Fractionation of non-animal protein hydrolysates for use in CHO (Chinese Hamster Ovary) cell media

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

This thesis presents a study on the enhancement of CHO cell growth by Yeast extract, Yeastolate, and Primatone fractions obtained by dead-end ultrafiltration. The total solid, peptide contents, antioxidant capacity and hydrophobicity of the fractions were evaluated.

The objective of this project was to evaluate the potential of sequential ultrafiltration as an effective, simple and economical method for the identification of CHO cell growth enhancement components in yeast extract and yeastolate (primatone).

The fractionation by sequential ultrafiltration (50 kDa membrane, 3 kDa membrane and 1 kDa membrane) of yeast extract (YE), yeastolate (YET), and primatone (PRI) showed different fouling and fractionation behaviour. Significant fouling was observed with the 50 kDa and 3 kDa membrane while negligible fouling was observed with the 1 kDa membrane. Similar and more significant fouling was observed with the 50 kDa membrane and for YE and PRI in comparison to YET. In contrast, more fouling was observed during the ultrafiltration with the 3kDa MWCO and for YE and YET in comparison to PRI. Finally a relatively constant permeate flux was obtained with the 1 kDa membrane, with PRI the highest and YET the lowest permeate flux. Different total peptide contents were present in the three feeds, 410, 327 and 300 mmol Phe-Gly equivalent/ g total solids for YE, PRI and YET respectively. In spite of different feed equivalent Phe-Gly, all three feeds contained a similar amount of equivalent Phe-Gly with molecular weight larger than 50 kDa, 15-19% of the initial feed stream. This was similar amount to the total solids content. The total peptide content of the retentate obtained for the 3kDa filtration indicated that YE and YET contained ~ 20% of equivalent Phe-Gly larger than 3 kDa but smaller than 50kDa. In contrast, PRI contained only 6% of equivalent Phe-Gly with such molecular weight. The retentate of the 1kDa filtration contained 55% of the feed equivalent Phe-Gly compared to 47%

for YE and 38% for YET ($p < 0.05$). All three feeds have similar total peptide content smaller than 1 kDa. For any given feed, the equivalent Phe-Gly was larger than 1 kDa but smaller than 3 kDa predominated. The total peptide content profile according to size coincides with the total solids distribution for all three feed types. This is the first study that reports on the total peptide content for YE, YET, and PRI subjected to ultrafiltration fractionation.

All three feeds and their fractions when freeze-dried had similar antioxidant capacity estimated by the FCR (Folin-Ciocalteu reagent) assay, ~ 40-50 mg Trolox/g sample.

The bioactivity of feed and fractions was measured as cell density for CHO (beta-IFN producers) in basal medium supplemented with a combination of the crude non-fractionated feed material and a specific fraction and grown in T25 flasks. PRI showed a similar growth enhancement effect for all fractions when compared to a culture supplemented with the crude non-fractionated. YE showed no growth enhancement for any of the fractions when compared to a culture supplemented with the crude non-fractionated YE. This observation need to be confirmed as a culture supplemented with the crude non-fractionated YE showed a very high growth stimulating effect which was much higher than PRI and YET at the same concentration. Finally, YET 3kDa retentate fraction displayed a 50 % growth enhancement effect. In conclusion, the fractions obtained from the two non-animal protein hydrolysates considered in this study, YE and YET showed limited CHO cell growth enhancement effect when compared to the non-fractionated material. Only the YET 3kDa retentate fraction displayed a good CHO cell growth enhancement effect. YET 3kDa represent an attractive serum substitute for its use in culturing CHO cells. PRI, an animal derived protein hydrolysate showed the best growth enhancement effect for all fractions produced in this study. These results suggest that YET has high potential as a media additive for the development of serum-free media which can promote cell growth and, in the future this work can contribute in production of therapeutic proteins markets.

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NOMENCLATURE

Roman letters

a	particle radius (m)
A	proportionality constant (mL/g)
A_M	membrane area (m^2)
c_B	solute concentration in bulk (kg/m^3 or g/mL)
c_R	protein concentration in the retentate (g/L)
c_F	protein concentration in the feed (g/L)
d	particle diameter (m)
F	Faraday constant (C/mol)
I	ionic strength (mol/m^3)
J_0	permeate flux at t = 0 ($L/m^2.h$ or m/s)
J_P	permeate flux ($L/m^2.h$ or m/s)
J_W	water flux ($L/m^2.h$ or m/s)
k	rate constant (s^{-1} or min^{-1})
l	length (m)
P_{in}	inlet pressure (Pa or psi)
P_{out}	outlet pressure (Pa or psi)
P_p	pressure on the permeate side (Pa or psi)
ΔP	axial pressure drop (Pa or psi)
R	gas constant (J/mol K)
R_F	resistance of fouling (m^{-1})
R_I	resistance of irreversible fouling (m^{-1})
R_M	clean membrane resistance (m^{-1})
R_R	resistance of reversible fouling (m^{-1})
R_T	total hydraulic resistance (m^{-1})
s	shape factor (-)
t	time (s or min)

T	temperature (K)
TMP	transmembrane pressure (Pa or psi)
u	electrophoretic mobility (m^2/V.s)
v	flow velocity (m/s)
VCR	volume concentration ratio (-)
V_F	initial feed volume (m^3)
V_P	cumulative permeate volume (m^3)
V_R	retentate volume (m^3)
YE	yeast extract
YET	yeastolate
PRI	primatone
MWCO	molecular weight cut off
RP-HPLC	reversed-phase high performance liquid chromatography
OPA	o-phthaldialdehyde
FCR	folin ciocalteu reagent
UF	ultrafiltration
PES	polyethersulfone
RC	regenerate cellulose
hTPO	recombinant human thrombopoietin
INF-γ	Interferon -γ

Greek letters

α	specific cake resistance (m/kg)
ε	porosity (-)
ε_m	medium permittivity (C^2/N m^2)
ζ	zeta potential (V or mV)
η	dynamic viscosity (Pa.s)
η_P	dynamic viscosity of permeate (Pa.s)
υ_h	specific hydrodynamic volume (mL/g)

κ^{-1}	thickness of electrical double layer (m)
μ	kinematic viscosity (m^2/s)
ρ	particle density (kg/m^3)
φ	volume fraction (-)

Chapter 1 **Introduction**

1.1 Research aim and outline

Animal cell cultures have been important on a laboratory-use scale for most of the last few decades. The initial need for human viral vaccines in the 1950s (particularly for poliomyelitis) accelerated the design of large-scale bioprocesses for mammalian cells (Maurice 2002).

In the last few years, there has been a rapid increase in the number and demand for glycoprotein produced from animal cell culture processes. Animal cells have complex nutrient requirements which are specific to a given cell line and glycoprotein. For the production of therapeutic proteins, a serum-free media is desirable because the use of serum has many drawbacks: undefined composition, high protein levels, risk of contamination by viruses, mycoplasma, Bovine Spongiform Encephalopathy, variable quality, and high cost. In contrast, serum-free media have many advantages: more defined components, a controlled cell culture environment, and low cost. Although much effort has been devoted in developing serum-free media for mammalian cells, such as low-cost hydrolysates contain undefined mixtures such as amino-acids, peptides, vitamins, and trace elements, sometimes using these low-cost hydrolysates result in decreased cell growth, because impurities interrupt the growth process (Zhang et al. 1994).

Since serum is a supplement of unknown composition, which could be contaminated with unwanted factors, there are scientific and safety reasons for omitting fetal bovine serum from culture media. Several media have been developed in which minimal or no animal derived components are present. But as yet, no standard serum free media has been developed to fulfill the nutritional requirements of each cell type.

A method is needed to separate and define the components providing growth enhancement from low-cost serum-free media ingredients. In this project (Figure 1.1), ultrafiltration was considered as a simple and low cost alternative for the fractionation of growth enhancing ingredients contained in non-animal derived hydrolysates such as yeast extract (YE) and yeastolate (YET), and primatone (PRI) as a source of animal derived protein hydrolysates. All the fractions

obtained by the ultrafiltration process were characterized for their total peptide content, antioxidant capacity and relative hydrophobicity.

The biological activity of CHO cell density produced using YE, YET, and PRI was also examined by measurement of the cell density for specific CHO cell line.

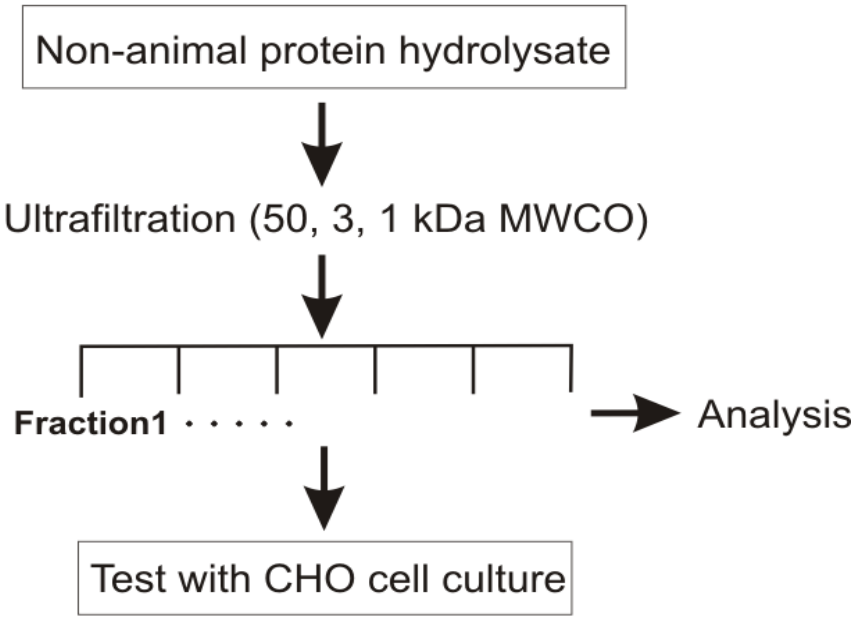

Figure 1.1 Schematic of approach

1.2 Structure of the thesis

Chapter 2 presents the objectives of this study. The background information on non-animal protein hydrolysates, dead-end ultrafiltration, antioxidant activity, RP- HPLC, CHO cell culture, and aggregation is provided in Chapter 3. The equipment and analytical techniques employed for this work are described in the materials and methods, Chapter 4. The experimental results and discussion are presented in Chapter 5 and 6. Chapter 7 contains the conclusions of this work and the recommendations for the future work.

Chapter 2 **Objectives**

The overall objective of this work was to fractionate by sequential membrane ultrafiltration two non-animal protein hydrolysates, yeast extract and yeastolate, and one animal protein hydrolysate, primatone and produce additives for CHO cell medium supplementation. Specific objectives were as follows:

- Characterize ultrafiltration membrane performance for hydrolysate fractionation (membrane fouling, total solids and total peptide retention).
- Evaluate antioxidant capacity and hydrophobicity of the fractionated hydrolysates
- Evaluate the growth enhancement capacity of the fractionated hydrolysates for CHO cell

Chapter 3 **Literature Review**

3.1 CHO Cell Culture

Chinese hamster ovary (CHO) cells are the most widely used for transfection, expression, and large-scale recombinant protein production. They have been adapted for growth in suspension culture and are well-suited for scale-up in stirred tank bioreactors. The advantages of CHO cells are to provide stable and accurate glycosylation, offer a post-translationally modified product and more accurate *in vitro* rendition of natural protein (Sheeley 1997). CHO cells are robust in culture and are able to produce a variety of recombinant glycoproteins at high levels on a large scale. There is tremendous work to produce pharmaceutical product trough the CHO cells: hTPO (Kaszubska et al 2000, Sung et al. 2004), IFN-γ (Burteau 2003).

There has been a rapid increase in the number and demand for approved biopharmaceuticals produced from animal cell culture processes over the last few years. Figure 3.1 shows the increase in the number of biopharmaceuticals approved for production by mammalian cell bioprocesses over the last 20 years with the actual and expected demand in kilograms. The sharp increase in the years 2000 is largely due to the number of approved therapeutic antibodies that are required at relatively high clinical does (Butler 2007).

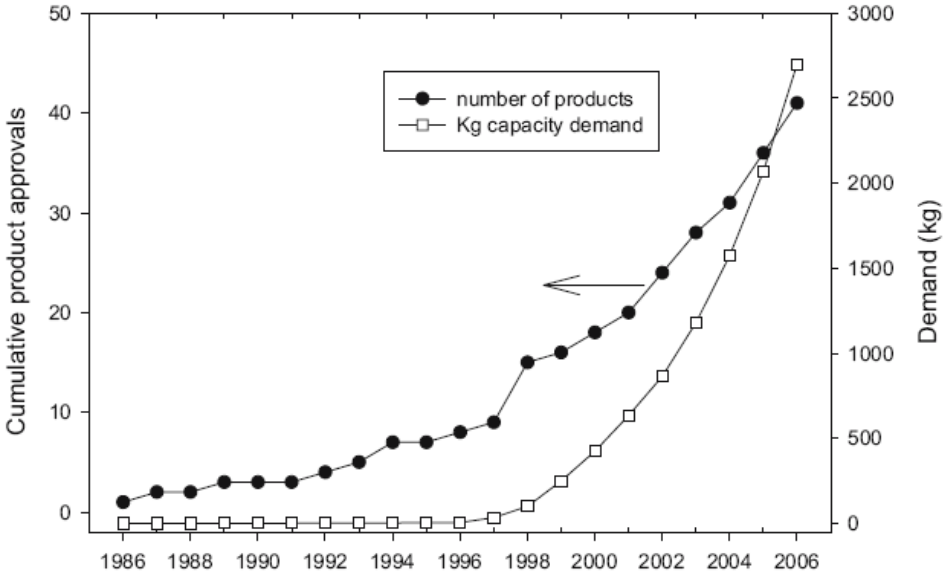

Figure 3.1 The demand for mammalian cell culture products (Molowa and Mazanet, 2003).

The commercial significance of these products as therapeutics is driving a vigorous interest for progress in process development using mammalian cell cultures. The high clinical dosage requirements for antibodies leads to large volume demands in biomanufacture (estimated to be >2000kg for all biotherapeutic proteins). Because of the high demand for individual antibodies, the cost efficiency in manufacture becomes extremely important (Butler2007).

However, different CHO cell clones often possess diverse nutritional requirements that are unique to each clone. As a result, medium optimization for CHO cells can be very challenging, often requiring the development of a custom medium for each particular clone.

3.1.1 Types of cell culture media

The basic nutrient requirement for mammalian cells was studied in 1955 by Eagle. In that study, mammalian cells can survive on glucose, amino acids, vitamins, and inorganic salts.

Classical cell culture medium contains glucose, amino acids, vitamins, trace metals, buffer, inorganic salts, and proteins to meet the nutrient demands and process-control demands of the mammalian cell culture. The composition of these components can affect cell growth, protein production, protein quality, and downstream protein purification. Depending on the application, supplements are often added, the most common one being animal-derived serum. Variation of the serum-containing medium has been developed.

Fetal bovine serum, in particular, is commonly used as a supplement to classical media (Gruber and Jayme, 1994; Jayme and Gruber 1994). Serum provides sterols, fatty acids, growth factors, protein stability, and protein transporters, trace metals, vitamins, and shear protection to mammalian cell cultures. Removing serum without addressing these functions could result in poor cell growth and performance. However, not all of these functions are critical for every cell line. Successful serum replacements reported in the literature should be explored in statistically design experiments to determine the main or interacting factors that are required for the process medium.

Removing the animal additives, serum, reduces the risk of viral contamination, increased process robustness (by reducing exposure to lot-to-lot variability of animal-source ingredients), reduces medium costs, and simplifies down-stream purification (Barnes 1987, McKeehan 1990). Especially, bovine spongiform encephalopathy (BSE) was a major factor in the move away from animal origin raw materials in the manufacture of human biotherapeutics. Lastly, serum introduces an unknown variable into the culture system.

Serum-free media are therefore a subset of all media, in which cells are capable of expanding and expressing a product in the absence of serum supplementation (Figure 3.2). To eliminate the use of serum, it is necessary to understand the many functions of serum, which include binding/carrier functions for lipids, hormones, growth factors, attachment factors, cytokines, and trace metals, as well as bulk protein functions like protease inhibition, shear protection and buffering (Jayme and Blackman, 1985). Early serum-free media often contained one or more serum function like albumin or fetuin, in addition to insulin, transferring, a source of lipids (often an animal-derived lipoprotein preparation), trace metal salts, and elevated levels of amino acids,

vitamins, and carbohydrates compared to classical media. Some literature reports on the development of serum-free medium by supplementing the medium with sterols and fatty acids. Cholesterol with methyl b-cyclodextrin (Gorfien et al. 2000), cis-unsaturated fatty acids (Butler and Huzel 1995, Liu et al. 2001), and ethanolamine and yeast extract (Liu et al. 2001) have been shown to result in increased growth and productivity in serum-free medium. Another method to increase the fatty acids for cells in serum-free medium is to supply insulin, which stimulates the biosynthesis of fatty acids (Komolov and Fedotor 1978). Medium supplement for the enhancement of growth are low molecular weight substance (Schlaeger 1996).

There is one report that the serum- free media (SFM) has more positive effect on mammalian cell culture than serum based media (Schlaeger 1996). Reduction or substitution of serum in culture media of mammalian cells (growth in suspension culture) has many well known advantages (Iscove 1984, Glassy 1988).

There are many studies suggesting that serum could be replaced by using low-cost commercial protein hydrolysates from animal tissues (peptones, tryptose), milk product (lactalabumin, casein), micro-organisms (yeast extract, Bacto-peptone) (Mitsuhashi and Maramorosch 1964, Jan 1994, Schlaeger 1996).

Cells often required a period of adaptation to SFM and it soon became apparent that a ‘universal SFM’ did not exist. As SFM came to be used more frequently for biological production applications, it also became apparent that the undefined nature of some of the medium constituents represented a potential cause of culture performance variability. Of greater concern was the potential for transmission of adventitious agents from the animal-derived medium components to patients receiving a biological therapeutic or prophylactic that had been produced in cultured cells. These factors have led to development of protein free media (PFM) and chemical defined media (CDM). PFM is a subset of SFM (Figure 3.2). It should be noted that there are many commercially available sources of cell culture media and not all suppliers use the same definitions.

PFM: Protein-Free Media contain no proteins, but may contain plant or yeast hydrolysates. Many are animal-origin-free. (GIBCO ®)

CDM: a formulation in which the chemical structure of each component is known. This is another potential source of confusion among different manufactures of cell culture media. Some manufacturers consider media that contain proteins like insulin or transferring to be chemically defined while others' definitions require that a CDM must also be protein-free (Gorfien 2007). Chemically fully defined means a medium that does not contain any complex additives such as lipid mixtures, hydrolysates, peptides or proteins or other, even recombinant, growth factors (Van der Valk 2004).

From the US 5512477 patent 1996, a serum-free eukaryotic cell culture medium supplement comprises carbon sources, vitamins, inorganic salts, amino acids and a protein digest. The medium supplement of the present invention enables the maintenance of mammalian cell cultures at cell densities equal to or greater than that obtained with batch culture methods while increasing longevity and productivity.

From the Robert 1975 in nature, the factors that control growth are probably polypeptide, hormones, or hormone-like materials, as well as a variety of low molecular weight nutrients. An understanding of the control of growth of mammalian cells will probably be found in the complex interactions of cells with these common types of materials.

