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SOME PROPERTIES OF PURIFIED *ASPERGILLUS SYDOWI* PLASMA COAGULASE

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Some enzymatic properties of the purified plasma coagulase from A. sydowi were studied. The enzyme was protected from thermal inactivation by addition of calcium ions and was stable within 35-65°C. Addition of calcium accelerated the coagulation of the dialysed plasma until it approached that of normal one. The protective effect of calcium was influenced remarkably by the pH-value of enzyme solution. EDTA inhibited A. sydowi plasma coagulase at pHs 5.5, 7.5 and 9.5, while Ca^{2+} protected the enzyme activity at all tested pHs. Hemoclar acted as anticoagulant, Ca^{2+} antagonised the inhibitory effect at low doses but failed at high ones. $Ca^{2+}>Li^{2+}>Mn^{2+}>Co^{2+}>Na^{+}>K^{+}>Cd^{2+}$ accelerated the enzyme reaction. Citrate, oxalate and salicylate were anticoagulants, heparin inactivated the enzyme completely. Glutathione had no detectable effect on plasma clotting. Vitamin K activated the enzyme activity, while all tested antibiotics were anticoagulants. The fungal enzyme coagulated human and rabbit plasma but not rat, guinea-pig and monkey's plasma. The clot was firm, insoluble in urea and soluble in NaOH.

Keywords: *Aspergillus sydowi; Enzymatic properties; Plasma coagulase; Coagulation.*

Introduction

A considerable amount of information is now available on the proteinases of fungi. Many surveys have been undertaken involving hundreds of species which have been screened for general proteinase avtivity on substrates such as casein or for more specific activities such as those of elastase, fibrinase enzyme or milk clotting enzymes (North, 1982; Meusso-doerffer et al., 1980; Kominami et al., 1981; Kula et al., 1979; Ansari and Stevens, 1983; Balasubramanian and Manocha, 1986; Danno and Yoshimura, 1967). Only a proportion of the enzymes detected have been subjected to more detailed characterization, among those were the plasma coagulating enzymes which have very little attention regarding their purification and characterization.

The aim of the present work was to study some properties of purified *A. sydowi* plasma coagulase.

Materials and Methods

Aspergillus sydowi Thom and Church plasma coagulase was prepared, and purified to homogenity according to Maksoud et al (1995).

Assay of plasma clotting activity:

Human plasma or standard human plasma (diluted 1:5 by distilled water) was used as a substrate. The activity of coagulase was determined according to the method described by Beesley et al. (1967) and adopted in our lab. The reaction mixture contained: 0.5 ml of 5 μM sodium borate buffer (pH 7), 0.5 ml plasma and enzyme extract. A fine glass rod was placed in the liquid of the reaction mixture and allowed to stand in an incubator at 37°C, until the clot was formed (usually overnight). The rod was rotated to collect the clot and pressed against the side of the tube to squeeze the liquid out of the clot, taking care to pick up on the rod any detached pieces of clot. The clot was washed with several portions of water, then dissolved in one ml of 5 mM NaOH (in a boiling water bath) until the clot was completely dissolved, then cooled and neutralized with sulfuric acid. The protein was measured using Lowry et al. method (1951). Unit of enzyme activity was defined as the amount of enzyme which catalyzes the formation of one mg clotted protein/24 hours.

The effect of metals, chemicals, vitamins and antibiotics on the enzyme activity was studied by preincubation with the enzyme at specified concentrations before addition of plasma as indicated in the text, tables and figures.

Results and Discussion

Thermostability :

Pure homogenous enzyme prepared as decribed by Maksoud et al. (1995) was used. Plasma coagulase is thermostable and lost about 55% of its activity after 15 minutes exposure to 70°C (Fig. 1). Complete inactivation was

attained at 80°C, while maximum activity was achieved at 50°C. Addition of $CaCl_2$ to plasma clotting enzyme solution protected it from inactivation up to 60°C. Furthermore about 4 fold increase in the plasma clotting activity was observed in presence of $CaCl_2$ at 35°C to 60°C.

