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Mestrado em Biotecnologia

Trabalho efetuado sob orientação de:

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Abstract

Fish allergy is an adverse immune response which affects ≈ 3% of the world population and is currently without cure. Parvalbumin (PARV) is the major allergen of fish allergies, eliciting IgE-mediated immune responses. It is a conserved protein in finfish species and highly abundant in the muscle, the largest portion of the food in fish. Two main PARV family clusters have the designations α and β, but only the β is a proven allergen. β-PARV is considered a pan-allergen because as it has a vital function, it is widely distributed in wide range of bony fish species and other animals. Attempts to neutralize the allergenicity of proteins in food have been taken, by testing innovative processing methods, such as the High Hydrostatic Pressure (HP). An eco-friendly technology able to preserve the freshness and nutritional benefits of food products, while prolonging their shelf-life. Although HP processing of fish has a relatively small effect on sensory parameters, evidence shows modifications in the proteome and protein solubility of HP treated sea bass (*Dicentrarchus labrax, dl*) fillets, but the potential influences on human health, regarding the allergenicity is poorly clarified. In this thesis, the effect of HP and/or storage time in the allergenicity potential of β-PARV from sea bass fillets was investigated. β-PARV was successfully purified from the sarcoplasmic protein fractions of HP (600Mpa and 5 min.) and control fish after isothermal storage for 1 and 11 days and was analyzed by biochemical and biophysical techniques and *in silico* analysis to predict potential changes in the structural conformation. The results show a prevalence of α-helix in dlβ-PARV isolated from unprocessed fish fillets and those exposed to HP and time of storage modified the secondary and tertiary protein structure. dlβ-PARV epitope reactive to IgE/IgE shared a significant portion of residues in a position predicted to be of α-helix. The results suggest that the structural changes caused by HP may alter the allergenicity potential of dlβ-PARV, since the allergenic epitope seems to depend on protein conformation.

Keywords: fish allergy; parvalbumin; allergenicity; processing; high-pressure immunoglobulin E (IgE); calcium-binding protein; continuous elution electrophoresis; protein structures

Resumo

O peixe é um dos recursos marinhos mais valiosos, sendo um factor vital para o crescimento económico e social de diversas nações e populações. A inclusão de peixe fresco num estilo de vida saudável é apoiada por diversos autores, principalmente porque são ricos em proteína. No entanto, a alergia ao peixe é uma resposta imune adversa, actualmente sem cura, cuja prevalência é cerca de 3% da população mundial, segundo dados da Organização das Nações Unidas para a Alimentação e a Agricultura. A parvalbumina (PARV) é o maior alérgeno em peixes, provocando respostas imunes mediadas por IgE e é abundante no músculo branco de muitos vertebrados. Existem dois grupos principais da família das PARV´s, α e β, mas apenas a β é comprovadamente alergénica. A β-PARV é uma proteína acídica, de 10 a 15 kDa e tem 3 domínios EF-hand, 2 dos quais ligam Ca^{2+}, factor importante na conformação dos epítopos IgE. Apesar de existirem diferentes isoformas em peixes, a sua estrutura tridimensional é bastante conservada, potenciando a reactividade cruzada por IgE. É considerada um pan-alérgeno porque como tem uma função vital, é amplamente distribuída numa ampla gama de espécies de peixes e outros animais.

O peixe fresco é um produto muito sensível e altamente perecível, contribuindo para grande parte do desperdício alimentar. Métodos inovadores de processamento (ex: ultra-som ou plasma frio) estão a ser testados de modo a terem um papel crucial no rendimento da produtividade dos actuais sistemas alimentares, especialmente na indústria de pescado. A Alta Pressão Hidrostática (APH), é uma tecnologia não-térmica, inovadora e ecológica, que se mostra muito atractiva para a indústria alimentar, dado que, a pressão inactiva microrganismos e metabolitos reesposáveis pela rápida degradação dos alimentos. O prolongamento do prazo de validade de peixe processado por APH já foi reportada em diferentes espécies, podendo triplicar o prazo de validade. Além disso, a APH é capaz de preservar a frescura e os benefícios nutricionais do pescado.

Estas novas tecnologias de processamento, incluindo a APH, têm também sido utilizadas como tentativas de neutralizar a alerginicidade das proteínas nos alimentos, mas o seu real impacto e consequências ainda não são muito claros. Uma delas é o facto de a pressão poder ser uma força desestabilizadora da estrutura e estabilidade das proteínas. As alergias a peixes ocorrem quando os anticorpos IgE se ligam aos epítopos no alérgeno do peixe ingerido, e o nível de exposição dos epítopos é influenciado pela

conformação tridimensional da proteína. Tem sido reportado que o processamento por APH é capaz de produzir eventos como a destruição de epítopos, formação de novos epítopos e neo-alérgenos ou mudanças conformacionais que mascaram ou expõem epítopos às enzimas gastrointestinais. O efeito da APH na β-PARV dos peixes tem sido reportado em diferentes espécies (como a corvina grande amarela *Larimichthys crocea*, a carpa prussiana *Carassius gibelio* ou o bacalhau-do-atlântico *Gadus morhua*) e apesar de se verificar alteração da estrutura do alergénio, esta parece ser no sentido de reduzir a sua alerginicidade, sustentando a relevância deste método na qualidade e segurança alimentar. No entanto, é de notar que este efeito pode não ser característico em todas as espécies de peixe ou não ser transversal a todos os pacientes alérgicos a peixe. Embora o processamento por APH tenha um efeito ténue nos parâmetros sensoriais e permita uma extensão da validade, estudos prévios mostraram modificação na solubilidade proteica e no proteoma total em filetes de robalo (*Dicentrarchus labrax*, dl) processados, mas o possível impacto no potencial de alerginicidade desta espécie de peixe é pouco conhecido.

Esta tese teve como principal objectivo, investigar o efeito do APH e/ou tempo de armazenamento no potencial de alerginicidade da proteína β-PARV de filetes de robalo. A β-PARV foi purificada de extractos proteicos da fracção sarcoplasmática do musculo branco de filetes de robalo, previamente processados (por AHP, 600 MPa durante 5 min.) e não processados (controlo, C) e armazenados isotermicamente durante 1 e 11 dias. Para atingir o objectivo, aplicou-se um conjunto de técnicas bioquímicas e biofísicas associadas à análise de proteínas [electroforese em gel poliacrilamida (SDS-PAGE/Nativo/2-DE), purificação por electroforese de eluição continua, *Western blot*, espectroscopia de massa (MALDI-TOF/MS), espectroscopia (fluorescência e dicroísmo circular-CD)] e também análises *in silico* de pesquisa de sequências, alinhamentos, previsão de características bioquímicas e biofísicas, bem como de previsão de estruturas de proteínas.

Os resultados indicam que o processamento por APH, mais do que o tempo de armazenamento, modificou o perfil do proteoma total e a solubilidade proteica da fracção sarcoplasmática do musculo do filete de robalo em comparação com o controlo. Proteínas de menor peso molecular surgem em maior diversidade após tratamento e proteínas de maior dimensão sofrem uma diminuição considerável. O anticorpo monoclonal anti-Parv19, reconhece a dlβ-PARV (algo nunca publicado até hoje). com o peso molecular esperado de 12kDa, independente da pressão ou tempo de armazenamento usado. A

purificação da dlβ-PARV a partir dos extractos das diferentes condições experimentais testadas (C1, C11, APH1, APH11) foi bem-sucedida, e o 2-DE revelou a existência vários spots, sugerindo que múltiplas isoformas existem ou modificações pós tradução ocorrem na proteína. As análises estruturais confirmaram a presença de estrutura terciária e secundária (maioritariamente de α-helix) na β-PARV não processada (C1). A remoção de cálcio da parvalbumina não aparenta afectar a reactividade com o anticorpo PARV-19 tanto com amostras controlo como em amostras processadas. O tratamento por AHP conduz á perda de estrutura secundária e terciária (APH1) sendo este efeito revertido durante o armazenamento (APH11), sugerindo que a alteração é reversível. Dado que o epítopo alergénico é de natureza conformacional, os resultados sugerem uma potencial recuperação da estrutura do epítopo e especulamos uma possível manutenção do índice de alerginicidade. Contudo, estudos adicionais serão necessários (ex, aplicação in vivo e/ou in vitro em linha celulares, ensaios de digestibilidade, etc.) para inferir sobre o real efeito no potencial de alerginicidade da dlβ-PARV sob as condições testadas.

Em resumo, o processamento por APH e o tempo de armazenamento (nas condições testadas) são dois factores que afectam as proteínas do músculo do robalo, mais concretamente a dlβ-PARV e poderão reflectir alterações na sua alerginicidade, mas são necessários mais estudos para confirmar esta relação.

Palavras-chave: alergia em peixe; parvalbumina; alerginicidade; processamento; alta pressão; imunoglobulina E (IgE); proteína com ligação a cálcio; electroforese por eluição contínua; estruturas proteicas

Abbreviations

2-DE – Two-Dimensional Gel Electrophoresis

C1 – Refers to control (non-treated) samples day 1

C11 – Refers to control (non-treated) samples day 11

CD – Circular dichroism spectroscopy

CEE – Continuous Elution Electrophoresis

dl – *Dicentrarchus labrax*

dlβ-PARV – β-parvalbumin from the *Dicentrarchus labrax*

EGTA – ethylene glycol-bis (2-aminoethyl ether) - N,N,N´,N´-tetraacetic acid

GdmCl - Guanidine Hydrochloride

HP – High pressure

HP1 – Refers to high-pressure samples after day 1 of storage

HP11 – Refers to high-pressure samples after day 11 of storage

HPP – High Pressure Processing

IgE – Immunoglobulin E

IgG – Immunoglobulin G

kDa – Kilodalton

MRE - Mean Residue Ellipticity

MS – Mass Spectrometry

MW – Molecular Weight

NCBI – National Center for Biotechnology Information

PARV – Parvalbumin

PBDe – Protein Data Bank in Europe

pI - Isoelectric Point

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SOA - State of the Art

TBS – Tris-Buffered Saline

TBST – TBS with 0.1% (v/v) Tween® 20 detergent

WB – Western Blot

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The green lines correspond to α-helix, the blue arrow is β-pleated sheets, and the faint lines represent random coil. (B) Three-dimensional conformation of Parvalbumin 4 (accession nº: ENSDLAP00005011508) showing the molecule surface using EzMol platform; (C) – Representation of the secondary structure prediction of Parvalbumin 4 (accession nº: ENSDLAP00005011508) using the EzMol platform. The Parvalbumin 4 (pvalb4-202) sequence was used to interrogate Phyre2 and the 3D structure model template with the best match was input in the EzMol platform. The colors were added manually to each residue to show relevant regions: Blue – PARV19 antibody epitope; purple – EF-hand domains; red – calcium-binding sites.

Figure 8 – Fluorescence emission spectra (λexc=280nm) of pure dlβ-PARV protein from the different experimental conditions tested (non-processed and 600 MPa/5 min pressurized samples and stored for up to 11 days, C1, C11, HP1 and HP11).

Figure 9 – Far-UV CD spectra of (1 μM) pure dlβ-PARV from different experimental conditions tested (non-processed and 600 MPa/5 min pressurized samples and stored for up to 11 days, C1, C11, HP1 and HP11). in 20 mM Tris-HCl, pH 7.5at 25 °C, scanned from 250 to 180 nm

Figure 10 – Calcium depletion assay. Coomassie blue stained native gel (12%) (A) and the corresponding Western blot (B) of the purified dlβ-PARV from the different experimental conditions (C1, C11, HP1, HP11) with or without Ca2+ depletion. The dlβ-PARV was identified with monoclonal anti-PARV19. This assay was used to assess if the absence of calcium affects antibody reactivity. 1 – intact dlβ-PARV; 2 - dlβ-PARV with 5mM EGTA; 3 – dlβ-PARV with 0.5mM CaCl2; 4 – dlβ-PARV with 5mM EGTA and 0.5mM CaCl2

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CHAPTER I

General Introduction and Objectives

1.1. Context in Biotechnology

The study of innovative processing methods applied to food products is an ongoing and important element of Biotechnology. The impact of processing on proteins and, consequently, in the human health is of great significance. Currently, there is an increasing demand for healthier food resources and more sustainable food systems. In this context there is a pressure to shift away from unsustainable terrestrial production to aquatic production, which has high potential for growth (EC, 2018). The emergence of novel protein sources from marine sources requires improvements in monitorization of their quality and safety. The present study is integrated in Blue Biotechnology because it involves the application of a food processing technology to a marine organism. The study is aimed at assessing the effect of an innovative processing method called High Pressure Processing (HPP), that is used to extend the shelf-life of highly perishable seafood, on the allergenicity of seafood products.

1.2. Seafood

1.2.1. Nutritional and socio-economic importance of seafood products

Nowadays, the seafood industry, responsible for the capture/culture through to marketing and selling of fish or fish products, is recognized as crucial for the development of many countries. According to the most recent FAO report, more than 175 million tonnes of fish with an estimated value of more than 400 billion dollars (US) were produced globally in 2018 and provided employment for more than 250 million people in the same year (FAO, 2020).

Fresh fish is considered one of the healthiest protein sources (it is rich in high quality protein, 20%) and this is one of the reasons that seafood products are in high demand. The nutritional benefits of fish are multiple. Fish flesh is lean, contains essential trace elements including iodine, it is a source of anti-inflammatory and antioxidant nutrients, and has a relatively high concentration of omega-3 polyunsaturated fatty acids (PUFA's), which cannot be produced by humans but are essential for normal growth and development (Hunter & Roberts, 2000; Zhang et al, 2019[b]). There is a well-recognized correlation between fish consumption and a decreased risk of coronary heart disease (Dale et al, 2019). Since fish consumption is also a major source of essential amino acids,

vitamins, and minerals, the inclusion of fish in the daily diet is highly recommended for a healthy life (FAO, 2020).

1.2.2. Aquaculture

In the recent years, aquaculture, defined as the controlled cultivation of aquatic organisms for food, has become a crucial engine for world seafood production. The aquaculture sector has been growing steadily, at 7.5% per year since 1970 and in 2018 production was over 82 million tons and was higher than fish coming from capture fisheries. Fish farming is important as it can improve human nutrition and contribute to food security. Seafood accessibility has expanded in countries with limited or no access to the sea or marine species, and this has contributed to reduce the price and make fish more accessible (FAO, 2020).

1.2.3. Productivity, safety, and quality

According to the FAO, every year more than 1.3 billion tonnes of food is wasted, and at the same time, more than seven hundred million people are in a situation of hunger. This paradox is a complex problem, but possible to solve. By developing a sustainable seafood industry, it can contribute to generate high quality protein in sufficient quantity to eliminate hunger and malnutrition, and simultaneously, increase the efficiency of food production system (FAO, 2020; FAO 2021).

The productivity of the seafood industry is highly affected by the inherent instability of fish, which leads to rapid degradation and spoilage of fresh fish. The short shelf-life of seafood is linked in part to their high-water content, promoting the growth of pathogenic microorganisms and their proteolytic action, which is directly correlated with food spoilage (Gram and Huss, 1996; Rosario et al, 2020). Since freshly caught or farmed fish is only stable for a relatively brief time compared to avian/mammal meat, there is a much higher likelihood of spoilage and waste. All these factors form the basis of current actions directed at the development or optimization of new efficient and beneficial conservation and processing methods for seafood products. However, the diversification of aquatic food and the introduction of innovative processing methods can have unpredicted effects, like for instance, enhancing or diminishing hypersensitivity reactions to seafood in humans (Achouri & Boye, 2013). The consequences of diversifying

processing methods on food and its potential to cause intolerance or allergic reaction needs further investigation.

