Purification and Characterization of Adenosine Diphosphatase from Human Umbilical Vessels

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Adenosine diphosphatase (ADPase) activity was solubilized with a non-ionic detergent, Tween 20, from human umbilical vessels and purified to homogeneity by diethylaminoethyl-Sepharose CL-6B, adenosine 5′-monophosphate-Sepharose 4B, and concanavalin A-Sepharose chromatography. The apparent molecular mass was 75 kDa. The purified enzyme hydrolyzed pyrophosphate bonds of nucleoside di- and triphosphates in the presence of calcium ion. It was insensitive to the adenosine triphosphatase (ATPase) inhibitors, oligomycin and ouabain, and sensitive to sodium azide. Therefore, we concluded that the ADPase activity in human umbilical vessels does not derive from ADPase degrading only ADP but from ATP diphosphohydrolase (EC 3.6.1.5). The broad substrate specificity and the sensitivity to various inhibitors and calcium ion are common to ATP diphosphohydrolase from bovine aorta. However, there might exist some structural difference around the active site, because the antiserum raised in rabbit against the bovine aorta enzyme scarcely inhibited the human umbilical enzyme.

Keywords ADPase; ATP diphosphohydrolase; apyrase; umbilical vessel; ecto-ATPase; endothelial cell; human

Introduction

An enzyme degrading adenosine diphosphate (ADP), adenosine diphosphatase (ADPase), has been found in various vascular tissues[1–4] and plasma.[5,6] Since ADP is involved in the aggregation of platelets on vascular tissue, the ADPase is throught to be important in protecting the vasculature from thrombus formation.[7] In our previous work we purified and characterized the ADPase from bovine aorta.[8] The enzyme degraded adenosine triphosphate (ATP) as well as ADP to produce adenosine monophosphate (AMP). We could not find an enzyme specific to ADP throughout the purification. Therefore, we concluded that the ADPase activity was derived from ATP diphosphohydrolase (EC 3.6.1.5). ATP diphosphohydrolase has been found in mammalian tissues,[9,10] plants,[11–14] and insects.[15–17] The enzyme has a broad substrate specificity and hydrolyzes pyrophosphate bonds of nucleoside di- and triphosphates in the presence of divalent cation. Vascular endothelial cells degrade extracellulary added ATP to adenosine with the reaction sequence ATP-→ADP-→AMP-→ adenosine by ecto-ATPase, -ADPase, and-5′-nucleotidase.[18] We found that the activities of ecto-ATPase and ecto-ADPase in bovine endothelial cells were not expressed by separated enzymes but by a sole enzyme, ATP diphosphohydrolase, from the experiments using various inhibitors and specific antibody raised against purified ATP diphosphohydrolase.[19] In this study ADPase activity was solubilized from human umbilical vessel and characterized to compare with bovine aorta ADPase.

Materials and Methods

Materials Diethylaminoethyl (DEAE)-Sepharose CL-6B, 5′AMP-Sepharose, and concanavalin A (ConA)-Sepharose were purchased from Pharmacia. Molecular mass standards for sodium dodecyl sulfate (SDS)-gel electrophoresis were obtained from LKB. Sodium azide, ouabain, oligomycin, and nucleoside di- and triphosphates were obtained from Sigma.

Preparation of Microsomes Wharton's jelly was removed from human umibilical cord. Vessels were washed with 0.9% NaCl for 48 h to remove blood. A solution containing 20 mM Tris–HCl (pH 7.4) and 0.25 M sucrose were added to vessels at a ratio of 1 : 4 (w/w), and homogenized with a Polytron homogenizer (Nihonseiki). The homogenate was centrifuged at $10000 \times g$ for 15 min. The supernatant was then ultracentrifuged at $105000 \times g$ for 90 min. The microsomal pellet was washed with 20 mM Tris–HCl (pH 7.4) buffer containing 0.15 M KCl and 10 mM ethylenediaminetetraacetic acid (EDTA). The pellet was resuspended in 100 mM bicarbonate buffer (pH 10.0) and stirred at 4 °C for 12 h. The suspension was centrifuged at $105000 \times g$ for 90 min. The pellet was used as microsomes in the following experiments.

