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Characterization of Recombinant Human Adipocyte-Derived Leucine Aminopeptidase Expressed in Chinese Hamster Ovary Cells[1]

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Adipocyte-derived leucine aminopeptidase (A-LAP) is a recently identified novel member of the M1 family of zinc-metallopeptidases. Transfection of the A-LAP cDNA into COS-7 cells resulted in the secretion of the enzyme. In this study, recombinant A-LAP was expressed in Chinese hamster ovary cells, purified to homogeneity and its enzymatic properties were characterized. The purified enzyme was active towards a synthetic substrate, L-leucyl-*p*-nitroanilide, yielding a V_{max} of 3.55 μmol/min/mg and a K_m of 1.28 mM, and was shown to be a monomeric protein with molecular mass of 120 kDa in solution. By monitoring the sequential N-terminal amino acid liberation, it was found that the enzyme hydrolyzes a variety of bioactive peptides, including angiotensin II and kallidin. Immunohistochemical analysis indicated that the enzyme is expressed in the cortex of the human kidney, where tissue kallikrein is localized. Taken together, these results indicate that A-LAP possesses a broad substrate specificity towards naturally occurring peptide hormones and suggest that it plays a role in the regulation of blood pressure through the inactivation of angiotensin II and/or the generation of bradykinin in the kidney.

Key words: aminopeptidase, angiotensin, bradykinin, kallidin, metallopeptidase.

Aminopeptidases hydrolyze the N-terminal amino acids of protein or peptide substrates. It is thought that they play important roles in protein maturation, activation, modulation and degradation of bioactive peptides, and the determination of protein stability (*1*). In addition, some aminopeptidases function as differentiation antigens and control cell proliferation and differentiation (*2–5*).

It is well known that human placenta and maternal serum contain several aminopeptidases that regulate the activity of peptide hormones such as oxytocin, vasopressin and angiotensins (*6*). Placental leucine aminopeptidase (P-LAP)/oxytocinase is one of these enzymes and is known to be secreted from the placenta and increase in maternal serum during pregnancy (*7*). It is therefore suggested that P-LAP plays an important role in the maintenance of pregnancy by degrading peptides hormones whose increase might have a significant effect on uterine tonus and uteroplacental blood flow (*8*).

Molecular cloning of human P-LAP revealed that the enzyme is a type II membrane-spanning protein belonging to the M1 family of zinc-metallopeptidases, which share the consensus $HEXXH(X)_{18}E$ motif essential for enzymatic activity (*9–11*). It is now clear that this growing family of mammalian zinc-containing aminopeptidases includes membrane-bound (*3*, *12*, *13*), cytosolic (*14*, *15*), and secretory proteins (*16*, *17*).

In an effort to identify an additional enzyme belonging to the family, we have succeeded in cloning a novel aminopeptidase, termed adipocyte-derived leucine aminopeptidase (A-LAP), which shows significant homology to P-LAP (*18*). As in the case with P-LAP, the deduced sequence of A-LAP contains the $HEXXH(X)_{18}E$ consensus motif and a hydrophobic region near the N-terminus that could function as an internal signal peptide and a membrane-spanning domain. However, our initial characterization of the enzyme revealed that the enzyme localizes mainly in cytoplasm when expressed in COS-7 cells.

To characterize the enzyme further with respect to its biochemical properties and physiological and/or pathological functions, we have established a large-scale production system of recombinant A-LAP and prepared a specific antibody. Re-examination of the processing of the enzyme in COS-7 cells resulted in the presence of the A-LAP protein mainly in the culture medium, suggesting that it can be secreted through proper secretory machinery. Enzymatic characterization revealed that A-LAP hydrolyzes a variety

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Abbreviations: P-LAP, placental leucine aminopeptidase; A-LAP, adipocyte-derived leucine aminopeptidase; Ang, angiotensin; PNGase F, peptide-*N*-glycosidase F; BFA, brefeldin A; Leu-pNA, L-leucyl-*p*-nitroanilide; CHO, Chinese hamster ovary; PVDF, polyvinylidene difluoride; TBS, Tris-HCl buffered saline; HRP, horseradish peroxidase.

of peptide hormones, including angiotensin (Ang) II and kallidin. These results suggest that A-LAP plays a role in the regulation of blood pressure through the inactivation of Ang II and the generation of bradykinin.

EXPERIMENTAL PROCEDURES

Materials—Peptide-*N*-glycosidase F (PNGase F) and endoglycosidase H were purchased from Boehringer-Mannheim. Brefeldin A (BFA) was from Wako Pure Chemicals (Osaka). L-Leucyl-*p*-nitroanilide (Leu-pNA) was from Nacalai Tesque (Kyoto). Angiotensin II (Ang II), kallidin, neurokinin A, neuromedin B, vasopressin and oxytocin were obtained from Peptide Institute (Osaka) and dynorphin A (fragment 1–8) from Sigma (St. Louis, MO).