1.3. Innovative food processing methods

With the increasing demand for productive food systems, new and innovative processing methods emerged in recent years. There has been a growing interest in a particular group of these techniques called non-thermal processing methods. The expenditure of energy in food preservation, avoids thermal fluctuations and, deterioration of the foodstuff, allowing the maintenance of its freshness and the main natural characteristics (Thakur & Nelson, 1998; Tapia De Daza et al, 1996). Some examples of emerging processing technologies that are being tested/applied are Ultrasound, Cold Plasma or High-Pressure Processing etc.. Each one has advantages and disadvantages, depending on the foodstuff to be used, but they possess a high potential to decrease the microbial activity in diverse types of food. However, more studies are required to optimize the decontamination efficiency without the need for the use of other non-thermal methods (Rosario et al, 2020; Thirumdas et al, 2017). In the present thesis I will focus on one processing method that has almost universal food applications, called High Pressure Processing (HPP).

1.4.High Pressure Processing (HPP)

1.4.1. Principles and mechanisms

HPP is a non-thermal method, first developed in 1889 (Hite, 1889), that uses physical High Pressure (HP) to inactive microorganisms present in food, via mechanical disruption of their cell membranes, and impairs the production of enzymes and metabolites (Thakur & Nelson, 1998; Tapia De Daza et al, 1996). The target-product is packed under vacuum and placed inside a high-pressure chamber filled with water. The pressure in the chamber is increased up to 1000 MPa applied to the water containing the product. Since the chamber is a confined environment and the pressure is constant, the isobaric principle states that the pressure transmitted will be identical in all directions (Zhang et al, 2019[a]). The increase in pressure at a constant temperature leads to a reduction in the entropy of the system and this could explain why this processing technique is able to preserve the freshness and nutritional benefits of food (Mozhaev et al, 1996; Meng et al, 2017).

1.4.2. Advantages and potential of application in seafood

HPP technology was not widely used until the industrialization of Japan in the 1990s and since then interest has risen in its use for seafood products (Oliveira et al, 2017). The only resource required is electricity, and it does not produce any waste or residues, and for this reason has been considered to be ecologically friendly (Thakur & Nelson, 1998). The versatility of HPP as a seafood processing method is one of its many advantages, since independently of the shape, size, or appearance, virtually any product can be treated. For seafood, the application of pressure ensures adequate decontamination, and this prolongs its shelf-life (Zhang et al, 2019[a]) without compromising the sensory parameters. Any harmful changes in fish flesh that may occur in terms of visual appearance, texture, and compounds due to HP treatment, need to be minimized by systematic investigation of the quality changes and validation of the processing parameters. Several reports and reviews have been published about the potential impact of HP on physical texture (Ramirez-Suarez & Morrissey, 2006; Schubring et al, 2003), visual aspects, lipid oxidation (Angsupanich & Ledward, 1998; Sequeira-Munoz et al, 2006), water content (Briones-Labarca et al, 2012; Campus et al, 2010), sensory properties (Erkan et al, 2010; Hurtado et al, 2000) and proteins.

1.4.3. Effect on proteins

Proteins are large molecules which are vital for the regulation of an organism's tissues and organs and are an important macronutrient in human nutrition. They consist of a polymer of amino acids linked together by peptide bonds (Alberts et al, 2002). There are four levels of protein conformation, namely: the primary structure, defined by the amino acids that constitute the polypeptide chain; the secondary structure, defined by effects between the atoms of the polypeptide chain, forming hydrogen bonds; the tertiary structure, defined as the tridimensional structure and for some proteins the quaternary structure, defined by the interaction between two or more polypeptides (Sanvictores et al, 2022; Rehman et al, 2022; Kerndt et al, 2022). The tridimensional protein structure is intimately associated with its function, and both can be altered by an external factor like HP for example. The effect of pressure on proteins has been extensively studied in the last decades in different types of seafood like shrimp, salmon, cod, and oysters (Oliveira et al, 2017). Pressure can be destabilizing and usually the tertiary structure is affected by

pressures above 200 MPa, and protein unfolding occurs when pressure reaches 500 MPa (Somkuti & Mueller, 2013). In thermal treatments, protein unfolding occurs and can lead to aggregation of polypeptide chains and is virtually irreversible since the internal energy of the system changes dramatically. Pressure only affects the volume of the system, and the occurrence of denaturation is often depicted as a transitional process between the two states of the protein, native and denatured. In other words, pressure induces denaturation but it is thought to be a reversible event, since protein refolding may occur after the release of the pressure. (Mozhaev et al, 1996; Somkuti & Smeller, 2013). Specific effects observed in proteins by HP are related with protein aggregation and the consequent loss of solubility when the pressure is above 300MPa (Oliveira et al, 2017).

From a nutritional point of view, seafood products are rich in protein and although consuming fish has a series of the aforementioned benefits, fish proteins are also one of the most common causes of food allergy. The effect of HP processing on allergens is unclear but since it can alter the conformation of proteins it may alter the allergenic potential of foods (Penas et al, 2011; Dhakal et al., 2014) including seafood (Zhang et al, 2019[a]; Zhang et al, 2017). The prevalence of allergies caused by HP-processed seafood products will depend on events like destruction of allergenic epitopes, formation of new epitopes and neo-allergens, conformational changes in the protein caused by HP may mask or expose epitopes to gastrointestinal enzymes and trigger an immune reaction (Somkuti & Smeller, 2013; Teodorowicz et al, 2017; Zhang et al, 2019[a]; Rahaman et al, 2016).

1.5. Seafood allergies and allergens

1.5.1. Food and allergenicity

A food allergy is an excessive response of the immune system to foods, caused by molecules called allergen. In foods the most common allergens are usually proteins which contain immunodominant sequences called epitopes. These regions can interact with B cells through their membrane receptors (antibody-mediated immunity) and initiate an allergic reaction. The interaction with an allergen induces B-cells to produce specific antibodies called immunoglobulin E (IgE). Another consequence is the production of memory B-cells which cause a much more rapid response to a second exposure to the allergen, this process is called sensitization (Cheng et al, 2022; Shakib et al, 2008;

Somkuti & Smeller, 2013; Vaughan et al 2013) and the main steps are illustrated in Fig. 1. The epitope in a protein may be a continuous sequence of aminoacids (linear) or formed by two sequences distant in a protein that are brough together in the tertiary structure (conformational). In the last case, the IgE reactivity can be influenced by structural changes in the protein (Tanabe, 2007; Valenta & Kraft, 2001)

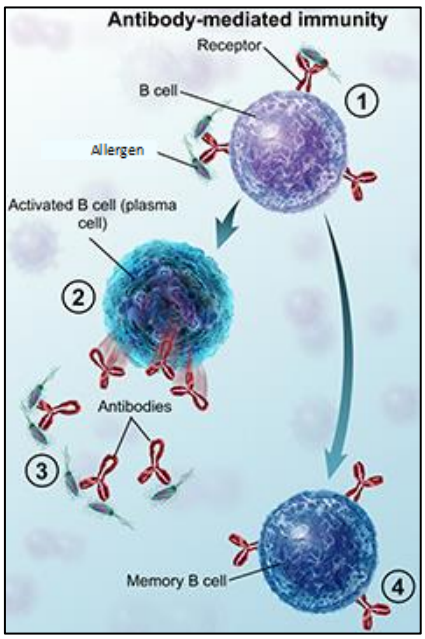

Figure 1 – Diagram of an allergic reaction, where the immune system of a hypersensitive individual treats an allergen as a pathogen. 1- The allergen interacts with the B cells; 2- the B cell is able to produce specific IgE: 3- The IgE targets the allergen; 4- Memory B cells are also produced, that will induce a faster IgE response, when a second exposure to the same allergen occurs (Image taken from: https://www.visiblebody.com/blog/et-tu-immune-system-4-seasonal-allergy-facts)

The consequence of the interaction of immunoglobulin with the epitopes in a protein will depend on the level of hypersensitivity of the individual. According to Zhang et al (2019) the most common symptoms are angioedema, gastrointestinal discomfort, and inflammation of the respiratory airways. In extreme cases allergic reactions can lead to death by anaphylaxis, a systemic acute event where several and very intense symptoms occur simultaneously (Cardona et al, 2020). It is also important to differentiate food allergy from food intolerance, that despite sharing some symptoms, are quite different since the latter is a non-immune adverse reaction (Muthukumar et al, 2020). Currently, there is no cure for food allergies, so avoidance of food that provoke an allergic reaction, by removing them from the diet of hypersensitive individuals is the best prevention. There is a downside to this control strategy for seafood allergies since the health benefits of seafood cannot be appreciated by these individuals (Jiménez-Saiz et al, 2015; Yuk et al, 2021). Thus, it could be advantageous to have alternative control strategies.

Food allergenicity is characterized by the potential of a food to induce sensitization and an immune reaction. In general, proteins in food with more reactive epitopes or more exposed epitopes will be more allergenic, but this depends on their physicochemical properties (Cheng et al, 2022). There have been attempts to neutralize the allergenicity of various food proteins, using different processing methods in different types of foods, but without much success to date and more work is required to overcome this challenge (Mondoulet et al., 2005; Besler et al, 2001).

1.5.2. Seafood allergens

Seafood is currently one of most common foods capable of causing allergic reactions, affecting around 3% of the world population. The prevalence of fish allergy is higher in countries where fish is more available and consumed, but in general it is rising due to the increase of fish consumption around the world. (Peters et al, 2017; Zhang et al, 2019[a]). Currently there are several proteins present in fish that are confirmed allergens. In Table I, a summary is provided of several species from different orders that are currently known to cause allergic reactions and contain at least one identified fish allergen. The most studied fish allergens are parvalbumin (PARV), enolase and aldolase. Aldolase and enolase are enzymes present in fish muscle and they are related to energy production. PARV was the first fish allergen identified, being the most relevant in eliciting an allergic reaction and it is present in fish muscle, in the form of two main isoforms beta(β) and alfa(α). Only β-PARV is proven to be allergenic in fish. Most of the fish consumed like salmon, tuna and cod present cross-reactivity, so β-PARV is considered an iso-allergen (Kuehn et al, 2014). β-PARV, the major allergen in fish is the focus of the present study.

Table I – Official entries for all fish allergens, according to the nomenclature database (http://allergen.org/) approved by the World Health Organization and the International Union of Immunological Societies (Accessed on 14/12/2021).

Order	Fish	Allergen name	Protein Identity
Clupeiformes	*Clupea harengus* (Atlantic herring)	Clu h 1.0101	Parvalbumin
		Clu h 1.0201	
		Clu h 1.0301	
	Sardinops sagax (Pacific pilchard)	Sar sa 1.0101	Parvalbumin
Cypriniformes	*Cyprinus carpio* (common carp)	Cyp c 1.0101	Parvalbumin
		Cyp c 1.0201	
Gadiformes	*Gadus callarias* (Baltic cod)	Gad c 1.0101	Parvalbumin
		Gad m 1.0101	Parvalbumin
		Gad m 1.0102	
		Gad m 1.0201	
	Gadus morhua (Atlantic cod)	Gad m 1.0102	Parvalbumin
		Gad m 2.0101	
		Gad m 3.0101	
Perciformes	*Lates calcarifer* (barramundi)	Lat c 1.0101	Parvalbumin
		Lat c 1.0201	
	Oreochromis mossambicus (tilapia)	Ore m 4.0101	Tropomyosin
	Thunnus albacares (yellowfin tuna)	Thu a 1.0101	Parvalbumin
		Thu a 2.0101	Enolase
		Thu a 3.0101	Aldolase
	Xiphias gladius (swordfish)	Xip g 1.0101	Parvalbumin
Pleuronectiformes	*Lepidorhombus whiffiagonis* (megrim)	Lep w 1.010	Parvalbumin
Salmoniformes	*Oncorhynchus keta* (Pacific salmon)	Onc k 5.0101	Vitellogenin
	Oncorhynchus mykiss (rainbow trout)	Onc m 1.0101	Parvalbumin
		Onc m 1.0201	
	Salmo salar (Atlantic salmon)	Sal s 1.0101	Parvalbumin
		Sal s 2.0101	Enolase
		Sal s 3.0101	Aldolase
Scorpaeniformes	*Sebastes marinus* (redfish)	Seb m 1.0101	Parvalbumin
		Seb m 1.0201	

1.5.3. The allergen Parvalbumin (PARV)

The major allergen in fish is parvalbumin (PARV), which is abundant in muscle, the part of the fish that is consumed. PARV proteins are classified into two main isoform groups: α and β, but only the β isoform has been shown to be allergenic. β-PARV, also known as oncomodulin, is considered a pan-allergen, which is a family of related proteins that have a vital function and so are widely distributed in nature, and it is present in the

muscle of a wide range of bony fish species and other animals. PARV is characterized by a well conserved sequence that shares more than 50% identity with sequence homologues from evolutionary distant fish species. So cross-reactivity is a common event, emphasizing the importance of avoiding all fish species in allergenic individuals. (Yuk et al, 2021; Kuehn et al, 2014; Kobayashi et al, 2016; Xu et al, 2021).

β-PARV is a small protein (~10-15 kDa) with a globular three-dimensional structure, which belongs to the EF-hand protein family. They have 3 EF-hand motifs, although only two are functional domains. In Fig. 2, the 3D structure of β-PARV from the Atlantic cod (*Gadus morhua*) is shown and provides an example of a species where the protein is a confirmed allergen. The native form of the protein is tightly packed, and it is predicted to have more than 60% of alpha-helix as secondary structure (represented by the spirals in Fig. 2) (Zhang et al, 2020; Moraes et al, 2014; Xu et al, 2021). PARV is a $Ca2^{+}$ binding proteins and due to the calcium chelator proprieties of the two EF-hand motifs, it makes them an important player in controlling intercellular calcium concentrations and, therefore, the relaxation or contraction of muscle. EF-hand motifs influence the structure of the protein, so calcium variations may influence the 3D structure and, consequently, the proteins allergenicity (Somkuti & Smeller, 2013; Pérez-Tavarez et al, 2021; Kuehn et al, 2014; Peters et al, 2017; Pérez-Tavarez et al, 2019; Permyakov et al, 2022).

