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QUARTERLY

Dedicated to Professor David Shugar on the occasion of his 80th birthday

Expression of small synthetic genes coding for hEGF, human epidermal growth factor, and CPTI II, serine proteinase inhibitor from *Cucurbitacea*, cloned in a novel expression/secretion vector in *Saccharomyces cerevisiae*

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Key words: yeast expression/secretion vector, cloning in *Saccharomyces cerevisiae*, serine proteinase inhibitor, human epidermal growth factor

Efficient synthesis of two small eukaryotic polypeptides of human and plant origin was carried out using a novel expression/secretion yeast vector, pYET. The yield was optimized in respect of the yeast strain, expression cassette construction, promoter regulation and culture conditions. Both cloned genes code for biotechnologically important proteins: human epidermal growth factor and a serine proteinase inhibitor from *Cucurbitacea*.

Cloning of short synthetic genes in *S. cerevisiae* with the purpose of efficient production of appropriate proteins calls for further development of reliable, stable, highly productive vector systems. In some instances the most valuable are secretion vectors, which direct the newly synthesized polypeptide outside the cell. During the secretory pathway this polypeptide undergoes proper folding due to formation of disulfide bridges. Bearing this in mind we constructed the novel shuttle vector, pYET, with regulated strong *CTA1* yeast promoter and a synthetic leader sequence [1], and used it for cloning two synthetic genes coding for small eukaryotic proteins, namely human epidermal growth factor (hEGF, 53 aa) [2], and the proteinase inhibitor (CPTI II, 29 aa), a member of the family of inhibitors isolated from seeds of *Cucurbitacea* [3, 4]. Both proteins in their native state form compact globular structures held by three disulfide bridges, and as such can be useful in protein folding studies. The human epidermal growth factor binds to susceptible cells through their EGF-receptor and stimulates their division. Numerous investigations of its biological activities suggested a potential therapeutic role for EGF. Clinical trials proved this to be true in ophtalmology, in treating skin injuries and stomach ulcers. A small plant proteinase inhibitor CPTI II belongs to the family of inhibitors derived from *Cucurbitacea* [3]. Their role in the native host is still a matter of controversy, however, they are active against various animal serine proteinases and have extremely high binding constants, of the range of 10^{11} M^{-1} [5]. When slightly modified, they inhibit human leukocyte elastase: this may be important for therapy.

Expression of appropriate genes and secretion of proteins to the surrounding medium

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Abbreviations: aa, amino acids; CPTI, *Cucurbita pepo* trypsin inhibitor; hEGF, human epidermal growth factor.

were optimized. Recombinant polypeptides were partially purified and their identity verified by several methods.

MATERIALS

Strains of microorganisms. The following strains were used:

Escherichia coli K12:

DH5aF'	*F'endA1 hsdR17(r_K^- m_K^+) supE44 thi-1 recA1 gyrA (Nal^r) relA1 Δ(lac-ZYA-argF)U169 (φ80dlocΔ(lacZ) M15)*
JM101	*F' traD36 $lacI^q$ Δ(lacZ)M15 $proAB^+$supE thi Δ(lac-proAB)*
MC 1066	*leuB trpC pyrF:Tn5(Km^r) r^- m^- araT lacX74 (lacZdem) strA*

Saccharomyces cerevisiae:

D11	*MATα ura3 leu2 his3*
BJ 5464	*MATα ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*
DCT 3-4D	*MATα ura3 leu1 leu2*
GC1-8b	*MATa ura3 leu2-3/112 trp1 ctal-2 ctt1-1*
OL1	*MATα ura3-251/328/372 leu2-3/112 his3-11/15*
WS21-1	*MATα leu2 his3 trp1 pep4*

The strain BJ 5464 was from the Yeast Genetic Stock Center, U.S.A., the remaining strains were from the collections of Institute of Biochemistry and Biophysics Polish Academy of Sciences.

Culture media. *E. coli* was grown in LB, and 2 x TY media, as indicated. For yeast cultures the following media were used: YNB, 0.68% composition of mineral salts and vitamins; YNB + CAA as above supplemented with 1% CAA; W_0, 0.68% composition of mineral salts and vitamins with 2% glucose; W_0 + CAA, as above, supplemented with 1% CAA; YP, 1% yeast extract with 1% peptone; YPG, as above, supplemented with 2% glucose. Media were supplemented with oleic acid to the concentration of 0.1, 0.2 and 0.5%, or with ethanol to 2% concentration, as indicated. Amino acids were added in concentrations of 10–20 μg/ml.