Purification of ADPase The solubilized fraction containing ADPase activity was dialyzed overnight against 20 mM Tris–maleate buffer (pH 6.5) containing 10% glycerol and 0.05% Tween 20 (buffer A). The dialyzate was applied to a column of DEAE-Sepharose CL-6B (20 × 1.6 cm) equilibrated with buffer A at a flow rate of 4.5 ml/h. Then the column was washed with buffer A. The fraction containing ADPase activity was eluted with a linear gradient of buffer A containing 0.05—0.2 M NaCl at the flow rate of 4.5 ml/h. The pooled fraction containing ADPase activity was dialyzed against 20 mM Tris–maleate buffer (pH 5.2) containing 10% glycerol, 0.05% Tween 20, and 2 mM $CaCl_2$ (buffer B). The dialyzate was concentrated by ultrafiltration and applied to a column of 5′AMP-Sepharose 4B (65 × 0.85 cm) previously equilibrated with buffer B at a flow rate of 2.3 ml/h. Then the column was washed with buffer B. The fraction containing ADPase activity was eluted with a linear gradient of buffer B containing 0—10 mM ADP at the flow rate of 2.3 ml/h.

ADPase Assay The activity was assayed by the method described previously.[8] Protein was measured by the method of Lowry *et al.*[20] with bovine serum albumin as a standard.

Preparation of Anti-ADPase–Antiserum ADPase was purified from bovine aorta by the method described previously.[8] One hundred micrograms of purified ADPase were injected into a rabbit. Booster injections of 100 μg of ADPase were performed after 1 and 2 weeks. The antiserum was obtained three weeks after the first injection.

Results

Solubilization of ADPase Activity The microsomal fraction of human umbilical vessels was prepared by the method described in Materials and Methods. Various detergents, such as Triton X-100, Rubrol-PX, Tween 20, cholate, and deoxycholate were tried in order to solubilize ADPase activity from the microsomes. Among the detergents tested, Tween 20 gave the best solubilization yield at 20.2% and a high specific activity of 2.66 unit/mg protein. In the following experiments ADPase activity was solubilized from the microsomes with a solution consisting of 1.0% Tween 20, 10% glycerol, 2 mM EDTA, and 50 mM Tris–maleate buffer (pH 9.0) by stirring for 24 h.

Purification of ADPase A summary of the purification is shown in Table I. The ADPase was purified about 41-fold with an overall yield of 2.6% after the step of 5′-AMP-Sepharose 4B column chromatography. The specific activity of the purified fraction was slightly lower than the enzyme purified from bovine aorta. The purity was checked

TABLE I. Purification of ADPase from Human Umbilical Vessels

Step	Total protein (mg)	Activity (U)	Specific activity (U/mg protein)	Purification (-fold)	Yield (%)
Microsomes	644	554	0.86	1	100
Solubilized microsomes	53.2	107	2.01	2.3	19.3
DEAE-Sepharose CL-6B	12.3	53	4.31	5.0	9.6
5'AMP-Sepharose 4B	0.42	14.8	35.4	41.3	2.6
ConA-Sepharose	0.10	3.7	37.0	43.0	0.7

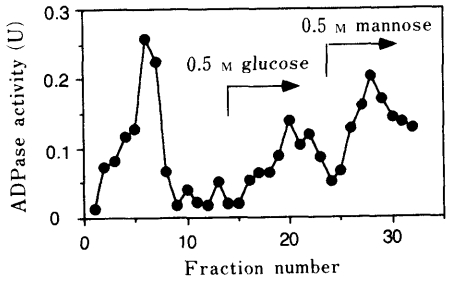

Fig. 1. ConA-Sepharose Chromatography of ADPase

The arrows indicate the beginning of column elution with buffer C containing 0.5 M glucose and 0.5 M mannose.

