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A synthetic operon containing 14 bovine pancreatic trypsin inhibitor genes is expressed in *E. coli*

Brigitte von Wilcken-Bergmann, Daniela Tils, Jürgen Sartorius, Ernst August Auerswald[1], Werner Schröder[1] and Benno Müller-Hill

Institut für Genetik der Universität zu Köln, Weyertal 121, 5000 Köln 41, and [1]Bayer AG, Institut für Biochemie, Postfach 10 17 01, 5600 Wuppertal 1, FRG

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A synthetic gene encoding the protein sequence of mature bovine pancreatic trypsin inhibitor (BPTI) has been cloned into a novel *E. coli* expression vector. After *in vitro* gene amplification by successive DNA duplications, more than 600 000 mostly inactive inhibitor molecules may be recovered from a single cell. After purification the inhibitory activity can be reconstituted almost completely. The specificity of BPTI for trypsin is abolished by a single amino acid exchange from lysine to isoleucine at position 15. The altered protein is shown to be an efficient inhibitor of human leukocyte elastase.

Key words: E. coli expression vectors/*in vitro* gene amplification/overproduction of BPTI/protein engineering/change of specificity of BPTI.

Introduction

Bovine pancreatic trypsin inhibitor (BPTI, or aprotinin) is a small, rather basic protein of 58 amino acids, which is purified from several bovine organs i.e. lung, pancreas or parotid glands (Kunitz, 1947; Fritz and Wunderer, 1983). BPTI inhibits trypsin most efficiently but it also acts as an inhibitor of chymotrypsin, plasmin and kallikrein® (Fritz and Wunderer, 1983). Its structure (Deisenhofer and Steigemann, 1975), function (Laskowski and Kato, 1980) and folding pathway (Creighton, 1978) have been studied intensively.

The recent analysis of cloned bovine DNA confirms that the mature inhibitor is processed from a larger precursor polypeptide by proteolytic cleavage of both termini (Laskowski and Kato, 1980; Anderson and Kingston, 1983) and that the bovine BPTI gene may be a member of a family of closely related proteins that have acquired different inhibitory specificities by few amino acid exchanges (Kingston and Anderson, 1986). Substitution of one amino acid may alter the substrate specificity of a protein. This has been shown for the lactose permease by Markgraf *et al.* (1985), and data concerning the serpins are reviewed by Carrel (1984). BPTI could be converted semi-synthetically into efficient human leukocyte elastase inhibitors by substituting valine, leucine, or methionine for the lysine residue at position 15 (Wenzel and Tschesche, 1981; Wenzel *et al.*, 1985). It was shown further that monosubstitution of lysine-15 by isoleucine, α-amino butyric acid, norvaline or norleucine yields inhibitors of leukocyte elastase (Tschesche *et al.*, 1985). Similarly, chymotrypsin is inhibited more efficiently than trypsin when tryptophan or phenylalanine are incorporated at position 15 of the inhibitor (Jering and Tschesche, 1976; Wenzel and Tschesche, 1984). Proteases and their specific inhibitors play an important role in several biological processes such as food digestion, blood coagulation and fibrinolysis, and some aspects of the immune response (Holzer and Tschesche, 1979). Diseases such as pulmonary emphysema or rheumatoid arthritis have been related to an imbalance between a proteinase and its specific natural inhibitor (Mittmann, 1972; Barrett, 1978; Menniger and Mohr, 1981). The clinical applications of BPTI and its properties as a therapeutic agent (Trasylol®) for the treatment of various diseases such as hyperfibrinolytic haemorrhage, acute pancreatitis, myocardial infarction, or traumatic haemorrhagic shock are reviewed in detail by Fritz and Wunderer (1983). Because of its comparatively small size, its stability and its frequent use as a model globular polypeptide (Wagner and Wuthrich, 1982; Karplus and McCammon, 1981) BPTI seems to be particularly well suited as a model system to study the possibilities of protein design as has been suggested by Marks and Anderson (1984).

Thus we have set up a system where a synthetic BPTI gene may be manipulated *in vitro* for fundamental scientific, as well as for practical purposes, and expressed in *E. coli* to yield the altered protein.

Results

In order to facilitate the cloning of the synthetic bovine pancreatic trypsin inhibitor (BPTI) gene we constructed new plasmid vectors by combining slightly modified DNA fragments from pBR322 (Sutcliffe, 1978), pKO60 (Besse *et al.*, 1986), bacteriophage fd11 (Beck *et al.*, 1978) and synthetic promoter sequences (Figure 1A). The upstream sequences and the −35 region of the synthetic promoter correspond to the bacteriophage T5 P25 promoter (von Gabain and Bujard, 1979). The Pribnow box and the downstream sequences are close to the consensus sequences (von Hippel *et al.*, 1984). These sequences (see Figure 3) proved to be a very efficient promoter as judged by the amounts of β-galactosidase synthesized under the control of this promoter (see Figure 4, lane 1).

