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A system for the synthesis of small functional peptides

Abstract We describe the synthesis and purification of two functional peptides, namely human insulin-like growth factor II (IGF-II) and *Xenopus laevis* magainin II in *Hansenula polymorpha* after their synthesis as hybrid proteins fused to the C terminus of endogenous amine oxidase. The hybrid genes, placed under control of the *H. polymorpha* alcohol oxidase promoter (P_{AOX}), were integrated into the genomic alcohol oxidase locus, yielding stable production strains. High-level synthesis of the fusion proteins, exceeding 20% of total cellular protein, was obtained when the transformed strains were grown in methanol-limited chemostat cultures; when expressed by itself, i.e. in the absence of the amine oxidase gene, IGF-II could not be recovered from crude cell extracts, probably as a result of rapid proteolytic degradation. Accumulation in peroxisomes did not significantly affect the IGF-II protein stability when expressed in the absence of the carrier protein. Apparently, fusion to the large (± 78 kDa) amine oxidase carrier particularly stabilizes the peptides and prevents them from proteolysis. After partial purification, the fusion partners were readily separated by factor Xa treatment.

Introduction

The methylotrophic yeast *Hansenula polymorpha* has been developed as an attractive host for the production of foreign proteins. High-level synthesis of such proteins was obtained by expressing their cognate genes from strong endogenous promoters e.g. the alcohol oxidase promoter (Buckholz and Gleeson 1991; Gellissen et al. 1992).

In methanol-grown cells of *H. polymorpha*, the key enzyme of methanol catabolism, alcohol oxidase is compartmentalized in peroxisomes (Veenhuis and Harder 1991). These organelles occupy a major part (up to 80%) of the cytoplasmic volume under these conditions and hence may provide a potential storage site for heterologous proteins. This approach is thought to be of particular advantage when cytosolic synthesis leads to foreign protein instability and/or causes undesirable effects on the viability of the host.

In order to verify this assumption, we have synthesized two small functional peptides in *H. polymorpha*, namely human insulin-like growth factor II (IGF-II) and *Xenopus laevis* magainin II (MAG-II), either as independent peptides or fused to peroxisomal amine oxidase (AMO) as carrier protein. IGF-II is a 67-amino acid single-chain polypeptide of 7.5 kDa, which structurally resembles insulin. Synthesis of IGF-I by *S. cerevisiae* has been shown to cause severe toxic effects on the host (Bayne et al. 1988; Shuster et al. 1989). Similar effects are expected for the structurally homologous IGF-II and this was therefore chosen as model peptide in our packaging approach. Magainin II is a 23-amino-acid peptide which is found in skin secretions of the African clawed frog *X. laevis* (Zasloff 1987; Moore et al. 1991). It belongs to the class of antimicrobial peptides that are synthesized by higher eukaryotes as a defence against microbial invasion. The precise action of these antibiotics is unclear but it is known that they rapidly permeabilize phospholipid vesicles (Grant et al. 1992). Because of this activity, biological production of these peptides in their mature and active form is thought to be impossible and hence it is taken as the second model protein. The results of this work are detailed in this paper.

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Materials and methods

Strains and cultivation

All strains used in this study are listed in Table 1. Transformants of *H. polymorpha* A16, carrying hybrid genes coding for AMO-IGF-II (GF37), AMO-MAG-II (GF70) or IGF-II-SKL (GF33) (where SKL is the peroxisomal targeting signal, Ser-Lys-Leu) under the control of the *H. polymorpha* alcohol oxidase promoter (P_{AOX}), were grown in carbon-source-limited chemostat cultures on mineral medium containing glucose (0.25% w/v) plus methanol (0.25% v/v) as carbon sources and ammonium sulphate (0.25% w/v) as the nitrogen source (van der Klei et al. 1991). Strain GF42 (P_{AOX} AMO-IGF-II-SKL) and GF38 (P_{AOX} IGF-II) were grown in shake flasks in mineral medium on methanol (0.5% v/v) as a carbon source and ammonium sulphate (0.25% w/v) as the nitrogen source (Veenhuis et al. 1979).

Molecular genetics and yeast transformation

All genetic manipulations were performed according to standard procedures (Sambrook et al. 1989). DNA fragments obtained by the polymerase chain reaction (PCR) and synthetic DNA fragments were sequenced using the T7 sequencing kit from pharmacia (Uppsala, Sweden). *E. coli* DH5α was used as host for plasmid amplification. *H. polymorpha* A16 was transformed by electroporation (Faber et al. 1994a). Forced integration of linearized plasmid DNA into the yeast genome was performed as described earlier (Faber et al. 1992, 1994b).

