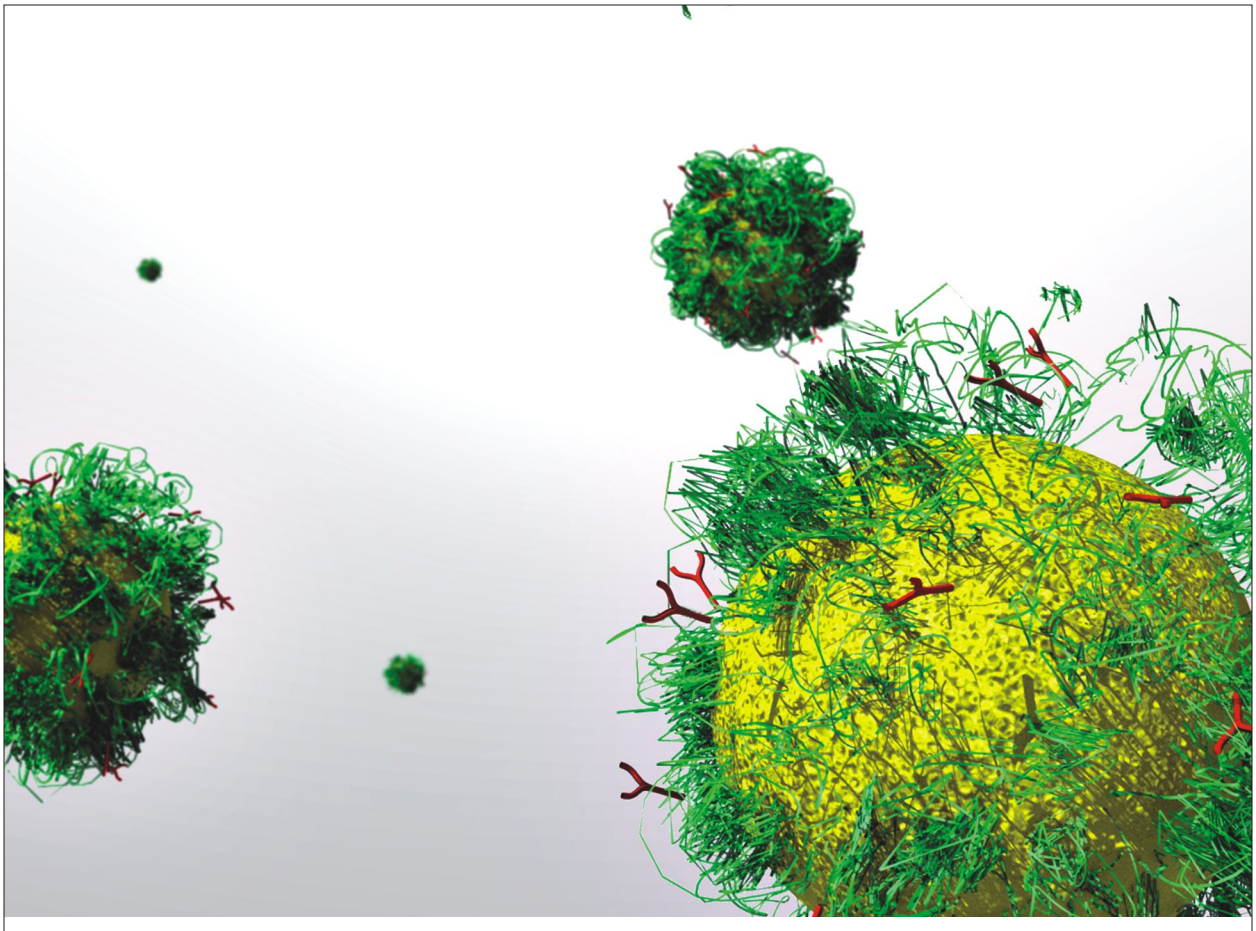

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Isolation, Partial Purification and Characterization of Angiotensin Converting Enzyme (ACE) from Rabbit (*Oryctolagus ciniculus*) Lungs