The BPTI gene was constructed from 14 synthetic complementary overlapping oligonucleotides to yield a double-stranded DNA segment of an overall length of 204 bp, which was cloned between the *Xba*I and *Hin*dIII sites of piWiT9 (Figure 1A). The synthetic sequences include a ribosomal binding site at an appropriate distance upstream of the initiation codon ATG. The coding sequence for mature BPTI was linked to the *lacZ* gene in phase by an intervening amber codon that is partially translated into a tyrosine in the recipient strain Su3. Thus colonies harbouring recombinant plasmids expressed a BPTI-β-galactosidase fusion protein and could be identified by their ability to hydrolyse X-Gal on indicator plates.

The DNA sequence was determined completely from both strands (Maxam and Gilbert, 1983); it proved that the synthetic oligonucleotides had been joined and cloned correctly. The amount of fusion protein was quantified by determining β-galactosidase activity in crude cell extracts (Miller, 1972). It

was ~50% of the β-galactosidase activity observed in the presence of a fully induced episomal wild-type *lac* operon, but this extract did not specifically inhibit trypsin. The reason for this failure was a rapid breakdown of the small peptide as demonstrated by pulse chase experiments. Figure 2 shows the results obtained with the *lon* mutants SG935 and SG936.

An attempt was then made to raise the rate of synthesis to a level above what was needed to saturate the intracellular proteolytic activities, and we used the unique *Xba*I site situated upstream of the ribosomal binding site, and the unique *Hin*dIII site downstream of the amber stop codon, to duplicate the BPTI gene. For this purpose plasmid DNA was digested with *Bam*HI and *Hin*dIII and the smaller fragment, containing the 3′ end of the tetracycline resistance genes, the origin of replication, the promoter and the BPTI gene was purified. A second sample of the same plasmid DNA was digested with *Bam*HI and *Xba*I to

