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A new member of the bacterial ribonuclease inhibitor family from *Saccharopolyspora erythraea*

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Abstract We have identified *Sti*, the gene of a ribonuclease inhibitor from *Saccharopolyspora erythraea*, by using a T7 phage display system. A specific phage has been isolated from a genome library by a biopanning procedure, using RNase Sa3, a ribonuclease from *Streptomyces aureofaciens*, as bait. Sti, a protein of 121 amino acid residues, with molecular mass 13 059 Da, is a homolog of barstar and other microbial ribonuclease inhibitors. To overexpress its gene in *Escherichia coli*, we optimized the secondary structure of its mRNA by introducing a series of silent mutations. Soluble protein was isolated and purified to homogeneity. Inhibition constants of complex of Sti and RNase Sa3 or barnase were determined at pH 7 as 5×10^{-12} or 7×10^{-7}, respectively.

Key words: Barstar; Barnase; Ribonuclease; Ribonuclease inhibitor; Phage display; mRNA secondary structure

1. Introduction

For some years, considerable interest has been focused on barnase and barstar, the extracellular ribonuclease and its protein inhibitor from *Bacillus amyloliquefaciens*, addressing problems of protein folding and protein recognition [1]. Many bacterial and fungal homologs of barnase have been identified, with essentially the same fold but with widely disparate sequences, providing clues as to just what elements of the sequence determine the fold. Presumably, homologs of barstar could be useful in the same manner. Barstar was unique until the first ribonuclease inhibitors from *Streptomyces aureofaciens* were cloned in 1998 [2]. Some putative ribonuclease inhibitors have been identified in genomes of bacteria within microbial genome sequencing projects and here we report the cloning and expression of another.

It is well known that barstar forms a very tight complex with barnase ($K_d = 10^{-14}$ M) as well as with barnase streptomycete homologs [3]. In spite of rather low sequence homology (22–27%) of streptomycete ribonucleases with barnase, there is a high structural similarity in their active sites. RNase Sa, the extracellular ribonuclease produced by *S. aureofaciens*, strain BMK, forms almost identical contacts with barstar as does barnase [4]. This has allowed the construction of plasmids in *Escherichia coli* containing the genes of both barstar and the streptomycete ribonucleases, with secretion of these ribonucleases on a scale of several tens of milligrams [3]. For another homolog of barnase, RNase St, the barstar gene provides some protection for *E. coli* from its gene expression but not enough to allow production of useful amounts of the enzyme [3]. RNase St [5] is secreted by an organism originally called *Streptomyces erythreus* but reclassified as *Saccharopolyspora erythraea* by Labeda [6]. The amino acid sequences of RNases Sa and St are about 20% identical to that of barnase and 40% to each other. A superimposition of tertiary structures of barnase with RNase St or RNase Sa reveals larger values of root mean square and maximum deviations for RNase St than for RNase Sa [3]. Additionally, since the ionic residues of barstar contributing to binding to ribonuclease are all negatively charged, it has been speculated that the high negative charge on RNase St ($pI = 3.9$) can cause a slower association rate with barstar (J. Sevcik, personal communication). As mutational and theoretical examinations of the barnase–barstar complex have implied, barstar's distribution of charged residues is well optimized for rapid and tight binding to barnase [7,8]. It has also been demonstrated that, in the case of barnase–barstar, electrostatic forces increase basal association rates by more than four orders of magnitude from 10^5 to 5×10^9 M^{-1} s^{-1} [9]. Based on theoretical and experimental work on the barnase–barstar complex, it seems likely that formation of a complex of RNase St and barstar is aggravated by unfavorable steric and electrostatic complementarities.

We have cloned and expressed the gene (*Sti*) of a ribonuclease inhibitor from *S. erythraea*. We demonstrate that Sti is a homolog of barstar and other microbial ribonuclease inhibitors. Its expression did not immediately lead to large scale production of RNase St in *E. coli* but it may be a step in that direction.