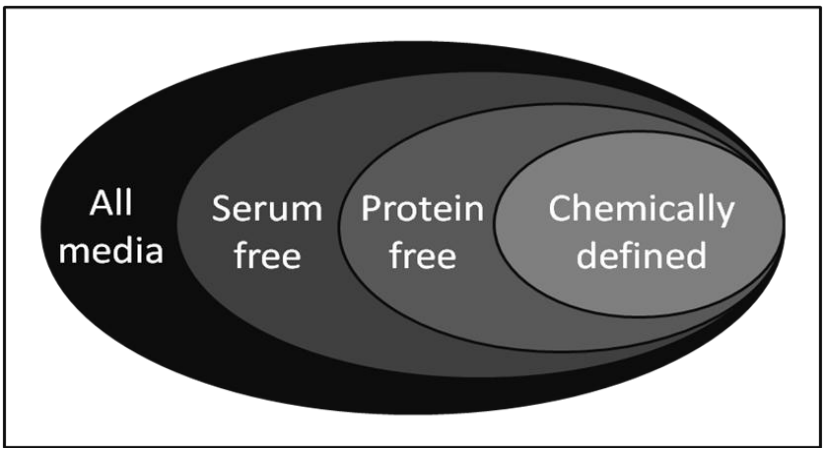

Figure 3.2 Different types of culture media.

3.2 Non-animal Protein Hydrolysates as additives

Non-animal hydrolysates have been reported to be attractive as additives for the development of serum-free media.

3.2.1 Yeast

Yeasts are a growth form of eukaryotic microorganisms classified in the kingdom Fungi. *Saccharomyces cerevisiae* is the one of the yeast species among 1,500 species. Yeast size can be varying greatly depending on the species, typically measuring 3 to 4 micrometers in diameter, although some yeast can reach over 40 micrometers.

Yeasts do not form a specific taxonomic or phylogenetic grouping. At present it is estimated that only 1% of all yeast species have been described. The term "yeast" is often taken as a synonym for *Saccharomyces cerevisiae*, however the phylogenetic diversity of yeasts is shown by their placement in both divisions Ascomycota and Basidiomycota. The budding yeasts ("true yeasts") are classified in the order Saccharomycetales.

The useful physiological properties of yeast have led to their use in the field of biotechnology. Fermentation of sugars by yeast is the oldest and largest application of this technology. Many types of yeasts are used for making many foods: Baker's yeast in bread production, brewer's yeast in beer fermentation, yeast in wine fermentation and for xylitol production. Yeasts are also one of the most widely used model organisms for genetics and cell biology. *Saccharomyces cerevisiae*, is used in baking as a leavening agent, where it converts the fermentable sugars present in the dough into carbon dioxide.

Growth conditions are following; yeasts are able to grow in food with low pH (5.0 or lower) and in the presence of sugars, organic acids and other easily metabolized carbon source. Yeasts will grow over a temperature range of 10 °C to 37 °C, with an optimal temperature range of 30 °C to 37 °C.

3.2.2 Yeast extract

Yeast extract are concentrate of the water-soluble portion of *Saccharomyces cerevisiae* cells that have been autolyzed. The autolysis is carefully controlled to preserve the naturally occurring B-complex vitamins. Yeast extract is considered a non-animal product and is used extensively for many non-animal formulations for bacterial, fungal, mammalian and insect cell culture.
Yeast extract have calcium, magnesium, potassium, sodium, chloride, sulfate, phosphate (BD element analysis catalogue 2007).

Yeast extract is the common name for various forms of processed yeast products that are used as food additives or flavours. They are often used in the same way that monosodium glutamate (MSG) is used, and like MSG, often contain free glutamic acids. The general method for making yeast extract for food products such as Vegemite and Marmite on a commercial scale is to add salt to a suspension of yeast making the solution hypertonic, which leads to the cells shriveling up. This triggers autolysis, where the yeast's digestive enzymes break their own proteins down into simpler compounds, a process of self-destruction. The dying yeast cells are then heated to

complete their breakdown, after which the husks (yeast with thick cell walls which would give poor texture) are separated.

Braz and Allen 1939, showed that the growth of several strains of streptococci and lactobacilli in milk culture was stimulated by the addition of yeast extract. These authors suggested that amino acids, which the yeast extract supplied, stimulated the cells to synthesize protein.

Smith 1973, yeast extract was fractionated on Sephadex G-25 into 7 fractions. The fraction most stimulatory to the growth of streptococcus lactis C10 contained over 70% of the amino N present in yeast extract and consisted of a wide variety of free amino acids and a small amount of peptide material.

Sung et al 2004, yeast hydrolysate was one of their serum free medium supplements for human thrombopoitin production of recombinant CHO cells. Among various hydrolysates tested, the positive effect of yeast hydrolysate on human thrombopoitin production was most significant.

Some works were carried out using different bioactive substance of YE. The beneficial effect of YE on the growth of lactic acid bacteria (LAB) is well established (Smith et al. 1975; Aeschlimann and von Stockar 1990). YE are an excellent source of the B-complex vitamins and are often used to supply these factors in bacteriological culture media (Difco product broacher 1984). They are also a reliable source of peptides and amino acids. Amino acids and peptides stimulate the growth of LAB and they have been shown to be partially responsible for YE stimulation of the growth of lactic cultures. Peptides can be superior to free amino acids as nitrogen sources for LAB because they can provide the cell with amino acids in a form that can be utilized more efficiently. Some commercial applications, microbiological media for example, require that YE produce clear solutions.

Gaudreau et al (1999) have compared the effect of ultrafitration of yeast extracts on their ability to promote lactic acid bacteria growth. Five yeast extracts (YE) with three from bakers' yeasts (A, B, E) while two were from brewers' yeasts (C, D) were fractionated by ultrafiltration (UF) with 1, 3, and 10kDa molecular weight cutoff membranes, concentrated by freeze-drying, and the resulting powders of yeast extract filtrates (YEF) were evaluated for their growth-promoting

properties on nine cultures of LAB. When Gaudreau was under the experiment, there was a need for growth-promoting properties evaluation of YE and YEF. An automated turbidimetric instrument was used to assess the effect of YE or YEF added to the base medium on the increase in turbidity of the cultures. The method described by Champagne et al. (1999) was used. Sterile media having various YE and YEF were prepared in test tubes, inoculated at 1% (v/v) from a fresh culture, and 350-µL samples were added aseptically to each microwell. Microplates were incubated for 24 h at 37°C for the lactobacilli as well as the pediococci, and at 22°C for the lactococci. The absorbency at 600 nm of each microwell was automatically recorded. There was an increase in α-amino nitrogen content of the YEF powders as the pore size of the UF membranes used to filter the YE solutions decreased. The source of YE had a much greater effect than UF on the growth of LAB. This was also the case for the YEF contents in total and α-amino nitrogen. Growth curves of the LAB showed that maximum growth rate (μ_{max}) data were on average 30% higher with bakers' YE than with brewers' YE, while maximum optical density (OD_{max}) values were on average 16% higher with bakers' YE. This could be related to the higher nitrogen content of the bakers' YE used in this study. Modification by UF of the YE had no significant influence on the growth of 4 of the 9 LAB strains. The three strains of *Lactobacillus casei* were negatively influenced by UF, as they did not grow as well in the media containing the YEF obtained after filtering with 1 and 3 kDa membranes. On a total solids basis, the 2.5 times retentates from the 10 kDa membrane gave, on average, 4% lower µmax and 5% lower ODmax values as compared to cultures where the corresponding YEF was used as medium supplement. This could also be partially related to the different nitrogen contents of the filtrates and retentate.

3.2.3 Yeastolate

Yeastolate is highly filterable, aqueous extract of Baker's or Brewer's yeast through autolysis. Yeastolate has been commonly used as a key component for microbial fermentations for many decades.

Yeastolate contains undefined mixture of amino acids, peptides, polysaccharides, vitamins, and minerals (Sommer et al 1996). Many efforts have been dedicated to screen yeastolate and to identify active components which support the cell growth and fermentation.

In Shen et al 2007, Yeastolate is effective in promoting growth of insect cell and enhancing production of recombinant protein, thus it is a key component in formulating serum-free medium for insect cell culture. However, yeastolate is a complex mixture and identification of the constituents responsible for cell growth promotion has not yet been achieved. This study used sequential ethanol precipitation to fractionate yeastolate ultrafiltrate (YUF) into six fractions (F1–F6). Fractions were characterized and evaluated for their growth promoting activities. Ultrafiltrated by using a Pall lab tangential flow system equipped with Omega membrane (10 kD MWCO).

3.2.4 Primatone

Primatone RL is another protein hydrolysate of a complex nature which supports mammalian cell growth. Primatone RL, and enzymatic digest of US meat, has been used extensively to reduce or replace fetal calf serum in vertebrate media for cultures of human and rodent cells, with no reduction in performance.

Primatone RL was originally described as a replacement for serum (Velez et al., 1986). The tryptic meat digest primatone RL is a low-cost medium supplement of a complex nature which serves as a source of amino acids, oligopeptides, iron salt, some lipids and other trace low molecular weight substance. Its addition to mammalian and insect cell culture media significantly improves the cell growth properties of many cell lines.

Schlaeger 1992 demonstrated that addition of the protein hydrolysate to a culture medium containing fatal calf serum, promotes growth depending on the medium used. The positive effect of PRI on cell growth was found in the presence of serum, and is even more pronounced in

serum-free cultures. Its addition to mammalian and insect cell culture media improves cell growth properties significantly.

At the early time, 1977, the primatone RL was expected as serum substitute on baby hamster kidney (BHK) cell culture. However the result of BHK cell numbers was almost half compared to serum based medium.

Gu 1997, indicates that serum-free media have been widely used in mammalian cell culture for therapeutic protein production, the effects of serum-substitutes on product quality have not been extensively examined. This study observed an adverse effect of Primatone RL, an animal tissue hydrolysate commonly used as a serum-substitute to promote cell growth, on sialylation of interferon-g (IFN-g) derived from Chinese hamster ovary (CHO) cell culture in both batch and fed-batch modes. However, the effect of serum-substitutes on product quality also demands consideration to ensure that product efficacy is not compromised. The results of these experiments have indicated that the use of Primatone RL can adversely affect sialylation of CHO-derived IFN-g in both batch and fed-batch modes. The observation of diminished sialylation at the optimal concentration of peptone for cell growth and product yield in the batch mode illustrates the potential trade off between product quantity and quality which must be evaluated in the use of serum-substitutes.

Schlaeger 1999 work, the growth promoting effects of Primatone RL are described in more detail using different mouse hybridomas, a mouse myeloma cell line, and human promyelocytic leukemia HL-60 cells. The positive effects on cell growth induced by primatone were observed in the presence of serum but were even more pronounced in serum-free culture. In addition the adaptation time from high serum to low (1%) or serum-free growth in the presence of Primatone is also significantly reduced. Primatone RL, when added to HL and DHI medium (chemically defined culture medium from Lonza), improves cell growth under low serum or serum-free conditions by increasing the maximum cell numbers and in particular the viability of the culture. The observed decrease in cell death (apoptosis) induction leads to a significant improvement in antibody (recombinant protein) production by increasing the volumetric yields during long-term batch culture.

3.2.5 Other plant protein hydrolysates

Many research proposed that soy protein hydrolysate, wheat gluten hydrolysate could be other option. Franek et al 2000 considered two plant enzymic hydrolysate, soy protein and wheat gluten that were separated using ultrafiltration to culturing mouse hybridoma ME-50. Individual peptide fractions of the hydrolysate were analyzed; the amino acid composition of the total hydrolysate was determined. Fractions were varied significantly in their growth promoting and production-enhancing activities. Other researchers considered the rapeseed hydrolysates as an alternative to serum in CHO cell culture media (Frages-Haddani et al, 2006). They collected the fraction using the 3 kDa and 1kDa MWCO ultrafiltration and nano-filtration. The smallest size (< 500Da) peptide fraction had most significant enhancement of CHO cell growth.

Chun et al 2007, investigated the effect of various hydrolysates was investigated on CHO cell media; one is being soy protein hydrolysate. The soy protein hydrolysate was filtered by 10, 5 and 3 kDa ultrafiltration. From this work we can see the effect of hydrolysate concentration of soy protein and effect of ultrafilterated hydrolysate on cell growth.

3.3 Ultrafiltration

3.3.1 General concept of ultrafiltration

Ultrafiltration uses polymeric membranes to retain macromolecules while allowing low molecular weight solutes and water to pass through the membrane under pressure. Ultrafiltration is widely used to concentrate (or dewater) a solution and remove low molecular weight impurities or buffer components.

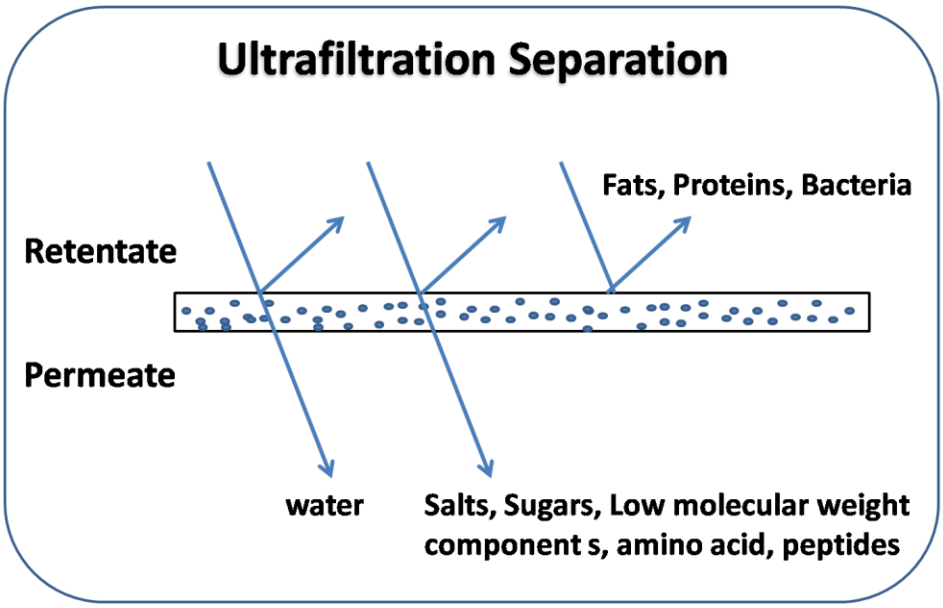

Figure 3.3 Pressure-driven membrane ultrafiltration separation characteristics.

3.3.2 Membrane properties

The pore size of ultrafiltration membrane is typically characterized by molecular weight cut-off (MWCO), which indicates the molecular weight of a component with 90% membrane retention. The key component of a membrane separation system is the membrane itself. A membrane is defined as a thin barrier or film through which solutions and solutes are selectively transported. Membrane must have: high hydraulic permeability, appropriate sieving property (selectivity), good mechanical durability, and good chemical and thermal stability, compatibility with substance being processed, excellent manufacturing reproducibility, and ease of manufacture (Michaels 1968).

3.3.3 Membrane material and chemistry

Ultrafiltration membranes are prepared from organic polymers or inorganic material (such as glass, metals and ceramics) (Kulkarni et al1992). Polymeric materials such as Polysulfone(PS), In Figure 3.4, polyethersulfone(PES), cellulose acetate(CA), regenerated cellulose, polyamides(PA), polyvinylidedefluoride(PVDF), polyacrylonitrile(PAN) are commonly used.

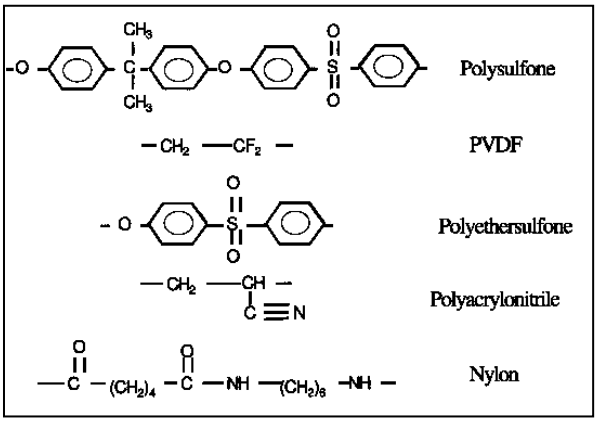

Figure 3.4 repeat units of various polymers used for preparing membrane

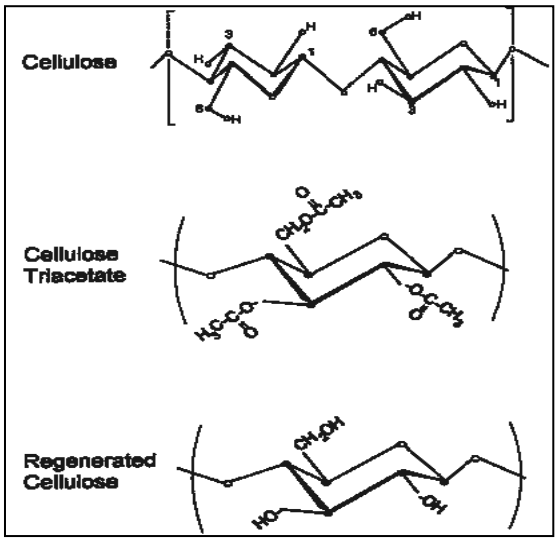

Figure 3.5 Structure of cellulose, cellulose triacetate (CT) and regenerated cellulose (RC) (adapted from Cheryan 1986)

Figure. 3.5 show the chemical structures of some of the organic polymeric material used for membrane preparation. Polysulfone, polyethersulfone and cellulose based membranes are more widely used than other types of polymeric membranes. Organic polymer based membranes are more popular than inorganic membranes, due to following reasons; inexpensive, easy to prepared, light and flexible to be moulded into various shapes and sizes, certain membrane types(hollow fibres) can only be prepared with organic polymers, and a wide range of membrane chemistry is available.

3.3.4 Ultrafiltration operation mode

There are two important operation modes in ultrafiltration. One is dead-end or conventional filtration and the other is cross-flow or tangential flow filtration (Figure 3.3). Some ultrafiltration equipment such as the pleated cartridges is operated in the dead-end mode, in which the feed is pumped directly toward the filter. There is one stream entering the filter module and only one

stream (called the permeate or filtrate) leaving the filter. The other UF modules are operated in the cross-flow mode, in which the feed is pumped tangentially to the membrane surface. On this mode of operation, there is one stream entering the module and two streams leaving the module which are called retentate and permeate. If the feed contains relatively high solids and the solid need to be recovered easily, cross-flow is advantageous in that it limits the build up of the solids on the membrane surface (shown in figure 3.6 as the ‘cake’).

In dead-end filtration the flow causes the build-up of the filter cake, which may prevent efficient operation. This is avoided in cross-flow filtration where the flow sweeps the membrane surface clean.

For dead-end filtration the thickness of the solids build up increases and the permeate flux decreases with time, ultimately reaching zero. In cross-flow filtration the feed can contain either a soluble or a solid solute, which becomes concentrated at the membrane surface; the permeate flux reaches a constant value at steady state.