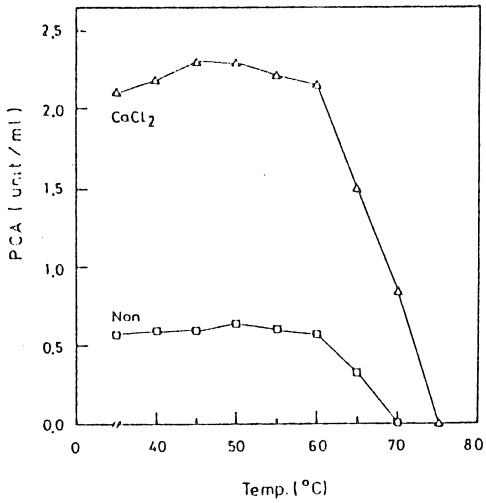

Fig.1. Thermostability of the purified plasma coagulase in presence and absence of $CaCl_2$ (10^{-2} M). Enzyme was exposed to different temperatures as indicated in presence and absence of $CaCl_2$, Residual activity was determined under standard assay conditions.

Investigating the effect of pure *A. sydowi* coagulase on dialyzed and normal human plasma in presence of different concentrations of calcium ions (Fig. 2) revealed that the enzyme activity declined in dialysed plasma by about 40% than normal one due to the leakage of

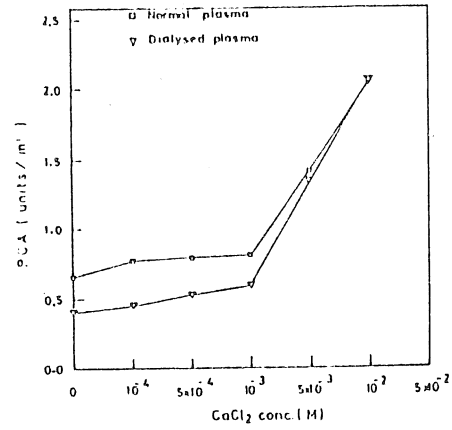

Fig.2. Effect of different concentrations of $CaCl_2$ on plasma coagulase using normal and dialyzed human plasma. Plasma was dialyzed against 0.05 M borate buffer, (pH 7) at 5°C for 48 hours.

Ca^{2+} during dialysis. Addition of Ca^{2+} at increasing concentration gradually compensated the diffused Ca^{2+} from plasma leading to the acceleration of the enzyme activity until it approached that of the normal plasma in presence of the highest experimental Ca^{2+} concentration. This experiment indicated that ionized calcium is the physiologically active portion in the process of blood coagulation and it is essential for stabilization of blood coagulation factors. Margueric (1977) claimed that fibrinogen in presence of Ca^{2+} resists denaturation at pH 5.0 and can resist proteolysis by fibrinase. Switzer and McKee (1980) found that Ca^{2+} stabilizes the thrombin activated factor VII.

The effect of Ca^{2+} and EDTA on the stability of *A. sydowi* was carried out using purified plasma coagulase which was dialyzed against 0.05 M borate buffer (pH 7) for 48 hours. The enzyme was mixed with the same volume of 0.05 M acetate buffer (pH 5.5 and 7.5) or 0.05 M sodium borate (pH 9.5) with or without 10^{-2} M calcium acetate or EDTA. The mixture (enzyme concentration 0.1 mg per ml) was incubated at 35°C for different time intervals (0-24 hours). The results shown and given on Fig. 3 and Table 1 revealed that EDTA, in all cases, completely inhibited the enzyme activity. Also incubation of the enzyme

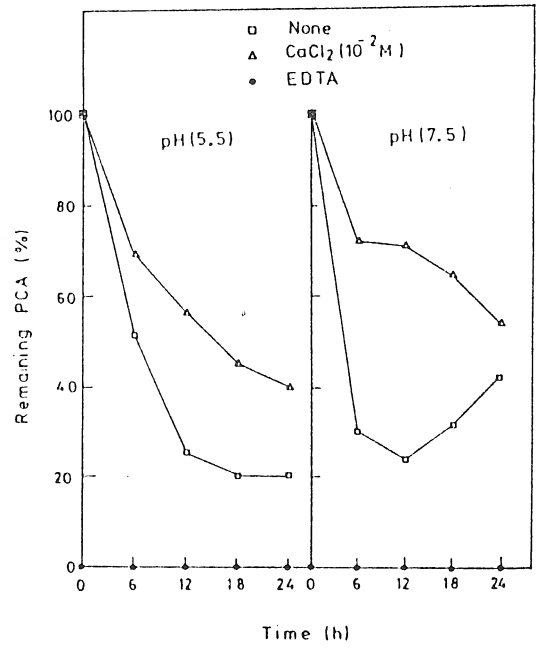

Fig.3. Effect of calcium ions and EDTA on the stability of purified plasma coagulase at pH 5.5 and 7.5. The experimental conditions are described in the text.

at pH 9.5 inactivated it completely in all treatments (Table 1). Ca^{2+} enhanced the enzyme activity by about 60% at pH 5.5 and 45% at pH 7.5 after 24 hours. Similarly, Danno and Yoshimura (1967) reported that addition of calcium ions to *A. sydowi* alkaline protease prevented its inactivation at pH 5.5 and 7.5, whereas at pH 9.0 calcium ions accelerated the rate of inactivation of the enzyme. Balasubramanian and Manocha (1986) reported that Ca^{2+} enhanced the proteinase activity of *Choanephora cucurbitarum*. It was not clear to them wheather Ca^{2+} activation was directly related to the enzymatic mechanism or was actually the result of stabilization of the enzyme against autolysis and denaturation on the glass surfaces.

