Opossum Serum α_1-Proteinase Inhibitor: Purification, Linear Sequence, and Resistance to Inactivation by Rattlesnake Venom Metalloproteinases[†,‡]

Joseph J. Catanese and Lawrence F. Kress*

Molecular and Cellular Biology Department, Roswell Park Cancer Institute, Buffalo, New York 14263

Received September 4, 1992; Revised Manuscript Received October 30, 1992

ABSTRACT: Opossum (*Didelphis virginiana*) serum was fractionated with $(NH_4)_2SO_4$ and then chromatographed on DEAE-Sepharose and phenyl-Sepharose. Affinity chromatography on a protein A–Sepharose–antibody column removed traces of opossum serum metalloproteinase inhibitors, and resulted in a homogeneous preparation of opossum α_1-proteinase inhibitor (α_1-PI). The inhibitor is a single-chain glycoprotein (17.7% carbohydrate) with an estimated M_r = 54 000. An opossum liver cDNA library was immunoscreened, and clones containing cDNA encoding for the open reading frame for opossum α_1-PI were isolated. The cDNA inserts contained nucleotide sequences corresponding to the amino-terminal and an internal peptide sequence of opossum α_1-PI which had been separately determined by protein sequence analysis. The entire inserts coded for a protein consisting of a 21-residue signal peptide and a 389-residue mature protein. Opossum α_1-PI shows 51–58% identity with other mammalian α_1-PI amino acid sequences, and the conserved residues expected for a member for the serpin family have been retained. The carbohydrate attachment sites and the reactive site residues (M-S) of opossum α_1-PI are identical to those of human α_1-PI. Opossum α_1-PI formed stable enzyme/inhibitor complexes with trypsin, chymotrypsin, and human neutrophil elastase, but did not react with thrombin or with snake venom serine proteinases. Opossum α_1-PI was inactivated by papain or *Pseudomonas aeruginosa* elastase, and electrophoretic analysis of the reaction products indicated limited proteolysis in the reactive site loop of the inhibitor. However, opossum α_1-PI retained essentially all its activity when incubated with crude rattlesnake venoms or purified rattlesnake venom metalloproteinases under conditions in which human α_1-PI was readily inactivated. The results are consistent with the hypothesis that opossum α_1-PI is susceptible to cleavage by nonvenom proteinases but is resistant to proteolytic inactivation by venoms of those snakes which it encounters in its environment.

α_1-Proteinase inhibitor (α_1-antitrypsin) (α_1-PI)[1] is the most abundant serine proteinase inhibitor found in human plasma. α_1-PI is a member of the serpin family, a group of proteins having significant sequence homology and consisting mostly of serine proteinase inhibitors from mammalian plasma. Although human α_1-PI inactivates numerous mammalian serine proteinases, its primary role is regulation of neutrophil elastase (Travis & Salvesen, 1983). Inactivation of neutrophil elastase occurs by formation of a stoichiometric enzyme/inhibitor complex accompanied by cleavage of the inhibitor at its reactive site bond (Johnson & Travis, 1978). This cleavage results in a conformational change in the inhibitor from a stressed to a relaxed form (Huber & Carrell, 1989). The complex is then recognized by cell-surface receptors (Perlmutter et al., 1990) and is rapidly cleared from the circulation (Mast et al., 1991).

Human α_1-PI can also be enzymatically inactivated without complex formation via limited proteolysis in the reactive site loop by proteinases from various sources (Johnson & Travis, 1977; Morihara et al., 1984; Desrochers & Weiss, 1988). Studies in this laboratory (Kress et al., 1979) have shown that metalloproteinases from rattlesnake (*Crotalus adamanteus*) venom enzymatically inactivate human α_1-PI by cleavage of a single bond in the inhibitor reactive site loop. Other human plasma serpins (antithrombin III, α_2-antiplasmin, C1-inhibitor) are also inactivated by snake venom metalloproteinases in a two-step process involving an initial noninactivating cleavage in the amino-terminal region of the inhibitor followed by an inactivating cleavage in the reactive site loop (Kress & Catanese, 1981; Kress et al., 1983). Since the opossum (*Didelphis virginiana*) is known to tolerate rattlesnake envenomation without developing the localized or systemic effects attributed to venom proteinases (Werner & Vick, 1977), it became of interest to study the effects of rattlesnake venom proteinases on opossum plasma serpins.

This report describes the purification, partial characterization, and primary sequence of opossum α_1-PI. Evidence is presented that opossum α_1-PI retains activity in the presence of rattlesnake venom proteinases under conditions in which human α_1-PI is rapidly inactivated.

EXPERIMENTAL PROCEDURES

Materials. Opossum (*Didelphis virginiana*) serum was obtained from Research Biogenics, Inc., Elgin, TX; lyophilized snake venoms were from Miami Serpentarium (*Crotalus atrox, C. adamanteus, Crotalus basiliscus*) or Sigma (*Bitis arietans*); DEAE-Sepharose, phenyl-Sepharose, and protein A–Sepharose were from Pharmacia; molecular weight standards and electrophoresis-grade purity reagents for PAGE were from Bio-Rad; bovine trypsin and α-chymotrypsin were from Worthington. Human neutrophil elastase was kindly provided by Dr. D. Johnson, East Tennessee State University; *Pseudomonas aeruginosa* elastase was kindly provided by Dr. K. Morihara, Kyoto, Japan. *C. atrox* α-protease (Kruzel & Kress, 1985), human α_1-PI (Kurecki et al., 1979), and *C. adamanteus* proteinase II (Kurecki et al., 1978) were prepared as described. BAEE, BAPA, and BTEE were from Sigma; casein (Hammarsten) was from BDH Chemicals; hide powder

† This research was supported by National Institutes of Health Grant HL22996.

‡The nucleotide sequence data reported in this paper have been submitted to the GenBank (L06824) and EMBL (Z18906) sequence databases.

[1] Abbreviations: SDS, sodium dodecyl sulfate; BTEE, benzoyl-L-tyrosine ethyl ester; BAPA, benzoyl-L-arginine *p*-nitroanilide; BAEE, benzoyl-L-arginine ethyl ester; PAGE, polyacrylamide gel electrophoresis; α_1-PI, α_1-proteinase inhibitor (α_1-antitrypsin).

0006-2960/93/0432-509$04.00/0

azure was obtained from Calbiochem or prepared in this laboratory (Rinderknecht et al., 1968). Dialysis tubing (Spectrapor) from Fisher was used according to manufacturer's instructions.