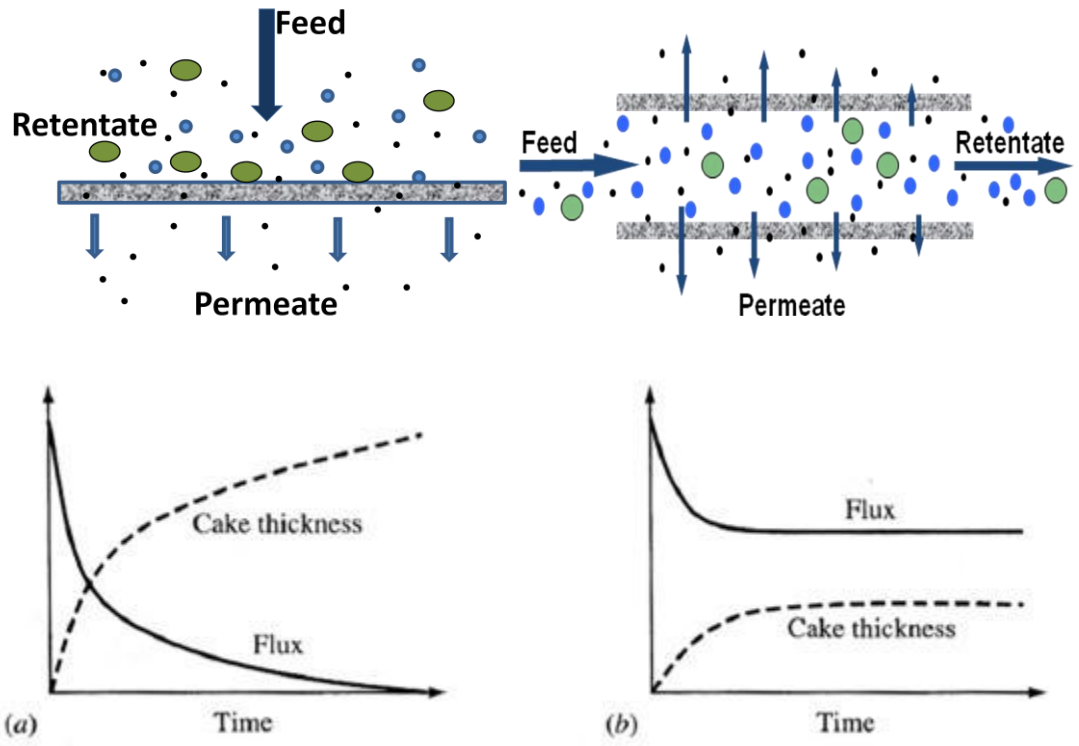

Figure 3.6 Schematic diagrams for (a) dead-end or conventional filtration and (b) cross-flow filtration. (Cross flow filtration adapted from Maja 2008)

3.3.5 Membrane fouling

Darcy's law can be used to estimate filtration resistance of the membrane:

$$J = \frac{\left(\frac{dV}{dt}\right)}{A_m} = \frac{TMP}{\eta(R_t)}$$

Equation 3.1

Where, *J* is the permeate flux (L/m^{2}h), TMP is the transmembrane pressure (N/m^2), and η is the permeate viscosity (N·s/m^2), R_t the total filtration resistance (m^{-1}), and A_m is the membrane area (m^2), V is the volume of the permeate (m^3), t is the time (s). Generally, the flux tends to decrease due to fouling and concentration polarization over time. Many models describing permeate flux decline have been developed. The resistance-in-series model explains the permeate flux decline caused by various filtration resistances including concentration polarization, and cake layer:

$$\frac{TMP}{\eta(R_m + R_c)} = \frac{TMP}{\eta(R_m + R_i + R_r)} \qquad \text{Equation 3.2}$$

Where the total filtration resistance R_t is composed of each filtration resistance caused by the membrane R_m, the irreversible fouling (R_i) and the reversible fouling (R_r). The irreversible fouling constitutes the fouling removed by chemical cleaning. The reversible constitutes the fouling removed by water cleaning. During filtration, particles are transported to the membrane surface by permeation drag. Concentration of peptides on the membrane surface reaches its maximum value after a short initial filtration and then the gel and cake layer starts to form. R_m was evaluated from clean water flux measurements at different transmembrane pressure.

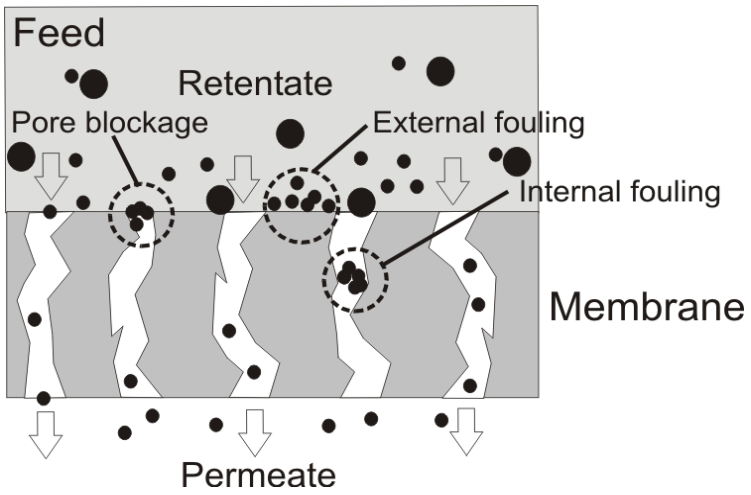

Figure 3.7 overall mechanisms of membrane fouling on and in the membrane

3.4 Characterization techniques

3.4.1 Peptide concentration

This method is based on the derivatization of the protein with *o*-phthalaldehyde (OPA), which reacts with the primary amines of the protein (i.e., NH_2-terminal amino acid and the α-amino group of the lysine residues). The sensitivity of the test can be increased by hydrolyzing the protein before it is tested. Hydrolysis makes the α-amino group of the constituent amino acids of the protein available for reaction with the *o*-phthalaldehyde reagent. The method requires very small peptide quantities.

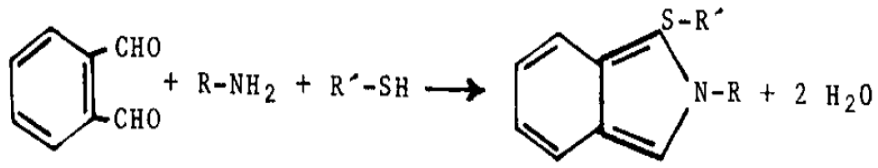

Figure 3.8 Chemical reaction of OPA assay

3.4.2 Hydrophobicity

The number, type and distribution of non-polar amino acid residues with the peptide determine its hydrophobic character. A separation method that is based on the hydrophobic character of peptides is hydrophobic interaction chromatography.

The high performance liquid chromatography (HPLC), especially at the reverse phase mode (RP-HPLC), showed to be efficient to separate peptides from protein hydrolysates and also to give some indications about their hydrophylicity and hydrophobicity (Lemieux et al., 1991) Some works were carried out using different experimental conditions of RP-HPLC for separating the

peptides from tryptic hydrolysates of casein, from β-lactoglobulin A, and the human growth hormone (Kalgahati & Horvath, 1987; Maa & Horvath, 1988).

The RP-HPLC was used to monitor the peptides produced at different stages of the hydrolysis process of whey proteins with commercial proteinases (Perea et al., 1993). Imbert & Nicolas (1993) proposed a technique of RP-HPLC for separating the two peptides obtained from κ-casein hydrolysis by rennet, A and B caseinomacropeptides, which are important for identifying the adulteration of milk or milk powder with whey. Also, RP-HPLC technique has been shown useful in separating active peptides from protein hydrolysates. Thus, the separation of a bioactive peptide prepared by a continuous hydrolysis of β-casein in a membrane reactor was carried out by RP-HPLC (Bouhllab et al., 1993). The elution profiles on a RP-HPLC column was also used in a collaborative study about the antioxidative activity of peptides from protein hydrolysates (Chen et al., 1995). Another utilization of RP-HPLC technique in protein hydrolysates analysis is related to the identification of the specificity of different proteases. Thus, the characteristic RP-HPLC patterns of k-casein cleavage were shown to be useful for characterizing the types of proteinases produced by different bacteria strains (Reid et al., 1994).

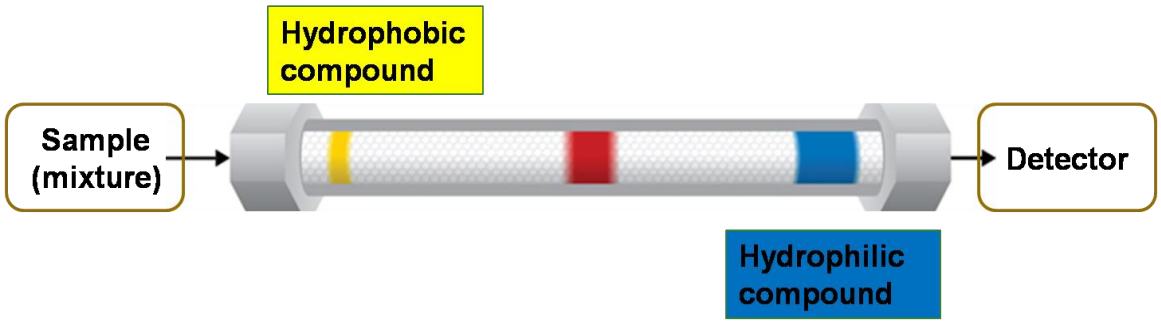

Figure 3.10 Principal of RP-HPLC with compounds

Reversible hydrophobic interaction between amino acid side chains on the peptide with the hydrophobic surface of packing material is the key principal of RP-HPLC.

3.4.3 Aggregation

Each peptide has a distinct and characteristic solubility in a defined environment and any changes to those conditions (buffer or solvent type, pH, ionic strength, temperature, etc.) can cause peptides to lose the property of solubility and precipitate out of solution. The environment can be manipulated to bring about the separation of peptides.

The objective of this work was to characterize the change in peptide solubility resulting from changing some physicochemical conditions in non-animal media supplements. Groleau 2003 was previously demonstrated that peptide-peptide interactions occured in a β-lactoglobulin (β-LG) tryptic hydrolysate, mainly driven by hydrophobic interactions, and that some peptides aggregated at acid pH. The aggregation of peptides during enzymatic hydrolysis of whey proteins has been observed in several studies (otte et al, 1997) in which aggregates were shown to consist of peptides of 2-6 kDa, predominantly linked together by non-covalent interaction. Groleau 2003 was undertaken to characterize peptide aggregates formed in a tryptic hydrolysate of β-LG under different physicochemical conditions, and to demonstrate the occurrence of peptide-peptide interactions in a peptide mixture. Turbidity (500nm) was used as a tool to follow the aggregation process, and peptide aggregates were isolated and identified by chromatography. The turbidity (500nm) of a 1% solution of tryptic peptides was measured at pH 3- 10, at 5, 25,50 C, in the presence of different salt concentrations (0, 0.5 and 1 M NaCl) in the presence of denaturing and reducing agents (6M urea, 5% SDS, or 5% β-mercaptoethanol), and under an electric field (isoelectric focusing).

3.4.4 Total antioxidant Capacity

Total antioxidant capacity works by measuring the amount of the substance being tested needed to inhibit the oxidation of the reagent. The reagent therefore measures the total reducing capacity of a sample.

On the basis of the chemical reactions involved, major antioxidant capacity assays can be roughly divided into two categories: (1) hydrogen atom transfer (HAT) reaction based assays and (2) single electron transfer (ET) reaction based assays. The ET-based assays involve one redox reaction with the oxidant (also as the probe for monitoring the reaction) as an indicator of the reaction endpoint. FCR assay is one of ET-based assays. Most HAT-based assays monitor competitive reaction kinetics, and the quantitation is derived from the kinetic curves. ORAC assay is one of HAT-based analysis. HAT-based methods generally are composed of a synthetic free radical generator, an oxidizable molecular probe, and an antioxidant. HAT- and ET-based assays are intended to measure the radical (or oxidant) scavenging capacity, instead of the preventive antioxidant capacity of a sample. Because the relative reaction rates of antioxidants (or substrates) against oxidants, particularly peroxyl radicals, are the key parameters for sacrificial antioxidant capacity, we will analyze antioxidation and its inhibition kinetics before in-depth analysis of the individual assays.

Chapter 4 **Materials and Methods**

4.1 Feed Solution preparation

BBL™ Yeast extract (No.211929, BD bionutrients, Mississauga), Difco™ TC yeastolate (Cat.No.255772, BD bionutrients, Mississauga) and primatone were obtained from Becton Dickinson (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Yeast extract (YE), yeastolate (YET), and primatone RL (PRI) were dissolved in deionized water (resistivity ~17.5 MΩ: Millipore Milli-Q system, Etobicoke, Canada). Each solution (100mL) containing 1% (w/v) of either YE, or YET, or PRI was stirred for 5 min prior to ultrafiltration. The pH of the solution was measured before ultrafiltration (2±16 pH, EW-35610, Cole-Parmer, Montreal, Canada).

4.2 Dead-end Ultrafiltration

Unstirred dead-end ultrafiltration was performed using an Amicon ultrafiltration cell (model 8200, Amicon Inc., Beverly, MA, USA), as in Figure 4.1. The maximum volume of ultrafiltration cell is 200ml and membrane area is $28.7\,cm^2$. The total volume of the feed was specific to the filtration step (Figure 4.2). Three types of membrane MWCO were used in this project: 50kDa, 3kDa, 1kDa.

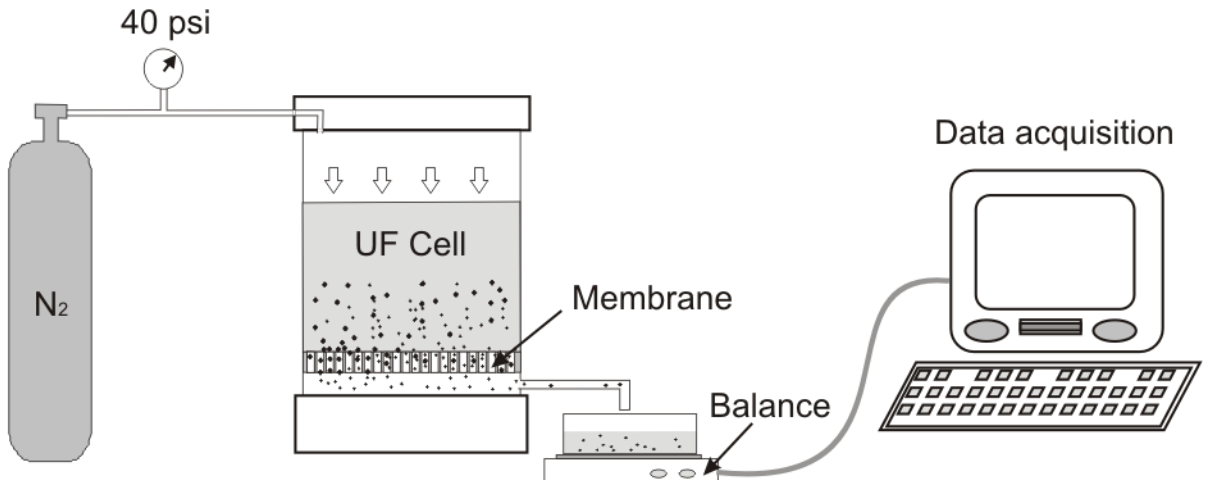

Figure 4.1 Schematic diagram of dead-end ultrafiltration experimental set-up

The 50kDa membrane was made of polyethersulfone (PES) (Omega Pall Corporation, Mississauga, Canada) and supplied as a flat sheet. The 3kDa and the 1kDa MWCO membranes were made of regenerated cellulose (RC) (Millipore Amicon Bioseparations, Etobicoke, Canada) and supplied as pre-cut membrane. The chemical structure of ployethersulfone and regenerated cellulose is shown in Appendix A. The sequence of the ultrafiltration is illustrated in Figure 4.2. Before each filtration, a new membrane was soaked in Nanopure water (resistivity ~17.5 MΩ-cm, Millipore Milli-Q system, Etobicoke, Canada) for 90 min with the skin (glossy) side down, and the water was changed three times to remove the glycerin coating (which was added by the manufacturer to prevent drying.) The water flux was measured as a function of transmembrane pressure, and used to calculate the membrane resistance (R_m) as shown in Appendix B. After measurement of the water flux, the feed solution was placed in the UF cell and the filtration was started under constant transmembrane pressure of 40 *psi* (276 *kPa*) at room temperature. Each filtration had different initial concentrations and volumes, and a final retentate volume and volume concentration ratio ($VCR=V_{feed}/V_{retentate}$) as indicated in Table 4.1. The mass of permeate was measured by an electronic balance (Adam Equipment Inc., Danbury, CT, USA), which was connected to a PC running Labview 7.1 and used to estimate the permeate flux.

Table 4.1 Summary of volume and VCR for each ultrafiltration step

	50 kDa MWCO	3 kDa MWCO	1 kDa MWCO
Feed	100ml	90ml	60ml
Retentate	10ml	30ml	30ml
Permeate	90ml	60ml	30ml
VCR	10	3	2

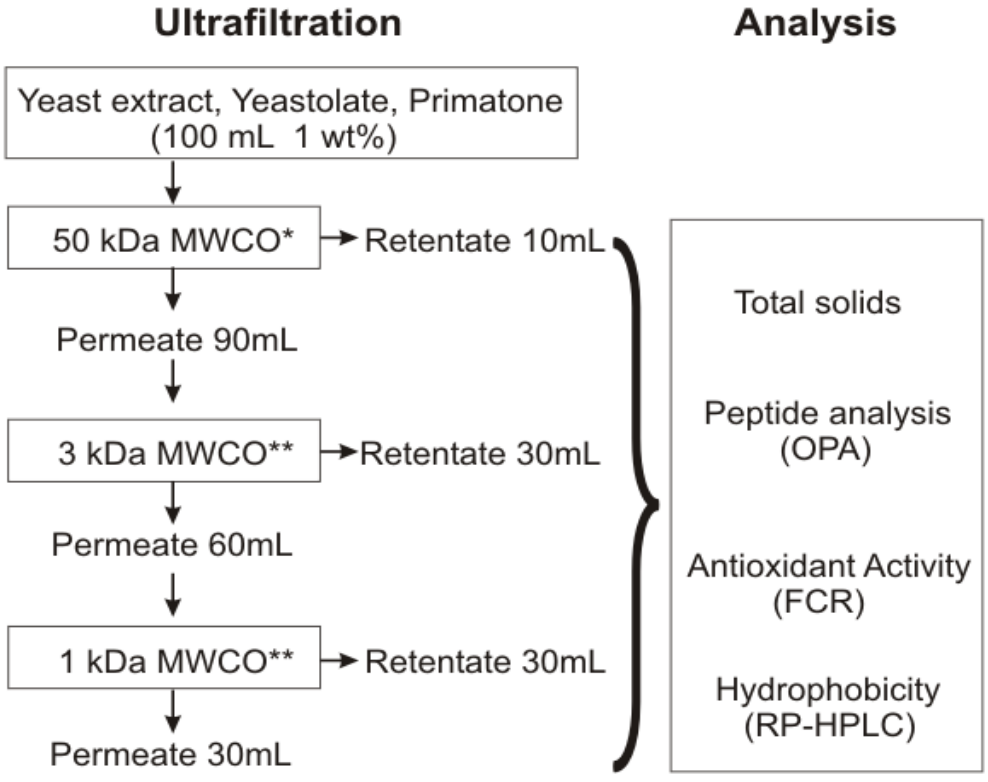

Figure 4.2 Schematic diagram of the ultrafiltration sequence

4.3 Total solids

The total solids content (water-soluble and non-water soluble components) of YE, YET, and PRI feed and fractions was determined by freeze drying of a prescribed volume of sample.

