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Investigating the proliferative effects of seven vegetable-derived protein hydrolysates on Bovine Skeletal Muscle Cells.

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Food Science

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Abstract

The cultured meat industry is rapidly growing and secured 1.38 billion$ in investments for 2021. Up to 95% of the total production cost is tied to the use of fetal bovine serum. Therefore, the most pressing issue for the industry is the generation of serum-free medias for sustained expansion of skeletal muscle cells. Discovering growth stimulators could directly be used in meat cultivation and be implemented in serum-free medias. The main goal of this study was to investigate the growth-promoting effects of seven vegetable-derived protein hydrolysates on bovine skeletal satellite/myoblast cells (MuSCs). Seven vegetable and one animal-derived protein concentrates were hydrolyzed with either alcalase or corolase. The hydrolysates were freeze-dried and characterized based on average molecular weight and molecular weight distribution with size exclusion chromatography. Furthermore, the hydrolysates were supplemented to MuSCs in normal serum conditions for 24, 48, and 96 hours. After incubation proliferation was measured with CyQuant proliferation assay to determine the supplementary effect of each hydrolysate. In the end, a DPP-IV assay was performed on hydrolysates to investigate DPP-IV inhibition. Size exclusion chromatography revealed the choice of raw material and enzyme affected the molecular weight and molecular weight distribution of peptides in the hydrolysates. The results show that Pea-isolate and pea-concentrate hydrolyzed with corolase significantly increased proliferation by 63.3% and 54%, respectively, after 96 hours of incubation. Whey protein concentrate hydrolyzed with alcalase significantly increased proliferation by 37.6%. Faba-bean hydrolysates significantly reduced cell proliferation, while Oat hydrolysate did not affect proliferation in a major way. DPP-IV assay showed hydrolysates from pea-isolate and Whey protein to have the strongest inhibition of DPP-IV. We suggest that Pea-isolate/concentrate and whey protein concentrate hydrolysates have serum replacement potential and require further investigation. They hold promising growth-stimulating effects on MuSCs and could be useful in the development of a serum-free media formulation.

Abbreviations

Muscle satellite cells (MuSCs)

Muscle derived stem cells (MDSCs)

Fetal bovine serum (FBS)

Chinese hamster ovary (CHO)

Dulbecco's Modified Eagle Medium (DMEM)

Molecular Weight (M_w)

Degree of hydrolysis (DH)

Plant protein hydrolysates (PPH)

Good cell manufacturing practice (GMP)

Growth factors (GF)

Monoclonal antibodies (mAbs)

Mitogen-activated protein kinase (AMPK)

Paired box protein 7 (Pax7)

Myoblast determination protein 1 (MyoD)

Myogenic factor 5 (myf5)

Epidermal growth factor (EGF)

Kunitz trypsin inhibitors (KTi)

Size exclusion chromatography (SEC)

Molecular weight distribution (MWD)

Mesenchymal stem cells (MSC)

Protein free media (PFM)

Interferon-gamma (IFN-γ)

Wheat protein (WP)

Rice protein (RP)

Phosphate-Buffer Saline (PBS)

Entactin-Collagen Laminin (ECL)

Mass spectrometry (MS)

Reverse phase liquid chromatography (RPCL)

Vascular endothelial growth factor (VEGF)

Transforming growth factors beta 1 (TGF-β1)

Interleukin-6 (IL6)

mothers against decapentaplegic homolog 2/3 (SMAD2/3).

Platelet-derived growth factor (PDGF)

Contents

1.0 Introduction

1.1 Background

Muscle-derived stem cells (MDSCs) are believed to be the progenitor cells of satellite cells, which are responsible for muscular regeneration (1). MDSCs are not restricted to the myogenic lineage and can differentiate into several cell types, such as osteocytes and adipocytes (2). The relative ease of harvesting and purifying MDSCs and their differentiating ability offer promising regenerative therapy for the treatment of heart failure and bone/cartilage healing.

To further the scientific advancement of regenerative therapy and produce pharmaceutical proteins for treating human diseases, mammalian cell cultures have become an important field in modern biotechnology (3). The biopharmaceutical industry has opened a heap of new therapeutics in developing vaccines, antibodies, interferons, and tissue engineering. In food science, an emerging new technology cultivated meat has the potential to transform protein production from farm to laboratory by offering higher efficiency, consistency, lower environmental impact, and increased animal welfare compared to conventional meat products (4). Competitive alternatives to the future of protein production are better utilization of plant protein. It is estimated that 50% of grains produced are fed to livestock, yet 854 million people suffer from hunger and malnutrition (5). Less developed countries could benefit from the reduced use of animal feed sustaining livestock. Production of cultivated meat may have the potential to work synergistically with better utilization of plant protein, as demand for meat continues to rise. Additionally, single-cell protein production implementing microbes in the human diet is another alternative, to meet the growing populations increasingly demanding protein needs (6).

The cultivation of meat and the production of therapeutic proteins demand increased growth and protein yield from mammalian cell cultures. At the same time, the economic cost of production must be reduced. The costliest part of growing mammalian cell cultures is due to fetal bovine serum (FBS) accounting for 95% of the total cost (7). FBS includes an undefined cocktail of growth factors necessary for in vitro cultivation of mammalian cell lines (8). However, it comes with the risk of infectious components and ethical issues and the supply is lower than the demand (7). The last decade increased attention has been put on substituting FBS. Scientists have been trying to replicate the properties of FBS for use in mammalian cell

cultures since the 1970s. Serum-free media (SFM) have been developed for cells producing therapeutic proteins, such as Chinese hamster ovary (CHO) cells (9). Unfortunately, muscle cells reduced/starved of serum have been previously reported to negatively impact proliferation and metabolic activity (10). It is of great interest to develop an SFM for muscle cells.

The first attempts to produce a serum-free media adopted animal-derived protein hydrolysates (11). Protein hydrolysates can be made through enzymatic protein hydrolysis (12). Enzymatic protein hydrolysis is the breakdown of proteins into peptides and free amino acids by peptidases. Several commercial enzymes are used today in producing protein hydrolysates for animal feed, food, and nutraceutical applications (13, 14). Protein hydrolysates are known to contain many constituents that the basal media use to cultivate mammalian cells, known as Dulbeccos Modified Eagle Medium (DMEM) (15). Depending on the raw material used protein hydrolysates can contribute with free amino acids, peptides, carbohydrates, vitamins, minerals, and other undefined components (16). It is suggested that hydrolysates not only contribute nutritionally, but low molecular weight (M_W) peptides can function as growth factors and higher M_W peptides as survival factors (17). Exploring a growth medium that replicates the benefits of FBS without the adverse effects of animal derivatives has increased the interest in plant protein hydrolysates (PPH). Plant protein is available in massive quantities and is relatively cheap. Therefore, further investigating plant protein hydrolysates applied on mammalian cell cultures are of great interest.

1.2 Aim of the thesis

The cultivated meat industry is quickly developing and in need of growth stimulators to allow upscaling of industrial cell systems. Additionally, good cell manufacturing practice (GMP) along with economical restraints accelerate the formulation of a SFM for sustained expansion of muscle cells. Therefore, this thesis investigates the proliferative effects of Peas, Faba-bean, Barley, Oats, and WPC80 hydrolyzed with alcalase or corolase on in vitro growth of bovine skeletal satellite cells (MuSCs).

1.3 Cultivated meat

The cultivated meat industry is growing at a tremendous pace. According to the report from the good food institute only four start-up companies in 2016 had the goal of commercializing cell-based meat (18). At the end of 2018 over 24 companies worldwide had the same purpose. Today there are 107 companies that has raised 1.38 billion$ investments in 2021, accounting for 71% of all time investments in cultured meat. This emerging technology has the potential to transform protein production by offering higher efficiency, lower environmental impact, and

increased animal welfare than conventional meat production (19). Limited life cycle assessments suggest that cultured meat could require 98% less land and 75% less water than traditional beef, while contributing 80-95%% fewer greenhouse gas emissions (4).

The commercialization of cell-based meat today still faces challenges regarding cost reduction and upscaling processes. Currently, the most effective cost factor is the production of cell culture medium (7). It is estimated that 95% of the total cost is contributed to the medium, whereas the most considerable portion is due to the high cost of FBS and commercial growth factors (GF). FBS is expensive, unsustainable, and contains inconsistent components (7). It brings the risk of infectious agents and is a concern for human health. Additionally, it reduces batch-to-batch reproducibility causing complications for upscaling processes. A possibility for upscaled industrial meat cultivation processing is shown (figure 1) (20).

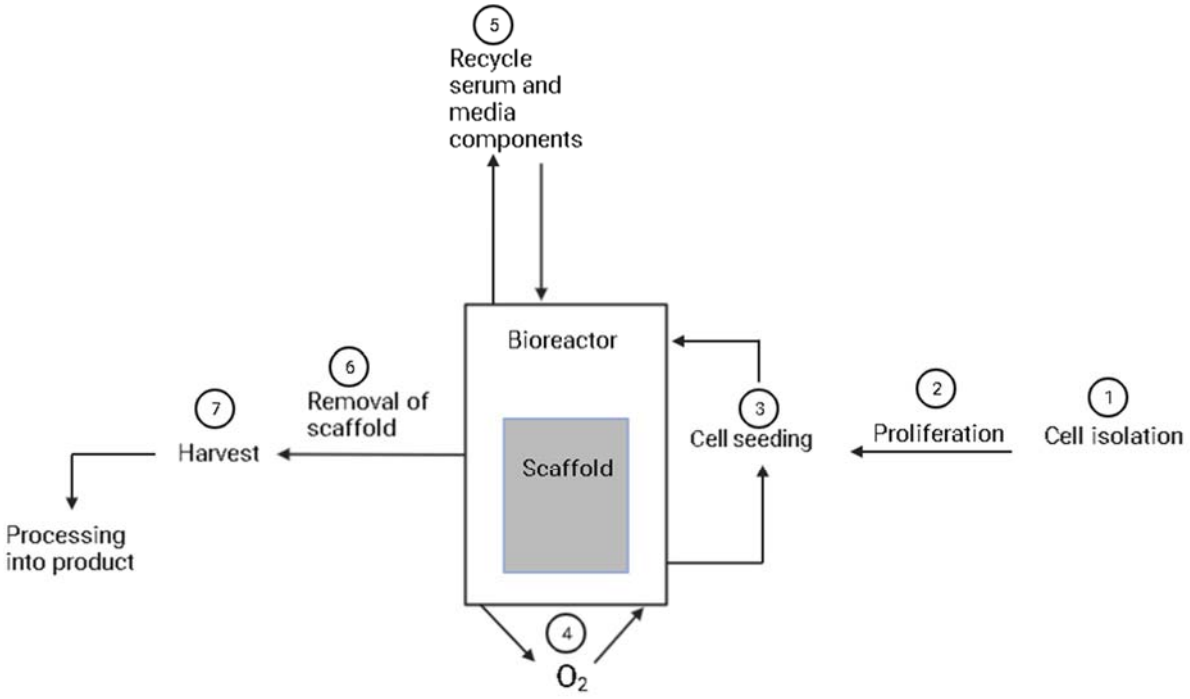

Figure 1. Flow chart of a potential cultured meat production system.

Figure 1 shows a flow chart of a potential cultured meat production system. 1: Cell isolation of wanted muscle cells, 2: Proliferation to increase viable cells for seeding, 3: Seeding cells onto scaffold inside the bioreactor, 4: Continuous O_2 supply through fluid loop, 5: Recycle serum and media components to reduce cost of production, 6: Removal of scaffold, 7: Harvesting cells.

Generating a serum-free media validated for sustained expansion of muscle cells remains the most pressing limitation in the field. It has become a part of GMP in research and food/pharmaceutical industry to develop SFM and move away from the use of FBS (7). A range of serum-free media has already been developed for mammalian cell lines with therapeutic applications in the pharmacological industry (3). These fields do not operate under the same economic constraints as the food and agriculture sectors. CHO cells applied in the pharmaceutical industry that produces monoclonal antibodies (mAbs) are considered large scale in the pharmacological industry. Nevertheless, they are many magnitudes smaller than the application of industrialized cultured meat production would have to be for achieving a cost-effective production. One possible approach is to increase the number of cells from a sample size by stimulating the proliferation of cells.

1.4 Myoblast myogenesis

To cultivate large quantities of meat for industrial purposes a small sample size of cells must be cultivated to massive quantities. MuSCs go through several growth stages that must be managed correctly to achieve a cost-effective production. This can be made possible by either massively increasing short term growth or by keeping cells from leaving the cell cycle, preventing fusing and differentiation into muscle fibers (see figure 2). (21). Skeletal muscle fibres consists of contractile myofibers that develop from their progenitor cells myoblasts, in a process called myogenesis (22).

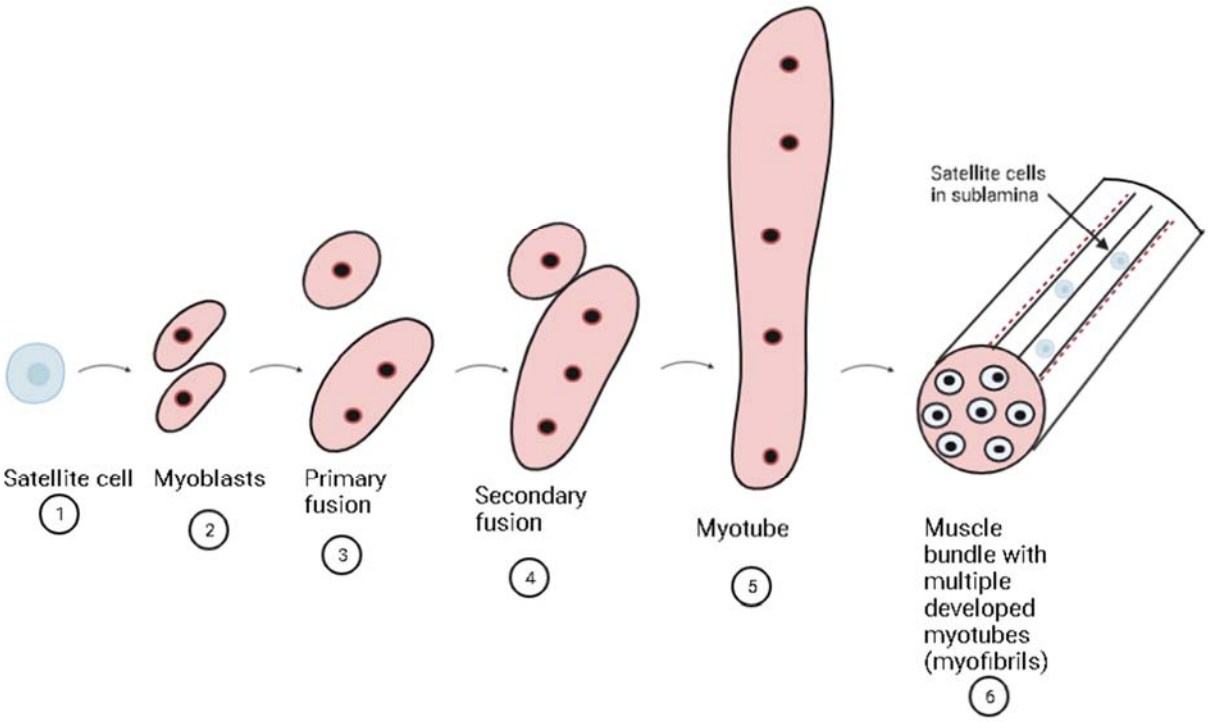

Figure 2. Myogenesis of mammalian skeletal muscle.

Figure 2 shows the myogenesis of mammalian skeletal muscle. 1: Upon physical or exercise induced injury satellite cells are activated to differentiate into myoblasts, 2: Myoblasts begin extensive proliferation, 3: Fusing into multinucleated myoblast, 4: Secondary fusion step, is the fusion into previously existing myotubes, 5: Several myoblasts have formed a Myotube, 6: Myotubes fuse with myofibrils increasing the size of the myofibril, at this point the myoblasts are considered differentiated into myofibrils.

Satellite cells lay dormant in the sublamina of the muscle. When the muscle is damaged, they fuse and increase the size of the myofiber, repairing damage. Satellite cells derive from their progenitor MDSCs. These cells have strong regenerative capabilities and evidence has been

accumulating proposing these cells as a true stem cell reservoir. Satellite cells have been shown to be activated by a mitogen (23). The mitogen is released as a result of muscle damage in myofibers during exercise or physical trauma. Activating the cell cycle progression of dormant satellite cells. This mitogen has later been shown to activate mitogen-activated protein kinase (MAPK) (24). MAPK are associated with increased transcriptional activity upregulating gene expression of several important transcription factors for muscle growth. Knockout of AMPK has been shown to result in a total loss of the transcription factor for paired box protein 7 (pax7), resulting in catastrophic effects on differentiation (25). Dormant satellite cells are characterized by their expression of Pax7, and non-expression of myoblast determination protein 1 (MyoD) and Myogenin. Pax 7 is an essential transcription factor for myogenic precursors, such as the transcription factors myogenic factor 5 (myf5) and MyoD (26, 27). Activated satellite cells are no longer dormant and have been shown to express MyoD and myf5, as early as 12h after injury. A previous study on mice saw that when MyoD is impaired myoblasts continued to proliferate but failed to differentiate and fuse into myotubes (27). In contrast myf5 inhibition has been shown to result in a myofiber hypertrophy phenotype in mice. These findings suggest that expression of MyoD is essential for differentiation in myoblasts, while myf5 expression has a crucial role in myoblast proliferation. The aforementioned observations suggest that satellite cells can enter different myogenic specifications based on whether MyoD or myf5 expression dominates.

Im summary it can be stated that Pax 7 decrease MyoD transcription and myogenin inhibit Pax 7 transcription. The ratio of Pax7 and MyoD determine the functions of satellite cells. A high amount of Pax7 to MyoD is observed in dormant satellite cells keeping them in the dormant state. A medium ratio of Pax7 to MyoD initiates proliferation, but still inhibits differentiation. A low ratio of Pax7 to MyoD allows differentiation. At the lowest concentration of Pax7 myogenin is observed terminating the differentiation process.

Terminal differentiation is marked by myoblasts fusing and forming myofibers (figure 2) (28). Most mammalian cells reside in the G0 phase of the cell cycle (29). When division is necessary cells enter the G1 phase and the synthesis phase begins. This phase typically lasts 12-24 hours for mammalian cells. Within this phase DNA in the nucleus is replicated. After the synthesis phase cells enter the G2 phase. Finally, in the mitosis phase DNA is packed into chromosomes and through several complex movements pull the cell apart into equal sets. The cell splits into two daughter cells with complementary chromosomes. The cell then goes back to the G0 phase and re-establishes homeostatic mechanisms.

Cells require certain growth factors and cytokines present in the serum to differentiate. When cells are devoid of serum, they usually do not pass through the G1/synthesis phase and halt the division progress. Some growth factors and cytokines directly stimulate cell division and are called mitogens (30). Others affect cell division by inhibiting cell cycle progression; these are called growth inhibitors. Growth factors and cytokines interact with the cell surface receptors and exert specific functions. One of these growth factors is epidermic growth factors (EGF). EGF binds to tyrosine kinase, which triggers the phosphorylation of tyrosine residues. Growth factor receptor-bound protein two is recruited to activate MAPK. MAPK further phosphorylates transcription factors that regulate the expression of cell cycle proteins to induce cell proliferation (31). Enzymatic hydrolysis of soy protein concentrate resulting in hydrolysates with peptides of low M_w have previously been observed to activate AMPK in muscle cells. However, if protein hydrolysates have mitogenic activity is unknown.

1.5 Enzymatic protein hydrolysis

To access the peptides in proteins they must first be liberated from the protein. As a result of hydrolysis proteins release peptides and free amino acids (16). Hydrolysis can be facilitated through microbial fermentation, acid/alkaline pH, or enzymatically. Extreme pH treatments such as Acid and alkali hydrolysis tend to give a product with reduced nutritional qualities (32). Especially alkaline treatment may cause amino acid modifications and crosslinks between proteins. The modification of L- amino acids may produce D- amino acids and racemic mixture DL- amino acids. D- amino acids can form toxic substances like lysino-alanine that have toxic effect on humans (33). This is due to lysine residues reacting with dehydroalanine a product of B-elimination from cysteine, serine, threonine, or phosphoserine. Therefore, microbial fermentation hydrolysis through Lactic acid bacteria or enzymatic hydrolysis is preferred in the making of hydrolysates intended for food products (12). Hydrolysis facilitated using enzymes can be completed under mild processing conditions. Such as, pH and temperature. Several studies have shown high reproducibility in the hydrolysate products when hydrolyzed enzymatically under similar conditions (34). There are several commercial enzymes approved as food-grade that may be applied on food products intended for human consumption.

During enzymatic protein hydrolysis the protease cleaves peptide bonds by adding an H_2O molecule to the reaction (12). The hydrolysis results in a C-terminal carboxylate ($-COO^-$) and an N-terminal amino group ($-NH_3^+$) at the point of cleavage. Breakdown of the peptide bond results in a conformation change in the primary structure of the protein and affects the secondary, tertiary, and quaternary structures (35). Proteases that cleave bonds between two

non-terminal amino acids are called endopeptidases. These are not restricted to terminal amino acids granting them a wide variety of cleavage sites. The proteases that release free amino acids from the N-terminal ($-NH_3^+$) are amino exopeptidases and generally release one, two, or three amino acids, while carboxy exopeptidases release free amino acids from the C-terminus ($-COO^-$) following the same pattern. Most proteases are highly substrate specific and will only cleave a peptide bond if the adjacent amino acids is also recognized. The catalytic site on proteases is flanked on one or both sides of the enzyme. This subsite is numbered from the catalytic site S1 to Sn toward the N-terminus and S1 to Sn′ toward the C-terminus (fig 3).

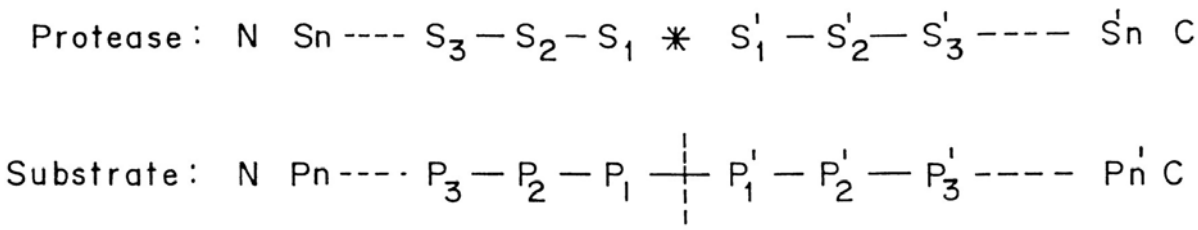

Figure 3. Active sites of proteases.

** Indicates the catalytic site of proteases. S1 to Sn and S1' to Sn' are the specific subsites of the enzyme. P1 to Pn and P1' to Pn' are the residues on the substrate accommodated by the subsites on the enzyme.*

To determine how many peptide bonds are cleaved during hydrolysis degree of hydrolysis (DH) is used. The most common methods for determining DH are the pH-stat and trinitrobenzenesulfonic acid. DH is defined as the proportion of total number of peptides bonds cleaved during hydrolysis and can be calculated: $\%DH = \frac{N}{Nt} x\ 100$

Where N is the number of cleaved peptide bonds and Nt is the number of peptide bonds present. The number of peptide bonds cleaved are dependent on many factors, such as enzyme applied, specificity of the peptidase to the protein chain of the raw material, and the presence of enzyme inhibitors.

1.5.1 Enzymes and enzyme inhibitors.

Alcalase 2.4L is a commercial enzyme from *bacillus licheniformis* containing three endopeptidases and one exopeptidase. It is commonly used in producing protein hydrolysates (36). It belongs to the family of subtilisins, which are serine endoproteases. Serine endopeptidase provides information about the catalytic structure for the triad of amino acids in the enzyme active site marked with * in figure 3. Serine serves as the nucleophilic amino acid at the active site. Alcalase is a nonspecific endoprotease and unlike many other enzymes that

cleave only peptide bonds between two specific amino acids, Adamson and Reynolds observed alcalase cleave peptide bonds when the amino acids Glu, Met, Leu, Tyr, Lys and Gln are positioned at P1 (37). Thus, alcalase can hydrolyze a wide variety of peptide bonds resulting in a strong tendency to give hydrolysates with many peptides of small size and hydrophobic characteristics.

Corolase 8000 or Thermomycolin/Thermomycolase is a thermotolerant serine endoprotease produced by the fungus *Malbranchea pulchella var. Sulfurea* (38). The fungal alkaline protease is ideal for hydrolyzing proteins under mildly alkaline conditions. It has an optimum temperature of 70°C in the presence of Ca^{2+}. The substrate specificity is broad if the P1 position contain a hydrophobic amino acid. Maurice et al. observed the specificity of thermomycolin towards synthetic ester substrates was Ala > Tyr > Phe >> Gly >> Leu> Trp > Val > Lys > Pro on the carboxyl side of the bond hydrolyzed (38). Compared to alcalase 2.4L corolase 8000 do not have exopeptidase activity.

1.5.2 Enzyme inhibitors

Enzyme inhibitors are present at a high concentration in legume seeds compared to other plant families (39). They have received much attention as antinutritional compounds due to reducing protein digestibility in humans by inhibiting digestive enzymes, such as trypsin and chymotrypsin. Most protease inhibitors react with proteases in a similar mechanism as substrates. Serine protease inhibitors form a family of homologous large glycoproteins that interact with a binding loop to the respective protease (40). Resulting in a temporary complex inhibiting the function of the protease. Legume seeds contain two major classes of proteins working as protease inhibitors: a) Kunitz, b) Bowman-Birk classes (39).

Kunitz trypsin inhibitors (KTi) inhibit trypsin through interaction with a single site on the inhibitor (41). The specificity of this inhibitor class is determined by two amino acid residues, arginine, and isoleucine. At the active site of the KTi these amino acids are essential for inhibiting functions. In other inhibitors arginine and serine are the active site residues and inhibiting serine proteases. Multiple Bowman-Birk protease inhibitors have been identified in pea seeds. What classifies bowman-birk inhibitors is the ability to inhibit two proteases at the same time (i.e., either trypsin/trypsin, trypsin/chymotrypsin, and Trypsin/Elastase (39). In peas trypsin/chymotrypsin are the major inhibitors (42). Bowman-birk inhibitors are extraordinary heat stable. This is contributed to the seven disulphide bonds, which renders them resistant to denaturation by boiling. It is necessary with harsher heat treatment than achieved during the enzymatic hydrolysis process to denature the inhibitor proteins. Raw

materials containing enzyme inhibitors is expected to yield a higher M_w after hydrolysis. This is due to reducing protease activity by forming a binding loop to the protease and as a result reduced breakdown of protein (40). When working with hydrolysates the M_w gives important information about the products created during the hydrolysis process. Size exclusion chromatography (SEC) is a standard method for investigating food-grade proteins/peptides and is used to determine the average M_w and molecular weight distribution (MWD).

