Use of membrane technology for concentration of bioactive peptides from geoduck (*Panopea zelandica*)

Bodhi I. T. M. Bettjeman

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Abstract

Enzymatic digestion of proteins generates biologically active peptides which can be concentrated by filtration with the appropriate membranes. This work uses a hydrolysate derived from the New Zealand geoduck *Panopea zelandica* and compares the effectiveness of membranes with varying molecular weight cut-offs (MWCOs) and surface charges for carrying out selective separations of the peptides. A membrane with a MWCO of 2.5 kDa was found to be most effective for obtaining the greatest differences between the permeate and retentate from the membranes when separating by size alone. For separating peptides based on charge the choice of membrane and pH was important. The results obtained suggested that each hydrolysate has different optimum conditions for achieving separation.

A challenge when analysing the effects of membranes on peptide mixtures is quantifying the changes that have occurred. The work presented here introduced a multivariate approach to interpretation of complete sets of HPLC data that had not previously been used for analysis of complex peptide mixtures. This approach was used to measure the effectiveness of the membrane separations, and allowed comparisons to be drawn between these separations and the peptide bioactivity as determined using the FRAP assay.

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Attestation of authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

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1 Introduction

Peptides with biological activity are a growing area of interest, as evidenced by the large number of papers that have been published investigating them (S.-K. Kim & Wijesekara, 2010; Korhonen & Pihlanto, 2006). A major challenge when preparing these potentially valuable molecules is the difficulty in separating the bioactive peptides from those without activity because of their physical and chemical similarities. A further challenge is understanding and describing the changes in the peptide mixtures after the separations have occurred. This thesis builds on previous work (Arrutia, Rubio, & Riera, 2016; Balchen, Reubsaet, & Pedersen-Bjergaard, 2008; Byun & Kim, 2001; Firdaous et al., 2010; Langevin, Roblet, Moresoli, Ramassamy, & Bazinet, 2012) exploring strategies for performing peptide separations using membrane technology, and also introduces novel approaches for understanding the effects of these membranes on the peptide mixtures.

Numerous authors have described separation of peptides using membranes based on membrane pore size characteristics (Bourseau et al., 2009; Jeon, Byun, & Kim, 2000; Picot et al., 2010; Ranamukhaarachchi, Meissner, & Moresoli, 2013). This approach uses a molecular sieving process to permit passage of the smaller peptides and reject the larger peptides. Other authors have described separation of peptides based on their charge characteristics (Arrutia et al., 2016; Arunkumar & Etzel, 2013, 2015; Butylina, Luque, & Nyström, 2006; Fernández, Suárez, Zhu, FitzGerald, & Riera, 2013; Fernández, Zhu, FitzGerald, & Riera, 2014; Garem, Daufin, Maubois, & Léonil, 1997; Pouliot, Wijers, Gauthier, & Nadeau, 1999; Xu & Lebrun, 1999). This approach takes advantage of the positive and negative charge carried by peptides under particular conditions, and

uses surface charged membranes to permit or reject their flow based on the charges.

These previous studies have used simplified models in order to describe the effects of the membrane processing. Some used mixtures with a limited number of peptides and were therefore able to monitor each peptide individually (Arrutia et al., 2016; Fernández et al., 2013; Fernández et al., 2014). Others used complex mixtures of peptides but a reductive approach to interpreting the data describing the separations (Bourseau et al., 2009; Butylina et al., 2006; Jeon et al., 2000; Miksik et al., 2001). The work presented here investigates an inclusive approach to interpreting the membrane separation data. This method has been described for interpretation of complex data elsewhere (Killeen et al., 2017) but this is the first known application for analysis of the membrane separations of complex peptide mixtures.

This work was carried out using geoduck (*Panopea zelandica*) as a substrate for protein hydrolysis, but the techniques described are applicable to any protein material that would have value as a hydrolysed product.

1.1 Membranes

Small molecules such as peptides can be separated by exploiting their molecular weight (Ferry, 1936), charge and polarity using either chromatographic techniques or membranes, such as in ultrafiltration or nanofiltration. Membrane separation technology has gained interest for processing biological materials because it can be scaled up for commercial applications, as indicated by the number of publications in this area (Balchen et al., 2008; Bazinet & Firdaous, 2009; Doyen, Beaulieu, Saucier, Pouliot, & Bazinet, 2011; Fernández et al., 2013;

Garem et al., 1997; Jeon et al., 2000; Martin-Orue, Bouhallab, & Garem, 1998; Pouliot et al., 1999; Wijers, Pouliot, Gauthier, Pouliot, & Nadeau, 1998).

Ultrafiltration and nanofiltration membranes have microscopic pores with varying molecular weight cut offs (MWCO) which largely determine their selectivity. Depending on the manufacturer, membrane material and method of manufacture these pores have different degrees of pore size variation. Although membrane manufacturers provide a nominal MWCO for their membranes these have been shown to have a degree of variation and are not absolute values (Butylina et al., 2006). Most membrane based processes use this size selectivity as the basis for whatever separation is desired. However, a number of publications (Fernández et al., 2013; Fernández et al., 2014) have shown that alternative selectivity can be achieved by exploiting the charge on the peptides. In these cases a membrane is selected that also carries a charge, and by modifying the pH of the solution the charge-states of certain peptides can be modified to affect the way in which they interact with the membranes, increasing or decreasing their transmission accordingly.

Numerous authors (Arunkumar & Etzel, 2014; Balchen et al., 2008; Bazinet & Firdaous, 2009; Doyen et al., 2011; Langevin et al., 2012) have investigated the use of membrane filtration in conjunction with an electric field (electrodialysis) to achieve fractionation of peptides according to their charge. This requires specialised equipment, but alternative methods of separation of peptides according to their charge were described by Fernandez and Garem (Fernández et al., 2013; Garem et al., 1997). These researchers used membranes with charged surfaces to improve the selectivity, and modified the charge interactions

of the peptides and the membranes by altering the pH. By varying the pH of the peptide solutions the researchers were able to change the permeability of certain peptides through the negatively charged membranes. Fernandez reported nearly complete rejection of acidic peptides at basic pH values due to these electrostatic repulsive forces and noted that the highest rate of peptide transmission occurred when the peptides were close to their isoelectric point.

Detailed information about the chemistry of the membranes is not readily available from the manufacturers. Specific information about the exact hydrophobicity or surface charge would have required the use of a goniometer (for hydrophobicity measurement) and zeta potential analyser (for surface charge analysis) which was outside of the scope of this project. The membranes chosen in this study were selected because they had a range of properties that would provide maximum opportunity for separating peptides. These membranes had variable MWCOs to compare the effects of differing pore sizes on peptide fractionation, and two membranes also carried a surface charge. These two were tested under acidic (pH 4), neutral (pH 7) and basic (pH 10) conditions to investigate their ability to selectively filter peptides on the basis of their charge.

1.2 Bioactive peptides

Peptides with biological activity can be generated by an organism for a specific biological function, as in the case of the antimicrobial peptides of eggs (Ibrahim, Sugimoto, & Aoki, 2000) and shellfish (Hubert, Noël, & Roch, 1996). They can also be generated by hydrolysis of proteins with proteolytic enzymes (proteases). Bioactive peptides can be produced from food proteins during normal digestive processes inside the body or by enzymatic treatment prior to consumption. Latent

bioactive peptides can be encrypted in longer proteins and liberated following hydrolysis of the parent protein (Korhonen & Pihlanto, 2006). Production of bioactive peptides by protein hydrolysis is an active area of research and has resulted in numerous publications (see Table 1).

Table 1. Examples of biologically active peptides (adapted from (Harnedy & FitzGerald, 2012)

Source	Component	Bioactivity	Reference
Cod	Frame	Antioxidant/ Ace inhibitory	(Jeon et al., 2000)
Herring (*Clupea harengus*)	Whole, Body, Head, Gonads	Antioxidant	(Sathivel et al., 2003)
Hoki (*Johnius belengerii*)	Skin	Antioxidant	(Mendis, Rajapakse, & Kim, 2005)
Hoki (*Johnius belengerii*)	Bone	Calcium binding	(Jung, Park, Byun, Moon, & Kim, 2005)
Hoki (*Johnius belengerii*)	Frame	Antioxidant	(S.-Y. Kim, Je, & Kim, 2007)
Hoki (*Johnius belengerii*)	Frame	Ca-binding	(Jung & Kim, 2007)
Pollock (Theragra chalcogramma)	Skin	Antioxidant	(S.-K. Kim et al., 2001)
Pollock (Theragra chalcogramma)	Skin	ACE inhibitory	(Byun & Kim, 2001)
Pollock (Theragra chalcogramma)	Frame	ACE inhibitory	(Je, Park, Kwon, & Kim, 2004)
Pollock (Theragra chalcogramma)	Frame	Antioxidant	(Je, Park, & Kim, 2005)
Pollock (Theragra chalcogramma)	Frame	Ca-binding	(Jung, Karawita, et al., 2006)
Sea Bream	Scale	ACE inhibitory	(Fahmi et al., 2004)
Snapper (*Priacanthus macracanthus*)	Skin	Antioxidant	(Phanturat, Benjakul, Visessanguan, & Roytrakul, 2010)
Snapper	Skin	Antioxidant	(Khantaphant & Benjakul, 2008)
Sole (*Limanda aspera*)	Skin	Antioxidant	(Giménez, Alemán, Montero, & Gómez-Guillén, 2009)
Sole (*Limanda aspera*)	Frame	Antioxidant	(Jun, Park, Jung, & Kim, 2004)

Sole (*Limanda aspera*)	Frame	Antioxidant	(Jun et al., 2004)
Sole (*Limanda aspera*)	Frame	Antihypertensive	(Jung, Mendis, et al., 2006)
Sole (*Limanda aspera*)	Frame	Anticoagulant	(Rajapakse, Jung, Mendis, Moon, & Kim, 2005)
Tuna	Frame	Antioxidant	(Je, Qian, Byun, & Kim, 2007)
Tuna	Frame	Antihypertensive	(S.-H. Lee, Qian, & Kim, 2010)
Yellowtail	Bone	Antioxidant/ Ace inhibitory	(Morimura et al., 2002)
Yellowtail	Bone	Antioxidant/ Ace inhibitory	(Ohba et al., 2003)Ohba et al. (2003)
Yellowtail	Scale	Antioxidant/ Ace inhibitory	(Ohba et al., 2003)Ohba et al. (2003)
Clam (*Meretrix lusoria)*	Muscle	ACE inhibitory	(Tsai, Chen, & Pan, 2008)
Krill (*Mesopodopsis orientalis*)	Fermented product	Antioxidant	(Faithong, Benjakul, Phatcharat, & Binsan, 2010)
Krill	Muscle	ACE inhibitory	(Kawamura, Takane, Satake, & Sugimoto, 1992)
Mussel (*Mytilus edulis*)	Fermented sauce	Antihypertensive	(Je, Park, Byun, Jung, & Kim, 2005)
Mussel (*Mytilus edulis*)	Fermented sauce	Antioxidant	Rajapakse, Mendis, Jung, et al. (2005)
Prawn (Penaeus japonicus)	Muscle	Antioxidant	(Suetsuna, 2000)
Oyster (*Crassostrea gigas*)	Fermented sauce	Antihypertensive	(Je, Park, Jung, Park, & Kim, 2005)
Oyster (*Crassostrea gigas)*	Muscle	Antimicrobial	(Liu et al., 2008)
Oyster (*Pinctada fucata martencii*)			(Katano et al., 2003)
Oyster (*Crassostrea talienwhanensis Crosse*)	Muscle	ACE inhibitory	(J. Wang et al., 2008)
Oyster (*Crassostrea gigas*)	Muscle	HIV-1 protease inhibitors	(T.-G. Lee & Maruyama, 1998)
Shrimp (*Penaeus aztecus*)	Head	Appetite suppressant	(Cudennec, Ravallec-Ple, Courois, & Fouchereau-Peron, 2008)
Shrimp (*Acetes chinensis)*	Whole shrimp	ACE inhibitory	(Hai-Lun, Xiu-Lan, Cai-Yun, Yu-Zhong, & Bai-Cheng, 2006)
Shrimp (Plesionika izumiae Omori)	Whole shrimp	Antihypertensive	(Nii, Fukuta, Yoshimoto, Sakai, & Ogawa, 2008)
Shrimp (Acetes vulgaris/Acetessp.)	Fermented product	Antioxidant	(Faithong et al., 2010)

Shrimp (Metapenaeus monoceros)	Shell waste	Antioxidant	(Manni, Ghorbel-Bellaaj, Jellouli, Younes, & Nasri, 2010)
Shrimp (Litopenaeus vannamei)	Cephalothorax	ACE inhibitory/ Antioxidant	(Benjakul, Binsan, Visessanguan, Osako, & Tanaka, 2009)
Squid (Dosidicus gigas)	Skin	Antioxidant	(Mendis, Rajapakse, Byun, & Kim, 2005)
Squid (Dosidicus eschrichitii Steenstrup)	Skin	Antioxidant	(Lin & Li, 2006)
Squid	Skin	Antioxidant	(Giménez et al., 2009)
Squid (Dosidicus gigas)	Muscle	Antioxidant	(Rajapakse, Mendis, Byun, & Kim, 2005)

The bioactivity of a hydrolysate is determined primarily by the protein source, but different hydrolysis methods have also been shown to affect the bioactivity of the products (Ahn, Je, & Cho, 2012). Hydrolysis by chemical means, using acids or bases, generally produces less bioactivity than when proteolytic enzymes are used because of the non-selective nature of this method. Protein hydrolysing enzymes (proteases) have different cleavage site specificity and therefore different proteases can generate various peptides with differing bioactive properties from the same protein material (Benjakul et al., 2009). Enzymes commonly used for generating protein hydrolysates include alcalase, flavourzyme, neutrase, pepsin, protamex and trypsin. In some applications the flavour of the hydrolysate is important, and the choice of enzyme has a large effect on this. Enzymes that leave a hydrophobic residue at the terminus of the peptide often generate bitterness (Jens Adler-Nissen, 1984). Another factor which can determine the bioactivity of a protein hydrolysate is the degree of hydrolysis, as measured by the pH change over time (Raghavan & Kristinsson, 2009).

Peptides derived from proteolytic hydrolysis of natural products have possible therapeutic applications (Ahn et al., 2012; Chakrabarti, Jahandideh, & Wu, 2014; Gauthier & Pouliot, 2003; Grienke, Silke, & Tasdemir, 2014; Sarmadi & Ismail, 2010; F. Shahidi & Zhong, 2008; Q. Wang et al., 2014). Separation of hydrolysates has the power to concentrate specific peptide components and enhance this bioactivity. This work aims to develop high value therapeutic products from under-utilised biological resources such as by-products of the marine industry.

A diverse range of bioactivities have been shown for many different peptides with the potential for many to be used as nutraceutical food supplements, or in some cases as medicines (Haefner, 2003). The range of bioactivities identified includes: antihypertensive properties (Haque & Chand, 2008; Hernández-Ledesma, del Mar Contreras, & Recio, 2011; Yamamoto, 1997), cholesterol lowering (Morimatsu et al., 1996; Nagaoka et al., 2001; Turpeinen et al., 2009), antioxidant (Ahn et al., 2012; Chakrabarti et al., 2014; Jia et al., 1996; Sarmadi & Ismail, 2010; Q. Wang et al., 2014), antimicrobial (Gobbetti, Minervini, & Rizzello, 2004; Liu et al., 2008), immunomodulatory effects (Mercier, Gauthier, & Fliss, 2004; Miyauchi, Kaino, Shinoda, Fukuwatari, & Hayasawa, 1997; Wong, Middleton, Montgomery, Dey, & Carr, 1998), anticancer (Azuma, Machida, Saeki, Kanamoto, & Iwami, 2000; Galvez, Chen, Macasieb, & de Lumen, 2001; Leung & Ng, 2007; Picot et al., 2006) , opioid like (Fukudome & Yoshikawa, 1993; Teschemacher, Koch, & Brantl, 1997) and mineral-binding activity (Meisel, 1998; Rutherfurd-Markwick & Moughan, 2005). In this work antimicrobial and antioxidant potential were investigated in the peptide fractions generated by membrane separations.

There is potential for peptides with antioxidative bioactivity to be used as dietary supplements to improve health. Free radicals and other reactive oxygen species are generated in the body as part of normal metabolic processes (Aruoma, 1998). These reactive molecules have been implicated in numerous human diseases, including cancer (Gey, 1993) heart disease (McMurray, McLay, Chopra, Bridges, & Belch, 1990) Parkinson's disease (Olanow, 1992) Alzheimer's disease (Varadarajan, Yatin, Aksenova, & Butterfield, 2000) and diabetes (Oberley, 1988). Further research is continuing to find links between free radicals and other diseases, particularly those diseases associated with aging (Liochev, 2013). The body has endogenous processes for reducing the damage that is caused by free radicals such as the enzyme superoxide dismutase (McCord, 2016) and by synthesising antioxidant compounds such as bilirubin (Boon, Hawkins, Coombes, Wagner, & Bulmer, 2015). The inclusion of additional antioxidant compounds from the diet has been shown to improve health (Ginter, Simko, & Panakova, 2013; Machlin & Bendich, 1987) and research suggests that dietary supplementation with antioxidant compounds can also improve health (Grodstein, Chen, & Willett, 2003; Zandi et al., 2004) so peptides with antioxidant activity have the potential to be used as health promoting supplements. The usual way to determine the antioxidant activity of a compound is to measure its ability to accept electrons in a chemical system, in a process that could be considered to be analogous to free radical quenching in a biological system. This can be tested using laboratory assays including the Ferric Reducing Antioxidant Power (FRAP) assay (Benzie & Strain, 1996) and the 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) antioxidant assay (Molyneux, 2004).

Some peptides have antimicrobial activity (Ganz & Lehrer, 1999; Joerger, 2003). There is concern about the overuse of antibiotics and the growing resistance of pathogenic microbes to these important drugs (Neu, 1992), increasing the interest in developing alternatives to the existing suite of compounds. One possible new source of antimicrobials is peptides. As well as the possibility of using antimicrobial peptides as medicines, they have potential to be used as food preservatives (Cleveland, Montville, Nes, & Chikindas, 2001).

1.3 Geoducks

The raw material chosen for this project was New Zealand geoduck (*Panopea zelandica*). This is a species of deep-water clam that is commercially harvested from Golden Bay, but also occurs in a number of other locations in New Zealand (Gribben, 2003). The volume of the fishery is currently relatively small, with a total allowable catch of just 40.5 mt/year being allocated through New Zealand's quota management system (MPI, 2014). The species has great potential for aquaculture and a similar species, *Panopea generosa*, is farmed in other countries (Brown & Thuesen, 2011). A number of publications have investigated the viability of farming *P. zelandica* (Gribben, Millar, & Jeffs, 2014; Viet Le, Alfaro, & King, 2014) and if this should develop as expected it will help to boost New Zealand's aquaculture exports to the NZ$1 billion forecast by 2025 (Carter, 2012). Aside from a brief investigation into the hydrolysis of *Panopea abrupta* (Wei, 2012), no literature could be found that investigated the bioactive peptides derived from either *P. generosa* or *P. zelandica*. This research therefore adds to the existing body of information about bioactive peptides from marine organisms.

1.4 Hydrolysis of Proteins

Hydrolysis of protein materials can be achieved in a number of ways (Kristinsson & Rasco, 2000; Fereidoon Shahidi, 2007) with each method having advantages and disadvantages. Chemical hydrolysis is fast and cheap but the resulting product is fully hydrolysed to amino acids so the hydrolysates generated do not contain bioactive peptides. This method is most commonly used to generate fertiliser from fish waste (Raa, Gildberg, & Olley, 1982; Vidotti, Viegas, & Carneiro, 2003). Autolytic digestion uses the proteolytic enzymes produced by an organism to digest its own tissues. This is the process used to make fish sauce and fish silage, where the whole fish is stored under specific conditions to allow the digestive enzymes to break down its own tissues. This process has the advantage of being cheap and simple, but the peptide mixtures obtained are highly variable due to the different proteases present. This variation in endogenous proteases can be attributed to differences in temperature (Goldspink, 1995), time of year (Pelletier, Guderley, & Dutil, 1993), sex (Figueiredo-Fernandes, Fontaínhas-Fernandes, Peixoto, Rocha, & Reis-Henriques, 2006) or geographic location (Pierce & Crawford, 1997) and the result is that a consistent product cannot be guaranteed. Finally, the protein material can be digested by the addition of exogenous proteases derived from some controlled source (fungal, bacterial, animal or vegetable). Although this has added expense related to the cost of the enzymes it has the advantage of providing some control over the digestion process. This helps to ensure product consistency and is therefore the method most commonly employed when trying to produce a mixture containing bioactive peptides.

An important consideration when generating a protein hydrolysate is to decide how far to permit the hydrolysis to proceed to avoid complete digestion of the proteins into amino acids. Ideally the digestion should proceed just long enough to have broken the proteins down into bioactive peptides, but no further. A number of publications describe methods for determining the degree of hydrolysis in a proteolytic digest (Jens Adler-Nissen, 1979; Kristinsson & Rasco, 2000; Panyam & Kilara, 1996; Fereidoon Shahidi, 2007). The investigators describe methods such as the depression of the freezing point, increase in solubility in trichloroacetic acid or the determination of the free amines resulting from peptide bond hydrolysis using trinitrobenzenesulfonic acid (TNBS) or o-phthaldialdehyde (OPA) based assays. Another way to monitor hydrolysis is by the pH-stat method (J. Adler-Nissen, 1986) which relies on the generation of acid during the hydrolysis of peptide bonds, resulting in a drop in pH. The volume of sodium hydroxide required to bring the solution back up to the original pH could then be used to determine the degree of digestion that has occurred. Most publications that have used the pH-stat method for monitoring the degree of hydrolysis have used a single protein and a single protease (Fernández et al., 2013; Fernández et al., 2014; Pouliot et al., 1999; Wijers et al., 1998). This is a simpler system than the digestion of whole tissue from an organism which is comprised of a complex mixture of proteins. The authors of the single protein, single enzyme publications were able to calculate how many proteolytic cleavage sites were present on the substrate protein amino acid sequence. Using this information they could determine how much acid would be generated by the complete digestion of a known quantity of protein. By recording the amount of NaOH consumed to

neutralise the acid that was generated by hydrolysis it was possible to determine a percentage of the maximum hydrolysis.

1.5 HPLC and chemometrics

Chemometric analysis is broadly defined as using a data-driven approach to find information from a chemical system. More generally the term is used to describe the application of analytical statistics to sets of data, usually using computers to perform the complex calculations. Numerous statistical methods can be applied depending on the type of data that is being analysed and the information that is desired from this data.

Chemometric analysis can be used in both descriptive and predictive applications, extracting meaningful information from complex data sets, and making associations between diverse properties of a data set. Chemometric data analysis is most commonly used for interpreting spectrographic data such as Near Infrared (NIR) and Raman Spectroscopy because the highly reproducible nature of this type of data makes it ideal for chemometric analysis, but it can also be applied to other types of data.

High Performance Liquid Chromatography (HPLC) analysis was chosen as the method to analyse the effects of the membrane processing. In HPLC peptides are grouped or separated according to their particular physicochemical properties depending on the chromatography mode selected. This is useful because it simplifies the information into a series of discreet peaks in a chromatographic trace.

HPLC analysis of hydrolysates generates highly complex chromatograms (e.g. Figure 7, page 42) which can be difficult to quantify, compare and interpret.

Principle Component Analysis (PCA) of HPLC data allows the complexity to be reduced to a series of points on a scores plot arranged according to how similar or different the samples are. In the Killeen paper (Killeen et al., 2017), discussed in more detail below, this simplification of the chromatographic GC data was achieved by importing the x-y vector data into a software package. Various pre-processing steps (described below) were completed and then the PCA was performed. Each data point in the chromatogram described the response of the detector at a point in time. The software plotted this information into a multi-dimensional graph, where more similar chromatograms were located closer together than less similar ones. Then the axis through the data that described the greatest variance was determined. This axis is termed a Principle Component (PC), and this particular one was the first principal component; PC1. After this a second axis, orthogonal to the first, could be determined that described the next greatest amount of variance between the samples. This was the second principal component, PC2. Plotting these principal components together on a graph, called a scores plot, represented the greatest variation in all of the data in a two dimensional format. Presenting the data this way is valuable because it allows the information to be interpreted in an intuitive manner. It also makes it easier to draw associations between different properties of a set of data (Esbensen, Guyot, Westad, & Houmoller, 2002).

Chemometric analysis of HPLC has been used in the past to interpret large data sets. Wang et al. (C.-Z. Wang et al., 2009) used PCA to interpret reverse phase (RP) HPLC of ginseng root extracts to identify samples that had been adulterated with other material. Mohler, Pripp and Piraino (Mohler Smith & Nakai, 1990; Piraino, Parente, & McSweeney, 2004) (Pripp, Shakeel Ur, McSweeney, & Fox,

1999) analysed the ethanol soluble fraction of cheese using RP-HPLC and interpreted the results using PCA. Mohler and Pripp both assigned the peaks for analysis manually and then performed the statistical analysis on this data, while Piraino used a fuzzy logic approach to automatically assign the peaks. An important point to note is that all of these authors used an approach in which only the most significant peaks in the traces were interpreted, but the smaller peaks were not. This was done by integration of the larger, more recognisable peaks and assigning them according to their retention time. This is a reductive approach and loses some of the detail present in the data, possibly leading to less informative PCA analysis.