Freeze drying was carried out in a lab scale freeze dryer (Thermo savant super modulyo Edwards pirani 501, Germany) equipped with rapid freezing and drying facilities. Permeate and retentate samples were pre-frozen with liquid nitrogen and freeze-dried under variable chamber pressure (15-5 $\times 10^{-3}$ mbar) and temperatures (-30-70° C). After freeze drying, each sample was weighed and the difference was reported as the solid contents for the corresponding sample volume. After measuring the solids mass, the freeze-dried samples were sent to University of Manitoba for their evaluation as CHO cell media supplements.

4.4 Total Peptide Content (OPA)

This method is based on the derivatization of the peptide with *o*-phthalaldehyde (OPA), which reacts with the primary amines of the peptide (i.e., N-terminal amino acid and the α-amino group of the lysine residues). The method requires very small quantities of the peptides.

Phenyl-Glycine solutions were prepared by dissolving Phenyl-Glycine (Sigma Aldrich, Mississauga, Canada) to concentrations 0, 250,500,750,1000mM in MilliQ water. A calibration curve was obtained with the above 5 Phe-Gly concentrations and was used to estimate the Phe-Gly equivalent concentration of a given sample.

Each sample solution contained 1.0wt/v% of lyophilized fractions which was within the range of the concentrations of the standard solutions. Deionized water was used as blank.

An OPA solution was prepared by dissolving 0.08g of ortho-phthalaldehyde (Sigma, Mississauga, Canada) in 2 ml of ethanol, then adding 50 ml of 1.481g sodium borate buffer (Fisher Scientific, Ottawa, Canada) and 20ml of 1g sodium dodecylsulfate (SDS, Fisher

Scientific, Ottawa, Canada), 200 μL of ß-mercaptoethanol (Fisher Scientific, Ottawa, Canada). At this time, the pH of the OPA solution was adjusted to 9.0 with HCl of analytical grade.
A prescribed volume (100 μL of Phe-Gly standard solution or 10 μL of test solution) was mixed with 1ml of OPA solution for 2min at room temperature. The absorbance of the final solution placed in a 1.5ml polymethyl methacrylate (PMMA) cuvette, was measured at 340nm and at room temperature (23±2℃), with a Cary 1 biosepectrophotometer (Varian, Winnipeg, Canada).

The relationship between the absorbance (intensity) and Phe-Gly concentration was linear for the above concentrations and was used to estimate the total peptide concentration of the sample. An example of a calibration curve and a sample calculation is presented in Appendix C.
Each assay was performed four times and a standard error (SE) was calculated for each data set.

4.5 Antioxidant capacity

The antioxidant capacity of the fractions was determined according to the FCR (Folin-Ciocalteu reagent) method (Stevanato et al 2004) with some modifications.
Trolox (0.0254g) was dissolved in ethanol (20mL) to make a standard solution. Portions of this trolox solution were diluted with the deionized water to obtain not fewer than five standard solutions with concentrations between 1-3mM and evenly spaced (Above 3mM, the calibration curve was non-linear).
The samples (1%wt/v) were dissolved in deionized water such that the antioxidant capacity would fall within the range of the concentrations of the Trolox standard solutions.
Deionized water was used as blank.
In a 4.5 volume PMMA cuvette, an aliquot of 20 μL of the sample to be analyzed or of the trolox solutions was added in series, 150 μL of Folin-Ciocalteu reagent (F5252, Sigma, Mississauga, Canada), 600 μL of Na_2CO_3 solution, and 2230 μL deionised water. After 2 hr incubation at room temperature and with cuvette cap, the absorbance increase was measured at 750nm. The

antioxidant capacity, expressed as a trolox equivalent (TE), was determined from the Trolox calibration curve. An example of calculation and calibration curve is presented in Appendix C.

4.6 Reversed-Phase (RP) HPLC

RP-HPLC analyses were performed using a HPLC system Varian (Mississauga, Canada) consisting of an injector, two pumps (Prostar 210 solvent module), a column heater (#4774, Biorad, Mississauga, Canada), auto sampler (module 410, Varian, Mississauga, Canada), and a UV/Visible detector (Prostar 325 UV-vis detector, Varian, Mississauga, Canada) adjusted at 214nm. The characteristics of the column are presented in Table 4.2. Data acquisition and analysis were done using the Prostar Chromatography software (version 6.0). The samples were analyzed with a symmetry® C18 column (3.9 x 150 mm, 5µm particle size, 100 Å pore size, Waters, Mississauga) at 45°C.

Elution was carried out with a gradient formed between the mobile phase A (N_2HPO_4 buffer, adjusted to pH 6.9 with ortho-phosphoric acid) and the mobile phase B (acetonitrile 60 v/v %). The following elution program was used: 0.1% mobile phase A increased to 4% over 20min at a flow rate of 1 ml/min; the injected samples were monitored at a 214nm wavelength. The sample volume injection was 10 µL.

The mobile phase was passed through a 0.2µm filter (cat 73520-996, Nalgen, Mississauga) and sonicated for degassing before use. Samples 1 % (wt/v) were prepared in the mobile A phase and filtered through 0.2 µm super® membrane low protein binding filters (PN 4602, life science, Mississauga) before injection.

Table 4.2 Reversed phase high pressure liquid chromatography column characteristics

	Name	Company	Inner diameter (mm) X Length (mm)	Packing particle size (µm)	Packing pore size (Å)
A	Symmetry ® C18	Waters	3.9 X150	5	100
B	Luna 3u C18(2)	Phenomenex	4.60X 100	3	100

4.7 Aggregation

A 10 %wt/v feed solution of YE, YET, and PRI was prepared with 1.01 g of feed and 10 mL water and the pH was adjusted to 4.0 with 1N HCl in order to induce acid aggregation. The solution was allowed to rest for 90 min at room temperature and then centrifuged at 329728.4 X g (Thermo WX Ultra 100, Missisauga) for 3hr (4°*C*) and the precipitate separated and lyophilized.

The peptide aggregation was calculated as:

$$\text{Aggregate}\,\% = 100 * \frac{Ci - Cf}{Ci} \qquad \text{Equation 4.1}$$

With initial mass (C_i) and final total solids mass (C_f) content

4.8 CHO cell growth enhancement

4.8.1 CHO Cell culture

CHO cells (beta-IFN producers) were inoculated at 10^5 cells/ml into 4 ml of medium contained in a 25 cm^2 T-flask. The basal medium (Biogro-CHO) was supplemented with the feed (crude-YE, YET, PRI) and its corresponding fractions (50kDa-re, 3kDa-re, 1kDa-re, and 1kDa-per) as will be detailed in section 4.8.3. Samples (0.1 ml) were removed each day for cell counting. Cultures were routinely maintained over 4 days. After that the cell viability decreased significantly. The work was conducted at University of Manitoba.

4.8.2 Cell counting

Culture samples were treated with 0.2 % trypan blue, (1:1 v/v) and the viable cells (unstained) were counted with a haemocytometer.

4.8.3 Growth enhancement assay

This was based upon the dependence of CHO growth on the feed hydrolysate (YE, YET, PRI) concentration in the Biogro-CHO basal medium. Conditions were selected such that growth was concentration dependent for crude non-fractionated YET. Briefly summarized (data not shown), the addition of YET up to 0.05% (w/v) to CHO cultures resulted in a concentration-dependent enhancement of cell growth. No growth was obtained in the absence of crude non-fractionated YET. Maximum cell yields were obtained at 0.05% (w/v) YET. The growth enhancement was consistent and reproducible over 5 culture passages. The cell yield was reduced to 50% maximum in crude non-fractionated YET of 0.01 to 0.025% (w/v).

At 0.05% (w/v) hydrolysate supplement, the growth of cells was over x 2 that at a concentration of 0.025% (w/v). As a consequence, fractions were tested by addition to the 0.025% (w/v) feed-supplemented media. Specific fractions were added to media containing the crude feed hydrolysate (0.025% (w/v)) of the same type. For example, Primatone fractions were added to media supplemented with the minimal levels of crude Primatone. A negative control consisted of the same volume of 0.025% (w/v) of the corresponding crude feed hydrolysate. The positive control consisted of the same volume of 0.05% (w/v) of the corresponding crude feed hydrolysate. The growth enhancement was normalized to compare between sample sets.

% growth enhancement = **(*X* – *N*)*100/ (*P* – *N*)** **Equation 4.3**

Where: X = Cell yield of sample

N = cell yield of negative control

P = cell yield of positive control

4.9 Statistical Analysis

The membrane lot to lot variability of 3kDa and 1kDa, membrane resistance was assessed at p= 0.05. The peptide contents and antioxidant capacity of the feed and the fractions with and without freeze-dring were compared using a two-tailed t-test as described by Montgomery 2001 to identify any significant differences.

Chapter 5 **Sequential Membrane Ultrafiltration Fractionation**

The volumetric permeate flux J_v, the volumetric filtration rate per unit membrane surface area and time expressed in $L/m^2 \cdot h$, represents the productivity of a membrane separation process. In typical ultrafiltration system operated at constant transmembrane pressure, the permeate flux will be affected by the following factors: properties of the membrane, the transmembrane pressure, the system hydrodynamics and the feed properties (concentration, composition, ionic strength, pH and size distribution). The characteristics of the fractions obtained for different membrane types and feed will be discussed with the objective of producing fractions with improved properties for the preparation of serum free medium supplements. Three different size-based membrane were characterized in section 5.1, permeate flux profile of each membrane with yeast extract (YE), yeastolate (YET), and primatone (PRI) in section 5.2. Fouling resistance analysis was investigated in section 5.3. The characterization of the freeze-dried fractions, total solids (5.4), total peptide concentration (5.5), antioxidant capacity (5.6) and hydrophobicity (5.7) will then be presented in the context of fractionation efficiency.

5.1 Membrane Characterization

The resistance of the clean membrane (R_m) was estimated and used subsequently to evaluate the fouling occurring during the filtration according to equation 4.2, and the method described in section 4.2. The global fouling resistance (R_g) was decomposed as the clean membrane resistance (R_m) and the total fouling resistance (R_t) according to equation 4.1. The resistance of the clean membrane measured with deionized water is presented in Table 5.1. The clean membrane resistance for the 3kDa ($4.3 \times 10^{13} m^{-1} \pm 0.49$, n=6) is statistically different ($p < 0.00002$) than for the 1kDa ($9.2 \times 10^{13} m^{-1} \pm 0.29$, n=6), both are regenerated cellulose membranes. This also indicates that these membranes possess a narrow pore size. In a different study for larger pore size PES

membranes, Skorepova (2007) reported that a 100kDa membrane had a lower resistance ($3.5x10^{11}m^{-1}$, n=19) than a 200kDa membrane ($4.5x10^{11}m^{-1}$, n=8). We did observe membrane to membrane variability between same-sized 3kDa membranes of the same manufacturer but from different lots. The 3kDa membrane resistance of lot A ($5.3x10^{13}m^{-1}$±0.4, n=3) and lot B ($3.3x10^{13}m^{-1}$±0.1, n=3) were statistically different ($p < 0.04$, n=3). On the other hand, no statistical difference was observed for different lots of the 1kDa clean membrane resistance (lot A ($9.3x10^{13}m^{-1}$±0.4, n=3) and lot B ($9.2x10^{13}m^{-1}$±0.4, n=3). The effect of these clean membrane resistance differences on the permeate flux will be discussed in section 5.2.

5.2 Permeate Flux Profile

After measuring the clean membrane resistance (R_m) with DI water, each feed was subjected to the sequential ultrafiltration process, 50 kDa, 3 kDa and 1 kDa (Figure 5.1), and the permeate flux (J_p) recorded. The performance of each filtration step and different types of feed will be discussed in the next sections.

5.2.1 Effect of membrane MWCO

The effect of the membrane MWCO, obtained from the permeate flux, is presented for each feed type in Figure 5.1. In general, a similar behavior was observed for all three feed types investigated in this study and a given filtration step. As expected, the permeate flux was much faster for the 50 kDa compared to both the 3 and 1 kDa membrane. For the 50 kDa filtration, the volume of the feed solution was 100 ml and the filtration was stopped when 90mL of permeate had been collected corresponding to VCR=10. The highest permeate flux, observed over the course of the filtration with the 50 kDa membrane (1600-50 $L/m^2 \cdot h$), indicated limited membrane fouling and limited retention of feed components. The total time for the filtration for all three feeds with the 50 kDa membrane was less than 20 minutes. The permeate flux for the 3 kDa and 1 kDa membrane was much lower for all three feeds (20-5 $L/m^2 \cdot h$) and the filtration took around 2 hours. The filtration with the 3 kDa membrane showed a decreasing flux over time

which indicates that fouling was occurring during the entire filtration operation. In contrast, a relatively constant permeate flux over time was observed for the filtration with the 1 kDa (5~8 $L/m^2 \cdot h$) and all three feeds which suggest negligible fouling.

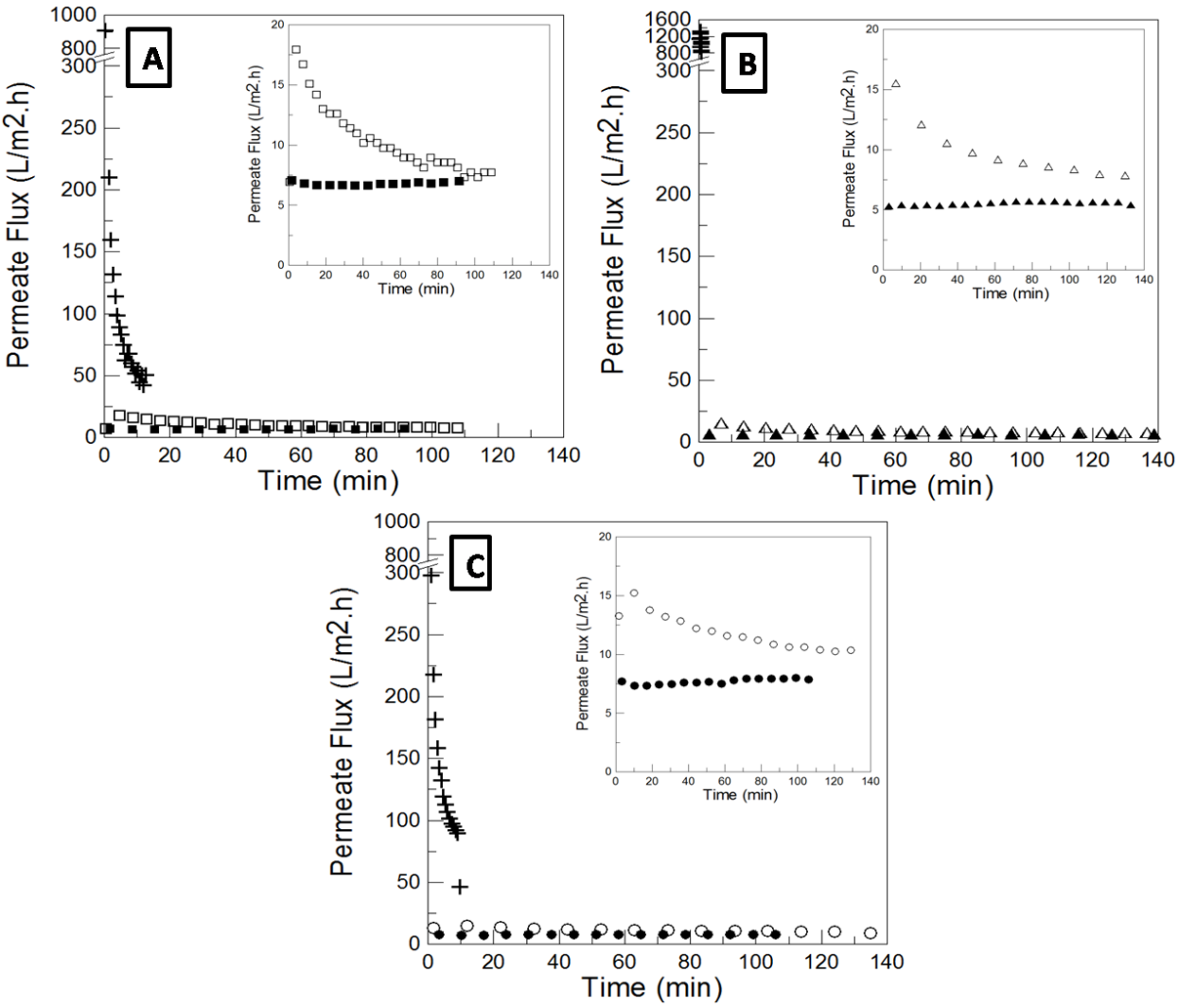

Figure 5.1 Permeate flux in unstirred dead-end sequential ultrafiltration at 276kPa and room temperature: A) YE (50kDa (+), 3kDa (□), and 1kDa (■) membrane); B) YET (50kDa (+), 3 kDa(Δ), and 1kDa(▲) membrane); C) PRI (50 kDa (+), 3kDa (○), and 1kDa (●) membrane); Insert shows the 3 and 1 kDa membrane filtration profile.

5.2.2 Effect of feed type

Figure 5.2 presents the effect of the feed type on the permeate flux for the 50 kDa membrane and VCR=10. YE and PRI had a similar permeate flux profile. In contrast, the permeate flux behavior for YET remained higher during the entire filtration in comparison to YE and PRI which is suggesting different fouling behavior and feed properties.

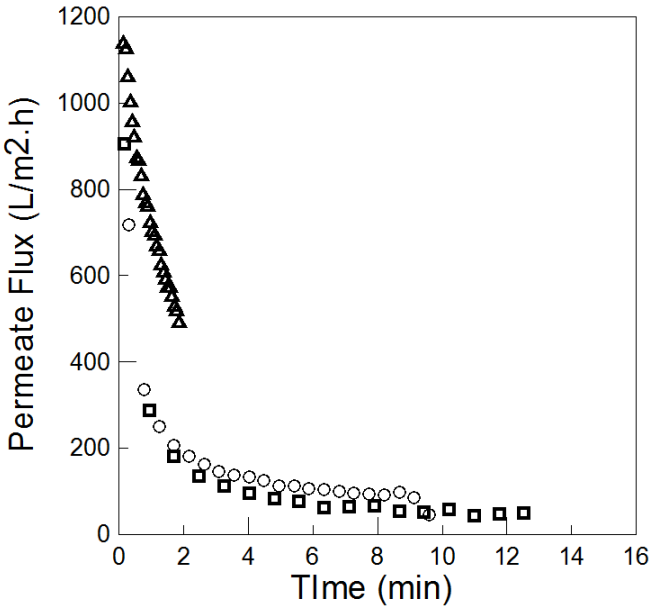

Figure 5.2 Permeate flux in unstirred dead-end ultrafiltration by 50kDa MWCO membrane to VCR 10: □ YE, Δ YET, and ○ PRI, 1wt% feed solution, initial volume 100ml, 276kPa at room temperature.

The permeate flux as a function of time for the 3kDa ultrafiltration of three feed types is shown in Figure 5.3. The initial feed solution was the permeate collected during the 50kDa filtration (90 mL). The filtration was stopped when 60mL of permeate had been collected corresponding to VCR=3. The flux increased initially (to 20 $L/m^2 \cdot h$), due to the pressurization of the cell to 276 kPa, followed by a rapid flux decline (to 10 $L/m^2 \cdot h$ and at 30min, $J_p/J_o=0.5$) and a subsequent

flux decrease until the end of filtration. The final flux was approximately 7.5 L/m^2·h (YE) and 8.7 L/m^2·h (YET), both lower permeate flux than for PRI (11 L/m^2·h). Champagne et al (1999) has shown that permeate flux of YE 10% (w/v) was 50 L/m^2·h operated by tangential filtration system with 3kDa MWCO. The permeate flux is higher than this study because of the tangential operation and that the solution was pre-filtered on a 8μm Whatman (No.2 filter paper).

