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Horse brain acylphosphatase: purification and characterization

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Two structurally different acylphosphatases found in horse brain were purified; they were not immunologically related. The molecular masses were almost identical and the kinetic parameters were rather similar. The data reported indicate that one of the purified brain acylphosphatases and an enzyme, previously isolated from horse muscle, are the same protein. The presence of this acylphosphatase form in the brain has not been reported before. The other acylphosphatase seemed to be the same as the enzyme which had been purified from calf brain and partially characterized by Diederich and Grisolia [(1969) J. Biol. Chem. 244, 2412–2417]. Furthermore, this enzyme seems to be identical to the acylphosphatase recently purified in our laboratory from human erythrocytes.

Acylphosphatase; Isoenzyme; Acyl phosphate; (Brain)

1. INTRODUCTION

We have previously reported on the existence of two distinct but related genes for acylphosphatase in human tissues [1]. Two acylphosphatase isoenzymes, one from skeletal muscle [2,3] and the other from erythrocytes [4], were isolated and their amino acid sequences determined [5]. About 56% of the amino acid positions of these enzymes were shown to be homologous. However, differences were found in the K_m and K_i values for some substrates and inhibitors [4]. The exact physiological significance of the two isoenzymes is not yet completely understood.

Acylphosphatase activity is present in brain extracts. Diederich and Grisolia [6] isolated an acylphosphatase from calf brain whose molecular mass, COOH-terminal residues and amino acid composition were similar to those of the human erythrocyte enzyme and different (except as regards molecular mass) from those of the skeletal muscle enzyme.

We investigated the possibility of the presence of both enzyme forms in horse brain. The confirmation of this point could raise other questions. Although apparently similar as regards substrate specificity and type of reaction catalyzed, as well as being evolutionarily related, they might perform different functions. Therefore, we decided to verify whether horse brain and erythrocyte acylphosphatase were identical and if the muscular form of acylphosphatase was also expressed in horse brain.

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Abbreviations: HPLC, high-pressure liquid chromatography; HBrI, horse brain acylphosphatase, muscular-type isoenzyme; HBrII, horse brain acylphosphatase, erythrocyte-type isoenzyme; Ho1, horse muscle acylphosphatase, mixed disulfide with glutathione form; Ho3, horse muscle acylphosphatase, S-S dimer; Mes, 2-*N*-morpholinoethanesulfonic acid; TFA, trifluoroacetic acid; PAGE, polyacrylamide gel electrophoresis

2. EXPERIMENTAL

CNBr-activated Sepharose 4B was purchased from Pharmacia, trypsin from Sigma, acetonitrile lichrosolv from Merck (Darmstadt), and nitrocellulose sheets from LKB. Benzoyl phosphate was synthesized according to Camici et al. [7]. Anti-horse muscle acylphosphatase antibodies were obtained and purified according to Berti et al. [8]; the immunoadsorbent used in the enzyme purification was prepared according to [3]. Anti-