Assay of Inhibitor Activity. Column fractions were assayed for trypsin inhibitory activity and column pools for various inhibitory activities as previously described (Kress, 1986). Inhibitor activity on human neutrophil elastase was assayed according to Brower and Harpel (1982) using succinyl-Ala-Ala-Ala-pNA (Sigma) as substrate. Inhibitor inactivation by papain was assayed according to Johnson and Travis (1977) and by *P. aeruginosa* elastase according to Catanese and Kress (1984); the reaction was monitored on SDS/PAGE as previously described (Kress & Catanese, 1981).

Physical and Chemical Properties. Molecular weight estimations on SDS/PAGE, sulfhydryl and carbohydrate content, extinction coefficient, and amino-terminal and internal peptide sequences for opossum α_1-PI were performed as previously described (Catanese & Kress, 1992).

cDNA Cloning. A Stratagene RNA isolation kit was used to obtain total RNA from opossum liver tissue that had been stored at −80 °C. All equipment and tubes were washed with DEPC-treated H_2O (Gilman, 1989). Poly(A)$^+$ mRNA was purified from total RNA (Pharmacia mRNA purification kit), and double-stranded cDNA was synthesized (Amersham cDNA synthesis system plus). An opossum liver cDNA library was constructed in λgt11 using the Amersham cDNA cloning system. The unamplified library was plated at a density of approximately 15 000 plaque-forming units per 150-mm LB plate and incubated at 42 °C for 3.5 h. Plates were then overlayed with dry nitrocellulose filters (BA 85, Schleicher & Schuell) previously saturated with 10 mM isopropyl β-D-galactoside and incubated at 37 °C for 3.5 h. Filters were then screened (Promega Protoblot immunoscreening system) using monospecific rabbit antiserum to opossum α_1-PI that had been treated with *Escherichia coli* extract (Promega) to reduce background color development. Immunopositive plaques were picked, replated, and rescreened until pure. Positive clones were used to prepare 10-mL plate lysates (Lech, 1989). Recombinant λgt11 DNA was purified using Lambdasorb phage absorbent (Promega), followed by phenol/$CHCl_3$ extraction and ethanol precipitation (Treco, 1989).

PCR. Recombinant inserts from the purified λgt11 DNA were amplified by the polymerase chain reaction in a Perkin-Elmer Cetus DNA thermal cycler using the Geneamp kit (Cetus) and λgt11 forward and reverse amplimers (15-mers) from Clontech; 200 μM (final) dNTP's and 0.2 μM (final) each primer were used in the amplification reaction. After an initial denaturation of 1.5 min at 94 °C, 40 cycles of the following cycler program were run: 1 min at 94 °C, 1 min at 60 °C, 2 min at 72 °C. A final elongation was performed at 72 °C for 7 min. Double-stranded PCR product was electrophoresed on a 3.0% Nusieve agarose (FMC) minigel in TBE buffer, excised, and purified using the Majic PCR Preps DNA purification system (Promega).

DNA Sequencing. Double-stranded DNA sequencing was performed on the amplified cDNA inserts by the dideoxy chain termination method (Sanger et al., 1977) using taq polymerase supplied with the fmol sequencing system (Promega) with 15-mer λgt11 forward and reverse primers (Clontech) and [γ-^{32}P]ATP (DuPont). Additional primers based on cDNA sequencing results were designed with the aid of Oligo version 4.0 primer software (National Biosciences) and were synthesized using an Applied Biosystems 380A DNA synthesizer, purified on a Poly-Pak (Glen Research) cartridge followed by chromatography on Mono Q HR 5/5 and ProRPC HR 5/10 using the (Pharmacia) FPLC system. The sequencing reactions were resolved on 6% polyacrylamide gels (Mizusawa et al., 1986) which were exposed after drying to X-OMAT-AR5 film (Kodak) at −80 °C for 18 h.

Sequence Homology. cDNA and amino acid sequences were used to search the Genbank (Release 71.0) and SWISS-PROT (Release 21.0) databases using the FASTA program (Pearson & Lipman, 1988) for sequence homology on the VAX computer (Devereux et al., 1984). The mammalian α_1-PI sequences with which opossum α_1-PI was compared are human (Long et al., 1984), baboon (Kurachi et al., 1981), rat (Chao et al., 1990), mouse (Sifers et al., 1990), sheep (Brown et al., 1989), rabbit/f (Saito & Sinohara, 1991), guinea pig/s (Suzuki et al., 1991), and bovine (Sinha et al., 1992).

Table I: Summary of Opossum α_1-PI Purification

	total protein (A_{280})	total inhibitor (units)	sp act. (units/A_{280})	yield (%)	purification factor
opossum serum (150 mL)	9324	69930	7.5	100	1
45–80% $(NH_4)_2SO_4$ ppt	3351	64004	19.1	92	2.5
DEAE-Sepharose	478	33173	69.4	47	9.2
phenyl-Sepharose	28[a]	22988	821.1	33	110

[a] This corresponds to 52.1 mg of protein using the $E(1\%/1\text{ cm}) = 5.37$ determined for pure opossum α_1-PI. By comparison, an equivalent amount of human plasma gives 53 mg of α_1-PI with a yield of 35% of the starting units (Kurecki et al., 1979).

RESULTS

Purification. A summary of the purification procedure is presented in Table I. Saturated $(NH_4)_2SO_4$ solution (118 mL) was added to opossum serum (145 mL) to achieve 45% saturation. The solution was stirred for 1 h at 4 °C, left overnight, and then centrifuged 20 min at 23000*g*, and the precipitate was discarded. Solid $(NH_4)_2SO_4$ (198 g/L) was added to the supernatant to achieve 80% saturation, the solution stirred 1 h, kept at 4 °C for 4 h, and centrifuged 20 min at 23000*g*, and the supernatant discarded. The precipitate was dissolved in 10.0 mL of 0.02 M sodium phosphate, 6.5, and dialyzed 72 h against three changes (1 L each) of buffer. The dialyzed solution (3351 A_{280} units)[2] was charged onto a 5.0 × 30 cm column of DEAE-Sepharose equilibrated with buffer, and eluted as shown in Figure 1. The material which inhibited trypsin was designated opossum α_1-PI. The opossum α_1-PI peak (478 A_{280} units) was pooled, and solid $(NH_4)_2SO_4$ was added to 1.0 M. The pool was dialyzed 48 h against 2.0 L of 0.1 M sodium phosphate/1.0 M $(NH_4)_2SO_4$, pH 7.0, charged on a 2.5 × 30 cm column of phenyl-Sepharose, and eluted as shown in Figure 2. Trypsin inhibitory activity was detected in two well-separated peaks. The material in the first peak also inhibited thrombin, but not chymotrypsin, and was designated opossum antithrombin III. The opossum α_1-PI (second activity peak) was pooled as shown, dialyzed for 48 h against three changes (2.0 L each) of 0.1 M NaH_2PO_4/0.25 M NaCl, pH 8.0, and concentrated to approximately 3.0 mg/mL using an Amicon YM-30 membrane. Inhibition of *C. atrox* venom metalloproteinases was noted in fractions 260–310 from the DEAE column. In order to remove traces of these inhibitors from the α_1-PI preparation, antiserum to opossum α1B-glycoprotein (Catanese & Kress, 1992) was

