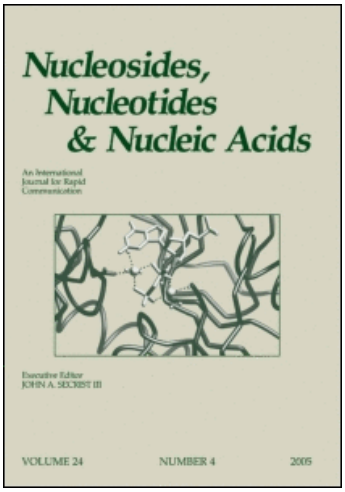

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Chemical Synthesis of Proteinase Inhibitor Genes

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CHEMICAL SYNTHESIS OF PROTEINASE INHIBITOR GENES

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Abstract: Genes coding for the two human cysteine proteinase inhibitors (cystatin C, stefin A) were obtained by enzymic ligation of chemically synthesized oligodeoxynucleotides. This article reports the results from the expression of these proteinase inhibitor genes in *E. coli* and from the isolation and characterization of the corresponding proteins.

Proteases play key roles in complex processes such as inflammation, phagocytosis and degradation, protein turnover, hemostasis and complement activation. In performing their physiological function they are controlled and regulated by their inhibitors. Our special interest focusses on the human cysteine proteinases and their roles in inflammation and cell invasiveness. Cysteine proteinase inhibitors selectively inhibit cysteine proteinases without affecting serine proteinases, which are vital for a variety of physiological functions.

Based on the complete protein sequences for the human cysteine proteinase inhibitors cystatin C and stefin A we deduced a synthetic DNA sequence using codons for highly expressed proteins in *E. coli*. According to the method of

Khorana[1]. 16 oligonucleotides (38 - 55 bp length) in the case of cystatin C and 10 oligonucleotides (46 - 80 bp length) in the case of stefin A, respectively, were obtained by automated DNA synthesis. Fragment assembly of the oligonucleotide building blocks and ligation into the cloning vector were performed in a single hot-pot reaction using standard methods[2].

Expression in *E. coli* was performed using derivatives of the commercially available vector pKK223-3 (Pharmacia) which carries the inducible *tac* promoter and a Shine-Dalgarno site upstream of a multi-purpose cloning site and, additionally, a ribosomal RNA transcription terminator downstream of this site[3]. Cystatin C and stefin A were expressed in high yields in *E. coli* JM105 with the vectors pMS103 and pMS200, both derived from the vector pKK223-3. This expression system allows protein secretion into the *E. coli* periplasm using a chemically synthesized 68-bp gene coding for the signal sequence of alkaline phosphatase at the 5´end (N-terminal end) of the structural genes. A similar construction has previously been described[4]. For detection and quantification of the cysteine proteinase inhibitors we used a specific enzyme assay system based on the inhibition of the plant cysteine proteinase papain[5] and furthermore an immuno assay using anti-cystatin C and anti-stefin A antibodies. Isolation and purification of the recombinant proteinase inhibitors (see TABLE 1) were performed following standard purification procedures based on CM-papain affinity chromatography[6], anion exchange chromatography on FPLC Mono Q column and HPLC "reversed phase" chromatography[2]. The purified recombinant proteins were characterized by amino acid sequence analysis using automated Edman degradation. TABLE 1 outlines the results of the expression. The results demonstrate that the alkaline phosphatase signal sequence construction does not lead to a homogeneous recombinant protein. The table lists all

TABLE 1: Isolation and purification of recombinant cysteine proteinase inhibitors from 1-L cultures of *E.coli* JM105/pMS103 (cystatin C) and JM105/pMS200 (stefin A).