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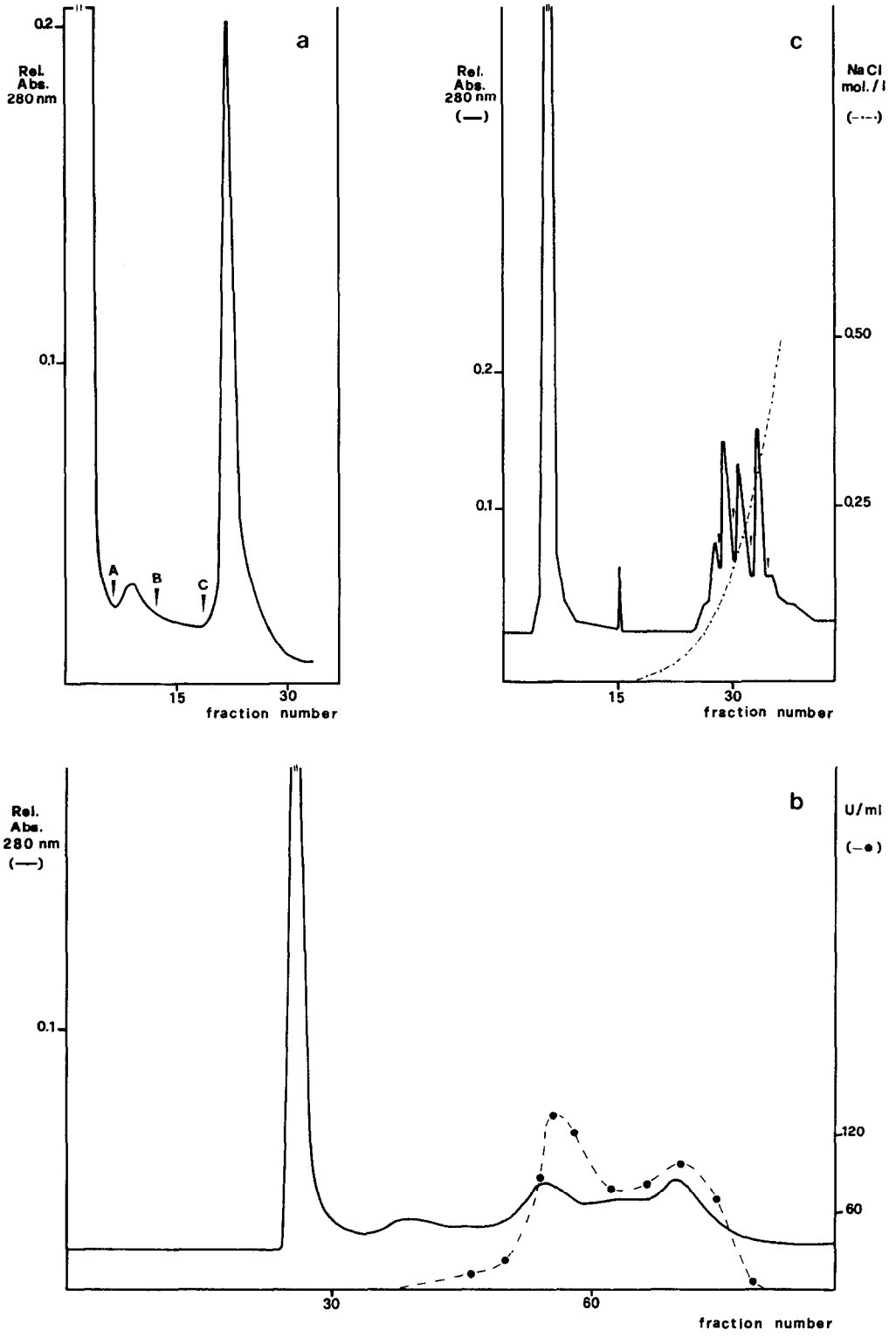

a
Rel. Abs. 280 nm
0.2
0.1
A
B
C
15
30
fraction number
c
Rel. Abs. 280 nm (—)
NaCl mol./l (-·-·)
0.2
0.1
0.50
0.25
15
30
fraction number
b
Rel. Abs. 280 nm (—)
U/ml (-●)
0.1
120
60
30
60
fraction number

Fig.1. (a) Affinity chromatography of the horse brain extract after isoelectric precipitation at pH 7.2. (A) 1.0 M NaCl wash, (B) 0.9% NaCl wash, (C) elution with 0.3 N acetic acid. (b) Elution pattern of Sephadex G-75M chromatography of the acylphosphatase activity after the affinity chromatography step. Column size, 2.5 × 55 cm; flow rate, 20 ml/h; elution, 0.05 N acetic acid/0.1 M NaCl; fraction volume, 2.5 ml. (——) Relative absorbance at 280 nm; (●---●) acylphosphatase activity. (c) HPLC purification of acylphosphatase activity eluted in Sephadex G-75M chromatography. Column, TSK CM 35W (LKB) (7.5 × 150 mm); elution, non-linear NaCl gradient (0–0.5 M) in 50 mM Mes-NaOH buffer, pH 6.5; flow rate, 48 ml/h. Arrows indicate the fractions containing acylphosphatase activity. (——) Relative absorbance at 280 nm; (-----) NaCl gradient.

