J. Biochem. 122, 779-787 (1997)

Alanyl Aminopeptidase from Human Seminal Plasma: Purification, Characterization, and Immunohistochemical Localization in the Male Genital Tract[1]

Kai Huang,* Shuji Takahara,* Toshiaki Kinouchi,† Masami Takeyama,‡ Tetsuo Ishida,§ Hisao Ueyama,* Katsuji Nishi,¶ and Iwao Ohkubo*,[2]

*Departments of Medical Biochemistry, §Biochemistry, and ¶Legal Medicine, Shiga University of Medical Science, Seta, Otsu, Shiga 520-21; †Department of Urology, The Center for Adult Diseases, Osaka, Higashinari-ku, Osaka 537; and ‡Department of Urology, Osaka Central Hospital, Osaka, Kita-ku, Osaka 537

Received for publication, May 23, 1997

Alanyl aminopeptidase (AAP) was purified to homogeneity from human seminal plasma. The calculated molecular weight of the purified enzyme was approximately 137,000±5,000 from light scattering, 140,000 (main) and 137,000 (minor) from non-denatured PAGE and 153,000 from SDS-PAGE in the absence or presence of 2-mercaptoethanol (2-ME). These findings suggest that the enzyme is monomeric in form in human seminal plasma. The enzyme hydrolyzed several amino acid 4-methyl-coumaryl-7-amide (MCA) substrates. The order of K_{cat}/K_m values of AAP at optimal pH (pH 7.5) was Ala- > Lys-Ala- ≥ Met- > Leu- > Phe- > Arg- ≥ Arg-Arg- > Lys- > Gly-MCAs. AAP was potently inhibited by bestatin, leuhistin, actinonin, amastatin, and 1,10-phenanthroline. These findings suggest that AAP is an aminopeptidase. We determined that the amino acid sequence of the first 22 residues of the enzyme was Ser[1]-Thr-Thr-Pro-Ser[5]-Ala-Ser-Ala-Thr-Thr[10]-Asn-Pro-Ala-Ser-Ala[15]-Thr-Thr-Leu-Asp-Gln[20]-Ser-Lys-. This sequence was completely coincident with that downstream of the transmembrane site of human intestinal alanyl aminopeptidase N (CD13). We also isolated cDNA encoding AAP from human prostate cDNA library, sequenced its structure, and confirmed human seminal plasma AAP to be identical with alanyl aminopeptidase N. We postulated that native human seminal plasma alanyl aminopeptidase is released into the seminal plasma after the specific site is cleaved by elastase or an elastase-like enzyme. The enzyme level in human seminal plasma determined by single radial immunodiffusion was 5.2±3.2 mg/100 ml (mean±SD, $n=20$) in individuals 20-47 years of age. AAP was immunohistochemically stained in the luminal site-cell membrane of epithelial cells in the prostatic gland and ductuli efferentes of the testis.

Key words: alanyl aminopeptidase, characterization, immunohistochemistry, purification, seminal plasma.

Membrane bound (aminopeptidase N or M) and cytosolic alanyl aminopeptidases [EC 3.4.14.2] in mammalian tissues and body fluids such as human placenta (*1*), human and porcine kidneys (*2, 3*), human and rat brains (*4, 5*), human pancreas (*6*), human skeletal muscle (*7, 8*), human maternal serum (*9*), bovine seminal vesicle secretion (*10*), and human seminal plasma (*11*) have been identified. These enzymes are thought to be physiologically involved in the breakdown of some oligopeptides or their fragments such as Met- and Leu-enkephalins, dynorphin-related peptides, and Met-Lys-bradykinin (*4, 12*). Furthermore, the entire amino acid sequences of human intestinal aminopeptidase N and rat kidney aminopeptidase M have been deduced from those of their cDNAs (*13-16*).

We showed that dipeptidyl peptidases II and IV (DPPs II and IV) are exopeptidases that co-exist in porcine seminal plasma, separated them by Sephacryl S-300 HR and zinc-chelate affinity column chromatography, and determined their physicochemical properties (*17, 18*). On the basis of the above data, we tried to purify DPP II from human seminal plasma using the substrate Lys-Ala-MCA. However, the purified enzyme had the physicochemical properties of an alanyl aminopeptidase (AAP). Although AAP is known to exist in seminal plasma (*10, 11*), little is known regarding its properties and histochemical localization. In the present study, we purified AAP from human seminal plasma. We then determined its molecular weight, kinetic parameters, N-terminal amino acid sequence, cDNA struc-

[1] This work was supported in part by Grants-in-Aid for Scientific Research (05670118 to I.O.) from the Ministry of Education, Science, Sports and Culture of Japan, and by a Grant (to I.O.) from the Japan Foundation for Applied Enzymology.

[2] To whom correspondence should be addressed. Tel: +81-775-48-2161, Fax: +81-775-48-2164, E-mail: ionfox@bellebsd.shiga-med.ac.jp

Abbreviations: AMC, 7-amino-4-methylcoumarin; MCA, 4-methyl-coumaryl-7-amide; MNA, 4-methoxy-2-naphthylamide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; 2-ME, 2-mercaptoethanol; E-64, *N*-(*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl)agmatin; DFP, diisopropylphosphofluoridate; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; PMSF, phenylmethylsulfonyl fluoride; PCMBS, *p*-chloromercuri-benzene-sulfonic acid.

ture, and immunohistochemical localization in the male genital tract.

MATERIALS AND METHODS

Materials—Semen was obtained from normal adult volunteers and centrifuged at 10,000×*g* for 20 min at 4°C. The seminal plasma obtained was stored at −30°C until use. E-64, diprotin A, antipain, and other proteinase inhibitors, and fluorogenic peptide substrates such as Ala-, Gly-, Lys-, Phe-, Pro-, Tyr-, and Leu-MCAs, and Lys-Ala- and Arg-Arg-MCAs were purchased from Peptide Institute (Osaka) or Bachem Feinchemikalien AG (Bubendorf, Switzerland). Q-Sepharose, Superdex 200, and standard proteins kits for molecular weight and isoelectric point determination were products of Pharmacia LKB Biotechnology AB (Uppsala, Sweden). Matrex Gel Red A was purchased from Amicon (Lexinton, MA, USA). Ala- and Lys-Ala-4-methoxy-2-naphthylamides and Fast Garnet GBC were also purchased from Sigma Chemical (St. Louis, MO, USA).

All other chemicals were of analytical grade.

Assay of Enzyme Activity—Enzyme activity was assayed fluorometrically by measuring (excitation, 380 nm; emission, 440 nm for AMC) the liberation of AMC at 37°C in a mixture containing 10 μl of 10 mM substrate, 100 μl of 0.5 M Tris-HCl buffer, pH 7.5, 10-20 μl of enzyme solution and Milli Q water (18 mΩ) in a total volume of 1 ml. After incubation for 30 min, 2 ml of 0.2 N acetic acid was added to the mixture to terminate the reaction. One unit of activity was defined as the amount of enzyme which is capable of hydrolyzing 1 μmol of substrate per minute. Protein was measured by absorbance at 280 nm in a 1-cm light path, and 1 mg of protein was defined as the concentration required to yield an absorbance of 20 ($E^{1\%}_{280nm}=20$) (see below), except that we used $E^{1\%}_{280nm}=10$ for the purification steps of AAP.