2. Materials and methods

2.1. Bacterial strains and vectors

S. erythraea, strain NRRL 2338, was used for genomic DNA isolation. T7 phage was grown on BLT5403 (Novagen). XL1-Blue MRF′ (Stratagene) was used for plasmid DNA preparation. BL21(DE3)-pLysS and Turner (DE3)pLacI (Novagen) were used for protein production. T7Select10-3b (Novagen) and pUC18 were used for construction of genomic library. pET28a and pET Blue-2 (Novagen) were used for overexpression of *Sti* gene in *E. coli*.

2.2. Construction of T7 phage display library

All DNA manipulations were performed using standard methods [10]. Genomic DNA was isolated and purified from *S. erythraea* ac-

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cording to Hopwood [11]. T7 phage DNA was isolated according to instructions of the manufacturer. The shotgun phage display library was constructed as follows: genomic DNA was fragmented randomly by sonication and fragments of 300–700 bp were isolated by preparative gel electrophoresis and made blunt-ended using mung bean nuclease. Phage vector was digested with the restriction enzyme *Sma*I and dephosphorylated with calf intestinal alkaline phosphatase. Ligation was carried out at 16°C overnight in presence of 5% PEG 8000. DNA was packaged in vitro using T7Select packaging extract (Novagen) and amplified before use in a liquid culture by infecting mid-log phase BLT 5403 *E. coli* cells. An average insert size and percentage of recombinant phages were determined by polymerase chain reaction (PCR) of a number of randomly chosen plaques with sense primer T3 5′-GACCAGATTATCGCTAAGTACGCAATG and the antisense primer T4 5′-GTTCACCGATAGACGCCAGAATGTC.

2.3. Phage affinity selection

Target ligands – barnase (extracellular ribonuclease from *B. amyloliquefaciens*) and RNase Sa3 (extracellular ribonuclease from *S. aureofaciens,* strain 3239) – were coupled to Iontosorb AV cellulose (Iontosorb) as described previously [2]. For phage binding experiments, 300 μl of affinity resin was packed into a column of 6 mm diameter. For the first round of biopannig 8 ml of phage lysate ($\sim$ order of 10^{10} pfu) was applied at a flow rate of 10 ml h^{-1}. The column was washed with 50 ml of 0.5 M NaCl, 20 mM Tris–HCl (pH 8) to remove non-specific binding phage and ribonuclease-bound phage was eluted by 1 ml of 0.5% sodium dodecyl sulfate (SDS) in the same buffer. Before subsequent amplification of phage, SDS was removed by precipitation with 1/10 volume of 10 M KAc on ice [12]. The column was washed with another 10 ml of elution buffer followed by 50 ml of washing buffer and used in next round. The entire procedure was repeated five more times; 2 ml of amplified phage was taken from previous rounds of panning.

2.4. DNA sequencing

The DNA sequences of phage inserts were determined after PCR amplification by automatic sequencing on an Applied Biosystems 373 Sequencer by using Big Dye chemistry (PE Applied Biosystems). Sequencing was performed in both directions.

2.5. Cloning the gene of inhibitor Sti from subgenomic plasmid library

This was carried out in order to confirm the 5′ and 3′ sequences surrounding the *Sti* structural gene and to avoid mutations introduced by PCR. *S. erythraea* genomic DNA was digested with a variety of restriction endonucleases and Southern blot analysis was performed using as a probe a PCR-amplified insert from a selected phage clone already identified by sequencing as a homolog of barstar. A 2.1 kb Sti fragment was cloned into pUC 18 vector and a positive clone was identified by hybridization with the ^{32}P-labelled probe.