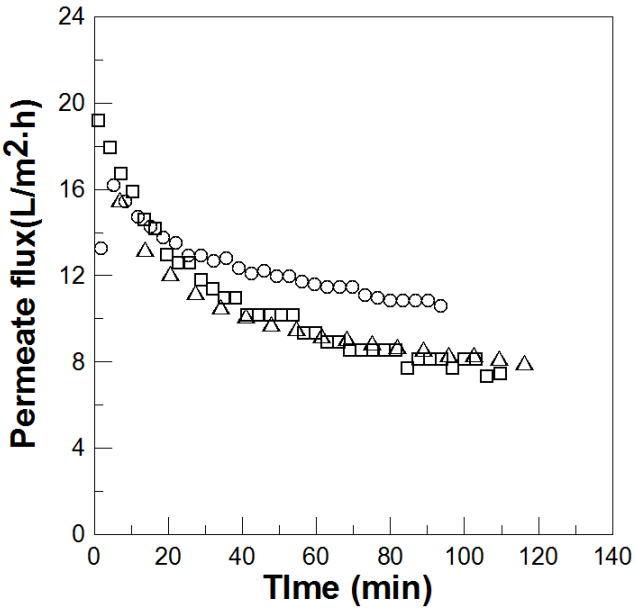

Figure 5.3 Permeate flux decline in unstirred dead-end ultrafiltration to VCR 3 using the 3 kDa membrane (Lot A): □ YE, Δ YET, and ○ PRI. Conditions: initial volume 90ml, 276kPa at room temperature.

The permeate flux for the 1kDa ultrafiltration of YE, YET, and PRI as a function of time is illustrated in Figure 5.4. The permeate flux remained relatively constant for the three types of feed solutions but at different permeate flux values. The filtration of YET took longer than YE and PRI because the permeate flux was ~5-6 L/m^2·h for YET compared to ~7-8 L/m^2·h for YE and PRI. The clean membrane resistances for the YE filtrations (8.1-8.5 x10^{13}m^{-1}) was lower

compared to the YET and PRI filtrations. Theoretically the lowest R_m values should give the highest flux rate; however, this was not observed for these experiments. Champagne et al (1999) has shown that permeate flux of YE 10% (w/v) was 30 $L/m^2 \cdot h$ operated by tangential filtration system with 1kDa MWCO. The feed solution pre-filtered on a 8μm Whatman (No.2 filter paper).

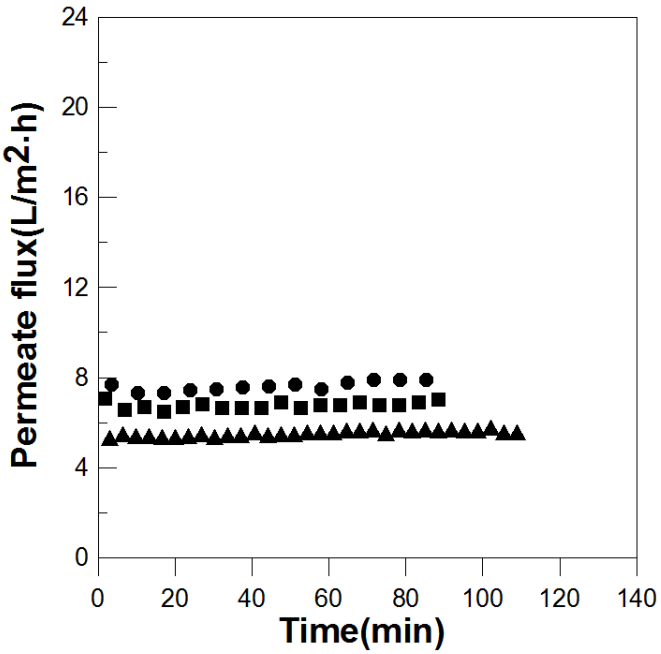

Figure 5.4 Permeate flux in unstirred dead-end ultrafiltration with 1 kDa membrane (Lot A) and VCR=2: ■ YE, ▲ YET, and ● PRI: Initial volume 60ml, 276kPa at room temperature.

5.2.3 Membrane to membrane variability

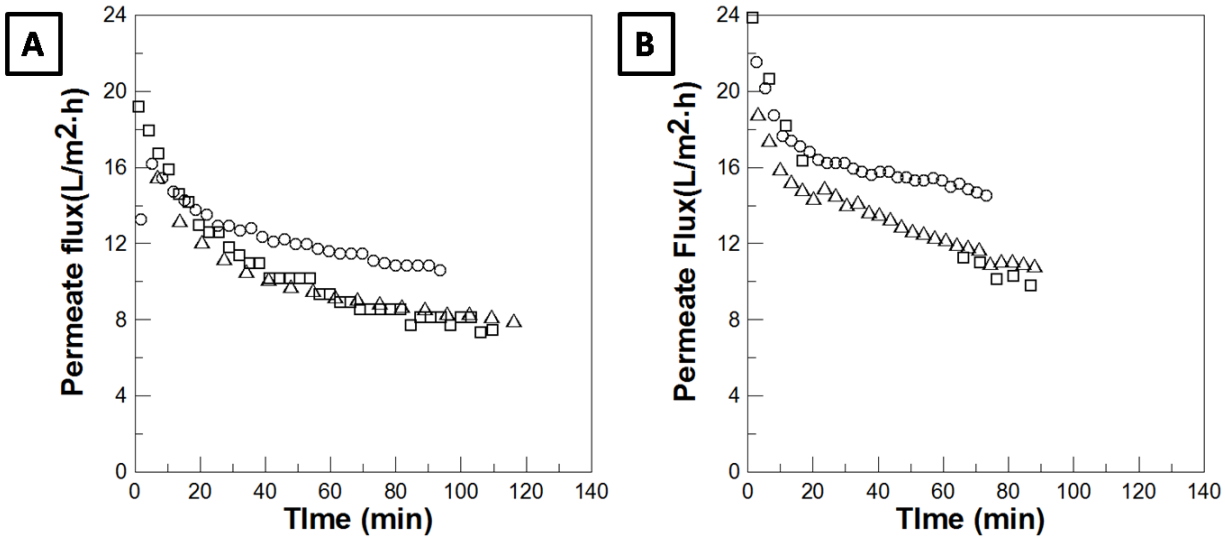

Figure 5.5 Permeate flux in unstirred dead-end ultrafiltration of 3KDa a) lot A b) lot B : □ YE, Δ YET, ○ PRI to VCR 3 , conditions: initial volume 90ml, 276kPa at room temperature.

The variability of the clean membrane resistance for the 3kDa membrane (identified from the measurements of the clean membrane resistance (section 5.1)) affected the permeate flux as illustrated in Figure 5.5. The contribution of this initial variability can be removed by looking at the normalized permeate flux, ie permeate flux at time t (J_p)/initial permeate flux at t=0 (J_o).
This approach was able to eliminate the membrane variability between different membrane lots as illustrated in Figure 5.6 where all filtrations for a given feed are collapsed in one curve.

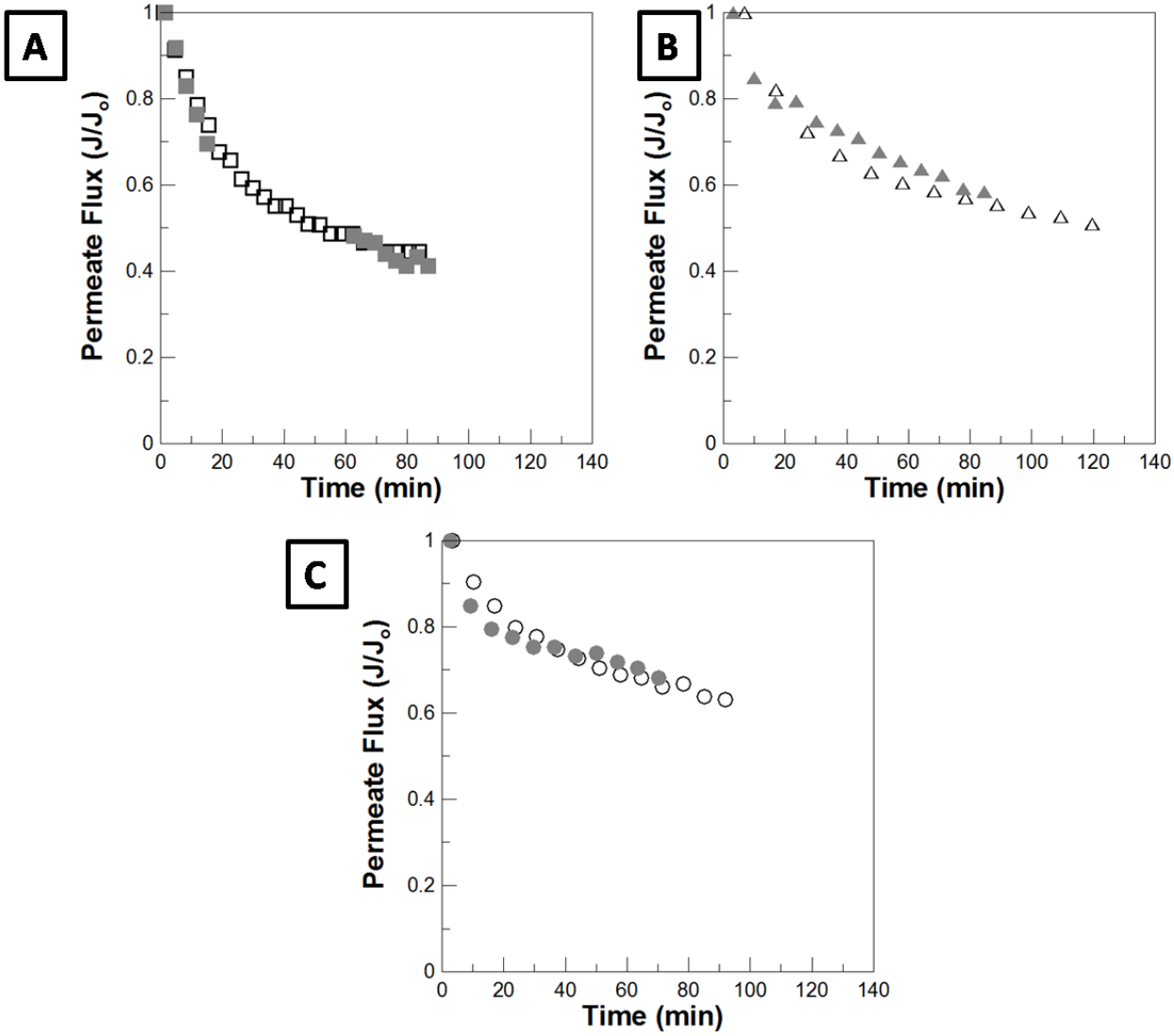

Figure 5.6 Permeate flux in unstirred dead-end ultrafiltration of 3kDa of YE, YET, PRI; A) □ YE lot A,■ YE lot B, B) ∆YET lot A, ▲ YET lot B, C) ○PRI lot A, ●PRI lot B to VCR 3 , conditions: initial volume 90ml, 276kPa at room temperature.

In contrast, the permeate flux profile of 1kDa was similar for membranes of different lots since these membranes had negligible clean membrane variability (Figure 5.7).

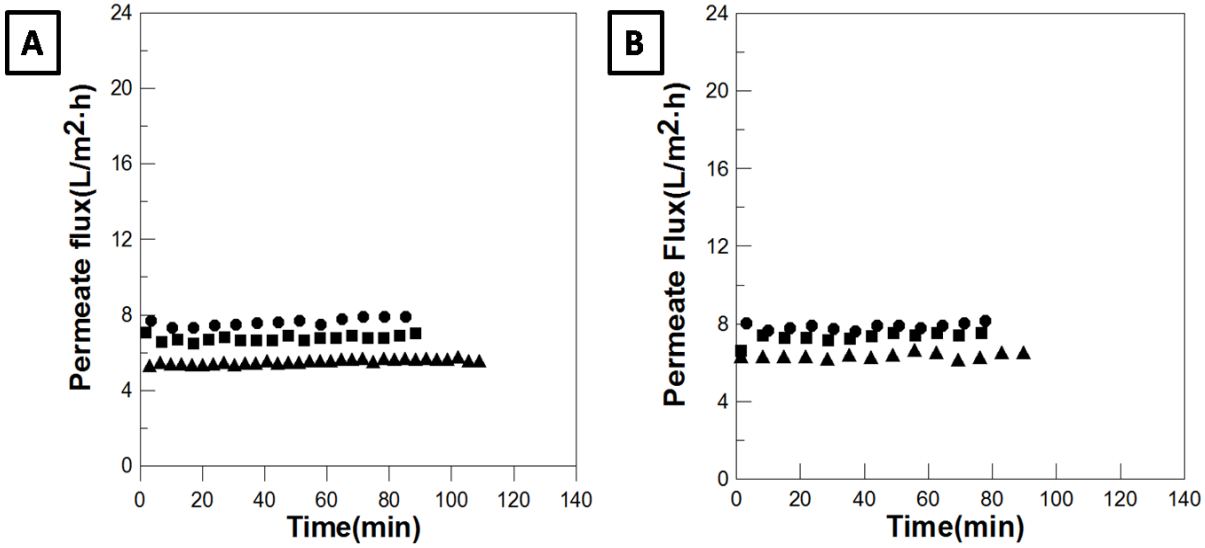

Figure 5.7 Permeate flux in unstirred dead-end ultrafiltration of 1kDa A) lot A B) lot B : ■ YE, ▲ YET, and ● PRI to VCR 2, conditions: initial volume 60ml, 276kPa at room temperature.

5.3 Fouling resistance analysis

To investigate whether the membrane fouling was caused by the different feed solutions and membrane MWCO, the global and individual fouling resistances were estimated and are reported in Table 5.1 for filtrations performed in duplicates.

The ratio of the sum of the reversible and irreversible fouling resistances to the membrane resistance was selected as an indicator of the relative fouling occurring during the filtration. The ratio is about 2 for YE and YET and the 3kDa membrane MWCO, an indication of the significance of fouling. In contrast, this ratio was 1.46 for PRI. For the 1kDa membrane MWCO, the ratio was ~1 or less which indicates that fouling was not significant when compared to the clean-membrane resistance.

Table 5.1 Global fouling resistance (R_G) evaluated from Eq. 3-2 with the contribution of membrane resistance (R_m) and the reversible and irreversible (R_r+R_i) fouling resistances for dead-end ultrafiltration

Feed	Membrane MWCO (kDa)	Permeate Flux * ($L/m^2 \cdot h$)	Global fouling resistance (R_G) (m^{-1})	Membrane resistance (R_m) (m^{-1})	Reversible and irreversible resistance (R_r+R_i) (m^{-1})	R_r+R_i/R_m
YE	3	8.65 ± 0.5	13.1×10^{13}	4.68×10^{13}	8.45×10^{13}	2.02
	1	7.26 ± 0.07	15.4×10^{13}	8.34×10^{13}	7.02×10^{13}	0.84
YET	3	9.08 ± 1.0	12.5×10^{13}	4.15×10^{13}	7.02×10^{13}	2.03
	1	6.06 ± 0.1	18.5×10^{13}	9.44×10^{13}	9.06×10^{13}	0.96
PRI	3	11.72 ± 1.6	10.2×10^{13}	4.09×10^{13}	6.12×10^{13}	1.46
	1	7.83 ± 0.04	14.2×10^{13}	9.36×10^{13}	4.88×10^{13}	0.52

*Measured at the end of the filtration, n=2; mean ±standard error

5.4 Total solids

Total solids analysis of the different streams provided information on the retention and correspondingly permeation efficiency of the filtration. The total solids content for 50, 3, and 1 kDa retentate and permeate is presented in Table 5.2. The three types of feed have similar total solids content recovered in the 50 kDa retentate fractions, ~14% of the initial solids of the feed stream. The distribution of total solids content differered for the subsequent 3 kDa and 1kDa fractionation steps. YE and YET were relatively similar, with equal distribution of total solids in the 3 fractions: fraction 1 (< 50kDa but > 3kDa), fraction 2 (< 3kDa but > 1kDa), fraction 3 (< 1kDa). In contrast, Primatone contained 14% total solids (< 50kDa but > 3kDa), 29% (< 3kDa but > 1kDa) and 33% (<1 kDa). The lower total solids content for PRI and fraction 1 (< 50kDa but > 3kDa) is reflected in the lower global fouling observed during the filtration. The higher

total solids content of fraction 2 (< 3kDa but > 1kDa) is also reflected in the higher global fouling observed during the filtration.

Table 5.2 Mass of total solids in feed and fractions, mean ±SE (n=2)

	Feed [g]	50kDa-retentate [g]	3kDa-retentate [g]	1kDa-retentate [g]	1kDa-permeate [g]	Total Loss [g]
YE	1.01	0.13±0.03	0.26±0.05	0.19±0.08	0.22±0.01	0.21
YET	1.01	0.15±0.02	0.22±0.04	0.24±0.01	0.28±0.01	0.12
PRI	1.01	0.14±0.04	0.14±0.10	0.29±0.01	0.33±0.08	0.11

5.5 Total peptide content

The total peptide content was estimated by the OPA assay as equivalent Phenyl Glycine (Phe-Gly). The total peptide content was evaluated for fresh and freeze-dried samples. There was no statistically significantly effect of freeze-drying on the Phe-Gly content (data not presented). Therefore the total peptide content is reported for the fresh samples in Tables 5.3 a, b, c. The feed had different equivalent Phe-Gly content for a similar total solids content. Yeast extract (YE) contained the highest amount of equivalent Phe-Gly which is statistically different than YET and PRI ($p<0.05$). In spite of different feed equivalent Phe-Gly, all three feeds contained a similar amount of equivalent Phe-Gly with molecular weight larger than 50 kDa, 15-19% of the initial feed stream. This was a similar amount as the total solids content presented in Table 5.2. The total peptide content of the retentate obtained for the 3kDa filtration indicated that YE and YET contained ~ 20% of equivalent Phe-Gly larger than 3 kDa but smaller than 50kDa. In contrast, PRI contained only 6% of equivalent Phe-Gly with such molecular weight. The retentate of the 1kDa filtration contained 55% of the feed equivalent Phe-Gly compared to 47%

for YE and 38% for YET ($p < 0.05$). Looking at the absolute content for each fraction and feed, the distribution according to molecular weight is summarized in Table 5.3 d. All three feeds have similar total peptide content smaller than 1 kDa. Differences are observed for all other fractions. But for any given feed, the equivalent Phe-Gly larger than 1 kDa but smaller than 3 kDa predominated. The total peptide content profile according to size coincides with the total solids distribution for all three feed types. This is the first study that reports on the total peptide content for YE, YET, and PRI subjected to ultrafiltration fractionation Indeed, different methods were used to assessed the α-Amino nitrogen (6g/100g of Total solid) and total nitrogen (10g/100g of Total solid) of the YE and fractions produced by tangential flow ultrafiltration were determined by titration following reaction with formaldehyde (Gaudreau et al, 1999).