Plasmid constructions

A schematic representation of the heterologous (hybrid) genes used in this study is shown in Fig. 1. The gene encoding mature human IGF-II was adapted by PCR, using the oligonucleotides listed in Table 2. With oligonucleotides 1 and 2, a *Xho*I site and the sequence encoding the factor Xa recognition site, -Ile-Glu-Gly-Arg-(-IEGR-), were introduced in front of the IGF-II coding sequence. In addition, a stop codon was introduced behind the codon for Glu[67], followed by a *Sac*I site. With oligonucleotides 1 and 3, a DNA fragment was obtained coding for IGF-II preceded by the *Xho*I/-IEGR- sequence and followed by a fragment encoding the C-terminal dodecapeptide of firefly luciferase, containing the SKL-COOH peroxisomal targeting signal (PTS1). A gene encoding IGF-II preceded by an *Nco*I restriction site and a translation start codon was synthesized by using oligonucleotides 2 and 4. A gene coding for IGF-II fused to the sequence encoding the C-terminal dodecapeptide of firefly luciferase
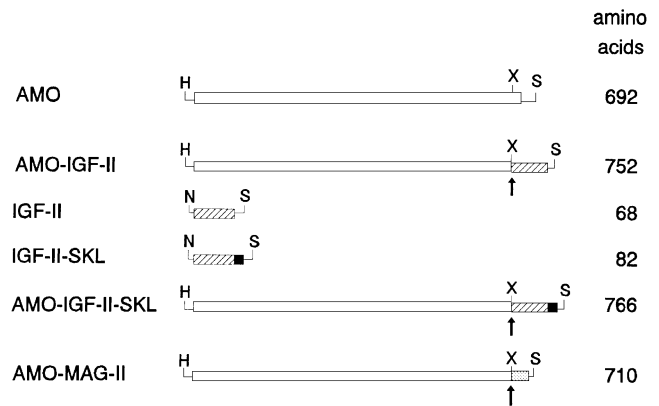

Fig. 1 Schematic representation of the gene constructs encoding heterologous proteins. The restriction sites used for gene construction and insertion into the expression vector pHIPX2 are indicated. *Open box* amine oxidase (*AMO*) sequences; *hatched box* insulin-like growth factor II (*IGF-II*) sequences; *solid box* sequence encoding the C-terminal dodecapeptide of firefly luciferase; *dotted box* magainin II (MAG-II) sequences. *Arrow* the factor Xa cleavage site. *H Hin*dIII, *N Nco* I, *S Sac*I, *X Xho*I

was synthesized by using oligonucleotides 3 and 4. A synthetic DNA fragment coding for the *Xho*I/-IEGR- sequence followed by the open-reading frame of magainin II (GIGKFLHSAKKFGKAF-VGEIMNS) was constructed using the oligonucleotides listed in Table 2.

The PCR fragments and the synthetic DNA fragment were cloned as *Xho*I and *Sac*I fragments in pOK12 (Vieira and Messing 1991) digested with the same restriction enzymes. After the sequence of the DNA inserts had been confirmed, they were cloned behind the amine oxidase gene and fused at the *Xho*I site present at the codon for Val[680] (Bruinenberg et al. 1989) and/or cloned behind the P_{AOX} in expression vector pHIPX2 (Faber et al. 1994b). This yielded pGF88 (pHIPX2 + IGF-II-SKL), pGF151 (pHIPX2 + AMO-

Table 1 *Hansenula polymorpha* strains used in this study. P_{AOX} alcohol oxidase promoter, *IGF-II* insulin-like growth factor II, *SKL* Ser-Lys-Leu, the peroxisomal targeting signal, *AMO* amine oxidase, *MAG-II* magainin II

H. polymorpha	Genotype	Reference
A16	*leu1.1*	Veale et al. 1992
GF16	P_{AOX} AMO	Faber et al. 1994c
GF33	pGF88 : P_{AOX} IGF-II-SKL	This study
GF37	pGF151 : P_{AOX} AMO-IGF-II	This study
GF38	pGF156 : P_{AOX} IGF-II	This study
GF42	pGF158 : P_{AOX} AMO-IGF-II-SKL	This study
GF70	pGF201 : P_{AOX} AMO-MAG-II	This study

Table 2 Oligonucleotides used in this study. *Italics*-Ile-Glu-Gly-Avg-: factor Xa cleavage site coding sequence; *triplets* IGF-II or magainin II coding sequence; **bold** C_{12} luciferase coding sequence; *underlined* translation stop codon; *lower case* translation start codon