Recent publications have used a more inclusive approach to PCA analysis of the raw X-Y chromatographic data. Killeen (Killeen et al., 2017) used the complete GC-FID chromatographic data from the analysis of lupulin glands of hops to generate a PCA scores plot that grouped hop varieties into their respective cultivars. This has been facilitated by the development of suitable pre-processing algorithms; particularly smoothing, standard normal variate (SNV) transformation, second derivative transformation and Correlation Optimised Warping (COW) (Tomasi, van den Berg, & Andersson, 2004; Vest Nielsen, Smedsgaard, & Frisvad, 1999). Chromatographic data, such as that obtained from HPLC or GC, does not generate perfectly reproducible data sets in the same way as spectroscopic analysis (NMR, Raman, FTIR etc.). The pre-processing algorithms described below are those used in the Killeen approach. These permit the chromatograms to be treated in the same way as spectral data, and the complete x-y data can be analysed to retain all of the information.

Smoothing (Figure 1, B) can be carried out to eliminate minor noise in the data. This is performed by generating a polynomial curve around each data point in a chromatogram using the points immediately adjacent, and then adjusting each point according to its respective curve to generate one continuous smooth line.

Standard normal variate transformation (Figure 1, C) is often carried out to eliminate differences related to sample loading. SNV is performed by normalising the y-axis data for each chromatogram against its average value, then standardising the average values of all of the chromatograms. This has the effect of putting each chromatogram on the same relative scale, even though there may be differences in the individual peak sizes.

A *second derivative* transformation (Figure 1, D) is a method that can be employed to eliminate arbitrary baseline variance. This works because only the rate of change in the raw data is used rather than the absolute value, and results in a data set where the end values of each trace line up at zero.

Correlation Optimised Warping (Figure 1, E) reduces some of the minor variation in the retention times of peaks which is inherent to chromatographic methods. The software carries out this process by dividing the chromatogram into segments and maximising the overlap of the retention times in each segment with a reference chromatogram. The degree of freedom for shifting peaks is determined by the “slack” that is defined for the transformation. A critical step in performing this transformation is the selection of an appropriate reference chromatogram that represents an average of all of the chromatograms.

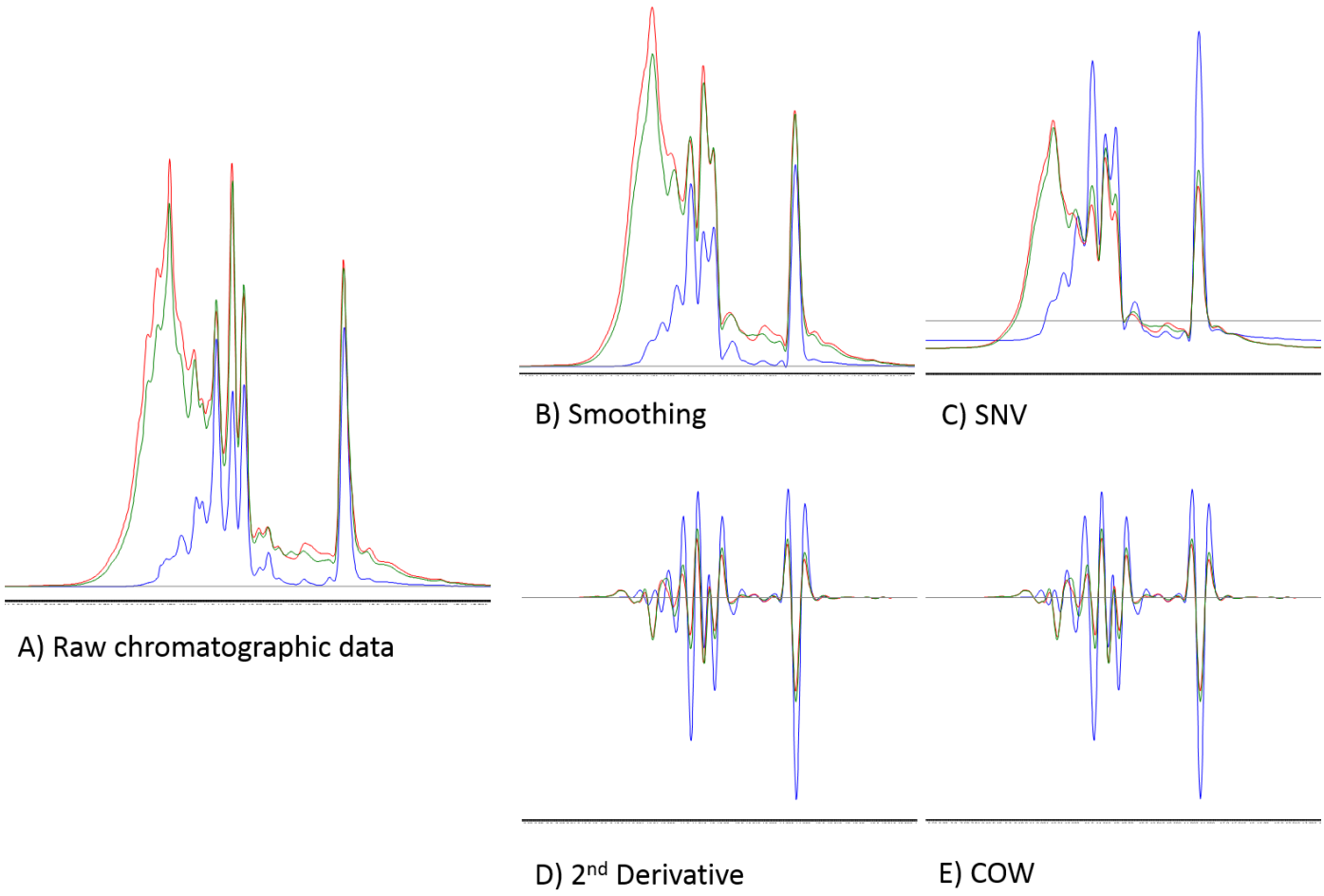

Figure 1. Effects of pre-processing transformations on the raw chromatographic data.

1.6 Summary

This project was designed to investigate membrane technology as a way of separating peptides. The raw material chosen was geoduck and peptide hydrolysis was investigated to determine a reproducible end point for two hydrolysate methods. Seven membranes were investigated for their ability to separate peptides by size. Two of these carrying a surface charge were also additionally tested with variable pH conditions to investigate their ability to separate peptides by charge. Both permeate and retentate samples were retained after the separations were complete. Membrane fractions were analysed

using SEC and RP HPLC coupled with principle component analysis (PCA). Membrane fractions were also tested for various biological activities, including anti-microbial activity and anti-oxidant activity. The results show that membranes can be used to successfully separate biologically active peptides present in complex mixtures.

2 Methods

2.1 Materials

2.1.1 *P. zelandica* samples

Geoduck (*P. zelandica*) samples were harvested offshore from Pakawau, Golden Bay, by PZL Harvesters Ltd as part of a population survey on the 28th and 29th August 2015. Samples were taken from the area indicated in Figure 2 and stored below 12°C during harvesting, below 5°C overnight, and then frozen on the day after collection. Depth of collection, shell length and flesh weight were recorded for each specimen.

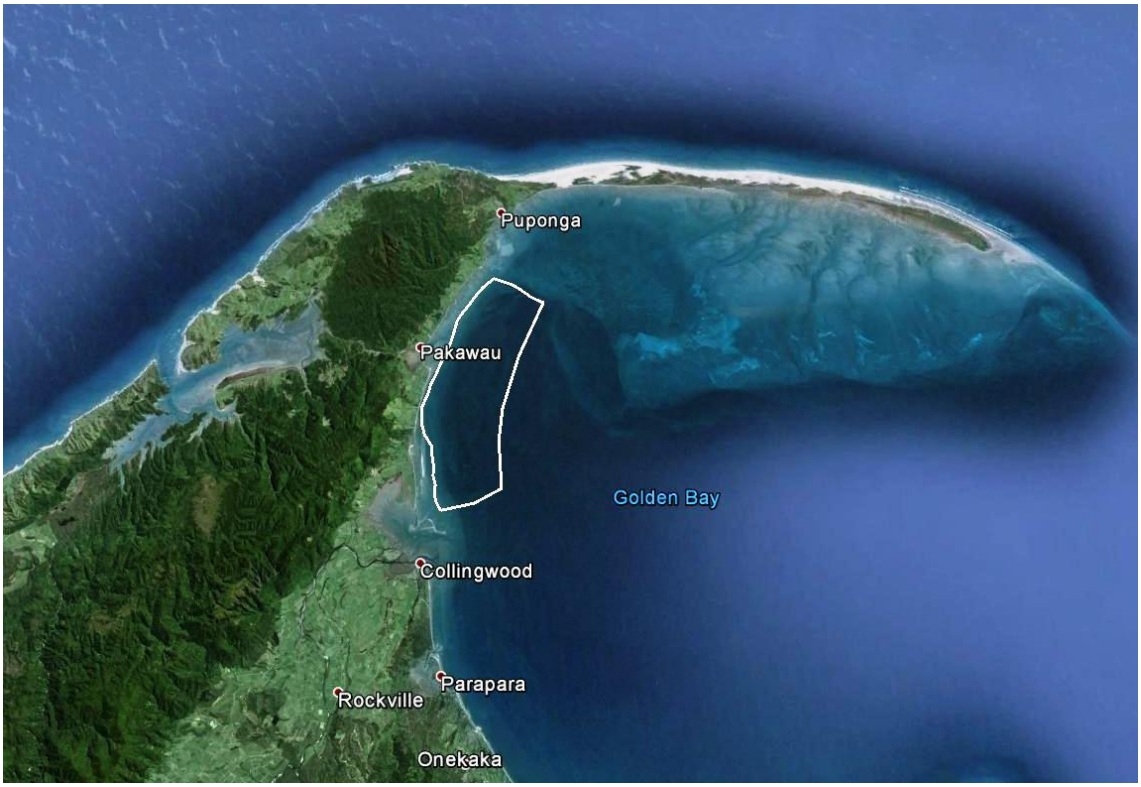

Figure 2. Satellite photograph of golden bay with the geoduck sampling area indicated by the white outline.

Geoduck samples were homogenised using a mincer (Everest model TEE32-2000, 2 mm diameter cutting die) and stored at -40°C prior to protease hydrolysis.

2.1.2 Proteases for hydrolysis

The proteases used for hydrolysis of the geoduck tissue were Enzidase 899® from Zymus International Ltd (Auckland, New Zealand) and Flavourzyme® from Novozymes (Bagsvaerd, Denmark).

2.1.3 Reagents and solvents for HPLC

All reagents and solvents were of HPLC grade. Trifluoroacetic Acid and Sodium Dodecyl Sulfate were from Sigma-Aldrich, St Lois, MO, USA. Acetonitrile was from Fisher Chemicals, Geel, Belgium. Potassium Phosphate was from Thermo-Fisher, Taren Point, NSW, Australia. Potassium Chloride was from Scharlau, La Jota, Barcelona, Spain.

2.1.4 Chromatography columns

All columns used for peptide investigations (RP, SEC, IEX, NP and HILIC) were from Phenomenex, Rosedale, Auckland, NZ.

2.1.5 Membranes

Ultrafiltration and nanofiltration membranes were supplied by Sterlitech^{TM}, WA, USA.

2.2 Hydrolysis of geoducks

2.2.1 Measuring the degree of hydrolysis

2.2.1.1 Titrimetric method 1 (J. Adler-Nissen, 1986)

A 5 g sample of homogenised geoduck flesh was suspended in 50 mL of deionised water and adjusted to pH 8.0 with 1 M NaOH. The sample was heated

to 50°C in a jacketed heater and Enzidase 899 (1% w/v) was added to initiate hydrolysis. Acid generated by protein hydrolysis was determined by the amount of a standardised solution of 0.01 M NaOH required to maintain a pH of 8.0. The reaction proceeded until no further space was available in the reaction cup. A negative control sample was analysed as above, but with the omission of the protease.

2.2.1.2 Titrimetric method 2 (modified from Adler-Nissen (J. Adler-Nissen, 1986))

To obtain information about longer digestion times a second pH titration was carried out using a burette. Homogenised geoduck flesh (50 g) was weighed into a 500 mL plastic screw top centrifuge cylinder and 50 mL of deionised water was added. The pH of the solution was adjusted to 7.5 with 1M NaOH. Samples for digestion had 250 μL of Enzidase 899 and 250 μL of flavourzyme added. The negative control samples had no enzyme added. Solutions were incubated at 50°C. To perform the titrations the samples were centrifuged at 6600 xg for 10 min and the supernatant was decanted into a beaker. The acid that was generated during hydrolysis was titrated against a standardised solution of 1 M NaOH using a burette and a pH meter (PHM92 Lab pH meter, Radiometer, Copenhagen). Titrated supernatant was returned to the centrifuge cylinder and digestion was continued. Titrations were performed at 1, 3, 4, 5, 22, 24, 26, 28, 46 and 52 h.

Samples (1.5 mL) were taken at each time-point for SDS-PAGE gel and HPLC analysis.

2.2.1.3 Time-course digests

Samples (2.0 g) of geoduck homogenate were weighed into 15 mL plastic centrifuge tubes. A 0.2 M phosphate buffer solution (pH 7.5) containing 2% Enzidase 899 was warmed to 50°C. To initiate hydrolysis 2 mL of the buffered enzyme was added to the geoduck sample and incubated at 50°C. At appropriate times tubes were transferred into a 95°C water bath for 15 min to inactivate the enzyme. Incubation times were 0, 5, 10, 20, 30, 40, 50, 60, 90,120, 150, 180, 240 and 300 min. This was performed with four solutions: buffer with 2% Enzidase 899; buffer with 2% flavourzyme; buffer with 1% Enzidase 899 and 1% flavourzyme, and buffer with no protease as a negative control.

2.2.1.4 HPLC analysis

Digested samples from the time-course (section 2.2.1.3) were centrifuged at 1920 xg for five min and the supernatant was filtered through a 0.2 µm filter. Samples were analysed by RP HPLC as described in section 2.3.5.1. The 215 nm traces were exported into The Unscrambler X 10.3 multivariate data analysis software (CAMO software, Oslo) and processed as described in section 2.3.6.

2.2.2 Dry weight analysis

Aluminium drying dishes were heated at 105°C overnight and then allowed to cool to room temperature in a desiccator. Samples of homogenised geoduck were weighed into the dry dishes and then samples were heated at 105°C for 24 h. Dried samples were allowed to cool in a desiccator before the weights were again recorded. Percentage dry weight was calculated as follows:

$$\%\ DW = \frac{DW}{WW} \times 100$$

Where dry weight (*DW*) is the weight of the dried tissue minus the weight of the drying dish, and wet weight (*WW*) is the weight of the wet tissue minus the weight of the drying dish.

2.2.3 Polyacrylamide gel electrophoresis (PAGE)

Samples of hydrolysate (100 µL) were mixed with 100 µL of tris-tricine gel running buffer and heated at 95°C for 5 min and 10 µL was loaded onto a Bio-Rad criterion 16.5% tris-tricine SDS-PAGE gel. Samples were run at 30 V for 1 h and then 85 V for the remaining time.

2.2.4 Preparation of Geoduck hydrolysate

2.2.4.1 Hydrolysis procedure:

Endopeptidase digest (GD1): Minced geoduck was defrosted, 4.4 kg was weighed into a 25 L stainless steel reaction vessel and 4.4 L of water was added. The pH was adjusted to 8.0 with 6 M NaOH. The reaction vessel was placed in a 50°C water bath and 44.0 mL of Enzidase 899 was added. The mixture was stirred at 300 rpm and the pH and temperature were measured. Sodium

hydroxide additions were made periodically to maintain a pH of 8. The reaction was stopped after 4 h by heating the mixture to >90°C for 10 min to deactivate the protease, and then cooled in a water bath.

Endopeptidase/exopeptidase digest (GD2): As described above, but at 2 h the pH was adjusted to 7.0 with conc HCl and 44 mL of flavourzyme was added. After a further 2 h the reaction was stopped as described above.

The hydrolysed geoduck mixture was strained through a muslin bag to remove any undigested material. The liquid hydrolysate was centrifuged using an Alfa-Laval LAPX-202 three phase separator fitted with a 55 mm fractionation disc and running at 8000 rpm to remove smaller particulates and lipid. The centrifuged hydrolysate was then filtered using a depth filtration apparatus fitted with a Seitz 900 filter with a fibracell/ celite filtration bed. This process was repeated with a Seitz 100 filter with a fibracell/ celite filtration bed. The clarified hydrolysate was passed through a 13 kDa molecular weight cut-off (MWCO) ultrafiltration column running at 20 psi. Finally the filtered hydrolysate was frozen to -20°C on trays and freeze-dried for long term storage. The filtered samples were loaded frozen into the freeze dryer and then the temperature was increased from -22°C to 10°C over 10 hr. The temperature was held at 10°C for 5 hr and then increased to 60°C over 10 hr. The temperature was then reduced to 20°C over 4 hr and maintained at this temperature until samples were unloaded from the freeze drier.

2.3 Membrane separations:

2.3.1 Preparation of hydrolysates for separation

The freeze dried hydrolysate (6 g) was dissolved in 180 mL of deionised water and the pH was adjusted appropriately using either 6 M NaOH or HCl. The pH was measured using a pH meter (pHM92 lab pH meter, Radiometer, Copenhagen) and the volume was made up to 200 mL to give a final hydrolysate concentration of 30 mg/mL.

In order to avoid any membrane fouling issues the hydrolysates were filtered prior to membrane fractionation using sequential filtration with 47 mm diameter cellulose acetate filters (pore sizes of 3 µm, 0.45 µm and 0.22 µm) fitted into a vacuum filtration apparatus (Millipore, MA, USA).

2.3.2 Size separations

For the experiments investigating whether the peptides could be selectively separated according to their size the hydrolysate samples were made up at pH 10. The solutions were then filtered through the seven membranes with various MWCOs shown in Table 2. A schematic representation of all the membrane separations is shown in Figure 3.

2.3.3 Charge separations

For the experiments investigating whether peptides could be selectively separated according to their charge each hydrolysate was made up at three different pH values; 4, 7 and 10. Each of these sets of solutions was then separated using the XT and V3 membranes (Table 2 and Figure 3).

*Table 2. Membranes that were compared to determine their suitability for separating peptide mixtures, including information about manufacturer, composition, and molecular weight cut-off (MWCO). * TFC: Polyamide- thin film composite. ** PES: Polyethersulfone. *** PVDF: Polyvinylidene difluoride.*

Membrane name	Manufacturer	Material	MWCO (Da)
DK	GE osmonics	Thin Film	150-300 Da
NFG	Synder	TFC*	600-800 Da
XT	Synder	PES**	1000 Da
GH	GE osmonics	Thin Film	2500 Da
MT	Synder	PES**	5000 Da
PW	Synder	PES**	10,000 Da
V3	Synder	Positively charged PVDF***	30,000 Da

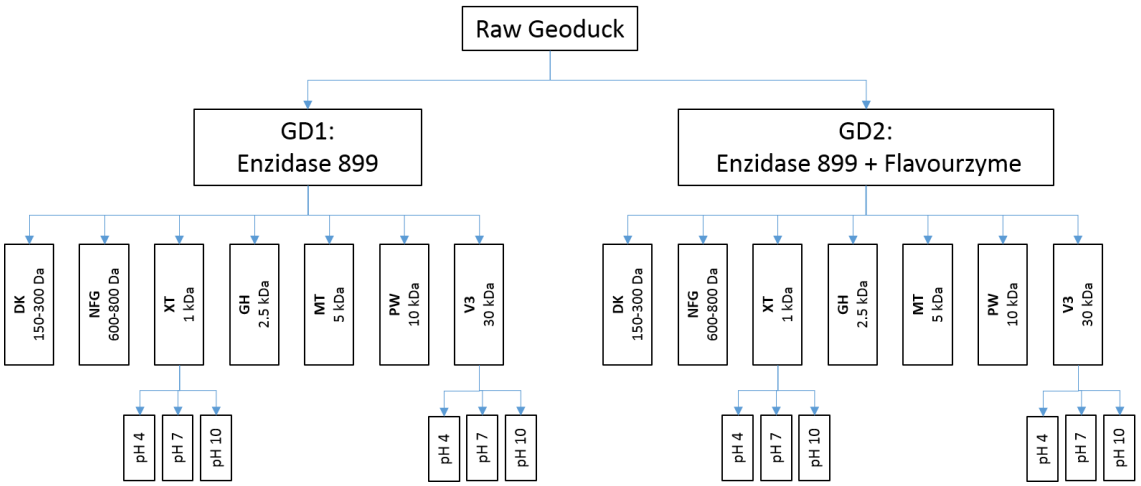

Figure 3. Experimental schematic for the membrane separations of the geoduck hydrolysates outlining the different enzyme treatments, membrane processing and pH modifications used.

2.3.4 Membrane separations of peptides

A pressurised stirred cell membrane filtration apparatus (HP4750 stirred cell, Sterlitech^{TM}, WA, USA) was assembled with the appropriate membrane installed. Membranes were soaked in deionised water for one hour prior to use. The pH-adjusted and filtered hydrolysate (200 mL) was transferred to the vessel and a measuring cylinder was placed under the permeate spout. Separation was performed with a constant pressure of 20 Bar and 400 rpm stirring. Permeates were collected as three lots of 50 mL and the time taken for each 50 mL to elute was recorded. Once 150 mL of permeate was obtained the remaining 50 mL of retentate was also collected. The conductivities of both the permeate and retentate samples were recorded. The permeate samples were pooled and then both the permeate and retentate samples were stored at -20°C. Once all the separations were complete the samples were freeze dried and weighed.

2.3.4.1 Determination of salt concentration

A conductivity meter (Cyberscan CON110, Eutech Instruments, Netherlands) was used to measure the conductivity of a range of NaCl standards from 1 mM to 1 M. A polynomial regression curve was generated from this data.

The conductivity of the peptide solutions were measured and recorded, and the relative NaCl concentrations were determined from the standard curve.

2.3.4.2 o-phthaldialdehyde (OPA) assay

A 0.8 mg/mL (6 mM) solution of o-phthaldialdehyde was made up in 0.1 M di-Sodium tetraborate decahydrate ($Na_2B_4O_7$) buffer. Mercaptoethanol (0.05% v/v) was added.

Peptide samples (10 µL) were aliquoted into a 96 well plate and 200 µL of the buffered OPA solution was added. The samples were reacted at room temperature for 20 min and then the absorbance was measured at 340 nm.

Samples of the freeze dried hydrolysates were used as the peptide standards to generate a calibration curve for determining the concentration of the membrane-separated samples.

2.3.5 HPLC analysis Methods

All chromatography was carried out on a Shimadzu LC-20 series HPLC, with UV detection by an SPD-M20A diode array detector (DAD) from Shimadzu, USA.

The samples were diluted to 20 mg/mL using the OPA assay results. The samples were filtered with a 0.2 µm filter and then 20 µL was injected (400 µg). In the case of samples less concentrated than 20 mg/mL the injection volumes were increased to ensure that 400 µg of material was run on the column.

2.3.5.1 RP HPLC elution conditions:

Reverse Phase (RP) chromatography was carried out with a Phenosphere Next C-18 column from Phenomenex. The column dimensions were 250 x 4.6 mm and the column packing was 5 µm beads with 120 Å pore size.

RP separations were performed at 30°C with a 1 mL/min flow rate. Solvent A was deionised water with 0.1% trifluoroacetic acid (TFA) and solvent B was acetonitrile with 0.1% TFA. The sample was loaded in 0% B, held at 0% B for 5 min and then the concentration was increased to 25% B over 45 min. The column was then flushed with 40% B for 5 min and re-equilibrated at 0% B for 5 min. The 215 nm wavelength data was recorded.

2.3.5.2 SEC HPLC elution conditions:

Size Exclusion Chromatography (SEC) was carried out with a Yarra SEC-2000 column from Phenomenex. The column dimensions were 300 x 7.8 mm and the column packing was 3 µm beads with 290 Å pore size.

SEC separations were performed using a 20 min isocratic flow of 100 mM phosphate buffer pH 6.8 + 0.5% SDS. Samples were run at 30°C using a 1 mL/min flow rate. The 215 nm wavelength data was recorded.

2.3.6 Chemometric analysis of HPLC data

The 215 nm data from the RP-HPLC and SEC-HPLC runs were exported from the Shimadzu Labsolutions software as ascii files and into The Unscrambler X 10.3 multivariate data analysis software (CAMO software, Oslo). The raw data required pre-processing to permit the software to make comparisons between the traces. Both the RP-HPLC and SEC-HPLC data were subjected to the same pre-processing steps: smoothing, Standard Normal Variate (SNV) transformation, second derivative transformation and Correlation Optimised Warping (COW).

Smoothing was performed using a Savitsky-Golay transformation model. Polynomial curves were generated from the data twenty points on either side of each individual point and combined into one continuous smoothed line.

For both the SEC and RP data sets the GD1 pH 10 input sample was selected as the reference for alignment of the peaks.

Principle component analysis was performed on the processed data and the results were displayed on scores plots.

In the case of the SEC data the complete chromatograms were used. In the case of the RP chromatograms the data was truncated to only include the data from between 14 min and after 58 min. This was done because during other investigations (not presented) it was found that the material eluting outside of this time period had an inconsistent elution profile, even within duplicate samples, and therefore had a disruptive effect on the PCA analysis.

