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PURIFICATION AND CHARACTERIZATION OF COAGULATING ENZYME FROM *ASPERGILLUS SYDOWI*

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A coagulase of Aspergillus sydowi has been purified to near homogenity from culture filtrate by treatment with $Ca_3(PO_4)_2$ gel, precipitation with ammonium sulphate, chromatographed on DEAE cellulose, DEAE Sephadex and Sephadex G-150. Fractionation of $Ca_3(PO_4)_2$ gel eluate on DEAE cellulose gave 5 active fractions with a low protein content whereas fraction of supernatant on DEAE Sephadex separates 3 active fractions (a, b and c). All the above active fractions coagulate both plasma and milk. The apparent molecular weight (for the three fractions a, b and c) was 65 000 Da as indicated by Sephadex G-100 gel filtration and SDS-PAGE. Fraction b was used as representative for characterization of coagulase. The purified enzyme had optimum temperature of 35°C, optimum pH 7 and 7.9 and Km values 3.439 mg/ml and 7.14 mg/ml for plasma and milk respectively.

Keywords: *Aspergillus sydowi; Coagulase; Plasma; Milk; Purification; Properties*

Introduction

Many studies on the properties of mold proteases have been carried out on purified preparations (Somkuti and Babel, 1968; Meusso-doerffer et al., 1980; Govind et al., 1981; Kominami et al., 1981; North, 1982; Klechkovskaya and Egorov, 1983; Ruchel et al., 1985; Balasubramanian and Manocha, 1986). Frequently, studies are mainly concerned with the milk-clotting activity of proteases (Sardinas, 1972; Etoh et al., 1992; Bayoumi, 1992; Jiao et al., 1992). Balasubramanian and Manocha (1986) purified an acid proteinase from mycelial extracts of *Choanephora cucurbitarum*. The enzyme hydrolyzes both bovine albumin and haemoglobin. Cinno and Tewari (1976) indicated that *Oidiodendron kalari*, in the yeast phase, produces a complex of at least six proteolytic enzymes with fibrinolytic activity. Multiple fungal proteolytic enzyme systems were also investigated by Bergkvist (1963), Hata et al. (1967), Lenny and Dalbec (1967) and Zuber (1970).

The aim of this investigation was the purification of plasma and milk by *A. sydowi* coagulase and studying some properties of the purified enzyme.

Materials and Methods

Microorganism and culture method

Aspergillus sydowi Thom and Church isolated from blood and whey by Abdel-Rahman et al. (1992) was used through this study. For optimum coagulase production the following semisynthetic medium was employed: 10.7 g/l blood powder, 11.0 g/l glucose monohydrate, 5 g/l whey powder, 200 µg/l K^+. The pH was adjusted to 6.5 and the incubation temperature was 37°C for 7 days under static conditions. The medium was dispensed in 350 ml quantities in 2000 ml flasks, sterilized and inoculated with 1 ml conidial suspension of *A. sydowi*.

Assay of milk clotting activity

The enzyme was assayed by incubating one ml of skim milk (El Naser Company, Egypt) at 40°C for 10 minutes. One ml of enzyme was added and the contents were thoroughly mixed. From time to time each tube was taken and held in an inclined position in order to detect the first sign of clotting. One unit of enyzme activity was that which clotted 1 ml milk in one minute at 40°C (Berridge, 1952).

Assay of plasma clotting activity

Human plasma or standard human plasma (diluted 1:5 by distilled water) was used as the substrate. The activity of coagulase was determined according to the method described by Beesley et al. (1967). The reaction mixture contained: 0.5 ml of 5 mM 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 liquid out of the clot, taking care to pick up on the rod any detached pieces of clot. The clot was washed in several portions of water, then placed in one ml of 5 mM NaOH in a bath of boiling water until the clot was completely dissolved, cooled and neutralized with sulphuric acid. The protein was measured using the method of Lowry et al. (1951). One unit of enzyme activity was defined as the amount of enzyme

which catalyzes the formation of one mg clotted protein/24 hours.

Purification of A. sydowi coagulase

Unless otherwise stated all purification steps were performed at 0-4°C. After each treatment the amount of protein was determined and plasma and milk clotting activities were assayed.

Crude extract

The crude extract was prepared by filtering the broth through four layers of guaze and then through prechilled puchner. The muddy matter was removed by centrifugation at 20.000 r.p.m. and the supernatant was designated as crude extract.

Heat treatment

The temperature of the crude extract was raised to 55°C with continuous stirring and kept at this temperature for 10 min, followed by cooling. The precipitate formed was removed by centrifugation and discarded.

Calcium phosphate gel treatment

Calcium phosphate gel (0.4% solid content) was prepared according to Tiselius et al. (1956). The gel was slowly added to the dark brown supernatant after heat treatment with continuous mixing. The mixture was allowed to stand for 3 hours followed by centrifugation. The adsorbed fraction was eluted with 2 M NaCl prepared in 0.05 M phosphate buffer, pH 7.4.

Ammonium sulphate precipitation

Solid $(NH_4)_2SO_4$ was added to each of supernatant and eluate of $Ca_3(PO_4)_2$ gel to bring the salt concentration to 80%. The mixtures were allowed to stand in cold (6°C) for 24 hours and the precipitate was collected by centrifugation at 20.000 r.p.m. for 10 min. The precipitated protein was dissolved in least amount of 0.05 M phosphate buffer pH 7.0. Ammonium sulphate fractions obtained from either calcium phosphate gel eluate or supernatant were dialyzed against the same buffer.

