METHOD DEVELOPMENT IN ASSESSING RECOMBINANT HUMAN GROWTH HORMONE EXPRESSION FROM *Pichia pastoris*

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METHOD DEVELOPMENT IN ASSESSING RECOMBINANT HUMAN GROWTH HORMONE EXPRESSION FROM *Pichia pastoris*

by

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PEMBANGUNAN KAEDAH DALAM MENILAI PENGEKSPRESAN HORMON PERTUMBUHAN MANUSIA REKOMBINAN DALAM *Pichia pastoris*

oleh

CHAI HUI LING

Tesis yang diserahkan untuk
memenuhi keperluan bagi Ijazah
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AOX	alcohol oxidase
APS	ammonium persulfate
BMGY	buffered glycerol-complex medium
BMMY	buffered methanol-complex medium
BSA	bovine serum albumin
CDW	cell dry weight
$CuSO_4$	copper sulfate
$CuSO_4 \cdot 5H_2O$	copper(II) sulfate pentahydrate
dH_2O	distilled water
ECL	electrochemiluminescence
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
g	gram
GB	glass bead
h	hour
HCl	hydrochloric acid
hGH	human growth hormone
HRP	horseradish peroxidase
IEF	isoelectric focusing
IEX	ion exchange
KCl	potassium chloride
kDA	kilo Dalton
K_2HPO_4	di-potassium hydrogen phosphate
KH_2PO_4	potassium dihydrogen phosphate
$KNaC_4H_4O \cdot 4H_2O$	potassium-sodium tartrate-tetrahydrate
KOH	potassium hydroxide
L	liter
mg	milligram
min	minute
mL	milliliter
MOX	methanol oxidase
Mut^+	methanol utilization plus

Mut^s	methanol utilization slow
NaCl	sodium chloride
Na_2CO_3	sodium carbonate
$NaHCO_3$	sodium hydrogen carbonate
Na_2HPO_4	di-sodium hydrogen phosphate
NaOH	sodium hydroxide
ng	nanogram
nm	nanometer
OD	optical density
OSF	osmotic shock fluid
PAGE	polyacrylamide gel electrophoresis
p*I*	isoelectric point
PMSF	phenylmethylsulfonyl fluoride
psi	pounds (pressure) per square inch
PVDF	polyvinyl-difluoride
Q	quaternary ammonium
Q FF	quaternary ammonium sepharose fast flow
rhGH	recombinant human growth hormone
rpm	rotation per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid
TEMED	N, N, N', N'-tetramethylethylenediamine
TMB	tetramethylbenzidine
Tris	tris[hydroxymethyl]aminomethane
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
YNB	yeast nitrogen base
YPES	yeast protein extraction solution

PEMBANGUNAN KAEDAH DALAM MENILAI PENGEKSPRESAN HORMON PERTUMBUHAN MANUSIA REKOMBINAN DALAM *Pichia pastoris*

ABSTRAK

Hormon pertumbuhan manusia rekombinan (rhGH) telah dihasilkan daripada strain *Pichia pastoris* GS115 2(5) yang telah mengalami pengubahsuaian genetik dalam proses pengkulturan dua-peringkat. Namun begitu, proses pengkulturan awal serta strategi pemulihan rhGH dari strain GS115 2(5) perlu dikaji. Matlamat kajian ini adalah untuk menilai proses hiliran terutamanya mengkuantifikasi dan memulih produk farmaseutikal tersebut. Peringkat pertama melibatkan pertumbuhan biojisim dalam medium penghasilan yang mengandungi gliserol sebagai sumber karbon tunggal. Peringkat kedua melibatkan pemindahan sel daripada peringkat pertama dan diaruhkan dengan metanol sebagai bahan pengaruh tunggal dalam medium pengkulturan. Pengekspresan rhGH telah dipastikan oleh kaedah elektroforesis gel, “Western-blot” dan “ELISA”. Keberkesanan pelbagai kaedah pemecahan sel seperti: manik kaca, “French press”, “Sonicator”, cecair pengesktrakan protein terubahsuai dan YPES telah dikaji. Dengan menggunakan kaedah pengisaran manik kaca yang telah dioptimumkan, proses fermentasi dijalankan dari 0 hingga 204 jam masa pengkulturan dengan pengaruhan metanol untuk mengkaji pengekspresan rhGH. Profil jisim kering sel dan pengekspresan rhGH tetap kekal pada 58 g/L dan 330 μg/L. Kesan kepekatan metanol dan rekabentuk kelalang ke atas pertumbuhan *P. pastoris* serta pengekspresan rhGH turut dikaji. Penulenan dan perolehan rhGH dilakukan secara kromatografi diikuti dengan resapan pasif daripada gel poliakrilamida, sebanyak 26.4% hasil akhir diperolehi. Titik isoelektrik rhGH pada p*I* 5.3 *pula* ditentukan melalui kaedah gel pemfokusan isoelektrik. Tahap

pengekspresan rhGH yang rendah telah mencetuskan pengesanan protease yang hadir dalam GS115 2(5) melalui protokol zymografi. Sistem “Sandwich ELISA” telah dibangunkan untuk kuantifikasi rhGH dengan menggunakan 1:1600 “capture antibody” dan 1:12800 “detection antibody”.

METHOD DEVELOPMENT IN ASSESSING RECOMBINANT HUMAN GROWTH HORMONE EXPRESSION FROM *Pichia pastoris*

ABSTRACT

Recombinant human growth hormone (rhGH) was expressed in genetically modified *Pichia pastoris* strain GS115 2(5) in a two-stage cultivation process. However, the preliminary cultivation process and purification strategies of rhGH from strain GS115 2(5) were still an open issue. The main aim of this research is to evaluate the downstream processes specifically to quantify and purify the pharmaceutical products. The culture was first grown in a glycerol-containing production medium as the sole carbon source prior to obtain high cell biomass and was transferred to the second stage where rhGH was induced with methanol as the sole inducer in the medium. rhGH expression from the cultures was confirmed with gel electrophoresis, Western blotting and ELISA. Different cell disruption methods such as glass beads, French press, sonicator, modified protein extraction fluid and YPES were evaluated in terms of efficiency. Fermentation process using methanol as sole carbon source from 0 hour to 204 hours was performed to determine the optimal induction period of the rhGH expression by using the optimized bead milling method. The profile of cell dry weight and the rhGH expression was maintained constantly at 58 g/L and 330 μg/L. Also, the effect of methanol concentration and flask design on the growth of *P. pastoris* and rhGH expression were investigated. The biopharmaceutical compound was purified with chromatographic methods and passive diffusion from polyacrylamide gel, with an overall recovery yield of 26.4%. Isoelectric point of rhGH at p*I* 5.3 was determined with isoelectric focusing gel. Low expression level of rhGH has prompted the detection of protease by using zymogram.