Expression of A-LAP in COS-7 Cells—The blunted *Eco*RI fragment of the human A-LAP1 cDNA was subcloned into the *Sma*I site of pSVL (Amersham-Pharmacia) (pSVL/A-LAP1). For transfection experiments, COS-7 cells were grown to confluency in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Then 2.5×10^6 cells per 100-mm dish were transfected with 20 μg of pSVL/A-LAP1 mixed with 80 μg of DOSPER (Boehringer Mannheim). Following transfection, the cells were incubated in serum-free medium (DMEM containing 10 mM HEPES, pH 7.4, 1 μg/ml of bovine insulin and 2 μg/ml bovine transferrin). After incubation, cells were separated from the culture medium and subjected to Western blot analysis (*18*).

Production of Recombinant A-LAP in Chinese Hamster Ovary Cells—The cDNA encoding the signal sequence of human trypsin II (*i.e.* MNLLLILTFVAAAVA) followed by the open reading frame of human A-LAP1 starting at Ala-37 was ligated into the *Eco*RI sites of pdKCR-dhfr(–) vector as described previously, and the expression plasmid, termed pdKCR-Trp(S)-ALAP1, was obtained (*19*, *20*). Chinese hamster ovary (CHO)-dhfr(–) cells were transfected with the plasmid using Lipofectin (Life Technologies) according to the manufacturer's instructions. The transfectants were selected in alpha minimum essential medium lacking ribonucleotides and deoxyribonucleotides supplemented with 10% fetal bovine serum; 68 clones were isolated by the limiting dilution method.

The transformed cells were grown to confluency in T-flasks (80 cm^2, Nunc) in Ham's F12 medium supplemented with 5% fetal bovine serum. The cells were then washed twice with PBS and incubated for 48 h with two changes of medium. After changing to serum-free medium (*i.e.* Ham's F12 medium containing 5 μg/ml bovine insulin and 1 μg/ml bovine transferrin), culture was continued for three more days, and the spent medium was collected for the survey of A-LAP activity.

Purification of Recombinant A-LAP—The clone 15 transformed cells were incubated in roller bottles (850 cm^2, Corning). The culture medium was concentrated on an ultrafiltration system, dialyzed against 50 mM Tris-HCl buffer (pH 8.0) and applied to a Q-Sepharose (Amersham-Pharmacia) column (2.5 × 20 cm) equilibrated in the same buffer. The column was then washed with the same buffer and eluted with a linear gradient of 0–0.4 M NaCl.

The A-LAP–containing fractions from the Q-Sepharose column were pooled and ammonium sulfate was added directly to a final 30% saturation. The sample was then applied to a phenyl-Sepharose 4B (Amersham-Pharmacia) column (2.5 × 10 cm) equilibrated in 50 mM Tris-HCl buffer (pH 8.0) containing 30% saturated ammonium sulfate. After loading, the column was washed with equilibration buffer and eluted with a linear gradient of 30–0% saturated ammonium sulfate.

The A-LAP fraction from the phenyl-Sepharose column was dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl and concentrated on a YM-membrane in an Amicon cell. The concentrate was then loaded onto a Sephacryl S200HR (Amersham-Pharmacia) column (2.5 × 100 cm) equilibrated in the same buffer.

The A-LAP fraction from the Sephacryl S200HR column was dialyzed against 50 mM Tris-HCl buffer (pH 8.0), applied to a Mono Q (Amersham-Pharmacia) FPLC column, and eluted with a linear gradient of 0–0.4 M NaCl. The active fraction was collected, concentrated and subjected to further characterization.

Western Blot Analysis—The test samples were separated by SDS-PAGE in 8% separating gels and transferred to polyvinylidene difluoride (PVDF) membranes (Pall). The membranes were blocked with Tris-HCl–buffered saline (pH 7.4) containing 5% skim milk for 1 h at room temperature, then incubated in Tris-HCl–buffered saline containing 0.05% Tween 20 (T-TBS) and 5% skim milk, and anti–A-LAP anti-serum diluted to 1/1,000 for 2 h at room temperature. The filters were washed four times with T-TBS and incubated for 1 h with horseradish peroxidase (HRP)–conjugated goat anti–rabbit IgG polyclonal antibody (Promega), diluted 1/5,000 in T-TBS containing 5% skim milk. After washing the filters three times with T-TBS, the blots were analyzed by an enhanced chemiluminescence method using an ECL plus Western blotting detection kit obtained from Amersham-Pharmacia. The results were visualized by fluorography using RX-U Fuji medical X-ray film.

Amino-Acid Sequence Analysis—Automated Edman degradation was carried out with an Applied Biosystems model 477A protein sequencer. Phenylthiohydantoin derivatives were identified by reverse-phase HPLC with an Applied Biosystems 120A on-line system.