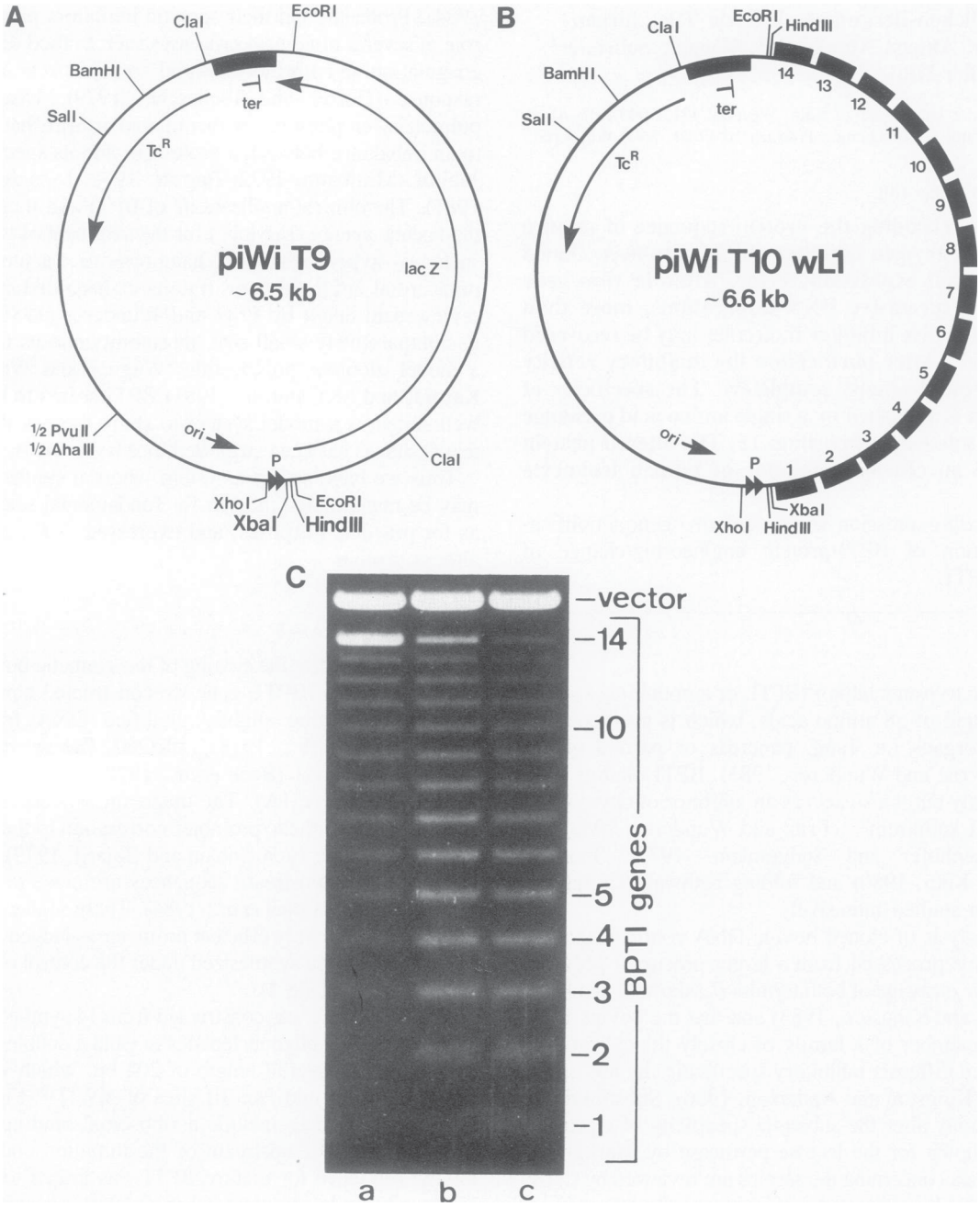

Fig. 1. (**A**) Physical map of the cloning vector piWiT9. The 332-bp *Sau*A fragment from bacteriophage fd11 which carries a transcription termination signal (**ter** ⊣) is shown black. It was linked to the 2.070-bp *Eco*RI-*Pvu*II fragment from pBR322 which confers resistance against tetracycline (Tc^R). The pBR322 derived origin of replication (**ori** →) was excised from pK060 with *Aha*III and *Xho*I, and the *lac*Z fragment extending from codon 6 to a few bp beyond the three termination codons was also taken from pK060. Synthetic promoter sequences ►► were inserted between the *Xho*I and the *Hin*dIII site. The *lacZ* gene is virtually not expressed at all from piWiT9 because it lacks an initiation codon for the start of translation. Some restriction sites are indicated. The unique *Xba*I site and the unique *Hin*dIII site which were used for the cloning and for the amplification of the synthetic inhibitor gene are emphasized by bold letters. Parallel to the cloning of the synthetic gene a small fragment providing a ribosomal binding site and a start codon for the *lacZ* gene was inserted between the *Xba*I and *Hin*dIII sites in order to allow a test of the quality of the synthetic promoter. This $lacZ^+$ derivative was named piWiT10 and used as a control plasmid (see Figure 3). (**B**) Physical map of the expression plasmid piWiT10wL1. The 14 inhibitor genes numbered 1 to 14 are shown black. For further details see Figure 2. All the other symbols are the same as in A. (**C**) 0.9% agarose gel showing piWiT10wL1 DNA digested completely with *Hin*dIII and partially with *BssH*II for different times (**a**) 15 min, (**b**) 35 min, (**c**) 75 min. *BssH*II cuts between every two inhibitor genes (see Figure 2).

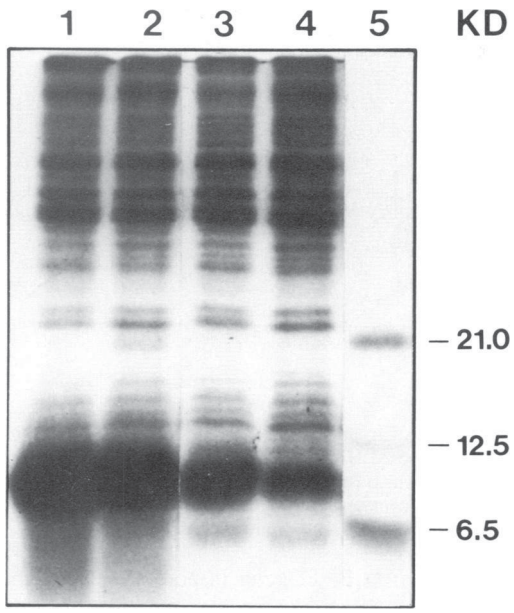

Fig. 2. Autoradiograph of a 17.5% SDS–polyacrylamide gel. SG935 (**lanes 1** and **3**) and SG936 (**lanes 2** and **4**) cells harbouring plasmids with two tandemly repeated BPTI genes were pulse labeled *in vivo* according to the protocol of McCarthy *et al.* (1985) using [^{35}S]-cysteine. The chase was 1 min (**lanes 1** and **2**) and 1 h (**lanes 3** and **4**). The samples of unfractionated cell lysates correspond to ~10^7 cells. **lane 5**: mol. wt standards.

yield a fragment which contained the BPTI gene again, the *lacZ* gene, the transcription termination signal, and the 5′ end of the tetracycline resistance genes. Both fragments were ligated in the presence of the synthetic adapter molecule

