UiT The Arctic University of Norway

Faculty of Biosciences, Fisheries and Economics

Bioactivity evaluation of underutilized marine raw materials for the management of metabolic syndrome

A focus on diabetes, inflammation, and oxidative stress

Njål Rauø

Master's thesis in Marine Biotechnology BIO-3901 June 2021

Acknowledgements

This thesis was conducted at Marbio, UiT, the Arctic University of Norway from August 2020 to May 2021.

First and foremost, I would like to thank my supervisors Jeanette Hammer Andersen and Espen Hansen for once again including me into the Marbio group, and for all the advice and guidance provided through this thesis. I would also like to express my gratitude to Chun Li, Kirsti Helland, Marte Albrigtsen, and Sara Ullsten-Wahlund for all the practical help with laboratory experiments, to Yannik Schneider for the close follow-up in practical and theoretical settings, and to the others at Marbio and Marbank for another great year. Finally, I would like to thank my family and friends for the support throughout this period, and my fellow office mates Daniel, Ella, and Heba for supporting each other through thick and thin.

Tromsø, May 2021

Njål Rauø

Abstract

Metabolic syndrome (MetS) is the medical term for a cluster of risk factors including obesity, high blood pressure, and high blood sugar, which increases the risk of developing stroke, heart disease, and type 2 diabetes. Marine raw materials have been described to exert various bioactive properties beneficial to human health, including anti-inflammatory, antioxidant, and antidiabetic abilities. In this study, protein hydrolysates of shrimp shell, cod head, sprat (*Sprattus sprattus*), and blue whiting (*Micromestistius poutassou*), biomass of the microalgae *Nannochloropsis salina* and *Tetraselmis chui*, and flash-fractions of 24 marine invertebrate extracts, were evaluated for anti-inflammatory, antioxidant, and antidiabetic bioactivities using a panel of bioassays. The refined shrimp peptide concentrate (RSPC) was characterized as active at the lowest concentration tested (0.5 mg/mL) in the antidiabetic DPP-IV inhibitory assay, and showed the higher antioxidative effect of the samples tested in the FRAP antioxidant assay. The rat insulinoma palmitate stress assay was developed to approach the apoptotic conditions of pancreatic beta cells in patients of type 2 diabetes, but could not determine whether samples exerted protective effects as standard test concentrations of palmitate and glucose could not be established. This study has given a thorough basis in the technical challenges met in investigating the bioactivity in complex marine samples, especially in disturbances in absorbance readouts due to strong color of the highly concentrated samples. Nevertheless, this study could be useful in guiding a better selection of which bioassays to be used and the adjustments needed in further evaluation of bioactivity in complex marine samples.

Table of Contents

Abbreviations

ACE	Angiotensin-converting enzyme
AIF	Anti-inflammatory
BSA	Bovine serum albumin
CHH	Cod head hydrolysate
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DPP-IV	Dipeptidyl peptidase IV
ELISA	Enzyme linked immunosorbent assay
EPA	Eicosapentaenoic acid
ESI	Electrospray ionization
FBS	Fetal bovine serum
FFA	Free fatty acid
FRAP	Ferric reducing ability of plasma
LPS	Lipopolysaccharides
MetS	Metabolic syndrome
MS	Mass spectrometer
n.d.	No date
NP	Natural product
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
RCPC	Refined cod peptide concentrate
RIN	Rat insulinoma
ROS	Reactive oxygen species
RSPC	Refined shrimp peptide concentrate
SDS	Sodium dodecyl sulphate
SM	Secondary metabolite
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
UHPLC	Ultra-high performance liquid chromatography

1 Introduction

1.1 Marine bioprospecting

Marine bioprospecting is an interdisciplinary field which takes on the investigation of marine raw materials for content of natural substances that may be of potential medical and economical value. Medical effects in natural resources have been essential knowledge in human history, where the use of plants and parts of animals has had a central role in the treatment of various illnesses. The principles of traditional medicine have laid the foundation for modern bioprospecting, which today enables researchers to investigate raw materials for compounds that may exert health-promoting effects, as well as the underlying molecular properties and biochemical interactions using techniques and equipment of high performance and precision.

1.1.1 Natural products

Molecules produced by living organisms are known as natural products (NPs), and may possess various biological activities based on the chemical, physical, and structural properties of the molecule. NPs are in general divided into two main classes: primary and secondary metabolites. In contrary to primary metabolites, secondary metabolites (SMs) are not directly involved in the normal growth, development, or reproduction of the organism. Instead, SMs make up for a chemical arsenal crucial to attract, deter, or kill other organisms and thus increase the likelihood of survival. Other NPs may protect against non-biological stressors, such as high light intensities or elevated temperatures, allowing the organism to adapt and thrive under such environmental conditions (Petersen, Kellermann, & Schupp, 2020).

Secondary metabolites have been found in prokaryotes and eukaryotes including bacteria, invertebrates, vertebrates, plants, and fungi, both in the oceans and on land (Torres & Schmidt, 2019). Such SMs include toxins, attractants, repellents, and dyes, and occupy a broad specter of functions advantageous to the organism. Many marine invertebrates including corals, bryozoans, sea cucumbers, and tunicates are sessile organisms and depend on alternative ways of attracting prey, and avoiding predators, microorganisms, and competitors compared to animals of more active lifestyles. Several marine invertebrates are known producers of SMs, some of which have been further developed into approved drugs, as well as clinical stage and preclinical stage drugs (Malve, 2016), and are therefore of high relevance in bioprospecting research. The development of chemicals potentially harmful to humans into drugs and

pharmaceuticals is made possible by optimizing the chemistry of these molecules. Modifications to molecular structures allows the redesigning of a lead compound for the purpose of enhancing its usefulness as a drug. This includes increasing potency, enhancing target site specificity, improving pharmacological parameters, or reducing toxicity. The cone snail *Conus magus* is a fish-hunting species producing a peptide toxin highly venomous to both pray and humans. The drug Ziconotide (Prialt) is a derivate of the ω-conotoxin, and is an analgesic agent used in extreme cases of chronic pain (McGivern, 2007).

1.1.2 The bioprospecting pipeline

The bioprospecting pipeline describes the processes involved in the development of a pharmaceutical, nutraceutical, or cosmeceutical, and may be divided into three main phases: (1) biodiscovery, (2) development, and (3) approval, marketing and sales (Calado et al., 2018). The biodiscovery phase takes on the initial steps of drug research, from sampling of raw material and species classification in the field, to hit discovery, assay development, and lead optimization. Several cycles of this pipeline might be needed to obtain reliable and reproducible results. Further development of a lead compound involves preclinical trials through several steps including animal studies, bioavailability optimization, and proof of concept, before implementing clinical studies in humans. Successful demonstration of concept, drug efficacy, effectiveness, and safety, lays the scientific basis for the final phase of drug development where a drug can be marketed and distributed.

In the pharmaceutical industry, discovery of novel compounds may often take basis in research on microorganisms, plants, or animals, where further drug development is based on hit discovery and subsequent hit-to-lead selection processes for isolation of an active substance, such as a secondary metabolite. The development of a pharmaceutical is a complex and more strictly regulated process in regards documentation of health effects, safety, and production, compared to that of a nutraceutical. Nutraceuticals can be defined as food-derived products with functions of both preventing and treating pathological conditions (Santini et al., 2018), and can be based on an extract or a mixture of compounds, which may potentially shorten the process from development to market. Omega-3 capsules and cod liver oil are based on rest materials from fisheries and are examples of nutraceuticals composed of several active ingredients. Existing discoveries on novel compounds from marine rest raw materials,

combined with an emerging focus on sustainability and utilization of resources, proposes a promising field for further research and potential novel nutraceutical discoveries.

1.1.3 Utilization of rest raw material from fisheries

The complete utilization of foods and raw materials has been an important mentality in the history of man as resource availability was season based and unpredictable. This mindset is still a necessity in underdeveloped countries where the access to food and clean water costs a considerably larger amount of time and energy than in the modern Western world, where resources are ubiquitous. Therefore, it is the responsibility of developed countries to invest in research to develop medical products and technology that can contribute to solving health issues and improve the life standard in less privileged parts of the world. The increased focus and interest in environment and sustainability has put pressure on industries to generate products from natural sustainable resources and reduce their carbon footprints. To achieve this, larger economical investments and adjustments will be needed. Such measurements might be difficult for smaller chains and companies to achieve in the early stages, requiring more drastic measures from brands of larger profit. Although often presented as an economic burden, the short-term expenses that might be required, would nevertheless be miniscule compared to the global consequences of ignoring the problem.

In the recent decade, complete utilization of raw materials has been common practice in the fishery industry where rest raw materials including blood, cutoffs, bones, and viscera have been used in generation of fish meal and fish oils, animal feed, and protein hydrolysates as byproducts additional to the main food productions. Despite contributing to reducing spoilage of raw material, most byproducts are of low value and might cause companies to refrain from investing in such measurements. Biotechnological studies have contributed to the discovery of potential processing techniques for extraction of high value compounds from rest material using enzymes or bacteria strains to metabolize wastes into molecules with useful functions.

The use of enzymes in industry has led to several improvements in the utilization of waste material. Methods that earlier required the use of chemicals, high temperatures, and other harsh conditions have now been replaced with the safer and more efficient use of enzymes. Enzymatic hydrolysis is a process in which enzymes catalyze the cleavage of chemical bonds in molecules with the addition of water. During the process, large chains of amino acids are cleaved into

smaller peptides and are extracted into the aqueous solution, whereas lipids accumulate into a separate phase. This principle has for long been a common technique used in fisheries for extraction of protein hydrolysates, as well as omega-3 fatty acids including eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) which are important for normal functioning of eyes, brain, heart, and triglyceride levels in the blood (Swanson, Block, & Mousa, 2012). Selection of enzyme, optimization of parameters including temperature and time of hydrolysis, as well as subsequent purification steps are all crucial for the end product, indicating that adjustments in such parameters may lead to the production of peptides with potentially novel bioactivities.

1.1.4 Microalgae

Microbial life in the sea is extremely diverse, and includes an abundance of bacteria, fungi, protozoa, and algae. Microalgae are tiny, single-celled photoautotrophic eukaryotes which are responsible for producing approximately half of the world's oxygen, and can be found in the sea, in freshwater and in brackish water. Microalgae serve a fundamental role in the marine food chain and are the unsung heroes behind the content of omega-3 fatty acids including DHA and EPA found in fatty fish, which accumulate their omega-3 reserves by consuming microalgae (Doughman, Srirama, & Sanjeevi, 2007). Microalgae are also known for production of several other types of valuable molecules including antioxidants, fatty acids, peptides, and enzymes (Barkia, Saari, & Manning, 2019).

1.1.5 Why look in the Arctic?

The Arctic is exposed to large seasonal shifts in the environment, and challenges organisms to adapt both extreme cold and months of darkness during winter, to warmer temperatures and constant daylight in the summer months. Despite inhospitable conditions, the Arctic houses a broad variety of thriving species who have been molded by evolution to adapt the extreme conditions by altering their physiology and metabolism. The resulting register of specialized SMs and proteins allows organisms to thrive in these environments, making the Arctic a potential keeper of undiscovered molecules of novel biological functions which may serve as leads in drug and product development.

Currents from the Atlantic and Pacific oceans carry nutrients to the cold and clean arctic waters and have provided optimal conditions for fish including cod and salmon to thrive in these areas. Cod has been an important product for consumption and export ever since the Viking era, and

has been a central resource in the history of Nordic countries as well as a highly valued food throughout the Mediterranean. Today, aquaculture and especially salmon farming has become a well-established industry alongside fisheries, with Norway being one of the leading nations in fish and sea food production globally, with the capacity of generating and exporting millions of high-quality meals every day throughout the year (Norwegian Seafood Council, 2020). Optimal conditions for marine life to thrive, supported by strict environmental and commercial regulations, as well as an increased focus on sustainability amongst producers and consumers are factors which contribute to production of sea food of highest quality.

Studying life in the Arctic may although be difficult as the region is hard to access. Expeditions in the northernmost part of the Arctic Ocean are challenged by thick ice and variable light conditions, making time a precious commodity. Nevertheless, bioprospecting in the Arctic has led to several important discoveries in drug development research, bioactivity studies, genomic mapping, species characterization, among others (Svenson, 2013).

1.2 Metabolic syndrome

Metabolic syndrome (MetS) is a collective name for a cluster of health conditions occurring together, reflecting overnutrition, sedentary lifestyles, and resultant excess adiposity (Cornier et al., 2008). The agreed-upon definition for MetS is the presentation of one or both of insulin resistance and excess body fat around the waist, which progress to be the initial cause of metabolic risk factors including high fasting blood sugar, high blood pressure, and abnormal cholesterol or triglyceride levels. MetS increases risk of heart disease, stroke, and type 2 diabetes, and represents a major clinical public health challenge worldwide (Figueiredo et al., 2018).

Lifestyle changes including increased physical activity and healthier diet are considered as the first line of treatment for MetS. Additional treatment strategies include pharmacological interventions and supplementary treatments such as nutraceuticals. Several nutraceuticals have been claimed to possess various health-improving effects such as reducing body fat, lowering fasting glucose, reducing pro-inflammatory markers, and lowering blood pressure (Patti et al., 2018). The dietary supplement PreCardix is a natural ACE-inhibitor clinically proven to lower blood pressure and is based on marine bioactive peptides extracted from shrimp shells. Additional underutilized marine resources including microalgae and rest raw materials from

fisheries may have favorable effects on metabolic health. Species of microalgae, such as *Chlorella vulgaris*, have been claimed to exhibit several beneficial effects against metabolic risk factors, including dyslipidemia, hyperglycemia, hypertension, and weight loss (Xia et al., 2017). Studies on marine protein hydrolysates have described promising *in vitro* antidiabetic effect in protein hydrolysates from salmon byproducts (Harnedy et al., 2018), as well as the ability to lower glucose and increase insulin level in mice in protein hydrolysates from blue whiting (Le Gouic, Harnedy, & FitzGerald, 2018).

1.3 Diabetes

Diabetes is a chronic, metabolic disease characterized by elevated levels of blood glucose, caused by a reduction or a complete loss of insulin production. Insulin is a hormone responsible for regulating the blood sugar by promoting the absorption of glucose from the blood into cells and tissues, and is secreted by pancreatic beta cells as a response to food intake and increased blood glucose levels. Without insulin, glucose cannot be moved from the blood stream to the cells, resulting in a high blood sugar and energy depletion in the cell. Unresolved diabetes may over time lead to serious medical consequences, and is one of the leading causes of death in the world. The WHO estimates that about 422 million people worldwide have diabetes, and 1.6 million deaths are directly attributed to diabetes each year.