Polyacrylamide Gel Electrophoresis—Samples were resolved by electrophoresis on 10% polyacrylamide slab gels in 25 mM Tris, 192 mM glycine, pH 8.3, and 0.1% SDS by the method of Laemmli (*19*). Proteins in the gel were stained with Coomassie Brilliant Blue R-250. To detect AAP activity, 10% polyacrylamide gels in the absence of SDS were also incubated at 37°C for 15 min in 100 mM Tris-HCl buffer, pH 7.5, containing 1 mM Lys-Ala-MNA or Ala-MNA, then the gel was stained with 0.15% Fast Garnet GBC in 2.1 M sodium acetate buffer, pH 4.2 (*20*).

Determination of Isoelectric Point—The isoelectric point of AAP was determined by chromatofocusing using a PhastSystem (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) in a pH gradient from 9 to 3 according to the manufacturer's instructions.

Determination of Molecular Weight—The apparent molecular weight (M_r) of AAP was determined by gel filtration chromatography on Superdex 200 (1.6×60 cm) equilibrated with 20 mM Tris-buffered saline, pH 7.5, at a flow rate of 0.75 ml/min in an FPLC system. The molecular weight of the purified AAP was calculated by comparison with the logM_r for standard proteins, thyroglobulin (M_r 669,000), ferritin (M_r 440,000), catalase (M_r 232,000), and aldolase (M_r 158,000).

We also determined the molecular weight by measuring low-angle laser light scattering combined with gel chromatography (*21, 22*). A TSK-GEL G3000SW$_{XL}$ column (7.8×300 mm), an LS-8000 low-angle laser light scattering photometer, a UV-8000uv-vis detector, and an RI-8000 differential refractometer, were all from Tosoh. The eluent buffer was 20 mM Tris buffered saline, pH 7.5, and the flow rate was 0.4 ml/min. The value of the specific refractive increment of the enzyme solution was assumed to be the same as that of the standard proteins. Molecular weight standards were bovine dopamine β monooxygenase, native tetramer (M_r 260,000), *Pseudomonas putida* catechol 2,3-dioxygenase (M_r 140,000), bovine dopamine β monooxygenase, denatured dimer (M_r 130,000), bovine serum albumin (M_r 66,300), and ovalbumin (M_r 43,500).

N-Terminal Amino Acid Sequencing—Purified APP (~300 pmol) was bound onto a Prospin membrane (Applied Biosystem, Foster City, CA, USA), then sequenced by automated Edman degradation using an Applied Biosystems Model 473 Protein Sequencer. Phenylthiohydantoin amino acids were identified by high-performance liquid chromatography (Applied Biosystem 120A analyzer).

Extinction Coefficient—We determined that the extinction coefficient ($E^{1\%}_{280nm}$) for APP from human seminal plasma was 20 based on absorbance at 205 and 280 nm according to van Iersel *et al.* (*23*).

Immunodiffusion—Single radial immunodiffusion was performed as described by Mancini *et al.* (*24*).

Immunohistochemistry—Most of the adult human tissues including testis, epididymis, seminal vesicle, and prostate were obtained at the time of autopsy within 12 h of death. They were all fixed in 10% neutral buffered formalin, then embedded in paraffin. Specimens of the tissues described above from 10 to 15 autopsies were immunochemically stained as described (*25*).

Purification of AAP from Human Seminal Plasma—All purification steps were performed at 4°C unless otherwise specified. At each step, the activities of AAP were measured in 50 mM Tris-HCl buffer, pH 7.5, using Ala- or Lys-Ala-MCAs.

Step 1. Dialysis: Human seminal plasma was dialyzed overnight against 20 mM Tris-HCl buffer, pH 8.0. The dialyzate was then centrifuged at 10,000×*g* for 45 min at 4°C and the supernatant was collected.

Step 2. Q-Sepharose column chromatography: The supernatant was applied at a flow rate of 50 ml/h to a Q-Sepharose column (2.5×20 cm) equilibrated with 20 mM Tris-HCl buffer, pH 8.0. The column was washed extensively with the equilibration buffer, then a linear gradient consisting of 500 ml of the same buffer and 500 ml of 20 mM Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl was applied. Fractions containing AAP and DPP II activities were pooled together because both enzymes eluted at essentially the same time. The pooled sample was dialyzed overnight against 20 mM Tris-HCl buffer, pH 7.5.

Step 3. Matrex Gel Red A column chromatography: The dialyzate was applied at a flow rate of 20 ml/h to a Matrex Red A gel column (1.5×20 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7.5. The pass-through fraction containing AAP and DPP II activities was collected. After washing the column with the same buffer, samples were eluted stepwise with the same buffer containing 1.0 M KCl. The collected fraction was dialyzed against 20 mM Tris-buffered saline, pH 8.0 for several hours.

Step 4. Zinc-Chelate Cellulofine column chromatography: The dialyzate was applied at a flow rate of 20 ml/h to a

Zinc-Chelate Cellulofine column (1.5×12 cm) equilibrated with 20 mM Tris-buffered saline, pH 8.0. After washing the column with the same buffer, non-adsorbed fractions containing AAP and DPP II activities were collected and concentrated by the addition of solid ammonium sulfate (75% saturation). The precipitate was dissolved in a minimum volume of 20 mM Tris-buffered saline, pH 8.0, and dialyzed overnight against the same buffer.

Step 5. Superdex 200 column chromatography in an FPLC system: Samples were applied at a flow rate of 0.5 ml/min to a Superdex 200 column (1.6×60 cm) equilibrated with 20 mM Tris-buffered saline, pH 8.0. Fractions containing both AAP and DPP II activities were collected, and NaCl was added at a final concentration of 3.0 M.

Step 6. Phenyl-Cellulofine column chromatography: The solution was applied at a flow rate of 20 ml/h to a Phenyl-Cellulofine column (1.5×8 cm) equilibrated with 20 mM Tris-HCl buffer, pH 8.0, containing 3.0 M NaCl. Non-adsorbed proteins were removed with the same buffer, then a decreasing salt gradient was applied with 80 ml of the above buffer and 80 ml of the above buffer without NaCl. Fractions containing both AAP and DPP II activities were collected and dialyzed overnight against 20 mM Tris-HCl buffer, pH 8.0 (Fig. 1A).