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ABSTRACT

A simple method was set up to isolate, partially purify and characterize angiotensin-converting enzyme from rabbit lungs. Angiotensin-converting enzyme was purified by a three-step method: Ammonium sulphate precipitation (30-70%), gel filtration chromatography (Sephadex G-75) and ion exchange chromatography (CM-sephadex). The enzyme activity was assayed by monitoring the rate of production of hippuric acid from the hydrolysis of Hippuryl-L-Histidine-L-Leucine by angiotensin-converting enzyme. From the results, the enzyme was purified 6.25-fold with a yield of 21% on CM-Sephadex column. The angiotensin converting enzyme from rabbit lung had a broad optimum pH range (8.0-8.3) and optimum temperature of 37°C. Initial velocity studies for the determination of kinetic constants with Hippuryl-L-Histidine-L-Leucine as substrate revealed a K_M and V_{MAX} of 1.8 and 0.4 μmol min^{-1}, respectively. The enzyme activity was not affected by Mg^{2+}, Ca^{2+}, Na^+ and K^+, while EDTA strongly inhibited it. In conclusion, this study has shown that though angiotensin-converting enzyme can be partially-purified from rabbit lungs via. a three-step purification protocol, the purification steps and assay conditions needs to be modified to obtain a higher yield and specific activity.

Key words: Angiotensin-converting enzyme, rabbit lung, hypertension

INTRODUCTION

According to the World Health Organization, hypertension can be defined as a chronic condition characterized by a systolic blood pressure greater than or equal to 140 mmHg and diastolic blood pressure greater than or equal to 90 mmHg. People with hypertension are often asymptomatic for years, hence, it is called a "silent killer" (Aftab, 1995). Hypertension is a risk factor for coronary heart disease, cardiovascular accidents, cardiac hypertrophy, aortic dissectionand renal failure. It is capable of accelerating atherogenesis and inducing changes favorable for aortic dissection and cerebrovascular haemorrhage (Holm *et al*., 2006). Studies have demonstrated that about 90-95% of cases of hypertension are primary (essential hypertension), while 5-10% is secondary (non essential hypertension). By definition, essential hypertension has no identifiable cause, while non-essential hypertension occurs as a result of an identifiable cause, which when treated can resolve without the need for antihypertensive medications (Lifton, 1996). Renal disease is the most common cause of secondary hypertension and most, if not all cases of essential hypertension is a result of disorders in renal function (Luke, 1993).

The Renin-Angiotensin Aldosterone System (RAAS) is an important regulator of blood pressure homeostasis in man. Functioning within the system is angiotensin-converting enzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1), which acts on the decapeptide angiotensin I to form angiotensin II, an active vasopressor. The enzyme also degrades the potent vasodilator, bradykinin to an inactive heptapeptide (Reeves and O'Dell, 1986; Schmieder *et al*., 2007). This has made the enzyme a target site for the treatment of hypertension, as inhibiting it, causes a decrease in angiotension II formation and diminishes angiotensin II-mediated aldosterone secretion from the adrenal cortex, leading to a decrease in water and sodium reabsorption and a reduction in extracellular volume (Klabunde, 2009).

The conversion of angiotensin I to angiotension II was initially assumed to take place within the circulatory system, however studies by Ng and Vane (1967) demonstrated that ACE activity in the plasma was insufficient to account for the rapid *in vivo* conversion. They also reported that the conversion of circulating angiotensin I to angiotension II occurred during passage through the lungs. Their report was confirmed by Stanley and Biron (1969) who demonstrated the importance of the lungs as a site of angiotensin-converting enzyme activity due to reduced angiotensin conversion observed in dogs on cardiopulmonary bypass. In another study, Cushman and Cheung (1971) investigated the angiotensin-converting enzyme activities of a large number of rat tissue homogenates and found high specific activities in the lungs and segments of the digestive tract. Other sites in which conversion of angiotensin I to angiotension II has been reported to occur include the renal vasculature of humans (Gocke *et al*., 1969), the vascular bed of the intestine and hind limb of dogs (Skeggs *et al*., 1956). Angiotensin-converting enzyme has also been isolated from human serum, with significantly higher concentrations found in the vascular bed of the lungs (Lanzillo and Fanburg, 1977).

