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A novel protein extracted from foxtail millet bran displays anti-carcinogenic effects in human colon cancer cells

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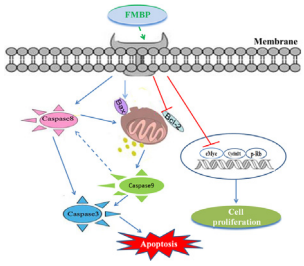
HIGHLIGHTS

- A novel 35 kDa protein, named FMBP, is extracted from foxtail millet bran.
- FMBP is highly homologous to peroxidase and possesses peroxidase activities.
- FMBP exhibits anti-colon cancer effects, but has lower toxicity in normal cells.
- FMBP inhibits cells proliferation in colon cancer by induction of G_1 phase arrest.
- FMBP induces caspase-dependent apoptosis in colon cancer cells.

GRAPHICAL ABSTRACT



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ABSTRACT

Millet is an important cereal food and exhibits multiple biological activities, including immunodulatory, antioxidant, antifungal and anti-hyperglycemia effects. Herein, we describe a novel 35 kDa protein with anti-cancer properties, named FMBP, which was extracted and purified from foxtail millet bran by cell-based screening. FMBP is highly homologous to peroxidase as revealed by mass spectrometry and gene sequencing analysis. Furthermore, *in vivo* anti-tumor results implicated that the novel protein FMBP had the ability to suppress xenografted tumor growth in nude mice. Mechanistically, FMBP is able to suppress colon cancer cell growth through induction of G_1 phase arrest and also causes a loss of mitochondrial transmembrane potential which results in caspase-dependent apoptosis in colon cancer cells. Notably, FMBP has much lower toxicity in normal colon epithelial cells. Taken together, FMBP has targeted anti-colon cancer effects and may serve as a therapeutic agent against colon cancer.

Abbreviations: CRC, colorectal cancer; FMB, foxtail millet bran; FBS, fetal bovine serum; MTT, 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide; DMSO, dimethylsulfoxide; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide; $\Delta\Psi m$, mitochondrial membrane potential; PBS, phosphate buffered saline; PMF, peptide mass fingerprint; PI, propidium iodide; PMSF, phenylmethanesulfonylfluoride; LCSM, laser confocal scanning microscope; PVDF, polyvinylidene fluoride membrane; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

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1. Introduction

Colorectal cancer (CRC) is the third most common cancer in males and the second in females worldwide (Center et al., 2009). An increased consumption of whole grain food has been suggested to reduce the risks of colon cancer (Chatenoud et al., 1998; Li et al., 2013; Lv et al., 2012; Omar et al., 2009). Foxtail millet (*Setaria italica*) which originated in China is now cultivated extensively around the world, including other parts of Asia, India, Africa, Canada and the United States (Austin, 2006; Liang et al., 2010; Taira, 1968). Numerous studies have demonstrated that foxtail millet contains abundant nutritional properties such as amylase inhibitors, glucosinolates, polyphenols and tannins (Elyas et al., 2002; Yamasaki et al., 2005). Foxtail millet has also been reported to exhibit a variety of biological activities, including anti-hyperglycemia (Anju and Sarita, 2010; Sireesha et al., 2011), antifungal (Joshi et al., 1998; Xu et al., 2011) and antioxidant effects (Amadou et al., 2011; Mohamed et al., 2012; Suma and Urooj, 2011).

Bran is a naturally rich by-product produced during the milling of cereal grains. It is the hard outer layer of cereal grains, containing abundant nutrition and lots of bioactive phytochemicals like alkaloids and phenolics which act as 'antinutrients' to prevent diseases (Patel, 2012). At present, several cereal brans have been widely recognized as antioxidant and anticancer agents. It has been reported that millet bran is rich in fractions containing the highest of antioxidant activities (Suma and Urooj, 2011). The phytochemicals, lipids and lignans in wheat bran, as well as the phytic acid from rice bran display strong inhibitory properties for colon cancer (Ferguson and Harris, 1999; Norazalina Saad and Ithnin, 2013; Qu et al., 2005; Reddy et al., 2000). Therefore, development of the medical functions of cereal bran will be very important.

Recently studies have indicated that peptides and proteins from cereal grain bran play very important roles in cancer prevention and treatment. Bioactive peptides derived from rice bran showed promising activities against colon, breast, lung and liver cancers (Kannan et al., 2008, 2009; Kannan et al., 2010). However, the studies on the bioactive fractions of millet bran are very limited, particularly concerning the biological macromolecules from foxtail millet bran which have anti-tumor activities. Herein, a 35 kDa protein, designated as FMBP, was extracted and purified from the aqueous extract of foxtail millet bran for the first time. It is homologous to peroxidase and appears the activities. Interestingly, this novel protein could specifically suppress proliferation and induce apoptosis in colon cancer cells but not in normal human colon epithelial cells. Consistently, it was able to significantly suppress the growth of xenografted DLD1 tumors in nude mice. Thereby, FMBP has the potential ability to develop as an effective therapeutic agent for patients with colon cancer.

2. Materials and methods

2.1. Reagents and antibodies

SP Sepharose XL ion exchange resin was purchased from GE Healthcare (Uppsala, Sweden). RPMI-1640 medium and fetal bovine serum (FBS) were obtained from GIBCO (Grand Island, NY). MTT and Hoechst 33342 were obtained from Sigma (St. Louis, MO). JC-1 and the pan-caspase inhibitor z-VAD-FMK were provided by Beyotime Institute of Biotechnology (Haimen, China). Annexin V-FITC apoptosis detection kit was purchased from Oncogene (San Diego, CA). The antibodies used in this study were as follows: Antibodies for Bax, Bcl-2, cyclinD1 were obtained from Bioworld Technology (Minneapolis, MN); Antibodies for c-Myc, caspase-3, caspase-9 and caspase-8 were obtained from Beyotime Institute of Biotechnology (Haimen, China); p-Rb was obtained from Cell Signaling Technology (Danvers, MA); Antibodies for GAPDH were obtained from Abmart (Arlington, MA).