1.5.3 Size exclusion chromatography

SEC is a convenient method for determining M_w of proteins and peptides and often used for validation of protein hydrolysis process. SEC separates the injected sample based on differences in hydrodynamical volume and size (43). The hydrolysate is injected into a column packed with porous particles of defined pore size. As the hydrolysate elutes through the column molecules that are too large to pass through the pores elute within the void volume of the column. The smaller molecules migrate into the pores of the stationary phase, thus getting a longer migration path and as a result they elute later. Consequently, high M_w peptides elute first from the column and low M_W peptides elute last (figure 4). As the eluate passes through the column a ultraviolet-detector measures absorbance at 214 nm creating a chromatogram. The chromatogram shows the intensity of the absorbance measurement on the Y-axis and elution time on the X-axis.

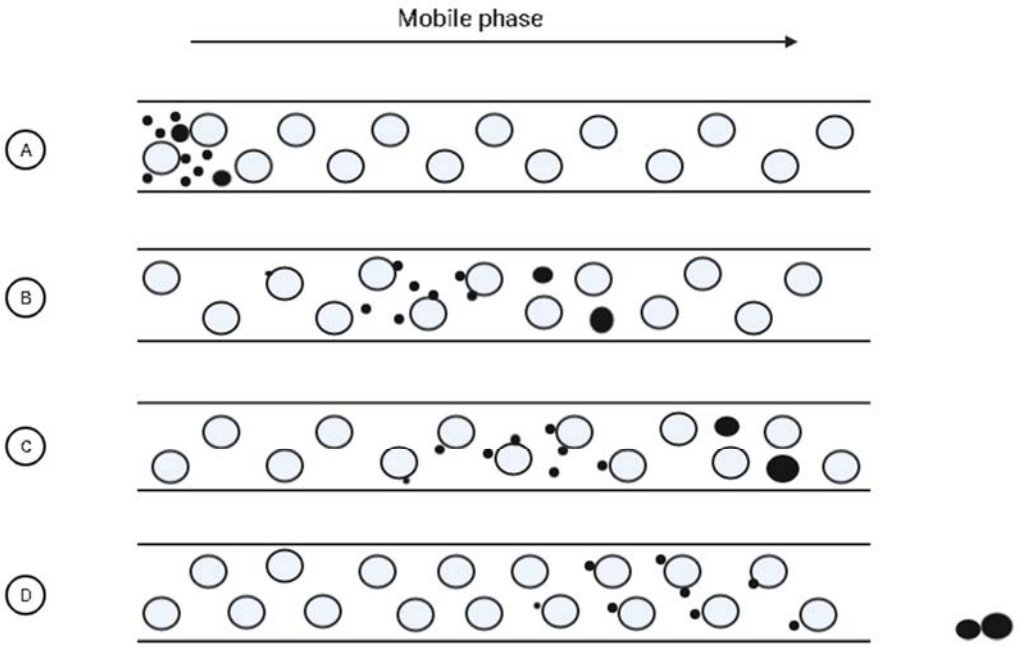

Figure 4. Visual representation of SEC separation of low and high M_W peptides.

Figure 4 shows: A: start of separation, B: smaller molecules migrate into porous particles, while larger molecules elute between regions packed with resin, C: Large molecules move faster through the column, D: Large molecules elute first, middle large particles are almost through the column, small molecules elute last.

When using BopSep-SEC-S2000 as a stationary phase, silica is the resin type (44). It has a particle size of 5 um and a pore size is 145 aangstrom. The stationary phase fractionates molecules based on their hydrodynamic volume. Glycosylated peptides with O-glycosylation or N-glycosylation are resistant to proteases likely due to the attached carbohydrate blocking access to the peptide core (45). These O-glycopeptides range between 300-400 kDa and may have low affinity for the column due to reduced charge, and likely affect the elution time of these peptides. Other interactions between the carbohydrate side chains, such as increased wall interactions or changes in the hydrodynamic volume of the glycopeptide could also explain deviations from other peptides. As SEC is a relative technique calibration for the column is needed. A standard curve with peptides of know M_w profiles must be used. The standard calibration is typically added in programmes such as PSS Win GPC that calculates the average M_w and the distribution of the samples.

1.6 Potential effect of protein hydrolysates.

Most food proteins contain bioactive peptides, but they are inactive within the protein sequence. They are released under the degradation of protein. Depending on the degree of hydrolysis proteins degrade into peptides and free amino acids. Oligopeptides contain roughly 2-50 amino acids, within this category di-, tri-, and penta- peptides are found. These peptides possesses pharmacological properties, and are named bioactive peptides (46). The bioactivity of peptides is mainly based on their amino acid composition and arrangement. The bioactive peptides found in protein hydrolysate have been reported in several studies to exert various biological functions, even beyond their nutritional value. Including antioxidant (47), antimicrobial (48), hypotensive activity (49), anticoagulant (50), cholesterol-lowering ability (51), hypoglycaemic effect (52), and antitumor activity (53), and cell proliferative enhancing effects (54).

1.6.1 Antioxidative properties of bioactive peptides

It is well established that the overproduction of reactive oxygen species (ROS) is implicated in cell death (55). ROS such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) can cause extensive damage to cell membranes and macromolecules. Peroxisomes, the organelle responsible for degrading fatty acids produce H_2O_2 as a by-product of the metabolism but are normally degraded by catalase. Under certain conditions H_2O_2 escapes degradation and causes higher levels of oxidative stress. Antioxidants counteract ROS effectively by scavenging them (56). Lunasin, a peptide derived from soybean, has previously been reported to protect Caco-2 cells from oxidative stress induced by H_2O_2 (57). The protective effect was hypothesised to be caused by the free radical scavenging activity, metal ion-chelating activity, and inhibitory effects on lipid peroxidation (58). Amino acids exhibit antioxidant activity with greater potency when incorporated into peptides. This has spiked a considerable interest in generating food-derived protein hydrolysates with antioxidative properties. Several studies have reported antioxidant activity from proteins deriving from animal and plant sources (57, 59, 60). Specifically, Alpha-Lactalbumin and Beta-Lactoglobulin from milk protein hydrolyzed with corolase generated antioxidant hydrolysates.

1.6.2 DPP-IV and the hypoglycaemic effect of bioactive peptides

Diabetes type 2 is a metabolic syndrome estimated to affect 462 million people worldwide (61). All forms of diabetes are characterized by elevated blood glucose due to insufficient or absolute release of insulin (62). Normal blood levels in humans contain 5mM glucose in homeostasis and insulin is secreted when the blood glucose concentration exceeds this limit (63). Insulin stimulate GLUT transporters, facilitating intracellular glucose uptake from the blood stream in

vivo (62). DPP-IV inactivates GLP-1 and GIP. DPPIV is a serine exopeptidase that cleaves proline and alanine from the N-terminus of GLP-1 and GIP. Removing the function of these hormones, thereby reducing the secretion of insulin. Thus, DPP-IV inhibitors are of interest when investigating glucose uptake.

For all cells except muscle cells insulin is necessary for cells to absorb glucose from the bloodstream. However, in muscle cells glucose can also enter the cell through facilitated diffusion via GLUT4, which is a glucose transporter that trans relocates from intracellular sites to the cell membrane upon contraction (64). In the precense of insulin peptide fractions from soy have been reported to increase glucose uptake (65). Cyril Roblet et al. observed enhancement of glucose uptake was correlated to activation of AMPK. AMPK is a cellular energy sensor that has been shown to increase glucose transport. It can be activated by adding phosphate groups. When the cellular levels of ATP are high, it activates AMPK, which again depletes ATP in the cell (64). Low levels of AMP stimulate myoblast differentiation, while high levels of AMPK inhibit fusion between cells.

1.7 Protein hydrolysates applied in animal cell cultures

Biopharmaceutical products play essential roles in the treatment of many diseases worldwide. According to Mordor intelligence (17), the biopharmaceutical industry is valued to 401.32 billion dollars. The production of monoclonal antibodies (mAbs) is quickly becoming the standard care for oncology and inflammation treatments; mAbs are immune system proteins that are artificially created in a lab (66) Most mAbs are produced through mammalian cell systems due to their ability to produce fully glycosylated and correctly folded proteins. (67). mAb sales for therapeutic use are expected to rise from 114 billion $ in 2021 to 179 billion $ in 2025. The goal for biopharmaceutical companies is to increase the quality and yield of the product while reducing costs. FBS is today essential for cultivating animal cell lines (7). It contributes to a good balance of known and unknown factors such as growth factors, hormones, and lipid components necessary for the survival and proliferation of cells. It is currently the limiting factor for cell line production, and the supply is lower than the demand. Additionally, animal components increase the risk of contamination with infectious agents. Therefore, it is of great importance to fully replace or reduce the amount of FBS in nutrient formulas applied in animal cell cultures.

In the 1880s, Robert Koch observed broth made with fresh beef serum-stimulated efficient growth in microorganisms. By adding Na+ and hydrolysates to the beef serum, the growth was increased further. This was the first known addition of hydrolysates to a culture media. The

work originated the well-known Petri dishes and agar used worldwide today. 80 Years later in 1959 Dulbeccos modified eagles medium (DMEM) a basal media for mammalian cell lines was introduced. It is typically used as a base to establish serum-free media (68). It supports the growth of many different mammalian cell lines. Cell lines successfully cultivated with DMEM include primary fibroblasts, neurons, glial cells, smooth muscle cells, HeLa, Cos-7, and PC-12. DMEM is used extensively all over the world for the cultivation of mammalian cells. FBS must be added to support the maintenance and growth of a mammalian cell culture growing on synthetic media. it is also common to add streptomycin and amphotericin B to reduce the risk of infections from bacteria and yeast/mold. But what are the exact components in FBS that incites growth? Hayashi and his group generated a hypothesis based on their previous experiments:

''*We have been led to this hypothesis by a series of experiments showing that serum depleted of certain hormones no longer supports the growth of cells unless the medium is supplemented with the hormones that were removed****''*** *(69)*

The group succeeded in growing a rat pituitary cell line in a defined serum-free medium supplemented with transferrin. This cell type secretes growth hormone and prolactin and is dependent on thyroid hormones for growth. The group managed to exclude the use of FBS when the medium was supplemented with Triiodothyronine (T3), thyrotropin-releasing hormone (TRH), transferrin, purified somatomedin, and the biologically active peptide of parathyroid hormone (PTH). These findings further supported their hypothesis that FBS contributes with vital hormones for mammalian cells. The results from demonstrated that it is possible to eliminate serum from the culture medium and discovered that the main functions of FBS in cell cultures is to provide hormones necessary for growth.

Animal-derived protein hydrolysates (ADPH) has been reported to have growth-promoting effects in mammalian cell cultures (11). Since 1977 chicken and fish-derived hydrolysates have been applied as serum substitutes or as growth enhancers. ADPH contains a mixture of peptides, amino acids, minerals, carbohydrates, and lipids that mimic the input of media formulations. Jan et al. observed the effect of ADPH on hybridoma cells (70). A defined medium containing FBS supplemented with meat protein hydrolysate resulted in 125-150% increases in antibody concentrations. The group used an equivalent amino acid mixture for comparison and saw only a 50% increase. There has been a discussion in the scientific community whether protein hydrolysates only contribute nutritionally. The results from the study further validate the

argument that protein hydrolysates not only bring nutritional value, but also exert growth-promoting effects in hybridoma cells.

Andreasen et al. investigated the growth-promoting effects of hydrolyzed by-products from the food industry and their growth-promoting effect on MuSCs. Like Jan et al., the group observed improved cell growth and metabolic activity by adding the hydrolysates to a serum-free media and compared them to cells in complete serum condition. The group observed pork plasma hydrolysates generated with alcalase increased metabolic activity by 110% and cell proliferation by 48%. When comparing the cell essay with the SEC results the group observed:

‘’*Hydrolysates rich in peptides with approximately 2-15 amino acids in length were shown to improve cell growth and metabolic activity*’’ (10)

The findings are consistent with other studies, suggesting that oligopeptides may function as growth factors (70, 71). Compared to normal serum conditions the study also demonstrates the detrimental effect of serum starvation on cell metabolism and proliferation. Serum reduction reduced metabolic activity by 18,4%, while a 52,2% reduction was observed in serum-free media. Cell proliferation was reduced by 17,3% and 46% respectively. Researchers are increasingly avoiding the use of serum with animal components. The removal of serum improves production consistency, resulting in less batch-to-batch variability, but it also speeds up and lowers the cost of downstream processing (3). Many scientists have set out on a quest to find simple, low-cost, and highly repeatable media for cultivating animal cells as an alternative to serum. Recombinant proteins, insulin (and other growth factors), hormones, lipids, and PPH are currently being used as serum substitutes in cell culture media formulation.

1.8 Plant-derived protein hydrolysate in animal cell culture

As the previous studies mentioned ADPH can increase proliferation in various cell lines. However, PPH have gained much attention. A concern when utilizing PPH in mammalian cell lines is the amino acid composition. Plant protein from legumes and cereal typically have low levels of Lysine and Methionine, therefore plant protein is considered to be of lower quality than animal derived protein (72) These amino acids are essential in the biosynthesis of carnitine, which takes place in the liver of all mammals in vivo. Carnitine is an important compound included in the transfer of fatty acids across mitochondrial membranes and concentrations have been reported to be 50 times greater in skeletal muscle cells. Removal of carnitine has been demonstrated to induce inhibition of proliferation in mouse C2C12 myoblast cells, thus

highlighting the importance of this compound and justify some doubts of applying PPH on animal cell cultures.

J. Lee et al. investigated the promotion of stem cell proliferation using various vegetable protein hydrolysates (73). The group found that pea and wheat hydrolysate supplementation increased cell proliferation by 25% and 20% in human mesenchymal stem cells (MSC) compared to a serum-free media. These findings show pea hydrolysates to increase proliferation in MSC when starved of FBS, suggesting growth promoting activities.

A considerable portion of the scientific research on plant protein hydrolysates in mammalian cell cultures has been used on cell lines capable of producing glycosylated proteins for pharmaceutical use. One of these cell lines is CHO. Ballez and coworkers cultivated CHO-320, which secretes recombinant interferon-gamma (IFN-γ), a cytokine crucial for adaptive immunity against viral, bacterial, and protozoan infections (71). The group claims to have developed a basal serum and protein-free medium (PFM) designed for CHO cell lines. The cells were previously adapted to cultivation in a PFM supplemented with plant protein hydrolysate. In suspension cultures FBS provides CHO-320 with cell growth and IFN-secretion, similar to those observed in serum-containing media when the PFS is enhanced with plant protein hydrolysates. The research investigated the addition of rice protein (RP) and wheat protein (WP) hydrolysates in CHO-320 cells both in suspension, with microcarriers and a bioreactor. Both RP and WP supported cell growth and IFN-γ secretion. In the suspension culture, adding plant protein hydrolysates increased IFN-γ secretion by 30% and cell density by 25%. Ballez et al. further conclude:

''plant protein hydrolysates could improve biosynthesis either on a nutritional basis or/and due to bioactive peptides acting as cell signal inducers.'' (71)

Another study proposed the effects observed could solely be of nutritional character (74). This hypothesis values protein hydrolysates as a cost-effective source of amino acids. The hypothesis is weakened, as it is now settled that a mixture of equivalent amino acid compositions could not match their effects (70). Ballez and coworkers argue that compounds in the hydrolysates exert biological stimulation of CHO-320 cell growth and recombinant IFN-γ secretion in a way that could not solely be nutritional. The group speculates that peptides in the hydrolysate interact with the surface of the cell membrane and that receptors involved in the stimulation of cell growth and protein synthesis are activated, with potentially higher uptake through peptide transporters.

The group observed several other interesting aspects during the experiment. With WP hydrolysate, lactate was consumed when glucose concentration was low in the media. This was not happening to the same degree in the PFS. Also, the concentration of amino acids Glu, Ser, Gly, Thr, Arg, Tyr, Val, Phe, Leu, and Ile increased at the end of the batch. The group propose two hypothesis to explain the observation.

1. Proteolytic cleavage of plant protein hydrolysates generates smaller peptides and free amino acids on the cell surface, or in the extracellular media. If transported by specific transporters and internalized in the cell, when cell viability dropped, it would have been observed that increased accumulation in the extracellular media. As the components of the cell would leak out. The hypothesis thereby assumes that hydrolysis of peptides is slower than the transport of the resulting amino acids (di-tri peptides). If not, amino acids would accumulate in the media, even in the exponential growth face.

2. After cell lysis, the intracellular proteases are released and induce an increase in proteolytic activity in the extracellular media. Thereby degrading peptides in the later phase. IFN-γ did not undergo any proteolytic degradation, as no bonds were observed with a lower M_W than the non-glycosylated form of IFN-γ.

Therefore, the group concludes that the existence of specific peptide transporters is present. As intracellular hydrolysis of peptides is slower than transporting. The authors conclude that CHO-320 cells employ peptide transporters to facilitate intracellular uptake of peptides of small size.

2.0 Materials and Method

2.1 Enzymatic protein hydrolysis

There are several ways to hydrolyze proteins: microbial fermentation, acid, alkali, and enzymatic hydrolysis are common practices. The intention of making a hydrolysate is to utilize it in food-grade products; thus, acid and alkali hydrolysis are unsuitable. Microbial fermentation offers a option for hydrolyzing proteins, however enzymatic hydrolysis was chosen for less batch-to-batch variability and control of the process.

A total of 16 protein hydrolysates were produced from seven vegetable protein concentrates and one animal-derived (table 1).

Table 1. Raw materials utilized in the hydrolysis of protein concentrates.

NAME	MATERIAL TYPE	MANUFACTURER
PI	Pea-protein isolate, 80% protein	Vestkorn, Norway
F55X	Pea-protein concentrate, 55% protein	Vestkorn, Norway
E1155X	Pea-protein concentrate, heat treated, 55% protein	Vestkorn, Norway
F67X	Faba bean-protein concentrate, 67% protein	Skjellfoss, Norway
F65X	Faba bean-concentrate, 65% protein	Skjellfoss, Norway
BARLEY	Barley-protein concentrate, 25% protein	Nofima, Cereal department, Norway
OATWELL	Oat-protein concentrate, 23% protein	Nofima, Cereal department, Norway
WPC80	Whey-protein concentrate, 80% protein	Tine, Norway

The protein concentrates were hydrolyzed with either Alcalase 2.4L or Corolase 8000 (table 2).

Table 2. Enzymes utilized in the hydrolysis of protein concentrates.

NAME	MATERIAL TYPE	MANUFACTURER
ALCALASE	Serine peptidase, Alcalase 2.4L	Novozymes, Denmark
COROLASE	Serine peptidase, Corolase 8000	Novozymes, Denmark
METLER TOLEDO XSR	Dual-range measurement weight	
200ML PYREX FLASK	Flask	
MQ WATER	dH_2O	
	Magnetic stirrer	
MENU MASTER	Microwave, menu master commercial	
WATER BATH		
IN BECKMAN COULTER AVANTI J.20	Centrifuge	
MILLIPORE VACUUM PUMP XF54230	Vacuum pump	
PAL ZEITS	Filter	

10g Of PI was weighted on a Metler Toledo XSR dual-range measurement weight and added to a 200 mL Pyrex flask. The process was repeated 1x resulting in two flasks with 10g of the same raw material. This was done for all protein raw materials. 200 mL of dH_2O was added to each flask. In the end Alcalase 2.4L or Corolase 8000 was added to the Pyrex flask. The enzyme concentration was calculated to be 0.5% of the protein content in the raw material (table 3).

10g raw material with 0.5% enzyme to protein concentration example:

Oatwell: 23% protein → 10g raw material → 2.3g protein → 2.3*0.005=0.0115g enzyme

Table 3. raw material and enzyme concentration for hydrolysis of raw materials.

NAME	PROTEIN CONCENTRATION (%)	AMOUNT OF ENZYME (G)
PI	80	0.04
F55X	55	0.275
E1155X	55	0.275
F67X	67	0.0335
F65X	65	0.0325
BARLEY	25	0.0125
OATWELL	23	0.0115
WPC80	80	0.04

The process was repeated with all eight raw materials. Prepared flasks were placed on a fisher 5000 magnetic stirrer at 11 RCF inside a water bath holding 53 °C for 90 minutes (figure 1). Coming out of the water bath, the flasks were put in a Menu master commercial microwave for 1 minute on strength 8 until boiling, then put in a 95 °C water bath for 15 minutes to ensure denaturation of the enzymes and stop the reaction. After cooling down to room temperature the samples were centrifuged in Beckman coulter Avanti J.20 centrifuge at 21952 RCF for 10 minutes. The supernatant was extracted and filtered through a Pall Zeits filter using a Millipore vacuum pump XF54230. The supernatant of Oatwell hydrolyzed with Alcalase turned into a gel and could not be filtrated through the Pal Zeits filter, the sample was lost. The filtrate from all other samples was stored in plastic containers at –40 °C.

Figure 5. Enzymatic hydrolysis of raw materials.

Figure 5 shows raw materials with added enzymes hydrolyzation process.

A simplified representation of the processing steps involved in the making of the protein hydrolysates is shown (figure 6).

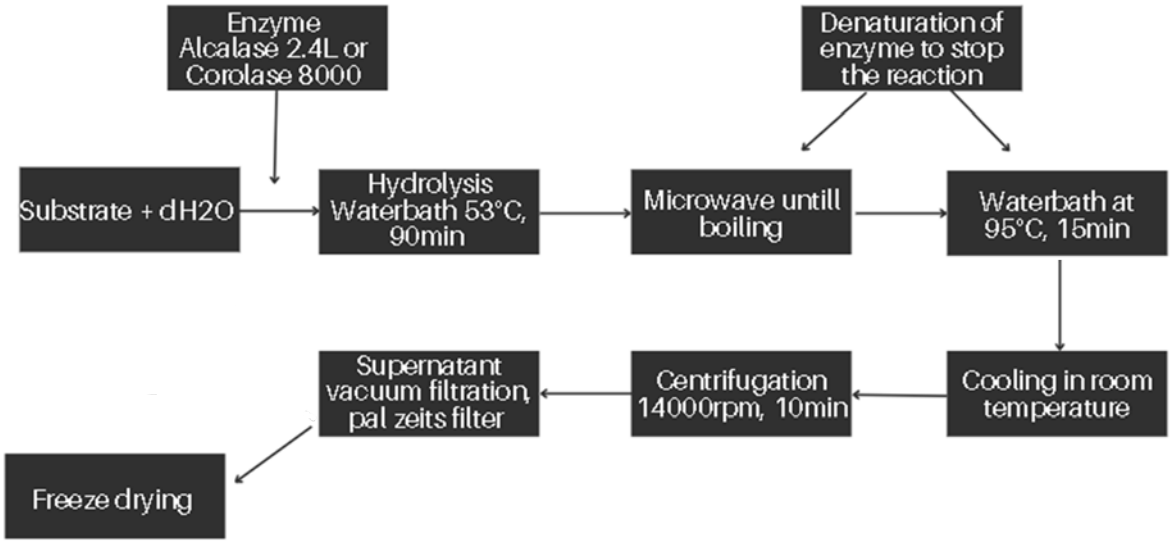

Figure 6. Flow chart of processing steps in the making of protein hydrolysates

Figure 6 shows the processing steps involved in the making of protein hydrolysates from start to finish.

2.2 Freeze-drying

Freeze drying of the supernatants was performed to standardize the concentration of the samples for further experiments. Freeze drying removes ice or other frozen solvents from materials through lyophilization/sublimation. Lyophilization or freeze-drying is a process in which water is removed under a vacuum (75). The vacuum allows the ice to go directly from a solid-state to vapor, without going through the liquid phase. The process involves three main steps: Freezing (the samples were already frozen), Primary drying (sublimation), and secondary drying. The method increases the stability of the dry powder and removes water without excessive heating to standardize the samples.

Frozen hydrolysates packed in plastic containers were taken out from –40 °C. The lid was perforated with a sharp sterile knife to make a hole for the gas to pass out. After approximately 10 minutes of being taken out from –40 °C they were placed in the Freeze dryer Martin Christ gamma 1-16 for 96 hours under –40 °C. Freeze drying was utilized to standardize the hydrolysates before adding them to cells and reduce batch-to-batch variability. After freeze-drying hydrolysates were transferred from plastic containers with a spatula into 50mL tubes.

2.3 Size exclusion chromatography

SEC was applied to gain information about the average M_W and MWD. SEC has previously been applied to separate peptides of different sizes (43). Even small peptides under 10,000 Da. Based on the elution time chromatograms were divided into four fractions, areas A, B, C, and D. Peptides within area A had an estimated average M_w of 27762 Da, B: 6767.3 Da, C: 2098.4 Da, and D: 723.7 Da. By dividing the average Mw of each zone by the M_w of each amino acid, an approximation of peptide length was done. The average Mw of all 22 amino acids is 113 Da (43). Additionally, the weight of the water molecule between amino acids forming the peptide bond at 18 Da was also included. Thus, a rough estimate of each amino acid in the peptide chain falls to 138 Da. The fractions can therefore display the percentage of peptides with approximate amino acid length. A: 277-76 amino acids, B: 76-27 amino acids, C: 22-8 amino acids, and D: 8-2 amino acids. Free amino acids are not detected at 214 nm and are not included.

Freeze-dried hydrolysates were resuspended in dH_2O at a 1 mg/mL concentration and vortexed to create a homogenous solution. Furthermore, 1.5 mL solution was added to 3 mL SEC vials and placed in the Ultimate 3000 HPLC with column Biosep SEC-s2000 from Phenomenex. The samples were injected at 15 µL and absorbance was measured at 214 nm, which is the standard absorbance for peptides. The mobile phase used to carry the mixture

down the column was a mixture of acetonitrile and ultrapute water in a proportion of 30 : 70, containing 0.05% trifluoroacetic acid. The isocratic elution time was 20 minutes. Between 17-20 min the mobile phase was changed to NaH_2PO_4 for column cleaning. For average M_w calculations a calibration curve of molecules with known M_w was applied (figure 3).

