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The proteolytic systems and heterologous proteins degradation in the methylotrophic yeast *Pichia pastoris*

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Abstract - The *Pichia pastoris* expression system has been successfully used for production of various recombinant heterogeneous proteins. The productivity of *P. pastoris* can be improved substantially by bioreactor cultivations. However, heterologous proteins degradation increases as well in high-cell density culture. Proteolytic degradation is a serious problem since the yeast has been employed to express recombinant proteins. In this review, some of the recent developments, as well as strategies for reducing proteolytic degradation of the expressed recombinant protein at cultivation, cellular and protein levels on the cytosolic proteasome, vacuolar proteases, and proteases located within the secretory pathway in *P. pastoris*, are reviewed.

Key words: *Pichia pastoris*, heterologous protein, degradation, proteases, proteasome.

INTRODUCTION

The methylotrophic yeast, *Pichia pastoris*, is widely used as host strain for the production of a variety of heterologous proteins (Cregg *et al*., 2000). There are several factors that account for its popularity, such as the strong and highly efficient alcohol oxidase (AOX) promoter, the stable integration of expression plasmids at specific sites in *P. pastoris* genome, the availability of host and vector as a commercial kit and the practical techniques for high-cell density culture. The productivity of *P. pastoris* in shake-flasks is normally low but can be improved greatly by bioreactor cultivations (Cregg *et al*., 2000). Unfortunately, the concentration of other cellular materials, such as proteases, increases as well in high-cell density culture. These materials can greatly reduce the desire protein production, mainly due to proteolysis of recombinant proteins in *P. pastoris* bioreactor cultures (Clare *et al*., 1991b; Brierley, 1998). Proteolytic degradation has been a perpetual problem since yeast has been employed to express recombinant proteins (Van Den Hazel *et al*., 1996). Yeast vacuoles contain various proteases whose levels vary according to the nutritional conditions. *Saccharomyces cerevisiae* is known to express several proteases, some of which have been well identified (Van Den Hazel *et al*., 1996). In comparison, the proteases in *P. pastoris* are not well characterised, although there have been several reports of proteolytic degradation of recombinant proteins produced in *P. pastoris* (Werten *et al*., 1999; Zhou and Zhang, 2002; Sinha *et al*., 2003). Some strategies have been used to overcome the proteolysis, including selection of protease deficient host strains (Gleeson *et al*., 1998; Goodrick *et al*., 2001), modification of the protein structure to resist the protease (Gustavsson *et al*., 2001), addition of protease inhibitors in bioreactor (Holmquist *et al*., 1997), changing the pH of the culture medium (Kang *et al*., 2000; Jahic *et al*., 2003a), supplementation of the medium with casamino acids and peptone (Clare *et al*., 1991b) and addition of protease inhibitors (Shi *et al*., 2003), have been suggested as means to reduce proteolytic degradation of the recombinant protein. However, no in-depth analysis on the conditions that promote proteolysis or the nature of the proteases acting on the desired protein is exactly known. The review, here, introduced the proteases found in *P. pastoris* and these recent developments, as well as strategies for reducing proteolytic degradation of the expressed recombinant protein on cultivation, cellular and molecular levels.

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PROTEASES IN *Pichia pastoris*

Since the proteases of yeast that were first identified and characterised were detected biochemically and proved to be of vacuolar (lysosomal) origin, an additional nonvacuolar set was defined genetically by mutations that caused incomplete proteolytic processing of precursors to killer toxin and the pheromone α-factor (Jones, 1991).

Intracellular proteases in yeast have been studied widely in the last decades and they were found have vital importance in the desired protein production at cellular lever. A lot of researches were carried out on *Saccharomyces cere-*

visiae, and numerous proteolytic enzymes have been found and their biochemical and genetical characterisation have been studied (Van Den Hazel *et al*., 1996; Flores *et al*., 1999). The same protease system occurs in *P. pastoris*. There are mainly three groups of proteases in yeast: cytosolic proteosome, vacuolar proteases and proteases located along with the secretory pathway (Jones, 1991).