2.6. Expression of the Sti gene in E. coli

Cloning restriction sites at 5′- end and 3′- end of *Sti* gene were created by PCR amplification of the gene using sense primer 5′-GAAGAAGAAGAACCATGGGCGGGTTGGAGGAACCCTCC carrying *Nco*I restriction site and antisense primer 5′-AAGAAGAAGAAGAAGCTTTCAGTTCCGGGTCAGGACCGC containing *Hin*dIII restriction site, which allowed in-frame insertion of the *Sti* gene into both pET28a and pET Blue-2 expression vectors. The amplified fragment was digested with *Nco*I and *Hin*dIII and ligated into the vector digested with the same restriction endonucleases to yield pETSti or pETBluSti, respectively. pETSti was transformed into BL21(DE3)-LysS *E. coli* strain; *E. coli* Turner(DE3)LacI was used for expression of Sti from pETBlueSti plasmid. LB medium supplemented with corresponding antibiotics was inoculated with a single colony and grown at 37°C until the absorbance value at 600 nm reached 0.5. Three ml of this culture was used to inoculate 100 ml of SB medium and grown to reach about 0.8 OD_{600}. The expression of protein was induced by adding IPTG to final concentration 1 mM and incubation extended at 37°C overnight.

2.7. Isolation and purification of recombinant Sti

Bacterial cells were harvested and soluble proteins were released by suspension of a cell pellet in BugBuster protein extraction reagent, supplemented with benzonase nuclease (Novagen) and incubated at room temperature for 20 min. Insoluble cell debris were removed by centrifugation. Nucleic acids were precipitated with streptomycin sulfate at final concentration 1.5%. The supernatant was clarified by centrifugation and diluted with an equal volume of 20 mM Tris–HCl, pH 7.2, 20 mM mercaptoethanol, 1 mM EDTA (buffer A), and applied onto HiPrep 16/10 Q XL Sepharose column (Amersham Biosciences), using HiTrap Sepharose Q FF 5 ml connected in series as a precolumn. After washing the column, Sti was eluted with a linear gradient of elution buffer (buffer A with 0.7 M NaCl). Fractions containing Sti inhibitor were collected, concentrated using Centriprep YM3 (Amicon) and applied further on 16/60 Superdex-75 size-exclusion column (Amersham Biosciences). Protein was eluted with 50 mM sodium bicarbonate, 20 mM mercaptoethanol.

2.8. Prediction of mRNA secondary structure

Analysis of potential secondary structure adopted by mRNA sequence was carried out using the program *mfold* [13].

2.9. Determination of inhibition constants

Determination of ribonuclease-inhibitor dissociation constants was performed basically as described previously [12]. In short, measurements of fluorogenic substrate (fluorescein-dArGdAdAd-TAMRA; Integrated DNA Technologies) hydrolysis were made using Perkin-Elmer LS-50B fluorescence spectrophotometer. Ribonuclease–barnase or RNAse Sa3 was combined with inhibitor at varying concentrations and the rate of substrate hydrolysis was measured by monitoring the increase in fluorescence (excitation 489 nm, emission 519 nm). The experimental data were fit to a series of curves computed according to the equation [14,15]:

$$v = v_0 \frac{[\mathrm{E}]-[\mathrm{I}]-K+\sqrt{([\mathrm{E}]-[\mathrm{I}]-K)^2+4[\mathrm{E}]K}}{2[\mathrm{E}]}$$

where v is the initial reaction velocity at inhibitor concentration [I], v_0 is the initial reaction velocity in the absence of inhibitor, [E] is concentration of ribonuclease and K, the dissociation constant, is the estimated parameter. Estimation of the dissociation constant K_d was done for every curve as determined from the best fit by non-linear regression.

3. Results and discussion

3.1. Cloning a gene of RNase St inhibitor

Our first attempts to clone the gene of RNase St inhibitor using heterologous DNA probes or degenerated synthetic oligonucleotide probes by low stringency hybridization failed (data not shown) as a consequence of rather low homology (Fig. 1) among the family of bacterial ribonuclease inhibitors. The most significant similarity in the multiple sequence alignment is observed in the small region, underlined in barstar, which is known to be responsible for binding to ribonuclease. The sequence of the rest of the inhibitor molecules appears to be more diverse. Phage display technology offers an alternative approach. Specific clones carrying an inhibitor gene can be selected from a whole genome library via an in vitro affinity selection of phage with the products of genes inserted into the phage genome displayed on its surface. Immobilized ribonuclease is used as the capturing ligand. We worked with a T7 phage display system which had already been successfully tested in our laboratory for protein folding studies [12]. A phage display library was constructed by shotgun cloning of fragmented chromosomal DNA from *S. erythraea*. The resulting library consisted of 1.2×10^6 clones and had a titer 2.5×10^{10} pfu ml^{-1}, which together was expected to display all proteins encoded by the sacchcaroplyspora genome in the form of polypeptides of corresponding size. Since RNase St was not available in quantities required for biopanning experiments, barnase and RNase Sa3, homologs of RNase St, were used as target ligands. Both enzymes, together with unrelated protein bovine serum albumin as a control, were immobilized on Iontosorb AV. These columns had performed well in af-

