Appl Microbiol Biotechnol (1995) 44:377–385 © Springer-Verlag 1995

ORIGINAL PAPER

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High-level secretion of hirudin by *Hansenula polymorpha* – authentic processing of three different preprohirudins

Received: 28 February 1995/Received revision: 19 April 1995/Accepted: 23 April 1995

Abstract A DNA sequence coding for a subtype of the hirudin variant HV1 was expressed in the methylotrophic yeast *Hansenula polymorpha* from a strongly inducible promoter element derived from a gene of the methanol metabolism pathway. For secretion, the coding sequence was fused to the *KEX2* recognition site of three different prepro segments engineered from the *MFα1* gene of *Saccharomyces cerevisiae*, the glucoamylase (*GAM1*) gene of *Schwanniomyces occidentalis* and the gene for a crustacean hyperglycemic hormone from the shore crab *Carcinus maenas*. In all three cases, correct processing of the precursor molecule and efficient secretion of the mature protein were observed. In fermentations on a 10-l scale of a transformant strain harbouring a *MFα1*/hirudin-gene fusion yields in the range of grams per litre could be obtained. The majority of the secreted product was identified as the full-length 65-amino-acid hirudin. Only small amounts of a truncated 63-amino- acid product, frequently observed in *S. cerevisiae*-based expression systems, could be detected.

Introduction

Variants of hirudin are potent inhibitors of blood coagulation originally isolated from the saliva of the medical leech *Hirudo medicinalis* (Bagdy et al. 1973; Walsmann 1991). These variants are 65- or 66-amino-acid polypeptides sharing a sulphonated tyrosine in position 63 and three disulphide bridges (Dodt et al. 1984; Seemüller et al. 1986). Hirudin specifically inhibits thrombin, an important physiological agonist of the arterial thrombotic process (Märki and Wallis 1990; Markwardt 1991a,b). Its high potency and specificity underly its potential as a therapeutic and diagnostic compound (Johnson et al. 1989; Markwardt 1991a,b). As a consequence, the development of effective recombinant expression systems has recently become a major target of genetic engineering after the respective gene sequences became available (Harvey et al. 1986; Loison et al. 1988). A preferred production system is based on the yeast *Saccharomyces cerevisiae* (Hinnen et al. 1994). In various constructs gene fragments coding for hirudin have been fused to a *S. cerevisiae*-derived prepro-α-factor leader segment (Märki et al. 1991) or to alternative sequences (Achstetter et al. 1992) for product secretion. As expected, the recombinant product was found to lack the sulphate residue in position 63. However, high levels of incorrectly processed and C-terminally degraded proteins have been encountered too. Improvements have been reported when protease-deficient mutants have been used for production (Hinnen et al. 1994).

The methylotrophic yeast *Hansenula polymorpha* has been developed as an expression system for heterologous proteins (Roggenkamp et al. 1986; Gellissen et al. 1992a–c; Gellissen 1994; Gellissen et al. 1994). As a facultative methylotroph this yeast species is able to use methanol as the sole energy and carbon source; other possible carbon sources are glucose or glycerol. Upon addition of methanol to culture media or in media supplemented with glycerol in low concentrations, key enzymes of methanol metabolism are strongly expressed. The strongly inducible promoter element of the cloned key enzyme gene for methanol oxidase (*MOX*) (Ledeboer et al. 1985) and a *MOX*-gene-derived terminator segment are used for heterologous gene expression, providing a production process in recombinant strains that can be induced by components of a culture medium.

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After uptake the transformation vector is stably integrated into the host's genome. Transformations result in a variety of strains harbouring different copy numbers of the integrated vector DNA in a head-to-tail arrangement. This stable multimeric integration of expression cassettes makes *H. polymorpha* an ideal host for a gene-dosage-dependent synthesis of foreign proteins (Gellissen et al. 1994).

For secretion, various leader sequences have been employed, among others that from the *S. occidentalis GAM1* gene (Gellissen et al. 1991) and the prepro sequence of the *MFα1* gene (Gellissen et al. 1994; Weydemann et al. 1995), which requires a two-step maturation of the resulting preproprotein equivalent to the situation in the baker's yeast.

In the following report we describe the successful engineering of recombinant strains secreting high levels of correctly processed hirudin using three different prepro structures for export targeting. In these constructs a hirudin sequence was fused to prepro segments derived and engineered from (a) the *S. cerevisiae MFα1* gene (Brake et al. 1984; Waters et al. 1988), (b) a suitable segment of the *S. occidentalis GAM1* gene (Dohmen et al. 1990) with an added *KEX2* recognition site and (c) a gene for a crustacean hyperglycaemic hormone (CHH) isolated from *Carcinus maenas* (Weidemann et al. 1989).

Materials and methods

Strains and media

Plasmid constructs were propagated in *E. coli* strain HB101 (Bolivar et al. 1977), the medium being supplemented with ampicillin (100 μg/l) when required for selection of transformants. The uracil-auxotroph mutant *H. polymorpha* strain RB11, deficient in oritidine-5′-phosphate decarboxylase (ura3) generated by the standard ethylmethane sulphate method, served as the standard host for the integration of the various plasmids constructed during the course of the present study. The strain was transformed as previously described (Dohmen et al. 1991).

Positive colonies were identified by culturing yeast clones under conditions of *MOX* promoter derepression (see later) and determining the presence of hirudin in the supernatants of the cultured cells by a thrombin-inhibition assay (Griesbach et al. 1985) using calibrated hirudin samples for standardization.