Sandwich ELISA system was developed to quantify the rhGH using 1:1600 capture antibody and 1:12800 detection antibody.

CHAPTER 1

INTRODUCTION

1.1 Research Background

In recent years, considerable effort and attention have been directed toward the use of yeasts, particularly *Saccharomyces cerevisiae,* for the expression of heterologous genes or cDNAs for both basic research and commercial applications. Many of the industrially significant proteins produced in yeast have been mammalian, frequently human proteins of medical or therapeutic importance.

Pichia pastoris, a methylotrophic yeast which feeds on methanol, has been widely reported as another favourite expression host for several kinds of heterologous protein expression. Several types of either recombinant proteins or fibrinolytic enzymes have been expressed in *P. pastoris* (Cereghino and Cregg, 2000). In a previous study, a system for expression of recombinant human growth hormone was developed by using *P. pastoris* GS115 2(5) as the host strain and pPIC3.5K as the expression vector (Loh, 2005). Human growth hormone (hGH) is a pituitary derived polypeptide secreted by the anterior lobe of the pituitary gland. It has a variety of biological activities including cell metabolism and protein synthesis (Kostyo and Isaksson, 1977). The hormone is playing a major role in the treatment of dwarfism, bone fractures and skin burns.

In this study, the expression of the recombinant from GS115 is reported. Cell disruption methods were compared and it is advantageous to have an understanding of the ultimate expression of the recombinant protein during the production phase. Therefore, fermentation of GS115 2(5) was carried out under different culture conditions, such as induced with different methanol concentration, comparing the effect of flask design on cell biomass and rhGH expression. Gel electrophoresis and Western-blot were used to detect the presence of the recombinant human growth hormone in the culture. Several downstream processing methods have been developed including the recovery of

rhGH and purification of the product, including the recovery of separated proteins by passive diffusion from SDS-PAGE. rhGH expressed was evaluated by assessment of their immunogenicity with ELISA. This requires the development of a reliable quantification assay for hGH.

1.2 Objectives of Study

A synthetic human growth hormone (hGH) gene was constructed by using single-step assembly polymerase chain reaction (PCR). The intracellular expression of rhGH in methylotrophic yeast, *P. pastoris* strain GS115 2(5) will be confirmed with electrophoresis methods. Overall, fermentation conditions, as well as the purification and quantification of rhGH from GS115 2(5) will be evaluated in this study.

For a successful proteomic analysis, a standard cell breakage method will be developed in terms of time-saving and cost efficiency. Besides, different cell disruption methods will be compared and their effect on the detection of rhGH will be investigated as well.

Preliminary investigation on the culture conditions of GS115 2(5) will be investigated in order to optimize the production of rhGH, including the regulation of *AOX* promoter in different concentration of inducer, metabolism of carbon sources and shake flask design on the growth and rhGH expression of the recombinant strain.

In the preparation of pure rhGH, the purification process represents the major manufacturing cost. Therefore, a small scale design of purification process is desired to recover the protein of interest. Chromatography method will be the preferable approach to separate the rhGH from other contaminants that differ in certain physico-chemical properties. Suitable column will be scouted out in terms of high selectivity and resolution operations. The purified hormone will be confirmed by gel electrophoresis, Western-blotting and ELISA.

To identify the isoelectric point of the recombinant protein, a modified isoelectric focusing gel purposely for small peptide will be carry out whereas zymography will be applied to detect the presence of protease in the crude cell lysate.

An immunodetection based system has been chose to detect the existence of the intracellular secreted rhGH in order to report the exactly amount of the recombinant

protein produced by *P. pastoris*. To achieve the highest sensitivity and specificity of the quantitative method, an indirect sandwich enzyme-linked immunosorbent assay (ELISA) which uses the polyclonal anti-hGH antibody as a capture and detection materials was developed, respectively.

CHAPTER 2

LITERATURE SURVEY

2.1 Human Growth Hormone

Human Growth Hormone (hGH), also known as somatotropin, is a single chain polypeptide hormone synthesized and secreted by cells of the anterior pituitary. It is localize in the somatotrope of the *pars distalis*, an acidophilic cell containing large numbers of granules of 300-400 nm. The predominant form of hGH is a protein comprised of 191 amino acids, with a molecular weight of 22 kDa and internally cross-linked by two disulfide bonds. The sequence of hGH based on strain pHGH31 and the structure is given in Figure 2.1 and Figure 2.2. The active core of the hormone involves the N-terminal two-thirds of the sequence; the remaining one-third of the sequence in the C terminal apparently stabilizes or protects the structure (Norman and Litwack, 1987). Its native structure resembles a globular protein with four α-helices connected by extended chains (Pearlman and Bewley, 1993).

The secretion of this hormone is under the control of the hypothalamic releasing hormones and sometimes directly or indirectly under neuronal control (Norman and Litwack, 1987). Once released from the somatotroph, GH circulates in the blood at levels greater than 3 ng per ml, with a total daily output of 1-4 mg.

Human growth hormone have been shown a significant role in conversion of body fat to muscle mass, growth of all tissues, tissue repair, cell replacement, bone strength and enzyme production (McKay and Leigh, 1992). GH interacts with receptors and causes the release of somatomedins into blood circulation. The somatomedins are growth stimulatory for various types of somatic cells and are of great importance in growth. Therefore, defective production of hGH causes the dwarfism syndrome in human being. Dwarfs in this category completely lack GH but not other pituitary hormones.