human erythrocyte acylphosphatase antibodies were obtained and purified as in [9], using the enzyme which had been purified according to Liguri et al. [4] as antigen. Affinity-purified goat anti-rabbit horseradish-conjugated antibodies and peroxidase color development reagent (containing 4-chloro-1-naphthol) were purchased from Bio-Rad. All other reagents were of analytical grade or the best commercially available.

Acylphosphatase activity was checked by a continuous optical test at 283 nm [10]; protein concentration was determined by the biuret method, by ultraviolet absorption, or by amino acid analysis. SDS-PAGE was performed as reported [3]. The purity of the enzymes was checked by urea-acetic acid electrophoresis [11]. Tryptic hydrolysis was performed as reported in [3]. Tryptic peptides were separated by reverse-phase HPLC as in [3] except that the dry hydrolysate was dissolved in 5% TFA in water before injection into the column. Amino acid analysis was performed according to [3].

The agarose gels for double immunodiffusion were cast on microscope slides using 2.5 ml of a 1.0% agarose solution in Tris-buffered normal saline (pH 7.2). 2-mm diameter wells were cut into the gel; each well was filled with 6.0 μl of either the enzyme or the antibody solutions: diffusion was carried out overnight at room temperature. Trans-blots to nitrocellulose paper, after urea-acetic acid electrophoresis of the enzymes, were performed in 0.7 N acetic acid at 160 mA constant current for 16 h at 4°C, using a Bio-Rad transblot apparatus. After blotting, the nitrocellulose sheets were saturated with 3.0% bovine serum albumin in 10.0% horse serum for 1 h, washed in 10 mM phosphate buffer (pH 7.4) containing 0.9% NaCl and 0.3% Tween 20 and incubated overnight in the same buffer in the presence of appropriate dilutions of antibody or antiserum. The sheets were then washed 3 times in the same buffer for an overall period of 30 min and exposed to an appropriate solution of horseradish peroxidase-labeled anti-rabbit antibodies diluted in the same buffer. The sheets were washed again as above indicated and exposed to horseradish peroxidase color development solution.

3. RESULTS

Acylphosphatase activity was extracted from horse brain as reported [6], the only difference in the procedure being the use of 2 kg tissue. We followed this purification procedure up to the step named fraction 2. The pH of fraction 2 was adjusted to 7.2 and the solution was centrifuged at 34000 × g for 60 min. The supernatant was applied to a column (2.5 × 15 cm) containing purified anti-horse muscle acylphosphatase antibodies bound to Sepharose 4B. Only about half of the acylphosphatase activity present in the supernatant was retained by the immunoadsorbent. This solution was kept apart for successive purification of the unbound activity. All succeeding operations were according to [3] with minor modifications.

Fig.1 shows the pattern of chromatographic steps of enzyme purification. Table 1 summarizes the main parameters of the HBrI purification steps.

Table 1

Purification of horse brain acylphosphatase immunobound form (HBrI)[a]

Step	Volume (ml)	Total activity[b]	Total protein (mg)	Spec. act. (U/mg)	Purification (-fold)	Yield (%)
Extract supernatant	4900	53900	18130	3.0	1.0	100.0
Isoelectric precipitation, pH 7.2	476	29036	4379	6.6	2.2	53.9
Affinity chromatography	60	9900	8.88	1115	371.6	18.4
Gel-filtration on Sephadex G-75	156	9360	2.50	3744	1300.0	17.4

[a] Starting from about 2 kg tissue

[b] Determined according to Manao et al. [3]