Gel Filtration—The molecular mass of A-LAP was estimated by gel filtration column chromatography on a TSK G3000SW (7.5 × 300 mm) column (Tosoh) and eluted with 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM Na_2SO_4 at a flow rate of 0.5 ml/min.

Ultracentrifuge Analysis—Molecular mass of A-LAP in solution was determined by sedimentation equilibrium experiments employing a Molecular Interaction analysis system (Optima-XLA, Beckman).

Measurement of the Leucine Aminopeptidase Activity of A-LAP—A-LAP activity was determined from the rate of increase in absorbance at 405 nm using Leu-pNA as a substrate (*21*). The reaction mixture containing the desired concentrations of A-LAP and substrate was incubated at 30°C for 2 min and the formation of product (*p*-nitroaniline, $\varepsilon = 9{,}920\ M^{-1}\ cm^{-1}$) was monitored at 405 nm using a spectrophotometer UV-2200 (Shimadzu).

Cleavage of Peptide Hormones by A-LAP—Peptide hormones (25 μM) were incubated with various concentrations of A-LAP at 37°C in 25 mM Tris-HCl buffer (pH 7.5). The reaction was terminated by the addition of 2.5% (v/v) formic acid. The generated peptides were separated by reverse-phase HPLC on an Inertsil ODS-2 (4 × 250 mm) column (GASUKURO Kogyo, Tokyo) using a Gilson HPLC

system with a Hewlett-Packard HP 1040A diode-array detector. Peptides generated from Ang II were eluted isocratically with 19.2% acetonitrile in 0.09% trifluoroacetic acid at a flow rate of 0.5 ml/min. As for kallidin, the generated peptides were loaded onto the column equilibrated in 8% acetonitrile in 0.09% trifluoroacetic acid and eluted with a two step gradient of 8% acetonitrile in 0.09% trifluoroacetic acid (0 min), 32% acetonitrile in 0.084% trifluoroacetic acid (12 min) to 80% acetonitrile in 0.075% trifluoroacetic acid (24 min) at a flow rate of 0.5 ml/min. The molecular masses of the peptides were determined by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) MS with a REFLEX mass spectrometer (Bruker-Franzen Analytik) using 2-mercaptobenzothiazole as the matrix (*22*).

Immunohistochemical Analysis—Formalin-fixed and paraffin-embedded biopsy specimens of human kidney remaining after diagnostic evaluation showing minimal histological abnormalities were obtained from the International Medical Center of Japan and used for A-LAP immunohistochemical staining. Paraffin sections, 2 μm-thick, were incubated with affinity-purified rabbit anti–human A-LAP polyclonal antibody (10 μg/ml) overnight at 4°C. HRP-conjugated goat anti–rabbit IgG (Promega) diluted 1/500 was used as the second antibody and 3,3′-diaminobenzidine (0.1 mg/ml) as an HRP substrate. The sections were counterstained with methyl green. For negative controls, 10 μg/ml of immunoglobulin purified from normal rabbit serum was used instead of the affinity-purified antibody (*23*, *24*).

RESULTS

Secretion of A-LAP Expressed in COS-7 Cells—In our previous work, we transfected COS-7 cells with the A-LAP expression vector and analyzed the subcellular localization of the enzyme by Western blot analysis employing the C15 antibody against the C-terminal amino acid sequence of the enzyme (*18*). A-LAP was detected mainly in the soluble fraction with a minimal amount in the membrane fraction of cell lysates. Since we could not detect the A-LAP protein secreted into culture medium, we concluded that A-LAP is a soluble protein localized predominantly in the cytosol.

However, when we prepared a rabbit polyclonal antibody against recombinant human A-LAP and re-examined the processing of the enzyme expressed in COS-7 cells, we observed that the bulk of the enzyme with a molecular mass of 111-kDa was secreted into the culture medium (Fig. 1). As reported previously (*18*), a band with a slightly lower molecular mass (105 kDa) was also detected in both the cell-associated soluble and membrane fractions. The difference was due to the structures of the sugar chains attached to the A-LAP proteins. By treatment with glycosidases, such as PNGase F and endoglycosidase H, it was shown that the secreted A-LAP contains complex-type sugar chains and cell–associated A-LAP high mannose and/or hybrid type *N*-linked sugar chains (data not shown). Treatment with BFA caused a significant decrease in the release of A-LAP into the medium (data not shown), indicating that A-LAP is secreted through a Golgi-mediated secretory pathway (*25*, *26*). We determined the N-terminal sequence of the secreted enzyme and two amino acid sequences corresponding to ^{37}ASPKRSDG and ^{41}RSDGTPF were obtained.