[2] An A_{280} unit is that amount of protein which if dissolved in 1 mL and read in a 1-cm light path at 280 nm will give an absorbance of 1.

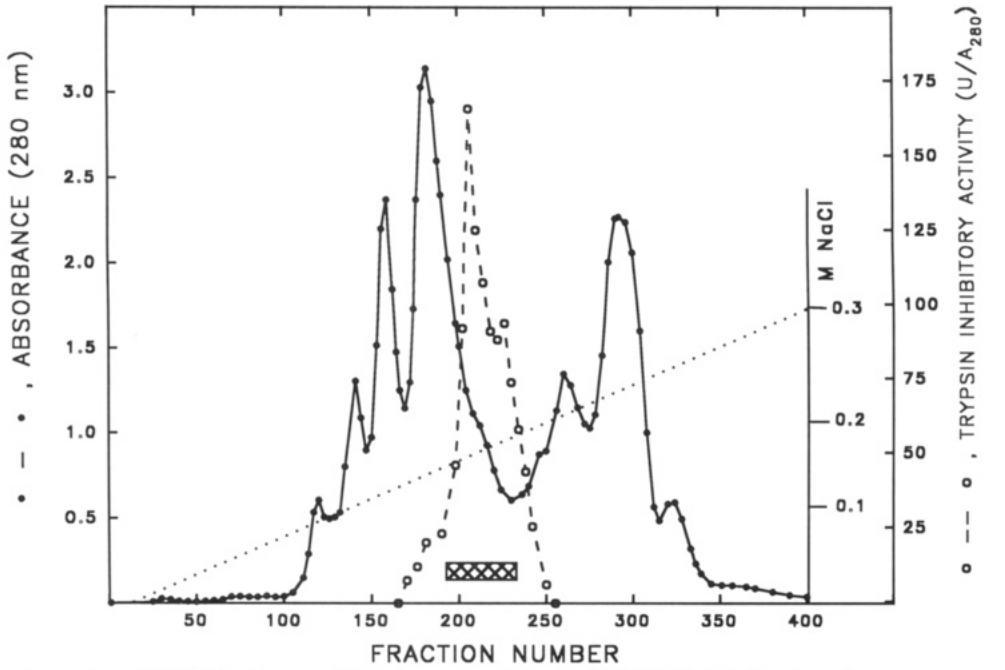


FIGURE 1: Chromatography on DEAE-Sepharose. 3351 A_{280} units of the 45–80% $(NH_4)_2SO_4$ fraction of opossum serum were charged on a 5.0 × 30 cm column of DEAE-Sepharose and eluted with 0.2 M sodium phosphate, pH 6.5, using a linear gradient from 0 to 0.3 M NaCl (2400 mL per bottle); 12-mL fractions were collected at a flow rate of 180 mL/h; (cross-hatched rectangle) α_1-PI pool.

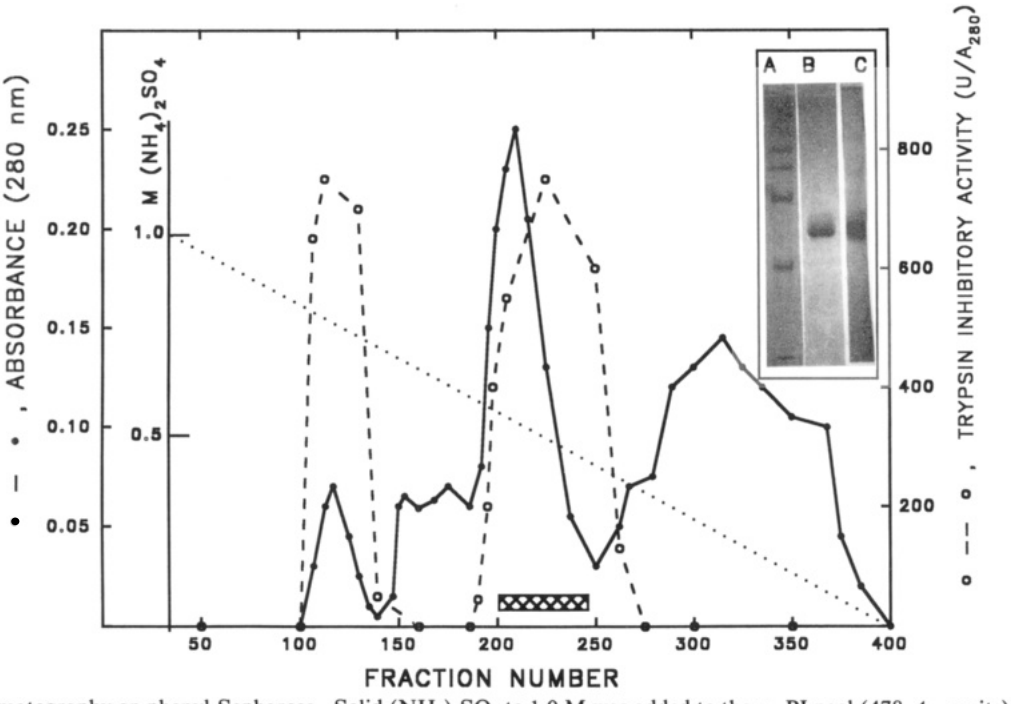


FIGURE 2: Chromatography on phenyl-Sepharose. Solid $(NH_4)_2SO_4$ to 1.0 M was added to the α_1-PI pool (478 A_{280} units) from the DEAE-Sepharose column. The pool was then dialyzed against 0.1 M sodium phosphate/1.0 M $(NH_4)_2SO_4$, pH 7.0, charged on a 2.5 × 30 cm phenyl-Sepharose column, and eluted with a decreasing linear gradient (900 mL per bottle) from 1.0 to 0 M $(NH_4)_2SO_4$ in buffer. At fraction 400, elution was continued with buffer only, and a slight amount of inactive material was removed. 4.5-mL fractions were collected at a flow rate of 90 mL/h; (cross-hatched rectangle) α_1-PI pool. (Insert) Electrophoretic analyses of α_1-PI pool after protein A–Sepharose affinity chromatography (see text): (A) SDS/PAGE, molecular weight standards; (B) SDS/PAGE, opossum α_1-PI. The α_1-PI band migrated between bovine serum albumin (66 kDa) and ovalbumin (45 kDa) at an estimated M_r = 54 000. (C) Opossum α_1-PI, disc electrophoresis, pH 8.8, anode at top.