Table 5.3 a Equivalent Phe-Gly content for 50kDa ultrafiltration, fresh sample, mean ±SE(n=4)

Extract	Feed (mmol)	Retentate (mmol)	Permeate (mmol)	Loss (mmol)	Recovery (%)*	
					Retentate	Permeate
YE	413.7±22.5	78.5±3.5	298.6±10.5	37	19	72
YET	303±8	47.5±1.5	262±3	6	15	86
PRI	330.1±1.6	62.7±0.5	267.2±2.9	1	18	80

* Phe-Gly (mmol) retentate/ Phe-Gly (mmol) feed

Table 5.3 b Equivalent Phe-Gly content for 3kDa ultrafiltration, fresh sample, mean ±SE (n=4)

Extract	Feed (mmol)	Retentate (mmol)	Permeate (mmol)	Loss (mmol)	Recovery (%)*	
					Retentate	Permeate
YE	298.6±10.5	82.8±3.5	215.7±12.0	1	20	72
YET	262.3±3.0	56.0±0.5	210.5±1.0	4	21	80
PRI	267.2±2.9	18.7±0.15	242.9±2.2	7	6	90

* Phe-Gly (mmol) retentate/ Phe-Gly (mmol) feed

Table 5.3 c Equivalent Phe-Gly content for 1kDa ultrafiltration, fresh sample, mean ±SE (n=4)

Extract	Feed (mmol)	Retentate (mmol)	Permeate (mmol)	Loss (mmol)	Recovery (%)*	
					Retentate	Permeate
YE	216±12	101±3	112.6±3.0	2	47	52
YET	210.5±1	81±1	111.4±0.3	19	38	52
PRI	243±2	134.7±0.7	98.6±10.4	10	55	40

* Phe-Gly (mmol) retentate/ Phe-Gly (mmol) feed

Table 5.3 d Equivalent Phe-Gly content distribution according to molecular weight, fresh sample, mean ±SE (n=4)

Extract	Feed [mmol]; (mmol/g total solids)	>50 kDa [mmol]; (mmol/g total solids)	<50 kDa; > 3kDa [mmol]; (mmol/g total solids)	<3kDa; > 1kDa [mmol]; (mmol/g total solids)	< 1kDa [mmol]; (mmol/g total solids)
YE	413.7±22.5; (410)	78.5±3.5; (604)	82.8±3.5; (318)	101.1±3; (532)	112.6±3 (512)
YET	303±8 (300)	47.5±1.5 (317)	56±0.5 (255)	80.6±1 (336)	111.4±0.3 (398)
PRI	330.1±1.6 (327)	62.7±0.55 (448)	18.7±0.15 (134)	134.7±0.7 (464)	98.6±10.35 (299)

5.6 Total antioxidant capacity

The antioxidant capacity of YE, YET, and PRI was measured by Folin-Ciocalteu's reagent (FCR) assay using 6-hydroxy-2,5,7,8-tetramethylchloroman-2-carboxylic acid (trolox) as a standard. The analysis conducted for fresh and freeze dried samples is presented in Table 5.4.

The total antioxidant capacity of YE, YET, and PRI ranged from 30 mg Trolox/g sample (YET 1kDa permeate) to 60 mg Trolox/g sample (YE 1kDa retentate), which are significantly different ($p < 0.05$). From the statistical analysis, freeze dried sample has a higher antioxidant capacity than fresh sample which is significantly different ($p < 0.05$) for all the YE fractions , the 50 kDa and 1 kDa fractions of YET and the 50 kDa, 3 kDa permeate and 1 kDa permeate for PRI. These results indicate that the antioxidant capacity will generally maintain for prolonged time, with freeze drying treatment.

When considering the freeze-dried samples, no real enhancement was observed as a result of ultrafiltration fractionation.

Table 5.4 Total antioxidant capacity of fresh and freeze dried fractions. Mean ± SE (n=4).

Sample		Feed [mg Trolox/ g sample]	50kDa-re [mg Trolox/ g sample]	3kDa-re [mg Trolox/ g sample]	1kDa-re [mg Trolox/ g sample]	1kDa-per [mg Trolox/ g sample]
YE	Fresh	49.2 ± 8.1	49.9± 9.2	56.2± 1.1	61.8± 1.9	41.3± 2.2
	Freeze dried	51.6 ±0.9	50.4± 2.6	46.9± 0.7	49.3± 1.8	47.8± 0.5
YET	Fresh	54.3 ± 1.6	50.1± 1.2	38.7± 8.3	44.6± 1.7	31.6± 1.4
	Freeze dried	42.8 ± 1.5	45.9± 2.4	44.1± 0.9	49.4± 0.4	45.9± 0.6
PRI	Fresh	55.2 ± 0.6	45.3± 3.9	50.7± 7.0	56.8± 1.0	47.4± 2.2
	Freeze dried	47.1 ± 0.6	45.6± 0.5	43.8± 0.3	46.9± 0.8	49.3± 0.6

There are a number of publications on the assessment of antioxidant capacity with different standards (garlic acid), assays (FCR, ORAC) and food products (wine, soy, sprout, onion, fruits) (Paixao 2007, Martin 2000, Wang 1996, Zielinska 2008). From the production of antioxidant capacity in YE and YET, they carefully controlled to preserve the naturally occurring B-complex vitamins and other vitamin mixture (Sommer 1996)

5.7 Hydrophobicity analysis by RP-HPLC

The relative hydrophobicity of the peptides and amino acids contained in feed, permeate, and retentate fractions was analyzed with Reversed Phase High Pressure Liquid Chromatography (RP-HPLC). The retention time gives a relative measure of the hydrophobicity of substances contained in a mixture. A procedure was first developed to identify the most suitable absorbance wavelength for detection and column for the separation of the components contained in samples without any pretreatment.

A comparison of the absorbance for YE feed solution was performed to identify the most appropriate wavelength for detection (Figure 5.8). Absorbance was observed at two wavelengths, 210 and 260 nm. From literature, ~260nm is used to detect protein while ~210 nm is used to detect small molecules such as amino acids or peptides (Tessier 2006). The next step was the comparison of the absorbance profile at 260 nm and 214 nm for YE separated with a C18 column (Figure 5.9). The absorbance at 214 nm provided a better more detailed resolution than the absorbance at 260 nm. The presence of a peak at 214 nm, near 210 nm, reflects small molecules which are of interest in this study and confirmed the choice of absorbance wavelength selected at 214 nm.

The next step was to investigate two types of columns. A mixture containting 5 different amino acids with different hydrophobicity characteristics (Alanine, Glycine, Tyrosine, Phenylalanine, and Tryptophan) was selected for this analysis(Figure 8.2 and 8.3 Appendix C). Only three of the five amino acids were resolved for both types of column investigated, C18 and C8. Alanine and glycine eluted at the same time and consisted of only one peak. As a consequence, the mixture

was reduced to three amino acids, tyrosine, phenylalanine and tryptophan and was used to analyze the chromatogram of YET (Figure 5.10). The retention time of the three individual amino acids when compared to the absorbance profile of YET feed (Figure 5.10) shows that YET contain Tyr, Phe, and Trp.

The hydrophobicity of each feed solution is illustrated in Figure 5.11 where three major peaks are identified for YE and YET and two major peaks for PRI.

The absorbance profile of the feed and the 1 kDa retentate and permeate fractions illustrated in Figure 5.12 does not reveal major differences for a given type of feed. This indicates that RP-HPLC of the untreated samples was unable to provide information on the relative hydrophobicity of the fractions.

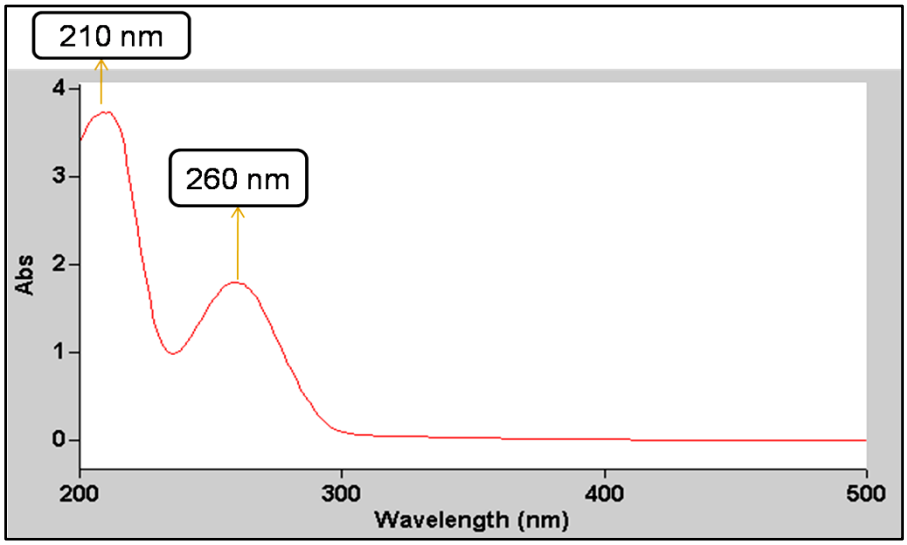

Figure 5.8 Scanning of absorbance for YE feed solution by spectrophotometry

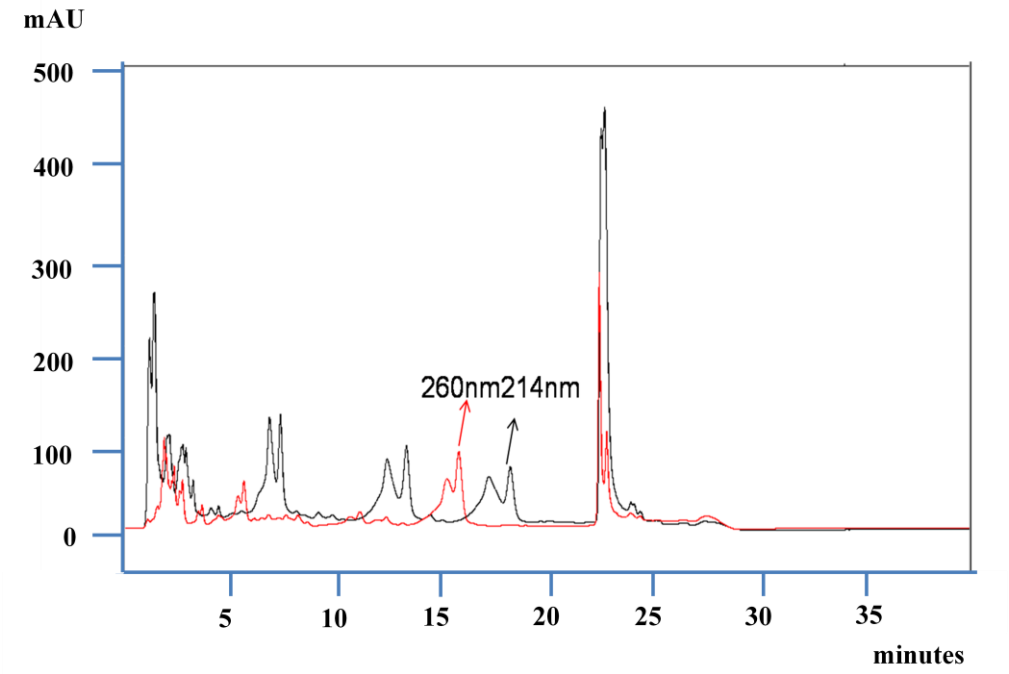

Figure 5.9 Chromatogram of YE feed solution with RP-HPLC C18 Column and detection at 260 nm and 214 nm

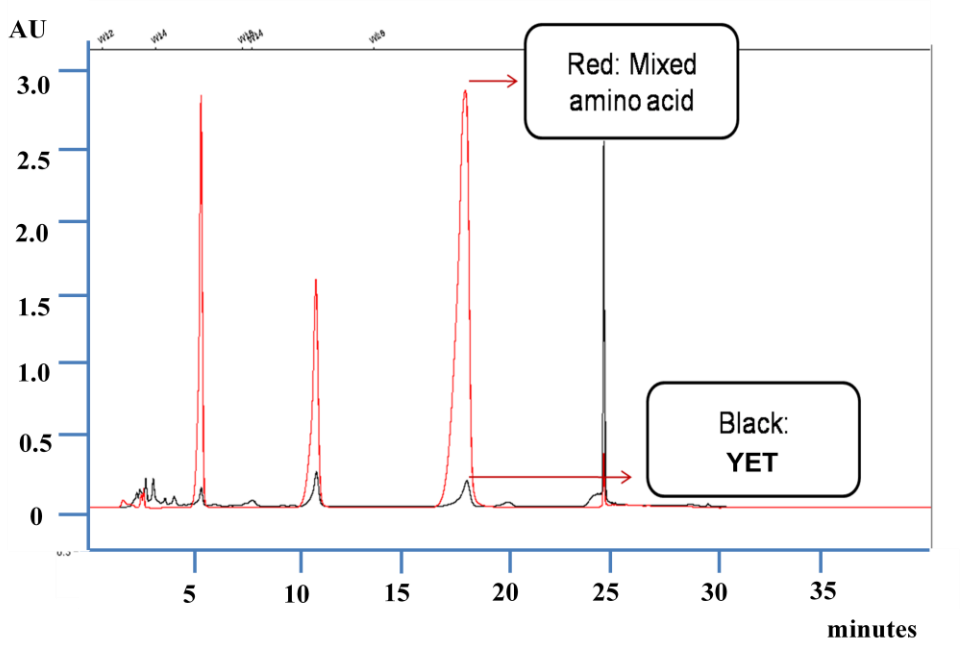

Figure 5.10 Comparison of the elution profile: Mixture of three amino acids (Tyrosine, Phenylalanine, and Tryptophan) and YET feed (component B; 1 % wt/v) with C8 column and detection at 214nm.

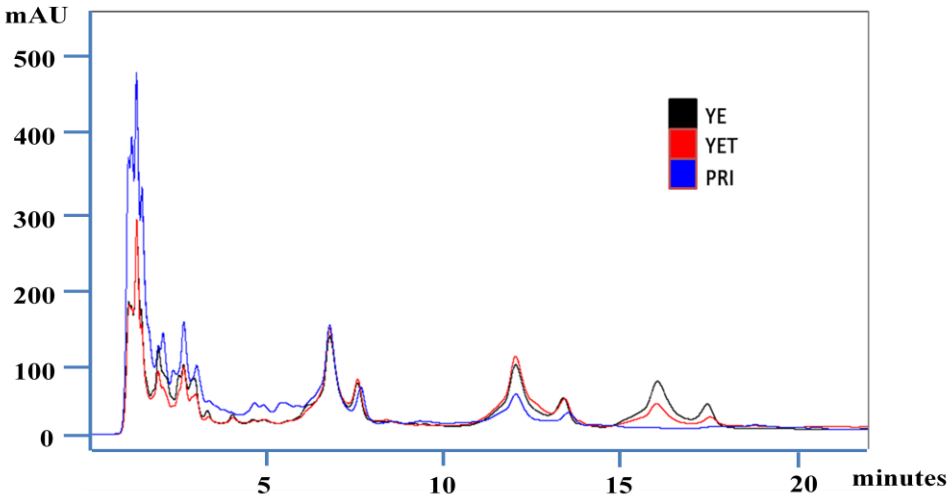

Figure 5.11 Comparison of YE, YET, and PRI feed (1% wt/v) analyzed by RP-HPLC C18 and detection at 214 nm

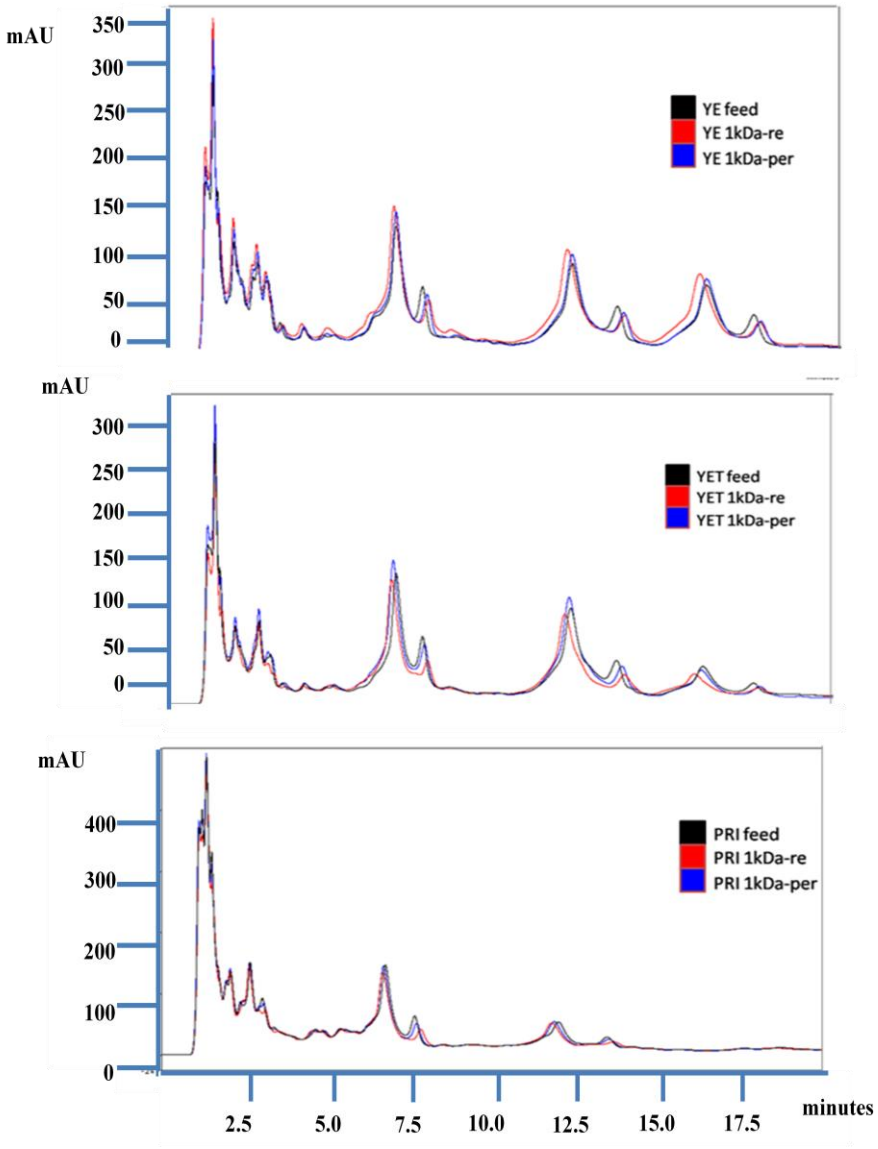

Figure 5.12 Comparison of the relative hydrophobicity for YE (A), YET (B), and PRI (C) feed and the corresponding 1 kDa fractions (1% w/v) with RP-HPLC C18 column and detection at 214 nm

5.8 Aggregation

The potential of acid precipitation to remove hydrophobic peptides contained in YE, YET and PRI was investigated. A very small amount of peptides, 1.5 wt%, were removed by acid

precipitation indicating that these feed contain a very small amount of hydrophobic peptides. This is quite small amount of aggregation than Shen 2007 work.

Chapter 6 **CHO cell growth enhancement**

In this chapter, the bioactivity of freeze-dried fractions obtained from YE, YET, and PRI subjected to different membrane ultrafiltration conditions (50, 3, and 1 kDa) is reported. The bioactivity was measured as cell density for CHO (beta-IFN producers) cell line cultured in T25 flasks with basal medium (Biogro-CHO) containing 0.025% of the fraction added to 0.025% of the corresponding crude non-fractionated feed hydrolysate. A negative control consisted of the same volume of 0.025% (wt/v) of the corresponding crude non-fractionated feed hydrolysate. The positive control consisted of the same volume of 0.05% (wt/v) of the corresponding crude non-fractionated feed hydrolysate.. The work was conducted at University of Manitoba. .