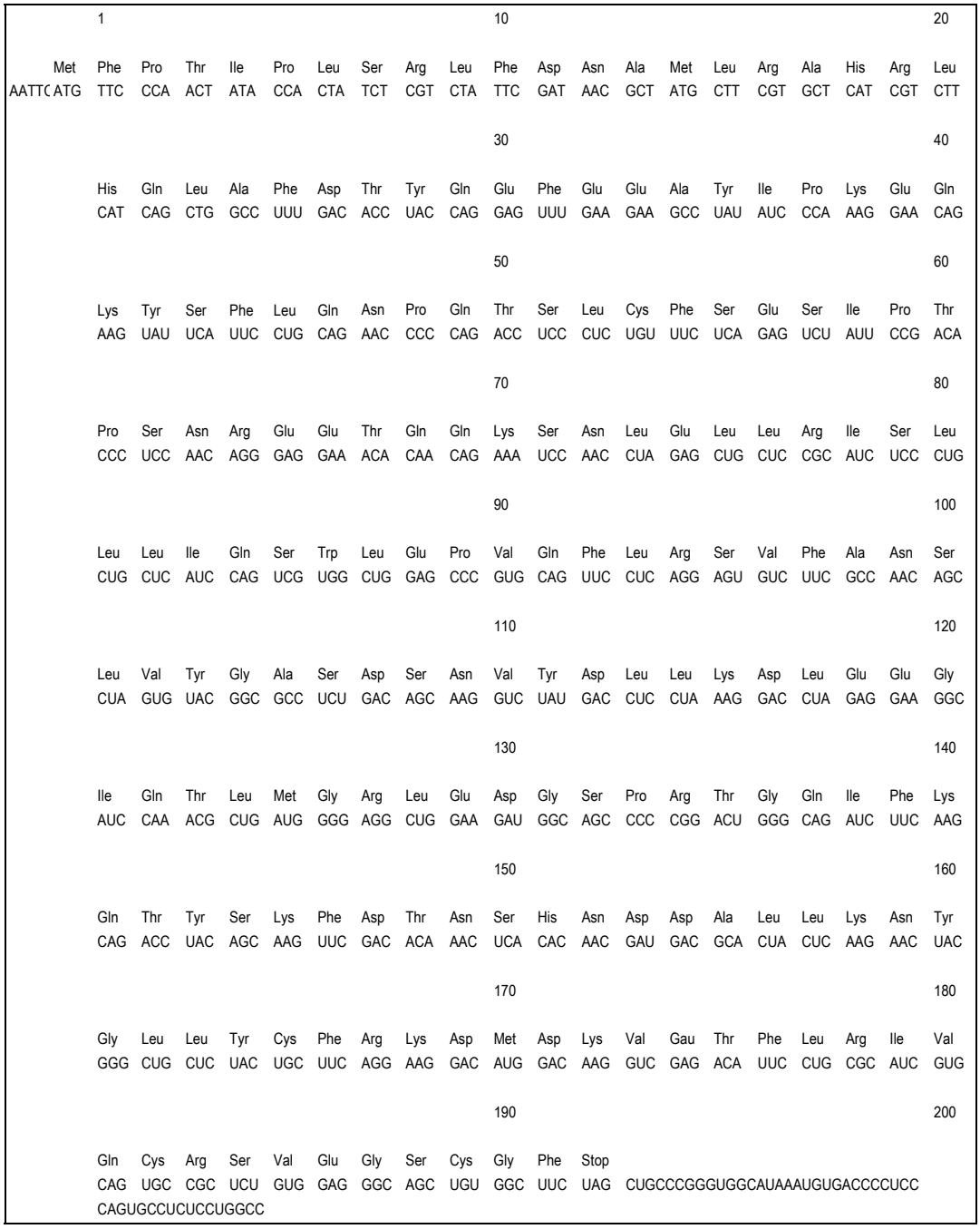

Figure 2.1 The amino acid and mRNA sequences of hGH from DNA sequencing of strain pHGH31 (Goeddel *et al.*, 1979).

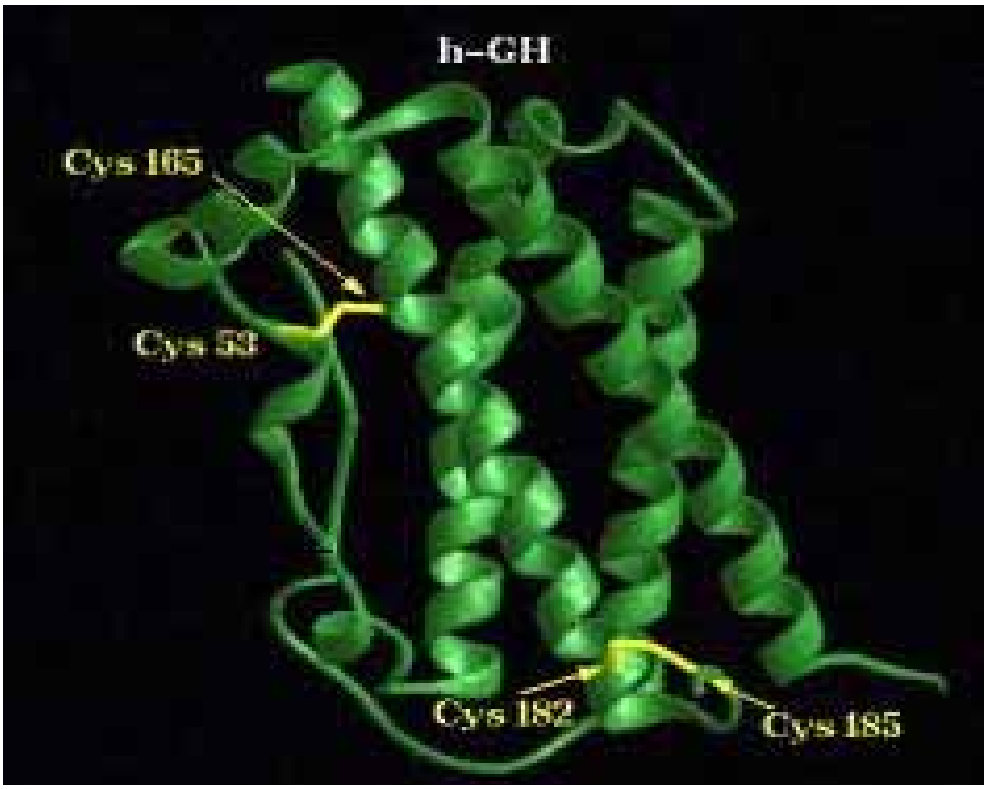

Figure 2.2 Structure of hGH

2.1.1 Recombinant human growth hormone

hGH is being secreted by human body throughout the lifespan but the secretion level was gradually decreased towards the constant level after aldolences period. Based on the worldwide statistic, a person is probably going to lose about 75% growth hormone that his or her body produced by age of 60 (http://www.advice-hgh.com/).