2.2. Preparation of foxtail millet bran

Seeds of foxtail millet were purchased from a local company (Tian-xia-gu Limited Company). These seeds were cleaned with double distilled water and pulverized in a plate mill to produce whole flour. The whole flour was sieved through a 44 μm mesh sieve to separate the bran rich fraction (Suma and Urooj, 2011). The bran fraction was stored in airtight containers.

2.3. Cell culture

The human colon carcinoma cell lines DLD1, SW480, HT-29 and human colon epithelial cell line FHC were obtained from the Chinese Type Culture Collection and cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) at 37 °C in 5% CO_2 incubator. All media were supplemented with 100 U/ml penicillin (Sigma) and 100 mg/ml streptomycin (Sigma).

2.4. Purification of FMBP and peroxidase activities analysis

For routine preparation, 100 g foxtail millet bran was soaked in deionized 0.02 M Tris–HCl buffer, pH8.0 (containing 0.85% NaCl and 1 mM PMSF) for 24 h, followed by centrifugation at 11,000 rpm, 30 min, 4 °C. The supernatant was collected, precipitated with acetone (V/V = 1:2) for 1 h at −20 °C, followed by centrifugation (11,000 rpm, 30 min, 4 °C). The precipitate was freeze-dried then dissolved in 0.02 M Tris–HCl buffer, pH 8.0 as the crude protein solution. The crude protein solution was precipitated with 30–80% saturated ammonium sulfate, followed by centrifugation (11,000 rpm, 30 min, 4 °C). The precipitate was re-dissolved in the same buffer and dialyzed. The dialyzed solution was centrifuged (11,000 rpm, 30 min, 4 °C) and loaded onto a SP cation-exchange column that had been pre-equilibrated with 20 mM Tris–HCl buffer (pH 8.0). Elution was with 40 and 100 mM NaCl in the same buffer. The eluted fraction of 100 mM NaCl was collected. The collected active fraction was incubated for 20 min at 80 °C and showed a single band on SDS-PAGE. The peroxidase activities of the various stages of purification were measured using the peroxidase detection kit (for plant peroxidase) purchased from Nanjing Jiancheng Biotechnology Co., Ltd.

2.5. Mass spectrometry analysis and protein identification

The purified target protein was resolved by 10% SDS-polyacrylamide gel. SDS-PAGE was performed using standard methods on the Bio-Rad Mini-ProteanII system (Imai and Mische, 1999). The target protein band was cut from SDS-polyacrylamide gel and sent to Shanghai Applied Protein Technology Co. Ltd, China, for protein identification. Protein identification was also performed using the methods of matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF, Applied Biosystems, USA) and peptide mass fingerprint (PMF).

2.6. MTT assay

DLD1, SW480, HT-29 and FHC cells were seeded in 96-well plates at 8×10^3 cells in 100 μl per well. These cells were allowed to grow for 24 h then FMBP at concentrations of 0, 0.025, 0.05, 0.075, 0.1 mg/ml were added into the appropriate wells, with the control being replaced with PBS. After 24, 48 and 72 h FMBP treatment, the old medium was removed and fresh medium added (100 μl/well). 20 μl MTT solution (5.0 mg/ml in phosphate-buffered saline) was added per well and then these cells were incubated for another 4 h at 37 °C. After removal of the medium, 150 μl of dimethyl sulfoxide was added to each well for 10 min to solubilize the purple formazan crystals. The absorbance was measured at 570 nm with an ELISA plate reader. Assays were performed in triplicate in three independent experiments. The percentage of Cell growth inhibition was calculated with the formula: (A_{570} Control − A_{570}Experiment)/A_{570} Control × 100%

2.7. Cell apoptosis and mitochondrial transmembrane potential ($\Delta\psi_m$) analysis

Apoptosis was observed by chromatin staining with Hoechst 33342 (Huang et al., 2012). DLD1 and FHC cells were seeded in laser confocal petri dishes (1×10^5 cells/dish) and allowed to grow for 24 h. Then the cells were treated with 0.1 mg/ml FMBP for 48 h. After washing with PBS, the cells were stained using 1 μl Hoechst 33342 (10 μg/ml) dye for 15 min. The cells nuclei were observed under laser confocal scanning microscope (LCSM). Flow cytometry investigation of FMBP-induced apoptosis in DLD1 and FHC cells was carried out using the Annexin V-FITC and propidium iodide (PI) staining method. Briefly, after incubation with 0.1 mg/ml FMBP for 48 h, cells were trypsinized and washed with PBS. Cells were then stained with 200 μl Annexin V solution (10 μl AnnexinV + 200 μl binding buffer) and 300 μl PI (5 μl PI + 300 μl binding buffer) in the dark (RT for 30 min.) Cell analysis was done using a FACSort flow cytometer. Measurement of changes of mitochondrial transmembrane potential ($\Delta\psi_m$) was performed in DLD1 cells. DLD1 cells were treated by the same procedure as above, but they were further stained with the dye JC-1 and analyzed using a FACSort flow cytometer.

2.8. Cell cycle analysis

Cell cycle was assessed using DLD1 cells with a cell cycle detection kit using FACScan. DLD1 cells were plated into 60 mm dishes (1×10^6 cells/dish) and incubated overnight. After 24 h, cells were treated with 0, 0.05, 0.1 mg/ml FMBP for 48 h and then trypsinized. These cells were washed with PBS and fixed in 70% ethanol at −20 °C for 12 h. The ethanol was subsequently removed after centrifugation and