)	254-260	2
847.700	847.427	-0.273	Keratin	P35908 Keratin, human (KRT2A)	(A)/QRSKEEA/(E)	361-367	3
1015.900	1015.542	-0.358	Keratin	P35908 Keratin, human (KRT2A)	(A)/LQQAKEDLA/(R)	449-457	6
1015.900	1015.542	-0.358	Keratin	P35908 Keratin, human (KRT2A)	(E)/ALQQAKEDL/(A)	448-456	6
1081.900	1081.531	-0.368	Keratin	P35908 Keratin, human (KRT2A)	(E)/AEALYHSKY/(E)	367-375	5
1082.000	1081.531	-0.468	Keratin	P35908 Keratin, human (KRT2A)	(E)/AEALYHSKY/(E)	367-375	5
1122.900	1122.502	-0.397	Keratin	P35908 Keratin, human (KRT2A)	(L)/TAERTSQNSE/(L)	244-253	3
1325.700	1325.765	0.064	Keratin	P35908 Keratin, human (KRT2A)	(E)/LNRVIQRLQGE/(I)	399-409	4
1547.500	1547.832	0.331	Keratin	P35908 Keratin, human (KRT2A)	(Q)/SKVDLLNQEIEFL/(K)	301-313	6
2012.900	2013.040	0.139	Keratin	P35908 Keratin, human (KRT2A)	(L)/EQQNQVLQTKWELLQQ/(M)	200-215	11
2984.500	2984.499	0.000	Keratin	P35908 Keratin, human (KRT2A)	(Q)/IHQSVTDTNVILSMDNSRNL DLDSIIA/(E)	324-350	4
3623.400	3623.690	0.290	Keratin	P35908 Keratin, human (KRT2A)	(F)/RGFSSGSAVVS GGSRSTSS FSCLSRHGGGGGGFGGGGF/(G)	20-58	5
362.200	362.167	-0.032	Keratin	P35527 Keratin, human (KRT9)	(L)/QSQ/(L)	385-387	1
362.200	362.192	-0.007	Keratin	P35527 Keratin, human	(Y)/ETL/(Q)	279-281	1

					(KRT9)			
483.200	483.187	-0.012	Keratin	P35527 Keratin, human (KRT9)	(Q)/DWY/(D)	187-189	1	
496.600	496.288	-0.312	Keratin	P35527 Keratin, human (KRT9)	(Y)/HNLL/(E)	455-458	1	
504.500	504.314	-0.186	Keratin	P35527 Keratin, human (KRT9)	(L)/SKKAA/(L)	389-393	1	
564.400	564.262	-0.137	Keratin	P35527 Keratin, human (KRT9)	(E)/VSSSGQ/(E)	356-361	0	
596.300	596.326	0.026	Keratin	P35527 Keratin, human (KRT9)	(L)/RHGVQ/(E)	374-378	0	
705.600	705.349	-0.251	Keratin	P35527 Keratin, human (KRT9)	(Q)/EELMAL/(K)	283-288	4	
713.600	713.274	-0.326	Keratin	P35527 Keratin, human (KRT9)	(F)/GGESGGSY/(G)	593-600	1	
731.600	731.405	-0.195	Keratin	P35527 Keratin, human (KRT9)	(Q)/ELNSRL/(A)	159-164	2	
731.600	731.405	-0.195	Keratin	P35527 Keratin, human (KRT9)	(Q)/ITDVRQ/(E)	423-428	0	
802.200	802.430	0.230	Keratin	P35527 Keratin, human (KRT9)	(L)/DKVQALE/(E)	169-175	3	
802.200	802.442	0.241	Keratin	P35527 Keratin, human (KRT9)	(Q)/ELNSRLA/(S)	159-165	3	
940.800	940.489	-0.311	Keratin	P35527 Keratin, human (KRT9)	(L)/DDFRIKF/(E)	237-243	1	
1060.100	1060.490	0.390	Keratin	P35527 Keratin, human (KRT9)	(A)/GGDGGILTANE/(K)	142-153	2	
1076.700	1076.558	-0.141	Keratin	P35527 Keratin, human (KRT9)	(E)/VQSSAKEVTQ/(L)	363-372	3	
				P35527 Keratin,				