5′ AGCTAATGAGCGCGC 3′
3′ TTACTCGCGCGGATC 5′

and transformed into *E. coli* BR17, a *RecA*$^-$ strain. Since the adapter molecules carry protruding ends complementary to the *Hin*dIII- and *Xba*I-generated single strands which are readily ligated but do not regenerate either site, the resulting new plasmid again has only one unique *Xba*I site upstream of the first BPTI gene, and one unique *Hin*dIII site downstream of the second BPTI gene. The series of reactions described above were repeated with this new plasmid DNA to yield another plasmid with four tandemly repeated BPTI genes, each preceded by its own ribosomal binding site. Prior to the next duplication the 3-kb *Eco*RI fragment containing the bulk of the *lacZ* gene was excised with *Eco*RI and deleted from the plasmid in order to reduce its overall size. After the third doubling of BPTI genes we happened to pick up a plasmid with seven BPTI genes instead of eight. Plasmids with odd numbers of BPTI genes were usually found among the products of the ligation reactions at varying frequencies. We attribute their rise to partial denaturation with subsequent false hybridisation during the melting of the seaplaque agarose (Maniatis *et al.*, 1982). We never observed any deviation from a given number of genes when these plasmids were propagated further uninduced in a *RecA*$^-$ strain.

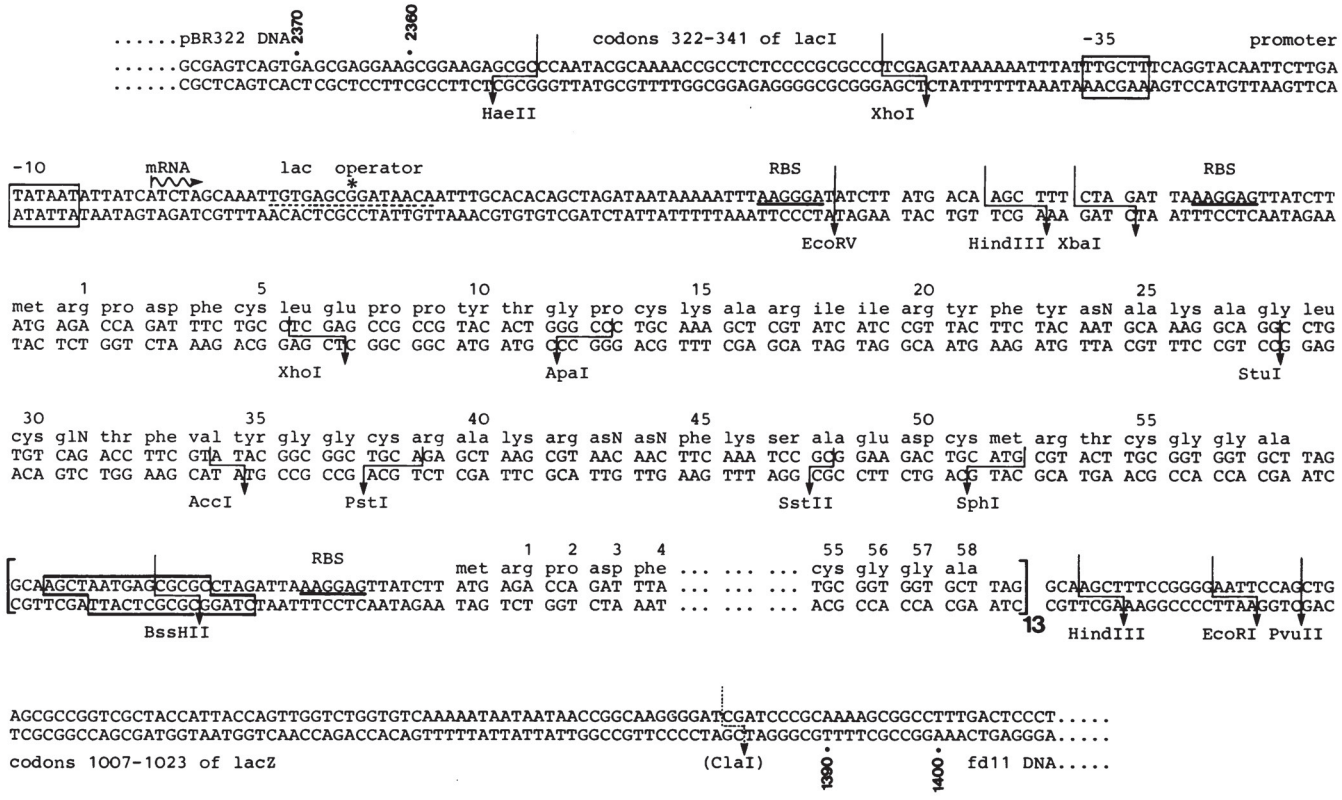

Fig. 3. DNA sequence of the right half of plasmid piWiT10wL1. Restriction enzyme cutting sites are indicated by arrows. The *Cla*I site in the bottom line is bracketed because the enzyme will not cut here unless the DNA is prepared from a *dam*$^-$ strain. The −10 and −35 promoter elements are shown in boxes. The presumed start of transcription is indicated by a wavy arrow. The *lac* operator sequences are shown by a dotted line between the DNA strands and by an asterisk above the central G/C pair. The ribosomal binding sites (RBS) are underlined. The protein sequence of the inhibitor is shown above its DNA. The adapter oligonucleotides used for joining the sticky *Hin*dIII and *Xba*I ends during the amplification of the genes are boxed. The 3183 bp from the *Xho*I site upstream of the promoter and the *Hin*dIII site downstream of the fourteenth inhibitor gene are entirely of synthetic origin. The numbering of the flanking pBR322 and fd11 DNA corresponds to the numbering of Sutcliffe (1978) and Beck *et al.* (1978).