Vectors. The following plasmids and phage M13 derivatives from IBB PAS collection were used for cloning in *E. coli*: pUC 18 and pUC 19 (both 2.7 kb), M13mp18 and M13mp19, (both 7.2 kb). pEMBL 18(+) (4.0 kb) was kindly offered by Dr P. Cegłowski, and M13mp18-KT was constructed in this work. It is a derivative of M13mp18 vector with the sequence coding for *Klyveromyces lactis* killer toxin inserted into *Sst*I/*Kpn*I sites. Yeast episomal plasmid, pJDB 219 (12.4 kb), was from Department of Genetics, Warsaw University, and YEp 352 (3.2 kb), from Department of Genetics of our Institute.

DNA preparations. The following synthetic oligodeoxynucleotides were purchased from the Molecular and Macromolecular Research Center, Łódź:

–a. coding for the fragment of pre-pro MFα: L1, L2

L1 5' C TCT TTG GAT AAA AGA

L2 5' A GTT TCT TTT ATC CAA AGA GGT AC

–b. coding for *hEGF*: 1-10

1. 5' AAC TCT GAC TCT GAA TGT CCA
2. 5' CA TTC AGA GTC AG
3. 5' TTG TCT CAC GACGGT TAC TGT TTG CAC GAC
4. 5' C ACC GTC GTG CAA GTC GTG CAA ACA GTA ACC GTC GTG AGA CAA TGG A
5. 5' GGT GTT TGT ATG TAC ATC GAA GCT TTG GAC AAG TAC GCT T
6. 5' GTA CTT GTC CAA AGC TTC GAT GTA CAT ACA AA
7. 5' GT AAC TGT GTT GTT GGT TAC ATC GGT GA
8. 5' TCT TTC ACC GAT GTA ACC AAC AAC ACA GTT ACA AGC
9. 5' A AGA TGT CAA TAC AGA GAC TTG AAG TGG TGG GAA TTG AGA TAA TAG
10. 5' TCGA CTA TTA TCT CAA TTC CCA CCA CTT CAA GTC TCT GTA TTG ACA

–c. coding for the signal peptide of the killer toxin of *K. lactis*:

5' C ATG AAT ATA TTT TAC ATA TTT TTG TTT TTG CTG TCA TTC GTT CAA GGT AC

5' C TTG AAC GAA TGA CAG CAA AAA CAA AAA TAT CTA AAA TAT ATT CAT GAGCT

–d. synthetic fragment coding for pre-pro MFα and CPTI II N-terminal five amino acids, synthesized in Sequencing and Oligonucleotide Synthesis Laboratory of our Institute:

5' C TCT TTG GAT AAA AGA CGT GTT TGT CCA AA

5' GAT CTT TGG ACA AAC ACG TCT TTT ATC CAA AGA GGT AC.

Analytical amounts of plasmid DNA were isolated from 2 ml overnight cultures of *E. coli* by alkaline lysis [6]. Preparations of plasmid DNA were obtained after ultracentrifugation in CsCl gradients or purified in the QIAGEN system, according to the manufacturers' instruction.

Enzymes. Restriction endonucleases were from Amersham, Boehringer, Fermentas and BRL. T4 kinase was from Boehringer, Klenow polymerase and T4 ligase from Amersham. All enzymes were used according to producers' suggestions. Rabbit polyclonal antibody against hEGF was supplied by Amersham.

Chemicals. The DNA sequencing kits were from Amersham or Pharmacia. The commercial preparation of recombinant hEGF and the silver staining kit were from Amersham. Sep-Pak C-18 was purchased from Waters, Bio-Rex 70 was from BioRad. Culture media were from Difco. All chemicals used were of the highest purity grade.

METHODS

Culture conditions. Liquid cultures were grown under vigorous shaking at 30°C. Cultures under glucose repression were started in 10 ml of W_0 medium inoculated with a single colony. After 48 h of growth these cultures were used to inoculate fresh medium to the approximate density of $A_{600} = 0.2$. Then, after 24 h of incubation, cells were centrifuged and suspended either in YNB + CAA or YP media supplemented with oleic acid to the concentrations of 0.1, 0.2 or 0.5%, or in YNB or YP media with 2% ethanol. Cells were cultured for the next 48 h (hEGF) or 120 h (CPTI II). Samples were taken and cellular density, the total number of cells compared to the number of the plasmid bearing cells were determined. In derepression experiments ethanol concentration was corrected each 24 h with 2% ethanol solution.