Cytosolic proteasome

Achstetter *et al.* (1984) reported purification of a large peptidase, which was proved to contain 10-12 no identical subunits and showed homology to the 20S cylindrical particles in many eukaryotic cells. The peptidases were named prosome, proteasome, multiprotease complex and multicatalytic protease, respectively. Proteasomes are large multicatalytic protease complexes, which fulfil central functions in major intracellular proteolytic pathways of the eukaryotic cell. The 20S proteasome, which was found in the cytoplasm and the nuclear of all eukaryote, is 700 kDa cylindrically shaped particle. The proteasome is composed of 14 different subunits (MW 22-25 kDa), which are arranged in a stack of 4 rings with 7-fold symmetry. The 26S proteasomes are an even larger proteinase complexes (about 1700 kDa), which degrade ubiquitinylated proteins in an ATP-dependent fashion *in vitro.* The 26S proteasomes are built up from the 20S proteasome, work as core particle with two additional 19S complexes on both ends of the 20S cylinder. Proteasomes are needed in stress-dependent and ubiquitin-mediated proteolysis (Hilt and Wolf, 1995). The cytosolic proteasome (multicatalytic proteinase system) is responsible for the selection and rapid degradation of short-lived proteins and proteins that are detrimental to the cell growth. The interplay between vacuolar and proteosome proteolysis is a key regulatory cell process, particularly in response to stress (Hilt and Wolf, 1992; Van Den Hazel *et al*., 1996).

Vacuolar proteases

Vacuolar proteases are responsible for a large fraction of the total cellular proteolysis in yeast, particularly under conditions of nutrient deprivation. The vacuolar uptake mechanisms of the proteins destined for degradation had, until recently, received little attention. These mechanisms, which include autophagocytosis and endocytosis, have been characterised (Van Den Hazel *et al*., 1996). The vacuolar proteases are involved in processes of cell differentiation, maturation of precursors and activation of zymogens of the vacuolar proteases, general protein degradation (in particular under nutritional stress), metabolism of exogenously supplied peptides, and other functions.

In yeast, a total of seven vacuolar proteases are known: two endoproteinases, proteinase yscA and proteinase yscB (PrB); two carboxypeptidases, carboxypeptidases Y and S (CpY, CpS); two aminopeptidases, aminopeptidases I and Co (ApI, ApCo) and Dipeptidyl aminopeptidase B (DPAP-B), DPAP-B were found in the vacuolar membrane (Jones, 1991; Knop *et al*., 1993).

Proteinase A

The aspartic protease proteinase A (PrA), encoded by the *PEP4* gene (Ammerer *et al*., 1986), is an aspartyl proteinase with similarity to the two-domain class of aspartyl proteinases that includes pepsin, renin, cathepsin D, and penicillopepsin (Woolford *et al*., 1986). PEP4 encodes a PrA precursor of 405 amino acids. A hydrophobic signal peptide is found at the N-terminus of the precursor, and this peptide is cleaved off upon translocation into the lumen of the endoplasmic reticulum (ER) (Klionsky *et al*., 1988). This glycoprotein of 42 kDa carries two asparagine-linked glycosyl side chains; its four cysteines are thought to form disulfide bonds. Three proteolytic cleavages occur during the posttranslational maturation of Pep4p. After removal of the signal sequence by signal peptidase, another 47 amino acids are removed from the NH, terminus late in the Golgi or in the vacuole. This intramolecular reaction is autocatalytic, since mutational change of either aspartate residue of the active site (Asn → Asn) results in nearly complete failure of processing (van den Hazel *et al*., 1995). The final cleavage removes 7 amino acids, it is catalysed by PrB (Klionsky *et al*., 1988).

