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Analysis of hydrolytic activity of phospholipase Cα from porcine retina on retinyl ester and phosphatidylcholine using non-denaturing two-dimensional electrophoresis and mass spectrometry

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Abstract

Hydrolysis of retinyl esters and phospholipids is important for visual functions of the animal retina. This study aimed to examine hydrolytic activity of an enzyme with native substrates such as retinyl esters and phospholipids responsible for this function in porcine retina. After cytosolic proteins were extracted from porcine retina, the proteins were separated using non-denaturing two-dimensional electrophoresis (2DE). Some major proteins and phospholipase Cα were identified by matrix-assisted laser desorption ionisation–time of flight–mass spectrometry (MALDI–TOF–MS) or electrospray ionisation–tandem mass spectrometry (ESI–MS/MS). The phospholipase Cα showed hydrolytic activities with not only α-naphtyl acetate but also with retinyl palmitate and phosphatidylcholine when effects of different substrates were investigated using enzyme activity staining on 2DE or MALDI–TOF–MS. Results indicated that hydrolytic activity of the enzyme with non-native and native substrates could be examined using a combination of non-denaturing 2DE and MALDI–TOF–MS.

Keywords: Retinyl palmitate; Phosphatidylcholine; Eserine; Phospholipase Cδ; Phospholipase Cα

1. Introduction

Enzymatic hydrolysis of retinyl ester and phospholipids play important roles in visual functions in the retina [1,2]. Especially, hydrolytic reaction from retinyl ester to retinol is necessary for vitamin A metabolism and transport in the retina [2]. In order to analyze hydrolytic activity with retinyl ester or other native phospholipds, it might be necessary that enzymes possessing hydrolytic activity are separated in non-denaturing conditions, and the native molecules such as retinyl ester and phosphlipids are hydrolyzed by the separated enzymes. We have reported that analysis of activity and mass spectrometric identification of enzymes using non-denaturing two-dimensional electrophoresis (2DE) after cytoplasmic proteins were extracted from mouse liver and bovine retina [3–5]. However, for analysis of hydrolytic activity catalyzed by enzymes, non-native chemicals such as α-naphtyl acetate are used. So, there is no information whether the separated enzymes catalyze hydrolysis of the native molecules such as retinyl ester and phosphlipids. Since it has reported that enzymatic hydrolysis of lipids such as phosphatidylcholine is investigated using matrix-assisted laser desorption ionisation–time of flight–mass spectrometry (MALDI–TOF–MS) [6], hydrolytic activity with phosphatidylcholine and retinyl palmitate can be investigated using MALDI–TOF–MS after cytosolic enzymes from the retina are separated using non-denaturing 2DE.

The present study showed that proteins extracted from porcine retina were separated by non-denaturing 2DE, and were identified using MALDI–TOF–MS or electrospray ionisation–tandem mass spectrometry (ESI–MS/MS). Furthermore, phospholipase Cα possessed hydrolytic activities with not only α-naphtyl acetate but also with retinyl palmitate and phosphatidylcholine after the separation by non-denaturing 2DE.

Abbreviations: MALDI–TOF–MS, matrix-assisted laser desorption ionization–time of flight–mass spectrometry; ESI–MS/MS, electrospray ionization–tandem mass spectrometry; PMF, peptide mass fingerprinting; Eserine, physostigmine

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Here, we indicated that hydrolytic activity of the enzyme with non-native or native substrates could be examined using a combination of non-denaturing 2DE and MALDI–TOF–MS.

2. Materials and methods

2.1. Sample preparation and 2DE

Reagents and chemicals were obtained as follows: acrylamide from Daiichi pure Chemical Co. Ltd. (Tokyo), ampholine pH 3.5–10 and pH 3.5–5 from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Bovine trypsin (sequence grade) from Roche (Mannheim). All other reagents were purchased from Sigma–Aldrich (Milwaukee) and Wako Pure Chemicals (Tokyo). Porcine retina was homogenized using a homogenizer (As one, Osaka) in 100 mM Tris–HCl buffer (pH 7.2), and the homogenate was divided into two fractions (cytosol and precipitate) by centrifugation for 5 min at 10,000 × *g*. Sucrose was added to the cytosol fraction. Protein concentration in each fraction was estimated from ultraviolet light absorption, on the assumption the absorbance at 280 nm of a 1 mg/ml solution was 1.0.

