Biochimica et Biophysica Acta, 657 (1981) 222—231

BBA 69178

ANGIOTENSIN CONVERTING ENZYME IN CULTURED ENDOTHELIAL CELLS AND GROWTH MEDIUM

RELATIONSHIPS TO ENZYME FROM KIDNEY AND PLASMA

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(Received February 26th, 1980)
(Revised manuscript received September 2nd, 1980)

Key words: Angiotensin converting enzyme; Cell-surface glycoprotein; Lectin binding; (Endothelial cell culture, Swine aorta)

Summary

We have previously reported that cultured endothelial cells from swine aorta possess angiotensin converting enzyme, (peptidyldipeptide hydrolase, EC 3.4.15.1) and release it into serum-free culture medium. The present work compares enzyme from these two sources, and from swine kidney and serum, with respect to antibody and lectin binding. Purified enzyme from swine kidney, and the activity in swine serum, cultured endothelial cells and culture medium bind similarly to rabbit antibodies prepared against the kidney converting enzyme. Enzyme from each of these sources was allowed to bind to an immobilized lectin (*Ricinus communis*), which binds to terminal galactose residues of glycoproteins. Increasing concentrations of galactose were used to remove enzyme from the lectin column and the distribution of enzyme activity in the galactose eluates was determined. The elution pattern was similar for kidney and endothelial cell enzyme, and different from the pattern found for both serum and medium enzyme. Neuraminidase treatment of either serum or medium enzyme altered the distribution of activity eluted to that found for endothelial cell or kidney enzymes. The effects of neuraminidase suggest that the difference in lectin binding between cell and medium enzyme reflects differences in the number of terminal sialic acid residues that cover galactose residues.

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Introduction

Angiotensin converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1) is a dipeptidyl carboxypeptidase [1]. Two physiologically important substrates for the enzyme are angiontensin I and bradykinin. The enzyme catalyzes the conversion of angiotensin I to angiotensin II, which is the most potent vasopressor known [2]. Bradykinin, a potent vasodepressor, is inactivated by the enzyme [3]. It has been demonstrated that it is located on the luminal side of the plasma membrane of vascular endothelium [4]. Enzyme activity has also been found in plasma [5], but the source of the circulating enzyme is still unknown.

It has been reported that the serum and tissue enzymes exhibit similar catalytic, physical and immunologic properties [6]. Their contents of mannose, fucose, galactose and *N*-acetylglucosamine are similar, but the sialic acid content of the serum enzyme is higher [6]. Das et al. [6], have suggested that the higher sialic acid content of the circulating enzyme may prevent its removal from the circulation by the hepatic lectin which has been postulated to initiate the catabolic phase for sialic acid-deficient serum glycoproteins.

We previously reported that dipeptidyl carboxypeptidase activity was found both in endothelial cells cultured from swine aorta and in serum-free spent culture medium [7]. In this paper, we further identify the activity found in our cultured cell system as angiotensin converting enzyme, using immunologic studies. We also compare enzyme from cultured cells, culture medium, kidney and serum with respect to their affinity for the lectin from *Ricinus communis*. The results suggest that the enzyme released into serum-free culture medium is like the serum enzyme in having a higher sialic acid content than enzyme associated with cells or tissue.

Materials and Methods

Materials. Hippuryl-histidyl-leucine (Hip-His-Leu) was purchased from Bachem Fine Chemicals, Torrance, CA. Immobilized lectin (*R. communis*, Type II) (1 mg lectin/ml bed volume of agarose), immobilized neuraminidase ($1 \cdot 10^{-3}$ unit/ml bed volume of agarose) and inactivated cells of *Staphylococcus aureus* (Cowen strain) were obtained from Sigma Chemicals, St. Louis, MO. Commercial products used in enzyme purification included DEAE-cellulose, DEAE-Sephadex, Sephadex G-200 (Sigma Chem., St. Louis, MO) Bio-Gel P-100 and hydroxyapatite (Bio-Rad, Richmond, CA). Swine kidney was obtained from a local slaughterhouse. Cultured endothelial cells from swine aorta were prepared as described before [8].