Proteinase B

The vacuolar endoprotease proteinase B (PrB), encoded by the *PRB1* gene, is a member of the subtilisin family of serine proteases (Moehle *et al*., 1987). The mature enzyme has a molecular mass of about 31-33 kDa. Cloning and sequencing of the structural gene forPrB (PrB1) revealed a surprisingly large open reading frame, coding for a precursor of at least 69 kDa (Moehle *et al*., 1987). This initial precursor is translocated into the ER. Subsequently, the signal peptide of about 20 amino acids and a large N-terminal propeptide of about 260 amino acids are removed, yielding a smaller precursor, proPrB(40-42 kDa). The removal of the N-terminal propeptide is presumably autocatalytic, as it does not occur in strains producing an active-site mutant form of PrB. This step occurs before delivery to the Golgi complex since it is not dependent on a functional Sec l 8p (Moehle *et al*., 1989; Hirsch *et al*., 1992). The large initial PrB precursor contains five acceptor sites for Asn-linked glycosylation. However, glycosylation occurs only at one site in the precursor, and the mature protein only contains O-linked glycosylation. The large N-terminal propeptide is required for correct biosynthesis of PrB, preventing N-linked glycosylation at other sites (Nebes and Jones, 1991). The large initial PrB precursor is transiently active in the ER and removes the N-terminal propeptide autocatalytically. This yields the smaller precursor, proPrB, which is subsequently inhibited by the non-covalently linked large propeptide. Upon arrival of this complex in the vacuole, the large propeptide is degraded, proPrB is C-terminally processed and PrB activity is unveiled (Nebes and Jones, 1991).

Carboxypeptidase Y

Carboxypeptidase Y (CPY), a serine protease encoded by the PRC1 gene, is the best characterised yeast protease. The biosynthesis, vacuolar sorting and processing of CPY have been studied extensively (Sorensen *et al*., 1994; Jung *et al*., 1999; Maeda *et al*., 2004). The initial precursor is encoded by PRC1 and consists of an N-terminal signal peptide, followed by a propeptide of 91 amino acids and the enzyme region of 421 amino acids. The signal peptide is removed upon entry into the lumen of the ER, and the inactive precursor (proCPY) becomes Asn-glycosylated at four positions. *In vitro* and *in vivo* studies of the folding of the CPY precursor have shown that the propeptide is required for folding of the enzyme (Ramos *et al*., 1994). Furthermore, a mutant form of CPY lacking all four glycosylation sites leaves the ER more slowly than wild-type

CPY, indicating that the carbohydrate side chains are required for efficient folding of the precursor. The proCPY transits further through the Golgi apparatus, where the carbohydrate side chains are elongated to a limited degree, resulting in a molecular mass of 69 kDa. Upon delivery to the vacuole, the propeptide of CPY is removed, yielding the active protease of 61 kDa. The half-time of maturation of CPY is about 6 min, most of which can be attributed to time of transport (Van Den Hazel *et al*., 1996).

Aminopeptidase Y

The recently characterised 70-kDa aminopeptidaseY (APY) is Asn-glycosylated at several positions (Yasuhara *et al*., 1994). Thus, it passes through the ER on its way to the vacuole, most likely as a soluble precursor. The structural gene for APY encodes a 537 amino acid precursor, which contains a 56 amino acid extension at the N-terminus as compared to the mature protein. The first 21 amino acids of the precursor resemble a signal peptide. The following 35 amino acids function as an inhibitory propeptide, since a 74 kDa inactive APY precursor is detected in a strain lacking PrA, PrB and other vacuolar proteases. Aminopeptidase Y may be identical to the previously described aminopeptidase Co, although the molecular mass of aminopeptidase Co was reported to be about 100 kDa (Achstetter *et al*., 1982).

