



Safety assessment of dehydration-responsive element-binding (DREB) 4 protein expressed in *E. coli*

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ABSTRACT

Dehydration-responsive element-binding (DREB) proteins are important transcription factors in plant responses and signal transduction. The DREB proteins can improve the drought and salt tolerance of plants, which provides an excellent opportunity to develop stress-tolerant genetically modified crops in the future. In the present study, a novel *TaDREB4* gene (GenBank Accession No: AY781355.1) from *Triticum aestivum* was amplified by PCR (polymerase chain reaction), and the recombinant plasmid pET 30a(+)/*TaDREB4* was successfully constructed. The fusion protein was induced by IPTG (isopropyl β-D-1-thiogalactopyranoside) and purified by the HisPrep™ FF 16/10 Column. The purity of the final purified *TaDREB4* protein was 93.0%. Bioinformatic analysis and digestive stability tests were conducted to assess the allergenicity of the *TaDREB4* protein, and acute toxicity tests were conducted in mice by oral administration of the *TaDREB4* protein (5000 mg/kg BW). The results indicated that there was almost no similarity between the *TaDREB4* protein and known allergens, and the protein was immediately degraded in simulated gastric and intestinal fluid within 15 s. In addition, no observed adverse effects were found in mice after 14 days. The results preliminary revealed that the protein is safe for human based on the current experiment.

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1. Introduction

Plants are sessile, and their growth and yield are strongly influenced by abiotic stress, such as drought, high salt content and temperature changes. Environmental stress presents a major challenge in our quest for sustainable food production because it reduces the potential yields by as much as 70% in crop plants (Agarwal et al., 2006). Plants respond to dehydration and low temperature with a number of physiological and developmental changes. In addition to changes in proteins and smaller molecules, many genes are also

induced by dehydration and low temperature. Several studies have revealed that the expression of stress-inducible genes in transgenic plants could enhance their stress tolerance (Steponkus et al., 1998; Holmberg and Billow, 1998).

Earlier studies have indicated that cis-acting dehydration-responsive element (DRE) is involved with gene induction by dehydration, high salt, and low temperature (Shinozaki and Yamaguchi-Shinozaki, 2000; Knight and Knight, 2001). The studies also showed that some dehydration-responsive element-binding (DREB) transcription factors could bind to DRE transcription and activate the transcription of genes driven by the DRE sequence. So, DREB transcription factors are important in the induction of a set of abiotic stress-related genes and imparting stress resistance to plants. Liu et al. (1998) and Stockinger et al. (1997) isolated DREBs from Arabidopsis using yeast one-hybrid screening and found that DREBs contain one conserved AP2/EREBP DNA-binding domain, which plays an important role in binding to the DRE element (Sakuma et al., 2002). In addition, a study suggested that DREB proteins can be separated into two groups (i.e., DREB1/C-repeat binding factor (CBF) and DREB2) that are involved in two separate signal transduction pathways under low temperature and dehydration, respectively (Agarwal et al., 2006).

Abbreviations: Bt, *Bacillus thuringiensis*; CAC, Codex Alimentarius Commission; DRE, dehydration-responsive element; DREB, dehydration-responsive element-binding; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; FAO, Food and Agriculture Organization; GMOs, genetically modified organisms; IPTG, isopropyl β-D-1-thiogalactopyranoside; OECD, Organization for Economic Co-operation and Development; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; WHO, World Health Organization; BW, body weight; aa, amino acid; PCR, polymerase chain reaction; CBF, C-repeat binding factor; LB, Luria-Bertani.

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Plants that are transfected with DREB genes may become more tolerant to stress because of the expression of DREB proteins. Thus, the DREB genes are powerful tools for genetic engineering because their overexpression can lead to the upregulation of an array of genes (Agarwal et al., 2006), which creates a new way to enhance the yield of crops. To date, several crops have been genetically engineered to express DREB genes, such as rice (Garladinne et al., 2011), tobacco (Ban et al., 2011), and wheat (Wang et al., 2006).

The first report regarding the safety of genetically modified organisms (GMOs) appeared in 1990 (IFBC, 1990), and several international organizations, such as the Food and Agriculture Organization/World Health Organization (FAO/WHO) and the Organization for Economic Co-operation and Development (OECD), have provided several recommendations to guarantee the safety of GMOs (Metcalf et al., 1996).