Further advances in molecular biology are bound to solve the problem mentioned as well as human dwarfism. Recombinant hormones were not available until 1977 when Itakura and co-workers (1977) produced the first recombinant somatostatin (Ribela *et al.*, 2003). Recent works on recombinant hGH expression have shown in study of Becker and Hsiung (1986) using *E. coli* as expression host; Franchi and colleagues (1991) using a recombinant *Bacillus subtilis* strain. Furthermore, an hGH analog namely hGHG120R, with only one of the amino acid been modified has shown a significance growth-suppressed phenotype in mice. This is one of the breakthroughs in the field of biotechnology where there is a significant conformation changes in the genetic engineered hGH molecules itself even with the only one amino acid difference from wild type hGH (Gu *et al.*, 1995).

Other hGH molecules include a single chain variant form with a molecular weight of 20 kDa (Lewis *et al.*, 1978), acetylated and deamidated 22K forms (Lewis *et al.*, 1981) and proteolytically cleaved two-chain forms (Chrambach *et al.*, 1973).

2.1.2 Isoforms of human growth hormone

Two major hGH splicing variants have been described: a 22 kDa protein and a 20 kDa protein. Basically, 22 kDa human growth hormone is the most abundant hGH isoform, constitutes approximately 80-90% of pituitary hGH (Baumann, 1991) and 20 kDa hGH serve as the second most abundant isoform. Both containing two binding sites and sequentially binds two molecules of growth hormone receptor (Hepner *et al.*, 2005). The 20 kDa variant formed by alternative splicing of mRNA and consequently lacks the amino-acid residues 32-46 of the 22 kDa hGH. This structural deficiency of 20 kDa hGH implies that the isoform shows diminished binding affinity for hGH receptor and can be a weaker agonist as compared with 22 kDa hGH. Although the affinity of 20 kDa hGH for the first hGH receptor binding is reduced to one-tenth, that for the second binding is increased ten-fold in comparison with those of 22 kDa hGH, indicating that 20 kDa hGH can be an effective hGH isoform in the presence of hGH binding proteins or hGH receptor extracellular domain (Uchida *et al.*, 1999).

At the earlier stage, Chrambach (1973) reported the major active forms of hGH are the proteolytically cleaved two-chain forms lacking 12 amino acids residue from 135-146 of 22K hGH, resulting from limited digestion by proteinases. Later, it was also reported by Lewis (1984) that the deletion mutant does exhibit several fold higher biological activity and potentates the growth promoting activity. Meanwhile, Grigorian and co-workers (2005) have isolated a relatively stable mercaptoethanol-resistant 45 kDa hGH using chromatography and preparative SDS-PAGE under reducing conditions. It was proved as the disulfide-linked hGH dimer held together by unusually stable disulfide bridges in the presence of 2-mercaptoethanol. They also successfully examined that the 22 kDa hGH is able to reassociate to form again 45 kDa hGH by removing the reductant by dialysis. Their work has led to the understanding of the structure of hGH isoforms is that it demonstrates that 45 kDa hGH is not a single chain polypeptide but is instead a homodimer of 22 kDa hGH monomers. Together with, the

45 kDa hGH dimer is held together by interchain disulfide bonds and not by divalent metal cation bridges.

Other hGH isoforms include glycosylated hGHs with molecular weights of 24 kDa and 12 kDa (Haro *et al.*, 1996), a 35-kDa hGH, oligomeric hGHs, and cleaved hGHs with molecular weights of 17 kDa and 5 kDa (Baumann, 1991).

2.1.3 Antibodies

Antibodies are immune system-related proteins also termed as immunoglobulins (Igs) indicating their role in adaptive immunity. Antibodies are divided into five distinct classes: IgG, IgM, IgA, IgD and IgE, based on their constant region structure and immune function. It is playing an important part in most of the rhGH research as the quantitative assay of the recombinant product is based upon the relation between antigen-antibody reactions (Tsushima *et al.,* 1999; Strasburger *et al.,* 1996).

The basic structure of all Ig molecules is composing of two identical light polypeptide chains and two identical heavy polypeptide chain linked together by disulfide bonds, one holding each light chain to each heavy chain, and two holding the heavy chains together. Each antibody has a specific affinity for a particular region of the antigen. This region is termed an epitope. The antigen-combining site or active site is a crevice between the variable regions of the light and heavy chain pair. The specificity of antibody is result from the molecular complementary between epitopes on the antigen molecule and amino acid residues present in the active site. According to Crowther (2001), a single antibody molecule has the ability to combine with a spectrum of different antigens and this phenomenon as multispecificity. Therefore, cross-reaction can occur when the antibody bind with antigen with similar structures and lead to false reading for the assay. According to Korz and colleagues (1995), different antibodies in immunoassays bind to a different spectrum of the growth hormone isoforms, the concentrations determined by immunoassay are likely to depend on the

particular antibody used. Consequently, specific antibodies as well as the concentration in used are chose against for the quantitation of recombinant produced.

2.2. Expression System

The choice of the expression system and an early assessment of process scalability issues have becoming the prime concern in order to minimize the risk factors associated with protein biopharmaceutical production. To date, microbial and mammalian cell expression systems are the most common systems being utilized. Even, efforts have been continued to be directed at transgenic (plant or animal) expression system. Nevertheless, recombinant proteins are still mostly expressed in microbial or mammalian cells on the basis of the nature of the target protein and also according to process scalability concerns. All of these systems have their own advantages and disadvantages.

2.2.1 *Escherichia coli*

Escherichia coli, used to be the mainstay system and, as a robust technology that is relatively inexpensive for protein production. The protein is synthesized and remains in the cytoplasm from which it must be isolated and purified. One of the major problems encountered when expressing proteins in prokaryotes is the difficulty in obtaining the soluble and active protein (Ubeidat and Rutherford, 2002). The bacteria expression system yielded a high abundance of protein, but the final protein was improperly folded and thus non-functional.