Carboxypeptidase S

Carboxypeptidase S (CpS), encoded by the CPS1 gene, is a metal ion-dependent carboxypeptidase (Jones, 1991). The soluble enzyme carboxypeptidase S (CPS) is synthesized as a membrane-associated precursor (proCPS) of 576 amino acids (encoded by *CPS1*), proCPS contains a single hydrophobic domain. The transport of proCPS is dependent on Secl8p and Sec7p, and proCPS receives Asn-linked glycosylation (two or three other five acceptor sites are glycosylated). Upon delivery of proCPS to the vacuole, a proteolytic cleavage between the membrane domain and the luminal domain liberates the enzyme (of 73 or 77kDa, depending on the degree of glycosylations) from the vacuolar membrane. The half-time of proCPS maturation is about 20 min (Spormann *et al*., 1991).

Aminopeptidase I

ApI, encoded by the LAP4 gene, is a metalloexopeptidase. It is a glycoprotein of 640 kDa and contains 12 subunits (Cueva *et al*., 1989). The LAP4 gene does not appear to encode a signal sequence of the type normally responsible for entry into the endoplasmic reticulum (Jones, 1991).

Aminopeptidase Co

ApCo is a 100-kDa metalloexopeptidase that requires Co^{2+} (Achstetter *et al*., 1982). Nothing has been reported about its gene or its synthesis.

Dipeptidyl aminopeptidase B

Dipeptidyl aminopeptidase B (DPAP-B), encoded by the DAP2 gene, is a membrane-bound vacuolar protease, DPAP-B is encoded by DAP2 and is not proteolytically processed upon arrival in the vacuole. The 841 amino acid enzyme consists of a short N-terminal cytoplasmic domain, followed by a single hydrophobic domain (starting at position 30 and functioning both as an ER-targeting signal and as a membrane anchor), and a luminal catalytic domain. Transport of DPAP-B to the vacuole occurs through the early compartments of the secretory pathway. In most molecules, six or seven of the eight consensus sites for Asn-linked glycosylation sites are glycosylated, whereas a small subpopulation of the molecules contains five or eight carbohydrate side chains (Roberts *et al*., 1989).

Proteases of the secretory pathway

The proteases of the secretory pathway, which are mainly located in the Golgi apparatus and the plasmatic membrane, act to process precursors to one or more secreted peptides (Flores *et al*., 1999).

Signal peptidase

Signal peptidase is an integral membrane protein that contains at least four subunits, one of which is glycosylated (Jones, 1991). The 18-kDa subunit is the product of the SECl1 gene, a not unexpected finding, since signal peptides of secreted proteins are not removed in the sed1 mutant at high temperature and the predicted sequence of Secllp shows marked similarity to that of one subunit of canine signal peptidase (Shelness and Blobel, 1990).

Kex2 endoprotease

Kex2 protease, encoded by the KEX2 gene, is an endoproteinase that cleaves on the COOH-terminal side of Lys-Arg or Arg-Arg paired basic residues (Germain *et al*., 1992). It is a serine protease of the subtilisin class and requires Ca^{2+} for activity. This glycoprotein carries both N-linked and O-linked sugars. Kex2 protease is an integral membrane protein, anchored by a sub-COOH-terminal transmembrane domain in a compartment thought to be the late Golgi (Germain *et al*., 1992). The *KEX2* gene was first identified by mutations that prevented production of killer toxin (killer expression) and caused sterility in cells of α mating type because of failure to produce the pheromone α-factor (Nakagawa *et al*., 1993). The α-factor precursor contains four copies of the pheromone peptide sequence separated by spacers. The processing is thought to be intramolecular and autocatalytic; and Lys-Arg sequences are found at residues 79-80 and 108-109, in a region that precedes the subtilisin homology, consistent with an autocatalytic cleavage mechanism (Jones, 1991; Nakayama *et al*., 1992; Nakagawa *et al*., 1993).

Kex1 carboxypeptidase

Kex1 carboxypeptidase, encoded by the KEX1 gene, is a serine protease (Shilton *et al*., 1996). It appears to be specific for basic residues. This glycoprotein contains *N*-linked sugars. Kex1 protease is an integral membrane protein. There are no indications of a zymogen form for this enzyme. Preliminary results suggest a late Golgi location. The KEX1 gene was first identified by mutations that prevented production of killer toxin. Unlike kex2 mutations, however, kex1 mutations have no effect on fertility of cells of a mating type. The fertility of kex1 mutants of α mating type depends on α-factor production solely from the COOH-terminal α-factor repeat, proving that Kex1 protease, in keeping with its substrate specificity, catalyses removal of the COOH-terminal Lys and Arg residues (Jones, 1991; Bussey, 2004).