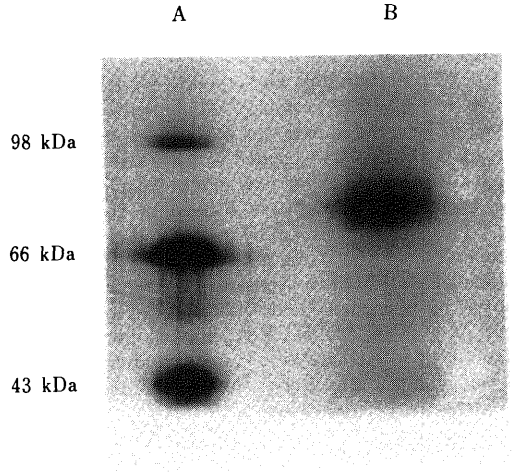

Fig. 2. SDS-PAGE of Purified ADPase

The purity was analyzed on 7.5% polyacrylamide gel electrophoresis with silver staining. Molecular mass standards (A) are 98 kDa of phosphorylase a, 66 kDa of bovine serum albumin, and 45 kDa of ovalbumin. The protein eluted from the ConA-Sepharose column with 0.5 M glucose was used as a sample (B).

by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). There were one major band with a molecular mass of 75 kilodaltons (kDa) and some minor bands (data not shown). We tried further purification to get a homogeneous preparation. In our preliminary experiments, we found three ADPase fractions having different affinities to ConA-Sepharose. Therefore, the enzyme sample was dialyzed against 100 mM Tris–HCl (pH 7.4) containing 10% glycerol, 0.05% Tween 20, 0.1 M NaCl, 1 mM $CaCl_2$, and 1 mM $ZnCl_2$ (buffer C). The dialyzate was applied to a column of ConA-Sepharose (9 × 0.7 cm) equilibrated in buffer C at the flow rate of 5 ml/h. The fraction containing ADPase activity

TABLE II. Substrate Specificity of ADPase

Substrate	Relative activity
ATP	1.1
ADP	1.0
GDP, UTP, IDP	0.9
GTP, CTP	0.7
UDP	0.6
CDP	0.5
AMP, GMP	0

Values are means of two experiments.

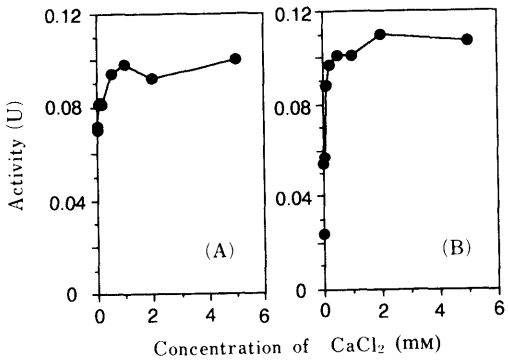

Fig. 3. Effects of Calcium Ion on ADPase (A) and ATPase (B) Activities

The enzyme was assayed in the presence of various concentrations of $CaCl_2$ with 2.5 mM ADP or 2.5 mM ATP. Values are means of two experiments.

was eluted with buffer C containing 0.5 M glucose, and then buffer C containing 0.5 M mannose. Figure 1 shows the results. The purity of second and third fractions eluted by glucose and mannose, respectively, was checked by SDS-PAGE. The second fraction gave a single band with silver staining, as shown in Fig. 2, but the third fraction still contained minor bands. The ADPase was purified about 43-fold and the overall yield was 0.7%, as shown in Table I.

Characterization of ADPase The homogeneous preparation was characterized in the following experiments. Table II shows the substrate specificity of the enzyme. Activities were measured using various nucleotides at a concentration of 5 mM and calculated from the amount of inorganic phosphate released for 1 h. The ADP hydrolysis rate, which was taken as 1.0, corresponds to 39.0 μmol Pi/min/mg protein. Although nucleotide monophosphate could not be hydrolyzed, the enzyme had nucleotide tri- and diphosphatase activities. Pyrophosphate, β-glycerophosphate, glucose-6-phosphate, and *p*-nitrophenylphosphate could not be the substrates of the enzyme (data not shown). The sensitivity of the enzyme to a divalent cation, Ca^{2+}, is shown in Fig. 3. The calcium ion was required to express the activity, because the addition of EDTA completely inhibited the activity (data not shown). The ATPase activity was more sensitive than the ADPase activity. Table III shows the effects of various inhibitors on the ADPase activity. Activities were measured in the presence of each inhibitor using 5 mM ADP as the substrate, and calculated from the amount of inorganic phosphate released for 1 h. The addition of ouabain and olygomycin, inhibitors of Na^+, K^+-ATPase and H^+-ATPase, respectively, did not inhibit the activity. On the contrary, the activity was inhibited by the addition of azide, which is known as an inhibitor

TABLE III. Effects of Inhibitors on ADPase Activity

Inhibitor	Inhibition (%)
Oligomycin (100 mg/ml)	0
Ouabain (1.0 mM)	2
Sodium azide (10 mM)	44

Values are means of two experiments.