General methods of DNA recombination. All recombination *in vitro* procedures were performed according to [7], if not stated otherwise. Yeast transformations were performed according to [8, 9]. DNA was sequenced manually by the dideoxy sequencing method with [α-^{32}P]dATP or [α-^{35}S]dATP. Matrices were prepared from appropriate derivatives of RF M13 recombinant vectors according to the procedure recommended by Amersham. Double stranded plasmid DNA was sequenced with the use of T7 DNA polymerase according to instructions from Pharmacia LKB. Derivatives of M13 phage were obtained from single plaques as described in "M13 cloning and sequencing handbook", Amersham, 1984.

Purification and determination of the activity of hEGF. Recombinant hEGF was isolated from culture supernatants by adsorption on Bio-Rex 70 according to [10], or with Sep-Pak according to producers' suggestions. SDS-PAGE was performed as in [11]. Final purification of hEGF was done by HPLC, according to [12]. Quantitative estimation of EGF in medium, based on its interaction with antibody, was performed by the "dot-immunoblot" assay. Briefly: a sample was applied on Millipore Immobilon-P membrane, and was incubated for 1 h in TBST buffer (150 mM NaCl, 10 mM Tris/HCl, pH 7.5, 0.3% Tween 20 plus 5% skim powdered milk). The rabbit EGF polyclonal antibody (10 mg/ml) was added. The membrane was incubated in the above solution under gentle shaking overnight at room temperature. Unbound antibody was washed off twice with large excess of TBST buffer and the membrane was incubated for 1 h with secondary antibody against rabbit IgG coupled with alkaline phosphatase. Then, the membrane was washed for 15 min, 4 times with TBST buffer and once with TBS buffer (100 mM Tris/HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). Colored spots were developed with a Boehringer kit. The reaction was stopped by washing with TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). Control experiments were run in parallel, including calibrating spots of known amounts of the commercial hEGF preparation. Blots were scanned and scans quantitated with the ImageQuant 3,3^{TM} program. Biological activity of hEGF, resulting in stimulation of DNA synthesis in human fibroblasts was estimated against appropriate controls [1].

Purification and determination of the activity of CPTI II. Preliminary purification of recombinant CPTI II was performed by affinity chromatography on Sepharose 4B coupled to trypsin [13]. The resulting preparation was further purified by HPLC. Inhibitory activity against trypsin was qualitatively determined

by electrophoresis on polyacrylamide gels [14] in the presence of edestin. The limit of detection of CPTI II by this method was 0.1 μg. Proteinase inhibition was quantitatively estimated by the BAPNA test [15].

RESULTS

Construction of the pYET vector

Construction of the yeast expression/secretion vector, pYET, has been described earlier [1]. In short: the strong regulated catalase A promoter (P_{CTA1}) of *S. cerevisiae* includes two positive promoter-controlling elements: the fatty acid-responsive sequence and the ADR1 transcription activator binding sequence. Gene expression directed by the *CTA1* promoter is derepressed by non-fermentable carbon sources, induced by fatty acids and is extremely sensitive to glucose repression [16, 17]. In pYET the above promoter region is followed by the *K. lactis* killer toxin leader sequence (KT) [18], the polylinker, and transcription termination and polyadenylation signals taken from 3'-end of *FLP* from 2 μ DNA. The 2 μ *S. cerevisiae* plasmid *ORI-STB* locus sequence and the *leu 2-d* gene recloned from pJDB219 plasmid, are responsible for high copy number and stable propagation in yeast of the pYET plasmid. The yeast *URA 3* gene is an auxotrophic selectable marker, and the f1 phage origin of replication (IG) makes possible propagation of the plasmid in a single-stranded form (Fig. 1).

Two variants of the vector were constructed which differ in their leader peptide sequence (Fig. 2).

pYET plasmid can be propagated in *E. coli*, due to a bacterial selection marker, (Amp^r), and the *ori* sequence, both derived from pEMBL 18 plasmid.

