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# Resistance of angiotensin I converting enzyme to hydrolysis by serine proteases

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Angiotensin I converting enzyme (EC 3.4.15.1, $A_1CE$)* is a key enzyme in the renin/angiotensin system that regulates blood pressure. $A_1CE$ has the dual function both of generating the pressor peptide hormone angiotensin II and, also, of inactivating the depressor peptide hormone bradykinin [1]. The enzyme is an acidic glycoprotein consisting of a single polypeptide chain of molecular weight 140,000 with an isoelectric point of 4.6 [2].

The kallikreins also are acidic glycoproteins previously thought to be specific for the proteolysis of kininogens. However, Sealey *et al.* [3] demonstrated that human urinary kallikrein was, in fact, capable of efficiently activating pro-renin. Thus, proteolysis by kallikreins is not limited to the release of kinins, and activation of pro-enzymes may be an important additional regulatory role for the kallikreins. Although human plasma kallikrein is selective and similar to human urinary kallikrein, it nevertheless has a somewhat broader substrate specificity than the urinary enzyme [4].

Nakahara [5] recently reported that $A_1CE$ was cleaved by human plasma kallikrein into enzymatically active subunits (mol. wt 180,000 and 95,000) with a total of 170% of the original $A_1CE$ activity, that $A_1CE$ is inactivated by trypsin, plasmin, or thrombin, and that $A_1CE$ is inhibited by Trasylol and soybean trypsin inhibitor. These results, if true, would be quite significant. They would indicate that kallikrein, the enzyme that generates bradykinin, activates $A_1CE$, one of the enzymes that destroys bradykinin, and thus is part of a balanced system regulating tissue levels of bradykinin. Other components of this system would include plasmin and thrombin. Nakahara hypothesized that kallikrein would ultimately reduce levels of bradykinin by generating enhanced bradykininase activity from $A_1CE$, while plasmin and thrombin would potentiate the effect of bradykinin through inactivation of $A_1CE$.

However, the data Nakahara presented to support his hypothesis were not unequivocal, since they were obtained using preparations of both $A_1CE$ and kallikrein that were highly contaminated with extraneous proteins. We therefore decided to investigate the kinetic and structural changes in homogeneous human plasma $A_1CE$ that are produced by kallikrein from both human plasma and urine and also by a select group of proteolytic enzymes of the serine protease family that have comparable specificity.

## *Materials and methods*

*Human plasma $A_1CE$.* The enzyme was purified from outdated human plasma as previously described [6]. At this stage, the $A_1CE$ specific activity is 40 units/mg using Hip-His-Leu as substrate [7]. The enzyme gives a single band when analyzed by SDS-PAGE [8].

*Human kallikrein.* The urinary enzyme was isolated by DEAE-cellulose, Trasylol-Sepharose affinity and Sephacryl S-200 gel chromatographies as reported previously [9, 10]. Kinin-generating and arginyl esterase specific activities of this preparation are the highest [9] of any preparation reported so far. Furthermore, the enzyme is pure by immunological criteria. The plasma enzyme was a gift from Dr. Y. Hojima, Scripps Clinic and Research Foundation, La Jolla, CA. It has a specific activity of 86 $\mu$moles$\cdot$min$^{-1}\cdot$mg$^{-1}$ against D-Pro-Phe-Arg-pNA and of 64 $\mu$moles$\cdot$min$^{-1}\cdot$mg$^{-1}$ against TAME.

*Proteolytic enzymes and inhibitors.* Human urokinase (Lot No. 98C-03401), porcine plasmin (77C-0053), bovine $\alpha$-chymotrypsin (105C-8120) and soybean trypsin inhibitor (T-9003) were purchased from the Sigma Chemical Co., St. Louis, MO. Human plasminogen (5280 HPG 59E 560), human thrombin (HTH-49), bovine trypsin (TRTPCK 0FA) and Trasylol (3210 PTI) were purchased from the Worthington Chemical Co., Nutley, NJ. A protease detection kit was from Bio-Rad Laboratories, Richmond, CA.

*Proteolysis of $A_1CE$.* A typical reaction mixture to determine the susceptibility of $A_1CE$ to proteolysis contained approximately 2 $\mu$g of $A_1CE$ as substrate and 0.5 $\mu$g or more of the proteolytic enzyme in a final volume of 0.25 ml of 0.1 M sodium phosphate buffer, pH 8.5. The control included $A_1CE$ and all other reagents except the proteolytic enzyme. The mixtures were incubated at 37° for up to 60 min. At selected time intervals, aliquots were removed and assayed for $A_1CE$ activity.

*Determination of enzyme activity.* $A_1CE$ activity was monitored by two methods. In method A, the radiolabeled substrate Hip-His-Leu was used according to Ryan *et al.* [11] in 0.05 M Hepes buffer, pH 8.0, containing 0.1 M sodium chloride and 0.75 M sodium sulfate. In method B, unlabeled Hip-His-Leu was used according to a modification of the method of Cushman and Cheung [12] as previously described [7]. Kallikrein activity was monitored using Pro-Phe-Arg-[$^3$H]BA [13] as substrate.