IGF-II

1 5′GGCCTCGAGA *ATC GAG GGC AGA* GCT TAC CGC CCC AGT GAG ACC3′

2 5′CGCGAGCTCTTA CTC GGA CTT GGC GGG GGT AG3′

3 5′CCGAGCTCGAGTTA **CAG CTT CGA CTT GCC GCC CTT CTT GGC CTT GAT CAG** CTG CAT CTC GGA CTT GGC GGG GGT AG3′

4 5′GAATTCC atg GCT TAC CGC CCC AGT GAG AC3′

Magainin-II

3754 5′TCG AGA *ATC GAG GGC AGA* GGC ATC GGC AAG TTC CTG CAC TCG GCC AAG AAG3′

3755 5′GA GTG CAG GAA CTT GCC GAT GCC *TCT GCC CTC GAT* TC3′

3756 5′TTC GGC AAG GCC TTC GTG GGC GAG ATC ATG AAC TCG TAAGAGCT3′

3757 5′CTTA CGA GTT CAT GAT ATC GCC CAC GAA GGC CTT GCC GAA CTT CTT GGC C3′

IGF-II), pGF156 (pHIPX2 + IGF-II), pGF158 (pHIPX2 + AMO-IGF-II-SKL) and pGF201 (pHIPX2 + AMO-MAG-II).

Biochemical methods

To prepare total cell lysates, cells were washed in 50 mM potassium phosphate buffer (pH 7.0, 0°C) and subsequently resuspended in loading buffer used for Sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE). After the addition of one-third volume of acid-washed glass beads, the mixture was vortexed four times for 2 min with intermittent cooling on ice for 5 min. The homogenate was incubated at 100°C for 5 min and subsequently centrifuged for 10 min at room temperature in an Eppendorf microfuge (14000 rpm). The resulting supernatant is referred to as total cell lysate and was loaded directly onto SDS/polyacrylamide gels. Crude cell extracts were prepared as described earlier (Waterham et al. 1992).

Total cell lysates and partially purified protein fractions (see below) were analysed by SDS-PAGE according to Laemmli (1970) using 7.5% and 15% gels. Gels were stained with Coomassie brilliant blue R-250 or subjected to immunoblotting, using the electrogenerated chemoluminescence Western blotting analysis system (Amersham) and polyclonal antibodies against amine oxidase (1: 5000 dilution), magainin II (1:5000 dilution) or monoclonal antibodies against rat IGF-II (1:1000 dilution) (Sanbio B.V., Uden, The Netherlands), which cross-react with human IGF-II. Transfer of proteins onto nitrocellulose (7.5% gels) or PVDF membranes (15% gels) after SDS-PAGE was performed according to Kyhse-Andersen (1984), using a semi-dry electroblotter (Ancos, Denmark). Relative amounts of protein in the total lysates were determined by densitometer scanning with the LKB Ultrascan XL enhanced laser densitometer. Partially purified fusion proteins (AMO-IGF-II or AMO-MAG-II) were obtained by differential centrifugation of crude cell extracts of the transformed strains at 4°C. The extract was centrifuged for 5 min at 2500 rpm. The supernatant was subjected to differential centrifugation subsequently at 5000 rpm (5 min), 7500 rpm (5 min), 10000 rpm (5 min) and 14000 rpm (15 min). The pellets were resuspended in 50 mM potassium phosphate buffer (pH 7.0).

Proteolytic cleavage of the fusion protein with factor Xa was performed as described in the protein fusion and purification system (Biolabs) except that 0.5% Tween 20 (or 0.5% Triton X-100) was added to the cleavage buffer. The biological activity of magainin II was determined by a modified plate assay (Zasloff 1987; Moore et al. 1991). E. coli strain DH1 was spread onto a LB plate. A dilution series of samples containing (a) pure magainin II (Bachem, Switzerland), (b) AMO-MAG-II fusion protein, (c) the cleaved fusion protein and (d) AMO-IGF-II fusion protein (used as negative control) were spotted on this plate and incubated overnight at 37°C.

Electron microscopy

Intact cells were prepared for electron microscopy as described by Keizer-Gunnink et al. (1992). Immunocytochemical experiments were performed on ultrathin sections of Unicryl-embedded cells using polyclonal antibodies raised against amine oxidase and dihydroxyacetone synthase and gold-conjugated goat anti-(rabbit Ig) antibodies.

Results

Transformation and strain selection

Prior to transformation, all expression plasmids were linearized to force targeted integration of the plasmid DNA into the genomic alcohol oxidase locus as described previously (Faber et al. 1992, 1994b). A normal expression level of the endogenous alcohol oxidase protein was maintained. Transformants were subjected to Southern blot analysis to determine the chromosomal configuration (data not shown). Five strains, designated GF33 (IGF-II-SKL), GF37 (AMO-IGF-II), GF38 (IGF-II), GF42 (AMO-IGF-SKL) and GF70 (AMO-MAG-II) were selected, which contained two to three copies of the respective expression plasmids in the genome. All strains grew well on various substrates, including methanol. However, the cell yield of GF70 (expressing AMO-MAG-II) in methanol-limited chemostat cultures was approximately 20% reduced, compared to identically grown GF37 and WT control cells.