Enzyme assay. Dipeptidyl carboxypeptidase activity was routinely measured by the fluorimetric method of Friedland and Silverstein [9]. The assay mixture contained 5 mM Hip-His-Leu/0.1 M sodium borate buffer, pH 8.3/0.3 M NaCl/ enzyme in a total volume of 0.25 ml. A unit of enzyme activity is defined as the amount catalyzing generation of 1.0 μmol His-Leu from Hip-His-Leu per min at 37°C. To assess the amount of hydrolysis due to other peptidases in the crude enzyme preparations, measurements were made in the presence and absence of SQ 20881 (1 μM), which is a specific nonapeptide inhibitor for

angiotensin converting enzyme [10]. Approx. 10% of measurable hydrolysis of Hip-His-Leu in cultured endothelial cells was due to other peptidases and could not be inhibited by SQ 20881. The amount of hydrolysis of Hip-His-Leu in any preparation which could not be inhibited by SQ 20881 was subtracted before total enzyme activity was calculated.

Enzyme preparation. Purified angiotensin converting enzyme was prepared from hog kidney cortex according to the method of Oshima et al. [11] except that no Co^{+2} was used in any procedure. For the preparation of enzyme activity from serum, 50 ml serum were prepared from fresh swine plasma. After dialysis against 0.0175 M potassium phosphate buffer (pH 6.9) the serum was applied to a DEAE-cellulose column (2 × 20 cm) equilibrated with the same buffer. 0.3 M NaCl in the same buffer was then used to elute the enzyme from the column. The final eluate of partially purified serum enzyme was concentrated to 10 ml by ultrafiltration with an Amicon PM-30 membrane. The concentrate was dialyzed against borate-buffered saline (0.01 M sodium borate/0.9% NaCl, pH 8.0). This procedure separates converting enzyme from other serum proteins, principally immunoglobulin G (IgG), which binds to the inactivated *Staphylococcus aureus* used in the antibody binding studies [12]. This partially purified serum enzyme was used for immunologic experiments. Serum enzyme was also partially purified using an immunoaffinity column (1.5 × 4 cm) which contained 6 mg rabbit IgG/ml bed volume of agarose. This preparation of serum enzyme was used for lectin affinity experiments. To prepare enzyme from cultured endothelial cells, $8 \cdot 10^7$ cells were collected by trypsinization and homogenized in 10 ml of borate-buffered saline containing 1% deoxycholate. After standing overnight at 4°C, the cell homogenate was centrifuged at 50 000 × *g* for 4 h. Over 90% of total enzyme activity was found in the supernate, which was then applied to a Bio-Gel P-100 column (1.5 × 60 cm). The column was eluted with borate-buffered saline, and the void volume collected and concentrated into 10 ml by ultrafiltration with an Amicon PM-30 membrane. Enzyme was also partially purified from culture medium. After the cultured cells became confluent, fresh medium without fetal calf serum was added and the cells were incubated for 6 days at 37°C as described before [7]. Culture medium was then collected and dialyzed against borate-buffered saline. The medium was concentrated to one-twentieth of the original volume by ultrafiltration with an Amicon PM-30 membrane and applied to a Bio-Gel P-100 column (1.5 × 60 cm). The column was eluted with borate-buffered saline and the void volume collected and reconcentrated by ultrafiltration with an Amicon PM-30 membrane. Medium and serum enzyme were treated where indicated with neuraminidase which was immobilized on agarose ($1 \cdot 10^{-3}$ unit/ml bed volume of agarose, 1 ml) in 1 ml borate-buffered saline, at 37°C, for 7 h with occasional agitation. The reaction mixture was transferred into a small column (0.5 × 1 cm) and the enzyme was eluted with borate-buffered saline.

Immunologic procedures. Purified swine kidney enzyme that exhibited a single protein band on standard gel electrophoresis was used to raise rabbit antibodies. The portion of the polyacrylamide gel which contained the enzyme was homogenized in borate-buffered saline. Before the injection the gel homogenate was mixed with equal volume of Maalox (final protein concentration: 50 μg/ml). Each rabbit received, in total, 250 μg protein. Each animal was