*Polyacrylamide gel electrophoresis.* It is conceivable that fragmentation of the $A_1CE$ molecule by proteolytic enzymes is not reflected in an observable parallel loss of $A_1CE$ activity. Therefore, it is important to monitor structural as well as enzymatic changes in $A_1CE$. The structural changes were monitored by PAGE in the presence of SDS by subjecting aliquots from both experimental and control samples to SDS-PAGE on 7% polyacrylamide gels as previously described [8].

## *Results and discussion*

In a recent publication, Nakahara [5] reported that human plasma $A_1CE$ is a substrate for human plasma kallikrein and related serine proteases. In contrast, our results indicate that human plasma $A_1CE$ is neither a substrate for human plasma kallikrein nor a substrate for human urinary kallikrein. In our experiments, no kinetic or structural changes of $A_1CE$ were observed after treatment with any of the serine proteases. Treatment of $A_1CE$ with human plasma kallikrein, human urinary kallikrein, human thrombin, porcine plasmin, human plasmin (generated *in situ* by the action of urokinase on plasminogen), bovine trypsin, and bovine $\alpha$-chymotrypsin for time intervals up to 60 min did not reduce the activity of $A_1CE$ to levels lower than those observed for controls (Table 1). The data with trypsin and $\alpha$-chymotrypsin support the results of Oshima *et al.* [14] who found that $A_1CE$ from hog kidney cortex was resistant to both of these enzymes.

Initially we observed loss of $A_1CE$ activity in the presence of human plasmin. However, when the plasmin was dialyzed prior to addition to $A_1CE$, the loss of $A_1CE$ activity did not occur. A low molecular weight contaminant, conceivably EDTA, was most probably responsible for the loss of $A_1CE$ activity. This rationale was proposed when we discovered that the urokinase used to activate the plas-

* Abbreviations: $A_1CE$, angiotensin I converting enzyme; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; KIU, kallikrein inhibitor units; DEAE, diethylaminoethyl; TAME, *p*-tosyl-L-arginine methyl ester; D-Pro-Phe-Arg-pNA, D-prolyl-L-phenylanalyl-L-arginyl-*p*-nitroanilide; Pro-Phe-Arg-[$^3$H]BA, L-prolyl-L-phenylanalyl-L-arginyl-[$^3$H]benzyl amide; and Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

Table 1. Effects of proteolytic enzymes on $A_1CE$ activity

| Proteolytic enzyme | Percent remaining $A_1CE$ activity | | |
|---|---|---|---|
| | Incubation time (min) | | |
| | 0 | 30 | 60 |
| Control | 100 | 98 | 90 |
| Plasma kallikrein (0.5 μg) | 100 | 93 | 101 |
| Urinary kallikrein (7.2 μg) | 100 | 102 | 95 |
| Thrombin (1 unit) | 100 | 90 | 92 |
| Porcine plasmin (5 μg) | 100 | 105 | 97 |
| Trypsin (5 μg) | 100 | 95 | 88 |
| α-Chymotrypsin (5 μg) | 100 | 95 | 93 |
| Human plasmin* (10 μg) | 100 | 103 | 98 |

* Generated *in situ* by the addition of urokinase to the plasminogen assay mixture.

minogen contained a high concentration of EDTA, a known $A_1CE$ inhibitor. Substantial plasmin activity in the urokinase-treated plasminogen preparation after dialysis was confirmed using the Bio-Rad protease detection kit.

The serine protease inhibitors Trasylol at concentrations of 700–1400 KIU and soybean trypsin inhibitor at concentrations of 10–200 μg per assay had no significant inhibitory effect on $A_1CE$. This is in marked contrast to the greater than 60% inhibition observed by Nakahara with concentrations of soybean trypsin inhibitor as low as 10 μg per assay. We observed similar results with Trasylol, once again in marked contrast to the results of Nakahara. These results can be rationalized if one assumes that the enzyme prepared by Nakahara was a serine protease, and that $A_1CE$ is not a serine protease.

The structural integrity of $A_1CE$ was monitored by SDS-PAGE. No change in the single polypeptide chain primary structure of $A_1CE$ was observed in the presence of the proteolytic enzymes. The molecular weight of $A_1CE$ by SDS-PAGE was 140,000 as previously reported [2]. A typical set of control and sample gels is shown for $A_1CE$ after treatment with human plasma kallikrein (Fig. 1). Identical results (not shown) were observed after treatment of $A_1CE$ with human urinary kallikrein, human thrombin, porcine plasmin, human plasmin, bovine trypsin, and bovine α-chymotrypsin. Although SDS-PAGE might not detect subtle changes in the primary structure of $A_1CE$, it most certainly has enough resolving power to detect the structural changes observed by Nakahara.

