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Preparation, Characterization and Application of High Specific Polyclonal Antibody Against Pancreatic Lipase Related Protein 1*

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Abstract Pancreatic acinar cells synthesize pancreatic lipase related protein 1 (PLRP1), which has a high degree of sequence and structural homology with pancreatic triglyceride lipase (PTL). PTL is required for efficient dietary triglyceride digestion, while the physiological role of PLRP1 has not been elucidated, although some investigations have shown that its expression level is changed under some physiological or pathological conditions. Specific antigenic peptides were fused to glutathione S-transferase (GST) and purified recombinant fusion protein was used to generate polyclonal antisera by immunization of rabbits. The antisera could detect the antigen as low as 0.6 ng and PLRP1 protein in 3 μg of mouse pancreatic juice extracts. The high specificity was verified in Western blot and immunohistochemistry analysis by using PLRP1 knockout (KO) mice as the control. Furthermore, it was showed that food intake could increase the exocrine secretion of PLRP1 into pancreatic juice. This implied that PLRP1 may fulfill dietary digestion function in the digestion track.

Key words specificity, pancreatic lipase related protein 1 (PLRP1), polyclonal antibody, GST fusion protein

Pancreatic triglycerides lipase (PTL) plays an important role in dietary fat absorption by hydrolyzing triglycerides into diglycerides and subsequently into monoglycerides and free fatty acids. The strategy to block fat absorption is useful for treatment of obesity[1]. The mouse pancreatic lipase related protein 1 (PLRP1) mRNA encodes a 473 amino acid polypeptide, including a 17-residue signal peptide, with the theoretical molecular mass of 50 ku. PLRP1 has 68% amino acid sequence identity with PTL, 63% identity with pancreatic lipase related protein 2 (PLRP2). PTL preferentially hydrolyse glyceride substrates, while PLRP2 shows more comprehensive lipase activities, such as phospholipase A1 and galactolipase activities[2, 3]. Their biochemical properties and various physiological roles have been reported so far[4~11]. However, no significant lipase activity was detected in PLRP1 either in the case of native or recombinant forms[5, 12, 13]. The PLRP1 and PTL genes have been found to be co-localized in the chromosomal region 10q24-q26 in humans[14], which is syntenic with chromosome 19 in the mouse[15]. This gene cluster probably resulted from the duplication of an ancestral gene[16].

Interestingly, lipase activity was observed when mutations were introduced in PLRP1 according to the key amino acids in PTL for lipase activity on triglycerides[17]. Although some data are now available on PLRP1 expression, tissue location[13, 18, 19], the physiological role of the protein still remains to be elucidated. To investigate the structure-function relationships and the physiological role of PLRP1, production of antibody with high specificity for PLRP1 is required.

In this paper, we describe the method used to

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raise the anti-PLRP1 polyclonal antisera by immunization of rabbits with the purified GST-antigenic peptides fusion protein. Then, a novel method was used to confirm the high specificity of the polyclonal antisera by using PLRP1 knockout (KO) mice as the negative control in Western blot and immunohistochemistry analysis. These results also indicated that PLRP1 was secreted into pancreatic juice. Furthermore, we showed that mouse secreted high-level of PLRP1 in pancreatic juice in response to food intake indicating that this protein may play a physiological role in dietary digestion.

1 Materials and methods

1.1 Materials

1.1.1 Plasmid vectors, bacteria strains were saved in our laboratory.

1.1.2 Reagent. Restriction endonuclease, T4 DNA ligase and Taq DNA polymerase were purchased from Takara Company Limited (Dalian, China). The primers were synthesized by Sangon (Shanghai, China).

1.1.3 Animals. Healthy New Zealand rabbits aged 4 months were purchased from the Animal Center of The Chinese Academy of Sciences and housed for additional 3 days before manipulation. PLRP1 knockout mice were generated in our laboratory by replacing the exon 1～8 with a neo cassette. Mice were kept in the conventional animal facility of the institute in accordance with institutional guidelines.