1107.700	1107.604	-0.095	Keratin	human (KRT9)	(L)/ASYLDKVVQAL/(E)	165-174	5
1325.700	1325.549	-0.150	Keratin	P35527 Keratin, human (KRT9)	(L)/LEGGQEDFESSGA/(G)	458-470	6
1325.700	1325.579	-0.120	Keratin	P35527 Keratin, human (KRT9)	(L)/NDMRQEYEQL/(I)	324-333	5
1325.700	1325.627	-0.073	Keratin	P35527 Keratin, human (KRT9)	(E)/DTKNRYCGQLQ/(M)	400-410	3
1400.500	1400.655	0.155	Keratin	P35527 Keratin, human (KRT9)	(F)/SASSLGGGFGGGSRGF/(G)	82-97	3
1549.500	1549.764	0.263	Keratin	P35527 Keratin, human (KRT9)	(A)/NEKSTMQELNSRL/(A)	152-164	4
2727.900	2728.382	0.482	Keratin	P35527 Keratin, human (KRT9)	(Q)/LQMIQEQISNLEAQITDVRQ EIE/(C)	409-431	11
3623.400	3623.851	0.451	Keratin	P35527 Keratin, human (KRT9)	(E)/VSSSGQEVQSSAKEVTQLRH GVQELEIELQSQL/(S)	356-388	15
362.200	362.167	-0.032	Keratin	P13645 Keratin, human (KRT10)	(L)/QSQ/(L)	380-382	1
362.200	362.192	-0.007	Keratin	P13645 Keratin, human (KRT10)	(E)/ITE/(L)	365-367	0
397.200	397.208	0.008	Keratin	P13645 Keratin, human (KRT10)	(Y)/SKY/(Y)	197-199	0
533.200	533.329	0.129	Keratin	P13645 Keratin, human (KRT10)	(L)/TLTKA/(D)	263-267	1
731.600	731.379	-0.220	Keratin	P13645 Keratin, human (KRT10)	(E)/QNRKDA/(E)	331-336	1
731.600	731.393	-0.206	Keratin	P13645 Keratin, human (KRT10)	(Q)/AQISALE/(E)	409-415	4
731.600	731.405	-0.195	Keratin	P13645 Keratin, human (KRT10)	(A)/LRQSVE/(A)	244-249	2

802.200	802.442	0.241	Keratin	Keratin, human (KRT10)	(A)/LRQSVEA/(D)	244-250	3
				P13645			
825.800	825.374	-0.426	Keratin	Keratin, human (KRT10)	(L)/AETEGRY/(C)	394-400	3
				P13645			
847.700	847.452	-0.248	Keratin	Keratin, human (KRT10)	(F)/NEKSKEL/(T)	341-347	2
				P13645			
1060.100	1059.604	-0.495	Keratin	Keratin, human (KRT10)	(L)/ALKQSLEASL/(A)	384-393	6
				P13645			
1060.100	1059.604	-0.495	Keratin	Keratin, human (KRT10)	(A)/LKQSLEASLA/(E)	385-394	6
				P13645			
1060.100	1060.502	0.401	Keratin	Keratin, human (KRT10)	(L)/AEQNRKDAE/(A)	329-337	4
				P13645			
1060.100	1060.502	0.401	Keratin	Keratin, human (KRT10)	(A)/EQNRKDAEA/(W)	330-338	4
				P13645			
1060.100	1060.542	0.442	Keratin	Keratin, human (KRT10)	(A)/YLKKNHEE/(E)	282-289	3
				P13645			
1076.700	1076.585	-0.115	Keratin	Keratin, human (KRT10)	(A)/RLAADDFFRL/(K)	228-236	4
				P13645			
1122.900	1122.492	-0.407	Keratin	Keratin, human (KRT10)	(Y)/EKHGNSHQGE/(P)	183-192	2
				P13645			
1122.900	1122.615	-0.284	Keratin	Keratin, human (KRT10)	(E)/IQTYRSLLE/(G)	446-454	4
				P13645			
1127.100	1126.669	-0.430	Keratin	Keratin, human (KRT10)	(E)/ADINGLRRVL/(D)	250-259	2
				P13645			
1325.700	1325.658	-0.041	Keratin	Keratin, human (KRT10)	(W)/FNEKSKELTTE/(I)	340-350	4
				P13645			
1400.500	1400.684	0.183	Keratin	Keratin, human (KRT10)	(L)/KKNHEEEMKDL/(R)	284-294	3
				P13645			
1549.500	1549.691	0.191	Keratin	Keratin, human (KRT10)	(L)/QQIRAETECQNTTE/(Y)	419-431	6