Dipeptidyl aminopeptidase A

DPAP-A, encoded by the STE13 gene which has been sequenced, is a membrane protein (Nothwehr *et al*., 1993).

The predicted polypeptide shows marked similarity to that of DPAP-B both in primary structure and in its gross topology (Jones, 1991). It is expected to be a type II glycoprotein, anchored in the Golgi by its sub-NH-terminal internal signal sequence, with a large lumenal domain and a small cytosolic domain.

Yeast aspartyl protease III (Yap3 protease)

A gene, YAP3, was described whose product, when overproduced, catalysed cleavage of the α-factor precursor on the COOH-terminal side of paired basic residues when Kex2 protease activity was absent (Egel-Mitani *et al*., 1990). Conceptual translation of YAP3 yields a protein with sequence similarity to the two-domain group of aspartyl proteases. Its topological features resemble those of Kex2 protease. It is predicted to have an NH-terminal signal sequence and a sub- COOH-terminal transmembrane anchor; and most of the protein should be within the compartmental lumen. In alignment with other aspartyl proteases, Yap3 protease has an extra 45 amino acids at its NH_2 terminus (excluding the predicted signal sequence). Interestingly, there are two Lys-Arg pairs in this 45 amino acid stretch. The second pair is at residues 66-67, within one amino acid of the NH-terminus (at 68 or 69) predicted by alignment (Bourbonnais *et al*., 1993).

CONTROLLING PROTEOLYSIS

The genetic background of *P. pastoris* host strain is reported to be related with transcription level, translation efficiency, secretory pathway, protein quality, plasmid stability and plasmid copy number. It is illustrated by the use of protease-deficient strains to improve the quality and yields of various heterologous proteins (Van Den Hazel *et al*., 1996). In general, the secreted recombinant proteins can potentially be proteolytically degraded in the culture medium by extracellular proteases, cell-bound proteases (Kang *et al*., 2000) and/or by intracellular proteases from lysed cells. Several problems due to proteolysis can be foreseen in the production of recombinant proteins: (a) decrease of product yield when the product is degraded; (b) loss of biological activity when the product is truncated; and (c) contamination of the product by degradation intermediates in downstream processing because of their similar physicochemical and/or affinity characteristics (Macauley-Patrick *et al*., 2005). In order to reduce the proteolysis, several strategies based on modification at the cultivation, cell, and recombinant protein level can be employed to control proteolysis in *P. pastoris*.

Cultivation-level strategies

Cultivation techniques can influence proteolysis of recombinant proteins. Recently, new bioreactor cultivation techniques have contributed to increase efficiency of production of heterologous proteins by controlling the proteolysis. We summarise recent advances in the cultivation of heterologous proteins with *P. pastoris*.

Different pH values were found to be optimal from the point of view of a recombinant protein's stability: in the production of recombinant hookworm anticoagulant peptide, optimal protein expression occurred between pH 6.8 and 7.0 to avoid proteolytic degradation (Inan *et al*., 1999); pH 6.0 was optimal in production of recombinant mouse epidermal factor (Clare *et al*., 1991b); pH 3.0 was optimal in the production of cytokine growth-blocking peptide (Koganesawa *et al*., 2002). It's known that *P. pastoris* is capable of growing across a relatively broad pH range (3.0-7.0) and this pH range does not affect the growth significantly, so it allows considerable freedom in adjusting the pH to one that is optimal for protease inactivation. The lower the cultivation pH is, the lower the activity of proteases is. At pH 4.0 in a fusion protein production, the serine protease activity was almost eliminated (Jahic *et al*., 2003a). In the production of recombinant gelatins, the proteolytic degradation of COL3A1 could be minimised by adjust pH to 3.0 to repress the extracellular proteases activity (Werten *et al*., 1999). However, different foreign protein and different yeast have different optimal pH, for example, the most favourable pH (7.2) for production of Fc fusion protein was significantly higher than the conventional recommended pH (Lin *et al*., 2007) since proteolysis of protein was also a possibility due to there could be a higher level of proteases at lower pH (Inan *et al*., 1999).