1.2 Methods

1.2.1 Construction of plasmids. Plasmid pGEXA was constructed previously in our laboratory by adding multiple cloning sites between *Bam*H Ⅰ and *Eco*R Ⅰ sites of pGEX-3x (Amersham Pharmacia Biotech, Uppsala, Sweden). The DNA fragment encoding the PLRP1 AD1 (aa 65～86) and AD2 (aa 465～473) was obtained by annealing of the complementary oligonucleotides listed as following: 5′ ATCCGCG-GCCGCACTGCTTTCCAGACTCTGCAGCTGTCT-GACCCGTCCACTATTGAAGCTTCTAACTTC 3′ and 5′ TTAGAATTCATTACATGGTGTCGGAAG-TTTTGCACGGCAGAGCAACCTGGAAGTTAGA-AGCTTCAATAG 3′, then digested with *Not* Ⅰ and *Eco*R Ⅰ, and inserted into the *Not* Ⅰ-*Eco*R Ⅰ sites of pGEXA to construct plasmid pGST-PLRP1-2D. In this vector, the PLRP1 AD1 and AD2 are fused in frame to the C terminus of the GST coding sequence. The veracity of the DNA constructs in this study was confirmed by DNA sequencing.

1.2.2 Expression and purification of proteins. *Escherichia coli* BL21 (DE3) was transformed with the expression vector pGST-PLRP1-2D, then the transformed cells were grown in Luria Bertani (LB) medium containing 100 mg/L ampicillin. Upon reaching mid-log phase, the culture was induced with 0.8 mmol/L isopropyl β-D-thiogalactopyranoside (IPTG) at 37℃ for 4 h. The cells were harvested by centrifugation and disrupted by sonication in phosphate buffered saline (PBS) solution. The cytoplasmic extract was filtered and subjected to Glutathione Sepharose 4B column (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. GST fusion protein 6HHA-GST was expressed and purified previously in our laboratory with 6 × His and HA tag at the N terminus of the GST.

1.2.3 Generation of polyclonal antibody. New Zealand white rabbit was immunized with the purified GST-PLRP1-2D protein. 200 μg of protein was emulsified in Complete Freund's Adjuvant (CFA) (Sigma-Aldrich, St. Louis, USA) and was administered by intradermal injection. Rabbits were given another two booster injections of 100 μg of protein with Incomplete Freund's Adjuvant (IFA) (Sigma-Aldrich) every other week. The rabbits were bled prior to immunization (pre-immune serum) and seven days after the last immunization (immune serum).

1.2.4 Enzyme-linked immunosorbent assay (ELISA). 96 wells polystyrene microtiter plates were coated with 100 μl/well purified protein solution at 1 mg/L overnight at 4℃, then blocked with 4% skim milk in PBS for 2 h at 37℃. After plates were washed three times with PBS, each well was incubated with 100 μl diluted antisera (dilution of 1∶3 200, 1∶6 400, 1∶12 800, 1∶25 600…1∶409 600) for 60 min at 37℃. Plates were washed four times with PBS-T (0.1% Tween 20), and each well was incubated with 100 μl goat anti-rabbit immunoglobulin-HRP conjugate (Sigma-Aldrich, 1∶10 000 dilution) for 60 min at 37℃. Plates were washed five times with PBS-T and 100 μl of TMB substrate solution was added into each well. After incubation at room temperature for 10 min, the reaction was stopped with the addition of 25 μl/well 2 mol/L H_2SO_4. The absorbance at 450 nm was measured with an ELISA plate reader (Bio-Rad, CA, USA). The titer of antibody was defined as the serum dilution at which the absorbance at 450 nm was half of the maximal value.

