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KININASE ACTIVITY IN HUMAN PLATELETS: CLEAVAGE OF THE Arg^1-Pro^2 BOND OF BRADYKININ BY AMINOPEPTIDASE P

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Abstract—A proline-specific peptidase aminopeptidase P (APP, EC 3.4.11.9) that cleaves the Arg^1-Pro^2 bond of bradykinin was isolated from human platelets by liquid chromatography. The enzyme was purified 557 times. The native molecule has a M_r of 223,000. Human platelet APP exists as a trimer with a subunit M_r of 71,000. The apparent K_m of platelet APP is 66 μmol/L for bradykinin and 47 μmol/L for the internally quenched fluorogenic substrate Lys (2,4-dinitrophenyl)-Pro-Pro-NH-CH_2-CH_2-NH-2-aminobenzoyl. 2HCl which is used for the routine determination of the enzyme activity. The optimum pH for hydrolysis of the fluorogenic substrate is 8.0, and the optimum temperature is 43°. Platelet APP is inhibited by 1,10-phenanthroline and activated by Mn^{2+}, thus confirming its metalloprotease nature. Cu^{2+}, Zn^{2+} and Hg^{2+} are strongly inhibitory. Inhibition by cysteine protease inhibitors suggests the presence of a thiol group essential for enzymatic activity. Serine protease inhibitors do not affect the enzyme activity.

While it is commonly accepted that platelets are active participants in the maintenance of hemostasis and in initiating tissue repair after injury, their role in biological processes such as natural defence, inflammation and peptide metabolism has received much less attention. We demonstrated recently the presence of aminopeptidase P (APP,‡ EC 3.4.11.9) in human platelets which suggests a role for platelets as scavengers for circulating peptides containing bonds susceptible to APP [1]. APP is an exopeptidase that occupies a special position in the group of aminopeptidases because of its unique ability to hydrolyse N-terminal peptide bonds with a penultimate proline residue [2–5]. A proline residue occurs at the penultimate position in several cytokines and in growth factors such as interleukin 1β and interleukin 2, lymphotoxin, granulocyte colony-stimulating factor, insulin-like growth factor, growth hormone, erythropoietin; in the tachykinin and kinin substance P and bradykinin; and in peptides involved in blood coagulation such as the Hageman factor, plasminogen and the fibrin α-chain. An N-terminal Xaa-Pro sequence can act as a structural element limiting susceptibility to proteolytic degradation since apparently only specialized exopeptidases can overcome such a sequence [2–5]. The specific catalytic action of APP may thus constitute a factor for regulating the biological activity of those peptides. Baker *et al.* [16] showed recently the importance of APP in the pulmonary inactivation of bradykinin in the perfused rat lung. Bradykinin is a humoral mediator of inflammation present in areas of tissue injury and has the capacity to produce cardinal signs of inflammatory response, such as vasodilatation, increase in capillary permeability and pain. Since it is known that blood cells such as macrophages and neutrophils [7, 8], which are principal cellular components of inflammation, are involved in the inactivation of bradykinin, we wondered whether platelets could exert kininase activity. Since bradykinin is one of the possible substrates for APP that is abundantly present in platelets, we purified APP from human platelets and showed that platelet APP may be important for the degradation of bradykinin.

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‡ Abbreviations: APP, aminopeptidase P; DFP, diisopropylfluorophosphate; DLPPEA, Lys-(2,4-dinitrophenyl)-Pro-Pro-NH-CH_2-CH_2-NH-2-aminobenzoyl.2HCl; DPP IV, dipeptidyl peptidase IV; DTT, dithiothreitol; PCMB, *para*-chloro-mercuribenzoate; PEP, prolyl endopeptidase; Phe-Pro-NHO-Nb.HCl, Phe-Pro-*O*-(nitrobenzoyl)-hydroxylamine; PMSF, phenylmethanesulfonyl fluoride; Z-Gly-Pro-MCA, *N*-benzyloxycarbonyl-4-methylcoumarinyl-7-amide; Z-Pro-prolinal, *N*-benzyloxycarbonyl-prolyl-prolinal; ACE, angiotensin-converting enzyme; CPN, carboxypeptidase N.

MATERIALS AND METHODS

Materials. Outdated platelet concentrates were provided by the Antwerp transfusion center. Columns, ExcelGels®, relative mobility markers and chromatography media were from Pharmacia Fine Chemicals (Uppsala, Sweden), except for the Bio-gel HT® calcium phosphate which was from Bio-Rad (Bio-Rad Laboratories, Richmond, U.S.A.). Triton X-100 was from the Aldrich Chemical Co (Sternheim, Germany). Di-isopropyl-fluorophosphate, bradykinin, des-Arg^1-bradykinin, and poly-L-proline (MW 6400) were from the Sigma Chemical Co (St Louis, MO, U.S.A.). All other reagents were of analytical grade from E. Merck,

(Darmstadt, Germany). For fluorometric measurements, the model RF-5000 fluorometer (Shimadzu Corp., Kyoto, Japan) was used. Colorimetric measurements were performed on a Hewlett-Packard 8540 UV/VIS diode-array spectrophotometer. All purification steps were performed on a Fast Protein Liquid Chromatography system (Pharmacia). HPLC analyses were performed with a M-45 solvent delivery system, a model 450 variable wavelength UV detector and a Data Module (all from Waters, Millipore, Brussels, Belgium). Peptide products were applied onto a Waters Novapack® C_{18} reversed phase column (5 mm i.d. × 10 cm) of 4-μm particle size.

