Analytical Biochemistry 383 (2008) 289–295

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Development of an indirect method for measuring porcine pancreatic lipase in human duodenal fluid

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ARTICLE INFO

Article history:
Received 3 July 2008
Available online 10 September 2008

Keywords:
Enzyme replacement therapy
Lipase
Lipase assay
ELISA
Pancreatic insufficiency

ABSTRACT

Patients with exocrine pancreatic insufficiency are usually treated with porcine pancreatic enzymes but the bioavailability of these enzymes in the gut remains a matter of discussion. In order to determine the duodenal availability of porcine pancreatic lipase (PPL) present in pancreatic extracts (PE) taken orally, we developed a method for quantifying PPL in samples containing both PPL and human pancreatic lipase (HPL). Total pancreatic lipase activity measurements using the pH-stat technique and tributyrin as substrate were combined with an HPL-specific ELISA. Based on the known specific activity of the purified HPL, its activity was deduced from the ELISA measurements, and the PPL activity was obtained by subtracting the HPL activity from the total pancreatic lipase activity. This assay was established and validated using various samples containing pure PPL and recombinant HPL or PE, mixed or not with human duodenal juice. Samples collected in vivo from patients treated with PE were also tested. It was found that PPL did not affect the HPL ELISA, and the indirect PPL assay gave a measurement accuracy of 6.6% with the samples containing pure PPL and 10% with those containing PE. This assay was also used successfully to discriminate between PPL and the endogenous HPL present in the duodenal contents of patients with severe pancreatic insufficiency treated with PE. This method might provide a useful means of assessing the availability of PEs at their site of action, in the absence of a PPL-specific ELISA.

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Chronic pancreatitis is characterized by exocrine pancreatic insufficiency [1] and to prevent the occurrence of malabsorption, patients with this condition are currently treated by prescribing enzyme replacement therapy [2]. The main drugs on the market at present are based on porcine pancreatic extracts, most of which come in the form of pH-sensitive enteric-coated pancreatin microspheres to prevent the enzymes from being inactivated by gastric acid. The use of these drugs of animal origin (which were classified in the United States simply as nutritional supplements until 2008) has been greatly restricted, however, because of the risk of viral or any other biological transmission to humans. New clinical investigations on the safety and the bioavailability of these enzymes at the site of action have been requested by the Food and Drug Administration before the marketing of these products as drugs can be authorized in the United States (see FDA *Guidance for Industry: Exocrine Pancreatic Insufficiency Drug Products*). At the same time, several recombinant enzymes, mainly lipases, are currently under investigation as candidates for replacing porcine pancreatic enzymes and improving the efficacy of pancreatic insufficiency treatments [3]. In order to assess the bioavailability of these various enzymes in the gastrointestinal (GI) tract, specific assays are required, and these assays have to be capable of discriminating between substitute enzymes and endogenous human enzymes.

Specific lipase assays have by now become available for determining the human digestive lipase content of samples collected from the GI tract. The respective levels of human gastric (HGL) and human pancreatic (HPL) lipase can be estimated, for instance, by performing discriminative activity measurements [4] or specific immunological tests [4–6]. A double-sandwich ELISA involving the use of polyclonal and monoclonal anti-HPL antibodies, with which HPL can be measured in biological fluids, has been developed by De Caro et al. [5] and further validated by Grandval et al. [7]. These assays have been used to quantify the rates of HGL and HPL secretion in healthy volunteers [4–6] and patients with chronic pancreatitis [8]. There exist, however, no comparable immunological methods for specifically quantifying porcine pancreatic lipase (PPL) in duodenal samples from patients with exocrine pancreatic insufficiency treated with porcine pancreatic extracts. In addition, with the cur-

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0003-2697/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2008.08.036

rently used pancreatic lipase assays, it is not possible to discriminate between HPL and PPL, since these enzymes hydrolyze the same triglyceride substrates and show similar specific activities.

The aim of this study was therefore to develop an indirect method of quantifying active PPL in the digestive tract, based on a combination of total lipase activity measurements and the previously developed ELISA for specifically measuring HPL. HPL activity can be deduced from the ELISA measurements, and PPL activity can be determined by subtracting the HPL activity from the total activity. This indirect method of measurement can be used to investigate the duodenal availability of the PPL from orally ingested pancreatic extracts and might provide informative data that could be analyzed together with the variable levels of steatorrhea observed during enzyme replacement therapy.

Materials and methods

Production and purification of recombinant HPL (rHPL)

rHPL was produced from transformants of the yeast *Pichia pastoris* using the commercially available constitutive expression system from Invitrogen, as described by Belle et al. [9]. The recombinant protein obtained was purified by performing cation-exchange chromatography on an SP–Sepharose column [9]. The production of rHPL was checked by performing amino acid analysis, MALDI-TOF mass spectrometry using Voyager DE-RP equipment (Perspective Biosystems, Inc.), and N-terminal sequence analysis using a Procise 494 sequencer to ensure that no proteolytic degradation had taken place and that the processing of the N-terminal signal peptide was correctly performed, resulting in the KEV N-terminal sequence characteristic of the mature protein. The protein mass concentration in a quality control sample of purified rHPL was determined by performing quantitative amino acid analysis.