Generally, the safety assessment of a GMO involves the following steps: characterization of the parent crop; characterization of the donor organism(s) from which any recombinant DNA sequences are derived, the transformation process, and the introduced recombinant DNA sequences; a safety assessment of the introduced gene products (proteins and metabolites); and a food safety assessment of whole food derived from the GM crop or the edible part of the GM crop (König et al., 2004).

To assess GMOs, especially GM crops, scientists need to focus on several aspects, such as nutrients, toxins, allergens and unintended effects. The assessment of allergenicity and toxicity often focuses on the exogenous proteins. Thus, when exogenous genes was transferred to other plants, the safety of the exogenous proteins is crucial to the safety of the new plant (Kuiper et al., 2003). Under the guidance of the FAO/WHO and the OECD, proteins such as *Bacillus thuringiensis* (Bt) protein (Herman et al., 2003; Xu et al., 2009; Cao et al., 2010), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) (Chang et al., 2003), 2S albumins (Murtagh et al., 2003), phosphinothricin acetyltransferase (Hérouet et al., 2005) and lysine-rich protein (He et al., 2009) have been assessed for allergenicity and toxicity. Some proteins do not show any toxicity or allergenicity (i.e., Bt, lysine-rich protein etc.), whereas other proteins result in toxicity and/or allergenicity (i.e., 2S albumins).

In the present study, we evaluated the safety of the *TaDREB4* gene and protein. Because the *TaDREB4* protein has a very low expression level in GMOs (König et al., 2004), it was expressed in *Escherichia coli* using the pET 30a(+) vector and purified with an affinity column. Bioinformatic analysis and a digestive stability test were conducted to assess the allergenicity of the *TaDREB4* protein, and an acute toxicity test was conducted in mice after an oral exposure.

2. Materials and methods

2.1. Materials

The pAHC25 plasmid was provided by Professor Youzhi Ma from the Institute of Crop Science, Chinese Academy of Agricultural Sciences.

2.2. Expression vector construction

The *TaDREB4* gene (GenBank Accession No: AY781355.1) was amplified from the pAHC25 plasmid using forward and reverse primers (5'-GCG GAT CCA TGA CCG TAG ATC GGA AG-3' and 5'-CCC AAG CTT TCA CAA CCC TTC GAA G-3') containing *Bam*HI and *Hind*III restriction sites (underlined). The amplified PCR product was purified on a gel and ligated into a pGEM-T Easy vector (Promega, WI, USA). The ligation product was used to transform *Escherichia coli* strain DH5 α (TIANGEN, Beijing, China). The transformants were selected by endonuclease digestion and sequencing. Then, the positive plasmids were digested by *Bam*HI and *Hind*III (Takara, Dalian, China) and ligated into PET 30a(+) (Novagen, WI, USA) with *Bam*HI and *Hind*III sites. Then, the recombinant plasmid encoding the *TaDREB4* protein was transferred into *E. coli* strain DH5 α . The transformants were selected by LB agar

plates containing 50 μ g mL⁻¹ kanamycin. The recombinant expression plasmids were verified by restriction digestion, and the positive clones were transformed into *E. coli* BL21 (DE3).

2.3. Expression of recombinant *TaDREB4* protein in *E. coli*

The transformed colony was cultured in LB (1% Bacto peptone, 0.5% yeast extract, 1% NaCl, pH 7.0) liquid medium containing kanamycin (50 μ g mL⁻¹) overnight at 37 °C with rotary shaking (200 rpm). Thirty mL of the cell culture was transferred to 1 L of fresh LB medium supplemented with kanamycin (50 μ g mL⁻¹). At OD₆₀₀ = 0.6–1.0, 0.05–1.0 mmol L⁻¹ IPTG was added to induce the expression of the recombinant protein at 28 and 37 °C. Every 2 h, 0.1 g of cells (wet weight) were collected and resuspended in 1 mL of TE buffer [10 mmol L⁻¹ Tris-HCl and 1 mmol L⁻¹ ethylenediaminetetraacetic acid (EDTA), pH 8.0]. The samples were then sonicated at 4 °C for 30 cycles (each cycle consisted of 10 s on and 10 s off). The cell fractions were separated by centrifugation at 10,000g for 5 min at 4 °C. The protein patterns were analyzed by SDS-PAGE.