Step 7. Resource Q column chromatography in an FPLC system: The fraction containing both enzymatic activities was applied at a flow rate of 0.6 ml/min to a column of Resource Q (bed volume: 1.0 ml) equilibrated with 20 mM Tris-HCl buffer, pH 8.0. After washing the column with the same buffer, a gradient consisting of 12 ml of the same buffer and 12 ml of the same buffer containing 0.4 M NaCl was applied. Fractions containing both AAP and DPP II activities were collected and dialyzed overnight against 20 mM Tris-HCl buffer, pH 8.0 (Fig. 1B). This preparation

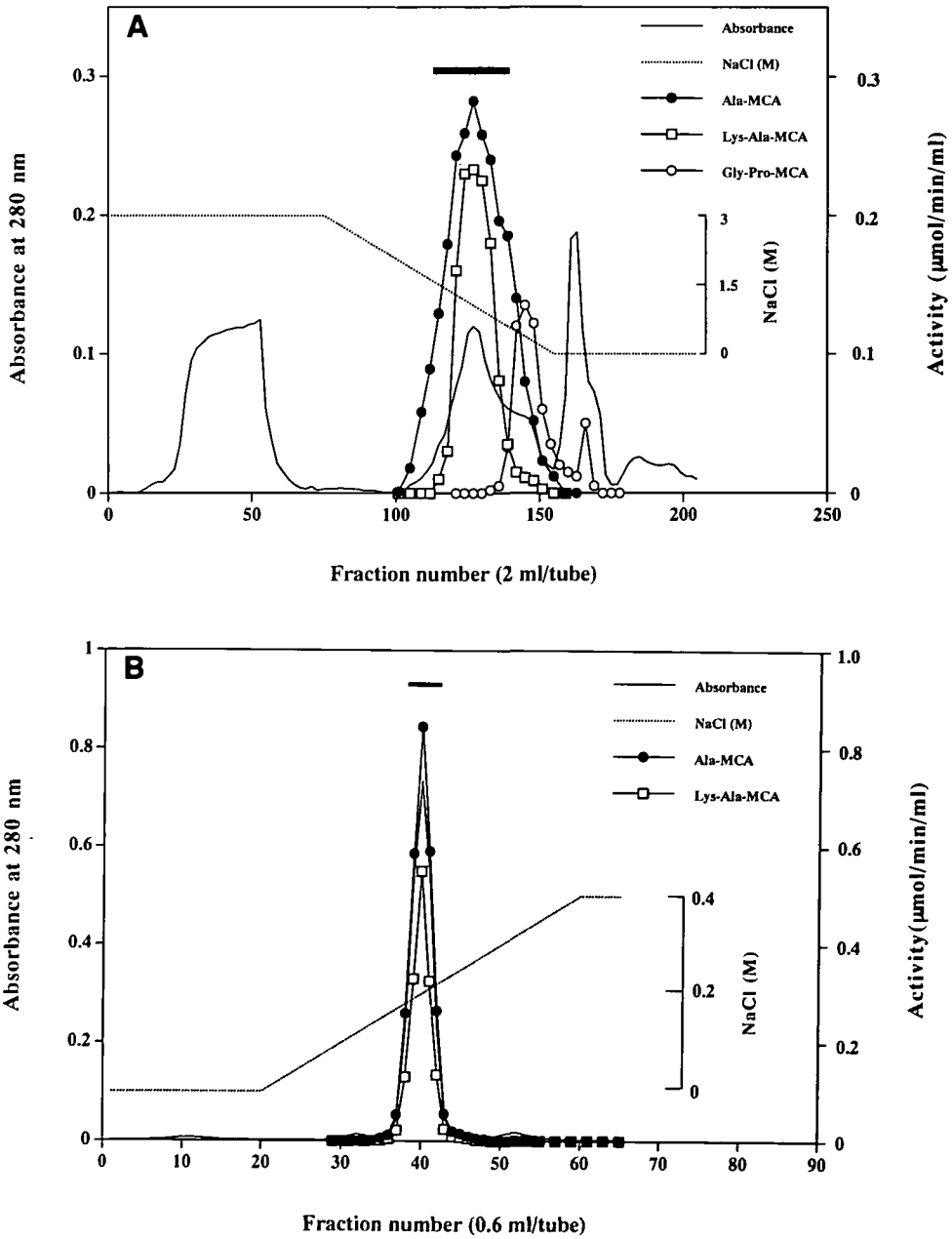

Fig. 1. **Purification of alanyl aminopeptidase (AAP) from human seminal plasma.** (A) Phenyl-Cellulofine column chromatography. Fractions containing AAP activity were collected. Bar indicates the fractions pooled. Absorbance at 280 nm, (–); AAP (● toward Ala-MCA and □ toward Lys-Ala-MCA); DPP IV (○ toward Gly-Pro-MCA). (B) Resource Q column chromatography in an FPLC system. Fractions containing AAP activity were collected. Bar indicates the fractions pooled. Absorbance at 280 nm, (–); AAP (● toward Ala-MCA and □ toward Lys-Ala-MCA).

was subsequently characterized in detail.

RESULTS

Enzyme Purification—Alanyl aminopeptidase (AAP) was purified from 70 ml of human seminal plasma by Q-Sepharose, Matrex Gel Red A, Zinc-Chelate Cellulofine, Superdex 200 (in FPLC), Phenyl-Cellulofine, and Resource Q (in FPLC) chromatography (Fig. 1, A and B). Table I shows typical results. Human seminal plasma AAP was purified approximately 250-fold with a 17.5% yield over dialyzed seminal plasma, and the enzyme at the final step migrated as a single band on SDS-PAGE in the absence and presence of 2-ME, and two bands (major band, $\geq 90\%$; minor band, $\leq 10\%$) on non-denatured PAGE (Fig. 2, A and B). The overall yield of AAP when calculated using $E^{1\%}_{280nm}=20$ was about 5 mg from 100 ml of human seminal plasma.

Molecular Weight, Activity Staining, and Isoelectric Point of the Purified Enzyme—The estimated molecular weight of the enzyme was 153,000 in the absence or presence of 2-ME on SDS-PAGE (Fig. 2A). However, its molecular weight was 140,000 (major band) and 137,000 (minor band) on non-denatured PAGE (Fig. 2B), and $137,000 \pm 5,000$ according to light scattering in the HPLC system. Accordingly, the native AAP is thought to be a monomer. Furthermore, both protein bands on non-denatured PAGE showed activity toward Ala-MNA and Lys-Ala-MNA (Fig. 2B). The intensity of AAP enzymatic activity was apparently parallel with the intensity of protein bands on non-denatured PAGE. The isoelectric point (pI) of the purified enzyme was 4.50.