adsorbed to protein A–Sepharose CL-4B and a 1.5 × 10 cm column poured. The α_1-PI pool from phenyl-Sepharose was charged onto the column and eluted with 0.1 M NaH_2PO_4/0.25 M NaCl, pH 8.0. The single flow-through peak containing α_1-PI activity was pooled, dialyzed extensively against 0.05 M Tris-HCl, pH 8.0, concentrated, and stored at –85 °C. Under these conditions, activity remained stable for at least 1 year, and no losses were noted with several freeze/thaw cycles.

Properties and Composition. The purified inhibitor migrated as a single band during electrophoresis in the presence and absence of SDS (Figure 2, insert). Only one band was detected in SDS gels run in the presence and absence of mercaptoethanol, indicating that opossum α_1-PI is composed of a single polypeptide chain. A molecular weight of 54 000 was estimated from the electrophoretic mobility of the inhibitor relative to standards of known molecular weight. Staining of gel bands with periodic acid–Schiff reagent indicated that

Table II: Nucleotide and Deduced Amino Acid Sequence of cDNA Coding for Opossum α_1-Proteinase Inhibitor[a]

Met Met Pro Ser Thr Leu	-16
GCT GTC CTA CCG AGT TTC TGC AGG AGT CTG AAG AAG GAT CTA ATG ATG CCT TCT ACT CTG	60
Ser Leu Cys Leu Met Leu Ala Gly Leu Cys Ser Leu Val Thr Ser His Leu Thr Glu Glu	5
TCA CTC TGC CTG ATG TTG GCT GGG CTA TGC AGC CTG GTC ACC AGC CAC CTA ACT GAG GAA	120
Ile Gln Ala Ser Asn Asp Thr Glu Asn Glu Tyr Ser Ser Thr Arg Arg Ile Ser Pro Tyr	25
ATC CAA GCC AGT AAT GAT ACT GAA AAT GAG TAT TCT TCC ACC AGG AGA ATT TCC CCT TAT	180
Met Thr Asp Phe Ser Ile Asp Phe Tyr Arg Leu Leu Val Ser Lys Ser Asn Thr Thr Asn	45
ATG ACT GAT TTT AGC ATC GAT TTT TAT AGA CTG CTG GTG TCT AAG TCC AAT ACC ACC AAC	240
Ile Phe Phe Ser Pro Ile Ser Ile Tyr Thr Ala Phe Thr Leu Leu Ala Leu Gly Ala Lys	65
ATA TTC TTC TCT CCT ATA AGT ATT TAC ACT GCC TTT ACC TTG CTG GCT CTT GGG GCC AAA	300
Ser Ala Thr Arg Asp Gln Ile Leu Thr Gly Leu Arg Phe Asn Arg Thr Glu Ile Ser Glu	85
TCA GCA ACT CGT GAT CAG ATC CTG ACG GGG TTA AGA TTC AAC CGT ACT GAG ATT TCA GAG	360
Glu His Ile Phe Glu Gly Phe Gln Gln Leu Leu Asn Thr Phe Asn Leu Pro Glu Asn Glu	105
GAA CAT ATT TTT GAA GGC TTC CAA CAG CTT CTT AAT ACA TTC AAC CTA CCT GAA AAT GAG	420
Leu Gln Leu Thr Thr Ser Asn Gly Leu Phe Ile Asp Lys Asn Leu Lys Leu Val Ala Lys	125
CTT CAA TTG ACC ACA AGC AAT GGC CTG TTT ATA GAC AAA AAT CTG AAA CTT GTA GCT AAA	480
Phe Leu Glu Asp Ser Lys Arg Leu Tyr Ala Ser Asp Thr Phe Ser Thr Asn Phe Glu Asp	145
TTT CTA GAA GAC AGC AAA AGA CTA TAT GCT TCT GAT ACC TTC TCT ACA AAT TTT GAA GAC	540
Asn Met Ala Ala Lys Lys Gln Ile Asn Asp Tyr Val Glu Lys Glu Thr Gln Gly Lys Ile	165
AAC ATG GCT GCC AAG AAA CAG ATC AAT GAC TAT GTA GAG AAG GAG ACC CAA GGA AAA ATA	600
Val Asp Leu Ile Gln Asn Leu Asp Ser Asn Val Val Phe Val Leu Val Asn Cys Ile Phe	185
GTG GAT TTG ATT CAA AAC CTG GAC TCT AAT GTC GTC TTT GTC CTG GTG AAT TGC ATT TTC	660
Phe Lys Gly Lys Trp Glu Lys Pro Phe Met Thr Glu Leu Thr Thr Glu Cys Pro Phe His	205
TTT AAA GGC AAG TGG GAG AAA CCC TTC ATG ACA GAG CTC ACT ACG GAA TGC CCC TTC CAC	720
Val Asp Ser Lys Thr Thr Val Pro Val Gln Thr Met Arg Arg Leu Gly Met Phe Asn Val	225
GTG GAT TCC AAA ACA ACT GTG CCA GTT CAA ACA ATG AGA CGC CTT GGC ATG TTC AAT GTG	780
Phe Tyr Asp Gln Asp Leu Ser Cys Trp Val Leu Lys Met Lys Tyr Met Gly Asn Ala Thr	245
TTT TAT GAT CAG GAC CTG TCC TGC TGG GTA TTG AAA ATG AAA TAT ATG GGA AAC GCA ACT	840
Ala Leu Phe Ile Leu Pro Asp Thr Gly Lys Ile Glu Lys Val Glu Asn Ala Leu Asn Lys	265
GCC CTT TTC ATA CTG CCA GAC ACA GGG AAG ATA GAA AAA GTG GAG AAT GCC CTG AAC AAA	900
Met Leu Phe His Lys Trp Thr Arg Asn Leu Lys Arg Arg Ala Ile Ser Leu Tyr Phe Pro	285
ATG CTG TTT CAC AAA TGG ACA CGC AAC TTA AAG CGC AGG GCA ATC AGT TTA TAT TTT CCA	960
Lys Val Ser Ile Ser Gly Asn Tyr Asp Leu Lys Ile Leu Arg Glu Leu Gly Ile Thr Asp	305
AAA GTT TCC ATC TCA GGA AAC TAT GAT CTG AAG ATA CTA CGT GAA CTG GGA ATC ACA GAT	1020
Val Phe Gly Ser Asn Ala Asp Leu Ser Gly Ile Thr Glu Glu Thr Asn Leu Lys Leu Ser	325
GTA TTT GGA AGT AAT GCG GAT CTC TCT GGA ATC ACA GAG GAA ACA AAC CTT AAG CTT TCC	1080
Gln Ala Val His Lys Ala Val Val Asn Ile Asp Glu Lys Gly Thr Glu Ala Ser Gly Ala	345
CAG GCT GTG CAC AAA GCT GTG GTG AAC ATT GAT GAG AAA GGA ACA GAG GCT TCA GGA GCC	1140
Thr Phe Ala Glu Gly Ile Pro Met Ser Ile Pro Pro Thr Val Glu Phe Leu Arg Pro Phe	365
ACT TTC GCA GAA GGA ATA CCT ATG TCC ATT CCC CCT ACC GTG GAA TTC TTG AGG CCT TTT	1200
Ile Phe Ile Ile Leu Glu Glu Asn Thr Lys Ser Val Leu Phe Met Gly Lys Val Met Asn	385
ATA TTT ATA ATT TTG GAA GAA AAC ACA AAG AGT GTA CTT TTC ATG GGA AAA GTT ATG AAT	1260
Pro Thr Gly Asn *	389
CCT ACT GGA AAT TAG CAT CTG CAG TCT CAG CCC CTC AAG ATC TCC GTG TTC TCT CGT CTC	1320
CTG CAG AAT AAA GAA GCA GCA TCC ATC TC	1349