Isolation of human platelets. Platelet concentrates were prepared from units of whole blood collected from healthy volunteers using citrate-phosphate-dextrose-adenine as anticoagulant following standard procedures. The units were centrifuged for 3.5 min at 3000 *g* at 20°. The platelet-rich plasma was transferred to transfer pockets and centrifuged for 15 min at 3000 *g* at 20°. The platelets were left as such in 50 μmol/L plasma for 1 hr. After they had been carefully resuspended, they were placed in an end-over-end agitator at 2 rpm at 22°. When outdated (max. 2 days), the platelet concentrates were centrifuged twice for 5 min at 250 *g* at 20° to sediment red and white cells. The supernatant was centrifuged at 2750 *g* for 15 min at 20°; The plasma was decanted and the platelet pellet was resuspended in phosphate-buffered saline (NaCl 8.0 g/L, KCl 0.2 g/L, KH_2PO_4 0.2 g/L, Na_2HPO_4 1.15 g/L, pH 7.3) containing 0.3% (w/v) trisodiumcitrate. The platelets were washed by repeating the centrifugation step twice and the final pellet was resuspended in 20 mmol/L Tris pH 7.4.

Preparation of platelet extract. Platelets were disrupted by freezing and thawing (−70°, +20°) and detergent treatment (1% Triton X-100), after which the unbroken cells were removed by centrifugation at 40,000 *g* for 20 min. The resulting supernatant was clarified by an ultracentrifugation step at 100,000 *g* for 120 min. The supernatant was used for further purification of the enzyme.

Enzyme assays. Aminopeptidase P activity was measured using the intramolecularly quenched fluorogenic substrate Lys (2,4-dinitrophenyl)-Pro-Pro-NH-CH_2-CH_2-NH-2-aminobenzoyl.2HCl (DLPPEA) [9]. Dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5) was determined fluorometrically using the substrate Gly-Pro-4-methoxy-β-naphthylamide [10]. Prolyl endopeptidase (PEP, EC 3.4.21.26) activity was measured using the substrate *N*-benzyloxycarbonyl-4-methylcoumarinyl-7-amide (Z-Gly-Pro-MCA), in 2 mmol/L EDTA, 2 mmol/L DTT, 1 mmol/L NaN_3 in 100 mmol/L phosphate buffer, pH 7.5. Hydrolysis of the substrate Pro-4-methoxy-β-naphthylamide in 50 mmol/L Tris pH 8.3, 2 mmol/L $MnSO_4$ was chosen for measurement of leucyl aminopeptidase (EC 3.4.11.1). One unit (U) of enzyme activity was defined as the amount of enzyme that cleaves one micromole of substrate per minute under the assay conditions.

Protein determination. Protein concentrations were measured according to the micro-assay conditions using the bicinchoninic acid detection reagent (Pierce Chemical Co., Rockford, IL, U.S.A.). Bovine serum albumin was taken as the standard.

Purification of aminopeptidase P. The platelet supernatant was applied on a DEAE Sepharose® fast flow column (2.6 × 13 cm) equilibrated with 20 mmol/L Tris pH 7.4, 70 mmol/L NaCl, 0.1% Triton X-100. Activity was eluted with a stepwise salt gradient of 135, 163 and 209 mmol/L NaCl in 20 mmol/L Tris pH 7.4, 0.1% Triton X-100 at a flow rate of 2 mL/min. After measurement of prolyl endopeptidase and APP activity, the active fractions were pooled, and DFP was added to a final concentration of 1 mmol/L. After 1 hr incubation at room temperature, the resulting fraction was dialysed against 20 mmol/L Tris pH 7.4, and applied on a Q-Sepharose HiLoad column (1.6 × 10 cm), equilibrated with 20 mmol/L Tris pH 7.4, 70 mmol/L NaCl, at a flow rate of 1 mL/min. Activity was eluted with a linear NaCl gradient from 70 to 350 mmol/L NaCl in the same buffer at 2 mL/min. Active fractions were pooled and applied at 0.3 mL/min on a Wheat Germ (1 × 6 cm) and a Concanavalin A Sepharose (1 × 10 cm) column, placed in series. The activity eluted with the equilibration buffer (20 mmol/L Tris pH 7.4, 500 mmol/L NaCl). The active fractions were pooled and dialysed against 10 mmol/L phosphate buffer, pH 7.5, before application on a Biogel HT calcium phosphate column (1.6 × 10 cm). The activity was eluted with a phosphate gradient from 10 to 50 mmol/L at a flow rate of 0.3 mL/min. The active fraction was concentrated by ultrafiltration on a Centriprep 10 concentrator and applied on a Sephacryl® S300 High Resolution gel-filtration column (1.6 × 70 cm) equilibrated with 20 mmol/L Tris pH 7.4. The enzyme was eluted at a flow rate of 0.2 mL/min and applied at the same flow rate on an Arginine Sepharose column (1.6 × 1 cm), equilibrated with the same buffer. Elution was performed by a linear NaCl gradient (0–163 mmol/L) in 10 column volumes at 0.3 mL/min. The active fractions were pooled and finally applied to a HR 5/5 Mono Q® column equilibrated with 20 mmol/L Tris pH 7.4, 70 mmol/L NaCl and eluted with a gradient of 70–350 mmol/L NaCl over 15 column volumes at a flow rate of 1 mL/min. The active fractions were pooled and stored at −70°.

Polyacrylamide disc gel electrophoresis. For further identification of the partially purified APP, disc gel electrophoresis of the active fraction was carried out in glass tubes (60 × 8.5 mm) with a separating gel of 7.5% acrylamide and 0.2% bisacrylamide containing 0.08 mol/L Tris pH 8.6. The electrode buffer consisted of 0.03 mol/L diethylbarbituric acid, 0.01 mol/L Tris pH 8.3. Bromophenol blue was used as a tracking dye. Electrophoresis was carried out at 150 V for 90 min. After electrophoresis, the gel was cut into 20 equal slices. Each slice was incubated for 60 min at 40° in 100 μL of 0.15 mmol/L DLPPEA in 0.2 mol/L Tris pH 8.0. After incubation, the gel slices were removed, and the remaining solution was diluted with 0.5 mL 0.2 mol/L Tris pH 8.0, 1 mmol/L DTT, 50 mmol/L EDTA to stop the reaction. Fluorescence was measured, and the gel slice that

yielded the highest activity was loaded on an SDS–PAGE.