The effect of the well known anticoagulant drug, hemoclar (pentosan polysulfate) on the activity of the pure *A. sydowi* coagulase was studied. It was found that pentosan inhibited plasma coagulation by the fungal enzyme (Fig. 4). The rate of inhibition was parallel to drug concentration. Addition of calcium ions could antagonise the inhibitory effect of the low doses of hemoclar while it failed at high doses.

Table 1. Effect of calcium ions and EDTA on the stability of *A. sydowi* plasma coagulase activity.

pH			Incubation time (h)				
			0.0	6.0	12.0	18.0	24.0
5.5	None	PCA (u/ml)	0.792	0.385	0.300	none	none
		Remaining activity	100.0	51.4	62.1	-	-
	EDTA (10^{-3} M)	PCA (u/ml)	none	none	none	none	none
	$CaCl_2$ (10^{-2} M)	PCA (u/ml)	0.328	0.228	0.185	0.149	0.132
		Remaining activity	100.0	69.5	56.4	54.4	40.2
7.5	None	PCA (u/ml)	0.762	0.579	0.563	0.521	0.436
		Remaining activity	100.0	24.0	26.1	31.7	42.8
	EDTA (10^{-3} M)	PCA (u/ml)	none	none	none	none	none
	$CaCl_2$ (10^{-2} M)	PCA (u/ml)	3.769	2.721	2.688	2.442	2.049
		Remaining activity	100.0	72.2	71.3	64.8	54.4
9.5	None	PCA (u/ml)	none	none	none	none	none
	EDTA (10^{-3} M)	PCA (u/ml)	none	none	none	none	none
	$CaCl_2$ (10^{-2} M)	PCA (u/ml)	Clumping	Clumping	Clumping	Clumping	Clumping

Barrowcliffe et al. (1986) reported that the mechanism of anticoagulation by hemoclar, in-vivo, was attributed to the release of hepatic triglyceride lipase (HTGL). In-vitro addition of HTGL to plasma markedly enhanced the anti-Xa clotting activity and caused a 70% inhibition to lipid peroxidase induced thrombin generation. They concluded that hemoclar prevents thrombin formation and hence inhibits coagulation.

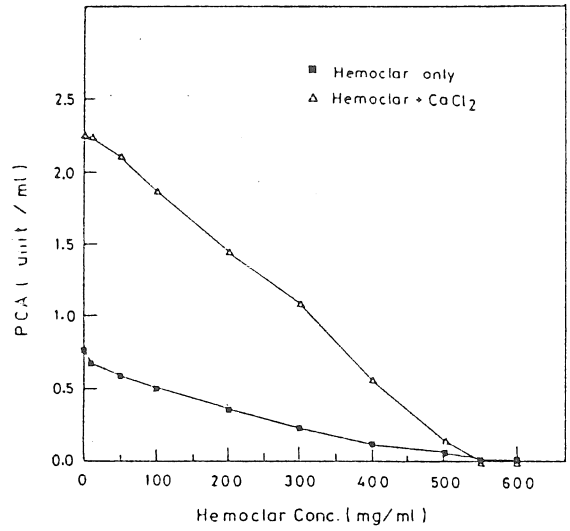

Fig.4. Effect of different concentrations of pentosan polysulfate (hemoclar) on PCA in presence and absence of $CaCl_2$ (10^{-2} M). Standard assay conditions were used except for the enzyme which was preincubated with hemoclar or hemoclar+$CaCl_2$ for 15 min.

Investigating the influence of several metal ions on enzyme activity (Table 2) revealed that Hg^{2+}, Cu^{2+} and Zn^{2+} immediately flocculated plasma protein (non enzymatically) at the time of addition. It was found that Ca^{2+}>Li^{2+}>Mn^{2+}>Co^{2+}>Na^{+}>K^{+}>Cd^{2+} were all promotive to the enzyme activity. In this connection, Klechkovaskaya et al. (1980) reported that plasma coagulase of *Aspergillus ochraceus* was inhibited by the ions of Cu, Ag, Pb, Zn and Hg (10^{-3} M) by 100, 91, 85, 50, 50, 38 and 25% respectively. Danno and Yoshimura (1967) found that Zn^{2+}, Ni^{2+} and Hg^{2+} were inhibitors to the homogenous milk

Table 2. Influence of metal ions and other chemicals on purified coagulase activity.

