Mem. Inst. Oswaldo Cruz, Rio de Janeiro, Vol. *86*, Suppl. II, 207-209, 1991

PROTEINASE INHIBITORS IN BRAZILIAN LEGUMINOSAE

C. A. M. SAMPAIO; M. L. V. OLIVA*; A. S. TANAKA & M. U. SAMPAIO

Departamento de Bioquímica, Escola Paulista de Medicina, Caixa Postal 20372, 04034 São Paulo, SP, Brasil
*Departamento de Química, Instituto de Química, UFMS, Campo Grande, MS, Brasil

Serine proteinase inhibitors, in the seeds of several Leguminosae from the Pantanal region (West Brazil), were studied using bovine trypsin, a digestive enzyme, Factor XIIa and human plasma kallikrein, two blood clotting factors. The inhibitors were purified from Enterolobium contortisiliquum *(Mr = 23,000),* Torresea cearensis *(Mr = 13,000),* Bauhinia pentandra *(Mr = 20,000) and* Bauhinia bauhinioides *(Mr = 20,000).* E. contortisiliquum *inhibitor inactivates all three enzymes, whereas the* T. cearensis *inhibitor inactivates trypsin and Factor XIIa, but does not affect plasma kallikrein; both* Bauhinia *inhibitors, on the other hand, inactivate trypsin and plasma kallikrein but only the* B. pentandra *inhibitor affects Factor XIIa. Ki values were calculated between 10^{-7} and 10^{-8} M.*

Key words: *Bauhinia bauhinioides* – *Bauhinia pentandra* – coagulation – *Enterolobium contortisiliquum* – proteinase inhibitor – *Torresea cearensis*

Proteinases are biologically controlled by various mechanisms. They may undergo inactivation, for example, by clearance by organs like liver, through specific receptors (Borges et al., 1986). Proteinases can be inactivated by proteolytic degradation, either specific or not (Laskowski & Kato, 1980). Furthermore, proteinase can be blocked by inhibitors, actually pseudo-substrates which display variable degrees of affinity towards the catalytic site of the enzymes (Travis & Salvesen, 1983).

Several plants are long known as rich sources of proteinase inhibitors, which can be taken as model compounds for inhibition of proteolytic enzymes of animal origin (Richardson, 1977).

We will report on the isolation of plant inhibitors which inactivate trypsin and selectively, some of the serine proteinases involved in the blood clotting cascade, plasma kallikrein and Factor XII (Kaplan & Silverberg, 1987).

MATERIALS AND METHODS

Bovine trypsin and chymotrypsin are Whortington products, human plasma kallikrein was isolated by a previous procedure (Oliva et al., 1982). Factor XIIa was kind a gift from Cuttler Laboratories. Sephadex G-150, DEAE-Sephadex A-50 and Sepharose CNBr-activated were Pharmacia products, acrylamide, bis-acrylamide were from Merck Darmstadt, acetyl-phenylalanine-arginine-p-nitroanilide (Ac-Phe-Arg-Nan) was synthesized by a procedure described elsewhere (Juliano & Juliano, 1985). The other chemicals were from the best quality available. Seeds were manually harvested from wild trees.

Purification – In a typical experiment, 15 g of cotyledons were homogenized in 150 ml of 0.15 M NaCl and precipitated with 80% acetone. The acetone fraction was dried under vacuum and dissolved in 0.05 M tris-HCl buffer, pH 8.0. The soluble fraction was applied to a column of trypsin-Sepharose (5.0 ml), prepared as described previously (Oliva et al., 1987), equilibrated with 0.05 M tris-HCl buffer, pH 8.0. The adsorbed inhibitor was eluted by acidification with 0.2 M KCl/HCl, pH 2.0. The inhibitors were submited to gel-filtration in an 1 x 100 cm Sephadex G-150 column, equilibrated with 0.05 M tris-HCl buffer, pH 8.0 and then chromatographed on a DEAE-Sephadex ion-exchange column (30-ml column, equilibrated with 0.05 M tris-HCl buffer, pH 8.0 and eluted with 0.05 to 0.3 M NaCl gradient).

