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Trypsin inhibitor gene cloned and expressed in *Saccharomyces cerevisiae*

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Trypsin inhibitors isolated from seeds of the *Cucurbitaceae* constitute a homologous family of squash serine protease inhibitors [1]. The physiological role of these inhibitors in plants is still a matter of discussion. When investigated *in vitro*, they show various biological activities, among them hormone-like effects on mouse fibroblasts in culture [2].

Because of their three-dimensional structure these polypeptides are convenient targets for conformational studies. Moreover, the biological activity of trypsin inhibitors could be modulated by modifications of the primary structure of their reactive site [3]. Preparation of these inhibitors from natural sources and their chemical synthesis are cumbersome and inefficient. Therefore application of recombinant techniques aimed at obtaining of large quantities of inhibitors from the above family seemed reasonable, the more so as in many instances the amino-acid sequence of a given polypeptide was known.

Investigations were undertaken on the *Cucurbita pepo* trypsin inhibitor (CPTI II)[1] which consists of 29 amino-acid residues cross-linked by three disulfide bridges. Its reactive peptide bond is located in the amino-terminal part of the molecule, between residues 5 (Lys) and 6 (Ile) [4].

We used two complementary oligonucleotides kindly supplied by Dr W. Mandecki which included the coding sequence for 29 amino acids of CPTI II with N-terminal methionine. Their sequence corresponded to the codons most frequently used by *S. cerevisiae* [5] flanked by the restriction sites for *Hin*dIII and *Sal*I. Both oligonucleotides were annealed, purified by gel electrophoresis and ligated to M13mp19 RF DNA. The final sequence was confirmed by the dideoxynucleotide method [6].

In the expression – secretion system two episomal yeast vectors, pYSV5 and pJK6, were used for recombination with the synthetic gene for CPTI II (Fig. 1). The gene was fused in-frame to the region coding for the secretion leader of α-factor, using the *Hin*dIII site located immediately downstream to the sequence coding for the processing signals Lys-Arg-(Glu-Ala)$_3$ (Fig. 2). Vectors: pJK6 and pYSV5 and their respective recombined derivatives were used to transform yeast strains (Table 1) to uracil or leucine prototrophy. The cloned gene was expressed under the control of two different promoters:

– i, in pJK6 vector it was *GAL1*, galactose inducible promoter (expression inducible up to one thousand times) [7];
– ii, in pYSV5 vector it was α-factor promoter, regulated by temperature in temperature sensitive *sir*3 yeast mutants [8].

Transformed cells were collected in various stages of growth and the supernatant was assayed for the presence of trypsin inhibiting activity by gel electrophoresis (Fig. 3). Three bands with different electrophoretic mobility were shown to inhibit trypsin. Such an activity was not found in cellular lysates.

[1] Abbreviation: CPTI, *Cucurbita pepo* trypsin inhibitor

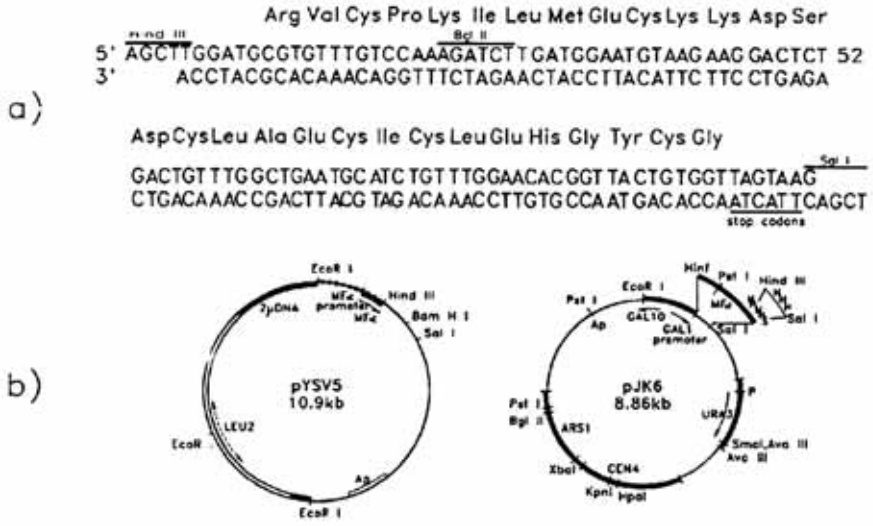

Fig. 1. Construction of CPTI II expression plasmids.
The 104 bp synthetic sequence coding for CPTI II (a) was inserted into expression vectors pJK6 and pYSV5 (b) *via* *Hin*dIII and *Sal*I sticky ends. The relevant regions of both vectors are shown schematically; thick and thin lines denote yeast and bacterial sequences, respectively