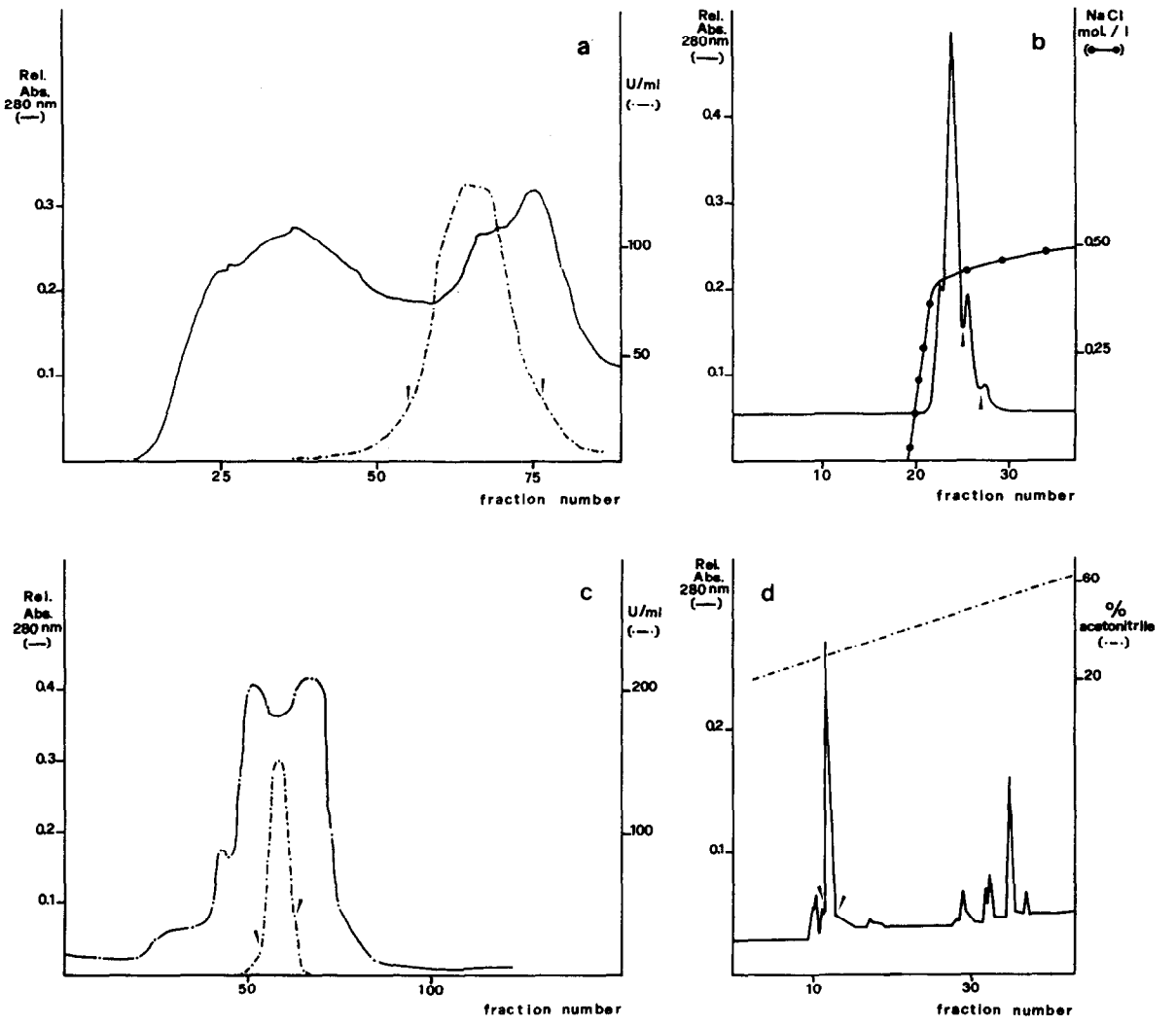


Fig.2. (a) CM Sephadex C25 chromatography of non-immunobound acylphosphatase activity. Column size, 2.5 × 25 cm; elution, linear NaCl gradient (0–0.5 M) in 20 mM acetate buffer, pH 5.8. (——) Relative absorbance at 280 nm; (·····) acylphosphatase activity. (b) HPLC purification of acylphosphatase activity eluted from ion-exchange chromatography. Column, TSK CM 35W (LKB) (7.5 × 150 mm); elution, non-linear NaCl gradient (0–0.5 M) in 50 mM Mes-NaOH buffer, pH 6.5. Arrows indicate the fractions collected. (——) Relative absorbance at 280 nm; (●——●) NaCl gradient. (c) Elution pattern of Sephadex G-75M chromatography of pooled fractions from HPLC chromatography. Column size, 2.5 × 60 cm; fraction volume, 2.5 ml. (—·—·—) Relative absorbance at 280 nm; (·····) acylphosphatase activity. The arrows indicate collected fractions. (d) HPLC purification by reverse-phase chromatography of material obtained from G-75 M chromatography. Column, Aquapore RP300 (250 × 4.6 mm, 10 μm). Solvent A: 10 mM TFA in water; solvent B: 10 mM TFA in acetonitrile. Flow rate: 1.5 ml/min. (——) Relative absorbance at 280 nm; (·····) acetonitrile concentration in effluent.

The supernatant, containing the acylphosphatase activity not bound by the immunoaffinity column, was applied once again to the immunoadsorbent to free it completely of HBrI. The solution was then adjusted to pH 5.8 and chromatographed on a (2.5 × 25 cm) CM-Sephadex C25 column in 20 mM acetate buffer; the column was eluted with an NaCl linear gradient (0–0.5 M in the same buffer) at a flow rate of 50 ml/h (fig.2a). Fractions containing acylphosphatase activity, equilibrated

Table 2

Physicochemical data of horse brain acylphosphatases, as compared with those relative to the enzymes from horse muscle and human erythrocytes

	HBrI		HBrII	Ho1[a]	Ho3[a]	Human erythrocytes[b]