Moreover, overexpression in heterologous proteins typically leads to the formation of inclusion bodies. In addition, they are unable to perform post-translational modification processes like proteolytic and glycosylation as eukarytos perform. For instance, Ge and co-workers (2005) reported that *Escherichia coli* were an unsuitable host cell in producing lumbrokinase. Besides forming inclusion bodies, an extra cysteine residue was formed, thereby requiring a renaturation process. This

contributed to the low enzymatic activity observed after the denaturation and renaturation processes. Bacterially expressed recombinant kringle 5 was also reported mainly insoluble in Zhou and co-workers' study (2004). The kringle 5 fragments of plasminogen have promise in anti-angiogenictherapy due to it potent inhibitory effect on endothelial cell proliferation. However, its low level expression and refolding yield has turned the research team to express the recombinant human kringle 5 in a modified strain of *Pichia pastoris*. Upon methanol induction, the expression level of the engineered strain in culture reached more than 300 mgL^{-1}. After purification process, yeast-expressed kringle-5 has a higher activity in anti-endothelial proliferation than bacterially expressed kringle-5.

Relatively, Castan and co-workers (2002) reported on the use of oxygen enrichment to achieve high productivity in *E. coli* processes with and without production of recombinant human growth hormone. *E. coli* was grown in batch cultivations where the air supply was enriched with either oxygen or carbon dioxide. They discovered that biomass concentration can be increased at approximately 40% oxygen enrichment in an *E. coli* fed-batch cultures.

Heterologous protein expression in *E. coli*, *S. cerevisiae* and *P. pastoris* were evaluated for their ability to rapidly, efficaciously and consistently produce single chain antibodies (scFv) for use in downstream proteomic applications by Miller and co-workers (2005). *E. coli* expression system was found to be the best among the host tested.

2.2.2 Insect cell expression system

Insect cell expression system is considered to be safe, easy to culture and gave a very high yield of protein but usually lead to cell lytic. Baculovirus-S2 system uses the popular and genetically well understood Drosophila S2 cells which do not appear to be lysed after infection. The protein was labelled with the His-tag originally to facilitate purification, but the yield was so high that it was not even necessary to

purify the medium. However, this tag makes it easy to detect the protein, thereby making verification of the experimental processes easier (Ota *et al.*, 1991). Silkworm larvae was chosen as the expression system in Sumathy and co-workers' study (1996) to reveal the expression of hGH by recombinant virus which harbouring the full length hGH gene. The protein was secreted into hemolymph of the larvae and isolated for biologically active assay as well as purified to homogeneity. Production and secretion of a bioactive hGH into the haemolymph of transgenic larvae was demonstrated by immunoblot analysis, ELISA and a proliferation bioassay. Stable expression of hGH was observed over 50 generations.

2.2.3 Mammalian cell expression system

Chinese hamster ovary (CHO) cell line has becomes the popular expression system for production of hGH, recently. Keane and colleagues (2002) investigated the effect of shear stress on recombinant CHO expressing rhGH. Purification of rhGH from Chinese hamster ovary (CHO) cell culture supernatant by Gladiflow large-scale electrophoresis is described in Catzel and co-workers' study (2003). Because of the difficulty of establishing and maintaining stable recombinant mammalian cell lines, this system, although effective, is costly. Gu and co-workers (1995) has selected mouse L cells to produce hGH analogs, namely hGHG120R, which acts as an hGH antagonist showing a significant growth-surpressed phenotype. The reason they choose mammalian cells as the expression system is the analogs is automatically secreted as an active polypeptide without any denaturation or refolding process as required in *E. coli*.

2.2.4 *Hansenula polymorpha*

*Hansenula polymorpha, a*lso known as *Pichia augusta* is one the best studied methylotrophic yeast species which share a common metabolic pathway that enables them to use methanol as a sole carbon source (Cereghino and Cregg, 2000).

Although the *H. polymorpha* methanol oxidase (*MOX*) gene and *P. pastoris* alcohol oxidase (*AOX*) genes are not regulated identically, they show some common features of their expression patterns. Both of them are fully repressed in ethanol and excess glucose in batch culture. Derepression will only occurs when the levels of glucose become very low (Klei *et al.*, 2006). However, *MOX* activity in *H. polymorpha* was observed during the exponential growth when cultured with sorbitol, glycerol, ribose and xylose (Egli *et al.,* 1982). In *H. polymorpha*, the enzymes can be induced either by methanol or by glycerol derepression. For instance, during growth of *H. polymorpha* on glycerol *MOX* levels as high as 60% of the values detected in methanol-grown cells can be obtained. An additional advantage of *H. polymorpha* is that this organism is thermotolerant which can grow at temperatures up to 47°C, which is beneficial in large scale fermentation because it reduces cooling costs and microbial contamination (Gellissen *et al.*, 2005).

2.2.5. *Pichia pastoris*

Since the early 1980s, yeasts have been used for the large scale production of intracellular and extracellular proteins of human, animal and plan origins (Romanos, 1995). Since the promoters controlling the expression of these genes are among the strongest and most strictly regulated yeast promoters, yeast is widely used as a superior host for a broad range of recombinant proteins (Zhang *et al.*, 2005). *Pichia pastoris*, a methylotrophic yeast has become an outstanding expression system for fundamental study and expression of recombinant proteins, recently. *P. pastoris* was initially chosen for the production of single-cell protein (SCP) due to its ability to achieve high cell density and product yield (Siegel and Brierley, 1989). It has been developed into a highly successful system for the expression of heterologous genes but yet, they have less-developed vector systems and strains and are more costly than bacterial methods.

Due to its advantages, *Pichia pastoris* has gradually become one of the most popular and important host organisms for the expression of heterologous proteins. Unlike other hosts, most of the enzymes involved in the methanol-utilization pathway are present at elevated levels in *Pichia pastoris* when grown in methanol-containing medium, compared to that grown on other carbon sources (Higgins and Cregg, 1998). *AOX* gene is not present in glucose-grown cells, but comprises as much as 30% of the total protein from *Pichia pastoris*. The cells themselves can be grown rapidly to high densities, and the level of product expression can be regulated by a simple manipulation of the medium.