2.4 Bioactivity testing

2.4.1 Zone of inhibition of microbial growth testing

Nine strains of bacteria were prepared for microbial inhibition testing (Table 3). These were recovered from -86°C storage and grown overnight in Brain Heart Infusion broth at the optimal temperatures outlined in Table 3. These cultures were then grown on *Colombia sheep blood agar* plates to obtain individual colonies. Colonies were used to inoculate 10 mL aliquots of Mueller Hinton (MH) broth and grown at optimal temperatures overnight. Cultures were centrifuged and washed twice with Salt Peptone Water (SPW) before re-suspension in SPW.

Colony forming unit (cfu) concentration was determined by performing drop plate enumeration.

For the temperature sensitive strains *P. phosphoreum* and *P. fluorescens* 10 mL of the inoculum was spread over the surface of room temperature MH agar plates as a bacterial lawn. For the other strains 10 mL of the inoculum was mixed with MH agar at 50°C and plated. Wells were made in the agar using a 6 mm core borer. Samples (35 µL) were pipetted into the wells. Membrane-separated samples were 200 mg/mL peptide. Distilled water was used as a negative control and chloramphenicol (30 µg) and colistin (10 µg) were used as positive controls. Salt solutions at 50 mg/mL, 100 mg/mL and 200 mg/mL were included as NaCl controls. Plates were grown for 24 h at the optimal temperatures and zones of inhibition were measured using callipers. Zone of inhibition was calculated as the width of the total zone of clearing minus the 6 mm well diameter. Samples were tested in triplicate.

Table 3. Bacterial strains used for anti-microbial screening and their optimal growth temperatures.

Name of bacteria	Optimal growth temperatures °C
Listeria monocytogenes-ScottA	37
Salmonella typhimurium	37
Escherichia coli 0157:H7	37
Staphylococcus aureus	37
Enterococcus faecalis	35
Morganella morganii (01A01)	37
Photobacterium phosphoreum	20
Pseudomonas fluorescens	25
Shewanella putrefaciens	30

2.4.2 Antioxidant activity assays

2.4.2.1 FRAP (Ferric Reducing Antioxidant Power) assay

The FRAP reagent consisted of 0.833 mM Tripyridyltriazine (TPTZ) + 1.66 mM ferric chloride in 250 mM acetate buffer pH 3.6. Trolox standard was dissolved in phosphate buffered saline at 250 mg/mL and a range of dilutions were prepared, from 3.125 mg/mL to 250 mg/mL. Twenty microliters of 20 mg/mL peptide samples and trolox standards were transferred to a 96 well plate in duplicate and 180 µL of the FRAP reagent was added. Plates were left to react for 20 min at room temperature and then the absorbance at 593 nm was recorded.

2.4.2.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay

The DPPH solution consisted of 0.025 mg/mL DPPH dissolved in methanol. Standards consisting of 0.4 mg/mL DL-α-tocopherol, 0.117 mg/mL quercitin, and 0.147 mg/mL ascorbic acid were made up in methanol and a series of dilutions of each standard was prepared, from undiluted to 1/10. Twenty microliters of 20 mg/mL peptide samples and diluted standards were transferred to a 96 well plate in duplicate and 180 µL of the DPPH reagent was added. Plates were left to react for 30 min at room temperature and then the absorbance at 515 nm was recorded.

3 Results and Discussion

3.1 Geoduck collection

Ninety four geoducks were collected from between 4.9 m and 10.1 m of water, with the average depth being 8.0 m. The average length of the shells was 109 mm and the average weight of the flesh was 205 g (Table 8, supplementary). These results were consistent with results published previously (Breen, Gabriel, & Tyson, 1991; Gribben & Creese, 2005).

3.2 Geoduck hydrolysis conditions

Significant effort was put into establishing appropriate conditions for the hydrolysis of the geoduck tissue because the amount of raw material was limited and difficult to replace. One of the most important factors was determining the appropriate degree of hydrolysis for the digests. The aim of determining the degree of hydrolysis was to find conditions that produced peptides of a useful size, and also to improve the reproducibility of the enzymatic digestions. A number of publications describe methods for determining the degree of hydrolysis in a proteolytic digest (Jens Adler-Nissen, 1979; Kristinsson & Rasco, 2000; Panyam & Kilara, 1996; Fereidoon Shahidi, 2007).

3.2.1 Degree of hydrolysis- titrimetric method

Adler-Nissen (J. Adler-Nissen, 1986) described a method for determining the degree of hydrolysis using a titrimetric method to monitor the acid production. This work involved a single protein substrate with a known amino acid sequence and a protease (trypsin) that had defined cleavage recognition sites on this sequence, so a complete digestion could be calculated theoretically. Using this

information the maximum possible cleavage could be calculated and the degree of hydrolysis could be deduced from the acid measured.

This principle was applied to try to determine a suitable point to stop the hydrolysis of the geoduck samples. The hydrolysis in this case was more complex because it used a whole organism which contained numerous proteins to be digested, and also used two proteases rather than one. Because of this it was not possible to calculate a maximum theoretical hydrolysis value based on known cleavage sites, so another approach was taken. The hydrolysis would be permitted to proceed until no further acid was produced. It was thought that this could be considered to be a complete hydrolysis and a “degree of hydrolysis” value for future digests could be calculated relative to this.

In the first experiment hydrolysis was performed in a pH-stat apparatus as described by Adler-Nissen (J. Adler-Nissen, 1986), but using minced geoduck as the protein substrate and Enzidase 899 as the protease. A negative control was also included that had no added protease (Figure 4).

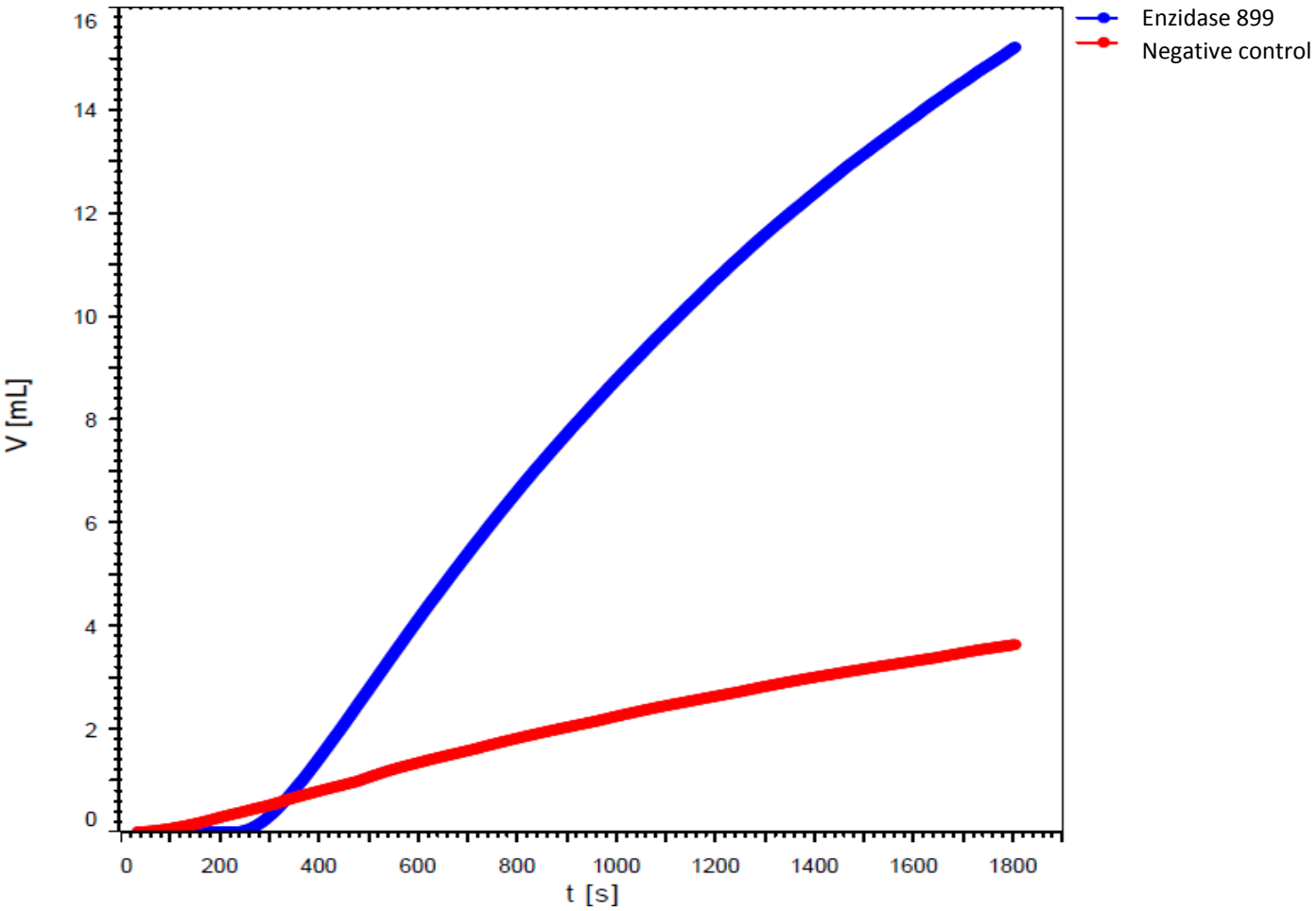

Figure 4. Titration curves showing relative rates of acid generation in geoduck samples with no added protease (negative control, red) and 1% protease (Enzidase 899, blue).

Two things were apparent from this experiment. First, the hydrolysis of the geoduck tissue required a larger reaction vessel than the small autotitrator cup to achieve complete hydrolysis. The autotitrator continued to add 0.01 M NaOH to maintain a pH of 8.0 until the reaction cup was full. Second, the negative control had a baseline level of proteolytic activity, indicating the presence of internally produced (endogenous) proteases. This would be consistent with endogenous enzymes acting on the proteins, which is not surprising considering that the geoduck tissue was a homogenate of the entire organism, including the digestive organs. The gut contains proteases that could act on the sample to generate acid without need of additional enzymes.

The rate of addition of 0.01 M NaOH required to maintain a pH of 8.0 was 0.1253 mL/ min (1.253 µM/ min) for the negative control and 0.6214 mL/ min (6.214 µM/ min) for the sample containing 1% Enzidase 899. This showed that the endogenous enzymes in the sample had approximately 20% of the activity of the protease-added sample. These results suggested that a certain amount of the hydrolysis was not controllable and highlighted the importance of adding sufficient proteases. This would minimise the contribution of the endogenous activity to the final product. A possible way to avoid this contribution would have been to heat the geoduck tissue prior to digestion, but other work (not reported here) has shown that cooked shellfish do not achieve efficient digestion, presumably because the tissues contract upon cooking and become inaccessible to the proteases.

While the pH-stat assay gave information about the relative rates of endogenous and exogenous enzyme activity, the hydrolysis endpoint was unable to be determined due to the size limitations of the reaction vessel. For this reason an alternative approach was investigated.

3.2.2 Degree of hydrolysis- recycled titrations

To try to determine an endpoint of digestion the hydrolysis was carried out on a larger scale and the acid that was produced was titrated using a burette containing 0.01 M NaOH. It was anticipated that after a sufficient amount of time had passed the hydrolysis would be complete and no further acid would be generated. Instead the production of acid remained steady for several days, with the final measurement being carried out at 52 h. (Figure 5)

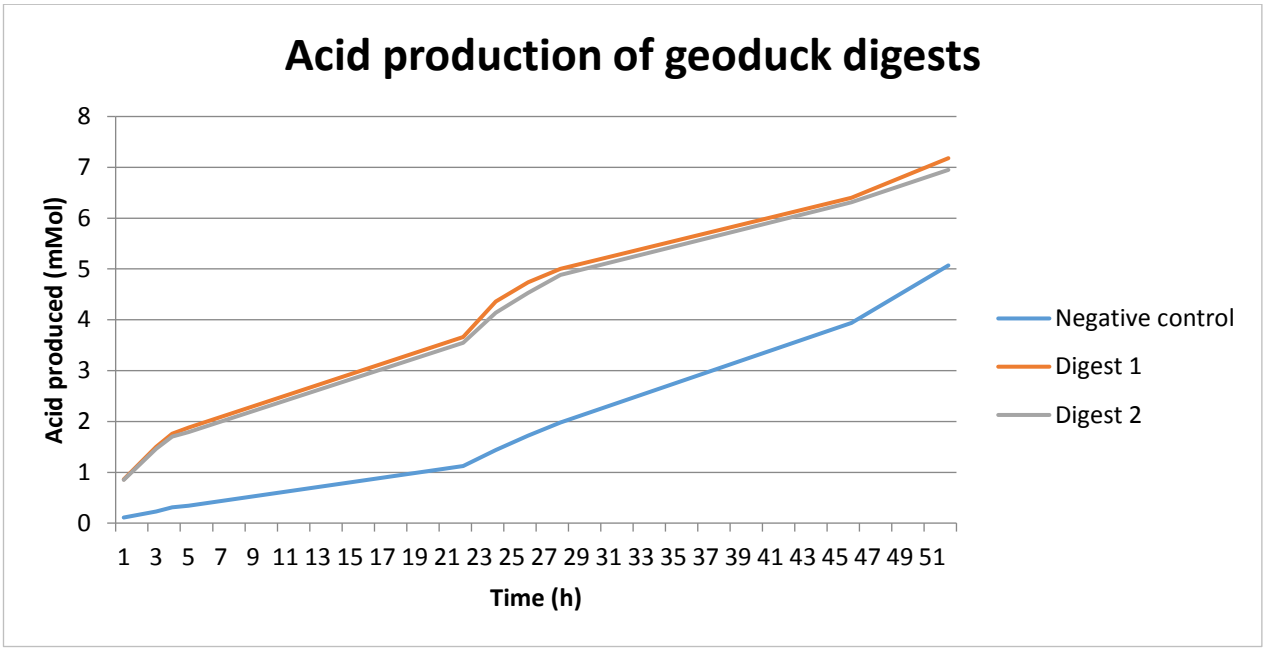

Figure 5. Acid produced by protein hydrolysis of geoduck tissue over 52 h as determined by NaOH titration. Negative control (blue) contains geoduck tissue with no added protease. Digest 1 (red) and digest 2 (green) are replicate samples containing 0.5% (w/v) Enzidase 899 and flavourzyme.

The failure of the digests to reach a determinable endpoint could have several explanations. The combined effect of all of the endogenous and exogenous proteases would mean that it is likely that nearly every peptide linkage could be vulnerable to hydrolysis if given sufficient time. The end result of this situation would be complete hydrolysis of the proteins into amino acids suggesting that limiting the time of the digestion is important.

A second possible explanation for why a digestion endpoint was unable to be determined was microbial activity, as indicated by the sulphurous odour of the final time points. The contribution of bacteria to the hydrolysis of the proteins complicated the situation by increasing the number of proteases acting on the geoduck proteins. In addition to standard peptide hydrolysis, bacteria are able to further metabolise amino acids into other products such as short-chain fatty acids, succinic acid, 8-amino valeric acid, and molecular hydrogen (Barker, 1981) . This

meant that once bacteria had become active the titrations would no longer be measuring just the hydrolysis of proteins, but also the products of complex microbial metabolism.

These results showed that determination of the endpoint of this enzyme hydrolysis was not possible using an acid-base titration method and by extension neither was it possible to determine the degree of hydrolysis after a certain time. This was because of the complex nature of the protein substrate with endogenous proteases and microbes from the digestive organs being included in the homogenised samples.

One useful observation was that the repeat samples (digest 1 and digest 2) tracked closely over time for the amount of acid produced which shows that, at least by this metric, the digests were consistent and repeatable (Figure 5).

3.2.3 SDS-PAGE analysis

Samples of the 52 hour hydrolysis time points were analysed by SDS-PAGE. (Figure 6). The negative control samples had no exogenous protease added while the sample labelled “digest 1” had 0.5% (w/v) Enzidase 899 and flavourzyme added. Despite the lack of added proteases the negative control sample still exhibited a breakdown of the high MW proteins into smaller peptides over time, consistent with the hypothesis that endogenous enzymes were active in the sample. In both samples the majority of the high MW material was gone after 22 hr. For the sample with protease added there was very little material larger than 6.5 kDa after five hours of hydrolysis which suggested that limiting the hydrolysis time would be necessary to ensure that the peptides were not fully broken down into amino acids.

An interesting observation was that the molecular weight band profiles of the two samples were different. The action of the endogenous enzymes in the negative control left two distinct bands between 31 and 45 kDa which were not apparent in the exogenous enzyme digest. Both samples had a band that was roughly 14.4 kDa, but the exogenous enzyme digest band was slightly lower suggesting a different molecular weight. These results indicate that the actions of the different enzymes did not only differ in their rate of activity, but also produced different peptides as would be expected.

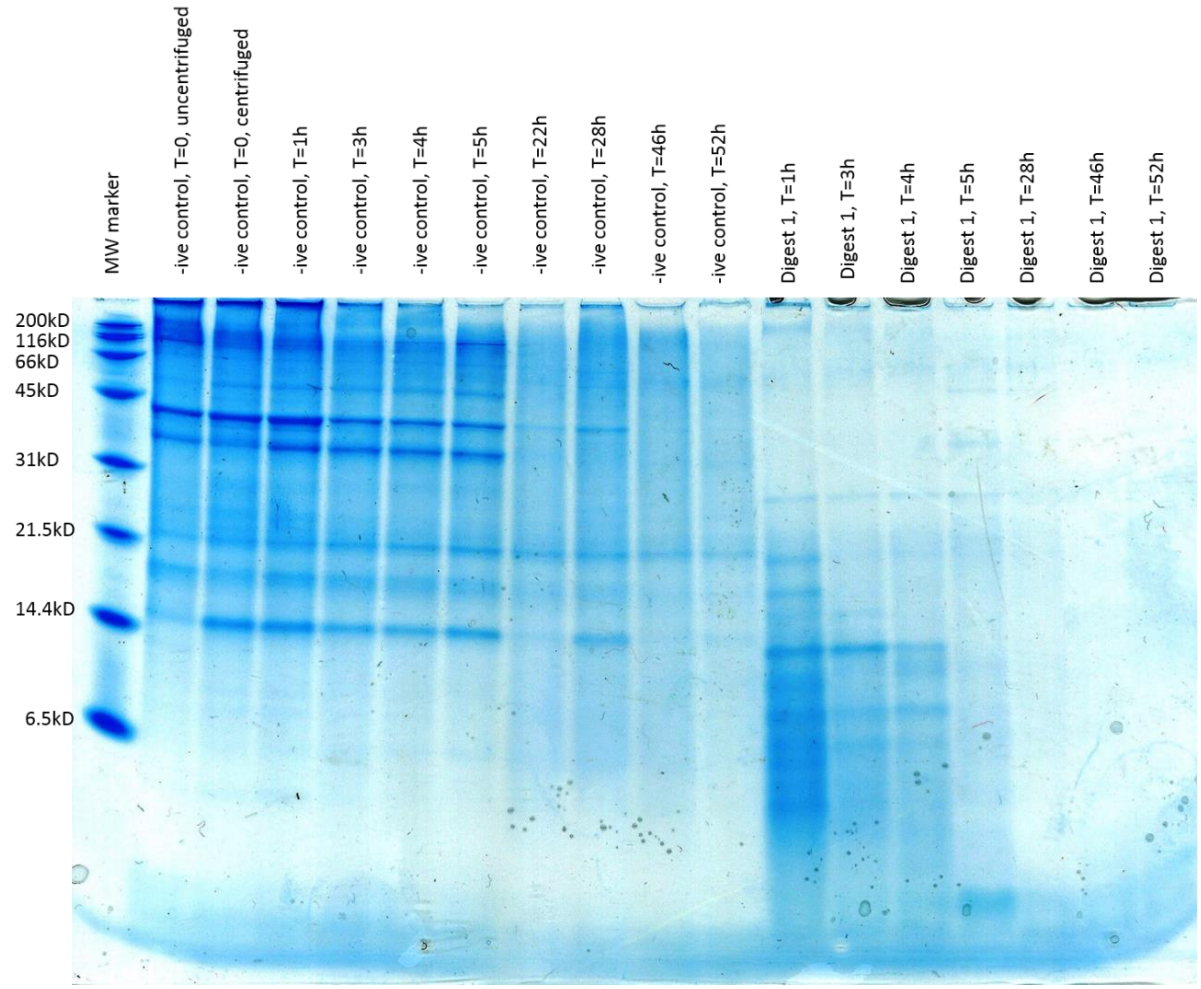

Figure 6. Samples of geoduck hydrolysates at various stages of digestion resolved on a Bio-Rad 16.5% tris-tricine SDS-PAGE gel. Negative control samples had no protease added. Digest 1 samples had 0.5% (w/v) Enzidase 899 and flavourzyme added.

Tris-tricine gels (16.5%) are the most suitable SDS-PAGE gels for separation of low MW proteins, but below 1 kDa even these gels are unable to separate the molecules (Schagger & Von Jagow, 1987). The significance of this is that after a certain level of hydrolysis no further distinction could be made between the peptides that had been generated and the entire sample migrated as a single band to the bottom of the gel. A method of analysis with greater resolution that SDS-PAGE is HPLC. The separation of the peptides in HPLC occurs under highly controlled temperatures and pressures and with strictly defined solvent compositions.

3.2.4 RP-HPLC of hydrolysates- long time points

A number of chromatographic modes were investigated, including Reverse Phase (RP), Size Exclusion (SEC), Normal Phase (NP), Weak Anion Exchange (WAX), Strong Cation Exchange (SCX) and Hydrophilic Interaction Chromatography (HILIC). RP and SEC were found to be the most suitable for peptide analysis, but SEC initially had issues related to column degradation. Therefore, to further characterise the hydrolysate samples RP-HPLC analysis of the time course samples was performed. Chromatograms were recorded at 215 nm and exported into The Unscrambler software (Figure 7). After appropriate pre-processing PCA was performed on the data. A scores plot (explained in section 1.5) which described 84% of the variance in the chromatographic data (72% from Principle Component 1 (PC 1) and 12% from PC 2) was generated from this analysis (Figure 8 and Figure 9).

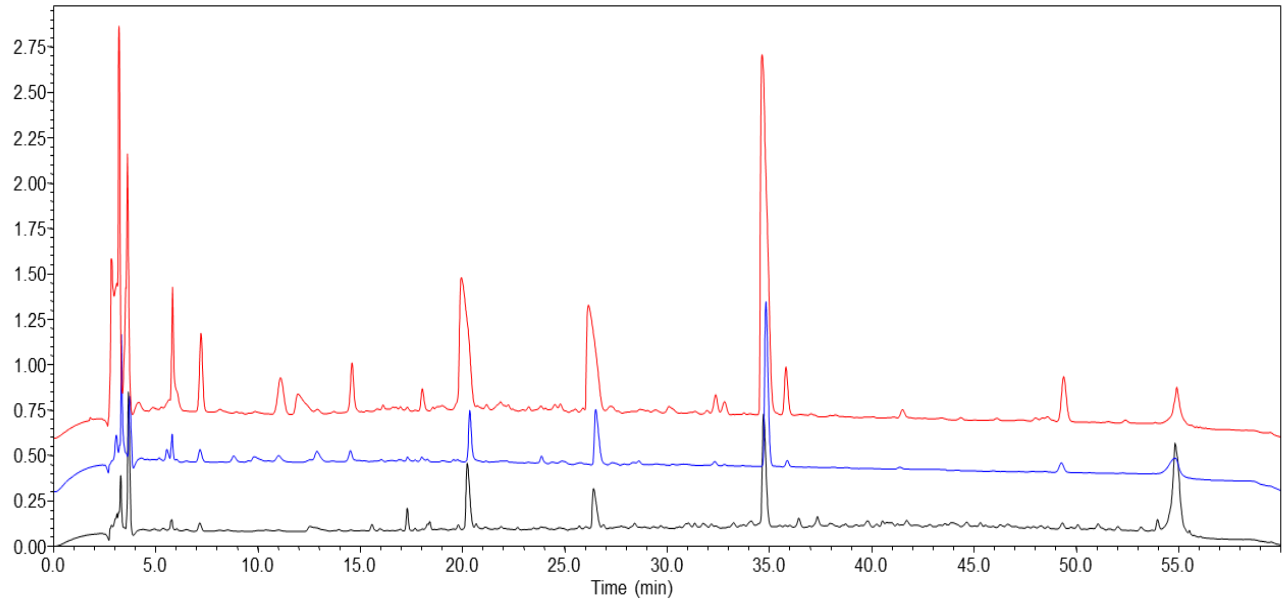

Figure 7. Overlays of 215 nm HPLC traces from geoduck digest time points after 1 hour (black), 22 h (blue) and 52 h (red). Samples were separated on a Phenosphere Next C18 reverse phase (RP) column. Buffer A was 0.1%TFA and buffer B was acetonitrile. The samples were loaded in 0% B and held for 5 min and then the gradient was increased to 25% B over 45 min. The concentration of B was increased to 40% B for 5 min and then run at 0% B for 5 min.