Production and intracellular location of the amine oxidase/small functional peptide fusion proteins

To obtain maximal expression levels of the AMO-IGF-II and AMO-MAG-II fusion genes, the transformants GF37 and GF70, respectively, were grown in methanol-limited chemostat cultures. In Coomassie-brilliant-blue-stained SDS/polyacrylamide gels containing total cell lysates of both transformants, an extra protein band of the expected size was present in addition to
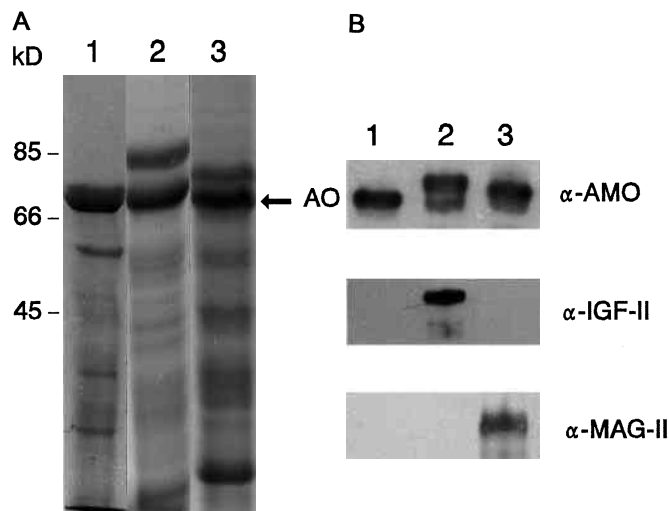

Fig. 2A, B SDS-PAGE analysis of total cell lysates of strain GF16 (lane 1 over-expression of amine oxidase), GF37 (lane 2 expression of AMO-IGF-II) and GF70 (lane 3 expression of AMO-MAG-II). Cells were grown in chemostat cultures on glucose/methanol/ammonium sulphate. A Coomassie-brilliant-blue-stained gel. Proteins with approximate sizes of 78 kDa (amine oxidase), 85 kDa (AMO-IGF-II) and 80 kDa (AMO-MAG-II) are clearly visible above the dominant alcohol oxidase band at 75 kDa. B Western blots of the lysates presented in A. The fusion proteins were recognized by antibodies against amine oxidase (α-AMO, lane 1), IGF-II (α-IGF-II, lane 2) and magainin II (α-MAG-II, lane 3)

those present in identical preparations of strain GF16, expressing amine oxidase by the P_{AOX} (Fig. 2A): an 85-kDa band for the AMO-IGF-II fusion protein and a 80-kDa band for the AMO-MAG-II fusion protein. In Western blots these bands were recognized by antibodies against amine oxidase and antibodies raised against the specific heterologous SFP, IGF-II and MAG-II respectively (Fig. 2B).

The levels of the AMO-IGF-II fusion protein were determined by densitometry of the Coomassie-brilliant-blue-stained SDS/polyacrylamide gels (Fig. 2A, lane 2), showing that AMO-IGF-II constituted approximately 21% of the total cellular protein. As is evident from Fig. 2A, comparable levels were obtained for AMO-MAG-II. Remarkably, no IGF-II protein was detected when it was expressed as a separate peptide in the absence of the amine oxidase carrier (see Fig. 1). Also IGF-II fused to a PTS1 signal (SKL), could not be detected by SDS-PAGE or Western blotting. Northern blot analysis, however, showed that the corresponding

transcript was synthesized. Therefore, we assume that IGF-II is rapidly degraded after its synthesis (data not shown).

The morphology of strains GF37 and GF70 and the subcellular location of the fusion proteins were studied by ultrastructural techniques. Since the N-terminal amine oxidase sequence present in the fusion proteins contains the topogenic information necessary for peroxisomal targeting (Faber et al. 1995b), we anticipated