These characteristics account for the success of *P. pastoris* in expressing various heterologous proteins. For example, gene-encoding lumbrokinase from earthworm was cloned and expressed in *P. pastoris* (Ge and co-workers, 2005). Recombinant wheat xylanase inhibitor (rTAXI-I) was expressed for first time in *P. pichia* after about a year (Fierens *et al.,* 2004) of its expression in *E. coli* (Fierens *et al.,* 2003). The results indicate that the biochemical properties and enzyme specificities are similar to those of the natural and non-glycosylated (rTAXI-I) produced by *E. coli*. These led to further analysis and ultimately, a large-scale production of the enzyme inhibitor. From the study of Ascacio and Barrera (2004), around 60% of biologically active recombinant canine growth hormone of the total secreted protein was produced by the fermented methanol utilization slow (Mut^s*)* expression system based on *P. pastoris*. Its contribution towards the production of vaccine can be seen from the study of Li and co-workers (2004), which the novel fusion protein act as the potential recombinant hepatitis B virus hepatitis E virus bivalent vaccine candidate was expressed successfully in *P. pastoris* with an expected molecular weight of approximately 32 kDa.

Expression systems are regulated at the level of transcription for target heterologous proteins. Commonly, these systems are used for transient production of the recombinant protein, which is induced after the growth phase, during which the

product formation is low. Often, after induction, the specific production rate increases to a maximum only for a short time and product synthesis continues only for one to three hours (Lin *et al.*, 2001).

2.2.5.1 Methylotrophy in *P. pastoris and m*ethanol-utilizing phenotypes

Pichia pastoris is one of the known genera of methylotrophic yeast which utilizes methanol as a sole carbon source. The methylotropic yeast *Pichia pastoris* usually utilizes alcohol oxidase (*AOX*) promoter to drive the expression of foreign gene. Recently, a continuous fermentation has been developed in *Pichia pastoris* with the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter (Cereghino *et al.*, 2002).

The general mode of methylotrophs to assimilate carbon is to convert three C1 molecules into a C3 compound via a cyclic pathway. It has been discovered that some of the enzymes, such as catalase and *AOX* are sequestered in a subcellular organelle called peroxisome (Graham *et al.*, 2000). These are two main enzymes that catalyse the oxidation of methanol, with catalase which decomposes hydrogen peroxide into water and oxygen (Figure 2.3) (Klei *et al.*, 2006). As shown in Figure 2.3, dihydroxyacetone synthase (DHAS) appears as the third enzyme which catalyses the first step of formaldehyde assimilation to form two C3 molecules, the dihydroxyacetone and glyceraldeyde-3-phosphate. The peroxisomal localization of *AOX*, catalase and DHAS is essential to allow methylotrophic yeast cells to grow on methanol (Veenhuis *et al.*, 2000).

Three methanol-utilizing phenotypes of *Pichia pastoris* have been used for the production of heterologous proteins (Inan and Meagher, 2001a; Inan and Meagher, 2001b). Regulation of methanol metabolism in methylotrophs is a very complex process including control of synthesis and activation of the corresponding enzymes as well as their degradation (Veenhuis *et al.,* 1983). Methanol utilization plus, Mut^+, is a phenotype with the insertion of the expression cassette into *HIS4* (histidinol dehydrogenase) locus where both the *AOX1* and *AOX2* genes are not disrupted. The

AOX1 promoter-Gene X expression cassette is inserted into the *Pichia* genome along with a *HIS4* gene for the selection of transformed cells in his^- host strain. However, a Mut^s strain (methanol-utilization slow) is generated when the expression cassette is inserted within the *AOXI* locus. Simultaneously, Mut^s phenotype of host strain can also be obtained by disruption of *AOX1* gene by gene insertion, for example, KM71 *(arg4his4 AOXI Δ:: SARG4)* strain. In the findings of Inan and Meagher (2001b), they observed that there was no induction or derepression of *AOX1* promoter without the presence of methanol, even the medium was containing other carbon sources such as alanine, mannitol, sorbitol and trehalose. These indicating that the *AOX1* promoter is regulated independently by methanol induction. Sakai and co-workers (1987) also suggested that methanol is the enhancer for alcohol oxidase activity as a growth substrate and reduced by glucose. Yet, growth yield on methanol is lower than that on glucose. A third host strain used for heterologous protein expression is the Mut^- (methanol-utilization negative) strain, defective in both *AOX1 and AOX2* genes, for example, MC100-3 *(arg4 his4 aox I Δ: : SARG4 aox2 Δ: : Phis4)* (Cregg *et al.,* 1989).