The Cushman and Cheung method of measuring ACE activity is a spectrophotometric assay which measures the rate of production of hippuric acid from Hippuryl-L-Histidyl-L-Lycine (HHL). A fluorescence method was developed by Setandreu and Toldra (2006), radiochemical (Huggins and Thampi, 1968), radioimmunoassay (Hollemans *et al*., 1969) and the automated ninhydrin method (Dorer *et al*., 1970). Of these methods, the spectrophotometric method of assay using ACE from rabbit lungs described by Cushman and Cheung (1971) has been shown to be reliable and easily reproducible hence, its adoption in several studies (Wang *et al*., 2008; Liu *et al*., 2009; Cheng *et al*., 2009). Also, there have been discrepancies in the physicochemical properties of ACE as a result of either the substrate (Bunning *et al*., 1983; Hooper *et al*., 1987; Andujar-Sanchez *et al*., 2003) or source of enzyme (Skeggs *et al*., 1956; Gocke *et al*., 1969; Hooper *et al*., 1987). This study therefore aims to isolate and partially purify ACE from rabbit lungs, investigate it's physical and chemical properties toward its possible use for ACE assays.

MATERIALS AND METHODS

Reagents: HEPES sodium salt, Hippuryl-L-Histidyl-L-Leucine (HHL), Sephadex G75, CM-Sephadex, ammonium sulphate and calcium chloride were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other reagents used were of analytical grade.

Animals: Three apparently healthy male rabbits (domestic White New Zealand rabbits) of about 6-8 months and weighing 1.9-2.2 kg were purchased from the Department of Anatomy, Faculty of Medicine, Ahmadu Bello University, Zaria, Kaduna State, Nigeria. They were allowed access to water and rabbit feed (Vital Feeds, Zaria, Kaduna State) *ad libitum*.

Experimental design: All experimental protocols were assessed and approved by the Animal Use and Care Committee of Ahmadu Bello University, Zaria, Kaduna State, Nigeria. The rabbits were sacrificed under anesthesia and lungs collected after dissection. The lungs were cleaned of fatty tissues, rinsed in normal saline (pH 9) and homogenized in cold trizma-HCl buffer (pH 7.8). The homogenate was centrifuged at 10000×g and 4°C for 15 min repeatedly until pelleting stopped. Supernatants were stored at -80°C, until required.

ACE activity assay: The ACE activity was determined as described by Cushman and Cheung (1971). Briefly, the sample (0.2 mL) was added to ACE solution (50 µL) and the reaction started by adding 0.2 mL of 5 mmol L^{-1} hippuric histidyl leucine. This was incubated at 37°C for 15 min. The reaction was terminated by adding 0.25 mL of 1.0 N hydrochloric acid and then 2.0 mL ethyl acetate to extract the hippuric acid formed by the action of ACE. This was centrifuged at 3600×g for 2 min and 1 mL of upper layer transferred into a microcentrifuge tube and heated by dry bath at 100°C for 15 min to remove ethyl acetate by evaporation. The resulting hippuric acid was dissolved in 3.0 mL of distilled water and the absorbance read at 228 nm using a spectrophotometer (Jenway 6100, Dunmow, Essex, U.K.). Activity in tissues was expressed in units which corresponds to 1 µmol of hippuric acid released by enzymatic hydrolysis of HHL per minute per milligram of tissue.

Determination of protein content: Protein content of the sample was determined by Biuret method using bovine serum albumin as standard. Briefly, 1 mL of the sample containing 50 mM HEPES buffer and 300 mM NaCl (pH 8.3) was dispensed into two test tubes. Biuret reagent (4 mL) was added to each test tube, mixed properly and incubated at 25°C for 30 min. The absorbance was read at 540 nm and concentration determined from the standard curve of bovine serum albumin.

Partial purification of ACE

Ammonium sulphate precipitation: The supernatant was transferred into an ice cold beaker kept chilled by placing on an ice tray and precipitated by adding ammonium sulfate at increasing concentrations (30-80%). The mixture was centrifuged at 10000×g for 15 min at 4°C and supernatant discarded leaving pellet. The pellet was reconstituted in 7 mL buffer solution and ACE activity and protein concentration determined.