2.4. MALDI-TOF MS/MS analysis of the *TaDREB4* protein

Protein bands were excised from the SDS-PAGE gel and washed with 200 μ L of 50 mmol L⁻¹ ammonium bicarbonate in 500 g L⁻¹ acetonitrile for 30 min. Then, the destained protein gel bands were dried and digested in 5 μ L of digestion solution (25 mmol L⁻¹ ammonium bicarbonate and 0.01 g L⁻¹ sequence-grade trypsin), and 25 μ L of digestion solution without trypsin was added to keep the gel pieces wet during digestion. After incubation at 37 °C for 12 h, the peptides were extracted with 20 μ L of 50 g L⁻¹ TFA for 1 h at 37 °C prior to incubation with 20 μ L of 25 g L⁻¹ TFA/500 g L⁻¹ acetonitrile for 1 h at 37 °C. The combined supernatants were evaporated in a vacuum concentrator and dissolved in 4 μ L of 5 g L⁻¹ aqueous TFA for MS/MS analysis (Xia et al., 2005).

The analysis was conducted on an ABI 4700 Proteomics Analyzer MALDI-TOF-MS/MS (Applied Biosystems, CA, USA). The peptide mass fingerprint that was obtained was compared with the theoretical amino acid sequence of the *TaDREB4* protein from NCBI.

2.5. Purification of recombinant *TaDREB4*

The cell pellet was suspended with 10 volumes of binding buffer (20 mmol L⁻¹ sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4). Lysozyme was added to a concentration of 0.2 mg mL⁻¹ and incubated at 37 °C for 30 min. After incubation, the sample was sonicated (99 cycles, 10 s on and 10 s off), and the suspension was obtained by centrifugation at 10,000g for 30 min at 4 °C. The soluble fractions were filtered through a 0.45 μ m filter before being purified with a column.

The *TaDREB4* protein was purified by a HisPrep™ FF 16/10 Column using a AKTA Basic 10 purifier (Amersham Biosciences, Uppsala, Sweden). The column was washed with 100 mL of distilled water (10 mL/min), washed again with 100 mL of elution buffer (20 mmol L⁻¹ sodium phosphate, 500 mmol L⁻¹ NaCl, 500 mmol L⁻¹ imidazole, pH 7.4) and equilibrated with 200 mL of binding buffer. The soluble sample was loaded onto the column using a syringe, and the column was then washed and eluted with elution buffer at 10 mL/min. Fractions containing pure target protein were collected in a tube, lyophilized and stored at -20 °C for further testing.

2.6. Bioinformatic analysis

The potential allergenicity and/or toxicity of transgenic proteins generated for consumption must be investigated before they are introduced into the food chain (Stadler and Stadler, 2003). Although in silico sequence analysis has some limitations, it is still the recommended method of the FAO/WHO. To assess the allergy-related safety of the *TaDREB4* protein, two database systems [i.e., AllergenOnline version 11.0 database (released in Feb 2012) using FASTA3 and NCBI Entrez database using BLASTP] were used to search any for any potential sequence matches to allergen proteins that might indicate the elevated risks of allergenic cross-reactivity (Randhawa et al., 2011). FASTA3 was performed on May 19, 2012, using full FASTA, which included a sliding window of 80 amino acid segments and eight amino acid identity searches. All of the BLASTP searches on the NCBI Entrez protein database were performed on May 19, 2012, with a filter of "allergen" and "toxin". If a protein shares greater than 70% identity over its length relative to an allergen, it is likely to be cross-reactive, whereas a protein with less than 50% identity is not likely to be cross-reactive (Aalberse, 2000). Codex (2003a,b) suggested that alignments of >35% identity over segments as short as 80 amino acids have potential cross-reactivity. The extent of similarity was evaluated by the expectation score (E score), where a high E score indicates a low degree of similarity between the query sequence and the sequence from the database (Randhawa et al., 2011).

2.7. In vitro digestion

The TaDREB4 protein was digested in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) (Sigma, USA) at 37 °C at a concentration of 0.25 mg mL⁻¹ or 0.1 mg mL⁻¹, respectively. The SGF contained 3.2 mg mL⁻¹ pepsin (Sigma, USA) with 34 mmol L⁻¹ NaCl and 0.7% HCl (pH 1.2). The SIF is defined as 50 mmol L⁻¹ potassium phosphate (pH 7.5) containing 10 mg mL⁻¹ pancreatin (Roesler and Rao, 2001). Aliquots were removed at intervals of 0 s, 15 s, 2 min, 30 min and 60 min. The SGF samples were mixed with the stop solution (0.2 mol L⁻¹ sodium bicarbonate) immediately after they were collected. The TaDREB4 protein was boiled with SDS sample buffer for 5 min, and the stability of the protein in SGF/SIF was observed by SDS–PAGE. The same procedure was performed with soybean trypsin inhibitor (STI) as a positive control and bovine serum albumin (BSA, for SGF) or α -casein (for SIF) as a negative control (Lu et al., 2007).