Purification and Characterization of Recombinant A-LAP Expressed in CHO Cells—To express recombinant A-LAP protein in CHO cells, we constructed an A-LAP expression vector consisting of the signal sequence of human trypsin II followed by the open reading frame of human A-LAP start-

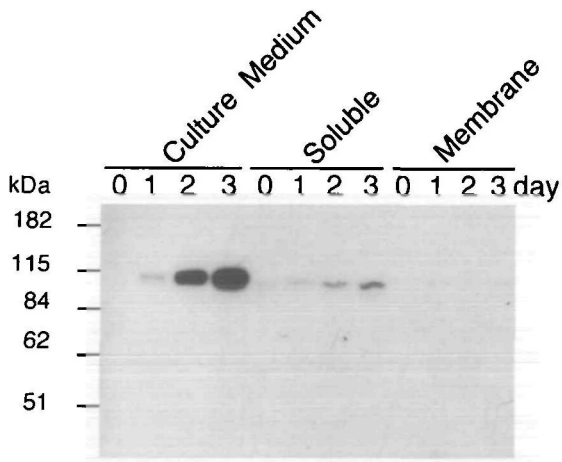


Fig. 1. **Secretion of A-LAP from COS-7 cells transfected with pSVL/A-LAP1.** After transfection, COS-7 cells were incubated for the indicated times and the culture medium was collected and concentrated. Cells were then lysed and fractionated into membrane and soluble (cytosolic) fractions. Western blot analysis was performed as described in "EXPERIMENTAL PROCEDURES."

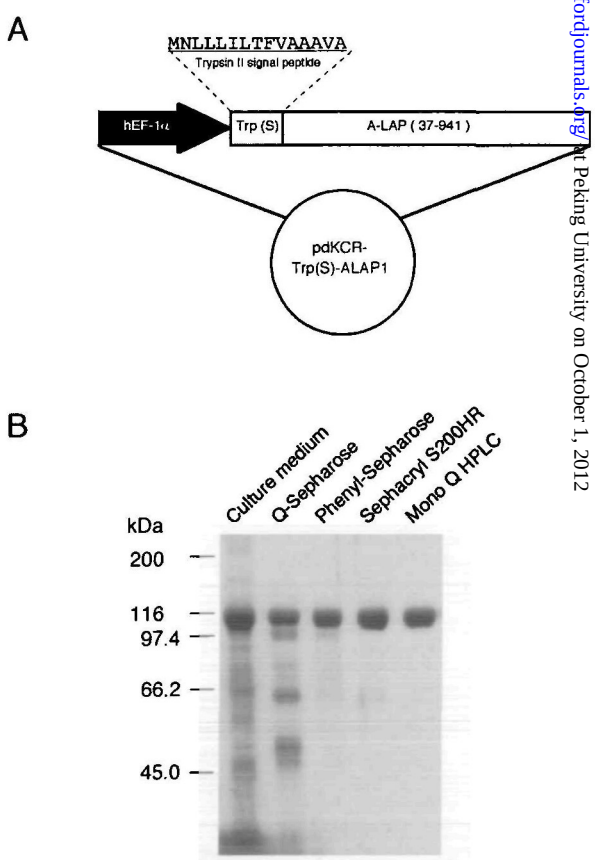


Fig. 2. **Purification of recombinant human A-LAP expressed in CHO cells.** A: Schematic representation of A-LAP expression vector, pdKCR-Trp(S)-ALAP1. B: Purification of recombinant A-LAP. A-LAP fractions from each column were analyzed by SDS-PAGE (8% gel).

ing at Ala-37 (Fig. 2A) (*20*).

After transfection with A-LAP cDNA into CHO cells, 68 clones were screened; clone 15 produced the highest A-LAP activity. Purification of A-LAP from the culture medium of clone 15 was performed as described above (Fig. 2B). The purified protein showed a single band with a molecular mass of 106 kDa on SDS-PAGE. As expected, PNGase F treatment decreased the apparent molecular mass of the protein to 93 kDa. But removal of *N*-linked sugar chains had no effect on the enzymatic activity (data not shown). The specific activity of A-LAP after the last step of purification was 192.0 nmol/mg/min with approximately 50% recovery and 5-fold purification (Table I).

We then determined the N-terminal amino acid sequence of the recombinant enzyme. A single amino acid sequence of 9 residues (RSDGTPFPW) was obtained, indicating the homogenous nature of the enzyme preparation. The result also indicates that four amino acids were deleted during enzyme preparation.

Figure 3 shows the elution profile of A-LAP from a TSK G3000SW column. Purified A-LAP eluted with aldolase, indicating that the molecular mass of the enzyme estimated from the gel filtration column chromatography was about 150 kDa. To determine the molecular mass of the enzyme in solution more precisely, we performed ultracentrifuge analysis. The molecular mass of A-LAP by sedimentation equilibrium experiments was calculated as 120 kDa. These results indicate that the enzyme exists in a monomeric form.