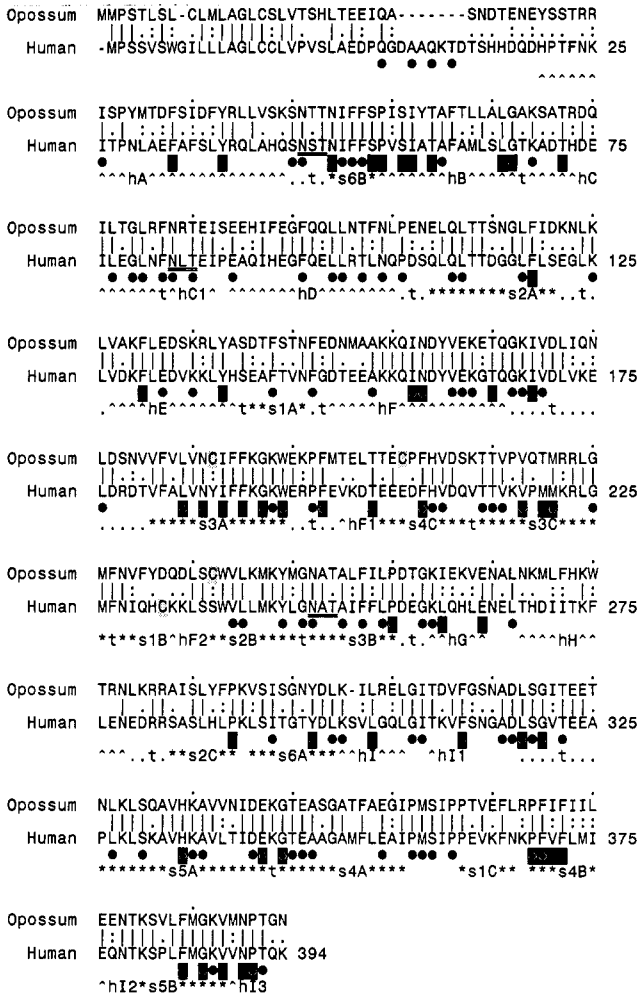
[a] Residue numbers in this table only refer to opossum α_1-PI. The nucleotide sequence was determined for both strands. Nucleotide residues 1–42 and 1272–1349 are noncoding. The locations of synthetic primers used to sequence in the forward direction are shaded, and those used to sequence the complementary strand are underlined. The amino acid sequences shown with shading were also determined by peptide analysis. The double-underline indicates the polyadenylation signal sequence. The asterisk indicates the stop codon.

opossum α_1-PI is a glycoprotein. Subsequent analysis (not shown) indicated a carbohydrate content of 17.7%, consisting of (residues per mole of α_1-PI) *N*-acetylneuraminic acid (11), glucosamine (15), galactosamine (3), and hexose (23). cDNA sequence analysis (see below) indicated three Cys residues in the mature protein. No free sulfhydryl groups were detected in native opossum α_1-PI or in inhibitor preparations which had been denatured by dialysis against 6 M guanidine hydrochloride.

Opossum α_1-PI cDNA. Library screening resulted in the isolation of five positive clones. The nucleotide and deduced amino sequences, for the longest full-length cDNA insert coding for opossum α_1-PI are shown in Table II. The nucleotide sequence for the complementary strand was also determined, and no differences were noted. The inhibitor consists of a 21-residue signal peptide and a 389-residue mature protein. Residues 1–16 and 106–125, as determined by protein sequence analysis, were identical to those deduced from cDNA, except for residue 10 which showed Asp in the sequenator analysis. cDNA analyses of the other positive clones all indicated Asn at this position. The difference between the amino acid sequence deduced from cDNA and that determined by sequenator after enzymatic digestion of the core molecule could be due to genetic polymorphism, since the protein sequence determinations were done on α_1-PI prepared from pooled opossum serum gathered in Texas, while the mRNA was from a single opossum cpatured in Pennsylvania.