Substrate Specificity and Kinetic Properties of the Purified Enzyme—As shown in Fig. 3, the time course of AAP reaction showed straight or convex curves for Ala-, Met-, and Lys-MCAs, but a concave curve for Lys-Ala-MCA, this latter indicating a two step reaction. As shown in Table II, the enzyme exhibited high activity toward the synthetic substrates Ala-, Met-, and Tyr-MCAs, moderate activity toward Leu- and Phe-MCAs, and weak activity toward Arg-, Lys-, and Gly-MCAs. Pro- and Pyr-MCAs were not hydrolyzed. It did not hydrolyze substrates with

TABLE I. **Purification of alanyl aminopeptidase from human seminal plasma.**

Step	Total absorbance (280 nm)	Total activity (units)	Specific activity (units/mg[a])	Yield (%)	Purification (-fold)
Dialysis	2,610	76.5	0.0293	100	1
Q-Sepharose	491	56.9	0.116	74.4	4
Matrex Red A	316	55.0	0.174	71.9	6
Zn-Chelate Cellulofine	155	54.4	0.351	71.1	12
Superdex 200	23.3	28.7	1.23	37.5	42
Phenyl-Cellulofine	2.7	14.9	5.52	19.5	188
Resource Q	1.8	13.4	7.44	17.5	254

[a]Protein was measured by absorbance at 280 nm in 1-cm light path, and 1 mg of protein was defined as the concentration required to yield an absorbance of 1.0 ($E^{0.1\%}_{280nm}=1.0$).

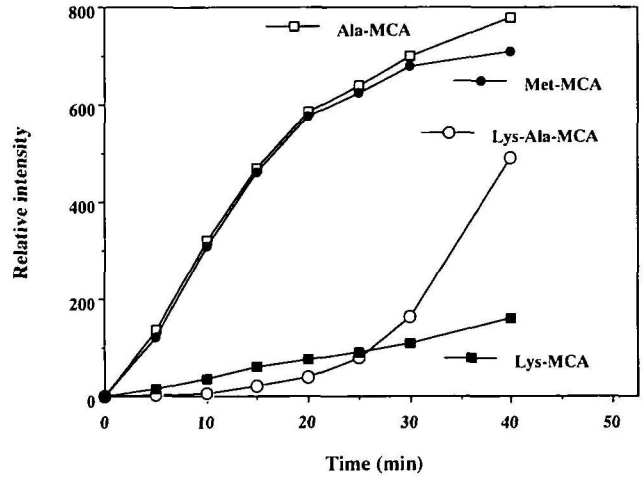

Fig. 3. **Time course of some MCA substrate hydrolyzing activities by purified human seminal plasma AAP.** Purified AAP (0.6 μg) was examined and its activity is expressed as relative intensity towards (□), Ala-MCA; (●), Met-MCA; (○), Lys-Ala-MCA; (■), Lys-MCA.

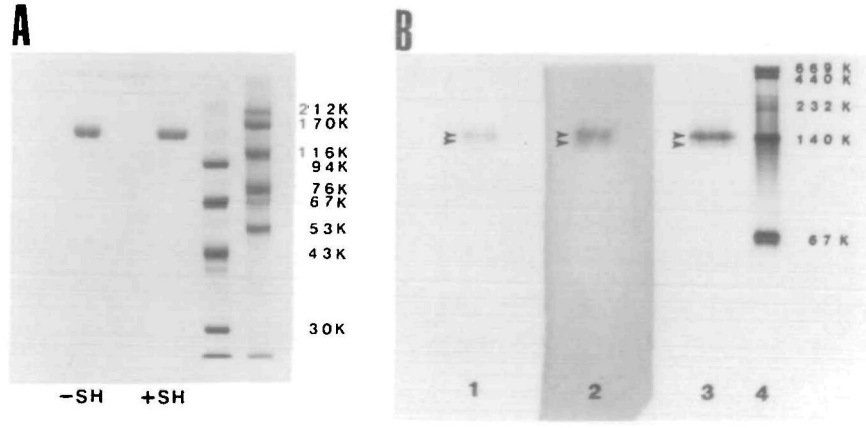

Fig. 2. **Polyacrylamide gel electrophoresis of AAP from human seminal plasma.** (A) Purified enzyme was resolved by electrophoresis on a 10% polyacrylamide gel in the presence of SDS, then the gel was stained with Coomassie Brilliant Blue R-250. Lane 1 (-SH) contained 3 μg of the purified enzyme, and Lane 2 (+SH) contained 3 μg of the purified protein in the presence of 2-ME. Lane 3 and 4 included the following standard proteins: myosin (M_r 212,000), α2-macroglobulin (M_r 170,000), β-galactosidase (M_r 116,000), phosphorylase *b* (M_r 94,000), transferrin (M_r 76,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), glutamic dehydrogenase (M_r 53,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,100) and α-lactalbumin (M_r 14,400). (B) Purified enzyme (2.5 μg) was resolved on a 10% polyacrylamide gel in the absence of SDS (lanes 1, 2, and 3). The gels were stained for AAP activity using Lys-Ala-4-methoxy-2-naphthylamide (lane 1) or Ala-4-methoxy-2-naphthylamide (lane 2) in combination with Fast Garnet GBC as described in "MATERIALS AND METHODS." Proteins in lanes 3 and 4 were stained with Coomassie Brilliant Blue R-250. Lane 4 included the following standard proteins: thyroglobulin (M_r 669,000), ferritin (M_r 440,000), catalase (M_r 232,000), lactate dehydrogenase (M_r 140,000), bovine serum albumin (M_r 67,000). Both major and minor bands (arrowheads) on lane 3 corresponding to arrowheads on lanes 1 and 2 are enzymatically active.

an N-blocked Gly-Pro sequence, such as Suc-Gly-Pro-MCA and Suc-Gly-Pro-Leu-Gly-Pro-MCA. Furthermore, the purified enzyme was found not to be an endopeptidase that would hydrolyze Z-Phe-Arg-MCA, Boc-Phe-Ser-Arg-MCA, and Suc-Ala-Ala-Ala-MCA substrates.

The K_m, K_{cat}, and K_{cat}/K_m values of AAP at optimal pH (pH 7.5) were 200 μM, 7.82 s^{-1}, and 39.1 $s^{-1} \cdot nM^{-1}$ for Ala-MCA, 230 μM, 7.52 s^{-1}, and 32.7 $s^{-1} \cdot nM^{-1}$ for Met-MCA, 250 μM, 8.33 s^{-1}, and 33.3 $s^{-1} \cdot nM^{-1}$ for Lys-Ala-MCA, and 250 μM, 5.45 s^{-1}, and 21.8 $s^{-1} \cdot nM^{-1}$ for Tyr-MCA, respectively. The K_m, K_{cat}, and K_{cat}/K_m values for other aminoacyl-MCAs are shown in Table III.