injected with 0.25 ml of antigen gel/Maalox mixture in each footpad, plus 0.25 ml subcutaneously in each of four locations along the back. The same procedure was repeated 3 weeks later. A further booster injection with 50 μg enzyme in 1 ml borate-buffered saline was given intravenously 1 week later. The harvest bleedings were begun 1 week after the final injection. The rabbit anti-enzyme IgG was isolated as described by MacDonald et al. [13]. The purified rabbit IgG was used to perform binding studies with converting enzyme activity from various sources. The reaction mixture for these binding studies contained $3.0 \cdot 10^{-3}$ units of enzyme activity and various amounts of rabbit anti-enzyme IgG in borate-buffered saline in a total volume of 0.2 ml. After the reaction mixture was incubated overnight at 4°C, 100 μl of a 20% cell suspension of inactivated *S. aureus* was added to absorb the antigen-antibody complex [12]. The suspension was agitated occasionally over a period of 30 min at 4°C and then centrifuged at $5000 \times g$ for 5 min. The unbound enzyme activity in the supernate was measured. The inhibition of enzyme activity by rabbit anti-enzyme IgG was also measured. Enzyme ($1 \cdot 10^{-3}$ units) was preincubated with various amounts of IgG in the enzyme assay buffer for 30 min at 4°C, and then substrate was added and the enzyme activity measured as usual.

Interaction of enzyme with lectin (R. communis). A small column of lectin immobilized on agarose (0.5 × 1 cm, 1 mg lectin/ml bed volume of agarose) was used to perform these experiments. Enzyme from various sources was placed on the column and allowed to incubate with the lectin for 2 h at 4°C. 1 ml borate-buffered saline was used to wash unbound protein from the column. 1 ml 0.025 M galactose in borate-buffered saline, borate-buffered saline alone, and 0.2 M galactose in borate-buffered saline were subsequently applied to the column. After addition of each eluting buffer, the column was allowed to equilibrate with that buffer for 2 h before the eluate was collected. The enzyme activity in the eluted fractions was measured in the presence of 0.2 M galactose.

Inhibition of enzyme activity by soluble R. communis lectin. Various amounts of lectin were preincubated with $3.0 \cdot 10^{-3}$ units of enzyme for 10 min in borate-buffered saline. Enzyme activity was measured as usual by addition of substrate to the enzyme lectin mixture.

Standard polyacrylamide slab gel electrophoresis was carried out as described by Baum et al. [14]. To determine the distribution of enzyme activity in the gel, the gel was sliced into 2-mm sections and each section was homogenized in 0.5 ml borate-buffered saline. The enzyme activity was measured in the gel homogenate. Protein concentrations were determined by using the method of Bradford [15].

Results

Dipeptidyl carboxypeptidase (angiotensin converting enzyme) prepared from swine kidney by the procedure of Oshima et al. [11], had a specific activity of 84 units/mg protein. It gave a single band on gel electrophoresis under nondenaturing conditions (Fig. 1, c). Coomassie blue staining and enzyme activity coincided in this location. Neuraminidase treatment of the enzyme did not change the mobility, indicating that this preparation of kidney converting