Chromatography on DEAE-cellulose

DEAE-cellulose was pretreated as recommended by Peterson and Sober (1962). The dialyzed ammonium sulphate fractions of $Ca_3(PO_4)_2$ gel supernatant was chromatographed on DEAE cellulose column (1.3 x 36 cm) and the exchanged material was eluted with stepwise gradient of 0.05-0.3 M NaCl prepared in the same buffer. 10 ml fractions were collected at a flow rate of 2 ml/min.

Chromatography on DEAE Sephadex

a- The pooled lyophilized active fractions obtained from the previous column was applied in small amount of phosphate buffer, pH 7.0, to DEAE Sephadex column (1.3 x 76 cm) which had been equilibrated with the same buffer.

b- The enzyme solution of $Ca_3(PO_4)_2$ gel eluate, concentrated with ammonium sulphate was dialyzed and applied to DEAE Sephadex column (1.3 x 70 cm). The exchanged material was eluted in succession by increasing NaCl molarity from 0.0-0.3 M. 10 ml fractions were collected at a flow rate of 2 ml/min.

Chromatography on Sephadex G-150

The pooled lyophilized fraction from DEAE Sephadex was applied to a Sephadex G-150 column (1.3 x76 cm) and elution was carried out using 0.05 M phosphate buffer (pH 7.0).

Protein determination

Protein concentration was routinely determined spectrophotometrically at 280 and 260 nm by the method of Segel (1968). In the presence of any interfering compound the procedure of Lowry et al. (1951) was followed.

Molecular weight determination

A- *Gel filtration*: The molecular weight of the purified enzyme was determined with the use of gel filtration on Sephadex G-100 column (Segel, 1968). A sample of the purified enzyme in 1 ml buffer was applied to the top of the column preequilibrated with 0.05 M phosphate buffer pH 7.0. The void volume (Vo) of the column was determined by the use of blue dextran. The column (30 x 1 cm) was calibrated using molecular weight standard protein (1 mg/ml) (bovine serum albumin 67 K.Da, pepsin 35 K.Da, carbonic anhydrase 29 K.Da and lysozyme 14.4 K.Da). The same run procedures were followed for enzyme peaks a, b and c.

The molecular weight of the purified enzyme was determined from the calibration curve of Sephadex G-100 gel between log molecular weight of the standard proteins versus relative elution volume (Ve/Vo) (Fig.1).

B- *Electrophoresis*: SDS-PAGE was carried out on 12.5% acrylamide gel according to the method of Laemmli (1970). The position of the protein

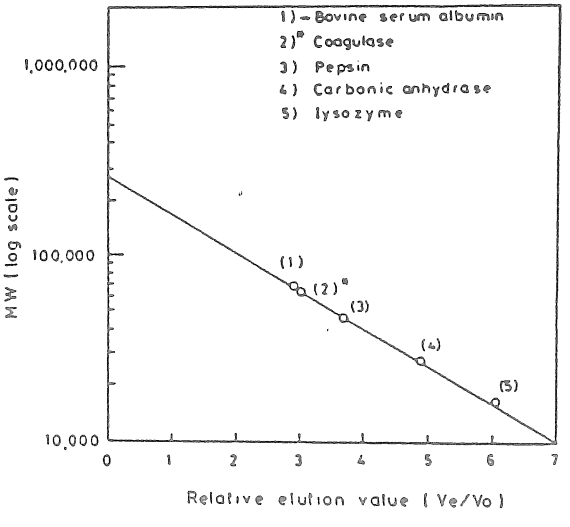

Fig.1. Estimation of the molecular weight of *A. sydowi* coagulase* by gel filtration

bands was determined by staining with silver nitrate (Morrissey, 1981). A protein sample (12 µl) containing 10-15 µg protein was mixed with 3 µl buffer and heated in boiling water bath for 5 min followed by centrifugation. Molecular mass marker proteins (phosphorylase B 97.4 K.Da, bovine serum albumin 66.2 K.Da, ovalbumin 45 K.Da, carbonic anhydrase 31 K.Da, soybean trypsin inhibitor 21.5 K.Da and lysozyme 14.4 K.Da) were used (Fig. 2).

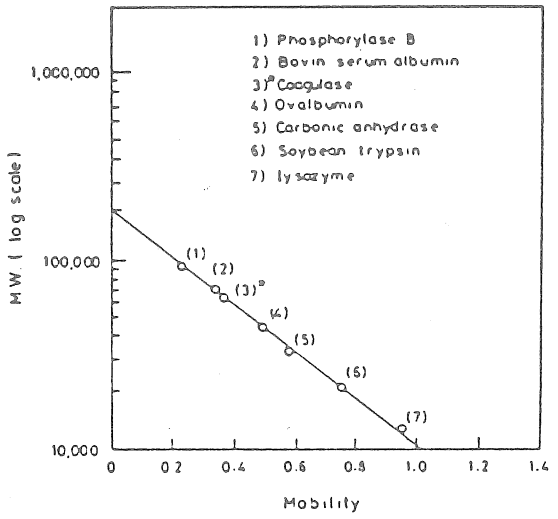

Fig.2. Estimation of the molecular weight of *A. sydowi* coagulase* by SDS-PAGE

Results and Discussion

A simple reproducible method for the preparation of pure homogenous *A. sydowi* coagulase was established. Although heat treatment (Table 1) led to no change in the clotting activity and the specific activity was slightly changed, this step was important to get rid of impurities present in the crude extract. Through calcium phosphate gel treatment the active enzyme appears in both supernatant and eluate. Concerning $(NH_4)_2SO_4$ for supernatant, 6 and 2.8 fold increase in specific clotting activity was manifested in plasma and milk respectively over the crude extract. For eluate the specific activity raised to 5.7 and 2.2 fold over crude extract for plasma and milk (Table 1). Purification on DEAE-Sephadex of calcium phosphate gel eluate (Fig. 3) reveals a five enzyme fractions with reproducable specific activities. However further purification of these fractions was difficult owing to their relatively low protein contents. Further purification of calcium phosphate gel supernatant on DEAE cellulose (Fig.4) revealed that *A. sydowi* coagulase was eluted in two peaks (within the first protein peak) designated as, (a) and (b) according to their elution order from the column. Fractionation on DEAE-