Sequence Homology. A search of the Genbank and SWISS-PROT databases with the nucleotide and amino acid sequences of opossum α_1-PI revealed the expected homology with members of the serpin family. The amino acid sequence of opossum α_1-PI showed the following identity with other mammalian α_1-PI sequences in the databank: human and baboon (58%); rat (57%); sheep and bovine (54%); mouse

Table III: Comparison of the Amino Acid Sequences of Human and Opossum α_1-Proteinase Inhibitor[a]



[a] Residue numbering, conserved residues in the serpin family, and structural notations are those for human α_1-PI according to Huber and Carrell (1989). In this alignment, the amino terminus of opossum α_1-PI is at H_{-3}-L. Cys residues are shaded; carbohydrate attachment sites are doubly-underlined; three gaps introduced in opossum α_1-PI during optimization by the computer program GAP are shown as dashes. The inhibitor reactive site is at M_{358}-S. The bonds in human α_1-PI which are cleaved during enzymatic inactivation are as follows: A_{350}–M, *C. adamanteus* proteinase II (Kress, 1986); F_{352}–L and M_{358}–S, papain (Mast et al., 1992); P_{357}–M, *P. aeruginosa* elastase (Morihara et al., 1984). (●) Identical in all nine mammalian α_1-PI sequences (see Experimental Procedures); (■) conserved residues in serpin family; (circle in solid box) identical in all α_1-PI sequences and also conserved in serpin family; ^h, helix; *s, strand of β-sheet; .t, turn.

(53%); rabbit (52%); and guinea pig (51%). A comparison of the amino acid sequences of opossum and human α_1-PI is shown in Table III. The carbohydrate attachment sequences and the reactive site bond (358M–S) are identical in both species. All conserved residues for the serpin family are present in opossum α_1-PI except M-220. This residue is M in all serpins, including all other mammalian α_1-PI's in the database. However, a T occurs at this position in opossum α_1-PI.

Inhibition Spectrum. Incubation of opossum α_1-PI with equimolar amounts of trypsin, chymotrypsin, or neutrophil elastase resulted in complete inhibition of proteolytic activity and formation of enzyme/inhibitor complexes stable to SDS/PAGE. The gel patterns (not shown) were similar to those previously noted with human α_1-PI and trypsin (Oda et al., 1977). Thrombin was not inhibited under the above conditions, either in the absence or in the presence of heparin. In addition, an excess of opossum α_1-PI had no effect on the activities of serine proteinases (BAEE substrate) in the following crude venoms which had been pretreated with EDTA to inactivate metalloproteinases: *C. adamanteus*, *C. atrox*, *C. basiliscus*, *B. arietans*, *Vipera ammodytes*, and *Dispholidus typus*. No proteolysis of opossum α_1-PI by the venom serine proteinases was noted in SDS/PAGE.

Table IV: Effect of Proteinases and Venom on Opossum and Human α_1-PI[a]

	α_1-PI act. remaining (%)	
venom/proteinase	opossum	human
C. atrox venom	100	11
C. atrox α-protease	100	7
C. adamanteus venom	100	0
C. adamanteus proteinase II	100	2
C. basiliscus venom	80	7
B. arietans venom	88	11
papain	0	0
P. aeruginosa elastase	0	0

[a] Opossum or human α_1-PI was incubated with crude venom or purified venom metalloproteinases at 23 °C in 0.05 M Tris-HCl/0.002 M $CaCl_2$, pH 8.0. Aliquots were assayed after 2-h incubation for residual α_1-PI inhibitory activity against bovine trypsin (BAPA substrate) (Kress, 1986). Final A_{280} ratios (inhibitor/proteinase) in the incubations were 7/1 for the venoms, 50/1 for the venom proteinases, 80/1 for papain, and 300/1 for *P. aeruginosa* elastase.

Resistance of Opossum α_1-PI to Proteolytic Inactivation. The effects of incubation with various proteinases on the inhibitory activity of opossum and human α_1-PI are shown in Table IV. In the presence of venom or proteinases of *C. adamanteus* and *C. atrox*, opossum α_1-PI retained all inhibitory activity against trypsin over a 2-h period, and electrophoretic analyses (not shown) of the reaction mixtures indicated that the 54-kDa inhibitor had remained intact during the incubation. In contrast, human α_1-PI under the same conditions lost essentially all activity, and electrophoretic analyses showed that the 54-kDa band corresponding to intact inhibitor had been converted to a 50-kDa modified form during a 2-h incubation period. This is the pattern expected for proteolytic cleavage in the reactive site loop of the human inhibitor as previously reported (Kress & Paroski, 1978). A 5-fold increase in the amount of the above venoms gave similar results for opossum α_1-PI. Under the latter conditions, human α_1-PI was totally inactivated after 30 min. In the presence of *C. basiliscus* and *B. arietans* venom (Table IV), opossum α_1-PI showed a slow loss of activity, and a band corresponding to modified inhibitor (50 kDa) was apparent during SDS/PAGE, indicating cleavage in the reactive site loop. The venoms and proteinases listed in Table IV were also incubated with large excesses of opossum α_1-PI and assayed for possible inhibition of the venom metalloproteinases. No decreases in proteolytic activity were observed during the 2-h incubations.

In order to test the effects of proteinases from sources other than snake venoms, opossum α_1-PI was incubated with papain or *P. aeruginosa* elastase, both of which are known to readily inactivate human α_1-PI (Johnson & Travis, 1977; Morihara et al., 1984). In the presence of these proteinases, opossum α_1-PI lost all inhibitory activity, and electrophoretic analyses showed that intact opossum α_1-PI (54 kDa) had been completely converted to a single modified species (50 kDa), indicating limited proteolysis in the inhibitor reactive site loop.

DISCUSSION

The opossum serum component described in this paper was originally designated α_1-PI on the basis of physical properties

and inhibition of trypsin, chymotrypsin, and neutrophil elastase. The linear sequence deduced from cDNA establishes its homology to known mammalian α_1-PI's. The sequence similarity between opossum α_1-PI and other α_1-PI sequences is striking in view of the fact that the opossum is the earliest known marsupial and is thought to have diverged from eutherian mammals approximately 100–135 million years ago (Slaughter, 1968; Clemens, 1968). The more limited identity of the amino-terminal region of opossum α_1-PI with other α_1-PI sequences is not unexpected since this region of the serpin molecule shows considerable variability (Huber & Carrell, 1989). The α_1-PI from an Australian marsupial, the tamar wallaby, also showed only limited positional identity in the amino-terminal region with other α_1-PI sequences (Patterson, 1991).