6.1 Primatone

In Figure 6.1 the CHO (beta-IFN producers) cell line cultured in basal medium (Biogro-CHO) supplemented with crude non-fractionated PRI (0.025 % wt/v) achieved a cell titre of about $124x10^4$ cells/ml which corresponds to the negative control. The positive control, the addition of crude non-fractionated PRI (0.05% wt/v), increased the maximal cell density by a factor of almost 2-fold. At day four, all four PRI fractions enhanced cell growth significantly compared to the negative control. However, there was no significant difference between the cell density for the four different types of PRI fractions.

The similar growth enhancement effects of the four fractions can not be linked to the differences of the total peptide content or the total peptide content/ mass of total solids (Table 5.3d), . For example, the 3kDa retentate has the lowest total peptide content (18mmol PheGly equivalent and 134 mmol PheGly equivalent/g total solids) while the 1kDa retentate (134mmol PheGly equivalent and 464 mmol PheGly equivalent/g total solids) has the highest total peptide content. A relationship exists for the similar cell growth enhancement of the four fractions and their similar antioxidant capacity (~45 mg Trolox/ g sample) and hydrophobicity analyzed by RP-HPLC in section 5.7.The cell growth-promoting effect is reflected by the difference between the

maximum cell density of the negative control and the maximum cell density normalized with the two controls according to equation 4.3, 38.9, 40.2, 34.5 and 45.7 % for the fraction larger than 50 kDa, the fraction smaller than 50 kDa but larger than 3 kDa, the fraction smaller than 3 kDa but larger than 1 kDa and the fraction smaller than 1 kDa, respectively.

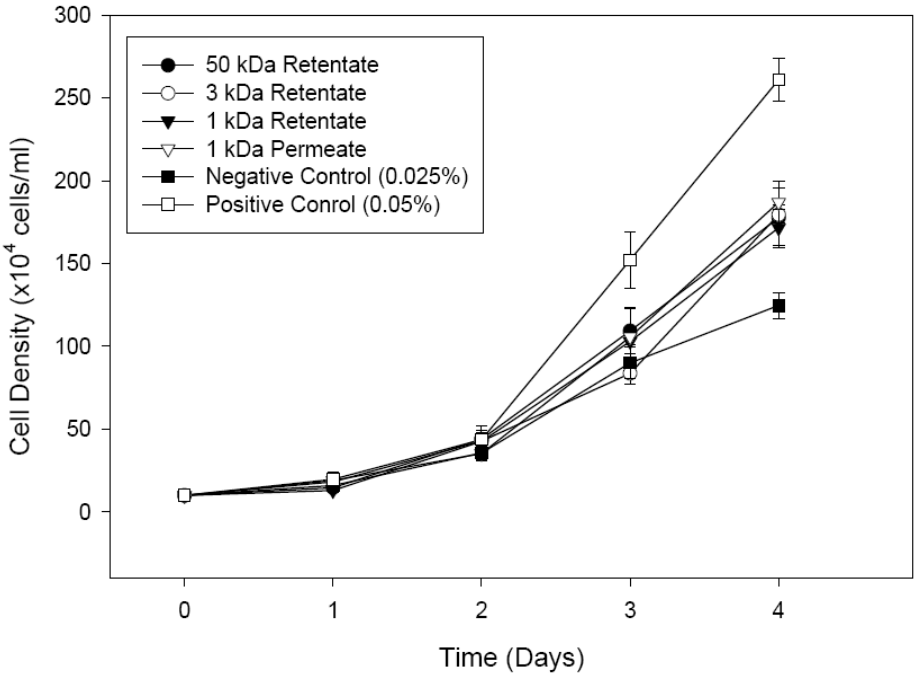

Figure 6.1 CHO cell growth curve in the serum-free basal medium supplemented with crude non-fractionated PRI and PRI fractions ; 50kDa retentate (●), 3kDa retentate (○), 1kDa retentate (▼), 1kDa permeate(∇), Negative control 0.025% (wt/v) crude non-fractionated PRI (■), Positive control 0.05% (wt/v) crude non-fractionated PRI (□) , the mean (±SE) of duplicate cultures at each of 3 culture passages (n=6).

6.2 Yeast Extract

The growth-promoting activity of YE and its fractions on CHO cells was tested in the basal medium ((Biogro-CHO) and compared to 0.025% (wt/v) of YE (negative control) and 0.05% (wt/v) of YE (positive control). Data in Figure 6.2 indicate that the growth of CHO cells in the basal medium with YE fraction (50, 3, and 1 kDa retentate and permeate) was very limited, and the maximum cell density was 217×10^4 cells/mL. The supplementation of the crude non-fractionated YE promoted the cell growth, with cell density of 222 $x10^4$ cells/ml for the negative control and cell density of 282 $x10^4$ cells/ml for the positive control. The supplementation of YE fractions was less growth-promoting compared to YE negative control suggesting that these fractions may contain growth inhibitory elements in higher amount than in the crude non-fractionated YE. One has to keep in mind that the YE negative control (222 $x10^4$ cells/ml) showed the highest growth enhancement compared to the negative control for YET ($122x10^4$ cells/ml) and PRI (124 $x10^4$ cells/ml). Further analysis would be required to confirm the growth effects of the negative control of YE.

The similar low growth patterns of the different fractions could not be related to the different amounts of total peptide estimated in the various fractions (Table 5.3d). For example, the total peptide content for the fraction smaller than 50 kDa but larger than 3 kDa (318mmol PheGly equivalent/g total solids) and the 50kDa-re (604mmol PheGly equivalent/g total solids) was different but the cell density was the same. The growth behavior of the different fractions could be related to the similar antioxidant capacity measured, ~50 mg Trolox/ g sample.

Previous study (Smith 1973) indicates that YE has small size of peptides which have potential growth ingredient. Also approximately 50% of amino acid is smaller than 3 kDa is occurs as important nutrients for vertebrate and invertebrate cells in culture. The concentration of free amino acids in a culture medium supplemented with wheat protein hydrolysate can differ during the growth phase (Hansen and Emborg, 1992).

It was reported that peptides of plant protein hydrolysate exerted specific effects, growth or production enhancing effects (Franek 2000, Franek and Katinger 2002).

Sung et al 2004, of these additives (yeast hydrolysate, soy hydrolysate, wheat gluten hydrolysate and rice hydrolysate), YE was most beneficial to human thrombopoietin (hTPO) production, resulting in a more than 11-fold increase in the maximum hTPO concentration. Furthermore, cell viability during the culture using YE supplemented serum-free medium was higher than that using other medium tested (soy, wheat gluten, and rice hydrolysate) and thereby rCHO cells, liberating proteases and glycosidase from dead cells, was least significant.

According to the production information provided by Quest International, total amino acid concentration in YE are not significantly different from those in other hydrolysate (wheat gluten and rice hydrolysate).

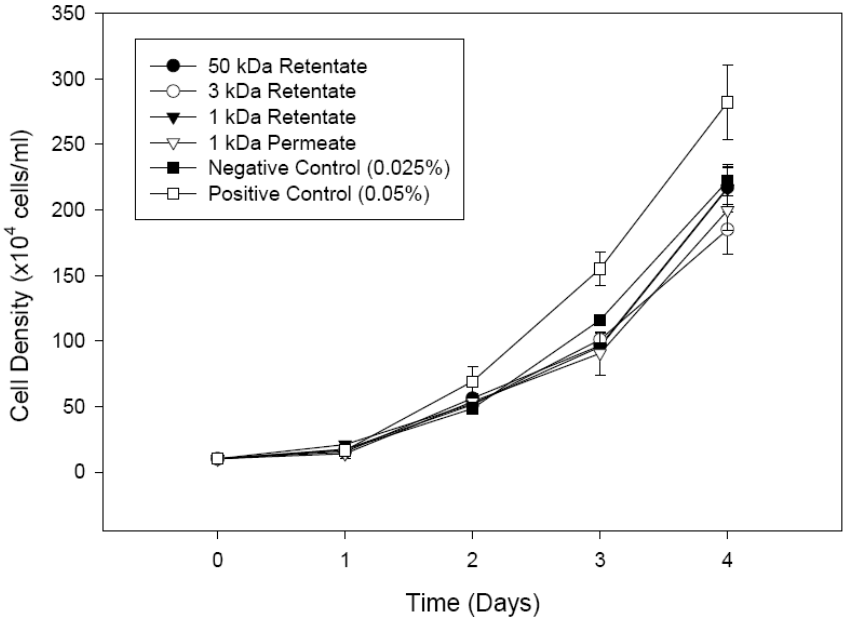

Figure 6.2 CHO cell growth curve in the serum-free basal medium supplemented with YE and YE fractions ; 50kDa retentate (●), 3kDa retentate (○), 1kDa retentate (▼), 1kDa permeate (∇), Negative control 0.025% (wt/v) YE (■), Positive control 0.05% (wt/v) YE (□), the mean (±SE) of duplicate cultures at each of 3 culture passages (n=6).

6.3 Yeastolate

The growth-promoting activity of YET and its fractions on CHO cells is presented in Figure 6.3. The growth of CHO cells in the basal medium supplemented with 0.025% (wt/v) YET (negative control) was 122×10^4 cells/mL and the maximum cell density was 219×10^4 cells/mL. The supplementation with crude non-fractionated YET (0.05% wt/v) or its fractions promoted the cell growth, and the maximum cell density was increased to 169, 144, and 135×10^4 cells/mL respectively in the cultures supplemented with 3kDa retentate, 1kDa retentate, and 1kDa permeate respectively. No growth enhancement effect was observed with the addition of the 50 kDa retentate. The cell growth-promoting effect of the added YET fractions is reflected by the cell density normalized with the two controls according to equation 4.3, 48.6, 22.6 and 13.7 % for the fraction smaller than 50 kDa but larger than 3 kDa, the fraction smaller than 3 kDa but larger than 1 kDa and the fraction smaller than 1 kDa, respectively.

The growth patterns of the different fractions obtained from YET could not be related to the different estimates of total peptide content (Table 5.3d). For example, the total peptide content for the fraction smaller than 50 kDa but larger than 3 kDa (255 mmol PheGly equivalent/g total solids) and the fraction smaller than 1 kDa (398 mmol PheGly equivalent/g total solids) were opposite to the effect of the growth enhancement. The growth behavior of the different fractions could not be related to the relatively similar antioxidant capacity measured, 42-49 mg Trolox/ g sample.

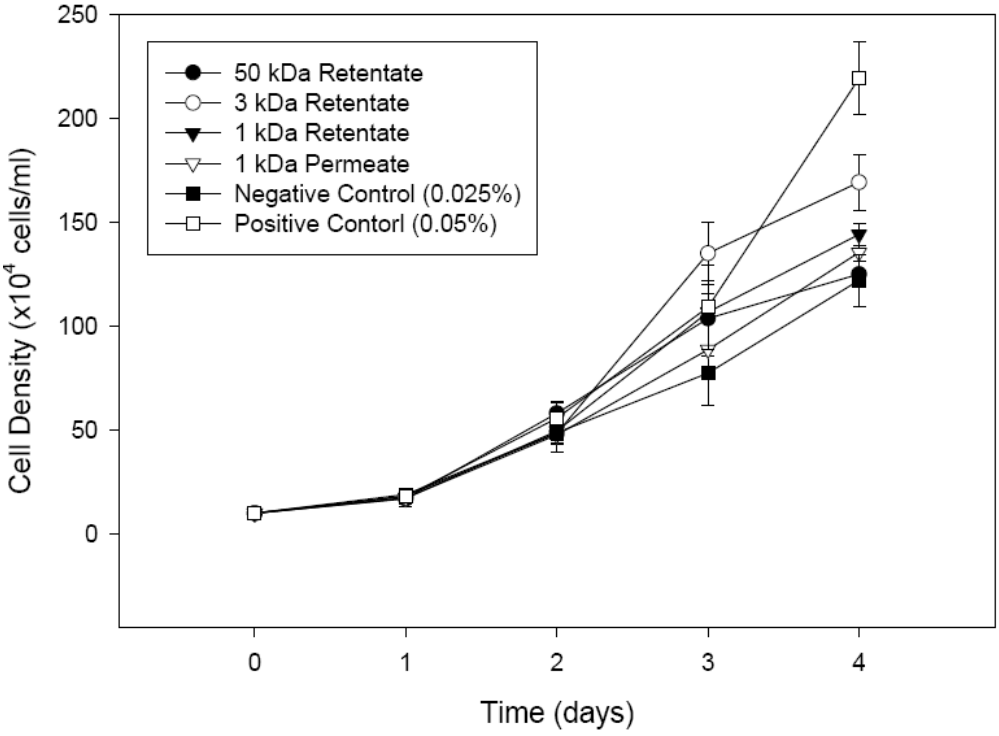

Figure 6.3 CHO cell growth curve in the serum-free basal medium supplemented with YET and YET fractions; 50kDa retentate (●), 3kDa retentate (○), 1kDa retentate (▼), 1kDa permeate (∇), Negative control 0.025% (wt/v) YET (■), Positive control 0.05% (wt/v) YET (□), mean (±SE) of duplicate cultures at each of 3 culture passages (n=6).

6.4 Comparison of the 1kDa ultrafiltation with PRI, YE, and YET

As the published studies on the growth promoting effects of hydrolysates indicate that the small peptides are the most active, the fractions produced during the last ultrafiltration step with the 1 kDa membrane for the three feed types will be discussed in this section. Figure 6.4a shows the growth profiles obtained by the supplementation of the 1kDa retentate factions from the three different sources. The growth enhancement of the 1 kDa retentate is highest for YE, with PRI as second and YET as the lowest effect. Figure 6.4b compares the growth profiles obtained from

the 1 kDa permeates from the three different sources. The 1 kDa permeate from Primatone and yeast extract had a significantly higher growth enhancement effect than Yeastolate ($p < 0.05$).

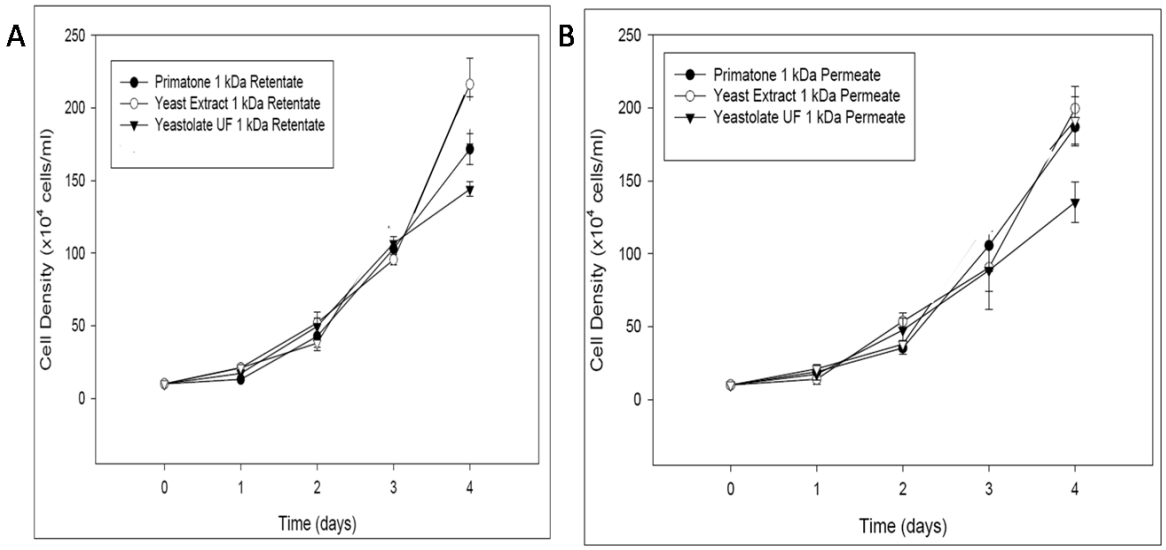

Figure 6.4 CHO cell growth curve in the serum-free basal medium supplemented with PRI, YE, and YET fractions; A) 1 kDa retentate: PRI (●), 1 kDa retentate YE (○), 1 kDa retentate YET 1kDa (▼); B) 1 kDa permeate PRI (●), 1 kDa permeate YE (○), 1 kDa permeate YET (▼). Mean (±SE) of duplicate cultures at each of 3 culture passages (n=6).

Considering all the fractions produced during the sequential ultrafiltration treatment, the relative CHO cell growth enhancement of the various fractions can be summarized as follows. The 1 kDa retentate and permeate fractions obtained from YET both showed lower growth when compared to the 3 kDa retentate. The relative growth enhancement of all four PRI fractions was high. The PRI 1 kDa permeate was the highest (45.7%). The relative growth enhancement of the 3 kDa retentate was high for both PRI (40.2%) and YET (48.6%). There was no apparent growth enhancement for any of the YE fractions. Note that the PRI positive control (261×10^4 cells/ml) was higher than the YET positive control (219×10^4 cells/ml) but lower than the YE positive control (282×10^4 cells/ml) and the negative control for YE gave a higher yield than the other

feed material at the same concentration. These differences may have also contributed to the differences observed for the fractions obtained by ultrafiltration.

Chapter 7 **Conclusions and Recommendations**

Enhancement of CHO cell growth by Yeast extract (YE), Yeastolate (YET), and Primatone (PRI) fractions with size-based ultrafiltration was investigated in this study. Three feed solutions (1% w/v) were prepared and subjected to sequential ultrafiltration (50kDa (polyethersulfone), 3kDa (regenerated cellulose), and 1kDa (regenerated cellulose)). Each filtration system was stopped at a specific volume concentration ratio (VCR), 10, 3, and 2, respectively. The membrane resistance and the global membrane fouling resistance were investigated. Feed and fractions (retentate and permeate) were collected and freeze dried for testing their biological activity on CHO cells (beta-IFN producers) and the total solids, total peptide content, antioxidant capacity and hydrophobicity were evaluated. Thermal stability was considered in peptide concentration and antioxidant activity by freeze-dry process.

The resistance of the clean membrane (R_m) was measured with deionized water; 3kDa ($4.3x10^{13}m^{-1}$±0.49, n=6) and 1kDa ($9.2x10^{13}m^{-1}$±0.29, n=6), as expected 1kDa membrane has higher resistance. For the 3kDa membrane, membrane to membrane variability of the same manufacturer was observed for different lots. The performance of the filtration for the three feed solutions and three membranes showed that the 50 kDa membrane has the highest permeate flux when compared to the 3 and 1 kDa membrane. The final permeate flux with the 3 kDa membrane of PRI (11 $L/m^2 \cdot h$) was higher than YE (7.5 $L/m^2 \cdot h$) and YET (8.7 $L/m^2 \cdot h$). The permeate flux with the 1 kDa membrane was relatively constant over time and similar for all three feeds, 6~8 $L/m^2 \cdot h$. The membrane to membrane variability of the 3kDa membrane for two different lots was observed, however this difference did not affect the type of fouling mechanism. The difference between membrane lots could be removed by considering the normalized permeate flux. Overall, similar filtration behavior with some fouling during the UF 3kDa for YE, YET but different for PRI.

The membrane fouling main factors were investigated by fouling resistance analysis. The ratio of the sum of the reversible and irreversible fouling resistances to the membrane resistance was

selected as indicator. This ratio for YE and YET and the 3kDa membrane was about 2 which showed that fouling was the dominant factor. In contrast, 1kDa membrane filtration had a ratio less than 1 which indicated that fouling was not significant compared to the clean membrane resistance.