Electrophoresis – The homogeneity and the molecular weight of the inhibitors were assessed by 10-20% SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Proteins in solution were estimated by absorbance at 280 nm.

Partially supported by FINEP and CNPq.

Inhibitory activity – It was measured as described previously (Oliva et al., 1987); the trypsin residual acivity was estimated by hydrolysis of 20 mM Tos.Arg.O.Me in a pH-stat (Oliva et al., 1982); and factor XIIa and human plasma kallikrein residual activity was measured using 1.0 mM Ac.Phe-Arg-p-nitroanilide (Oliva et al., 1987). Ki and inhibitor concentration were determined using a slow-tight binding mechanism model (Knight, 1986).

RESULTS

The affinity chromatography profile for purification of *E. contortisiliquum* inhibitor, that was eluted with KCl/HCl, pH 2.0, is shown in Fig. 1. The overall yield of the purification process was between 40 and 60% and a 30 to 100-fold purification was achieved. All studied inhibitors were eluted as a single peak in both systems. Molecular weights determined by SDS-electrophoresis of the inhibitors are shown in Table I.

A typical inhibition curve of trypsin by *E. contortisiliquum* inhibitor can be seen in Fig. 2. The same kind of curve is seen for human plasma kallikrein and Factor XII fragment. The discrimination of the inhibitor activities can be seen in Table II, that shows the Ki values for the inhibition of the studied enzymes.