Fig. 3A–D Ultrastructural analysis of the AMO-IGF-II-producing strain GF37 grown in chemostat cultures on glucose/methanol/ammonium sulphate (A–C). Cells contain many peroxisomes together with large cytosolic proteinaceous aggregates (∗) (**A**). These protein aggregates are intensively labelled in immunocytochemical experiments using anti-AMO, indicating that the fusion protein is not imported into peroxisomes (**B**). After incubation with anti-(dihydroxyacetone synthase), only peroxisomes are labelled (**C**). Cytochemical demonstration of endogenous amine oxidase in peroxisomes of cells of strain GF37, grown on methanol/methylamine (Ce^{3+} + methylamine) (**D**). *N* nucleus *P* peroxisome. *Bars* 0.5 μm
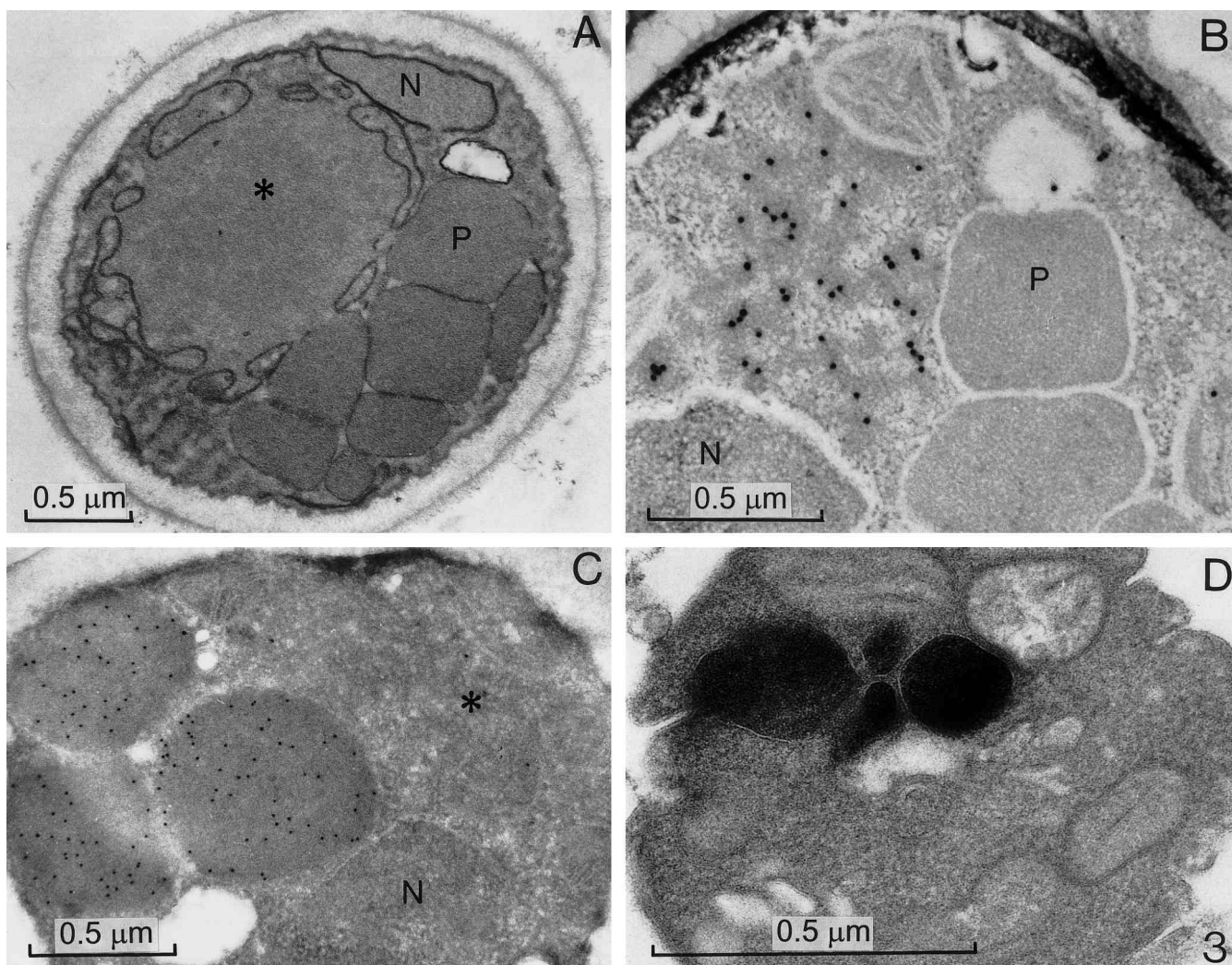
that the fusion product would accumulate, at least partly, in these organelles. However, in strain GF37 the AMO-IGF-II fusion protein was located in the cytosol in a loose aggregate, which frequently occupied a major part of the cytoplasm (Fig. 3A). This was evident from immunocytochemical experiments, which revealed that these structures were densely labelled using anti-AMO antibodies; peroxisomes invariably were not labelled (Fig. 3B). In strain GF70, expressing AMO-MAG-II, proteinaceous aggregates were not observed. Instead, strong proliferation of interconnected membranes had occurred; since these membraneous networks were continuous with the nuclear membrane, they are considered to be derived from the endoplasmic reticulum (Fig. 4A). In addition, in many cells focal thickenings of the cell wall were observed (Fig. 4B). Immunocytochemically, using anti-AMO antibodies, specific labelling was observed on the membraneous networks but not on the cell wall outgrowths, indicating that the fusion protein was associated with these membranes (Fig. 4C); as with AMO-IGF-II, no peroxisomal labelling was observed. In both strains the import of homologous peroxisomal matrix proteins (e.g. alcohol oxidase, dihydroxyacetone synthase and catalase) was not affected as was evident from immunocytochemistry

(shown for dihydroxyacetone synthase in strain GF37; Fig. 3C).