Proteins in fractions (100–500 μg) were subjected to microscale non-denaturing 2DE as previously reported [3,4]. IEF was done on rod gels (length, 35 mm; i.d., 1.3 mm.) containing 4% acrylamide (0.2% Bis), 2% ampholine pH 3.5–10, 1% ampholine pH 3.5–5, 0.05% ammonium persulfate, 0.029% TEMED. The electrode solutions were 0.04 M NaOH (cathode) and 0.01 M H_3PO_4 (anode). Following IEF, the IEF gel was placed on top of the second-dimension slab gel, which was then run on a 4–17% acrylamide linear gradient (0.2–0.85% Bis gradient) or 8–17% acrylamide linear gradient (0.4–0.85% Bis gradient) containing 0.1% sodium dodecyl sulfate (SDS). After equilibration, size separation was performed in the electrode buffer 0.05 M Tris and 0.38 M glycine (pH 8.3) or the electrode buffer containing 0.1% SDS. Gels were stained with 0.1% CBB, 7% (v/v) acetic acid and 50% (v/v) methanol for 15 min and were destained in 20% (v/v) methanol and 7% (v/v) acetic acid for 2 h.

2.2. Detection of hydrolytic activity with α-naphtyl acetate, retinyl palmitate and phosphatidylcholine

Once the obtained proteins in the cytosol were separated by non-denaturing 2DE, hydrolytic activity with α-naphtyl acetate was analyzed using a previously described method [3,4]. The 2DE gel was incubated in 10 ml 0.2M phosphate buffer (pH 7.1) containing 0.2 ml 1% α-naphtyl acetate and 4 mg Fast-red TR salt. In order to analyze the positions of hydrolytic activity on non-denaturing 2DE or non-denaturing IEF/SDS-PAGE, CBB staining was performed following detection of hydrolytic activity on the gel. To investigate changes in hydrolytic activity with α-naphtyl acetate in the presence of inhibitors, the 2DE gel was incubated in a solution containing 1.0 mM physostigmine (eserine). Following eserine treatment, the 2DE gel was incubated in the solution mixture to analyze activity. For analysis of hydrolysis of retinyl palmitate and phosphatidylcholine by MALDI–TOF–MS, several regions of interests (81 mm^2) of esterase activity were excised from the non-denaturing 2DE gel, and areas were divided into 9.0 mm^2 pieces. Proteins in each piece were incubated with 50 μl of 10 mg/ml retinyl palmitate or 50 μl of 20 mg/ml phosphatidylcholine for 30 min at 37 °C. After the incubation, all the liquid was collected. Then, 1 μl of the liquid was mixed with 1 μl of a solution containing saturated α-cyano 4-hydroxycinnamic acid, 0.1% trifluoroacetic acid and 60% acetonitrile. Mass analysis was done using MALDI–TOF–MS (Voyager DE PRO; Applied Biosystems, Framingham) in a positive ion reflector mode. To calibrate mass in the spectrum, angiotensin II (*m/z* 1047.19) was used as external standard.

2.3. Protein identification by ESI–MS/MS and MALDI–TOF–MS

To identify proteins, protein spots were excised from the gel, and digested in gel using trypsin, as a described previously [7–11]. The polypeptides were analyzed by nano ESI mass spectrometry or MALDI–TOF–MS. For analysis by ESI mass spectrometry, the capillary was attached to a nanospray ion source on the electrospray quadrupole time-of-flight instrument (QSTAR) from Applied Biosystems (Framingham, MA, USA). After MS/MS spectra were obtained, peptide sequences were identified using Bioanalyst™ (Applied Biosystems, Foster city, CA, USA). Peptide sequences were then aligned using the PepSea™ server (Toronto, Canada) against the NCBInr. database (2004-10-19) to identify proteins. For analysis using MALDI–TOF–MS, the obtained polypeptides were mixed with α-cyano 4-hydroxycinnamic acid. Mass analysis was performed using MALDI–TOF–MS, operating in the positive ion reflector mode. To calibrate mass in the spectrum, some peaks of digested trypsin (*m/z* 2163.057, 2273.160, 906.505 and 1153.574) were used as internal standards. Spectra were analyzed using mass values for monoisotropic peaks that were used for searches (Mascot, http://www.matrixscience.com/) against the MSDB. database. The database was searched using the following parameters: taxonomy (other mammalia), trypsin digest (one missed cleavage allowed), cysteine modified by carbamidomethylation, mass tolerance 80 ppm using internal calibration, and oxidation of methionines. Criteria used to accept identification included the extent of sequence coverage, the number of matched peptides, and the probabilistic score (probability of a random match was required to be <0.05).