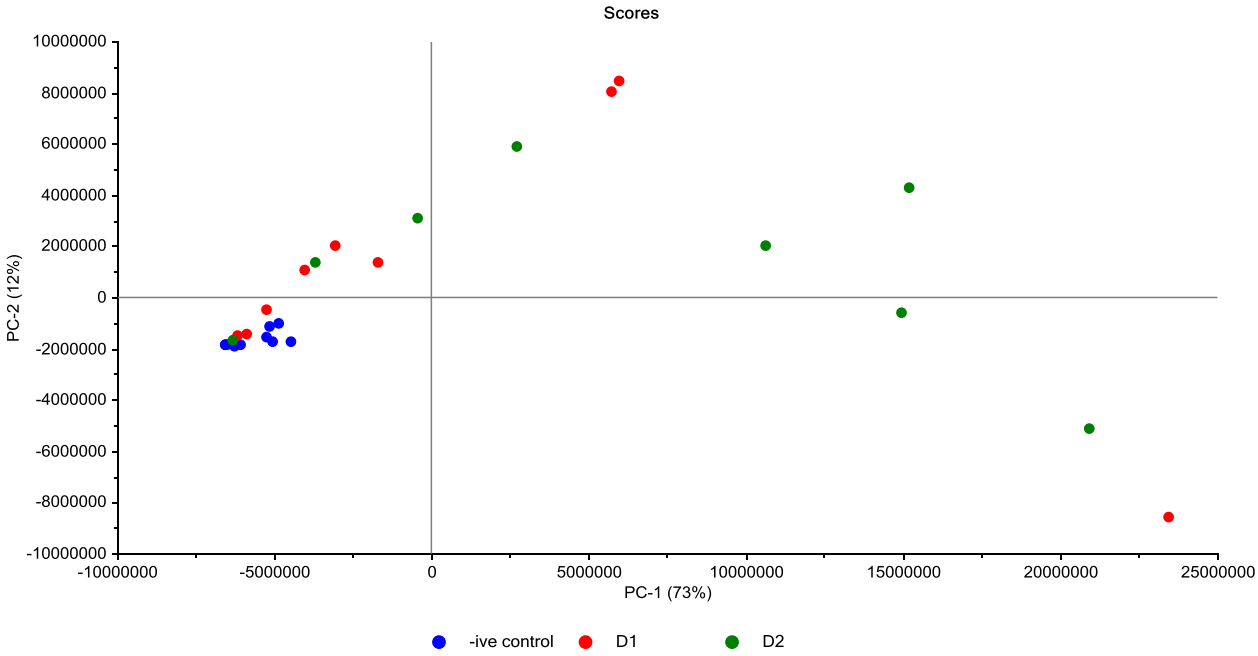

Figure 8. Principle Component Analysis (PCA) scores plot illustrating the arrangement of the samples according to enzyme treatment. The plot was generated from the 215 nm data of the Reverse Phase (RP) separations of the hydrolysates. Negative control samples (blue) digest 1 (red) and digest 2 (green) samples each have time points taken at 1, 3, 4, 5, 22, 28, 46, and 52 h.

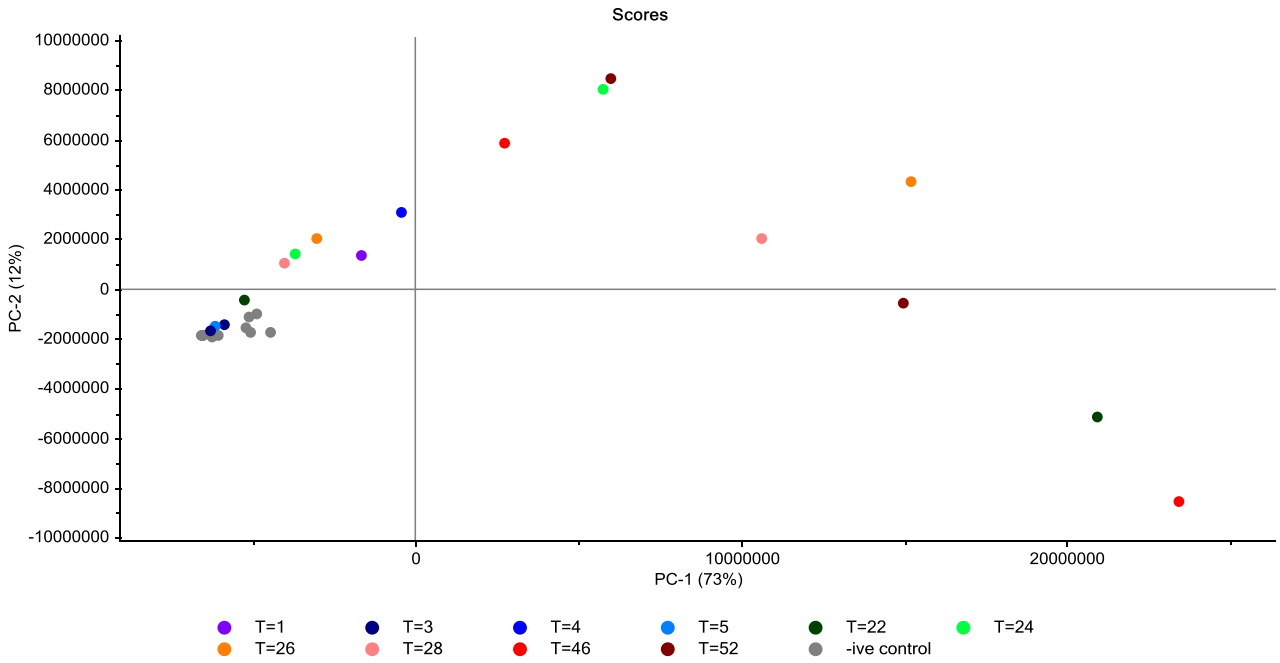

Figure 9. Principle Component Analysis (PCA) scores plot illustrating the arrangement of the samples according to time. The plot was generated from the 215 nm data of the Reverse Phase (RP) separations of the hydrolysates. Digestion times are indicated in the legend. Negative control samples are grey.

Figure 7 highlights the complexity of the data that needed to be analysed and the importance of the Unscrambler software in elucidating useful information from this data. This figure contains only three out of the 46 chromatograms, and making meaningful comparisons would have been too cumbersome and difficult to do manually. Manual comparisons would have taken a lot of time and effort and had a high probability of missing subtle variances between samples. Once the traces were digitised PCA could be applied to allow the samples to be grouped according to trends deciphered by the software, as discussed in the introduction (section 1.5).

The negative control samples clustered together, indicating that they had a high degree of similarity to each other (Figure 8). Conversely, the duplicate digest treatments were more spread out, indicating that they differed from both the

negative control samples and each other. However, when these samples were labelled by digestion time (Figure 9) a pattern began to emerge. Samples that were digested for shorter times (1, 3, 4 or 5 h) grouped near to the negative control samples. Samples taken at 22 h or longer were more widely distributed across PC1. One interpretation of this result is that after 22 h the digests were sufficiently complete so that no further trends could be discerned. This would mean that sometime before 22 h the degree of hydrolysis could be effectively considered to be 100% complete. For a better indication of when the hydrolysis was approaching effective completion a second experiment with shorter time points was necessary.

An interesting observation in Figure 9 is that there was a trend up and then down on the PC2 axis that seemed to be related to the PC1 variance. It is not clear why this was, but it could indicate a second compositional change that was occurring in the samples.

3.2.5 RP-HPLC with chemometric analysis- short time points

A set of geoduck digests were performed with four digest conditions and over five h. The four digest conditions were negative control (no added protease), Enzidase 899 (1% Enzidase 899 added), flavourzyme (1% flavourzyme added) and mix (0.5% Enzidase 899 and 0.5% flavourzyme added). Incubation times were 0, 5, 10, 20, 30, 40, 50, 60, 90,120, 150, 180, 240 and 300 min. Samples were separated using RP-HPLC and the 215 nm data was transferred into The Unscrambler software. PCA was performed and a scores plot which described 80% of the variance (61% from PC1 and 19% from PC2) was generated (Figure 10).

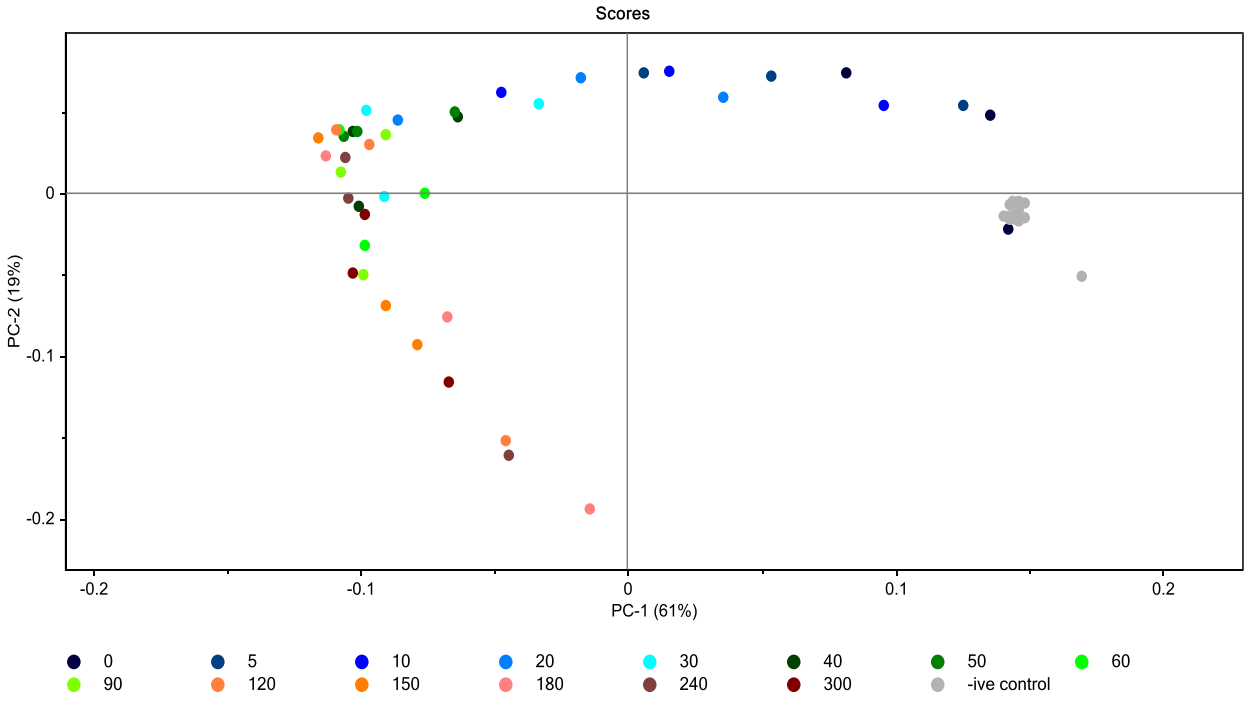

Figure 10. Principle Component Analysis (PCA) scores plot illustrating the arrangement of the samples according to time. Plot was generated from the 215 nm traces of the Reverse Phase (RP) separations of the hydrolysates. Digestion times are indicated in the legend. Negative control samples are in grey.

Using the shorter digestion times for analysis the software was able to extract greater detail about the progress of the hydrolysis (Figure 10). A trend can be seen on the PC1 axis where the samples that were taken after longer digestion times have moved to the left, away from the negative control samples. After approximately 120 min the trend changes, and the longer time points move vertically on the PC2 axis. The PC1 and PC2 axes are influenced by different peaks in the HPLC traces which suggests that the composition of the peptide mixtures is changing differently with time.

The time dependant peptide composition changes are visible in the loadings plots for this data set (Figure 11A and 11B). Both PC1 and PC2 were strongly influenced by the large peak eluting at 35 min, the peak eluting at 20 min affected

the PC1 loading more than the PC2 loading, and the peaks eluting at 26 and 55 min were more influential on the PC2 loadings.

The change in the progression of the samples from the PC1 axis to the PC2 axis with time is consistent with a two stage hydrolysis. This could suggest a situation where there were a large number of more accessible peptide bonds that could be cleaved quickly by the proteases, leading to a rapid change in the peptide profile. It is possible that this would then be followed by a second stage of hydrolysis of less accessible peptide bonds. No clear trends are apparent between the 180, 240 and 300 min samples and they are evenly dispersed along the PC2 axis, suggesting that by this stage the hydrolysis by exogenous enzymes was largely complete. However, as concluded earlier, the mixture would continue to hydrolyse if permitted because of the action of endogenous proteases and microbes. From these results it was determined that 240 min of digestion was a suitable hydrolysis time for obtaining a complex mixture of peptides from the raw geoduck material. This peptide mixture could then be taken forward for the other stages of this project; membrane fractionation, HPLC analysis and bioactivity testing.

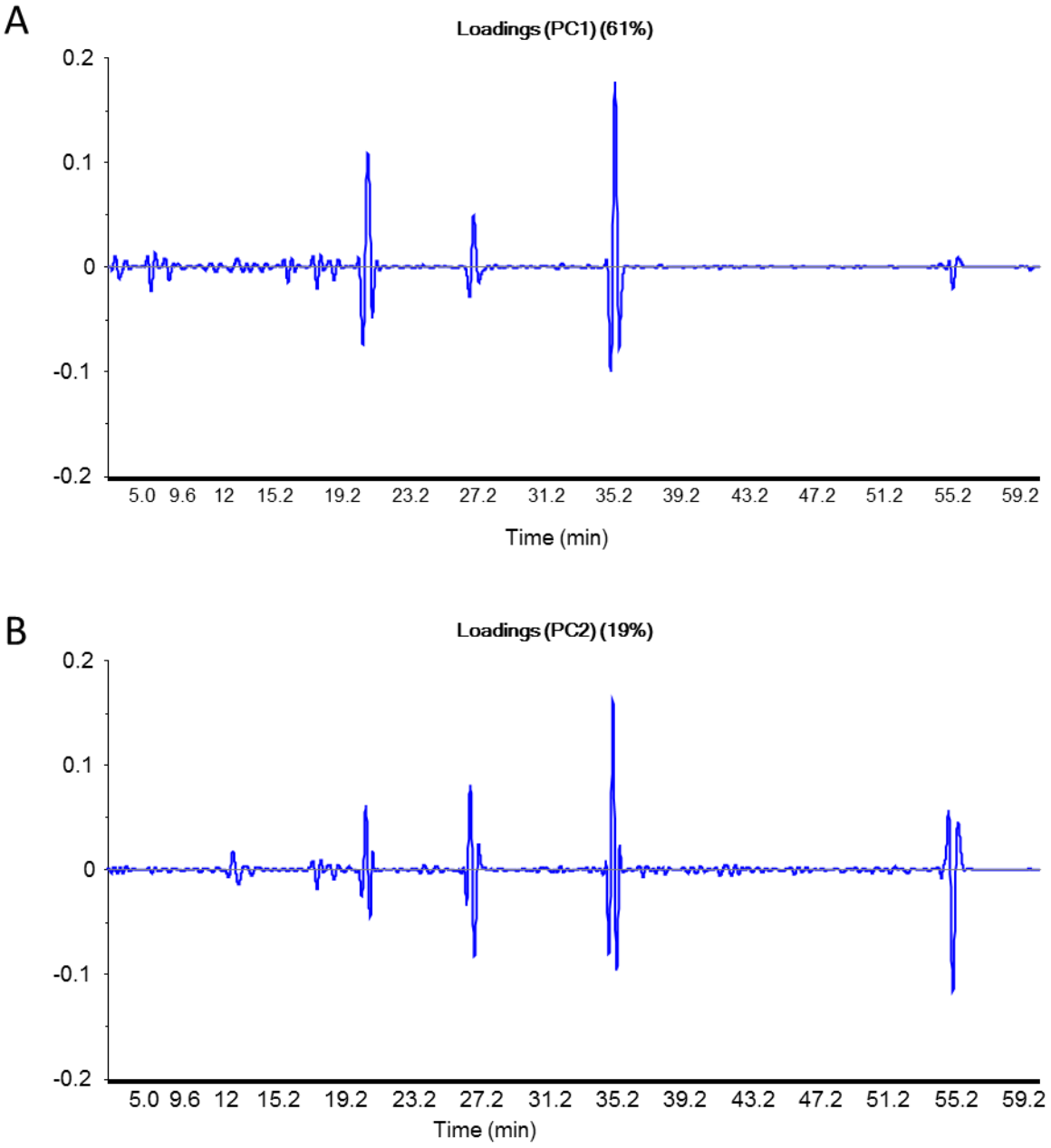

Figure 11. PCA loadings plots showing the contribution of the different HPLC peaks on the PC1 (A) and PC2 (B) axes.

3.3 Geoduck hydrolysate preparation and storage

Geoduck hydrolysis was performed for 240 min with two different enzyme systems as described in the methods. The yields were 278.8 g of freeze dried GD1 hydrolysate (endo-peptidase digest) and 276.4 g of freeze dried GD2 hydrolysate (endo- and exo- peptidase digest).

Samples were taken at various times during the digests to track the progress of the hydrolysis and the effects of the filtration. These samples were run on 16.5% Tris-tricine gels (Figure 12 and Figure *13*). In both digests the samples taken at different times during the hydrolysis indicated a gradual shift from larger, high MW proteins into smaller low MW peptides as the digestion proceeded. The samples after depth filtration also showed a depletion of medium and high MW material. This was more distinct in the GD2 gel (Figure *13*). The GD1 gel (Figure 12) appeared to have very high MW material in this sample, which may have been indicative of contamination, or post filtration aggregation which was not reversible by the addition of SDS and β-mercaptoethanol in the gel loading buffer. It was unlikely that this material was aggregated prior to filtration as it would have been too bulky to pass through the depth filter so presumably the aggregation occurred during storage. Freeze drying and ultrafiltration had no effect on the samples as these lanes look the same as the depth filtered samples. The predominant band in these final samples was from material that was smaller than 1.5 kDa, which were the hydrolysed peptides.

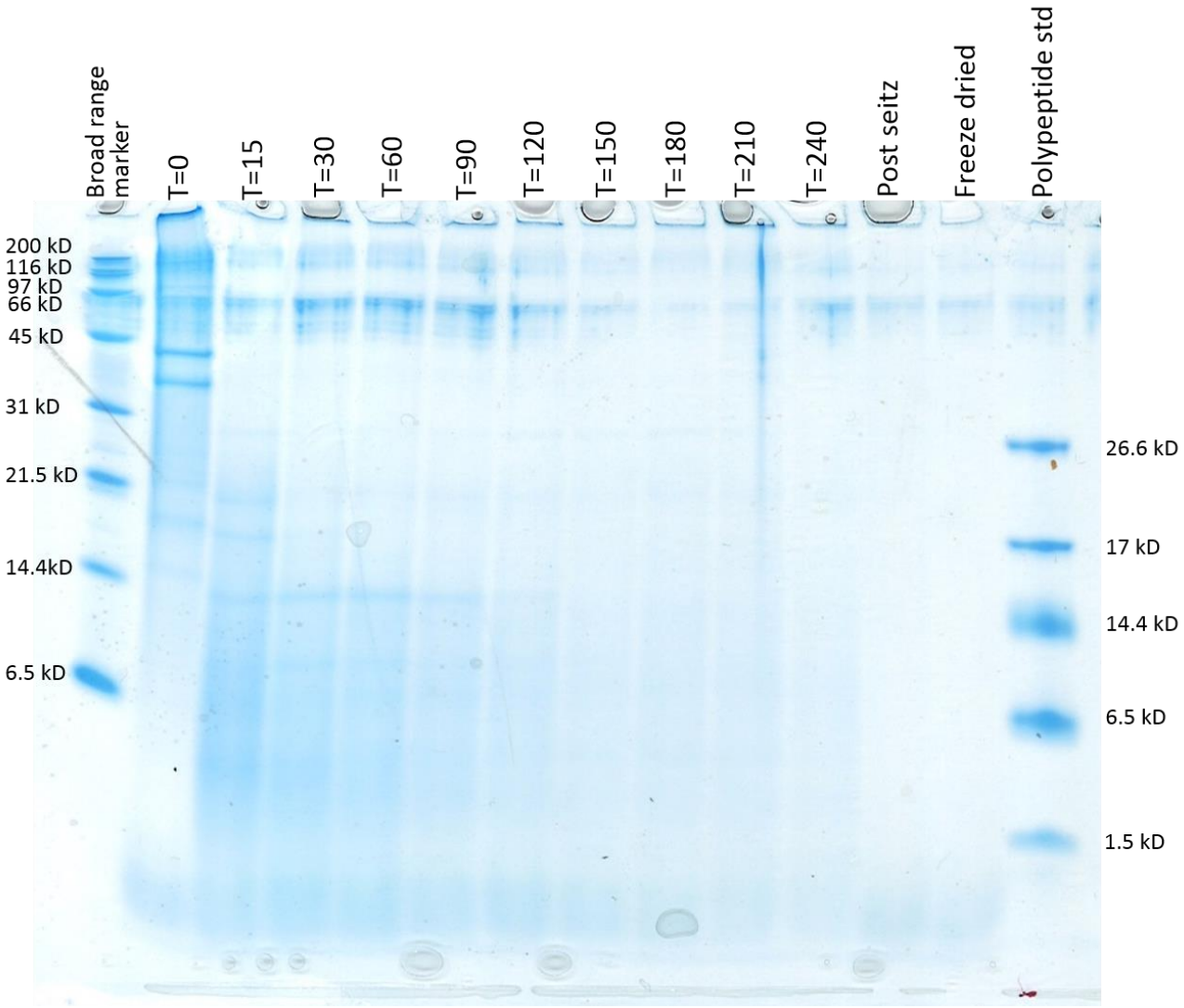

Figure 12. 16.5% PAGE gel of geoduck hydrolysis at various times with Enzidase 899 (GD1).

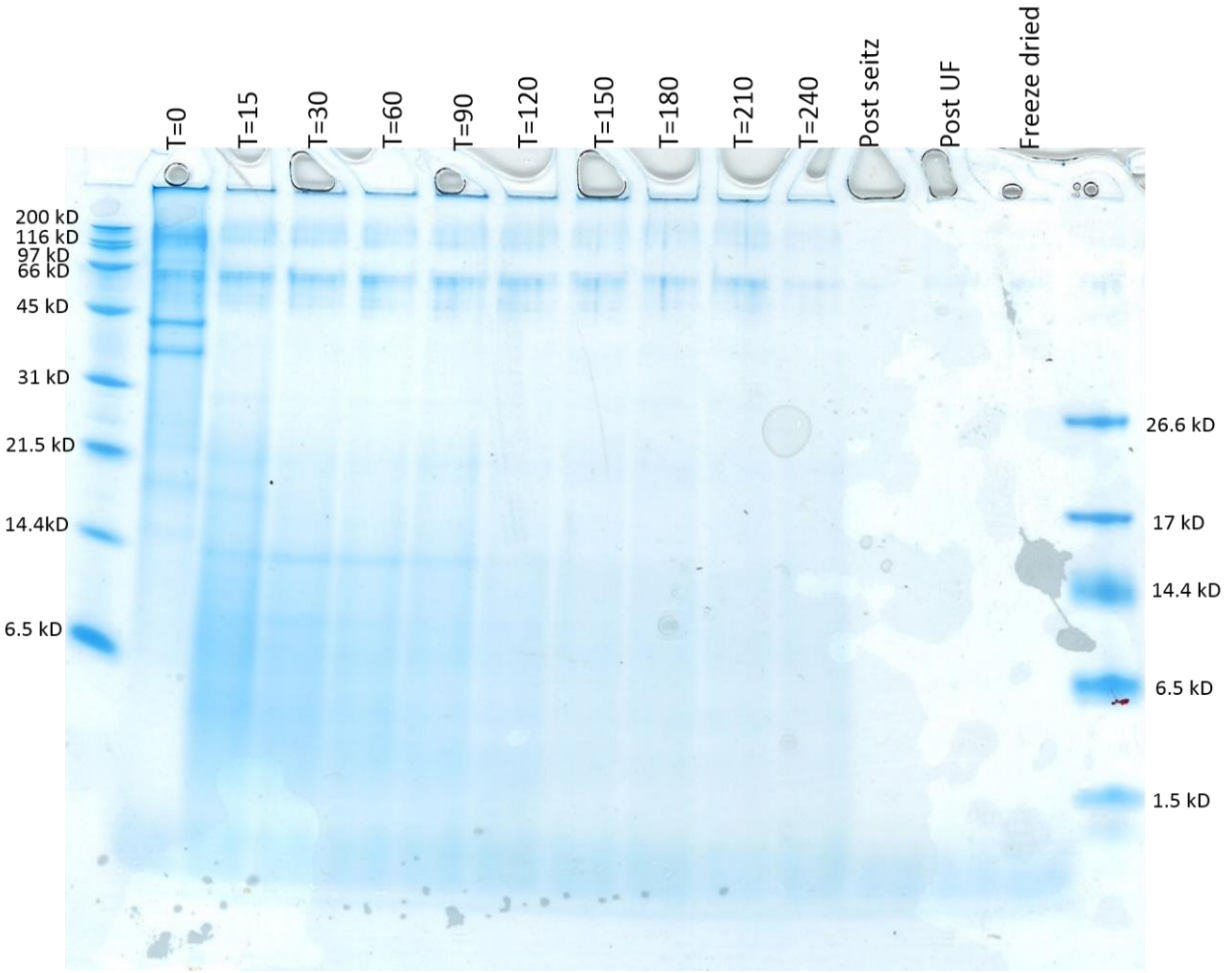

Figure 13. 16.5% PAGE gel of geoduck hydrolysis at various times with Enzidase 899 and Flavourzyme (GD2).