Effects of Inhibitors, Metal Ions, and Other Reagents on the Purified Enzyme—We examined the effects of various standard protease inhibitors against AAP (Table IV). AAP activity was potently inhibited by bestatin, leuhistin, actinonin, amastatin, and 1,10-phenanthroline, and moderately by EDTA, arphamenine A, and puromycin. However, it was resistant to the other protease inhibitors tested including benzamidine (1 mM), DFP (1 mM), AEBSF (1 mM), α-PMSF (1 mM), E-64 (0.1 mM), antipain (0.1 mM), chymostatin (0.1 mM), leupeptin (0.1 mM), pepstatin (0.1 mM), elastatinal (0.1 mM), and phosphoramidon (0.1 mM). These findings showed that the AAP from human seminal plasma belonged to the aminopeptidase family. The enzyme activity of AAP was not inhibited by diprotin A (Ile-Pro-Ile), a substrate analog for DPP IV. The enzyme activity was also not affected at concentrations ranging from 0.1 to 5 mM by such sulfhydryl reagents as 2-ME, iodoacetic acid, iodoacetamide, *N*-ethylmaleimide, and PCMBS. As shown in Table V, Cd^{2+}, Cu^{2+}, and Zn^{2+} completely inhibited the enzyme activity and Hg^{2+} moderately inhibited it (Table V).

Optimal pH and Enzyme Stability—The hydrolytic activity of AAP for Ala-, Met-, and Lys-Ala-MCA was assayed in the pH range from 2.5 to 10.5. The activity towards these three substrates was optimal at pH 7.5. Furthermore, to examine the effect of pH on stability, AAP was incubated at various pH values for 24 h at 4°C. The enzyme was stable over the pH range 6.0 to 10.0. In 20 mM Tris-buffered saline, pH 7.5, AAP was stable for at least

TABLE II. **Substrate specificity of alanyl aminopeptidase from human seminal plasma.** Assay was proceeded at 37°C in 50 mM Tris-HCl buffer, pH 7.5, in the presence of 0.6 μg of the enzyme and 100 μM substrate.

Substrate	Enzyme activity (%)
Ala-MCA	100
Met-MCA	98
Tyr-MCA	80
Leu-MCA	64
Phe-MCA	53
Arg-MCA	29
Lys-MCA	16
Gly-MCA	14
Pro-MCA	0
Pyr-MCA	0
Lys-Ala-MCA	25
Arg-Arg-MCA	33
Gly-Pro-MCA	0
Pro-Phe-Arg-MCA	0
Boc-Phe-Ser-Arg-MCA	0
Z-Phe-Arg-MCA	0
Suc-Ala-Ala-Ala-MCA	0
Suc-Leu-Leu-Val-Tyr-MCA	0
Suc-Gly-Pro-MCA	0
Suc-Gly-Pro-Leu-Gly-Pro-MCA	0

TABLE III. **Kinetic parameters of AAP from human seminal plasma.** K_{cat} values were calculated assuming that 137 mg of protein represents 1 μmol of enzyme.

Substrate	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m ($s^{-1} \cdot nM^{-1}$)
Ala-MCA	200	7.82	39.1
Met-MCA	230	7.52	32.7
Tyr-MCA	250	5.45	21.8
Leu-MCA	333	6.42	19.5
Phe-MCA	270	4.50	16.7
Arg-MCA	160	2.36	14.8
Lys-MCA	100	1.25	12.5
Gly-MCA	200	1.10	5.5
Lys-Ala-MCA	250	8.33	33.3
Arg-Arg-MCA	50	0.85	14.1

TABLE IV. **Effect of aminopeptidase inhibitors on AAP activity.** Assay was proceeded at 37°C in 50 mM Tirs-HCl buffer, pH 7.5, in the presence of 0.6 μg of the enzyme and 100 μM Ala-MCA.

	Concentration (μM)	Residual activity (%)
Control	None	100
Bestatin	100	4
Leuhistin	100	0
Actinonin	100	0
Amastatin	100	0
Arphamenine A	500	58
Puromycin	100	33
EDTA	2,000	58
1,10-Phenanthroline	1,000	0

TABLE V. **Effect of divalent cations on AAP activity from human seminal plasma.** Assay was proceeded at 37°C in 50 mM Tris-HCl buffer, pH 7.5, in the presence of 0.6 μg of the enzyme and 100 μl of 10 mM Ala-MCA.

Cation	Concentration (mM)	Residual activity (%)
Control	1	100
$CaCl_2$	1	95
$MgCl_2$	1	100
$BaCl_2$	1	95
$SrCl_2$	1	97
$ZnCl_2$	1	4
$CdCl_2$	1	2
$MnCl_2$	1	84
$CoCl_2$	1	60
$NiCl_2$	1	63
$CuCl_2$	1	4
$HgCl_2$	1	29

Fig. 4. **N-terminal amino acid sequence of AAP purified from human seminal plasma.** The wavy underlines correspond to transmembrane sequences. Dashes indicate gaps introduced into the sequences. Common residues among AAP, HuAPN, and RtAPM are shown in filled boxes. The sequences of HuAPN and RtAPM are quoted from Olsen *et al.* (*13*) and Look *et al.* (*16*), and Malfroy *et al.* (*15*), respectively.

