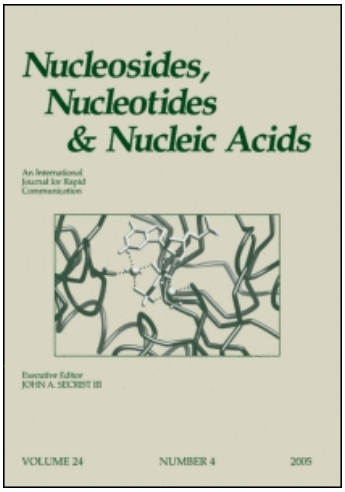

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Display Of Ribonuclease T1 On The Surface Of Bacteriophage M13

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DISPLAY OF RIBONUCLEASE T1 ON THE SURFACE OF BACTERIOPHAGE M13

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ABSTRACT: In this work the expression of functional ribonuclease T1 on the surface of the filamentous *Escherichia coli* phage M13 is described. Ribonuclease T1 was fused to the phage coat protein pIII and functionally displayed on the phage surface as shown by a negative staining zymogram and RNase indicator plates.

Phage display technology provides a powerful tool in protein engineering studies. By the expression of peptides and proteins on the surface of filamentous phages large pools of peptides and randomized proteins can be screened for certain binding properties. The linkage of DNA fragments coding for these peptides either to gIII, the gene of the viral coat protein III, or to the gene gVIII encoding major coat protein pVIII, leads to viral fusion proteins, which are assembled upon hyperinfection with helper phages. Recombinant phages, displaying peptides on the surfaces can be isolated by affinity chromatography (biopanning) and the corresponding peptide sequence can be determined directly by DNA sequencing. Thus, the display of random peptides has been used in selecting ligands of defined binding properties[1,2], the display of single chain antibodies led to recombinant antibodies against certain antigens[3,4] and recently the expression of randomized enzyme led to variants with new enzymatic properties[5].

In this work we describe the expression of active ribonuclease T1 (RNase T1; EC 3.1.27.3) on the surface of the filamentous *Escherichia coli* phage M13. RNase T1 is one of the most extensively studied model enzymes with respect to protein stability and -folding, enzymatic action and structure (for review see 6). We fused the RNase T1 gene to the surface exposed N-terminal domain of pIII (T1::gIII). RNase activity of the fusion

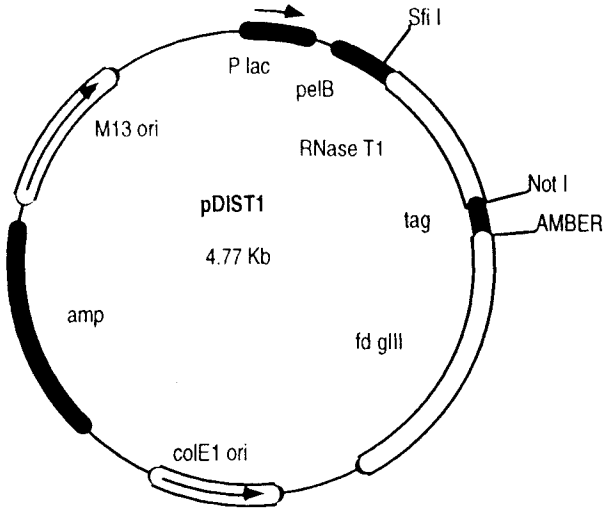

FIG. 1: Map of the phagemid vector pDIST1. P lac, lac promoter/operator region; pelB leader, leader peptide of the pectate lyase; tag, c-myc peptide tag; fd-gene III; amber, UAG amber codon; gene of the viral coat protein III; colE1 ori, origin of replication of the *E. coli* ColE1 plasmid; amp, ampicillin resistence gene; M13 ori, origin of replication of M13 genome.

protein T1::pIII was shown by a zymogram assay[7]. Furtheron we showed that RNase T1 displayed by recombinant phages exhibits RNase activity by the use of RNase indicator plates[8]. This work offers the possibility to get further insights into protein-nucleic acid interactions.

RESULTS AND DISCUSSION

Construction of phagemid vector pDIST1

To clone the RNase T1 gene upstream of gIII contained in the phagemid vector pHEN1[9], flanking *Sfi*I and *Not*I restriction sites were introduced by PCR. Amplification was performed with *Vent* DNA-polymerase and 100 ng of the RNase T1 gene containing

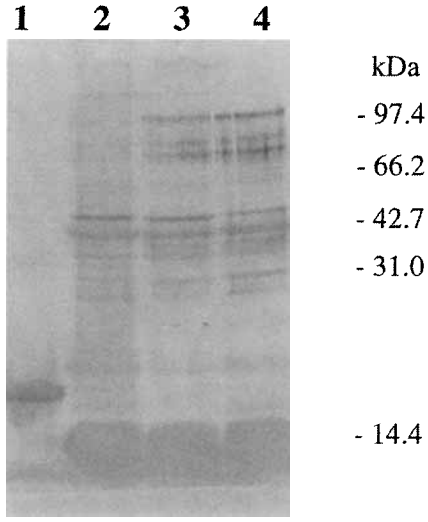

FIG. 2: Western blot analysis of fusion protein RNase T1::pIII. Phage particles displaying RNase T1 were denatured and their coat proteins separated by SDS-PAGE. Proteins were blotted onto nylon membranes and probed with rabbit polyclonal anti-RNase T1 antibodies. Purified RNase T1 (lane 1), M13 phages (lane 2), M13 phages displaying RNase T1 (lanes 3,4). The resulting fusion protein RNase T1::pIII has a theoretical molecular weight of 71 kDa.