Table 1. Purification scheme of *A. sydowi* coagulase(s)

Procedure	Total volume (ml)	Total protein (mg)	Total activity (u/ml)		S.A.*		Recovery %	
			PCA	MCA	PCA	MCA	PCA	MCA
1) Crude	1850.00	4766.00	2436.0	9866.0	0.51	2.06	100.00	100.00
2) Heat	1845.00	4686.00	2398.0	9834.0	0.51	1.99	98.40	99.70
3) Calcium phosphate gel								
A) Supernatant	2185.00	2091.00	1874.0	1748.0	0.89	0.83	76.90	17.70
B) Eluate	530.00	559.00	435.0	848.0	0.77	1.52	17.90	8.60
4) $(NH_4)_2SO_4$ for								
A) Supernatant	32.50	209.00	647.7	1274.0	3.00	5.90	26.60	12.90
B) Eluate	9.85	136.40	375.0	611.0	2.85	4.62	15.40	6.19
5) Column chromatography of supernatant (A)								
i) DEAE cellulose	5.00	135.00	322.0	793.0	2.38	5.87	13.20	8.00
Peak (a)		24.10	142.9	563.0	5.90	23.40	5.90	5.70
Peak (b)		21.60	180.0	229.5	8.30	10.60	7.40	2.30
ii) DEAE -Sephadex	5.00	78.00	263.0	620.0	3.37	7.95	10.80	6.30
Peak (a)		3.60	37.5	173.0	10.40	48.00	1.50	1.80
Peak (b)		50.60	190.0	422.0	3.70	8.40	7.80	4.30
Peak (c)		3.80	34.3	25.0	9.00	6.60	1.40	0.30
iii) Sephadex G-150	3.00	24.00	170.0	-	3.40	-	7.00	-
6) Column chromatography of eluate (B) on DEAE-Sephadex	3.00	86.00	289.0	383.0	3.36	4.46	11.86	3.82
Peak (a)		16.30	50.3	213.0	3.08	13.06	2.06	2.15
Peak (b)		1.83	16.1	4.0	8.79	2.19	0.66	0.04
Peak (c)		3.78	58.9	27.0	15.58	7.14	2.40	0.37
Peak (d)		4.47	88.5	113.4	19.79	25.37	3.63	1.15
Peak (e)		7.16	28.8	12.0	4.02	1.68	1.18	0.12

*S.A.= Specific activity (unit/mg protein)

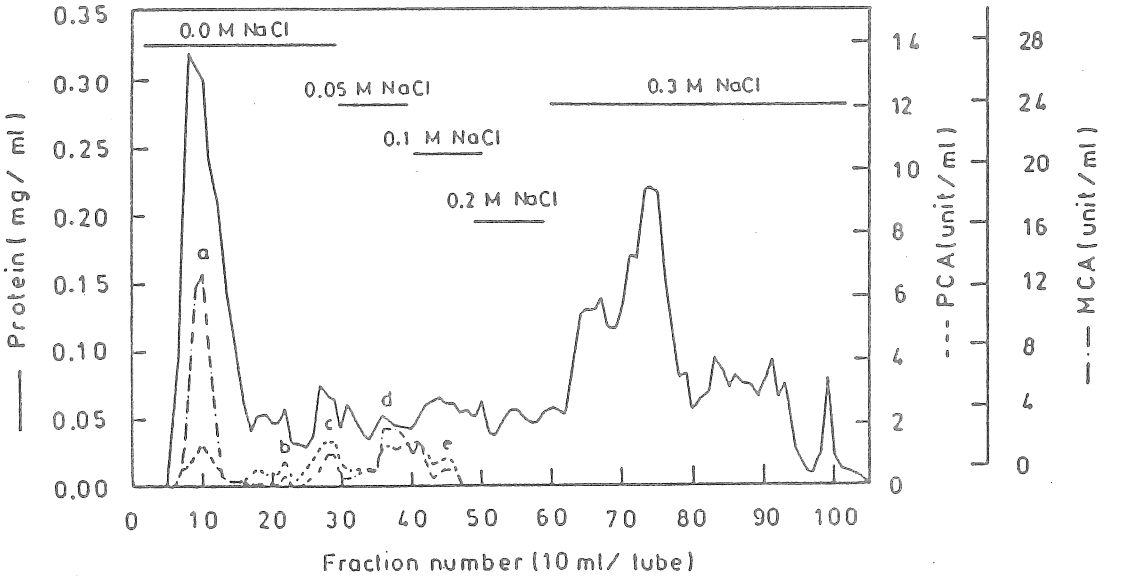

Fig.3. Typical elution profile for the behaviour of *A. sydowi* coagulase of calcium phosphate gel eluate on DEAE-Sephadex