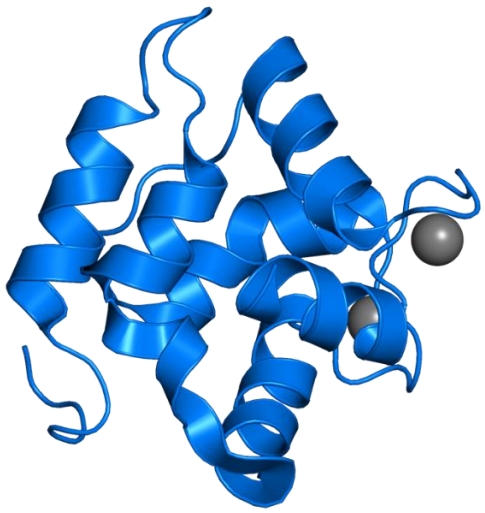

Figure 2 – Representation of the resolved 3D structure of β-PARV from the Atlantic cod *Gadus morhua* (UniProt ID: Q90YK9; PBDe ID: 2mbx). The black spheres represent the calcium ions interacting with the EF-hand motifs. Retrieved from the RCSB Protein Data Bank (https://www.rcsb.org/)

The official database of allergens includes 21 PARVs from 12 fish species from different orders, and the number rises dramatically if all the different isoforms identified in fish are included (Kuehn et al, 2014). The allergenicity of β-PARV develops with the increase in the concentration of this protein in muscle, the only tissue in which it is found in vertebrates. In fish, β-PARV is more prevalent in large-sized migratory species (Kubota et al, 2016; Kobayashi et al, 2016). The allergenicity of the main allergen in fish is mainly due to its acidic properties and resistance to denaturation and proteolytic digestion (Bugajska-Schretter, et al 1998). Since β-PARV in fish is stable in a great thermal amplitude, it maintains its allergenic properties, even when it is present in an acidic environment (Tong et al, 2018). This raises the question about whether HP is a sufficiently intense treatment and can influence the allergenicity of this major fish allergen. The effects of pressure on β-PARV are emphasized in a limited number of studies. Liu et al (2010) and Liu et al (2012) did not observe any change in β-PARV allergenicity in two different fish species (silver carp *Hypophthalmichthys molitrix* and largemouth bass *Micropterus salmoides*). The main conclusion of Somkuti et al (2012) while studying the effect of pressure on cod (*Gadus morhua*) parvalbumin was the extreme stability of this allergen, due to the two Ca2+ binding sites. Harsh pressure conditions are needed to cause a change in β-PARV structure. Nevertheless, there are reports that HP tends to reduce the allergenicity of fish β-PARV, a conclusion reached by Zhang et al (2020) in the Large yellow croaker (*Larimichthys crocea*) and by Liu et al, 2009 in the Prussian carp (*Carassius gibelio*). Currently there are no studies of the effects of pressure on the allergenicity of β-PARV in the European sea bass (*Dicentrarchus labrax*), one of the most important aquaculture products in the Mediterranean region (Vandeputte et al, 2019) and this is a gap that the present thesis explores.

1.6. Objectives of the thesis

This thesis explores the effect of an innovative HP processing method on proteins present in the white muscle of the European sea bass (*Dicentrarchus labrax),* one of the most farmed species in Mediterranean. The laboratory-based analysis reported in this thesis are under preparation for publication and for this reason the thesis starts with a more extensive consideration of the state of the art (SOA) and is followed by the presentation of the thesis work in the form of a scientific article. For this reason, some overlap exists between the general SOA and the introduction of the scientific manuscript

that reports the methods, results and discussion. The thesis and manuscript herein is focused on the potential of HP to alter the structure and/or the allergenicity of β-PARV from European sea bass white muscle. The workflow follows three main activities: 1) the identification of the effect of HP on the total proteome profile of the sea bass, 2) purification of the processed and non-processed β-PARV and 3) characterization of the structural modifications of the β-PARV that was purified.

CHAPTER II

EFFECTS OF HIGH-PRESSURE PROCESSING (HHP) ON THE STRUCTURE AND POTENTIAL OF ALLERGENICITY OF β-PARVALBUMIN FROM THE EUROPEAN SEA BASS (*Dicentrarchus labrax*)

EFFECTS OF HIGH-PRESSURE PROCESSING (HHP) ON THE STRUCTURE AND POTENTIAL OF ALLERGENICITY OF β-PARVALBUMIN FROM THE EUROPEAN SEA BASS (*Dicentrarchus labrax*)

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1. Introduction

Seafood is currently one the most valuable marine resources in the world, contributing to economic and social growth. The addition of fresh fish to the diet is part of a healthy lifestyle that is supported by many authors, due to the numerous nutritional advantages (Hunter & Roberts, 2000; Zhang et al, 2019[b]; Dale et al, 2019; FAO, 2020), including their role as an excellent source of high biological value in protein. A drawback is the allergenic potential of fish currently for 2.9% of the world population. It is expected to grow with the exponential growth of the population and also due to the increase in fish consumption caused by the need of substitute proteins from terrestrial systems (Schrama et al, 2022; FAO, 2020).

Parvalbumin (PARV) induces IgE mediated allergic reactions and is known as a major allergen in fish. The 12 kDa protein belongs to the EF-hand protein family and can bind to Ca^{2+} ions, an important function for fish movement. PARV regulates the concentration of intercellular calcium and influences the relaxation or contraction of the muscle, according to the swimming demands of the fish (Yuk et al. 2021; Somkuti & Smeller, 2013; Pérez-Tavarez et al, 2021; Kuehn et al, 2014; Pérez-Tavarez et al, 2019). The PARV family is divided into the two major isoform clusters: α and β. Both are present in the muscle of a broad range of bony fishes, but only the β-PARVs are proven to be allergenic so far. Numerous evidence of cross-reactivity between fish species, mainly caused by PARV, have been reported in the last decades, and since there is no cure for fish allergy, it is recommended the avoidance of all fish in the diet of sensitized individuals (Yuk et al, 2021; Kuehn et al, 2014; Kobayashi et al, 2021).

The need for sustainable and effective food systems is on the rise. According to FAO, 1/3 of the world food production is wasted every year, while 10% of the world population is starving (FAO, 2020). Innovative processing methods (e.g., ultrasound or cold plasma (Rosario et al, 2020 Thirumdas et al, 2017)) can play a vital role in shifting the productivity yield, especially in the seafood industry because fresh fish is a highly perishable food product. High Pressure Processing (HPP) in an innovative method that has been extensively studied in recent years, including in fish (Thakur & Nelson, 1998; Mozhaev et al, 1996; Zhang et al, 2020). It is an attractive non-thermal method for the food industry since the mechanical pressure efficiently inactivates microorganisms and

metabolites present in the product and does not produce any waste, and can be applied to products of any shape, size, or appearance (Zhang et al, 2019[a]; Thakur & Nelson, 1998; Tapia De Daza et al, 1996). Shelf-life extension of pressure-treated fish has been already reported in distinct species, for up to three times the expected time (Tsironi et al, 2015, Rode et al, 2016). Although HPP treatment of fish has a relatively small effect on sensory parameters, studies have shown a significant modification in the total proteome and protein solubility of HPP treated sea bass (*Dicentrarchus labrax*), one of the most farmed species in the Mediterranean (Tsironi et al, 2015; Anjos et al 2019). However, the potential consequences of HPP fish processing on human health, regarding the allergenicity is poorly clarified.

Pressure can be a destabilizing force in the protein cohesion, especially in its structure and conformation. Somkuti & Mueller (2013) stated that 200 MPa pressures are enough to affect the tertiary structure and above 500 MPa causes protein unfolding. At this point, it is logical to think of the possible effect that pressure may have on the protein allergenicity. Fish allergies occur when the antibodies from the immune system react with epitopes on the allergen, and the level of exposure of the allergenic epitopes are influenced by the tridimensional conformation of the protein. HP processing is capable of producing events like the destruction of epitopes, formation of new epitopes and neo-allergens, or even conformational changes that may mask or expose the epitopes to gastrointestinal enzymes (Somkuti & Smeller, 2013; Teodorowicz et al, 2017; Zhang et al, 2019[a]; Rahaman et al, 2016).

In fish PARV, the pressure effect has been studied in many species such as the Large yellow croaker (*Larimichthys crocea*) (Zhang et al, 2020), the Prussian carp (*Carassius gibelio*) (Liu et al, 2019) or the Cod (*Gadus morhua*) (Zhang et al, 2019[a]). The cited publications, report that HP tends to reduce the allergenicity of PARV, supporting the value of HP to improve the quality and safety of seafood products. In the present thesis, the effect of HP processing on proteins of the white muscle sarcoplasmic fraction of the European sea bass (*Dicentrarchus labrax, dl)* fillets will be accessed, and is focused on dl- β-PARV. This study will be addressed to the potential of HPP to change the structure and/or the potential of allergenicity of the dlβ-PARV and includes 3 main tasks: 1) the identification of the effect of HP on the proteome profile of sea bass fillets;

2) purification of the processed and non-processed dlβ-PARV and 3) the characterization of the structural modifications of the purified dlβ-PARV.

2. Materials and methods

2.1 Raw materials – sea bass fillets

Thirty-two fillets (weight: 110 ± 10 g, length: 20 ± 2 cm) of European sea bass (*Dicentrarchus labrax, dl*) were supplied by Nireus Aquaculture, Greece. Fish were filleted within 2-3 hours of capture and preserved in polystyrene boxes at 0 °C in flaked ice while transferred to the Laboratory of Food Chemistry and Technology (NTUA, Greece).

2.2 *Experimental set up (HP processing/storage time) and sampling*

The equipment used and the methodology followed for the HP processing, storage time and sampling procedures were as previously described (Tsironi et al, 2015; Anjos et al 2019). Fish fillets were wrapped in pouches (two per pouch) composed by a multilayer (PP-PE) packaging material. The packaged fish consisted of processed (HP samples) and none processed control samples (C) as indicated in the overview of the experimental design (Fig. 3). HP processed fish fillets (n = 16) were vacuum-packed and treated in-pack at 600 MPa at 25 °C for 5 min. Pressure force and pressure time parameters were chosen to maximize the potential effect of HP on the sea bass samples (Tsironi et al., 2015). Immediately after processing, fish fillets (n = 8) were stored in isothermal conditions at 2 °C for 11 days (HP11 samples), to simulate the conditions of the commercial cold chain and the remaining fillets (n = 8) were immediately sampled (HP1 samples). The non-processed (control) fillets were, immediately sampled (n = 8) at 1 day (C1 samples) and the remaining fillets (n = 8) were stored aerobically in non-sealed pouches, simulating conventional aerobic retail display facilities. Both control and HP-treated fillets, were stored in high-precision low-temperature incubators Sanyo MIR 153 (Sanyo Electric, JP), always in isothermal conditions at 2 °C (± 0.2 °C) closely supervised with miniature data-loggers (COX TRACER ®, US). Samples with an approximate size of 1 cm^3 (≈ 350 mg) of white muscle were collected from fillets at two different time points, 1 day (C1 and HP1) and 11 days (C11 and HP11) (Fig. 3), and immediately stored at -80 ºC.

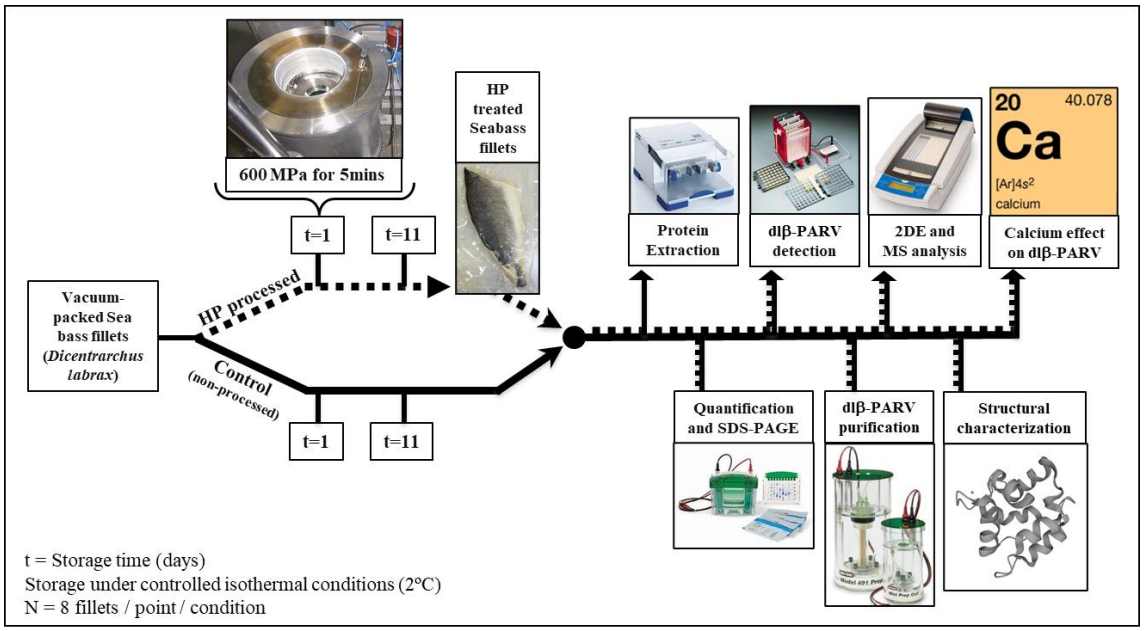

Figure 3 – Schematic overview of the experimental design and associated analysis. All the procedures represented by the boxed images in the scheme are described in the main sections of the manuscript. The image labeled "Structural characterization" was extracted from the Uniprot website and shows the resolved secondary structure of Atlantic cod (Gadus morhua) β-PARV, a recognized fish allergen by the Allergen Nomenclature Sub-committee (WHO/IUIS) (http://www.allergen.org/viewallergen.php?aid=708).

2.3 Sarcoplasmic protein extracts preparation

For each experimental condition (C1, HP1, C11, HP11), sarcoplasmic enriched protein extracts were prepared from white muscle of individual sea bass fillets (n= 8 individuals per group). The methodology used was based on (Pazos et al 2015) and (Anjos et al 2019) with small modifications. In brief, muscle samples (≈ 350 mg/each) were reduced to a powder in liquid nitrogen using a mortar and pestle. Approximately 350 mg of 0.5 mm zirconia silica beads (Biospec, USA) were mixed with ± 125 mg of powdered sample in refrigerated 2 mL tubes (SARSTEDT®, DE). Sarcoplasmic protein extraction buffer containing, 10mM Tris buffer pH 7.2 supplemented with 1x Protease inhibitor (Sigma-Aldrich™, US), was added to each tube in a ratio of 1:4 (1 g tissue/4 mL of buffer) and the samples were mechanically disrupted in a TissueLyser II homogenizer (QIAGEN®, NL) using 2 cycles of 20 s with a frequency of 30 s^{-1} (see the general set-up under "protein extraction" in Fig. 3). The soluble white muscle sarcoplasmic protein fraction was separated by centrifugation at 28,000 g for 15 min at 4 °C (Eppendorf™, DE), aliquoted and stored at -80 °C for further analysis.

2.4 Total protein quantification and SDS-PAGE analysis

The soluble sarcoplasmic enriched protein extracts of HP processed, and control fillets were quantified using the Bradford Assay with a bovine serum albumin standard set (#5000206, BioRad™, US) as recommended by the manufacturer, in a Tecan Infinite M200 Microplate Reader (Tecan™, CH). Sea bass white muscle sarcoplasmic fraction protein profiles were analyzed through 1D polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE, 15% resolving/5% stacking) following the Laemmli method (Laemmli, 1970) and using a PageRuler™ Prestained Protein Ladder (Thermo Scientific™, US). 20 µg of total protein was mixed with 5 x SDS sample buffer (250 mM tris-HCl pH 6.8, 10% (v/v) SDS, 50% (w/v) glycerol, 0.15 % (w/v) bromophenol blue) and 500 mM DTT (freshly added), and thermally denatured at 95°C for 5 min. The electrophoresis was carried out in a Mini-PROTEAN Tetra Cell (BioRad™, US) at 25 mA constant current and at 4 °C (the equipment is shown in Fig. 3 in the image labeled "Quantification and SDS-PAGE"). Gels were stained with Coomassie Brilliant Blue (R-250) Dye and imaged with the Imaging System Azure c600 (Azure Biosystems, US).