The synthetic gene coding for hEGF and its recombination with pYET plasmid

The *hEGF* gene was designed using preferred yeasts codons, and assembled by enzymatic ligation of 10 synthetic fragments (Fig. 2). Two recombinant plasmids: pYET-EGF1 and pYET-EGF2 were obtained. The first version, pYET-EGF1, was constructed and described in [1]. The gene was fused in frame with the killer toxin leader sequence and ligated to the pYET vector. The second construction, pYET-EGF2, started with ligation of the chemically synthesized, double-stranded DNA linker, coding for the last five amino acids of the pre-pro MFα-1 leader sequence, to the *hEGF* gene. The ligation product was cloned in M13mp18-KT vector in its *KpnI/SalI* sites, downstream to the killer toxin leader (KT) coding sequence. From the resulting plasmid the *SstI/SalI* fragment (coding for KT:α-factor:*EGF* gene) was fused in frame to pYET vector cleaved with the same enzymes.

Construction of the pYET-CPTI II plasmid

The procedure for assembling of the synthetic gene coding for CPTI II was described earlier [4]. The gene was cloned in M13mp19 vector in its *HindIII/SalI* restriction sites. The synthetic double stranded oligonucleotide block coding for five C-terminal amino acids of pre-pro MFα1 leader and five N-terminal amino acids of CPTI II (Fig. 2, 4) was ligated to the 80 bp fragment of the *CPTI II* gene recovered from polyacrylamide gel after digestion of the plasmid M13mp19-CPTI II with *BglII/SalI*. The 110 bp product recovered from polyacrylamide gel was in turn ligated to the restriction fragment M13mp18-KT after its digestion with *KpnI/SalI*, downstream to the sequence coding for *K. lactis* killer toxin leader. The constructed ligation product was sequenced. The 158 bp *SstI/SalI* insert from the above plasmid was isolated from an agarose gel and ligated to pYET vector digested with *SstI/SalI*. This construct makes possible synthesis of CPTI II with its native NH_2 end [19].

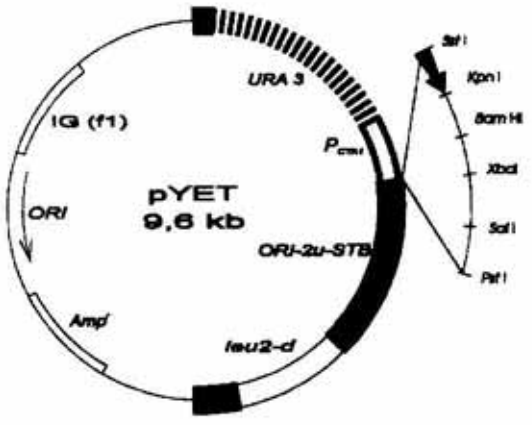

Fig. 1. Physical map of the pYET plasmid.
The arrow preceding the polylinker corresponds to the leader peptide sequence.