Dialysis: Salt components were separated from proteins by dialysis in a membranous dialysis bag. The dialysis membrane was immersed in a beaker containing 250 mL of HEPES buffer (pH 8.3) and dialyzed for 24 h with a change of buffer (Craig, 1967).

Gel filtration: The dialyzed proteins were subjected to gel filtration with Sephadex G75 (1.5×15 cm) equilibrated with HEPES buffer (pH 8.3). The protein content and ACE activity of each fraction was also determined.

Ion exchange chromatography: The fraction with the highest ACE activity was introduced into a column (1.5×40 cm) packed with resin (CM-Sephadex) and equilibrated with the HEPES buffer (pH 8.3). Elution was done stepwise with 0.0-0.5 M NaCl solution.

Characterization of enzyme

pH and temperature-dependent studies: A pH dependent assay was performed by varying the pH from 6-10. A temperature-dependent study was also conducted by determining the enzyme activity at varying incubation temperatures ranging from 20-60°C.

Effect of monovalent and divalent metal ions on ACE activity: ACE activity was assayed as described above in the presence of chloride salts of the following monovalent and divalent metal ions: Sodium, Potassium, Magnesium, Calcium as well as EDTA each at a concentration of 2 mM.

Initial velocity studies: This was done by incubating the enzyme with varying concentrations of the substrate, Hippuryl-L-Histidyl-L-Leucine (2, 5, 7 9 and 10 mM) to obtain values of initial velocity V_o. The Michealis constant (K_M) and maximum velocity (V_{MAX}) were determined from Lineweaver-Burk's plot.

RESULTS

The purification profile of ACE is summarized in Table 1 below. The specific activity of the crude enzyme was 0.049 U mg^{-1}. Precipitation of the crude enzyme increased the specific activity to 0.08 U mg^{-1} at a recovery of 69.2%. Upon dialysis, the activity of the enzyme decreased (0.198 μmol min^{-1}) while specific activity increased and about 60% yield. Gel filtration and ion exchange chromatography gave a specific activity of 0.8 and 5.0 U mg^{-1} of protein at a purification fold of 5.0 and 6.25, respectively. A final recovery of 21% was obtained after ion exchange chromatography.

Precipitation with ammonium sulphate from 30-40% saturation increased ACE activity but decreased it at 50%. There was a gradual increase in the enzyme activity above 50% precipitation. However, the highest activity was obtained at 70% ammonium sulphate saturation (Fig. 1).

Figure 2 shows the elution profile of the enzyme obtained from gel filtration chromatography. The enzyme activity increased from the first to the fourth fraction and then decreased gradually until activity was lost in fractions 14-18. A sharp increase in enzyme activity was observed in fractions 20-27 while it decreased from fractions 28-30. Fractions 3-5 had the least protein content, while fractions 23-28 had the highest protein content (Fig. 2).

During ion exchange chromatographic separation of ACE, there was a steady increase in activity, with the peak at 0.3 mM NaCl. Subsequent decrease in activity was seen as the concentration increased (Fig. 3). The temperature-dependent profile of ACE increased gradually until an optimal peak was obtained at 37°C. Further increase in temperature led to a decrease

Table 1: Purification profile of angiotensin converting enzyme from rabbit lungs

Purification step	Protein (mg mL^{-1})	Total activity (μmol min^{-1})	Specific activity (U mg^{-1})	Purificationfold	Yield (%)
Crude	10.300	0.501	0.049	1.00	100.0
$(NH_4)_2SO_4$	4.600	0.343	0.080	1.60	69.2
Dialysis	1.200	0.198	0.160	2.00	57.7
Sephadex G-75	0.006	0.048	0.800	5.00	24.2
IEC	0.002	0.010	5.000	6.25	21.0