2.8. Acute toxicity test

2.8.1. Administration and dosing

Healthy male and female KM mice weighting 18–22 g were obtained from Vital River Laboratories Animal Technology Co. Ltd. (Beijing, China). The mice were acclimated for 1 week before the study. The mice were divided into three groups, and each group consisted of 12 mice (six males and six females). The relative humidity and the temperature of the animal rooms were maintained at 40–72% and 22 ± 3 °C, respectively. The mice were maintained on a 12 h light/dark cycle with air changes 15 times per hour. The mice had free access to water and feed.

The purified TaDREB4 protein (93% purity) was diluted with redistilled water, and the concentration of TaDREB4 protein was adjusted to 200 mg mL⁻¹. BSA was used as the negative control, and water was used as the blank control. After a 16 h fasting period, one group received BSA protein, another group received TaDREB4 protein (5000 mg kg⁻¹ BW) and the control group received a comparable volume of water by gavage. The mice were continuously observed for general behavioral changes, signs of toxicity and mortality for 1 h after treatment. After 1 h, the mice were intermittently observed for every 4 h, and thereafter over a period of 24 h (Twaij et al., 1983). The mice were further observed for up to 14 days following treatment for behavioral changes and signs of toxicity. On day 15, all of the mice were anesthetized by carbon dioxide inhalation and killed by exsanguination to collect blood samples and to conduct gross and histopathological examinations.

2.8.2. Blood biochemistry and hematology assays

Blood samples were taken from the eyeball veniplex and centrifuged. The following biochemical parameters were detected using a Hitachi 7020 automatic biochemical analyzer (Hitachi, Tokyo, Japan): phosphorus (P), alanine aminotransferase (ALT), cholesterol (CHO), total protein (TP), urea (BUN), albumin (ALB), alkaline phosphatase (ALP), creatinine (CR), glucose (GLU), lactate dehydrogenase (LDH) and glutamyltranspeptidase (GGT).

Blood samples for the hematology analysis were stabilized by EDTA sodium salt. The following parameters were assayed using a HEMAVET 950FS animal blood cell counter (Drew Scientific, Dallas, USA): erythrocyte count (RBC), hematocrit (HCT), the mean cell volume (MCV), white blood cells (WBC), the mean cell hemoglobin (MCH), platelet count (PLT), neutrophils (N), lymphocytes (L), monocytes (M), eosinophils (E), basophils (B) and the mean cell hemoglobin concentration (MCHC) (Xu et al., 2009).

2.8.3. Organ weights, gross necropsy and histopathology

All of the mice were dissected, and the following organs were excised sequentially: brain, thymus, heart, lungs, liver, spleen, adrenals, kidneys and ovaries or testes. All of the organs were weighed and immediately fixed in 4% buffered formaldehyde for histological processing. Paraffin-embedded tissues were sectioned (4–6 μ m thick) and stained with standard hematoxylin and eosin for examination with light microscopy.

2.8.4. Statistics

The data from the treatment group were compared with the water control and the BSA control for each gender. The homogeneity variance was analyzed using the Statistical Product and Service Solutions (SPSS Inc., Chicago, USA). When $p < 0.05$, a step-down analysis was conducted with the least squares differences (LSD) method. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Expression and purification of TaDREB4 protein

3.1.1. Construction of the expression vector

The 1041 bp TaDREB4 gene (GenBank Accession No: AY781355.1) was successfully amplified from the pAHC25 vector (Fig. 1). The PCR products were initially cloned into the pGEM-T

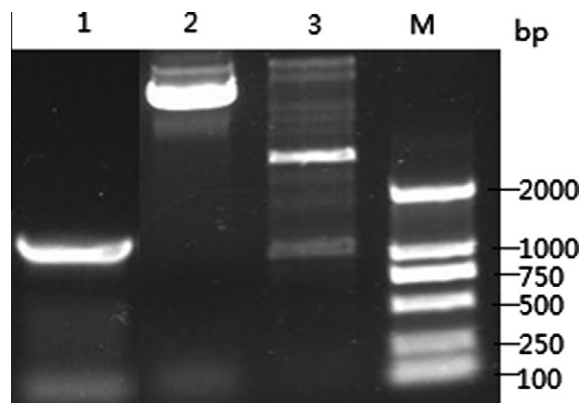


Fig. 1. Cloning of the TaDREB4 gene by PCR and endonuclease digestion. Lane 1, PCR product of TaDREB4; Lane 2, the pET 30a(+)/TaDREB4 vector; Lane 3, endonuclease digestion the pET 30a(+)/TaDREB4 vector.