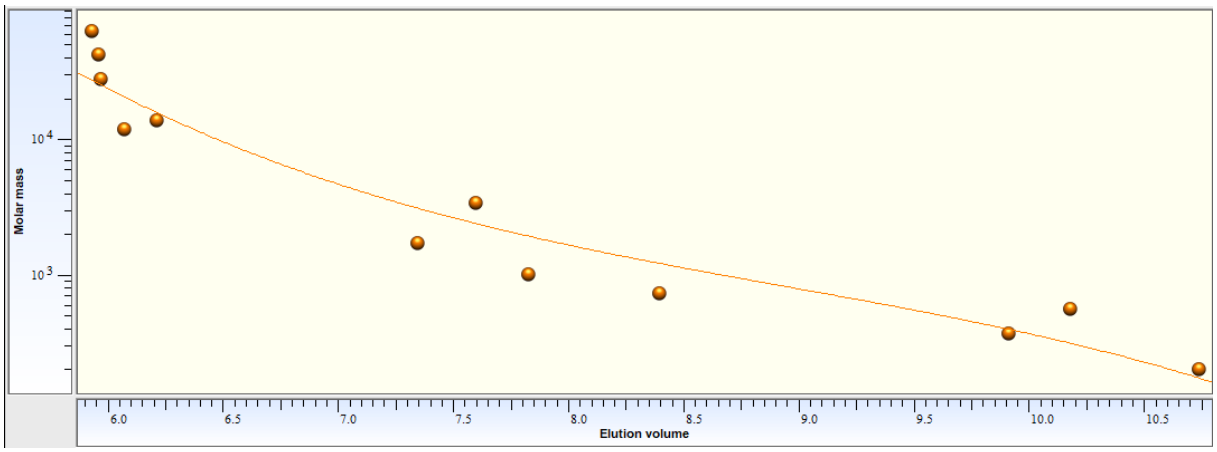

Figure 7. Calibration curve applied for analysis of SEC data.

Figure 7 shows the elution volume on the x-axis and the molar mass on the Y-axis. From left to right standard samples with known M_w (1-12 as shown in table 7) are represented in this figure as spheres.

Molecules with a known Mw from the calibration curve were utilized to calibrate the M_w of peptides in the protein hydrolysate with the software PSS WinGPC (table 8).

Table 8. Molecules, Mw, RT(I), RT(J), RT(K), Mean RT, SD, Log Mw and MeanRT for molecules of known Mw used for calibration.

	MOLECULES	MOLECULAR WEIGHT (G/MOL)	RT(I)	RT(J)	RT(K)	MEAN RT	SD	LOGMW	MEANRT
1	Bovine Serum Albumin	66000	5,9248	5,9248	5,9332	5,9276	0,0048	4,82	5,9276
2	Albumin from chicken egg white	44287	5,9498	5,9582	5,9582	5,9554	0,0048	4,646	5,9554
3	Carbonic anhydrase	29000	5,9665	5,9665	5,9665	5,9665	0,0000	4,462	5,9665
4	Lysosyme	14300	6,2082	6,2082	6,2165	6,2110	0,0048	4,155	6,2110
5	Cytochrome c from bovine heart	12327	6,0665	6,0665	6,0748	6,0693	0,0048	4,091	6,0693
7	Insulin Chain B Oxidized from bovine pancreas	3496	7,5915	7,5998	7,5998	7,5970	0,0048	3,544	7,5970
8	Renin Substrate Tetradecapeptide porcine	1759	7,3415	7,3415	7,3498	7,3443	0,0048	3,245	7,3443
9	Angiotensin II human	1046	7,8165	7,8248	7,8248	7,8220	0,0048	3,02	7,8220
10	Bradykinin Fragment 1-7	757	8,3915	8,3915	8,3998	8,3943	0,0048	2,879	8,3943
11	[D-Ala2]-Leucine enkephalin	570	10,1748	10,174	10,174	10,174	0,0000	2,756	10,1748
12	Val-Tyr-Val	379	9,9082	9,9082	9,9082	9,9082	0,0000	2,579	9,9082
13	L-Tryptophan	204	10,7332	10,733	10,733	10,733	0,0000	2,31	10,7332

Table 8 shows the known standard molar mass and sample names represent the data points used in the calibration curve utilized for data analysis of the SEC data. Molecules marked in orange was used for multi area settings.

2.4 Supplementing vegetable peptides to proliferating bovine muscle cells. 24h, 48h, and 96h treatment.

To investigate the proliferating effects of the hydrolysates, they were supplemented to bovine skeletal muscle cells (MUSCS) under normal-serum conditions. Coating, thawing, seeding, and supplementation of the hydrolysates to cells were done inside a Scan LAF bench, to reduce the risk of microbial contamination (76).

2.4.1 Creating cell proliferation media for MuSCs.

500 ML of DMEM(1X) + GlutaMAX –1 (+) 1g/L D-glucose, (+) Pyruvate was supplied by Nofima from Gibco. 10 mL FBS (2% serum) was added to the DMEM flask along with 10 mL Ultroser G, 2.5 mL streptomycin (10.000 units/ mL) and 2.5 mL fungizone (250 ug/mL amphotericin).

2.4.2 Coating

It was essential for further work with cells that they were attached to the flask surface. Entactin-Collagen Laminin (ECL) is a cell culture attachment factor applied widely for research. Coating of incubation flasks was done by adding 175 µL of 10 mg/mL ECL in two straight lines inside the flask and a cell scraper was used to evenly distribute the coating reagent. The flask was incubated for 1 hour at 37 °C. For the 96-well plates the following volumes shown were applied (table 9). The plates were gently tipped back and forth to spread the coating mix following incubation for 1 hour at 37°C. Before adding cells the coating was removed using Integra vacusafe with an attached Pasteur pipette and the wells were washed with 100uL Phosphate-Buffer Saline (PBS) with a multi pipette.

Table 9. ECL + DMEM coating.

Article	Surface area per well	Coating reagent (ECL)	DMEM
96-well plate	0.3 cm^2	0.9 µL	100 µL

2.4.3 Thawing and expanding cells.

Myoblasts/satellite cells from bovine sirloin harvested according to Andreassen et al (10) were taken out of the cryo-tank 15 minutes before the experiment. 5 mL of preheated proliferation

media was added to the frozen cells in cryotubes in droplets. After the addition of proliferation media cells and media were pipetted gently several times to ensure a homogenous solution. The mixture of cells and medium was centrifuged at 34 RFC for 5 minutes in an Eppendorf centrifuge 5430. After centrifugation, the supernatant was removed to get rid of toxic DMSO. The cell pellet was resuspended in 5 mL proliferation media, first 1 mL to distribute the cells evenly and additionally 4 mL. 100 uL from the cell suspension was counted using Nucleocounter NC-202. Cells were then seeded on pre-coated flasks, the coating followed the same procedure as stated earlier. 20 mL proliferation media was added to the flask. Cells were then incubated for 72 hours at 37℃.

2.4.4 Seeding cells

When cells had reached confluency the proliferation medium was removed. The flask was washed twice with 10 mL PBS to remove excess media. After washing with PBS 5 mL pre-heated 0.25% trypsin-EDTA (Canada), was added to the flask, following incubation at 37 ℃. The flask was incubated for a maximal of 10 minutes, while checking the cells in a microscope every 3 min to observe the detachment cells. Trypsinization induces cell dissociation and the proteolytic enzyme breaks down proteins. Thereby dissociating adherent cells from the flask. When most of the cells had detached 5mL proliferation media was added and cells were transferred to a 15 mL falcon tube. The falcon tube was centrifuged for 5 min at 34 RFC. The supernatant was removed carefully and the cell pellet was resuspended in 1 mL proliferation media. 100 mL from the cell suspension was added to an Eppendorf tube and counted using Nucleocounter NC-202 (table 10). The number of cells for each well was decided to be 500 before the experiment. The remaining cells were incubated back into the coated flask with 20 mL of proliferation media to repeat the experiment on the next batch of hydrolysates 48h later. Due to a low cell concentration when preparing for batch 6 coated 96-well plates were stored for three days at 4 ℃, while incubating new cells from cryo. The cells were allowed to attach and proliferate for 24h before stimulation with hydrolysates.

Table 10. Batch number, incubation time, cell count, viability, and number of passages after thawing from cryobank.

BATCH NUMBER + INCUBATION TIME AFTER CRYO	CELL COUNT, CELLS/ML	VIABILITY (%)	NUMBER OF PASSAGES BEFORE THE EXPERIMENT
1, 72H	2.20E+05	91.7%	3
2, 120H	1.79E+05	86.8%	3
3, 72H	4.65+05	93.9%	3
4, 120H	3.05E+05	86.7%	3
5, 72H	6.25+05	92.1%	3
6, 120H	6.33E+05	96.5%	3
7, 72H	4.25E+05	92.6%	3
8, 120H	2.32E+05	87.4%	3
9, 72H	6.33E+05	94%	3
10, 120H	2.96E+05	92%	3

Table 10 shows the batch number, incubation time after cryo, cell count, viability and number of passages before being applied in the experiment.

2.4.6 Supplementing MUSCS with vegetable hydrolysates

Enzymatically hydrolyzed freeze-dried hydrolysates (table 11) were weighed out at 50mg and resuspended in 5mL PBS creating a stock solution 10 mg/mL). Followed by vortexing until a whirlpool was visible and sustained for 15 seconds using a fisher scientific vortex to make a homogenous solution.

Table 11. Name of hydrolysates after enzymatic hydrolysation, and enzyme used.

Name	***Enzyme***
PI-A	Alcalase
PI-C	Corolase
F55X-A	Alcalase
F55X-C	Corolase
E1155X-A	Alcalase
E1155X-C	Corolase
F67X-A	Alcalase
F67X-C	Corolase
F65X-C	Corolase
Barley-A	Alcalase
Barley-C	Corolase
OBG28-C	Corolase
WPC80-A	Alcalase
WPC80-C	Corolase

Table 11 shows the new name of the hydrolysate and enzyme used for hydrolysis. The hydrolysates receive -A, or -C based on hydrolysation with alcalase or corolase for further reference. Example: PI hydrolyzed with alcalase: PI-A. PI hydrolyzed with corolase: PI-C.

9 mL PBS was added to a 15 mL falcon tube and added 1mL of stock solution, creating a 10-fold dilution. For the next dilution 1 mL of 10^{-1} was used. The process was repeated eight times (10^{1}-10^{-7}). Resuspended hydrolysates were further diluted in Eppendorf tubes before supplementing to culture. 100 µL resuspended hydrolysate (10 mg/mL) and 900 mL proliferation media were added to an Eppendorf tube resulting in 1mg/mL of hydrolysate in DMEM. Other dilutions followed the same pattern (10^{0}-10^{8}). Before supplementing hydrolysates to the cell culture old proliferation media was removed. 100 µL of hydrolysate mixture was added in triplicates to respective wells. A visual representation of cells supplemented with hydrolysates in a 96-well plate is presented (figure 8). Three identical plates were supplemented, which were incubated for 24, 48, and 96 hours.

Figure 8. Representation of 96-well plates.

Hydrolysate 1 1mg/ml	**Hydrolysate 1** 1mg/ml	**Hydrolysate 1** 1mg/ml	**Hydrolysate 2** 1mg/ml	**Hydrolysate 2** 1mg/ml	**Hydrolysate 2** 1mg/ml	**Hydrolysate 3** 1mg/ml	**Hydrolysate 3** 1mg/ml	**Hydrolysate 3** 1mg/ml	**DMEM**	**WPC80 Alcalase** 1mg/ml	**PBS/DMEM**
Hydrolysate 1 0,1mg/ml	**Hydrolysate 1** 0,1mg/ml	**Hydrolysate 1** 0,1mg/ml	**Hydrolysate 2** 0,1mg/ml	**Hydrolysate 2** 0,1mg/ml	**Hydrolysate 2** 0,1mg/ml	**Hydrolysate 3** 0,1mg/ml	**Hydrolysate 3** 0,1mg/ml	**Hydrolysate 3** 0,1mg/ml	**DMEM**	**WPC80 Alcalase** 0,1mg/ml	**PBS/DMEM**
Hydrolysate 1 0,01mg/ml	**Hydrolysate 1** 0,01mg/ml	**Hydrolysate 1** 0,01mg/ml	**Hydrolysate 2** 0,01mg/ml	**Hydrolysate 2** 0,01mg/ml	**Hydrolysate 2** 0,01mg/ml	**Hydrolysate 3** 0,01mg/ml	**Hydrolysate 3** 0,01mg/ml	**Hydrolysate 3** 0,01mg/ml	**DMEM**	**WPC80 Alcalase** 0,01mg/ml	**PBS/DMEM**
Hydrolysate 1 0,001mg/ml	**Hydrolysate 1** 0,001mg/ml	**Hydrolysate 1** 0,001mg/ml	**Hydrolysate 2** 0,001mg/ml	**Hydrolysate 2** 0,001mg/ml	**Hydrolysate 2** 0,001mg/ml	**Hydrolysate 3** 0,001mg/ml	**Hydrolysate 3** 0,001mg/ml	**Hydrolysate 3** 0,001mg/ml	**DMEM**	**WPC80 Alcalase** 0,001mg/ml	**PBS/DMEM**
Hydrolysate 1 0,0001mg/ml	**Hydrolysate 1** 0,0001mg/ml	**Hydrolysate 1** 0,0001mg/ml	**Hydrolysate 2** 0,0001mg/ml	**Hydrolysate 2** 0,0001mg/ml	**Hydrolysate 2** 0,0001mg/ml	**Hydrolysate 3** 0,0001mg/ml	**Hydrolysate 3** 0,0001mg/ml	**Hydrolysate 3** 0,0001mg/ml	**DMEM**	**WPC80 Alcalase** 0,0001mg/ml	**PBS/DMEM**
Hydrolysate 1 0,00001mg/ml	**Hydrolysate 1** 0,00001mg/ml	**Hydrolysate 1** 0,00001mg/ml	**Hydrolysate 2** 0,00001mg/ml	**Hydrolysate 2** 0,00001mg/ml	**Hydrolysate 2** 0,00001mg/ml	**Hydrolysate 3** 0,00001mg/ml	**Hydrolysate 3** 0,00001mg/ml	**Hydrolysate 3** 0,00001mg/ml	**DMEM**	**WPC80 Alcalase** 0,00001mg/ml	**PBS/DMEM**
Hydrolysate 1 0,01ug	**Hydrolysate 1** 0,01ug	**Hydrolysate 1** 0,01ug	**Hydrolysate 2** '0,01ug	**Hydrolysate 2** 0,01ug	**Hydrolysate 2** 0,01ug	**Hydrolysate 3** 0,01ug	**Hydrolysate 3** 0,01ug	**Hydrolysate 3** 0,01ug	**DMEM**	**WPC80 Alcalase** 0,01ug	**PBS/DMEM**
Hydrolysate 1 0,001ug	**Hydrolysate 1** 0,001ug	**Hydrolysate 1** 0,001ug	**Hydrolysate 2** 0,001ug	**Hydrolysate 2** 0,001ug	**Hydrolysate 2** 0,001ug	**Hydrolysate 3** 0,001ug	**Hydrolysate 3** 0,001ug	**Hydrolysate 3** 0,001ug	**DMEM**	**WPC80 Alcalase** 0,001ug	**PBS/DMEM**

Figure 8 shows the placement of hydrolysates, DMEM, WPC80-A, and PBS/DMEM wells. Wells containing DMEM, WPC80-A, and PBS/DMEM are identical on all plates.

The same procedure was applied to all hydrolysates. Following supplementation of hydrolysates to the cell culture each plate was incubated at 37 ℃ for 24 hours, 48 hours, or 96 hours. After the incubation period of each plate the media was removed and each well was washed with PBS. The plates were then frozen at -80℃ until analyzing cell proliferation using CyQuant proliferation Assay.

2.5 CyQuant proliferation-assay

CyQuant proliferation assay is a fluorescence-based method for studies involving the passage of cells over a period of time (77). It is commonly used in studies examining cell proliferation and cell growth inhibition. The dye produces a large fluorescence enhancement upon binding to cellular nucleic acids that can be measured at 480 nm and 520 nm.

Aluminium foil was placed around tubes containing CyQuant GR cell-lysis buffer to protect them from light. 96-well plates were taken out of –80 ℃ storage and thawed for 10 minutes. The assay has a linear detection range extending from 50-50.000 cells in 200 µL. Therefore, 200 mL CyQuant GR cell-lysis buffer was added to each well and incubated at room

temperature protected from light for 3 minutes. After incubation quantified cell proliferation was measured by Synergy H1 microplate reader from Bionordica. The plate was placed inside the microplate reader and measured absorbance at 480 nm and 520 nm. The process was repeated for all plates.

2.6 DPP-IV Assay

DPPIV assay provides a convenient fluorescence-based method for screening DPP4 inhibitors. The assay uses the fluorogenic substrate Gly-Pro-Aminomethylcoumarin (AMC) to measure DPP4 activity (78). Cleavage of the peptide bond by DPP-IV releases a free AMC group causing a fluorescence change that can be analyzed using an excitation wavelength of 350-360 nm and emission of 450-465 nm.

DPPIV assay kit was taken out of the freezer. The assay buffer was thawed for 20 minutes before the experiment. 5 mL essay buffer was diluted in 45 mL water. The inhibitor samples (freeze-dried hydrolysates) were weighted at 50 mg and added to 5mL mQ water making a 10mg/mL stock for all 14 hydrolysates. The position of inhibitors is shown (figure 9). 240 µL substrate (H-Gly-Pro-AMC) was diluted in a 5.76 mL assay buffer and vortexed. The Sitagliptin bottle was added 500uL of assay buffer (1mM stock) and vortexed. The enzyme was thawed on ice and added 480 uL assay buffer to each enzyme vial and vortexed. The content of the two bottles was combined making 960 µL of enzyme solution.

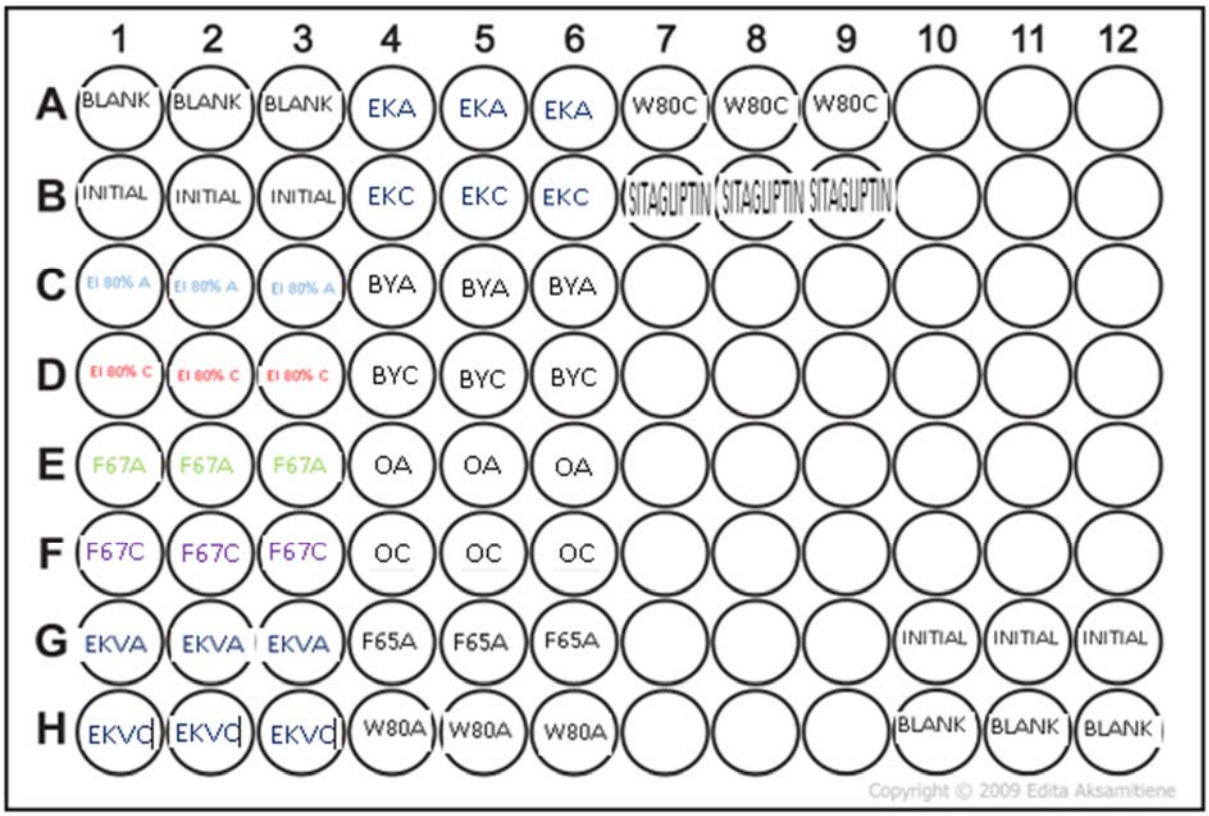

Figure 9. Representation of 96-well plate from DPP-IV assay kit

Figure 9 shows the position of blanks, initial activity, and substrate wells in the DPP-IV assay kit.

For the assay a white 96 well-plate from the assay kit with small wells was used. 30 µL of buffer was added to all the wells using a multi pipette. 20 µL of buffer was added to all background wells. 10 µL of buffer was added to initial activity wells. 10 µL sitagliptin was added to all sitagliptin wells. 10 µL inhibitors were added to corresponding wells. 10 µL of DPP4 was added to all wells except the background wells. 50 µL substrate was added to all wells. The plate was incubated at 37 °C giving the enzyme optimal conditions for the reaction. Fluorescence was measured in a plate reader: excitation = 355 nm, emission= 455 nm. The average fluorescence of initial activity, blank, and inhibitor wells was determined. The fluorescence of the blank wells was subtracted from the initial activity and inhibitor wells. Percent inhibition for each compound was determined by:

$$\% \text{ Inhibition} = \left[\frac{\text{Initial Activity - Inhibitor}}{\text{Initial Activity}} \right] \times 100$$

2.7 Data analysis

2.7.1 Statistical analysis of 96-well plates

The average of replicated wells was normalized to the average of the control (DMEM/PBS) giving a percentage difference in 24, 48, and 96h plates. The percentages were utilized to perform a one-way ANOVA test to look for significant findings. As the ANOVA test does not report which pairs of means are different Dunnett's multiple comparison tests were used to compare all cells with supplemented hydrolysates to the control, to identify pairs with significant differences and determine the statistical significance of the difference. The P-value of individual groups compared to control cells (DMEM/PBS) uses the adjusted p-value from Dunnets multiple comparison test. The strength of the p-value is represented in stars.

3.0 Results

Enzymatic protein hydrolysis of seven vegetable protein concentrates and one animal-derived was done with Alcalase and Corolase. The hydrolysates were freeze-dried and further analyzed by SEC. Results from the CyQuant proliferation assay are presented to show the potential effect of adding vegetable hydrolysate to bovine skeletal muscle cells. In the end, the hydrolysates were screened for DPP-IV-inhibition activity.

3.1 Size exclusion chromatography demonstrated differences in M_w and peptide distribution in protein hydrolysates.

Areas A, B, C, and D (figure 10) split the chromatogram based on elution time to highlight the absorbance intensity of peptides within each area. Moving from area A- D peptide size decreases. An approximation of peptide size within A: 277-76 amino acids, B: 76-27 amino acids, C: 22-8 amino acids, and D: 8-2 amino acids.

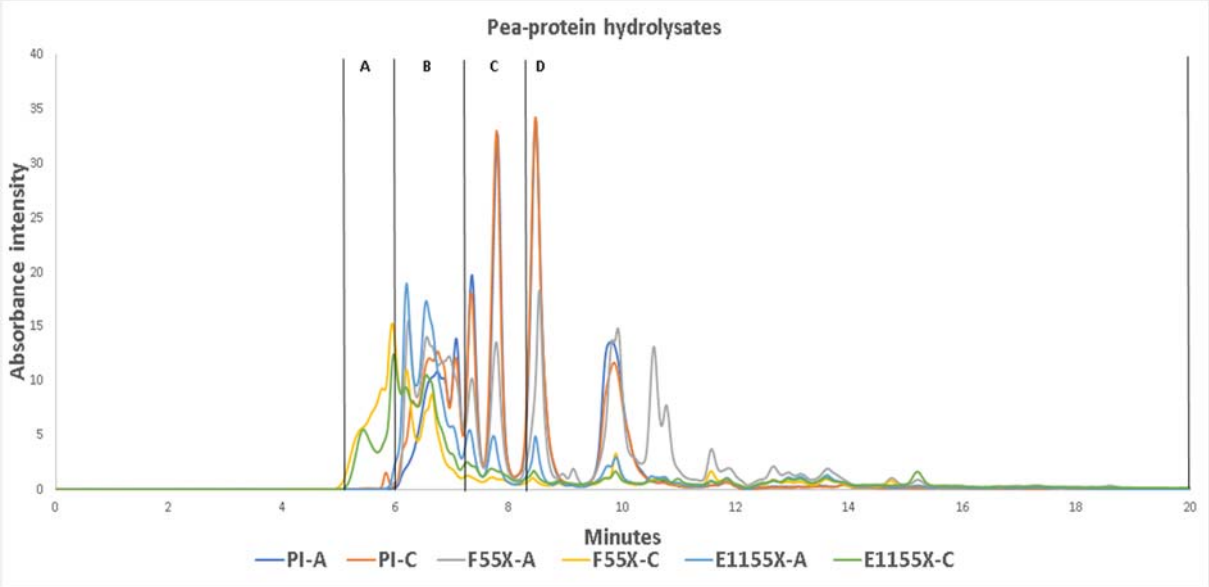

Figure 10. Size exclusion chromatography demonstrated differences in M_w and peptide distribution in pea hydrolysates.

Figure 10 shows each Pea hydrolysates retention time between 4 and 20 minutes, and absorption intensity at 214 nm. The chromatogram is split into four sections based on the time of elution: A, B, C, and D. Area A: 5-6min, Area B: 6-7.3min, Area C: 7.3-8.4, and Area D: 8.4-20 min.