3. Results and discussion

3.1. Activity analysis and identification of phospholipase Cα after separation by 2DE

Non-denaturing 2DE patterns of proteins in the cytosol fraction (a) and detection of hydrolytic activity with α-naphtyl acetate (b, c) are shown in Fig. 1. Several spots exhibiting hydrolytic activity with α-naphtyl acetate were detected at p*I* 5.8–6.2/150 kDa (arrowhead) and p*I* 5.8–6.2/85 kDa (arrow), were observed in the 2DE pattern (Fig. 1b). Activities were

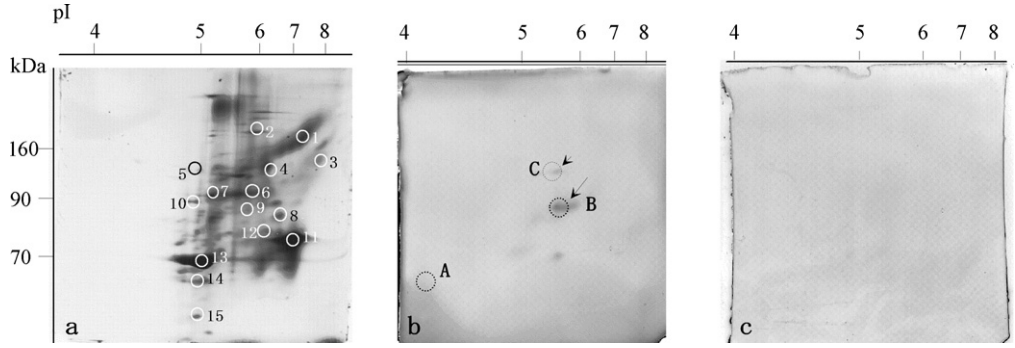

Fig. 1. CBB staining of cytosolic proteins (a), hydrolytic activity with α-naphtyl acetate (b) and hydrolytic activity on α-naphtyl acetate in the presence of 1.0 mM eserine (c) after proteins were separated using non-denaturing 2DE. Spots in non-denaturing 2DE are labeled 1–15 to indicate spots analyzed by MALDI–TOF–MS or ESI–MS/MS (a). Activity spots of hydrolytic activity with α-naphtyl acetate (arrowhead, arrow in b): Dotted circles (A, B and C) on 2DE indicate excised gels with changes of substrates.

inhibited by 1.0 mM eserine (Fig. 1c). Eight spots on non-denaturing 2DE were tentatively identified by peptide mass finger-printing using MALDI–TOF–MS, and seven spots on non-denaturing 2DE were identified by peptide sequence tag using ESI–MS/MS (Table 1). Alpha enolase (spot 4), ceruloplasmin (spot 7), phosholipase Cα (spot 9), crystalin β(spot 12) and annexin V (spot 15) were identified in other mammalian (bovine, mouse, human and rabbit) homologues because the amount of information on porcine proteins (*Sus scrofa* 6058 entries in NCBInr. 2005.0106) is small. A spot at p*I* 6.0/85 kDa exhibiting hydrolytic activity on α-naphtyl acetate was identified as phosholipase Cα by ESI–MS/MS (Fig. 1a and Table 2). It has been reported that phosholipase Cα is enzymatic processing fragment of phosphoinositide-specific phospholipase Cδ isoform [12]. After the enzymatic cleavage of the phospholipase Cδ, α-naphtyl acetate might be hydrolyzed by this enzyme.