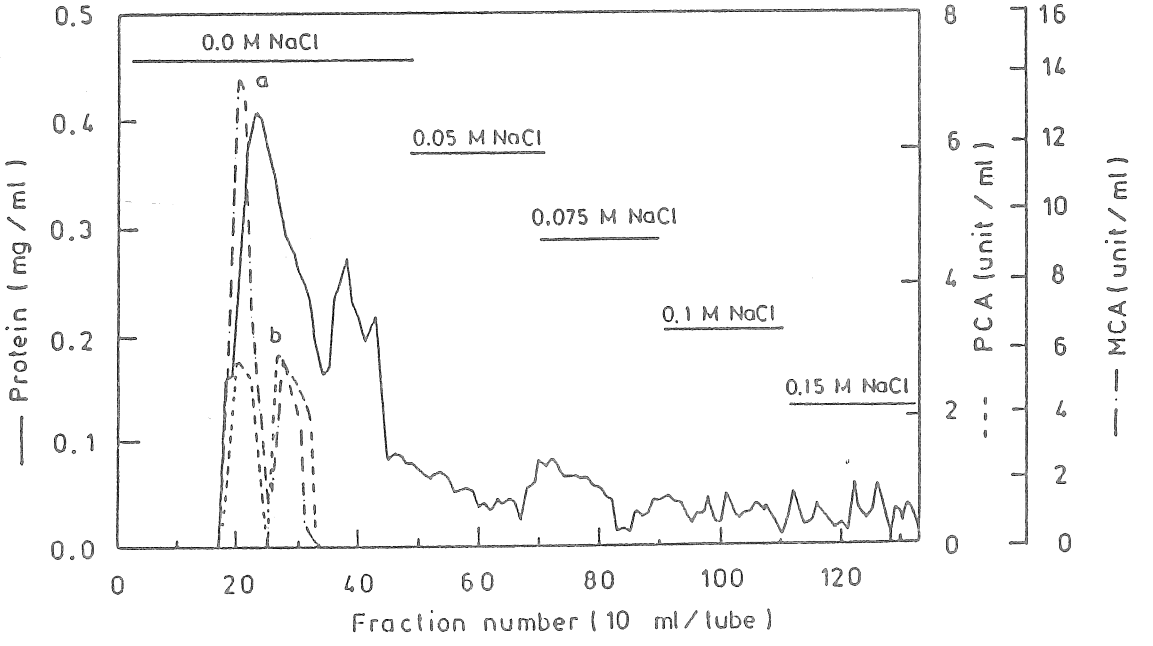

Fig.4. Typical elution profile for the behaviour of *A. sydowi* coagulase of calcium phosphate gel supernatant on DEAE-cellulose

Sephadex (Fig. 5) led to the separation of three distinct fractions designated as (a, b) and (c) according to their elution order from the column. Fraction (b) showed the highest protein level, so it was used for further purification on Sephadex G-150 (Fig.6) The specific activity of the final preparation was found to be 7.8 and 16.5 for plasma and milk, respectively. During the purification of *A. sydowi* extracellular coagulase, only several purification

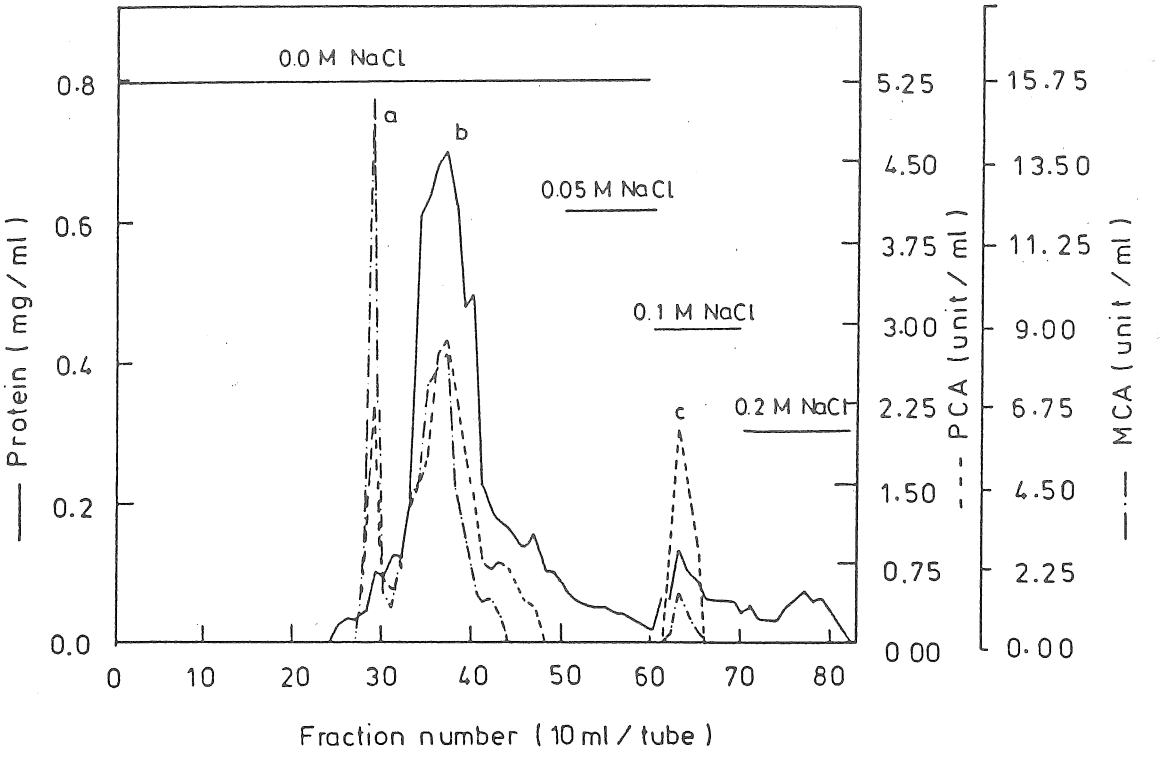

Fig. 5. Typical elution profile for the behaviour of *A. sydowi* coagulase of calcium phosphate gel supernatant on DEAE-Sephadex