1.2.5 Extraction of pancreatic juice. Mice were

euthanized by cervical dislocation. 50 mg of pancreas was removed and soaked in 500 μl physiological saline (PBS) solution overnight at 4℃ containing 1 mmol/L phenyl-methyl sulfonyl chloride (PMSF), then centrifuged at 14 000 *g* for 10 min at 4℃. The supernatant was removed and the protein concentration was determined using the Bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Haimen, China).

1.2.6 Preparation of pancreas lysates. Mice pancreas were homogenized in RIPA Lysis Buffer (Beyotime Biotechnology, Haimen, China) containing 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), then the lysates were centrifuged at 10 000 *g* for 5 min at 4℃, and the protein content in the supernatants was determined by using the Bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Haimen, China).

1.2.7 Western blotting. Proteins were electrophoretically separated on 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). The blotted membrane was blocked in 1× TBS containing 0.1% Tween (TBST) and 5% nonfat dry milk for 1 h at room temperature with gentle, constant agitation. After incubation with primary antibody (1∶10 000 dilution) at 4℃ overnight, the membrane was washed three times with TBST buffer followed by incubating with goat anti-rabbit (Sigma-Aldrich, 1∶10 000 dilution) or anti-mouse HRP-conjugated IgG (Sigma-Aldrich, 1∶10 000 dilution) for 1 h at room temperature with agitation. The membrane was then washed 3 times with TBST buffer, and the proteins of interest on immunoblots were detected by using an enhanced chemiluminescence detection system (Sigma-Aldrich).

1.2.8 Immunohistochemistry. Pancreas from mouse were immersed in OTC embedding compound (Tissue-Tek) and frozen at –20℃. Sections with 10 μm in thickness were prepared and fixed in acetone at –4℃ for 10 min. After washing with PBS, the slides were treated with Histostain-Plus Kit (Zymed, CA, USA). Before the slides were loaded with primary antibody, the antisera (100 μl) were incubated with purified GST fusion protein 6HHA-GST (0.2 g/L) and Glutathione Sepharose 4B beads for 2 h to remove non-specific antibody.

1.2.9 In study of mouse PLRP1 levels in fasted and fed states. Male mice aged 4 months were divided into two groups and performed separately. One group of mice (n=3) were fed ad libitum and had free access to food and water, while the other group (n =3) were fasted for 9 h before the experiment.

2 Results

2.1 Identification of specific antigenic peptides

To minimize potential antibody cross-reactivity, multiple sequence alignment[20] was performed among the mouse PLRP1 protein, mouse PLRP2 protein, and PTL protein sequence. The result showed that their sequences are highly conservative, and hence indicated that they belong to pancreatic lipase family(Figure 1a). Two regions of PLRP1 (aa 65～86 and aa 465～473) show less homology among PLRP1, PLRP2 and PTL, so the corresponding amino acid sequences were ligated and fused in-frame to the C terminus of the GST (Figure 1b).

2.2 Expression and purification of fusion proteins

In order to produce the antigen, the recombinant plasmid pGST-PLRP1-2D was transformed into *E. coli* BL21(DE3) to express fusion protein GST-PLRP1-2D. SDS-PAGE analysis showed that a band with molecular mass of approximately 30 ku, which was consistent with the expected molecular mass of GST-PLRP1-2D fusion protein, was observed in the extract of transformed bacteria after IPTG induction. The expression level of expected protein was approximately 30% of total cellular proteins(Figure 2a). After affinity purification with glutathione Sepharose 4B column, the recombinant fusion protein GST-PLRP1-2D was obtained at approximately 85% purity as detected by 12% SDS-PAGE (Figure 2b).

2.3 Polyclonal antisera showed high detection sensitivity for antigen and PLRP1 protein in pancreatic juice extracts

We obtain the anti-PLRP1 polyclonal antisera by a rapid immunization protocol on rabbits within five weeks. ELISA was performed using purified protein GST-PLRP1-2D and 6HHA-GST as the antigens to determine the antibody titer. The antisera displayed a high titer of 120 000 against GST-PLRP1-2D, more than the titer of 90 000 against protein 6HHA-GST, and the pre-immune sera did not recognize the antigen GST-PLRP1-2D (Figure 3a). The antisera could detect the antigen as low as 0.6 ng (Figure 3b) and the 50 ku band which matched the protein size of PLRP1 in 3 μg of mouse pancreatic juice extracts (Figure 3c). The result also showed that PLRP1 was a secreted protein which presented in pancreatic juice.