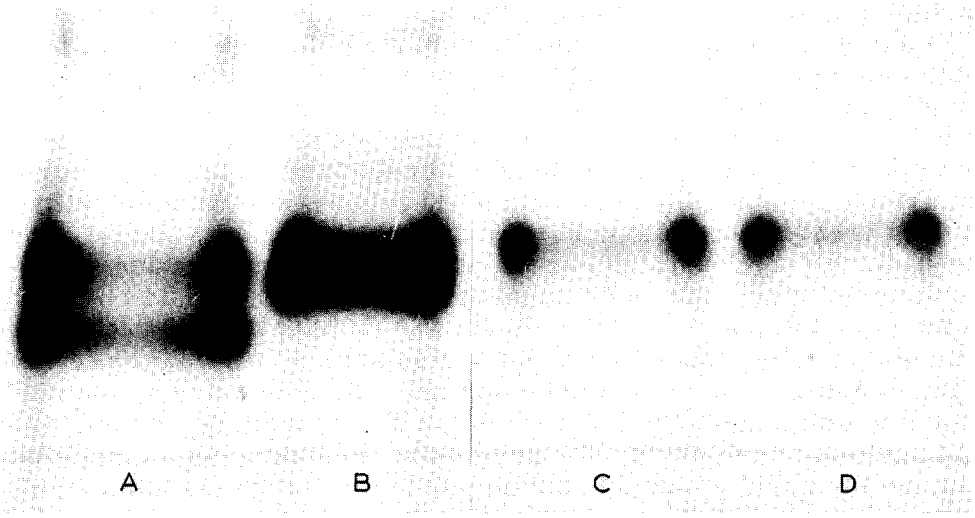

Fig. 1. Polyacrylamide gel electrophoresis under nondenaturing conditions of purified kidney converting enzyme. Electrophoresis was carried out on a 20 X 20 cm slab gel containing a 5—20% acrylamide gradient [14]. The photograph shows the top 4 cm of the gel. (A) 3 μg enzyme preparation with two bands coincident with enzyme activity. (B) 3 μg enzyme preparation as used in (A), but treated with neuraminidase. (C) 2.5 μg enzyme from another preparation. (D) Same as (C) but treated with neuraminidase.

enzyme has a relatively low content of terminal sialic acid residues (Fig. 1, c and d). In another preparation of purified enzyme, however, we observed two bands of enzyme activity on gel electrophoresis under nondenaturing conditions. After the neuraminidase treatment, the faster migrating of these two bands was shifted to a slower migrating band not exactly coincident with the other slower migrating band (Fig. 1, a and b). A preparation of kidney enyzme which showed a single band on gel electrophoresis was used for antibody preparation and for the experiments described below.

Rabbit anti-enzyme IgG was purified through $(NH_4)_2SO_4$ precipitation and DEAE-cellulose chromatography [13]. Purified enzyme from swine kidney, and the enzyme activity in swine serum, cultured endothelial cells and serum-free culture medium bind similarly to rabbit antibodies prepared against the purified kidney enzyme (Fig. 2). The affinities of cell-associated and medium enzyme, as well as enzyme from swine serum, for the antibody raised to swine kidney enzyme are the same. Enzyme activity was also fully inhibited by anti-enzyme IgG present in the enzyme assay mixture (Fig. 3), although a much higher concentration of IgG is required for maximum inhibition than for maximum binding. Again the extent of inhibition of enzyme activity from various sources is approximately the same.

The pH optima for the enzyme activities from cultured endothelial cells and the serum-free culture medium are at 8.8 and 8.4, respectively (Fig. 4). Medium enzyme treated with neuraminidase, however, had a pH optimum at 8.8 (Fig. 4). This result suggests that enzyme associated with cells has a lower sialic acid content than enzyme which has been released into the culture medium.

To explore further for differences in the carbohydrate composition between

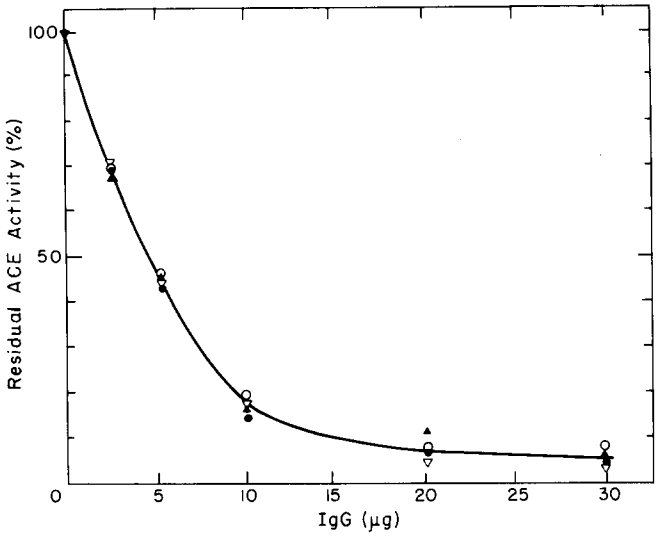

Fig. 2. Binding of converting enzyme to rabbit anti-enzyme immunoglobulin (IgG). Incubation mixture (200 μl) as described in Materials and Methods contained the indicated amounts of rabbit IgG. ○———○, serum enzyme; ▲———▲, kidney enzyme; ●———●, enzyme from cultured endothelial cells; ▽———▽, enzyme released into the culture medium by cultured endothelial cells. ACE, angiotensin converting enzyme.

