Journal of Pharmaceutical and Biomedical Analysis 71 (2012) 179–182

Contents lists available at SciVerse ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

Enzymolysis of high density lipoprotein with a combination of membrane-immobilized esterase and trypsin

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ARTICLE INFO

Article history:
Received 11 April 2012
Received in revised form 24 July 2012
Accepted 29 July 2012
Available online 4 August 2012

Keywords:
HDL
Carboxylesterase
Trypsin
Electrophoresis
MALDI-TOF MS
6,9-Diamino-2-ethoxyacridine

ABSTRACT

Apolipoprotein A-1 (apo A-1), a major component of high density lipoprotein (HDL), was efficiently digested by membrane-immobilized trypsin after HDL was treated with membrane-immobilized esterase. Compared to treatment with membrane-immobilized trypsin alone, the relative amounts of apo A-1 polypeptides, *m*/*z* 1723.78 and *m*/*z* 1568.82, increased by 2.7- and 3.9-fold, respectively, when HDL was treated with membrane-immobilized esterase and trypsin. Furthermore, the efficient digestion of apo A-1 by trypsin was inhibited when HDL was treated with membrane-immobilized esterase in the presence of an esterase inhibitor, 6,9-diamino-2-ethoxyacridine (acrinol). The data indicate that the lipid components of lipoproteins are released by membrane-immobilized esterase. This method can be used to investigate the structure and function of other apolipoproteins.

1. Introduction

Lipoproteins contain different kinds of lipids. For example, high density lipoprotein (HDL) is mainly composed of apolipoproteins such as apolipoprotein A-1 (apo A-1) [1]. The conformation and lipid-binding site of apo A-1 have been examined [2]. Lipid-free apo A-1 is a more accessible substrate for matrix metalloproteinase than lipid-bound apo A-1 [3]. To investigate the structure and function of HDL apolipoprotein components, it is necessary to remove lipids from the lipoproteins without destroying protein structure and function. We previously reported that phosphatidylcholine, a type of lipid, is hydrolyzed by a membrane-immobilized esterase identified as carboxylesterase 1 [4–6]. Furthermore, esterase treatment of the lipids exposes apolipoproteins, which can then be digested by trypsin. Proteins can be rapidly and efficiently digested by proteolytic enzymes such as trypsin and chymotrypsin immobilized on membranes [7–9]. Therefore, if enzymes such as esterase and trypsin are immobilized on membranes, enzymolysis of macromolecules such as HDL can be efficiently performed on the surface of the membrane. On the other hand, it has been reported that esterase activity is inhibited by an inhibitor, 6,9-diamino-2-ethoxyacridine (acrinol) [10]. Thus, the efficient digestion of apo A-1 by trypsin can be inhibited, even after treatment of HDL by membrane-immobilized esterase in the presence of acrinol, because the HDL lipids are not released.

We describe the efficient digestion of the apo A-1 component of HDL by membrane-immobilized trypsin following lipid removal by membrane-immobilized esterase after production of membrane-immobilized enzymes. Furthermore, digestion of apo A-1 by trypsin was inhibited when HDL was treated with the membrane-immobilized esterase in the presence of acrinol. This method can also be used for the investigation of structure and function of other apolipoproteins.

2. Materials and methods

2.1. Materials and preparation of membrane-immobilized enzymes

Acronym of polyvinylidene difluoride (PVDF) membrane was purchased from Millipore (Bedford, MA, USA). Acrylamide and carrier ampholyte (Pharmalyte, pH 3–10) were purchased from Kishida Chemicals (Osaka, Japan) and GE healthcare (Uppsala, Sweden), respectively. HDL was purchased from Meridian Life Science Inc. (Cincinnati, OH, USA). Adrenocorticotropic hormone (ACTH), Ponceau S, α-cyano-4-hydroxycinnamic acid and bovine pancreatic trypsin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Bovine trypsin (sequence grade) was purchased from

Abbreviations: 2-DE, two-dimensional electrophoresis; PVDF, polyvinylidene difluoride; MALDI-TOF MS, matrix assisted laser desorption/ionization time of flight mass spectrometry; HDL, high density lipoprotein; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Fast Red TR salt, 4-chloro-2-methylbenzene diazonium salt.