Opossum and human α_1-PI's are comparable in molecular size and carbohydrate content. However, human α_1-PI contains a single Cys residue which is present as a mixed disulfide with cysteine or glutathione (Travis & Salvesen, 1983). In contrast, cDNA analysis of opossum α_1-PI indicates Cys residues at C-187 in strand 3A, C-206 in strand 4C, and C-237 in strand 2B (human α_1-PI numbering, Table III). Cys residues also occur at positions 187 and 237 in heparin cofactor II, and at position 237 in mouse and human α_1-antichymotrypsin, and protein C inhibitor (Huber & Carrell, 1989). In all other α_1-PI sequences, there is a Cys residue at position 232. There are four Cys residues in sheep and bovine α_1-PI (Brown et al., 1989; Sinha et al., 1992) and two in rabbit α_1-PI (Saito & Sinohara, 1991). An analysis of the three-dimensional structure of human α_1-PI has indicated that a disulfide bond can be accommodated without significant structural perturbation (Huber & Carrell, 1989). However, it has not been determined whether the Cys residues in opossum α_1-PI are present as a disulfide bond and one mixed disulfide or entirely as mixed disulfides. Either interpretation would be in agreement with the observed lack of sulfhydryl groups in opossum α_1-PI.

Table III indicates the extent of identity or similarity between opossum and human α_1-PI. A comparable pattern (not shown) is apparent for the alignment of the opossum α_1-PI sequence with other mammalian α_1-PI sequences (rat, mouse, sheep, rabbit/f, baboon, guinea pig/s, and bovine) presently in the databank. The most variable region is in the amino terminal and helix A (residues 20–44). Shorter regions of variability occur from residues 199 to 207 (helix F1 and strand 4C) and from residues 229 to 234 (strand 1B and helix F2). These segments are near the reactive site loop (strand 4A) in models of the intact α_1-PI molecule (Engh et al., 1990). The sequence from residues 268 to 288 (helix H and strand 2C and the turn between the two) shows nearly as much variability as the amino-terminal portion of the molecule, and no serpin family conserved residues occur in this segment. This region of opossum α_1-PI has an excess of positively charged residues, as is also true for mouse α_1-PI. The other mammalian α_1-PI sequences resemble human α_1-PI in having a slight net positive charge in this segment.

Opossum α_1-PI also has regions which show a high degree of identity with human α_1-PI (Table III) and with other α_1-PI sequences. Residues 49–54 (NIFFSP) in strand 6B are present in all α_1-PI sequences and also in thyroxine binding globulin and protein C inhibitor. Strand 6B is in a buried and apolar segment of the molecule, and is strongly conserved in the serpin family (Huber & Carrell, 1989). Residues 315–319 (ADLSG) and 344–347 (GTEA) are also identical in all α_1-PI sequences. The longest segment of identical or similar residues in opossum and human α_1-PI sequences occurs in helix F and the wide turn into strand 3A (positions 153–173). Ninety percent of the residues in this region are identical in these two species, and 43% are identical in all α_1-PI sequences. However, only four serpin family conserved residues occur in this region. The 5-residue segment from positions 167 to 171 (GKIVD) is also highly conserved in all α_1-PI sequences. Similarly, positions 73–118 contain no serpin family conserved residues, but the α_1-PI sequences show 35% identity in this segment of the molecule. Of the residues in strand 3A (positions 181–194), 43% are identical in all α_1-PI sequences, and in strand 5A (positions 330–342), 46% of the residues are identical. These strands of the β-sheet have been shown to arrange themselves in a parallel fashion in models of intact α_1-PI (Engh et al., 1990). A Cys residue (C-187) occurs in strand 3A of opossum α_1-PI and also in heparin cofactor II, whereas all other α_1-PI sequences have Tyr in this position.

The α_1-PI reactive site loop consists of strand 4A (residues 344–362) which is inserted in an antiparallel fashion between strands 3A and 5A in the inhibitor (Huber & Carrell, 1989; Engh et al., 1990). This strand contains only one serpin family conserved residue (G-344). However, the opossum and human α_1-PI sequences show 79% identity in this strand, and for all α_1-PI sequences, 43% of the residues in strand 4A are identical. Four residues differ in the opossum and human α_1-PI reactive site loop, three of which are of smaller molecular size in opossum α_1-PI (T-351, A-353, G-355). The alignment of all α_1-PI sequences indicates that in the reactive site loop the P7, P6, and P4 residues are the most variable. These may be contact points between α_1-PI and neutrophil elastase during enzyme/inhibitor complex formation, since inhibitor contact residues are known to be hypervariable (Laskowski et al., 1987). Residues 280 and 305 are also highly variable, as previously noted in a comparison of six mammalian α_1-PI sequences (Saito & Sinohara, 1991).

The inactivation of opossum α_1-PI in the presence of papain or *P. aeruginosa* elastase (Table IV) was similar to that previously observed for human α_1-PI in the presence of these proteinases (Johnson & Travis, 1977; Mast et al., 1992; Morihara et al., 1984). The 50-kDa inactivated opossum inhibitor was not subject to further proteolytic attack, indicating that inactivation had occurred by limited proteolysis in the reactive site loop. Inactivation of opossum α_1-PI presumably occurred at the same sites as in human α_1-PI, since the Pro–Met and Met–Ser bonds cleaved by these proteinases during inactivation are present in both inhibitor species (Table III). Venoms from *C. basiliscus* and *B. arietans* were the most active of those tested for human serpin inactivation (Kress, 1988). Opossum α_1-PI also showed some loss of activity in the presence of these venoms (Table IV), but the rate of inactivation was slower than that noted with human α_1-PI.

In contrast, opossum α_1-PI retained full activity in the presence of rattlesnake (*C. atrox* and *C. adamanteus*) venom, or metalloproteinases from these venoms, under conditions in which human α_1-PI was readily inactivated. There was no evidence in SDS/PAGE of any proteolysis of intact opossum α_1-PI during the 2-h incubation under the conditions given in Table IV or when the amount of these venoms was increased 5-fold. The conservation in opossum α_1-PI of a reactive site sequence which resists proteolytic inactivation by rattlesnake venoms has survival value for the animal. The geographic distribution and habitat of the opossum overlap those of *C. adamanteus* (Eastern diamondback rattlesnake) and *C. atrox* (Western diamondback rattlesnake) (Jurgelski, 1974; Russell,

1979). It is known from observations in the field and from laboratory experiments involving snakebite or direct injection of venom that the opossum can tolerate rattlesnake venom without noticeable effect (Kilmon, 1976; Werner & Vick, 1977). The resistance of opossum α_1-PI to venom metalloproteinases would account in part for the opossum's ability to survive rattlesnake bite. This property enables the major plasma serpin to remain active and to regulate any localized or systemic endogenous serine proteinases activated by the venom. Various animals in addition to the opossum are known to survive rattlesnake envenomation (Perez et al., 1979; De Wit & Weström, 1987; Weissenberg et al., 1991), and the sera of these species may also contain serpins which resist proteolytic inactivation by venom metalloproteinases.