SDS–PAGE. Analytical SDS–PAGE was performed in polyacrylamide gradient gels (ExcelGel, 8–18%) containing 0.1% SDS. Samples were diluted twice in 50 mmol/L Tris pH 7.5, 2% SDS, 10 mmol/L DTT and 0.2 g/L Bromophenol blue and boiled for 3 min before application to the gel. Electrophoresis was performed at a constant current of 25 mA for 75 min. The proteins were visualized by silver staining according to Heukeshoven and Dernick [11] with slight modifications [12]. As relative mobility markers, α-lactalbumin (M_r 14,400), trypsin inhibitor (M_r 20,100), carbonic anhydrase (M_r 30,000), ovalbumin (M_r 43,000), albumin (M_r 67,000) and phosphorylase b (M_r 94,000) were used.

Determination of pH optimum and temperature optimum. The pH optimum of APP was determined using buffers each containing 50 mmol/L citric acid, acetic acid, piperazine, histidine, imidazole, Tris and ethanolamine; pH adjustments were made using HCl or NaOH at room temperature. For the determination of the temperature optimum for the hydrolysis of DLPPEA, the activity of the purified sample was measured according to the standard procedure, but the incubation temperature was varied.

Determinations of pH stability and temperature stability. The pH stability was determined by preincubating aliquots of the purified enzyme for 60 min at 37° in buffers each containing 15 mmol/L citric acid, potassium monobasic phosphate, 5,5-diethylbarbituric acid and boric acid. The pH adjustments were made with NaOH at room temperature. To determine the temperature stability, aliquots of the purified enzyme were preincubated at 37, 40, 45 and 50° for 2–120 min. after preincubation, all samples were stored on ice until measurement by the standard assay procedure.

Estimation of relative molecular mass. The relative molecular mass of the native purified enzyme was estimated by gel filtration using a Sephacryl S 300 High Resolution column. Enzyme solution (0.5 mL) was loaded on the column and eluted with 10 mmol/L Tris pH 7.4, 50 mmol/L NaCl at a flow rate of 0.3 mL/min. Thyroglobulin (M_r 669,000), ferritin (M_r 440,000), catalase (M_r 232,000) and aldolase (M_r 158,000) were used as relative molecular mass standards. The relative molecular mass of aminopeptidase P in platelet extracts was determined under the same conditions but with 20 mmol/L Tris pH 7.4 (N = 2) or with 20 mmol/L Tris pH 7.4, 150 mmol/L NaCl as elution buffer.

Effect of inhibitors and bivalent metal ions. To evaluate the effect of Mn^{2+} and Co^{2+}, the activity of the purified enzyme was measured using the standard assay with the following modifications: the metallobuffer manganese-citrate was omitted from the substrate solution, and $MnCl_2$ and $CoCl_2$ were added at concentrations ranging from 10^{-1} to 10^{-7} mol/L.

To measure the effect of protease inhibitors and other bivalent ions, the sample was preincubated in 0.2 mol/L Tris pH 8.0, 10 mmol/L trisodiumcitrate and 2.6 mmol/L $MnSO_4$ with different inhibitors. The concentrations of the inhibitors in the preincubation solution were double as compared with the concentration during assay. As a control, deionized water was used instead of inhibitor. After preincubation for 20 min at 37°, 30 μL of 0.8 mmol/L DLPPEA was added to 30 μL of the preincubated sample. The mixture was incubated for 20 min at 40°. The reaction was stopped by adding 2.25 mL 0.2 mol/L Tris, pH 8.0, 50 mmol/L EDTA, 1 mmol/L DTT.

Influence of reducing and chaotropic agents. The purified enzyme was incubated with urea, mercaptoethanol and guanidinium-chloride in concentrations ranging from 0.5 to 6.5 mol/L during 90 min at ambient temperature, after which the standard assay procedure was followed for determination of the remaining activity.

Substrate specificity. Hydrolysis of poly-L-proline by the purified APP was determined as follows: a stock solution of poly-L-proline (1.7 mmol/L) was prepared by dissolving the polymer in ice-cold water. Mn^{2+}-citrate reagent was prepared fresh before the assay by mixing 2.5 mL of 0.05 mol/L 5,5-diethylbarbituric acid pH 8.6, 0.5 mL of 0.4 mol/L trisodiumcitrate, 0.5 mL of 0.1 mol/L NaOH and 0.5 mL of 0.1 mol/L manganous chloride. Ninhydrin reagent was prepared by dissolving 3 g ninhydrin in a mixture of glacial acetic acid (60 mL) and phosphoric acid (6 mol/L, 40 mL), by warming at 70°. A mixture of 25 μL of the poly-L-proline stock solution, 10 μL 0.01 mol/L EDTA, 750 μL 0.05 mol/L 5,5-diethylbarbituric acid pH 8.6 and 200 μL Mn^{2+}-citrate reagent was placed at 40° and 15 μL enzyme solution is added. After incubation for 30 min, the reaction was stopped by adding 2.5 mL ninhydrin reagent. The solution was cooled with ice-cooled water. To determine the amount of proline formed, 2.5 mL of glacial acetic acid was added and the solution was heated at 100° for 30 min. After cooling, the intensity of the formed red color was measured. The amount of proline formed was calculated from a calibration curve constructed with 1–75 μg proline.

To determine the apparent K_m for the substrate DLPPEA, enzyme (5 μL) was incubated with substrate concentrations ranging from 0.02 to 0.2 mmol/L in 0.2 mol/L Tris pH 8.0, 7.0 mmol/L trisodiumcitrate and 1.9 mmol/L manganese sulfate (50 μL), and incubated for 5–20 min at 40°. To remain within the linearity of the method, the purified sample was diluted with water to a final activity of 14 U/L. The hydrolysis was terminated by adding 2.25 mL 0.2 mol/L Tris pH 8.0, 1 mmol/L DTT, 50 mmol/L EDTA. The fluorescence of the samples was measured at an excitation wavelength of 320 nm and an emission wavelength of 410 nm. All measurements were duplicated and three independent determinations were made. The K_m value was calculated using a Cornish-Bowden plot [13].