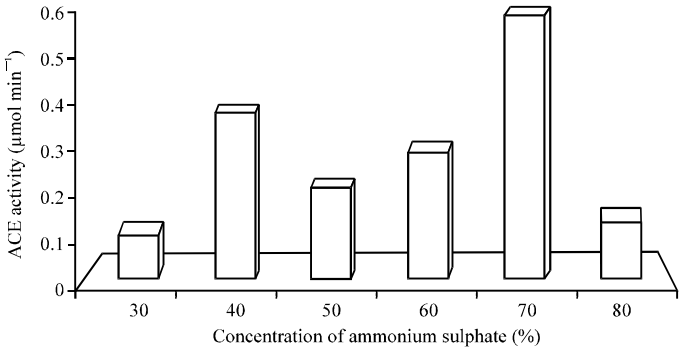

Fig. 1: Ammonium sulphate precipitation chart of angiotensin converting enzyme

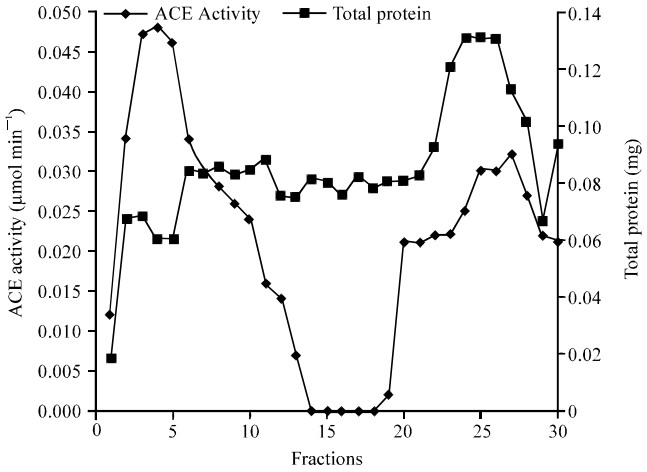

Fig. 2: Elution profile of angiotensin-converting enzyme obtained after Sephadex G-75 gel filtration chromatography

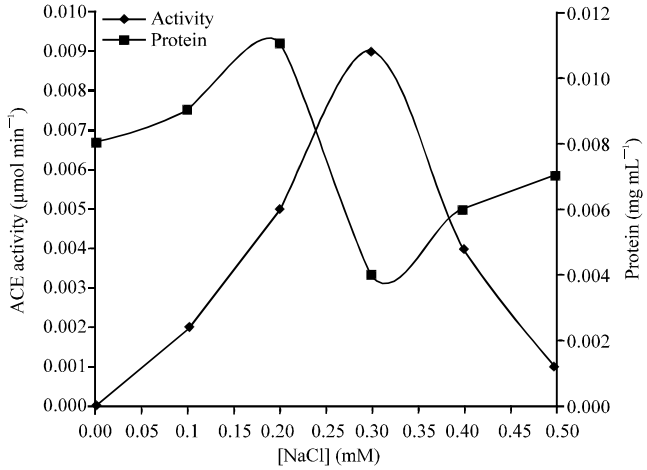

Fig. 3: Profile of angiotensin-converting enzyme obtained after ion exchange chromatography

in activity of the enzyme (Fig. 4). The partially purified enzyme was most active between pH 8-8.3 (Fig. 5) with complete cessation of activity at pH 10. From Fig. 6, it was observed that Na^+, Mg^{2+},