ACKNOWLEDGMENT

We thank Mr. Al Cairo, Biopolymers Facility, Roswell Park Cancer Institute, for synthesizing the nucleotide primers.

REFERENCES

Brower, M. S., & Harpel, P. C. (1982) *J. Biol. Chem. 257*, 9849.
Brown, W. M., Dziegielewska, K. M., Foreman, R. C., Saunders, N. R., & Wu, Y. (1989) *Nucleic Acids Res. 17*, 6398.
Catanese, J. J., & Kress, L. F. (1984) *Biochim. Biophys. Acta 789*, 37.
Catanese, J. J., & Kress, L. F. (1992) *Biochemistry 31*, 410.
Chao, S.; Chai, K. X., Chao, L., & Chao, J. (1990) *Biochemistry 29*, 323.
Clemens, W. A. (1968) *Evolution 22*, 1.
Desrochers, P. E., & Weiss, S. J. (1988) *J. Clin. Invest. 81*, 1646.
Devereux, J., Haeberli, P., & Smithies, O. (1984) *Nucleic Acids Res. 12*, 387.
De Wit, C. A., & Weström, B. R. (1987) *Comp. Biochem. Physiol. 86A*, 1.
Engh, R. A., Wright, H. T., & Huber, R. (1990) *Protein Eng. 3*, 469.
Gilman, M. (1989) in *Current protocols in molecular biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K., Eds.) pp 4.1.2–4.1.6, John Wiley & Sons, New York.
Huber, R., & Carrell, R. W. (1989) *Biochemistry 28*, 8951.
Johnson, D., & Travis, J. (1977) *Biochem. J. 163*, 639.
Johnson, D., & Travis, J. (1978) *J. Biol. Chem. 253*, 7142.
Jurgelski, W., Jr. (1974) *Lab. Anim. Sci. 24*, 376.
Kilmon, J. A., Sr. (1976) *Toxicon 14*, 337.
Kress, L. F. (1986) *J. Cell. Biochem. 32*, 51.
Kress, L. F. (1988) in *Hemostasis and Animal Venoms* (Pirkle, H., & Markland, F. S., Jr., Eds.) pp 335–348, Marcel Dekker, New York.
Kress, L. F., & Paroski, E. A. (1978) *Biochem. Biophys. Res. Commun. 83*, 649.
Kress, L. F., & Catanese, J. J. (1981) *Biochemistry 20*, 7432.
Kress, L. F., Kurecki, T., Chan, S. K., & Laskowski, M., Sr. (1979) *J. Biol. Chem. 254*, 5317.
Kress, L. F., Catanese, J. J., & Hirayama, T. (1983) *Biochim. Biophys. Acta 745*, 113.
Kruzel, M., & Kress, L. F. (1985) *Anal. Biochem. 151*, 471.
Kurachi, K., Chandra, T., Degen, S. J. F., White, T. T., Marchioro, T. L., Woo, S. L. C., & Davie, E. W. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6826.
Kurecki, T., Laskowski, M., Sr., & Kress, L. F. (1978) *J. Biol. Chem. 253*, 8340.
Kurecki, T., Kress, L. F., & Laskowski, M., Sr. (1979) *Anal. Biochem. 99*, 415.
Laskowski, M., Jr., Kato, I., Kohr, W. J., Park, S. J., Tashiro, M., & Whatly, H. E. (1987) *Cold Spring Harbor Symp. Quant. Biol. 52*, 545.
Lech, K. (1989) in *Current protocols in molecular biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K., Eds.) pp 1.12.1–1.12.2, John Wiley & Sons, New York.
Long, G. L., Chandra, T., Woo, S. L. C., Davie, E. W., & Kurachi, K. (1984) *Biochemistry 23*, 4828.
Mast, A. E., Enghild, J. J., Pizzo, S. V., & Salvesen, G. (1991) *Biochemistry 30*, 1723.
Mast, A. E., Enghild, J. J., & Salvesen, G. (1992) *Biochemistry 31*, 2720.
Mizusawa, S., Nishimura, S., & Seela, F. (1986) *Nucleic Acids Res. 14*, 1319.
Morihara, K., Tsuzuki, H., Harada, M., & Iwata, T. (1984) *J. Biochem. 95*, 795.
Oda, K., Laskowski, M., Sr., Kress, L. F., & Kowalski, D. (1977) *Biochem. Biophys. Res. Commun. 76*, 1062.
Patterson, S. D. (1991) *Comp. Biochem. Physiol. 100B*, 439.
Pearson, W. R., & Lipman, D. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2444.
Perez, J. C., Pichyangkul, S., & Garcia, V. E. (1979) *Toxicon 17*, 601.
Perlmutter, D. H., Joslin, G., Nelson, P., Schasteen, C., Adams, S. P., & Fallon, R. J. (1990) *J. Biol. Chem. 265*, 16713.
Rinderknecht, H., Geokas, M. C., Silverman, P., & Haverback, B. J. (1968) *Clin. Chim. Acta 21*, 197.
Russell, F. E. (1979) *Handb. Exp. Pharmacol. 52*, 978–996.
Saito, A., & Sinohara, H. (1991) *J. Biochem. (Tokyo) 109*, 158.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.
Sifers, R. N., Ledley, F. D., Reed-Fourquet, L., Ledbetter, D. H., Ledbetter, S. A., & Woo, S. L. C. (1990) *Genomics 6*, 100.
Sinha, D., Bakhshi, M. R., & Kirby, E. P. (1992) *Biochim. Biophys. Acta 1130*, 209.
Slaughter, B. H. (1968) *Science 162*, 254.
Suzuki, Y., Yoshida, K., Honda, E., & Sinohara, H. (1991) *J. Biol. Chem. 266*, 928.
Travis, J., & Salvesen, G. S. (1983) *Annu. Rev. Biochem. 52*, 655.
Treco, D. A. (1989) in *Current protocols in molecular biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K., Eds.) pp 2.1.1–2.1.7, John Wiley & Sons, New York.
Weissenberg, S., Ovadia, M., Fleminger, G., & Kochva, E. (1991) *Toxicon 29*, 807.
Werner, R. M., & Vick, J. A. (1977) *Toxicon 15*, 29.