cell-associated enzyme, and enzyme released into the medium, each form was allowed to bind to a column of *R. communis* lectin, Type II, which had been immobilized in agarose. This lectin has been shown to bind to terminal galactose residues in glycoproteins [16]. The enzyme was then dissociated from the lectin using 0.025 M and 0.2 M galactose. The fractions of bound enzyme which were eluted with each galactose concentration were compared for

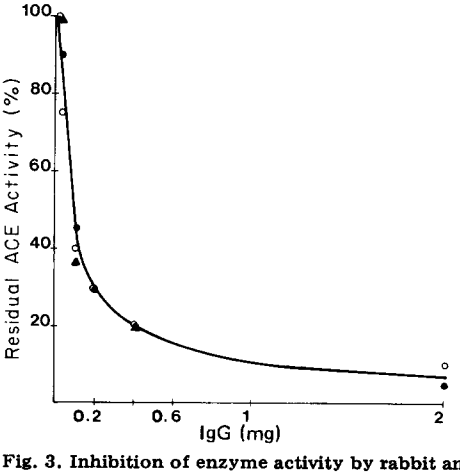

Fig. 3. Inhibition of enzyme activity by rabbit anti-enzyme IgG. Enzyme ($3 \cdot 10^{-3}$ unit) was preincubated with the indicated amounts of rabbit anti-enzyme IgG and residual activity was assayed as described in Materials and Methods. ●———●, kidney enzyme; ○———○, serum enzyme; ▲———▲, medium enzyme. ACE, angiotensin converting enzyme.

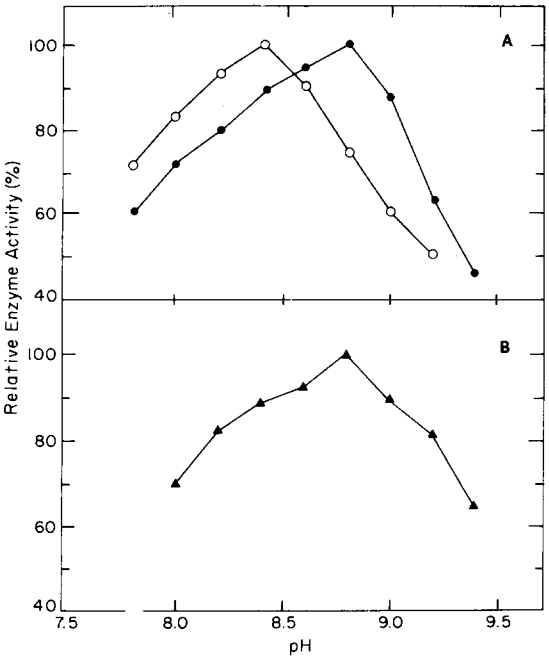

Fig. 4. pH optimum of converting enzyme. The buffer used in this experiment contained 0.1 M potassium phosphate/0.1 M boric acid/0.3 M NaCl, adjusted to the appropriate pH with 0.5 M NaOH. Activity is reported as percent of maximum observed. (A) ○———○, enzyme from culture medium; ●———●, neuraminidase-treated medium enzyme; (B) ▲———▲, enzyme from cultured endothelial cells.

TABLE I

BINDING OF ENZYME FROM CULTURED CELLS, KIDNEY, CULTURE MEDIUM AND SERUM TO IMMOBILIZED LECTIN FROM *R. COMMUNIS*

Enzyme (approx. $1 \cdot 10^{-3}$ units of activity) from each source was placed on a column of immobilized lectin and eluted with borate-buffered saline, 0.025 M galactose in borate-buffered saline and 0.2 M galactose in borate-buffered saline as described in Materials and Methods. Enzyme from each source was tested twice; values from both experiments are shown, together with the average distribution ratio for each source. Up to 5% of applied enzyme activity did not bind to the lectin column and was present in the first borate-buffered saline fraction.

Enzyme source	Experiment	% of applied enzyme activity		A/B	Average of A/B
		Fraction A 0.025 M galactose	Fraction B 0.20 M galactose		
Cultured cells	I	32	63	0.51	0.56
	II	36	61	0.60	
Kidney cortex	I	25	72	0.34	0.40
	II	30	70	0.46	
Serum-free culture medium	I	62	36	1.7	1.6
	II	61	39	1.6	
Serum	I	61	35	1.7	1.6
	II	60	38	1.6	
Serum-free culture medium (N) *	I	30	67	0.45	0.50
	II	34	61	0.55	
Serum (N) *	I	37	58	0.64	0.57
	II	32	64	0.50	

* N, treated with neuraminidase.