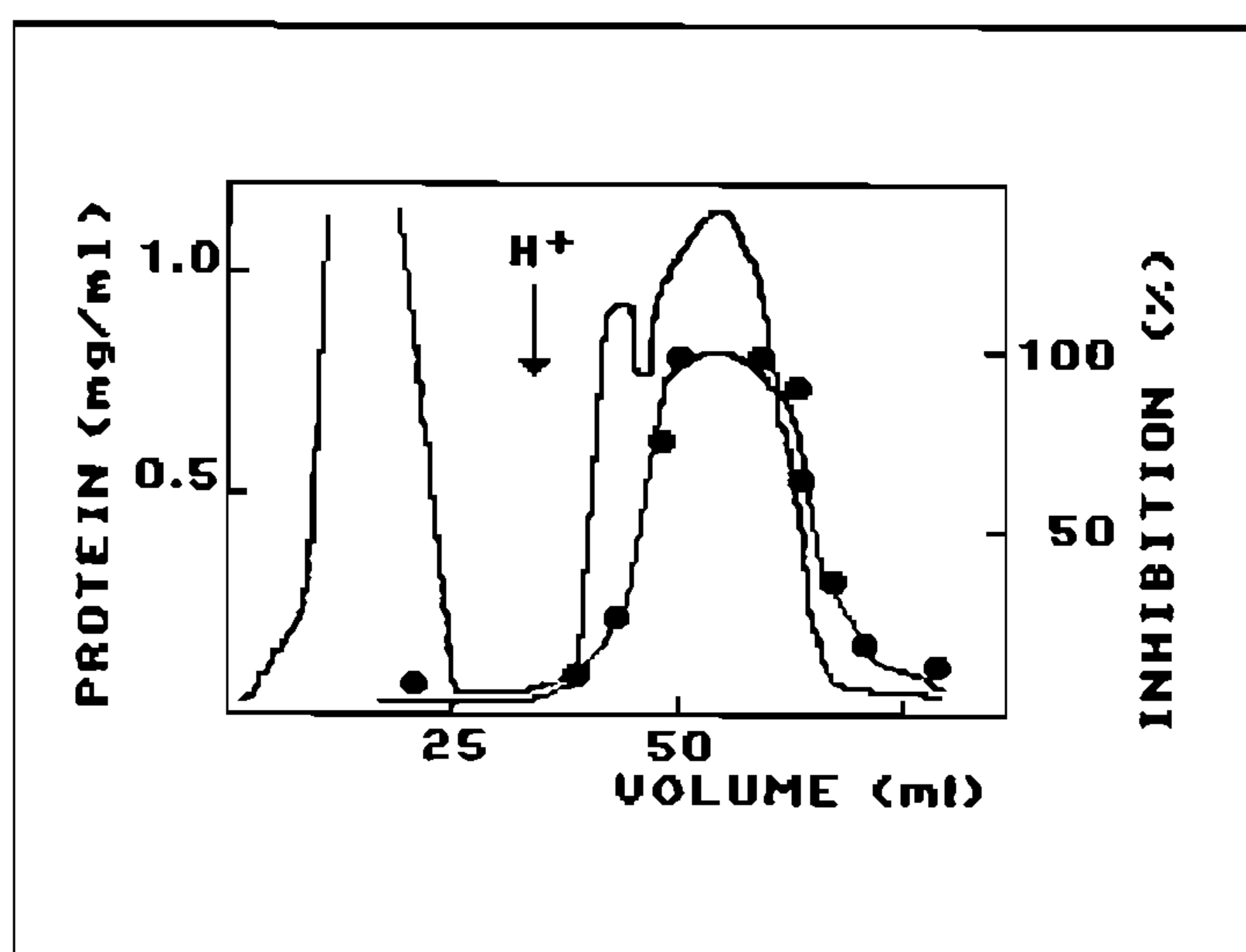

Fig. 1: trypsin-sepharose affinitty chromatography of *Esterolobium contortisiliquum* trypsin inhibitor – Column (1 x 6 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. Sample: 209 mg acetone powder in 10 ml in equilibrium buffer. Elution with 0.2 M KCl/HCl, pH 2.0. (——) protein estimated by absorbance at 280 nm. (–•–•–) inhibitory activity (100 µl against 5 µg trypsin in 0.1 M tris-HCl buffer, 0.02M $CaCl_2$, pH 8.0). Trypsin remaining activity was measured by the hydrolysis of 20 mM Tos-Arg.O.Me, pH 8.0, 30 °C, in a pH stat.

TABLE I

Molecular weight estimation, based upon SDS-polyacrylamide gel electrophoresis (10-20% gradient)

Inhibitor source	Molecular weight	
	Non-reduced	Reduced
E. contortisiliquum	23,000	17,000/ 8,000
T. cearensis	13,000	13,000
B. pentandra	21,000	21,000
B. bauhinioides	21,000	21,000