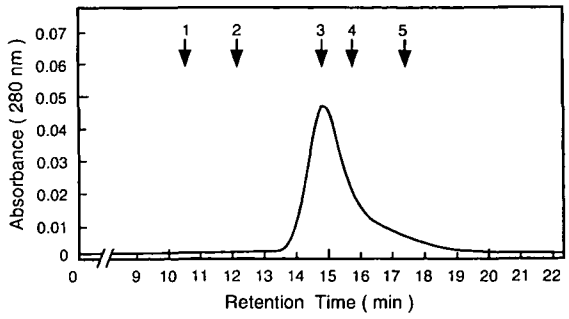
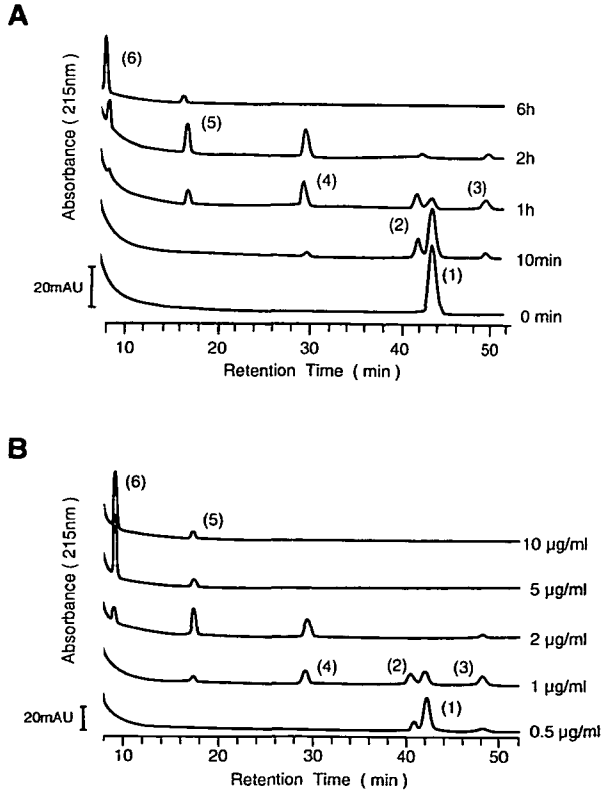


Fig. 3. **Gel filtration column chromatography (TSK G3000SW) of purified A-LAP.** Molecular markers (Pharmacia) used were thyroglobulin (669 kDa, 1), ferritin (440 kDa, 2), aldolase (158 kDa, 3), albumin (67 kDa, 4), and ovalbumin (43 kDa, 5).

TABLE I. **Purification of recombinant A-LAP.**

	Protein (mg)	Total activity (nmol/min)	Total activity (%)	Specific activity (nmol/mg/min)	Purification (fold)
1. Conditioned medium	329.7	12,735.7	100.0	38.6	1.00
2. Q-Sepharose	166.3	11,270.9	88.5	67.8	1.76
3. Phenyl-Sepharose	53.9	9,832.7	77.2	182.5	4.73
4. Sephacryl S200HR	34.9	6,366.8	50.0	182.6	4.73
5. Mono Q	32.9	6,300.0	49.6	192.0	4.97



C

(1) Asp-Arg-Val-Tyr-Ile-His-Pro-Phe (Angiotensin II)

(2) Arg-Val-Tyr-Ile-His-Pro-Phe (Angiotensin III)

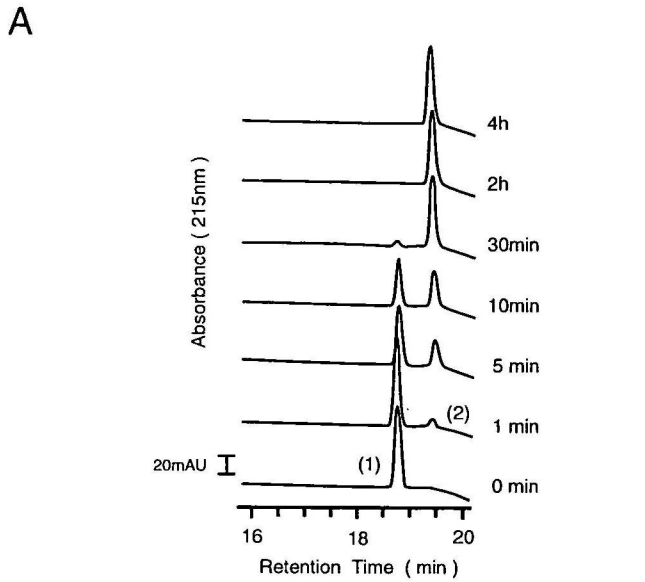
(3) Val-Tyr-Ile-His-Pro-Phe (Angiotensin IV)

(4) Tyr-Ile-His-Pro-Phe

(5) Ile-His-Pro-Phe

(6) His-Pro-Phe

Fig. 4. **Cleavage of Ang II by recombinant A-LAP.** A: Ang II (25 μM) was incubated with A-LAP (10 μg/ml) at 37°C for the indicated times. B: Ang II (25 μM) was incubated with various concentrations of A-LAP at 37°C for 6 h. After the reaction was terminated by adding 2.5% (v/v) formic acid, the generated peptides were loaded onto an HPLC column and separated as described in "EXPERIMENTAL PROCEDURES." C: Structures of Ang II–derived peptides. After separation on an HPLC column, the peptides eluted were collected and their structures were determined by MALDI-TOF MS.