The PI hydrolysates from the different enzymes resulted in different MWD. PI-A had the highest peak in area C, while PI-C the highest peak was in area D. The MWD for F55X was different using different enzymes. When using alcalase the highest peak was seen in area D,

with two smaller peaks in area C. When using corolase the highest peak was seen in the border in area A, with a lesser peak in area B. F55X-C hydrolysate had the highest peak in area A and a lesser peak in area B. The MWD for E1155X was different using different enzymes, when using alcalase the two highest peaks were seen in area B, followed by two small peaks in area C. When using corolase the highest peak was seen in area A, with a lesser peak in area B. Percentage of distribution is shown (figure 11).

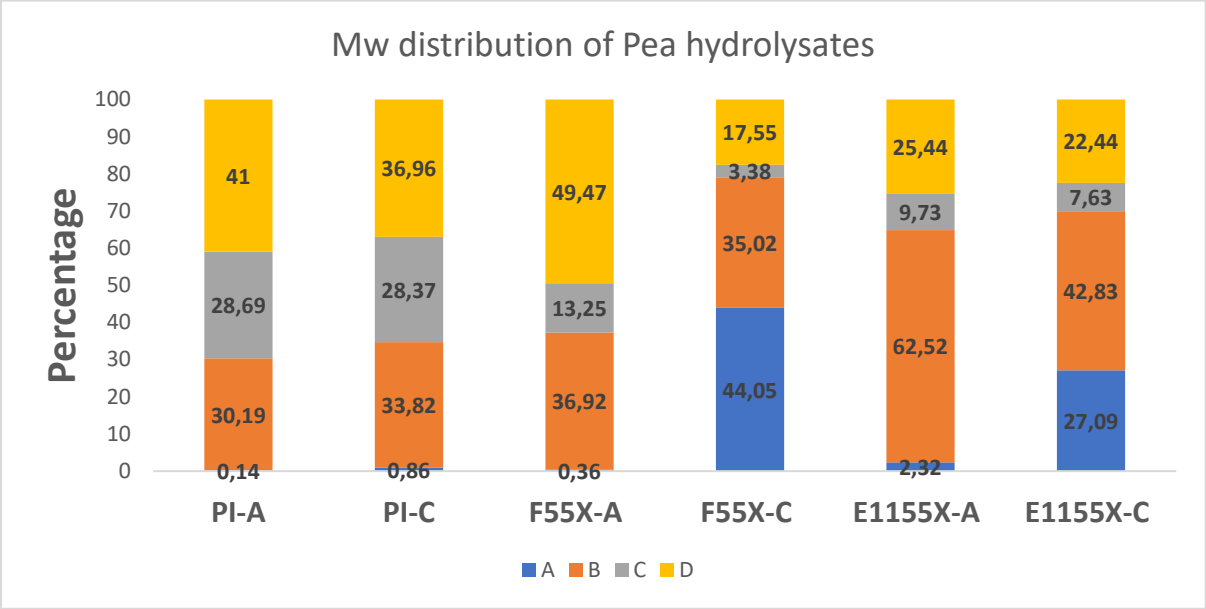

Figure 11. M_w distribution of peptides in Pea-protein hydrolysates.

Figure 11 shows the percentage distribution of the M_w for Pea hydrolysates in areas A, B, C, and D. The areas describe the time the peptide fraction elutes through the column. Area A: 5-6 min, Area B: 6-7.3 min, Area C: 7.3-8.4 min, and Area D: 8.4-20 min.

Out of the pea-protein hydrolysates, F55X-A had the highest number of peptides in area A at 44%, and E1155X-C the second-highest at 27%. The remaining pea-protein hydrolysates had under 3% of peptides in area A. Pea-protein hydrolysates had a similar MWD of peptides within area B independent from the enzyme used, ranging from 30-43%. The exception is E1155X-A containing 62.52% of peptides within area B. PI-A and PI-C had the highest number of peptides within area C at 28.69% and 28.37%. The remaining pea-protein hydrolysates contained a low amount of peptides within area C, ranging from 3-13%. F55X-A contained the highest number of peptides within area D at 49.47%. While PI-A and PI-C also contained a high number of peptides within area D at 41%, and 36.96%. Chromatogram for Faba-bean hydrolysates is hown (figure 12).

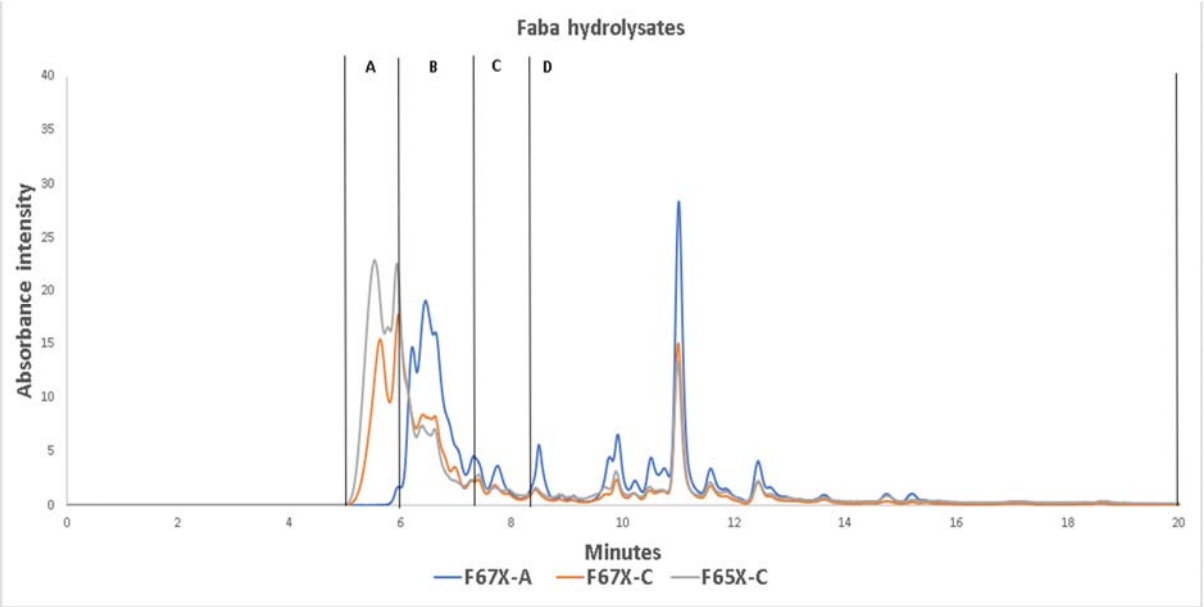

Figure 12. Size exclusion chromatography demonstrated differences in M_w and peptide distribution in Faba-bean hydrolysates.

Figure 12 shows all Faba hydrolysates retention times between 4 and 20 minutes, and absorption intensity at 214 nm. The chromatogram is split into four sections based on the time of elution: A, B, C, and D. Area A: 5-6min, Area B: 6-7.3min, Area C: 7.3-8.4, and Area D: 8.4-20 min.

The MWD for F67X was different using different enzymes, when using alcalase the highest peak was seen in area D, with a lesser peak in area B. When using corolase the highest peak was seen on the border of areas A and B, with two lesser peaks in areas A and D. The MWD for F65X using different enzymes could not be established due to insufficient sample material with alcalase for SEC analysis. When using corolase F65X had two equally large peaks in area A and a lesser peak in area D. Percentage of distribution is shown (figure 13).

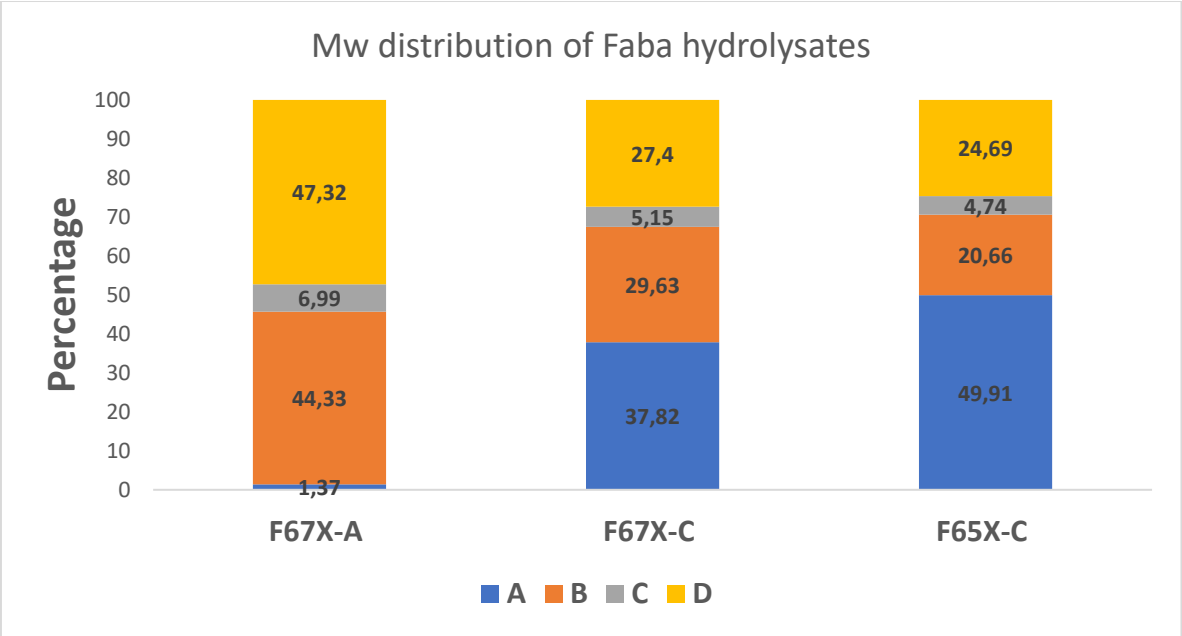

Figure 13. Mw distribution of peptides in Faba-protein hydrolysates.

Figure 13. shows the percentage distribution of the M_w for Faba hydrolysates in areas A, B, C, and D. The areas describe the time the peptide fraction elutes through the column. Area A: 5-6 min, Area B: 6-7.3 min, Area C: 7.3-8.4 min, and Area D: 8.4-20 min.

Out of the faba hydrolysates, F65X-C had the highest number of peptides in area A at 49.91%, while F67X-C also had a considerable number of peptides in area A at 37.82%. F67X-A had the highest number of peptides within area B at 44.33%. None of the faba-protein hydrolysates had a large number of peptides in area C. F67X-A had the highest percentage of peptides within D at 47.32%. Chromatogram for Barley, Oats and WPC80 is shown (figure 14).

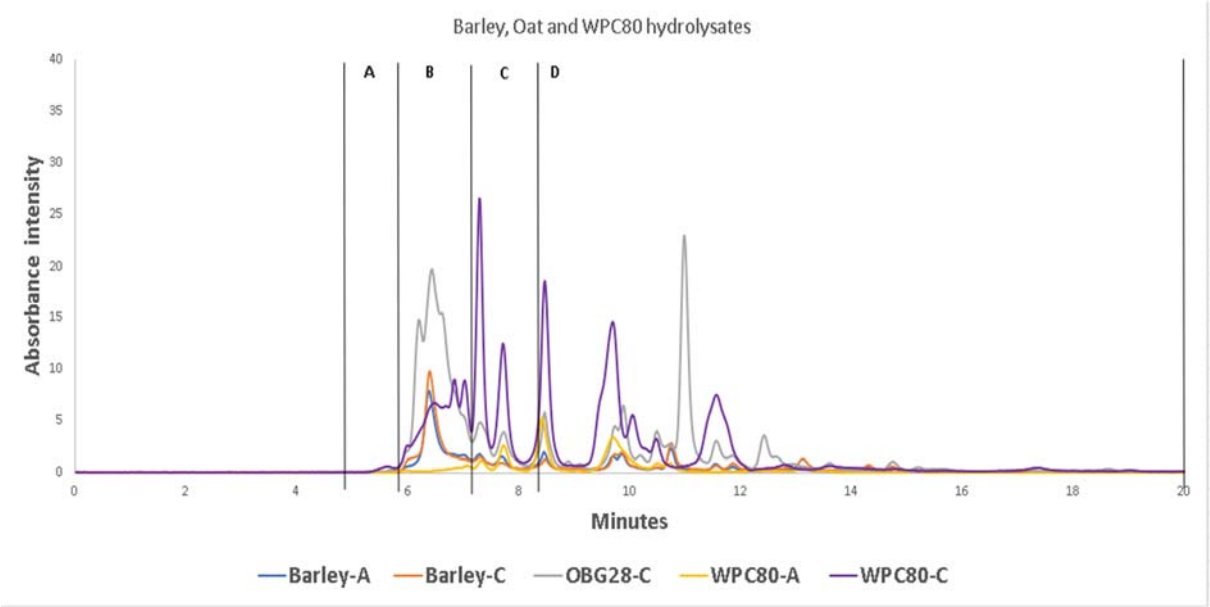

Figure 14. Size exclusion chromatography demonstrated differences in Mw and peptide distribution in Barley, Oat, and WPC80 protein hydrolysates.

Figure 14 shows Barley, Oat, and WPC80 hydrolysates retention time between 4 and 20 minutes, and absorption intensity at 214 nm. The chromatogram is split into four sections based on the time of elution: A, B, C, and D. Area A: 5-6min, Area B: 6-7.3min, Area C: 7.3-8.4, and Area D: 8.4-20 min

The MWD of Barley was not different using different enzymes, alcalase and corolase had their highest peaks in area B, with an additional small peak on the border of areas C and D. The MWD of OBG28 could not be observed due to alcalase forming a gel, that could not be analyzed by SEC. OBG28 when using corolase had the highest peak in area D, with a lesser peak in area B. The MWD of WPC80 was different using different enzymes, using alcalase yielded no high peaks, but several minor peaks in areas B, C, and D. Using corolase had the highest peak on the border of areas B and C, with three additional large peaks in area D. Percentage of distribution is shown (figure 15).

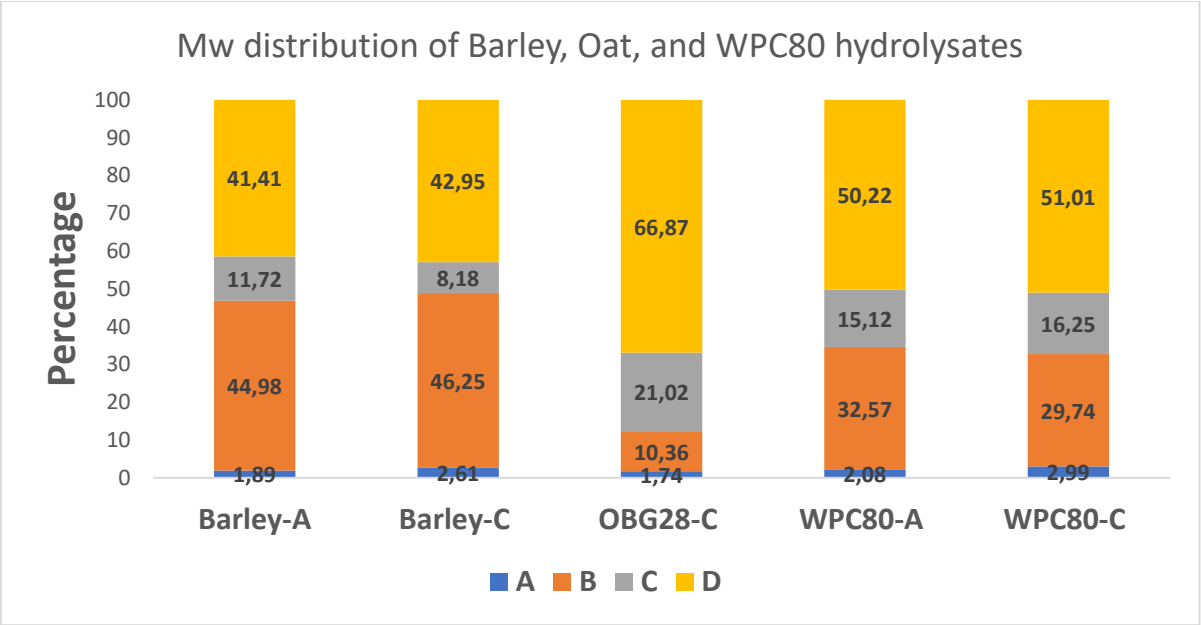

Figure 15. Mw distribution of peptides in Barley, Oat, and WPC80 hydrolysates.

Figure 15 shows the percentage distribution of the Mw for Barley, Oat, and WPC80 hydrolysates in areas A, B, C, and D. The areas describe the time the peptide fraction elutes through the column. Area A: 5-6 min, Area B: 6-7.3 min, Area C: 7.3-8.4 min, and Area D: 8.4-20 min.

None of the Barley, Oat, or WPC80 hydrolysates had a major percentage of peptides within area A. Out of these hydrolysates, Barley independent from the enzyme used had the highest number of peptides within area B at 44.98%, and 46.25%. OBG28-C had the highest number of peptides within area C at 21%. Additionally, OBG28-C had the highest number of peptides in area D at 66.87%. Barley and WPC80 also had a high number of peptides within area D ranging from 41-51%. The average M_w of all protein hydrolysates is shown (figure 16).

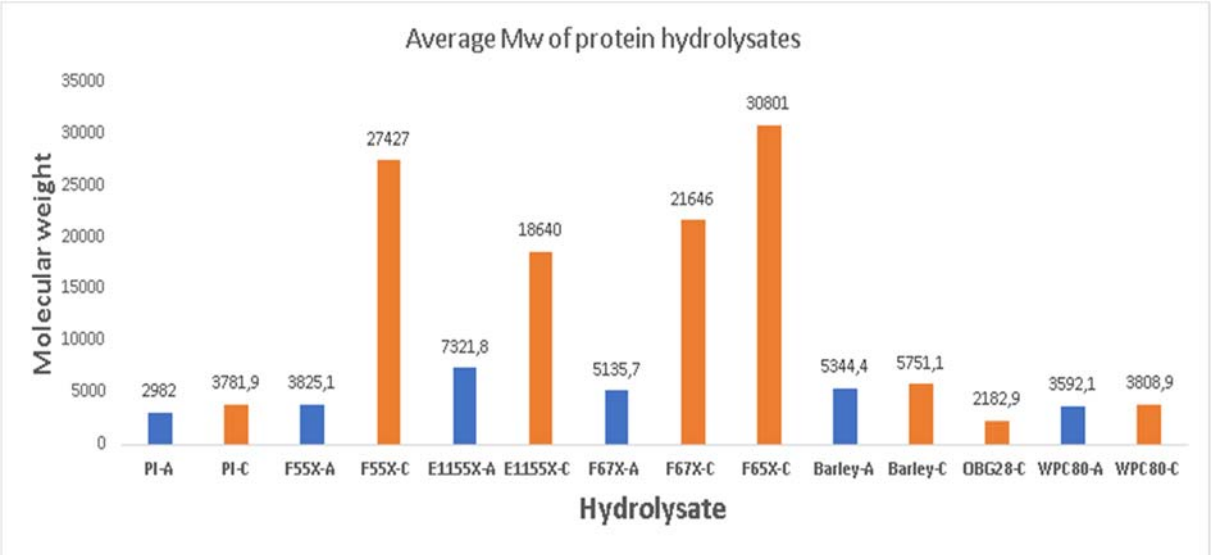

Figure 16. The average Mw of the protein hydrolysates was lower when hydrolyzed with alcalase compared to corolase.

Figure 16 shows the average Mw of redissolved protein hydrolysates. Blue colour shows substrate hydrolyzed with Alcalase. Orange colour shows substrate hydrolyzed with Corolase.

Hydrolysis using alcalase yielded a lower M_w for all substrates than corolase. The faba-protein hydrolysate F65X-C had the highest M_w at 30801 Da, while the oat-protein hydrolysate OBG28-C had the lowest M_w at 2182.9 Da. Barley with alcalase show no noticeable difference in M_w compared corolase. E1155X with alcalase show a lower M_w compared to corolase, 7321.8 Da, and 18640 Da respectively. PI with alcalase shows no noticeable difference in M_w compared to corolase. F55X with alcalase shows lower Mw compared to corolase, 3825.1 Da, and 27427 Da, respectively. F67 with alcalase shows a lower Mw compared to corolase, 5135.7 Da, and 21646 Da, respectively. WPC80-A did not have noticable difference in M_w compared to WPC80-C.

3.2 Quantifying cell proliferation using DNA based CyQuant proliferation Assay

To investigate the growth-promoting effects of several vegetable protein hydrolysates on bovine skeletal muscle cells, hydrolysates were supplemented to the cell culture medium for 24, 48, and 96 hours. Consecutively, cell proliferation was quantified using DNA based CyQuant proliferation assay.

3.2.1 Investigating the proliferating effects of pea hydrolysates on MuSCs

PI-C and E1155X-C hydrolysates show to be the most effective growth stimulators of pea-protein hydrolysates in this study. Interestingly, both hydrolyzed with corolase. F55X-C was

the only pea hydrolysate to show a reducing effect on proliferation. The proliferative effect of supplementing Pea hydrolysates are shown (figure 17, 18, 19, 20, 21, 22)

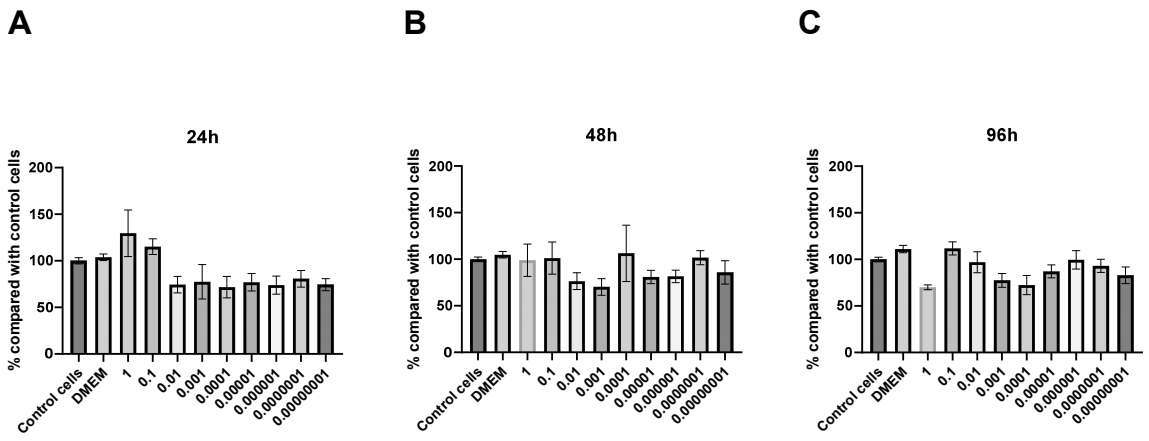

Figure 17. Additive effect of PI-A on the proliferation of bovine skeletal muscle cells.

*Figure 17 shows the mean cell proliferation in bovine skeletal muscle cells after the incubation period for control cells, and cells added PI-A hydrolysate. The X-axis display the concentration of added hydrolysate in mg/mL. A:[P=0.0001], B:[P=0.0268], and C:[P=0.001]. The number of * represents the statistical significance rating.*

One-way Anova revealed there was a statistically significant difference in bovine skeletal muscle cell proliferation between control cells, and cells supplemented with PI-A hydrolysates. Adding 1 mg/ml of PI-A hydrolysate to MuSCs increased cell growth after 24 h of stimulation, while longer incubation times had the opposite effect, and we could see a reduction in cell growth compared with control cells. Adding lower concentrations of PI-A reduced cell growth both short-term and after a longer incubation period.

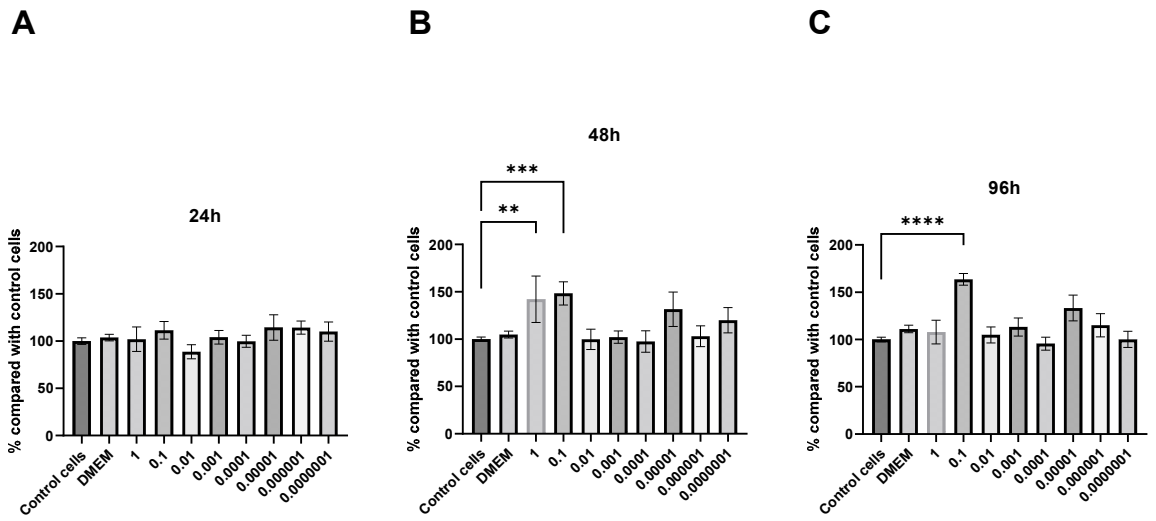

Figure 18. Additive effect of PI-C on the proliferation of bovine skeletal muscle cells.

Figure 18 shows the mean cell proliferation in bovine skeletal muscle cells after the incubation period for control cells, and cells added PI-C hydrolysate. The X-axis display the concentration of added hydrolysate in mg/mL. A:[P=0.7846], B:[P= 0.0002], C:[P= <0.0001]. *The number of * represents the statistical significance rating.*

One-way ANOVA revealed there was no statistically significant difference in bovine skeletal muscle cell proliferation between control cell, and cells supplemented PI-C hydrolysate after 24 hours, but was significant after 48, and 96 hours. Adding PI-C hydrolysate at a concentration of 0.1 mg/mL to MuSCs increased cell growth after 24, 48, and 96 hours of stimulation, while longer incubation times had a better effect. Adding 1, and 0.1 mg/mL of PI-C hydrolysate significantly increased cell growth by 42.11% and 48.34% after 48 hours of stimulation. After 96 hours of stimulation, the growth persisted at a concentration of 0.1 mg/mL, increasing cell growth by 63.6%.