2.5 ***dlβ-PARV detection by Western blot***

Western blot was used to detect dlβ-PARV in the white muscle sarcoplasmic protein fractions isolated from C and HP processed samples. The protocol used was an adaptation of the method of Hilger et al (2004) as described in Anjos et al. (2022) with optimization of the method for specific detection of sea bass β-PARV immunodetection. In brief, protein samples (20 µg) were separated by SDS-PAGE and were transferred to 0.2 µm nitrocellulose membrane (BioRad™, US) using a TE 22 Mini Tank Transfer Unit (Cytiva™, US) during 1h, at 4 ºC under constant current at 300 mA (the equipment is shown in Fig. 3 in the image labeled "dlβ-PARV detection"). Transfer of the proteins from the polyacrylamide gel to the nitrocellulose membrane was confirmed by Ponceau S staining (Sigma Aldrich, USA). Membranes were then stored in the blocking buffer 3 % (w/v) skim milk diluted in 1x Tris buffered saline (20 mM Tris-base, 150 mM NaCl pH 7.6, TBS) with 0,1 % (v/v) Tween® 20 detergent (1x TBST) overnight at 4 ºC. The protein extract immobilized on the membranes was incubated with a monoclonal anti-parvalbumin antibody (PARV19, mouse ascites fluid, Sigma-Aldrich™, US) diluted 1:5000 in 1x TBS, for 4 hours at room temperature. Detection of the primary antisera was carried out by incubating the membrane for 1 h at room temperature with an anti-mouse

IgG (whole molecule) – peroxidase conjugated antibody (produced in rabbit, Sigma-Aldrich™, US), diluted 1:80.000 in 1x TBS. As reaction controls, duplicate membranes were incubated with secondary antibody only. Blotted antigens were detected by chemiluminescence using an ECL™ Prime Western Blotting Detection Kit (GE Healthcare™, US) according to the manufacturer´s instructions. The chemiluminescence signaling was captured using an Azure c600 Imaging System (Azure Biosystems™, US) and were analyzed with AzureSpot software (version 14.2, Azure Biosystem™., US). The detection times varied depending on the intensity of the signal.

2.6 dlβ-PARV purification

dlβ-PARV was purified from the sea bass white muscle sarcoplasmic protein extracts of C and HP processed samples (both at 1 and 11 days) by CEE using a Model 491 Prep-Cell (Bio-Rad™, Portugal) following the general procedures described by the manufacturer and the optimizations for equipment operation described in (Anjos et al., 2019), the equipment is shown in Fig. 3 in the image labeled "dlβ-PARV purification"). Protein extracts prepared from samples from the same experimental condition were pooled (n=8). Between 8 and 28 mg of total protein (≈ 3 ml) was used for purification after it was mixed with 5x concentrated sample buffer (250 mM tris-HCl pH 6.8, 50 % (w/v) glycerol, 0.15 % (w/v) bromophenol blue). A preparative semi-native PAGE system was applied with the following conditions: 1) the resolving gel was composed of 15% polyacrylamide in 0.375 M Tris-HCl, pH 8.8 with a height of 7.5-8.4 cm and the stacking gel was 4 % polyacrylamide in 0.125 M Tris-HCl, pH 6.8 with a height of 1.5 cm; 2) both gels were prepared in a 37 mm diameter cylinder and were SDS free; 3) the running buffer used was 0.025 M Tris-HCl; 0.19 M glycine; 0.1% SDS pH 8.3. The electrophoresis was run at 4 ºC during 20 h, with the first 13 h run at 2W/20 mA until the ion front reached the end of the gel column and in the remaining 7 h the running conditions were set at 12W/48mA. Protein fractions that were collected into the elution chamber were then collected into 1.5 ml/fractions at 4 ºC in 1x elution buffer (20 mM Tris-HCl, pH 7.5). The fraction collection used a peristaltic pump (LKB P-1, Pharmacia™, SE) to drives the eluted sample into the fraction collector (FRAC-100, Pharmacia™, SE) that was prepared with 11.5ml polypropylene tubes (Sarstedt™, DE). Around three hundred fractions were collected and stored at -20°C until further analysis.

The eluted proteins were detected by analytical SDS-PAGE gel (15%) and Coomassie blue or silver staining using the Silver Stain Plus Kit (BioRad™, US). The presence of dlβ-PARV, was confirmed in the eluted fractions by WB as described previously. Pure dlβ-PARV fractions were pooled and concentrated >10 x using Amicon® Ultra-15 Centrifugal Filter Units (Merck™, DE) through centrifuge cycles of 1h at 5000 rpm at 4 ºC. In addition, other protein fractions were pooled to create a library of isolated sea bass muscle sarcoplasmic proteins, including fractions of semi purified PARV protein and other contaminating proteins with a similar MW and eluting in the same fractions. The final protein concentration was determined using the method described by Gill & Hippel (1989) based on UV absorption at 280 nm and on the molar extinction coefficient estimated according to the following equation $\varepsilon M_{, Gdm.Cl} = a\ \varepsilon_{M, Tyr} + b\ \varepsilon_{M, Trp} + c\ \varepsilon_{M, Cys}$ (a, b and c are replaced by the number of respective residues present in the sequence). The determined values were used to estimate the yield of the purification from muscle to isolated protein. The purified and concentrated dlβ-PARV samples were aliquoted and stored at -80°C. The process of purification was repeated whenever more purified dlβ-PARV was needed to conduct the biophysical experiments.

2.7 Two-dimensional gel electrophoresis and mass spectrometry analysis

A two-dimensional gel electrophoresis (2DE) and WB coupled to mass spectrometry were performed to characterize the purified dlβ-PARV (Magdeldin et al, 2014). For isoelectric focusing, an Immobiline® DryStrip (pH 4-7 linear, 11cm, GE Helthcare™, US) was rehydrated in rehydration buffer (8M UREA, 2% (w/v) CHAPS, 0.5 % IPG buffer and 0.002% bromophenol blue in electrodeionised water (Elix® water purification system, Merck Millipore), and 2.8% (w/v) DTT) containing 10µg of semi-purified dlβ-PARV (C1) for 15 hours. The strip was focused on an Ettan™ IPGphor™ 3 IEF System (Sigma-Aldrich™, US), for 30 min at 300V, 30 min at 1000 V, 1:20 h at 5000 V and 25 min at 5000 V until total voltage hour (Vh) reached 20,000 Vh (the equipment is shown in Fig .3 in the image labeled “2DE and MS analysis”). The strips were equilibrated in two steps: 15 min in equilibration buffer (50 mM tris-HCl pH 8.8, 6M Urea, 87% (v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol blue in electrodeionised water (Elix® water purification system, Merck Millipore) with 0.1 % (w/v) DTT and 15 min in equilibration buffer with 0.25% (w/v) iodoacetamide. The

second dimension was performed in a 15 % SDS-PAGE gel during 1:25 h at 25 mA constant and 4 °C. One of the gels was stained with Coomassie Brilliant Blue (R-250) and an image captured using an Imaging System, Azure c600 (Azure Biosystems™, US) and the replicate gel was used for transfer to nitrocellulose membrane and used for WB analysis following the procedure described in section 2.5.

The protein identification was carried out by Matrix-Assisted Laser Desorption/Ionization – Time of Flight Mass Spectrometry (MALDI-TOF/TOF). The visible spots in the Coomassie Blue stained 2D gel were excised immersed in water electrodeionised water (Milli-Q® water purification system, Merck Millipore) and analyzed by mass spectrometry by a service provider. In summary, after trypsin digestion the peptide data was acquired in positive reflector MS and MS/MS modes using a 5800 MALDI-TOF/TOF (AB Sciex, ES) mass spectrometer and using TOF/TOF Series Explorer Software v.4.1.0 (Applied Biosystems, US). For MS/MS analysis, the twenty-five most intense precursor ions from the MS spectra were chosen for analysis. Protein Pilot Software v.4.5 (ABSciex, ES) with the Mascot search engine (MOWSE algorithm), was used to analyze the raw MS and MS/MS data. The search parameters were as follows: monoisotopic peptide mass values were considered, maximum precursor mass tolerance (MS) of 50 ppm and a maximum fragment mass tolerance (MS/MS) of 0.3 Da. The search was performed against the predicted proteins from the sea bass genome database (assembly dicLab v1.0c with annotation from July 2013 and protein prediction from May 2015; file diclab1_pep.faa.gz downloaded from http://seabass.mpipz.mpg.de/DOWNLOADS/ in March 2016; Tine et al, 2014). The fixed modifications were set to be carboxyamidomethylation of cysteine, and the oxidation of methionine and N-Pyro Glu of the N-terminal Q were set as variable modifications. Protein identification was only conformed when considerable protein homology scores were achieved and at least one peptide was fragmented with a substantial individual ion score ($p<0.05$).

2.8 dlβ-PARV in silico biochemical and structural characterization

The genome of *D. labrax* available from ENSEMBLE (http://www.ensembl.org) was queried using the β-PARV protein named Parvalbumin 1 (pvalb1-201; accession nº ENSDLAP00005003855) to find possible isoforms of dlβ-PARV. Multiple sequence

alignment of the deduced amino acid sequences of dlβ-PARV isoforms and two fish species β-PARV (Atlantic cod and salmon, Saptarshi et al (2014)) were carried out and edited using GeneDoc v. 2.7.0. The editing and visualization parameters permitted amino acid sequence similarity of β-PARV to be compared and permitted identification of conserved motifs, domains and IgE or IgG epitopes by comparison with annotated β-PARV sequences and combining searches in NCBI using CD search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), Ensemble genomes (http:// www.ensembl.org), Uniprot, (http://www.uniprot.org), PROSITE (http://prosite. expasy.org/cgi-bin/prosite/ScanView.cgi) and the literature (Kobayashi et al., 2016). The consensus sequence for calcium (Ca)-binding sites were manually identified by sequence similarity with Ca-binding b-hairpin loops characteristic of the EF-hand motif as well as conserved Cys. dlβ-PARV amino acid identity and physico- chemical properties (such as theoretical Mw, pI, computed extinction coefficient and instability index) were determined using ProtParam software (https://web.expasy.org/protparam). In the present study we only report the *in-silico* analysis of the dlβ-PARV isoform sequences identified by MALDI-TOF, which were predicted to be present in purified sample.

The secondary structure and the three-dimensional structure homology model of the dlβ-PARV, were obtained using the online platform for protein structure prediction Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index; Kelley et al, 2015). The sequence of the major dlβ-PARV allergen representative isoform (Accession nº ENSDLAP00005011508) in FASTA format was chosen for the analysis and was the most intense spot in 2D gel electrophoresis and was robustly assigned to PARV by the MALDI-TOF analysis. Phyre2 uses C-score (confidence score) to evaluate the quality of models, typically in the range of [100 to 0], where 100 represents the highest confidence. The predicted dlβ-PARV model obtained in an intensive mode with the highest C-score and identity % values were retrieved and the generated PDB file was edited in the web server for molecular visualization EzMol (http://www.sbg.bio.ic.ac.uk/ezmol/), to highlight the most relevant protein regions.

2.9 Structural characterization of dlβ-PARV

Conformational changes of the purified dlβ-PARV from the different experimental conditions tested (C and HP samples, at day 1 and 11) were determined by

fluorescence spectroscopy, circular dichroism (CD) spectroscopy and Ca^{2+} depletion assays.

2.9.1 *Fluorescence emission spectroscopy*

Fluorescence spectroscopy was used to analyse the change in the tertiary structure of dlβ-PARV isolated from muscle exposed to the different experimental conditions assessed (C1, C11, HP1, HP11). Fluorescence spectra were recorded on a Fluoromax-4 spectrofluorometer (Horiba, JP) using a rectangular quartz cell with a 1 cm path length. The protein samples (1µM with or without 6M GdmCl) were excited at 280 nm and the emission spectrum recorded between 300 and 500 nm using a 10/5 nm slit width at room temperature (25°C). The baseline was set using elution buffer (20 mM Tris-HCl, pH 7.5) and all the baseline values obtained were subtracted from the raw sample values to correct for the background (adapted from Pain 1996) Scans were obtained with the FluorEssence™ software (v. 3.9, Horiba, Japan) and normalized to the maximum emission wavelength for comparison.

2.9.2 *Circular dichroism spectroscopy (CD)*

CD was used to analyze and compare the changes in secondary structure of the dlβ-PARV from the different experimental conditions tested (C1, C11, HP1, HP11). Measurements were performed in a JASCO J-815 CD spectropolarimeter (Jasco, JP) using a quartz cell with a path length of 0.1 cm (Hellma, Germany) and the Far-UV spectra were recorded between 250 – 190 nm with a scanning speed of 50 nm/min and a bandwidth of 2 nm. Pure dlβ-PARV in elution buffer (20 mM Tris-HCl, pH 7.5) was used in a final concentration of 1 µM. CD measurements were the average of three consecutive CD scans that were automatically corrected for the baseline spectrum of the elution buffer and the spectra were also normalized to the maximum emission wavelength. Results were expressed using the following equation:

$$[\theta]\mathrm{MR} = \frac{100 \text{ x } \theta}{\mathrm{C} \text{ x } \mathrm{N} \text{ x } \mathrm{I}}$$

where [θ]MR is mean residue ellipticity in deg cm2/dmol, θ is the ellipticity measured in mdeg, C is the molar concentration of the protein in g/mol, N is the number of residues, and I is the path length in cm (Applied Photophysics, 2021). The database Dichroweb

(http://dichroweb.cryst.bbk.ac.uk) was used to compare the spectra of dlβ-PARV and estimate the percentage of secondary structure types (see Annex II).

2.9.3 *Calcium depletion assay*

Since β-PARV is a Ca^{2+} binding protein and the conformation of IgE binding epitopes are maintained by Ca^{2+} binding, a calcium depletion assay was performed to verify if Ca^{2+} affects the protein conformation and how it changes in samples from the different experimental conditions tested (C1, C11, HP1, HP11). The methodology followed was as in Kuehn et al, (2010), with some modifications. In brief, each pure dlβ-PARV (10 µg) from C1, HP1, C11 and HP11 was incubated for 30 min with agitation at 4ºC, under 4 different conditions (1 – intact dlβ-PARV; 2 – dlβ-PARV with 5 mM EGTA; 3 – dlβ-PARV with 0.5 mM $CaCl_2$, and 4 – dlβ-PARV with 5 mM EGTA and 0.5 mM $CaCl_2$) and fractionated in a native PAGE (12%). The samples were mixed with 20% (v/v) of 5x native loading buffer (250 mM Tris-HCl pH 6.8, 50 % (w/v) glycerol and 0.15% (w/v) bromophenol blue) and the electrophoresis run in native running buffer (2.5 mM tris base, 19.2 mM glycine in electrodeionised water (Elix® water purification system, Merck Millipore) at 25 mA constant per gel for 1:10 h. A WB analysis following the same procedure as previously described (*see* 2.5) was performed, the main modification was the use of a nondenaturing transfer buffer (25 mM Tris Base, 150 mM Glycine, 10% (v/v) methanol in electrodeionised water (Elix® water purification system, Merck Millipore)). The images were captured with the Azure c600 Imaging System (Azure Biosystems™, US).

3 Results

3.1 Effects of HP processing and/or storage time on the global proteome and on dlβ-PARV detection

As a first approach, the global proteome of the white muscle sarcoplasmic fractions of sea bass fillets from different experimental conditions, non-processed and 600 MPa/5 min pressurized samples and stored for up to 11 days (C1, C11, HP1 and HP11) were analyzed to evaluate the effect of the HP and/or storage time. The solubility of the total protein was evaluated, and HP caused a 6-fold reduction in solubility (Fig. 4A). In Fig. 4B, the effect of HP on the sarcoplasmic protein profile is clear compared to the control samples. SDS-PAGE revealed an increase in abundance of proteins with low molecular weight (<35 kDa) in the processed samples, while the high molecular weight bands were evident and increased in quantity in control samples across the storage time. HP treated samples had a significant reduction in the content of high molecular weight proteins (<35kDa), since only three main bands are visible above 35 kDa. The target of the present study was the sea bass sarcoplasmic dlβ-PARV (dotted rectangle in Fig. 4B), which is a protein of low MW (~ 12 kDa), that has been described as a major allergen of the muscle in different fish species (Kobayashi et al, 2016).