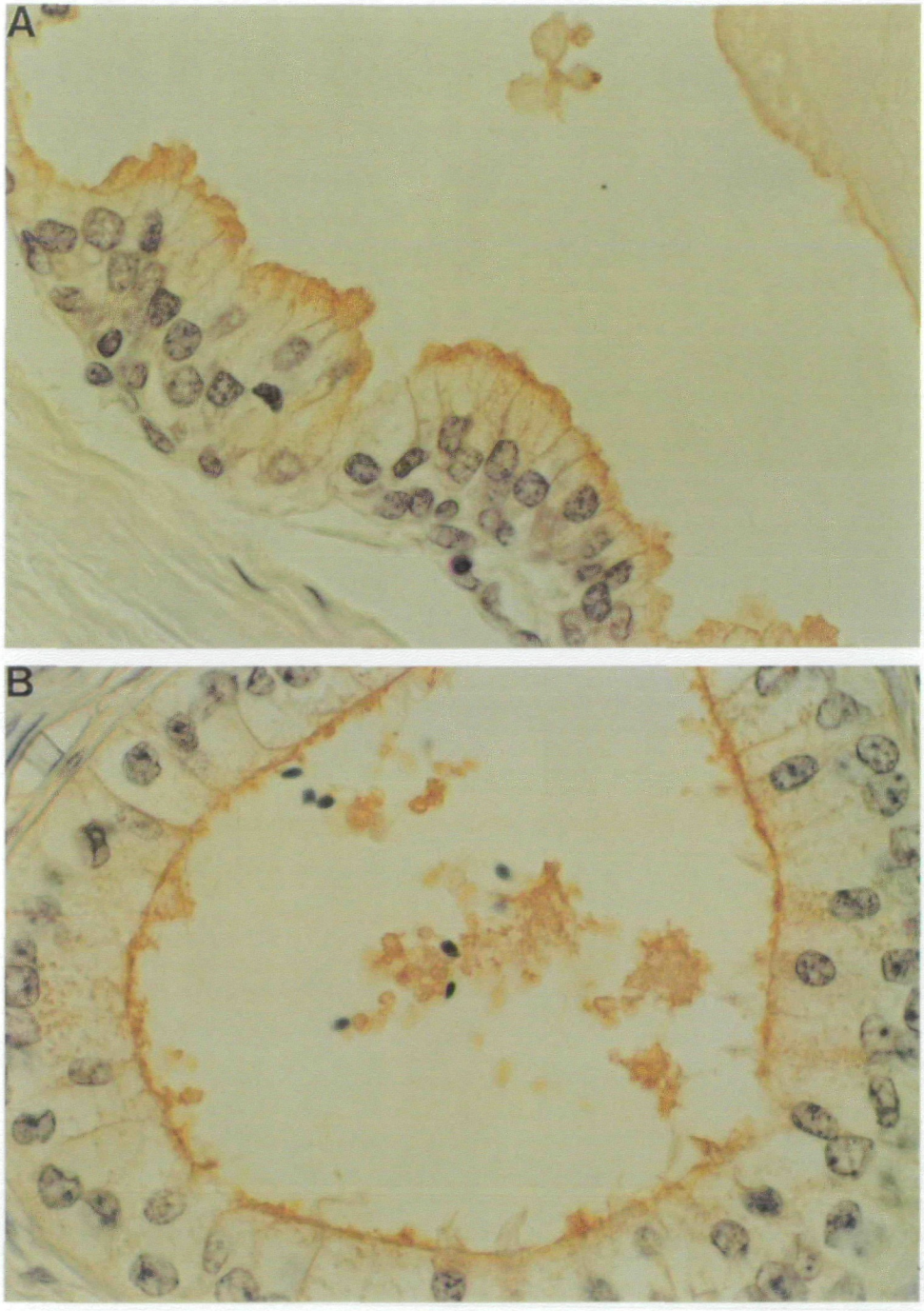

Fig. 5. **Immunohistochemical localization of AAP in prostate (A) and the ductuli efferentes of the testis (B).** (A) Luminal site-cell membrane of the epithelial cells of the prostatic gland is positively stained. ×400. (B) Positive staining in luminal site-cell membrane of the epithelial cell of the ductuli efferentes of the testis. ×400.

four months at −30°C. It was also resistant against several repeated cycles of freezing and thawing.

We examined the effect of temperature on AAP stability. The enzyme was stable for at least 30 min up to 50°C.

Determination of the N-Terminal Amino Acid Sequence—As shown in Fig. 4, the amino acid sequence of the first 22 residues of the purified AAP from human seminal plasma was Ser^{1}-Thr-Thr-Pro-Ser^{5}-Ala-Ser-Ala-Thr-Thr^{10}-Asn-Pro-Ala-Ser-Ala^{15}-Thr-Thr-Leu-Asp-Gln^{20}-Ser-Lys. Although two bands of the protein were observed on non-denatured PAGE (Fig. 2B), only serine was detected at the N-terminal amino acid of the protein. The yield for residues 1-22 in each cycle was 64, 133, 128, 114, 38, 107, 25, 90, 58, 55, 50, 40, 35, 10, 45, 34, 28, 37, 21, 15, 2, and 6 pmol.

Isolation and Cloning of cDNA Encoding AAP from Human Prostate—We screened a λgt 11 cDNA library constructed from human prostate with anti-human seminal plasma AAP rabbit IgG (see below). Two overlapping clones (AP-1 and -2) covering approximately 3 kb were obtained from a total of 800,000 plaques. AP-1, composed of 1,000 bp, started from the nucleotide C^{261} of human intestinal alanyl aminopeptidase N (CD13) (*16*). The above N-terminal amino acid sequence determined by Edman degradation started from $T^{262}C^{263}C^{264}$ (serine). On the other hand, AP-2 composed of 2,600 bp, started from the

nucleotide C^{840} of the aminopeptidase N (*16*), and contained a stop codon (TAG), 3′ noncoding sequence, polyadenylation signal (AATAAA) and poly(A) tail. The nucleotide and predicted amino acid sequences of AAP cDNA were completely coincident with human intestinal alanyl aminopeptidase (data not shown) (*16*).

Immunohistochemistry of the Male Genital Tract—To determine the immunohistochemical localization of AAP in the male genital tract, we purified an anti-human seminal plasma AAP rabbit IgG by ammonium sulfate fractionation (0-35% saturation) and DEAE-Sephacel column chromatography. The purified antibody maximally inhibited 70% of the activity of purified AAP (data not shown). As shown in Fig. 5, A and B, the enzyme was intensely stained in the luminal site-cell membrane of epithelial cells in the prostate and the ductuli efferentes of the testis. These data showed that AAP in human seminal plasma was released from these tissues in the male genital tract.

DISCUSSION

In the present study, we purified AAP to homogeneity from human seminal plasma by Q-Sepharose, Matrex Gel Red A, Zinc-Chelate Cellulofine, Superdex 200 (in FPLC), Phenyl-Cellulofine, and Resource Q (in FPLC) chromatography (Fig. 1, A and B). The calculated molecular weight of the purified enzyme was 137,000±5,000 from light scattering in the HPLC, 140,000 and 137,000 from non-denatured PAGE and 153,000 from SDS-PAGE in the absence or presence of 2-mercaptoethanol (2-ME) (Fig. 2, A and B). The molecular weights of the enzyme determined by SDS-PAGE were similar to those (M_r 130,000-160,000) of the purified human aminopeptidases with a pH optimum of pH 7.5 (*26-28*). These findings suggested that the enzyme from human seminal plasma is a monomer. The isoelectric point (pI) of the purified enzyme was 4.5 according to chromatofocusing using the PhastSystem. This pI value was significantly lower than those (5.2 and 5.6) of AAPs from human placenta (*1*) and bovine seminal vesicle secretion (*10*).

As shown in Table II, AAP rapidly hydrolyzed the substrates Ala-MCA and Met-MCA, moderately hydrolyzed Leu- and Phe-MCAs and weakly hydrolyzed Arg-, Arg-Arg-, Lys-, and Lys-Ala-MCAs at pH 7.5. However, it did not hydrolyze Pro- and Pyr-MCAs, or endopeptidase substrates such as Boc-Phe-Ser-Arg-MCA, Z-Phe-Arg-MCA, Suc-Ala-Ala-Ala-MCA, and Suc-Leu-Leu-Val-Tyr-MCA. Thus, the enzyme releases one amino acid residue from the N-termini of peptides with broad specificity as described (*2, 3*). The enzyme hydrolyzed substrates for dipeptidyl peptidase II (Lys-Ala-MCA). However, it did not hydrolyze the substrates Gly-Pro-MCA for dipeptidyl peptidase IV or Pro-Phe-Arg-MCA for kallikrein. These findings suggest that the enzyme does not show substrate specificity for proline at the N-termini of peptides. The two step reaction using Lys-Ala-MCA (Fig. 3) seemed to be dependent on its substrate specificity.

The K_m, K_{cat}, and K_{cat}/K_m values of aminoacyl-MCAs are shown in Table III. The catalytic efficiency ($K_{cat}/K_m = s^{-1}\cdot nM^{-1}$) of AAP toward Ala-MCA (39.1) was the highest, followed by Lys-Ala-MCA (33.3) and Met-MCA (32.7).