Easy vector before being ligated into the PET 30a(+) vector. Both of these processes were selected by endonuclease digestion and sequencing (data not shown). The results showed that the TaDREB4 gene was correctly inserted into the PET 30a(+) expression vector.

3.1.2. Optimization of the expression conditions

The TaDREB4 protein was induced at 28 °C and 37 °C (Fig. 2B and C) with IPTG (0.1 mmol L⁻¹). We also used different concentrations of IPTG (0.05–1.0 mmol L⁻¹) at 37 °C to induce expression (Fig. 2A). The percentage of the exogenous protein was displayed under the lanes of the Fig. 2. As shown in Fig. 2, the band for the target protein (346 aa or approximately 38 kDa) was observed at approximately 55 kDa, which was larger than predicted. The expression of the induced protein was compared with uninduced recombinant strains. The amount of induced protein increased with time at both 28 °C and 37 °C, but the correlation was not obvious after 4 h. The optimized expression of the TaDREB4 protein was induced with 0.2 mmol L⁻¹ IPTG at 37 °C for 4 h.

The TaDREB4 protein was purified by a HisPrep™ FF 16/10 Column using the AKTA Basic 10 purifier (Fig. 3A), and SDS–PAGE showed that the protein reached 93% purity after purification (Fig. 3B).

3.1.3. Identification of the target protein by MALDI-TOF-MS/MS

As shown in Fig. 4, six peptide fragments (WVAEIR, GGPENTQCGFR, LWLGTFTPAEDAAR, DAEAAAAAATPFEIPALQPGR, TNFPAHPAQAPAVPALAAIEGVVR and STSEEDVFPELEPISSLPDGEAD GFDIEELLR) with 118 amino acids, which covered approximately 34.1% of the protein sequence, were identical to the theoretical amino acid sequence of the TaDREB4 protein. The MS/MS result also showed the expressed protein was the TaDREB4 protein (data not shown).

In the SDS–PAGE, the apparent molecular mass of the target protein was higher than its theoretical molecular mass, which was calculated based on the amino acid composition. A similar result has also been observed in other studies (Niu and Guiltinan, 1994; DeMaria and Rewer, 1996) and may be a result of the SDS–PAGE procedure. In the presence of the anionic detergent sodium dodecyl sulfate, the separation of proteins by electrophoresis is dependent on the molecular weights of their polypeptide chains (Shapiro et al., 1967), and the apparent molecular weights of the proteins may be affected by several factors, such as the amino acid composition, the isoelectric point of the protein and the space structure (Kaufmann et al., 1984).

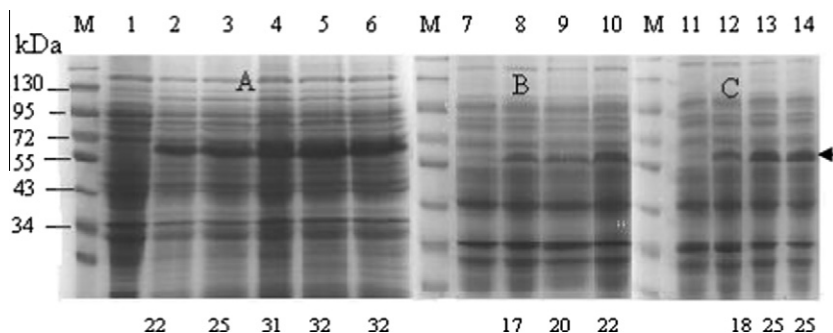


Fig. 2. The protein expressed with different IPTG concentrations and temperatures. Lanes 1, 7 and 11 are the uninduced recombinant strains. (A) Lanes 2–6 are cells induced with 0.05, 0.1, 0.2, 0.5 and 1.0 mmol L⁻¹ IPTG, respectively. (B) Lanes 8–10 are cells induced for 2 h, 4 h and 6 h, respectively, at 28 °C. (C) Lanes 12–14 are cells induced for 2 h, 4 h and 6 h, respectively, at 37 °C.