Test-tubes (3 ml) and shake-flask (50 ml) cultures were grown at 37°C in a synthetic medium (Gellissen et al. 1994) supplemented with 1% (w/v) glycerol as carbon source for culture growth and at lower levels for heterologous gene expression (*MOX* promoter derepression). When recombinant strains were grown on a 10-l scale a Biostat ER-10 fermenter (Braun, Melsungen, Germany) was charged with 7 l of this medium supplemented with 3% (w/v) glycerol. After steam sterilization the medium was inoculated with 1 l of an overnight culture of the recombinant strain grown in shake flasks as described above. Glycerol was added in pulses to maintain a concentration of 1.5% (w/v). Then, after 25 h, upon consumption of glycerol a pO_2-controlled feed was initiated to maintain glyerol concentrations between 0.05% and 0.4% (w/v). Glycerol was added as a 600-ml 50% (w/v) solution to 1 l of the synthetic medium. The culture conditions were maintained as follows: pH 3.8, 30°C, aeration rate 10 l/min; stirrer speed 500 rpm.

Glycerol concentrations of fermenter samples were determined by gas chromatography, and the hirudin content by HPLC analysis and by the methods described before.

Isolation and analysis of nucleic acids

Plasmid DNA was prepared from *E. coli* cultures by the alkaline extraction method (Birnboim and Doly 1979). Total yeast DNA was isolated from 50-ml cultures grown in yeast nutrient broth supplemented with 2% glucose, according to Sherman et al. (1986). DNA fragment separation and Southern analysis followed standard protocols (Maniatis et al. 1982). Restriction enzymes, ligases and DNA-modifying enzymes were obtained from Boehringer Mannheim, Germany, and employed according to the manufacturer's instructions.

Gas chromatography

Gas-chromatographical quantification of glycerol was carried out on a Shimadzu GC-14A gas chromatograph equipped with an integrater (C-R4AX; Shimadzu, Duisburg, Germany), a 15-m-long SPB-5 column with a 1.5-mm thick layer, and an inner diameter of 0.53 mm (Supelco, Bad Homburg, Germany), and a flame-ionization detector. Centrifuged and sterile-filtered fermentation samples were added as 50-μl aliquots into 1 ml of 0.01% (v/v) 1-butanol. For separation of samples (sample volume 1 μl) the column was heated to an initial temperature of 100°C, which was increased to 250°C at a rate of 15°C/min (injector temperature: 320°C; detector temperature: 320°C). Helium served as the carrier gas. A solution of 5 g/l (w/v) glycerol served for standardization.

HPLC analysis

Centrifuged and sterile-filtered fermentation samples were added as 50-μl aliquots into 1 ml degased buffer A [5% v/v CH_3CN; 0.1% v/v (TFA); 250 mM $NaClO_4$]. 100-μl aliquots of these samples were analysed on a Shimadzu C-R4AX Chromatopac equipped with a controller (421A), a solvent-delivery module (114 M), a variable-wavelength detector (165), adjusted to 214 nm, and an Altex 210 A valve (all from Beckman, München, Germany). Samples were separated on a 5-μm Spherisorb C8 column (20 mm × 4.6 mm; Phase-Separation) eluted with a mixture of buffer A and buffer B (80% v/v CH_3CN; 0.09% v/v trifluoroacetic acid (TFA), the percentage of buffer B increasing from an initial 10% to 35% at a 2%/min rate. The flow rate was adjusted to 1 ml/min.

Protein analysis

Secreted proteins were separated on 15% sodium dodecyl sulphate/polyacrylamide gels according to Laemmli (1970) and visualized by Coomassie staining. The N terminus of hirudin isolates was determined by automated liquid-phase sequence determination (Needleman 1978) on a pulse liquid-phase peptide sequenator 477A (Applied Biosystem, Warrington, England).

Hirudin purification

For hirudin purification from fermentation cultures cells were removed by cross-flow filtration through a Pelicon filtration unit (Millipore, Eschborn, Germany) employing a 0.2-μm membrane (Sartorius, Göttingen, Germany). Supernatants were adjusted to

pH 3.0 and diluted in 20 mM citrate buffer pH 3 to 6 mS/cm. The diluted samples were adsorbed to a SP-Sepharose FF column (Pharmacia, Uppsala, Sweden) equilibrated in 20 mM citrate buffer pH 3. Crude hirudin was eluted with 500 mM NaCl in 20 mM citrate buffer pH 3 and then applied to two subsequent reversed-phase separations on an XK-26 column (Pharmacia, Uppsala, Sweden) filled with 50 ml of Lichroprep RP-18 (Merck, Darmstadt, Germany). In both separation runs the column was equilibrated in 0.1% (v/v) TFA (buffer A) and hirudin was eluted with a mixture of buffer A and buffer B (0.1% v/v TFA in 40% v/v isopropanol), the percentage of buffer B increasing from an initial 0% to 50%. In the second separation step the percentage of buffer B increased at a lower rate. The hirudin fractions were combined and desalted by gel filtration on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden).

Results

Plasmid construction

A 500-base (0.5-kb) DNA fragment containing a synthesized *MF*α1-leader/hirudin gene (subtype of HV1) fusion was cloned into the multiple cloning site of plasmid pMOX conveniently located between a *MOX* promoter and a *MOX* terminator element thus constituting an expression cassette for the secretion of hirudin. The construction results in plasmid pMOX-MF-Hir (see Fig. 1A). For construction of the plasmid pMOX-GAM-Hir a synthesized DNA fragment was used coding for amino acids 1–72 of the *GAM1* gene from *S. occidentalis* (Dohmen et al. 1990) and an amino acid linker with the sequence His-Pro-Leu-Glu-Lys-Arg, thus constituting a *KEX2* recognition site for processing of prepro structures (Julius et al. 1984). The sequence was first fused to the hirudin segment and cloned into the vector pUC18. The resulting fusion was isolated as a 0.5 kb 5′*Eco*RI-*Bgl*II3′ fragment and cloned into the pMOX plasmid as described before (Fig. 1C). Accordingly, a synthetic DNA fragment coding for the prepro leader sequence of crustacean hyperglycaemic hormone (CHH) from the shore crab *C. maenas* (Weidemann et al. 1989) was fused to the hirudin segment and cloned into pUC18. Again, the fusion was isolated and inserted as 0.5-kb fragment into the multiple-cloning site of pMOX, as described before, resulting in the plasmid pMOX-CHH-Hir (Fig. 1B). The derived primary sequence of the three prepro fragments created is shown in Fig. 2.