1.3.1 Diabetes type 1

In type 1 diabetes, beta cells are recognized as foreign elements and are attacked and destroyed by the immune system. Destruction of insulin-producing beta cells initiates a cascade of physiological changes in the body. Loss of insulin causes blood glucose levels to increase, known as *hyperglycemia*, while simultaneously depleting cells of energy. Absence of glucose causes muscle and fat tissues to be broken down for energy, resulting in weight loss and hunger. As glucose is an osmotic active molecule, high blood glucose will further draw water from cells and cause dehydration. Over time, untreated hyperglycemia may eventually lead to diabetic ketoacidosis, causing nausea and vomiting. The brain being highly sensitive to homeostatic changes, serious consequences including mental status changes and cerebral edema may occur if left untreated (Shastry & Bhatia, 2006). Although the mechanisms behind the auto-immune destruction of beta cells in type 1 diabetes are unknown, the development of various autoimmune diseases have been linked to chronic unresolved inflammation (Duan, Rao, & Sigdel, 2019).

1.3.2 Diabetes type 2

In diabetes type 2 the body does not produce enough insulin or cells do not react to insulin, a condition known as insulin resistance. Insulin resistant cells cannot easily take up glucose from the blood for energy. To compensate, beta cells in the pancreas in number and size to produce more insulin. Over time, beta cells are exhausted by the constant overactivity, resulting in a decrease in size and eventually death of beta cells (Cerf, 2013). This again leads to decreased insulin levels and hyperglycemia as a follow. As some insulin is still in circulation in the blood, patients of diabetes type 2 are usually not in danger of developing diabetic ketoacidosis compared to patients of type 1 diabetes (Evans, 2019). Instead, a more likely complication to be developed in patients of type 2 diabetes is hyperosmolar hyperglycemic state (Pasquel & Umpierrez, 2014). The high concentration of glucose in the blood causes water to diffuse out of the cells and into the blood stream, causing increased urination and dehydration of the cells. As in type 1 diabetes, mental status changes may occur as one of many serious consequences in extreme cases of dehydration.

1.3.3 Complications

Common for type 1 and type 2 diabetes are elevated levels of blood glucose, which, if left untreated, may damage capillaries, and affect nerves, organs, and normal healing processes in the body. Retinopathy, nephropathy, and neuropathy are three well known complications of prolonged, untreated diabetes (Nathan, 1993). Diabetic neuropathy is a type of nerve damage often recognized by decreased sensation in toes and fingers, as well as malfunctions in the autonomic nervous system, affecting the digestive system, urinary tract, and heart. Nerve damage combined with poor blood supply can lead to ulcers, typically on the feet, where minor cuts and wounds may lead to infection. In severe cases, an infection can spread to the bone or lead to tissue death, where amputations may be necessary to prevent further damage. Poor blood supply will damage the kidneys and may lead to nephrotic syndrome where the kidneys' ability to filter blood diminishes over time. Abnormal blood flow to the eye and retina may eventually lead to retinopathy and blindness if left untreated. According to the WHO, nearly 3 % of global blindness can be attributed to diabetic retinopathy. Diabetes also increases medium and large arterial wall damage, and subsequent atherosclerosis. As blood vessels become damaged, the body will try to protect further damage by depositing cholesterol. However, excess cholesterol deposits may cause narrowing of blood vessels, increasing the risk of heart attacks and strokes

which are the major causes of morbidity and mortality in patients with diabetes (Katakami, 2018).

1.3.4 Treatments

As of today, type 1 diabetes cannot be prevented, and patients of type 1 diabetes will require life-long treatment of insulin injections. In patients of type 2 diabetes, risks and severity of the condition can be reduced by exercise, weight loss, and diet, although insulin or other blood-sugar lowering treatments may be needed. Metformin is an orally administrated drug considered as the first-line medication for treatment of type 2 diabetes (Holman, 2007). Metformin is used to decrease the hepatic glucose production and increase target cell insulin sensitivity. Second-line medications including GLP-1 analogs and DPP-IV inhibitors have shown to stimulate insulin secretion, delay gastric emptying, and increase satiety. GLP-1 analogs must be administrated subcutaneously to avoid degradation in the gut, and may often cause side effects such as nausea, vomiting, and constipation. In comparison, DPP-IV inhibitors are orally available, and have been shown to cause fewer side effects (Lacroix & Li-Chan, 2012). Species of red algae, sponges, and anemones (Lauritano & Ianora, 2016), as well as fish protein hydrolysates (Harnedy-Rothwell et al., 2020) have shown inhibitory activities of the DPP-IV enzyme, making marine organisms a targeted field in screening for antidiabetic properties.

1.4 Inflammation and oxidative stress

Although the complicated regulatory networks involved in the pathophysiology of diabetes are still not completely understood, studies suggest that both inflammation processes and oxidative stress may be involved the development of diabetes (Lauritano & Ianora, 2016).

Inflammation is the immune system's protective response to various forms of harm including infection, injury, or loss of tissue homeostasis, and can be classified as either acute or chronic. Acute inflammation can be defined by the characteristic symptoms including swelling, redness, heat, pain, or loss of function. Acute inflammation can be induced by several factors such as infection, physical damage, or allergens, and lasts often for a relatively short period of time as cellular and molecular processes normally minimize impending injury or infection efficiently (Chen et al., 2017). In comparison, chronic inflammation is referred to as slow, long-term inflammation, and may last up to several months or years. Persistent infection, virus infection, or persistent injury such as obesity, stress, poor diet, smoking, or other long-time exposure to

toxic agents are factors known to act detrimental on tissues and organs and may lead to chronic inflammation. Diabetes, cardiovascular disease, allergies, and autoimmune diseases are some of the conditions that may be mediated by chronic inflammation (Pahwa, Goyal, Bansal, & Jialal, 2020).

Oxidative stress is a phenomenon caused by an imbalance between production and accumulation of oxygen reactive species (ROS) in cells and tissues and the ability of a biological system to detoxify these reactive products (Pizzino et al., 2017). ROS are natural products of various biological processes in the body, although environmental factors such as UV, radiation, pollutants, or drugs contribute to greatly increase ROS production. Oxidative stress has been linked to the development of various diseases, including Alzheimer's disease, Parkinson's disease, cancer, and diabetes type 2 (Pizzino et al., 2017). Fruits and vegetables are major sources of antioxidants, including vitamin C, vitamin E, beta-carotene, flavonoids, and polyphenols (Betteridge, 2000). As vitamin supplements have become an established product in pharma, recent studies have described both anti-inflammatory and antioxidative properties in underutilized materials of marine origin, and reflects an interest in investigating their bioactive potential. Thus, marine raw materials may represent a potential source to encountering bioactive molecules and subsequent development into nutraceuticals.

2 Aim of the thesis

The aim of the thesis was to investigate underutilized marine resources for ingredients that could be used in the management of metabolic syndrome (MetS) in humans. The samples involved in this study included biomass of microalgae and protein hydrolysates of fish wastes, provided through the EU-funded project SuReMetS.

The main objective for the project was to screen the marine samples in a selection of *in vitro* bioassays to evaluate antidiabetic, antioxidant, and anti-inflammatory activity.

Due to the ongoing Covid-19 pandemic, delays in the delivery of the samples from the European partners were experienced. Thus, additional samples were added to this thesis, including protein hydrolysates of shrimp shell and cod head provided by Marealis Innovation AS, Tromsø, and flash-fractions of marine invertebrates provided by Marbank, UiT.

The SuReMetS project: From Sustainable Resources to novel marine nutraceuticals for the management of Metabolic Syndrome

An EU-funded project aiming to 1) identify enzymes that can be used to process biomass with high efficiency at low temperatures, and 2) extract value from marine resources by identifying novel marine ingredients.

3 Method background

3.1 Bioassays

An *in vitro* bioassay is a replica of a given chemical or biological process that may be used to measure the impact of a substance added to the process, such as toxicity against cancer cells, inhibitory effect against an enzyme, antioxidative effects, among others. The field of bioassay-guided bioprospecting uses bioassays to examine organic material from various origins for bioactive compounds that may be of advantage.

3.1.1 FRAP antioxidant assay

An antioxidant assay allows for samples to be investigated for potential antioxidative abilities. The FRAP (ferric reducing ability of plasma) assay is a chemical assay used to measure the antioxidative activity of samples by reduction of the Fe^{III}-TPTZ complex. At low pH, the complex is reduced to Fe^{II} and develops a strong blue color with maximum absorption at 593 nm. In the presence of an antioxidant, the conditions will promote reduction of the complex and thus lead to color formation. Results are presented with reference to Trolox, a water-soluble analog of vitamin E. The FRAP method is retrieved from Benzie and Strain (1996).

3.1.2 Inhibition of the DPP-IV enzyme

The enzyme dipeptidyl peptidase IV (DPP-IV) plays a major role in glucose metabolism by the rapid degradation of incretins such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP). Incretins are a group of metabolic hormones secreted by the gut as a response to food ingestion, and exert antidiabetic activity through the enhancement of glucose-dependent insulin secretion and suppression of glucagon release and appetite. Existing diabetes drugs cover various markers of glucose metabolism including DPP-IV inhibition (gliptins), resistance to DPP-IV degradation (incretin mimetics), enhancement of insulin production and glucose sensitivity in beta cells (sulfonylureas), inhibition of glucose reabsorption (thiazolidinediones), among others, but do also entail several side effects and discomforts.

In the DPP-IV inhibitory assay, samples are evaluated for antidiabetic activity based on their ability to inhibit the enzymatic degradation of the Gly-Pro-p-nitroanilide substrate. DPP-IV preferentially cleaves N-terminal dipeptides from peptides with proline, alanine, or hydroxyproline at the penultimate position (Mulvihill & Drucker, 2014), where cleaving of the

Gly-Pro-p-nitroanilide substrate will release the nitroanilide complex and generate a yellow color to the solution.

3.1.3 Protection of beta cells

Glucose and free fatty acids (FFAs) are essential sources of energy within the cells. After a meal, pancreatic beta cells produce and secrete insulin which regulates the metabolism of fats, carbohydrates, and protein by promoting the absorption of glucose from the blood into liver, fat, and skeletal muscle cells. FFAs exert both positive and negative effects on pancreatic beta cell survival and insulin secretory function, depending on concentration, duration, and glucose abundance (Nemecz et al., 2019). In type 2 diabetes, chronic exposure to elevated levels of glucose and FFAs leads to insulin resistance in cells and tissues, causing beta cell dysfunction and eventually beta cell apoptosis by persistent overactivity and cellular stress (Cnop et al., 2005). In type 1 diabetes, beta cells are recognized as foreign and destroyed by the immune system. Although the mechanisms involved are still not clear (Szablewski, 2014), beta cell destruction is thought to occur mainly through activated macrophages and T cells by cytokine-mediated induction of apoptotic pathways (Sarkar et al., 2009). Cytokines are signaling molecules secreted by cells of the immune system and are responsible for initiating various immunological responses in the body. In type 1 diabetes, beta cells are destroyed by an autoimmune response, where IL-1β and IFN-γ, as well as TNFα, bind to cell surface receptors and initiate intracellular protein cascades which eventually leads to cell death (Rabinovitch & Suarez-Pinzon, 1998). These actions may although be inhibited by molecules interacting with the receptors, proteins, or cytokines involved in the apoptotic cascade (Chou et al., 2010).

In the RIN assays, cells of the rat insulinoma RIN-m5F cell line are used to mimic the various conditions observed in patients of diabetes type 1 and type 2. An insulinoma refers to a tumor of beta cells that produces excess insulin, a condition that may lead to severely low blood sugar levels after a meal, known as *postprandial hypoglycemia*. In research context, cells of the RIN-m5F insulinoma cell line can be exposed to stress factors such as cytokines or FFAs to induce apoptosis *in vitro*. These conditions allow sample substances to be investigated for protective effect against markers of the various apoptotic signaling pathways.

3.1.3.1 RIN cytokine stress assay

In the RIN cytokine stress assay, cells of the RIN-m5F cell line are exposed to the cytokines IFN-γ and IL-1β to induce pancreatic cell death as observed in patients of type 1 diabetes. The

cytokines bind to cell surface receptors which induce various intracellular protein cascades, ultimately leading to apoptosis. IFN-γ and IL-1β, as well as tumor necrosis factor (TNF, TNFα), act synergetic in inducing apoptosis by enhancing the activation and recruitment of various necrosis factors, as illustrated in figure 1. Whereas a combination of all three cytokines causes a too strong apoptotic action on cells in the RIN assay, the apoptotic effect caused by the combination of IFN-γ and IL-1β is sufficient to allow sample substances to be evaluated for potential inhibitory effect against beta cell apoptosis.

IFN-γ largely signals through a Janus kinase (JAK)–signal transducer and activator of transcription (STAT)-mediated signaling pathway. The two Janus kinases JAK1 and JAK2 involved in the JAK-STAT signaling pathway play an important role in transmitting signals induced by type I and II interferon interactions. Deucravacitinib (BMS-9861965) is a highly selective allosteric inhibitor (IC_{50} = 1.0 nM) of JAK1 and was used as a positive control in the RIN cytokine stress assay.

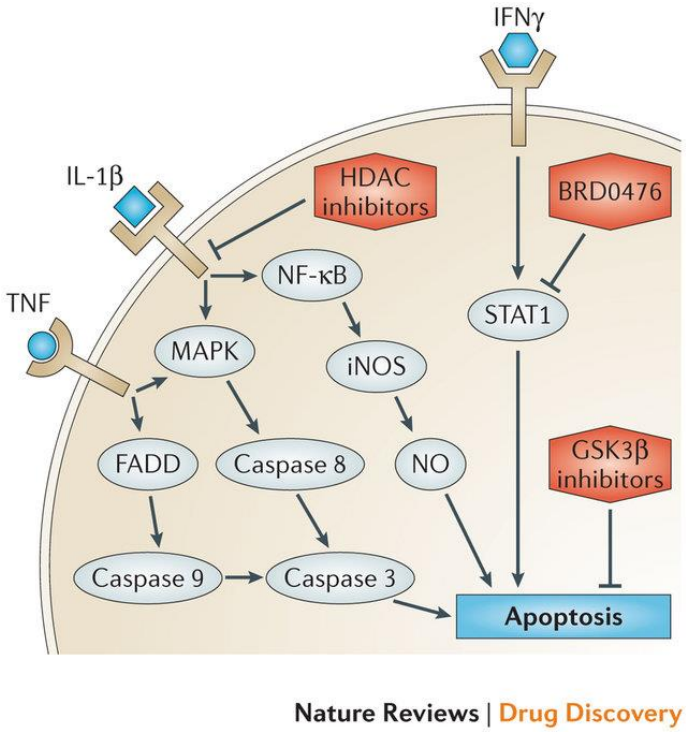

Figure 1. Induction of apoptotic signaling pathways by IFN-γ, IL-1β, and TNFα receptor-ligand interactions. IFN-γ induced apoptosis is mediated through JAK-STAT signaling. Binding of IL-1β induces an increase in nitric oxide (NO) which ultimately induces cell death. The MAPK pathway is activated by both IL-1β and TNFα, which leads to apoptosis by caspase activity. Whereas IFN-γ not being detrimental to the cell individually (Gysemans, Callewaert, Overbergh, & Mathieu, 2008), simultaneous binding of IFN-γ, IL-1β, and TNFα will cause a strong apoptotic action. Inhibitors of apoptotic signaling pathways may interact with receptors or other cascade proteins and prevent cell death. Retrieved from (Vetere, Choudhary, Burns, & Wagner, 2014).