Several reports have indicated that medium composition influences heterologous protein expression in yeast by affecting cell growth and viability (Shiba *et al*., 1998; Chen *et al*., 2000; Kang *et al*., 2000) or the secretion of extracellular proteases (Sreekrishna *et al*., 1997; Chung and Park, 1998). Inclusion of L-asparagine as the sole nitrogen source was found to promote secretion of mouse α-amylase in a protease deficient strain of *S. cerevisiae* (Chen *et al*., 2000), whereas yeast extract, casamino acids, or EDTA appears to enhance protein accumulation by *P. pastoris*. In some cases, L-arginine was used to inhibit the extracellular trypsinlike proteases (Sreekrishna *et al*., 1997). 0.4 M L-arginine, 5 mM EDTA, or 2% casamino acids in the BMMY induction medium could increase a single-chain antibody (scFv) production approximately 3-5-fold, with the addition of casamino acids resulting in the highest levels of expression. By addition of some amino acid-rich supplements (e.g. peptone, casamino acids) in the culture medium, which possibly act as excess alternative and competing substrates for one or more proteases, and these supplements can also repress protease induction caused by nitrogen starvation. By adding amino acid-rich supplements, the stability of product can be improved, and this was reported by several research papers (Clare *et al*., 1991a; Werten *et al*., 1999; Macauley-Patrick *et al*., 2005).

During the production of recombinant human antithrombin (rAT) by fermentation of *P. pastoris*, the formaldehyde produced by methanol oxidation in the culture broth may decrease the heparin cofactor activity of recombinant human antithrombin. Replacing the methanol with glycerol as the carbon source improved the specific heparin cofactor activity from 20 to above 40% of that of plasma-derived human antithrombin (Kuwae *et al*., 2005). In the glycerol-limited fed-batch fermentation, rAT is expressed at 100 mg/l under the control of truncated mutated *AOX2* promoter.

The cultivation performed with standard medium resulted in accumulation of lipid-like substances which caused major problems in the downstream processing and encouraged aggregation of recombinant protein (Brady *et al*., 2001). Jahic and coworkers developed a modified cultivation technique which included the induction of the medium salt concentration. The medium conductivity was kept constant at low value (about 8 mS/cm) by salt feeding (Jahic

et al., 2006). The modified cultivation technique resulted in lower cell death and consequently lower concentration of proteases and other contaminating proteins in the culture broth in the one-step recovery and purification of a fusion protein. In the production Fc fusion protein with fed-batch fermentation, the salt supplementation at induction was the most significant factor that affect the protein titer (Lin *et al*., 2007). Salt supplementation negatively affect the protein titer, the reason is that high salt concentration in medium induces the secreted proteins to form aggregates, eliminating them from proper protein. These researches indicate that use low salt medium with a concentrated salt feed and conductivity control can improve the yield and quality of the product.