Similar results were obtained with cells grown in the presence of methylamine, a condition which is known to induce the PTS2 import machinery maximally (Faber et al. 1994b). These results indicate that, despite the presence of peroxisomal targeting information, the hybrid proteins are not correctly sorted, presumably as a result of the addition of the heterologous peptides to the amine oxidase C terminus. However, normal peroxisomal import of AMO-IGF-II was accomplished when the hybrid protein was subsequently fused to the C-terminal dodecapeptide of firefly luciferase containing the PTS1 sequence, SKL (Fig. 4D). Apparently, the

Fig. 4A–C Ultrastructural analysis of the AMO-MAG-II-producing strain GF70 grown in thermostat cultures on glucose/methanol/ammonium sulphate. In the cells a strong proliferation of membranes (**A**) and/or focal thickenings of the cell wall (**B**) were observed. Immunocytochemically, using anti-AMO, membrane structures were specifically labelled (**C**). The low labelling intensity is characteristic for these cells and probably reflects the fact that the antigenic epitopes are, at least partly, hidden in the membranes. **D** In methanol-grown cells of strain GF42, the AMO-IGF-II-SKL hybrid protein is located in peroxisomes (anti-AMO). *N* nucleus, *P* peroxisome. *Bars* 0.5 μm, or stated otherwise
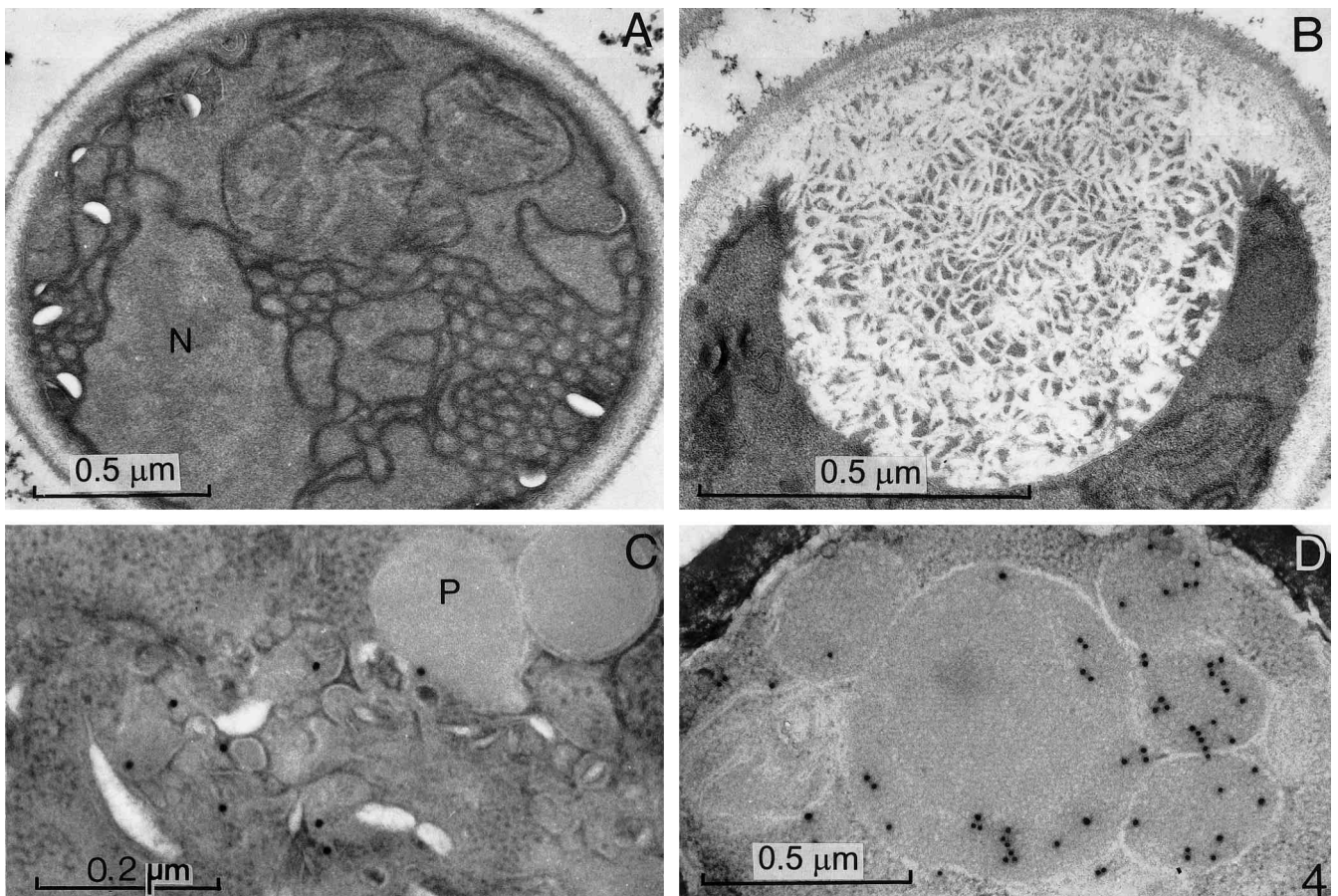
amine oxidase fusion proteins are structurally not amenable to import via the PTS2 import pathway.