B

(1) Kallidin (Lysyl-Bradykinin) Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

(2) Bradykinin Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg

Fig. 5. **Conversion of kallidin to bradykinin by recombinant A-LAP.** A: Kallidin (25 μM) was incubated with A-LAP (2 μg/ml) at 37°C for the indicated times. The generated peptides were loaded onto an HPLC column and separated as described in "EXPERIMENTAL PROCEDURES." B: Structures of kallidin-derived peptides determined by MALDI-TOF MS.

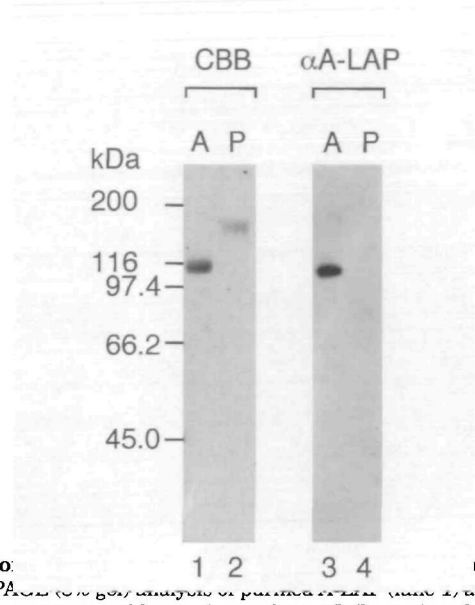


Fig. 6. **Detectio** **ti-body.** A: SDS-PA[illegible] and P-LAP (lane 2). B: Western blot analysis of A-LAP (lane 3) and P-LAP (lane 4) by anti–A-LAP polyclonal antibody.

In our previous work, we characterized some enzymatic properties of A-LAP employing the protein immunoprecipitated with C15 anti–A-LAP antibody (*18*). Here we further characterized the enzymatic properties of purified A-LAP using synthetic substrate, Leu-pNA since it was shown that among synthetic substrates tested the enzyme shows a preference for leucine. We found that the pH profile of the enzyme activity revealed a neutral optimal pH of 7.5, with a relatively sharp pH dependence; the Lineweaver-Burk plot yielded a first order graph indicating a V_{max} of 3.55 μmol/min/mg and a K_m of 1.28 mM.

Cleavage of Peptide Hormones by A-LAP—We next searched for A-LAP–mediated degradation of natural bioactive peptides. We first examined the enzyme activity toward oxytocin and vasopressin, both of which have an N-terminal cysteine in disulfide linkage with an internal cysteine and are good substrates for P-LAP (*22*). No detectable activity was observed to these hormones (data not shown), indicating that the substrate specificity of A-LAP is different from that of P-LAP.

Figure 4 shows the cleavage of Ang II by A-LAP. Analysis of the degradation products determined by MALDI-TOF MS showed the sequential release of N-terminal amino acids, and the final product detected on the column was de-[Val-Tyr-Ile] Ang IV (*i.e.* His-Pro-Phe). Conversion of Ang II to biologically inactive Ang IV through Ang III was rapid with the appearance of Ang IV observed within 10 min. A dose response study indicated that during 6 h of incubation, a maximum response was obtained at enzyme concentrations above 5 μg/ml. As expected, amastatin (100 μM) and $ZnCl_2$ (30 μM) inhibited the degradation of Ang II (data not shown).

When kallidin was incubated with A-LAP, rapid conversion to bradykinin was observed (Fig. 5). Once converted to bradykinin, no further N-terminal amino acid release was observed during the incubation period. As little as 1 μg/ml of the enzyme was enough for the maximum reaction. These results indicate that while A-LAP inactivates Ang II, it can also mediate the final process of the bradykinin generating pathway (*27*).

We also surveyed the A-LAP–catalyzed cleavage of other bioactive peptides and found that several neuronal peptides, such as dynorphin A, neurokinin A, and neuromedin B, are cleaved by the enzyme (data not shown). These results suggest that although A-LAP shows an apparent preference for leucine among synthetic substrates tested, it possesses a broad substrate specificity towards natural substrates.

Immunohistochemical Localization of A-LAP in the Human Kidney—To elucidate the possible involvement of A-LAP in the regulation of blood pressure, we examined the expression of the protein in the kidney. For this purpose we employed an affinity-purified antibody to rule out the possibility that the antibody also recognizes the most closely related enzyme, P-LAP. As shown in Fig. 6, the antibody recognizes A-LAP but not P-LAP, confirming the specific recognition of the antibody.