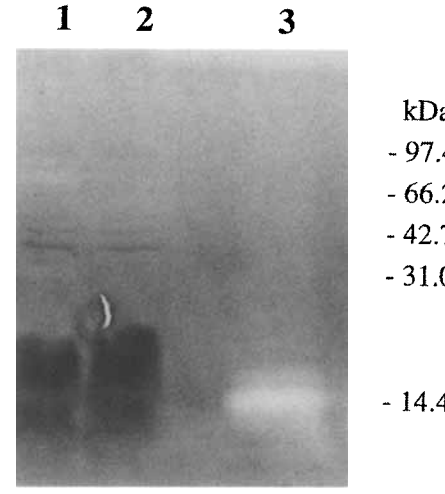

FIG. 3: Detection of RNase activity of RNase T1::pIII by zymogramm assay. To determine RNase activity of fusion protein, a zymogram was performed. Phage proteins were separated by SDS-PAGE in a polyguanylic acid containing gel, renaturated after separation and stained with toluidine blue. RNase activity is represented by white bands on the gel. M13 phages displaying RNase T1 (lane 1), M13 phages (lane 2), purified RNase T1 (lane 3).

expression/secretion vector pA2T1[10] as template and the oligonucleotides SfiI (5´ GCG GCC CAG CCG GCC ATG GCC GCA TGC GAC TAC ACT TGC GG 3´) and NotI (5´ TCG CGG CCG CTG TAC ATT CAA CGA AGT TGT TAC C 3´) as primers. PCR was carried out with an initial denaturation step (5 min at 98 °C), followed by 25 cycles with 1 min at 98 °C, 1 min at 45 °C and 1 min at 72 °C. To complete polymerization, a final extension step (5 min at 72 °C) followed. Reactions were carried out under conditions recommended by the manufacturer. The resulting PCR fragment was purified, digested with *Sfi*I / *Not*I and cloned in pre-cut pHEN1 vector by standard cloning

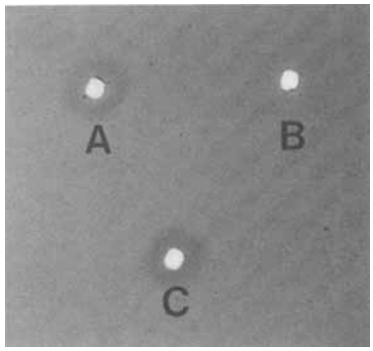

FIG. 4: RNase activity of assembled phage particles displaying RNase T1. To analyze phage particles for RNase activity, phages were applied to RNase indicator plates. Active particles show a red halo on the RNA and toluidine blue containing blue plates. Phages displaying RNase T1 (A), M13 phages (B), RNase T1 (C).

techniques[11] yielding pDIST1 (FIG. 1). The correct sequence of the amplified RNase T1 gene, was proven by double strand DNA sequencing.

Display of fusion protein T1::gIII on phage particles

Escherichia coli XL1-Blue cells transformed with phagemid pDIST1 were grown in 2xYT medium containing 150 µg ampicillin per ml and 0.001 % thiamine at 37 °C to a OD_{600} of 0.6. Phagemid-containing cells were superinfected with M13K07 helper phage at a multiplicity of infection of 100 and grown after supplementing 70 µg kanamycin per ml at 37 °C for 15 hours with vigorous agitation. Cells were removed by several centrifugation steps and phages were obtained by precipitation after the addition of 0.25 volumes of 3.5 M NaCl / 20 % polyethylene glycol 8000 solution. The phage yield was 1-2 x 10^{10} plaque forming units per ml culture volume.

Recombinant phages were analyzed for the expression of fusion protein T1::pIII by western blot analysis (FIG. 2). RNase activity of the fusion protein was detected by a zymogram assay[7] using polyriboguanylic acid as substrate (FIG. 3). Both assays showed

that there was no presence of RNase T1 in the phage preparation resulting from the non-suppression of the amber codon between RNase T1 and gIII gene. In order to show that assembled phages still exhibit RNase activity, this preparation was analyzed on RNase indicator plates[8] (FIG. 4). The expression of functional RNase T1 on the surface of filamentous phage described in this work will allow us to screen large amounts of randomized RNase T1 variants by the use of the phage display technique to find variants with altered specificities and enzymatic mechanisms using affinity materials like non-cleavable polyribonucleic acids or transition state analogues.

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