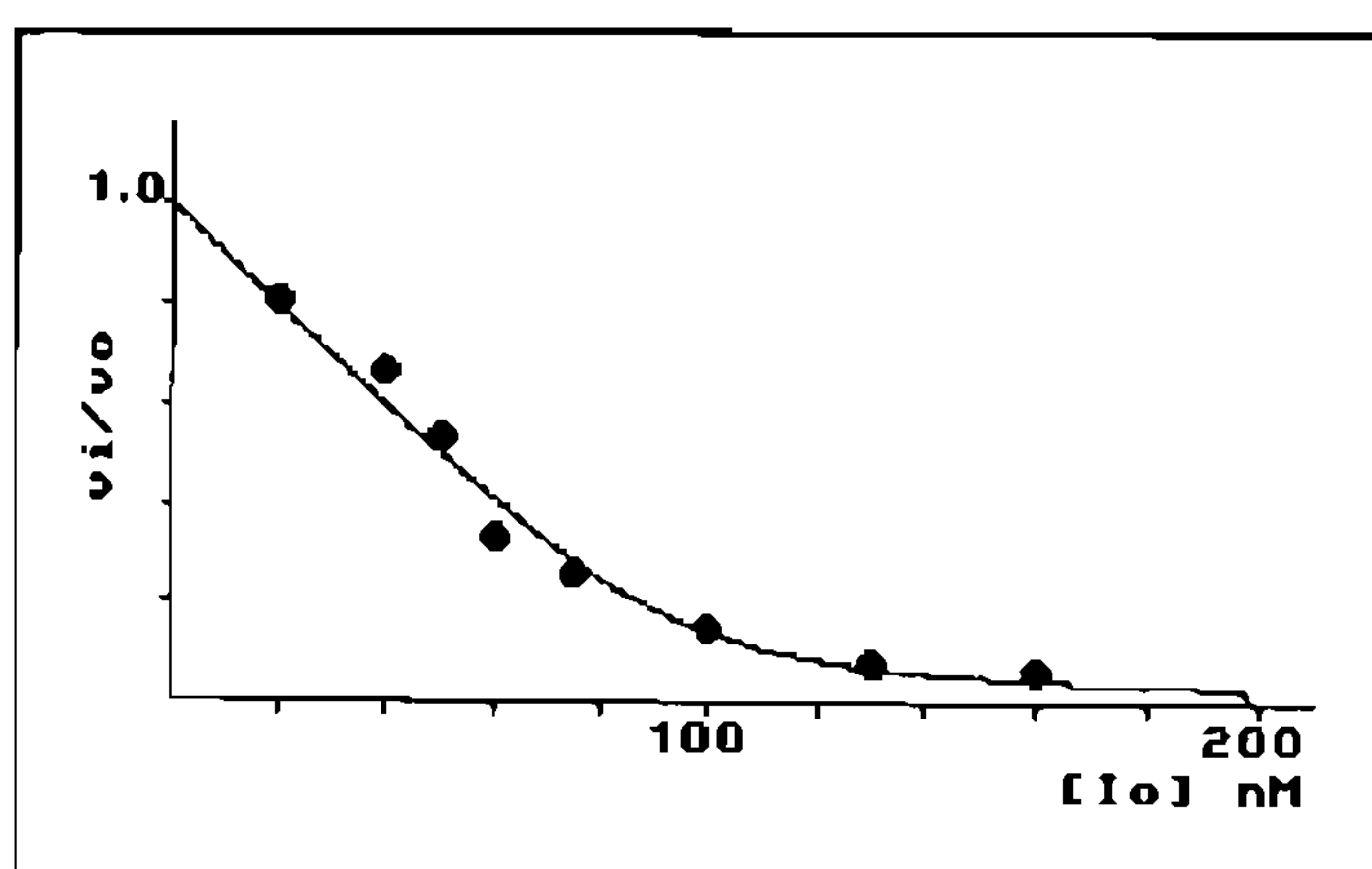

Fig. 2: trypsin inactivation curve by *Enterolobium contortisiliquum* inhibitor – Bovine trypsin (83 nM, NPGB-titrated) was pre-incubated with purified *E. contortisiliquum* inhibitor (Io), for 10 min, at 30 °C, in 1.0 ml 0.1 M tris-HCl buffer, 0.02 mM $CaCl_2$, pH 8.0 and assayed residual activity was followed by hydrolysis of 20 mM Tos.Arg.O.Me, in a pH stat.

TABLE II

Ki values (M) for serine proteinase plant inhibitors

Inhibitors source	Ki (M)			
	Trypsin 10^{-8}	Chymo-Trypsin 10^{-7}	HuPK 10^{-8}	F-XIIa 10^{-7}
E. contortisiliquum	0.3	1.2	0.5	1.5
T. cearensis	0.1	2.4	NI	0.6
B. pentandra	2.7	2.3	1.0	0.8
B. bauhinioides	1.8	2.9	2.3	NI

NI: no inhibition; HuPK: human plasma kallikrein; F-XIIa: Factor XIIa fragment.

With respect to the ability to affect blood clotting tests, prothrombin time and activated partial thromboplastin time (APTT) were assayed in the presence of inhibitors, with normal human plasma (Lourenço et al., 1989). Fig. 3 shows the effect of these inhibitors on clotting tests.