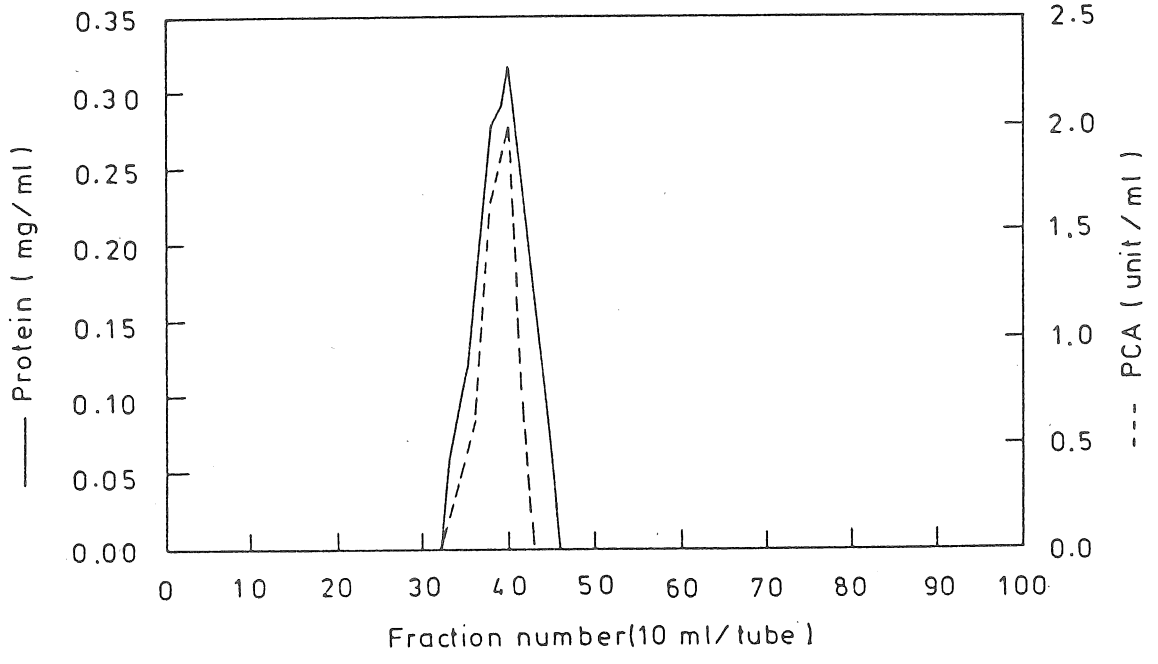

Fig. 6. Typical elution profile for the behaviour of *A. sydowi* coagulase of calcium phosphate gel supernatant on Sephadex G-150

fold increases (7 and 4 for plasma and milk respectively) were obtained over the crude extract. These indicated a high coagulase content in the original extract relative to other proteinaceous compounds. This may be expected because the species used were selected on the basis of their high coagulating activity by Abdel-Rahman et al. (1992).

The present data showed that *A. sydowi* coagulase produces a complex of at least three clotting isoenzymes which differ in their affinity to clot plasma and milk. The phenomenon that coagulase enzyme was assayed when the fungus was firstly cultivated on the synthetic Stefanini medium indicated that at least one of the three enzymes fractionated during purification is constitutive and the others are induced. This finding is matching with several other investigators (Cino and Tewari, 1976; Bergkvist, 1963; Dernby, 1971; Hata et al., 1967; Lenny and Dalbec, 1967; Zuber, 1970) who have characterized multiple fungal proteolytic enzyme systems. North and Harwood (1979) described eight fungal types of proteinases from *Dictyostelium discoideum* which can be distinguished from one another on the basis of their sensitivity to various inhibitors.

It is quite difficult to compare the different purification schemes reported by different investigators for the preparation of coagulases from different sources. This is mainly because different units were used in calculating the specific activity and the great variation in the initial specific activites of the starting crude material. However, a purification scheme for preparation of a homogenous enzyme with 3.4 purification folds over crude for *Mucor pusillus* milk coagulase were recorded by Smokuti and Babel (1968), 18 purification folds for *Aspergillus candidus* proteinase by Nasuna and Ohara (1972), 4 purification folds for protease of *Choanephora cucurbitarum* by Balasubramanian and Manocha (1986), 4.5 purification folds for *A.sydowi* protease by Danno and Yoshimura (1967) and 5.7 purification folds for *Oidiodendron kalari* collagenase by Cino and Tewari (1976).

Characterization of pure *A. sydowi* coagulase was carried out on fraction (b) obtained from Sephadex G-150 of calcium phosphate gel supernatant (Table 1). The determination of the apparent molecular weight of *A. sydowi* coagulase shows that it is 65 K.Da by using gel filtration and SDS-PAGE methods (Figs1, 2 and Plate 1) which is higher than the range of 30-45 K.Da reported for other fungal proteinases (North, 1982). Two years later, North and Whyte (1984) purified two proteinases from cell extract of *Dictyostelium discoideum* and found that the apparent molecular weight for one of them was 58 K.Da and for the other 30 K.Da as indicated by SDS-PAGE. They suggested that the high molecular weight enzyme was presumably a glycoprotein and it is possible that the presence of a larger carbohydrate moiety may be responsible for the higher molecular weight. Barrett and McDonald (1980) found that mammalian cathepsin D has a molecular weight of about 42 K.Da. Nasuno and Ohara (1972) recorded a molecular weight of about 23 K.Da for a homogenous *Aspergillus candidus* alkaline proteinase.