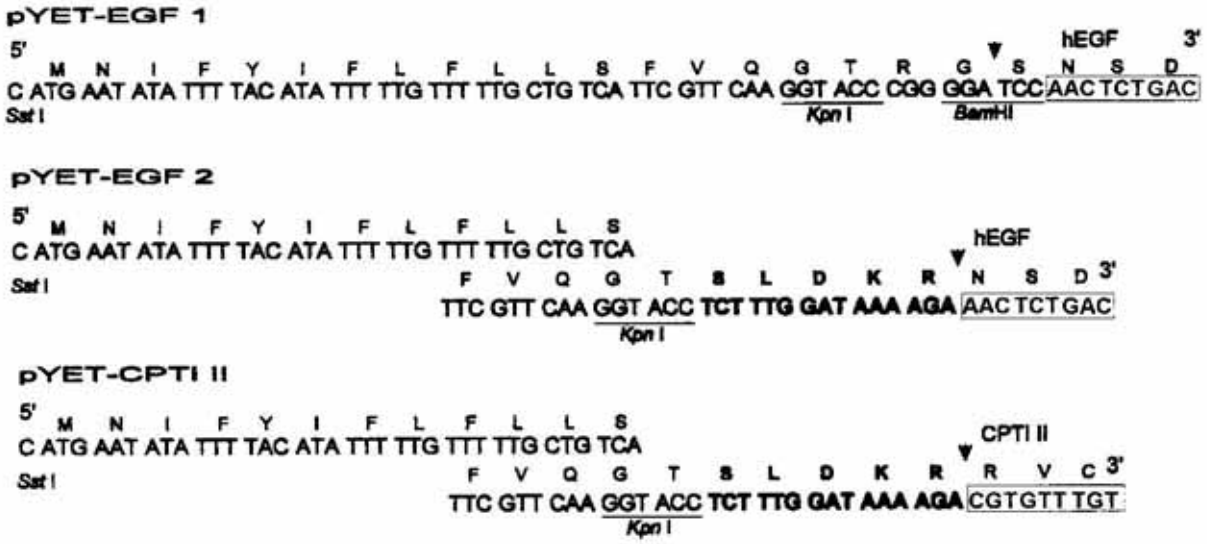

Fig. 2. Nucleotide and respective amino-acids sequences for the signal peptide flanking the 5' end of the cloned genes in two pYET variants.
Nucleotides coding for the sequence recognized by Kex2p endopeptidase are marked in bold-face and the cleavage site by an arrow.

Stability of pYET vector and of its recombined forms

The vector itself as well as the recombined pYET plasmids were stable when transformed to the recipient strains: OL1, BJ5464 (both genes), GC1-8b, WS21 (*hEGF* gene), D11, DCT30-4D (*CPTI II* gene), cultured in non-selective YP medium. As an example quantitative data for growing pYET and pYET-EGF in selective and nonselective media and pYET-CPTI II under promoter derepression are shown (Tables 1, 2)

Optimization of the yield of recombinant proteins

The yield in culture supernatants of hEGF was estimated by the dot-immunoblot test, and that of CPTI II on polyacrylamide:edestin gels.

The genotype of the recipient strain is important for the yield of recombinant proteins. Among the investigated yeast strains (see Materials)

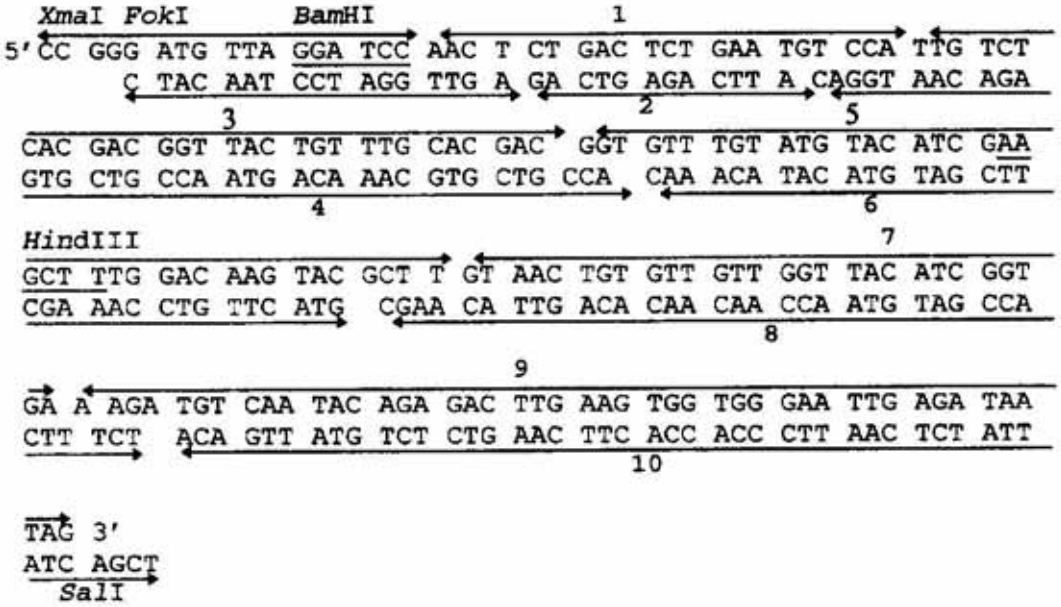

Fig. 3. Nucleotide sequence of the synthetic hEGF gene.
Arrows mark indicate the length of respective oligonucleotides.