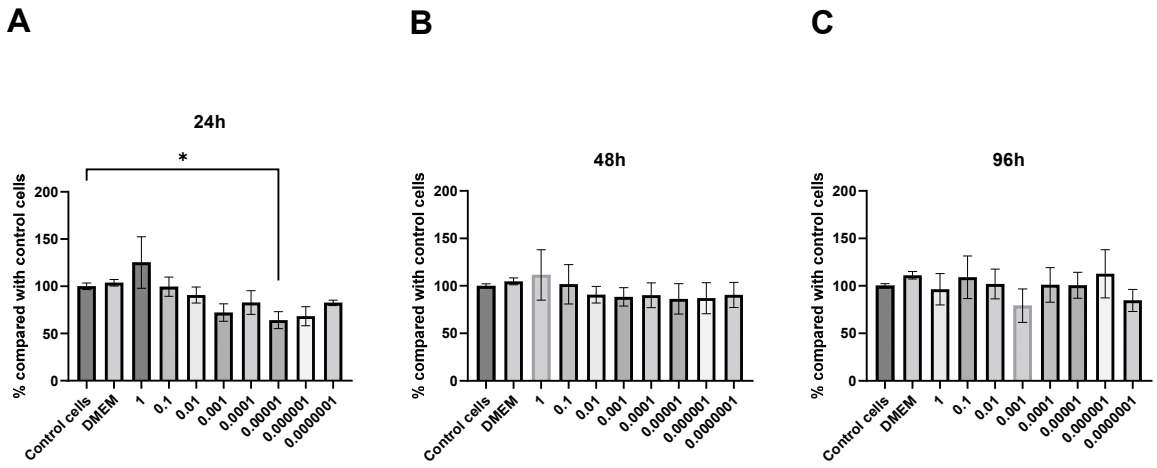

Figure 19. Additive effect of F55X-A hydrolysate on the proliferation of bovine skeletal muscle cells.

*Figure 19 shows the mean cell proliferation in bovine skeletal muscle cells after the incubation period for control cells, and cells added F55X-A hydrolysate. The X-axis display the concentration of added hydrolysate in mg/mL A:[P= 0.0009], B[P= 0.5346], C:[P= 0.2364]. The number of * represents the statistical significance rating.*

One-way ANOVA revealed there was a statistically significant difference in bovine skeletal muscle cell proliferation between control cells, and cells supplemented with F65X-C hydrolysate after 24, and 96 hours, but not after 48 hours. Adding 1 mg/mL of F55X-A hydrolysate to MuSCs increased cell growth by 25% after 24 hours of stimulation, and lower concentrations of F55X-A hydrolysate reduced cell growth significantly. F55X-A reduced cell growth in the short term but did not have a significant impact on growth following 48, and 96 hours of stimulation.

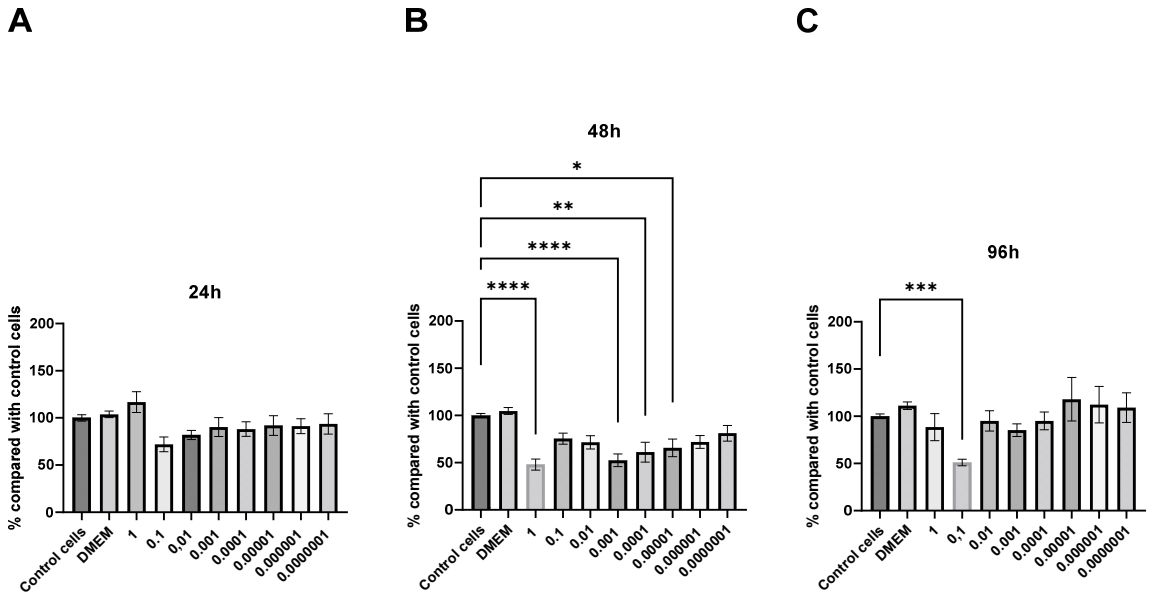

Figure 20. Additive effect of F55X-C hydrolysate on the proliferation of bovine skeletal muscle cells.

Figure 20 shows the mean cell proliferation in bovine skeletal muscle cells after the incubation period for control cells, and cells added F55X-C hydrolysate. The X-axis display the concentration of added hydrolysate in mg/mL. A:[P= 0.0911], B:[P= 0.0001], C:[P= 0.0002]. *The number of * represents the statistical significance rating.*

One-way ANOVA revealed there was not a statistically significant difference in bovine skeletal muscle cell proliferation between control cells, and cells supplemented with F55X-C hydrolysate after 24 hours but was significant after 48, and 96 hours. Adding 1 mg/mL F55X-C hydrolysate to MuSCs increased growth by 16.7% after 24 hours of stimulation, while longer incubation times had the opposite effect. Adding F55X-C hydrolysate at 1 mg/mL reduced cell growth by 51.9% after 48 hours of stimulation. The effect dissipated after 96 hours. Adding F55X-C hydrolysate at 0.1 mg/mL reduced cell growth by 50% after 96 hours of stimulation. At this time, the lower concentrations increased cell growth.

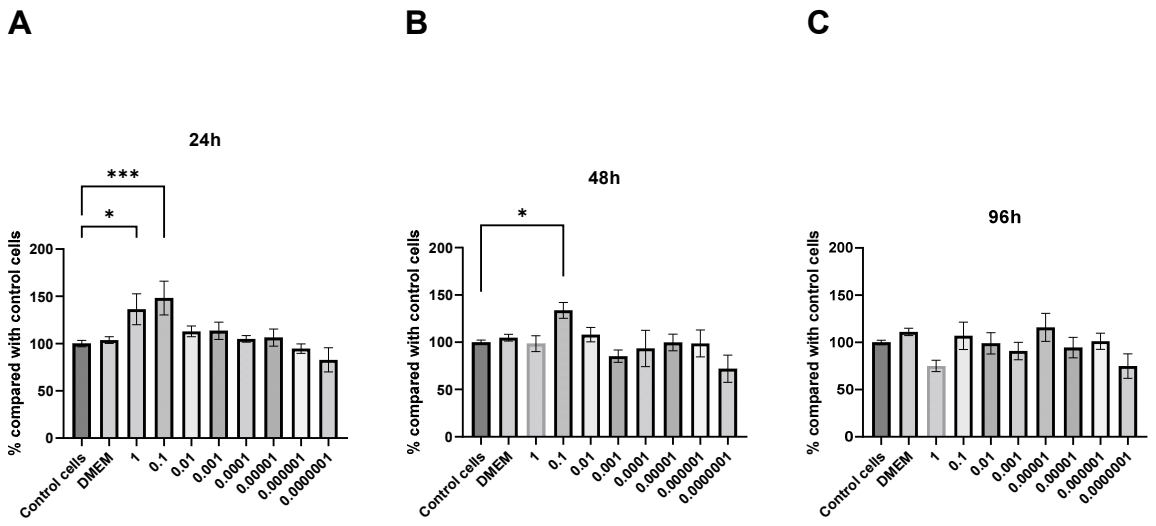

Figure 21. Additive effect of E1155X-A hydrolysate on the proliferation of bovine skeletal muscle cells.

Figure 21 shows the mean cell proliferation in bovine skeletal muscle cells after the incubation period for control cells, and cells added E1155X-A hydrolysate. The X-axis display the concentration of added hydrolysate in mg/mL. A:[P= 0.0013], B:[P= 0.0164], and C:[P= 0.0117]. *The number of * represents the statistical significance rating.*

One-way ANOVA revealed there was a statistically significant difference in bovine skeletal muscle cell proliferation between control cells, and cells supplemented with E1155X-A hydrolysate. Adding 1, and 0.1 mg/mL of E1155X-A hydrolysate on MuSCs significantly increased growth by 36.4%, and 48.2% after 24 hours of stimulation. Following 48 hours of stimulation of E1155X-A hydrolysate 0.1 mg/mL significantly increased cell growth by 33.7%, however, after 96 hours of stimulation, the growth-promoting effect had dissipated.

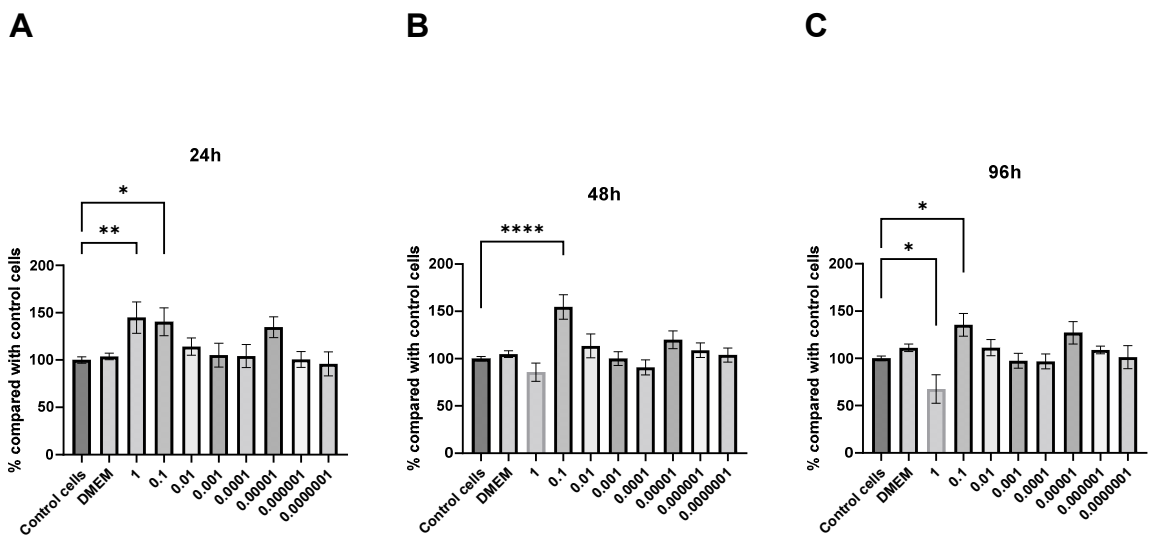

Figure 22. Additive effect E1155X-C hydrolysate on the proliferation of bovine skeletal muscle cells.

Figure 22 shows the mean cell proliferation in bovine skeletal muscle cells after the incubation period for control cells, and cells added E1155X-C hydrolysate. The X-axis display the concentration of added hydrolysate in mg/mL. A:[P= 0.0010], B: [P= 0.0002], and C: [P= 0.003]. *The number of * represents the statistical significance rating.*

One-way ANOVA revealed there was a statistically significant difference in bovine skeletal muscle cell proliferation between control cells, and cells supplemented with E1155X-C hydrolysate. Adding E1155X-C hydrolysate increased cell growth at several concentrations after 24 hours of stimulation. Longer incubation times show a similar effect. At a concentration of 0.1 mg/mL, the growth-promoting effects were lasting through 24, 48, and 96 hours of stimulation by 44.8%, 54%, and 35%, being statistically significant in all time frames.

3.2.2 Investigating the proliferating effects of Faba-bean hydrolysates on MuSCs

Faba bean hydrolysates did not increase proliferation in MuSCs. A strong reducing effect on proliferation was observed when supplementing with F67X-C hydrolysate. The proliferative effects of supplementing Faba-bean hydrolysates to MuSCs are shown (figure 23, 24, 25).

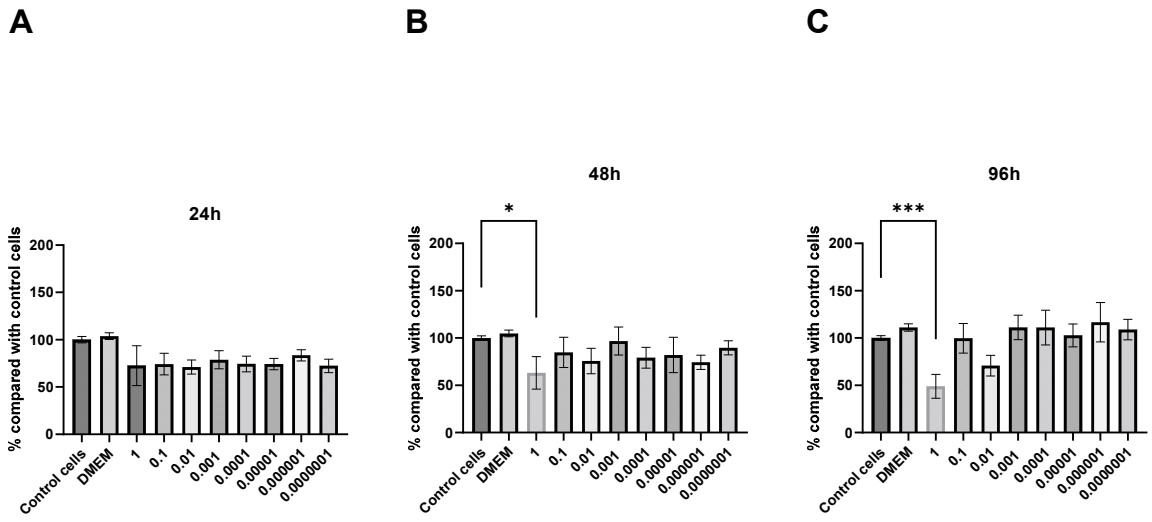

Figure 23. Additive effect of F67X-A on the proliferation of bovine skeletal muscle cells.

Figure 23 shows the mean cell proliferation in bovine skeletal muscle cells after the incubation period for control cells, and cells added F67X-A hydrolysate. The X-axis display the concentration of added hydrolysate in mg/mL. A:[P= 0.0003], B:[P= 0,0013], C:[P= 0.0001]. *The number of * represents the statistical significance rating.*

One-way ANOVA revealed there was a statistically significant difference in bovine skeletal muscle cell proliferation between control cells, and cells supplemented with F67X-A hydrolysate. Adding F67X-A hydrolysate reduced cell growth at all concentrations after 24 and 48 hours of stimulation. At 1 mg/mL cell growth was significantly reduced by36.9%, while longer incubation time further decreased cell growth at this concentration to 51.1%. Adding lower concentrations of F67X-A hydrolysate reduced cell growth short term but increased again after 96 hours of stimulation.

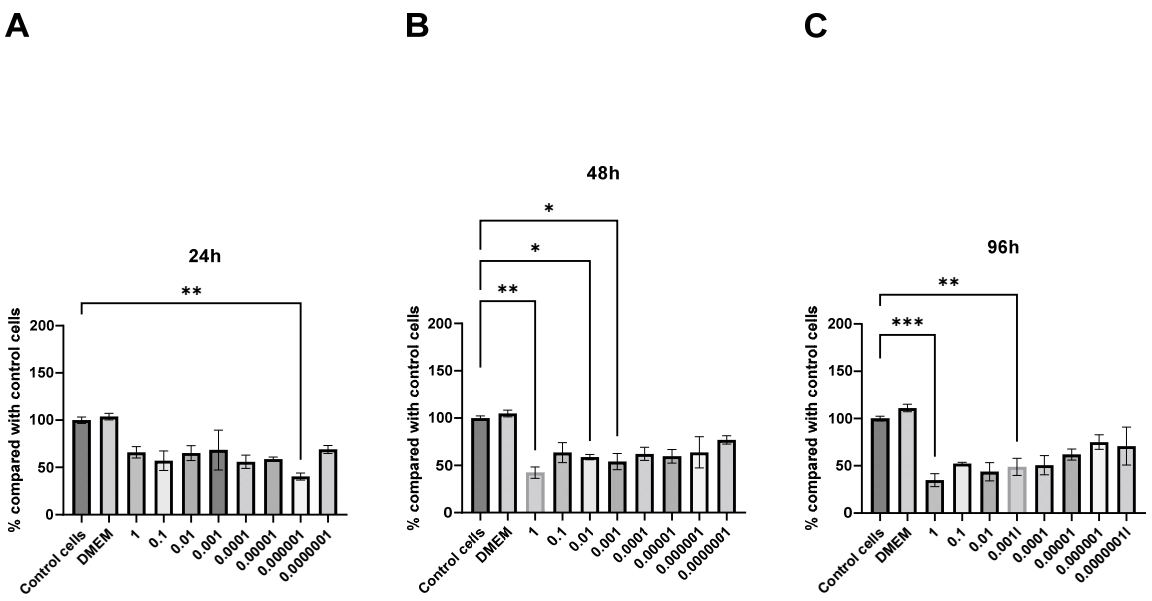

Figure 24. Additive effect of F67X-C hydrolysate on the proliferation of bovine skeletal muscle cells.

Figure 24 shows the mean cell proliferation in bovine skeletal muscle cells after the incubation period for control cells, and cells added F67X-C hydrolysate. The X-axis display the concentration of added hydrolysate in mg/mL. A:[P= 0.0001], B:[P= 0.0001], and C:[P= 0.0001]. *The number of * represents the statistical significance rating.*

One-way ANOVA revealed there was a statistically significant difference in bovine skeletal muscle cell proliferation between control cells, and cells supplemented with F67X-C hydrolysate. Adding F67X-C hydrolysate reduced cell growth significantly after 24, 48, and 96 hours of stimulation at all concentrations. At a concentration of 1 mg/mL the highest reduction in cell growth was observed after 48, and 96 hours of stimulation, by 57.8%, and 61.1% respectively. Adding lower concentrations of F67X-C hydrolysate reduced cell growth both short-term and after a longer incubation period.

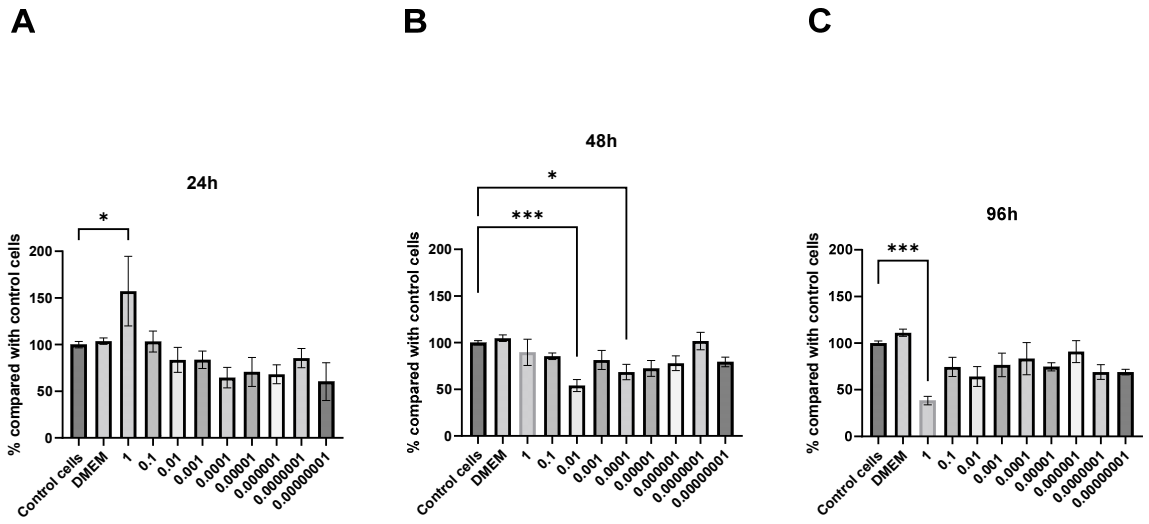

Figure 25. Additive effect of F65X-C hydrolysate on the proliferation of bovine skeletal muscle cells.

Figure 25 shows the mean cell proliferation in bovine skeletal muscle cells after the incubation period for control cells, and cells added F65X-C hydrolysate. The X-axis display the concentration of added hydrolysate in mg/mL A:[P= 0.0001], B:[P= 0.0001], and C:[P= 0.0001]. *The number of * represents the statistical significance rating.*

One-way ANOVA revealed there was a statistically significant difference in bovine skeletal muscle cell proliferation between control cells, and cells supplemented with F65X-C hydrolysate. Adding 1 mg/mL F65X-C hydrolysate to MuSCs significantly increased cell growth by 57,3% after 24 hours of stimulation, while longer incubation times had the opposite effect. Adding lower concentrations of F65X-C reduced cell growth both short-term and after longer incubation periods.

3.2.3 Investigating the proliferating effect of Barley, Oats, and WPC80 hydrolysates on MuSCs.

Barley hydrolysates had a strong reducing effect on proliferation in MuSCs, although the effect was reversed for the lower concentration after 96 hours. The same applies to Oat hydrolysate. WPC80 increased proliferation in MuSCs.The proliferative effect of supplementing Barley, Oats, and WPC80 hydrolysates are shown (figure 26, 27, 28, 29, 30).

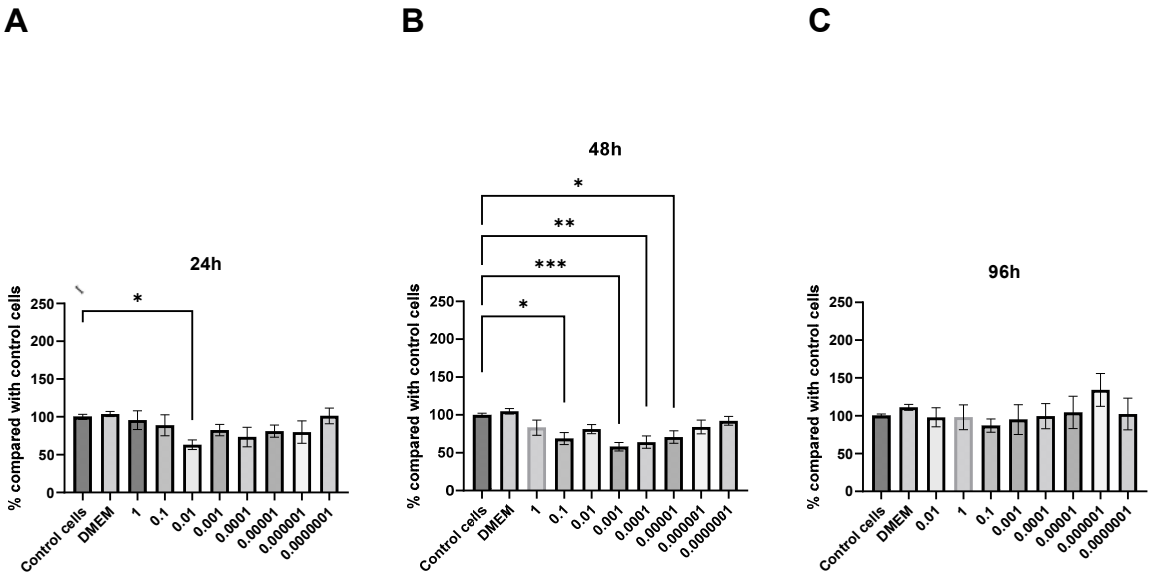

Figure 26. Additive effect of Barley-A on the proliferation of bovine skeletal muscle cells.

Figure 26 shows the mean cell proliferation in bovine skeletal muscle cells after the incubation period for control cells, and cells added Barley-A hydrolysate. The X-axis display the concentration of added hydrolysate in mg/mL. A:[P= 0.0084], B:[P= 0,0001], C:[P= 0.1626]. *The number of * represents the statistical significance rating.*

One-way ANOVA revealed there was a statistically significant difference in bovine skeletal muscle cell proliferation between control cells, and cells supplemented Barley-A hydrolysates after 24, and 48 hours, but not 96 hours. Adding 0.01 mg/mL barley-A hydrolysate to MuSCs significantly reduced cell growth after 24 hours of stimulation. After 48 hours of stimulation, several concentrations significantly reduced cell growth, while after 96 hours the reducing effect of Barley-A hydrolysate can be seen to diminish.

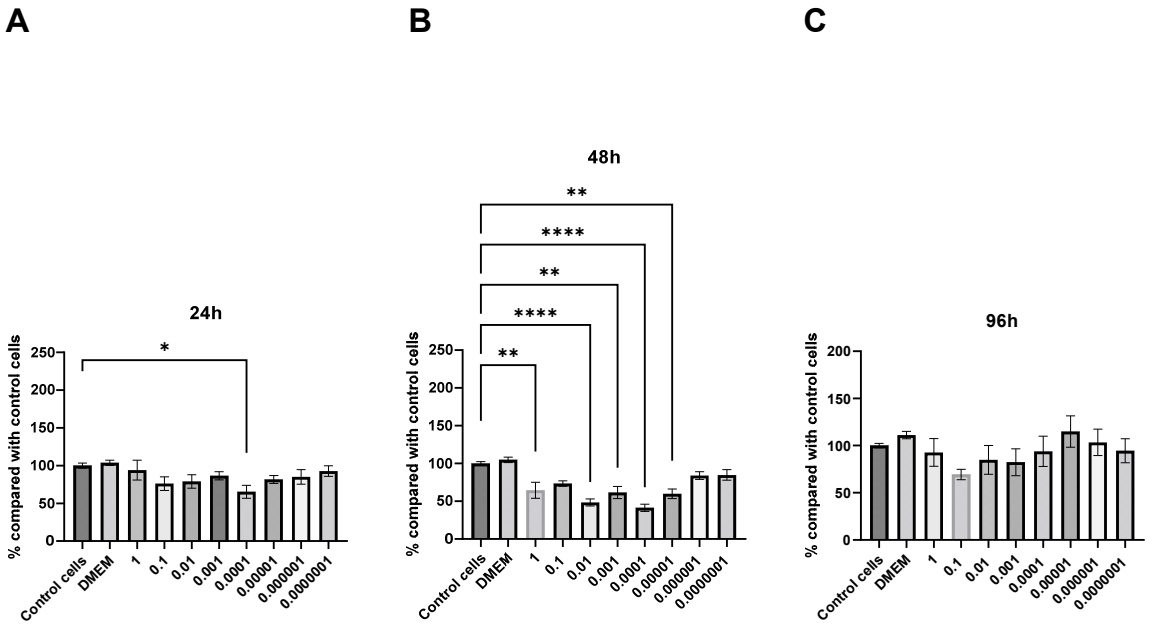

Figure 27. Additive effect of Barley-C hydrolysate on the proliferation of bovine skeletal muscle cells.