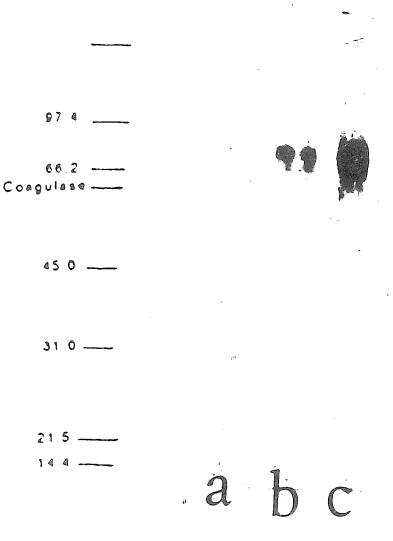

Plate 1. SDS-Polyacrylamide Gel Electrophoresis of *A. sydowi* coagulase (Fraction a, b and c). Identical patterns were obtained under reducing or non-reducing conditions. The position of marker proteins in K.Ds are indicated by arrows. Gels were stained by silver staining as detailed in the materials and methods.

In plasma and milk clotting activity, a direct relation was found between the quantity of protein and the enzyme activity (Figs 7 and 8). Linear increase in enzyme activity was recorded up to 35 µg enzyme protein.

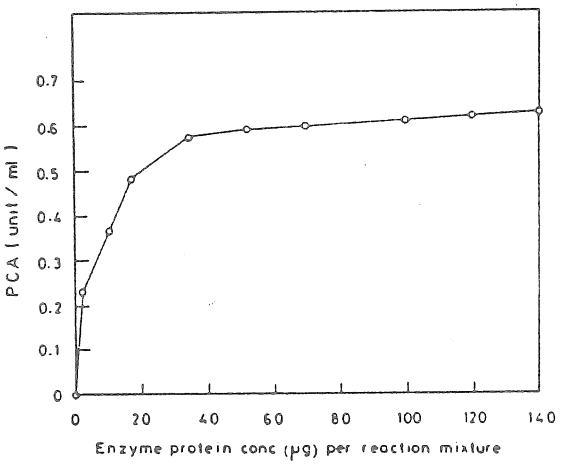

Fig. 7. Dependence of reaction rate on plasma coagulase concentrat ion. The experiment was carried out under standard assay condition in which the enzyme concentration was varied.

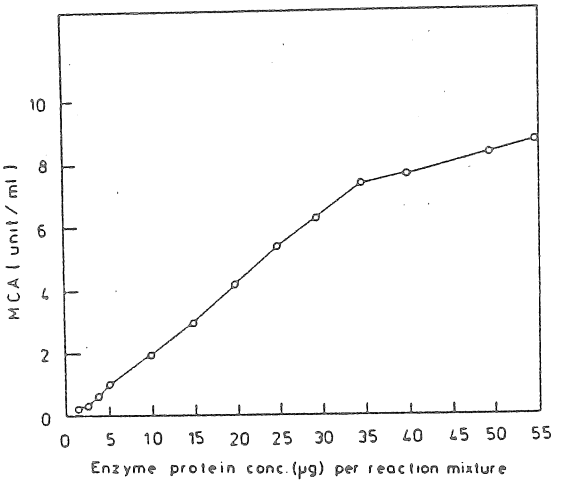

Fig. 8. Dependence of reaction rate on milk coagulase concentration. The experiment was carried out under standard assay conditions in which the enzyme concentration was varied.