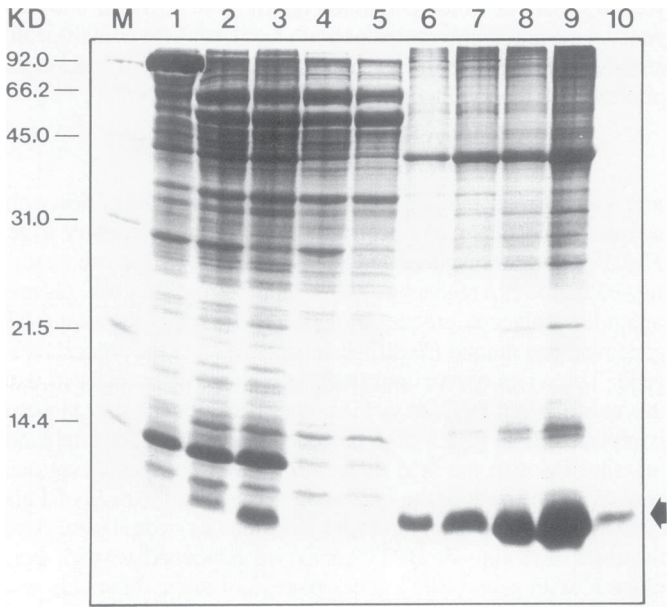

Fig. 4. 15% reducing SDS-polyacrylamide gel stained with Coomassie Blue. **M** 1 : mol. wt standards; **lanes 1–3**: complete cell lysates corresponding to 2×10^8 cells each. The host is *E. coli* BR17 carrying (**lane 1**) a β-galactosidase overproducing plasmid (piWiT10, see legend to Figure 1A), (**lane 2**) the BPTI plasmid piWiT10wL1, and (**lane 3**) the altered Ile_{15}BPTI producing plasmid piWiT10wi7). **Lanes 4** and **5** show the soluble protein fractions of the lysates of lanes 2 and 3 respectively. **Lanes 6** and **7**: pellets from 10^9 (6) and 2×10^9 (7) cells of *E. coli* BR17 harbouring plasmid piWiT10wL1. The pellets have been washed with 0.5% SDS and 2 M urea. **Lanes 8** and **9** same as **6** and **7** but with plasmid piWiT10wi7. **Lane 10**: 2.5 μg commercial BPTI. The inhibitor band is indicated by an arrow.

Table I. Comparison of trypsin and elastase inhibition

Inhibitor	50% Inhibition	
	Trypsin	Elastase
Commercial BPTI	250 ng	–[a]
Bacterial BPTI	300 ng	–[a]
Ile_{15}BPTI	–[a]	200 ng

2 μg trypsin and 0.5 μg elastase were used for the inhibition assays.
[a]There was no measurable inhibition in the presence of 10 μg of the respective inhibitors.

When 14 BPTI genes had been assembled in this manner (Figure 1, B and C) cell extracts were examined on protein gels after silver staining (Bürk *et al.*, 1983). There was a faint band co-migrating with commercial BPTI, which seemed to be slightly more intensive in the extract prepared from the cells harbouring the BPTI producing plasmid than in the control extract which did not contain any BPTI. We then altered the sequences between the promoter and the first structural gene of this artifical operon, because we had observed that the length and composition of the 5′ transcribed sequences could have a dramatic effect upon the expression of β-galactosidase or the BPTI–galactosidase fusion protein. The various mRNA leader sequences that have been studied in connection with different genes will be published and discussed elsewhere. The sequence which gives the highest yield with the BPTI genes is presented in Figure 3. It contains the natural *lac* operator sequences and a short open reading frame

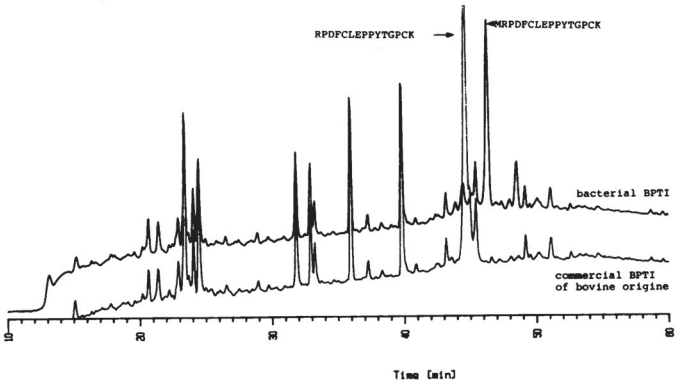

Fig. 5. Comparison of tryptic peptides of bacterial BPTI and commercial BPTI. The superimposed HPLC elution profiles are identical except for the N-terminal peptide which carries an additional methionine in case of the bacterial BPTI. For comments on the small extra peak in the bacterial BPTI peptide profile between 48 and 49 min see Discussion.

upstream of the first BPTI gene in addition to the initial promoter sequences. In the presence of these sequences β-galactosidase expression dropped to about 20–25% of the yields obtained in their absence, in contrast to the BPTI synthesis (Figure 4).