Most *Pichia* fermentation is run at an optimum temperature of 30 °C, and it was known that temperature above 32 °C could be harmful to protein expression and could lead to cell death. High cultivation temperature results in cell death, which will intern result in cell lysis and higher protease activity in fermentation media. On the contrary, low cultivation temperature can improve yields of recombinant protein, possibly due to higher stability of the recombinant protein at low temperatures, release of less proteases from dead cells, and less folding problems at low temperatures (Hong *et al*., 2002). Li *et al*. (2001) have shown that decrease of process temperature from 30 °C to 23 °C increased the yield of herring antifreeze proteins from 5.3 mg/l to 18.0 mg/l, and increased cell viability as well. Lin and coworkers found that low temperature can prevent excess protein degradation during induction phase in the production of Fc fusion protein (Lin *et al*., 2007). Jahic and coworkers (2003a) showed that using a decay temperature profile during induction phase decreases both protease activity and cellular lysis. They obtained much higher concentration of a fusion protein by applying a temperature-limited fed-batch (TLFB) technique than by the traditional methanol fed-batch technique (Jahic *et al*., 2003b). In TLFB, the common methanol limitation is replaced by temperature limitation, in order to avoid oxygen limitation at high-cell density cultivation. Compared with the methanol fed-batch technique, a lower cell death rate was obtained in the TLFB process, resulting in a lower protease activity in the culture supernatant for the benefit of lower temperature and higher AOX activity.

The specific growth rate has also been used to reduce proteolysis. This can be controlled by the addition of excess methanol (2-10 g/l). Proteolytic degradation of hirudin was greatly reduced when the specific growth rate was kept below the maximum, and was particularly obvious when the specific growth rate was maintained at 0.02-0.047 h^{-1}. This corresponded with a methanol concentration of 3.09 g/l, i.e. methanol was kept at growth rate-limiting quantities (Zhou and Zhang, 2002).

Addition of specific protease inhibitors to the culture medium may be also an option. Shi *et al*. (2003) identified three types of proteases present in *P. pastoris* culture expressing scFv targeted against *Mamestra configurata* serpins. These were aspartic, cysteine and serine-type proteases. Total protease activity was reduced by 53% when a serine protease inhibitor was added to the culture medium and by 30% when an aspartic protease inhibitor was used. However, on an industrial scale, use of specific protease inhibitors could prove to be cost-prohibitive.

The combination of one or all of these cultivation-level strategies would prove to be an effective way to obtain intact recombinant proteins from *P. pastoris* with minimum proteolytic degradation. The work described in this section demonstrates the necessity for process optimization at all levels, not only to achieve maximum growth and protein production, but also to obtain maximal amounts of the desired protein in an intact and bioactive form.

Cell-level strategies

Several protease-deficient strains, such as SMD1163 (*his4 pep4 prb1)*, SMD1165 (*his4 pep4)*, and SMD1168 (*his4 pep4)*, have been found to be effective in enhancing the yield and the quality of various heterologous proteins (Cereghino and Cregg, 2000). These strains were impaired on the genes encoding proteinase A (*PEP4*) and/or proteinase B (*PRB1*) (Sreekrishna *et al*., 1997). Proteinase A is necessary for the activation of vacuolar proteases, such as carboxypeptidase Y and proteinase B. Proteinase B has about half the activity of the processed enzyme before being activated by proteinase A. Therefore, *pep4* mutants eliminate the activity of proteinase A and carboxypeptidase Y, and partially reduce proteinase B activity. The *prb1* mutants eliminated activity of proteinase B, whereas *pep4 prb1* double mutants showed a significant reduction or elimination of all three of these protease activities (Jahic *et al*., 2003a). In fermenter cultures of *P. pastoris*, the combination of high cell densities and lysis of a small percentage of cells results a relatively high concentration of these vacuolar proteases in the culture broth (Cereghino and Cregg, 2000). These protease-deficient strains, combined with other strategies to reduce proteolysis, have been invaluable in the production of insulin-like growth factor-I (Brierley, 1998), ghilanten (Brankamp *et al*., 1995) and laccase (Jonsson *et al*., 1997). Cereghino and Cregg (2000) pointed out that protease-deficient strains are not as vigorous as wild-type strains with respect to *PEP4*, that they have lower viability, exhibit lower specific growth rates and are more difficult to transform, although no evidence was given to support this view. Brankamp *et al*. (1995) reported higher secretion levels of a recombinant ghilanten and higher cell growth rates using a protease-deficient strain (SMD1168) than those obtained using a 'protease-normal' strain. However, since the protease-normal strain had a slow methanol utilization phenotpye (Mut^s), it can not be concluded that the protease-deficient strain performed any better with regard to recombinant protein and biomass production levels than if the comparison had been made with a methanol plus phenotype (Mut^+) strain. Comparison of protease-deficient and wild-type strains for the production of herring antifreeze protein demonstrated that, while the wild-type strain began product secretion a day earlier than the protease deficient strain, a higher yield of intact product was obtained after purification using the protease-deficient strain (12.8% as opposed to 4% from the wild-type) (Li *et al*., 2001). This was probably due to the lack of proteolytic degradation of the product using the protease-deficient strain. It is difficult to ascertain the usefulness of protease-deficient strains for the production of foreign proteins from the results summarised above, and they should perhaps only be used, as Cereghino and Cregg (2000) suggested, when all other methods for reducing proteolysis have been exhausted.