A band with the expected size of dlβ-PARV is visible in representative sample of the 4 experimental groups. A specific and single immunoreactive dlβ-PARV protein signal with a MW of the expected size was detected using a monoclonal antibody PARV-19 in sea bass sarcoplasmic extracts, in all experimental conditions (Fig. 4C). The WB results confirmed the presence of dlβ-PARV, but the chemiluminescence signal intensity of dlβ-PARV was apparently weaker in C1 samples, which was not totally evident from the SDS-PAGE analysis (Fig 4B). Processed samples have similar protein profiles in the Coomassie blue stained gels, but HP1 had a more intense immunoreactive signal based on the results presented in Fig.4C, which may suggest that the treatment causes unfolding of the protein and unmasked hidden epitopes. The chemiluminescence signal intensity of the HP1 sample, was corroborated by the intensity of the Coomassie blue stained band in SDS-PAGE (Fig. 4B). Both control samples (C1 and C11) had a similar band intensity in the Coomassie blue stained polyacrylamide gel, but after 11 days in storage there was a slight increase in the signal detected by WB, so in this particular case, the storage time

seems to disturb the dlβ-PARV. It is important to highlight that no aggregation or degradation of the protein was observed.

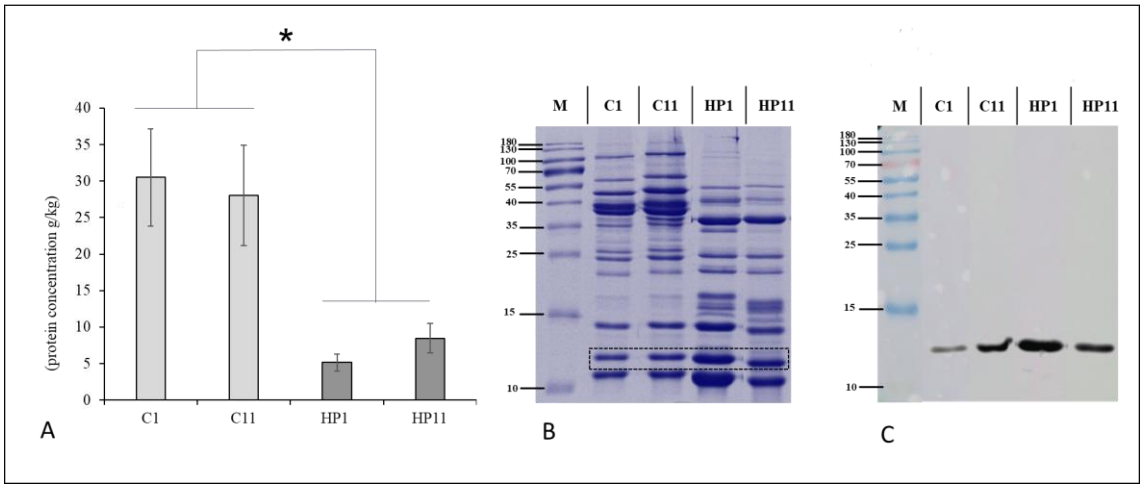

Figure 4 – Panel illustrating the results for the effects of HP and storage time on the proteome of the white muscle sarcoplasmic fraction of sea bass fillets and dlβ-PARV detection by immunoblotting. A) Bradford protein quantification of soluble sarcoplasmic proteins (mean ± SEM of n = 8 individuals per treatment). * Indicates significant differences ($p < 0.05$) in HP treated samples compared to the control samples. B) Coomassie blue stained 1D SDS-PAGE (15 %) of soluble sarcoplasmic proteins from 8 pooled individual samples (20 µg per lane) per experimental condition. C) Western blot under reducing conditions detecting sea bass dlβ-PARV using the antibody PARV-19. M: Molecular weight marker (kDa); Dot square: expected MW for dlβ-PARV

3.2 Analysis of the purified dlβ-PARV

dlβ-PARV was successfully purified from the sea bass white muscle sarcoplasmic protein extracts (n = 8 pooled fillets/experimental condition) and from all the individually experimental conditions (C1, C11, HP1, HP11), by CEE. To our knowledge this is the first time that this technology was used for the purification of PARV. The yield of the purification process, which relates mg of purified dlβ-PARV and mg of total sarcoplasmic protein extracted, (Annex I) was 9.31% (C1), 13.95% (HP1), 31.81% (C11) and 32.15% (HP11). After purification and concentration, the isolation of the dlβ-PARV protein was considered to be successful since it was the most representative band in the purified proteins analyzed by SDS-PAGE and is visible as a strong band across all the experimental conditions (Fig. 5A). Additional residual protein bands were also detected in the purified protein fractions.

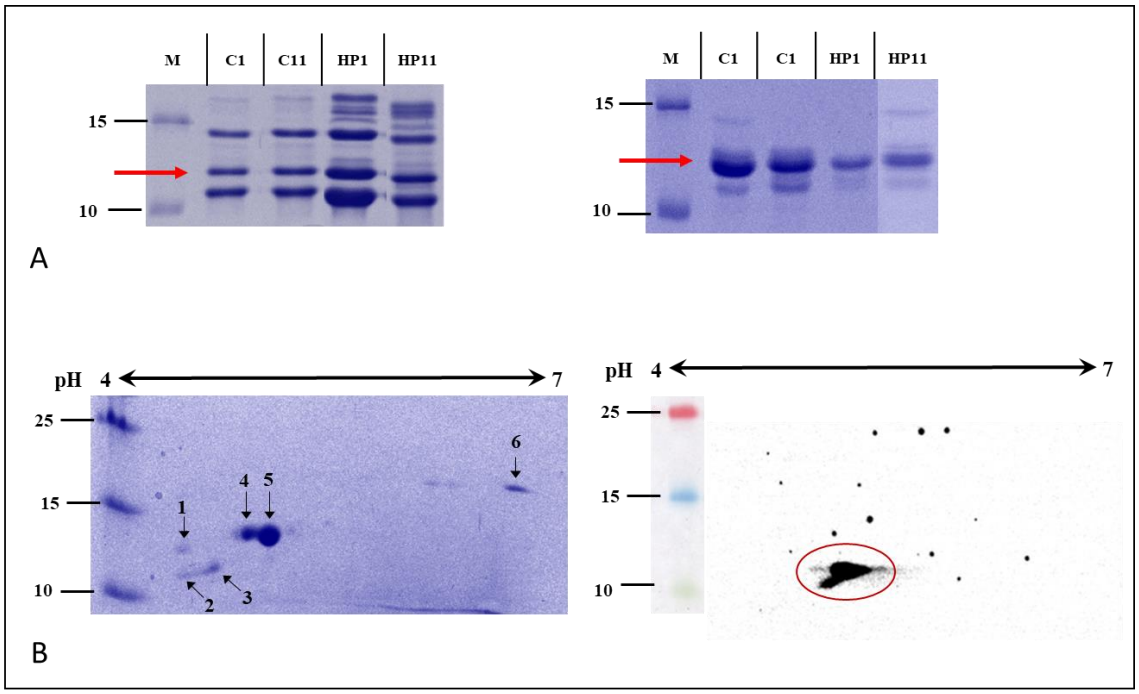

Figure 5 – – Purification and analysis of dlβ-PARV from all the experimental conditions tested. A) Coomassie blue stained 1D SDS-PAGE (15 %) of pooled (n = 8) extracts before and after dlβ-PARV purification by continuous elution electrophoresis. The red arrows indicate the band of interest. M: Molecular weight marker (kDa); B) Two-dimensional gel electrophoresis (2D) stained with Coomassie blue on the left-hand side and Western blot of the purified dlβ-PARV from the sarcoplasmic fraction of the control sample (C1) using the monoclonal anti-PARV19 (on the right). Excised spots sent for analysis are indicated by black arrows and numbers (1-6). Different potential dlβ-PARV isoforms gave a positive chemiluminescent signal and are indicated within the red circle.

Since fish were used for the study and they have undergone a whole genome duplication event (Glasauer & Neuhauss, 2014) and taking into consideration that several β-PARV isoforms have been identified in different fish species (Pérez-Tavarez et al, 2021), we investigated the existence of dlβ-PARV isoforms in the purified protein fraction of the C1 sample by 2DE and WB coupled to mass spectrometry. The 2D gel revealed the complex nature of the isolated dlβ-PARV (Fig. 5B), several protein spots were identified by 2D SDS-PAGE in the expected vicinity of the β-PARV protein, which had different intensities, sizes, and isoelectric points (IP's). Around six protein spots (1, 2, 3, 4, 5, 6) were detected by Coomassie blue staining but fewer (4, 5) were immunoreactive with the monoclonal antibody PARV-19 (Fig. 5B). It was predicted that β-PARV isoforms, should have a molecular weight around 10-15 kDa, which corresponded to the region of the polyacrylamide gel where most of the protein spots were localized. It was clear that protein spot number 5 was the most abundant and

immunoreactive in the Western blot, as shown in Fig.5B. For instance, the protein spot assigned to number 6, besides not being reactive, was much bigger than the predicted size and IP for dlβ-PARV.

Table II – Results from mass spectrometry analysis of protein spots excised from a 2-DE gel (see Fig. 5B). The table lists the protein spots identified by MALDI-TOF/TOF (MS/MS) using the mass peak spectra of the tryptic peptides used to search the sea bass database with the MASCOT search engine via Protein Pilot Software v. 4.5 (ABSciex).

Spot	Acession Number [c]	Sequence coverage (%)	Mascot Score [a]	P-value [b]	Identified peptides
1	DLAgn_00189240_1	14	123	5,017 E^{-13}	K.LFLQNFK.A, K.AFFIIDQDK.S
2	DLAgn_00099060_1	16	92	6,307 E^{-10}	K.YKDFFAK.V, K.LFLQNFSAGAR.A
3	DLAgn_00099060_1	10	41	7,948 E^{-05}	K.LFLQNFSAGAR.A
4	DLAgn_00189240_1	14	73	5,017 E^{-08}	K.LFLQNFK.A, K.AFFIIDQDK.S
5	DLAgn_00189240_1	14	105	3,168 E^{-11}	K.LFLQNFK.A, K.AFFIIDQDK.S

a) Protein scores greater than 57 are significant ($p<0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits. b) p-value calculated as $10^{-0.1*score}$. c) The accession numbers are from genome assembly of *Dicentrarchus labrax* (dicLab v1.0c; available in: http://seabass.mpipz.mpg.de/)

The identity of the spots observed in Fig. 5B corresponding to the purified dlβ-PARV from the sarcoplasmic fraction of the control sample (C1) was confirmed by MALDI-TOF/TOF analysis. Table II summarizes the MS results. The MASCOT blast search revealed that the amino acid sequence for spots 1, 2, 4 and 5, had the best match with the sequences predicted for dlβ-PARV alternative isoforms or modified protein forms. The MS fingerprint spectra of spots 1, 2, 3, 4 and 5 were composed of the tryptic peptides identified in Table II. The protein spot 3 had a protein score smaller than 57, so the result

was not significant, and the protein was not assigned to dlβ-PARV. Spots 1, 2, 4 and 5 had hits for PARV in the Ensembl database. Spots 1 and 4 were identified as the same sequence for Parvalbumin 4 (pvalb4 - 202, accession nº ENSDLAP00005011517), both with 14 % sequence coverage. Protein spot 5 had a hit for Parvalbumin 4 (pvalb4 – 201, accession nº ENSDLAP00005011508) with 14 % coverage and protein spot 2 was identified as the PARV sequence with the accession nº ENSDLAP00005045785, covering 16 % of the sequence. In the 2D and the corresponding WB (Fig. 5B), the most intense protein spot with a strong signal (spot 5) was confirmed by MS to be the most prevalent (indicated as the Parvalbumin 4 from *Dicentrarchus labrax* in Ensembl database, with the accession code ENSDLAP00005011508).

3.3 dlβ-PARV in silico characterization

All the β-PARV transcripts from *Dicentrarchus labrax* extracted from the Ensembl database and two β-PARV from bony fish species were biochemically characterized in silico. Atlantic cod (Gadus morhua) and Atlantic salmon (Salmo salar) were chosen for comparative sequence analysis because β-PARV is an allergen well characterized in those fish species (Van Do et al, 2003; Lindstrøm et al, 1996). In Table III the main biochemical and biophysical proprieties of the different 13 PARV isoforms from the *D. labrax* (retrieved from the Ensembl database) are summarized. The theoretical values obtained for the isoelectric point (pI) and molecular weight (MW) of the predicted isoforms of dlβ-PARV were estimated to be between 4.45 and 6.19 for the pI, and between 11.25 and 17.19 kDa for the MW.

The extinction coefficient had values with significant differences among the different isoforms because of the variable number of Trp, Tyr and Cys residues, or their absence. It was not possible to determine extinction coefficients for all sequences since one of them did not possess any of the mentioned residues (when only one or two of the 3 residues were present, the extinction coefficient was computed with a significant error (>10%)). The dlβ-PARV isoforms predicted by MS to be present in the purified fraction (shown with red boxes in Table III) contained 1 Tyr, 0 Trp and 11 Phe (pvalb4-201), 0 Tyr, 0 Trp and 10 Phe (pvalb4-202) and 1 Tyr, 0 Trp and 10 Phe (ENSDLAP00005045785). The Instability Index values of the isolated dlβ-PARV isoforms purified varied between 21.77 and 45.60 with three of the isoforms being considered unstable molecules.

Table III – Biochemical proprieties of the all the PARV isoforms purified from *D. labrax* and two PARV sequences from two other bony fish species, using the ProtParam webserver (https://web.expasy.org/protparam/). *D. labrax* isoforms were used to search the Ensembl database, and the two bony fish species sequences were procured in the UniProt database. The protein spots identified in the MS analysis (Table II) in *D. labrax* genome, match with the protein name and accession number highlighted in the red boxes.