Generation of hirudin-secreting *H. polymorpha* strains

After rounds of transformation with the various constructs described before, colonies secreting hirudin were isolated and sequentially grown in selective and rich medium to obtain mitotically stable transformants bearing the integrated foreign DNA. Out of several hundred clones obtained, examples representative for the different constructs were selected for a more detailed analysis.

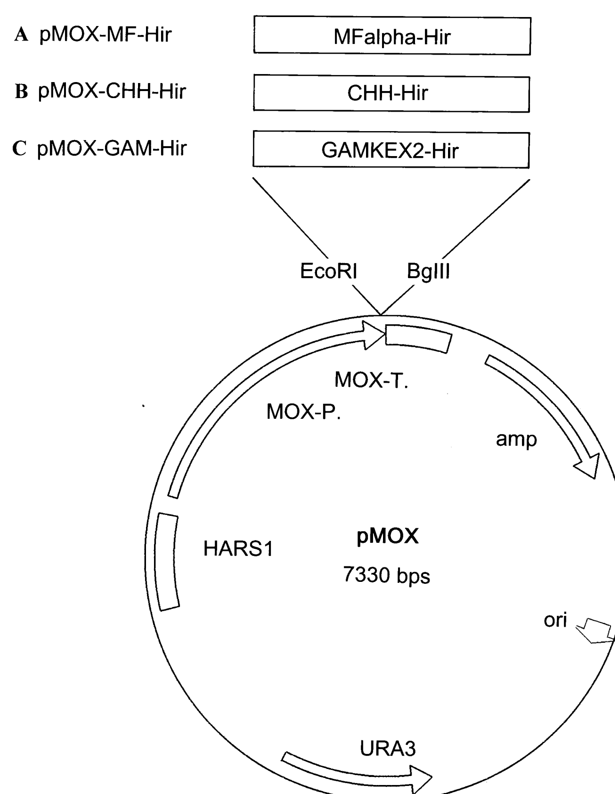

Fig. 1 Physical map of the various expression vectors for hirudin used in this study. The various hirudin/secretion leader fusions were inserted as *Eco*RI/*Bgl*II fragments into the multiple-cloning site of the *H. polymorpha* expression/integration vector pMOX separating a *MOX* promotor (*MOX-P*) and a *MOX* terminator (*MOX-T.*) sequence. The plasmids contain the following additional components: *ori* and a *bla* (*amp*^{R}) gene for selection and propagation in *E. coli*, and a *Hansenula* autonomously replicating sequence (*HARS1*) and a *URA3* gene (*URA3*) for propagation and selection in the uracil-auxotrophic *H. polymorpha* host strain RB11. The resulting plasmids are pMOX-MF-Hir (containing the hirudin sequence fused to a prepro segment of *MF*α*1*), pMOX-CHH-Hir (containing the hirudin sequence fused to a prepro segment of a crustacean hyperglycaemic hormone gene) and pMOX-GAM-Hir (containing the hirudin sequence fused to a modified prepro segment of the *S. occidentalis GAM1* gene)

Examination of genomic DNA by Southern hybridization identified several strains with differing copy numbers of the correctly integrated expression cassette. To estimate the copy number, DNA was restricted with *Sal*I/*Bgl*II, transferred to a nitrocellulose filter and hybridized to a 32P-labelled *MOX* promotor probe. The signal intensity of calibrated DNA dilutions could be compared to that of a calibrated standard of the untransformed host containing the genuine single-copy *MOX*-gene alone, and provided an estimation of heterologous gene content. In the example documented in Fig. 3 pMOX-MF-Hir transformants are analysed and found to contain forty copies of the expression cassette integrated into the genome in one case, and even more than that in the other example. In both types of

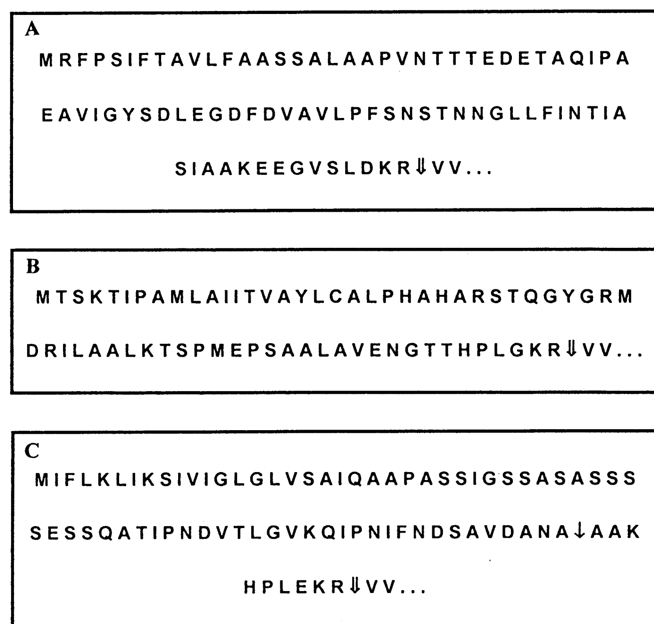

Fig. 2A–C Primary structure of the three secretory leader elements used in this study. **A** Prepro MF*α1* leader sequence, **B** *Carcinus maenas* prepro CHH leader sequence, **C** modified *Schwanniomyces occidentalis* GAM1 leader. The open arrow indicates the KEX2 claevage site in the three sequences, the small arrow in C indicates the beginning of a KEX2 spacer added to the N-terminal GAM1 sequence to create a prepro segment