3.1.3.2 RIN palmitate stress assay

In type 2 diabetes, chronic exposure to elevated levels of glucose and FFAs may lead to insulin resistance in cells and tissues, causing beta cell dysfunction and eventually beta cell apoptosis by persistent activity and cellular stress (Cnop et al., 2005). Palmitic acid is the most common saturated fatty acid found in animals, plants, and microorganisms (Gunstone, 2007). Exposure of human pancreatic cells to palmitic acid has been described to cause oxidative stress, endoplasmic reticulum (ER) stress, inflammation, and apoptosis (Nemecz et al., 2019). In the RIN palmitate stress assay, RIN-m5F beta cells are exposed to high concentrations of glucose and palmitate to mimic the conditions observed in patients of type 2 diabetes, allowing samples to be evaluated for protective abilities against beta cell apoptosis. Imatinib (STI571) is a tyrosine kinase inhibitor described to show positive effects in antidiabetic studies. Although the molecular *in vivo* mechanisms underlying the beneficial effects of Imatinib are not well known, as illustrated in figure 2, observations describe counteracting effects against high-fat diet induced insulin resistance and hyperglycemia in rats (Hägerkvist, Makeeva, Elliman, & Welsh, 2006). Imatinib was used as a positive control in experiments of the RIN palmitate stress assay.

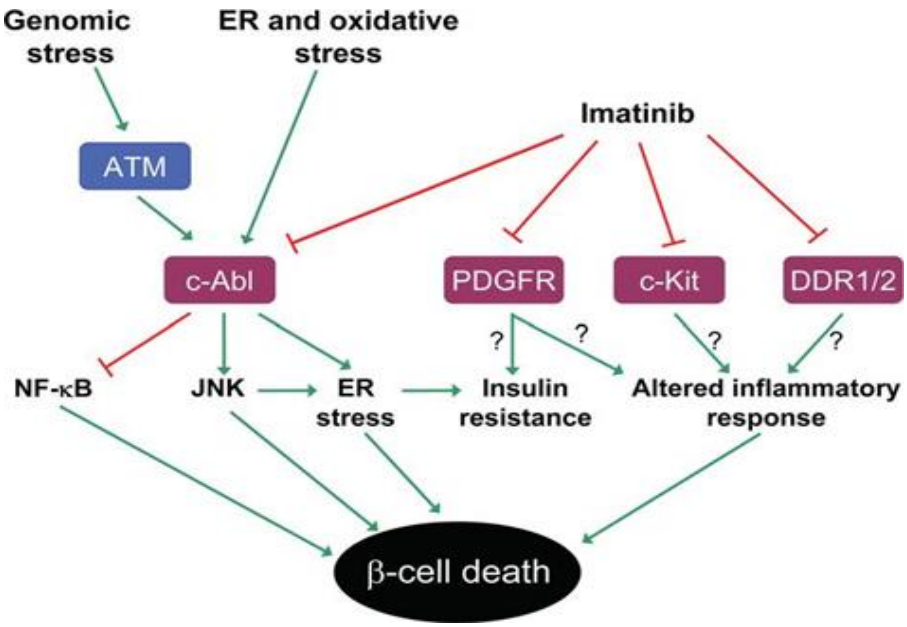

Figure 2. Oxidative stress and endoplasmic reticulum (ER) stress may cause death in beta cells through activation of the c-Abl tyrosine kinase and subsequent signaling pathways. Chronic exposure to high concentrations of glucose and free fatty acids have been described to cause beta cell death through oxidative and cellular stress. Imatinib is a known inhibitor of the tyrosine kinases c-Abl, PDGFR, c-Kit and DDR1/2, and promotes survival of beta cells through down-regulation of pro-apoptotic responses (Welsh, n.d.). Although all mechanisms are not totally understood, Imatinib has been shown to exert antidiabetic action in both animal models (Hägerkvist, Jansson, & Welsh, 2008) and in patients of type 2 diabetes (Veneri, Franchini, & Bonora, 2005).

3.1.4 Anti-inflammatory assay

One of the several important cell types that make up the immune system is the macrophage. When an antigen is recognized by a macrophage, various cytokines and chemokines are produced and secreted to alert and recruit other cells and components of the immune system to the site of stimulus. Tumor necrosis factor (TNFα) is one pro-inflammatory cytokine produced by macrophages.

THP-1 is a human monocytic cell line derived from an acute monocytic leukemia patient. In the anti-inflammatory (AIF) assay, THP-1 monocytes were differentiated to adherent macrophages by stimulation by phorbol 12-myrisate 13-acetate (PMA). Adhered macrophages were stimulated with LPS to induce production of TNFα, allowing samples to be evaluated for inhibitory activity against TNFα secretion, attenuating further inflammatory responses.

AIF result evaluation using the ELISA assay

Results from the anti-inflammatory assay were analyzed using the ELISA assay. The sandwich *enzyme linked immunosorbent assay* (ELISA) is a method for measuring levels of cytokines such as TNFα in a sample. In measuring levels of TNFα, specialized ELISA microtiter plates are initially coated with TNFα specific antibody, before adding samples which act as an antigen and bind to the adhered antibody. Finally, a biotin-conjugated TNFα antibody is then added, creating an antibody-antigen-antibody sandwich. Further addition of para-nitrophenyl phosphate (pNPP) substrate together with a phosphatase enzyme initiates a coloring process as a result of enzymatic reactions. The strength of the color is proportional to the levels of TNFα in the solution, allowing the amounts of TNFα to be determined using absorbance measurements.

3.2 Chemical characterization of compounds

Ultrahigh-performance liquid chromatography (UHPLC) is a technique used for separating molecules of a sample based on their physical and chemical properties. The sample is injected into a liquid mobile phase and transported through a column packed with a porous, solid material, known as the stationary phase, which retards the compounds in the sample. The surface of the packing material is modified with different functional groups, giving it defined properties with respect to physiochemical properties such as polarity. Differences in molecular properties of the analytes, such as charge, polarity, or size, will therefore affect the degree of

interaction with the stationary phase, causing analytes to elute at different times. A detector, such as a UV detector or a mass spectrometer (MS), detects the analytes as they elute, and generates a graph known as a chromatogram. The chromatogram represents the intensity of analytes (y-axis) as a function of retention time (x-axis), where each analyte can be visualized as a peak in the chromatogram. While the chromatogram may provide quantitative data about an analyte, mass spectra can also be used to retrieve qualitative information, as it will give information of the molecular mass of the analyte. A high-resolution MS (HRMS) has the capacity of separating masses that are very close to each other, allowing the accurate mass to be used to calculate the elemental composition of the analyte.

3.3 SDS-PAGE

SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) is an electrophoretic method using voltage to separate proteins or peptides in an analyte based on their mass. First, the analyte is prepared with SDS causing peptides to become negatively charged based on their number of amino acids. The analyte is further loaded onto the polyacrylamide gel submerged in a buffer in the electrophoresis chamber. When voltage is applied, the negatively charged peptides start to migrate through the gel towards the positively charged anode, each with different speed depending on their mass. Subsequent peptide staining allows the different proteins to be visualized as bands in the gel. The mass of the peptides in the analyte can be determined by comparison with a protein standard containing proteins of known masses.

4 Workflow

All laboratory experiments in this project were conducted at Marbio, University of Tromsø. Marbio is an analytical platform for screening, isolation, and identification of bioactive natural products from marine organisms through measurements in bioactivity assays and chromatographic and spectroscopic analyses. The workflow of this project is illustrated in figure 3.

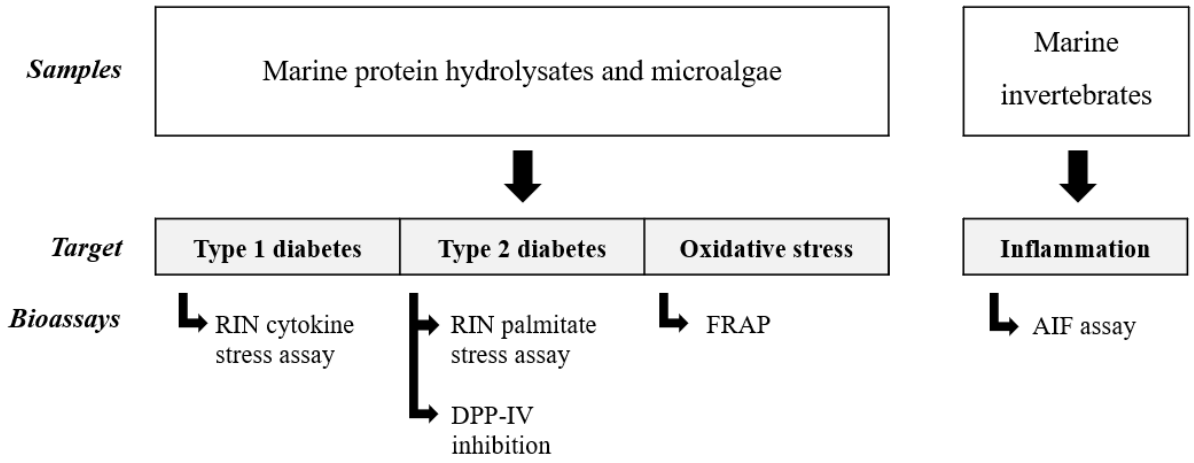

Figure 3. An overview of the workflow in the project. Marine protein hydrolysates and microalgae were evaluated for antioxidant and antidiabetic effects by applying a panel of bioassays. As part of the initial practice in laboratory work, 192 flash fractions of marine invertebrate extracts were evaluated for anti-inflammatory (AIF) activity.

4.1 Samples

The samples in this project consisted of refined peptide concentrates of cod head (RCPC) and shrimp shell (RSPC), cod head protein hydrolysate (CHH), protein hydrolysates of sprat (*Sprattus sprattus*), blue whiting (*Micromesistius poutassou*), and freeze-dried samples of the microalgae *Tetraselmis chui* and *Nannochloropsis salina*, presented in table 1. Additional samples are presented in table 2, and included twenty-four marine invertebrate extracts from Marbank that were separated into eight flash-fractions each.

Table 1. An overview of the marine protein hydrolysates and microalgae investigated in this project.

Sample name	Description	Origin	Latin name	Provider
RCPC	Refined cod peptide concentrate	Cod head	*Gadhus morhua*	Marealis Innovation AS
RSPC	Refined shrimp peptide concentrate	Shells, shrimp	*Pandalus borealis*	Marealis Innovation AS
CHH	Cod head hydrolysate	Cod head	*Gadhus morhua*	Marealis Innovation AS
Sprat	Sprat hydrolysate	Sprat	*Sprattus sprattus*	Bio-marine Ingredients Ireland Ltd.
Blue whiting	Blue whiting hydrolysate	Blue whiting	*Micromesistius poutassou*	Bio-marine Ingredients Ireland Ltd.
N. salina	Freeze-dried microalgae biomass		*Nannochloropsis salina*	BlueBioTech GmbH, Germany
T. chui	Freeze-dried microalgae biomass		*Tetraselmis chui*	BlueBioTech GmbH, Germany

*Table 2. An overview of the marine invertebrates investigated in this project. * Two extracts were of this species. ** Three extracts were of this species.*

Phylum	**Class**	**Genus**	**Species**
Annelida	Polychaeta	*Brada*	*Inhabilis*
Bryozoa	Gymnolaemata	*Securiflustra*	*Securifrons*
Echinodermata	Stelleroidea	*Crossaster*	*Paposus*
Echinodermata	Stelleroidea	*Ophiocten*	*Sericeum*
Mollusca	Gastropoda	*Beringius*	*Turtoni*
Mollusca	Gastropoda	*Buccinum*	*Fragile*
Mollusca	Gastropoda	*Buccinum*	*Glaciale**
Mollusca	Gastropoda	*Buccinum*	*Hydrophanum*
Mollusca	Gastropoda	*Buccinum*	*Polare***
Mollusca	Gastropoda	*Buccinum*	*Scalariforme**
Mollusca	Gastropoda	*Buccinum*	*Undatum*
Mollusca	Gastropoda	*Euspira*	*Pallida*
Mollusca	Gastropoda	*Neptunea*	*Despecta*
Mollusca	Gastropoda	*Onchidiopsis*	*Sp.*
Mollusca	Gastropoda	*Plicifusus*	*Kroeyeri*
Mollusca	Gastropoda	*Volutopsius*	*Norvegicus*
Porifera	Demospongiae	*Axinella*	*Sp.*
Porifera	Demospongiae	*Polymastia*	*Grimaldii*
Porifera	Demospongiae	*Polymastia*	*Thielei*
Sipuncula	-	*Phascolion*	*Strombus*

4.2 Bioactivity studies

The bioactivity studies in this project involved biochemical and cellular *in vitro* bioassays to evaluate the samples for antidiabetic, antioxidative, and anti-inflammatory activity.

As part of the initial laboratory practice, flash fractions of marine invertebrates were tested in the cellular anti-inflammatory assay for evaluation of inhibitory activity against secretion of TNFα by LPS induced THP-1 cells.

A panel of *in vitro* bioassays was further applied to evaluate marine hydrolysates and microalgae for antioxidative and antidiabetic abilities, including the antidiabetic assays dipeptidyl peptidase IV (DPP-IV) inhibitory assay, rat insulinoma (RIN) cytokine stress assay, RIN palmitate stress assay, and the FRAP (ferric reducing ability of plasma) antioxidative assay.

4.3 Chemical analysis

Chemical analysis of protein hydrolysates of sprat (*Sprattus sprattus*) and blue whiting (*Micromesistius poutassou*) was performed to investigate and compare the composition of peptides and amino acids in the samples. Analyses were performed through high resolution electrospray ionization mass spectrometry (HRESIMS) using positive (ESI+) and negative ionization modes (ESI-). The degree of hydrolysis and the size distribution of peptides in the five protein hydrolysates were investigating using SDS-PAGE.

5 Material and methods

5.1 FRAP (ferric reducing ability of plasma) assay

Equipment

Blank 96 well plates	NUNC	Cat. no 732-2717
Plate reader DTX880 with 593 nm filter		

Media and reagents

HCl 37 %	Merck KGaA	Cat. no 100317
Iron-3-chloride hexahydrate ($FeCl_3$*$6H_2O$)	Merck KGaA	Cat. no 103943
Acetic acid	Sigma	Cat. no A6283
Sodium acetate-3-hydrate		
2,4,6-tripyridyl-s-triazine (TPTZ)	Sigma Aldrich	Cat. no 93285
Trolox	Sigma Aldrich	Cat. no 238812
Methanol	VWR International	Cat. no 20964

Preparations of reagents

Acetate buffer pH 3.6

Dissolved 3.01 g sodium acetate-3-hydrate in 16 mL acetic acid. Diluted in water to a total volume of 1000 mL, and adjusted pH to 3.6.