Table 1
Cytosol proteins extracted from pig retina (Fig. 1a), after the proteins were separated by nondeanaturing 2DE

Spot number	Protein identity	Swiss-Port name or Accession number	Peptide matches	Sequence coverage (%)	Average error between obtained masses and matches peptides (ppm)	Theoretical p*I* (kDa)	Estimated p*I* (kDa) on non-denaturing 2DE	Organism[a]	Methods
1	IgG heavy chain precursor	AAD38418	6	18	12	6.8/53	7.5/160	P	PMF
2	Glutamate-ammonia ligase	S41452	10	28	21	6.3/43	6.0/180	P	PMF
3	Glutathione transferase	S13780	6	42	43	7.3/23	8.0/150	P	PMF
4	Alpha enolase	ENOA BOVIN	9	28	7	6.4/47	6.2/140	B	PMF
5	Interphotoreceptor retinoid binding protein	Q28991	7	26	47	4.8/45	5.1/140	P	PMF
6	Transferrin	S01384	24	40	12	6.9/79	6/90	P	PMF
7	Ceruloplasmin	gi|6978695				5.4/122	5.1/90	R	MS/MS
8	Triosephospate isomerase	gi|2501308				7.5/7	6.8/85	P	MS/MS
9	Phospholipase Cα	gi|303618				6.6/57	6.0/85	H	MS/MS
10	Malate dehydrogenase	gi|47523114				6.5/37	5.2/87	P	MS/MS
11	Hemoglobin	1QPWB	8	80	10	6.8/16	7.5/75	P	PMF
12	Crystallin β B2	gi|6681035				7.0/23	6.1/80	M	MS/MS
13	Albumin	ABPGS	9	21	21	5.9/71	5.0/70	P	PMF
14	Peroxiredoxin 2	gi|1717797				4.5/14	4.9/68	P	MS/MS
15	Annexin V	gi|284588				4.8/36	5.0/55	B	MS/MS

P: procine, B: bovine, R: rabbit, M: mouse, H: human; PMF: peptide mass fingerprinting.
[a] Mammalian homologue.

Table 2
Identification analysis of spot 9 (Fig. 1a) using ESI-MS/MS, after the proteins were separated by nondenaturing 2DE

Protein (species)	Accession number	Start–end residue	Sequence	Measured mass (Da)
Phospholipase C-alpha (human)	gi1303618	21–38	LAAASDVLELTDDNFESR	1638.44
		95–104	YGVSGYPTLK	1082.49
		259–271	DLLIAYYDVDYEK	1618.48
		449–460	GFPTIYFSPANK	1340.44
		472–482	ELSDFISYLQR	1369.47

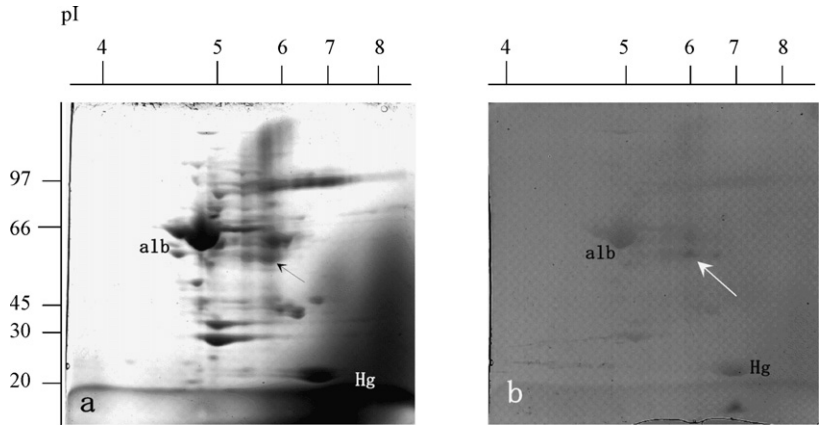

Fig. 2. CBB staining of cytosolic proteins (a), hydrolytic activity on α-naphtyl acetate (b) after proteins were separated using non-denaturing IEF and SDS-PAGE. A spot (arrow) at p*I* 5.8–6.2/60 kDa possessing hydrolytic activity with α-naphtyl acetate in the presence of SDS (b). alb, albumin: Hg, hemoglobin.

The molecular mass of phosholipase Cα is 60–65 kDa, however, the molecular mass of the spot was 85 kDa when proteins were separated under non-denaturing condition (Fig. 1a and Table 1). When proteins were separated by SDS-PAGE after the separation using non-denaturing IEF (Fig. 2), the spot at p*I* 5.8–6.2/60 kDa retained hydrolytic activity with α-naphtyl acetate (arrow in Fig. 2b). These results indicate that the spot at p*I* 5.8–6.2/85 kDa on the non-denaturing 2DE is thought to slowly migrate in the electrode buffer (pH 8.3) because the protein is not enough negative charge, comparing to other acidic proteins such as albumin (p*I* 5.0/70 kDa). Further, since it is said that phosholipase C interacts other protein such as myelin basic protein [13], the spot at p*I* 5.8–6.2/85 kDa on the non-denaturing 2DE is thought to possess phosholipase Cα and other molecules. Therefore, it is possible that interaction between phosholipase Cα and other proteins has effect on the migration of the spot of phosholipase Cα under non-denaturing condition.