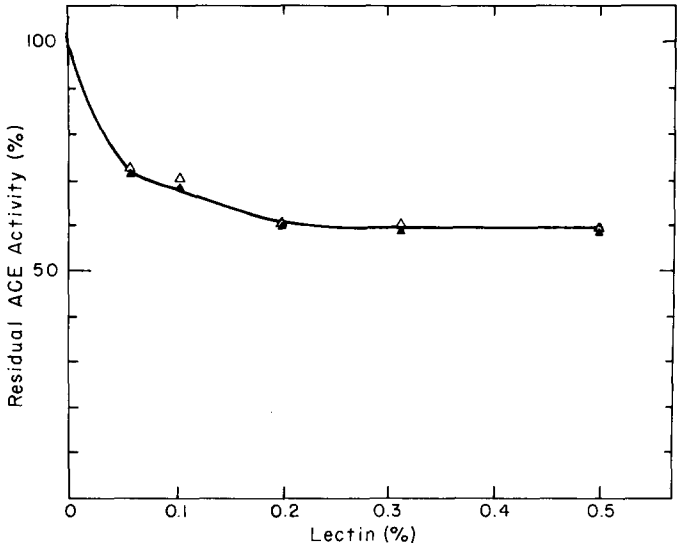

Fig. 5. Inhibition of enzyme activity by soluble *R. communis* lectin. Enzyme ($3 \cdot 10^{-3}$ unit) was preincubated with the indicated amounts of lectin and residual enzyme activity was assayed as described in Materials and Methods. △———△, enzyme from culture medium; ▲———▲, neuraminidase treated enzyme from culture medium. ACE, angiotensin converting enzyme.

enzyme from cultured cells and culture medium, kidney and serum. The results are shown in Table I. The enzyme distribution in the eluted fractions of the lectin column can be divided into two groups. The first group, with 60—62% of the enzyme activity in the 0.025 M galactose fraction, included enzymes from serum and serum-free culture medium. In the second group, including enzymes from kidney and cultured endothelial cells, 61—72% of the enzyme activity was in the 0.2 M galactose fraction. After treatment with neuraminidase, serum-free medium and serum enzymes exhibited similar behavior to the cell and tissue enzymes. The inhibition of enzyme activity from culture medium by soluble lectin was also examined (Fig. 5). Up to 40% of medium enzyme activity could be inhibited by lectin. There was no difference in the extent of inhibition for the native and neuraminidase-treated medium enzymes.

Discussion

The purified dipeptidyl carboxypeptidase from swine kidney gave a single band on gel electrophoresis under nondenaturing conditions. Neuraminidase treatment did not change the mobility, indicating that this enzyme has a relatively low content of terminal sialic acid residues (Fig. 1). However, in one preparation of purified enzyme, two forms of enzyme activity with different contents of sialic acid were observed on gel electrophoresis under nondenaturing conditions (Fig. 1). This observation is consistent with the report of Oshima et al. [11], that there are two forms of converting enzyme with different contents of sialic acid in swine kidney cortex. It is possible, that individual differences, from animal to animal, in degree of sialylation of angiotensin converting

enzyme account for the observation of separable pools in some batches of purified enzyme and not in others.

Johnson and Erdös [17] reported that cultured endothelial cells from human umbilical vein convert angiotensin I to angiotensin II, and this activity was partially inhibited by antibody to human lung converting enzyme. Previously, we reported that a peptidase capable of converting Hip-His-Leu to His-Leu is found in both cultured arterial endothelial cells and in serum-free culture medium incubated over these cells [7]. The binding studies of anti-enzyme IgG to enzyme activity from various sources reported here, show unequivocally that the dipeptidyl carboxypeptidase activity in our cultured endothelial cell system is due to angiotensin converting enzyme (Figs. 2 and 3). The affinity of IgG for enzyme from kidney, serum, cultured cells and serum-free medium is essentially the same, that is, any differences among the forms in the polypeptide chain or in conformation are more subtle than can be detected using a mixed antibody population.

