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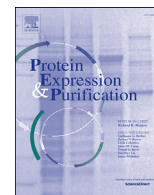

Development of monoclonal antibodies and immunochromatographic lateral flow device for rapid test of alanine aminotransferase isoenzyme 1

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A B S T R A C T

Background: Alanine aminotransferase (ALT) has been used as a sensitive marker for liver injury in people and in preclinical toxicity studies. But measurement of ALT isoenzymes, ALT1 and ALT2, was reported to be of more diagnostic value. The aim of this study is to develop an ideal pair of anti-ALT1 monoclonal antibodies (MAbs) of high specificity and affinity, and subsequently prepare a Immunochromatographic lateral flow device (LFD) for rapid test of ALT1 in human serums.
Methods: The complete coding sequence of ALT1 gene (1500 bp) was cloned from human hepatoma G2 cells (HepG2) and inserted into the expression vector pET-32a(+). ALT1 recombinant protein was routinely prepared by *E.* coli BL21 (DE3) expression and Ni^{2+} affinity purification. Balb/c mice were immunized with purified ALT1 and the splenocytes were fused with Sp2/0 myeloma cells. The positive clones, verified by indirect enzyme-linked immunosorbent assay (ELISA) using purified ALT1, were subcloned to single clones by limiting dilution process. A MAb pair was selected from the obtained MAbs according the sandwich ELISA pairing results and then used for lateral flow device (LFD) production. After evaluation of the sensitivity and specificity, the LFD strips were employed to test human serum samples with known ALT activity levels.
Results: ALT1 recombinant protein was expectedly prepared by expression and purification. A total of 8 stable clones that produced antibodies specifically recognizing ALT1 protein were developed. After sandwich ELISA pairing, an ideal pair of anti-ALT1 MAbs, designated as BD7 and DG3, were selected and proved to be of high specificity, titer and affinity. Based on the MAb pair, LFD strips specifically for ALT1 rapid test were subsequently prepared. The detection threshold of the LFD strips was 12 U/L. No cross reaction was found.
Conclusions: The ALT1 LFD with high sensitivity and specificity was successfully developed. It is valuable for testing ALT1 protein in human sera and can be a beneficial complement for traditional ALT test.
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1. Introduction

Alanine aminotransferase (ALT) [EC 2.6.1.2., also known as glutamate pyruvate transaminase, GPT] is the enzyme that catalyzes the reversible transfer of the α-amino group of alanine to the α-ketogroup of ketoglutaric acid to generate glutamate and pyruvate. It plays an important role in the intermediary metabolism of glucose and protein. ALT has been used as a sensitive marker for liver injury in people and in preclinical toxicity studies. Serum ALT activity is significantly increased in a variety of liver conditions [1–4]. However, the molecular basis for the elevation of serum ALT activity is still unknown. Serum ALT elevation is also observed in extrahepatic diseases, such as muscle diseases, obesity and pancreatitis [5,6], as well as in some people without any overt

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evidence of clinical diseases [7,8]. On the other hand, serum ALT is not elevated in some patients with histopathologically confirmed liver impairment [9,10]. Thus, interpretation of serum ALT activity data can sometimes be challenging in clinical diagnostics and in preclinical drug toxicity evaluation.

Recently two ALT isoenzymes, ALT1 and ALT2, have been identified in humans. Early studies show that, in mRNA and protein levels, ALT1 is expressed highly in kidney and moderately in liver, muscle and heart, while ALT2 is expressed highly in fat, muscle, kidney and brain and moderately in liver [11]. At the subcellular level, ALT1 is a cytoplasmic protein and ALT2 a mitochondrial protein. ALT1 is only located in the cytosol and endoplasmatic reticulum (ER), whereas ALT2 is mainly localized to the mitochondrial fraction and ER [12]. The difference of ALT isoenzymes in structure, tissue and subcellular distributions suggests that the two isoenzymes may have different biological functions and may be related to different clinical conditions [12,13]. Therefore, measurement of ALT isoenzymes may be of more diagnostic value than the currently-used ALT activity assay, which presumably measures a total activity of ALT1 and ALT2.

Though much work has been done on ALT isoenzymes, the protein tests of ALT1 and ALT2 still basically rely on their polyclonal antibodies, whose specificities are questionable [12]. To date, the report on development of ALT1 monoclonal antibody (MAb) has not been seen yet. In this study, we described the development of a pair of highly sensitive and specific MAbs targeting ALT1, and the lateral flow device (LFD) for rapid test using this MAb pair. The LFD strips can test the ALT1 levels rapidly with high specificity and sensitivity.

2. Materials and methods

2.1. Cells and animals

E. coli strains (DH5α and BL21 (DE3)) and human hepatoma G2 cells (HepG2) were preserved in our laboratory. Mouse myeloma cells (Sp2/0) were provided by the Institute of Hepatitis Study of the Chongqing University of Medical Sciences. Balb/c mice were obtained from the Animal Centre of the Chongqing University of Medical Sciences and were housed in an environmentally controlled room at 23 ± 1.5 °C with a 12 h light/12 h dark cycle.