Activities	10^{-4} M		10^{-3} M	
	PCA (u/ml)	Activity change %	PCA (u/ml)	Activity change %
Metals				
• $CaCl_2$	2.46	402.00 (+)	2.85	482.0 (+)
• $CdSO_4$	1.24	153.00 (+)	2.12	332.6 (+)
• $CoCl_2$	2.08	324.00 (+)	1.34	173.5 (+)
• KCl	1.45	196.00 (+)	1.37	180.0 (+)
• $LiSO_4$	2.30	369.00 (+)	2.23	210.0 (+)
• NaCl	1.87	282.00 (+)	1.25	219.0 (+)
• $MnSO_4$	2.15	338.00 (+)	1.72	251.1 (+)
• $CuSO_4$	Clumping		Clumping	
• $HgCl_2$	Clumping		Clumping	
• $ZnSO_4$	Clumping		Clumping	
Chemicals				
• K-oxalate	none	-	none	-
• K-citrate	none	-	none	-
• Na-salycilate	0.44	10.20 (-)	0.27	159.5 (-)
• $Na(CH_3COO)_2$	Clumping		Clumping	
• Glutathione	0.50	2.04 (+)	0.52	2.6 (+)
• EDTA	none	-	none	-
• Heparin	none	-	none	-

Control for PCA=0.49
(+), (-):Activity increase or decrease than control

clotting protease of *A. sydowi*, while Ca^{2+} markedly protected it from inactivation. Balasubramanian and Manocha (1986) found that Hg^{2+}, Fe^{2+} and Zn^{2+} inhibited the protease activity of *Chanephora cucurbitarum*, while Ca^{2+} and Cu^{2+} activated it by 2 and 4 folds respectively.

Addition of some chemicals revealed that potassium citrate and potassium oxalate at 10^{-4} and 10^{-3} M concentrations were complete plasma anticoagulants (Table 2). Aspirin (Na- salicylate) inhibited the coagulation of plasma and marked inhibition was detected by increasing aspirin concentration. Lead acetate resulted in immediate flocculation of plasma protein (non-enzymatically) at the time of addition. EDTA and heparin inactivated the *A. sydowi* coagulase completely. Glutathione had no detectable effect on plasma clotting activity.

Lotter and Horstmann (1967), Rammell (1962) and Wood (1959) recommended oxalate and citrate as anticoagulants to plasma. Difco (1966) and BBL (1968) laboratories recommended the use of EDTA as anticoagulant since it is not utilized by bacteria and also it eliminates false positive coagulation of plasma by citrate, oxalate and acetate utilizing organisms. Levin et al. (1984) stated that aspirin inhibits the activity of vascular plasminogen activators in-vivo and concluded that aspirin may have an antifibrinolytic effect in man.

The observation that EDTA completely inhibits *A. sydowi* coagulase activity suggests that this enzyme is a metalloenzyme. In accordance with the present result, Klechkovaskaya et al. (1980) reported that the plasma coagulase of *Aspergillus ochraceus* was entirely inhibited with EDTA. Cino and Tewari (1976) found that *Oidiodendron kalari* protease was irreversibly inactivated by EDTA. The same conclusions were reported by Danno and Yoshimura (1967) for *A. sydowi* protease and Rippon and Hoo (1971) for *Trichophyton schonleinii* protease. Oppositely, Balasubramanian and Manocha (1986) found that EDTA did not inhibit the protease activity of *Choanephora cucurbitarum* and suggested that this enzyme was not a metalloenzyme.

The observation that sulfhydryl reagents such as glutathione failed to influence *A. sydowi* coagulase indicated that free sulfhydryl group was not required for enzyme activity. This finding agrees with those of Somkuti and Babel (1968), Bergkvist (1963) and Fukumoto et al. (1967). Conversely, the necessity of sulfhydryl group for yeast proteinase was described by Kominami et al. (1981) and Balasubramanian and Manocha (1986).