To determine the kinetic constants of bradykinin for aminopeptidase P, enzyme (10 μL) was incubated at 40° for 10 min with bradykinin concentrations ranging from 0.015 to 0.4 mmol/L in 0.2 mol/L Tris pH 8.0 with 7.0 mmol/L trisodiumcitrate and 1.9 mmol/L manganese sulfate (90 μL). The hydrolysis of bradykinin was terminated by the addition of 60 μL of 7% (v/v) perchloric acid, adjusted to

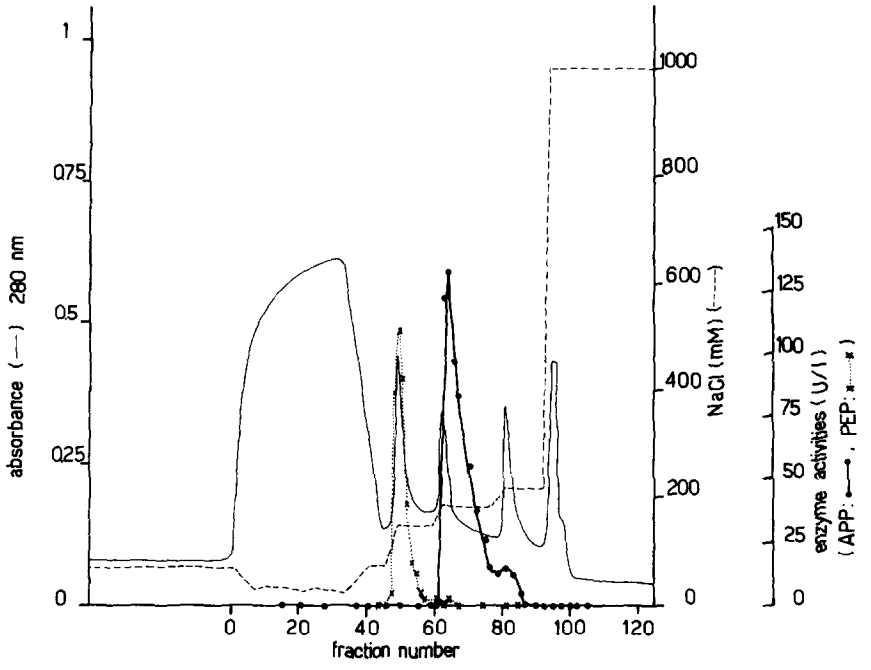

Fig. 1. Purification of APP by chromatography on DEAE Sepharose fast flow. Platelet extract, clarified by ultracentrifugation, was applied on a DEAE Sepharose fast flow column equilibrated with 20 mmol/L Tris pH 7.4, 0.1% Triton X-100. The column was washed with the same buffer until the A_{280} returned to the baseline. Activity was eluted with a stepwise salt gradient of 135, 163 and 209 mmol/L NaCl in 20 mmol/L Tris pH 7.4, 0.1% Triton X-100 at a flow rate of 2 mL/min.

pH 3.25 with NaOH. The sample was centrifuged for 5 min in an Eppendorf centrifuge. Fifty microlitres of the supernatant were used for injection onto the column. The products of the reaction were separated by HPLC using a Novapack C_{18} reversed-phase column. The peptide products were eluted with an isocratic system of 16% acetonitrile in 60 mmol/L phosphate buffer, pH 3.25, using a flow rate of 1 mL/min at ambient temperature. Peaks were detected at 205 nm and identified by co-elution with peptide standards. A standard curve of the des-Arg^1-bradykinin was made by injecting known amounts of des-Arg^1-bradykinin (0.05–1.5 μg), prepared under the same conditions as described above. The correlation between peak heights versus des-Arg^1-bradykinin concentration was adequate (r = 0.998).

RESULTS

Purification of APP

Using the fluorometric assay based on the cleavage of the Lys (2,4 dinitrophenyl)-Pro bond in the internally quenched substrate DLPPEA for monitoring the activity, APP was purified from platelet extract. Due to the presence of the Pro-Pro bond, DPP IV cannot cleave this substrate [3]. PEP activity was monitored using Z-Gly-Pro-MCA as the substrate in the fractions after the first anion exchange chromatography. The highest Z-Gly-Pro-MCA cleaving activity eluted before the activity peak of APP (Fig. 1). Fractions containing the highest PEP activity were concentrated and assayed with the APP substrate, which did not result in the formation of the fluorescent product. After DEAE Sepharose fast flow, the APP activity eluted in a single peak at a NaCl concentration of 163 mmol/L. Addition of DFP, which inhibits DPP IV as well as PEP, to the pooled active fractions did not result in a change in APP activity, also proving the specificity of the substrate. On the strong anion exchanger Q-Sepharose HiLoad the enzyme activity eluted between 230 and 300 mmol/L NaCl. The serial elution from Concanavalin A and Wheat Germ Sepharose did not result in an additional purification of the enzyme but—as was determined in former similar purification procedures using DPP IV-rich samples without addition of DFP—was effective in the removal of DPP IV, which would interfere with further experiments on the degradation of peptides, since DPP IV acts on the same biological substrates as APP [2]. The purification on calcium phosphate resulted in an 8-fold enrichment of the enzyme preparation. Additional purification on Arginine Sepharose and Mono Q resulted in a final enzyme preparation which was enriched 557 times compared with the clarified platelet extract (Table 1). No DPP IV, PEP or leucyl aminopeptidase activity, which were all present in the platelet extract, could be measured in the purified sample. No DPP IV or PEP activities were measured in the fraction containing APP obtained by the same purification procedure without prior addition of DFP to the sample. This confirms the effective removal of DPP IV and PEP by the above purification procedure.