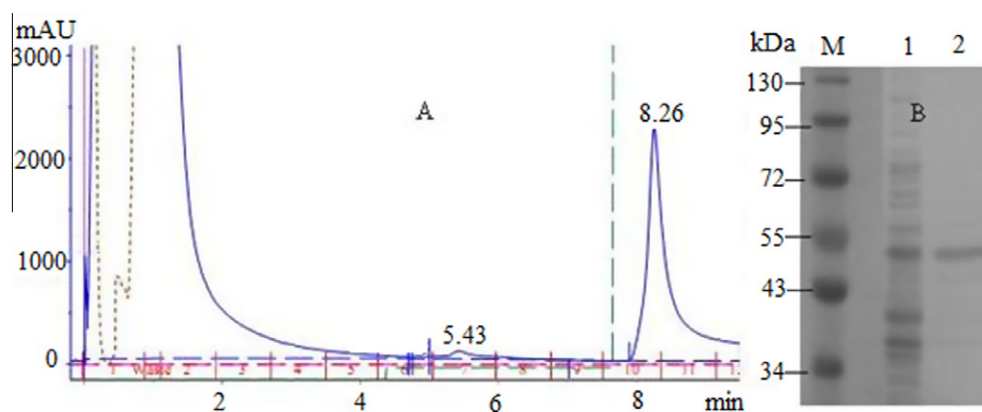


Fig. 3. Protein purification. (A) TaDREB4 protein purified by the AKTA Basic 10 purifier. The purified protein solution in the 10th and 11th tubes, which corresponds to the highest peak at 8.26 min, was collected for SDS–PAGE detection. (B) Lane 1 is the total protein from *E. coli*, and Lane 2 is the purified protein.

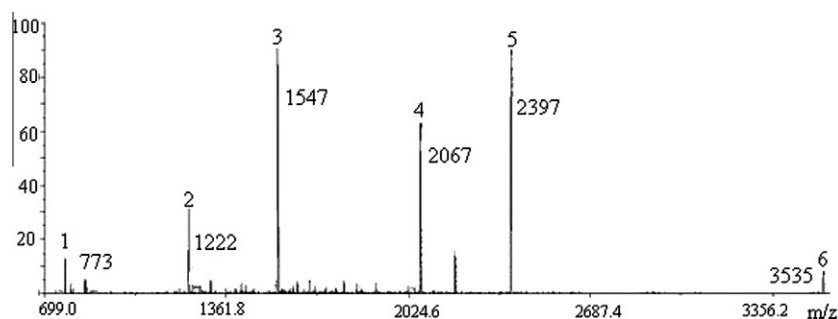


Fig. 4. MALDI-TOF-MS analysis of recombinant TaDREB4 protein. Six peptide fragments corresponding to the six peaks were identical with the theoretical amino acid sequence of TaDREB4 protein, with 34.1% sequence coverage.

The MALDI-TOF-MS/MS results indicated that the recombinant protein was the expected protein and can be used for the safety assessment.

3.2. Bioinformatic alignment of the TaDREB4 protein

The TaDREB4 protein only showed significant alignments with a few known allergens when we searched in the AllergenOnline database (Table 1). The maximum percent identity that was found in the full length search was 28.7% identity with pollen allergen, which was below the level that is likely to indicate cross-reactivity. As described by Aalberse (2000), a protein sharing more than 70% identity over its length relative to an allergen is likely to be cross-reactive, whereas proteins with less than 50% identity are not

Table 1

Summary of the results using FASTA for TaDREB4 gene.

GI No.	Source	Allergen	E-score	% Identity
113561	<i>Poa pratensis</i>	Pollen allergen	2.3	28.7
169971	<i>Glycine max</i>	Glycinin precursor	2.5	25.5
258588247	<i>Prunus dulcis</i>	Pru du amandin	2.5	27.5
190684063	<i>Triticum aestivum</i>	Dehydrin	4.3	25.4

likely to be cross-reactive. The identity results of the FASTA3 alignments of every possible 80 aa segment of the TaDREB4 encoded protein revealed that there were no matches greater than 35% identity over 80 amino acids. The eight amino acid short-sequence identity search showed two 8 mer matches with the thioredoxin h1 protein from *Zea mays* (EAAAAAAT, AAAAAATP).

The *E* score values in the FASTA3 search were all more than 1.0. A high *E* score indicates a low degree of similarity between the query sequence and the sequence from the database. The BLASTP on the NCBI search results also revealed that there were no significant alignments between the target protein and allergenic proteins. In conclusion, no significant alignments were found between TaDREB4 and allergenic proteins using two search tools.