This alteration raised the BPTI synthesis from levels insufficient to be seen on protein gels, to more than 2% of the total cellular protein (Figure 4, lane 2). With 32 such genes (Figure 4, lane 3) the amount of protein is almost doubled, but with seven or eight genes no band can be seen neither in complete cell extracts nor in the insoluble fraction (data not shown).

The BPTI synthesized in *E. coli* was found to be predominantly insoluble when the cells were lysed with lysozyme (Figure 4). Inactive BPTI was obtained almost pure after washing cell debris twice with 2 M urea (Figure 4). It was then dissolved in 6 M guanidinium hydrochloride and renatured according to the procedure of Creighton (1985, see also Materials and methods). Bacterial BPTI was obtained in pure form after perchloric acid precipitation and affinity chromatography on trypsin sepharose. The amino acid composition was determined (data not shown) and agreed well with the predicted values and the protein sequence of the first twenty residues was verified. A comparison of peptide maps of bovine and bacterial BPTI is shown in Figure 5 (Mayes, 1985).

Inhibition of trypsin was measured (Nakajima *et al.*, 1979) and found to be almost as efficient as with bovine BPTI (Table I). The yields were 1 μg soluble BPTI per 10^9 cells, with 5 μg renatured BPTI from the insoluble material. This corresponds to at least 2% of the total cellular protein or 600 000 molecules per cell.

Making use of the unique *Apa*I and *Stu*I sites within the DNA sequence of the synthetic BPTI gene we exchanged the lysine codon at position 15 (Figure 3) for an isoleucine codon, amplified the modified gene by five successive duplications as described above, and analysed the gene product. The Ile_{15}BPTI produced from piWiT10wi7 migrates slightly faster on SDS–polyacrylamide gels (Figure 4). A similar behaviour has been reported for semi-synthetically produced Val_{15} BPTI (Wenzel *et al.*, 1985). The purified Ile_{15}BPTI no longer inhibits trypsin, but it is an efficient inhibitor of human leukocyte elastase (Table I).

Discussion

From the known amino acid sequence (Kassel *et al.*, 1965) of bovine pancreatic trypsin inhibitor (BPTI) we derived a cor-

responding DNA sequence (Figure 3) using, preferentially, codons that occur frequently in highly expressed genes of *E. coli* (Grosjean and Fiers, 1982). However, we did not hesitate to depart from this rule whenever we could introduce a unique restriction site. Thus we used the AGA codon for Arg_{39} in order to generate the *Pst*I site (Figure 3). We also used the AGA codon for Arg_1 in order to enhance ribosome binding (Scherer *et al.*, 1980) since Robinson *et al.* (1984) have reported that translation is not impaired by a cluster of four such unfavourable codons so long as the protein production does not exceed 14% of the total cellular protein.

Seven unique restriction sites distributed throughout the gene, in addition to the flanking *Xba*I and *Hin*dIII sites, allow the alteration of the primary structure of the gene by exchanging small restriction fragments for freshly prepared oligonucleotides. Thus nearly all amino acids may be exchanged, and the influence of codon usage as well as the function of sequences in the 5′ noncoding region can be investigated. We have introduced an isoleucine at position 15 and obtained an altered inhibitor which has lost its affinity for trypsin, but efficiently and specifically inhibits human leukocyte elastase (Table I), an enzyme which has been implicated to cause severe damage to lung tissue when released in excess (Cochrane *et al.*, 1983; Gadek *et al.*, 1979). It remains to be seen whether this engineered elastase inhibitor will be of therapeutic use for the treatment of pulmonary emphysema.