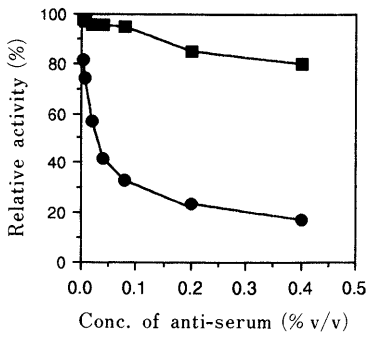

Fig. 4. Effects of Anti-serum Raised against Bovine Aorta ATP Diphosphohydrolase on Human Umbilical ADPase

Various concentrations of the anti-serum were added to human umbilical enzyme (■) and bovine aorta enzyme (●). Values are means of two experiments.

of mitochondrial ATPase.

Sensitivity to anti-serum raised in a rabbit against bovine aorta ADPase was examined in order to know the structural relationship between the human umbilical cord and bovine aorta enzymes. Figure 4 shows the results. The anti-serum strongly inhibited bovine aorta ADPase activity, but the extent of inhibition was very small in human umbilical cord ADPase. The preimmune serum did not affect either activity at a concentration of 0.4%.

Discussion

In this paper we describe the purification of human umbilical ADPase activity to clarify its physiological role in the blood vessel wall. After solubilizing the activity from umbilical vessel microsomes with Tween 20, the ADPase was purified to homogeneity by DEAE-Sepharose CL-6B, 5′-AMP Sepharose 4B, and ConA-Sepharose column chromatography.

The purified enzyme had broad substrate specificity, as seen in the bovine aorta enzyme.[8] Besides ADP, pyrophosphate bonds of nucleoside di- and triphosphate were hydrolyzed in the presence of divalent cation. No enzyme specific to ADP could be found throughout the purification. When we divided the vessels into artery and vein and purified ADPase from each type of vessel, both enzyme preparations had the same broad substrate specificity. The purified enzyme had no pyrophosphatase, alkaline phosphatase, or non-specific phosphatase activities. Taking also the sensitivity to various inhibitors (Table III) into consideration, we concluded that the ADPase activity was derived from ATP diphosphohydrolase (EC 3.6.1.5), as is the case with bovine aorta.

There were three fractions having different affinities to ConA-Sepharose, as shown in Fig. 1. Both umbilical vein and artery had ADPase activity, and in our previous study, we showed that in bovine smooth muscle cells as well as endothelial cells.[19] Therefore, although no difference could be found in substrate specificity, sensitivity to Ca^{2+} ion, and K_m value for ADP between the enzyme eluted by glucose and the enzyme eluted by mannose (data not shown), it is possible that isozymes having a different structure of oligosaccharides might exist in umbilical vessels.

There is a difference of sensitivity to calcium ion between ADPase and ATPase activities of the enzyme. ADPase activity needed a smaller amount of calcium to express the activity than ATPase did. The same result was obtained in the bovine aorta enzyme.[8] As for substrate specificity and sensitivity to various inhibitors, human umbilical vessel and bovine arorta aorta enzymes had the same characteristics. However, anti-serum raised in a rabbit against the bovine aorta enzyme scarcely inhibited human umbilical enzyme. It appears that there are some structural differences around active sites between the enzymes.