3.3. Degradation in simulated digestive fluids

SDS–PAGE was used to detect the digestion of the TaDREB4 protein in SGF and SIF. Fig. 5 shows that TaDREB4 protein was rapidly digested within 15 s in both the SGF and the SIF. The results of the positive and negative controls agreed with our expectations (data not shown).

3.4. Acute toxicity test of TaDREB4 protein in KM mice

In the acute toxicity experiment, none of the mice that were dosed with BSA (negative controls, 5000 mg kg^{−1}·BW) or water (blank controls, 20 mL kg^{−1}·BW) showed any visible signs of systemic toxicity during the study. In addition, no mortality or toxic effects were observed in the mice after acute oral administration of TaDREB4 protein (5000 mg kg^{−1} BW).

3.4.1. Clinical observations and body weight

We did not observe any treatment-related adverse effects in the clinical appearance of the mice. For example, the body weights of the male and female mice in the TaDREB4 group were comparable with the control groups (Table 2).

3.4.2. Organ weights, gross necropsy and histopathology

No group-related alterations or statistically significant differences were observed in the organ weights of the male and female mice treated with TaDREB4 protein compared with the control groups (Table 2). Although we observed marked differences in the liver weights in both TaDREB4 males and females compared with the controls, there were no histopathological changes. The relative value of the hearts of TaDREB4 females was significantly different from the negative controls, and the relative value of the ovaries was significantly different from the blank controls. These differences were all observed in females and were only different from the negative or blank control. Considering some of the main functions of the liver are to remove toxins from the body, we carried out the pathological analysis seriously. However, no specific pathological phenomena were observed. These differences were considered to be random and unrelated to the TaDREB4 protein.

The blood hematology and biochemistry results are shown in Tables 3 and 4. For the hematology results, small but statistically significant differences were found between the male and female

TaDREB4 protein groups and the control groups. The eosinophil (*E*) value of the female TaDREB4 protein group was significantly different from the BSA group but not the water group. Interestingly, this phenomenon was not observed in the male mice; thus, the eosinophil results were not considered to be related to the TaDREB4 protein. For the serum biochemistry results, the differences in ALP and GGT were only found in females or males, respectively; thus, changes in ALP and GGT were considered to be unrelated to TaDREB4.

4. Discussion

An increasing number of reports have demonstrated that many plant genes are induced by drought, high salt and cold temperatures. The encoding products of the genes induced by drought, high salt and cold can be classified into two groups: functional proteins (e.g., LEA proteins, osmotin and proline synthetase) and transcription factors (e.g., MYC, MYB and DREB) (Liu et al., 2000). The present study demonstrated that DREB transcription factors can control the expression of many genes involved in the responses to environmental stress. For the ideal and multiple effects of the DREB transcription factors, it could be more effective than single functional genes.

Although the transcription factors offers enormous benefits, GMOs also need to be assessed to ensure that the food products are as safe as traditional food. Several international organizations and countries have developed guidelines to investigate the safety of GMOs (OECD, 2000, 2003; European Commission, 2003a; US FDA, 1992). The majority of biotechnology applications have resulted in the expression of new proteins in GMOs. With the development of biotechnology, most new proteins that have been introduced in GMO's are from other organisms. Humans may have not been exposed to them before, so the new proteins are likely to exist certain potential risk. Under the circumstances, allergenic and potential toxicity of these proteins must be considered (Metcalf, 2005).

Although DREB transcription factors have been widely used, safety assessments about DREBs have rarely been reported; thus, the present study is important.

The FAO/WHO recommends that an important first step in the investigation of recombinant proteins that are introduced into plants is the screening of the amino acid sequences of recombinant proteins for similarity to sequences of known allergens. In addition, the FAO/WHO recommends screening for toxicity. Generally speaking, a protein that shares more than 70% identity over its length relative to an allergen is likely to be cross-reactive, and proteins that have less than 50% identity are not very likely to be cross-reactive (Aalberse, 2000). When a newly expressed protein shares more than 35% identity in a segment of 80 or more amino acids with a known allergen, the protein is likely to cross react with