3.2. Analysis of hydrolytic activity with retinyl esters and phospholipids after separation by non-denaturing 2DE

Fig. 3 shows MALDI–TOF–MS spectra of phosphatidylcholine (a) and its digested fragments (b) after phosphatidylcholine was incubated with excised gels containing proteins at positions of spots A and B (Fig. 1b) before staining. As shown in Fig. 3b, peaks at *m/z* 761 [M + H$^+$] and 783 [M + Na$^+$] were not detected, whereas relative intensity of peaks at *m/z* 496 and 524 [M + H$^+$] (arrows in Fig. 3b) clearly increased when phosphatidylcholine was incubated with excised gels containing proteins at positions of spots B (Fig. 1b), in comparison with Fig. 3a. It has been reported that the same MS peaks at *m/z* 496 and 524 [M + H$^+$] of lysophosphatidylcholine were obtained when pure phosphatidylcholine was digested by cholesterol esterase [6]. So, these peaks could represent lysophosphatidylcholine digested by phosholipase Cα in the non-denaturing 2DE gel. Similar hydrolytic activities were obtained when phosphatidylcholine was incubated with excised gels containing proteins at positions of spots C in Fig. 1b (data not shown).

In Fig. 4a, the peak of retinyl palmitate at *m/z* 525 [M + H$^+$] (arrowhead in Fig. 4a) was obtained when retinyl palmitate was

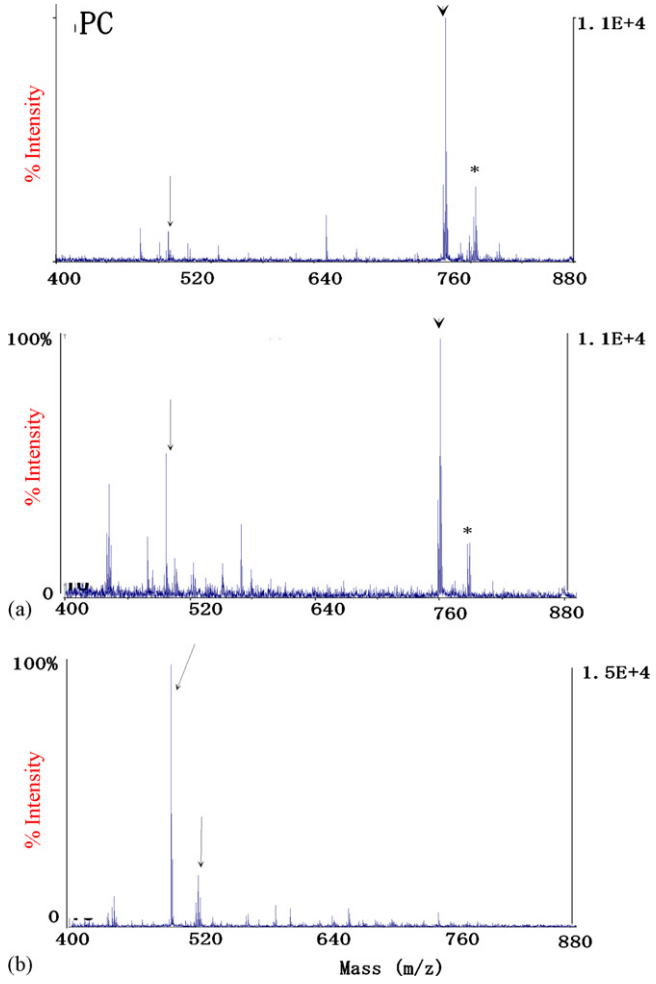

Fig. 3. MALDI–TOF–MS spectra of phosphatidylcholine after phosphatidylcholine (purchased PC) was incubated with excised gels containing proteins at position of spots A (a) and B (b) in Fig. 1b before staining. Peaks of phosphatidylcholine are shown at *m/z* 761 [M + H$^+$] and 783 [M + Na$^+$] (arrowhead and (*), respectively). Peaks at *m/z* 496 and 524 [M + H$^+$] (arrows) represents lysophosphatidylcholine.