The physiological role of the ADPase activity in ATP diphosphohydrolase is not known. Recently, we found that ecto-ADPase and-ATPase in bovine aorta endothelial cells are not separate enzymes but are expressed by one enzyme, ATP diphosphohydrolase.[19] Lin and Guidotti cloned and sequenced a complementary deoxyribonucleic acid (cDNA) coding rat liver plasma membrane ecto-ATPase.[21] The enzyme belonged to a member of the immunogloblin superfamily. Aulivillius determined partial amino acid sequence of a cell adhesion molecule in rat hepatocytes, Cell-CAM 105, and found that the sequence agreed with that of ecto-ATPase.[22] In our preliminary experiment the anti-serum raised against bovine aorta inhibited adhesion of cultured bovine aorta endothelial cells. It is also reported that rat liver ecto-ATPase is a substrate for tyrosine kinase activity of the insulin receptor.[23] Therefore, ATP diphosphohydrolase, which is responsible for ecto-ATPase activity in the vessel wall, might be important in signal transduction through cell–cell adhesion.

References

1) J. M. Dawson, N. D. Cook, S. B. Coade, H. Baum, and T. J. Peters, *Biochim. Biophys. Acta*, **856**, 566 (1986).
2) D. R. Cooper, G. P. Lewis, G. E. Liebermann, H. Webb, and J. Westwick, *Thromb. Res.*, **14**, 901 (1979).
3) A. P. Heyns, D. J. Van den Berg, G. M. Potgieter, and F. P. Retief, *Thromb. Diath. Haemorrh.*, **32**, 417 (1974).
4) G. E. Liebermann and G. P. Lewis, *Artery*, **8**, 374 (1980).
5) I. Holmsen and H. Holmsen, *Thromb. Diath. Haemorrh.*, **26**, 177 (1971).
6) D. J. Richardson, G. P. Smith, T. W. Meade, P. Langley, and T. J. Peters, *Clin. Chim. Acta*, **121**, 87 (1982).
7) M. A. Barradas, D. P. Mikhailidis, and P. Dandona, *Int. J. Gynecol. Obstet.*, **31**, 15 (1990).
8) K. Yagi, Y. Arai, N. Kato, K. Hirota, and Y. Miura, *Eur. J. Biochem.*, **180**, 509 (1989).
9) D. Lebel, G. G. Poirier, S. Phaneuf, P. St.-Jean, J. F. Laliberte, and A. R. Beaudoin, *J. Biol. Chem.*, **255**, 1227 (1980).
10) A. F. Knowles, R. E. Isler, and J. F. Reece, *Biochim. Biophys. Acta*, **731**, 88 (1983).
11) A. Traverso-Cori, H. Chaimovich, and O. Cori, *Arch. Biochem. Biophys.*, **109**, 173 (1965).
12) L. Tognoli and E. Marre, *Biochim. Biophys. Acta*, **642**, 1 (1981).
13) F. Vara and R. Serrano, *Biochem. J.*, **197**, 637 (1981).
14) M. Mancilla, A. M. Kettlun, M. A. Valenzuera, and A. Traverso-Cori, *Phytochemistry*, **23**, 1397 (1984).
15) J. M. C. Ribeiro, J. J. F. Sarkis, P. A. Rossingnol, and A. Spielman, *Comp. Biochem. Physiol.*, **79B**, 81 (1984).
16) J. J. Sarkis, J. A. Guimaraes, and J. M. Ribeiro, *Biochem. J.*, **233**, 885 (1986).

17) M. J. Mant and K. R. Parker, *Br. J. Haematol.*, **48**, 601 (1981).
18) E. L. Gordon, J. D. Pearson, and L. L. Slakey, *J. Biol. Chem.*, **261**, 15496 (1986).
19) K. Yagi, M. Shinbo, M. Hashizume, L. S. Shimba, S. Kurimura, and Y. Miura, *Biochem. Biophys. Res. Commun.*, **180**, 1200 (1991).
20) O. H. Lowry, N. J. Rosebrogh, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
21) S. H. Lin and G. Guidotti, *J. Biol. Chem.*, **264**, 14408 (1989).
22) M. Aurivillius, O. C. Hansen, M. B. Lazrek, E. Bock, and B. Obrink, *FEBS Lett.*, **264**, 267 (1990).
23) R. N. Margolis, M. J. Schell, S. I. Taylor, and A. L. Hubbard, *Biochem. Biophys. Res. Commun.*, **166**, 562 (1990).