	Low molecular mass	High molecular mass				
pH optimum	5.3	5.3	6.0	5.5	5.3	5.5
K_m (benzoyl phosphate) (mM)	0.95	1.11	0.14	2.00	1.10	0.08
K_i (P_i) (mM)	0.98	0.84	0.10	1.70	1.60	0.30
Inhibition type	comp.	comp.	comp.	comp.	comp.	comp.
K_i (Cl^-) (mM)	35.0	38.0	45.0	41.0	25.0	51.7
Inhibition type	comp.	comp.	comp.	comp.	comp.	comp.
Molecular mass (kDa)	12.6	25.1	12.5	12.6	24.2	11.5

[a] From Manao et al. [3]
[b] From Liguri et al. [4]

comp., competitive

with 50 mM Mes-NaOH buffer (pH 6.5), were applied to a (7.5 × 150 mm) HPLC TSK CM 35W column (LKB), and eluted with an NaCl gradient in the same buffer, as indicated in fig.2b. Fractions containing the enzyme activity were concentrated and chromatographed on a (2.5 × 60 cm) Sephadex G-75 M column in 0.05 N acetic acid/0.1 M NaCl at a flow rate of 20 ml/h (fig.2c). The pool of fractions containing acylphosphatase activity was chromatographed on an Aquapore RP300 HPLC column (10 μm, 0.46 × 25 cm); the column was eluted with a 0.01 M TFA/acetonitrile linear gradient at a flow rate of 90 ml/h (fig.2d). Urea-acetic acid–PAGE of the enzymes purified according to the two procedures above demonstrated that HBrI and HBrII were essentially pure (not shown). SDS-PAGE of the purified enzymes revealed the presence of two molecular forms of HBrI, with different apparent molecular masses (12.6 and 25.1 kDa, respectively) (not shown). In addition, the higher molecular mass band disappeared upon incubation of samples with 2-mercaptoethanol, giving rise to a band of molecular mass identical to that of the lower molecular mass component (as in the case of horse muscle acylphosphatase [3]). As for HBrII, an apparent molecular mass of about 12.6 kDa was calculated by SDS-PAGE. Table 2 compares the molecular masses and some kinetic parameters of HBrI and HBrII, with those previously determined for the horse muscle and human erythrocyte acylphos-