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http://dx.doi.org/10.1016/j.jpba.2012.07.032

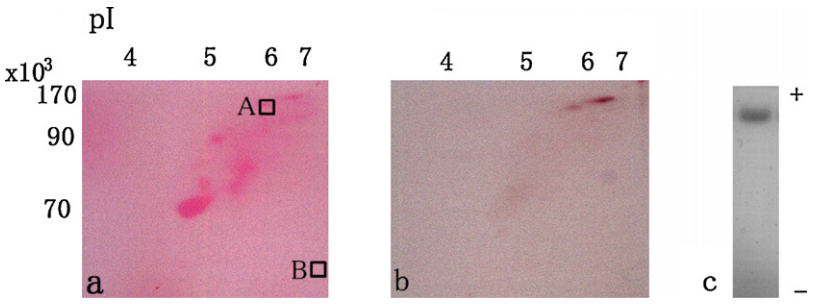

Fig. 1. Ponceau S staining in 0.1 M acetate buffer solution (pH 5.1) (a) followed by esterase activity staining, (b) after separation of cytosolic proteins from mouse liver by non-denaturing 2-DE and membrane blotting. Ponceau S staining in 0.1 M Tris–HCl buffer solution (pH 7.0) after separation of bovine pancreas trypsin by non-denaturing electrophoresis, and membrane blotting (c). Excised spot A possesses esterase activity and spot B dose not possess esterase activity. Proteins are migrated toward an anode in the 2-DE (a and b), whereas trypsin is migrated toward a cathode of non-denaturing electrophoresis (c).

Roche (Mannheim, Germany). All other reagents were purchased from Wako Pure Chemicals (Osaka, Japan) or Nacalai Tesque (Osaka, Japan).

Mouse livers (Swiss Webster) were purchased from Rockland Inc. (Gilbertsville, PA, USA) and homogenized in Tris–HCl buffer (pH 7.2; 0.1 M). The homogenate was centrifuged for 5 min at 10,000 × *g* to obtain the cytosolic fraction. Sucrose was added to the liver cytosolic fraction at a concentration of 40% (w/v). Proteins in the cytosolic fraction (100–300 μg) were subjected to microscale non-denaturing two-dimensional electrophoresis (2-DE) by a method previously reported [11]. To immobilize proteins on PVDF membranes, proteins were transferred to PVDF membranes by a semi-dry type transblotting apparatus using electrode buffers of Tris (0.05 M) – glycine (pH 8.3; 0.38 M) with a constant current of 23 mA per gel for 4 h [12]. To detect proteins on the PVDF membrane, membranes were soaked in 0.5% Ponceau S in 10 mL of acetate buffer (pH 5.1; 0.1 M). For preparation of membrane-immobilized trypsin, sucrose was added to bovine pancreatic trypsin to a concentration of 40% (w/v). Trypsin (20 μg) was then subjected to non-denaturing electrophoresis on a 5% acrylamide (0.25% Bis) gel containing Tris–HCl (pH 6.9; 0.13 M). The electrode buffer consisted of Tris (0.05 M) – glycine (pH 8.3; 0.38 M). Because trypsin is a type of basic proteins, it migrates toward a cathode. When the top and bottom of the gel were connected to anode and cathode, respectively, trypsin was migrated from the top to the bottom of the gel. The protein was then transferred to a PVDF membrane on the cathode using a semi-dry-type blotting apparatus to immobilize it. The immobilized enzyme was detected by staining with 0.001% Ponceau S in 10 mL of Tris–HCl buffer (pH 7.0; 50 mM) and destained with H_2O. To analyze esterase activity, membranes were incubated in 10 mL of phosphate buffer (pH 7.1; 0.2 M), containing 0.2 mL of 1% α-naphthylacetate and 4 mg of 4-chloro-2-methylbenzene diazonium salt (Fast Red TR salt). Regions of membrane containing esterase or trypsin enzymes were excised and used to digest HDL samples.