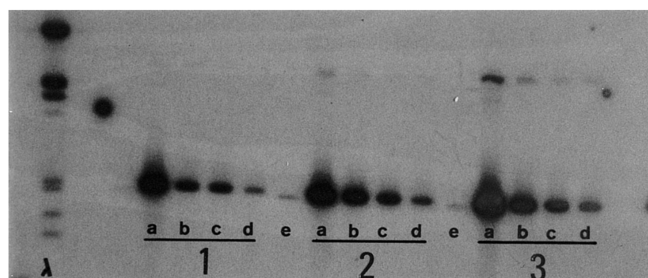

Fig. 3 Copy-number determination of the heterologous DNA. Genomic DNA from three selected recombinant strains (*1, 2, 3*) was digested with *Sal*I/*Bgl* II. Standardized dilutions of the various restriction samples were separated through 0.8% agarose gels, transferred to nitrocellulose filters and hybridized to a ^{32}P-labelled *MOX* promotor fragment. Copy number was estimated by comparing the intensity of the resulting hybridisation signals with that of the DNA from the untransformed host strain RB11. From these dilutions strain 1 was estimated to contain some 40 copies, whereas the remaining two harbour the expression cassette for hirudin in a yet higher copy number. *1–3* Analysis of *Sal*I/*Bgl*II-digested DNA from three selected clones: *a* undiluted sample, *b* 1:10 dilution, *c* 1:20 dilution, *d* 1:40 dilution, *e* sample from host strain RB11 (*1e* undiluted; *2e* 1:1 dilution), *f* *Sal*I/*Bgl*II digest of plasmid pMOX-MF-Hir. *λ* Size marker (*Eco*RI/*Hin*dIII digest of *λ* DNA)

Table 1 Characteristics of selected strains secreting hirudin. Fermentations were on a 2-l scale and continued for 36 h under conditions for *MOX* promoter derepression

Strain	Copy number	N terminus	DSH65 (%)
pMOX-MF-Hir	40	13% DSH66	70
pMOX-GAM-Hir	10	Properly processed	88
pMOX-CHH-Hir	10	Properly processed	90

transformants resulting from the introduction of pMOX-CHH-Hir and pMOX-GAM-Hir, respectively, a gene dosage of between one and ten copies could be determined (see Table 1). The integrated DNA was found to be mitotically stable after passaging for more than 80 generations (data not shown). For further studies we focused on the analysis of a single transformant of each construct, now designated according to the respective plasmid used for transformation.

The selected strain pMOX-MF-Hir contains some forty copies, and strains pMOX-CHH-Hir and pMOX-GAM-Hir both harbour ten copies of the hirudin expression cassette (Table 1).

Expression and properties of hirudin secreted from the three different strains

In a comparative analysis the three strains were fermented on a 2-l scale as described. The synthesis of hirudin was promoted by reducing the glycerol concentration in the culture broth, resulting in derepression of the *MOX* promoter. After 36 h under *MOX* promoter derepression culture supernatants were harvested and analysed for hirudin content. The highest accumulation of hirudin was observed in the culture of the strain pMOX-MF-Hir, and was at gram levels, whereas the remaining strains pMOX-GAM-Hir and pMOX-CHH-Hir produced less than 1 g, roughly reflecting the difference in hirudin gene dosage (Table 1). Nevertheless, all constructs achieved an efficient secretion, confirming that all three prepro structures are recognized by the secretory system of the methylotrophic host. A comparative HPLC analysis revealed that, in all three cases, the correctly processed full-length DSH65 form represents the major product of the secreted hirudin, ranging from 70% (pMOX-MF-Hir) to 90% (pMOX-CHH-Hir) of the overall hirudin yield. However, the hirudin constituency differs among the three samples. In the pMOX-MF-Hir culture a DSH66 species contributes 13%, which is not found in the two remaining supernatants. Determination of its N-terminal amino acids proved its identity as a species with an N-terminal extension of a single amino acid (data not shown), all other species represent molecules with a correctly processed N terminus. Minor amounts of C-terminally truncated molecules were observed, namely DSH63 and a yet unknown species. DSH64, described as a major contaminant of full-length