Table 1. Purification of APP from human platelets

	Activity (U)	Protein (mg)	Sp. act. (mU/mg)	Purification factor	Yield (%)
Thrombocyte extract	23.0	2796	8.39		
DEAE Sepharose	21.4	386	55.5	6.6	91
Q-Sepharose HiL.	16.6	99	167.5	20	77
Wheat Germ + Con A Sepharose	15.7	94	168.1	20	67
Hydroxyl Apatite	12.6	9.3	1359	161	54
Centriprep 10	5.1	3.5	1439	171	32
Sephacryl S300	5.8	3.1	1858	221	25
Arg-Sepharose	5.7	2.8	2031	242	24
Mono Q Sepharose	4.2	0.92	4672	557	18

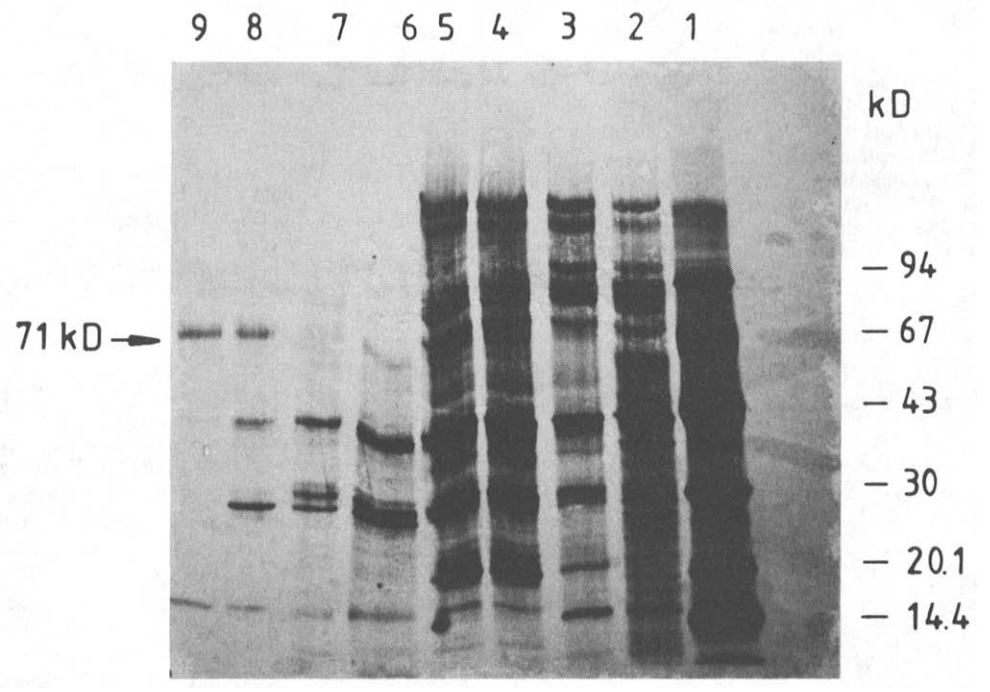

Fig. 2. SDS–PAGE of APP after different steps of purification. Reduced and denatured samples were separated by SDS–PAGE and silver stained. Lane 1: relative mobility markers: phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin; lane 2: platelet extract; lane 3: Active fraction after DEAE Sepharose fast flow; lane 4: active fraction after Q-Sepharose HiLoad; Lane 5: active fraction after Con A and Wheat Germ Sepharose; Lane 6: active fraction after Hydroxyl Apatite; lane 7: active fraction after Sephacryl S300 HR; lane 8: active fraction after Arginine Sepharose; lane 9: active fraction after Mono Q Sepharose.

The enzyme remained stable at −70° for 4 weeks. After 6 months of storage, the activity of the enzyme preparation was 60% of its original value.

On SDS–PAGE, the purified sample showed a major band on ExcelGel with a M_r of 71,000. Some minor bands from contaminating proteins with lower relative mobility were also still present (Fig. 2). Further analytical purification of the enzyme was performed by native PAGE and this resulted in a final catalytically active enzyme preparation which appeared in one single band on SDS–PAGE.

Enzymatic and physicochemical properties

The purified enzyme has a sharp optimum pH at 8.0 and the enzyme was stable between pH 6 and 8 (Fig. 3). The optimum temperature for hydrolysis of DLPPEA is 43°. The enzyme was stable at 37° for 20 min, slowly denaturing thereafter. After 2 hr of incubation at 37°, 80% of its original activity was measured. The enzyme rapidly denatured at 45° (Fig. 4). The relative molecular mass of the purified APP determined by gel filtration was 223,000, which is similar to the relative molecular mass found for APP in the crude platelet extracts (M_r = 217,000, range 214,000–219,000, N = 3). The enzyme was strongly activated by Mn^{2+} and less so by Co^{2+}. The optimal concentration for activation by Mn^{2+} was situated between 0.5 and 1 mmol/L (Fig. 5). APP

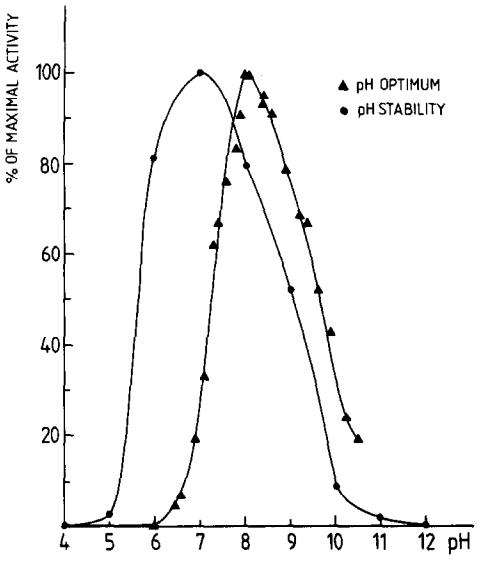

Fig. 3. Effect of pH on APP activity. The pH stability was determined by preincubating aliquots of the purified enzyme for 60 min at 37° in buffers with varying pH. Remaining activity was measured according to the standard assay [9]. The optimum pH was determined according to the standard assay using buffers with varying pH.