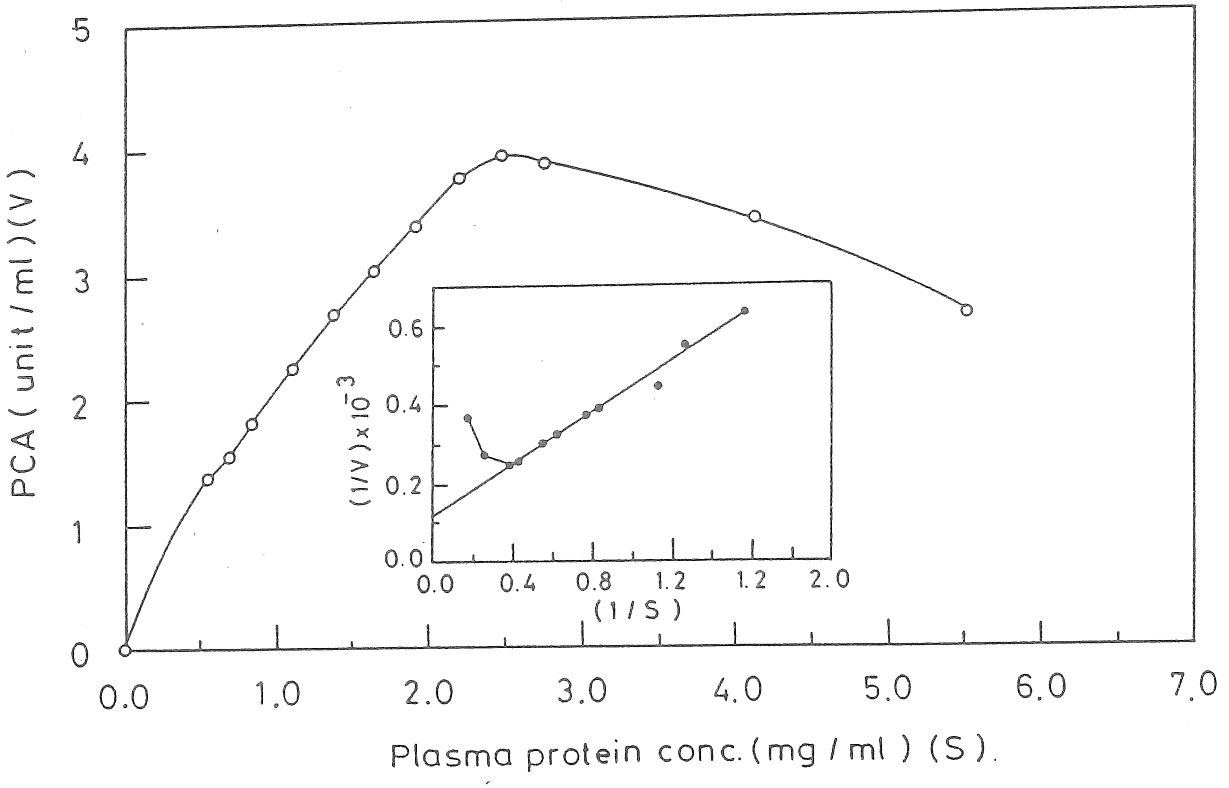

Fig. 9. Effect of substrate concentration on purified *A. sydowi* plasma coagulase. Standard assay conditions were used except for standard plasma protein which was varied as indicated. The inset shows the double receprocal plot for plasma protein, all points are derived from Fig. 9.

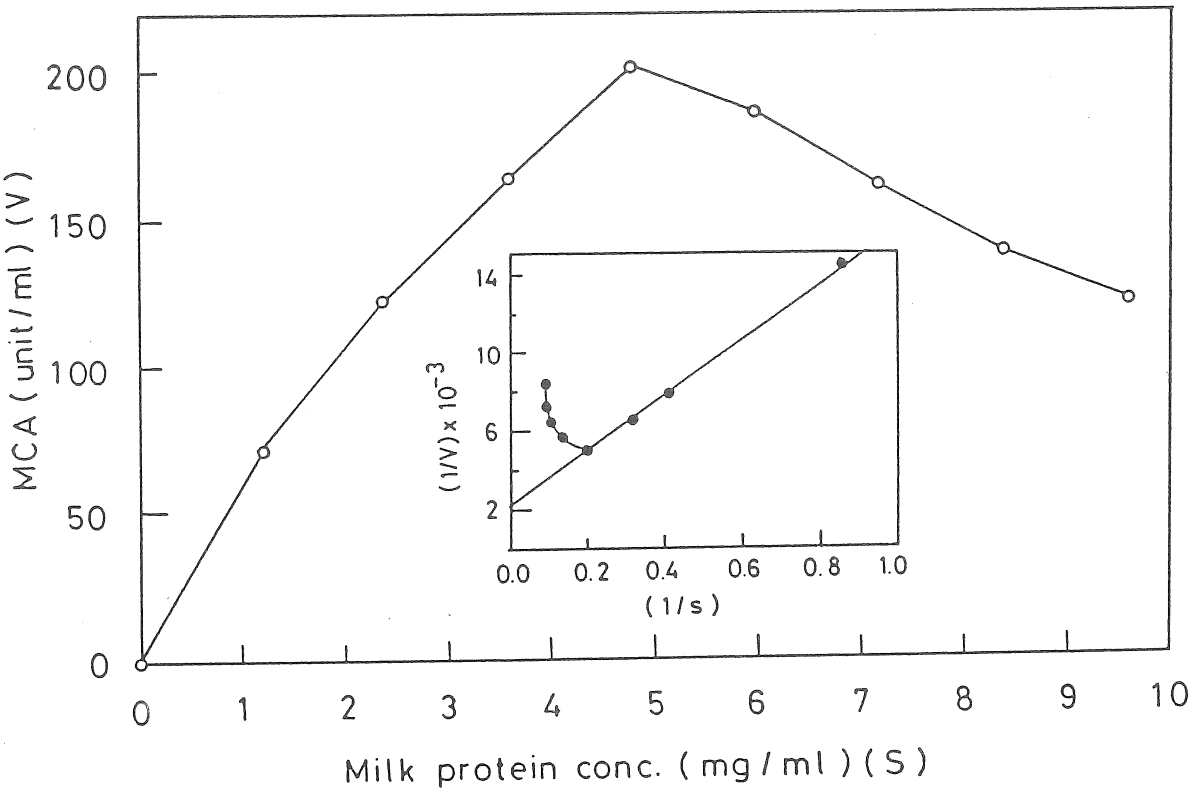

Fig. 10. Effect of milk protein concentration on purified *A. sydowi* coagulase. Standard assay conditions were used except for the milk protein, which was varied as indicated. The inset shows the double receprocal plot for milk protein, all points are derived from Fig. 10.

The Michaelis-Menten constant (Km) for*A. sydowi* plasma and milk coagulases were 2.439 mg/l and 7.140 mg/l, respectively (Figs 9 and 10). The K_m constant for other fungal proteases were 0.357% for *Mucor pusillus* milk clotting protease (Somkuti and Babel, 1968) and 2.8 mg/l for *Choanephora cucurbitarum* protease (Balasubramanian and Manocha, 1986). The K_m for human plasma clot by the action of thrombin ranged from 0.14 to 0.56 m mol/l (Fenton et al., 1986).