2.2. Digestion by membrane-immobilized trypsin and MALDI-TOF MS analysis

HDL (25 μL of 1.5 μg/μL solution) was first incubated with either membrane-immobilized esterase (Fig. 1a, spot A) or membrane without esterase enzyme (Fig. 1a, spot B) for 4 h at 37 °C. Following this, HDL digestion products were incubated with membrane-immobilized trypsin for 0–6 h at 37 °C. To inhibit esterase activity on the membrane, HDL was incubated with membrane-immobilized esterase (Fig. 1a, spot A) in the absence or presence of 0.1 mM acrinol, or without membrane-immobilized esterase (Fig. 1a, spot B) in the absence of 0.1 mM acrinol for 4 h at 37 °C. Following these treatments, HDL digestion products were incubated with membrane-immobilized trypsin for 6 h at 37 °C. The obtained polypeptides were collected and analyzed by MALDI-TOF MS. One microlitre of the liquid containing polypeptides was mixed with 1 μL of a solution containing saturated α-cyano 4-hydroxycinnamic acid, 0.1% trifluoroacetic acid and 70% acetonitrile. The sample mixture and matrix was placed on a stainless steel sample plate (sample plate for Voyager DE PRO; Applied Biosystems, Framingham, MA, USA) and dried. Mass analysis was performed using MALDI-TOF MS (Voyager DE PRO; Applied Biosystems) in positive ion reflector or linear mode. Monoisotopic or average peak of ACTH 18-39 (*m*/*z* 2465.1989) or (*m*/*z* 2466.72)

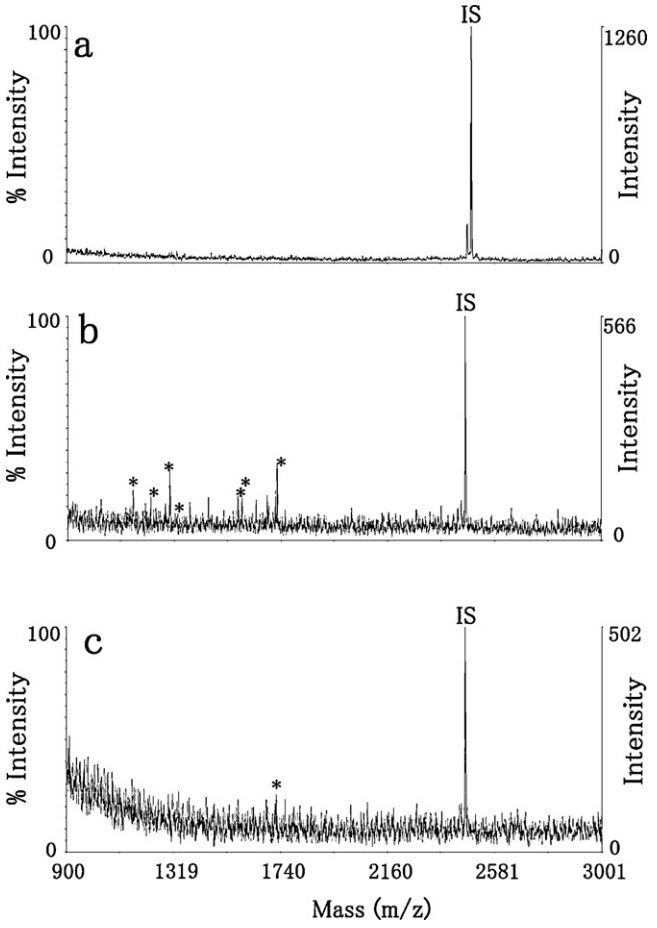

Fig. 2. MALDI-TOF MS spectra of HDL polypeptides following incubation with membrane-immobilized trypsin for 0 h (a) or 6 h (b and c) at 37 °C following incubation with (b, spot A in Fig. 1a) or without (c, spot B in Fig. 1a) membrane-immobilized esterase for 4 h at 37 °C. * Polypeptides from *homo sapiens* apo A-1. IS indicates internal standard of ACTH 18-39 (*m*/*z* 2465.1989).