The total solids and total peptide contents and the antioxidant capacity of the feed and the fractions were estimated. YE and YET fractions possessed relatively similar profiles, with equal distribution of total solids in the size-based fractions. In general, the total peptide content measured as equivalent PheGly was ~37 %< 1kDa<~30 %< 3kDa<~25 %< 50kDa<~8 % of three materials approximately. The total antioxidant capacity of the freeze-dried samples was quite similar for all fractions, ~ 40-50 mg Trolox/g sample. The hydrophobicity of YE and YET was similar but different than PRI. Differences in the hydrophobicity between the feed and the fractions could not be observed with the approach considered in this study where the samples were directly analyzed by reversed-phase HPLC. Finally, a negligible amount of hydrophobic components (1.5 wt %) could be removed by acid precipitation.

The bioactivity of feed and fractions was measured as cell density for CHO (beta-IFN producers) in basal medium supplemented with a combination of the crude non-fractionated feed material and a specific fraction. PRI showed a similar growth enhancement effect for all fractions when compared to a culture supplemented with the crude non-fractionated. YE showed no growth enhancement for any of the fractions when compared to a culture supplemented with the crude non-fractionated YE. This observation need to be confirmed as a culture supplemented with the crude non-fractionated YE showed a very high growth stimulating effect which was much higher than PRI and YET at the same concentration. Finally, YET 3kDa retentate fraction displayed a 50 % growth enhancement effect.

In conclusion, the fractions obtained from the two non-animal protein hydrolysates considered in this study, YE and YET showed limited CHO cell growth enhancement effect when compared to the non-fractionated material. Only the YET 3kDa retentate fraction displayed a good CHO cell growth enhancement effect. YET 3kDa represent an attractive serum substitute for its use in culturing CHO cells. PRI, an animal derived protein hydrolysate showed the best growth

enhancement effect for all fractions produced in this study. These results suggest that YET has high potential as a media additive for the development of serum-free media which can promote cell growth and, in the future this work can contribute in production of therapeutic proteins markets.

Future work should consider growth experiments that include supplementation with only the individual fractions up to 0.05% in media in T25 flasks and analysis of the amino acids profiles to evaluate the amino acid consumption by the cells. Also alternative methods for hydrolysate fractionation such as nanofiltration, which separates components according to size and charge, should be investigated. We can investigate other types of antioxidant capacity assays such as the ORAC assay which is based in hydrogen atom transfer (HAT) mechanism. This study did not find differences of hydrophobicity between the feed and the fractions through RP-HPLC of the untreated samples. Sample pre-treatment and different solvent and gradients should be investigated to improve the separation of the components. .

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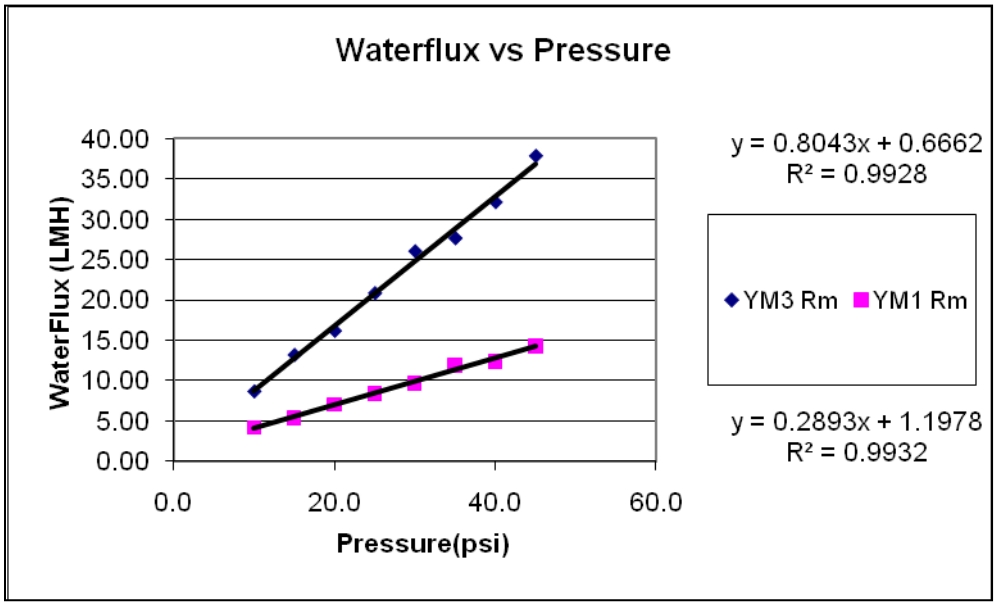
Appendices

Appendix A

Calculation of fouling resistance



- Estimation of total membrane resistance (R_T)

$$J_P = \frac{TMP}{\eta_P R_T}$$

- Estimation of reversible (R_R) and irreversible (R_I) membrane fouling

$$R_T = R_M + R_F$$

R_M: clean membrane resistance

$$R_F = R_R + R_I$$

Sample Calculation

1. Using Waterflux to find membrane resistance Rm

Pressure (psi)	Pressure (Pa= N/m^2)	Permeate mass (g)	Time (s)	Flow rate (kg/s)	Permeate Flux (kg/m2s)	Permeate Flux (L/m2h)	Average Permeate Flux (L/m2h)	R_m (1/m) at 25 C	Average R_m (1/m) at 25 C
10.00	6.89E+04	0.05	22.94	2.18E-06	7.59E-04	2.74E+00	2.79E+00	1.02E+14	1.00E+14
10.00	6.89E+04	0.05	21.78	2.30E-06	8.00E-04	2.89E+00		9.65E+13	
10.00	6.89E+04	0.05	22.78	2.19E-06	7.65E-04	2.76E+00		1.01E+14	
10.00	6.89E+04	0.05	21.19	2.36E-06	8.22E-04	2.97E+00		9.39E+13	
10.00	6.89E+04	0.05	24.31	2.06E-06	7.17E-04	2.59E+00		1.08E+14	
15.00	1.03E+05	0.05	12.87	3.89E-06	1.35E-03	4.89E+00	5.01E+00	8.55E+13	8.35E+13
15.00	1.03E+05	0.05	11.97	4.18E-06	1.46E-03	5.25E+00		7.95E+13	
15.00	1.03E+05	0.05	12.32	4.06E-06	1.41E-03	5.11E+00		8.18E+13	
15.00	1.03E+05	0.05	13.34	3.75E-06	1.31E-03	4.72E+00		8.86E+13	
15.00	1.03E+05	0.05	12.38	4.04E-06	1.41E-03	5.08E+00		8.22E+13	

Factors	Values
Membrane area	0.00287 m^2
Water density	997.13 kg/m^3
Water viscosity	8.9E-04 Pa X second

Example; use the first law of column data to find R_m

R_m= (Pressure X Water density)/(Permeate Flux X Water viscosity)

= (6.89E+04 Pa X 997.13 kg/m^3)/(7.59E-04 kg/m^2s X 8.9E-04 Pa·s) = 1.02 E+14m^{-1}

Rm= 1.02 E+14m^{-1}

2. Using feed solution to find Total resistance (the same method of R_m)

Rt =(Pressure X Feed solution density)/(Permeate Flux X Feed solution viscosity)

*Feed solution is 1wt/v%, assume that density and viscosity of feed solution are the same as Water

R_t= 1.42 E+14m^{-1}

R_t=R_m+R_f, R_f= 4E+13

Appendix B

Calculation of FCR assay

The linear regression method, the absorbance of the solutions can be plotted from the standard solutions versus the antioxidant concentrations, and the standard curve best fitting the plotted points determined (Eq3.1). From the standard curve so obtained and the absorbance of the test solutions, the concentration of antioxidant in the test solution established.

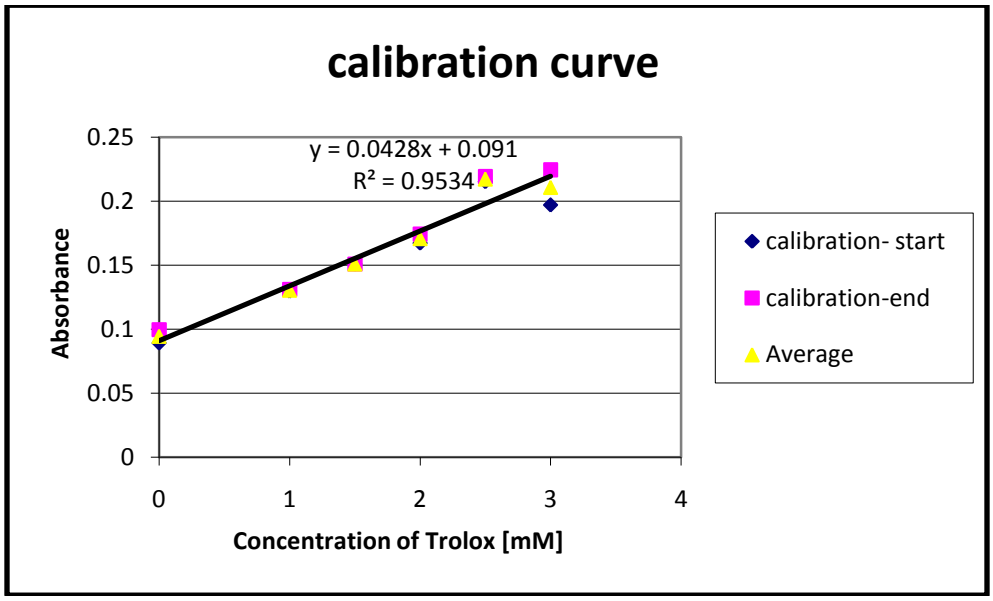
Calibration curve: $y = ax + b$

*a-slop, b-intercept

Concentration of Trolox mM : (Absorbance-intercept)/slop of calibration

Trolox mg/ g of samples: $\text{Trolox mM} \times \text{Trolox MW} \times (\text{Volume of sample}) / (\text{mass of sample})$

Equation 8.1



To calculate Phe-Gly concentration from calibration curve

*Slop-0.0428, Intercept- 0.091
*Absorbance- 0.333
*Trolox MW- 250.29 g/mol

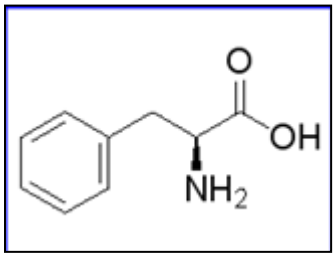
Concentration of Trolox mM : (0.333-0.091)/0.0428= 5.65mM

Trolox g/mol of sample = (((5.56*10^-3)*250.29*20)/202)*1000= 137.78 g/mol of Trolox

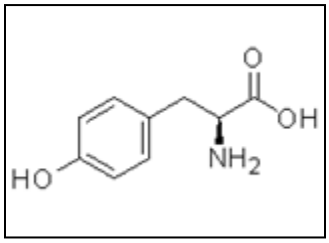
Appendix C

Standard amino acid profile

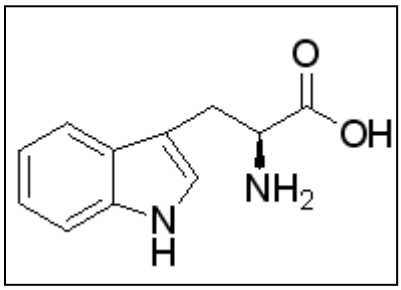
1. L- Phenylalanine , $C_9H_{11}NO_2$, FW165.2



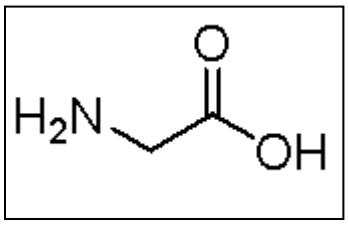
2. L-Tyrosine , $C_9H_{11}NO_3$, FW 181.2



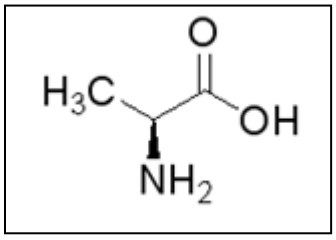
3. L-Tryptophan $C_{11}H_{12}N_2O_2$, FW 204.2



4. Glycine, NH_2CH_2COOH, FW 75



5. Alanine , $C_3H_7NO_2$, FW 89



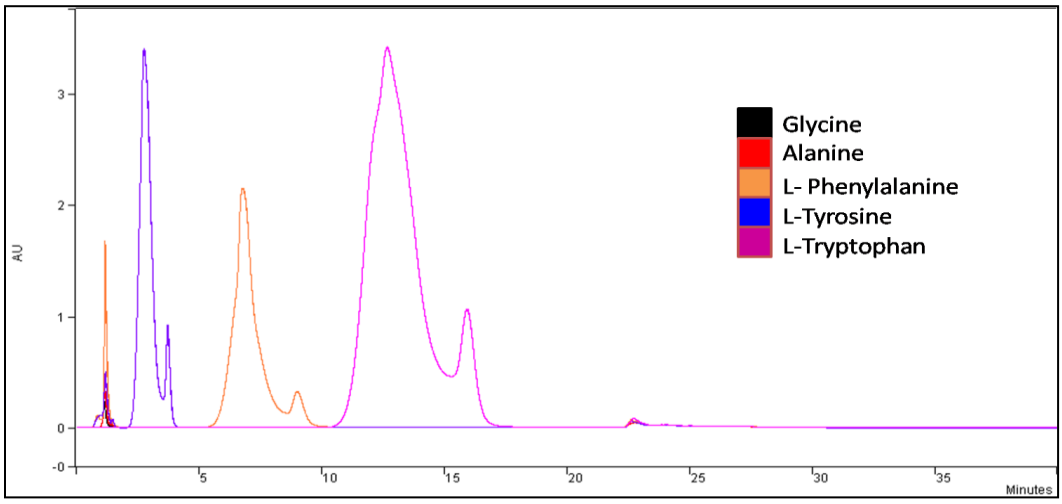


Figure 8.2 Amino acid profiles with RP-HPLC C18 column and detection at 214nm (Glycine and Alanine was not detected)

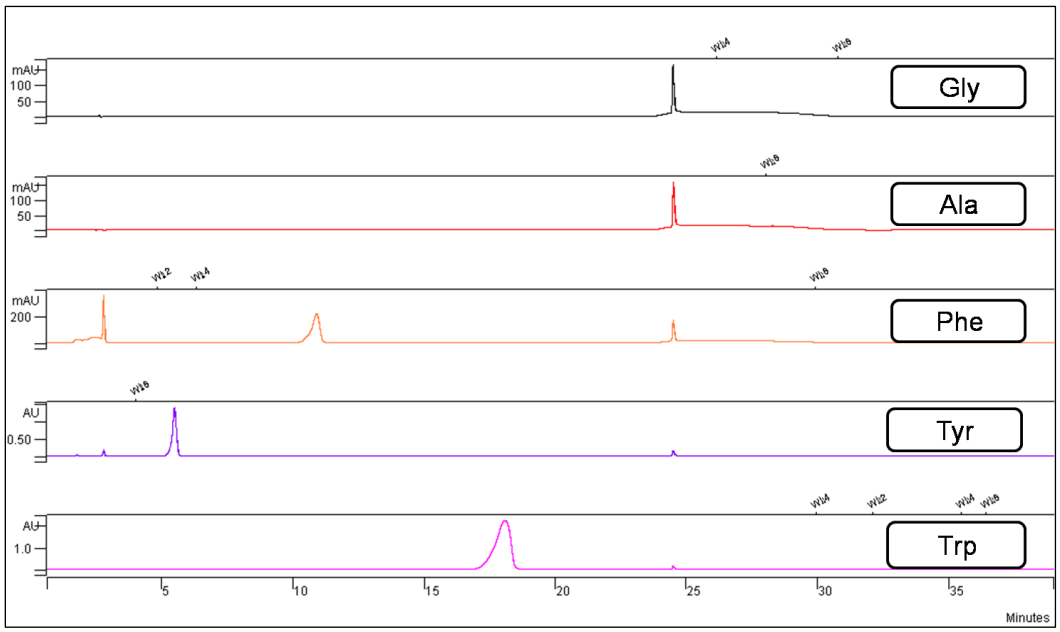


Figure 8.3 Marking samples of amino acid profile with RP-HPLC C8 column at 214nm

The growth curve of CHO cell in the serum-free basal medium supplemented with PRI and PRI fractions over 3 passages, mean (±SE) of duplicate cultures at each of 3 culture passages (n=6), cell density (X 10^4 cells/mL).

	DAY 1	DAY 2	DAY 3	DAY 4	%growth ehancement
50 kDa Re	14.67	44.00	109.33	177.67	38.9
3 kDa Re	18.17	42.83	83.67	179.50	40.2
1 kDa Re	13.00	42.67	103.00	171.67	34.5
1 kDa Per	19.00	35.33	105.67	187.00	45.7
Negative Control	16.00	35.67	90.00	124.67	
Positive Control	19.67	43.67	152.17	261.00	

The growth curve of CHO cell in the serum-free basal medium supplemented with YE and YE fractions over 3 passages, mean (±SE) of duplicate cultures at each of 3 culture passages (n=6), cell density (X 10^4 cells/mL).

	DAY 1	DAY 2	DAY 3	DAY 4	%growth enhancement
50 kDa re	15.33	56.33	96.50	217.00	- ve
3 kDa re	17.33	51.33	101.00	185.00	-ve
1 kDa re	21.00	52.00	95.50	216.33	-ve
1 kDa per	14.00	53.67	90.50	199.67	-ve
Negative Control	17.00	48.33	116.00	222.00	
Positive Control	16.50	69.00	155.00	282.00	

The growth curve of CHO cell in the serum-free basal medium supplemented with YET and YET fractions over 3 passages, mean (±SE) of duplicate cultures at each of 3 culture passages (n=6), cell density (X 10^4 cells/mL).

	DAY 1	DAY 2	DAY 3	DAY 4	%growth enhancement
50 kDa Re	17.00	58.00	103.67	125.00	3.1
3 kDa Re	18.33	48.33	135.00	169.17	48.6
1 kDa Re	17.17	49.50	106.67	144.00	22.7
1 kDa Per	17.33	47.67	88.67	135.33	13.7
Negative Control	19.00	48.67	77.33	122.00	
Positive Control	18.00	55.67	109.00	**219.00**	

Characterization of YET and its fractions followed by ultrafiltration

YET Extract	Feed	>50 kDa (retentate)	<50 kDa; > 3kDa	<3kDa; > 1kDa	< 1kDa
Total solid [g]	1.01	0.15±0.02	0.22±0.04	0.24±0.01	0.28±0.01
Phe-Gly [mmol]	303±8	47.5±1.5	56±0.5	80.6±1	111.4±0.3
Antioxidant [mg Trolox/g sample]	42.81.5	45.9± 2.4	44.1± 0.9	49.4± 0.4	45.9± 0.6

The growth curve of CHO cell in the serum-free basal medium supplemented with YE, YET and PRI 1 kDa retentate and permeate fractions over 3 passages, mean (±SE) of duplicate cultures at each of 3 culture passages (n=6), cell density (X 10^4 cells/mL).

A	DAY 1	DAY 2	DAY 3	DAY 4
PRI 1 kDa Re	13.00	42.67	103.00	171.67
YE 1 kDa Re	21.00	52.00	95.50	216.33
YET 1 kDa Re	17.17	49.50	106.67	144.00
B	DAY 1	DAY 2	DAY 3	DAY 4
PRI 1 kDa Per	19.00	35.33	105.67	187.00
YE1 kDa Per	14.00	53.67	90.50	199.67
YET 1 kDa Per	17.33	47.67	88.67	135.33