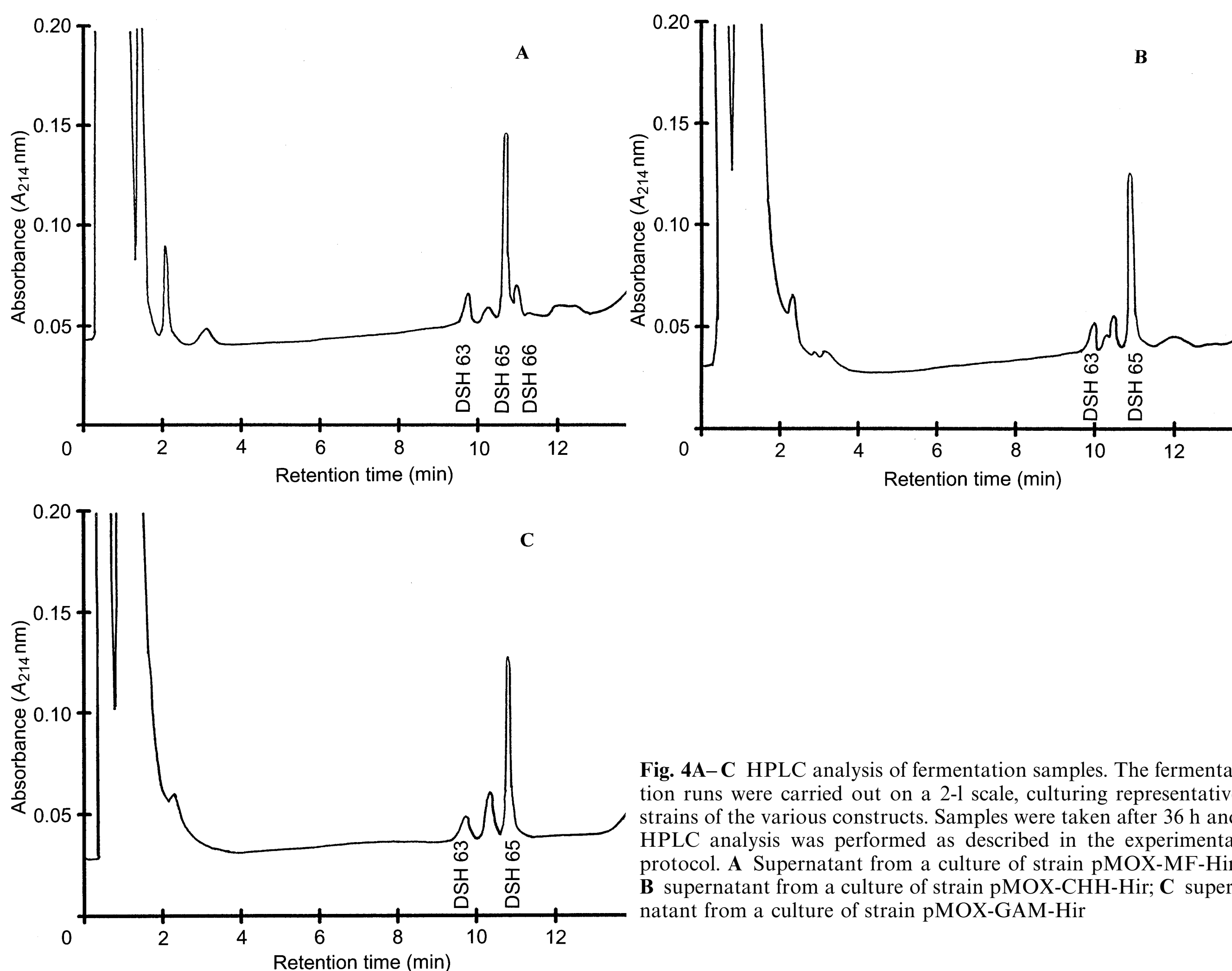

Fig. 4A–C HPLC analysis of fermentation samples. The fermentation runs were carried out on a 2-l scale, culturing representative strains of the various constructs. Samples were taken after 36 h and HPLC analysis was performed as described in the experimental protocol. **A** Supernatant from a culture of strain pMOX-MF-Hir; **B** supernatant from a culture of strain pMOX-CHH-Hir; **C** supernatant from a culture of strain pMOX-GAM-Hir

S. cerevisiae-derived hirudin, could not be detected (Fig. 4; see also the protein analysis in Fig. 6). The extent of C-terminal truncation was strongly dependent upon the design of the fermentation process. Conditions were identified that minimize the appearance of degraded molecules.

Fermentation studies on a 10-l scale

Strain pMOX-MF-Hir, which exhibited the highest productivity was selected for further fermentation runs on a 10-l scale. In the analysis documented in Fig. 5 pMOX-MF-Hir was cultured following a one-carbon-source mode. First the cells were grown in 3% glycerol to a dry weight of 25 g/l. Then, upon consumption of the carbon source, the glycerol was kept at low levels to promote heterologous gene expression. After a total fermentation time of 70 h the hirudin accumulated in the culture broth in gram amounts (Fig. 5).

Purification of DSH65 hirudin accumulated in the culture medium.

The low abundance of contaminating secretory proteins of the *H. polymorpha* host enabled the relatively simple purification procedure schematically described in Fig. 6. After removal of the yeast cells the supernatant was subjected to three consecutive chromatographic steps. The resulting isolate was free of contaminating proteins and hirudin degradation products (see Fig. 7).

Discussion

One major attraction in choosing *H. polymorpha* as a system for heterologous gene expression is the potential of this microorganism to secrete large amounts of proteineaceous products into the medium (Gellissen et al. 1994). Secretion provides an attractive mechanism

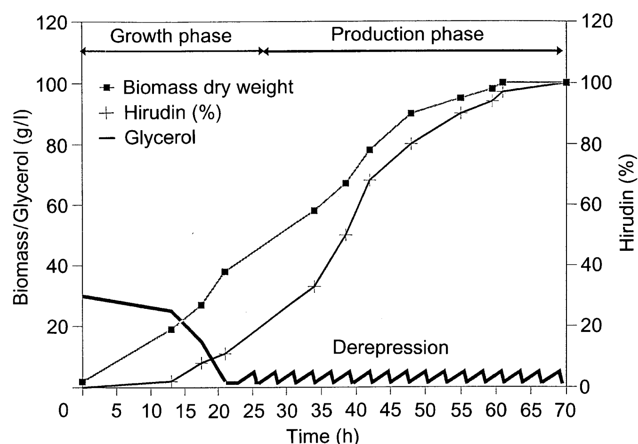

Fig. 5 Fermentation of strain pMOX-MF-Hir on a 10-l scale. A fermentation run was carried out as described in the experimental protocol. The fermentation started with 3% glycerol at the beginning. After consumption of the carbon source, a pO_2-controlled feed was initiated after 25 h, resulting in a glycerol concentration between 0.05% and 0.3% (w/v). Timed fermentation samples were taken and analysed for hirudin content in the supernatant and other fermentation parameters. After a fermentation time of 70 h, cells were harvested and hirudin was purified from the supernatant. Hirudin content is given as a relative percentage of the final titre, which was determined to be much more than 1 g

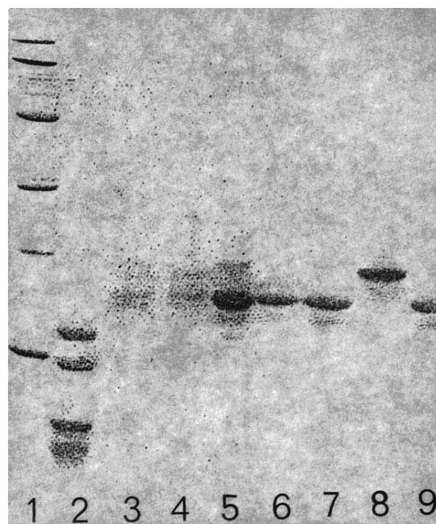

Fig. 7 Analysis of proteins of yeast culture supernatants secreting hirudin. Denatured protein samples were separated through 15% SDS-PAGE gels and visualized by Coomassie blue staining. *1* Pharmacia low-molecular-mass markers corresponding to 94, 67, 43, 30, 20.1 and 14.4 kDa respectively; *2*, Pharmacia low-low-molecular-mass markers corresponding to 17.2, 14.6, 8.2, 6.4 and 2.5 kDa respectively; *3* and *4* supernatants from two independent fermentations of strain pMOX-MF-Hir; *5* hirudin isolate after a first purification step (cation-exchange chromatography); and 6, isolate after the second purification step (reversed-phase chromatography) (procedure details see Materials and methods); *7*, *9* DSH65 standard; *8*, DSH66 standard. During incomplete processing, the hirudin possesses an N terminal 1-amino-acid extension derived from the MFα1 leader