40 mM HCl

Diluted 646 µL 37 % HCl in water to a total volume of 200 mL.

FRAP-reagent

Fe-solution

Dissolved 25,7 mg iron-3-chloride hexahydrate in 5 mL water.

TPTZ-solution

Dissolved 15,6 mg 2,4,6-tripyridyl-s-triazine in 5 mL 40 mM HCl.

Mixed 5 mL Fe-solution, 5 mL TPTZ-solution, and 50 mL acetate buffer pH 3.6. Incubated at 37°C for 10 minutes before use.

Trolox for standard curve

Dissolved 31,25 mg Trolox in 1,25 mL methanol. Diluted 100 µL Trolox-solution in 9,9 mL water to obtain a 1:100 dilution, and prepared following dilutions of 250, 125, 62.5, 31.25, and 15.625 µM in volumes of 1 mL.

Procedure

Added 5 µL sample, 15 µL water, and 150 µL FRAP-reagent to all wells in the Nunc-plates. Samples were tested in two parallels. Plates are incubated for 30 minutes at 37°C before reading plates at 593 nm using the DTX880 plate reader.

5.2 Antidiabetic assays

5.2.1 DPP-IV inhibitory assay

Reagents

100 mM Tris-HCl pH 8.0		
Sodium acetate 1 M pH 4.0		
Gly-Pro-p-nitroanilide 160 mM	Merck	Cat. no G0513-25MG
DPP-IV 0,01 Unit/mL	Merck	Cat. no 317640-250MIU-M
Diprotin A (Ile-Pro-Ile)	Sigma	Cat. no I9759-25MG

Instruments

Incubator cabinet, 37°C
Envision® micro plate reader, 405 nm wavelength

Procedure

A working solution of Gly-Pro-p-nitroanilide was prepared by diluting 160 mM stock 1:100. Diprotin A was diluted in Tris-HCl to 1 mg/mL and used as positive control.

Samples were added in triplicates in volumes of 25 µL before adding an equal amount of 1.6 mM Gly-Pro-p-nitroanilide to each well. The plate was incubated for 10 min at 37°C and added 50 µL DPP-IV solution to each well before incubated for another 60 minutes at 37°C. The reaction was stopped by adding 100 µL of 1 M sodium acetate to each well. Absorbance was measured at 405 nm using the Envision microplate reader.

5.2.2 Cell work and preparations of the RIN-m5F cell line

Growth medium

RPMI 1640, low endotoxin	Biochrom	Cat. no FG1385
FBS		
L-glutamine		
Sodium pyruvate	Biochrom	Cat. no L0473
Penicillin-streptomycin	Sigma	Cat. no P4333

Reagents

Trypan blue 0,4 %	Sigma	
Phosphate buffered saline (PBS)	Sigma	Cat. no D8537
Trypsin		

Equipment

Centrifuge
Counting chamber
Light microscope
Incubator cabinet, 37°C, 5 % CO_2

Preparing new cell batch

Cell suspension was stored in cryotubes in liquid nitrogen. Transferred cryotube of cell suspension to prewarmed Milli-Q (MQ) water for thawing. Transferred cell suspension to 10 mL medium and centrifuged at 150 g for 5 min. Removed supernatant and resuspended cell pellet in 15 mL medium. Incubated cell suspension for seven days before splitting and seeding.

Cell culture

Cells of the RIN-m5F cell line were cultured in a 75 cm^2 tissue culture (TC) flask in 15 mL RPMI medium of 10 % FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and penicillin-streptomycin solution to concentrations of 100 U penicillin/mL and 100 µg streptomycin/mL.

Counting cells

The number of living cells was counted using a counting chamber and a light microscope. A volume of cell suspension was diluted in Trypan blue 1:1 before counting. Dead cells were colored by Trypan blue. The cell count was used to estimate the concentration of cells in suspension.

Splitting cells

Cell density was observed using a light microscope. Removed medium from flask and washed with 10 mL PBS. Added 3 mL trypsin solution and incubated at 37^{o}C for 3-4 minutes. Added 10 mL prewarmed medium and centrifuged cell solution at 150 g for 5 minutes. Supernatant was discarded before resuspending cell pellet in 10 mL medium. Counted cells and prepared cell solution of $2x10^5$ cells/mL. New cell passage was prepared by transferring 1-2 mL cell solution based on cell density to medium to a total volume of 15 mL. Medium was changed after 72 h and 120 h of incubation after splitting. Cells were split every seven days.

Seeding cells

Cell solution of $2x10^5$ cells/mL was seeded in 96-well microtiter plates of 100 µL/well, and incubated at 37^{o}C, 5 % CO_2 for 72 h for cells to attach.

5.2.3 RIN cytokine stress assay

Cell line

RIN-m5F Rat insulinoma cell line; ATCC CRL-11605

Equipment

Blank 96 well plates	NUNC	732-2717
Incubator cabinet, 37°C, 5 % CO_2		

Reagents

RIN-m5F growth medium		
20 ng/mL human hIL-1β stock		
20 ng/mL IFN-γ stock		
BSA	Sigma	Cat. no A2153
DMSO	Sigma	Cat. no D4540
Deucravacitinib (BMS-9861965)		
CellTiter 96® Aqueous One Solution	Promega	Cat. no G3582

Adding samples and reading results

Removed medium and added 100 µL medium to edge and cell control wells, and 90 µL medium to wells where samples and DMSO control be added. Samples and DMSO control were added in volumes of 10 µL. Incubated plates for 24 h before adding 5 µL cytokine mix consisting of 1:1:48 hIL1β, IFN-γ, and 0,1 % BSA in PBS to sample wells. Plates were incubated for 72 h before adding 10 µL Aqueous One Solution to wells, incubated for 90 minutes and read in a DTX 880 plate reader.

Procedure

After 72 h of incubation, samples were added to cells and incubated for 24 h. Sample wells and cytokine control wells were added 5 µL 0,1 % BSA cytokine mix. Incubated for 72 h before adding 10 µL Aqueous One Solution to wells. Incubated for 90 minutes and read plates using a DTX 880 plate reader.

5.2.4 RIN palmitate stress assay

Equipment

Heat element		
Blank 96 well plates	NUNC	Cat. no 732-2717
DTX 880 plate reader		

Reagents

RIN-m5F growth medium		
Sodium palmitate	Sigma-Aldrich	Cat. no 408-35-5
Glucose solution 200 g/L	ThermoFisher	Cat. no A2494001
Ethanol 96 %	VWR	Cat. no 20823.362
CellTiter 96® Aqueous One Solution	Promega	Cat. no G3582

Procedure

The initial experiment involved determining a suitable test concentration of palmitate through testing of a dilution series of 500 to 1000 µM palmitate, with each concentration supplemented with 25 mM glucose based on the studies by Alnahdi, John, and Raza (2019). In subsequent experiments of evaluating protective effects of samples, cells were exposed to concentrations of 800 µM palmitate and 25 mM glucose, added to wells in a 1:9 ratio. To obtain a final concentration of 800 µM palmitate, an 890 µM concentration of palmitate was prepared and added to wells. Cells were incubated with samples for 24 h before discarding supernatant and re-adding sample and 890 µM palmitate with 25 mM glucose. In a final experiment, the concentration of palmitate was lowered to 300 µM as based on studies by Alnahdi et al. (2019).

Palmitate was prepared in 50 % EtOH to a concentration of 100 mM based on the methods of Wang and Welsh (2014). The 100 mM stock was heated to approximately 75^{o}C before diluting in 1 % BSA RIN-m5F growth medium. All concentrations of palmitate were supplemented with 25 mM glucose. After 72 h of incubation post seeding, cells were added samples and incubated for 24 h. Removed medium from sample wells and added 90 µL 890 µM palmitate stock to wells before adding 10 µL sample. Incubated for 72 h before adding 10 µL Aqueous One Solution to wells. Incubated for 90 minutes and read plates using a DTX 880 plate reader.

5.3 Anti-inflammatory assay

Cell line

THP-1 Human monocyte, acute monocytic leukemia; ATCC no TIB-202; ECACC no 88081201

Equipment

Light microscope
Incubator cabinet 37^{o}C, 5 % CO_2
Centrifuge
Counting chamber

Media/reagents

RPMI 1640, low endotoxin	Biochrom	Cat. no FG1385
FBS, ultralow endotoxin	Biowest	Cat. no S1860-500
L-glutamine	Biowest	Cat. no X0551-100
Gentamycin	Biochrom	Cat.no A 2712
Trypan blue 0,4 %	Sigma	
PMA, stock solution 1 mg/ml	Sigma	Cat. no P1585
PBS	Sigma	Cat. no D8537
DMSO	Sigma	Cat. no D4540
LPS	Sigma	Cat. no L2630
CellTiter 96® Aqueous One Solution	Promega	Cat. no G3582

5.3.1 Preparations of the THP-1 cell line

THP-1 cell line

Cells of the THP-1 cell line were cultured in a 175 cm^2 TC flask in 40 mL growth medium supplied with 10 % FBS, 2 mM L-glutamine, and 10 µg/mL gentamycin.

Counting cells

The number of living cells was counted using a counting chamber and a light microscope. A volume of cell suspension was diluted in Trypan blue 1:1 before counting. Dead cells were colored by Trypan blue. The cell count was used in estimating the concentration of cells in the suspension.

Splitting cells

Cell culture was transferred from the TC flask to a centrifuge tube and centrifuged at 150 g for 5 minutes. Cell pellet was resuspended in 25-30 mL growth medium, and counted cells using a counting chamber. New cell passage was cultured in a concentration of 2-3x10^5 cells/mL in 50-60 mL growth medium and incubated at 37°C, 5 % CO_2. Cells were split every 3-4 days.

Preparation of cell plates

For seeding cells into wells of microtiter plates, a 10^6 cells/mL cell suspension was prepared and added 50 ng/mL PMA to induce differentiation of monocytes to adherent macrophages. PMA cell suspension was added to wells of microtiter plates in volumes of 100 µL, and incubated for 48 h at 37°C, 5 % CO_2. To confirm cell adhesion, cells were observed using a light microscope. Removed medium and washed wells with PBS, before adding 100 µL PMA-free medium to each well. Incubated cells for 24 h at 37°C, 5 % CO_2.

5.3.2 Adding samples

Removed medium from wells of prepared microtiter plates containing cells. For testing of flash fractions at 50 µg/mL, 85 µL medium and 5 µL 1 mg/mL sample was added to wells. Edge wells and cell control were added 100 µL medium. Positive control consisted of 1 ng/mL LPS. Plates were incubated for 1 h at 37°C, 5 % CO_2 before adding 10 µL 10 ng/mL LPS solution to

sample wells. Incubated plates for 6 h at 37ºC, 5 % CO_2 before storing at -80ºC until performing the ELISA assay.

Preparations of flash fractions

Freeze dried flash fractions were added 6,2 µL DMSO and placed in shaker for 2 h to dissolve. Fractions were further diluted in MQ water to 1 µg/µL 2.5 % DMSO before placed in shaker for 1 h until homogenous.

5.3.3 Result evaluation using the ELISA assay

Equipment

Nunc Maxisorp 96F-well ELISA plate	VWR	Cat. no 735-0083
Aquamax4000 plate washer		
DTX 880 plate reader		
Robotic system		

Reagents

TRIZMA base	Sigma	Cat. no 93352
NaCl	Sigma	Cat. no S5886
Tween20	Sigma	Cat. no P1379
BSA	Sigma	Cat. no A2153
pNPP substrate 5 mg	Sigma	Cat. no S0942
pNPP substrate 40 mg	Sigma	Cat. no P5994
0,1 M Glycine buffer pH 10.4		
ExtrAvidin®-Alkaline Phosphatase	Sigma	Cat. no E2636
Anti-Human TNF-alpha Purified	eBioscience	Cat. no 14-7348-85
Anti-Human TNF-alpha Biotin	eBioscience	Cat. no 13-7349-85
Human TNF-alpha recombinant protein	eBioscience	Cat. no 14-8329-63

Buffer solutions

Glycine buffer

A 0.1 M glycine buffer was prepared by adding 7,51 g glycine, 1 mL of 1 M $MgCl_2$, and 136 g ZnCl3 to 1 L of MQ water. Adjusted pH to 10.4.

TBS buffer

A 10x tris-buffer solution (TBS) was prepared by adding 12,1 g TRIZMA base and 88 g NaCl to 1 L of MQ water. Adjusted pH to 7.4 and diluted 1:10 before use. The 1x concentration of TBS was further used to prepare the following buffers:

Washing buffer	TBS pH 7.4 added 0,05 % Tween20
Blocking buffer	TBS pH 7.4 with 2 % BSA
Assay diluent	TBS pH 7.4 with 1 % BSA

Controls

Human TNFα standard curve in 1:2 dilution for concentrations 2000-31,2 pg/mL and blank (assay diluent), as well as LPS- and cell control from cell assays were prepared for each tray.

Procedure

Coated Nunc Maxisorp 96F-well ELISA plates with antibody by diluting *anti-human TNF-alpha purified* antibody solution in 1 x TBS pH 7.4 to 2 µg/mL, adding 100 µL to each well before incubating in refrigerator overnight.

Washed ELISA plates using the Aquamax4000 plate washer. Added 200 µL blocking buffer to each well and incubated in room temperature for 1 h with shaking. Rewashed plates. Prepared a 1:2 dilution series of TNF standard with 2000 pg/mL TNFα as the highest concentration and assay dilution (0 pg/mL TNFα) as the lowest concentration, with a total volume of 100 µL in each well in two parallels. Transferred samples and controls from microtiter plates tested in the anti-inflammatory assay to coated ELISA plates. Samples added LPS were diluted 1:20 in assay diluent, cell controls were diluted 1:2. Incubated ELISA plates for 2 h in room temperature with shaking. Rewashed plates. Diluted Anti-Human TNF-alpha Biotin in assay diluent to 3 µg/mL, adding 100 µL to each well. Incubated for 1 h at room temperature with shaking. Rewashed

plates. Diluted ExtrAvidin®-Alkaline Phosphatase 1:20000 in assay diluent, adding 100 µL to each well. Incubated for 30 minutes at room temperature with shaking.

Prepared 1 mg/mL pNPP substrate in 0.1 M glycine buffer pH 10.4 and let dissolve for minimum 15 minutes. Washed ELISA plates using ELISA SOAK WASH program in Aquamax4000. Added 100 µL pNPP substrate solution to each well and incubated for 45 minutes at room temperature without shaking. Plates were read in the DTX880 plate reader at 405 nm.

