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Lipoamidase (lipoyl-X hydrolase) from pig brain

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Although the optimum substrate for lipoamidase (lipoyl-X hydrolase) has not yet been determined, it is known that lipoamidase activity, as determined by hydrolysis of the synthetic substrate lipoyl 4-aminobenzoate (LPAB), is widely distributed in pig brain tissues, i.e. in the cerebrum, cerebellum and medulla. Over 95% of the enzyme activity is present in the membrane subfractions, indicating that brain lipoamidase is an integral membrane protein enzyme. To elucidate the chemical nature and the optimum substrate of the abundant lipoamidase in the brain, we isolated it from the membrane subfractions. After an 8-step purification procedure, brain lipoamidase was purified 601-fold and identified as a 140 kDa glycoprotein by SDS/PAGE. A mechanistic study to determine K_m and $V_{max.}$ values was carried out using various synthetic compounds. Lipoyl-lysine, which is generally believed to be a naturally occurring substrate of lipoamidase, was first compared with biotinyl-lysine, because these two vitamins have reactive sulphur atoms and are similar in molecular mass and structure. The K_m for lipoyl-lysine was 333 μM, whereas biotinyl-lysine was not hydrolysed. Stringent specificity for the lipoyl moiety is demonstrated, as expected. Dipeptides of amino acid-lysine structures were studied, and dipeptides of aspartyl- and glutamyl-lysine hydrolysis occurred at high K_m (3 mM) values. Thus lysine in the moiety is not very effective as an optimum substrate. The chemical bond structures of the amide bond (lipoyl-lysine) and peptide bond (aspartyl-lysine) were hydrolysed. Next, the ester bond compound was tested, and it was observed that lipoylmethyl ester was hydrolysed at high specificity. These findings indicate that this enzyme has broad specificities with respect to bond structure; it therefore is a unique hydrolase having stringent specificity for lipoic acid and relatively broad specificity for the chemical bond and the X moiety. Various inhibitors were tested; a few reagents, such as organic mercurials, di-isopropylfluorophosphate, 1,10-phenanthroline, sodium azide and angiotensin-converting enzyme inhibitor exhibited some inhibition (not more than 60%). Thus the active centre of this enzyme is a complex type. Although ATP is not hydrolysed and the lowest K_m value is exhibited by the synthetic substrate reduced form LPAB (12 μM), some other compounds may still be expected to be hydrolysed by this unique and abundant brain lipoamidase.

INTRODUCTION

Lipoamidase (lipoyl-X hydrolase) was first described as a hydrolytic enzyme which cleaves protein-bound-forms of lipoic acid, lipoyl-lysine and lipoyl amides such as lipoamide and lipoyl-4-aminobenzoate (LPAB) [1,2]. This enzyme was found in a bacterial strain (*Streptococcus faecalis* 10C1) [1,2] and in bakers' yeast [3]. Recently, we found that the enzyme activity is widely distributed in mammalian samples such as human serum [4], human breast milk [5] and guinea pig liver [6]. Guinea pig liver lipoamidase was found to be an integral membrane glycoprotein enzyme [6]. We have also recently achieved isolation of breast milk lipoamidase and guinea pig liver lipoamidase [5,6]. The reason for such a wide distribution of lipoamidase in animal tissues and in body fluids (serum and milk) has not yet been elucidated; however, this distribution is analogous with that of the enzyme acetylcholine esterase. Lipoamidase activity, like acetylcholine esterase, is widely present in the pig brain.

Reports of lipoate-responsive neuronal diseases [7–10] suggest that lipoic acid plays an important role in the human brain. However, no descriptions of the content of lipoic acid or lipoamidase activity in brain tissues has been given. We therefore studied the subcellular distribution of lipoamidase in the brain, and performed chemical characterization of brain lipoamidase after isolation from brain membranes. Four kinds of hydrolase activities exist, i.e. amidase (amide bond), esterase (ester bond), protease (peptide bond) and carbohydrase (ether bond). A substrate specificity study was performed on the purified enzyme with various substrates, and the results indicated that this enzyme hydrolysed amide, ester and peptide bonds. Hence this enzyme cannot be categorized as either an amidase or an esterase. Some of the characteristics of this unique enzyme are also described.

MATERIALS AND METHODS

Chemicals and reagents

Di-isopropylfluorophosphate (DFP), phenylmethanesulphonyl fluoride (PMSF), *p*-chloromercuribenzoate (PCMB), *p*-hydroxymercuribenzoate (PHMB), di-

Abbreviations used: LPAB, lipoyl 4-aminobenzoate; PAB, *p*-aminobenzoic acid; PCMB, *p*-chloromercuribenzoate; PHMB, *p*-hydroxymercuribenzoate; PMSF, phenylmethanesulphonyl fluoride; DFP, di-isopropylfluorophosphate; NP-40, Nonidet P-40; WGA, wheat-germ agglutinin; DTNB, dithiobis(2-nitro)benzoic acid; h.p.a.l.c., high-performance affinity liquid chromatography; h.p.g.p.c., high-performance gel-permeation chromatography; PAS, periodate-Schiff.