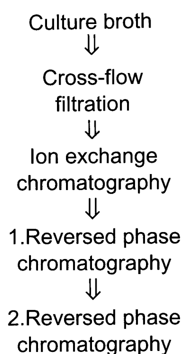

Fig. 6 A flow diagram of the purification of hirudin

for product recovery without having to break cells, especially since the methylotrophic host secretes genuine polypeptides in low abundance only. Accordingly, a relatively simple combination of chromatographic procedures can be employed for purification of secreted hirudin, mainly to exclude incorrectly processed molecules from the desired isolate.

As a unicellular eukaryotic organism, yeasts share, to a large extent, the basic characteristics of the secretory pathway found in all organisms ranging from fungi to mammals. As a consequence, a heterologous primary translation product can be processed correctly irrespective of the source of the leader sequence employed for secretion (Hadfield et al. 1993; Gellissen et al. 1994). Such a leader may comprise a short 20-amino-acid sequence targeting the polypeptide to the endoplasmatic reticulum, which is cleaved off upon entry into the compartment by a signal peptidase (Novick et al. 1981; Schekman 1985). Alternatively, it may consist in a prepro segment. For processing of the latter, further proteolytic cleavage of the imported propolypeptide is required by a dibasic protease of the Golgi apparatus equivalent to the *S. cerevisiae* endopeptidase F (product of the *KEX2* gene) (Waters et al. 1988).

A variety of leader sequences has been employed in the growing number of yeast expression systems following the general design of the two basic types, many of them originating from a heterologous source (Hadfield et al. 1993; Hinnen et al. 1994; Gellissen et al. 1994).

However, exceptions are known, and not all heterologous leader sequences function in yeasts. For instance, those of leech echistatin and human tissue plasminogen activator do not direct secretion in *S. cerevisiae* (Hoffmann and Schultz 1991). Furthermore, correct processing might be hampered by structural or steric features of the respective newly created processing sites, resulting in the inaccurate removal of a leader sequence by the two types of proteases involved. Examples of such inaccuracies are available for both basic

leader types, among others for aprotinin fused to a MFα1 prepro leader (Vedvick et al. 1991), and they can be predicted to some extent by empirical rules (Garnier et al. 1978; von Heijne 1986). For hirudin secretion in *S. cerevisiae*, mainly MFα1 has been used (Hinnen et al. 1994) though alternative structures have been reported (Achstetter et al. 1992).

In the study presented we employed prepro segments, which seemed more suitable to us to direct secretion of smaller products, like hirudin, from a *H. polymorpha* host. In addition to the 85-amino-acid MFα1 leader frequently used in yeast bioengineering (Hinnen et al. 1994), we chose a 66-amino-acid *C. maenas*-derived CHH leader (Weidemann et al. 1989) and a modified 78-amino-acid *S. occidentalis*-derived GAM1 leader (Dohmen et al. 1990) as preprohirudin components. In the case of the GAM1 leader we have designed a *KEX2* recognition site using computer modelling according to the empirical rules cited before (Garnier et al. 1978). They all efficiently direct secretion of the heterologous compound. Closer inspection and comparison of the secreted products reveals that expression of all three prepro sequences results in correctly processed N termini of the derived mature proteins. However, the traditional MFα1 leader appears to be less favourable in this specific case as compared to the newly designed structures, since a hirudin species with a 1-amino-acid extension of the N terminus (DSH66) contributes 13% to the overall hirudin yield. In contrast, only hirudin with a correctly processed N terminus is secreted from the alternative strains. Thus, the CHH and the GAM1 leader constitute attractive new elements for industrial bioengineering.

C-terminal truncation has been reported for several recombinant products secreted from a *S. cerevisiae* host, among others for a human atrial natriuretic peptide (Vlasuk et al. 1986) and epidermal growth factor (George-Nascimento et al. 1988). It is also observed for hirudin resulting in truncated proteins of 64 and 63 amino acids respectively (Hinnen et al. 1994). This degradation is mainly due to the activity of carboxypeptidase yscα and carboxypeptidase Y (yscY) assigned to the vacuole (Achstetter and Wolf 1985; Stevens et al. 1986; Heim et al. 1994). However, especially in strains secreting heterologous proteins at very high levels, most of the proteolytic activity of the latter is found in the medium providing evidence for a considerable release of this enzyme under these circumstances (Stevens et al. 1986). The yield of intact hirudin can be improved by using mutant strains with a reduced vacuolar enzyme content (Hinnen et al. 1994).

In hirudin-producing *H. polymorpha* strains similar oberservations can be made. To some extent degradation products can be identified in all three strains examined, but apparently C-terminal proteolysis is much less pronounced compared to the situation in *S. cerevisiae*. The overall share of the full-length DSH65 varies between 70% and 90% depending on the strain and the fermentation conditions applied. The main degradation product is the truncated DSH63 and a yet unknown hirudin species eluted by HPLC between the intact DSH65 and the truncated DSH63 form, together contributing some 10% to the overall hirudin yield. In contrast to the findings in recombinant *S. cerevisiae* strains, a DSH64 species could only be detected in trace amounts.