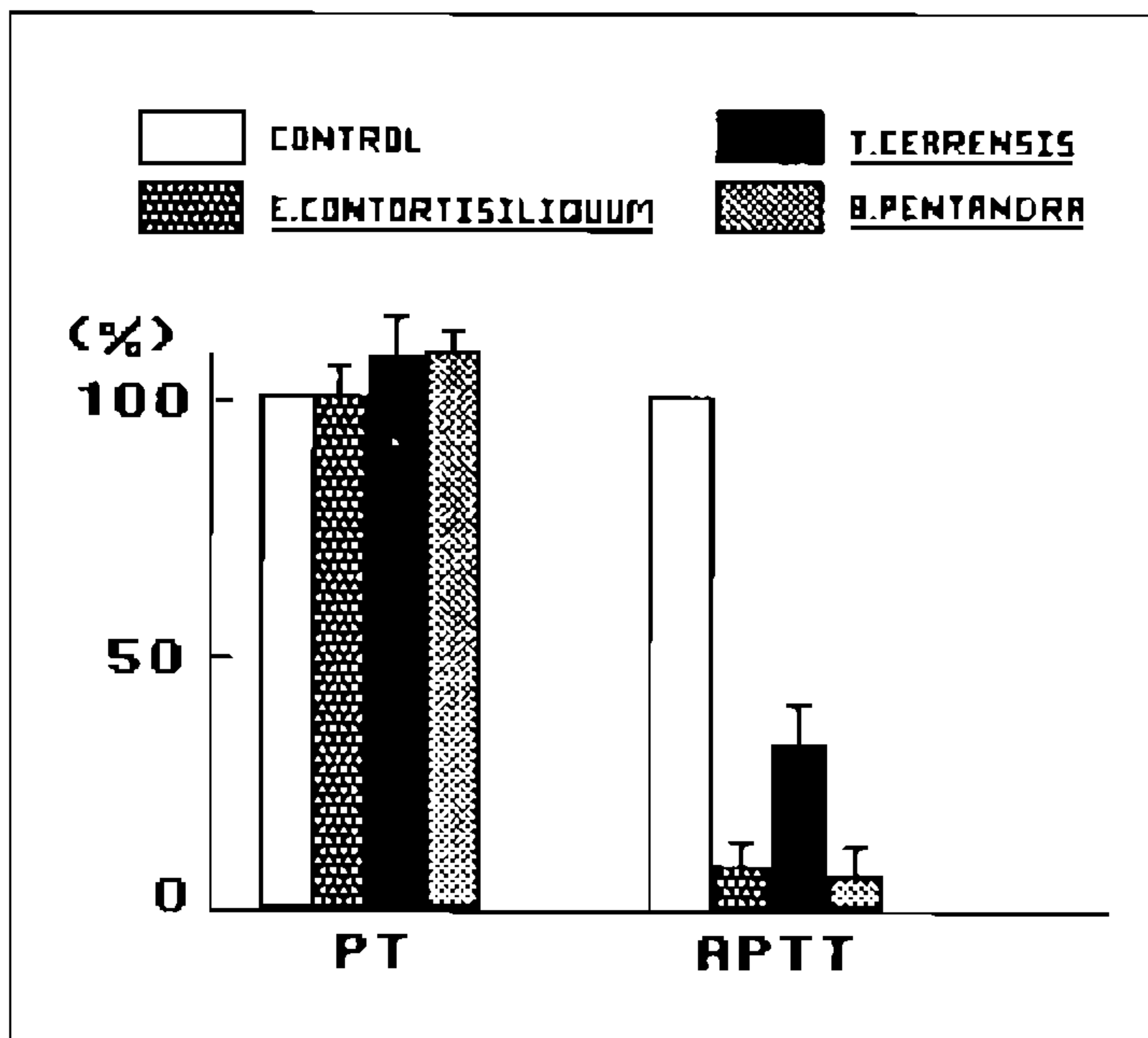

Fig. 3: effect of inhibitors on blood clotting tests – Prothrombin time (PT) was determined by the method of Quick. Activated partial thromboplastin time (APTT) was performed using cephalin and kaolin. Excess of the inhibitors were used (between 200 and 300 μg/ml, final concentration). Inhibition was expressed as the ratio between clotting time (sec) in the absence and in the presence of the inhibitors.