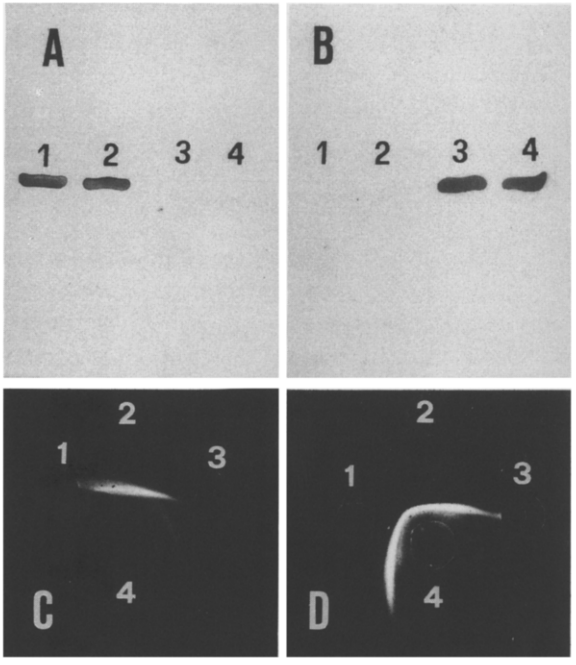


Fig.3. (A,B) Immunoblot analysis of HBrI and HBrII. (1) HBrI, (2) horse muscle acylphosphatase, (3) HBrII, (4) human erythrocyte acylphosphatase. The nitrocellulose sheets were incubated with anti-horse muscle acylphosphatase purified antibodies (A) or anti-human erythrocyte acylphosphatase antiserum (B). (C,D) Double immunodiffusion of HBrI (lower molecular mass form) and HBrII, in the presence of either human erythrocyte (C) or horse muscle acylphosphatase (D). (C) Well 1, HBrI; well 2, human erythrocyte acylphosphatase; well 3, HBrII; well 4, anti-human erythrocyte acylphosphatase antiserum. (D) Well 1, HBrI; well 2, horse muscle acylphosphatase; well 3, HBrII; well 4, anti-horse muscle acylphosphatase purified antibodies.

Table 3

Amino acid analysis of horse brain acylphosphatases[a]