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hydrolipoic acid, lipoamide, biotinyl 4-aminobenzoate, biotinyl-lysine (biocytin), 1,10-phenanthroline, bestatin, phosphoramidon, angiotensin-converting enzyme inhibitor, *N*-succinyl-L-proline, diethyl pyrocarbonate, 5-hydroxytryptamine–agarose and dipeptides were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Nonidet P-40 (NP-40), DL-lipoic acid and GlcNAc were from Nacalai Tesque Co., Kyoto, Japan. *p*-Aminobenzoic acid (PAB), L-lysine hydrochloride and 2-mercaptoethanol were from Kanto Chemical Co., Tokyo, Japan. Aprotinin was from Takara Shuzo Co., Kyoto, Japan. Epoxy-activated aminohexyl (EAH)-Sepharose 4B was from Pharmacia Fine Chemicals, Uppsala, Sweden. Agarose-bound wheat-germ agglutinin (WGA) and agarose-bound concanavalin A were from Vector Laboratories, Inc., Burlingame, CA, U.S.A. A serotonin high performance affinity liquid chromatography (h.p.a.l.c.) column was obtained from Seikagaku–Kogyo Co., Tokyo, Japan. *N*-Glycanase was from Genzyme Co., Boston, MA, U.S.A. Glycopeptidase F from *Flavobacterium meningosepticum* and β-galactosidase from *Escherichia coli* were from Boehringer–Mannheim Yamanouchi, Tokyo, Japan. Ceruloplasmin was prepared as described in [5].

Specimens

Pig brains were obtained from Clea Japan Inc., Tokyo, Japan. Unstripped pig brains (lot no. 29410) were also obtained from Pel-Freez Biologicals, Rogers, Arkansas, U.S.A. The specimens were stored at −80 °C.

Synthesis of lipoyl 4-aminobenzoate (LPAB) and lipoyl derivatives

LPAB and lipoyl-L-lysine were synthesized as previously described [4,11]. Reduced-form lipoic acid (dihydrolipoic acid) and reduced-form LPAB (dihydrolipoyl 4-aminobenzoate) were synthesized by reduction with sodium borohydride, essentially as described by Wagner *et al.* [12], and the content of the synthesized reduced product was determined by h.p.l.c. [11]. Biotin methyl ester and lipoyl methyl and ethyl esters were synthesized by simply incubating biotin or lipoate at 37 °C for 2 h (ethyl ester at 60 °C) with corresponding anhydrous alcohols in the presence of a catalytic amount of HCl.

Assay of enzyme activity

Lipoamidase activity was determined by the h.p.l.c. fluorimetric method developed by us [4,13]. Succinate dehydrogenase was determined by the method described by Arrigoni & Singer [14], and (Na+K)-activated ATPases was determined by Tashima's method [15]. A Hitachi U-3200 spectrophotometer was used.

Tissue distribution of lipoamidase activity

Tissue homogenates were prepared in a phosphate buffer (1 mM, pH 7.4) containing 0.32 M-sucrose. Tissues were cut into pieces using a scalpel and homogenized mechanically using a Potter–Elvehjem glass homogenizer.

Subcellular distribution of lipoamidase activity

Differential centrifugation was carried out in 0.32 M-sucrose solution containing 1 mM-sodium phosphate (pH 7.4) and 0.2 mM-EDTA, essentially as described by Albers *et al.* [16]

Purification of pig brain lipoamidase

All purification procedures except for h.p.a.l.c. were performed at 4 °C. Pig brain (80 g) was homogenized in the 0.32 M-sucrose containing 1 mM-sodium phosphate, pH 7.4 (800 ml). The homogenate was centrifuged at 105000 *g* for 90 min, and the resultant precipitate was dispersed in 0.05 M-phosphate buffer (pH 6.0) containing 0.2 mM-EDTA, 10% glycerol and 1% NP-40 (600 ml). The dispersed membrane fractions were mixed with 500 ml of CM-cellulose and filtered through a gauze cloth. The filtrate (1.2 litres) was diluted to one-fifth of its original concentration and adjusted to 1 mM-EDTA, 10% glycerol and 0.5% NP-40 (pH 8). A portion (2 l) of the diluted filtrate was loaded on to the DEAE-cellulose column (40 cm × 1.9 cm) and developed with a linear gradient of NaCl (from 0 to 0.5 M; total vol. 500 ml). The enzyme-containing fractions were collected and concentrated by an ultrafiltration membrane (PM-10, Amicon Co., Danvers, MA, U.S.A.). This step was repeated twice. The concentrated DEAE-cellulose-enriched enzyme was then separated by a gel-permeation chromatography column of Sephadex G-200 (100 cm × 1.9 cm). The mobile phase was 0.05 M-sodium phosphate buffer (pH 6.8) containing 1 mM-EDTA, 0.1% NP-40 and 10% glycerol. The enzyme-containing fractions were collected and concentrated as above. This step was repeated eight times. The concentrated enzyme was then separated on a gel-permeation chromatography column of Sepharose CL-4B (100 cm × 1.9 cm) with the mobile phase as above except for a non-ionic detergent (NP-40) concentration of 0.5%. This gel-permeation chromatography was repeated five times, and the enzyme fractions were collected for concentration by PM-10 ultrafiltration membranes. The concentrated enzyme solution (25 ml) was dialysed against 2 l of a 0.5% NP-40 solution containing 5 mM-sodium phosphate (pH 7.0), 10% glycerol and 100 mM-NaCl. The dialysed enzyme solution was loaded on to a WGA–agarose affinity column (40 mm × 10 mm internal diam.). The bound enzyme was then specifically eluted by a linear gradient of 0–0.3 M-GlcNAc. The enzyme was eluted at a GlcNAc concentration of 0.06 M. The enzyme fractions were concentrated and dialysed against the 0.5% NP-40 solution containing 5% acetone, 5% glycerol, 1 mM-EDTA, 1 mM-mercaptoethanol and 5 mM-sodium phosphate (pH 7.0). This solution was loaded on to a serotonin-h.p.a.l.c. column (150 mm × 4.0 mm internal diam.; Honen Corporation, Tokyo, Japan) and eluted with a linear gradient of 5–50 mM-phosphate for 30 min at a flow rate of 1.0 ml/min. The h.p.l.c. system used incorporated an LKB 2150 pump (Pharmacia). The sample-loading apparatus used was an EIE-005 injector (Senshu Scientific Co., Tokyo, Japan) equipped with a stainless steel tube (75 mm × 7.5 mm internal diam.), which enabled sample loading in a volume of up to 2 ml. The enzyme-containing solutions were concentrated and stored at −80 °C for chemical and mechanistic studies. An outline of the purification is given in Table 3.