2.2. ALT1 cDNA cloning and vector construction

Total RNA, extracted from HepG2 cells using RNA isolation kit (Qiagen), was converted to cDNA using RT-PCR Kit (Takara). The complete coding sequence of ALT1 gene (1500 bp) was amplified from the resultant cDNA by the polymerase chain reaction (PCR, forward 5′-cggaattcacttctggtcctgccacctc-3′, reverse 5′-cccaagctttgccacacagctttattaggg-3′). The PCR reaction systems were 30 μL, including 10 × PCR buffer 3 μL, dNTP 3 μL, forward primer 1.5 μL、reverse primer 1.5 μL, $MgC1_2$ 1.6 μL、cDNA 2 μL、Taq DNA polymerase 0.2 μL and ddH_2O_2 17.3 μL. The Thermal cycler was programmed with an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of 94 °C for 40 s, 60 °C for 40 s, 72 °C for 40 s and a final extension at 72 °C for 10 min. The resultant PCR products were excised by HindIII/EcoRI and ligated into HindIII/EcoRI -cut pET32a(+) vector (Novagen), followed by transforming the ligation reactions into JM109. The positive plasmids, which were verified by PCR (forward 5′-cggaattcacttctggtcctgccacctc-3′, reverse 5′-cccaagctttgccacacagctttattaggg-3′) and HindIII/EcoRI excision, were sequenced to confirm no cloning-introduced mutations.

2.3. Bacterial expression of ALT1 recombinant protein

The plasmid pET32a(+)-ALT1, isolated from the host DH5α cells using Plasmid mini preparation kit (Qiagen), was transformed into BL21 (DE3). A positive clone was selected on LB solid medium containing ampicillin (60 μg/mL) and inoculated into l L LB liquid medium containing ampicillin (60 μg/mL). Cultures were grown at 37 °C with 220 rpm shaking until an OD600 of about 0.6, at which time isopropyl-β-D-thiogalactopyranoside (IPTG) was added (1 mM, final concentration) to induce expression of ALT1 recombinant protein. Growth was continued at 37 °C with 220 rpm shaking for an additional 4 h. Cells were harvested by centrifugation at 4000 × g and 4 °C for 10 min.

2.4. Purification of ALT1 recombinant protein

Cell pellets were resuspended in 50 ml lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5% glycerol, 2 mM Tris-2-carboxyethyl phosphine, and 0.05% Triton X-100) containing 10 mg lysozyme (50,000 U/mg, Sigma Chemicals, St. Louis, MO). The cell suspension was incubated for 30 min at 37 °C. After incubation, the suspension was sonicated with a Branson ultrasonic disintegrator (VWR ScientiWc Products, Chicago, IL) for 5 min at 50% duty cycle. Cell lysate was centrifuged at 12,000 × g for 30 min at 4 °C, and the supernatant was stored at 4 °C for further use. The His-tagged ALT1 recombinant protein was purified under native conditions by Ni^{2+}-chelating column chromatography (1.5 ml) according to the Handbook from Qiagen. The Ni^{2+}-chelating column was pre-equilibrated with lysis buffer. The unbound proteins were removed with wash buffer (40 mM imidazole, 8 M urea, PBS) and the recombinant protein was eluted in elute buffer (500 mM imidazole, 8 M urea, PBS). All fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was determined by Bradford assay with bovine serum albumin as a standard and using absorbance at 562 nm.

2.5. ALT activity assays

ALT activity was measured at 37 °C with Laspec Alpha-1506 analyzer according to the following principle: pyruvate produced from the transmination of alanine is correlated with the LDH-NADH reaction. NADH is oxidized to NAD with a decrease in the absorbance at 340 nm.The rate of NADH decrease is directly proportional to that of formation of pyruvate and thus the ALT activity. One unit of ALT activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol per liter of NAD per minute under assay conditions.

2.6. Immunization procedures

Eight-week-old female Balb/c mice were immunized with purified ALT1 recombinant protein. Each mouse was immunized 4 times with 100 μg of ALT1 protein every two weeks. The first immunization was performed using complete Freund's adjuvant (Sigma–Aldrich). Incomplete Freund's adjuvant (Sigma–Aldrich) was used for subsequent immunizations. Three days before cell fusion, blood was collected by a vertical incision of the tail vein, followed by determination of antibody titers by indirect ELISA. The mouse with the highest titer was injected intravenously with 50 μg of ALT1 protein (without any adjuvant) and was used for fusion three days later [14].

2.7. Hybridoma cell production

Mouse myeloma Sp2/0 cells, used as fusion partners, were cultured and propagated in RPMI-1640 culture medium (Giboco) and 10% fetal bovine serum (FBS) (Hyclone). Three days after the

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