1549.500	1549.730	0.230	Keratin	P13645 Keratin, human (KRT10)	(A)/DLEMQIESLTEEL/(A)	268-280	7
2012.900	2013.097	0.197	Keratin	P13645 Keratin, human (KRT10)	(Q)/SVEADINGLRRVLDELTL/(T)	247-264	6
2727.900	2728.253	0.353	Keratin	P13645 Keratin, human (KRT10)	(L)/NNMRSQYEQLAEQNRKDAEA WF/(N)	319-340	12
2727.900	2728.346	0.446	Keratin	P13645 Keratin, human (KRT10)	(L)/DKVRALEESNYELEGKIKEW YE/(K)	162-183	11
2984.500	2984.323	-0.176	Keratin	P13645 Keratin, human (KRT10)	(Y)/GGLGGFGGGSFRGSYGSSSF GGSYGGIFGGGSF/(G)	75-107	7

Peptides resulting from protease autolysis:

PEPA_PIG (P00791)

Pepsin A precursor (EC 3.4.23.1)

Sus scrofa (Pig).

User mass	DB mass	Δ mass (daltons)	peptide	position	modifications	missed cleavages
397.200	397.208	0.008	(A)/SKY/(F)	50-52		0
483.200	483.245	0.044	(L)/SITY/(G)	131-134		0
533.200	533.257	0.056	(L)/TGPTSA/(I)	281-286		0
564.400	564.303	-0.097	(W)/VPSVY/(C)	99-103		0
596.300	596.329	0.028	(E)/LSITY/(G)	130-134		1
731.600	731.393	-0.206	(Q)/GLVSQDL/(F)	203-209		2
1057.900	1057.520	-0.379	(L)/VSQDLFSVY/(L)	205-213		3
1057.900	1057.575	-0.325	(F)/DRANNKVGLA/(P)	373-382		2
1076.700	1076.493	-0.206	(E)/TEPGSFLYY/(A)	165-173		4
1082.000	1082.427	0.427	(F)/NPDDSSSTFEA/(T)	116-125		2

Matching peptides for specific cleavage:

User mass	DB mass	Δ mass (daltons)	peptide	position	modifications	missed cleavages
317.200	317.182	-0.018	(Q)/NAL/(I)	404-406		1
317.200	317.218	0.018	(F)/KGL/(V)	20-22		0
360.300	360.213	-0.087	(L)/VEL/(L)	529-531		1
360.300	360.213	-0.087	(A)/LVE/(L)	528-530		1
362.200	362.156	-0.044	(L)/VDE/(P)	380-382		0
362.200	362.192	-0.007	(E)/LTE/(F)	46-48		1
362.200	362.192	-0.007	(A)/TLE/(E)	355-357		1
414.200	414.202	0.002	(L)/FTF/(H)	506-508		1
414.200	414.271	0.071	(Y)/VPKA/(F)	497-500		0
414.200	414.271	0.071	(E)/GPKL/(V)	571-574		0
496.600	496.324	-0.275	(E)/HVKL/(V)	39-42		0
504.500	504.266	-0.233	(F)/DEKL/(F)	502-505		1
504.500	504.278	-0.222	(A)/SLRE/(T)	79-82		1
514.200	514.230	0.029	(E)/YGFQ/(N)	400-403		2