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sti           SGLEEPSADV SAVKAAEDAE RRGAAAHVLD GSELLSKRAA LDGIAAVLDF P-EWAGRNLD  59
barstar       ---------- ---------- ----KKAVIN GEQIRSISDL HQTLKKELAL P-EYYGENLD  35
yrdF          ---------- ---------- ----RKIIID GRDFENIEVL HDDLKDKLDF P-DYYGRNLD  35
sai14         ---------- ---------- ---TVTYVID GFEIDTLEDF WDVVGQAIGV D-GYFGHNLD  36
sai20         ---------- ---------- TDNELIVDLR GRQIETLNDF WDAVSEPCGL P-EWFGRNLD  39
s.coelicolor  ---------- ---------- ------ITID VSEVTDERTL HVLLKRELGF P-DFYGMNWD  33
yersinia      ---------- ---------- ----VKVVFD FNHIPDLPAF YRAFSQNFAL S-EDFGANLD  35
neisseria     ---------- ---------- ----QLEII- GSKIYTEQDF HNQISKIFSI Q-DYYGNNLD  34
clostridium   LNIVKIYHFS YKRSYYEPYE NVSYETYYLD GNNITTYESF FCALGEAING PGGYYGTDEL 204
clostridium2  ---------- ---------- ----KIVTID GKEFKDKDNL HRILKKELKF P-EYYGKNLD  35

sti           ALYDCLTDLS WLPE-GEHVL IWSGYQALAD YD--PKAYRK ISAVLKEASE TSFCG----- 111
barstar       ALWDCLTGWV EYP----LVL EWRQFEQSKQ LT--ENGAES VLQVFREAKA EG--------  81
yrdf          ALWDCLTGWV DLP----LTL VLKNFEFSNT FL--GSYADD VLEVIQEAQE ELK-------  82
sai14         AFADCLGGGY GTPDDGDYVI EWRHHELSRR RLGCPETVRQ LELRLARVHP TNR-------  89
sai20         AWSDTIETRG ISEVIDSHDI LVVHVDQRGL FEGHRREADV LADTFDGEQN QLIVHG----  95
s.coelicolor  AFWDSITGLV SIP----THV RFVGWDQLAA GV--PRGAAM LRRALDDYQA TYR-------  80
yersinia      ALWDVVTGEI ALP----VEI EFVHFSRRHQ RR-----FAA IVLLLEEAEE ELAG---R--  81
neisseria     ALWDLLSTNV ERP----ITL VWKDAMFSKN QL--ENIFIE IVNVLERVKK QDEDYGFE--  86
clostridium   NILDCLTGGF KACAP--FKL VWRNHKIALK NLSITEWENK IERNKKENSK IFEDDYFHSK 262
clostridium2  ALWDCLTGET KMP----LKI VWKNFNDSKG YL--GQYAER TAQVLLRVEE YFN-------  82