Purification of native porcine pancreatic lipase

The native PPL was purified as described by Rovery et al. [10].

Production and purification of anti-HPL polyclonal antibodies

After a rabbit was immunized with native antigen (4 mg of native HPL purified from human pancreatic juice [11]), the serum was collected and then half-diluted with PBS buffer (phosphate-buffered saline: potassium phosphate 10 mM, NaCl 150 mM, pH 7.4). The proteins were precipitated with saturated ammonium sulfate (final concentration 50%). After a 30-min centrifugation at 10,000 rpm, the pellets containing the immunoglobulins were suspended in the PBS buffer and dialyzed against the same buffer for one night at 4 °C. The dialyzed solution was then incubated for 4 h at 4 °C on a column containing Affi-Gel 10 (Bio-Rad) coupled to 10 mg of HPL. The antibodies were eluted using an HCl–glycine buffer 0.2 M, pH 2.3. Each fraction obtained was immediately neutralized using a Tris–HCl buffer 2 M, at pH 9 (150 μl/ml). The absorption of the eluted proteins was measured at 280 nm. The purified antibodies were dialyzed against a PBS buffer for one night at 4 °C before being concentrated and aliquotted. The aliquots were stored at −20 °C.

Production and purification of anti-HPL monoclonal antibody (mAb)

The anti-HPL mAb 146-40 was generated by fusion of mouse spleen cells immunized with native HPL purified from human pancreatic juice [11] and mouse myeloma (P3X63Ag8,653). The hybrid cells (hybridomas) were cloned using limited dilution methods. The hybridoma, which produces mAb, was injected into the peritoneum of a mouse. The resulting ascitic liquid contained the mAb, which was purified by immunoaffinity chromatography on a column of protein A–Sepharose CL-4B (Pharmacia). The antibodies obtained were biotinylated using the procedure described by Guesdon et al. [12]. The anti-HPL mAb 146-40 belongs to the IgG1 immunoglobulin subclass. The antigenic determinant of this antibody is located in the N-terminal domain of the HPL [13,14].

Immunoblotting

The immunoreactivity of the antibodies was determined using immunoblotting procedures. The HPL and the PPL were separated by denaturing 12% polyacrylamide gel electrophoresis [15] before being transferred onto a nitrocellulose membrane. This membrane was saturated with a PBS–Regilait 3% buffer (w/v) for 1 h and washed with the same buffer. The membrane was then incubated with the antibodies in PBS buffer, Regilait 3% (w/v), and Tween 20 (0.05% v/v). The secondary antibody, which was a rabbit anti-IgG antibody from mouse coupled to the alkaline phosphatase diluted to 1/2000, was incubated for 1 h with the membrane in the PBS buffer, Regilait 3% (w/v), Tween 20 (0.05% v/v). The immunoreactivity was revealed using a 5 mM substrate solution of bromo-4-chloro-3-indoyl phosphate dissolved in 0.1 M Tris–HCl, pH 9.5, containing 0.1 M NaCl, 1 mM $MgC1_2$, and 3.5 mM tetrazolium blue.

Preparation of samples containing both purified PPL and rHPL

Three series of solutions were prepared in PBS buffer containing a fixed mass concentration of PPL (60 μg/ml) and a variable amount of rHPL (0, 5.93, 11.80, 23.68, or 47.40 μg/ml). For each series, a control series containing only rHPL at the same concentration was also produced. The PPL concentration chosen was expected to be in the PPL concentration range in the duodenal contents during clinical experiments, based on the total amounts of pancreatic extracts administered and the test meal volume.

Preparation of samples containing both porcine pancreatic extracts and rHPL

The contents of a commercial capsule of Creon (Solvay Pharmaceuticals, Hannover, Germany) were used as the source of porcine pancreatic extracts (PE). Pancreatin (delipidated pancreatic powder) and excipients contributed to 60 and 40% w/w of the solid mixture, respectively. PPL mass concentration in these formulated pancreatic extracts was estimated to be 1.56% w/w, based on pancreatic lipase activity measurements performed with the tributyrin substrate (124,000 U/g of formulated pancreatic extracts) and a specific activity of 8000 U/mg for PPL. By combining this pancreatin sample with rHPL, the four series of samples obtained contained a set mass concentration of formulated pancreatic extract (i.e., 3.5 mg/ml final concentration, corresponding to 55 μg/ml of PPL) and a variable amount of rHPL (10.20, 17.86, or 25.52 μg/ml). These mixtures were prepared in PBS buffer in the presence and absence of protease inhibitors.

Preparation of duodenal samples containing both porcine pancreatic extracts and rHPL

Using a pool of duodenal samples obtained in a previous study from patients with pancreatic insufficiency [8], a solution of duodenal fluid supplemented with formulated porcine pancreatic extract (final concentration 3.5 mg/ml) was prepared. One milliliter of this solution was added to a mixture of 1 ml glycerol (Sigma) and 40 μl protease inhibitor solution. The protease inhibitor solu-

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