The $A_1CE$ assay system used by Nakahara with the synthetic substrate Hip-His-Leu included cobalt ions. Cobalt ions are not necessary for human plasma $A_1CE$ activity. They are known to induce a cryptic $A_1CE$-like activity in guinea pig and hog plasma that is mediated by an enzyme with a molecular weight above 400,000 and that does not require chloride ion for activity and, therefore, is not a true $A_1CE$ [15]. Possibly such an enzyme also exists in human plasma. Furthermore, both bradykininase and $A_1CE$ activity were measured using relatively non-specific bioassays. Thus, it is quite possible for kallikrein to have activated one or more intermediate proteases which then inactivated bradykinin by cleaving any of its peptide bonds and not necessarily the $Pro^7$-$Phe^8$ bond susceptible to $A_1CE$ cleavage. Although all $A_1CE$s are bradykininases, not all bradykininases are $A_1CE$s. Intermediate proteases—activated by either trypsin, plasmin, or thrombin—could also be associated with the inactivation of $A_1CE$.

Also, we question the accuracy of the molecular weight of 180,000 reported by Nakahara for $A_1CE$ subunit-1. This value is incompatible with previously published data indicating that $A_1CE$ is a single polypeptide chain with a molecular weight of approximately 140,000 [2, 8, 16–18].

The differences between our data and those reported by Nakahara might be explained by assuming either the presence of at least two $A_1CE$-like enzymes in human plasma or an influence of extraneous proteins on $A_1CE$ catalytic activity in heterogeneous preparations.

In summary, in a recent publication, Nakahara [5] reported that human plasma $A_1CE$ is a substrate for some proteolytic enzymes. He found that $A_1CE$ was cleaved in the presence of human plasma kallikrein to two enzymatically active subunits with a sum total of 170% of the original bradykininase activity as measured by bioassay. Both subunit-1 (mol. wt 180,000) and subunit-2 (mol. wt 95,000) had $A_1CE$-like, as well as bradykininase, activity. Also, Nakahara found that $A_1CE$ is a substrate for trypsin, plasmin, and thrombin, can be cleaved to inactive fragments by these enzymes, and is inhibited by Trasylol and soybean trypsin inhibitor. However, the $A_1CE$ employed in that study was not pure. Therefore, we performed similar experiments using a pure preparation of human plasma $A_1CE$ with human plasma kallikrein and, in addition, human urinary kallikrein. The effects on $A_1CE$ of other proteolytic enzymes, including trypsin, α-chymotrypsin, plasmins, and human thrombin, as well as inhibition by Trasylol and soybean trypsin inhibitor, were studied. $A_1CE$ activity was monitored using the synthetic substrate Hip-His-Leu. Our results indicate that $A_1CE$ activity was not affected by either kallikreins, trypsin, α-chymotrypsin, plasmins, or human thrombin. The structure of $A_1CE$, monitored by SDS-PAGE, was not altered by any of the proteolytic enzymes. In addition, $A_1CE$ was not inhibited by 700–1400 KIU Trasylol or 10–200 μg soybean trypsin inhibitor. Possible explanations for the difference of these data from those of Nakahara include the presence of at least two $A_1CE$-like enzymes in human plasma and an influence of extraneous proteins on $A_1CE$ catalytic activity in heterogeneous preparations.

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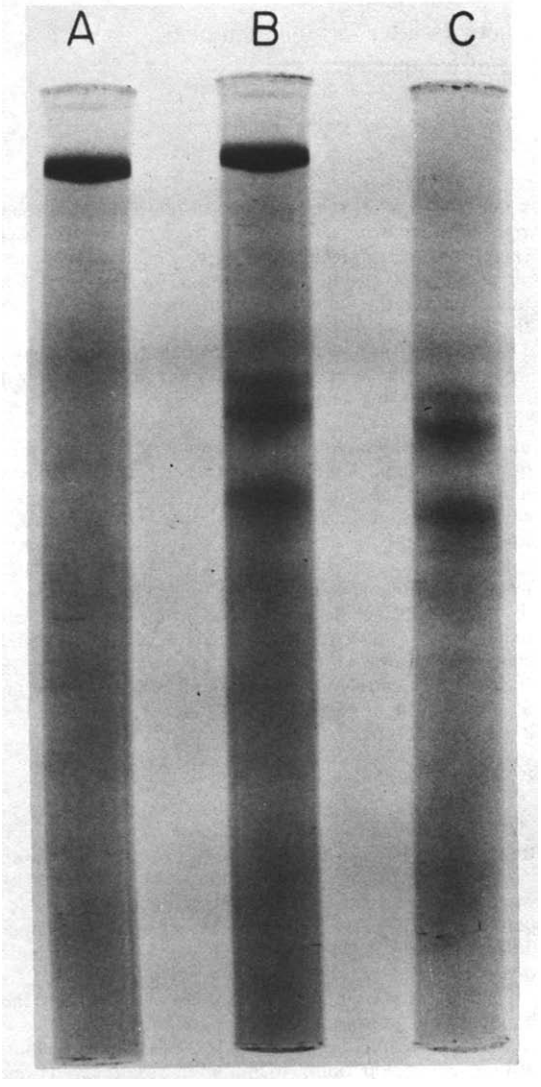


Fig. 1. SDS-PAGE of $A_1CE$ after treatment with human plasma kallikrein. The 7% monomer gels were prepared as previously described [8]. The power setting was 8 mA/tube for 4 hr. Key: (A) untreated $A_1CE$; (B) $A_1CE$ incubated with human plasma kallikrein; and (C) human plasma kallikrein.

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