Figure 7 shows immunohistochemical localization of A-LAP in the cortex of the human kidney. A-LAP immunoreactivity was detected in the distal tubules and collecting ducts. Since the availability of human kidney is limited, we also examined the immunohistochemical localization of the enzyme in the kidneys of Wistar Kyoto rats and BALB/c mice. A-LAP immunoreactivity was detected in the distal tubules of the cortex and the collecting ducts of the cortex and medulla (data not shown).

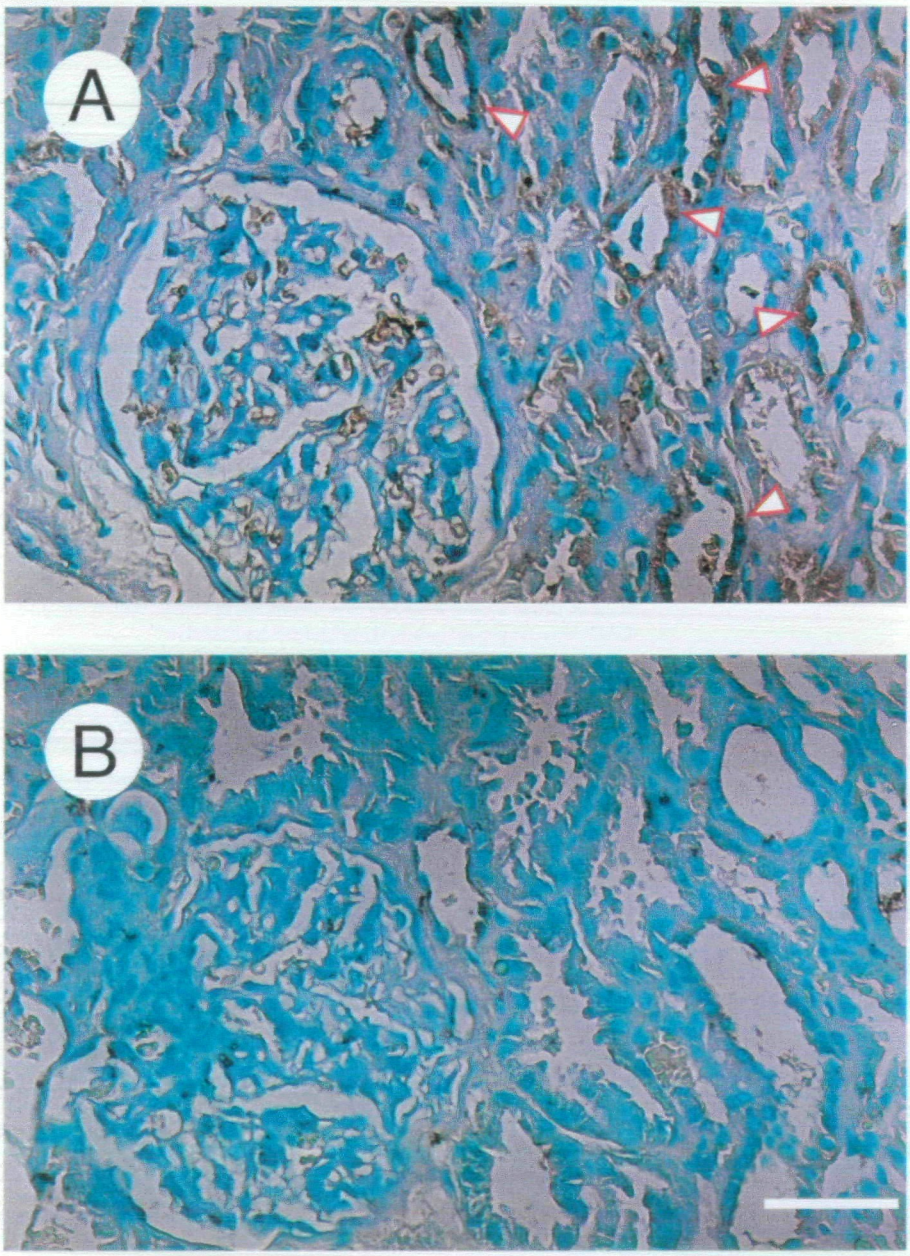


Fig. 7. **Immunohistochemical localization of A-LAP in the human renal cortex.** After incubation with 10 μg/ml of affinity-purified anti–A-LAP antibody (A) or 10 μg/ml of normal rabbit immunoglobulin (B), bound antibody was detected using HRP-conjugated anti–rabbit IgG and 3,3′-diaminobenzidine. The sections were counterstained with methyl green. Arrowheads indicate A-LAP immunoreactivity found in the distal tubules. Bar = 50 μm.