3.3.1 Dry weight analysis

Dry weight analysis was performed in triplicate on the homogenised geoduck and the dry weight percentage was determined to be 18.5% (+/- 0.03) (Supplementary Table 9). The wet weight of the minced geoduck in each digest was 4.4 kg, equivalent to 814 g of dry material for hydrolysis. This yielded 278.8 g and 276.4 g of GD1 and GD2 freeze dried hydrolysate, respectively. The freeze dried hydrolysates therefore represent 34% of the input weight showing that the proteases were able to solubilise approximately one third of the available solids during hydrolysis.

3.4 Membrane separations

3.4.1 Introduction

The membrane separations performed in this research are grouped into two classes: size separations, selecting peptides on the basis of their molecular weight; and charge separations, selecting peptides on the basis of their charge under particular conditions.

In the size experiments a pH of 10 was selected. This was because most papers (Jeon et al., 2000; Pouliot et al., 1999; Tessier, Harscoat-Schiavo, & Marc, 2006) report that a basic pH for carrying out size separations is advantageous as this pH has fewer issues related to membrane fouling (pH 10 for Jeon, pH 9 for Pouliot, pH 9 for Tessier). A pH of 10 was selected in order to maintain consistency with the charge separations.

A third possible mode of separation was also considered that would exploit the hydrophobicity of the peptides. A number of ultra-hydrophilic membranes with highly charged surfaces are available commercially for removing oil from waste water. It was thought that these membranes could potentially be used to fractionate peptides by selectively rejecting hydrophobic peptides and allowing passage of more hydrophilic ones. Preliminary experiments (not reported) with these membranes showed that they were not currently a feasible option for investigation as the MWCO of these membranes is too large (50 kDa) to permit selectivity of peptides (<10 kDa), and all of the material passed rapidly through the membrane. It is possible that in the future these membranes could be produced with smaller pore sizes and could then be an option for further investigation.

Information about the conductivity and peptide concentration from the OPA assay (Table 10 and Table 11, supplementary data) were used to determine the percentage of the total amount of each of these components that had passed through the membrane (Table 4). Information about the permeation rate (flux) (Table 5) was also obtained during the membrane separations. This data could be used to make comparisons between the membranes which would be helpful for future membrane-related decisions.

The maximum percentage of permeation of the peptide was 75% because only 150 mL of the 200 mL input was allowed to pass through the membrane. Therefore, for the purposes of these experiments, a sample with approximately 75% permeation can be considered to have had zero rejection.

Table 4. Percentage of NaCl and peptide in the permeate and retentate samples as determined by conductivity and OPA assay. Membrane fraction code is in the format: hydrolysate type (GD1 or GD2), membrane type, pH (10, 7 or 4) and permeate/retentate/input. Eg. "GD1DK10 Perm" is the permeate from the GD1 hydrolysate separated using the DK membrane when the separation was performed at pH 10.

Sample name	MWCO of membrane	% distribution of NaCl	% distribution of peptide
GD1DK10 Permeate	150-300 Da	18.2	0.9
GD1DK10 Retentate	150-300 Da	81.8	99.1
GD1NFG10 Permeate	600-800 Da	55.2	14.3
GD1NFG10 Retentate	600-800 Da	44.8	85.7
GD1XT10 Permeate	1 kDa	70.5	54.6
GD1XT10 Retentate	1 kDa	29.5	45.4
GD1GH10 Permeate	2.5 kDa	55.4	21.9
GD1GH10 Retentate	2.5 kDa	44.6	78.1
GD1MT10 Permeate	5 kDa	65.8	27.1
GD1MT10 Retentate	5 kDa	34.2	72.9
GD1PW10 Permeate	10 kDa	76.0	62.9
GD1PW10 Retentate	10 kDa	24.0	37.1
GD1XT7 Permeate	1 kDa	71.7	63.8
GD1XT7 Retentate	1 kDa	28.3	36.2
GD1XT4 Permeate	1 kDa	69.3	41.6

GD1XT4 Retentate	1 kDa	30.7	58.4
GD1V310 Permeate	30 kDa	70.2	54.0
GD1V310 Retentate	30 kDa	29.8	46.0
GD1V37 Permeate	30 kDa	75.0	74.0
GD1V37 Retentate	30 kDa	25.0	26.0
GD1V34 Permeate	30 kDa	74.9	73.4
GD1V34 Retentate	30 kDa	25.1	26.6
GD2DK10 Permeate	150-300 Da	35.0	28.0
GD2DK10 Retentate	150-300 Da	65.0	72.0
GD2NFG10 Permeate	600-800 Da	61.2	40.5
GD2NFG10 Retentate	600-800 Da	38.8	59.5
GD2XT10 Permeate	1 kDa	74.3	14.8
GD2XT10 Retentate	1 kDa	25.7	85.2
GD2GH10 Permeate	2.5 kDa	60.2	23.3
GD2GH10 Retentate	2.5 kDa	39.8	76.7
GD2MT10 Permeate	5 kDa	68.7	50.2
GD2MT10 Retentate	5 kDa	31.3	49.8
GD2PW10 Permeate	10 kDa	77.5	34.4
GD2PW10 Retentate	10 kDa	22.5	65.6
GD2XT7 Permeate	1 kDa	72.4	40.6
GD2XT7 Retentate	1 kDa	27.6	59.4
GD2XT4 Permeate	1 kDa	74.2	75.6
GD2XT4 Retentate	1 kDa	25.8	24.4
GD2V310 Permeate	30 kDa	72.5	71.8
GD2V310 Retentate	30 kDa	27.5	28.2
GD2V37 Permeate	30 kDa	73.8	69.5
GD2V37 Retentate	30 kDa	26.2	30.5
GD2V34 Permeate	30 kDa	69.1	54.4
GD2V34 Retentate	30 kDa	30.9	45.6

Table 5. Membrane permeation rates (flux)

	Total time (min)	Permeate volume (mL)	Average flow (mL/min)
GD1DK10	510	76	0.15
GD1NFG10	390	135	0.35
GD1XT10	135	150	1.11
GD1GH10	415	135	0.33
GD1MT10	189	150	0.79
GD1PW10	84	150	1.79
GD1XT7	103	150	1.46
GD1XT4	175	150	0.86
GD1V310	92	150	1.63
GD1V37	0.7	150	214.29
GD1V34	0.8	150	187.50
GD2DK10	466	112	0.24
GD2NFG10	418	145	0.35
GD2XT10	268	151	0.56
GD2GH10	647	142	0.22
GD2MT10	441	150	0.34
GD2PW10	42	150	3.57
GD2XT7	326	150	0.46
GD2XT4	168	150	0.89
GD2V310	53	150	2.83
GD2V37	21	150	7.14
GD2V34	156	150	0.96

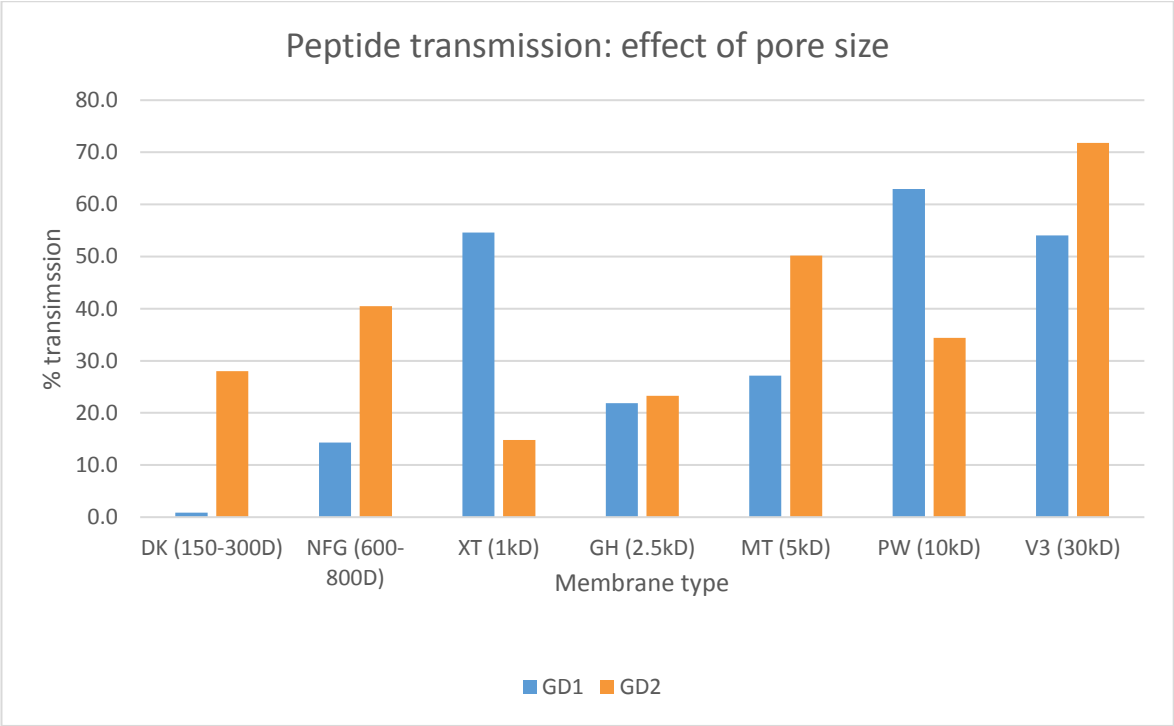

Figure 14. Effect of pore size on peptide transmission through membranes with differing pore size characteristics.

Figure 14 illustrates the effects of pore size on the permeability of the peptides and demonstrates that with the smallest pore sizes the amount of peptide that was permitted to pass through the membranes was variable but in general was proportional to the pore size properties of the membranes. In a number of situations this did not hold true, suggesting that factors other than just pore size were at play such as the physicochemical interactions of the peptides and the membranes.

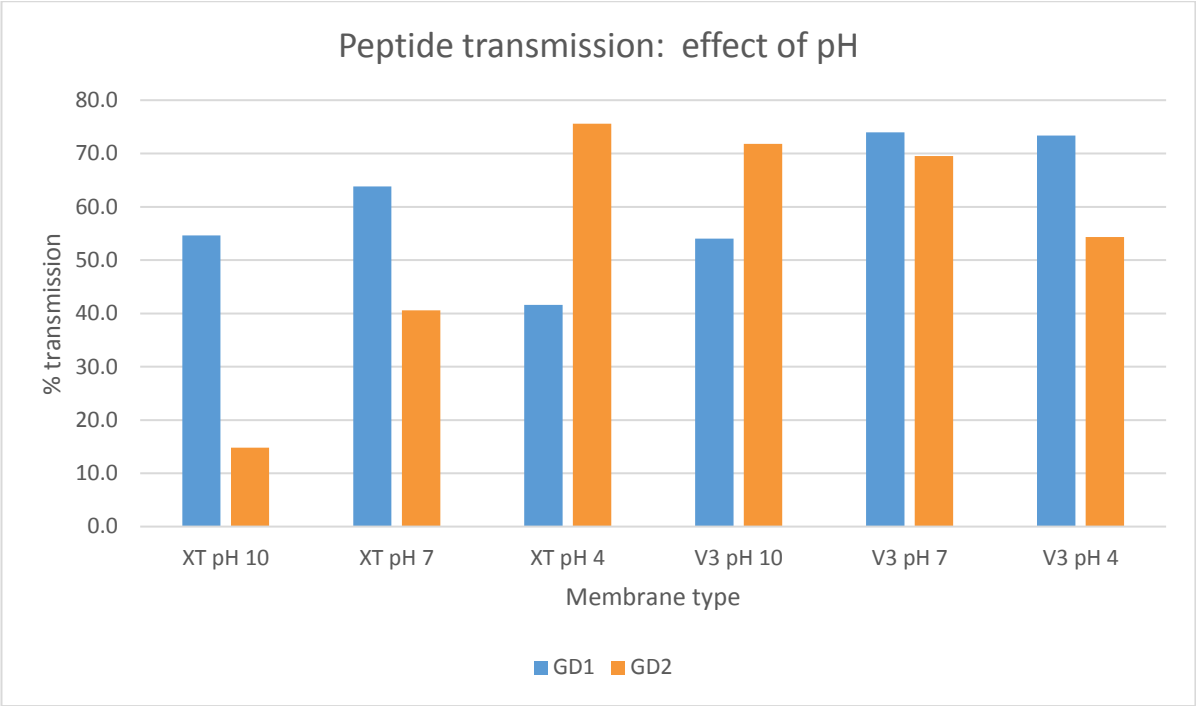

Figure 15. Effect of pH on peptide transmission through two membranes (XT membrane: 1 kDa MWCO, -ve charge. V3 membrane: 30 kDa MWCO, +ve charge).

Figure 15 illustrates the effects of altering the pH of the peptide solutions when membranes with charged surfaces are used, and demonstrates that the pH affects the permeability of peptides. In particular the permeability of the GD2 hydrolysate was reduced under neutral and basic conditions when filtering through the XT membrane, and the GD1 hydrolysate was less permeable under acidic conditions with the XT membrane.

3.5 Multivariate analysis of HPLC results

3.5.1 Comparison of hydrolysates

Samples were separated with RP and SEC HPLC as described in the methods. The differences between the two input samples (GD1 and GD2) were able to be seen by direct comparison of the traces in Figure 16 and Figure 17.

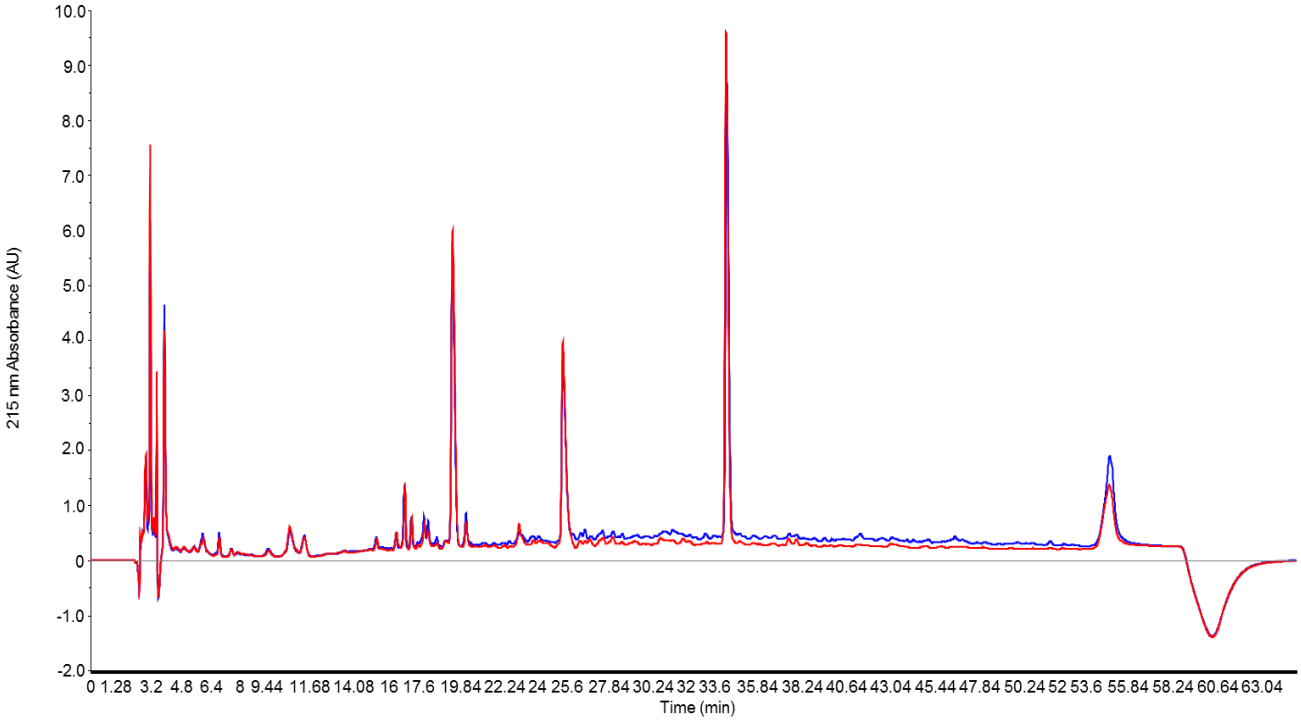

Figure 16. RP HPLC chromatograms of GD1 (blue) and GD2 (red) overlaid for comparison.

RP chromatography (Figure 16) showed that there was more material eluting later in the GD1 analysis than the GD2 analysis. This suggested that the GD1 hydrolysate had more hydrophobic material than the GD2 hydrolysate. The GD1 hydrolysate was prepared with only the endopeptidase Enzidase 899, while the GD2 hydrolysate was prepared with Enzidase 899 and also the exopeptidase flavourzyme. Exopeptidases such as flavourzyme have been shown to have specificity towards hydrophobic amino acids (Raksakulthai & Haard, 2003), which is consistent with the results observed in the RP analysis i.e. the GD2 hydrolysate

had more of the hydrophobic amino acids hydrolysed by flavourzyme and therefore the GD2 peptides are less hydrophobic.

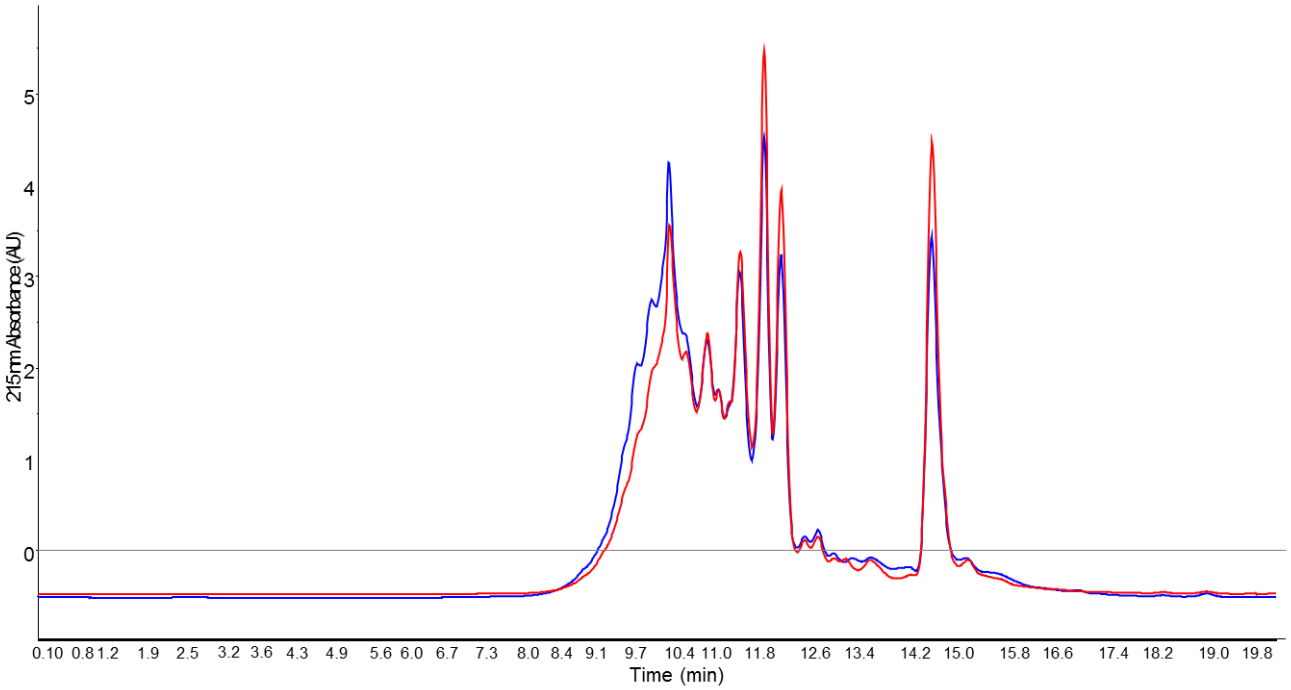

Figure 17. SEC HPLC chromatograms of GD1 (blue) and GD2 (red) overlaid for comparison.

SEC chromatography indicates that the relative amounts of high and low molecular weight material is different between the two hydrolysates (Figure 17). GD1 has more material eluting earlier in the trace, indicating a greater proportion of high molecular weight material than the GD2 hydrolysate. This is also consistent with the method of hydrolysis. The exposure to the additional protease in the GD2 digest means that more protease recognition sites were available for cleavage in this digest, leading to greater hydrolysis and smaller peptides.

3.5.2 Principle Component Analysis (PCA)

The chromatographs of the 215 nm absorbance information from the RP-HPLC and SEC-HPLC separations of the input samples, the permeates and the retentate samples were processed using The Unscrambler multivariate analysis software. The data was pre-processed as described in the methods section, except that for the RP samples an additional step was included to exclude data from before 14 min and after 58 min. This was done because during other investigations (not presented) it was found that the material eluting outside of this time period had an inconsistent elution profile, even within duplicate samples, and therefore had a disruptive effect on the PCA analysis.

Table 6. Reference table of peptide separations and numbers for interpretation of PCA plots (Figure 18 to Figure 28). Membrane fraction code is in the format: hydrolysate type (GD1 or GD2), membrane type, pH (10, 7 or 4) and permeate/retentate/input. Eg. "GD1DK10 Perm" is the permeate from the GD1 hydrolysate separated using the DK membrane when the separation was performed at pH 10.

Sample number	Membrane fraction	Sample number	Membrane fraction
1	GD1DK10 Perm	24	GD2DK10 Retentate
2	GD1DK10 Retentate	25	GD2NFG10 Perm
3	GD1NFG10 Perm	26	GD2NFG10 Retentate
4	GD1NFG10 Retentate	27	GD2XT10 Perm
5	GD1XT10 Perm	28	GD2XT10 Retentate
6	GD1XT10 Retentate	29	GD2GH10 Perm
7	GD1GH10 Perm	30	GD2GH10 Retentate
8	GD1GH10 Retentate	31	GD2MT10 Perm
9	GD1MT10 Perm	32	GD2MT10 Retentate
10	GD1MT10 Retentate	33	GD2PW10 Perm
11	GD1PW10 Perm	34	GD2PW10 Retentate
12	GD1PW10 Retentate	35	GD2XT7 Perm
13	GD1XT7 Perm	36	GD2XT7 Retentate
14	GD1XT7 Retentate	37	GD2XT4 Perm
15	GD1XT4 Perm	38	GD2XT4 Retentate
16	GD1XT4 Retentate	39	GD2V310 Perm
17	GD1V310 Perm	40	GD2V310 Retentate
18	GD1V310 Retentate	41	GD2V37 Perm
19	GD1V37 Perm	42	GD2V37 Retentate
20	GD1V37 Retentate	43	GD2V34 Perm
21	GD1V34 Perm	44	GD2V34 Retentate
22	GD1V34 Retentate	45	GD1 pH 10 input
23	GD2DK10 Perm	46	GD2 pH 10 input

Figures 18, 20, 22, 25 and 27 are of the PCA scores plot from the RP-HPLC data, but with the individual samples coloured differently according to the treatment/ condition. Figures 19, 21, 23, 26 and 28 are of the PCA scores plot from the SEC-HPLC data.

These figures illustrate the selective effect of the membranes on the hydrolysate samples. For both the RP and SEC data the intersection of the lines is at the centre of the plots and is very close to where the input samples (numbered 45 and 46) have been placed. Permeate and retentate samples that are in this region of the plots are more similar to the unprocessed input samples, indicating that the membranes involved have been less effective at separating these samples. Points which are located further away from the centre show that these are less similar to the input samples, and that the membranes used in these separations have been more effective.

3.5.3 Permeates and retentates

Figure 18 and Figure 19 show that the PCA of the HPLC data is able to distinguish between the permeate and retentate samples. For both data sets the permeate samples tend to group towards the positive values on the PC1 axis (to the right), and the retentate samples tend to group towards the negative values on the PC1 axis (to the left).

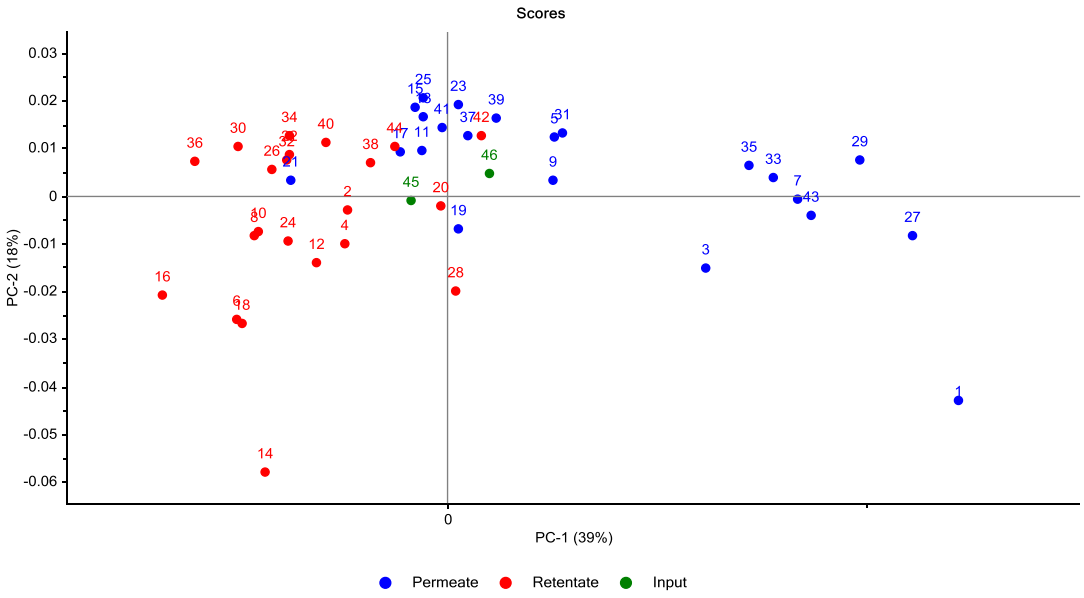

Figure 18. PCA of RP HPLC data from the membrane separations with samples marked according to whether they are input (green), permeate (blue), or retentate (red).