Table 1
Stability of pYET and pYET-EGF1 plasmids in S. cerevisiae strains

Strain	Number of colonies grown on solid medium		Stability of plasmids (%)
	selective (W_0 - ura)	nonselective (YPG)	
GC(pYET-EGF1)	8.9×10^7	1.0×10^8	89%
GC(YET)	8.9×10^7	1.0×10^8	89%
OL1(pYET-EGF1)	1.0×10^8	1.1×10^8	90%
OL1(pYET)	1.1×10^8	1.2×10^8	91%
WS(pYET-EGF1)	1.0×10^8	1.1×10^8	90%
WS(pYET)	9.8×10^7	1.4×10^8	89%

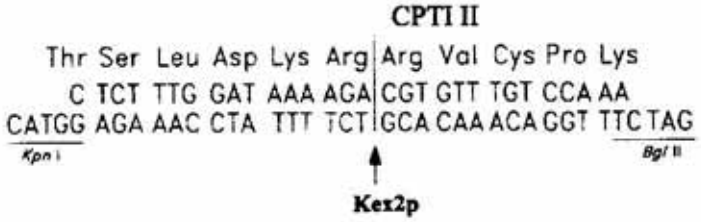

Fig. 4. Nucleotide sequence of the synthetic DNA fragment linking in frame the sequence coding for killer toxin leader with that coding for five N-terminal amino-acids sequence.
Restriction sites and the site cleaved by Kex2p peptidase are marked.

OL1 strain was shown to be the most efficient in transformation yield, rate of growth and cell density at the end of the logarithmic phase. For both recombinant proteins similar yields were obtained both in OL1 and BJ5464 strains, the latter is deficient in vacuolar proteases.

The culture medium plays a significant role in yield determination; of YNB, YNB + CAA and YP, the last medium gave the best result.

The mode of promoter regulation was crucial for the yield: oleic acid, a potential transcription inducer, even at the lowest, 0.1% concentration, slowed down cellular growth and lowered the yield of expression of a given recombinant protein. Thus, in the system studied, ethanol was recommended for transcription derepression.

Culture growth under transcription derepression. CPTI II was stable in supernatant and the best yield was attained after 120 h of derepression. According to earlier reports concerning instability of EGF in YNB+CAA culture media [20] maximum EGF yield was observed after 48 h of derepression.

Both leaders used, namely killer toxin leader or hybrid combination of killer toxin and α-factor leaders, were efficient and the products were correctly processed in the secretory pathway.

The stability of *medium pH* did not play any marked role in determining the final yield of

Table 2
Stability of the recombined pYET-CPTI II plasmid in S. cerevisiae strains cultivated for 120 h in nonselective YP medium, under conditions resulting in derepression of the transcription. Prior to derepression cells were cultivated in selective YNB medium

Strain	Number of colonies grown on solid medium	
	selective (W_0 - ura)	nonselective (YPG)
OL1(pYET-CPTI II)	5.1×10^8	5.2×10^8
BJ5464 (pYET-CPTI II)	5.0×10^8	4.8×10^8

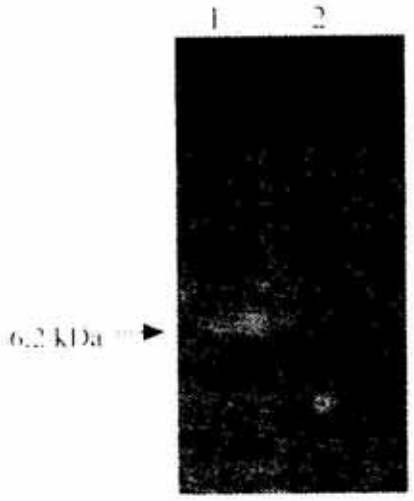

Fig. 5. Polyacrylamide gel electrophoresis of recombinant hEGF under denaturing conditions, stained with silver kit.
Lane 1, Molecular weight markers from BRL; Lane 2, hEGF purified on Bio-Rex, followed by HPLC.

expression and secretion of the respective recombinant proteins.

Maximal yield (4.3 mg of EGF per 1 l of supernatant) was obtained for OL1(pYET-EGF2) strain cultivated up to the density of 1.2×10^8 cells/ml (48 h) in YP medium supplemented with 2% ethanol. Maximum yield (3.2 mg of purified CPTI II per 1 l of supernatant was obtained for OL1(pYET-CPTI II) strain cultivated for 120 h in YP medium supplemented with 2% ethanol (the final density of 5×10^8 cells).