Figure 27 shows the mean cell proliferation in bovine skeletal muscle cells after the incubation period for control cells, and cells added Barley-C hydrolysate. The X-axis display the concentration of added hydrolysate in mg/mL. A:[P= 0.0091], B:[P= 0,00001], C:[P= 0.0081]. *The number of * represents the statistical significance rating.*

One-way ANOVA revealed there was a statistically significant difference in bovine skeletal muscle cell proliferation between control cells, and cells supplemented with Barley-A hydrolysate. Adding Barley-A hydrolysate to MuSCs significantly reduced growth after 24, and 48 hours of stimulation. After 48 hours of stimulation a 48%, and 36% significant reduction in growth was observed. After 96 hours of stimulation, the growth reducing the effect of Barley-C hydrolysate was seen to slightly dissipate.

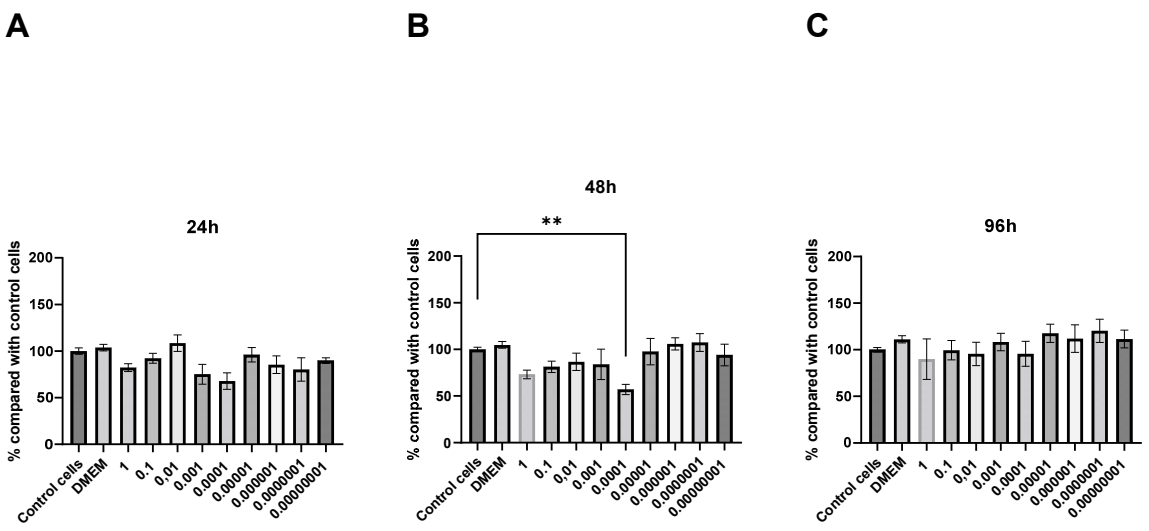

Figure 28. Additive effect of OBG28-C hydrolysate on the proliferation of bovine skeletal muscle cells.

Figure 28 shows the mean cell proliferation in bovine skeletal muscle cells after the incubation period for control cells, and cells added OBG28-C hydrolysate. The X-axis display the concentration of added hydrolysate in mg/mL. A:[P= 0.0281], B: [P= 0.0013], C: [P= 0.2628]. *The number of * represents the statistical significance rating.*

One-way ANOVA revealed there was a statistically significant difference in bovine skeletal muscle cell proliferation between control cells, and cells supplemented with OBG28-C hydrolysate after 24, and 48 hours, but not after 96 hours. Adding OBG28-C hydrolysate to MuSCs decreased cell growth after 24, and 48 hours of stimulation, while a longer incubation time had the opposite effect.

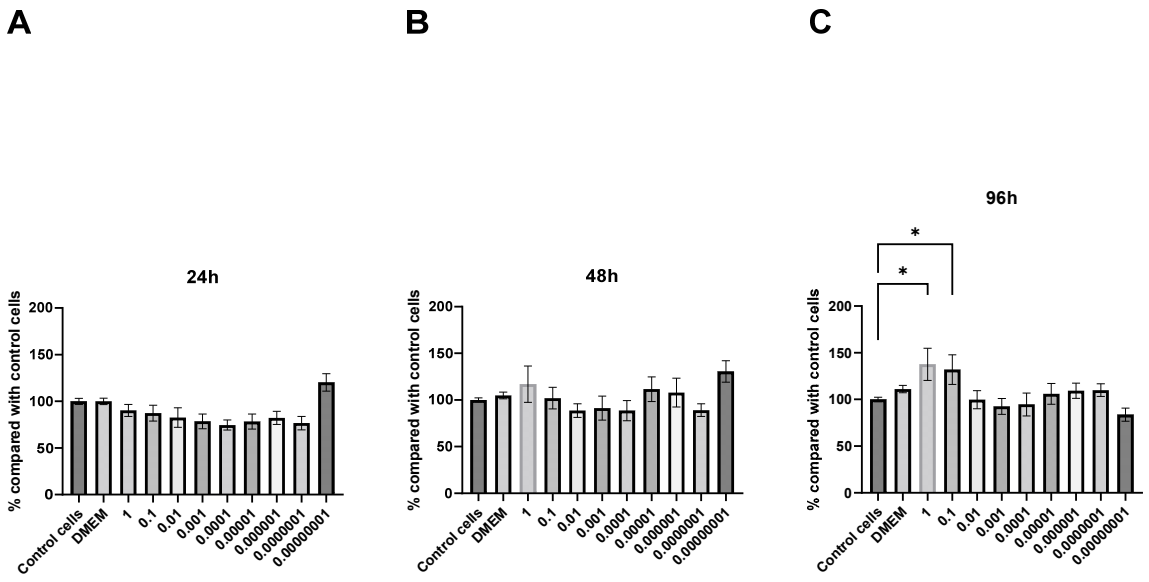

Figure 29. Additive effect of WPC80-A hydrolysates on the proliferation of bovine skeletal muscle cells.

*Figure 29 shows the mean cell proliferation in bovine skeletal muscle cells after the incubation period for control cells, and cells added WPC80-A hydrolysate. The X-axis display the concentration of added hydrolysate in mg/mL. A:[P= 0.0005], B: [P= 0.2612], C: [P= 0.0046]. The number of * represents the statistical significance rating.*

One-way ANOVA revealed there was a statistically significant difference in bovine skeletal muscle cell proliferation between control cells, and cells supplemented with WPC80-A hydrolysate after 24, and 96 hours, but not after 48 hours. Adding the lowest concentration of WPC80-A hydrolysate increased cell growth by 20.2% after 24 hours of stimulation. The effect persisted through 48 hours, increasing cell growth by 30.5%. The growth-promoting effect dissipated after 96 hours of incubation Adding 1, and 0.1 mg/mL WPC80-A hydrolysate did not increase growth short term, however, it did increase growth in the long term. At 96 hours of incubation, these concentrations significantly increased cell growth by 37.6% and 31.9% respectively.

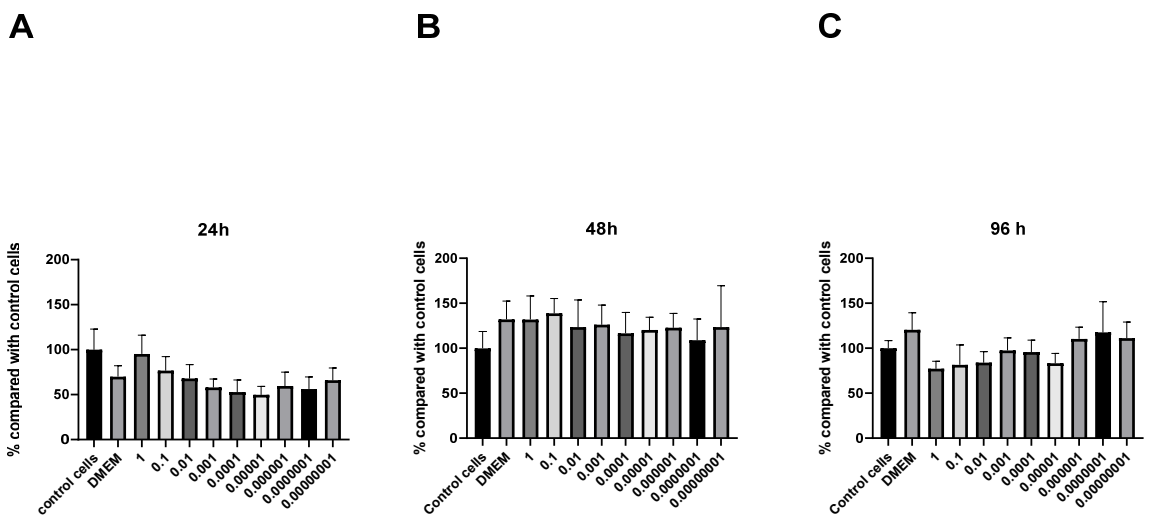

Figure 30. Additive effect of WPC80-C hydrolysates on the proliferation of bovine skeletal muscle cells.

Figure 30 shows the mean cell proliferation in bovine skeletal muscle cells after the incubation period for control cells, and cells added WPC80-C hydrolysate. The X-axis display the concentration of added hydrolysate in mg/mL. A:[P= 0.0001], B:[P= 0.1419], C:[P= 0.0001]. *The number of * represents the statistical significance rating.*

One-way ANOVA revealed there was a statistically significant difference in bovine skeletal muscle cell proliferation between control cells, and cells supplemented with OBG28-C hydrolysate after 24, and 48 hours, but not after 96 hours. Adding OBG28-C hydrolysate to MuSCs significantly reduced cell growth after 24 hours of stimulation, compared to control cells. While 48 hours of stimulation increased cell growth significantly. After 96 hours of stimulation WPC80-C hydrolysate reduced cell growth, except for the lower concentrations.

3.4 DPPIV-inhibition assay

To investigate the DPPIV-inhibiting properties of the protein hydrolysates, a DPPIV assay was performed (table 11).

Table 11. DPPIV inhibiting effect of protein hydrolysates.

Inhibitor	Average inhibition (%)	SD
SITAGLIPTIN	102.18	0.32
PI-A	35.23	3.99
PI-C	32.17	1.91
F55X-A	13.21	0.18
F55X-C	31.15	4.25
E1155X-A	23.11	0.69
E1155X-C	21.99	3.87
F67-A	23.26	2.05
F67-C	19.8	6.8
F65-C	17.6	4.16
Barley-A	13.64	5.36
Barley-C	12.59	2.8
OBG-28-C	22.34	2.26
WPC80-A	27.25	5.09
WPC80-C	25.12	4.83

Table 11 shows the name of the inhibitor, % inhibition, and SD calculated from the DPPIV inhibitor screening assay kit. Positive control: Sitagliptin

All protein hydrolysates had a percentage of DPPIV-inhibiting activity (table 11). PI hydrolysates had the highest inhibition at 35% and 32%, along with F55X-C hydrolysate. While WPC80 hydrolysates had the second-highest inhibition. Barley hydrolysates showed the lowest inhibition at 12.5%

4.0 Discussion

This study aimed to investigate the growth-promoting effects of seven vegetable-derived protein hydrolysates on the proliferation of MuSCs. Can hydrolysates derived from Pea, Faba-bean, Barley, Oats, and Whey concentrates increase cell proliferation in MuSC? The data suggest that hydrolysates deriving from Pea and WPC80 have growth-promoting effects in MuSC. In contrast, other hydrolysates reduced or had little effect on proliferation.

4.1 Choice of raw material and protease affected the M_w of the hydrolysates.

SEC provides vital information about the M_w and MWD of hydrolysates. This method is commonly used for analyzing protein and peptides for food-grade products. In this study, DH was not measured. Based on previous work from Aase Kristoffersen et al. DH is indirectly correlated with M_w (44). Hence, for further discussion, hydrolysates with low M_w will be referred to as high DH, and hydrolysates with high M_w may be referred to as low DH. A previous study has demonstrated that hydrolysates with oligopeptides between 2-15 amino acids have improved cell growth and metabolic activity in MuSCs (10). To quantify the percentage of peptides within this range MWD was used.

This study suggests that the choice of raw material and protease is essential for the hydrolysates DH and MWD. Raw materials hydrolyzed with alcalase yielded an average M_w under 5400 Da, except E1155X at 7321 Da (figure 16). Alcalase 2.4L is a nonspecific serine endopeptidase containing three endopeptidases and one exopeptidase (37). Therefore, it cleaves various peptide bonds and has a strong tendency to give hydrolysates with peptides of small size and hydrophobic characteristics. Alcalase has been applied on many protein substrates such as tuna, whey, potato, soy, and others, repeatedly yielding a high DH (79-81). The results from this study concur with these findings. Alcalase is an excellent mix of proteases for hydrolysing food-grade proteins to a high DH. Nevertheless, the raw material still influences the DH, as seen with E1155X, and minor variations in average M_w between the hydrolysates.

Raw materials hydrolyzed with corolase exhibited a more complicated MWD. Not all raw materials achieved a high DH with this enzyme. This can be seen from the average M_w (figure 16). A possible explanation could be attributed to corolase encountering low substrate specificity. During hydrolysis, as proteins are degraded to peptides, all possible cleavage sites may have been used. At this point in the hydrolysis, the exopeptidase activity of alcalase is the only protease with a high substrate specificity, continuing further degradation of peptides. The lack of exopeptidase activity may be a limiting factor for corolase on these raw materials.

However, if low substrate specificity was the limiting factor, it would be expected to see the same trend on PI. PI, F55X, and E1155X all derive from pea protein from the same distributor. As such, the amino acid composition of pea proteins is expected to be equivalent. PI had a high DH with both enzymes (figure 16). Based on this observation, low substrate specificity was probably not the limiting factor during hydrolysis. PI has a lower amount of carbohydrate and a higher protein content compared to the other pea raw materials. The legume-derived raw materials from pea and faba-bean containing 55-67% protein (F55X, E1155X, and F67X) achieved a lower DH with corolase than alcalase. A possible explanation could be the enzyme inhibitors present in the raw material.

Interestingly, E1155X had a higher DH than other raw materials of the same protein content hydrolyzed with corolase (figure 16). We speculate an inactivation of serine protease inhibitors during heat treatment could explain these results. Enzyme inhibitors are present at a high concentration in legume seeds compared to other plant families (39). Legume seed enzyme inhibitors can be classified into a-amylase and protease inhibitors (39). The protease inhibitors can further be classified into Kunitz and Bowman-Birk classes (41). Trypsin and chymotrypsin inhibitors are the most prevalent. These trypsin and chymotrypsin inhibitors are serine-protease-inhibitors.

Serine-proteinase-inhibitors form a family of homologous, large glycoproteins compromised of about 400 amino acid residues (40). They form a binding loop to the protease similarly to substrate-protease. The inhibitor must be recognized by the S1 pocket, containing the catalytic triad of amino acids determining the substrate specificity of the protease. Retaining the folded three-dimensional shape is essential for the glycoprotein to be recognized by the protease and exert inhibitory functions. The number of disulfide bonds substantially affect the structural stability of proteins (82). Mession J-L et al. observed dissociation of legumin oligomers and their rearrangements via hydrophobic interactions and sulfhydryl/disulfide bonds exchange reactions during the heat-treatment of pea-protein, giving rise to mainly high M_w aggregates of random structure. This study observes that heat-treatment of pea protein prior to hydrolysis yield a higher DH with corolase than other raw materials of similar protein content. We suggest the observed phenomenon can be explained by the denature of glycoproteins in the raw material, making them unrecognizable for the protease they inhibit.

To our knowledge the only protease inhibitors observed in peas are trypsin and chymotrypsin inhibitors (39). These enzymes are serine proteases with structures similar to alcalase and corolase. It is possible that glycoproteins inhibiting trypsin and chymotrypsin could inhibit

these enzymes as well. Further research investigating the presence of serine inhibitors and their mechanisms could prove useful for further studies on legume-derived hydrolysates.

4.1.2 Weaknesses in the MWD profile and recommendation for further peptide analysis.

The MWD is an area from the chromatogram with a percentage of peptides within it. The MWD suggests that the choice of the enzyme was not of great significance for the DH and peptides generated for hydrolysates from PI, Barley, Oats, and WPC80 raw materials (figure 16A, 16C). However, based on the chromatograms we can see this is not true. Weaknesses in the MWD can be seen from the respective chromatograms. Chromatogram for PI show differences between alcalase and corolase in intensity and elution time (figure 10, 14). The MWD of PI-A adheres to the peaks in area B and partially in C. Upon inspection of area C PI-C is shown to have a higher intensity when peptides elute in this area and in area D. Thus, indicating different peptide sequences between the enzymes used. The MWD is similar, however the chromatogram shows differences in peptide size. The same principle applies to WPC80. The chromatogram of Barley hydrolyzed with alcalase or corolase do exhibit similarities (figure 14). Supporting the MWD profile of this raw material.

PI hydrolysates regardless of enzyme used achieved a high DH (figure 16). Comparison of these findings with a previous study found alcalase and corolase to yield a high DH on PI (83). Garcia Arteaga et al. used an SDS-page analyzis observing the M_w of pea-protein isolate hydrolyzed with alcalase 2.4L, and corolase 7089. The SDS-page used could not identify peptides under 6.5 kDa. For alcalase they observed present bands between 97-6.5 kDa with the strongest bands at 6.5 kDa and corolase 7089 between 50-6.5 kDa. Alcalase 2.4L is identical to the enzyme used in the present study. The same could not be said for corolase 7089, which originates from *Bacillus subtilis*. The enzyme corolase 8000 originates from the fungus Malbranchea pulchella var. sulfurea. The enzyme to substrate ratio (E/S) in the present study correlates to Garcia Aertega et al at 0.5% E/S. This study applied enzyme to protein ratio. Nevertheless, PI contain 80% protein and the differences in M_w between the studies was minimal. Oats hydrolyzed with corolase show the highest DH out of all the raw materials (figure 16). The differences in M_w and MWD between Oats hydrolyzed with alcalase and corolase could not be compared, as oats hydrolyzed with alcalase turned into a gel and was not eligible for SEC. Oats hydrolyzed with alcalase have previously been reported to form gels (84). The abundant amino acid Glu in oats are preferred by alcalase and a high DH with this enzyme is therefore expected. Alcalase is known to generate peptides of low Mw and

hydrophobic characteristics. The formation of a gel is mainly supported by hydrophobic interactions as the attractive forces of hydrophobic interactions aggregate, leading to the formation of a gel (80).

To further discuss the peptides in the hydrolysates more information on the peptide sequence in necessary. To reveal the amino acid composition of peptides in this study, a coupled Mass spectrometry (MS) with reverse phase capillary liquid chromatography (RPLC) is recommended. RPLC contains a column filled with silica particles conjugated to carbon chains (85). Peptides passing through the column are exposed to organic solvents. The hydrophilic peptides with a positive charge move through the column first followed by negatively charged hydrophobic peptides later. The peptides eluting first are sprayed into the mass spectrometer and are heated, evaporating into ions. As the ions pass through the mass spectrometer they are fragmented by their mass-to-charge ratio and measured. Based on this information they are fragmented into sections. The fragmentation of individual peptides collides with gas particles removing the amino acid on the N-terminal of the peptide. Finally, peptide sequencing software analyses the product comparing it to peptide databases.

4.2 Supplementation of Pea hydrolysate significantly increased proliferation in MuSCs under normal-serum conditions.

The cultivation of meat may become a valuable protein source for humans in the future. Challenges regarding replacement/reduction of serum is of the essence. Sustainable growth stimulators must be found to replace/reduce the amount of serum needed. This study therefore investigated the proliferative effects of seven vegetable hydrolysates on MuSCs.

The most promising Pea-protein hydrolysates were PI-C and E1155X-C. The data from this study shows supplementation of 0.1 mg/mL PI-C hydrolysate significantly increased cell proliferation in MUSCSs by 63.3% after a 96-hour incubation period (figure 18). Pea-hydrolysate is previously known to exert growth-promoting effects in mammalian cells such as human mesenchymal stem cells (73). After three-day incubation period with normal-serum conditions, J. Lee et al. observed a 20% increase in proliferation on MSC supplemented pea-hydrolysate. Based on a cytokine analysis the group suggested that pea-hydrolysate stimulates intracellular synthesis of vascular endothelial growth factors (VEGF), interleukin 6 (IL-6), and transforming growth factors beta 1 (TGF-β1) in MSC.

A previous study has shown the high specificity of VEGF (86) as the proliferation induced by this growth factor almost exclusively relates to vascular endothelial cells. Therefore, it lacks

mitogenic activity for other cell types and can likely be excluded as the cause for the proliferating effects seen in MuSCs supplemented PI-C hydrolysate. IL-6 has been shown to stimulate platelet-derived growth factors (PDGF) in vascular smooth muscle cells (87). The results from Ikeda et al. suggest that the proliferative effect of IL-6 is dependent on the production of endogenous PDGF. In another study the proliferating effect of PDGF on mouse skeletal muscle cells was investigated (30). Yablonka et al. demonstrated that PDGF exerts mitogenic effects on mouse skeletal muscle cells. In the presence of PDGF fewer cells differentiated and more cells were available for the next round of cell division. If PDGF suppresses differentiation another mitogen must be present to induce proliferation. It is likely that PDGF can regulate myoblast proliferation and differentiation, and has a role in increasing the number of myoblasts during skeletal muscle regeneration. TGF-β1 and myostatin the two most important regulators for muscle growth have been shown to impact myoblast proliferation and differentiation by activating distinct pathways in mouse myoblasts (88). Primarily TGF-β1 stimulates myoblast proliferation through mothers against decapentaplegic homolog 2/3 (SMAD2/3). SMAD2/3 is a direct mediator of TGF-β1(89) and is known to exert specific biological functions during myogenesis. It is also shown that myoblast proliferation is increased by changing the localization of proliferating cell nuclear antigen, which increases cell division and prevents cell cycle exit. (90, 91). It is possible that PI-C hydrolysate stimulates myoblast proliferation through TGF-β1 and its activation mechanism through SMAD proteins. Nevertheless, it must be mentioned that the increase in TGF-β1 seen in MSCs does not necessarily correspond to MuSCs. For further research a cytokine analysis is recommended to determine whether TGF-β1 is elevated in MuSCs after supplementation of PI-C.

Earlier work investigating the proliferating effect of yeast extract and pork plasma hydrolysates on MuSCs have reported peptides with approximately 2-15 amino acids in length improve cellular growth and metabolic activity (10). The MWD of PI-C reveal 36.95% of peptides are within 2-8 amino acids (area D, figure 11) and 28.37% of peptides are within 8-22 amino acids. The results seen with PI-C agree with the current literature. However, PI-A show similar MWD without the proliferative effects (figure 11). It is well established that different enzymes produce different protein hydrolysates even from the same raw material (34). The peptides generated from alcalase did not exhibit proliferative effects on any of the raw materials except for WPC80. But corolase did on PI and E1155X. The bioactive mechanisms of peptides are not fully understood, without peptide isolation or an amino acid

analysis of PI hydrolysate the mechanisms of these peptides are hard to identify. Nevertheless, it is possible the amino acid composition generated in hydrolysates with alcalase do not incite the proliferative effects seen with corolase. For further research on PI-C hydrolysates, peptide isolation and characterization is recommended.

The statistically significant 63.3% increase in proliferation from PI-C looks promising, on the other hand the limitation of this study is seen in the number of data points available. The findings cannot necessarily be generalized. Still, it brings insight into a potential hydrolysate with promising growth-promoting capabilities. Unfortunately, this study did not investigate the serum-replacement potential of this hydrolysate. Further research might benefit from exploring the effect of PI-C hydrolysate in a serum reduced/starved media on MuSCs. Determining the mechanisms behind the major increase in growth from PI-C hydrolysate on MuSCs could prove beneficial for meat cultivation and tissue engineering companies. Finally, pharmaceutical companies that produce monoclonal antibodies intended for therapeutic means, could also benefit from this growth stimulator. The proliferative stimulating effects could yield a more cost-effective protein harvest from other mammalian cells. Further exploration of mechanisms on growth promoting hydrolysates on MuSCs was not investigated in this study. For further research a quantitative polymerase chain reaction (qPCR) is recommended to measure change in the expression of Pax7, MyoD and Myogenin.

4.3 Supplementation of Faba bean hydrolysates significantly reduced cell proliferation in MuSCs.

All faba-bean hydrolysates at a 1 mg/mL concentration reduced cell proliferation in MUSCSs at 24, 48, and 96 hours (figures 23, 24, 25). This study suggests that faba-bean hydrolyzed with alcalase and corolase have antiproliferative effects on MUSCSs. We hypothesize the effect may be due to the high content of polyphenols in faba-beans (92). Polyphenols are secondary metabolites of plants. There are more than 8000 polyphenolic compounds that have been identified in plant species (93). They primarily occur in conjugated forms with a sugar residue linked to hydroxyl groups. It has previously been reported that polyphenols from plants exert antiproliferative effects on several cancer cell types (94, 95). The antiproliferative effects of polyphenols deriving from red wine have also been reported on rat aortic smooth muscle cells (96). Iijima K et al. observed the total polyphenolic fraction from red wine to have a potent inhibitory effect on the proliferation and DNA synthesis of rat aortic smooth muscle cells. The group saw polyphenols downregulating the expression of cyclin A mRNA and cyclin A promoter activity. In mammalian cells cyclin A is expressed during the synthesis

phase and mitosis. It is essential to initiate DNA replication and to restrict replication to only once per cycle (97). Polyphenols from red wine have been reported to decrease the binding of nuclear proteins to the activating transcription factor site in the cyclin A promoter, thereby downregulating the mRNA levels of transcription factors and cAMP-responsive element-binding protein. The decreased expression of cyclin A promoter may be the cause to the antiproliferative effects observed when supplementing faba-bean hydrolysates to MuSC.