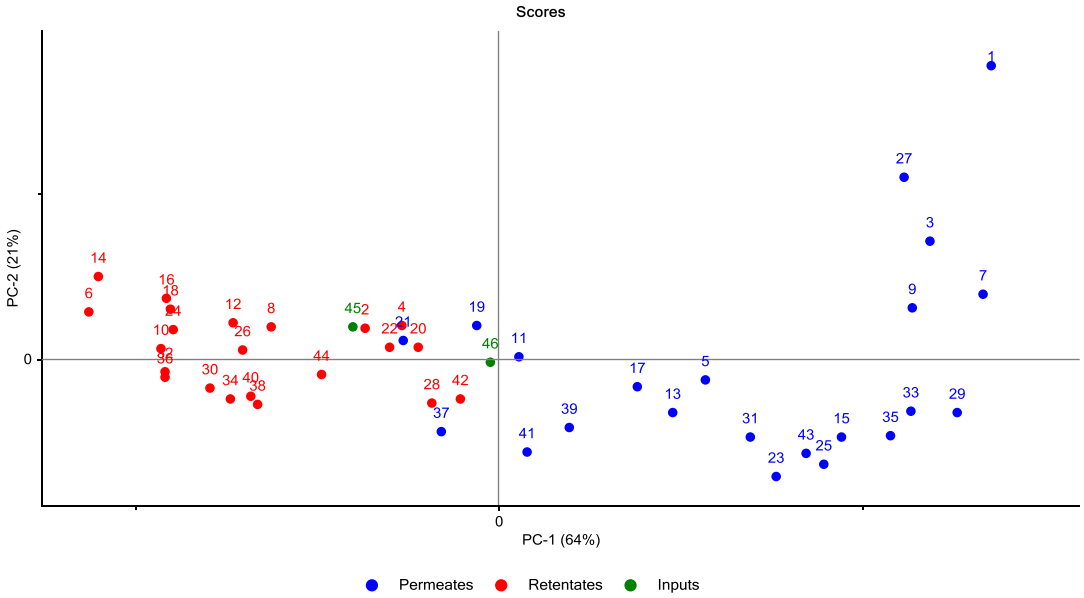

Figure 19. PCA of SEC HPLC data from the membrane separations with samples marked according to whether they are input (green), permeate (blue), or retentate (red).

In Figure 20 and Figure 21 the points are coloured according to which enzyme treatment was used to generate the hydrolysate, blue for GD1 (endopeptidase) and red for GD2 (endo-exopeptidase). Separation along the PC2 axis can been seen for both the RP and SEC plots, although more clearly for the SEC data. This

indicates that the different proteases affected the peptide composition enough to be seen on the scores plot. The difference was not attributable to any variation in peptide concentration as concentration effects were excluded during the SNV part of the pre-processing of the data.

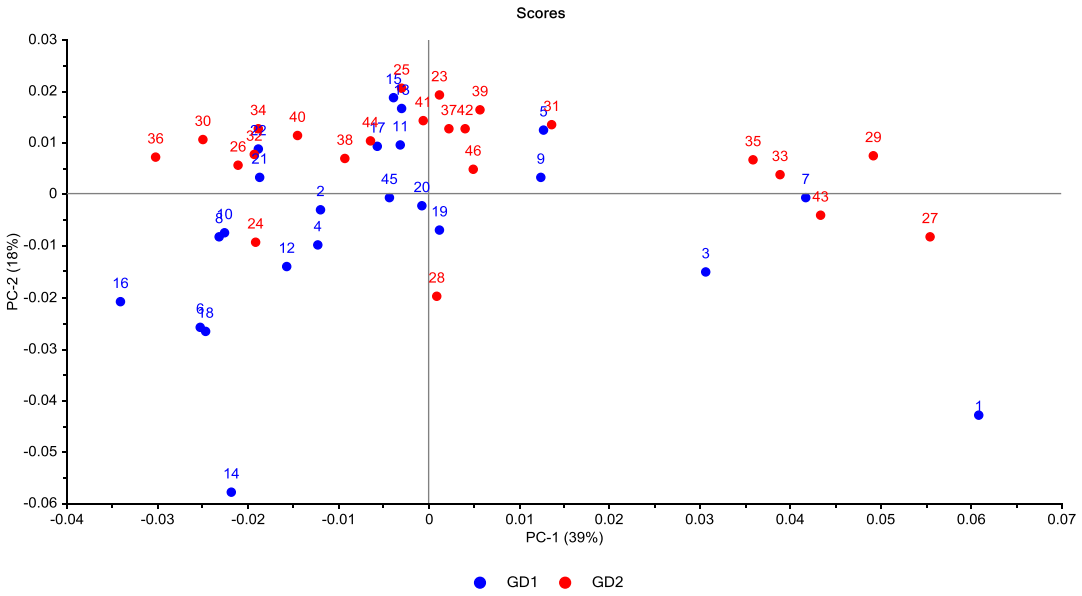

Figure 20. PCA of RP HPLC data from the membrane separations with samples marked according to the enzyme treatment. GD1/ Enzidase 899 (blue) and GD2/ Enzidase 899+flavourzyme (red).

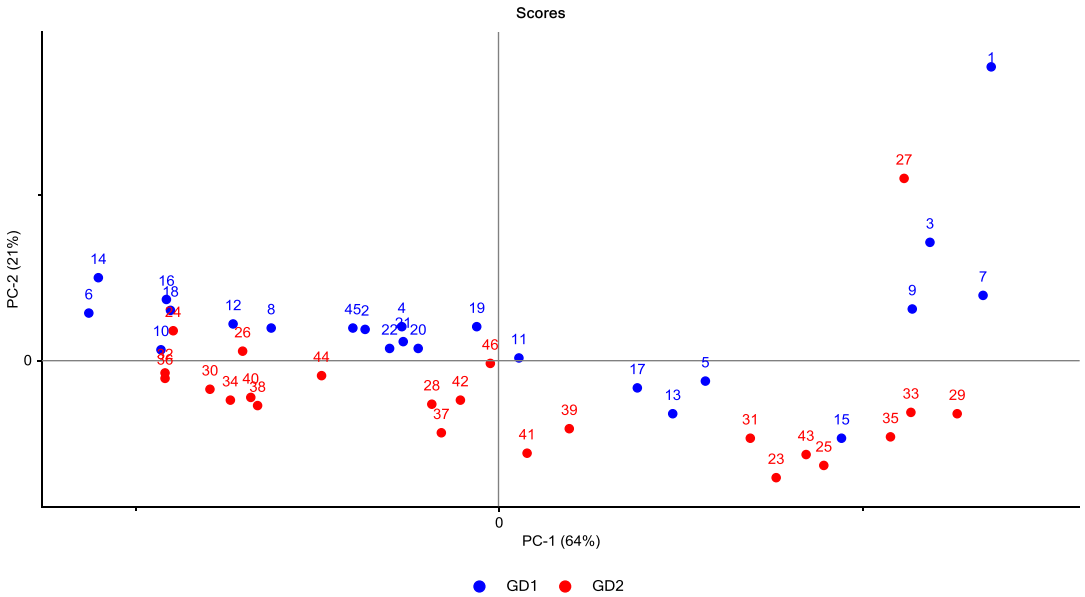

Figure 21. PCA of SEC HPLC data from the membrane separations with samples marked according to the enzyme treatment. GD1/ Enzidase 899 (blue) and GD2/ Enzidase 899+flavourzyme (red).

This result shows that the PCA analysis can arrange samples according to the hydrolysate type. It is possible that an analysis of numerous hydrolysates generated from diverse enzymes and substrates could be used to build a predictive PCA model. Hydrolysates with greater similarity would be clustered together which could be a potential method for inferring similarities in their properties, including bioactivity.

3.5.4 Membrane pore size characteristics

Figure 22 and Figure 23 show the scores plots with the points coloured according to which membrane was used for the separation. Only separations performed at pH 10 were highlighted to ensure that equivalent samples were compared, with separations performed at pH 4 and 7 coloured grey. As discussed earlier the permeate and retentate samples separated along the PC1 axis. The PC1 data from Figure 22 and Figure 23 were therefore used to generate a second graph (Figure 24) which provided a quantitative representation of the effectiveness of the membranes in separating the peptides. The y-axis values in this graph represents the difference between PC1 values of the permeate and retentate.

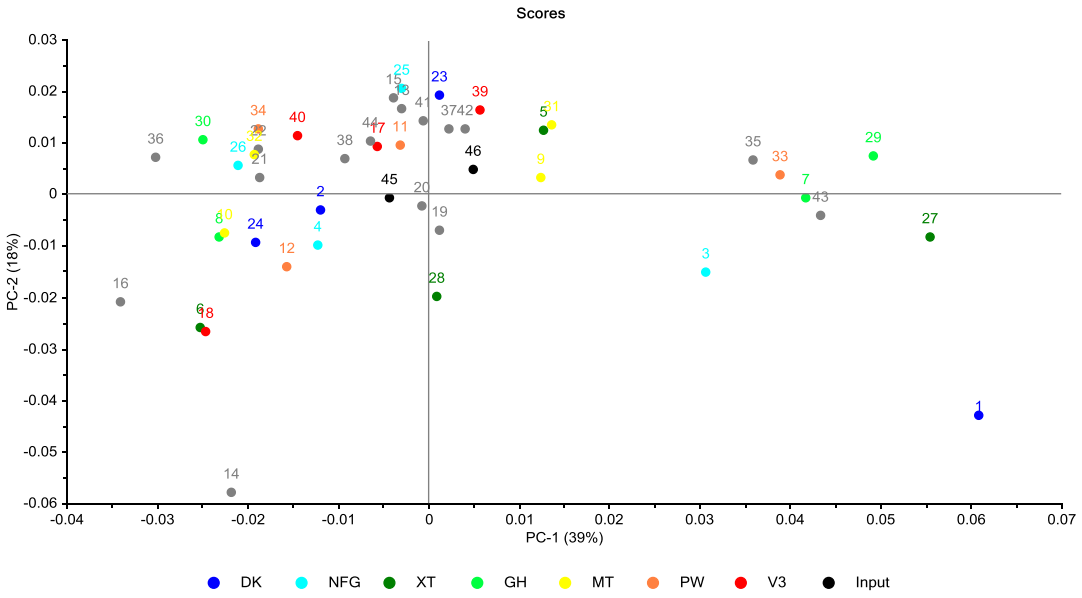

Figure 22. PCA of RP HPLC data from the membrane separations with samples marked according to the membrane used. All coloured samples were separated at pH 10. Samples separated at pH 4 or 7 are coloured grey. MWCOs of membranes: DK 150-300 Da; NFG 600-800 Da; XT 1 kDa; GH 2.5 kDa; MT 5 kDa; PW 10 kDa; V3 30 kDa.

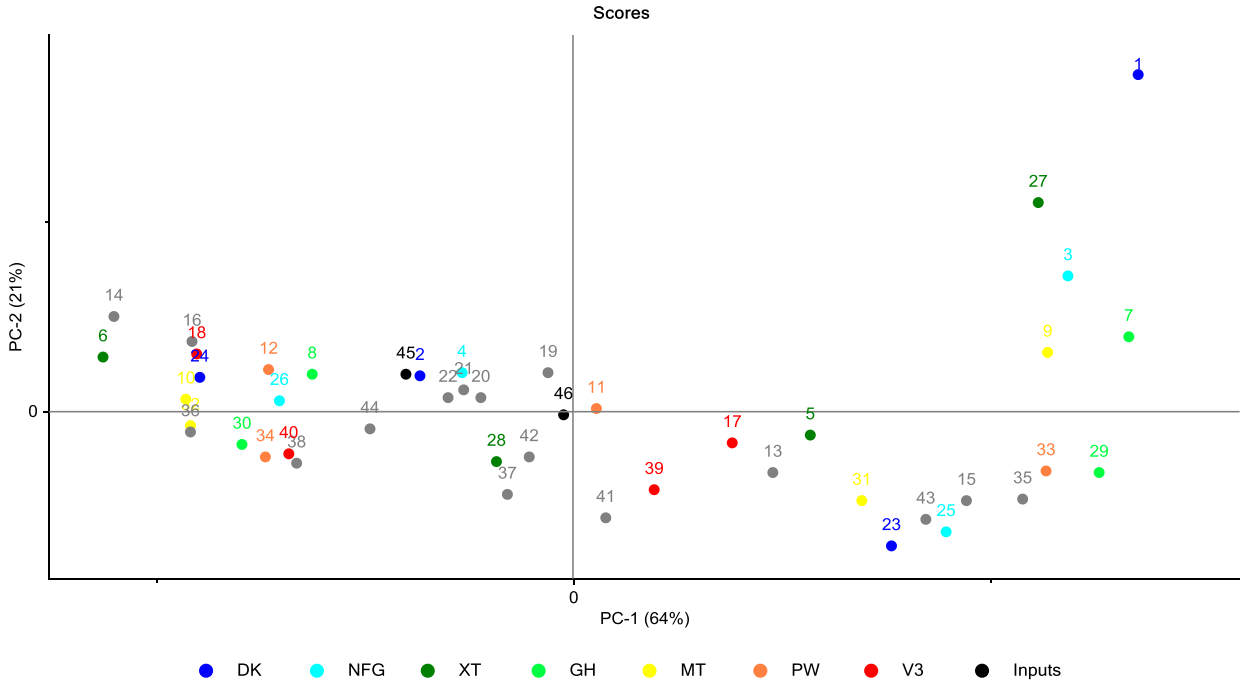

Figure 23. PCA of SEC HPLC data from the membrane separations with samples marked according to the membrane used. All coloured samples were separated at pH 10. Samples separated at pH 4 or 7 are coloured grey. MWCOs of membranes: DK 150-300 Da; NFG 600-800 Da; XT 1 kDa; GH 2.5 kDa; MT 5 kDa; PW 10 kDa; V3 30 kDa.

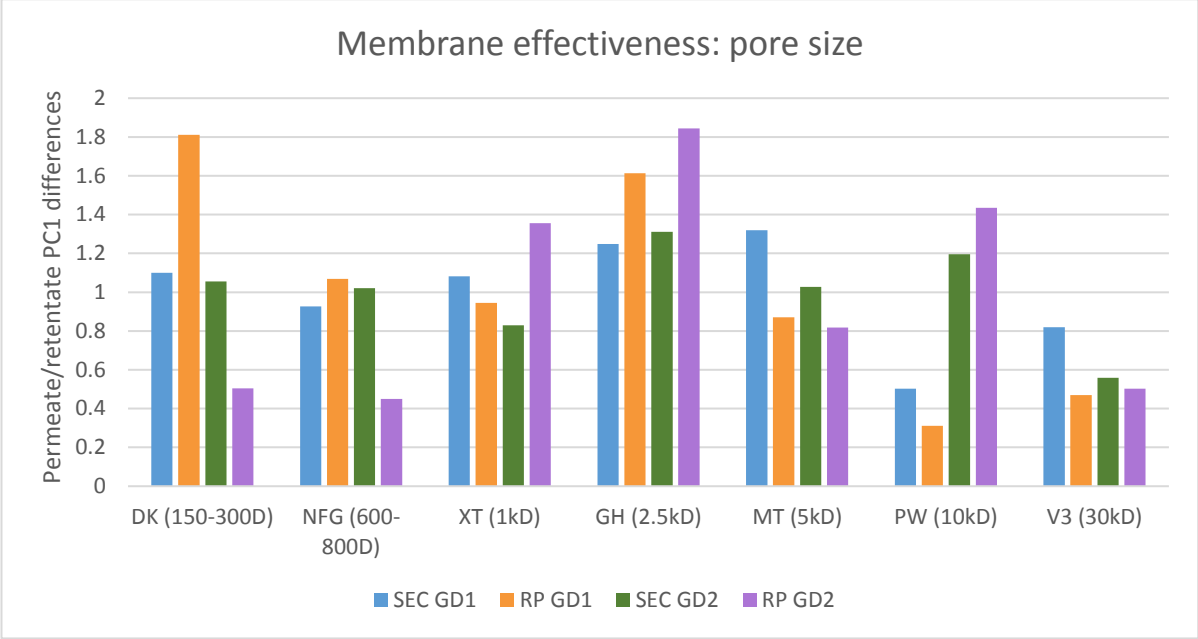

Figure 24. Membrane effectiveness as related to membrane MWCO. The difference between the PC1 scores of the permeate and retentate samples is indicative of the effectiveness of the separation. Membrane separations are all from the experiments performed at pH 10. Data is arranged by chromatography mode used and hydrolysate type: SEC of GD1 (blue), RP of GD1 (orange), SEC of GD2 (green) RP of GD2 (purple).

The results of the separation varied depending on the hydrolysate and the membrane used. The GD1 hydrolysate had good separation when processed with the DK membrane (150-300D MWCO) but poor separation with the PW membrane (10 kDa MWCO) while the GD2 hydrolysate was the opposite. The V3 membrane performed poorly with both hydrolysates, presumably because the 30 kDa MWCO membrane had pores too large to reject enough of the peptides to change the composition.

In general it appears that the three membranes with a moderate pore size; XT (1 kDa), GH (2.5 kDa) and MT (5 kDa), were the most consistently effective at achieving separation of the mixtures with both of the hydrolysates. Both GD1 and GD2 had similar changes in permeate/retentate composition when processed with these membranes. Further, the most effective membrane of these three was the GH membrane, with a MWCO of 2.5 kDa.

This result indicates that the average molecular weight of the peptides in these hydrolysates was approximately 2.5 kDa. In this case, when using the GH membrane approximately half of the peptides would have been small enough to pass through the membrane while the other half was rejected. This situation would create the greatest possible difference between the permeate and retentate samples. A membrane with an excessively small pore size would reject most of the peptides, only allowing the very smallest molecules through. This would produce a permeate with a higher concentration of only a very small population of peptides, and a retentate with a peptide population that was largely unchanged. Conversely, a membrane with a pore size that was so large that it would only reject the largest peptides would produce a retentate that had elevated concentrations of a small population of peptides, and a permeate with a peptide population that was largely unchanged.

This model explains why the 1 kDa, 2.5 kDa and 5 kDa membranes are the most consistently effective, but not why the two hydrolysates behave differently with the 150-300D, 600-800D and 10 kDa membranes. Lack of detailed information about the membranes makes it difficult to confidently comment on this, but it is notable that the GD1 hydrolysate appears to have greater separation than the GD2 peptide on the two smaller membranes, which are both described as “Thin Film” membranes, while the GD2 hydrolysate has greater separation on the larger membrane which is made of polyethersulfone (PES). It is possible that as well as separating the peptides by size, these membranes are also separating the peptides by their charge, as discussed below.

3.5.5 Membranes with charged surfaces at various pHs

The XT membrane was made of polyethersulfone (PES) which carries a negative charge at pH values of 4 or higher (Fernández et al., 2013; Susanto & Ulbricht, 2005), while the V3 membrane was a polyvinylidene difluoride (PVDF) membrane with a positively charged surface modification. Detailed information about this modification was not available from the manufacturer, but a paper investigating surface charge modifications of PVDF membranes (Breite, Went, Prager, & Schulze, 2015) found that it was possible to create membranes that maintained a positive charge at pH 10 and lower. Therefore, for the purposes of this discussion the XT membrane will be treated as having a negative charge and the V3 membrane will be treated as having a positive charge under all of the conditions tested.

Figures 25 to 28 illustrate the effect that pH had on the separations when using two different membranes. In Figure 25 and Figure 26 the samples that were separated using the negatively charged XT (1 kDa MWCO) membrane were coloured according to the pH that the separations were carried out at, while the remainder of the points were left grey.

Figure 27 and Figure 28 have the equivalent data displayed, but for the positively charged V3 (30 kDa MWCO) membrane. As for the size separation data, the PC1 data from Figure 25 to Figure 28 were used to generate a second graph (Figure 29) which provided a measurable and interpretable representation of the effectiveness of the membranes in separating the peptides. The y-axis values in this graph represented the difference between PC1 values of the permeate and

retentate. A larger difference was representative of a greater variation in the samples and therefore a more effective membrane for separating the peptides.

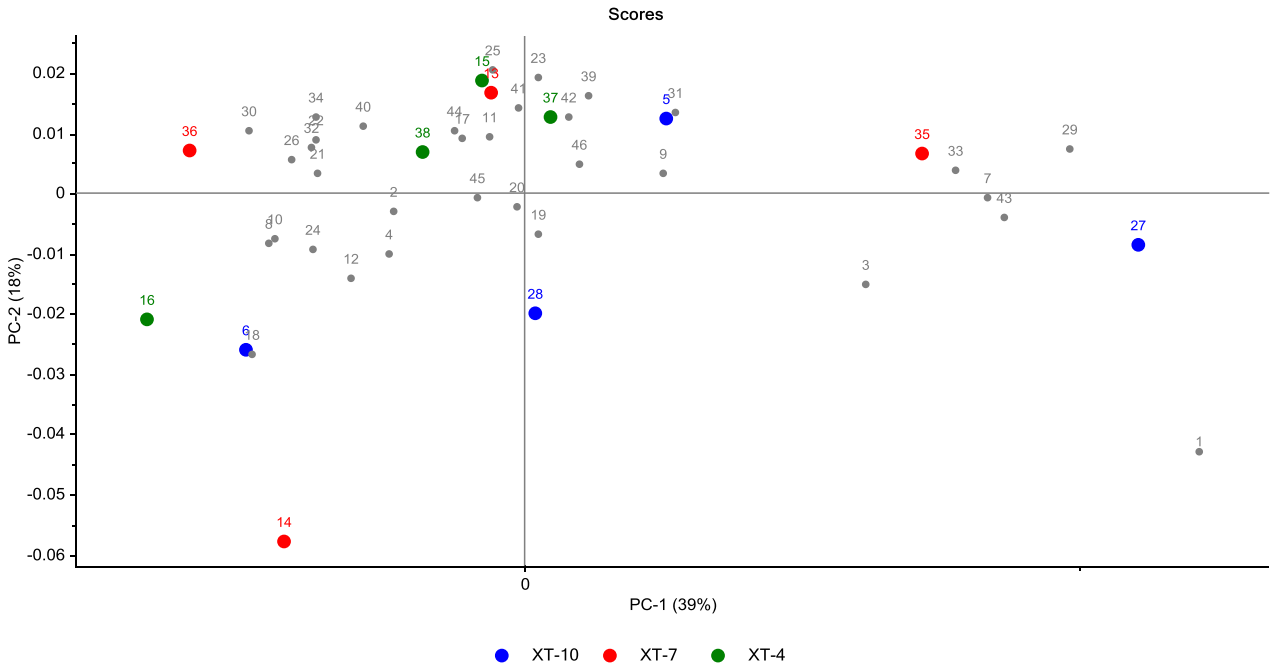

Figure 25. PCA of RP HPLC data from the membrane separations with samples marked according to the pH of the solution during separation. The coloured samples were separated with an XT (1 kDa MWCO) membrane at pH 10 (blue), pH 7 (red) and pH 4 (green). Remaining points (grey) are samples that were separated with uncharged membranes.

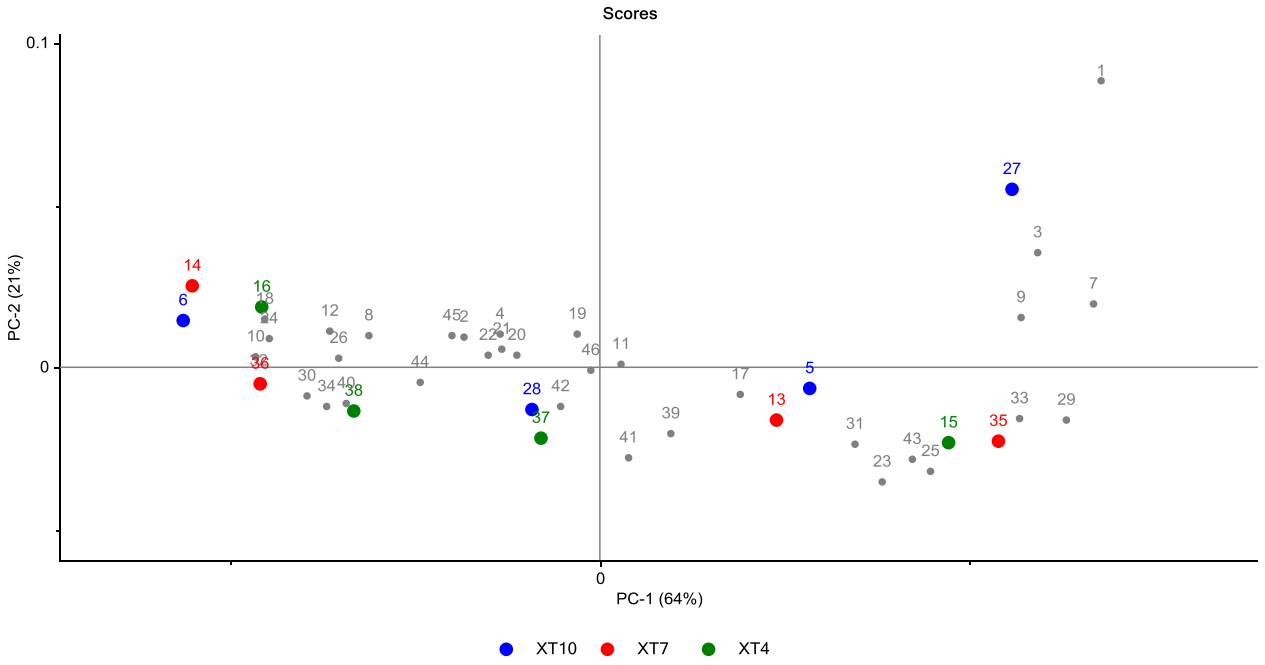

Figure 26. PCA of SEC HPLC data from the membrane separations with samples marked according to the pH of the solution during separation. The coloured samples were separated with an XT (1 kDa MWCO) membrane at pH 10 (blue), pH 7 (red) and pH 4 (green). Remaining points (grey) are samples that were separated with uncharged membranes.