Organism	Name	Acession number (Ensembl or UniProt)	MW (kDa)	PI	Extinction coefficients	Instability Index
European sea bass (*D. labrax*)	Parvalbumin 1 (pvalb1-201)	ENSDLAP00005003855	11,57	4,47	1615	33,40 (stable)
	Parvalbumin 4 (pvalb4-201)	ENSDLAP00005011508	13,44	5,79	1615	27,42 (stable)
	Parvalbumin 4 (pvalb4-202)	ENSDLAP00005011517	11,32	5,63	125	30,66 (stable)
	Parvalbumin 4 (pvalb4-203)	ENSDLAP00005011532	15,44	5,14	5875	28,36 (stable)
	Parvalbumin 5 (pvalb5-201)	ENSDLAP00005011559	11,25	4,47	1615	45,60 (unstable)
	Parvalbumin 6 (pvalb6-201)	ENSDLAP00005008682	11,89	4,68	1490	22,78 (stable)
	Parvalbumin 6 (pvalb6-202)	ENSDLAP00005008706	13,56	6,19	1490	21,77 (stable)
	Parvalbumin 7 (pvalb7-201)	ENSDLAP00005004149	11,98	4,54	Unable to calculate	23,37 (stable)
	Parvalbumin 8 (pvalb8-201)	ENSDLAP00005011473	11,86	4,54	250	47,62 (unstable)
	Parvalbumin 9 (pvalb9-201)	ENSDLAP00005028704	17,19	4,63	20065	40,63 (unstable)
	–	ENSDLAP00005045797	12,84	4,72	2980	32,39 (stable)
	–	ENSDLAP00005045785	11,49	4,45	1490	23,74 (stable)
	–	ENSDLAP00005045810	11,94	4,92	5500	38,69 (stable)
Atlantic cod (Gadus morhua)	Parvalbumin beta	Q90YK9	11,55	4,58	0	22,30 (stable)
Atlantic salmon (Salmo salar)	Parvalbumin beta 1	Q91482	11,88	4,95	250	21,78 (stable)

Saptarshi et al (2014) compared fish PARV between various species from distinct orders, in different parameters. One of the achievements was the successful linkage between the immunological cross-reactivity of PARV and the corresponding phylogenetic nature of the species based on the amino acid sequence and using a consensus sequence, the authors predicted the monoclonal anti-Parv19 epitope. A multiple sequence alignment with all the dlβ-PARV (including the sequences identified in the MS analysis as belonging to the purified protein), the two PARV sequences from the two other bony fish species and with the described domains/motifs based on literature searches (Saptarshi et al 2014) is shown in Fig. 6. The monoclonal anti-PARV19 epitope is a region of 20 aminoacids, located between the 13th residue (Lys) and the 39th residue (Gly) in the protein and is boxed within a blue outline. The level of conservation was similar between the sea bass sequences and the other fish sequences. The identified epitope region was also conserved across all compared sequences as observed in Fig 6. Less conserved regions are observed in the N-terminal region and in the very divergent C-terminal region of the molecules. The 4 known IgE epitopes are identified within green boxes and two of them share a part of the monoclonal anti-PARV19 epitope (1st to 18th residues and 24th to 45th). Which means the potential IgE reactivity could be shared with the monoclonal anti-PARV19. The two Cys residues that form a cysteine bond are indicated (19th and 34th residues) and are conserved except in 3 out of 13 sea bass sequences The EF-hand motif regions are highly conserved and the calcium binding sites (marked within red boxes) are very conserved regions, which confirms the overall stability of this protein.

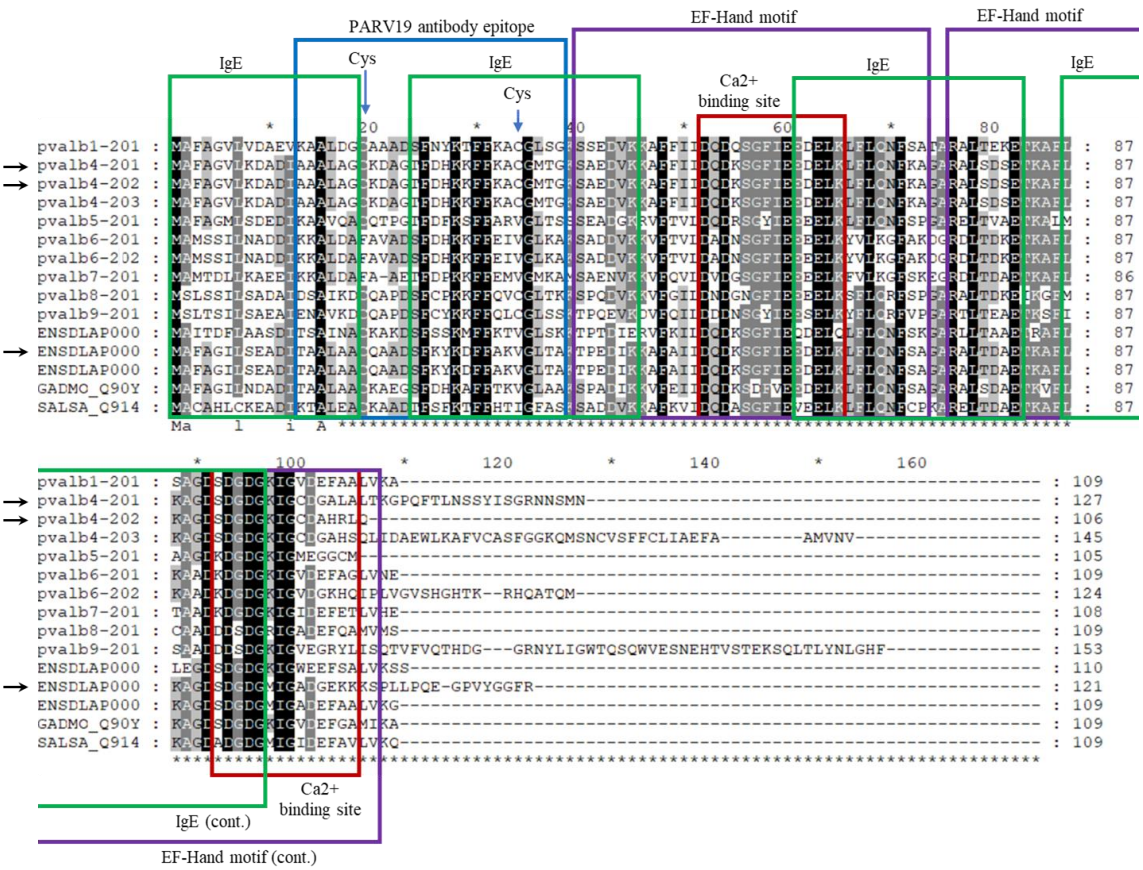

Figure 6 – Multiple sequence alignment of the 13 dlβ -PARV sequences retrieved from the Ensembl database and two β-PARV sequences from bony fish species. GADMO_Q90Y is from the Atlantic cod (Gadus morhua) with the UniProt accession number Q90YK9, and SALSA_Q914 is from the Atlantic Salmon (Salmo salar) with the UniProt accession number Q91482. Conservation is shown using shading, where the most conserved residues are darker and less conserved are lighter. The regions marked within boxes are based on the results of Saptarshi et al (2014). The two vertical arrows indicate the cysteine residues, and the horizontal arrows indicate the dlβ-PARV sequences revealed in the MS analysis.

To the best of our knowledge, the *D. labrax* β-PARV three-dimensional structure has not yet been resolved. The MS results revealed one dlβ-PARV isoform to be more abundant in the white muscle sarcoplasmic fraction than the remaining predicted isoforms, namely the Parvalbumin 4 isoforms (pvalb4-202, accession nº: ENSDLAP00005011517 and spot nº5 in Fig.5). The best 3D model template, belonging to the carp species Cyprinus carpio within PBD ID 1B8L, from the Phyre2 fold library (http://www.sbg.bio.ic.ac.uk/phyre2/html/flibview.cgi?pdb=d1b8la) was chosen to be edited in the EzMol web site (http://www.sbg.bio.ic.ac.uk/~ezmol/).

According to the output result (Fig. 7A), Parvalbumin 4-201 has α-helix as the main secondary structure, and it corresponds to more than 50% of the molecular

secondary structure, with a high level of confidence (<70%). The Phyre2 server does not predict β-turns or β-bends (which are treated as coils), or π-helices and 3_{10}-helices (which are merged into the α-helix category). Only the 25th (Phe) and the 26th (Asp) amino acid seem to form a β-strand and this structure accounted for only 2% of the molecule. The rest of the protein is most likely to be random coil, representing 42% of the sequence, disordered regions accounts for 25% of the protein and the weakest α-helix regions are often associated with disordered regions Fig. 7A.

The three-dimensional model and secondary structure of the dlβ-Parvalbumin 4-201 (Parvalbumin 4- 201, accession nº: ENSDLAP00005011508) is shown in Fig. 7B and Fig. 7C, respectively. Phyre2 compares the input sequences with multiple 3D model templates, and the best match was the template d1b8la (in PBD with the ID 1B8L), with 84% sequence coverage, having the best C-Score (99.9%) and identify (76%). The Parv19 antibody epitope (in blue in fig. 7C) is mainly composed of alfa-helix secondary structure which explains in part the stability of the allergenicity even when tissues or protein extracts are exposed to harsh conditions.

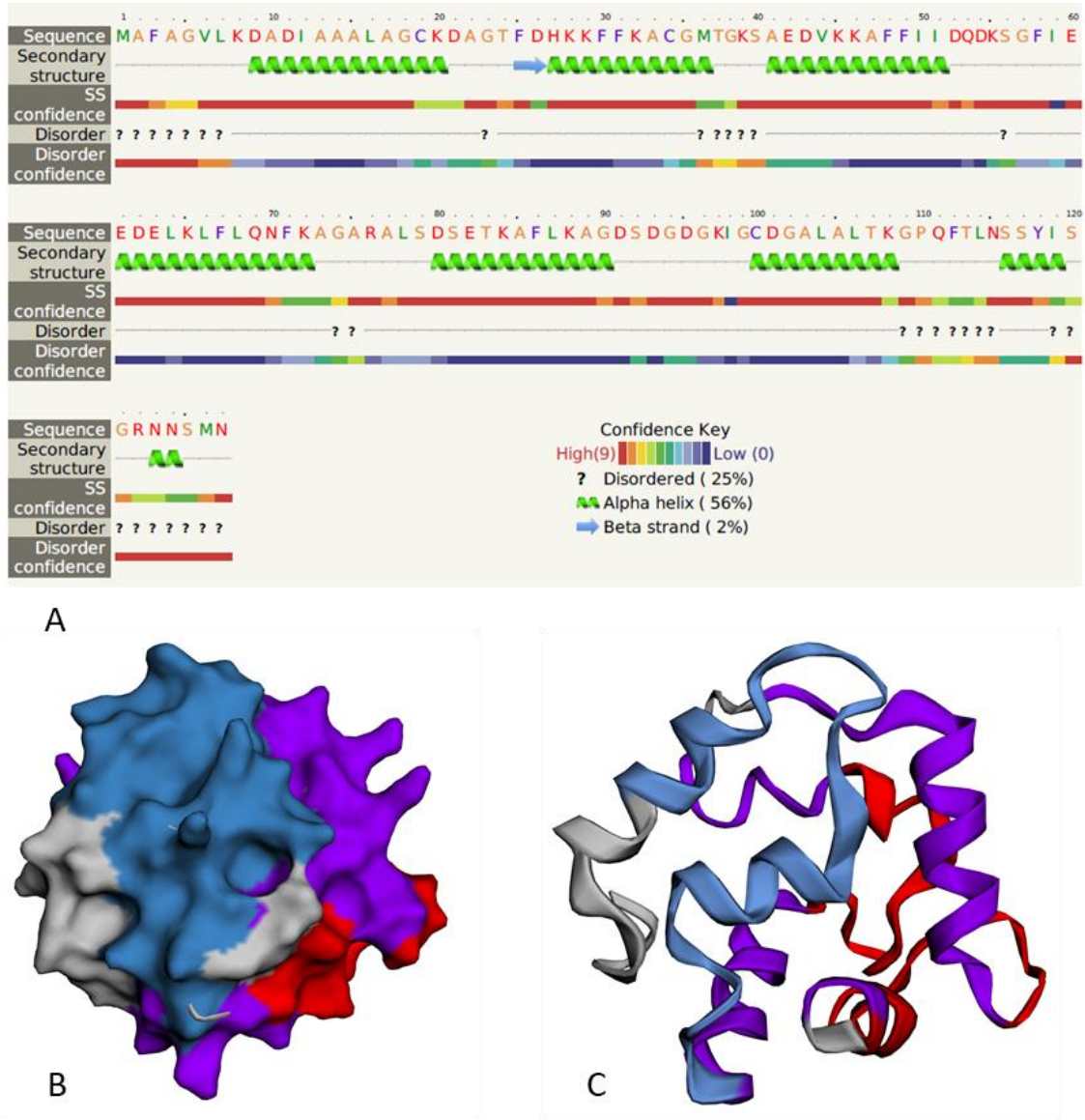

Figure 7 – (A) Predicted secondary structure for the dlβ-PARV isoform sequence Parvalbumin 4 (accession nº: ENSDLAP00005011508) analyzed in the Phyre2 software. It was possible to map each amino acid to their role in the protein secondary structure. The green lines correspond to α-helix, the blue arrow is β-pleated sheets, and the faint lines represent random coil. (B) Three-dimensional conformation of Parvalbumin 4 (accession nº: ENSDLAP00005011508) showing the molecule surface; (C) – Representation of the secondary structure prediction. The structural images in (B) and (C) were created based on the template "d1b8la_" (PBD ID: 1B8L) from the Phyre2 fold library (http://www.sbg.bio.ic.ac.uk/phyre2/html/flibview.cgi?pdb=d1b8la_) and using the EzMol server. The Parvalbumin 4 (pvalb4-202) sequence was used to interrogate Phyre2 and the 3D structure model template with the best match was input in the EzMol platform. The colors were added manually to each residue to show relevant regions. Blue – PARV19 antibody epitope; purple – EF-hand domains; red – calcium-binding sites.

*3.4 Effects of HP processing and/or storage time on dl*β*-PARV protein structure*

3.4.1 Pressure and storage time influences tridimensional conformation

Intrinsic fluorescence emission (arising from tryptophan, tyrosine, and phenylalanine in proteins) was used to analyse how the tertiary structure of dlβ-PARV changed in response to HP processing and/or with storage time in line with the experimental conditions assessed. Zero tryptophans, one tyrosine and eleven phenylalanine residues were predicted in the dlβ-PARV sequence under analysis (assuming the more abundant dlβ-PARV isoform was Parvalbumin 4-201, accession nº: ENSDLAP00005011508 corresponding to the protein spot nº5 in 2DE results in Fig. 5). The remaining 2 dlβ-PARV isoforms detected by MS in the C samples, were predicted to possess, respectively, 1 and 0 tryptophan's, 0 and 0 tyrosine's and, 10 and 10 phenylalanine's, a content of aromatic amino acids that even if less abundant than in Parvalbumin 4-201 permit structural analysis by intrinsic fluorescence emission.

dlβ-PARV proteins from C1, C11, HP1 and HP11 samples were excited at 280 nm to measure the tyrosine emission spectrum between 300 and 500 nm using a 10/5 nm slit width. dlβ-PARV had a tertiary structure with an emission maximum of 339 nm (C1), 341 nm (C11), 351 nm (HP1), and 345 nm (HP11), indicating some burying of tyrosine residues mainly in C samples (Fig. 8). In the C samples the storage time did not affect the tertiary structure of dlβ-PARV. However, a shift in emission maximum was seen immediately after high pressure processing (HP1) from 339 to 351 nm in comparison with C1, indicating that pressure tends to promote the unfolding of dlβ-PARV. Eftink M. R. (1994) states that, generally, proteins that suffer denaturation shift the maximum of fluorescence intensity to a wavelength equal or higher than 350 nm. The outcome of the exposure of dlβ-PARV isolated from the C1 and HP1 samples to GdmCl (6M) was indicative of tertiary structure unfolding and a strong maximum emission shift occurred from, 339 to 354 nm and from 351 to 355 nm, indicating that the protein was susceptible to chemical unfolding (results not shown). The interesting observation in Fig. 8 is the spectra of the control and HP sample, since HP1 had a maximum emission at 351 nm, while HP11 had a maximum emission at 345 nm, which was more similar in the latter case to the C samples. The results of the intrinsic fluorescence emission spectroscopy indicate that HP processing causes a change in the tertiary structure but that after 11 days of storage the native conformation of the protein is reacquired.