Polyphenols are also present in peas (98). To evaluate the credibility of our hypothesis, a difference would be observed when comparing the proliferating effect of F55X to E1155X as the latter is a heat-treated protein concentrate. Heat treatment is an effective method of increasing the protein digestibility of raw materials containing polyphenols by denaturing the protein and removing its function (99). Therefore, if polyphenols exhibit antiproliferative effects on MuSCs it should be observed that the heat-treated protein concentrate would give a higher increase in proliferation. E1155X independently of protease applied increased proliferation to a higher degree than F55X (figure 19, 20, 21, 22) supporting the hypothesis. For further research on legume-derived hydrolysate supplemented to mammalian cell cultures heat treatment may be an option to reduce the antiproliferative effects of polyphenols.

4.4 Protein hydrolysates showed DPP-IV inhibiting effects.

Antidiabetic agents such as sitagliptin and metformin inhibit DPP-IV. These compounds have been shown to increase glucose uptake in muscle cells following pharmacological stimuli (100). The glucose uptake is associated with AMPK and is believed to incite an insulin-independent absorption mechanism. Peptides deriving from chicken and flaxseeds hydrolysates have been shown to inhibit DPP-IV. Additionally, these hydrolysates have been shown to increase glucose uptake in muscle cells (101, 102). We are inclined to suspect that the hydrolysate PI-C, which had the highest inhibition of DPP-IV may stimulate AMPK in MuSCs. A DPP-IV assay was performed on the hydrolysates in this study to investigate DPP-IV inhibiting activity. All hydrolysates showed some DPP-IV inhibiting activity ranging from 13.21% - to 35.23% inhibition.

PI-C hydrolysate showed a 32.17% inhibition on DPP-IV (table 8). DPP-IV inhibition from pea-protein hydrolysate have previously been reported (103). As well as DPP-IV inhibition from other vegetable derived protein hydrolysates, such as flaxseed and soy (65, 102). It has previously been reported that low M_w peptide fractions between 300-400 kDa from chicken hydrolysate hydrolyzed with corolase increase glucose uptake in MuSCs (101). The MWD of

PI-C hydrolysate show 41% of peptides within area D containing peptides with an estimated 2-8 amino acids, matching the Mw seen from the chicken hydrolysate study.

The findings of Fitzgerald et al. report an increased inhibition of DPP-IV in pea-protein hydrolysate compared to the raw material (104). A possible explanation may be the high amount of Trp containing peptides released during hydrolysis. Several Trp-containing peptides have been shown to have DPP-IV-inhibiting activities (104). Generally, a Trp residue at the N-terminus or position two of the peptide is believed to incite these functions. In the present study WPC80-A and WPC80-C showed a 27.25% and 25.12% inhibition of DPP-IV (table 8). This agrees with the current literature. A recent study observed DPP-IV inhibition from milk protein hydrolysates (105). Several peptides from milk hydrolysates have been observed to be competitive inhibitors of DPP-IV. It has been suggested that milk-derived protein hydrolysates act as DPP-IV substrates. Di-peptide Trp-Val seems to be able to make a direct interaction on the active site of DPP-IV (106). Further supporting the findings from Fitzgerald et al. that Trp-containing peptides inhibit DPP-IV. It is debated whether hydrolysates deriving from the casein or whey fraction of milk has the strongest DPP-IV inhibition. The results from these studies differ on this subject.

Supplementing soy and chicken-hydrolysates to muscle cells has previously been shown to enhance intracellular AMPK (65, 101). AMPK is associated with increased glucose uptake through non-insulin-dependent mechanisms in muscle cells. High levels of AMPK inhibit cell-cycle exit and therefore, differentiation. It is possible that supplementing protein hydrolysates in this study increases the activation of AMPK in a similar manner to soy/chicken hydrolysate, allowing the cells to proliferate for another cell cycle. If so, this could explain the connection between the major growth promoting effects observed from the proliferation assay. Further investigation into the DPP-IV inhibiting properties of plant protein hydrolysates is necessary to unravel their mechanisms and explore the use of food-derived hydrolysates as growth stimulators in mammalian cells.

4.4.1 WPC80 hydrolysates inhibited DPP-IV

In the present study WPC80-A showed a 27.25% inhibition on DPP-IV. It has been previously reported that WPC80 hydrolysates have DPP-IV inhibiting qualities (107). Konrad B et al. reported that WPC80 hydrolyzed with a serine protease below three kDa generally had the greatest potency. The fraction that exhibited the highest inhibition of DPP-IV was WPC80 peptides within the 3-10 kDa range. Interestingly, this correlates with the results seen in this study. WPC80-A had an average molecular weight of 3592.1 Da (fig 11) with 50.22%

of peptides within section D (figure 15), indicating a high number of peptides with low Mw. Other studies have also confirmed WPC80 to contain peptides with DPP-IV inhibitory activities (108, 109). Silveira et al. reported peptide fractions isolated from tryptic hydrolysate of WPC80 function as an effective DPP-IV inhibitor. These findings correlate with the studies on plant protein hydrolysates, stating that a possible explanation for the DPP-IV inhibiting activities is connected to Trp residues in the N-terminal of the peptides.

5.0 Conclusion

This study aimed to investigate if seven vegetable-derived and one animal-derived protein hydrolysates could stimulate increased growth in MuSCs. SEC revealed a completely different peptide distribution on the same raw material when different enzymes were applied. Heat-treatment of raw material prior to hydrolysis also affected the peptide distribution. Hydrolysates in this study showed DPP-IV inhibition. The results suggest that pea-protein concentrate/isolate hydrolyzed with corolase and WPC80 hydrolyzed with alcalase stimulate proliferation in MuSCs. Interestingly, these hydrolysates also had the strongest inhibition of DPP-IV. According to the MWD the findings concur with previous work, stating that peptides between 2-15 amino acids increase cell proliferation in MuSCs. Further findings suggest that Faba-bean and Barley hydrolysates independently of enzyme used reduced cell proliferation. Oat hydrolysate did not affect proliferation in a substantial way. To our knowledge no previous vegetable-derived protein hydrolysates have been shown to increase proliferation in MuSCs, but increased glucose uptake from soy hydrolysate have been reported. This study provides future researchers with promising growth stimulating hydrolysates. The most promising hydrolysates from this study are possible candidates to be used for upscaling processes by stimulating the proliferation stage of industrial meat cultivation. This study did not investigate the hydrolysates method of action on cells, or its serum replacement potential. Further investigation of the hydrolysates mode of action and determinating specific peptide sequences responsible for the growth promoting effects observed in this study would prove useful. Fractionation of peptides with a favorable M_w is suggested as an efficient way of increasing the desirable qualities of peptides. Identifying singular components in the hydrolysate responsible for the growth promoting effects may contribute with components to the development of a SFM for MuSCs. Thereby allowing cells to proliferate for another cell cycle. However, this study did not monitor gene expression and can only make a guess as to what mechanisms are involved. For further research qPCR is recommended to measure the expression of Pax7, MyoD and Myogenin in MuSCs after supplementation of growth promoting hydrolysates.

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Supplementary information, CyQuant proliferation assay

PI-A	Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Below threshold?	Summary	Adjusted P Value
24 hours						
	Control cells vs. DMEM	-3,78	-18,45 to 10,89	No	ns	0,9966
	Control cells vs. 1 mg/ml	-29,55	-79,87 to 20,78	No	ns	0,626
	Control cells vs. 0.1 mg/ml	-15,15	-45,34 to 15,05	No	ns	0,8028
	Control cells vs. 0.01 mg/ml	25,62	-4,576 to 55,82	No	ns	0,1566
	Control cells vs. 0.001 mg/ml	22,54	-7,652 to 52,74	No	ns	0,2972
	Control cells vs. 0.0001 mg/ml	28,22	-1,973 to 58,42	No	ns	0,0838
	Control cells vs. 0.00001 mg/ml	23,01	-7,185 to 53,21	No	ns	0,2715
	Control cells vs. 0.000001 mg/ml	26,07	-4,122 to 56,27	No	ns	0,1412
	Control cells vs. 0.0000001 mg/ml	19,29	-10,91 to 49,48	No	ns	0,511
	Control cells vs. 0.00000001 mg/ml	25,61	-10,68 to 61,90	No	ns	0,3711
48 hours						
	Control cells vs. DMEM	-4,831	-19,96 to 10,30	No	ns	0,988
	Control cells vs. 1 mg/ml	1,081	-52,41 to 54,57	No	ns	>0,9999
	Control cells vs. 0.1 mg/ml	-1,156	-33,25 to 30,94	No	ns	>0,9999
	Control cells vs. 0.01 mg/ml	23,63	-8,465 to 55,73	No	ns	0,315
	Control cells vs. 0.001 mg/ml	29,8	-2,292 to 61,90	No	ns	0,0879
	Control cells vs. 0.0001 mg/ml	-6,344	-38,44 to 25,75	No	ns	0,9994
	Control cells vs. 0.00001 mg/ml	19,08	-13,02 to 51,17	No	ns	0,6098
	Control cells vs. 0.000001 mg/ml	18,45	-13,65 to 50,54	No	ns	0,6535
	Control cells vs. 0.0000001 mg/ml	-1,641	-33,74 to 30,46	No	ns	0,9999
	Control cells vs. 0.00000001 mg/ml	14,22	-24,35 to 52,80	No	ns	0,9655

96 hours						
	Control cells vs. DMEM	-11,14	-24,42 to 2,148	No	ns	0,1671
	Control cells vs. 1 mg/ml	30,02	-15,55 to 75,59	No	ns	0,4644
	Control cells vs. 0.1 mg/ml	-11,81	-35,92 to 12,31	No	ns	0,8212
	Control cells vs. 0.01 mg/ml	3,001	-21,11 to 27,12	No	ns	0,9996
	Control cells vs. 0.001 mg/ml	22,49	-1,627 to 46,60	No	ns	0,0852
	Control cells vs. 0.0001 mg/ml	27,51	3,392 to 51,62	Yes	*	0,0147
	Control cells vs. 0.00001 mg/ml	12,86	-11,26 to 36,97	No	ns	0,7382
	Control cells vs. 0.000001 mg/ml	0,4806	-23,63 to 24,60	No	ns	>0,9999
	Control cells vs. 0.0000001 mg/ml	6,975	-17,14 to 31,09	No	ns	0,9924
	Control cells vs. 0.00000001 mg/ml	17,02	-10,33 to 44,36	No	ns	0,5439
PI-C	**Dunnett's multiple comparisons test**	**Mean Diff,**	**95,00% CI of diff,**	**Below threshold?**	**Summary**	**Adjusted P Value**
24 hours						
	Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Below threshold?	Summary	Adjusted P Value
	Control cells vs. DMEM	-3,78	-17,09 to 9,533	No	ns	0,992
	Control cells vs. 1 mg/ml	-1,984	-34,91 to 30,94	No	ns	0,9999
	Control cells vs. 0.1 mg/ml	-11,36	-44,29 to 21,57	No	ns	0,971
	Control cells vs. 0.01 mg/ml	11,26	-21,67 to 44,19	No	ns	0,9726
	Control cells vs. 0.001 mg/ml	-4,076	-37,00 to 28,85	No	ns	0,9996
	Control cells vs. 0.0001 mg/ml	0,2727	-32,66 to 33,20	No	ns	>0,9999
	Control cells vs. 0.00001 mg/ml	-14,42	-47,35 to 18,51	No	ns	0,8857
	Control cells vs. 0.000001 mg/ml	-14,31	-47,24 to 18,62	No	ns	0,8901
	Control cells vs. 0.0000001 mg/ml	-10,06	-42,99 to 22,86	No	ns	0,9876
48 hours						
	Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Below threshold?	Summary	Adjusted P Value

	Control cells vs. DMEM	-4,831	-17,98 to 8,317	No	ns	0,9573
	Control cells vs. 1 mg/ml	-42,11	-75,64 to -8,591	Yes	**	0,0051
	Control cells vs. 0.1 mg/ml	-48,34	-81,86 to -14,82	Yes	***	0,0007
	Control cells vs. 0.01 mg/ml	0,2061	-33,32 to 33,73	No	ns	>0,9999
	Control cells vs. 0.001 mg/ml	-2,106	-35,63 to 31,42	No	ns	0,9998
	Control cells vs. 0.0001 mg/ml	2,555	-30,97 to 36,08	No	ns	0,9998
	Control cells vs. 0.00001 mg/ml	-31,58	-65,10 to 1,943	No	ns	0,0786
	Control cells vs. 0.000001 mg/ml	-3,106	-36,63 to 30,42	No	ns	0,9997
	Control cells vs. 0.0000001 mg/ml	-19,98	-53,51 to 13,54	No	ns	0,5869
96 hours						
	Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Below threshold?	Summary	Adjusted P Value
	Control cells vs. DMEM	-11,14	-23,46 to 1,188	No	ns	0,1043
	Control cells vs. 1 mg/ml	-7,957	-38,44 to 22,53	No	ns	0,9942
	Control cells vs. 0.1 mg/ml	-63,63	-94,11 to -33,14	Yes	****	<0,0001
	Control cells vs. 0.01 mg/ml	-4,852	-35,34 to 25,64	No	ns	0,9995
	Control cells vs. 0.001 mg/ml	-13,21	-43,70 to 17,28	No	ns	0,8919
	Control cells vs. 0.0001 mg/ml	4,44	-26,05 to 34,93	No	ns	0,9996
	Control cells vs. 0.00001 mg/ml	-33,33	-63,81 to -2,838	Yes	*	0,023
	Control cells vs. 0.000001 mg/ml	-15,04	-45,53 to 15,45	No	ns	0,798
	Control cells vs. 0.0000001 mg/ml	-0,09946	-30,59 to 30,39	No	ns	>0,9999
Barley-A	**Dunnett's multiple comparisons test**	**Mean Diff,**	**95,00% CI of diff,**	**Below threshold?**	**Summary**	**Adjusted P Value**
24 hours						
	Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Below threshold?	Summary	Adjusted P Value
	Control cells vs. DMEM	-3,78	-17,48 to 9,921	No	ns	0,9929

	Control cells vs. 1 mg/ml	4,318	-29,57 to 38,20	No	ns	0,9996
	Control cells vs. 0.1 mg/ml	11,04	-22,84 to 44,93	No	ns	0,98
	Control cells vs. 0.01 mg/ml	36,83	2,948 to 70,72	Yes	*	0,0242
	Control cells vs. 0.001 mg/ml	17,38	-16,50 to 51,27	No	ns	0,7613
	Control cells vs. 0.0001 mg/ml	26,67	-7,221 to 60,55	No	ns	0,2273
	Control cells vs. 0.00001 mg/ml	18,86	-15,03 to 52,75	No	ns	0,6723
	Control cells vs. 0.000001 mg/ml	20,13	-13,75 to 54,02	No	ns	0,5908
	Control cells vs. 0.0000001 mg/ml	-1,269	-35,16 to 32,62	No	ns	>0,9999
48 hours						
	Control cells vs. DMEM	-4,831	-16,26 to 6,599	No	ns	0,905
	Control cells vs. 1 mg/ml	16,78	-12,36 to 45,93	No	ns	0,6308
	Control cells vs. 0.1 mg/ml	31,2	2,058 to 60,34	Yes	*	0,0279
	Control cells vs. 0.01 mg/ml	18,68	-10,46 to 47,82	No	ns	0,49
	Control cells vs. 0.001 mg/ml	42,02	12,88 to 71,16	Yes	***	0,0007
	Control cells vs. 0.0001 mg/ml	35,85	6,705 to 64,99	Yes	**	0,0066
	Control cells vs. 0.00001 mg/ml	29,19	0,04550 to 58,33	Yes	*	0,0494
	Control cells vs. 0.000001 mg/ml	15,86	-13,28 to 45,00	No	ns	0,6989
	Control cells vs. 0.0000001 mg/ml	7,881	-21,26 to 37,02	No	ns	0,9933
96 hours						
	Control cells vs. DMEM	-11,14	-25,99 to 3,711	No	ns	0,2825
	Control cells vs. 1 mg/ml	2,019	-34,71 to 38,75	No	ns	0,9999
	Control cells vs. 0.1 mg/ml	13,05	-23,68 to 49,77	No	ns	0,9652
	Control cells vs. 0.01 mg/ml	2,091	-34,64 to 38,82	No	ns	0,9999
	Control cells vs. 0.001 mg/ml	4,964	-31,76 to 41,69	No	ns	0,9996
	Control cells vs. 0.0001 mg/ml	0,4494	-36,28 to 37,18	No	ns	>0,9999

	Control cells vs. 0.00001 mg/ml	-4,507	-41,23 to 32,22	No	ns	0,9996
	Control cells vs. 0.000001 mg/ml	-34,2	-70,93 to 2,523	No	ns	0,0851
	Control cells vs. 0.0000001 mg/ml	-2,331	-39,06 to 34,40	No	ns	0,9998
Barley-C	Dunnett's multiple comparisons test	Mean Diff,	95,00% CI of diff,	Below threshold?	Summary	Adjusted P Value
24 hours						
	Control cells vs. DMEM	-3,78	-16,91 to 9,349	No	ns	0,9914
	Control cells vs. Bygg K1 mg/ml	13,5	-18,98 to 45,97	No	ns	0,9131
	Control cells vs. Bygg K 0.1 mg/ml	5,854	-26,62 to 38,33	No	ns	0,9994
	Control cells vs. Bygg K 0.01 mg/ml	23,72	-8,752 to 56,19	No	ns	0,3152
	Control cells vs. Bygg K 0.001 mg/ml	20,92	-11,55 to 53,40	No	ns	0,4822
	Control cells vs. Bygg K 0.0001 mg/ml	34,55	2,081 to 67,03	Yes	*	0,0295
	Control cells vs. Bygg K 0.00001 mg/ml	18,21	-14,26 to 50,69	No	ns	0,6631
	Control cells vs. Bygg K 0.000001 mg/ml	14,91	-17,57 to 47,38	No	ns	0,8554
	Control cells vs. Bygg K 0.0000001 mg/ml	7,088	-25,38 to 39,56	No	ns	0,9974
48 hours						
	Control cells vs. DMEM	-4,831	-16,06 to 6,398	No	ns	0,8959
	Control cells vs. Bygg K1 mg/ml	35,54	6,915 to 64,17	Yes	**	0,0059
	Control cells vs. Bygg K 0.1 mg/ml	26,56	-2,067 to 55,19	No	ns	0,0874
	Control cells vs. Bygg K 0.01 mg/ml	51,65	23,02 to 80,28	Yes	****	<0,0001
	Control cells vs. Bygg K 0.001 mg/ml	38,42	9,787 to 67,04	Yes	**	0,0022
	Control cells vs. Bygg K 0.0001 mg/ml	58,61	29,98 to 87,24	Yes	****	<0,0001
	Control cells vs. Bygg K 0.00001 mg/ml	40,2	11,58 to 68,83	Yes	**	0,0011
	Control cells vs. Bygg K 0.000001 mg/ml	16,01	-12,62 to 44,64	No	ns	0,6672
	Control cells vs. Bygg K 0.0000001 mg/ml	15,25	-13,38 to 43,88	No	ns	0,7224
96 hours						

	Control cells vs. DMEM	-11,14	-24,62 to 2,344	No	ns	0,1776
	Control cells vs. Bygg K1 mg/ml	30,59	-2,756 to 63,94	No	ns	0,0943
	Control cells vs. Bygg K 0.1 mg/ml	15,15	-18,19 to 48,50	No	ns	0,8622
	Control cells vs. Bygg K 0.01 mg/ml	7,265	-26,08 to 40,61	No	ns	0,9974
	Control cells vs. Bygg K 0.001 mg/ml	17,65	-15,70 to 50,99	No	ns	0,7292
	Control cells vs. Bygg K 0.0001 mg/ml	6,159	-27,19 to 39,50	No	ns	0,9994
	Control cells vs. Bygg K 0.00001 mg/ml	-14,97	-48,32 to 18,37	No	ns	0,8702
	Control cells vs. Bygg K 0.000001 mg/ml	-3,407	-36,75 to 29,94	No	ns	0,9997
	Control cells vs. Bygg K 0.0000001 mg/ml	5,42	-27,93 to 38,77	No	ns	0,9995
F67X-A	**Dunnett's multiple comparisons test**	**Mean Diff,**	**95,00% CI of diff,**	**Below threshold?**	**Summary**	**Adjusted P Value**
24 hours						
	Control cells vs. DMEM	-3,78	-17,36 to 9,799	No	ns	0,9926
	Control cells vs. 1 mg/ml	27,5	-6,089 to 61,08	No	ns	0,1863
	Control cells vs. 0.1 mg/ml	25,85	-7,732 to 59,44	No	ns	0,252
	Control cells vs. 0.01 mg/ml	28,92	-4,669 to 62,50	No	ns	0,1407
	Control cells vs. 0.001 mg/ml	21,17	-12,42 to 54,76	No	ns	0,5122
	Control cells vs. 0.0001 mg/ml	25,65	-7,938 to 59,23	No	ns	0,2613
	Control cells vs. 0.00001 mg/ml	25,76	-7,829 to 59,34	No	ns	0,2564
	Control cells vs. 0.000001 mg/ml	16,49	-17,10 to 50,07	No	ns	0,8024
	Control cells vs. 0.0000001 mg/ml	27,62	-5,962 to 61,21	No	ns	0,1818
48 hours						
	Control cells vs. DMEM	-4,831	-17,80 to 8,133	No	ns	0,9535
	Control cells vs. 1 mg/ml	36,86	3,808 to 69,91	Yes	*	0,0189
	Control cells vs. 0.1 mg/ml	15,21	-17,84 to 48,26	No	ns	0,8538
	Control cells vs. 0.01 mg/ml	24,33	-8,723 to 57,38	No	ns	0,3059

	Control cells vs. 0.001 mg/ml	3,167	-29,89 to 36,22	No	ns	0,9997
	Control cells vs. 0.0001 mg/ml	20,94	-12,11 to 54,00	No	ns	0,5054
	Control cells vs. 0.00001 mg/ml	17,9	-15,15 to 50,96	No	ns	0,7041
	Control cells vs. 0.000001 mg/ml	25,61	-7,439 to 58,67	No	ns	0,2444
	Control cells vs. 0.0000001 mg/ml	10,35	-22,70 to 43,41	No	ns	0,9852
96 hours						
	Control cells vs. DMEM	-11,14	-24,85 to 2,578	No	ns	0,1944
	Control cells vs. 1 mg/ml	51,06	17,14 to 84,99	Yes	***	0,0004
	Control cells vs. 0.1 mg/ml	0,224	-33,70 to 34,15	No	ns	>0,9999
	Control cells vs. 0.01 mg/ml	29,16	-4,766 to 63,08	No	ns	0,1422
	Control cells vs. 0.001 mg/ml	-11,13	-45,06 to 22,79	No	ns	0,9791
	Control cells vs. 0.0001 mg/ml	-11,06	-44,98 to 22,87	No	ns	0,98
	Control cells vs. 0.00001 mg/ml	-2,737	-36,66 to 31,19	No	ns	0,9998
	Control cells vs. 0.000001 mg/ml	-16,74	-50,67 to 17,18	No	ns	0,7978
	Control cells vs. 0.0000001 mg/ml	-8,923	-42,85 to 25,00	No	ns	0,994
F67X-C	**Dunnett's multiple comparisons test**	**Mean Diff,**	**95,00% CI of diff,**	**Below threshold?**	**Summary**	**Adjusted P Value**
24 hours						
	Control cells vs. DMEM	-3,78	-17,18 to 9,619	No	ns	0,9924
	Control cells vs. 1 mg/ml	31,64	-14,32 to 77,59	No	ns	0,3941
	Control cells vs. 0.1 mg/ml	34,04	-11,92 to 80,00	No	ns	0,2989
	Control cells vs. 0.01 mg/ml	42,91	-3,042 to 88,87	No	ns	0,0836
	Control cells vs. 0.001 mg/ml	34,82	-11,14 to 80,78	No	ns	0,2713
	Control cells vs. 0.0001 mg/ml	44,08	-1,878 to 90,04	No	ns	0,0689
	Control cells vs. 0.00001 mg/ml	41,33	-4,625 to 87,29	No	ns	0,1076
	Control cells vs. 0.000001 mg/ml	59,67	13,71 to 105,6	Yes	**	0,0034

	Control cells vs. 0.0000001 mg/ml	30,97	-14,99 to 76,92	No	ns	0,4231
48 hours						
	Control cells vs. DMEM	-4,831	-16,51 to 6,850	No	ns	0,9173
	Control cells vs. 1 mg/ml	57,77	16,47 to 99,07	Yes	**	0,0012
	Control cells vs. 0.1 mg/ml	36,45	-4,854 to 77,75	No	ns	0,1214
	Control cells vs. 0.01 mg/ml	41,31	0,01115 to 82,62	Yes	*	0,0499
	Control cells vs. 0.001 mg/ml	46,06	4,754 to 87,36	Yes	*	0,0189
	Control cells vs. 0.0001 mg/ml	37,85	-3,455 to 79,15	No	ns	0,0952
	Control cells vs. 0.00001 mg/ml	40,4	-0,9027 to 81,70	No	ns	0,0595
	Control cells vs. 0.000001 mg/ml	36,27	-5,033 to 77,57	No	ns	0,1253
	Control cells vs. 0.0000001 mg/ml	23,11	-18,19 to 64,41	No	ns	0,6692
96 hours						
	Control cells vs. DMEM	-11,14	-23,26 to 0,9852	No	ns	0,0935
	Control cells vs. 1 mg/ml	51,21	9,626 to 92,79	Yes	**	0,0065
	Control cells vs. 0.1 mg/ml	65,13	23,54 to 106,7	Yes	***	0,0002
	Control cells vs. 0.01 mg/ml	47,69	6,108 to 89,27	Yes	*	0,0143
	Control cells vs. 0.001 mg/ml	56,25	14,67 to 97,84	Yes	**	0,002
	Control cells vs. 0.0001 mg/ml	49,41	7,832 to 91,00	Yes	**	0,0098
	Control cells vs. 0.00001 mg/ml	38,05	-3,535 to 79,63	No	ns	0,096
	Control cells vs. 0.000001 mg/ml	24,92	-16,67 to 66,50	No	ns	0,5825
	Control cells vs. 0.0000001 mg/ml	29,19	-12,39 to 70,77	No	ns	0,3679
F65X-C	**Dunnett's multiple comparisons test**	**Mean Diff,**	**95,00% CI of diff,**	**Below threshold?**	**Summary**	**Adjusted P Value**
24 hours						
	Control cells vs. DMEM	-3,78	-18,17 to 10,61	No	ns	0,9965
	Control cells vs. 1 mg/ml	-57,31	-106,7 to -7,962	Yes	*	0,0121