Protein content

Protein concentrations were assayed by Lowry's method [17] or by using a BCA protein assay kit from Pierce Chemical Co., Rockford, CA, U.S.A. BSA was used as a standard protein.

SDS/PAGE

SDS/PAGE was carried out essentially according to Laemmli [18]. A polyacrylamide concentration of 6% was used. Proteins were detected by Coomassie Brilliant Blue R-250, periodate-Schiff (PAS) stain or silver stain [5].

Amino acid analysis

Amino acid composition was determined by means of an h.p.l.c. amino acid analyser [6].

Ultracentrifugation analysis in glycerol gradient

Ultracentrifugation analysis using a glycerol gradient for assessment of the molecular mass of lipoamidase was performed essentially as described by Ortiz *et al.* [19]. The molecular mass markers used were γ-globulin (155 kDa), BSA (68 kDa) and lysozyme (14.4 kDa).

N-Glycanase treatment

N-Glycanase and glycopeptidase F treatment of lipoamidase was performed by incubating 2.3 μg of lipoamidase in 0.05 ml of reaction mixture (0.1 M-sodium phosphate buffer, pH 7.0) at 37 °C for 17 h with or without 3 μl of glycopeptidase. The samples were then analysed by SDS/PAGE

Inhibitor tests

Purified lipoamidase was incubated as follows. Serine inhibitors (DFP and PMSF), which modify active centre serine residue(s) by alkyl-phosphorylation or sulphonylation, were dissolved to 50 mM in dimethyl sulphoxide. Then 0.5, 0.05 and 0.005 mM concentrations of DFP and PMSF were prepared by dilution with distilled water. Inhibitors (0.01 ml) were mixed with 0.01 ml of purified enzyme and were stood for 15 min at 23 °C. The reaction was started by addition of 0.08 ml of substrate-containing buffer (pH 7.0). This mixture and the control reaction mixture without inhibitor were incubated at 37 °C for 0.5 or 1 h, and enzyme activity was determined as described above. Other inhibitors were dissolved in distilled water.

Kinetic studies

Michaelis constants (K_m) of lipoamidase were determined by assessing the reaction rates at substrate concentrations of about $K_m/3$, $K_m/2$, K_m, $2K_m$ and $3K_m$ (μM). K_m values for lipoylmethyl ester, biotinylmethyl ester and lipoylethyl ester were determined in the reaction mixture containing 5% acetone. The reaction rate was determined by the h.p.l.c. fluorimetric method [13] for LPAB, reduced-form LPAB and biotinyl-PAB. The reverse-phase h.p.l.c. method [6] was used at 330 nm u.v. (specific for lipoic acid; dithiolane ring structure) for lipoamide, lipoylmethyl ester and lipoylethyl ester, and at 215 nm for biotinylmethyl ester. An h.p.l.c. amino acid analyser was used for lipoyl-lysine. K_m and $V_{max.}$ values were determined by means of double-reciprocal plots [20]. K_i values for oxidized-form lipoic acid and reduced-form lipoic acid were determined as follows. Reaction rates were determined as above at competitive inhibitor concentrations of 0, 5 or 10 mM. Double-reciprocal calculations were performed, and K_m and K_i were also determined. Lipoic acid, dihydrolipoic acid and biotin were dissolved in distilled water and neutralized with 3 M-NaOH.

RESULTS AND DISCUSSION

Tissue distribution of lipoamidase in pig brain

As shown in Table 1, pig brain tissues were studied for lipoamidase activity. The cerebellum has been found to have higher activities of lipoamidase than other brain tissues (cerebrum and medulla).

In rat brain tissues, lipoamidase activity was at a higher level in the cerebrum than in other brain tissues, as shown in Table 1. Furthermore, the cerebrum had higher lipoamidase activity than the liver (21.9 pmol/min per mg of protein). In guinea pig tissues, the liver exhibited higher enzyme activity (50.2 pmol/min per mg of protein) than other tissues, e.g. the cerebellum (23.4 pmol/min per mg of protein), kidney (19.2 pmol/min per mg of protein), cerebrum (13.6 pmol/min per mg of protein) and heart (1.4 pmol/min per mg of protein). The human body fluids, serum and breast milk, contained high lipoamidase activity. Human breast milk had the highest specific activity (73 ± 28 pmol/min per mg of protein [5]) among the specimens studied, and human serum also exhibited relatively high lipoamidase activity (23 ± 16 pmol/min per mg of protein [4]).