Step	Cystatin C yield			Stefin A yield		
	mg	IU	%	mg	IU	%
total cell extract (analytical) #1)	6.4	1300	100	8	1900	100
periplasmic lysis (preparative)	4	810	62	4.2	1000	52
CM papain affinity chromatography	2	405	31	2	475	25
FPLC anion-exchange chromatography	-	-	-	0.5	120	6.2
HPLC "reversed phase" chromatography	1.6 #2)	324	25	0.4 #3)	95	5

The designation of the N-terminal amino acid of the various polypeptides refers to the general nomenclature of signal peptide expression systems.

#1) Overall yields were calculated from values measured for samples containing 1 A_{578} of bacteria.

#2) Recombinant cystatin C represents a mixture of the following N termini: Gly^{-3}, Ser^{+1}, Leu^{+9} and Gly^{+12}.

#3) Recombinant stefin A represents a mixture of Ala^{-3} and Gly^{-2} N termini.

characterized recombinant cysteine proteinase inhibitor polypeptides obtained with the periplasmic expression systems pMS103 and pMS200.

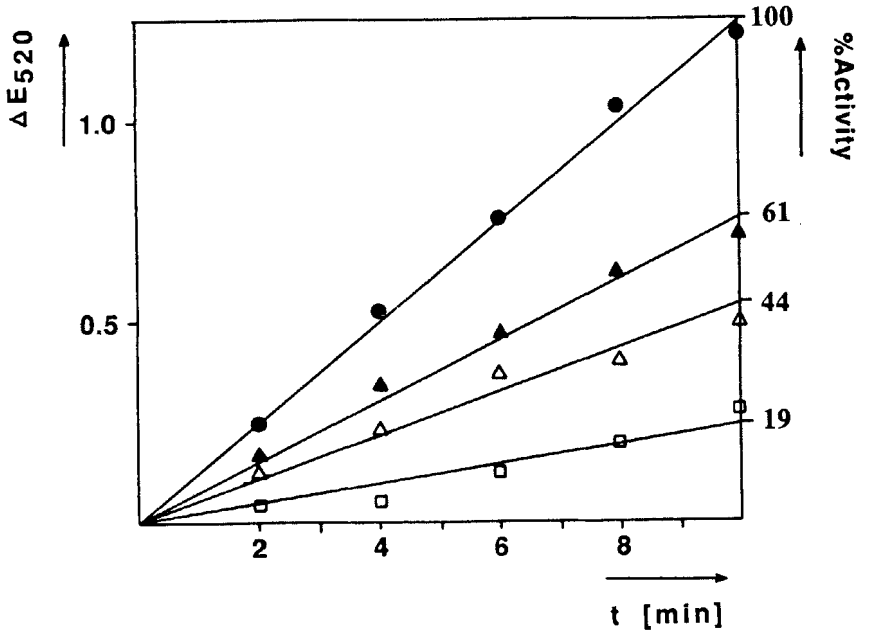

FIG. 1: Inhibition of papain by recombinant inhibitor stefin A. Constant amounts of the cysteine proteinase papain (6.45 pmol) were incubated with increasing amounts of recombinant inhibitor stefin A. Papain concentration was determined by titration with E_{64}[5].

●—● Substrate hydrolysis by papain without recombinant stefin A after 10 min taken as 100 %.

Substrate hydrolysis by papain in the presence of

▲—▲ 2.7 pmol recombinant stefin A;

△—△ 3.6 pmol recombinant stefin A;

□—□ 5.4 pmol recombinant stefin A.

The protein chemical characteristics of the recombinant proteinase inhibitors are similar to those of their natural counterparts, stefin A exhibiting closer relations than cystatin C. Recombinant stefin A has the same isoelectric point as its human analogue and the inhibition constant with papain also in the same picomolar range. The inhibition of papain by recombinant stefin A is shown in FIG. 1. For the

estimation of the inhibition constant we applied concentrations as determined by Edman degradation.

It was possible to express the human cysteine proteinase inhibitors cystatin C and stefin A in *E. coli*, using the alkaline phosphatase periplasmic expression vectors pMS103 and pMS200. The recombinant proteins differ from their human polypeptides, having additional or less N-terminal amino acids.

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