sti           --RTFTAVLT RN-------- ---------- ---------- ---------- 121
barstar       --CDITIILS ---------- ---------- ---------- ----------  89
yrdf          --DEFKIIIE ---------- ---------- ---------- ----------  90
sai14         --EQVAAELA AARAGKGPTV FDWLVDIIEE RHPGGLRLA- ---------- 126
sai20         --PDWPAATD ---------- ---------- ---------- ---------- 103
s.coelicolor  --PEFFAQYA ---------- ---------- ---------- ----------  88
yersinia      --LHFNVVDE HIV------- ---------- ---------- ----------  92
neisseri      --EKFNYILE ---------- ---------- ---------- ----------  94
clostridium   PEKSFFDTII EIFVEHKITV MPLFYRVISS RGNNTAFVSK AWSDYVVYI 322
clostridium2  --GKFKVEIQ ---------- ---------- ---------- ----------  90
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Fig. 1. Amino acid sequences of barstar ribonuclease inhibitor homologs. Sti – from *Saccharopolyspora erythraea*; barstar – from *Bacillus amyloliques*; yrdf – from *Bacillus subtilis*; sai14 and Sai20 – from *Streptomyces aureofaciens*; s. coelicolor – putative inhibitors from *Streptomyces coelicolor*; yersinia – from *Yersinia pestis*; clostridium and clostridium 2 – from *Clostridium acetobutylicum*. Conserved amino acid residues are expressed by shaded letters. Underlined amino acid of barstar indicate the chief contact region with barnase.

finity purification of ribonuclease inhibitors from *S. aureofaciens* [2]. Since a positive control experiment, using phage with displayed barstar diluted to 10^{-6}, showed a significant enrichment of binding phage in population of phages eluted from affinity columns (62%) after only six rounds (data not shown), panning was repeated six times. The phage recovered after the final round were plated out to obtain individual clones for sequencing. The displayed fragment was amplified by PCR and sequenced. Although pilot experiments with yrdF – ribonuclease inhibitor from *Bacillus subtilis* (data not shown) – confirmed observations that a DNA fragment can be displayed on the phage surface even if there is a shift in reading frame between the inserted DNA and the capsid phage protein [16,17], the sequences were translated in three possible frames and a search for homology between putative protein and the family of bacterial inhibitors was performed using the program Clustal W. Among 30 randomly picked phage after six rounds of selection on barnase, none showed any homology with RNase inhibitors, although some of the phage were identical, suggesting that some sort of selection had taken place. On the other hand, two out of eight clones randomly picked from a pool of phage selected on RNase Sa3 were identical and their translated sequence shows significant homology with other ribonuclease inhibitors (Fig. 1).

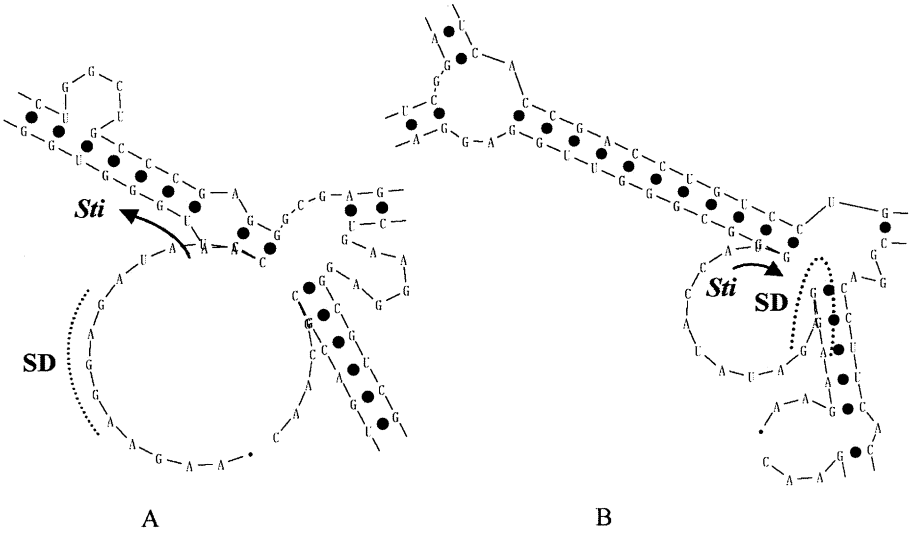

Fig. 2. Proposed mRNA secondary structure of *Sti* gene variants in the initiation region of protein synthesis. A: Wild-type *Sti* gene with Shine-Dalgarno sequence (SD). Shine-Dalgarno sequence and initiation codon (ATG) are marked. Stability of helical structure in this region is $\Delta G = -22.3$ kcal/mol. B: Structure of mRNA upon introduction of silent mutations into *Sti* gene. Stability of helical region is $\Delta G = -10.5$ kcal/mol.