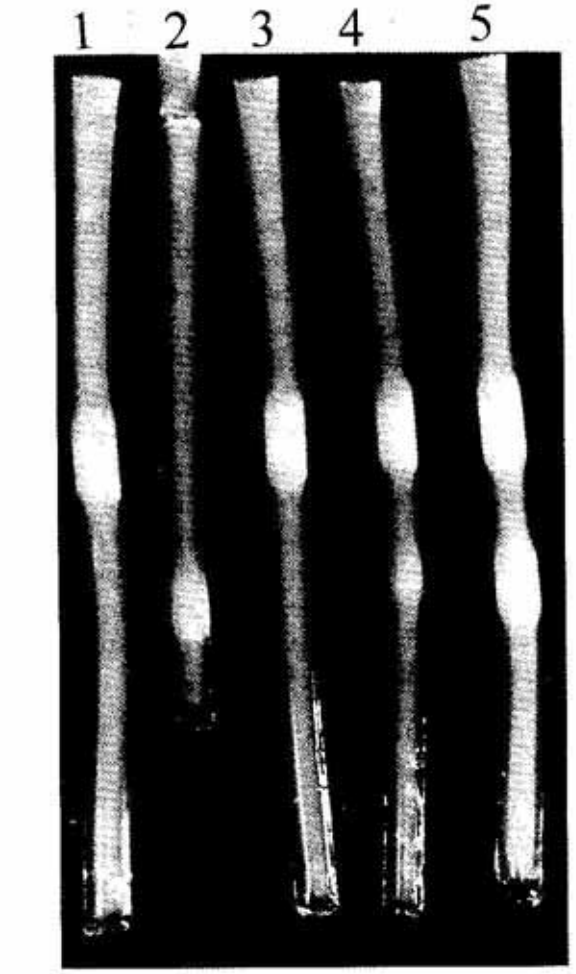

Fig. 7. Electrophoretic test for CPTI II identification on polyacrylamide gels
1, 100 µl of YP supernatant of 120 h culture of OL1(pYET-CPTI II) strain; 2, 15 µl of peak 1 fraction (as on Fig. 5); 3, 8 µl of peak 2 fraction (as on Fig. 5); 4, 10 µl of the preparation of recombinant CPTI II partly purified by affinity chromatography; 5, 15 µl of peak 1 fraction mixed with 8 µl of peak 2 fraction.

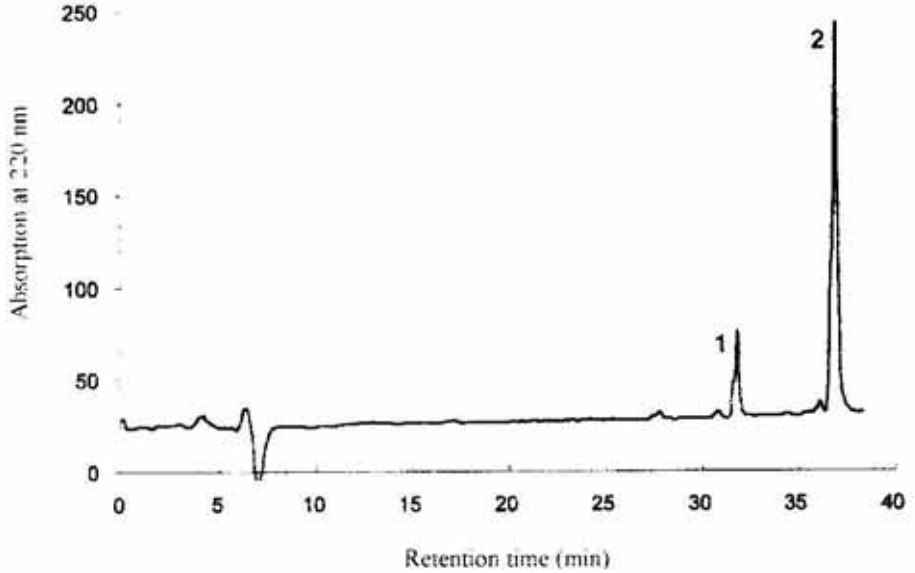

Fig. 6. HPLC elution profile of recombinant CPTI II partly purified by trypsin-affinity chromatography. Peak 1: CPTI II cut with trypsin, peak 2: unmodified CPTI II.