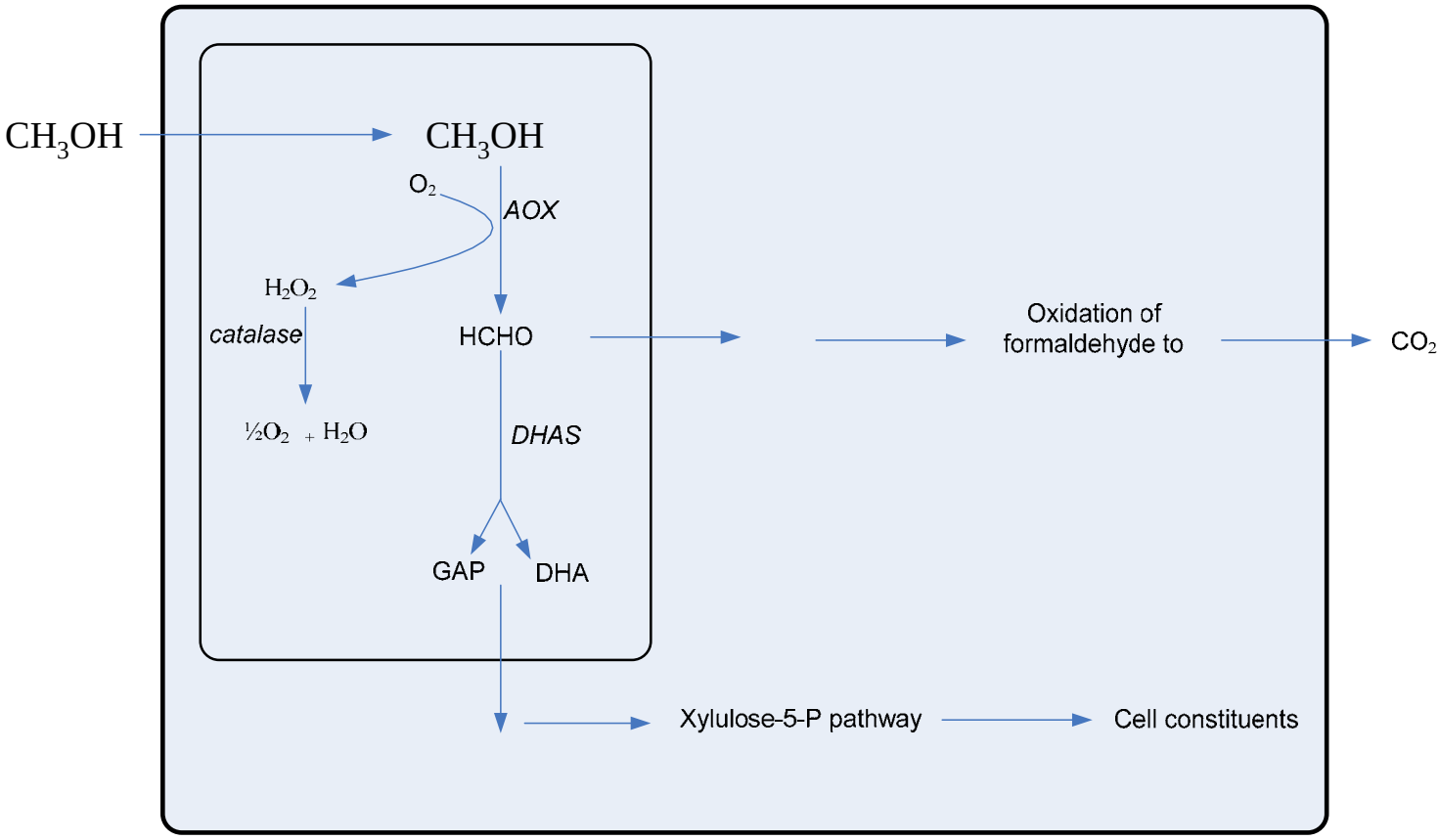

Figure 2.3 Schematic representation of the compartmentalization and the function of peroxisome in methanol metabolism in yeast.

2.3 Fermentation Conditions

Fermentation process development is conducted not only to optimize production in high density media. Parameters will be also chosen with regard to the genetic stability, scalability of the process and overall process robustness. For the production of recombinant proteins in *P. pastoris*, minimum two stages are involved. The first stage is normally to promote cell growth on a non-fermentable carbon source whereas second stage is initiated by adding inducer to induce the expression system upon the production of heterologous proteins. It has been shown earlier that glycerol and methanol are the two main carbon sources for the fermentation of recombinant *P. pastoris* (Chen *et al.,* 1997; Chung *et al.,* 2000). Even so, the presence of glycerol in the medium will repress the *AOX* promoter and thus inhibit the expression of heterologous gene. Hence, the expression of recombinant protein is usually initiated by methanol after glycerol depletion occur (Brierley *et al.*, 1990).

Productivity of the process is often to be the most interest part for the design of industrial production processes. Productivity can be improved with the help of a high growth rate and high feed rate of the limiting substrate. In fed-batch processes, the cell productivity and final cell concentration are limited by the rate of substrate consumption and the rate of maintenance (Castan *et al.*, 2002).

2.3.1 Fermentation by-products

By-products are usually produced during the glycerol fed-batch phase and the mixed-feed induction phase of high cell density fermentation, regardless of the phenotype of the yeast strains or phenotype. It also been reported by Vollbreeht (1982) that restricted oxygen supply resulted the excretion of by-products which is undesired. The most common by-products produced were ethanol, acetate, succinate and lactate. *P. pastoris* was able to grow on ethanol as sole carbon and energy source. With a carbon sources mixture, diauxic growth pattern was observed with glycerol was preferred over ethanol, followed by acetate and methanol (Inan and Meagher, 2001a).

Nevertheless, some of these by-products have certain effects during the induction phase.

Upon the reports of Chiruvolu and co-workers (1999), accumulation of residual ethanol does occurred during *Pichia* fermentation when a high feed-rate of glycerol was used even though glycerol is a non-fermentable carbon source and *Pichia pastoris* is not considered a fermentative yeast. Nevertheless, the actual mechanism pathway was still remaining unexplored. Due to this, Inan and Meagher (2001a) assumed that ethanol is metabolized to acetaldehyde and then to acetate, which is assimilated into acetyl-CoA as described for *Hansenula polymorpha* (Sulter *et al.*, 1991) and *Pichia methanolica* (Tolstorukov *et al*., 1989). The rate-limiting step in ethanol utilization appears to be the assimilation of acetate, based on the accumulation of acetate after the depletion of ethanol.

As mentioned earlier, ethanol and acetate as the by-products of *Pichia* fermentation has a great impact on the expression of β-galactosidase and methanol utilization. Presence of ethanol has repressed the *AOX1* promoter and inhibited the methanol induction in a Mut^+ strain for at least 3 hours during shake flask fermentation. However, high concentration of acetate in 200 mg per liter is able to delay the methanol utilization and β-gal expression for up to 7 hours cultivation (Inan and Meagher, 2001a). Nonetheless, the mechanism of ethanol repression in *P. pastoris* has not yet been studied thoroughly.

2.4 Recovery of Intracellular Recombinant Protein

2.4.1 Cell disruption

Cell disruption technique involves both mechanical and non-mechanical methods to disrupt microorganisms have been extensively studied in most of the literatures. According to Engler (1994), the disruption of cells is one of the most important steps for biopharmaceutical technology, especially for the isolation and

preparation of intracellular products. Improper disruption methods can affect target protein authenticity and activity. Examples of non-mechanical methods include common methods like osmotic shock fluids and sonication (Ribela *et al.*, 2000). Common mechanical method to disrupt cells is the bead mill, where mechanical forces are directly applied to cells, causing them to lyse. A vortexing cell in the presence of acid-washed glass beads is another mechanical disruption procedure that is commonly employed to release intracellular proteins from yeast cells (Canales *et al.*, 1998). Although disruption by glass-beads does a good job in small scales, this method is inappropriate in large scales.