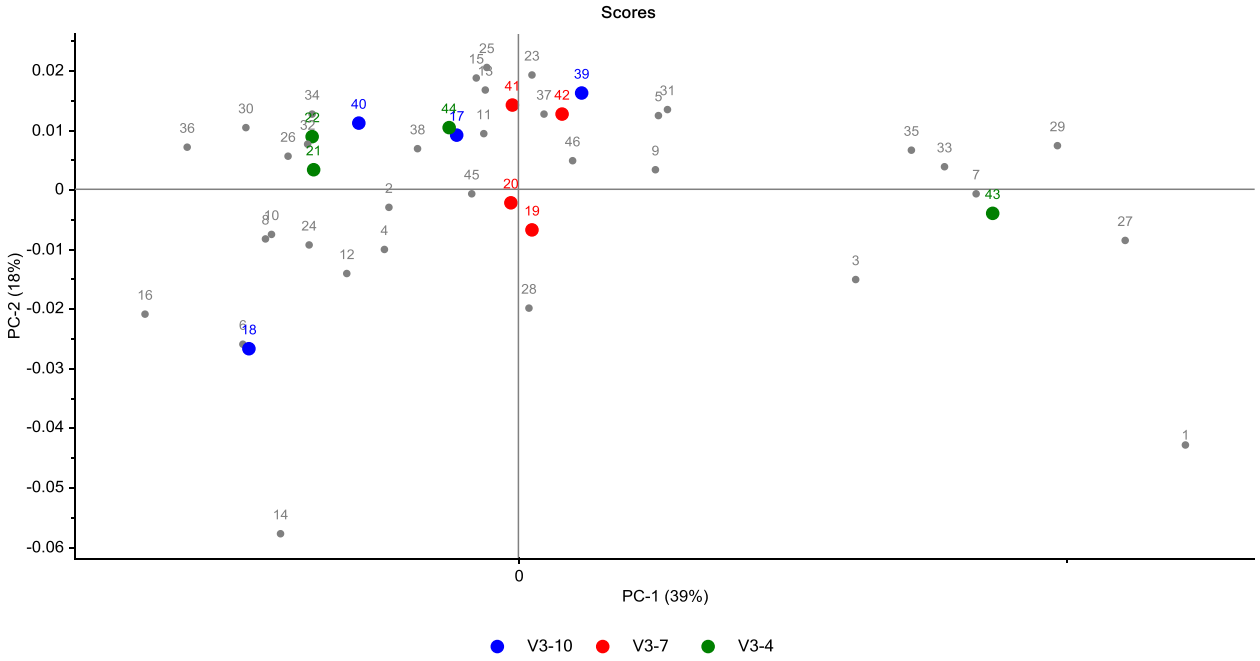

Figure 27. PCA of RP HPLC data from the membrane separations with samples marked according to the pH of the solution during separation. The coloured samples were separated with a V3 (30 kDa MWCO) membrane at pH 10 (blue), pH 7 (red) and pH 4 (green). Remaining points (grey) are samples that were separated with uncharged membranes.

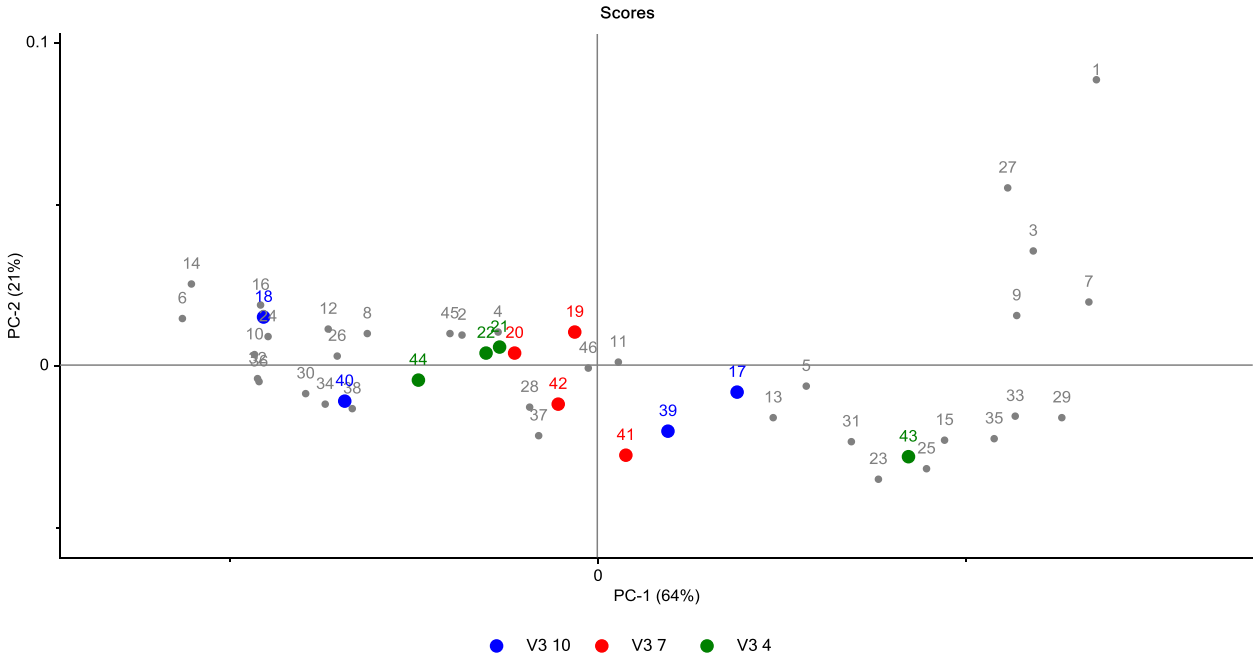

Figure 28. PCA of SEC HPLC data from the membrane separations with samples marked according to the pH of the solution during separation. The coloured samples were separated with a V3 (30 kDa MWCO) membrane at pH 10 (blue), pH 7 (red) and pH 4 (green). Remaining points (grey) are samples that were separated with uncharged membranes.

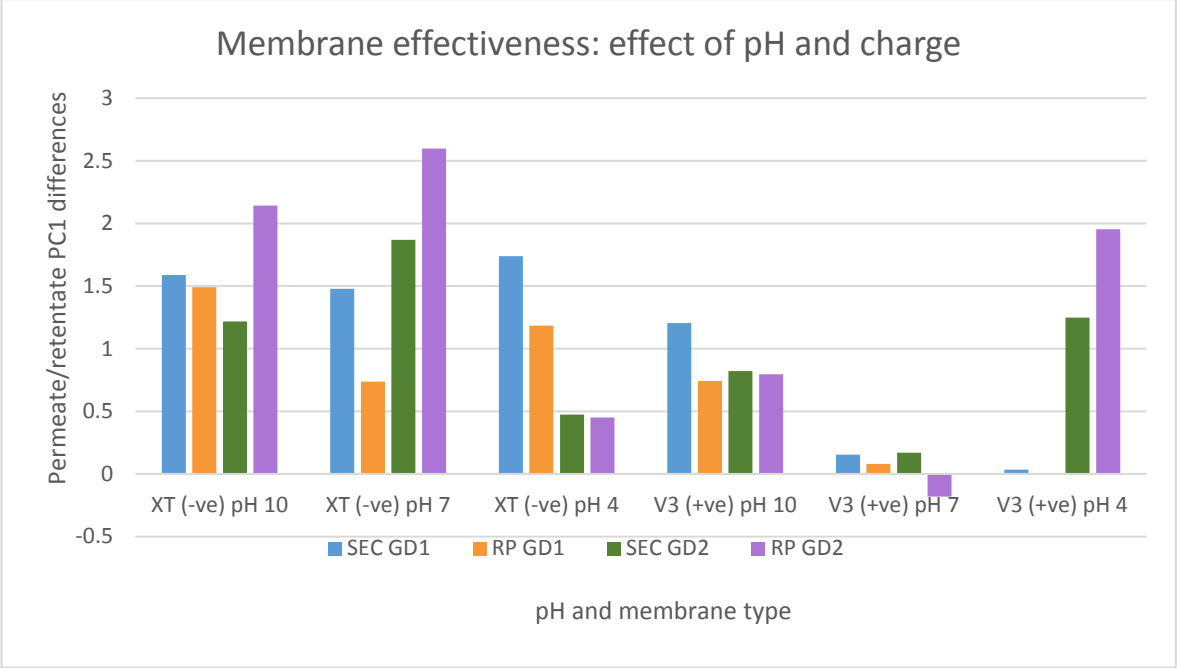

Figure 29. Effect of the membrane charge and pH of the solution on the effectiveness of the membrane separations. PC1 values from the PCA of the separations carried out at different pHs were used to calculate the effectiveness of the separations as related to charge. Retentate value subtracted from permeate value indicates the effectiveness of separation.

Figure 29 shows that the choice of the pH and type of membrane can influence the effectiveness of a peptide separation. The most obvious result from this graph was the very low difference for both GD1 and GD2 when they were filtered with the V3 membrane at pH 7. This was a consistent result with both the SEC and RP chromatography analysis, and shows that this condition was ineffective for peptide separation. This was presumably because all of the peptides were fully permeable under these conditions and no compositional changes occurred.

The V3 membrane (30kDa MWCO, +ve charge) at pH 4 gave no difference for the GD1 hydrolysate but a high response for the GD2 hydrolysate, indicating that there was some physicochemical difference between these samples affecting their interactions with the membranes. At pH 10 this difference was not observed, and both hydrolysates behaved equivalently. This will be discussed in more detail below.

With the XT membrane (1 kDa MWCO, -ve charge) both GD1 and GD2 had good separation at pH 10 and 7. At pH 4 GD2 had reduced separation, but GD1 still had effective separation. The fact that the XT membrane overall gave greater separation than the V3 membrane was likely to be related to its smaller pore size. As seen above, the XT membrane was one of the most effective tested and the V3 membrane was the least effective because its large pores were unable to reject all but the largest peptides.

It was possible to make some inferences about the peptide composition of the hydrolysates based on the results obtained from the charge separations, particularly from the V3 membrane. In the case of this membrane, the better separations were solely because of increased peptide rejection as it was clear that this membrane does not separate effectively by size. However, in the case of the XT membrane the interpretation is less clear. This membrane was already shown to be effective at separating peptides by size, so an increase in rejection (i.e. allowing fewer peptides to pass through the membrane) because of charge has the potential to either increase or decrease the effective separation depending on the peptide mixture.

Peptides can be classified as being acidic, basic or neutral. Generally, at pH values higher than 5 acidic peptides are negatively charged and at pH values below 8.5 basic peptides are positively charged. Neutral peptides have isoelectric points (pI) somewhere between 5 and 8.5 and can be positively or negatively charged depending on the pH of the solution. As described by Fernandez (Fernández et al., 2013) and Tsuru (Tsuru, Shutou, Nakao, & Kimura, 1994), under basic conditions acidic peptides are rejected by negatively charged

membranes because of repulsive electrostatic interactions with the negative surface groups, while under acidic conditions they are transmitted through the membranes more easily because they have no charge to be repelled. It might be expected that basic peptides would be more permeable under acidic conditions because the positive charge that they carry would draw them into the membrane and facilitate transmission. This is not the case however, as the influence of the Donnan effect (Donnan, 1995) causes the counter-ion concentration in the retentate to increase. This creates an electrical potential across the membrane which prevents further permeation of the basic peptides. The highest permeability then, only occurs when the peptides carry no charge. The opposite situation is also true for positively charged membranes, where basic peptides are rejected under acidic conditions because of their positive charge and acidic peptides are rejected under basic conditions because of the Donnan effect.

The results observed in Figure 29 are consistent with the GD1 hydrolysate having more hydrophobic and acidic groups than the GD2 hydrolysate. In the interactions with the V3 membrane, the GD2 peptides are separated more efficiently than the GD1 peptides at pH 4, both hydrolysates are not affected by the membrane at pH 7 and they are both affected moderately at pH 10. At pH 4 the GD1 hydrolysate would have hydrophobic and acidic groups all carrying no charge, and the peptides could pass freely through the membrane. Conversely, the basic residues on the GD2 membrane would be positively charged at this pH and would be repelled by the positively charged membrane. At pH 7 the net charge on GD2 peptides would still be positive but less so than at pH 4. The permeability of these peptides would be increased which would account for the reduced efficiency observed for this separation. Finally, at pH 10 GD1 neutral and

acidic peptides would be carrying a negative charge and be repelled by the positively charged V3 membrane because of Donnan effects. The basic GD2 peptides would carry no charge at this pH and the separation would be reduced.

3.6 Bioactivity analysis

3.6.1 Zone of inhibition bioactivity analysis

Nine strains of bacteria were chosen to be screened against for anti-microbial activity. These were selected based on the fact that they are problematic for humans, either as pathogens or food spoilage bacteria. A peptide mixture that could inhibit the growth of these bacteria would therefore have value for food preservation or as a potential lead for drug discovery. Each membrane fraction was tested in duplicate for antimicrobial activity against the nine strains of bacteria and the petri dishes were inspected for signs of inhibition of growth. No inhibition of microbial growth was observed for any of the peptide samples tested. The antibiotic samples that were used for the positive controls did exhibit clear zones of inhibition, proving that the assay was working correctly.

These results were unexpected, as previous work (unpublished) showed high levels of antimicrobial activity when using marine protein hydrolysates.

3.6.2 Antioxidant bioactivity analysis

3.6.2.1 DPPH radical-scavenging assay

Standard curves were generated from the antioxidant standards which were used to determine the equivalent amount of antioxidant compounds present in the peptide mixtures.

All values obtained using the DPPH assay were negative. This is consistent with the peptides becoming insoluble in the methanol used to dissolve the DPPH. Precipitated peptides would increase the 515 nm absorbance leading to a higher absorbance than the starting blue DPPH solution, and generating the negative results. Using this solvent system it is therefore not possible to analyse peptides

for antioxidant activity. DPPH is not readily soluble in any solvents that are compatible with peptides (Choo, Birch, & Stewart, 2009) and therefore this assay is not suitable for investigation of water soluble bioactive peptides.

3.6.2.2 FRAP free radicle scavenging assay

A standard curve was generated using a solution of Trolox made up in phosphate buffered saline at pH 7.4. The membrane separated peptide samples were then tested and Trolox equivalent values were determined (Figure 30)

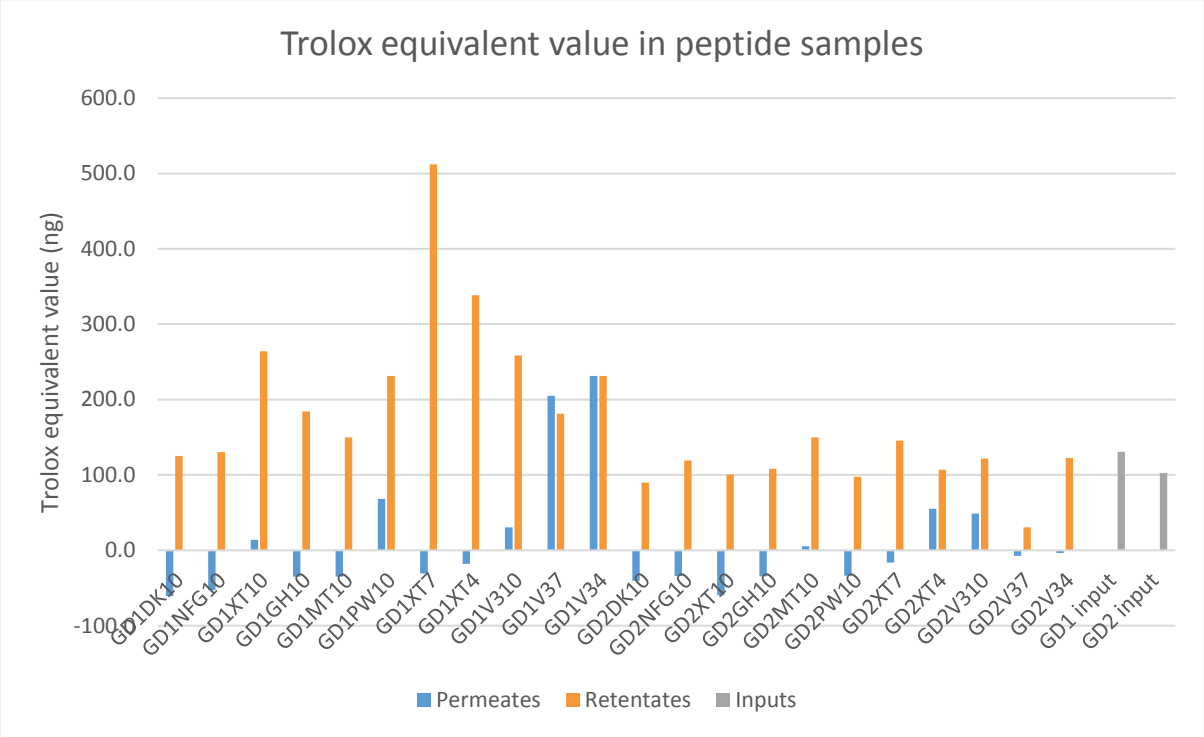

Figure 30. Trolox equivalent values in membrane separated peptides from the FRAP assay.

Figure 30 shows that there are marked differences in antioxidant activity between the permeate and retentate samples. In most cases the permeate samples had very low or negative values, while all of the retentate samples had positive values of varying intensity. The input samples also had positive values.

It is notable that the two instances where the antioxidant activity was equivalent in the permeate samples were with GD1V37 and GD1V34. These two samples were separated using the V3 membrane, with a pore size of 30 kDa. The next smallest pore size was the PW membrane (10 kDa MWCO) which permitted the passage of some of the active component, and then the 5 kDa MWCO MT membrane rejected all of it. This suggested that the antioxidant component was smaller than 30 kDa and able to pass easily through the V3 membrane, but larger than 5 kDa and unable to pass the MT membrane.

It is also notable that the antioxidant component only passed through the V3 membrane at the pH of 10, and was rejected at pH 4 and 7. This suggested that the antioxidant peptide had a high molecular weight and a pI greater than 7. At pH 4 and 7 this peptide would have carried no charge and been able to pass through the large pores of the positively charged V3 membrane. At pH 10 the peptide would have become negatively charged and been prevented from crossing the membrane because of Donnan effects. Because of its high molecular weight this peptide was rejected by all of the membranes with pore sizes smaller than 30 kDa.

For GD2 the antioxidant peptide does not appear to be as permeable with any of the membranes as for GD1. It is possible that the reduced hydrophobic amino acids in the GD2 peptides (as demonstrated in Figure 16) because of the action of the exopeptidase flavourzyme has made the peptides relatively more polar and therefore less permeable. The antioxidant activity of the GD2 peptides also appears to be lower than GD1 which may also be related to the loss of hydrophobic peptide residues. Other authors have described the importance of

hydrophobic amino acids to the bioactivity of antioxidant peptides (Peña-Ramos, Xiong, & Arteaga, 2004).

The GD1 hydrolysate samples had greater variability in antioxidant activity than the GD2 hydrolysate samples and seemed to have equivalent or greater activity. Particularly notable is the GD1XT10 retentate sample, with equivalent antioxidant activity to 500 ng of Trolox. This sample was the Enzidase 899 only geoduck hydrolysate, separated using the 1 kDa MWCO XT membrane at a pH of 10.

3.6.3 Relating HPLC data with bioactivity

One of the aims of this project was to develop methods that could be used to observe peptide separations by membrane treatment, and to relate these separations to changes in bioactivity. The data from the PCA analysis of HPLC data described above can be compared with the results of the FRAP assay in order to achieve this goal (Figure 31 and Figure 32). The differences between the permeate and retentate values were calculated from both the FRAP results and from the PCA data from the HPLC analyses (RP and SEC). The scale of the two sets of data that were to be compared were very different, so each set was normalised to bring it into a comparable scale. The size separations, all carried out at pH 10 and using membranes with variable MWCOs, are compared in Figure 31. The charge separations, using the negatively charged XT membrane and the positively charged V3 membrane at pH 4, 7 and 10, are compared in Figure 32. The relationships between the two HPLC-derived data sets and the FRAP bioactivity assay were also calculated (Table 7).

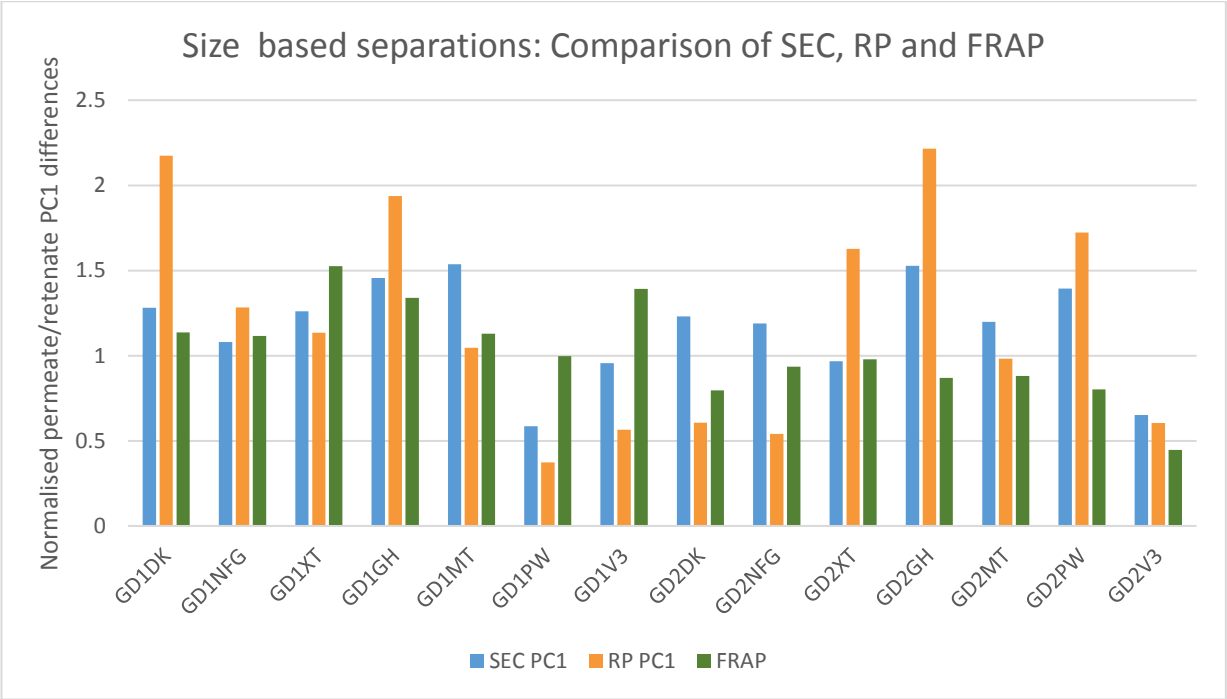

Figure 31. HPLC PCA1 data vs FRAP bioactivity results comparing membranes separating by pore size. Differences between the permeate and retentate PC1 scores data were calculated from the SEC-HPLC and RP-HPLC PCA analyses to give SEC PC1 (blue) and RP PC1 (orange) information. Differences between the permeate and retentate trolox equivalents were calculated from the FRAP assay results to give the FRAP data (green). All results were normalised against an average of the data in order to put them on a comparable scale.