6 Results

6.1 Evaluation of anti-inflammatory activity in marine invertebrates

The purpose of the anti-inflammatory assay was to encounter substances which inhibited LPS-induced expression of TNFα. The anti-inflammatory assay was part of the initial practice in performing bioassays and cell work. Flash fractions (192) from twenty-four marine invertebrate extracts were tested for anti-inflammatory effect at a concentrations of 50 µg/mL, of which seven fractions were characterized as active (≥50 % inhibition) or questionable (40 to 50 % inhibition) as presented in table 3. The marine bryozoan *Securiflustra securifrons* exerted 100 % inhibition of TNFα expression but was concluded cytotoxic based on activity in parallel bioactivity testing on the A2058 melanoma cell line. Active and questionable fractions were retested in the second screen in concentrations of 50, 25, 10 and 1 µg/mL without reproducing inhibitory effects (data not shown).

*Table 3. An overview of active, questionable, and inactive flash fractions of marine invertebrates screened in the anti-inflammatory assay. Fractions exerting ≥50 % inhibition were defined as active (A), whereas fractions exerting 40-50 % were defined as questionable (Q). Inactive fractions (-) exerted less than 40 % inhibition. *Two extracts of 8 fractions each were of this species. **Three extracts of 8 fractions each were of this species.*

Species	Flash fractions							
	1	2	3	4	5	6	7	8
Axinella sp.	-	-	-	-	-	-	-	-
Beringius turtoni	-	-	-	-	-	-	-	-
Brada inhabilis	-	-	-	-	-	-	-	-
Buccinum fragile	-	-	-	-	-	-	-	-
*Buccinum glaciale**	-	-	-	Q	-	-	-	-
Buccinum hydrophanum	-	-	-	-	-	-	-	-
*Buccinum polare***	-	-	-	A	-	-	-	-
*Buccinum scalariforme**	-	-	-	A	-	-	-	-
Buccinum undatum	-	-	-	-	-	-	-	-
Crossaster paposus	-	-	-	-	-	-	-	-
Euspira pallida	-	-	A	-	-	-	-	-
Neptunea despecta	-	-	-	-	A	-	-	-
Onchidiopsis sp.	-	-	-	-	-	-	-	-
Ophiocten sericeum	-	-	-	-	-	-	-	-
Phascolion strombus	-	-	-	-	-	-	-	-
Plicifusus kroeyeri	-	-	-	Q	-	-	-	-
Polymastia grimaldii	-	-	-	-	-	-	-	-
Polymastia thielei	-	-	-	-	-	-	-	-
Securiflustra securifrons	-	-	-	-	A	-	-	-
Volutopsius norvegicus	-	-	-	-	-	-	-	-

6.2 Evaluation of bioactivities in marine hydrolysates and microalgae

Further bioactivity screening involved the five protein hydrolysates of RCPC, RSPC, CHH, sprat, and blue whiting, and the freeze-dried material of the two microalgae *N. salina* and *T. chui*, presented in table 1. The protein hydrolysate samples were evaluated for antioxidative properties in the FRAP assay, and antidiabetic properties in the DPP-IV inhibitory assay, RIN palmitate stress assay, and the RIN cytokine stress assay. Due to the challenges experienced in the strong color of samples, the biomasses of *N. salina* and *T. chui* were excluded from the results of the FRAP assay and DPP-IV inhibitory assay. To demonstrate the disturbances generated during absorbance measurements, samples of microalgae were included in results from the RIN cytokine stress assay.

6.3 RIN cytokine stress assay

The RIN cytokine stress assay was applied to approach the immunological mechanisms involved in the development of type 1 diabetes, and evaluate if samples could prevent cytokine-induced apoptosis. Cells of the rat insulinoma cell line RIN-m5F were incubated with sample for 24 h before adding the cytokines IL-1β and IFN-γ. An increased survival of cells compared to the cytokine control would indicate the presence of such protective effect.

The results presented in figure 4 are based on three parallels, tested in one biological replicate. As no effect was shown in lower concentrations of these samples in previous screening, hydrolysates of sprat and blue whiting and the biomasses of *N. salina* and *T. chui* were tested at concentrations of 10 mg/mL. As samples of RCPC, RSPC, and CHH had not been tested previously in this assay, all samples were included in testing at concentrations of 0.1 and 1 mg/mL. As presented in figure 4, none of the samples showed any protective effect at 0.1 mg/mL. At 1 mg/mL, biomass of *T. chui* represents 30 % cell survival, but was considered a false positive due to high color in the sample which interfered with the readouts. The four samples tested at 10 mg/mL were all highly colored, and thus, the cell survival at this concentration was considered to be due to disturbances in absorbance readouts.

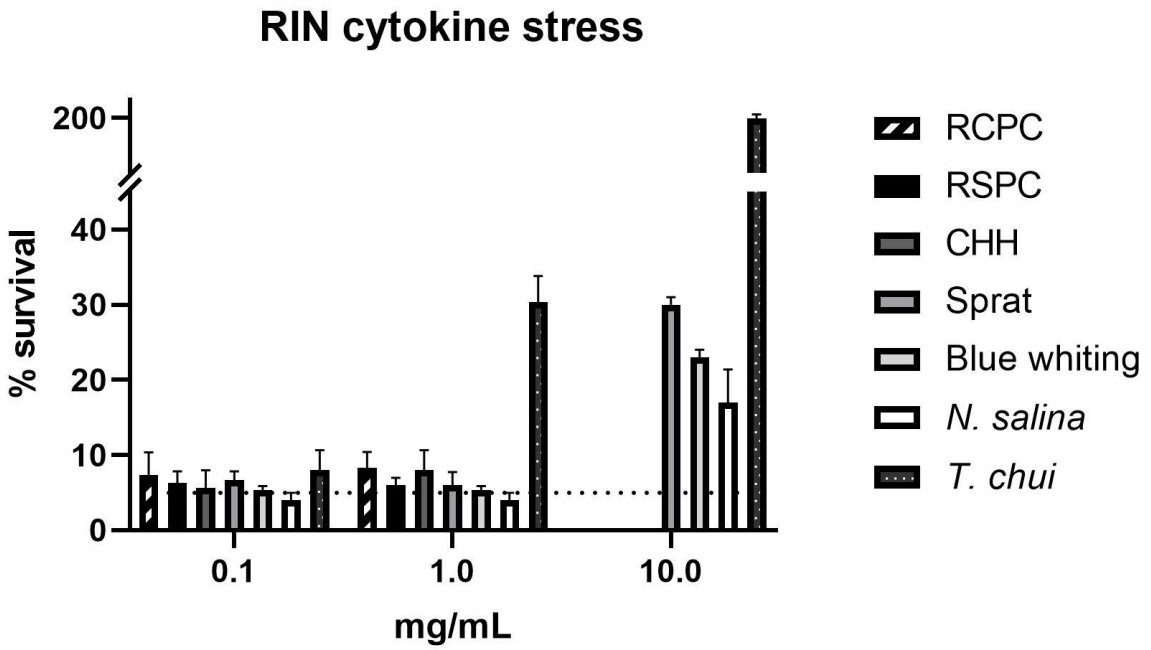

Figure 4. Protein hydrolysates of cod (RCPC), shrimp shell (RSPC), cod head (CHH), blue whiting, and sprat, as well as freeze-dried biomass of the microalgae Nannochloropsis salina *and* Tetraselmis chui*, were evaluated for protective abilities against apoptosis of beta cells induced by 20 ng/mL concentrations of the cytokines IL-1β and IFN-γ. The dotted line represents 5 % survival of cells observed in the cytokine control. The four latter samples were tested in concentrations of 10 mg/mL, where strong sample colors generated false indications of cell survival. The 1 mg/mL concentration of* T. chui *was also of strong color and produced similar outcome. Thus, none of the samples were considered to promote survival of beta cells.*

6.4 FRAP assay

In the FRAP assay, samples were evaluated for antioxidative potential by reduction of the Fe^{III}-TPTZ complex to Fe^{II}. In the presence of an antioxidant, an intense blue color is generated. The degree of reduction and antioxidative ability is measured using absorbance measurements, where results are further compared to the Trolox antioxidant reference and presented as µM Trolox equivalents (TE). Figure 5 illustrates the antioxidative activity of the five samples at concentrations 0.5, 1, 2.5, and 5 mg/mL, based on three technical replicates. At the two highest concentrations, the hydrolysates present different degrees of antioxidative effect, whereas in the two lower concentrations, very similar activity can be observed. Of the five protein hydrolysates, RSPC showed the higher antioxidative ability in all four concentrations equivalent to approximately 9, 16, 33, and 53 µM TE, respectively.

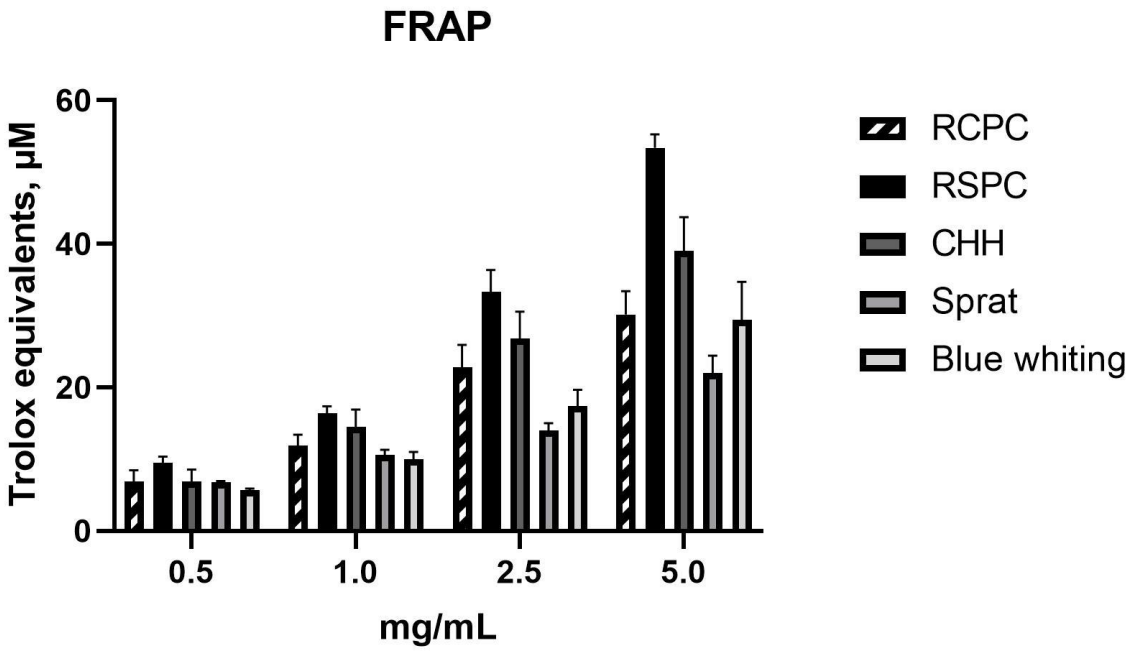

Figure 5. Marine protein hydrolysates were evaluated for antioxidative effect in the FRAP (ferric reducing ability of plasma) assay. The antioxidative strength was measured in units of µM Trolox equivalents (TE) by the Trolox equivalent antioxidant capacity (TEAC) standard, based on three technical replicates.

6.5 DPP-IV inhibitory assay

In the DPP-IV inhibitory assay, samples were evaluated for the ability to inhibit degradation of the Gly-Pro-p-nitroanilide substrate by the DPP-IV enzyme. Enzymatic degradation of the Gly-Pro-p-nitroanilide substrate generates a darker yellow color to the solution, whereas inhibition of the DPP-IV enzyme prevents the color reaction from proceeding. The negative control containing substrate and enzyme allows complete degradation and represents 0 % inhibition. The inhibitory activity of analytes was calculated in relation to the negative control as described in equation 1, and further compared to the 100 % inhibitory activity exerted by the DPP-IV inhibitor Diprotin A, as described in equation 2.

Equation 1 $$Inhibitory\ activity\ _{sample} = \frac{OD_{negative\ control} - OD_{sample}}{OD_{negative\ control}}$$

Equation 2 $$\%\ Inhibition = \frac{Inhibitory\ activity\ _{sample}}{IInhibitory\ activity\ _{Diprotin\ A}} * 100\ \%$$

However, equation 1 relies on that the OD value of the sample is lower than that of the negative control for the inhibitory activity of an analyte to be correct. As the different concentrations of

the samples were colored, this interfered with the readout and subsequent calculations. As a measurement, a parallel control plate containing sample, substrate, and Tris-HCl buffer in replacement for enzyme was prepared in a subsequent experiment. Absorbance measurements of the control plate allowed noise from the sample color to be subtracted from the assay plate, providing a more accurate measure of the inhibitory effect of the sample. A sample was considered active when exerting 50 % inhibition of the DPP-IV enzyme. As presented in figure 6, eleven of the twenty samples and concentrations were characterized as active based on the average of three technical replicates. At 2.5 mg/mL, all five hydrolysates were characterized as active, whereas RSPC was the only sample active at 0.5 mg/mL showing 64 ± 12 % inhibitory effect. RSPC was determined active at all four test concentrations, whereas RCPC and CHH were active down to concentrations of 1 mg/mL.

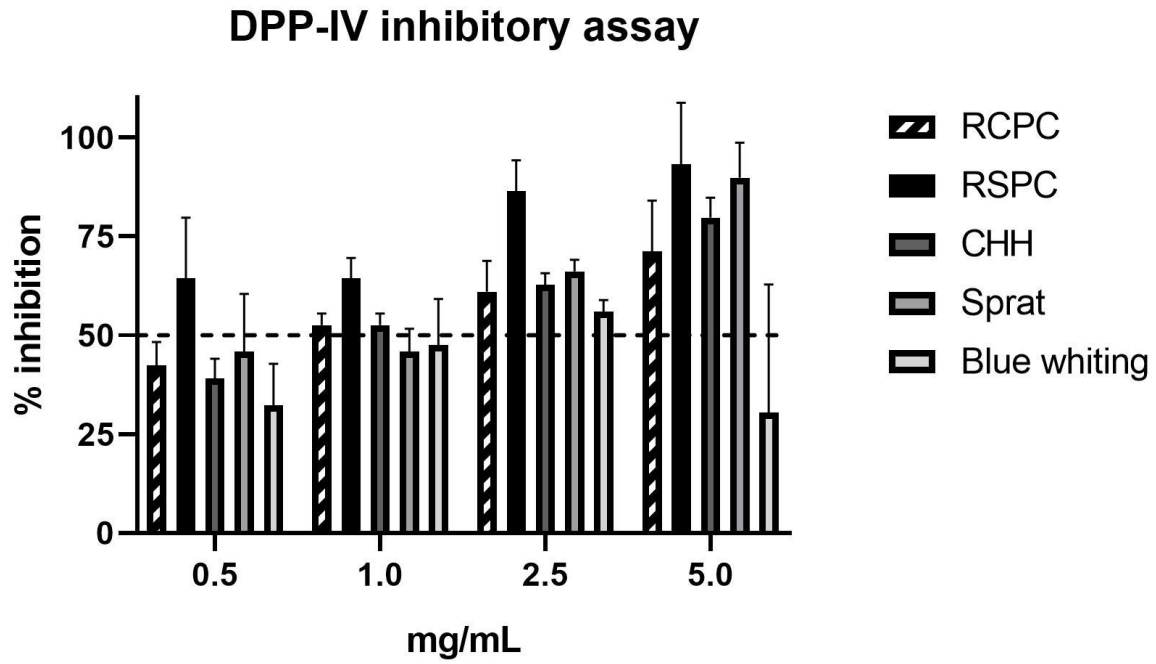

Figure 6. Protein hydrolysates of cod (RCPC), shrimp shell (RSPC), blue whiting, cod head (CHH), and sprat, were evaluated for inhibitory effect of the DPP-IV enzyme. Samples were compared to the effect of the DPP-IV inhibitor Diprotin A (1 mg/mL), representing 100 % inhibition. Samples exerting ≥50 % inhibition were characterized as active. All four concentrations of RSPC exerted above 50 % inhibition. Eleven of the twenty samples and concentrations were characterized as active based on the average of three technical replicates.