705.600	705.378	-0.222	(L)/VVSTQTA/(L)	575-581	1
705.600	705.382	-0.218	(F)/YAPPELL/(Y)	149-154	4
705.600	705.382	-0.218	(Y)/APELLE/(Y)	150-155	4
705.600	705.414	-0.185	(E)/KVLTSAA/(R)	187-193	1
713.600	713.419	-0.180	(F)/AVEGPKL/(V)	568-574	2
713.600	713.492	-0.108	(F)/KGLVLIA/(F)	20-26	2
825.800	825.450	-0.349	(E)/TYVPKAF/(D)	495-501	2
847.700	847.289	-0.411	(A)/DESHAGCE/(K)	56-63	2
884.700	884.451	-0.248	(A)/KDAFLGSF/(L)	322-329	3
940.800	940.514	-0.286	(L)/VLIASFQY/(L)	23-30	4
1015.900	1015.488	-0.412	(E)/KSHCIAEVE/(K)	285-293	2
1015.900	1015.507	-0.393	(Y)/SRRHPEYA/(V)	334-341	2
1015.900	1015.513	-0.386	(Y)/FYAPELLY/(Y)	148-155	6
1060.100	1060.473	0.372	(A)/KTCVADESHA/(G)	51-60	2
1081.900	1081.568	-0.332	(L)/SQKFPKAEF/(V)	219-227	4
1107.700	1107.343	-0.357	(E)/TYGDMADCCE/(K)	83-92	2
1107.700	1107.533	-0.167	(E)/YSRRHPEY/(A)	333-340	2
1107.700	1107.547	-0.153	(F)/DEKLFTFHA/(D)	502-510	4
1122.900	1122.652	-0.248	(L)/PKLKPDNTL/(C)	113-122	1
1127.100	1126.662	-0.438	(E)/EHFKGLVIA/(F)	17-26	4
1127.100	1127.486	0.385	(E)/RMPCTEDYL/(S)	444-452	2
1127.100	1127.594	0.494	(E)/VEKDAIPENL/(P)	292-301	3
1325.700	1325.674	-0.026	(Y)/QEAKDAFLGSFL/(Y)	319-330	7
1549.500	1549.663	0.162	(Q)/NCDQFEKLGEGYGF/(Q)	390-402	6
2012.900	2013.021	0.121	(A)/LTPDETYVPKAFDEKLF/(T)	490-506	7
2231.800	2232.039	0.238	(L)/IKQNCQDFEKLGEYGFQNA/(L)	387-405	9
2481.900	2482.105	0.205	(A)/PELLYYANKYNGVFQEECCQA E/(D)	151-171	12
2727.900	2727.421	-0.479	(E)/FVEVTKLVTDLTQVHKECCH GDLL/(E)	227-250	6
2727.900	2728.335	0.435	(L)/AKYICDNQDTISSKLKECCD KPLL/(E)	260-283	6
2984.500	2984.563	0.062	(F)/LYEYSRRHPEYAVSVLLRLA KEYE/(A)	330-353	13

Matching peptides for unspecific cleavage:

User mass	DB mass	Δ mass (daltons)	peptide	position	modifications	missed cleavages
697.400	697.253	-0.146	(A)/TLEECC(A)	355-360		2
697.400	697.356	-0.044	(N)/FVAFVD(K)	550-555		2
697.400	697.424	0.024	(L)/PKLKPD(P)	113-118		0
697.400	697.424	0.024	(P)/KLKPD(P)	114-119		0
802.200	802.373	0.172	(R)/HPEYAVS(V)	337-343		2
802.200	802.394	0.194	(K)/PKATEEQ/(L)	536-542		3
802.200	802.409	0.209	(S)/LHTLFGD(E)	66-72		2
802.200	802.430	0.230	(H)/KPKATEE/(Q)	535-541		2
802.200	802.457	0.256	(K)/AWSVARL/(S)	212-218		3
802.200	802.478	0.278	(C)/KVASLRE/(T)	76-82		2
1400.500	1400.684	0.183	(L)/VDEPQNLIKQNC(D)	380-391		3
1400.500	1400.764	0.264	(H)/KSEIAHRFKDLG(E)	4-15		3

1547.500	1547.669	0.169	(V)MENFVAFVDKCCAA/(D)	547-560	5
1547.500	1547.691	0.190	(E)/RNECFLSHKDDSP(D)	98-110	2
1547.500	1547.737	0.236	(G)DLLECADDRADLAK(Y)	248-261	6
1547.500	1547.781	0.280	(A)/EDKGACLLPKIETM(R)	171-184	4
1547.500	1547.789	0.289	(K)ADEKKFWGKYLY/(E)	128-139	6
1547.500	1547.818	0.317	(K)HLVDEPQNLIKQN(C)	378-390	4
1547.500	1547.843	0.343	(K)VLTSARQLRCAS(I)	188-201	4
1894.600	1894.886	0.285	(K)NYQEAKDAFLGSFLYE/(Y)	317-332	10
1894.600	1894.930	0.329	(S)LGKVGTRCCTKPESERM(P)	429-445	2
1894.600	1894.937	0.336	(R)RPCFSALTPDETYVPKA/(F)	484-500	5
1894.600	1894.994	0.394	(K)PILLEKSHCIAEVEKDAI(P)	281-297	6
1894.600	1894.994	0.394	(P)LLEKSHCIAEVEKDAIP(E)	282-298	6
2465.000	2465.202	0.201	(E)/CCQAEDKGACLLPKIETMRE KV(L)	167-188	7
2465.000	2465.343	0.343	(L)/VELLKHKPKATEEQLKTVME N(F)	529-549	8
3623.400	3623.663	0.263	(L)/TADFAEDKDVCKNYQEAKDA FLGSFLYEYSR(R)	305-335	15
3623.400	3623.882	0.481	(G)LVLIAFSQYLQCCPFDEHVK LVNELTEFAKT(C)	22-52	16

Peptides are displayed with the adjoining residues before cleavage greyed out and in parentheses.

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