	Control cells vs. 0.1 mg/ml	-3,345	-38,93 to 32,24	No	ns	0,9997
	Control cells vs. 0.01 mg/ml	16,41	-19,18 to 51,99	No	ns	0,8722
	Control cells vs. 0.001 mg/ml	16,27	-19,32 to 51,86	No	ns	0,878
	Control cells vs. 0.0001 mg/ml	35,44	-0,1432 to 71,03	No	ns	0,0517
	Control cells vs. 0.00001 mg/ml	29,25	-6,334 to 64,84	No	ns	0,1874
	Control cells vs. 0.000001 mg/ml	31,75	-3,840 to 67,33	No	ns	0,1153
	Control cells vs. 0.0000001 mg/ml	14,35	-21,24 to 49,94	No	ns	0,9406
	Control cells vs. 0.00000001 mg/ml	39,64	-9,713 to 88,99	No	ns	0,2117
48 hours						
	Control cells vs. DMEM	-4,831	-16,44 to 6,774	No	ns	0,9281
	Control cells vs. 1 mg/ml	10,36	-30,67 to 51,39	No	ns	0,9968
	Control cells vs. 0.1 mg/ml	14,58	-15,01 to 44,16	No	ns	0,8221
	Control cells vs. 0.01 mg/ml	45,98	16,40 to 75,57	Yes	***	0,0002
	Control cells vs. 0.001 mg/ml	18,5	-13,70 to 50,71	No	ns	0,657
	Control cells vs. 0.0001 mg/ml	31,49	1,900 to 61,07	Yes	*	0,0291
	Control cells vs. 0.00001 mg/ml	27,6	-1,990 to 57,18	No	ns	0,0852
	Control cells vs. 0.000001 mg/ml	22,03	-7,554 to 51,62	No	ns	0,3018
	Control cells vs. 0.0000001 mg/ml	-1,696	-31,28 to 27,89	No	ns	0,9999
	Control cells vs. 0.00000001 mg/ml	20,62	-20,41 to 61,65	No	ns	0,8044
96 hours						
	Control cells vs. DMEM	-11,14	-23,80 to 1,520	No	ns	0,1259
	Control cells vs. 1 mg/ml	61,55	18,13 to 105,0	Yes	***	0,0009
	Control cells vs. 0.1 mg/ml	25,53	-5,775 to 56,84	No	ns	0,1955
	Control cells vs. 0.01 mg/ml	35,75	4,440 to 67,06	Yes	*	0,0145
	Control cells vs. 0.001 mg/ml	23,34	-7,966 to 54,65	No	ns	0,3001

	Control cells vs. 0.0001 mg/ml	16,62	-14,69 to 47,92	No	ns	0,7497
	Control cells vs. 0.00001 mg/ml	25,3	-6,005 to 56,61	No	ns	0,2051
	Control cells vs. 0.000001 mg/ml	9	-22,31 to 40,31	No	ns	0,9929
	Control cells vs. 0.0000001 mg/ml	31,01	-0,3011 to 62,32	No	ns	0,054
	Control cells vs. 0.00000001 mg/ml	31,05	-12,36 to 74,47	No	ns	0,3548
F55X-A	**Dunnett's multiple comparisons test**	**Mean Diff,**	**95,00% CI of diff,**	**Below threshold?**	**Summary**	**Adjusted P Value**
24 hours						
	Control cells vs. DMEM	-3,78	-17,98 to 10,42	No	ns	0,9937
	Control cells vs. 1 mg/ml	-25,17	-60,30 to 9,961	No	ns	0,3399
	Control cells vs. 0.1 mg/ml	0,3679	-34,76 to 35,50	No	ns	>0,9999
	Control cells vs. 0.01 mg/ml	9,253	-25,88 to 44,38	No	ns	0,994
	Control cells vs. 0.001 mg/ml	27,86	-7,270 to 62,99	No	ns	0,2191
	Control cells vs. 0.0001 mg/ml	17,2	-17,93 to 52,33	No	ns	0,8047
	Control cells vs. 0.00001 mg/ml	35,86	0,7323 to 70,99	Yes	*	0,0422
	Control cells vs. 0.000001 mg/ml	31,66	-3,475 to 66,78	No	ns	0,1062
	Control cells vs. 0.0000001 mg/ml	17,49	-17,64 to 52,62	No	ns	0,7897
48 hours						
	Control cells vs. DMEM	-4,831	-18,66 to 9,002	No	ns	0,9687
	Control cells vs. 1 mg/ml	-11,53	-46,80 to 23,74	No	ns	0,9796
	Control cells vs. 0.1 mg/ml	-1,69	-36,96 to 33,58	No	ns	0,9999
	Control cells vs. 0.01 mg/ml	9,307	-25,96 to 44,57	No	ns	0,9939
	Control cells vs. 0.001 mg/ml	11,6	-23,67 to 46,86	No	ns	0,9788
	Control cells vs. 0.0001 mg/ml	9,826	-25,44 to 45,09	No	ns	0,9926
	Control cells vs. 0.00001 mg/ml	13,74	-21,53 to 49,01	No	ns	0,9396
	Control cells vs. 0.000001 mg/ml	12,99	-22,28 to 48,26	No	ns	0,9566

	Control cells vs. 0.0000001 mg/ml	9,45	-25,82 to 44,72	No	ns	0,9935
96 hours						
	Control cells vs. DMEM	-11,14	-26,09 to 3,813	No	ns	0,2908
	Control cells vs. 1 mg/ml	20,84	-16,14 to 57,82	No	ns	0,6572
	Control cells vs. 0.1 mg/ml	-1,016	-38,00 to 35,96	No	ns	>0,9999
	Control cells vs. 0.01 mg/ml	3,64	-33,34 to 40,62	No	ns	0,9997
	Control cells vs. 0.001 mg/ml	-9,145	-46,12 to 27,83	No	ns	0,9967
	Control cells vs. 0.0001 mg/ml	-2,059	-39,04 to 34,92	No	ns	0,9999
	Control cells vs. 0.00001 mg/ml	-0,6583	-37,64 to 36,32	No	ns	>0,9999
	Control cells vs. 0.000001 mg/ml	-12,76	-49,74 to 24,22	No	ns	0,971
	Control cells vs. 0.0000001 mg/ml	15,31	-21,67 to 52,29	No	ns	0,915
F55X-C	**Dunnett's multiple comparisons test**	**Mean Diff,**	**95,00% CI of diff,**	**Below threshold?**	**Summary**	**Adjusted P Value**
24 hours						
	Control cells vs. DMEM	-3,78	-16,98 to 9,417	No	ns	0,9917
	Control cells vs. 0,01 mg/ml	18,13	-14,52 to 50,77	No	ns	0,675
	Control cells vs. 1 mg/ml	-16,74	-49,38 to 15,90	No	ns	0,7618
	Control cells vs. 0.1 mg/ml	28,08	-4,563 to 60,72	No	ns	0,1414
	Control cells vs. 0.001 mg/ml	9,744	-22,90 to 42,38	No	ns	0,9895
	Control cells vs. 0.0001 mg/ml	11,9	-20,74 to 44,54	No	ns	0,9593
	Control cells vs. 0.00001 mg/ml	8,088	-24,55 to 40,73	No	ns	0,9966
	Control cells vs. 0.000001 mg/ml	8,735	-23,91 to 41,38	No	ns	0,9936
	Control cells vs. 0.0000001 mg/ml	6,472	-26,17 to 39,11	No	ns	0,9994
48 hours						
	Control cells vs. DMEM	-4,831	-16,24 to 6,582	No	ns	0,9042
	Control cells vs. 1 mg/ml	51,89	22,80 to 80,99	Yes	****	<0,0001

	Control cells vs. 0.1 mg/ml	24,48	-4,621 to 53,57	No	ns	0,161
	Control cells vs. 0.01 mg/ml	28,44	-0,6626 to 57,53	No	ns	0,0599
	Control cells vs. 0.001 mg/ml	47,48	18,38 to 76,58	Yes	****	<0,0001
	Control cells vs. 0.0001 mg/ml	38,76	9,664 to 67,86	Yes	**	0,0024
	Control cells vs. 0.00001 mg/ml	34,21	5,112 to 63,31	Yes	*	0,0109
	Control cells vs. 0.000001 mg/ml	28,04	-1,061 to 57,13	No	ns	0,0666
	Control cells vs. 0.0000001 mg/ml	18,83	-10,27 to 47,92	No	ns	0,4768
96 hours						
	Control cells vs. DMEM	-11,14	-24,69 to 2,413	No	ns	0,1824
	Control cells vs. 0,1mg/ml	49,05	15,54 to 82,57	Yes	***	0,0006
	Control cells vs. 0.01 mg/ml	5,058	-28,46 to 38,58	No	ns	0,9996
	Control cells vs. 1 mg/ml	11,58	-21,94 to 45,10	No	ns	0,9708
	Control cells vs. 0.001 mg/ml	14,8	-18,72 to 48,32	No	ns	0,8807
	Control cells vs. 0.0001 mg/ml	5,022	-28,50 to 38,54	No	ns	0,9996
	Control cells vs. 0.00001 mg/ml	-17,97	-51,49 to 15,55	No	ns	0,7153
	Control cells vs. 0.000001 mg/ml	-12,25	-45,77 to 21,27	No	ns	0,9586
	Control cells vs. 0.0000001 mg/ml	-9,081	-42,60 to 24,44	No	ns	0,9933
E1155X-A	**Dunnett's multiple comparisons test**	**Mean Diff,**	**95,00% CI of diff,**	**Below threshold?**	**Summary**	**Adjusted P Value**
24 hours						
	Control cells vs. DMEM	-3,78	-17,46 to 9,903	No	ns	0,9928
	Control cells vs. 1 mg/ml	-36,44	-70,29 to -2,601	Yes	*	0,0264
	Control cells vs. 0.1 mg/ml	-48,2	-82,05 to -14,36	Yes	***	0,0009
	Control cells vs. 0.01 mg/ml	-12,9	-46,75 to 20,94	No	ns	0,9467
	Control cells vs. 0.001 mg/ml	-13,54	-47,38 to 20,31	No	ns	0,9297
	Control cells vs. 0.0001 mg/ml	-4,932	-38,78 to 28,91	No	ns	0,9996

	Control cells vs. 0.00001 mg/ml	-6,345	-40,19 to 27,50	No	ns	0,9994
	Control cells vs. 0.000001 mg/ml	5,258	-28,59 to 39,10	No	ns	0,9995
	Control cells vs. 0.0000001 mg/ml	17,19	-16,66 to 51,03	No	ns	0,7712
48 hours						
	Control cells vs. DMEM	-4,831	-17,18 to 7,520	No	ns	0,9382
	Control cells vs. 1 mg/ml	1,533	-29,96 to 33,02	No	ns	0,9999
	Control cells vs. 0.1 mg/ml	-33,71	-65,20 to -2,222	Yes	*	0,0279
	Control cells vs. 0.01 mg/ml	-8,125	-39,62 to 23,37	No	ns	0,9946
	Control cells vs. 0.001 mg/ml	14,79	-16,70 to 46,28	No	ns	0,839
	Control cells vs. 0.0001 mg/ml	6,516	-24,97 to 38,01	No	ns	0,9978
	Control cells vs. 0.00001 mg/ml	0,2905	-31,20 to 31,78	No	ns	>0,9999
	Control cells vs. 0.000001 mg/ml	1,193	-30,30 to 32,68	No	ns	>0,9999
	Control cells vs. 0.0000001 mg/ml	28,04	-3,450 to 59,53	No	ns	0,1146
96 hours						
	Control cells vs. DMEM	-11,14	-23,56 to 1,282	No	ns	0,1096
	Control cells vs. 1 mg/ml	24,89	-8,546 to 58,33	No	ns	0,292
	Control cells vs. 0.0001 mg/ml	-15,92	-49,36 to 17,51	No	ns	0,8289
	Control cells vs. 0.1 mg/ml	-7,03	-40,47 to 26,41	No	ns	0,9977
	Control cells vs. 0,01 mg/ml	0,9946	-32,44 to 34,43	No	ns	>0,9999
	Control cells vs. 0,001 mg/ml	9,123	-24,31 to 42,56	No	ns	0,9932
	Control cells vs. 0.00001 mg/ml	5,422	-28,01 to 38,86	No	ns	0,9995
	Control cells vs. 0.000001 mg/ml	-1,281	-34,72 to 32,15	No	ns	>0,9999
	Control cells vs. 0.0000001 mg/ml	24,98	-8,454 to 58,42	No	ns	0,2876
E1155X-C	**Dunnett's multiple comparisons test**	**Mean Diff,**	**95,00% CI of diff,**	**Below threshold?**	**Summary**	**Adjusted P Value**
24 hours						

	Control cells vs. DMEM	-3,78	-17,81 to 10,25	No	ns	0,9934
	Control cells vs. 1 mg/ml	-44,85	-79,56 to -10,15	Yes	**	0,0036
	Control cells vs. 0.1 mg/ml	-40,38	-75,09 to -5,674	Yes	*	0,0122
	Control cells vs. 0.01 mg/ml	-14,26	-48,97 to 20,45	No	ns	0,9185
	Control cells vs. 0.001 mg/ml	-5,188	-39,90 to 29,52	No	ns	0,9996
	Control cells vs. 0.0001 mg/ml	-4,238	-38,95 to 30,47	No	ns	0,9996
	Control cells vs. 0.00001 mg/ml	-34,67	-69,37 to 0,04109	No	ns	0,0505
	Control cells vs. 0.000001 mg/ml	-0,5654	-35,27 to 34,14	No	ns	>0,9999
	Control cells vs. 0.0000001 mg/ml	4,098	-30,61 to 38,81	No	ns	0,9997
48 hours						
	Control cells vs. DMEM	-4,831	-16,65 to 6,989	No	ns	0,9208
	Control cells vs. 1 mg/ml	14,32	-15,82 to 44,46	No	ns	0,8299
	Control cells vs. 0.1 mg/ml	-54,7	-84,84 to -24,56	Yes	****	<0,0001
	Control cells vs. 0.01 mg/ml	-13,46	-43,60 to 16,68	No	ns	0,8737
	Control cells vs. 0.001 mg/ml	-0,04143	-30,18 to 30,10	No	ns	>0,9999
	Control cells vs. 0.0001 mg/ml	9,298	-20,84 to 39,44	No	ns	0,9867
	Control cells vs. 0.00001 mg/ml	-19,88	-50,02 to 10,26	No	ns	0,4507
	Control cells vs. 0.000001 mg/ml	-8,967	-39,10 to 21,17	No	ns	0,9897
	Control cells vs. 0.0000001 mg/ml	-3,838	-33,98 to 26,30	No	ns	0,9996
96 hours						
	Control cells vs. DMEM	-11,14	-23,55 to 1,275	No	ns	0,1092
	Control cells vs. 1 mg/ml	32,44	1,737 to 63,14	Yes	*	0,0314
	Control cells vs. 0.1 mg/ml	-35,45	-66,15 to -4,744	Yes	*	0,0133
	Control cells vs. 0.01 mg/ml	-11,33	-42,03 to 19,38	No	ns	0,9562
	Control cells vs. 0.001 mg/ml	2,577	-28,13 to 33,28	No	ns	0,9997

	Control cells vs. 0.0001 mg/ml	3,242	-27,46 to 33,95	No	ns	0,9997
	Control cells vs. 0.00001 mg/ml	-27	-57,71 to 3,698	No	ns	0,1237
	Control cells vs. 0.000001 mg/ml	-8,901	-39,60 to 21,80	No	ns	0,991
	Control cells vs. 0.0000001 mg/ml	-1,251	-31,95 to 29,45	No	ns	0,9999
OBG28-C	**Dunnett's multiple comparisons test**	**Mean Diff,**	**95,00% CI of diff,**	**Below threshold?**	**Summary**	**Adjusted P Value**
24 hours						
	Control cells vs. DMEM	-3,78	-17,11 to 9,550	No	ns	0,9933
	Control cells vs. 1 mg/ml	17,68	-28,04 to 63,40	No	ns	0,9543
	Control cells vs. 0.1 mg/ml	7,751	-25,22 to 40,72	No	ns	0,9972
	Control cells vs. 0,01 mg/ml	-8,601	-41,57 to 24,37	No	ns	0,9965
	Control cells vs. 0.001 mg/ml	24,82	-8,147 to 57,79	No	ns	0,2876
	Control cells vs. 0.0001 mg/ml	32,14	-0,8265 to 65,11	No	ns	0,0612
	Control cells vs. 0.00001 mg/ml	3,917	-29,05 to 36,89	No	ns	0,9997
	Control cells vs. 0.000001 mg/ml	14,55	-18,42 to 47,52	No	ns	0,8992
	Control cells vs. 0.0000001 mg/ml	19,7	-13,27 to 52,67	No	ns	0,6057
	Control cells vs. 0.00000001 mg/ml	10,16	-35,56 to 55,88	No	ns	0,9976
48 hours						
	Control cells vs. DMEM	-4,831	-16,91 to 7,244	No	ns	0,9435
	Control cells vs. 1 mg/ml	26,78	-15,91 to 69,48	No	ns	0,5391
	Control cells vs. 0.1 mg/ml	18,66	-12,13 to 49,45	No	ns	0,5867
	Control cells vs. 0.01 mg/ml	13,4	-17,39 to 44,18	No	ns	0,9071
	Control cells vs. 0.001 mg/ml	16,09	-14,70 to 46,88	No	ns	0,7661
	Control cells vs. 0.0001 mg/ml	42,95	12,16 to 73,74	Yes	**	0,0011
	Control cells vs. 0.00001 mg/ml	2,441	-28,35 to 33,23	No	ns	0,9998
	Control cells vs. 0.000001 mg/ml	-5,907	-36,69 to 24,88	No	ns	0,9994

	Control cells vs. 0.0000001 mg/ml	-7,472	-38,26 to 23,31	No	ns	0,997
	Control cells vs. 0.00000001 mg/ml	5,976	-36,72 to 48,67	No	ns	0,9996
96 hours						
	Control cells vs. DMEM	-11,14	-24,14 to 1,861	No	ns	0,1481
	Control cells vs. 1 mg/ml	10,03	-34,55 to 54,62	No	ns	0,9975
	Control cells vs. 0.1 mg/ml	0,5114	-31,64 to 32,66	No	ns	>0,9999
	Control cells vs. 0.01 mg/ml	4,486	-27,67 to 36,64	No	ns	0,9996
	Control cells vs. 0.001 mg/ml	-8,276	-40,43 to 23,88	No	ns	0,9966
	Control cells vs. 0.0001 mg/ml	4,351	-27,80 to 36,50	No	ns	0,9996
	Control cells vs. 0.00001 mg/ml	-17,65	-49,80 to 14,51	No	ns	0,7124
	Control cells vs. 0.000001 mg/ml	-11,95	-44,10 to 20,20	No	ns	0,9647
	Control cells vs. 0.0000001 mg/ml	-20,25	-52,41 to 11,90	No	ns	0,533
	Control cells vs. 0.00000001 mg/ml	-11,49	-56,07 to 33,10	No	ns	0,9966
WPC80-A	**Dunnett's multiple comparisons test**	**Mean Diff,**	**95,00% CI of diff,**	**Below threshold?**	**Summary**	**Adjusted P Value**
24 hours						
	Control cells vs. DMEM	-0,02079	-14,04 to 13,99	No	ns	>0,9999
	Control cells vs. WPC80 1 mg/ml	9,698	-13,93 to 33,33	No	ns	0,9281
	Control cells vs. WPC80 0.1 mg/ml	12,56	-9,951 to 35,06	No	ns	0,6816
	Control cells vs. WPC80 0.01 mg/ml	17,37	-5,134 to 39,88	No	ns	0,2532
	Control cells vs. WPC80 0.001 mg/ml	21,5	-1,010 to 44,00	No	ns	0,0715
	Control cells vs. WPC80 0.0001 mg/ml	25,35	2,843 to 47,86	Yes	*	0,0168
	Control cells vs. WPC80 0.00001 mg/ml	21,68	-0,8249 to 44,19	No	ns	0,067
	Control cells vs. WPC80 0.000001 mg/ml	17,75	-4,761 to 40,25	No	ns	0,2291
	Control cells vs. WPC80 0.0000001 mg/ml	23,33	0,8234 to 45,84	Yes	*	0,0369

	Control cells vs. WPC80 0.00000001 mg/ml	-20,27	-82,04 to 41,49	No	ns	0,9838
48 hours						
	Control cells vs. DMEM	-4,831	-18,73 to 9,063	No	ns	0,9769
	Control cells vs. WPC80 1 mg/ml	-16,84	-49,85 to 16,16	No	ns	0,7868
	Control cells vs. WPC80 0.1 mg/ml	-1,941	-31,42 to 27,54	No	ns	0,9998
	Control cells vs. WPC80 0.01 mg/ml	11,43	-18,05 to 40,90	No	ns	0,9523
	Control cells vs. WPC80 0.001 mg/ml	8,761	-20,71 to 38,24	No	ns	0,9919
	Control cells vs. WPC80 0.0001 mg/ml	11,39	-18,08 to 40,87	No	ns	0,9533
	Control cells vs. WPC80 0.00001 mg/ml	-11,52	-40,99 to 17,96	No	ns	0,95
	Control cells vs. WPC80 0.000001 mg/ml	-7,94	-37,42 to 21,54	No	ns	0,9962
	Control cells vs. WPC80 0.0000001 mg/ml	10,87	-18,60 to 40,35	No	ns	0,9654
	Control cells vs. WPC80 0.00000001 mg/ml	-30,55	-90,31 to 29,22	No	ns	0,7853
96 hours						
	Control cells vs. DMEM	-11,14	-24,98 to 2,702	No	ns	0,209
	Control cells vs. WPC80 1 mg/ml	-37,61	-69,50 to -5,711	Yes	*	0,0102
	Control cells vs. WPC80 0.1 mg/ml	-31,9	-60,39 to -3,419	Yes	*	0,0177
	Control cells vs. WPC80 0.01 mg/ml	0,2605	-28,22 to 28,74	No	ns	>0,9999
	Control cells vs. WPC80 0.001 mg/ml	7,45	-21,03 to 35,93	No	ns	0,9965
	Control cells vs. WPC80 0.0001 mg/ml	5,333	-23,15 to 33,82	No	ns	0,9994
	Control cells vs. WPC80 0.00001 mg/ml	-5,924	-34,41 to 22,56	No	ns	0,9993
	Control cells vs. WPC80 0.000001 mg/ml	-9,349	-37,83 to 19,13	No	ns	0,9852
	Control cells vs. WPC80 0.0000001 mg/ml	-9,977	-38,46 to 18,51	No	ns	0,9756
	Control cells vs. WPC80 0.00000001 mg/ml	16,24	-41,51 to 73,99	No	ns	0,9933
WPC80 -C	**Dunnett's multiple comparisons test**	**Mean Diff,**	**95,00% CI of diff,**	**Below threshold?**	**Summary**	**Adjusted P Value**
24 hours						

	control cells vs. DMEM	30,05	8,846 to 51,26	Yes	**	0,0016
	control cells vs. 1mg/mL	4,833	-16,37 to 26,04	No	ns	0,9939
	control cells vs. 0,1mg/mL	23,38	2,180 to 44,59	Yes	*	0,0234
	control cells vs. 0,01mg/mL	32,02	10,81 to 53,22	Yes	***	0,0007
	control cells vs. 0,001mg/mL	42,12	20,91 to 63,32	Yes	****	<0,0001
	control cells vs. 0,0001mg/mL	47,22	26,01 to 68,42	Yes	****	<0,0001
	control cells vs. 0,00001mg/mL	50,29	29,08 to 71,49	Yes	****	<0,0001
	control cells vs. 0,000001mg/mL	40,46	19,26 to 61,67	Yes	****	<0,0001
	control cells vs. 0,0000001mg/mL	43,77	22,56 to 64,97	Yes	****	<0,0001
	control cells vs. 0,00000001mg/mL	34,12	12,91 to 55,32	Yes	***	0,0003
48 hours						
	Control cells vs. DMEM	-32,11	-67,93 to 3,713	No	ns	0,1108
	Control cells vs. 1 mg/mL	-31,85	-67,67 to 3,975	No	ns	0,1168
	Control cells vs. 0,1 mg/mL	-38,7	-74,52 to -2,872	Yes	*	0,0257
	Control cells vs. 0,001 mg/mL	-23,49	-59,31 to 12,34	No	ns	0,476
	Control cells vs. 0,0001 mg/mL	-26,14	-61,97 to 9,679	No	ns	0,3261
	Control cells vs. 0,00001mg/mL	-16,52	-52,34 to 19,31	No	ns	0,8752
	Control cells vs. 0,000001 mg/mL	-20,25	-56,07 to 15,58	No	ns	0,6791
	Control cells vs. 0,0000001 mg/mL	-22,72	-58,54 to 13,10	No	ns	0,5234
	Control cells vs. 0,00000001 mg/mL	-8,874	-44,70 to 26,95	No	ns	0,9985
	Control cells vs. 0,00000001 mg/mL	-23,37	-59,19 to 12,46	No	ns	0,4833
96 hours						
	Control cells vs. DMEM	-20,45	-44,57 to 3,672	No	ns	0,1357
	Control cells vs. 1 mg/mL	22,82	-1,308 to 46,94	No	ns	0,0726
	Control cells vs. 0,1 mg/mL	18,48	-5,644 to 42,60	No	ns	0,2169

	Control cells vs. 0,001 mg/mL	16,1	-8,025 to 40,22	No	ns	0,357
	Control cells vs. 0,0001 mg/mL	2,453	-21,67 to 26,58	No	ns	0,9996
	Control cells vs. 0,00001mg/mL	4,326	-19,80 to 28,45	No	ns	0,9993
	Control cells vs. 0,000001 mg/mL	16,8	-7,325 to 40,92	No	ns	0,3108
	Control cells vs. 0,0000001 mg/mL	-10,4	-34,52 to 13,72	No	ns	0,8179
	Control cells vs. 0,00000001 mg/mL	-17,5	-41,62 to 6,622	No	ns	0,2686
	Control cells vs. 0,00000001 mg/mL	-11,27	-35,39 to 12,85	No	ns	0,7502

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