6.6 RIN palmitate stress assay

The RIN palmitate stress assay was developed in this project to "mimic" the *in vivo* conditions in patients of type 2 diabetes. The principle of the RIN palmitate stress assay was to investigate samples for protective abilities of beta cells exposed to high concentrations of palmitate and glucose. The development of the assay was based on studies by Wang and Welsh (2014) which describes preparations of palmitate in 50 % EtOH, and Alnahdi et al. (2019) which included the use of 25 mM glucose in similar experiments on RIN cells. This study involved an initial experiment on determining an appropriate palmitate test concentration, shown in figure 7, as well as subsequent experiments where samples were added to investigate their protective potential against the concentrations of palmitate and glucose. In subsequent testing, no effect could be observed in any samples or concentrations, nor in the Imatinib control, when exposing cells to 800 µM palmitate and 25 mM glucose. In attempting to observe effect of samples, the concentration of palmitate was lowered to 300 µM as described in the study by Alnahdi et al. (2019). As the 300 µM palmitate control, the 10 µM Imatinib control, and samples tested at 0.1 and 1 mg/mL all provided 50-70 % survival compared to the cell control, screening in the palmitate stress assay was terminated (data not shown).

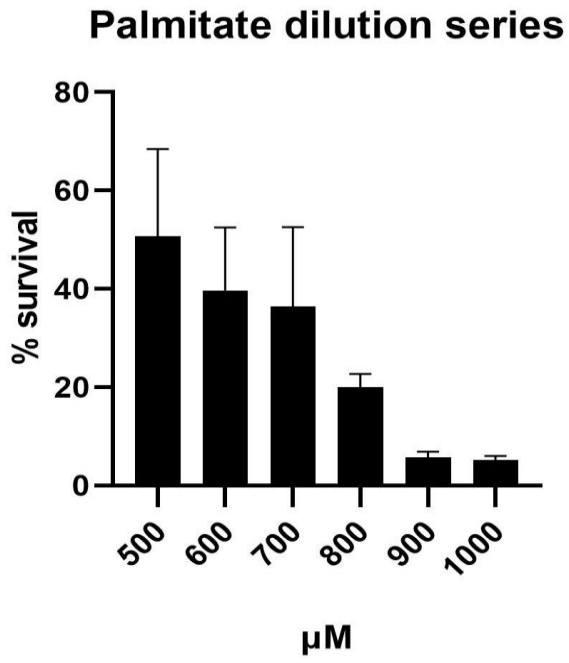

Figure 7. A dilution series of palmitate based on nine technical replicates was initially tested to determine a standard test concentration used in subsequent experiments of the RIN palmitate stress assay. All concentrations were supplemented with 25 mM glucose. The concentration of 800 µM was chosen due to the adequate apoptotic effect observed.

6.7 Chemical analysis

To evaluate the degree of hydrolysis of the five protein hydrolysates, analysis through gel electrophoresis using SDS-PAGE was performed. As seen in figure 8, distribution of bands accumulated at around 3.5 kDa, and the hydrolysates were therefore determined as completely hydrolyzed.

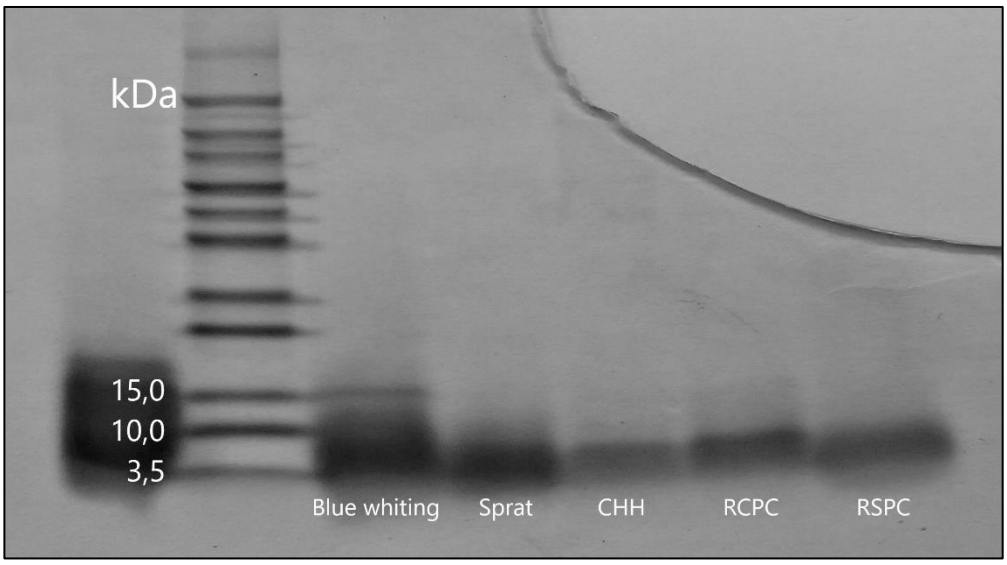

Figure 8. SDS-PAGE separates proteins based on their molecular weight and was used to evaluate the degree of hydrolysis in the five protein hydrolysates. The Novex® Sharp Pre-stained Protein Standard covers a range of 3.5-260 kDa and was used as ladder. The gel provides information on the approximate distribution of sizes of peptides present in a sample. The bands of all five hydrolysates are distributed around 3.5 kDa, indicating that the peptides in the samples are of this estimated size.

To compare the molecular composition of the protein hydrolysates of sprat (*Sprattus sprattus*) and blue whiting (*Micromesistius poutassou*), UHPLC in the combination with mass spectrometry using positive and negative electrospray ionization (ESI) was applied.

As the two hydrolysates were described to be of similar proximal compositions, similar chromatograms were expected. Figures 9 and 10 illustrate a comparison of the chromatograms of blue whiting and sprat obtained from ESI+ and ESI-, respectively. The similarity in peaks and the distribution in retention times in the two pairs of chromatograms illustrate similar chemical composition in the two hydrolysates.

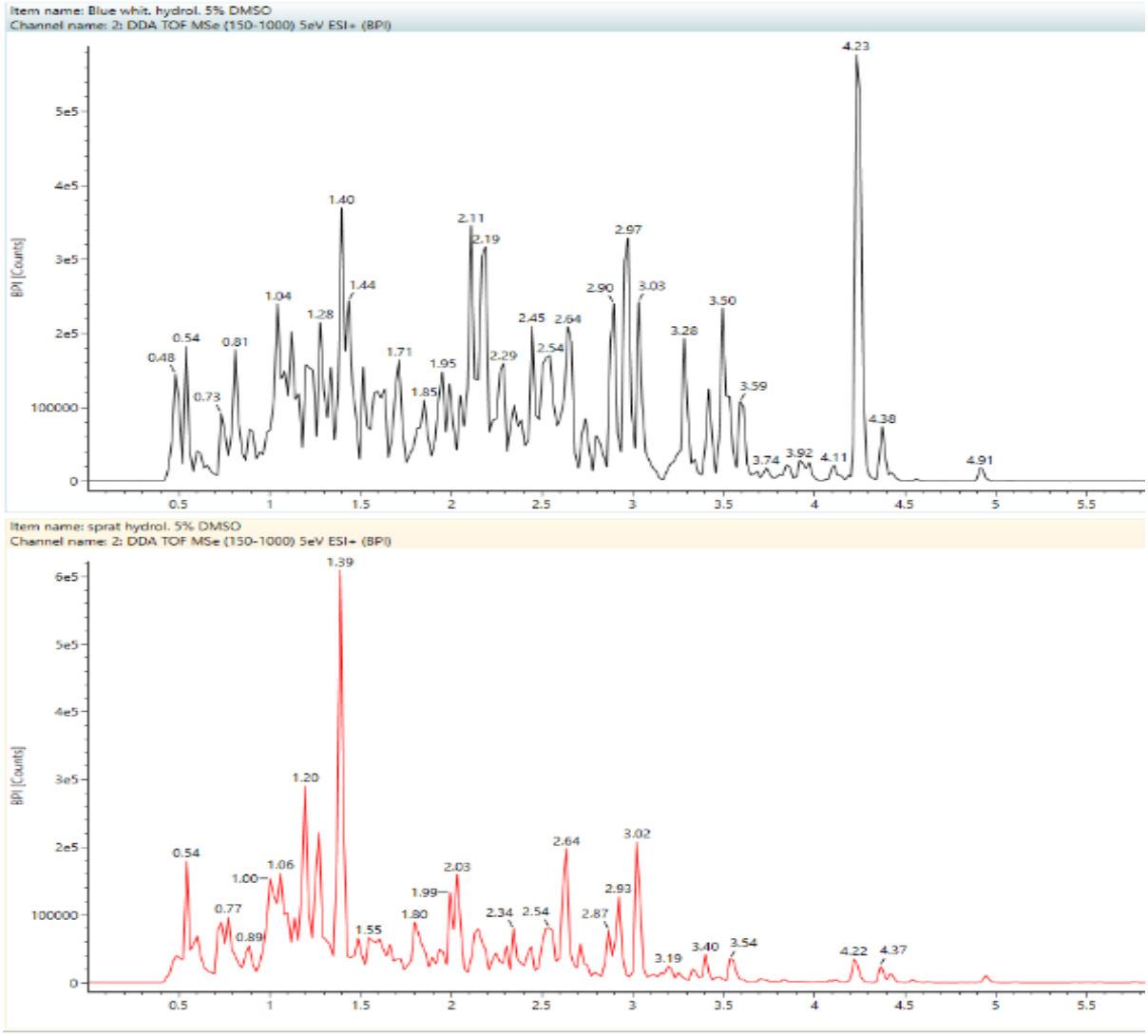

Figure 9. Comparison of the ESI+ chromatograms of protein hydrolysates of blue whiting (upper) and sprat (lower) illustrates similar qualitative presentation of positively charged compounds within 5 minutes of retention time, although different quantities of these compounds.

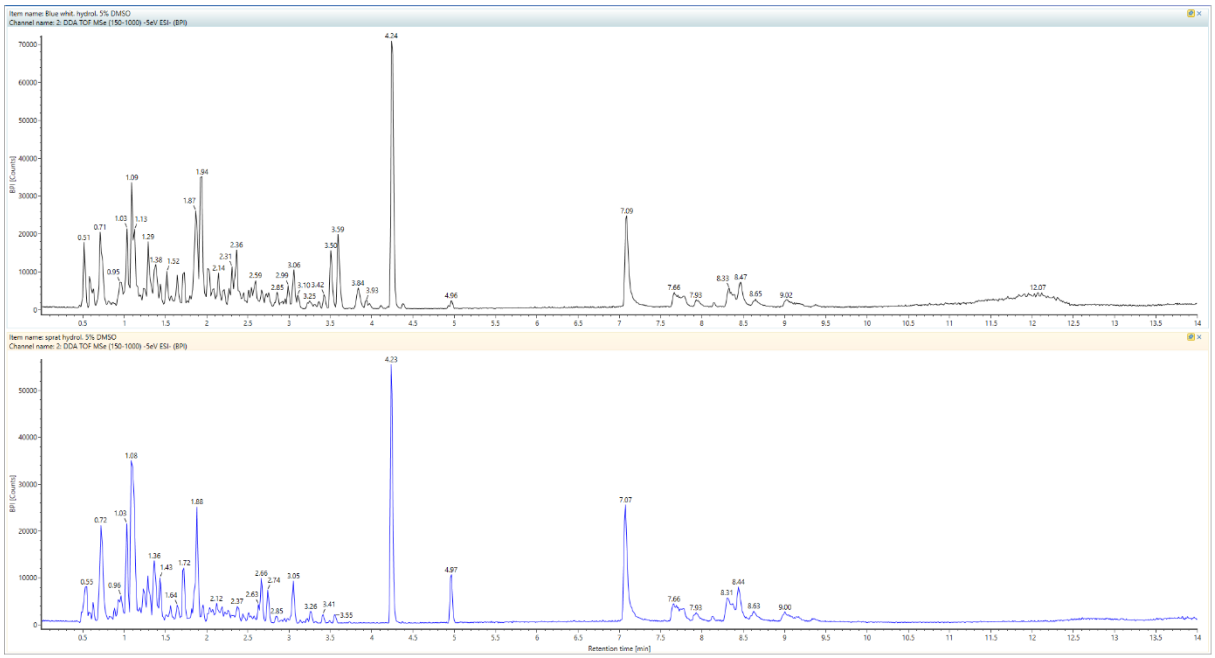

Figure 10. Comparison of the ESI- chromatograms of protein hydrolysates of blue whiting (upper) and sprat (lower) illustrates a highly similar qualitative and quantitative presentation of negatively charged compounds in the two hydrolysates. In addition to the compounds detected within the first five minutes which also could be seen in the ESI+ chromatograms, a cluster of compounds was detected between seven and nine minutes of retention time.

7 Discussion

The overall aim of this project was to investigate underutilized marine resources for ingredients that could be used in the management of metabolic syndrome (MetS) in humans. Samples included protein hydrolysates of various species and materials, including cod head (RCPC and CHH), shrimp shell (RSPC), sprat (*Sprattus sprattus*), blue whiting (*Micromesistius poutassou*), and biomass from the two microalgae *Nannochloropsis salina* and *Tetraselmis chui*. Additional experiments included evaluation of anti-inflammatory activity in 192 flash fractions of twenty-four marine invertebrate extracts. Samples were selected in cooperation with the industry partners involved in the SuReMetS project, with a special focus on underutilized species and the availability of biomass for potential scale-up.