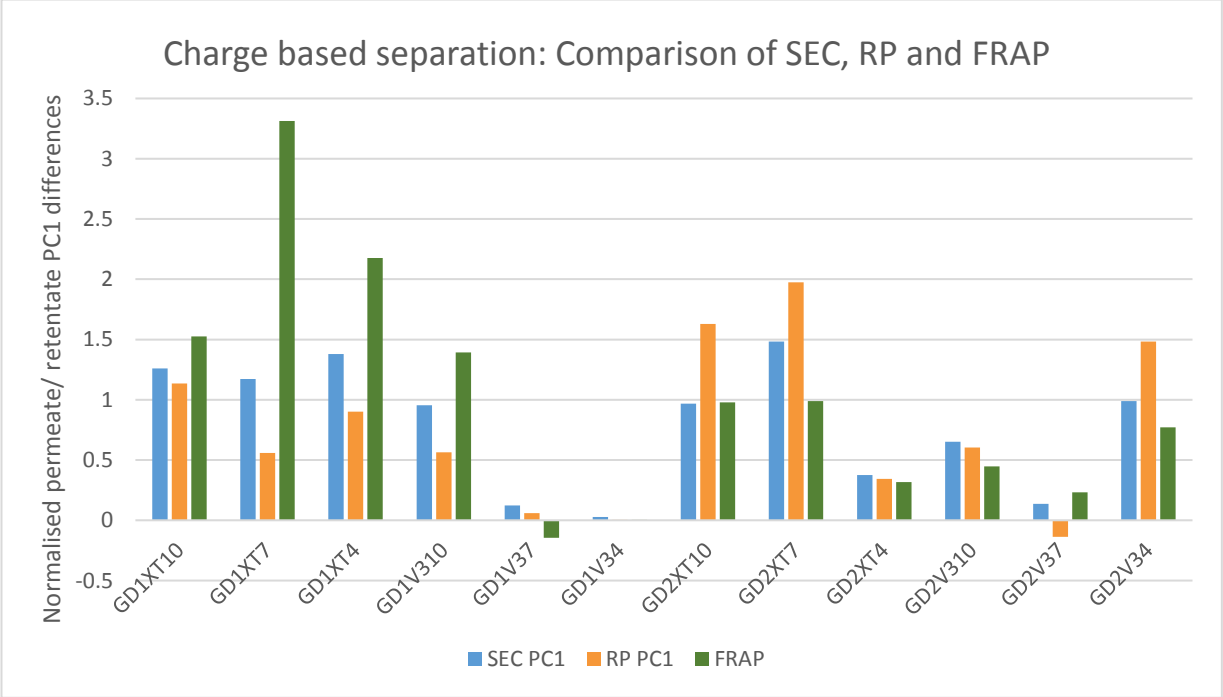

Figure 32. HPLC PCA1 data vs FRAP bioactivity results comparing membranes separating by charge. Differences between the permeate and retentate PC1 scores data were calculated from the SEC-HPLC and RP-HPLC PCA analyses to give SEC PC1 (blue) and RP PC1 (orange) information. Differences between the permeate and retentate trolox equivalents were calculated from the FRAP assay results to give the FRAP data (green). All results were normalised against an average of the data in order to put them on a comparable scale.

Table 7. R^2 correlation values showing the relationship between the data extracted from the HPLC analyses and the FRAP bioactivity data.

	Separation by size	Separation by charge
SEC data: RP data	R^2=0.3856	R^2=0.6526
SEC data: FRAP data	R^2=0.079	R^2=0.5566
RP data: FRAP data	R^2=0.0247	R^2=0.0911

Figure 31 and Figure 32 suggest that there is some relationship between the data extracted from the RP and SEC chromatograms and the FRAP data. The R2 values calculated for these data sets in Table 7 show that only some of these relationships are at a level high enough to be considered significant. The two HPLC data sets have a moderate correlation (R2=0.3856) in the size separation samples, and a higher degree of correlation in the charge separation samples (R^2=0.6526). This is a reassuring result which shows that the two different chromatographic techniques are responding to the differences in peptide composition in hydrolysates treated with the membranes.

Figure 32 and Table 7 also show that there is a high degree of correlation between the SEC data set and the FRAP results (R^2=0.5566). The remaining correlations were weak, having R2 values lower than 0.1.

4 Conclusion

This project successfully demonstrated that ultrafiltration membranes could be used to fractionate complex mixtures of peptides. In addition, it also showed that HPLC analysis coupled with multivariate data analysis is a powerful technique for interpreting and measuring the differences occurring in these separations.

The purpose of this project was to investigate the ability of membrane technology to selectively separate peptides from within protein hydrolysates. In order to achieve this goal methods were developed that could be used to quantify the effectiveness of these separations. Additionally, methods were developed to identify a suitable level of hydrolysis when generating the hydrolysates.

Several methods for determining suitable hydrolysis conditions were investigated. The assays that monitored the progress of the hydrolysis by the generation of acid demonstrated a number of things. Firstly, the rate of acid production appeared to be largely reproducible, as the duplicate hydrolysis reactions used for the time course assay tracked very closely in the titrations. Secondly, the raw geoduck used as the protein substrate contained endogenous proteases with approximately 20% of the activity of the added proteases. This result was observed in both of the pH titration experiments and was also indicated on the PAGE gels as a decrease in high molecular weight material with time. It was determined that in order to maintain reproducibility between batches of hydrolysate it would be necessary to ensure that sufficient exogenous protease was added, otherwise the action of the endogenous enzymes would dominate the hydrolysis and determine the product properties.

HPLC analysis coupled with chemometric interpretation of the data showed that the hydrolysis appeared to have a rapid initial stage and then a slower secondary stage. This was shown by the two-stage change in the peak profiles of the HPLC analysis. The HPLC/ chemometric analysis also confirmed that a suitable time for hydrolysis was 240 min. This enabled sufficient hydrolysis without complete degradation of the proteins into amino acids.

The dry weight analysis of the homogenised geoduck and the hydrolysed geoduck showed that approximately 34% of the available input solids had been solubilised during the hydrolysis.

Analysis of the hydrolysate samples by RP-HPLC and SEC-HPLC showed that the GD1 hydrolysate was larger and more hydrophobic than the GD2 hydrolysate. This is consistent with the expected behaviour of single and multiple protease systems. The GD1 hydrolysate was prepared using only the single endopeptidase Enzidase 899, while the GD2 hydrolysate was prepared using Enzidase 899 followed by the exopeptidase Flavourzyme. Flavourzyme has different recognition sites on protein substrates and therefore a greater number of cleavages would have occurred in the GD2 hydrolysis, leading to the generation of smaller peptide fragments. In addition, Flavourzyme specifically targets hydrophobic residues at the ends of peptides which would lead to less of these amino acids being included in GD2 hydrolysate, reducing the hydrophobicity.

Chemometric evaluation of the RP-HPLC and SEC-HPLC data demonstrated a number of things. The HPLC-chemometric analytical approach was capable of distinguishing between the permeate and retentate samples from the membrane

separations, and to quantify the effectiveness of these separations by the degree of similarity to the input hydrolysates used. This method was also able to differentiate between the two different enzyme treatments, and points to the possibility of the development of a predictive peptide model that could group peptides with similar properties.

The results obtained indicated that for the hydrolysates used, the most effective membranes were those with mid-range pore sizes, between 1 kDa and 5 kDa, with the most effective being the 2.5 kDa membrane. In addition, the permeate-retentate differences showed that an appropriate choice of membrane and pH conditions can be used to optimise the separation of the peptides by their charge characteristics.

Bioactivity assays on the membrane fractions using the FRAP assay identified a component present in both GD1 and GD2 that was mostly impermeable to the membranes. The only membranes that did not reject this substance were the 30 kDa V3 membrane at pH 4 and pH 7, suggesting that the unknown compound had a high molecular weight (between 10 kDa and 30 kDa) and a pI greater than 7.

Overall the results of this project demonstrate that ultrafiltration and nanofiltration membranes are a viable method of concentrating bioactive peptides present in protein hydrolysates. This project also introduced a novel analytical method that had not previously been applied to HPLC of hydrolysates, and demonstrated that it could be used to identify the changes that had occurred during membrane processing. This work builds on the existing body of knowledge in this area and

has the potential to contribute to the development of commercial peptide products from protein-rich sources.

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6 Supplementary data

Table 8. Depth of collection, shell size and flesh weight of individual geoducks used in this study.

	Depth (m)	Shell size (mm)	Flesh weight (g)
1	11.2	95	135
2	11.2	117	220
3	11.2	102	245
4	11.2	118	250
5	11.2	103	150
6	11.2	100	220
7	11.2	98	140
8	6.9	79	50
9	6.9	97	135
10	6.9	119	225
11	6.9	123	220
12	6.9	113	235
13	6.9	122	240
14	6.3	95	125
15	6.3	87	65
16	6.3	126	220
17	6.3	96	145
18	6.3	124	255
19	6.3	124	285
20	6.3	141	270
21	6.3	73	35
22	6.3	114	165
23	6.3	131	300
24	7.4	117	320
25	7.4	109	330
26	7.4	115	270
27	7.4	112	200
28	7.8	104	280
29	6.4	112	255
30	6.4	121	325
31	6.4	92	150
32	6.4	80	95
33	6.4	81	115
34	9.1	102	305

35	9.1	91	155
36	9.1	109	205
37	9.1	117	225
38	9.1	105	210
39	9.2	99	205
40	9.2	103	150
41	9.2	119	290
42	8.9	117	230
43	8.9	113	225
44	8.9	119	185
45	8.9	112	260
46	8.9	108	200
47	8.9	127	380
48	8.9	116	395
49	9.9	113	175
50	9.9	116	310
51	8	111	180
52	8	116	230
53	8	114	185
54	8	116	190
55	8	118	225
56	8	129	215
57	8	110	140
58	8	109	195
59	8	107	170
60	8	109	145
61	9.8	102	125
62	9.8	114	235
63	9.8	95	105
64	9.8	105	205
65	9.8	105	185
66	9.8	102	195
67	9.8	120	210
68	9.8	120	205
69	9.8	103	130
70	9.4	101	220
71	9.1	101	165
72	9.1	115	234
73	9.1	107	150
74	9.1	90	135
75	7	120	265
76	7	117	290
77	8.8	101	155
78	8.8	114	195

79	8.8	105	180
80	8.8	109	185
81	8.8	115	175
82	8.8	112	225
83	8.8	111	175
84	8.8	110	250
85	4.9	113	235
86	4.9	108	210
87	4.9	113	255
88	4.9	104	210
89	4.9	115	220
90	4.9	113	124
91	4.9	115	225
92	4.9	114	190
93	4.9	99	160
94	4.9	102	195
Averages	8.0	109	205

Table 9. Dry weight analysis of homogenised geoduck tissue.

Sample	Tare (g)	Wet weight + dish (g)	Wet weight (g)	Dry weight + dish (g)	Dry weight (g)	% dry weight	Average	Std dev.
1	16.6256	21.0281	4.4025	17.4399	0.8143	18.5		
2	16.7073	20.7436	4.0363	17.4558	0.7485	18.5		
3	16.6888	19.4515	2.7627	17.2015	0.5127	18.6	18.5	0.03

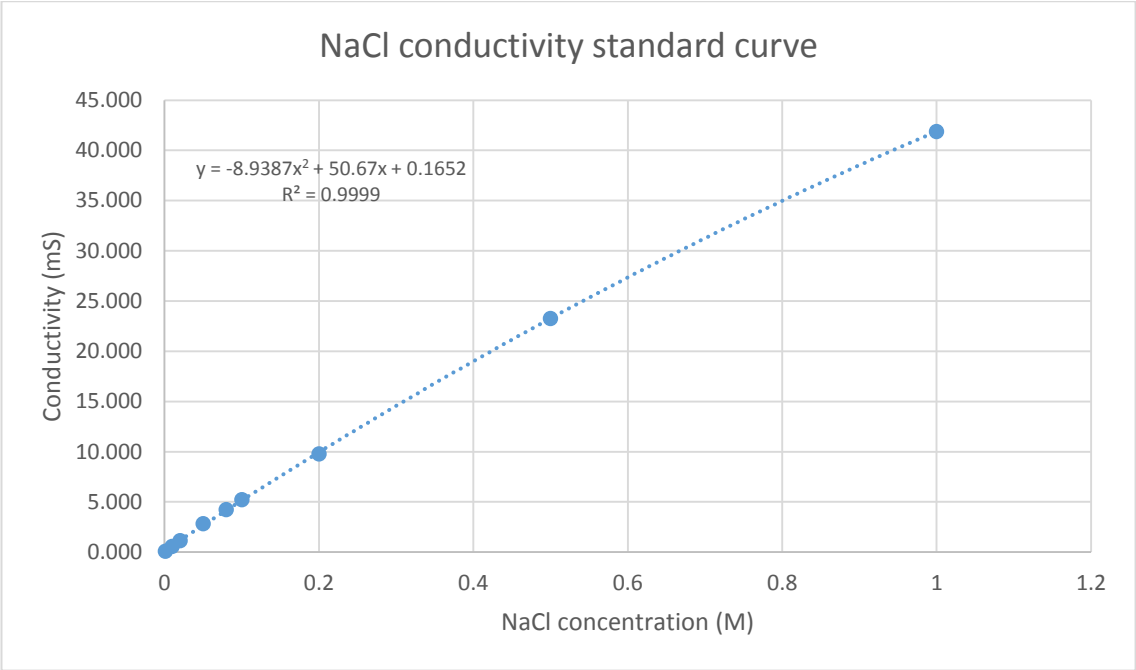

Figure 33. NaCl conductivity standard curve.

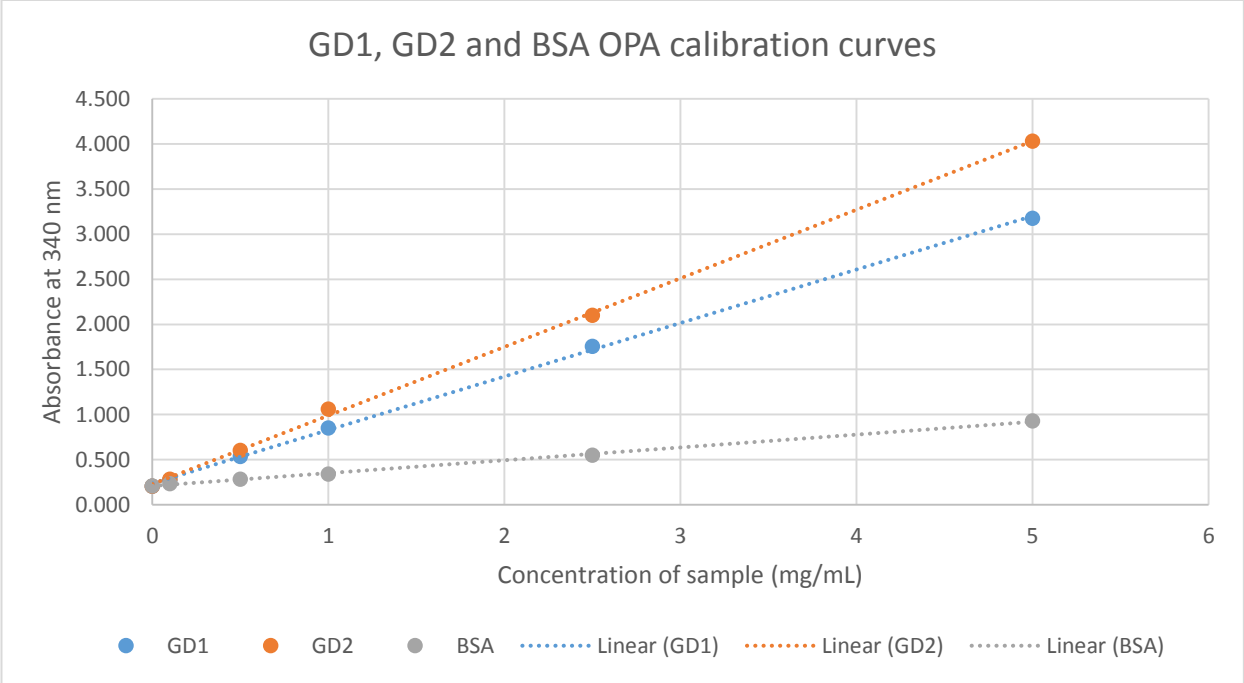

Figure 34. OPA assay standard curves for concentrations of GD1, GD2 and BSA. Note the very different slope for the BSA relative to the hydrolysates illustrating why it is not a suitable standard for these samples.

Table 10 . NaCl distribution in permeate and retentate samples as determined by conductivity.

Sample name	Volume (mL)	Conductivity (mS)	NaCl concentration (mg/mL)	Total NaCl (mg)	% distribution of NaCl
GD1DK10 Permeate	76	3.08	3.36	255.0	18.2
GD1DK10 Retentate	124	8.12	9.25	1147.6	81.8
GD1NFG10 Permeate	135	5.05	5.62	759.0	55.2
GD1NFG10 Retentate	65	8.30	9.47	615.6	44.8
GD1XT10 Permeate	150	5.76	6.45	967.9	70.5
GD1XT10 Retentate	45	7.92	9.01	405.7	29.5
GD1GH10 Permeate	135	5.22	5.83	786.7	55.4
GD1GH10 Retentate	65	8.52	9.74	632.8	44.6
GD1MT10 Permeate	150	5.57	6.24	935.7	65.8
GD1MT10 Retentate	50	8.53	9.75	487.4	34.2
GD1PW10 Permeate	150	6.10	6.86	1028.9	76.0
GD1PW10 Retentate	37.5	7.61	8.64	324.2	24.0
GD1XT7 Permeate	150	4.77	5.30	795.3	71.7
GD1XT7 Retentate	50	5.60	6.27	313.5	28.3
GD1XT4 Permeate	150	5.15	5.74	861.6	69.3
GD1XT4 Retentate	50	6.76	7.63	381.7	30.7
GD1V310 Permeate	150	6.01	6.75	1012.5	70.2
GD1V310 Retentate	50	7.58	8.61	430.4	29.8
GD1V37 Permeate	150	4.93	5.48	822.6	75.0
GD1V37 Retentate	50	4.93	5.49	274.4	25.0
GD1V34 Permeate	150	5.70	6.38	957.4	74.9
GD1V34 Retentate	50	5.74	6.43	321.7	25.1
GD2DK10 Permeate	112	4.41	4.89	547.7	35.0
GD2DK10 Retentate	88	10.04	11.58	1019.0	65.0
GD2NFG10 Permeate	145	5.63	6.30	913.3	61.2
GD2NFG10 Retentate	55	9.18	10.53	579.3	38.8
GD2XT10 Permeate	151	6.46	7.28	1099.2	74.3
GD2XT10 Retentate	37.5	8.84	10.12	379.5	25.7
GD2GH10 Permeate	142	5.76	6.46	917.2	60.2
GD2GH10 Retentate	58	9.11	10.45	606.0	39.8
GD2MT10 Permeate	150	6.01	6.75	1013.1	68.7
GD2MT10 Retentate	42.5	9.46	10.87	462.1	31.3

GD2PW10 Permeate	150	6.65	7.50	1125.6	77.5
GD2PW10 Retentate	40	7.22	8.18	327.2	22.5
GD2XT7 Permeate	150	4.71	5.23	784.9	72.4
GD2XT7 Retentate	40	6.62	7.47	298.7	27.6
GD2XT4 Permeate	150	5.46	6.11	916.4	74.2
GD2XT4 Retentate	40	7.05	7.98	319.1	25.8
GD2V310 Permeate	150	6.16	6.93	1039.5	72.5
GD2V310 Retentate	50	6.97	7.88	394.2	27.5
GD2V37 Permeate	150	4.89	5.44	816.2	73.8
GD2V37 Retentate	50	5.20	5.80	290.1	26.2
GD2V34 Permeate	150	5.40	6.04	905.3	69.1
GD2V34 Retentate	50	7.16	8.11	405.4	30.9

Table 11. Peptide distribution in permeate and retentate samples as determined by OPA assay.

Sample name	Volume (mL)	Peptide concentration by OPA (mg/mL)	Total peptide by OPA (mg)	% distribution of peptide
GD1DK10 Permeate	76	0.6	49	0.9
GD1DK10 Retentate	124	45	5585	99.1
GD1NFG10 Permeate	135	6.5	883	14.3
GD1NFG10 Retentate	65	81.3	5286	85.7
GD1XT10 Permeate	150	21.3	3188	54.6
GD1XT10 Retentate	45	58.8	2647	45.4
GD1GH10 Permeate	135	10.6	1427	21.9
GD1GH10 Retentate	65	78.3	5091	78.1
GD1MT10 Permeate	150	14.9	2238	27.1
GD1MT10 Retentate	50	120.2	6011	72.9
GD1PW10 Permeate	150	21.8	3266	62.9
GD1PW10 Retentate	37.5	51.3	1923	37.1
GD1XT7 Permeate	150	30.4	4562	63.8
GD1XT7 Retentate	50	51.7	2584	36.2
GD1XT4 Permeate	150	18.6	2786	41.6
GD1XT4 Retentate	50	78.2	3911	58.4
GD1V310 Permeate	150	21.5	3221	54.0
GD1V310 Retentate	50	54.8	2740	46.0
GD1V37 Permeate	150	31.4	4712	74.0
GD1V37 Retentate	50	33.2	1659	26.0
GD1V34 Permeate	150	26.7	4004	73.4
GD1V34 Retentate	50	29	1451	26.6

GD2DK10 Permeate	112	18.8	2110	28.0
GD2DK10 Retentate	88	61.6	5418	72.0
GD2NFG10 Permeate	145	18.3	2646	40.5
GD2NFG10 Retentate	55	70.8	3893	59.5
GD2XT10 Permeate	151	2.1	321	14.8
GD2XT10 Retentate	37.5	49.2	1845	85.2
GD2GH10 Permeate	142	11	1569	23.3
GD2GH10 Retentate	58	89.2	5175	76.7
GD2MT10 Permeate	150	20.8	3123	50.2
GD2MT10 Retentate	42.5	73	3102	49.8
GD2PW10 Permeate	150	12.2	1823	34.4
GD2PW10 Retentate	40	87	3480	65.6
GD2XT7 Permeate	150	17.2	2577	40.6
GD2XT7 Retentate	40	94.4	3775	59.4
GD2XT4 Permeate	150	29.3	4394	75.6
GD2XT4 Retentate	40	35.5	1420	24.4
GD2V310 Permeate	150	21.1	3169	71.8
GD2V310 Retentate	50	24.9	1246	28.2
GD2V37 Permeate	150	25.9	3889	69.5
GD2V37 Retentate	50	34.1	1705	30.5
GD2V34 Permeate	150	21.6	3247	54.4
GD2V34 Retentate	50	54.5	2726	45.6

Table 12. Equivalent concentrations of antioxidants in membrane separated peptide samples.

		Vit E equivs (µg)	Quercitin equivs (µg)	Vit C equivs (µg)
1	GD1DK10 Perm	-5.603	-2.138	-2.673
2	GD1DK10 Retentate	-5.438	-2.075	-2.596
3	GD1NFG10 Perm	-5.795	-2.212	-2.763
4	GD1NFG10 Retentate	-5.331	-2.034	-2.545
5	GD1XT10 Perm	-5.528	-2.110	-2.638
6	GD1XT10 Retentate	-5.375	-2.051	-2.566
7	GD1GH10 Perm	-5.698	-2.175	-2.718
8	GD1GH10 Retentate	-5.329	-2.033	-2.544
9	GD1MT10 Perm	-5.634	-2.150	-2.688
10	GD1MT10 Retentate	-5.336	-2.036	-2.548
11	GD1PW10 Perm	-5.486	-2.094	-2.618
12	GD1PW10 Retentate	-5.319	-2.030	-2.540
13	GD1XT7 Perm	-5.375	-2.051	-2.566
14	GD1XT7 Retentate	-5.681	-2.168	-2.710
15	GD1XT4 Perm	-5.224	-1.993	-2.495

16	GD1XT4 Retentate	-5.283	-2.016	-2.522
17	GD1V310 Perm	-5.486	-2.094	-2.618
18	GD1V310 Retentate	-5.290	-2.019	-2.526
19	GD1V37 Perm	-6.062	-2.314	-2.889
20	GD1V37 Retentate	-5.171	-1.973	-2.470
21	GD1V34 Perm	-5.814	-2.219	-2.773
22	GD1V34 Retentate	-5.025	-1.917	-2.401
23	GD2DK10 Perm	-5.642	-2.153	-2.691
24	GD2DK10 Retentate	-5.547	-2.117	-2.647
25	GD2NFG10 Perm	-5.151	-1.966	-2.461
26	GD2NFG10 Retentate	-5.297	-2.021	-2.529
27	GD2XT10 Perm	-5.564	-2.124	-2.655
28	GD2XT10 Retentate	-5.358	-2.045	-2.558
29	GD2GH10 Perm	-5.567	-2.124	-2.656
30	GD2GH10 Retentate	-5.338	-2.037	-2.549
31	GD2MT10 Perm	-5.477	-2.090	-2.614
32	GD2MT10 Retentate	-5.251	-2.004	-2.508
33	GD2PW10 Perm	-5.610	-2.141	-2.677
34	GD2PW10 Retentate	-5.414	-2.066	-2.584
35	GD2XT7 Perm	-5.617	-2.144	-2.680
36	GD2XT7 Retentate	-5.331	-2.034	-2.545
37	GD2XT4 Perm	-5.382	-2.054	-2.569
38	GD2XT4 Retentate	-5.394	-2.059	-2.575
39	GD2V310 Perm	-5.535	-2.112	-2.641
40	GD2V310 Retentate	-5.431	-2.072	-2.592
41	GD2V37 Perm	-5.634	-2.150	-2.688
42	GD2V37 Retentate	-5.952	-2.272	-2.838
43	GD2V34 Perm	-5.297	-2.021	-2.529
44	GD2V34 Retentate	-5.445	-2.078	-2.599
45	GD1 pH4	-5.108	-1.949	-2.440
46	GD1 pH7	-5.690	-2.172	-2.714
47	GD1 pH10	-5.489	-2.095	-2.620
48	GD2 pH4	-5.239	-1.999	-2.502
49	GD2 pH7	-5.593	-2.135	-2.669
50	GD2 pH10	-5.428	-2.072	-2.591