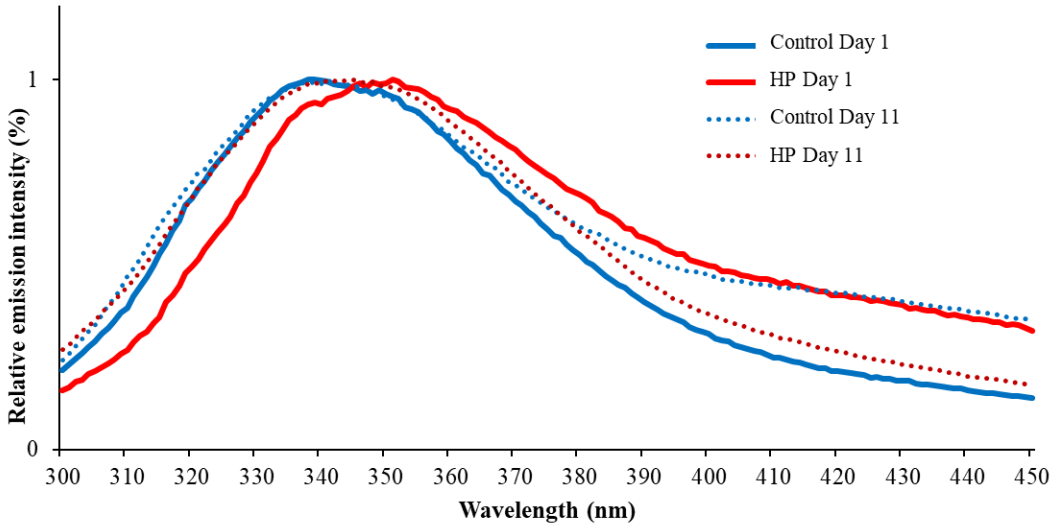

Figure 8 – Fluorescence emission spectra (λ_{exc}=280nm) of pure dlβ-PARV protein from the different experimental conditions tested (non-processed and 600 MPa/5 min pressurized samples and stored for up to 11 days, C1, C11, HP1 and HP11).

3.4.2 Secondary structure effects caused by HP processing and storage time

Far-UV CD spectra indicated that the dlβ-PARV protein possessed secondary structure in the control and pressurized sample. Two negative peaks (at 208 nm and at 222 nm) and one positive peak (190 nm) were observed (Fig. 9). According to Adler A.J. et al (1973) this is a typical spectrum for a protein with α-helix as the main secondary structure. The differences between the spectra are in the intensity of the peaks. C1 had the bigger values of MRE suggesting a greater prevalence of α-helix in dlβ-PARV isolated from both the control and HP-treated fish fillets. C1 and C11 maintained the same spectrum, but the MRE values were reduced to almost the half in C11 compared to C1. When the spectra from the processed samples were compared dlβ-PARV isolated from HP11 fish fillets had higher peaks of emission for the α-helix than dlβ-PARV isolated from the HP1 fish fillets, which suggests the protein folding recovered after the initial unfolding 1 day after HP processing and acquired the native state 11 days after storage (Fig.8). Generally, dlβ-PARV isolated from HP1 had a similar spectrum but lower MRE values than C1 and HP11. This could be interpreted as a loss of secondary structure of dlβ-PARV isolated from fish fillets exposed to high pressure. HP11 had similar MRE values to the C1 sample, in the 3 peaks described for α-helix, the predominant secondary structure of dlβ-PARV predicted in the 3D model (Fig.7A). If the loss of secondary

structure of dlβ-PARV exposed to HP and the recovery of the native structure after 11 days storage is associated with a shift in protein allergenicity remains to be determined.

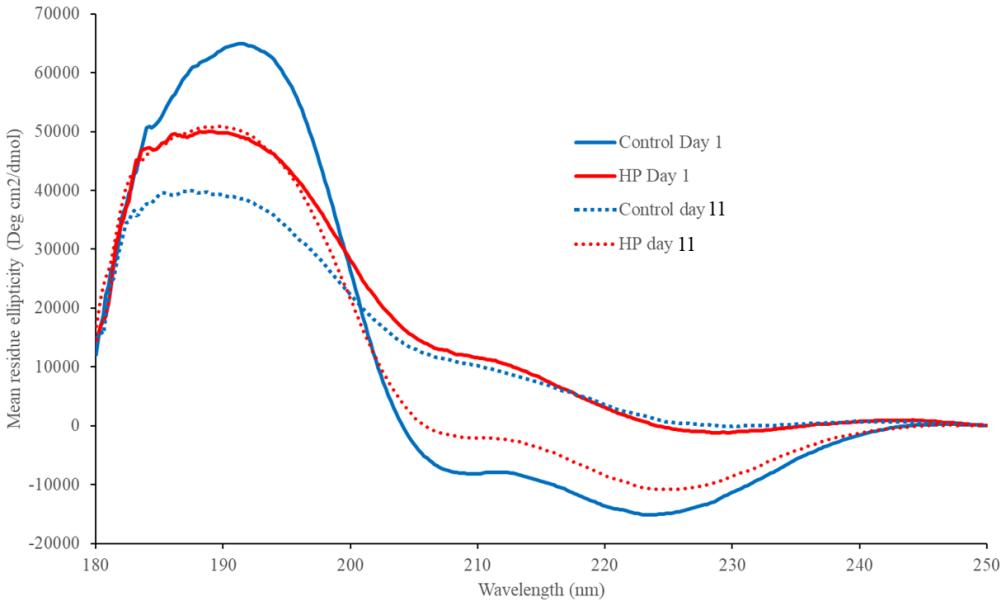

Fig. 9 – Far-UV CD spectra of (1 μM) pure dlβ-PARV from different experimental conditions tested (non-processed and 600 MPa/5 min pressurized samples and stored for up to 11 days, C1, C11, HP1 and HP11). in 20 mM Tris-HCl, pH 7.5at 25 °C, scanned from 250 to 180 nm.

The Far-UV CD spectra of pure dlβ-PARV isolated from fish muscle exposed to different experimental conditions (C1, C11, HP1 and HP11) was compared using the web server Dichroweb (http://dichroweb.cryst.bbk.ac.uk), and the algorithms SELCOM3 AND CONTILL but the results obtained did not agree with the experimentally obtained spectra. (Annex II - For each condition and algorithm, the sum of all values is 1, for C1 and HP11, β-strand is said to be the main secondary structure, an outcome that goes against the predicted results according to bibliography).

3.4.3 Effect of calcium in the dlβ-PARV structure

To access the effect of calcium on the dlβ-PARV conformation and how it changed after HP processing and/or time of storage a calcium depletion assay was performed as shown in Fig. 10A and B. For each of the four experimental conditions (C1, HP1, C11, HP11), four different incubation conditions were performed, (based on Kuehn et al, (2010)). In Fig. 10, intact samples refer to samples without any treatment, serving

as a control. For the depletion the calcium, EGTA was used because is a good calcium chelator (Miller & Smith, 1984) since it is able to dissociate the calcium ions from the proteins. $CaCl_2$ was added to confirm the presence of calcium because it is unknown whether the metal is present in the intact samples. In Fig. 10A, a clear protein band was observed for dlβ-PARV isolated from C1 fish samples, and the isolated protein had a similar pattern in the presence or absence of Ca^{2+}. A shift in the position of the protein in SDS-PAGE towards a lower molecular weight was observed under Ca^{2+} depletion by EGTA, which is coherent with a shift in protein conformation. A similar transition in dlβ-PARV conformation was visible for the protein isolated from fish muscle exposed to the other experimental conditions, HP1, C11 and HP11, although the detection of multiple protein bands, was indicative of an additional effect on the protein of HP and storage time at 1 and 11 days (Fig. 10A).

The binding ability of dlβ-PARV to the monoclonal antibody anti-parvalbumin clone PARV19 (known to have Ca^{2+} dependent epitope, Bugajska-Schretter et al, 2000) across the four incubation conditions for all experimental conditions is shown in Fig. 10B. For both the unprocessed samples, there is no apparent effect on the intensity of the signal of the time of storage on the dlβ-PARV immunoreactivity under calcium depletion. Comparison of the intensity of the signal of the samples at day 1, revealed C1 had a single immunoreactive protein as seen in the Coomassie blue stained gel (Fig. 10A). However, in the samples of HP day 1, although a single band was detected in the Coomassie blue stained gel (Fig. 10A), the immunoreactive signal for the dlβ-PARV isolated from C11, HP1 and HP11 did not have discrete bands but was characterized by a smear (Fig. 10B). This outcome suggests that HP and storage affect the EF hand motifs in dlβ-PARV, which are responsible for the calcium ion binding.

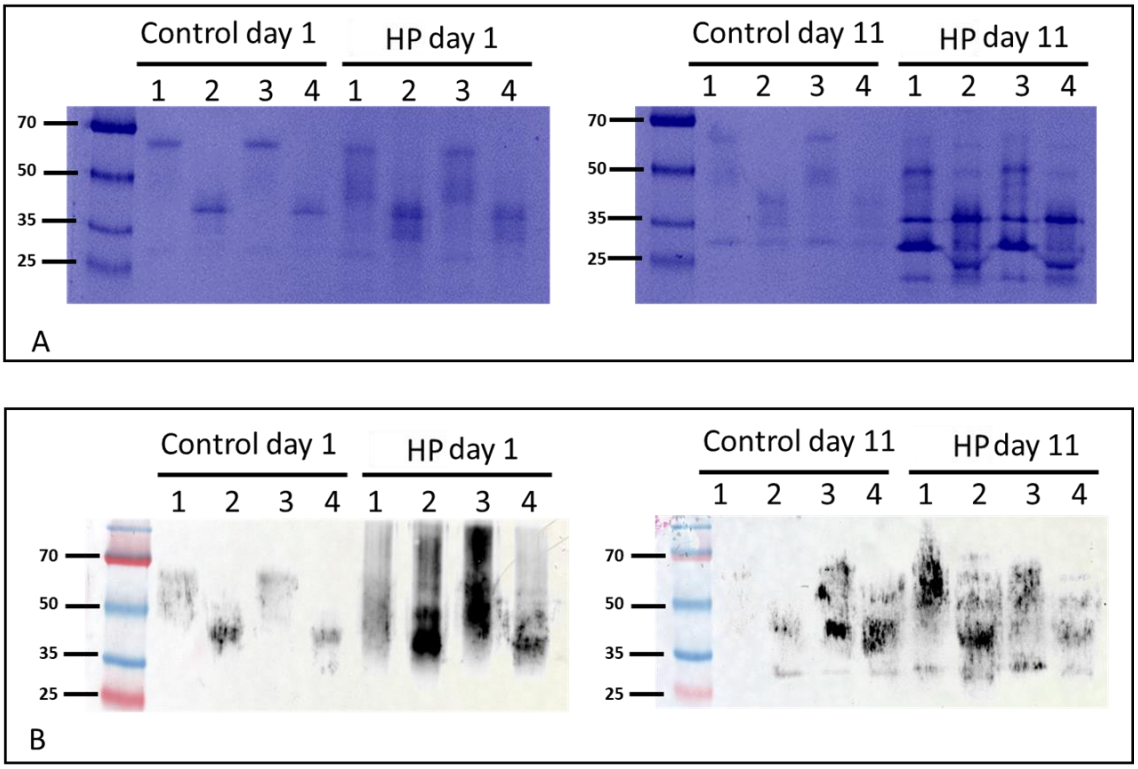

Figure 10 – Calcium depletion assay. Coomassie blue stained native gel (12%) (A) and the corresponding Western blot (B) of the purified dlβ-PARV from the different experimental conditions (C1, C11, HP1, HP11) with or without Ca^{2+} depletion. The dlβ-PARV was identified with monoclonal anti-PARV19. This assay was used to assess if the absence of calcium affects antibody reactivity. 1 – intact dlβ-PARV; 2 - dlβ-PARV with 5mM EGTA; 3 – dlβ-PARV with 0.5mM CaCl2; 4 – dlβ-PARV with 5mM EGTA and 0.5mM CaCl2.

4. Discussion and conclusion

Fish is a valuable source of high-quality protein in human diet, and its consumption is expected to increase with the growth of world population combined with the need for alternative protein sources. However, fish consumption constitutes a potential risk of allergenicity, bigger than meat consumption (Wilson & Platts-Mills, 2018) and so far 70-95% of the recorded cases are elicited by fish muscle β-PARV. Since fresh fish is highly perishable novel processing technologies are being investigated as a means to extend the shelf life and reduce waste and at the same time improve the quality and safety of fish products. Sea bass (*D. labrax*) is one of the most produced and consumed species in the Mediterranean area, but few studies exist about the feasibility of processing technologies to prolong shelf-life and there are even fewer studies about its allergenicity potential. This highlights the need for optimized standard operating procedures for the development of high quality and safe sea bass products. For these reasons, the present thesis was dedicated

to studying the effects of high-pressure processing and/or storage time on the structure and allergenicity potential of β-PARV, a putative allergen, in sea bass fillets. The experimental conditions tested were non-processed and 600 MPa/5 min pressurized samples that were stored for up to 11 days (C1, C11, HP1 and HP11) under refrigerated conditions to simulate conditions experienced in a supermarket. The values of pressure intensity and storage time were chosen based on previous studies (Tsironi et al, 2015; Anjos et al., 2019), in which sea bass fillets were shown by sensorial and microbiological analysis to be acceptable for consumption under these operating procedures.

As a first approach, comparison of protein profiles via solubility and SDS-PAGE analysis allowed an assessment of the impact of HP and/or storage time on the global proteome of the sarcoplasmic fraction of the white muscle of sea bass fillets. SDS is an anionic detergent that makes proteins negatively charged after binding, causing the molecular weight to be the principal factor influencing their behaviour and position in the polyacrylamide gel during electrophoresis (Nowakowski et al, 2014). This factor is crucial for WB analysis, because the signal given by the chemiluminescent reaction is expected to appear as a single protein band in the position of the predicted molecular weight of β-PARV. One band does not necessarily correspond to a monomeric form as there is the likelihood of the protein to be present as protein aggregates.

The results indicated that HP, and not storage time, induced a significant reduction in protein solubility in the muscle samples compared to the control muscle samples (Fig. 4A). Since proteins with a less-compact structure are likely to more soluble (Sathe et al, 2018) the reduced protein solubility observed may be the first evidence of protein unfolding induced by pressure. Similarly, the proteome profiles of the white muscle soluble sarcoplasmic fraction were affected by HP treatment but not storage time (Fig. 4B). Low molecular weight protein bands that were poorly detectable in the protein extracts of the control muscle were clearly detectable in HP processed samples, while the high MW protein bands disappeared from the protein extracts of those samples (HP processed). These results may indicate that pressure may have caused the breakdown of some proteins into smaller peptides. Other possible effects of HP that explains the reduced protein solubility may be protein denaturation and the formation of protein aggregates in fish muscle, which is a well-known and irreversible consequence of thermal treatments. However, it has been suggested that in pressure processing protein denaturation is

unlikely to happen (Somkuti et al (2013) and our spectroscopy results with the soluble proteins agree with this, although of course insoluble proteins would not be represented in the fish muscle extracts. In fluorescence emission spectroscopy and Far-UV CD spectroscopy both secondary and tertiary structures observed in the Control samples were lost in the HP treatments at day 1 but recovered after 11 days of storage. However, with only these data, it was not possible to access how closely identical the recovery of structural proprieties when compared to the original protein conformation. Future studies will be required to explore this further.