According to Shepard and colleagues (2002), high pressure homogenization is the most commonly employed method for large scale disruption of cells and recovery of intracellular recombinant proteins in the biopharmaceutical manufacture industry. However, this practical application is limited by several characteristics intrinsic to the method. First, high pressure homogenization is non-selective; structural elements of the cell are disintegrated and essentially the entire contents of the cytoplasm and cellular organelles are released. Second, the target protein is subjected to the same severe mechanical stresses that cause cell disruption, which may affect the recovery and activity of the target protein (Fish *et al.*, 1983). Recently, it has been shown that free-radicals are formed at the impingement plate of the high pressure homogenizer (Lander *et al.*, 2000). The generation of free radicals may lead to the degradation of sensitive biological molecules including recombinant proteins.

Chai and co-workers (2005) has worked on the different cell breakage methods with *P. pastoris* strain GS115 2(5) expressing rhGH. The effect of the chemical and mechanical lysis processes on the quality of the cell extract was assessed on the basis of total protein released and the existence of the target protein on SDS gel electrophoresis as well as Western-blotting. Non-mechanical solutions offer a gentle disruption method as opposed to harsh mechanical cell breakage. Conversely, the soluble proteins need to be concentrated since dilution of sample cannot be avoided

during the process. Yet, vortexing with glass beads is the most effective disruption method on small sample volumes.

2.4.2 Purification of recombinant hGH

Protein purification processes always the most complex and time-consuming step for a recombinant protein production. In order to obtain high purity and recovery of recombinant product, purification process has to be designed carefully and effectively. As mentioned in the study of Catzel and co-workers (2003), to achieve the high levels of purity, a number of purification processes need to be incorporated into the downstream process. Therefore, steps of purification process have been described in order to achieve higher accumulative yield of rhGH from the expression system selected (Itakura *et al.*, 1977).

Several of separation techniques have been used to purify hGH from fermentation broths, including ultrafiltration, salt precipitation, size exclusion chromatography, ion exchange and reverse-phase high performance liquid chromatography. There is no denying the fact that most of the downstream process applied to purify the recombinant hGH was performed using chromatographic methodology. In a combination of anion exchange chromatography and gel filtration method, Patra and co-workers (2000) successfully obtained 99% purity of monomeric rhGH from *E. coli* with an overall yield of 50%. However, purification steps involving ammonium sulphate precipitation, Sephadex G-25 desalting, and IEX chromatograhphy (using Q-Sepharose High Performance medium, purchased from Amersham Pharmacia) was used to purify the secreted porcine somatotropin in *P. pastoris* by Ouyang and colleagues (2003).

2.4.2.1 Ion exchange chromatography (IEX)

Ion exchange chromatography is based on the p*I* value and attraction between opposite charges on the proteins and an insoluble material. Proteins bind to ion

exchangers by electrostatic forces between the proteins' surface charges (mainly) and the dense clusters of charged groups on the exchangers (Scopes, 1982). The charges of the ion exchangers are balanced by counterions such as chloride ions or buffer ions. Briefly, by passing the sample down a charged resin in a column, the biomolecule will bind to the ion exchangers when they carry an oppositely charge to that of the ion exchanger. Those certain proteins with their characteristic surface charges at a specific pH are held back on the resin more strongly than others, resulting in a high degree of separation (Robson and Garnier, 1986). Nevertheless, the binding occurred is reversible where the adsorbed proteins are eventually deabsorbed from the ion exchanger as the pH of the buffer changes. Typically, there are two types of ion exchanger: cation exchanger and anion exchanger. For an anion exchanger, a positive buffering ion, such as Tris (pK_a) 8.2, is often used and usually with Cl^- as the counterion.

Recombinant hGH, a recombinant fusion protein with a molecular weight of 22 kDa, have been purified from the periplasmic fraction of *E. coli* by Becker & Hsiung (1986) using anion exchange chromatography followed by gel filtration method. In their study, one solvents-exchange step on gel filtration column prior to lyophilization was carried out. The specific activity of hGH after this purification step was estimated to be greater than 90%. By using the same type of expression host, Oliveira and co-workers (1999) were purified rhGH from *E. coli* periplasmic space using a six steps purification procedure. The focus of the study is on the hormone recovery yields and maximum contaminant host cell elimination (ECP). The research team had recovered a final yield of more than 40% by careful optimization of each purification step and with respect to hGH recovery and ECP elimination.

2.4.2.2 Desalting

Dialysis is used both for removing excess low-molecular weight solute and simultaneously introducing a new buffer solution, sometimes just water, to the sample.

However, complete removal of salts from a sample cannot achieve in one dialysis simply because at equilibrium what was originally inside the dialysis tube now is distributed throughout the buffer and the dialysis tube. Consequently, changing the buffer at least once is needed, and to be speed up the process, stirring of the buffer and movement of the tube should occur.

According to (Scopes, 1982), dialysis is a convenient step to be carried out overnight as it needs no attention, and equilibrium is reached by morning. However, due to the sensitivity of some protein samples to temperature where most preparations are still done at low temperatures or shorter time needed to reach the equilibrium at 25°C; the possibility of proteolytic degradation during dialysis may make its use undesirable. Before gel filtration techniques became widely used, dialysis was a routine standard procedure, involved in almost all enzyme purifications. Although it is simple and requires little equipment, it has two major disadvantages: the need to change dialysate perhaps at awkward hours and the relative slowness of the method. Except when it would be a case of carrying out dialysis overnight compared with not doing anything overnight, the rapidity and complete separation achieved by gel filtration makes this a much preferable method, especially on a small scale.

2.4.2.3 Elution of proteins by diffusion

With protein elution approach, the protein of interest can be extracted out of the polyacrylamide gel and then recover it. Usually, by gel excision and the desired protein is eluted directly into a free solution or liquid phase (Jeno and Horst, 1996). Instead of forming individual sample wells, a single well comb spanning the width of the polyacrylamide gel is applied to form a single sample well since only one type of protein is loaded into the well. The sample capacity of the gel is variant depending on the thickness of the gel itself (Hames and Rickwood, 1990). Different types of elution buffers were recommended either in online articles or journals and books. Typically,