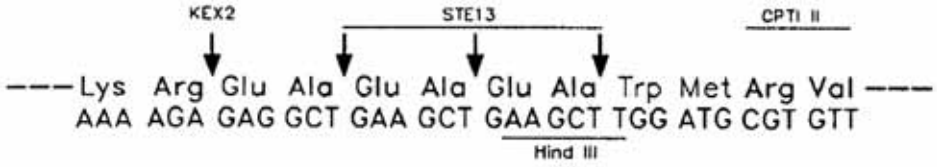

Fig. 2. Nucleotide and corresponding amino-acid sequences at the fusion junction between MF_α leader sequence and the sequence of CPTI II gene.
Previously established and possible cleavage sites for endopeptidase KEX2 and dipeptidyl aminopeptidase STE13 are indicated [9]

Table 1

Genotypes of Saccharomyces cerevisiae strains employed: a. Transformed with pJK6 and pJK6-CPTI II; b. Transformed with pYSV5 and pYSV5-CPTI II.

The lithium acetate method was used in the *S. cerevisiae* transformation procedure [10]. Transformed cells were grown in uracil or leucine minimal media supplemented with Casamino acids, with 3% glucose as a carbon source, to a density of 5×10^7 - 1×10^8 cells/ml at 30°C. JRY188 cells were grown at the restrictive temperature of 37°C. For induction, cells were spun down and the respective pellets: a, washed with sterile water and resuspended to the same density in the induction medium containing 3% galactose as a carbon source; b, resuspended to the same density in fresh medium without Casamino acids and grown at the permissive temperature (25°C, JRY188/pYSV5-CPTI II). The WS21-1/pYSV-5-CPTI II strain was not thermally induced (it was grown at 30°C)

	Strain	Genotype
a	AH 201	*Matα, his3Δδ, ura3, trp1*
	OL 1	*Matα, leu 2-3, 112, his 3-11-15, ura 3 251,372,328*
	D 11	*Matα, ura3, leu 2, his 3*
	DCT 30-4D	*Matα, leu 1, leu 2, ura 3*
b	JRY 188	*Matα, sir 3-8, leu 2-3,2-112, ura3-52, his 4, rme, (trp+/–)*
	WS 21-1	*Matα, leu 2, his 3, trp 1, pep 4*

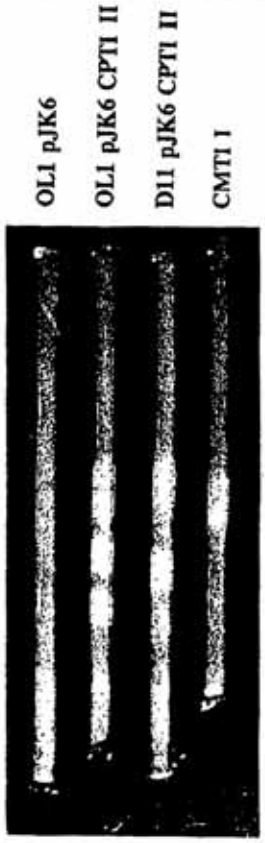

Fig. 3. Qualitative electrophoretic assay for trypsin inhibition.
Electrophoresis of culture samples (1.5 ml, freeze-dried and dissolved in 80 µl of water) was performed on 7.5% polyacrylamide gels supplemented with edestin (final concentration 0.1%). Gels were soaked in trypsin solution (1 mg/100 ml of 50 mM Tris). White bubbles (undigested edestin) indicate positions of the trypsin inhibitor on the gel

It could be suggested that the multiple products of gene expression in the described system originated from their incorrect processing by endoproteinases involved in maturation of yeast proteins (cf. Fig. 2). Besides, due to the *Hin*dIII restriction site used in construction of the recombined vector, and because of the presence of Met codon on 5′ end of the coding sequence, there are most likely two additional amino-acid residues at the N-terminus of the expressed polypeptide.

Nevertheless these possible modifications did not appreciably influence the inhibitory activity of the cloned protein. This activity was quantitatively determined in fractions eluted from the affinity column packed with the anhydrotrypsin-Sepharose 4B derivative, to which the supernatant from the induced yeast culture was applied (Fig. 4).

The amount of the secreted trypsin inhibitory activity was dependent on the transformed strain, expression vector, culture medium and the stage of culture growth, at which gene expression is induced. Maximal productivity (estimated as 100 µg of active protein per 1 l of culture) was achieved with D11 and OL-1 strains (see Table 1), transformed with the pJK6-CTPI II plasmid, induced for 96 h in the selective minimal medium supplemented with Casamino acids. Owing to *URA 3* marker, the stability of the recombined plasmid in this enriched medium was high (20% of seggregation after 14 generations).