Subcellular distribution of cerebrum lipoamidase

The homogenates of pig cerebrum were differentially centrifuged and fractionated. As shown in Table 2, lipoamidase activities were mainly (over 90%) present in membrane subfractions, i.e. in the nuclear, mitochondrial and microsomal fractions. The pattern of subcellular distribution of lipoamidase activity was similar in cerebrum and cerebellum; however, microsomal activity was the highest in medulla tissue (Table 2). The nerve-terminal-button fraction seemed to have higher lipoamidase activity than did myelin membranes (J. Oizumi & K. Hayawaka, unpublished work). These results indicate that the pig brain lipoamidase was present as an integral membrane protein.

Dispersal of membrane subfractions

To assess the dispersal of membrane lipoamidase, the neutral detergents Triton X-100 and NP-40 were tested for their effects on enzyme activity. Neither detergent influenced lipoamidase activity at concentrations of up

Table 1. Lipoamidase activity in brain tissue homogenates from pig rat and guinea pig

Tissue homogenates were prepared, using a Potter–Elvehjem-type glass homogenizer, in 0.32 M-sucrose containing 1 mM-sodium phosphate buffer (pH 7.0). Wet weights of cerebrum, cerebellum, and medulla were 80, 11 and 9.7 g respectively in pig brain; 0.4, 0.3 and 0.3 g respectively in rat brain; and 1.8, 0.7, and 0.5 g respectively in guinea pig brain.

Tissue Species...	Lipoamidase activity (pmol/min per mg of protein)		
	Pig	Rat	Guinea pig
Cerebrum	14.4	23.7	13.6
Cerebellum	26.8	18.5	23.4
Medulla	7.6	11.1	9.7

Table 2. Subcellular distribution of pig cerebrum lipoamidase activity as compared with (Na + K)-activated ATPase and succinate dehydrogenase (SDH)

Ouabain-sensitive (Na + K)-ATPase and SDH activities were determined as described in the Materials and methods section. ND, not detectable.

Fraction	Tissue...	Lipoamidase (pmol/min per mg of protein) Cerebrum	Cerebellum	Medulla	ATPase (nmol/min per mg of protein)	SDH (nmol/min per mg of protein)
Homogenate		15.8	26.8	7.6	381	5.86
Nucleus (P1)		11.0	21.1	4.6	392	3.62
Mitochondria (P2)		15.5	28.4	4.8	513	9.61
Microsome (P3)		16.0	22.6	10.0	733	0.95
Soluble (S3)		2.0	2.2	2.0	ND	ND

to 1%. However, 45% and 35% inhibition of lipoamidase activity was observed for Triton X-100 and NP-40 respectively at 2%. Other detergents, such as digitonin, digitoxicin, desoxycholate and cholate, all strongly inhibited (by approx. 100%) the enzyme activity at 0.1%. Therefore we chose NP-40 at a concentration less than 1% for dispersal of the membrane lipoamidase.

Dispersal by organic solvent was also tested. Acetone is a suitable solvent for dissolution of many lipid compounds, and Scouten *et al.* [21] employed a 10%