Purification of the fusion proteins

The fact that the AMO-IGF-II fusion protein was present in proteinaceous aggregates facilitated its puri-fication. Highly purified fusion protein (more than 90%) could be obtained by differential centrifugation of a crude extract of methanol-grown GF37 cells (Fig. 5A). The insoluble (14000 rpm pellet) fraction comprised approximately 50% of the total AMO-IGF-II fusion protein synthesized in these cells. The same procedure applied to methanol-grown GF70 also yiel-ded partially purified AMO-MAG-II, probably because of the partial enrichment of the complex mem-branous structures in the 14000 rpm pellet (Fig. 5B).

Subsequently both fractions containing either the insoluble (lanes 6, Fig. 5A, B) or soluble (lanes 7, Fig. 5A, B) fusion proteins were subjected to factor Xa digestion to release the heterologous peptides from the amine oxidase carrier protein. After 4 h of incubation of the insoluble fraction in the presence of factor Xa at
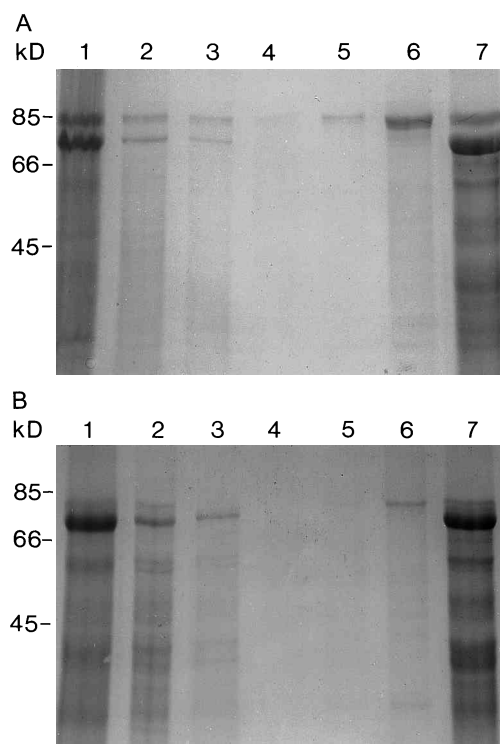

Fig. 6A, B Factor Xa treatment of fractions containing partially purified fusion protein. Starting material was a 30000 *g* pellet frac-tion which also contained, besides the fusion protein, alcohol oxidase. **A** Part of a Coomassie-brilliant-blue-stained gel (7.5% polyacrylamide). Protein fractions were loaded before (*lanes 1, 3*) and after (*lanes 2, 4*) 4 h of incubation with factor Xa. *Lanes 1, 2* fractions from strain GF37 (AMO-IGF-II), *lanes* 3, 4 fractions from strain GF70 (AMO-MAG-II). *Arrowhead* the position of alcohol oxidase and the amine oxidase carrier protein after factor Xa treat-ment. **B** Western blot of fractions 1 and 2 from **A**. These fractions were loaded on a 15% polyacrylamide gel and, after transfer to PVDF membranes, decorated with monoclonal antibodies against rat IGF-II. The reacting band, seen in *lane 2*, shows the same electrophoretic mobility as pure human IGF-II used as a control (not shown)

30°C, over 90% of the AMO-IGF-II or AMO-MAG-II fusions was effectively digested (Fig. 6A) and the het-erologous peptide was released from the carrier protein (shown for IGF-II, Fig. 6B). Similar results were obtained with the soluble fractions (data not shown).

The AMO-MAG-II hybrid protein, produced by *H. polymorpha*, displayed antimicrobial activity. This could be clearly demonstrated in a plate assay, using *E. coli* DH1 as the indicator strain. After the plates had been spotted with various amounts of 14000 rpm supernatants, containing the soluble part of AMO-MAG-II, clear halos readily appeared at the sites where these fractions had been spotted (data not shown).