With respect to pH profiles, in case of plasma clotting activity a broad pH optimum around pH 7.5 in borate buffer was recorded (Fig. 11). Concerning milk coagulase, the maximum activity was at pH 7.0 in phosphate buffer (Fig12). Different pH optima were recorded for microbial coagulases as 6.8, 5.8, 3.5, 3 and 2.5 for milk clotting proteases of *Byssochlamys fulva, Aspergillus niger, Mucor pusillus, Choanephora cucurbitarum* and *Rodotorula glutinis*, respectively (Sun, 1976;

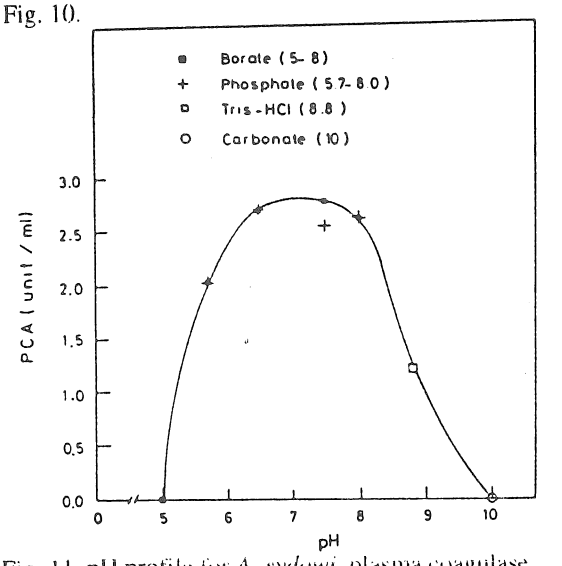

Fig. 11. pH profile for *A. sydowi* plasma coagulase. Standard assay conditions were used except for the pH value, which was varied as indicated.

Abdel-Fattah and Mabrouk, 1971; Iwasaki et al., 1967; Balasubramanian and Manocha, 1986; Kamada and Murao, 1972). Concerning plasma

clotting enzymes, the optimum pHs were 8-10, 8 and 6.5 for *Aspergillus ochraceous* (Klechkovaskaya et al., 1980), rat (Kato et al., 1987) and *Oidiodendron kalari* (Cino and Tewari, 1976) respectively.

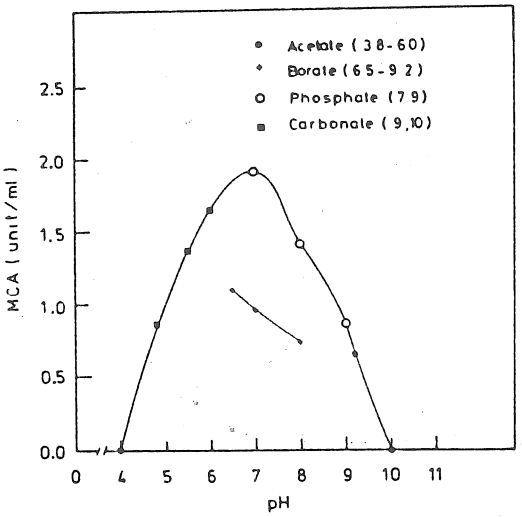

Fig.12. pH profile for purified *A. sydowi* milk coagulase. Standard assay conditions were used except for the pH value, which was varied as indicated.

The optimum temperature of *A. sydowi* was around 35°C for both plasma and milk at pH 7.5 (Figs 13 and 14). The enzyme activity was reduced by about 55% and 92% for plasma and milk, respectively at 70°C. Martin et al. (1980) found that the optimum activity of crystalline chymosin and *Mucor pusillus* milk clotting protease exhibited optimal temperature around 49, 55°C respectively, whereas, the optimum temperature for *Mucor miehei* milk clotting protease was higher than 63°C. Foda et al. (1976) found that maximum activity of milk clotting enzyme from *Aspergillus flavus* was at 25°C, the activity was destroyed by heating to a temperature higher than 50°C. Klechkovskaya et al. (1980) reported that the proteolytic enzyme of *Aspergillus ochraceus* had maximum plasma coagulating activity at 45°C. The enzyme was entirely inactivated at 55°C within 20-30 minutes.

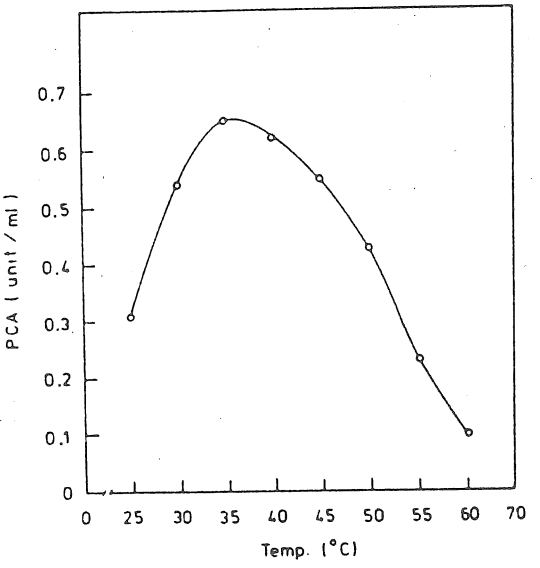

Fig.13. Effect of temperature on the activity of *A. sydowi* plasma coagulase

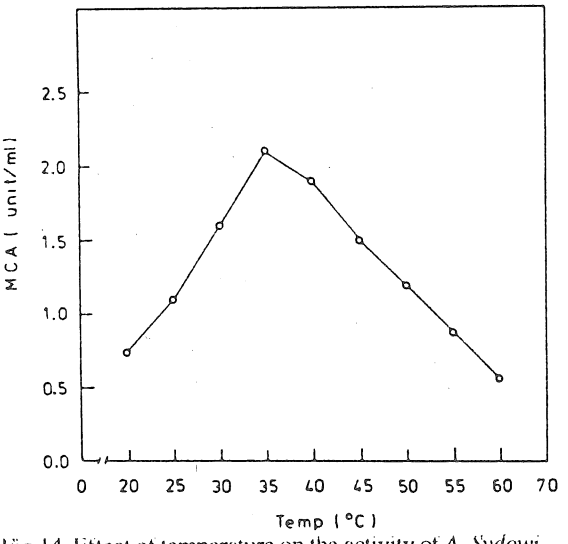

Fig.14. Effect of temperature on the activity of *A. Sydowi* milk coagulase

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