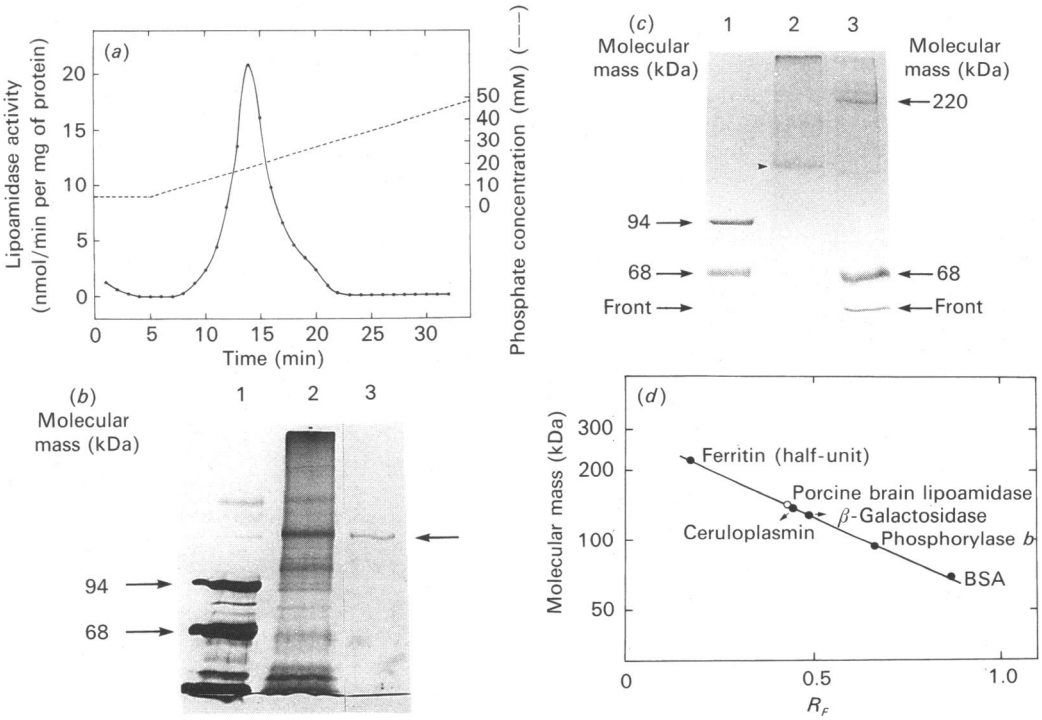

Fig. 1. Purification and molecular mass determination of lipoamidase

(*a*, *b*) Final purification step by serotonin-h.p.a.l.c. (*a*) Chromatogram h.p.l.c. An LKB-2150 h.p.l.c. pump and a serotonin-h.p.a.l.c. column (150 mm × 4.0 mm int. diam.) were used, with a flow rate of 1.0 ml/min. The phosphate concentration was increased linearly from 5 to 50 mM in 30 min. Other details were as described in the Materials and methods section. (*b*) Membrane proteins as detected by SDS/PAGE (6% acrylamide concentration). Proteins were silver stained. Lane 1, LMW Calibration Kit (Pharmacia) (94 kDa, phosphorylase *b*; 68 kDa, BSA); lane 2, WGA step enzyme (1.8 μg of protein) before loading on to the serotonin-h.p.a.l.c. column; lane 3, lipoamidase peak of serotonin-h.p.a.l.c. separation (fraction 14; 0.02 μg of protein). (*c*, *d*) Molecular mass determination by denaturing SDS/PAGE of purified pig brain lipoamidase. (*c*) Purified lipoamidase. Lane 1, LMW Calibration Kit (Pharmacia) (94 kDa, phosphorylase *b*; 68 kDa, BSA); lane 2, purified brain lipoamidase (2.3 μg of protein); lane 3, HMW Calibration Kit (Pharmacia) [220 kDa, ferritin (half unit); 68 kDa, BSA]. An acrylamide concentration of 6% was used. Protein was detected by Coomassie Brilliant Blue R-250 staining. (*d*) Molecular mass estimation by linear plotting of SDS/PAGE data. Ceruloplasmin (135 kDa) and β-galactosidase (115 kDa) were also employed. A logarithmic plot of molecular mass versus R_F was obtained. Pig brain lipoamidase molecular mass was determined to be 140 kDa.

Table 3. Summary of purification of membrane lipoamidase from pig brain

Step	Volume (ml)	Protein (mg/ml)	Activity (pmol/min per mg of protein)	Purification (-fold)	Recovery (%)
1. Homogenate	600	9.74	14.2	1.00	100
2. Membrane fraction	400	9.14	27.8	1.96	89.0
3. CM-cellulose	1000	2.83	27.8	1.96	68.4
4. DEAE-cellulose	800	1.38	47.0	3.31	45.1
5. Sephadex G-200	320	2.58	55.7	3.92	40.0
6. Sepharose CL-4B	450	0.73	122	7.89	26.7
7. WGA affinity	256	0.23	238	16.8	12.2
8. Serotonin affinity	14	0.11	8530	601	11.3

acetone solution in the purification step for lipoamide dehydrogenase (EC 1.6.4.3) from pig heart. Therefore we also tested the effect of acetone on lipoamidase activity. The residual activities were 92, 96, 110, 103 and 100% of the original at acetone concentrations of 10, 7.5, 5, 2.5 and 0% respectively. Thus we used 5% acetone solution system in the final serotonin-h.p.a.l.c. step.

Purification of porcine brain lipoamidase

The dispersed membranes were processed by the 8-step purification procedure described in the Materials and methods section. A typical example of a final separation serotonin-h.p.a.l.c. column is shown in Fig. 1(*a*) and the efficiency of the column is also shown (Fig. 1*b*). The presence of 0.5% NP-40, 5% glycerol and 1 mM-2-mercaptoethanol was essential for full recovery of enzyme from the h.p.a.l.c. column. After the final serotonin-h.p.l.c. step, a single protein band was obtained in an SDS/PAGE system (6% polyacrylamide gel) (Figs. 1*b* and 1*c*), and 601-fold purification was achieved. An outline of purification with respect to enzyme activity is also shown in Table 3.

Hydroxyapatite h.p.l.c., DEAE-5PW h.p.l.c. and lipoyl–EAH-Sepharose affinity chromatography [22] were tested and found not to be very effective in purification of the enzyme, although the reason for this has not been elucidated fully. However, hydrophobic interactions were so strong that suitable adsorption–desorption conditions might not have been achieved. Serotonin-affinity resin was reported to adsorb specifically sialic-acid-containing glycoprotein [23]; however, the possibility of direct interaction of serotonin with the serotonin receptor molecule, which is present in brain membranes, has yet to be explored. The points of similarity between lipoamidase and serotonin receptor remain to be elucidated, although we have not yet been able to purify the serotonin receptor.