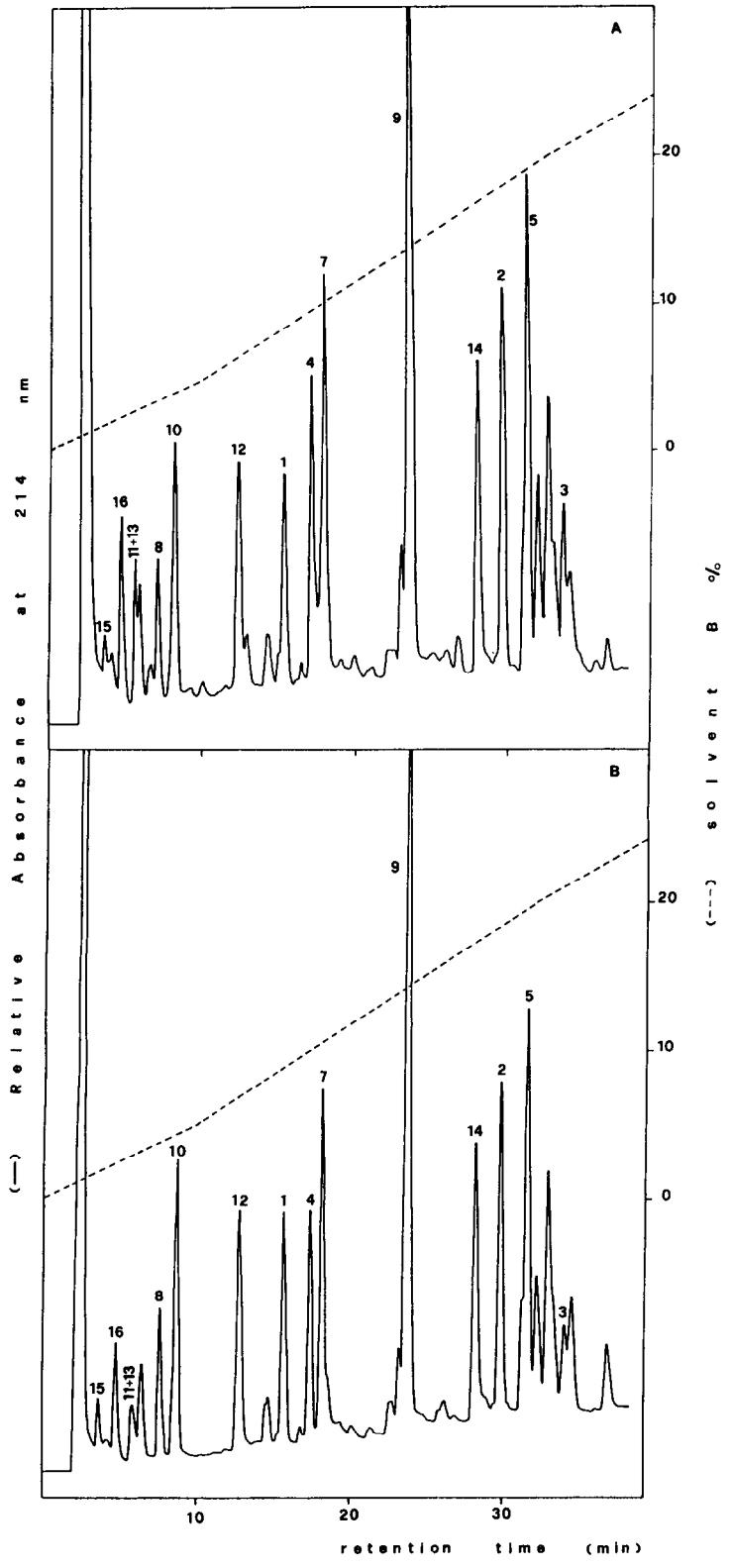
	Brain		Horse[b] muscle	Human[c] erythro-cytes	Calf[d] brain
	Immuno-bound form (HBrI)	Non-immuno-bound form (HBrII)			
Aspartic acid	8.6	9.6	8	10	9.5
Threonine	6.0	5.2	6	5	6.2
Serine	12.4	6.7	13	5	5.3
Glutamic acid	10.4	13.5	10	14	15.0
Proline	2.8	2.2	3	2	4.0
Glycine	8.3	9.4	8	10	10.0
Alanine	3.5	5.0	3	3	5.0
Cysteine	1.1[e]	0.7[e]	1		
Valine	11.2	9.7	12	9	9.5
Methionine	1.8	1.2	2	1	1.4
Isoleucine	3.0	5.5	3	6	4.5
Leucine	3.4	6.7	3	6	6.8
Tyrosine	3.7	2.8	4	2	3.0
Phenylalanine	4.0	4.8	4	5	4.9
Lysine	9.0	9.8	9	10	9.7
Histidine		2.0		3	3.0
Arginine	6.5	4.5	7	4	3.7
Tryptophan[f]	1.8	1.3	2	2	2.7

[a] Calculated as mol residue/mol enzyme
[b] Residues present in the main chain of horse muscle acylphosphatase according to its amino acid sequence [5]
[c] Residues present in the human erythrocyte acylphosphatase according to its amino acid sequence [4]
[d] Recalculated from data reported by Diederich and Grisolia [6]
[e] Determined as cysteic acid
[f] Determined according to Edelhoch [13]

phatases. The low and high molecular mass forms of HBrI showed kinetic parameters which were very similar to those calculated for the two enzyme forms previously purified from horse skeletal muscle [3]. On the other hand, the kinetic parameters of HBrII appeared quite different from those of HBrI but similar to those elicited by the human erythrocyte acylphosphatase.