The low expression level of the recombined gene could be due to specific inhibition of the KEX 2 processing protease by the expression product, a serine protease inhibitor. KEX 2 protease belongs to the subtilisin serine protease family, and CPTI II inhibits subtilisin, however, to a low extent. To test the above assumption, the level of secretion of α-factor by yeast strains recombined with CPTI II gene was estimated.

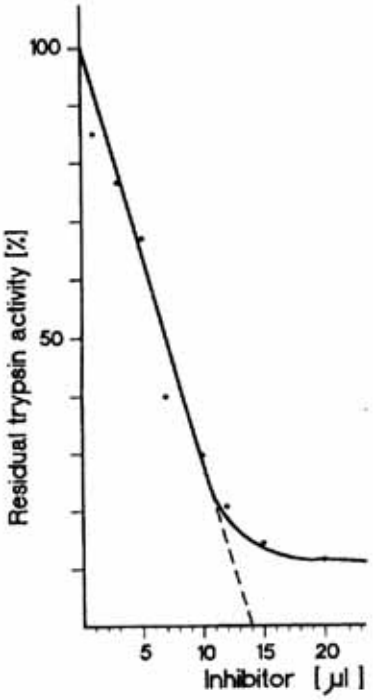

Fig. 4. Trypsin inhibition by recombinant CPTI II.
Bovine trypsin (2 µg) was mixed with various amounts of recombinant CPTI II, partially purified from the culture medium by affinity chromatography. The residual trypsin activity was assayed using Nα-benzoyl-DL-arginine-*p*--nitroaniline as substrate

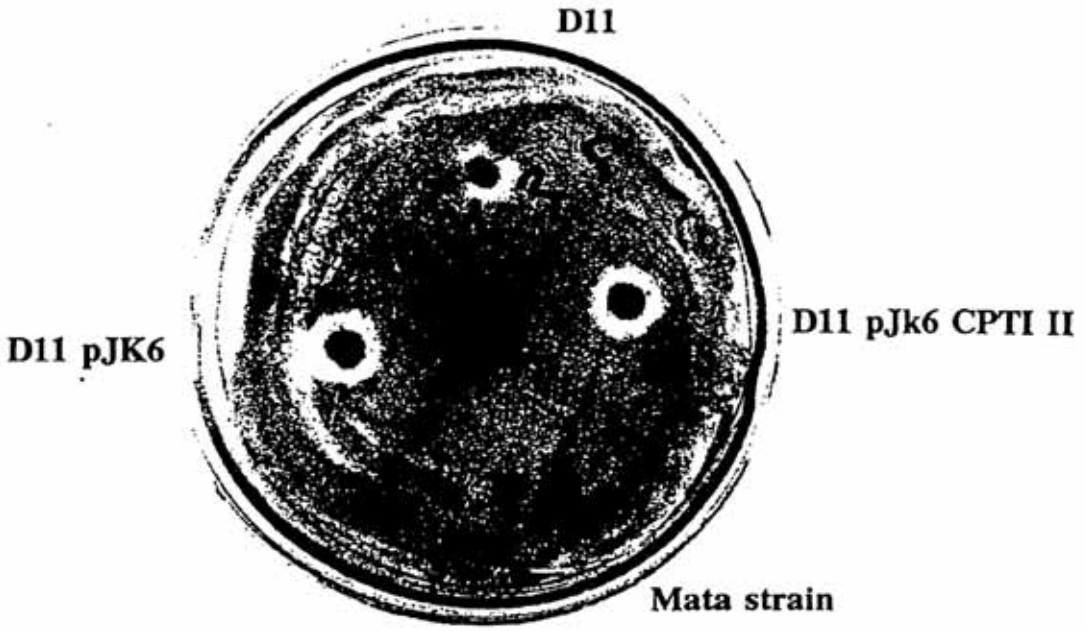

Fig. 5. Secretion of the mature α-factor by yeast strains, D11/pJK6-CPTI II, and D11/pJK6, as visualized by formation of halos on the plate.
MATa M171-C *ura3 his3 leu2 sst2* cells, supersensitive to α-factor, were spread onto a YPGal (pH 3.5) plate. Strains to be tested were spotted on the same plate. Zones of growth inhibition were visible after 2 days incubation at 30^0C [11]

It was found that in these strains the level of mature α-factor production was not appreciably changed (Fig. 5). This suggests that KEX 2 protease is not significantly inhibited by the recombinant trypsin inhibitor.

The synthetic gene for the small plant polypeptide, exhibiting various biological activities, was cloned and expressed in *S. cerevisiae*. The work is in progress in our laboratory with the aim to improve the level of expression and to achieve synthesis of a homogeneous product.

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