On investigating the influence of some vitamins on the activity of *A. sydowi* pure coagulase (Table 3) it was found that the effect of vitamins on plasma clotting activity was concentration dependant, in which low doses (10^{-5} M) inhibited plasma coagulation while high concentrations (10^{-4} M) accelerated it. Vitamin K, on the other hand, enhanced coagulation by the fungal enzyme at both experimental concentrations. Abdel-Fattah and Mabrouk (1971) found that ascorbic acid inhibits milk clotting enzyme activity of *Aspergillus niger*. The role played by vitamin K in enhancing coagulase activity from different sources has been reported by several investigators (Moslet and Hansen, 1992; Mosterd and Thijssen, 1992; Matsuura et al. 1989; Bobichon and Bouchet, 1987; Schentag et al. 1987).

Studying the effect of some antibiotics on purified *A. sydowi* coagulase activity

Table 3. Effect of some vitamins on PCA of the purified coagulase enzyme.

Activities / Vitamins	10^{-5} M PCA g (u/ml)	10^{-5} M Activity change (%)	10^{-4} M PCA (u/ml)	10^{-4} M Activity change (%)
Pyridoxin-HCl	0.330	19.5-	0.47	14.6+
Pyridoxal-5-phosphate	0.364	11.2-	0.53	29.2+
Riboflavine	0.081	80.2-	0.53	29.3+
Thiamine-HCl	0.387	5.6-	0.47	14.9+
Vitamin K	0.530	29.3+	0.88	114.6+

Control for PCA=0.41 u/ml.
(+), (-): Activity increase or decrease than control

(Table 4) revealed that penicillin, actinomycin D and streptomycin act as anticoagulants. Akiyama et al. (1988) found that aztreonam, cefoperazon, ampicillin and cephalosporins prolonged the blood coagulation time and decreased the normal prothrombin in a dose-dependent manner. Lipsky (1988) observed adverse effect of tetrazole antibiotics on blood coagulation (Table 4).

Table 4. Effect of some antibiotics on PCA.

Activities / Antibiotics	100 unit/ml PCA (u/ml)	100 unit/ml Activity change (%)	1000 unit/ml PCA (u/ml)	1000 unit/ml Activity change (%)
Actinomycin D	0.41	14.0-	0.40	16.7-
Penicillin	0.44	8.0-	0.41	14.6-
Streptomycin	0.38	20.0-	0.37	22.9-

Control for PCA=0.48 u/ml.
(+), (-): Activity increase or decrease than control.

The influence of different plasma sources on the activity of *A. sydowi* pure coagulase revealed that the fungal enzyme could coagulate human and rabbit plasma while it failed to coagulate plasma from guineapig, rat and monkey (Table 5). Addition of calcium ions to plasma stimulated the coagulating activity in human and rabbit plasma only. This indicates that the plasma clotting factors vary among animal species leading to differences in coagulating behaviour. Burrows and Moulder (1968) and Orth et al. (1971) stated that most coagulase enzymes clotted human, pig, rabbit and horse plasma but not rat, chicken, guinea pig, bovine or sheep plasma. Orth et al. (1971) showed also that the coagulation reaction factor (CRF) was highest in human plasma with a decreasing concentration in pig, rabbit, horse and lowest in bovine, chicken and sheep. Tager and Drummond (1965) recommended the following plasma criteria for coagulase testing: It must contain sufficient concentration of CRF and fibrinogen and be fairly free of fibrinolytic activity and inhibitors. They also observed low fibrinolysis when rabbit, human, sheep and horse plasma were used.

Table 5. Effect of different plasma sources on PCA.

Source of plasma	PCA (u/ml)	PCA (u/ml) + $CaCl_2$
Human	0.733	2.000
Guiea-pig	-ve	-ve
Rat	-ve	-ve
Monkey	-ve	-ve
Rabbit	0.03	0.100

Finally the solubility of the plasma clot resulting from *A. sydowi* coagulase in urea (Table 6) was examined. It was completely insoluble in 5 and 10 M urea, while it was completely soluble in 0.25 M NaOH. This finding is in accordance with the conclusion of Laki and Lorand (1948) who reported that the fibrin clot obtained from blood plasma is insoluble in 5 M urea solution, whereas the clot obtained from pure fibrinogen is readily soluble. They ascribed the insolubility of the plasma clot to the presence of a factor present in normal plasma which introduces firm bonds between fibrin molecules during clotting rendring it rendening in urea. Loewy et al. (1961) succeeded in purifying this factor and suggested that the Laki-Lorand Factor (LLF) possibly converts the intramonomer disulfide bonds to intermonomer disulfide bonds.

Table 6. Solubility of plasma clot in urea and sodium hydroxide.

Reagent	Protein (mg)
5 M urea	0.016
0.25 mM NaOH	0.253
10 M urea	0.024
0.25 mM NaOH	0.256

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