Fig.3A and B shows the immunoblot analysis of HBrI and HBrII as compared with horse muscle and human erythrocyte acylphosphatase. The sheets were incubated with either purified anti-horse muscle acylphosphatase antibodies (A) or anti-human erythrocyte acylphosphatase antiserum (B). At high levels of sensitivity, the immunoblot shows a cross-reaction between anti-horse muscle acylphosphatase antibodies and HBrI (A) and between anti-human erythrocyte enzyme antiserum and HBrII (B). Fig.3C and D shows double immunodiffusion of HBrI (lower molecular mass form) and HBrII, respectively, in the presence of either anti-human erythrocyte acylphosphatase antiserum or anti-horse muscle acylphosphatase purified antibodies. In fig.3C a reaction of partial identity between HBrII and human erythrocyte acylphosphatase is observed in the presence of anti-erythrocyte acylphosphatase antiserum. Differences in one or more epitopes of

Fig.4. HPLC-reverse phase separation of tryptic peptides from horse muscle (A) and horse brain (HBrI, lower molecular mass form) (B) acylphosphatase. Column and elution conditions were as reported in the legend to fig.3.


A
B
9
20
10
0
5
7
2
14
4
10
12
1
16
11+13
8
15
3
(—) Relative Absorbance at 214 nm
(---) solvent B %
10
20
30
retention time (min)

the two enzymes, probably due to species differences, allowed for the formation of a spur. HBrI did not react with the anti-human erythrocyte antiserum. In fig.3D, HBrI and horse muscle acylphospatase are not distinguished by anti-horse muscle acylphosphatase purified antibodies. In fact, a reaction of immunological identity can be seen. On the other hand, HBrII did not react with these antibodies. Fig.3C and D shows the immunoblot analysis of HBrI and HBrII as compared with horse muscle and human erythrocyte acylphosphatase, which had been incubated with either purified anti-horse muscle acylphosphatase antibodies or anti-human erythrocyte acylphosphatase antiserum.

Table 3 lists the amino acid compositions of HBrI (lower molecular mass form) and HBrII as compared with that of horse skeletal muscle acylphosphatase. In addition, table 3 reports on the amino acid content of human erythrocyte acylphosphatase taken from sequence data [4,5]. The amino acid composition of HBrI was almost the same as that resulting from the sequence of the carboxymethylated derivative of horse skeletal muscle acylphosphatase [5]. The amino acid compositon of HBrII was similar to that determined for both the human erythrocyte and the bovine brain enzymes previously isolated [4,6].

The fingerprints of the tryptic peptides of horse skeletal muscle acylphosphatase and HBrI (lower molecular mass form), performed as indicated above, showed two profiles which were very similar (fig.4). The amino acid compositions of the corresponding peaks (not reported) were almost identical.

4. DISCUSSION

The data reported demonstrate the existence of at least two acylphosphatases in horse brain. The calf brain acylphosphatase which had been previously isolated [6] is probably one of these enzymes, as there is great similarity between its amino acid composition and that of HBrII. The two amino acid compositions are also very similar to that determined for the acylphosphatase recently isolated from human erythrocytes and sequenced in our laboratory [4]: the few differences in amino acid composition may be due to species difference. The kinetic parameters of the erythrocyte acylphosphatase are very similar to those determined for HBrII, further confirming the close similarity of these enzymes [4].

Our results indicate the presence of an acylphosphatase not previously described in horse brain. It differs from HBrII and erythrocyte acylphosphatase in amino acid composition, immunological behavior, and kinetic parameters. Our data on the molecular features, as well as on the kinetic and immunological properties of this enzyme indicate that it is the acylphosphatase previously purified from horse skeletal muscle [3]. Finally, our results show that the products of the genes for the two acylphosphatase isoenzymes can be present at the same time and almost to the same extent in total brain.

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