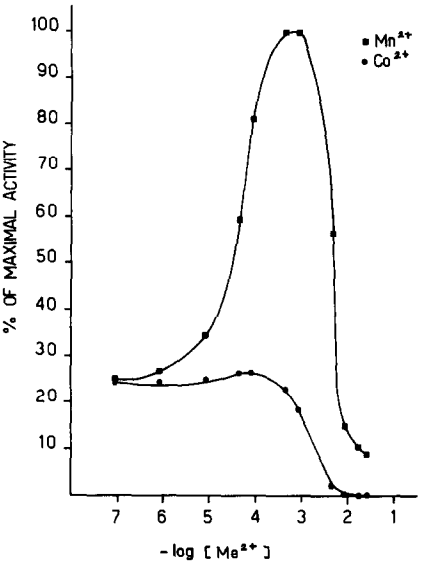

Fig. 5. Effect of Mn^{2+} and Co^{2+} ions on APP activity. The activity of the purified enzyme was measured using the standard assay [9] with the following modifications: the metallobuffer manganese-citrate was omitted from the substrate solution, and $MnCl_2$ or $CoCl_2$ were added at concentrations ranging from 10^{-1} to 10^{-7} M.

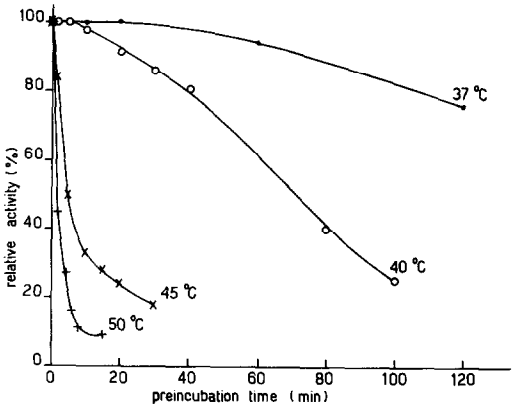

Fig. 4. Thermal stability of purified APP. Enzyme was preincubated at different temperatures as indicated and subsequently assayed according to the standard assay [9].

was inhibited by Cu^{2+}, Zn^{2+} and Hg^{2+}, and by the chelating agents, 1,10 phenanthroline and EDTA (in the absence of Mn^{2+}), and was not affected by the serine protease inhibitors PMSF and DFP, thus demonstrating its metalloprotease nature (Table 2). The strong inhibition by PCMB, reacting with free SH groups, indicates the presence of a thiol group essential for enzymatic activity. The bovine lung trypsin inhibitor aprotinin partly inhibited DLPPEA cleavage by APP, as a competitive substrate with N-terminal Arg-Pro-Asp- sequence. The specific irreversible mechanism-based inhibitor of DPP IV, 4-Phe-Pro-NHO-Nb.HCl [14], did not affect the APP activity, while the specific reversible inhibitor of PEP, Z-Pro-prolinal, inhibited the APP activity slightly at 0.3 mmol/L. The metalloproteinase inhibitor amastatin completely inhibited the enzyme at 0.1 mmol/L. Bestatin proved to be a poor inhibitor of APP considering the K_i values for other aminopeptidases, which are in the nanomolar range. The reducing agents mercaptoethanol and DTT slightly activated the enzyme at a 1 mmol/L concentration. Treatment of the enzyme with mercapto-ethanol, urea, or guanidiniumchloride at concentrations ranging from 0.5 to 6.5 mol/L resulted in a total loss of activity at a concentration of 4, 2, and 2 mol/L, respectively.

Substrate specificity

The apparent K_m for the substrate DLPPEA, calculated using a Cornish-Bowden plot [13], was 47 μmol/L (SD 2 μmol/L, N = 3) (Fig. 6).

We failed to demonstrate poly-L-proline cleaving activity with human platelet APP. Even after 6 hr incubation at 40°, no formation of free proline could be detected. The bradykininase activity of APP was determined at substrate concentrations ranging from 15 to 400 μmol/L. Incubation times varied from 5 to 15 min to determine initial velocities. The substrate conversion remained below 20% in all the determinations. The Michaelis–Menten constant was calculated using a Cornish-Bowden plot [13]. Results indicate a K_m of 66 μmol/L (SD 8 μmol/L, N = 6) (Fig. 7). In all the experiments, bradykinin (retention time 10.0 min) was cleaved into only one degradation product (retention time 13.2 min) co-eluting with the peptide standard des-Arg^1-bradykinin, which

Table 2. Effect of inhibitors and metal ions on platelet APP activity

Compound	Concentration (mM)	Residual activity (% of control)
Protease inhibitors		
o-Phenanthroline	10	0
	1.0	12
EDTA	10	100 (10*)
PCMB	0.1	0
	0.001	1
PMSF	1.0	93
DFP	1.0	98
Aprotinin	0.026	75
Z-Pro-prolinal	0.3	82
4-Phe-Pro-NHO-Nb.HCl	0.1	100
Bestatin	1.0	10
	0.1	100
Amastatin	1.0	0
	0.1	0
Mercapto-ethanol	1.0	109
Dithiothreitol	1.0	112
Metal ions		
Cu^{2+}	1.0	0
	0.1	7
Zn^{2+}	0.1	1
Hg^{2+}	1.0	0
Ca^{2+}	1.0	100

Inhibitors were preincubated with enzyme and buffer for 20 min at 37° before addition of substrate. The given concentrations are final concentrations in the incubation solution. After incubation with substrate for 30 min at 40°, the remaining activity was compared with a control preincubated without inhibitor.
* Measured without $MnSO_4$ and trisodiumcitrate.