The excellent productivity of the engineered *H. polymorpha* strains further attests to the potential of this system for a commercially superior hirudin production process. The strain pMOX-MF-Hir was optimized for yields in gram ranges in fermentations being scaled up at present to an industrial scale of several thousand litres. The selected strain harbours some 40 copies of the heterologous expression cassette stably integrated into the genome. The lower productivity of the strains with the CHH and the GAM1 constructs is probably the result of the expression cassettes being present in low copy numbers. Since the production capability for a given protein is correlated, to some extent, to the dosage of the respective gene in the expressing host, it appears most likely that yet unidentified high-copy transformants can be selected for both strain types exhibiting favourable productivity properties comparable to the strain with the MFα1 leader. The resulting strains will excel and show even better characteristics combining excellence of productivity and processing.

The hirudin-secreting strains were fermented to high densities and high product yields when glycerol was used as the only carbon source. This option of a single-carbon-source fermentation mode for fermenting *H. polymorpha* strains is unique among the methylotrophic yeasts, providing an industrial process in which the use of methanol is avoided. The application of a derepression mechanism has so far been restricted to recombinant *H. polymorpha* strains where the *FMD* promoter was employed for heterologous gene expression. In the particular constructs used in this study it was possible to derepress the *MOX* promoter under restrictively low glycerol conditions. In *Pichia pastoris*, a second methylotrophic yeast, recombinant gene expression strictly depends on the presence of methanol for the production of heterologous proteins (Vedvick et al. 1991).

Hirudin, as the most potent thrombin inhibitor described so far, can be used as a laboratory or diagnostic compound for examination of unclotted blood samples under physiological conditions. Most important, it offers new opportunities in medicine as a potential therapy for thrombotic disorders (Markwardt 1991a,b).

H. polymorpha provides several advantages as the production organism for a compound considered for administration to humans. The mitotically stable

introduction of foreign genes creates strains suited to a reproducible fermentation process under non-selective conditions using inexpensive culture media. Broad risk-assessment studies confirm the ecological safety of recombinant strains (Tebbe et al. 1994a, b). The characteristics and the performance of the hirudin-producing strains described in this publication attest, together with previous examples, that the *H. polymorpha* expression system meets, to a large extent, the general demands and prerequisites for an industrial application where a system of choice should produce an optimal amount of authentic bioactive material in a reproducible and stable production process.

A hirudin production process based on *H. polymorpha* has been approved by governmental authorities according to the German gene technology law (Weydemann et al. 1995).

Acknowledgement The authors gratefully acknowledge the technical assistance of H. Ervens and G. Volkmar. We thank Dr. M. Hermans for helpful discussion and her help in calculating fermentation parameters.

References

Achstetter T, Wolf, DH (1985) Hormone processing and membrane bound proteases in yeast. EMBO J 4:173–177

Achstetter T, Ngyen-Juilleret M, Findeli A, Merkamm M, Lemoine Y (1992) A new signal peptide useful for secretion of heterologous proteins from yeast and its applications for synthesis of hirudin. Gene 110:25–31

Bagdy D, Barabas E, Graf L (1973) Large scale preparation of hirudin. Thromb Res 2:229–238

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1521

Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heyneker HL, Boyer HW, Crosa JA, Falkow S (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95–113

Brake AJ, Merryweather JP, Coit DG, Heberlein UA, Masiarz FR, Mullenbach GT, Urdea MS, Valenzuela P, Barr PJ (1984) α-factor-directed synthesis and secretion of mature foreign proteins in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 81: 4612–4616

Dodt J, Müller HP, Seemüller U, Chang JY (1984) The complete amino acid sequence of hirudin, a thrombin specific inhibitor. FEBS Lett 165:180–184

Dohmen RJ, Strasser AWM, Dahlems U, Hollenberg CP (1990) Cloning of the *Schwanniomyces occidentalis* glucoamylase gene (*GAM1*) and its expression in *Saccharomyces cerevisiae*. Gene 95:111–121

Dohmen RJ, Strasser AWM, Höhner CB, Hollenberg CP (1991) An efficient transformation procedure enabling long-term storage of competent cells of various yeast genera. Yeast 7:691–692

Garnier J, Osguthospe DJ, Robson B (1978) Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. J Mol Biol 120:97–120

Gellissen G (1994) Heterologous gene expression in C_1 compound-utilizing yeasts. In: Murooka Y, Imanaka T (eds) Recombinant microbes for industrial and agricultural applications, Dekker, NewYork, pp 787–796

Gellissen G, Janowicz ZA, Merckelbach A, Piontek M, Keup P, Weydemann U, Hollenberg CP, Strasser AWM (1991) Heterologous gene expression in *Hansenula polymorpha:* efficient secretion of glucoamylase. Biotechnology 9:291–295

Gellissen G, Weydemann U, Strasser AWM, Piontek M, Janowicz ZA, Hollenberg CP (1992a) Progress in developing methylotrophic yeasts as expression systems. Trends Biotechnol 10:413–417

Gellissen G, Janowicz ZA, Weydemann U, Melber K, Strasser AWM, Hollenberg CP (1992b) High-level expression of foreign genes in *Hansenula polymorpha*. Biotechnol Adv 10:179–189

Gellissen G, Melber K, Janowicz ZA, Dahlems U, Weydemann U, Piontek M, Strasser AWM, Hollenberg CP (1992c) Heterologous protein production in yeasts. Antonie van Leeuwenhoek Int J Gen Mol Microbiol 62:79–93

Gellissen G, Hollenberg CP, Janowicz ZA (1994) Gene expression in methylotrophic yeasts. In: Smith A (ed) Gene expression in recombinant microorganisms, Dekker, New York, pp 195–239

George-Nascimento C, Gyenes A, Halloran SM, Merryweather JP, Steimer KS, Masiarz FR, Randolph A (1988) Characterization of recombinant human epidermal growth factor produced in yeast. Biochemistry 27:797–802

Griesbach U, Stürzebecher J, Markwardt F (1985) Assay of hirudin in plasma using a chromogenic thrombin substrate. Thromb Res 37:347–350

Hadfield C, Raina KK, Shashi-Menon K, Mount RC (1993) The expression and performance of cloned genes in yeasts. Mycol Res 97:897–944