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AAGAAGGAGATATACCATGGGTGGTTTAGAAGAGCCCTCCGCTGACGTCAGCGCCGTCAA  60
AGCCGCCGAGGACGCCGAGCGACGCGGCGCGGCGGCGCACGTCCTGGACGGTTCGGAGTT 120
ATTAAGCAAGCGCGCGGCGCTCGACGGCATCGCCGCGGTCCTGGACTTCCCGGAGTGGGC 180
GGGCCGCAACCTCGACGCCCTCTACGACTGCCTCACCGATCTGAGCTGGCTGCCCGAGGG 240
CGAGCACGTGCTGATCTGGTCCGGCTACCAGGCACTGGCCGACTACGACCCGAAGGCCTA 300
CCGGAAGATCAGCGCGGTGCTGAAGGAGGCGTCGGAGACCAGCTTTTGCGGCCGGACGTT 360
TACCGCGGTCCTGACGCGCAAC                                        382
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Fig. 3. Silent mutations (shaded bases) introduced into the *Sti* gene by site-directed mutagenesis. Dotted underline marks Shine-Delgarno sequence, and solid underline marks the start codon.

Using the gene (*Sti*, GenBank accession number AY374309) for this inhibitor homolog as a Southern hybridization probe, we re-isolated and sequenced the gene from a subgenomic plasmid library, ensuring that no errors had been introduced during PCR or phage replication and that we had the correct 5′ sequence. Several potential translation initiation codons were identified, and the GTG codon which is preceded by the plausible Shine-Dalgarno sequence GAAGGAG [18] was assumed to be the translation start of the *Sti* gene. Codon usage of the *Sti* gene is consistent with patterns found in the related *Streptomycetes* genes, with 95% of codons having G or C in the third position and an overall G+C content of 70.8%. Analysis and comparison of both sequences, that from the displayed fragment and that from the positive clone from the plasmid library, confirmed that the whole Sti protein was displayed on the phage surface.

3.2. *Overexpression of* Sti *gene – alteration of mRNA secondary structure*

Using newly constructed restriction sites, the amplified *Sti* gene was placed into the *E. coli* pET 28a expression vector that contains a T7 promoter, which is activated by the production of T7 polymerase. The level of Sti production was monitored by checking the ability to inhibit barnase or RNase Sa3 and by SDS–polyacrylamide gel electrophoresis (PAGE). However, neither test revealed a presence of inhibitor protein upon induction. Variation in cultivation conditions (*E. coli* strain, temperature, medium, time and concentration of IPTG) did not affect the expression level of inhibitor. We also tried the pTrc99A expression vector, which contains IPTG inducible Trc promoter, without any success (data not shown). We then considered the possibility that our difficulty might be due to the low efficiency of translation of *Sti* gene's mRNA in *E. coli*. That the problem was rare codon usage was rejected, since there are only three codons in our gene which are rarely used in *E. coli* and these were not near the 5′ end where they tend to be most damaging [19–21].

Another cause of low efficiency of translation of heterologous genes in *E. coli*, as shown in numerous reports [22–25], is formation of secondary structures in the mRNA, especially when such structure includes parts of the Shine-Dalgarno sequence and/or the start codon. We did identify, using the *mfold* program [13], such a potential structure in the start region of the *Sti* mRNA. A complementary sequence, which can form a very stable structure with the N-terminal region of the inhibitor gene with minimum free energy −22.3 kcal/mol, partially covering the translation initiation codon, was detected down the coding sequence (Fig. 2). To attenuate this helix structure by preventing base pairing, we consecutively introduced silent mutations in different positions downstream from the translation start site of the *Sti* gene (Fig. 3). Finally Sti protein was efficiently produced from an improved gene with all the alterations shown. The difference was even more dramatic after we subcloned the same gene into a pET Blue-2 vector, a high copy version of pET vectors (Fig. 4). Since we did not find any significant differences at the transcription level between wild-type and improved genes as assayed by Northern hybridization, we conclude that the presence of unfavorable mRNA secondary structure strongly suppresses the expression rate of Sti protein from its wild-type gene in *E. coli*. Presumably the source organism has mechanisms to ameliorate this problem, which it must face more often because of the high G/C content of its DNA.