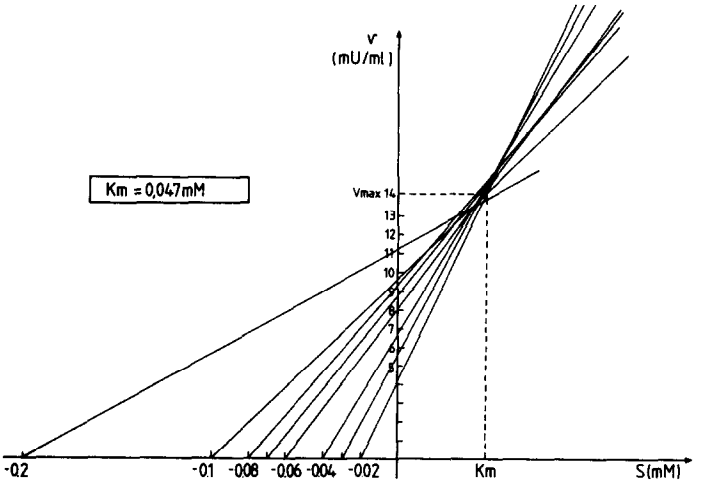

Fig. 6. Cornish-Bowden plot of the enzymatic velocity (v) against the concentration of DLPPEA (S) to determine the Michaelis–Menten constant K_m. Enzyme activities were measured with substrate concentrations ranging from 0.02 to 0.2 mmol/L, according to the standard assay [9].

indicates that the purified APP was devoid of other kininase activity. In a separate experiment, the purified APP was incubated for 20 min at 40° with 0.5 mmol/L bradykinin in the absence and presence of 1 mmol/L DFP. No qualitative or quantitative difference in the cleavage product could be detected.

DISCUSSION

We demonstrated recently the presence of aminopeptidase P in human platelets [1]. In order to study its characteristics and physiological function, we have purified this enzyme from human platelets

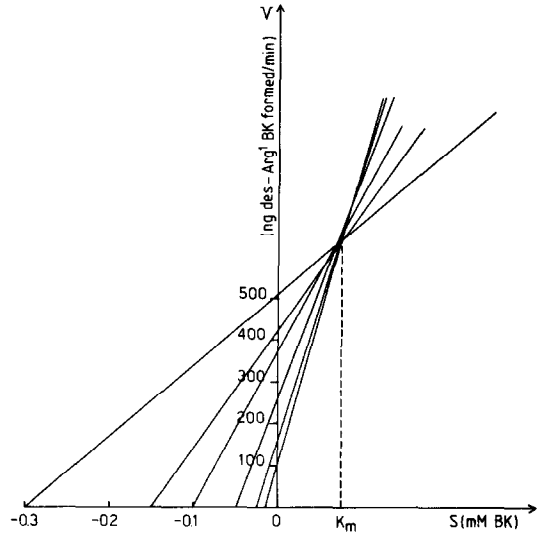

Fig. 7. Cornish-Bowden plot of the enzymatic velocity (v) against the concentration of bradykinin (S) to determine the Michaelis–Menten constant K_m. Separation and quantitation of bradykinin (BK) and des-Arg^1-bradykinin (des-Arg^1 BK) were assessed by HPLC on a Waters Novapack C_{18} reversed phase column, as described in Materials and Methods.

for the first time. The most striking feature of APP is its specificity for N-terminal X-Pro bonds [2]. Indeed, APP cleaves the Lys-Pro and Arg-Pro bond in the respective substrates DLPPEA and bradykinin. The use of the intramolecularly quenched substrate DLPPEA for monitoring the enzyme activity during purification rules out substrate cleavage by all proline-specific peptidases except APP [2, 15]. Our experiments allowed us to prove that prolyl endopeptidase is unable to hydrolyse this substrate. The bacterial APP (APP from *Escherichia coli* B and APP II from *E. coli* HB101) hydrolyses the N-terminal Pro-Pro bond with a high efficiency [16, 17]. Mammalian APP, however, hydrolyses the Pro-Pro bond very slowly compared with other X-Pro bonds [18, 19]. We could not demonstrate cleavage of this bond in poly-L-proline by human platelet APP. Failure to cleave the Pro-Pro bond was also reported for the rat intestinal microvillar APP [20]. It is as yet unclear whether the specific activity of both our and the intestinal APP preparation was too low to demonstrate the Pro-Pro cleavage or that other preparations of mammalian APP were contaminated by other interfering aminopeptidases. In agreement with earlier observations on mammalian APP, human platelet APP has an optimum pH of around 8.0 [18, 21], requires Mn^{2+} ions for optimal activity [9, 20, 22, 23] and is a metalloenzyme [3, 9]. In contrast with the rat lung enzyme, which is not affected by bestatin and amastatin at 1 mmol/L concentrations [24], the human platelet enzyme is strongly inhibited under these conditions.

Furthermore, although both human platelet and rat brain APP exhibits the same subunit M_r of 71,000, the rat brain enzyme forms dimers or trimers in solution depending on the salt concentration [19] while the only active form of platelet APP is a trimer, with a native M_r of 223,000.

Both brain and platelet APP show high affinity for bradykinin. While it has been demonstrated that in the blood stream bradykinin is rapidly converted by angiotensin-converting enzyme (ACE, EC 3.4.15.1) and carboxypeptidase N (CPN, EC 3.4.17.3), and that several cellular blood elements such as neutrophils and macrophages exert bradykinin-inactivating activity [7, 8, 25], little information on the possible involvement of other proteases or of platelets in kinin conversion is available. We have demonstrated previously [1] that the relative distribution of the kininases in serum differs significantly from their distribution in platelets. While ACE and CPN are present at high specific activities in serum, they are very poorly represented in platelets. APP, however, has a much higher specific activity in platelets than in serum. The results of the present study demonstrate that purified platelet APP, free of contamination by other protease activity, is capable of hydrolysing bradykinin. These data, the affinity of APP for a wide variety of biologically active peptides [19] and the presence of APP in endothelium [26], in serum [9] and in platelets [1], indicate an important role for APP in the metabolism of circulating peptides. Human platelets, as well as neutrophils and macrophages, may have a function in peptide metabolism and, more specifically, in the degradation of bradykinin from sites where it is accumulated. The development of a specific inhibitor for APP might provide a useful tool for establishing the involvement of human platelets in functions other than hemostasis and thrombosis, such as peptide metabolism and inflammation. Since APP may be important in the degradation of bradykinin analogs resistant to the activity of both ACE and CPN, specific inhibitors may potentiate the action of B1 analogs and B2 agonists [27]. In addition, considering the wide clinical use of ACE inhibitors and knowing that APP, like ACE, inactivates bradykinin but does not participate in the metabolism of angiotensin I, it might be of interest to consider the clinical value of specific APP inhibitors.