Molecular mass characteristics of brain lipoamidase

SDS/PAGE analysis (denaturing conditions) gave a value for the molecular mass of lipoamidase of 140 ± 2.5 kDa, present as a single polypeptide (Fig. 1*b*, lane 3; Fig. 1*c*, lane 2). Gel-permeation chromatographic analyses in non-denaturing conditions were performed (Fig. 2). Sephadex G-200 gel-permeation chromatography indicated that lipoamidase activity was eluted at a retention volume of 140 ± 6.3 kDa (Fig. 2*a*). Sepharose CL-4B gel-permeation chromatography also indicated a molecular mass of $140(\pm 7)$ kDa (Fig. 2*b*). High-performance gel-permeation chromatography (h.p.g.p.c.) analysis using silica-based propyl-diol-type gel (TSK gel 3000-SW; Toso Co., Tokyo, Japan) demonstrated a molecular mass of 130 ± 8.4 kDa with a carrier of 0.5% NP-40 solution containing 0.1 M-sodium phosphate (pH 6.8) (Fig. 2*c*). The h.p.g.p.c. column could not elute the enzyme activity at 0.1% NP-40, and the concentration of 0.5% NP-40 was necessary to elute the enzyme. However, possible strong hydrophobic interaction of enzyme and h.p.g.p.c. gel support has resulted in an unsymmetrical peak shape even at this high (0.5%) concentration of NP-40.

Ultracentrifugation analysis with a linear glycerol gradient (5–30%) caused the enzyme activity to sediment at a molecular mass of 160 ± 19 kDa (Fig. 2*d*).

Glycoprotein nature of lipoamidase

Purified lipoamidase was electrophoresed on SDS/PAGE and stained with PAS staining (Fig. 3*a*). Lipoamidase was found to be specifically stained by the PAS (Fig. 3*a*, lane 2, indicated by the arrow), because only ovalbumin was stained, whereas BSA and phosphorylase *b* were not detected by PAS (Fig. 3*a*, lane 1). This strongly indicates that lipoamidase contains carbohydrate moieties.

Glycopeptidase treatment was performed with *N*-glycanase and glycopeptidase F; the results are shown in Fig. 3(*b*). Treatment with both glycopeptides shifted the enzyme protein towards the direction of slightly smaller size (Fig. 3*b*, lanes 3 and 4; 140 to 132 kDa). The bands (33 kDa) which appeared after glycopeptidase treatment were caused by the added *N*-glycanase and glycopeptidase F.

Amino acid composition of brain lipoamidase

The amino acid composition of purified pig brain lipoamidase was analysed. The results are shown in Table 4, along with the data for guinea-pig liver lipoamidase, human milk lipoamidase and the acetylcholine receptor of *Electrophorus electricus* [24]. The amino acid composition of pig brain lipoamidase is very similar to that of guinea pig liver lipoamidase, and both lipoamidases are integral membrane protein enzymes [6]. The hydrophilicity, as defined by Capaldi & Vanderkooi [25], was also similar to that of liver lipoamidase. The amino acid composition and hydrophilicity of human milk enzyme are rather different