DISCUSSION

Trypsin is inhibited by all inhibitors, as one would expect, and the Ki values fall in the range of 10^{-9} to 10^{-8} M. Chymotrypsin, like trypsin, is also inhibited, but with much lower affinity in some cases. Human plasma kallikrein was not inhibited by *T. cearensis* inhibitor and Factor XII fragment was not inhibited by *B. bauhinioides* inhibitor. Both *Torresea* and *Bauhinia* inhibitors do not differ much in their ability to inhibit trypsin or chymotrypsin. Comparing both *Bauhinia* inhibitors, it is interesting to observe that they differ remarkably in the inhibition of Factor XII, since only the *B. pentandra* species affect this blood clotting enzyme.

Plant inhibitors are customarily divided into two major types: one is Kunitz type and the second is Bowman-Birk type. Kunitz type inhibitors are in the Mr range of 20,000 and contain a single inhibition center located on a single polypeptide chain. Besides the molecular weight differences, these two types of inhibitor differ in some other structural aspects, concerning the degree of homology among them, as well the number of disulfide bridges in their non-reduced molecules (Richardson, 1977).

The trypsin inhibitors, isolated from *E. contortisiliquum, B. pentandra* and *B. bauhinioides*, belong to the Kunitz type. On the other hand, the inhibitor purified from *Torresea cearensis* (Mr =13,000) belongs to the Bowman-Birk type, although no duplicity of inhibition center was ever detected in our preparations, as it has been described for other inhibitors of this type (Richardson, 1977).

REFERENCES

BORGES, D.; SAMPAIO, C. A. M.; LLOSA, P. & PRADO, J. L., 1986. The liver is the main organ to clear plasma and tissue kallikreins from rat plasma *in vivo*. *Adv. Exp. Med. Biol., 198:* 229-233.

JULIANO, M. A. & JULIANO, L., 1985. Synthesis and kinetic parameters of hydrolysis by trypsin of some acyl-arginyl-p-nitroanilides and peptides containing arginyl-nitroanilides. *Brazilian. J. Med. Biol. Res., 18:* 435-445.

KAPLAN, A. P. & SILVERBERG, M., 1987. The coagulation-kinin pathway of human plasma. *Blood, 70:* 1-15.

KNIGHT, C. G., 1986. The characterization of enzyme inhibition, p. 23-51. In A. J. Barret & G. Salvesen, (eds). *Proteinase Inhibitors*. Elsevier, Amsterdam.

LAEMMLI, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227:* 680-685.

LASKOWSKI, Jr, M. & KATO I., 1980. Protein inhibitors of proteinases. *Ann. Rev. Biochem., 49:* 593-626.

LOURENÇO, D. M.; SAMPAIO, M. U.; KERBAUY, J. & SAMPAIO, C. A. M., 1989. Estimation of plasma kallikrein in sickle cell anemia, and its relation to the coagulation and fibrinolytic systems. *Adv. Med. Exp. Biol., 247B:* 553-557.

OLIVA, M. L. V.; GRISOLIA, D. M.; SAMPAIO, M. U. & SAMPAIO, C. A. M., 1982. Properties of a highly purified human plasma kallikrein. *Agents and Actions, 9:* 52-57.

OLIVA, M. L. V.; SAMPAIO, M. U. & SAMPAIO, C. A. M., 1987. Serine- and SH-proteinase inhibitors from *Enterolobium contortisiliquum* beans. Purification and preliminary characterization. *Brazilian J. Med. Biol. Res., 20:* 767-770.

RICHARDSON, M., 1977. Proteinase inhibitors of plants and micro-organisms. *Phytochemistry, 16:* 159-169.

TRAVIS, J. & SALVESEN, G., 1983. Human plasma inhibitors. *Ann. Rev. Biochem., 52:* 655-709.