Fig. 1　The comparison of predicted amino acid sequences of mouse PLRP1, PLRP2(Q4VBW7) and PTL(Q6P8U6) and identification of specific antigenic peptides

(a) Multiple sequence alignment results show that the protein encoded by mouse PLRP1 protein has an amino acid sequence identity of 63% and 66% with the protein encoded by mouse PLRP2 and PTL gene, respectively. (b) Diagram showing the GST-PLRP1-2D fusion protein. AD1: Antigen determinant 1; AD2: Antigen determinant 2.

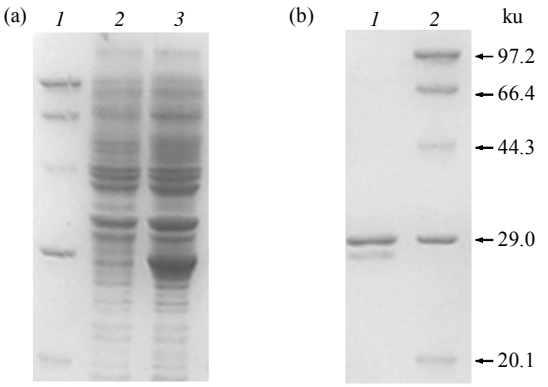

Fig. 2　Expression and purification of GST-PLRP1-2D fusion protein

(a) GST-PLRP1-2D protein expressed in *E. coli*. *1*: Molecular mass marker; *2*: BL21(DE3) transformed with pGST-PLRP1-2D as a control; *3*: BL21 (DE3) transformed with pGST-PLRP1-2D and induced by isopropyl β-D-thiogalactopyranoside. (b) SDS-PAGE analysis of purity of GST-PLRP1-2D fusion protein. *1*: Purified protein pGST-PLRP1-2D; *2*: Molecular mass marker.

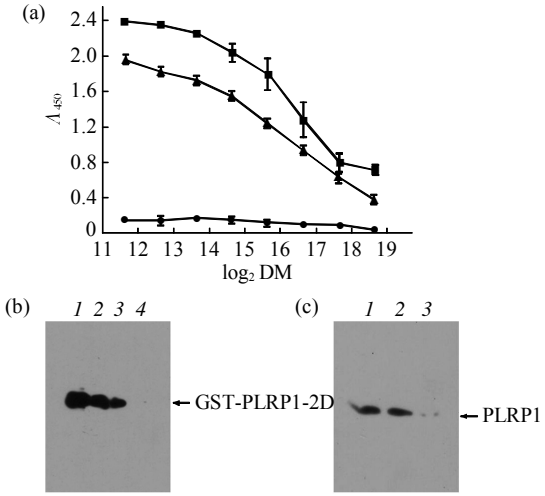

Fig. 3　Assessment on the detection sensitivity of the antisera

(a) Determination of the antibody titer by ELISA. The antiserum titer was defined as the serum dilution at which the absorbance at 450 nm was half of the maximal value. Data are shown as the $\bar{x} \pm s$. n=3; DM: Dilution multiple.■—■: Immune sera against GST-PLRP1-2D; ●—●: Pre-immune sera against GST-PLRP1-2D; ▲—▲: Immune sera against 6HHA-GST. (b) The test of antigen detection sensitivity using purified protein pGST-PLRP1-2D as the antigen. *1*: 15 ng; *2*: 3 ng; *3*: 0.6 ng; *4*: 0.1 ng. (c) Western blot analysis of PLRP1 in pancreatic juice extracts. *1*: 5 μg; *2*: 3 μg; *3*: 1 μg.