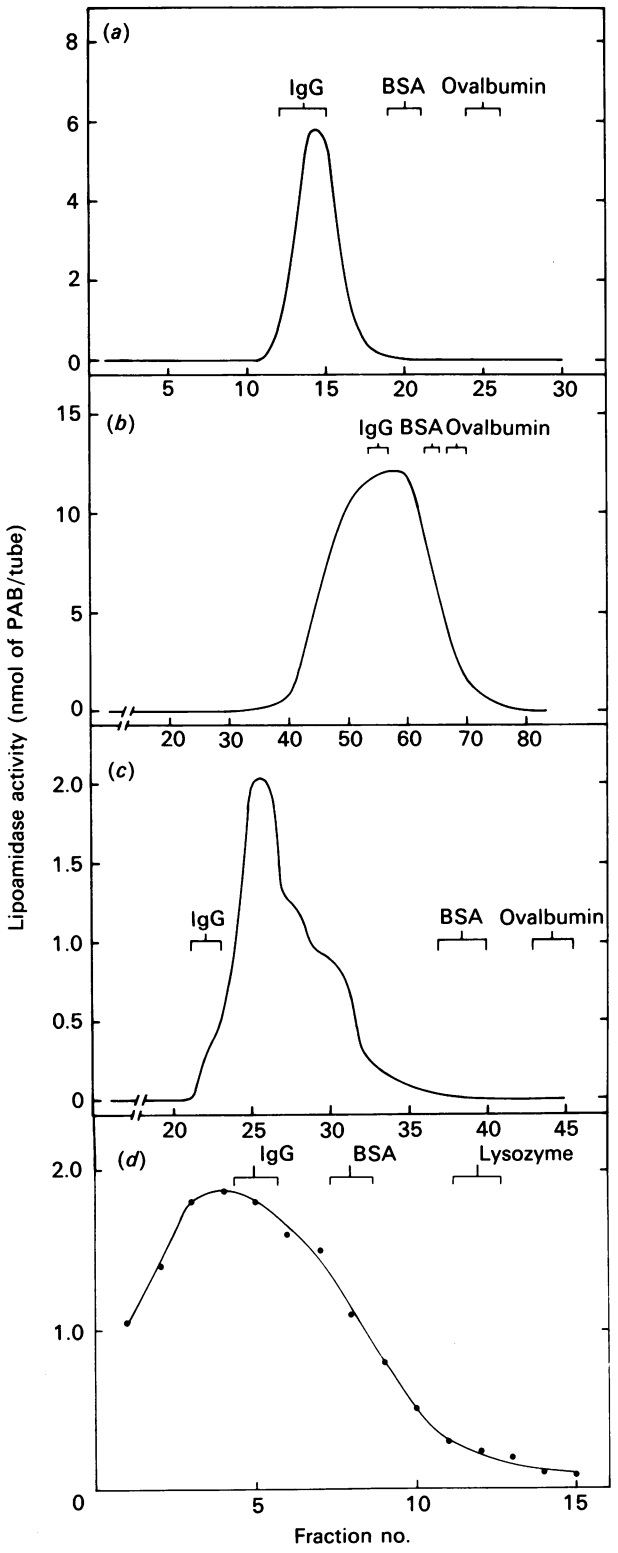

Fig. 2. Molecular mass estimation of lipoamidase

(*a–c*) Non-denaturing gel-permeation chromatography. (*a*) Sephadex G-200 (0.1% NP-40); (*b*) Sepharose CL-4B (0.5% NP-40); (*c*) h.p.g.p.c. of TSK gel 3000-SW (0.5% NP-40). (*d*) Molecular mass estimation by ultracentrifugation on linear glycerol gradient (5–30%). Fractionation was performed from top to bottom. Centrifugation was performed at 50000 $g_{av.}$ for 17 h. Other details are described in the Materials and methods section.

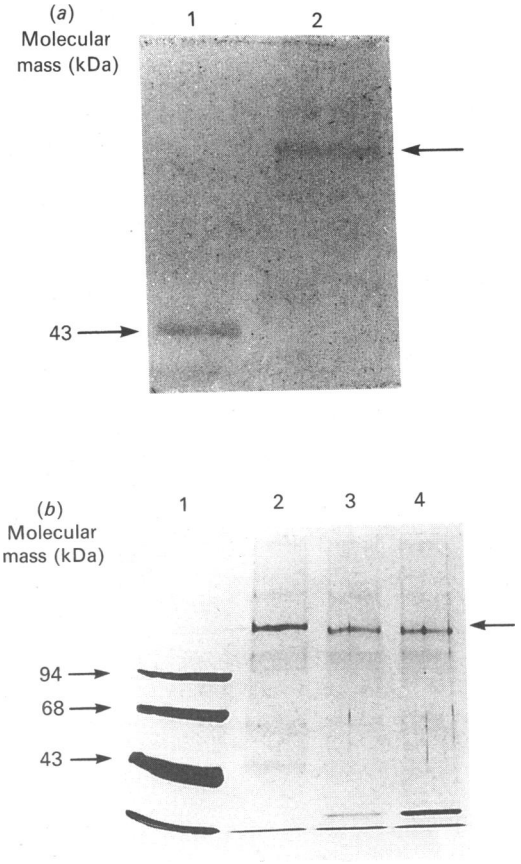

Fig. 3. Glycoprotein nature of brain lipoamidase

SDS/PAGE (7.5% acrylamide) was performed. (*a*) PAS stain. Lane 1, LMW Calibration Kit [only ovalbumin (43 kDa) was stained]; lane 2, brain lipoamidase (2.6 μg of protein). (*b*) Effect of glycopeptidase on lipoamidase as detected by silver stain. Lane 1, LMW markers (94 kDa, phosphorylase *b*; 68 kDa, BSA; 43 kDa, ovalbumin); lane 2, brain lipoamidase (untreated); lane 3, *N*-glycanase-treated enzyme; lane 4, glycopeptidase-F-treated enzyme.

from those of animal tissue enzyme. Unexpectedly, the amino acid composition of acetylcholine receptor from *Electrophorus electricus* is similar to that of brain lipoamidase.

pI, pH and thermostability characteristics

The pI of lipoamidase was determined by isoelectric focusing electrophoresis, and a value of 5.4 was obtained. The values of guinea pig liver and human milk lipoamidases were 5.7 [6] and 4.2 [5] respectively. The pH optimum of brain lipoamidase is a relatively broad range of pH 6.0–7.5. Relatively sharp pH optimums at pH 7.8, 8.0, 7.0 and 7.0 have been obtained for lipoamidases from bacteria [2], guinea pig liver [6], human serum [4] and human milk [5] respectively. Approx. 35% of original activity remained after heat treatment at 60 °C for 15 min. Bakers' yeast [3] and human milk [5] lipoamidase exhibited complete inactivation after heat treatment at 58 °C for 5 min and at 60 °C for 5 min respectively.

Kinetic properties

K_m values for LPAB and reduced-form LPAB were 31

Table 4. Amino acid composition of purified pig brain lipoamidase compared with enzymes from other sources

The amino acid composition was determined as described in the Materials and methods section, and is expressed as mol %. Means of three determinations are shown. Cys and Trp have not yet been determined, because the Cys value was highly variable and Trp was degraded in 6 M-HCl.

		Composition (mol %)			
Amino acid	Source...	Pig brain	Guinea pig liver*	Nicotinic AchR†	Human milk‡
Asx		9.3	9.4	11.5	11.4
Thr		6.3	5.2	5.6	7.5
Ser		6.4	7.1	6.3	5.0
Glx		12.1	12.1	10.3	6.6
Pro		4.7	6.8	5.7	16.8
Gly		8.5	10.3	5.9	9.3
Ala		8.4	9.1	5.8	8.9
Val		7.6	6.3	8.7	8.0
Met		1.6	2.1	2.0	0.5
Ile		5.9	3.7	6.5	4.1
Leu		9.1	10.7	10.6	6.9
Tyr		2.9	0.9	4.2	3.1
Phe		4.4	5.0	5.7	3.9
Lys		6.3	5.5	4.6	4.8
His		1.7	2.9	2.2	1.1
Arg		4.7	4.0	4.2	2.2
Hydrophilicity§		46.8%	46.2%	44.7%	38.6%