The most severe impediment that usually obstructs the production of small foreign proteins in *E. coli* is the fact that these are liable to be degraded quickly by the host proteases. Such foreign peptides have been protected successfully against proteolytic attack by fusing them to *E. coli* proteins (Maniatis *et al.*, 1982; Kempe *et al.*, 1985) or to each other (Shen, 1984; Kempe *et al.*, 1985). However, such a strategy is only applicable as long as there are means to cleave the fused peptides and gain the desired product intact. In our case, pulse-chase experiments (Figure 2) had indicated that BPTI synthesized at a high rate from two copies of the gene was equally rapidly degraded. We therefore employed a different strategy which should be applicable to a wide range of small proteins, especially those which cannot be cleaved intact from a fusion because they contain all the amino acid residues in their sequence which can be used for specific cleavage at the fusion points. Lee *et al.* (1984) have reported previously on the cloning of up to four tandem repeats of an interferon gene and pointed out the economy of such a procedure. We have amplified the synthetic BPTI gene in order to accelerate the synthesis until the production of BPTI was so much faster than the proteolytic breakdown that the product could accumulate and finally reach an intracellular concentration, where we hoped it would precipitate and thus be inaccessible to the host proteases. Similarly temperature-sensitive runaway replicons have been used to enrich cell extracts for unstable proteins (Brent and Ptashne, 1981). A further enhancement could be envisaged by combining both strategies. From our yields we have calculated a final cellular concentration of 0.5 mM for the BPTI peptide which is a 50-fold excess over the concentration of β-galactosidase monomers in a fully induced wild-type *E. coli*. These values make it highly probable that the insoluble fraction of the inhibitor recovered from the sediment indeed reflects the degree of intracellular precipitation of the overproduced peptide.

The use of two different restriction sites and a synthetic adapter, which after ligation destroys both sites, greatly facilitates the accumulation of more than four genes. In our case the ribosomal binding site was an integral part of the gene segment. Alternatively it might be positioned on the adapter molecule. Our adapter carries additional termination codons in phase (TAATGA, see Figure 3) because the single amber codon proved to be slightly leaky in terminating transcription. The additional peak in the tryptic map of the bacterial BPTI at 48−49 min (Figure 5) is most probably due to trace amounts of C-terminal peptide lengthened by three residues.

Plasmid piWiT10wL1, as well as its analogue piWiT10wi7 which carries 32 Ile_{15}BPTI genes, is completely stable as long as it is propagated in any *RecA*$^-$ *lacI*^{Q} strain in the absence of the inducer IPTG. Plasmid piWiT10wL1 which carries 14 BPTI genes is also reasonably stable when grown in the presence of IPTG in *E. coli* BR 17, the strain that gives the highest protein yields; after >60 generations less than 5% of the plasmid DNA exhibited a reduction in size that would correspond to a loss of one or two BPTI genes. Under the same conditions plasmid piWiT10wi7 with 32 Ile_{15}BPTI genes was considerably less stable, the protein yield dropped significantly after 20 generations in fully induced state. We do not yet know whether the selective pressure against the Ile_{15}BPTI synthesis originates from overstraining the synthesis capacity of the cells or from a toxic quality of the Ile_{15}BPTI peptide, which is absent from normal BPTI.

During the last years data has been gathered which will contribute to an understanding of mRNA translational efficiency (Scherer *et al.*, 1980; Hui *et al.*, 1984; Schoner *et al.*, 1984; Stanssens *et al.*, 1985). We can add another item to this collection. We have constructed a strong promoter which caused an extremely high level of β-galactosidase synthesis (Figure 4, lane 1), but it did not suffice to express the BPTI gene to such an extent that the product could be detected on protein gels. The additional sequences introduced into the 5′ untranslated region of the mRNA which significantly enhanced the BPTI expression had an inverse effect when combined with the *lacZ* gene. These observations indicate that the translational efficiency of a mRNA does not so much depend on the sequences upstream of the initiation codon but on the interaction of the mRNA leader *and* the coding sequences of the gene.

Materials and methods

Bacterial strains

E. coli Su3 is *lac-pro met*$^-$ *arg*$_{am}$ *Sup*F *thi*$^-$ and was kindly provided by J.Miller. *E. coli* SG 935 and SG 936 are F$^-$, *lac*(am), *trp*(am), *pho*(am), *Sup*C(t^s), *rps*L, *mal*(am), *htp*R(am), *tsx*::Tn*10*, *lon*(am) (Buell *et al.*, 1985). *E. coli* BB1.8 which is *mal*$^-$ and λ^R and was obtained from P.Starlinger. BB1.8 was chosen because of its high efficiency of protein synthesis in comparison to about a dozen other *E. coli* strains (Weidemann, 1985). A *RecA*$^-$ allele was introduced by standard procedures (Miller, 1972) into this strain which was designated BR17.

Plasmids

pBR322 (Sutcliffe, 1978), pKO60 (Besse *et al.*, 1986) and pUR278 (Rüther and Müller-Hill, 1983) have been described. Bacteriophage fd11 DNA (Beck *et al.*, 1978) was a gift of B. Gronenborn.