3.3. *Purification and characterization of Sti inhibitor*

Sti protein was fully soluble and purified by two sequential procedures – anion exchange chromatography on HiPrep Q XL Sepharose and size exclusion chromatography on Superdex-75. We obtained highly purified protein as judged by SDS–PAGE. One purification yielded approximately 40–60 mg of pure protein from 1000 ml culture. Inhibition constants K_d for the complexes Sti–RNase Sa3 and Sti–barnase were estimated by enzyme titration with inhibitor as described in Section 2 as 5×10^{-12} or 7×10^{-7}, respectively (Fig. 5). Interaction of Sti–Sa3 is almost five orders of magnitude tighter than that between Sti and barnase, the Sti–barnase complex being the weakest observed among all such pairs. As noted above, we can assume that differences in the shape of active sites between RNase St and barnase and in the distribution of their charged residues on the surface cause low affinity of barstar to RNase St as indicated by the poor protection provided by barstar against the toxic effects of RNase St. Since

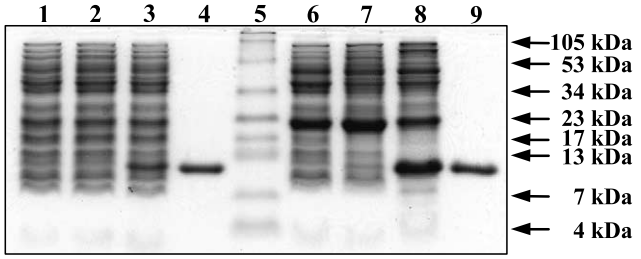

Fig. 4. SDS–PAGE of recombinant Sti protein. Lane 1: bacterial lysate of *E. coli* BL21 transformed with pET28a; lane 2: bacterial lysate of *E. coli* BL21 transformed with pETSti; lane 3: bacterial lysate of *E. coli* BL21 transformed with pETStimut6; lane 4: purified Sti protein; lane 5: Multimark, molecular mass protein standards (Invitrogen); lane 6: bacterial lysate of *E. coli* Turner transformed with pETBlue-2; lane 7: bacterial lysate of *E. coli* Turner transformed with pETBlueSti; lane 8: bacterial lysate of *E. coli* Turner transformed with pETBlueStimut6; lane 9: purified Sti protein.

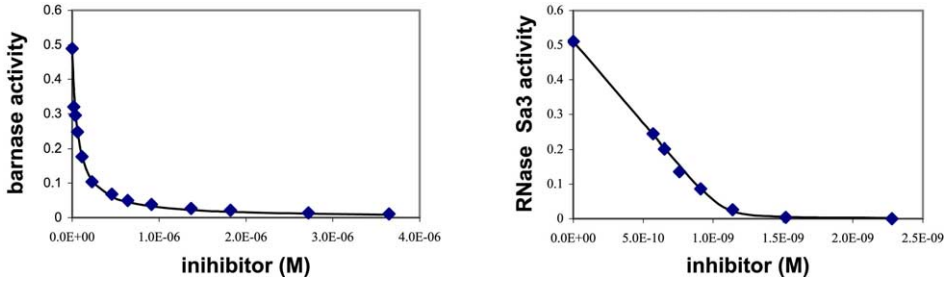

Fig. 5. Inhibition of barnase and RNAse Sa3 by Sti. Curves were generated according to the Morrison equation [14,15]. $K_d = 7 \times 10^{-7}$ M for the barnase–Sti complex, $K_d = 5 \times 10^{-12}$ M for the RNase Sa3–Sti complex.

Sti, as a natural inhibitor of RNase St, has evolved to inhibit this particular ribonuclease, it is not surprising that the reciprocal combination, barnase to Sti, is also weakly bound for the same reasons.

Our attempts to improve expression of RNase St in the presence of Sti inhibitor have been disappointing. Production of active protein has been increased two to three times (data not shown), but yield is still rather low and there are apparently some other intricacies in addition to the toxicity of this enzyme. Work on this is in progress.

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