As shown in Table IV, AAP activity was potently inhibited by 1,10-phenanthroline, bestatin, leuhistin, actinonin, amastatin, and moderately by EDTA and arphamenine A. The enzyme was moderately sensitive, with $IC_{50} = 50\ \mu M$, to puromycin (data not shown). However, it was resistant to other protease inhibitors such as DFP, E-64, pepstatin, elastatinal, and phosphoramidon. The enzyme activity was also not affected at 1 or 5 mM concentrations of the sulfhydryl reagents. These findings also indicated that an SH-residue is not involved in the expression or regulation of the activity of AAP. As shown in Table V, Cd^{2+}, Cu^{2+}, and Zn^{2+} completely inhibited, and Hg^{2+} moderately inhibited AAP activity (Table V). Other investigators have reported that Hg^{2+}, Cd^{2+}, and Cu^{2+} potently inhibited AAP activity (*3, 7, 8*). These findings and the inhibition by EDTA and 1,10-phenanthroline also indicate that a metal-binding region(s) on AAP is important for the expression or regulation of the enzyme activity. This notion is also supported by the amino acid sequence data of aminopeptidase N (*13, 16*).

The amino acid sequence of the first 22 residues of the purified AAP from human seminal plasma was completely coincident with that downstream (amino acid residues number 48 to 70 including one gap in Fig. 4) of the N-termimal transmembrane site of human intestinal aminopeptidase N (*13, 16*). We confirmed that the AAP from human seminal plasma belongs to the aminopeptidase family, but it is not identical with puromycin-sensitive AAP (puromycin-sensitive enkephalin-degrading aminopeptidase) according to the above data and the N-terminal amino acid sequence (*29, 30*). We also observed a major and a minor band of the purified AAP on non-denatured PAGE, which were stained with Coomassie Brilliant Blue (Fig. 2B). Both protein bands were also stained with substrates, Ala- and Lys-Ala-MNAs, and the intensity of the activity staining was apparently parallel to that of protein bands stained with Coomassie Brilliant Blue (Fig. 2B). Moreover, we detected only one amino acid, serine, as the N-terminal amino acid of the enzyme. These findings indicate that the minor band may correspond to the truncated AAP in its C-terminal region. Furthermore, by using a polyclonal antibody, we isolated a cDNA encoding this enzyme, determined its nucleotide sequence, and predicted the amino acid sequence of AAP (data not shown). The sequence was identical to that described by Olsen *et al.* and Look *et al.* (*13, 16*). Accordingly, judging from the upstream amino acid sequence of the cleavage site (Pro^{45}-Val^{46}-Ala^{47}), the enzyme was apparently attacked by elastase or an elastase-like enzyme, then released into seminal plasma from the cell membrane in the human male genital tract tissues (Fig. 4). DPP IV also penetrates the cell membrane and is released into the seminal plasma after cleavage of the hydrophobic N-terminal transmembrane domain by chymotrypsin- or pepsin-like enzymes (*17*). Immunohistochemical staining revealed that the luminal cell membrane of epithelial cells of the prostatic gland and the ductuli efferentes of the testis were potently stained with anti-human seminal plasma AAP rabbit IgG (Fig. 5, A and B). From the above data, we determined the enzyme level in human seminal plasma by single radial immunodiffusion using the newly determined extinction coefficient ($E^{1\%}_{280nm} = 20$) (*24*). The enzyme level was 5.2±3.2 mg/100 ml (mean±SD, $n = 20$) in normal adults 20-47 years of age. This finding suggested that AAP constitutes 0.5-1.0% of the total protein in human seminal plasma.

Human intestinal aminopeptidase N is identical to myeloid plasma membrane glycoprotein CD 13 (*16*). It has been postulated that the enzyme functions in the hydrolytic inactivation of regulatory peptides such as enkephalin that are involved in signal transduction at the cell membrane (*31, 32*). Furthermore, it is also indicated that aminopeptidase N is a major receptor for the enteropathogenic coronavirus TGEV (transmissible gastroenteritis virus) and human coronavirus 229E (HCV-229E) (*33, 34*). Thus, aminopeptidase N may play important roles in the entry of coronavirus into and its replication inside cells. On the other hand, Agrawal and Vanha-Perttula (*10*) speculated that possible substrates for aminopeptidases, including AAP, are located on a non-uniform spermatozoan surface. NagDas and Bhattacharyya (*35, 36*) reported that the AAP activity level in seminal plasma is significantly higher in non-obstructive azoospermic patients and in men who produce poorly- or non-coagulating ejaculates than normal controls. It is likely that there is some relationship between seminal plasma AAP and sterility in men. However, the real physiological function of AAP in seminal plasma remains unknown.

We thank Mr. Giyozan Yamasaki (Central Research Laboratory, Shiga University of Medical Science) for excellent technical assistance in sequencing of the N-terminal amino acids of human seminal plasma AAP.