* According to our result in ref. [6].
† Calculated from the data of nicotinic acetylcholine receptor (AchR) from *Electrophorus electricus* by Chang *et al.* [24].
‡ J. Oizumi & K. Hayakawa, unpublished work.
§ Defined according to the method of Capaldi & Vanderkooi [25].

and 12 μM respectively (Table 5). Competitive inhibition by lipoic acid and reduced-form lipoic acid (dihydrolipoic acid) was also observed. K_i values for lipoate and dihydrolipoate were 1.8 and 0.40 mM respectively. Competitive inhibition by biotin was not observed up to a concentration of 20 mM. Milk lipoamidase had a K_m value for LPAB of 25 μM, and a K_i value for lipoate of 0.15 mM [5].

Substrate specificity

To elucidate the optimum substrate, we studied the substrate specificity of pig brain lipoamidase with various substrates. These results are also shown in Table 5. Interestingly, reduced-form LPAB was hydrolysed at a rate approx. three times higher than that for LPAB. As shown in Table 5, the K_m value for reduced-form LPAB was the lowest (12 μM) of the compounds tested.

Table 5. Substrate specificity of pig brain lipoamidase

Purified enzyme (5.6 μg/0.1 ml of reaction mixture was used. Means of three determinations ± S.D. are shown. ND, not determined.

Substrate	K_m (μM)	$V_{max.}$ (nmol/min per mg of protein)	$10^{-3} \times k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)
LPAB	31 ± 2.6	8.5 ± 0.6	0.639
Reduced-form LPAB	12 ± 1.8	25.5 ± 1.2	4.96
Lipoamide*	502 ± 46	98.9 ± 13	0.460
Lipoyl-lysine†	333 ± 38	3.4 ± 0.22	0.0024
Aspartyl-lysine†	2857 ± 212	0.3 ± 0.02	0.0003
Lipoylmethyl ester*	62 ± 3.7	166.0 ± 11	6.25
Lipoylethyl ester*	37 ± 1.4	129.2 ± 13	8.16
Biotinmethyl ester‡	2500 ± 230	1.0 ± 0.12	0.0009
Biotinyl-lysine†	ND	> 0.001	ND
Biotinyl-PAB	ND	> 0.001	ND

* Determined by measurement of lipoic acid with a specific u.v. detection at 340 nm [11].
† Determined by using an h.p.l.c.-amino acid analyser.
‡ Determined by measurement of biotin in the same system as in * with detection at u.v. 215 nm.

Table 6. Effects of thiol and serine inhibitors on pig brain lipoamidase

Inhibitor	Concentration (μM)	Residual activity (%)
None	0	100
PMSF	0.5	123
	5.0	69
	50.0	36
DFP	0.5	103
	5.0	70
	50.0	44
PCMB	0.5	117
	5.0	94
	50.0	50
PHMB	0.5	100
	5.0	54
$HgCl_2$	0.5	100
	5.0	53
	50.0	42
2,2'-Dipyridyl disulphide	100	47
	1000	34
DTNB	100	52
	1000	46
$CuCl_2$	100	107
	500	39
$ZnCl_2$	100	109
	500	57

Lipoyl-lysine was considered to be a cellular substrate for this enzyme. X-lysine-type dipeptides were tested, but only aspartyl-lysine ($K_m = 2.9 \pm 0.2$ mM, $V_{max.} = 263 \pm 23$ pmol/min per mg of protein) and glutamyl-lysine ($K_m = 5.0 \pm 0.8$ mM, $V_{max.} = 284 \pm 36$ pmol/min per mg of protein) were hydrolysed. Other X-lysine dipeptides, such as Gly-Lys, Val-Lys, His-Lys and Lys-Lys, were not hydrolysed. These results indicate that lysine residue does not contribute effectively as optimum substrate.

As a measure of specificity, $k_{cat.}/K_m$ values were calculated (Table 5). The lipoyl-ethyl and -methyl esters were found to be the most specific substrates when the $k_{cat.}/K_m$ ratio was used as a measure of specificity. Biotinyl-lysine, biotinyl-PAB and biotinmethyl ester were also studied (Table 5). The results indicated specific recognition of the lipoyl moiety as compared with the biotinyl moiety. ATP (phosphodiester bond) was not hydrolysed. Although the optimum substrate was reduced-form LPAB or lipoyl ethyl-ester, the possibility remains that other substrates might be hydrolysed in brain tissues.

Inhibitor tests

As shown in Table 6, the enzyme exhibited the characteristics of both serine- and thiol-type hydrolases. Other compounds such as diethyl pyrocarbonate (modifier of active His residue) produced slight (20%) inhibition at 1 mM. Angiotensin-converting enzyme inhibitor and *N*-succinyl-L-proline inhibited LPAB hydrolysis by 37% and 16% respectively at 1 mM. Bestatin (aminopeptidase inhibitor), phosphoramidon (putative enkephalinase inhibitor, metalloproteinase inhibitor) and aprotinin (serine protease inhibitor) did not inhibit enzyme activity at 2.5 mM; however, 1,10-phenanthroline (metalloproteinase inhibitor) inhibited it slightly (31%) at 1 mM. These results indicate that the active centre of this enzyme is of a complex type.

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