2.4 Confirmation of specificity of polyclonal antibody using PLRP1 knockout (KO) mice

To confirm the specificity of the antibody against PLRP1, we used PLRP1 knockout mice as a control in which the gene was deleted by homologous recombination. PLRP1 protein was detected in the Western blot assay of pancreas lysates from wild-type mice, but not in knockout mice (Figure 4a). Furthermore, PLRP1 was only detected in pancreatic juice extracts from wild-type mice (Figure 4b). Immunohistochemistry staining of mouse pancreas with the polyclonal anti-PLRP1 antibody showed positive signal in pancreatic acinar cells from wild-type mice using PLRP1 knockout mice as the negative control (Figure 4c). These results verified the specificity of antisera with no cross-reactivity.

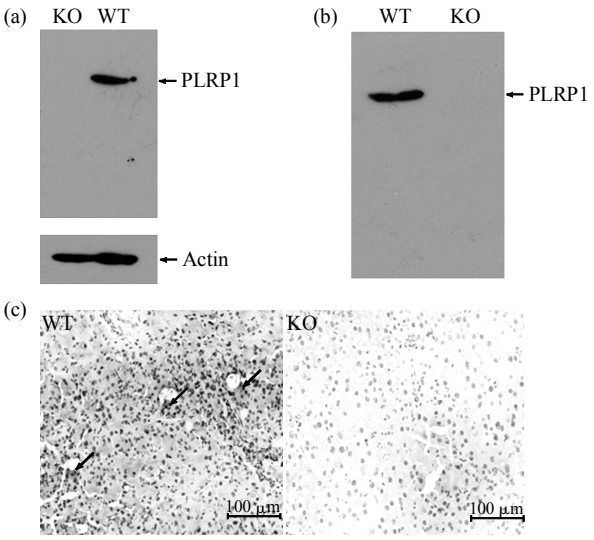

Fig. 4 Verification of the specification of polyclonal antisera using PLRP1 knockout (KO) mice as the control

(a) Western blot analysis of PLRP1 expression in pancreas from wild-type mice using PLRP1 knockout (KO) mice as the control. WT, pancreas lysate protein (150 μg) from wild-type mice; KO, pancreas lysate protein (150 μg) from knockout mice. (b) Western blot analysis of PLRP1 secretion into pancreatic juice. WT, pancreatic juice extracts (20 μg) from wild-type mice; KO, pancreatic juice extracts (20 μg) from knockout mice. (c) Immunohistochemistry analysis of PLRP1 expression in pancreas from wild-type mice and PLRP1 knockout (KO) mice. WT, pancreas from wild-type mice; KO, pancreas from knockout mice. The arrows indicated the pancreatic acinar cells with positive signal.

2.5 Increased secretion of PLRP1 into pancreatic juice in response to food intake

Pancreatic juice contains all kinds of enzymes which are important for dietary digestion. Food intake can stimulate exocrine secretion of pancreatic juice. As shown in Figure 5a, the total protein levels in pancreatic juice extracts when mice were fasted were about 1 g/L, whereas it was about 1.6 g/L in fed state. We also showed the levels of PLRP1 in pancreatic juice extracts(Figure 5b) and pancreas lysate(Figure 5c) were higher when mice in fed state than in fasted state. The results suggested that food intake could increase the exocrine secretion of PLRP1 into pancreatic juice, where it may fulfill the biological function.