Identification of recombinant proteins

On SDS-polyacrylamide gels the recombinant hEGF migrated according to its predicted molecular mass of 6 kDa (Fig. 5). The purified preparation reacted with the specific antibody and promoted division of human fibroblasts [1]. The identity of recombinant CPTI II was confirmed by biological activity tests: in supernatants on polyacrylamide:edestin gels, due to its specific retention on a trypsin-Sepharose column, by a specific BAPNA test for partly purified preparations and by HPLC separation (Fig. 6). The material obtained in two peaks was analyzed by electrophoresis in polyacrylamide:edestin gels. Peak 1 (15%) was found to correspond to inhibitor cut with trypsin in its active center, still retaining its inhibitory activity. Peak 2 (85%) corresponds to the native ("virgin") form of inhibitor [21] (Fig. 7).

DISCUSSION

Expression of recombinant genes in *S. cerevisiae* can be influenced by various conditions which are often unpredictable *a priori* [22–26], and attainment of high levels of production of recombinant proteins is still a matter of trial and error. Novel vectors for cloning foreign genes should be investigated in various recipient strains, under various conditions of growth and regulation.

The expression/secretion *S. cerevisiae* vector recently constructed in our laboratory [1] was used for cloning and expression of two genes coding for small proteins. Both constructed synthetic genes included codons preferred by yeast. Both polypeptides have in common their small size and three disulfide bonds, making their structures compact and globular.

Such structures could be formed only in the oxidizing environment of specific cellular compartments, therefore the *S. cerevisiae* secretion system, enabling transport of the newly synthesized polypeptides outside the cell, was used. The secretion system was also recommended previously for those proteins which are secreted from cells of the native host (i.e. EGF).

Prior to our work the *hEGF* gene was cloned and its expression investigated in *E. coli* [27], *S. cerevisiae* [28], and *Pichia pastoris* [29]. The reported yield in *S. cerevisiae* was in the range of 4–10 mg per 1 liter of culture.

Information about cloning small serine protease inhibitors is scarce. Chen *et al.* [30] reported cloning of the gene coding for the methionine-less mutein of the squash serine protease inhibitor, TTI, with the yield of 2 mg per 1 liter. The recombinant protein, though bearing three additional amino acids on its N-end, retained its inhibitory activity.

Prior to construction of a pYET vector we had used other, available expression/secretion yeast vectors ([4] and unpublished results for hEGF): the multi-copy (2 μ) number plasmid pYSV5, and the low-copy number (ARS/CEN) vector, pJK6. Derivatives of pJK6 (pJK6-CPTI II and pJK6-EGF) were stable in nonselective media, whereas pYSV5 derivatives segregated.

Moreover, the expression cassette from pJK6-EGF plasmid was introduced to the chromosome-integrating vectors pRS303, pRS305, pFL34, and the auxotrophic yeast strain OL1 was transformed with recombinant plasmids.

In all experiments performed with the above vectors, under various experimental conditions, the yield of recombinant products was lower than the yield obtained in this work, and the biologically active products were often heterogeneous in size, what was probably due to the imperfect posttranslational proteolytic modifications.

The gene coding for CPTI II was recently cloned in *E. coli* but the protein was obtained in a low yield (60 μg/l), and only when a secretion vector was used [31].

Thus, the data presented in this work clearly show that of all investigated systems, the novel pYET vector offered the best yields in production of small proteins containing disulfide bonds.

The most important feature of the constructed novel vector is that it is stable in rich media, and may be used for production of recombinant proteins without selection pressure. In rich media cells grow to high density, and the produced proteins are less prone to proteolysis by vacuolar enzymes, which are induced in the cell under nitrogen starvation in minimal media [20].

Due to its two selectable markers, pYET can be grown under selection for uracil and leucine; the defective promoter of the *leu-2d* gene ensures a high plasmid copy number, which in

some instances results in high expression of the recombinant gene located on the plasmid.

The yield of recombinant proteins secreted by *S. cerevisiae* was shown in the past to be highly variable, however, the yield of small heterologous proteins was in most instances in the range of several milligrams per liter [20, 27, 30].

We thank Dr Marek Skoneczny for providing us with promoter *CTA1*.

Note added in proof: when this article was being edited, a paper describing efficient synthesis in *E. coli* of a mutated CMTI II, the trypsin inhibitor devoid of the internal methionine, was published — Bolewska, K., Krowarsch, D., Otlewski, J., Jaroszewski, Ł. & Bierzyńska, A. (1995) Synthesis, cloning and expression in *E. coli* of a gene coding for the Met8 → Leu8 CMTI II — representative of the squash inhibitors of serine proteinases. *FEBS Lett.* **377**, 172–174.

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