Protein-level strategies

If a linker between the domains of a fusion protein contains an amino acid sequence recognized by native proteases, it could be particularly sensitive to degradation. Accordingly, the amino acid sequence can be deleted if it is not essential for the function of the protein. Gustavsson and coworkers (2001) designed stable linker peptides for a cellulose-binding domain lipase fusion protein in order to decrease proteolysis. The fusion product was produced at levels of around 10 mg/l and the activity of the cellulose binding domain was found to be lower when the linker peptide chain was shorter than when the linker was longer. Paradoxically, those fusion products with shorter linker lengths were less susceptible to protease activity, and so a trade-off between high product activity and high protease activity on the one hand, and lower product activity coupled with lower protease activity on the other, would have to be made if this particular approach were to be considered.

FUTURE ASPECTS

Proteolytic degradation of secreted recombinant proteins can be caused by degradation of the cell membrane due to cell lysis in high cell density fermentation. Yeast cells are known to be stressed by starvation, change of carbon sources, heat and pH changes, or toxic chemicals. Proteins damaged by oxidative stress and heat-shock response also elicit a proteolytic response (Hilt and Wolf, 1992). In this regard, proteins damaged by oxidative stress are a probability since methanol metabolism demands high oxygen and a by-product is hydrogen peroxide. Excess production of the vacuolar proteases resulted in a secretion to cytosol and eventually to the culture medium. Since, the recombinant proteins are also produced during the methanol induction period, the presence of proteases was deleterious to the success of the process. Further researches to understand the nature of the proteases and the circumstances which promote proteolysis of recombinant protein with a view to improvement of the recombinant protein production are critical important. Protease profiling using DNA microarray chips provides a general view of the protease transcriptome, but messenger RNA expression levels do not reflect protease protein abundance or activity, which can be determined with protease-specific and protease-activity protein chips. Substrate chips analyse the net proteolytic potential of the entire functional protease degradation towards a particular substrate without identifying the active proteases that are involved. This is important information, as the net cleavage of a particular substrate determines the biological response (López-Otín and Overall, 2002).

One of the most important recent contributions to the understanding of vacuolar proteolysis is the identification and characterisation of novel pathways of import to the vacuole, such as autophagocytosis, and the import of aminopeptidase I. Thus, both for the vacuolar proteases and for their substrates, many different pathways of import have now been identified, indicating that there must be a multitude of gene products involved invacuolar delivery. Many questions concerning the specificity of the various import pathways and the *cis*- and trans-acting factors involved are still unsolved (Van Den Hazel *et al*., 1996).

CONCLUSIONS

The use of *P. pastoris* for the production of heterologous proteins is highly effective. Proteolytic degradation has been a serious problem since this yeast was employed to express recombinant proteins. Three groups of proteases: the cytosolic proteasome, vacuolar proteases, and proteases located within the secretory pathway exist in *P. pastoris*. Several strategies based on modification at the cultivation, cell, and recombinant protein level can be employed to control proteolysis in *P. pastoris*. However, more researches are needed to understand the nature of the proteases and the circumstances which promote proteolysis of recombinant protein with a view to improvement of the recombinant protein production.

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