REFERENCES

1. Hahn, T., Graf, R., Öney, T., and Desoye, G. (1994) Effects of the synthetic glucocorticoid triamcinolone acetonide on vasoactive hydrolases of the human placenta in vitro. *Placenta* **15**, 377-388
2. Mantle, D., Lauffart, B., McDermott, J., and Gibson, A. (1990) Characterization of aminopeptidases in human kindey soluble fraction. *Clin. Chim. Acta* **187**, 105-114
3. Itoh, C. and Nagamatsu, A. (1995) An aminopeptidase activity from porcine kidney that hydrolyzes oxytocin and vasopressin: purification and partial characterization. *Biochim. Biophys. Acta* **1243**, 203-208
4. Mantle, D., Lauffart, B., Perry, E.K., and Perry, R.H. (1989) Comparison of major cortical aminopeptidase activity in normal brain and brain from patients with Alzheimer's disease. *J. Neurol. Sci.* **89**, 227-234
5. Iribar, C., Esteban, M.J., Martinez, J.M., and Peinado, J.M. (1995) Decrease in cytosolic aspartyl-aminopeptidase but not in alanyl-aminopeptidase activity in the frontal cortex of the aged rat. *Brain Res.* **687**, 211-213
6. Sidorowicz, W., Jackson, G.C., and Behal, F.J. (1980) Multiple molecular forms of human pancreas alanine aminopeptidase. *Clin. Chim. Acta* **104**, 169-179
7. Lauffart, B. and Mantle, D. (1988) Rationalization of aminopeptidase activities in human skeletal muscle soluble extract. *Biochim. Biophys. Acta* **956**, 300-306
8. Mantle, D., Hardy, M.F., Lauffart, B., McDermott, J.R., Smith, A.I., and Pennington, R.J.T. (1983) Purification and characterization of the major aminopeptidase from human skeletal muscle. *Biochem. J.* **211**, 567-573
9. Lalu, K., Lampelo, S., and Vanha-Perttula, T. (1986) Characterization of three aminopeptidases purified from maternal serum. *Biochim. Biophys. Acta* **873**, 190-197
10. Agrawal, Y. and Vanha-Perttula, T. (1986) Alanyl aminopeptidase of bovine seminal vesicle secretion. *Int. J. Biochem.* **18**, 725-729
11. NagDas, S.K. and Bhattacharyya, A.K. (1988) Purification and characterization of human seminal plasma aminopeptidase. *Ital. J. Biochem.* **37**, 148-164
12. Hiroi, Y., Endo, Y., and Natori, Y. (1992) Purification and properties of an aminopeptidase from rat-liver cytosol. *Arch. Biochem. Biophys.* **294**, 440-445
13. Olsen, J., Cowell, G.M., Kønigshøfer, E., Danielsen, E.M., Møller, J., Laustsen, L., Hansen, O.C., Welinder, K.G., Engberg, J., Hunziker, W., Spiess, M., Sjöström, H., and Norén, O. (1988) Complete amino acid sequence of human intestinal aminopeptidase N as deduced from cloned cDNA. *FEBS Lett.* **238**, 307-314
14. Watt, V.M. and Yip, C.C. (1989) Amino acid sequence deduced from a rat kidney cDNA suggests it encodes the Zn-peptidase aminopeptidase N. *J. Biol. Chem.* **264**, 5480-5487
15. Malfroy, B., Kado-Fong, H., Gros, C., Giros, B., Schwartz, J.C., and Hellmiss, R. (1989) Molecular cloning and amino acid sequence of rat kidney aminopeptidase M: a member of a super family of zinc-metallohydrolases. *Biochem. Biophys. Res. Commun.* **161**, 236-241
16. Look, A.T., Ashmun, R.A., Shapiro, L.H., and Peiper, S.C. (1989) Human myeloid plasma membrane glycoprotein CD 13 (gp 150) is identical to aminopeptidase N. *J. Clin. Invest.* **83**, 1299-1307
17. Ohkubo, I., Huang, K., Ochiai, Y., Takagaki, M., and Kani, K. (1994) Dipeptidyl peptidase IV from porcine seminal plasma: purification, characterization and N-terminal amino acid sequence. *J. Biochem.* **116**, 1182-1186
18. Huang, K., Takagaki, M., Kani, K., and Ohkubo, I. (1996) Dipeptidyl peptidase II from porcine seminal plasma: purification, characterization, and its homology to granzymes, cytotoxic cell proteinases (CCP 1-4). *Biochim. Biophys. Acta* **1290**, 149-156
19. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
20. Ogata, S., Misumi, Y., and Ikehara, Y. (1989) Primary structure of rat liver dipeptidyl peptidase IV deduced from its cDNA and identification of the NH2-terminal signal sequence as the membrane-anchoring domain. *J. Biol. Chem.* **264**, 3596-3601
21. Hayashi, Y., Matsui, H., and Takagi, T. (1989) Membrane protein molecular weight determined by low-angle laser light-scattering photometry coupled with high-performance gel chromatography in *Methods in Enzymology* (Fleischer, S. and Fleischer, B., eds.) Vol. 172, pp. 514-528, Academic Press, San Diego
22. Bhat, M.A., Ishida, T., Horiike, K., Vaidyanathan, C.S., and Nozaki, M. (1993) Purification of 3,5-dichlorocatechol 1,2-dioxygenase, a nonheme iron dioxygenase and a key enzyme in the biodegradation of a herbicide, 2,4-dichlorophenoxy acetic acid (2,4-D), from *Pseudomonas cepacia* CSV90. *Arch. Biochem. Biophys.* **300**, 738-746
23. van Iersel, J., Jzn, J.F., and Duine, J.A. (1985) Determination of absorbance coefficients of purified proteins by conventional ultraviolet spectrophotometry and chromatography combined with multiwavelength detection. *Anal. Biochem.* **151**, 196-204
24. Mancini, G., Carbonara, A.O., and Heremans, J.F. (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry* **2**, 235-254
25. Nishi, K., Fukunaga, T., Yamamoto, Y., Yamada, M., Kane, M., Tanegashima, A., Rand, S., and Brinkmann, B. (1992) ABH-related antigens in human male genital tract. A histochemical examination. *Int. J. Leg. Med.* **105**, 75-80
26. See, H. and Reithmeir, R.A. (1990) Identification and characterization of the major stilbene-disulphonate- and concanavalin A-binding protein of the porcine renal brush-border membrane as aminopeptidase N. *Biochem. J.* **271**, 147-155
27. Favaloro, E.J., Browning, T., and Facey, D. (1993) CD 13 (GP 150; aminopeptidase-N): predominant functional activity in blood is localized to plasma and is not cell-surface associated. *Exp. Hematol.* **21**, 1695-1701
28. Menrad, A., Speicher, D., Wacker, J., and Herlyn, M. (1993) Biochemical and functional characterization of aminopeptidase N expressed by human melanoma cells. *Cancer Res.* **53**, 1450-1455
29. Dyer, S.H., Slaughter, C.A., Orth, K., Moomaw, C.R., and Hersh, L.B. (1990) Comparison of the soluble and membrane-bound forms of the puromycin-sensitive enkephalin-degrading aminopeptidase from rat. *J. Neurochem.* **54**, 547-554
30. Constam, D.B., Tobler, A.R., Rensing-Ehl, A., Kemler, I., Hersh, L.B., and Fontana, A. (1995) Puromycin-sensitive aminopep-

tidase. Sequence analysis, expression, and founctional characterization. *J. Biol. Chem.* **270**, 26931-26936
31. Matsas, R., Stephenson, S.L., Hryszko, J., Kenny, A.J., and Turner, A.J. (1985) The metabolism of neuropeptidase phase separation of synaptic membrane preparations with Triton X-114 reveals the presence of aminopeptidase N. *Biochem. J.* **231**, 445-449
32. Bowes, M.A. and Kenny, A.J. (1987) An immunohistochemical study of endopeptidase-24.11 and aminopeptidase N in lymphoid tissues. *Immunology* **60**, 247-253
33. Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L.K., Sjöström, H., Norén, O., and Laude, H. (1992) Aminopeptidase N is a major receptor for the entero-pathogenic coronavirus TGEV. *Nature* **357**, 417-420
34. Yeager, C.L., Ashmun, R.A., Williams, R.K., Cardellichio, C.B., Shapiro, L.H., Look, A.T., and Holmes, K.V. (1992) Human aminopeptidase N is a receptor for human coronavirus 229E. *Nature* **357**, 420-422
35. NagDas, S.K., Bhattacharyya, A., and Bhattacharyya, A.K. (1984) Aminopeptidase in human seminal plasma. *Andrologia* **16**, 451-457
36. NagDas, S.K. and Bhattacharyya, A.K. (1987) Enzyme activities of human seminal plasma having different states of coagulation. *Int. J. Fertil.* **32**, 86-89