Harvey RP, Degryse E, Stefani L, Schamber F, Cazenave JP, Courtney M, Tolstoshev P, Lecocq JP (1986) Cloning and expression of a cDNA coding for the anticoagulant hirudin from the bloodsucking leech, *Hirudo medicinalis*. Proc Natl Acad Sci USA 83:1084–1088

Heijne G von (1986) A new method for predicting signal sequence cleavage sites. Nucleic Acids Res 14:4683–4690

Heim J, Takabayashi K, Meyhack B, Märki W, Pohlig G (1994) C-terminal proteolytic degradation of recombinant desulfatohirudin and its mutants in the yeast *Saccharomyces cerevisiae*. Eur J Biochem 226:341–353

Hinnen A, Buxton F, Chaudhuri B, Heim J, Hottiger T, Meyhack B, Pohlig G (1994) Gene expression in recombinant yeast. In: Smith A (ed) Gene expression in recombinant microorganisms, Dekker, New York, pp 121–193

Hoffmann KJ, Schultz LD (1991) Mutations of the alpha galactosidase signal peptide which greatly enhance secretion of the heterologous proteins by yeast. Gene 94:105–111

Johnson PH, Sze P, Winant R, Payne PW, Lazar JB (1989) Biochemistry and genetic engineering of hirudin. Semin Thromb Hemost 15:302–315

Julius D, Brake A, Blair L, Kunisawa R, Thorner J (1984) Isolation of the putative structural gene for the lysine-arginine endopeptidase required for processing of yeast prepro-α-factor. Cell 37:1075–1083

Laemmli U (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Ledeboer AM, Edens L, Maat J, Visser C, Bos J, Verrips CT, Janowicz, ZA, Eckart M, Roggenkamp RO, Hollenberg CP (1985) Molecular cloning and characterization of a gene coding for methanol oxidase in *Hansenula polymorpha*. Nucleic Acids Res 13:3063–3082

Loison G, Findelli A, Bernard S, Nguyen-Juilleret M, Marquet M, Riehl-Bellon N, Carvallo D, Guerra-Santos L, Brown SW, Courtney M, Roitsch C, Lemoine Y (1988) Expression and secretion of biologically active leech hirudin. Biotechnology 6:72–77

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, NY

Märki WE, Wallis RB (1990) The anticoagulant and antithrombotic properties of hirudins. Thromb Haemost 64:344–348

Märki WE, Grossenbacher H, Grütter MG, Liersch MH, Meyhack B, Heim J. (1991) Recombinant hirudin: Genetic engineering and structure analysis. Semin. Thromb Hemost 17:88–93

Markwardt F (1991a) Hirudin and derivatives as anticoagulant agents. Thromb Haemost 66:141–152

Markwardt F (1991b) The comeback of hirudin as an antithrombotic agent. Semin Thromb Haemost 17:79–82

Needleman SB (ed) (1978) Protein sequence determination Springer, Berlin

Novick P, Ferro S, Schekman R (1981) Order of events in the yeast secretory pathway. Cell 25:205–215

Roggenkamp RO, Hansen H, Eckart M, Janowicz ZA, Hollenberg CP (1986) Transformation of the methylotrophic yeast *Hansenula polymorpha* by autonomous replication and integration vectors. Mol Gen Genet 202:302–308

Schekman R (1985) Protein localization and membrane traffic in yeast. Annu Rev Cell Biol 1:115–143

Seemüller U, Dodt J, Fink E, Fritz H (1986) Proteinase inhibitors of the leech *Hirudo medicinalis* (hirudins, bdellins, eglins). In: Barrett AJ, Salvesen G (eds) Proteases. Elsevier, Amsterdam, pp 337–359

Sherman F, Fink GR, Hicks JB (1986) Methods in yeast genetics. Cold Spring Harbor Press, Cold Spring Harbor, NY

Stevens TH, Rothman JH, Payne GS, Schekman R (1986) Gene dosage-dependent secretion of yeast vacuolar carboxypeptidase Y. J Cell Biol 102:1551–1557

Tebbe CC, Vahjen W, Munch JC, Feldmann SD, Ney U, Sahm H, Gellissen G, Amore R, Hollenberg CP (1994a) Verbundprojekt Sicherheitsforschung Gentechnik. Teil 1. Überleben der Untersuchungsstämme und Persistenz ihrer rekombinanten DNA. Bioengineering 6/94:14–21

Tebbe CC, Vahjen W, Munch JC, Meier B, Gellissen G, Feldmann SD, Sahm H, Amore R, Hollenberg CP, Blum S, Wackernagel W (1994b) Verbundprojekt Sicherheitsforschung Gentechnik. Teil 2. Mesokosmenuntersuchungen und Einfluß der Habitatbedingungen auf die Expression, Überdauerung und Übertragung des Aprotinin-Gens. Bioengineering 6/94:22–26

Vedvick T, Buckholz RG, Engel M, Urcan M, Kinney J, Provow S, Siegel RS, Thill GP (1991) High-level secretion of biologically active aprotinin from the yeast *Pichia pastoris*. J Ind Microbiol 7:197–202

Vlasuk GP, Bencen GH, Scarborough RM, Tsai PK, Whang JL, Maak T, Camargo MJF, Hirsher SW, Abraham JA (1986) Expression and secretion of biologically active human natriuretic peptide in *Saccharomyces cerevisiae*. J Biol Chem 261: 4789–4796

Walsmann P (1991) Isolation and characterization of hirudin from *Hirudo medicinalis*. Semin Thromb Hemost 17:83–87

Waters MG, Evans EA, Blobel G (1988) Prepro α-factor has a cleavable signal sequence. J Biol Chem 263:6209–6214

Weidemann W, Gromoll J, Keller R (1989) Cloning and sequence analysis of cDNA for precursor of a crustacean hyperglycemic hormone. FEBS Lett 257:31–34

Weydemann U, Keup P, Gellissen G, Janowicz ZA (1995) Ein industrielles Herstellungsverfahren von rekombinantem Hirudin in der methylotrophen Hefe *Hansenula polymorpha*. Bioscope 3:8–15