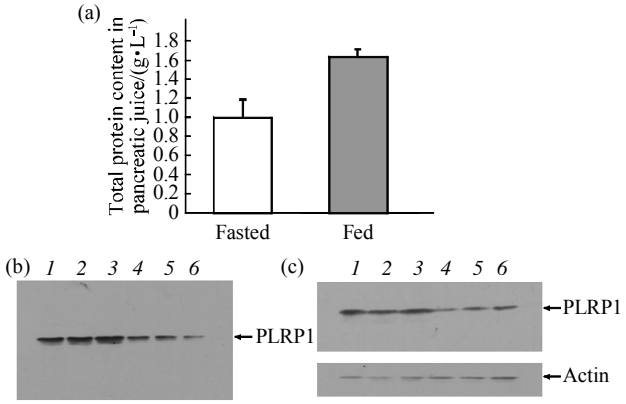

Fig. 5 Increased secretion of PLRP1 into pancreatic juice in response to food intake

(a) Determination of total protein contents in pancreatic juice in fed and fasted states. n=3, * $P < 0.05$. (b) Western blot analysis of mouse PLRP1 levels in pancreatic juice. Each lane were loaded 5 ng of pancreatic juice extracts. *1*～*3*: Mice were fed; *4*～*6*: Mice were fasted. (c) Western blot analysis of mouse PLRP1 levels in pancreas lysate with pancreatic actin as the contol. *1*～*3*: Mice were fed; *4*～*6*: Mice were fasted.

3 Discussion

Antibodies are widely used in many fields such as biochemistry and cell biology. In many instances, an antibody may be required to display a high degree of specificity for its target antigen. It can be especially difficult when the target protein is structurally similar to other proteins. So, confirmation of the specificity of antibodies is necessary for immunological assay. Here, we described a novel method to verify the antibody's specificity by using gene knockout mice as the negative control.

In this paper, we successfully selected two specific antigenic regions from mouse PLRP1 protein sequence by homologically sequence comparison of PLRP1 to PTL and PLRP2. The two antigenic fragments were linked directly and fused in frame to the C terminus of GST to be expressed in *E. coli*. Antisera against mouse PLRP1 was raised by immunization of rabbits with the purified recombinant protein. Western blot assay was employed to verify the quality of the antisera and a specific band of 50 ku was

visible when the mouse pancreatic total protein lysate was loaded. The specificity of the antisera was further confirmed by using the knockout mice as a control in which the PLRP1 gene was disrupted.

The results from Western blot analysis also showed that the PLRP1 was secreted into the pancreatic juice. Furthermore, food intake can increase the level of PLRP1 in pancreatic juice indicating it may fulfill dietary digestion function in the digestion track. Although its physiological function has not been clearly defined, some investigations have reported that the alteration expression of this gene may be involved inflammation, obesity, hyperlipidemia, atherosclerotic lesion and prostate cancer[21~23]. Production of the specific anti-PLRP1 antibody provides us a useful tool for further investigation of the function of PLRP1.

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抗小鼠 PLRP1 多克隆抗体的制备以及特异性鉴定和应用 *

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摘要　小鼠胰泡细胞表达和分泌一种被称为胰甘油三脂酶(PTL)的脂肪酶，主要参与食物来源的甘油三脂的消化吸收，胰泡细胞同时也表达胰脂肪酶相关蛋白 1(PLRP1)，它同 PTL 具有很高的同源性．为了研究 PLRP1 的生物学功能，需要制备其抗体．应用谷胱甘肽 S- 转移酶(GST)表达系统表达了 GST 融合蛋白，亲和纯化后用以免疫新西兰大白兔，获得了抗 PLRP1 的多克隆抗体．免疫印迹分析表明，该多克隆抗血清能检测出 0.6 ng 的融合蛋白抗原以及在 3 μg 的小鼠胰液提取物中检测出 PLRP1 蛋白．在证明抗体的特异性方面，尝试了一种新的方法：用 PLRP1 基因剔除的小鼠作为阴性对照，通过免疫印迹和免疫组化实验证明了该多克隆抗血清具有很高的特异性．进一步的研究发现，进食能促进胰腺中 PLRP1 的外分泌，这表明 PLRP1 可能在食物的消化过程中具有一定的生物学功能．

关键词　特异性，胰脂肪酶相关蛋白 1，多克隆抗体，GST 融合蛋白
学科分类号　Q78

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