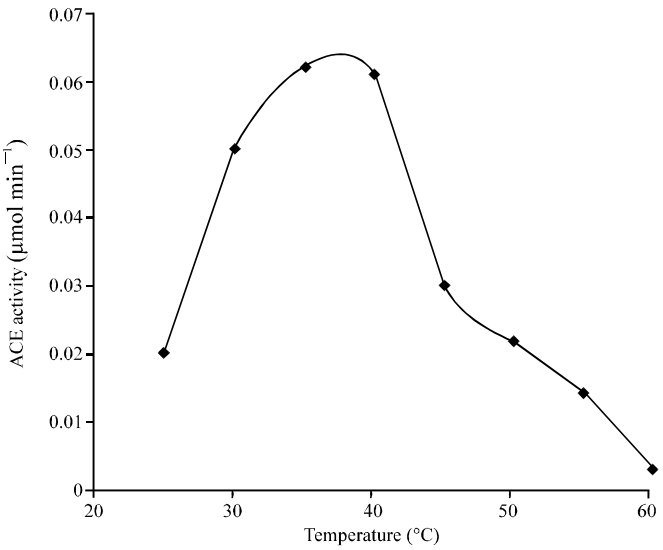

Fig. 4: Effect of temperature on angiotensin-converting enzyme activity

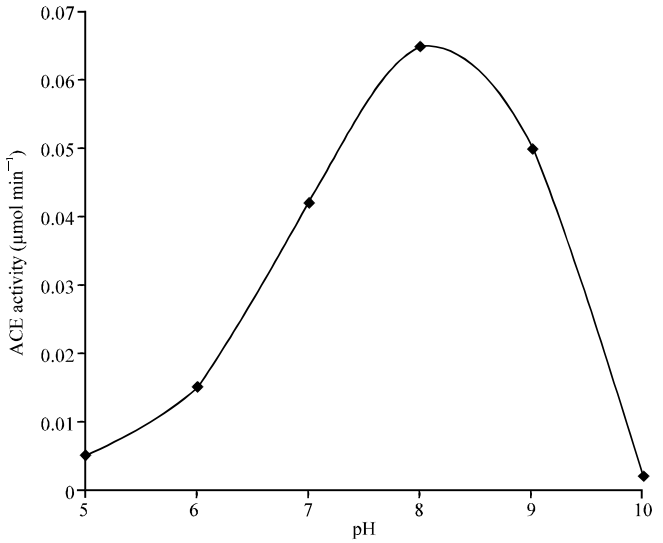

Fig. 5: Effect of pH on angiotensin-converting enzyme activity

Ca^{2+} and K^{+} had no effect on the activity of the enzyme, while EDTA strongly inhibited the enzyme with complete loss of activity. Figure 7 presents the double reciprocal plot of ACE with K_M and V_{MAX} of 1.8 mM and 0.42 µmol min^{-1}, respectively.

DISCUSSION

Angiotensin converting enzyme has been purified and from different sources by different methods (Erdos and Yang, 1967; Hooper *et al.*, 1987). The difficulty in obtaining a substrate specific for the enzyme (Huggins and Thampi, 1968; Hollemans *et al.*, 1969) prompted most researchers to depend on the simpler, substrate specific and easily reproducible spectrophotometric method described by Cushman and Cheung (1971). In the present study, the activity of ACE

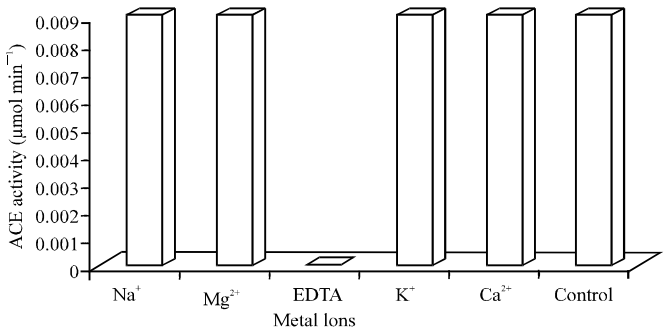

Fig. 6: Effect of monovalent and divalent metal ions on angiotensin-converting enzyme activity

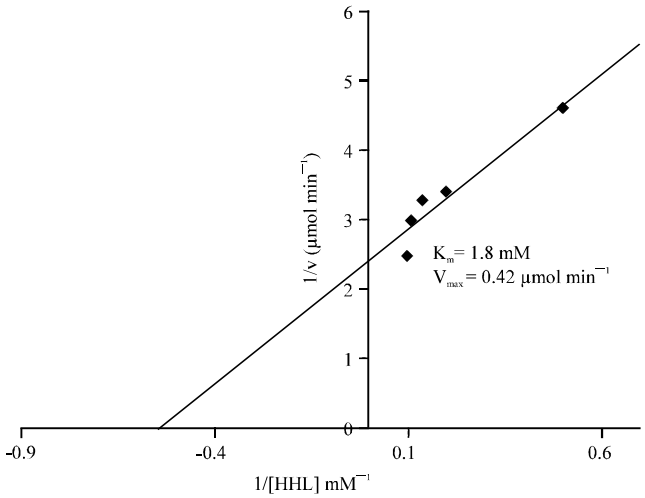

Fig. 7: Lineweaver Burk's plot of angiotensin-converting enzyme from rabbit lungs