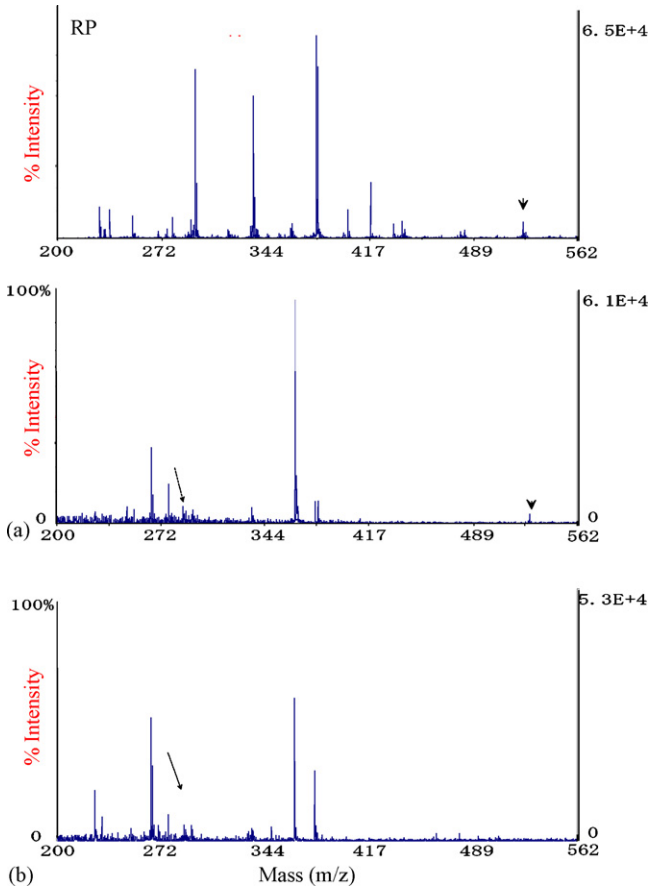

Fig. 4. MALDI–TOF–MS spectra of retinyl palmitate after retinyl palmitate (purchased RP) was incubated with excised gels containing proteins at positions of spots A (a) and B (b) in Fig. 1b. Peaks of retinyl palmitate (arrowhead) and retinol (arrow) are indicated at 525 [M + H$^+$] and 287.5 [M + H$^+$], respectively.

incubated with the excised gel containing proteins at the position of spot B (Fig. 1b) before staining. As shown in Fig. 4b, the peak at *m/z* 525 [M + H$^+$] was not detected when retinyl palmitate was incubated with the excised gel containing proteins at positions of spot B (Fig. 4b) before staining. On the other hand, relative intensity of the peak of retinol at *m/z* 287.5 [M + H$^+$] (arrow in Fig. 4b) clearly increased when retinyl palmitate was incubated with the excised gel containing proteins at the positions of spot B (Fig. 4b) before staining, compared with Fig. 4a. From these results, we concluded that retinyl palmitate could be hydrolyzed to retinol by phosholipase Cα, and hydrolytic activity was obtained. Similar hydrolytic activities were obtained when retinyl palmitate was incubated with excised gels containing proteins at positions of spot C in Fig. 1b (data not shown). Since it is reported that phosphatidylinositol-4, 5-bisphosphate phospholipase C is in bovine retina [14], it might be also important roles in porcine retina. After enzymatic cleavage of phospholipase C, the enzyme is thought to catalyze non-specific hydrolysis of α-naphtyl acetate, phosphatidylcholine and retinyl palmitate. Sanghani et al. [15] reported that some non-specific esterases possess hydrolytic activity for retinyl ester in rat liver. Retinyl ester can be hydrolyzed by the fragment of phospholipase C in the retina, and this hydrolysis may be important for vitamin A metabolism and transport in the retina.

4. Conclusions

Present study indicates that hydrolytic activity of the enzyme with non-native or native substrates could be examined using a combination of non-denaturing 2DE and MALDI–TOF–MS. And, this method can be applied to screening substrate changes catalyzed by other enzymes.

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