Bioactivity studies in this project have involved the use of *in vitro* biochemical and cellular bioassays, including the TNFα anti-inflammatory assay, the FRAP antioxidant assay, the antidiabetic assays dipeptidyl peptidase IV (DPP-IV) inhibitory assay, the rat insulinoma (RIN) cytokine stress assay, and the RIN palmitate stress assay. The bioassays were chosen based on their relevance towards the various conditions that are involved in metabolic syndrome, including diabetes type 1 and 2, as well as factors linked to the development of diabetes type 2, including oxidative stress and inflammation. Cells are biological entities whose function and survival depend on a vast number of cellular and molecular processes and interactions. In addition, environmental factors such as temperature, moisture, air, and exposure time during the different cell works, may differ in between runs and cause additional variation in survival of cells. During the months of performing the anti-inflammatory assay, RIN cytokine stress assay, and RIN palmitate stress assay, cell survival and their sensitivity to samples varied considerably, making it challenging to reproduce results. In addition to the challenges met through working with RIN cells, the palmitate stress assay has been an experimental assay developed with basis in literature and through several attempts and adjustments of the protocol. This shows the importance of including controls, and repeating assays in several biological replicates. Improvements of the assays, or replacement with more robust assays will be needed to better evaluate the antidiabetic ability of these samples.

7.1 Evaluation of anti-inflammatory activity in marine invertebrates

Marine invertebrates are known to produce an array of secondary metabolites (SMs) including toxins, attractants, repellents, and dyes, and contribute to increasing the organisms' likelihood of survival and reproduction. Bioactivity studies on marine invertebrates have encountered additional functions of such SMs that may be of benefit to humans, including antidiabetic, antioxidative, and anti-inflammatory abilities, among others. Although different species of marine invertebrates have been found to produce SMs with anti-inflammatory abilities, many screening studies have tested crude extracts without any further chemical characterization.

In the anti-inflammatory bioassay performed in this project, seven of the 192 flash fractions were characterized as active in the first screen but could not be reproduced in the second screen. The fraction of *Securiflustra securifrons* was characterized as active in the first screen but was excluded from the second screen due to being determined as cytotoxic in previous studies. The challenge in reproducibility could be due to a highly sensitive assay which requires precise techniques in cultivation of cells and preparation of samples and reagents. Further investigation of these fractions was not pursued within this project.

7.2 Evaluation of bioactivities in marine hydrolysates and microalgae

Compared to bioactivity screening of fractionated extracts, such as flash fractions, complex samples such as crude protein hydrolysates and microalgae biomass challenge the standard methods for evaluation of bioactivity. In a protein hydrolysate, the activity of a potential active peptide may be thinned out by the high number of peptides, requiring higher test concentrations to observe activities high enough to be considered relevant. However, very high concentrations of most substances will eventually be toxic to cells. Another consequence of higher test concentrations is the accumulation of the natural pigments in a sample. High concentrations of microalgae are of especially strong color due to the content of photosynthetic pigments such as chlorophylls and carotenoids (Alam, 2019), which may disturb absorbance measurements and generate data which do not reflect on the bioactivity of such samples.

A focus in this project was to discover novel areas of use for crude protein hydrolysates from algae and various marine rest raw materials without further purification steps. Although the purpose of fractionation is of facilitating intent, purification of peptide concentrates may also

lead to loss of synergistic effects and instead be of negative outcome. While enzymatic hydrolysis may be performed in full scale and produce larger quantities of protein hydrolysates, purification is a time-consuming step which may be of additional cost to the process. The exclusion of purification steps has although brought other complications when evaluating mentioned materials in mentioned bioassays. Whereas bioactivity studies on secondary metabolites (SMs) may involve test concentrations in units of µg/mL, samples have in this project been tested at concentrations of mg/mL in order to observe biological effects. The main issue in increasing test concentrations was experienced in absorption measurements as samples were colored to various extent based on the origin of the material and their concentrations, which subsequently disturbed readout values. Alternative approaches were performed and initiated in solving this issue in the DPP-IV inhibitory assay, including absorbance measurements of samples parallel to the result readouts, and replacement of substrate with fluorescent substrate.

Additional issues included precipitation of microalgae samples due to incomplete rupture of cell walls. Although sonication did aid in dissolving microalgae biomass, precipitation still occurred in both preparations and under assay conditions. Incomplete rupture of cell walls indicates incomplete access to sarcoplasmic proteins and other intracellular molecular content that may possess biological activity. The presence of particles in a sample may also affect the accuracy in concentrations, e.g. when preparing dilutions, as well as disturbing absorbance measurements in result evaluations. Biomass of the two microalgae *N. salina* and *T. chui* were highly colored in all concentrations tested, including 0.5, 1.0, 2.5, and 5.0 mg/mL, and caused interference in the readout and result evaluation in all assays.

7.3 RIN cytokine stress assay

In type 1 diabetes, pancreatic beta cells are destroyed by the immune system due to an autoimmune response. Several immune cells, including macrophages, dendritic cells, B cells, and T cells, have been shown to be involved in the pathogenesis of autoimmune beta cell destruction (Yoon & Jun, 2005). The rat insulinoma (RIN) cytokine stress assay is a recently established bioassay at Marbio and was included in this project to evaluate samples for protective effect against cytokine-induced apoptosis of RIN cells. As presented in figure 4, no protective effect was observed in any of the samples. Samples showing increased survival of cells were of the highest concentration (10 mg/mL), as well as the 1 mg/mL sample of *T. chui,*

but since these samples were highly colored, they were considered as false positives. This was confirmed by using microscopy observations, where the 10 mg/mL concentration of all samples were determined as toxic to cells. Thus, none of the samples were considered as protective against cytokine-stressed RIN beta cells.

7.4 FRAP assay

In the FRAP assay, samples were evaluated for antioxidative potential by their ability to reduce the chemical Fe^{III}-TPTZ complex. The degree of antioxidative ability was determined using absorbance measurements and presented in units of µM Trolox Equivalents (TE).

Due to the difficulties in measuring individual antioxidant components of a complex mixture, Trolox equivalency is used as a benchmark in such measurements. Thus, the antioxidative ability of samples in the FRAP assay was not determined using threshold values. Interpretation of results may be aided by comparison to the antioxidative ability of material of similar origin, or to material known for its antioxidative potential. As presented in figure 5, the sample presenting the highest µM TE in the FRAP assay was the 5 mg/mL concentration of RSPC. While presenting a TEAC of 53,50 ± 1,36 µM TE, these results are likely to be biased due to the color present in the higher concentrations. In comparison, the 0.5 mg/mL dilution of the RSPC sample was of considerably weaker color and resulted in 9,67 ± 0,59 µM TE, which, based on the calculations presented in equations 3 and 4, equivalents to 19,34 µmol TE/g dw,

Equation 3 $$9{,}67\ \mu M\ TE * 170\ \mu L = 0{,}001644\ \mu mol\ TE$$

Equation 4 $$\frac{0{,}001644\ \mu mol\ TE}{0{,}085\ mg} = 0{,}01934\ \mu mol\ TE/mg\ = 19{,}34\ \mu mol\ TE/g$$

where 9,67 µM refers to the Trolox equivalent (TE) exerted by the 0.5 mg/mL RSPC sample; 170 µL the total volume in the well; and 0,085 mg the mass of 0.5 mg/mL RSPC in the well.

At 0.5 mg/mL, the antioxidative effects the hydrolysates of RCPC, CHH, sprat, and blue whiting were very similar, equivalenting to approximately 6 µM TE or 12 µmol TE/g dw. In comparison, Le Gouic, Harnedy, and FitzGerald (2018) describe antioxidative activities of protein hydrolysates from skin of seabass (*Lates calcarifer*) with different degrees of hydrolysis to 2.57 and 2.65 µmol TE/g dw in FRAP experiments, as well as Huang, et.al. (2012) describe an antioxidative capacity of 149.8 µmol TE/g dw in blueberries, although using different antioxidant assays (DPPH and ABTS). However, comparing results from different studies may

be difficult due to different equipment and protocols, and does not necessarily provide a correct comparison of materials and their bioactive potential.

As an alternative to FRAP, the ORAC assay could be used to measure the antioxidative potential of samples using fluorescence measurements. This way, color disturbances could be avoided, providing more accurate measurements of the antioxidative potential of these samples.

7.5 DPP-IV inhibitory assay

In the DPP-IV inhibitory assay, samples were evaluated for antidiabetic activity by inhibition of the DPP-IV enzyme. The five samples presented high inhibitory activity in the concentrations of 2.5 and 5 mg/mL, as well as three of five hydrolysates being active in the 1 mg/mL concentration. The low activity and high standard deviation observed in the 5 mg/mL concentration of blue whiting could be due to pipetting errors. RSPC was characterized as active in all four concentrations. The results presented in figure 6 are based on the absorbance measurements of the assay plate and a control plate where enzyme was replaced with buffer. Subtraction of OD values of the control plate from the OD values of the assay plate allowed background noise to be removed, and the inhibitory effect of samples to be estimated.

To relate the inhibitory effects of samples to a known reference, these results were presented in comparison to the effect of the known DPP-IV inhibitor Diprotin A. As the inhibitory effect of Diprotin A may vary between biological replicates, an alternative way of presenting results would be to compare the inhibitory effect of samples to the maximum reaction of enzymatic degradation of the Gly-Pro-p-nitroanilide substrate. In the results presented in figure 6, the inhibitory activity of Diprotin A exerted 100 % inhibition, but only 82 % inhibition compared to the maximum reaction in the negative control, indicating that the inhibitory effect of samples compared to the maximum assay reaction is 82 % of the effect presented in figure 6.

Although a total of eleven of twenty samples and concentrations were characterized as active based on these results, retests will be necessary to determine if the results are valid. The two lowest concentrations, 0.5 and 1.0 mg/mL, were of little observable color in all five hydrolysates, and may therefore provide more reliable results compared to the two highest concentrations. The concentrations of 2.5 and 5.0 mg/mL of RSPC and other fish hydrolysates were of notable color and may therefore lead to higher variance when calculating ΔOD values. As the high concentrations are strong in color, these samples challenge the use of equation 1 to

calculate inhibitory effects. As samples of higher concentrations may exert higher inhibitory activity than the 1 mg/mL Diprotin A control, the use of the negative control in calculations of results would provide a better definition of the range of inhibitory effect in the system. Also, determining a standard for presentation of results is important to enable samples from various experiments using the DPP-IV assay to be comparable. Nevertheless, using a fluorescent substrate in replacement of the Gly-Pro-p-nitroanilide substrate, color disturbances and subsequent estimations of the inhibitory effect of samples could be avoided.

7.6 RIN palmitate stress assay

In combination of performing the RIN cytokine stress assay, the RIN palmitate stress assay was developed to cover type 2 diabetes. In figure 7, a dose-response curve was observed as cell survival decreased with increased dose of palmitate. Based on these results, the concentration of 800 µM palmitate was selected as test concentration due to the adequate apoptotic effect observed. In subsequent experiments, solutions of sample and palmitate were added to wells in a 1:9 ratio. To obtain a final concentration of 800 µM palmitate, an 890 µM concentration of palmitate was prepared and added to wells. Cells were incubated with samples for 24 h before discarding supernatant and re-adding sample and 890 µM palmitate with 25 mM glucose.

In one of the experiments, wells added 800 µM palmitate were observed to contain a grainy texture compared to the cell and DMSO controls. This may indicate precipitation of palmitate either when added to wells, or during preparations of dilutions. The palmitate solution was prepared by dissolving sodium palmitate in 50 % EtOH to a concentration of 100 mM, and required heating up to 75^{o}C for the solution to dissolve completely and to remain stable when removed from the heat source. In comparison, the protocol described by Wang and Welsh (2014) includes heating of the palmitate solution to 45^{o}C. Due to the high dilution ratio when further diluted in growth medium, it is unlikely that the palmitate dilutions have been of too high temperature before being added to cells, affecting cell survival negatively.

In comparing these experiments to literature, Wang and Welsh (2014) describe exposure of cells to 500 µM palmitate, and Alnahdi et. al. (2019) describe exposure of RIN-5F cells (ATCC CRL-2058) to concentrations up to 300 µM palmitate and up to 25 mM glucose. Thus, there would be reason to assume that the concentration of 800 µM palmitate used in experiments was too high, and that the initial testing of palmitate dilution series was flawed. In one experiment

it was decided to use 300 µM palmitate, but no difference in cell survival could be observed. Therefore, no protective effect could be detected in experiments of the RIN palmitate stress assay. Further experiments would include selecting suitable concentrations of palmitate and glucose before evaluating protective effect of samples. Unfortunately, this was not possible to achieve within the time frame.

7.7 Chemical characterization

SDS-PAGE was applied to evaluate the degree of hydrolysis in the protein hydrolysates. Figure 8 illustrates the band distribution of samples at around 3.5 kDa, indicating complete hydrolysis and that the hydrolysates are composed of small peptides. As the distribution of bands in the gel is meant to determine the content of peptides and proteins of different sizes, the SDS-PAGE does not provide accurate information about the sizes of the different components.

Chemical analysis using UHPLC-MS is a precise and effective technique often used in analysis of bioactive samples. MS may provide data on the chemical composition of a sample which further can be used to potentially identify single bioactive compounds, or to characterize the chemical nature of a mixture of compounds, such as accurate measurements of the size distribution of peptides in a hydrolysate. As the protein hydrolysates of sprat and blue whiting exerted similar effects in bioactivity testing, and were described by industrial partners to be of similar proximal composition, it was of interest to compare the content of peptides in the two hydrolysates. The chromatograms generated using ESI+ illustrated a qualitatively similar content of compounds, although differences in the quantity of these compounds.

7.8 Future perspectives

The initial data collected of these samples shows the need for proper assays and assay readouts to properly investigate their bioactive potential. As the main challenge experienced was the strong color of highly concentrated samples, this problem could be avoided by integration of fluorescence substrates to perform measurements of fluorescence instead of absorbance. As the results presented in this thesis are based on only one biological replicate, interpretations must be done cautiously. Nevertheless, these results may although serve as a pointer for further bioactivity studies on material of similar origin. The RSPC protein hydrolysate exerted both antidiabetic and antioxidative properties, and will therefore be fractionated using Flash chromatography to further investigate the bioactive peptides of the hydrolysate. As the chemical

composition of protein hydrolysates highly depend on hydrolyzation parameters including enzymes, temperature, and duration of hydrolysis, adjustments of such parameters may generate hydrolysates which contain peptides whose activity can be beneficial for the management of MetS. Some of the samples tested in this project will be hydrolyzed with alternative conditions and further tested for bioactivity. Utilization of underutilized marine raw materials is advantageous from both an economic, environmental, and ethical perspective. The development of nutraceuticals from rest raw materials from fisheries is of emerging interest, where several *in vitro* studies describe different bioactive properties, including antidiabetic, antioxidative, and anti-inflammatory activities in fish protein hydrolysates. A recent *in vivo* study by Jensen et al. (2020) investigated the effect of low doses of cod protein hydrolysate on the glucose regulation and lipid metabolism in patients of MetS, without demonstrating a strong effect *in vivo*. Nevertheless, the study reflects an interest in investigating the bioactive potential of underutilized marine materials. As adjustments in the design of the study by Jensen et al. (2020) could be implemented, research on fish hydrolysates and the effect on metabolic markers may still be considered as a prospect for future studies. Thus, *in vitro* bioactivity studies of underutilized marine materials may lead to novel discoveries and lay an important scientific foundation to continue into preclinical and clinical studies.