DISCUSSION

To elucidate the biological significance of A-LAP, we have for the first time produced recombinant human A-LAP in CHO cells and characterized its enzymatic nature.

cDNA cloning of human A-LAP revealed that the enzyme carries a significantly hydrophobic region near the N-terminus that could function as an internal signal peptide and a membrane-spanning domain (*18*). Indeed it has been reported that the A-LAP gene is located on chromosome 5q15-21, relatively close to P-LAP (*28*, *29*), suggesting a recent gene duplication. However, we have shown in this study that the majority of A-LAP is secreted into the culture medium when expressed transiently in COS-7 cells. In our initial study, we could neither immunoprecipitate nor detect by Western blot analysis the secreted enzyme in the presence of albumin in the culture medium (*18*). Removal of the albumin made it possible to detect the enzyme in the medium.

At present, the interrelationship between the secreted and cell-associated soluble forms of A-LAP is unclear. However, it is conceivable that differential usage of translation initiation sites might cause the appearance of two populations. It has been reported that puromycin-sensitive aminopeptidase might be translated preferentially at the second methionine codon of the open reading frame, resulting in the loss of a signal peptide and the cytosolic localization of the enzyme (*14*). By analogy, we suggest that the cytosolic localization of A-LAP is attributable to the preferential translation from Met-13, which is located in the middle of the putative signal peptide and is flanked by a Kozak consensus-like sequence. On the other hand, it is likely that translation of the secreted form of A-LAP is initiated at

Met-1, retaining the N-terminal hydrophobic sequence and entering into the BFA-sensitive secretory pathway. Alternatively, it is also possible that the apparent cytosolic localization of A-LAP is due to the weak interaction of the enzyme with intracellular membranes. In a future study, it is necessary to elucidate in detail the secretion mechanisms of A-LAP.

Although aminopeptidases belonging to the M1 family (*i.e.* aminopeptidase A, aminopeptidase N, and P-LAP) are usually homodimeric (*2*, *22*), ultracentrifuge analysis revealed that A-LAP is a monomeric protein. The implications for this difference are unclear at present, but it is possible that the failure to form dimers causes the low affinity of the enzyme for the synthetic substrate, Leu-pNA. To elucidate the structural features in detail, we are now planning to determine the three-dimentional structure of the enzyme by X-ray crystallography.

In the previous work, we measured the hydrolytic activity of A-LAP towards various synthetic substrates and reported its restricted substrate specificity (*18*). However, its broad specificity is apparent when tested against various natural peptide hormones. Considering that A-LAP could be secreted under some biological conditions, a variety of bioactive peptides might be metabolized by the enzyme. Our results also imply that, as in the case of P-LAP (*22*), it is difficult to estimate the susceptibility of the given hormones to the enzyme from the data obtained using artificial substrates.

To survey natural substrates, we examined several peptides for cleavage by the enzyme. When Ang II was treated with A-LAP, sequential cleavage of N-terminal peptide bonds was observed, confirming the aminopeptidase activity of the enzyme towards natural peptides for the first time. We also found that kallidin is converted into bradykinin by the enzyme, indicating that A-LAP can mediate the final step of bradykinin generation. It is well established that tissue kallikrein converts low molecular weight kininogen to kallidin (*27*). Although it is obvious that further studies are required to elucidate the physiological role of the enzyme, our present data suggest that A-LAP is one of the long sought kinin-converting enzymes (*30*) and plays some roles in the regulation of blood pressure. It should be mentioned here that several neuronal peptides such as neurokinin A and neuromedin B are also susceptible to the enzyme, suggesting that it has some functional roles in the brain.

Immunohistochemical analyses revealed that A-LAP protein is indeed produced in the kidney where Ang II and bradykinin are produced and play roles in the regulation of blood pressure. More closely, it was shown that A-LAP immunoreactivity is detected in the distal tubules of the cortex and the collecting ducts of the cortex and medulla of the rodent kidney. Since the distal tubule is known to be the dominant site of tissue kallikrein expression (*31*, *32*), it is quite possible that kallikrein and A-LAP react sequentially with low molecular weight kininogen to form bradykinin.

Growing evidence indicates the biological significance of aminopeptidases belonging to the M1 family of metallopeptidases. Employing highly selective aminopeptidase A and aminopeptidase N inhibitors, Reaux *et al.* recently elucidated the important roles of these two enzymes in the brain renin-angiotensin system (*33*, *34*). It has also been reported that puromycin-sensitive aminopeptidase gene–deficient mice show dwarfism and display increased anxiety and analgesia (*35*). P-LAP is believed to repress the labor-inducible effect of oxytocin, thus maintaining normal pregnancy. In this paper, we report the production and characterization of recombinant human A-LAP. Our data suggest the potential role of the enzyme in the kidney in the regulation of blood pressure. Moreover, we found that the expression of cell-associated A-LAP protein is transiently increased during the differentiation of 3T3-L1 preadipocytes into adipocytes, tempting us to speculate that the enzyme also plays a role in cell differentiation. Further studies, including the development of a specific A-LAP inhibitor and the generation of A-LAP gene–deficient mice, are required to determine the physiological and/or pathological functions of the enzyme.

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