Das et al. [6] have shown that angiotensin converting enzymes purified from rabbit serum and rabbit lung are immunologically and catalytically similar but differ in carbohydrate composition. The enzyme from serum has a higher sialic acid content. Oshima et al. [11] have reported that enzyme with a higher sialic acid content has a lower pH optimum. The pH optima found for enzyme from cultured cells and from serum-free medium, and the shift from 8.4 to 8.8 of the optimum for medium enzyme treated with neuraminidase, are consistent with the proposal that the enzyme released from the cells has a higher sialic acid content than that which remains associated with cells.

The lectin-binding studies show that angiotensin converting enzyme associated with cells or tissue has more terminal galactose residues than the serum enzyme and enzyme released from cultured cells into the medium. Since the galactose content of serum and tissue enzyme are comparable, but the serum enzyme has more sialic acid [6], the differences in lectin binding probably reflect differences in the number of terminal sialic acid residues that cover galactose residues. Further evidence for this interpretation is supplied by the observation that neuraminidase treatment of either serum or medium enzyme alters the distribution of activity eluted from the lectin column to that found for cell or tissue enzyme (Table I). In the studies of the inhibition of medium enzyme activity by soluble lectin, there was no difference in the extent of inhibition for the native and neuraminidase-treated medium enzymes (Fig. 5). However, the neuraminidase treatment for medium enzyme increased the binding affinity of enzyme for lectin (Table I). These data show that the galactose residues newly exposed by neuraminidase treatment are in sites which have little effect on catalytic activity.

It has been reported that the serum survival time of a number of plasma glycoproteins is markedly shortened when their terminal sialyl residues are removed by treatment with neuraminidase [18]. The significance of our findings is that the cultured endothelial cells release into the medium a form of angiotensin converting enzyme which is suitable for long life in the circulation.

Acknowledgements

This work was supported by grants HL 22486, HL 24660 and RR-07048 from the National Institues of Health. L.L.S. is an Established Investigator of the American Heart Association. The authors thank Ellen. S. Dickinson for preparation of the endothelial cell cultures.

References

1 Erdös, E.G. (1975) Circ. Res. 36, 247—255
2 Helmer, O.M. (1957) Am. J. Physiol. 188, 571—577
3 Dorer, F.E., Kahn, J.R., Lentz, K.E., Levine, M. and Skeggs, L.T. (1974) Circ. Res. 34, 824—827
4 Ryan, J.W., Ryan, U.S., Schultz, D.R., Whitaker, C., Chung, A. and Dorer, F.E. (1975) Biochem. J. 146, 497—499
5 Skeggs, L.T., Marsh, W.H., Kahn, J.R. and Shumway, N.P. (1954) J. Exp. Med. 99, 275—282
6 Das, M., Harley, J.L. and Soffer, R.L. (1977) J. Biol. Chem. 252, 1316—1319
7 Hayes, L.W., Goguen, C.A., Ching, S.F. and Slakey, L.L. (1978) Biochem. Biophys. Res. Commun. 82, 1147—1153
8 Magargal, W.W., Dickinson, E.S. and Slakey, L.L. (1978) J. Biol. Chem. 253, 8311—8318
9 Friedland, J. and Silverstein, E. (1976) Am. J. Clin. Pathol. 66, 416—424
10 Cheung, H.S. and Cushman, D.W. (1973) Biochim. Biophys. Acta 293, 451—463
11 Oshima, G., Nagasawa, K. and Kato, J. (1976) J. Biochem. 80, 477—483
12 Kessler, S.W. (1975) J. Immunol. 115, 1617—1624
13 MacDonald, A.B., Fraser, C.E.O. and Rubin, A.S. (1978) J. Immunol. 34, 137—147
14 Baum, S.G., Horwitz, M.S. and Maizel, J.V. (1972) J. Virol. 10, 211—219
15 Bradford, M.M. (1976) Anal. Biochem. 72, 248—254
16 Nicolson, G.L. and Blaustein, J. (1972) Biochim. Biophys. Acta 266, 542—547
17 Johnson, A.R. and Erdös, E.G. (1977) J. Clin. Invest. 59, 684—695
18 Morell, A.G., Gregoriadis, G., Scheinberg, I.H., Hickman, J. and Ashwell, G. (1971) J. Biol. Chem. 246, 1461—1467