8 Conclusion

Underutilized marine raw materials were evaluated for bioactivities using a panel of antidiabetic, antioxidant, and anti-inflammatory bioassays. Of the 192 flash fractions of twenty-four marine invertebrate extracts, seven fractions were characterized as active, without being further investigated. The RIN palmitate stress assay was developed to approach the apoptotic conditions of pancreatic beta cells in patients of type 2 diabetes, but could not determine whether samples exerted protective effects as standard concentrations of palmitate and glucose could not be established. The refined shrimp peptide concentrate (RSPC) was characterized as active at the lowest concentration tested (0.5 mg/mL) in the DPP-IV inhibitory assay. Of the five hydrolysates, RSPC also showed the higher antioxidative effect in the FRAP assay. The results from *in vitro* bioactivity screening obtained in this project may therefore indicate potential antidiabetic and antioxidative effects of RSPC and other marine protein hydrolysates *in vivo*, although further *in vitro* studies are needed to validate these results. This study has given a thorough basis in the technical challenges met, and could be useful in guiding a better selection of which bioassays to be used in further bioactivity studies, especially when evaluating the effects in complex marine samples.

Works cited

Alnahdi, A., John, A., & Raza, H. (2019). N-acetyl cysteine attenuates oxidative stress and glutathione-dependent redox imbalance caused by high glucose/high palmitic acid treatment in pancreatic Rin-5F cells. *PloS one, 14*(12), e0226696. doi:10.1371/journal.pone.0226696

Barkia, I., Saari, N., & Manning, S. R. (2019). Microalgae for High-Value Products Towards Human Health and Nutrition. *Marine drugs, 17*(5), 304. doi:10.3390/md17050304

Benzie, I. F. F., & Strain, J. J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. *Anal Biochem, 239*(1), 70-76. doi:10.1006/abio.1996.0292

Betteridge, D. J. (2000). What is oxidative stress? *Metabolism, 49*(2, Supplement 1), 3-8. doi:https://doi.org/10.1016/S0026-0495(00)80077-3

Calado, R., Leal, M. C., Gaspar, H., Santos, S., Marques, A., Nunes, M. L., & Vieira, H. (2018). How to Succeed in Marketing Marine Natural Products for Nutraceutical, Pharmaceutical and Cosmeceutical Markets. In P. H. Rampelotto & A. Trincone (Eds.), *Grand Challenges in Marine Biotechnology* (pp. 317-403). Cham: Springer International Publishing.

Cerf, M. E. (2013). Beta cell dysfunction and insulin resistance. *Frontiers in endocrinology, 4*, 37-37. doi:10.3389/fendo.2013.00037

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., . . . Zhao, L. (2017). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget, 9*(6), 7204-7218. doi:10.18632/oncotarget.23208

Cnop, M., Welsh, N., Jonas, J.-C., JÖRns, A., Lenzen, S., & Eizirik, D. L. (2005). Mechanisms of pancreatic β-cell death in type 1 and type 2 diabetes : Many differences, few similarities. *Diabetes, 54*, S97-S107.

Cornier, M.-A., Dabelea, D., Hernandez, T. L., Lindstrom, R. C., Steig, A. J., Stob, N. R., . . . Eckel, R. H. (2008). The metabolic syndrome. *Endocrine reviews, 29*(7), 777-822. doi:10.1210/er.2008-0024

Doughman, S., Srirama, K., & Sanjeevi, C. (2007). Omega-3 Fatty Acids for Nutrition and Medicine: Considering Microalgae Oil as a Vegetarian Source of EPA and DHA. *Current diabetes reviews, 3*, 198-203. doi:10.2174/157339907781368968

Duan, L., Rao, X., & Sigdel, K. R. (2019). Regulation of Inflammation in Autoimmune Disease. *J Immunol Res, 2019*, 7403796-7403792. doi:10.1155/2019/7403796

Evans, K. (2019). Diabetic ketoacidosis: update on management. *Clinical medicine (London, England), 19*(5), 396-398. doi:10.7861/clinmed.2019-0284

Figueiredo, P. S., Inada, A. C., Fernandes, M. R., Arakaki, D. G., Freitas, K. d. C., Avellaneda Guimaraes, R. d. C., . . . Hiane, P. A. (2018). An Overview of Novel Dietary Supplements and Food Ingredients in Patients with Metabolic Syndrome and Non-Alcoholic Fatty Liver Disease. *Molecules, 23*(4), 877. doi:10.3390/molecules23040877

Gunstone, F. D., John L. Harwood, Albert J. Dijkstra. (2007). *The Lipid Handbook*. Retrieved from http://aevnmont.free.fr/SACH-BOOKS/Organic%20Chemistry/Gunstone_The%20Lipid%20Handbook%203rd%20ed.pdf

Harnedy-Rothwell, P. A., McLaughlin, C. M., O'Keeffe, M. B., Le Gouic, A. V., Allsopp, P. J., McSorley, E. M., . . . FitzGerald, R. J. (2020). Identification and characterisation of peptides from a boarfish (Capros aper) protein hydrolysate displaying in vitro

dipeptidyl peptidase-IV (DPP-IV) inhibitory and insulinotropic activity. *Food Research International, 131*, 108989. doi:https://doi.org/10.1016/j.foodres.2020.108989
Harnedy, P. A., Parthsarathy, V., McLaughlin, C. M., O'Keeffe, M. B., Allsopp, P. J., McSorley, E. M., . . . FitzGerald, R. J. (2018). Atlantic salmon (Salmo salar) co-product-derived protein hydrolysates: A source of antidiabetic peptides. *Food Research International, 106*, 598-606. doi:https://doi.org/10.1016/j.foodres.2018.01.025
Holman, R. (2007). Metformin as first choice in oral diabetes treatment: the UKPDS experience. *Journ Annu Diabetol Hotel Dieu*, 13-20.
Hägerkvist, R., Jansson, L., & Welsh, N. (2008). Imatinib mesylate improves insulin sensitivity and glucose disposal rates in high-fat diet fed rats. *Clinical science (London, England : 1979), 114*, 65-71. doi:10.1042/CS20070122
Hägerkvist, R., Makeeva, N., Elliman, S., & Welsh, N. (2006). Imatinib mesylate (Gleevec) protects against streptozotocin-induced diabetes and islet cell death in vitro. *Cell Biol Int, 30*(12), 1013-1017. doi:10.1016/j.cellbi.2006.08.006
Jensen, C., Fjeldheim Dale, H., Hausken, T., Hatlebakk, J. G., Brønstad, I., Lied, G. A., & Hoff, D. A. L. (2020). Supplementation with Low Doses of a Cod Protein Hydrolysate on Glucose Regulation and Lipid Metabolism in Adults with Metabolic Syndrome: A Randomized, Double-Blind Study. *Nutrients, 12*(7), 1991. doi:10.3390/nu12071991
Katakami, N. (2018). Mechanism of Development of Atherosclerosis and Cardiovascular Disease in Diabetes Mellitus. *Journal of atherosclerosis and thrombosis, 25*(1), 27-39. doi:10.5551/jat.RV17014
Lacroix, I. M. E., & Li-Chan, E. C. Y. (2012). Dipeptidyl peptidase-IV inhibitory activity of dairy protein hydrolysates. *International Dairy Journal, 25*(2), 97-102. doi:https://doi.org/10.1016/j.idairyj.2012.01.003
Lauritano, C., & Ianora, A. (2016). Marine Organisms with Anti-Diabetes Properties. *Mar Drugs, 14*(12), 220. doi:10.3390/md14120220
Le Gouic, A. V., Harnedy, P. A., & FitzGerald, R. J. (2018). Bioactive Peptides From Fish Protein By-Products. In J.-M. Mérillon & K. G. Ramawat (Eds.), *Bioactive Molecules in Food* (pp. 1-35). Cham: Springer International Publishing.
Malve, H. (2016). Exploring the ocean for new drug developments: Marine pharmacology. *J Pharm Bioallied Sci, 8*(2), 83-91. doi:10.4103/0975-7406.171700
McGivern, J. G. (2007). Ziconotide: a review of its pharmacology and use in the treatment of pain. *Neuropsychiatric disease and treatment, 3*(1), 69-85. doi:10.2147/nedt.2007.3.1.69
Mulvihill, E. E., & Drucker, D. J. (2014). Pharmacology, physiology, and mechanisms of action of dipeptidyl peptidase-4 inhibitors. *Endocrine reviews, 35*(6), 992-1019. doi:10.1210/er.2014-1035
Nathan, D. M. (1993). Long-Term Complications of Diabetes Mellitus. *New England Journal of Medicine, 328*(23), 1676-1685. doi:10.1056/nejm199306103282306
Nemecz, M., Constantin, A., Dumitrescu, M., Alexandru, N., Filippi, A., Tanko, G., & Georgescu, A. (2019). The Distinct Effects of Palmitic and Oleic Acid on Pancreatic Beta Cell Function: The Elucidation of Associated Mechanisms and Effector Molecules. *Front Pharmacol, 9*, 1554-1554. doi:10.3389/fphar.2018.01554
Norwegian Seafood Council. (2020). Norwegian seafood exports top NOK 107 billion in 2019. Retrieved from https://en.seafood.no/news-and-media/news-archive/norwegian-seafood-exports-top-nok-107-billion-in-2019/

Pahwa, R., Goyal, A., Bansal, P., & Jialal, I. (2020). *Chronic Inflammation*. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK493173/

Pasquel, F. J., & Umpierrez, G. E. (2014). Hyperosmolar hyperglycemic state: a historic review of the clinical presentation, diagnosis, and treatment. *Diabetes Care, 37*(11), 3124-3131. doi:10.2337/dc14-0984

Patti, A. M., Al-Rasadi, K., Giglio, R. V., Nikolic, D., Mannina, C., Castellino, G., . . . Toth, P. P. (2018). Natural approaches in metabolic syndrome management. *Archives of medical science : AMS, 14*(2), 422-441. doi:10.5114/aoms.2017.68717

Petersen, L.-E., Kellermann, M. Y., & Schupp, P. J. (2020). Secondary Metabolites of Marine Microbes: From Natural Products Chemistry to Chemical Ecology. In S. Jungblut, V. Liebich, & M. Bode-Dalby (Eds.), *YOUMARES 9 - The Oceans: Our Research, Our Future: Proceedings of the 2018 conference for YOUng MArine RESearcher in Oldenburg, Germany* (pp. 159-180). Cham: Springer International Publishing.

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., . . . Bitto, A. (2017). Oxidative Stress: Harms and Benefits for Human Health. *Oxidative medicine and cellular longevity, 2017*, 8416763-8416763. doi:10.1155/2017/8416763

Santini, A., Cammarata, S. M., Capone, G., Ianaro, A., Tenore, G. C., Pani, L., & Novellino, E. (2018). Nutraceuticals: opening the debate for a regulatory framework. *British journal of clinical pharmacology, 84*(4), 659-672. doi:10.1111/bcp.13496

Sarkar, S. A., Kutlu, B., Velmurugan, K., Kizaka-Kondoh, S., Lee, C. E., Wong, R., . . . Pugazhenthi, S. (2009). Cytokine-mediated induction of anti-apoptotic genes that are linked to nuclear factor kappa-B (NF-κB) signalling in human islets and in a mouse beta cell line. *Diabetologia, 52*(6), 1092-1101. doi:10.1007/s00125-009-1331-x

Shastry, R. M., & Bhatia, V. (2006). Cerebral edema in diabetic ketoacidosis. *Indian Pediatr, 43*(8), 701-708.

Svenson, J. (2013). MabCent: Arctic marine bioprospecting in Norway. *Phytochemistry Reviews, 12*(3), 567-578. doi:10.1007/s11101-012-9239-3

Swanson, D., Block, R., & Mousa, S. A. (2012). Omega-3 Fatty Acids EPA and DHA: Health Benefits Throughout Life. *Adv Nutr, 3*(1), 1-7. doi:10.3945/an.111.000893

Szablewski, L. (2014). Role of immune system in type 1 diabetes mellitus pathogenesis. *Int Immunopharmacol, 22*(1), 182-191. doi:10.1016/j.intimp.2014.06.033

Torres, J. P., & Schmidt, E. W. (2019). The biosynthetic diversity of the animal world. *J Biol Chem, 294*(46), 17684-17692. doi:10.1074/jbc.REV119.006130

Veneri, D., Franchini, M., & Bonora, E. (2005). Imatinib and Regression of Type 2 Diabetes. *The New England journal of medicine, 352*, 1049-1050. doi:10.1056/NEJM200503103521023

Vetere, A., Choudhary, A., Burns, S., & Wagner, B. (2014). Targeting the pancreatic ??-cell to treat diabetes. *Nature reviews. Drug discovery, 13*. doi:10.1038/nrd4231

Wang, X., & Welsh, N. (2014). Bcl-2 maintains the mitochondrial membrane potential, but fails to affect production of reactive oxygen species and endoplasmic reticulum stress, in sodium palmitate-induced β-cell death. *Ups J Med Sci, 119*(4), 306-315. doi:10.3109/03009734.2014.962714

Welsh, N. (n.d.). Role of tyrosine kinases in ß-cell apoptosis and diabetes. Retrieved from https://www.mcb.uu.se/res/groups/nw/projects/project1/

Xia, E.-Q., Zhu, S.-S., He, M.-J., Luo, F., Fu, C.-Z., & Zou, T.-B. (2017). Marine Peptides as Potential Agents for the Management of Type 2 Diabetes Mellitus-A Prospect. *Marine drugs, 15*(4), 88. doi:10.3390/md15040088

Yoon, J. W., & Jun, H. S. (2005). Autoimmune destruction of pancreatic beta cells. *Am J Ther, 12*(6), 580-591. doi:10.1097/01.mjt.0000178767.67857.63

Appendix

As part of the initial practice in laboratory work, additional samples were evaluated for bioactivity by screening in various bioassays. Fourteen protein hydrolysates from cod (*Gadhus morhua*), cod head, cod skin, and shrimp shells, produced using different enzymes and parameters of hydrolyzation, were provided by Marealis Innovation AS. The protein hydrolysates were tested in the DPP-IV inhibitory assay, FRAP (ferric reducing ability of plasma) antioxidative assay, and the rat insulinoma (RIN) cytokine stress assay, but did not exert activity in any of the assays. In addition, three isolated compounds investigated at Marbio, including the chlorinated metabolite chlovalicin B isolated from the Arctic marine basidiomycete *Digitatispora marina*, were evaluated for protective effects against cytokine-mediated apoptosis in the RIN cytokine stress assay. The testing of the three MBCs was terminated due to limitations in material and time.

The Marealis RSPC (refined shrimp peptide concentrate) tested in this study was sampled during participation at Marealis' full-scale production at the bioprocess facility Biotep Nofima in Kaldfjord, Tromsø, January 2021.