isolated from rabbit lung was determined as described by Cushman and Cheung (1971). The first purification step, which is ammonium sulphate precipitation of the crude enzyme, gave the highest activity at 70% saturation. This does not conform to results by Quassinti *et al.* (1998) who demonstrated a higher activity at 65% saturation. Although our result was higher, it was no doubt close to theirs and the difference may be due to assay conditions. Ammonium sulphate was used because it has been reported to have no effect on the active site or biological activity of the enzyme. It is also highly soluble and changes in temperature have been shown to have little or no effect on its solubility (Dixon and Webb, 1964). The high specific activity (0.16 U mg^{-1}) and purification fold (2.0) of the crude ACE after dialysis may be due desalting and removal of low molecular weight contaminants. The specific activities obtained after gel filtration was higher than 0.05 µmol min^{-1} mg^{-1} obtained by Sharma and Singh (1988) using sephadex G-200. Also, previous studies by Friedland *et al.* (1981) obtained a lower specific activity (1.59 µmol min^{-1} mg^{-1}) using DEAE-Cellulose. The increase in specific activity obtained after the three purification steps may be due to the removal of other synergistically interacting components of the lungs (Sallau *et al.*, 2008). This result is lower than 30.9 and 21.6 U mg^{-1} reported by Cushman and Cheung (1971) and Bunning *et al.* (1983), respectively.

The high ACE activity and least protein content of fractions 3 and 4 after gel filtration, may be attributed to the high concentration of ACE in both fractions. Also, the general pattern of the graph reflects the presence of an isozyme. This is in agreement with studies by Kelley and Chrin (1988) who demonstrated the presence of more than one form of lung ACE with differing characteristics. Also, Rice *et al.* (2004) reported that ACE exists in two forms: somatic ACE, which is transcribed from exons 1-26 and testicular ACE, transcribed from exons 13-26.

The optimal pH of the partially-purified rabbit lung ACE (8.0-8.3) was found to be consistent with previous work (Cushman and Cheung, 1971) and has been shown to enhance the enzymatic reaction by the general base catalytic mechanism (Cushman and Cheung, 1971). The inability of Na^+, Ca^{2+}, Mg^{2+} and K^+ ions at 2 mM to affect ACE activity and the strong inhibition by EDTA is similar to results obtained by Dorer *et al.* (1970) showing that the enzyme is a metalloenzyme. Other reports by Bunning *et al.* (1983) demonstrated the effect of Zn^{2+} at the active site of the enzyme, by inhibiting the enzyme with the O-phenanthroline, chelating agent, to give a metal-free apoenzyme which was virtually inactive. Subsequent re-addition of Zn^{2+} to the apoenzyme fully restored its activity to that of the native enzyme, demonstrating that ACE is a metalloenzyme that requires Zn^{2+} for its catalytic activity. The Michaelis-Menten constant, K_M (1.8 mM) obtained for the partially purified enzyme was closely related to the 1.6 mM obtained from human serum ACE by Friedland *et al.* (1981) but lower than that reported by Cushman and Cheung (1971) from rabbit lung acetone powder extract. In other studies, Sharma and Singh (1988) using the same substrate (HHL) got a K_M of 20 mM, while Ronca-Testoni (1983) using furanacryloyl-L-phenylalanine and glycyl-glycine as substrate obtained a K_M of 0.31 mM. The fairly low K_M obtained in the present result is an indication of moderately high affinity of the enzyme for the substrate.

In conclusion, this study has shown that ACE, partially purified from rabbit lung via., a three-step purification protocol: Ammonium sulphate precipitation, gel filtration chromatography and ion exchange chromatography, has some similar physicochemical properties as reported in other studies. However, the low specific activity (5.0 U mg^{-1}) and final percentage recovery (21%) demonstrates the need for proper modification of the purification steps.

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