Enzymes and chemicals

The restriction endonucleases, *T4* DNA ligase, polynucleotide kinase, and DNA polymerase I, large fragment, were purchased from Bethesda Research Laboratories (Neu-Isenburg, FRG), Boehringer (Mannheim, FRG) or New England Biolabs (Bad Schwalbach, FRG) and employed as recommended by the manufacturers. [^{32}P]-nucleotides were obtained from Amersham Buchler (Braunschweig, FRG); 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and isopropyl-β-D-thiogalactoside (IPTG) from Bachem Fine Chemicals (Torrance, USA). The chemicals for DNA synthesis were purchased from Applied Biosystems (Pfungstadt, FRG) and the chemicals for DNA sequence analysis (Maxam and Gilbert, 1983) have been listed previously (Büchel *et al.*, 1980).

Methods

The methods for the construction of plasmids are standard techniques (Maniatis *et al.*, 1982). Synthetic oligonucleotides were prepared automatically on an Applied Biosystems 380A DNA synthesizer. Full-length molecules were purified from preparative denaturing polyacrylamide gels after ethidium bromide staining. When only two complementary oligonucleotides were to be cloned they were used without adding 5′ phosphates. For the simultaneous cloning of up to 14 oligonucleotides, the 5′ ends were phosphorylated and ^{32}P was incorporated into a tenth of each oligonucleotide sample prior to a second polyacrylamide gel electrophoresis, and the labelled bands were then cut from the gels. Removing the majority of unphosphorylated oligonucleotides by a second gel purification prior to cloning greatly enhances the yields when more than two oligonucleotides are to be cloned simultaneously.

Transformation of *E. coli* was carried out according to the method of Hanahan (1983). *E. coli* proteins were analysed essentially as described by Marston *et al.* (1984) with the following modifications worked out by Weidemann (1985): cells from 3ml overnight cultures were harvested by a short centrifugation and resuspended in 122 μl 40 mM Tris-HCl, 5 mM EDTA, pH 8 containing 0.3 mg/ml lysozyme. After 30 min at room temperature 18 μl 8% sodium deoxycholate were added, after further 10 min the suspension was mixed with 150 μl 10 mM $MgCl_2$, 20 mM Tris−HCl, pH 8 and 10 μg DNaseI/ml, which was allowed to react for about 20 min. The sample was then either mixed with 600 μl reducing sample buffer (Laemmli, 1970), containing 8M urea and incubated at 37°C for 1 h or fractioned first by 10 min centrifugation in an Eppendorf centrifuge. The pelleted cell debris was then washed with 40 mM Tris-HCl, 5 mM EDTA, pH 8 containing 0.5% SDS or 2 M urea before it was dissolved in sample buffer and incubated at 37°C as above. Aliquots of the samples were then analysed on polyacrylamide gels as described (Laemmli, 1970). Pulse-chase experiments were performed as described by McCarthy *et al.* (1985).

Active inhibitor was purified from the soluble fraction of the lysate by perchloric acid precipitation followed by affinity chromatography on trypsin sepharose (Fritz *et al.*, 1970). For the preparation of renatured BPTI by solid state folding (Creighton, 1985) cells from 5 l overnight culture were harvested, disrupted in a French Press, and centrifuged for 20 min at 20 000 r.p.m. The pellet was dissolved in 10 ml buffer A [8 M urea, 1 mM bis-(hydroxyethyl)-disulfide, 1 mM 2-mercaptoethanol, 50 mM Tris−HCl, 1 mM EDTA, pH = 8.2] and reduced for 1 h at 50°C under nitrogen. The solution was then applied to an Econo column (25×10 mm, Biorad, München, FRG) filled with ~10 ml CM Sepharose Fast Flow (Pharmacia, Freiburg, FRG) which was equilibrated with buffer A. The column was then developed by a linear gradient of 100 ml buffer A and 100 ml buffer B, which is identical to buffer A except that it contains no urea. The column was washed with buffer C (50mM Tris−HCl, 1 mM EDTA, pH = 8.2) until the baseline was stable. Native BPTI was then eluted from the column with a second linear gradient formed from 100 ml buffer C and 100 ml buffer C which contained 0.6M NaCl.

Purified inhibitor was used for amino acid analysis (Benson and Hare, 1975) carried out on a Biotronic LC 5000 Analyser from Biotronic (Maintal, FRG). Protein sequence analysis was performed on a gas phase sequenator from Applied Biosystems (Pfungstadt, FRG) according to Hewick *et al.* (1981). The phenylthiohydantoin derivatives were analysed on a cyano-HPLC column from DuPont (Wilmington, USA) as described by Beyreuther *et al.* (1983). Peptide mapping has been described (Mayes, 1985).

Trypsin inhibition was assayed (Erlanger *et al.*, 1961) using N-benzoyl-DL-arginine-*p*-nitroanilide (BANA) from Merck (Darmstadt, FRG). Human leukocyte elastase from Elastin Products Company (Pacific, USA) and methoxysuccinyl-L-alanyl-L-alanyl-L-prolyl-L-valine-*p*-nitroanilide from Bachem (Budendorf, Switzerland) were used to assay leukocyte elastase inhibition (Nakajima *et al.*, 1979).

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