Most patients allergic to fish have anti-β-PARV IgE that cross react with the protein from different fish species. Currently there is no antibody available for specific detection of fish allergens. The PARV-19 antibody has been reported to bind to a range fish parvalbumins and the literature does not have much to say about its specificity in terms of fish species. Authors like Saptarshi et al (2014) or Gajewski et al (2009) propose anti-PARV-19 for the detection of fish parvalbumin since the immunoreactivity of this monoclonal antibody has already been shown for a wide range of fish parvalbumins. In the present study the major allergen in fish muscle, β-PARV (Fig. 4C) was identified by WB in sea bass muscle using anti-PARV-19 and revealed a single immunoreactive protein band of the expected size (12kDa), in all protein extracts from fish exposed to different experimental conditions tested (C1, C11 HP1 and HP11). This is the first time that the immunoreactivity of anti-β-PARV-19 has been demonstrated with European sea bass (*Dicentrarchus labrax*) dl β-PARV. This indicates that the monoclonal antibody PARV-19, recognized the allergenic epitope in sea bass β-parvalbumin, an expected outcome based on the high conservation of sea bass protein with the allergen epitope previously identified between fish species (Kuehn et al., 2014). Furthermore, PARV-19 has previously been shown cross-react with PARV in different fish species, including Pacific and horse mackerel, red sea bream, sardine, carp, catfish, cod (Kobayashi, et al. 2006), although it fails to detect the allergen in the yellowfin tuna. In our preliminary results we speculate that HP (particularly at 1 day) and storage time may affect the structure of dlβ-PARV, and its allergenicity as revealed by the differences in the immunoreactive β-PARV signal intensity in comparisons with C1 (Fig.4C). This may indicate that in fish muscle exposed to high pressure the conformation of dlβ-PARV changed and the allergenic epitope became slightly more available to react with the

antibody. Shriver et al (2011) previously proposed that modifications in protein conformation caused by HP, was likely to modify protein allergenicity, regarding IgE-reactive conformational epitopes.

The target allergen, dlβ-PARV was purified from sea bass white muscle by preparing sarcoplasmic protein extracts for individual samples from all experimental conditions (C1, C11, HP1, HP11), using CEE (PREP-CELL, Bio-Rad). This is a challenging procedure that has not been extensively used it enables the purification of one or more proteins from a complex mixture based on their mobility in an electric field. The Prep-Cell system was used to purify dlβ-PARV as there was a good separation from similar molecular weight proteins in the extracts. Our protein has a relatively low MW which allowed to be one of first proteins to be eluted and the purification was quicker. A challenge in successful protein purification is to avoid physical or chemical modifications, a relevant point for the present study since the objective was to assess the potential effect of the processing method on the protein characteristics. Additionally, according to Einarsdóttir et al (2007), CEE is a good replacement for traditional chromatography since the method produced a good yield and did not cause loss of immunological activity (of the purified hormones).

Several studies have described fish β-PARV purification from muscle using a variety of methods, but they incorporate steps such as heat treatment, salt precipitations or harsh protein extraction methods (Bugajska-Schretter et al, 2000; Kobayashi et al, 2006; Li et al, 2014), which may directly interfere with our objective of identifying allergenic epitopes in the native protein. For these reasons, semi-native PAGE without thermal denaturation or reducing conditions were used to avoid unwanted structural changes that might affect protein conformation and allergenicity. The dlβ-PARV protein fraction with the correct MW was successfully purified from white muscle of all the experimental conditions (Fig. 5A). In addition to the most representative band of dlβ-PARV, additional protein bands with residual levels of the target protein were observed (Fig. 5A) and were later shown to be alternative dlβ-PARV isoforms.

The fish species that are usually consumed tend to be teleosts, which underwent a specific whole genome duplication, acquiring and retaining multiple copies of some genes

(Glasauer & Neuhauss, 2014). According to the literature, several different gene and protein isoforms of β-PARV have been identified in different fish species (Pérez-Tavarez et al, 2019, 2021). To assess the complexity of the PARV isoforms in sea bass muscle extracts in the present study we purified and characterized protein fractions of dlβ-PARV from C1 by 2DE and Western blot coupled to mass spectrometry. Proteins with the same molecular weight but different PI's, and vice versa, can be separated using two-dimensional electrophoresis (Magdeldin et al, 2014). In Fig. 5B the control protein extracts were separated in the two dimensions and then stained with Coomassie blue, which revealed 6 protein spots. From visual observation, protein spots 1, 4 and 5 have the same approximate size as predicted for PARV but different PI's and it was hypothesized that they might represent different isoforms of the protein. Protein spots 2 and 3 had a slightly different MW from PARV but may also represent alternative isoforms. Protein spot 6 had a very different PI and MW from PARV and so was not assigned as putative PARV isoform. Spot 5 is, undoubtedly, the most intense spot and was immunoreactive with the antibody Parv19. 2DE revealed another protein spot with strong Coomassie blue staining that was located in proximity to protein spot 5. However, the results of WB revealed that only the protein in spot 5 was immunogenic with the monoclonal antibody Parv19. However, the specificity of the antibody used is for the allergenic form of PARV and this may mean that it does not detect all isoforms of PARV. For this reason, MS analysis was used to determine the number of putative PARV isoforms by analyzing the 9 isolated gel spots that run-in proximity to dlβ-PARV in 2D SDS PAGE (Table II). The goal was to establish PARV isoform complexity in sea bass muscle. The analysis of the proteins identified in 2DE revealed at least three PARV isoforms (spots 1, 2, 4 and 5). Spots 1 and 4 were identified as the same PARV isoform sequence and this may happen when the peptide(s) identified in the MS was (were) not big enough to differentiate two similar sequences. Protein spot 5 was the most intense spot in the 2D gel and was strongly detected in the WB and MS confirming its identity as dlβ-PARV and based on the overall results the protein in spot 5 was the most common PARV isoform in the control samples.

In silico analysis predicted at least 13 β-PARV genes in the sea bass genome that had similar values of MW and PI. Nevertheless, it is important to remember that the *Ensembl* database for the sea bass was constructed using the transcriptome as a template for the

prediction of the protein sequences. MS results predict that 3 different isoforms of dlβ-PARV are present in the muscle of the sea bass (control sample). Based on the MS analysis two isoforms of Parvalbumin 4 and one unnamed isoform appeared to be present in the control sample of sea bass. More specifically, the Parvalbumin 4 (pvalb4-202; accession nº ENSDLAP00005011508) sequence is predicted to be present in protein spot 5 of the 2D gel (fig.5B). In summary, the dlβ-PARV purified fraction from the sea bass white muscle is a mixture of different dlβ-PARV isoforms, and if they arise due to different amino acid sequences or posttranslational modifications was unclear.

The results of Saptarshi et al (2014) on the allergenic epitope of salmon PARV were used to predict this same region in sea bass PARV, more specifically in the three PARV isoforms found in the MS results (Fig. 6). This comparison is valid according to Kobayashi et al (2016) findings, where IgE reactivity were accessed in purified PARVs of nine species, and it was concluded that the IgE-binding epitopes in PARV are present in highly conserved regions of the sequence. The sequence of the identified antigenic epitope (in Atlantic salmon) is almost identical with the antibody epitope, so it is possible to associate PARV allergenicity with the PARV-19 antibody reactivity. In the sea bass muscle Parvalbumin 4 (pvalb4 – 202) was confirmed to be the PARV isoform in spot 5 in 2DE (in fig.5B) and was the most strongly immunoreactive protein with the monoclonal Parv19 antibody. This, therefore, suggests that this is the allergenic protein in the complex mixture of β-PARV isoforms that were identified in sea bass muscle.

Food processing is imprinted in the diet of modern societies, and the nature and magnitude of the processing methods affect the physicochemical changes in protein structure. Conformational epitopes are associated with the 3D protein structures, which tend to be more sensitive to processing methods that cause protein denaturation and aggregation. In this way food allergenicity might be influenced by structural changes in proteins caused by processing and the masking or generation of sites that represent IgE epitopes. Several 3D protein models of fish β-PARV have been resolved, *in silico* or by physical approaches, however at our knowledge the dlβ-PARV 3D protein conformation has not been determined so far. In general fish β-PARV is characterized by alfa-helix secondary structure (Kuehn et al, 2014). The secondary structure of the most common PARV isoform was predicted using Phyre2 software (Kelley, L. et al, 2015) and served

as the basis for planning and interpreting the results of the biophysical studies. The *in-silico* 3D model of the dlβ-PARV (the dlβ-PARV sequence, the most prevalent in the purified protein sample (pvalb4 – 202/spot nº5 in Fig.5B)) was constructed based on the fish model 1B8L from cod and features of 56% α-helix as the main secondary structure (Fig.7A and B), which agrees with the previous reports on the native structure of fish PARV (Pérez-Tavarez et al, 2019; Moraes et al, 2014). Moreover, a very exposed epitope region recognized by anti-PARV19 was identified (see Fig. 7B (purple region)), which explains the positive immunoreactivity of dlβ-PARV with the monoclonal antibody PARV19 used in our study (Saptarshi et al 2014; Gajewski et al, 2009).

Protein structure can be monitored thanks to several spectroscopic techniques with different applications and aftermath. Regarding the determination of tertiary structure, the array of methods is more limited but still diverse. From all of them, the technique with more sensibility is the fluorescence spectroscopy, since samples with concentrations less than 1 nm can produce reproducible signal (Johnson 2005). It has the advantage of being nondestructive and allowing the monitoring any kinetic changes through time. This technique explores the intrinsic fluorescent nature of the 3 fluorophores (in proteins are the aminoacids tyrosine, tryptophan, and phenylalanine), which produces a emission spectra when excited at certain excitation wavelength. Tryptophan is generally the most common fluorescent residue and the most sensitive to the protein environment (Lakowicz, 2006), but complex protein containing several tryptophans in the sequence hamper often the perception of tyrosine emission spectra (Johnson, 2005). The object sample of the present study seems to be a complex mixture of different dlβ-PARV isoforms [the most abundant pvalb4-202, (with 0 Trp, 0 Tyr, 10 Phe), and the pvalb4-201 (with 0 Trp, 1 Tyr, 11 Phe)] and we cannot discard the contribution of all for spectroscopic analysis. However, for the calculations (when needed) it was assumed the more abundant dlβ-PARV isoform (Parvalbumin 4-202, accession nº: ENSDLAP00005011517 and spot nº5 in Fig.5B). In our results it is clear the presence of four typical spectra for fluorescence spectroscopy (Fig.8). C1 sample presents a spectra that peaks at 339nm which confirms the non-denatured state of the proteins and 11 days (C11) after storage slight protein denaturation is evident since the spectra peak is 341nm. The shift in the spectra peak is a consequence of the proteins in the stored fish gradually loosing conformation as the muscle deteriorates. The most interesting results appear when HP1 and HP11 spectra

were compared. HP caused denaturation of dlβ-PARV since the peak of the spectra shifted from 339nm (C1) to 351nm. But after 11 days of storage the dlβ-PARV started to regain the native structure since the peak of the spectra returned to 345nm but not to 339nm. The main result of the fluorescence experiment is the loss of tertiary structure after pressure treatment and the partial regain after 11 days of storage. There was recovery of secondary structure but not enough to be equal to the initial one. Protein denaturation is generally considered to be irreversible because huge quantities of energy are required to reverse the process, but in pressure-induced denaturation this does not appear to be the case. According to Mozhaev et al (1996) and Somkuti & Smeller (2013) the reversibility of the process is possible, and the fluorescent spectrometry results seem to confirm it: 11 days of storage, after the release of pressure, some reversal of the change in structure of dlβ-PARV occurs. But at this point, it is not possible to say dlβ-PARV regains the same 3D structure that existed before pressure was applied and if the allergenicity potential was modified. Considering the secondary structure of the dlβ-PARV, the results obtained by CD spectroscopy analysis are in concordance with the previous hypothesis that pressure causes a loss of structure and with the fluorescence spectroscopy results. The dlβ-PARV spectra obtained for the C1 sample, was a typical spectrum of a protein enriched in α-helix, consolidating the results of the 3D model predictions (Fig. 7A). 11 days of storage caused loss of secondary structure for the control sample, despite the tertiary structure remaining more or less unchanged. Comparing C1 and HP1, it is possible to observe that pressure causes a decrease in the secondary structure of dlβ-PARV, but that some reacquisition of structure occurred after 11 days of storage. In summary, HP under the tested conditions (600MPa/5 min) changes the structural conformation of dlβ-PARV but after 11 days of storage at 4ºC the folding state seems to recover. At present, the recovery of the structural proprieties 11 days after pressure treatment in dlβ-PARV has not previously been reported.

It has been described that Ca^{2+} has the ability to stabilize the structural conformation of β-PARV and in this way plays an important role in β-PARV IgE-binding properties. In the present study we investigated the role of Ca^{2+} in the structural integrity of the dlβ-PARV and possible consequences for the allergenicity. Calcium is an important structural element of β-PARV since its binds to the EF-hand and stabilizes the molecule. Storage

time does not seem to affect calcium binding since PARV-19 reactivity was similar in both control and processed samples (Fig.10B). Since the calcium binding region shown in Fig. 6 is predicted to be in a different site from the PARV-19 epitope, it is unlikely to directly affect antibody reactivity and allergenicity. Somkuti et al (2012) showed pressure treated cod parvalbumin did not have reduced IgE binding when calcium levels were modified. The results with cod parvalbumin are in concordance with our experimental results since the PARV-19 antibody was immunoreactive with both control (C1, C11) and HP-treated samples (HP1, HP11, Fig. 10B).

In conclusion, the purification of dlβ-PARV was achieved, and structural studies revealed multiple isoforms of dlβ-PARV in muscle, as well as a loss of structural properties after pressure treatment. HP processing and the storage time both affected the white muscle proteome of the sea bass and, in particular, dlβ-PARV. The pressure used induced structure changes, specifically a loss of secondary and tertiary structure. With this, changes in the allergenicity of the dlβ-PARV are highly likely to occur after HP treatment, but more studies such as digestibility studies, cell-line allergenicity experiments or even clinical trials with hypersensitive individuals are needed to confirm this hypothesis. The connection between some structural recovery after 11 days of storage and the allergenicity also needs to be evaluated, to establish the characteristics of the regains in the structure.

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Annexes

Annex I – Yield of the purification process of the dlβ-PARV by continuous elution electrophoresis. The protein concentration was measured by Bradford assay The Yield is the % purified dlβ-PARV in the total sarcoplasmic protein quantity

	Total soluble sarcoplasmic proteins		Purified dlβ-PARV		
Samples ID	Concentration (mg/ml)	Total Quantity (mg)	Concentration (mg/ml)	Quantity (mg)	Yield (%)
Control day 1	9,521	28,563	2,421	2,662	9,31
HP day 1	8,574	19,7202	2,118	2,753	13,95
Control day 11	3,483	13,5837	1,967	4,327	31,81
HP day 11	2,931	7,9137	1,694	2,541	32,15

Annex II – Output results from the Dichroweb analysis algorithms SELCON3 and CONTINLL according to the appropriated wavelength ranges. The data from the CD measurements was corrected and uploaded to the Dichroweb server (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml)

	SELCON3				CONTINLL			
	α-Helix	β-Strand	Turns	Unordered	α-Helix	β-Strand	Turns	Unordered
Control day 1	0,067	0,438	0,189	0,299	0,054	0,342	0,176	0,299
HP Day 1	0,674	0	0,109	0,213	0,102	0,382	0,218	0,213
Control day 11	0,674	0	0,109	0,213	0,102	0,382	0,218	0,213
HP day11	0,067	0,438	0,189	0,299	0,102	0,381	0,218	0,299