Discussion

In this paper we describe the use of the methylotrophic yeast *H. polymorpha* as a possible host for the hetero-logous expression of small functional peptides. In recent years, microbiological production of small pep-tides has gained interest as a low-cost alternative for chemical synthesis. However, the development of effi-cient heterologous expression systems has met several problems, ranging from unexpectedly low yields due to intracellular proteolytic degradation of the foreign gene product to undesirable effects of the heterologous prod-ucts on the viability of the cells. In part, these problems could be overcome by the selection of suitable produc-tion strains by classical mutagenesis, as for instance in the case of IGF-I produced by baker's yeast (Shuster et al. 1989). We have followed a different strategy to

Fig. 5A, B SDS-PAGE of protein fractions obtained after differen-tial centrifugation of crude extracts of strain GF37 (**A**) and GF70 (**B**). *Lanes 1* total cell extract; *lanes 2* 2500 rpm pellet; *lanes 3* 5000 rpm pellet; *lanes 4* 7500 rpm pellet; *lanes 5* 10000 rpm pellet; *lanes 6* 14000 rpm pellet; *lanes 7* 14000 rpm supernatant. As can be seen in *lanes 6*, the 14000 rpm pellet fraction is enriched in the specific fusion protein
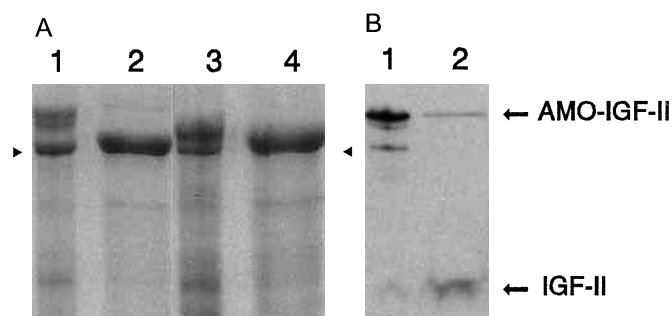
synthesize small peptides by fusing them to the endogenous amine oxidase, for which we had already established a high level of expression in *H. polymorpha* (Faber et al. 1994b). Furthermore, this approach allowed us to study the possible advantage of the accumulation of the hybrid proteins in peroxisomes.

The two proteins tested, human insulin-like growth factor-II (IGF-II) and magainin II (MAG-II) from *X. laevis*, were synthesized at high levels (about 20% of the total protein) in fusion with amine oxidase. Unexpectedly, neither of the fusion proteins was imported into peroxisomes. As shown before (Faber et al. 1993, 1995b), amine oxidase is a typical PTS2 protein, the import of which is not affected by small deletions of the C terminus. Our data lead us to conclude that the addition of IGF-II and magainin II to the C terminus of amine oxidase specifically interfered with proper targeting via the PTS2 import machinery. This view is based on the findings that (a) the PTS2 import machinery functions efficiently in the transformed strains, since homologous amine oxidase was normally imported, and (b) the AMO-IGF-II hybrid protein was correctly imported when a PTS1 (SKL) was subsequently fused to the C terminus of the hybrid protein. This indicates that IGF-II, in principle, can be imported.

Attempts to synthesize IGF-II as the single peptide in the same expression system failed. Even when fused to a PTS1 sequence (SKL), IGF-II was not detected in crude extracts. As transcription of the heterologous genes was observed, judged from Northern blot analysis (data not shown), the protein is probably rapidly degraded after synthesis. Although the AMO-IGF-II fusion protein did not accumulate in peroxisomes, significant amounts of IGF-II were produced in fusion with the amine oxidase carrier protein. Apparently IGF-II is less susceptible to proteolysis in this hybrid form. Therefore, compartmentalization of the protein was not essential to avoid proteolytic degradation of the heterologous fusion protei.

The 23-amino-acid peptide magainin II from *X. laevis* was also synthesized to high levels in the same expression system. As for AMO-IGF-II, partial purification and factor Xa cleavage of the fusion protein were readily achieved. However, the morphological response to AMO-MAG-II synthesis in *H. polymorpha* was remarkably different. First, strong endoplasmic reticulum proliferation was observed. Since the fusion protein appeared to be associated with these structures, this suggests that the magainin part of the hybrid protein drives its insertion into the membrane. Secondly, often a focal cell wall thickening was observed. Similar structures have often been observed in mycoparasitic systems where they are considered to reflect a response of the cell to a toxic substance or enzyme (Persson et al. 1985). By analogy, the cell wall proliferation in *H. polymorpha* may be a response to the high intracellular antibiotic concentration. The antimicrobial activity of the AMO-MAG-II fusion protein was indeed maintained, as was demonstrated in a qualitative plate assay. Although this activity was not lethal for the *H. polymorpha* cells, it may very well explain the lower cell yields observed for the cells synthesizing AMO-MAG-II.

In conclusion, we have developed a suitable system for the biological synthesis of small functional peptides in the methylotrophic yeast *H. polymorpha*. The heterologous peptides were synthesized fused with an endogenous carrier protein but could be readily released from this partner by proteolytic (factor Xa) treatment.

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