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REFERENCES

1. Scharpé SL, Vanhoof GC, De Meester IA, Hendriks DF, van Sande ME, Muylle LM and Yaron A, Exopeptidases in human platelets: an indication for proteolytic modulation of biologically active peptides. *Clin Chim Acta* **195**: 125–132, 1990.

2. Yaron A, The role of proline in the proteolytic regulation of biologically active peptides. *Biopolymers* **26**: S215–222, 1987.
3. McDonald JK and Barrett AJ, *Mammalian Proteases. A Glossary and Bibliography*, Vol. 2. Academic Press, New York, 1986.
4. Walter R, Simmons WH, Yoshimoto T, Proline-specific endo- and exopeptidases. *Mol Cell Biochem* **30**: 111–127, 1980.
5. Mentlein R, Proline residues in the maturation and degradation of peptide hormones and neuropeptides. *FEBS Lett* **234**: 251–256, 1988.
6. Baker CRF, Little AD, Little GH, Canizaro PC and Behal FJ, Kinin metabolism in the perfused ventilated rat lung. I: Bradykinin metabolism in a system modeling the normal, uninjured lung. *Circ Shock* **33**: 47–47, 1991.
7. Nagaoka I and Yamashita T, Possible involvement of aminopeptidase, an ectoenzyme, in the inactivation of bradykinin by intact neutrophils. *Biochim Biophys Acta* **847**: 67–76, 1985.
8. Nagaoka I and Yamashita T, Possible involvement of angiotensin I-converting enzyme in the inactivation of bradykinin by intact macrophages. *Biochim Biophys Acta* **886**: 231–242, 1986.
9. Holtzman EJ, Pillay G, Rosenthal T and Yaron A, Aminopeptidase P activity in rat organs and human serum. *Anal Biochem* **162**: 476–484, 1987.
10. Scharpé S, De Meester I, Vanhoof G, Hendriks D, van Sande M, Van Camp K and Yaron A, Assay of dipeptidyl peptidase IV in serum by fluorimetry of 4-methoxy-2-naphthylamine. *Clin Chem* **334**: 2299–2301, 1988.
11. Heukeshoven J and Dernick R, Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* **6**: 103–112, 1985.
12. Pharmacia LKB Biotechnology, ExcelGel Precast gels and buffer strips for horizontal SDS electrophoresis. Instruction manual.
13. Cornish-Bowden A and Wharton CW, *Enzyme Kinetics*. IRL Press, Oxford, 1988.
14. Demuth HU, Neumann U and Barth A, Reactions between dipeptidyl peptidase IV and diacyl-hydroxylamines: mechanistic investigations. *J Enzyme Inhibit* **2**: 239–248, 1989.
15. Hendriks D, De Meester I, Umiel T, Vanhoof G, van Sande M, Scharpé S and Yaron A, Aminopeptidase P and dipeptidyl peptidase IV activity in human leukocytes and in stimulated lymphocytes. *Clin Chim Acta* **196**: 87–96, 1991.
16. Yaron A and Mlynar D, Aminopeptidase-P. *Biochem Biophys Res Commun* **32**: 658–663, 1968,
17. Yoshimoto T, Murayama N and Tsuru D, A novel assay method for aminopeptidase P and partial purification of two types of the enzyme in *Escherichia coli*. *Agric Biol Chem* **52**: 1957–1963, 1988.
18. Dehm P and Nordwig A, The cleavage of prolyl peptides by kidney peptidases. Partial purification of a "X-Prolyl-Aminopeptidase" from swine kidney microsomes. *Eur J Biochem* **17**: 364–371, 1970.
19. Harbeck HT and Mentlein R, Aminopeptidase P from rat brain. Purification and action on bioactive peptides. *Eur J Biochem* **198**: 451–458, 1991.
20. Lasch J, Koelsch R, Ladhoff A-M and Hartrodt B, Is the proline-specific aminopeptidase P of the intestinal brush border an integral membrane enzyme ? *Biomed Biochim Acta* **45**: 833–843, 1986.
21. Orawski AT, Susz JP and Simmons WH, Aminopeptidase P from bovine lung: solubilization, properties, and potential role in bradykinin degradation. *Mol Cell Biochem* **75**: 123–132, 1987.
22. Hooper NM, Hryszko J and Turner AJ, Purification and characterization of pig kidney aminopeptidase P. A glycosyl-phosphatidylinositol-anchored ectoenzyme. *Biochem J* **267**: 509–515, 1990.
23. Fleminger G and Yaron A, Sequential hydrolysis of proline-containing peptides with immobilized aminopeptidases. *Biochim Biophys Acta* **743**: 437–446, 1983.
24. AchutaMurthy PN, Orawski AT and Williams HS, Partial purification and properties of aminopeptidase P from rat lung. *Fed Proc Fed Am Soc Exp Biol* **44**: 876: 1985.
25. Erdos EG, Kininases. In: *Handbook of Experimental Pharmacology* (Ed. Erdos EG), Vol. 25, Suppl. I, pp. 427–487. Springer, Heidelberg, 1979.
26. Vanhoof G, De Meester I, Yang QR, Vanden Berghe D, Van Camp K, Yaron A and Scharpé S, Aminopeptidase P activity in human endothelium, epithelium and fibroblasts. *Eur J Cell Biol* **53** (Suppl 31): 35, 1990.
27. Ward PE, Chow A and Drapeau G, Metabolism of bradykinin agonists and antagonists by plasma aminopeptidase P. *Biochem Pharmacol* **42**: 721–727, 1991.