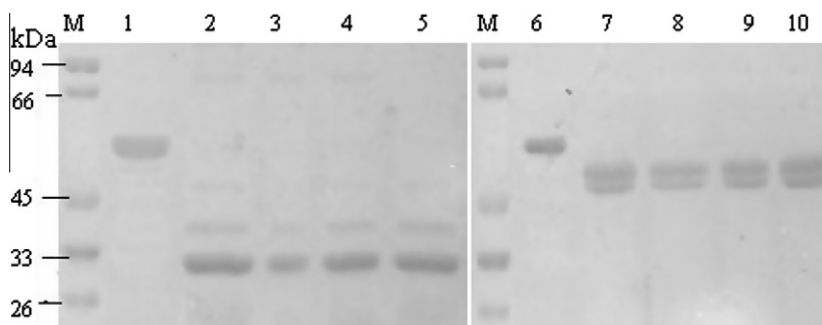


Fig. 5. The digestive stability in simulative gastric and intestinal fluids. Lanes 1–5, the TaDREB4 protein was digested in SIF for 0 s, 15 s, 2 min, 30 min and 60 min, respectively. Lanes 6–10, the TaDREB4 protein was digested in SGF for 0 s, 15 s, 2 min, 30 min and 60 min, respectively.

Table 2Body and organ weights (the mean value $\pm$ SD) ($n = 6$).

Groups	Male			Female		
	Water	BSA	TaDREB4	Water	BSA	TaDREB4
Final body weight (g)	32.00 $\pm$ 1.23	32.68 $\pm$ 1.60	33.80 $\pm$ 2.12	26.68 $\pm$ 1.32	26.28 $\pm$ 2.58	26.06 $\pm$ 1.96
Brain (%)	1.38 $\pm$ 0.12	1.34 $\pm$ 0.06	1.36 $\pm$ 0.09	1.68 $\pm$ 0.13	1.79 $\pm$ 0.11	1.77 $\pm$ 0.09
Heart (%)	0.59 $\pm$ 0.06	0.60 $\pm$ 0.17	0.60 $\pm$ 0.13	0.59 $\pm$ 0.01	0.64 $\pm$ 0.06	0.54 $\pm$ 0.03 ^b
Liver (%)	4.17 $\pm$ 0.13	4.08 $\pm$ 0.25	4.86 $\pm$ 0.25 ^{a,b}	3.99 $\pm$ 0.56	4.32 $\pm$ 0.37	4.91 $\pm$ 0.45 ^{a,b}
Spleen (%)	0.28 $\pm$ 0.03	0.30 $\pm$ 0.04	0.31 $\pm$ 0.05	0.32 $\pm$ 0.07	0.35 $\pm$ 0.07	0.37 $\pm$ 0.07
Lungs (%)	0.59 $\pm$ 0.08	0.64 $\pm$ 0.06	0.63 $\pm$ 0.11	0.78 $\pm$ 0.12	0.72 $\pm$ 0.10	0.77 $\pm$ 0.08
Kidneys (%)	1.51 $\pm$ 0.13	1.42 $\pm$ 0.07	1.46 $\pm$ 0.14	1.23 $\pm$ 0.07	1.24 $\pm$ 0.21	1.33 $\pm$ 0.12
Thymus (%)	0.21 $\pm$ 0.05	0.25 $\pm$ 0.05	0.23 $\pm$ 0.05	0.27 $\pm$ 0.06	0.27 $\pm$ 0.04	0.25 $\pm$ 0.07
Adrenals (%)	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01	0.03 $\pm$ 0.01
Testes/ovaries (%)	0.72 $\pm$ 0.08	0.65 $\pm$ 0.06	0.65 $\pm$ 0.06	0.06 $\pm$ 0.02	0.09 $\pm$ 0.02	0.10 $\pm$ 0.02 ^a

^a $p < 0.05$ for the experimental group compared with the blank control group.^b $p < 0.05$ for the experimental group compared with the negative control group.**Table 3**Hematology mean values $\pm$ SD ($n = 6$).

Groups	Male			Female		
	Water	BSA	TaDREB4	Water	BSA	TaDREB4
WBC ($10^9/L$)	3.13 $\pm$ 1.10	4.01 $\pm$ 1.51	3.39 $\pm$ 0.79	3.59 $\pm$ 1.08	1.87 $\pm$ 0.58 ^c	3.05 $\pm$ 1.34
RBC ($10^{12}/L$)	6.71 $\pm$ 1.13	6.14 $\pm$ 1.09	6.38 $\pm$ 1.18	5.23 $\pm$ 0.96	4.46 $\pm$ 1.23	4.62 $\pm$ 1.35
HCT (%)	40.12 $\pm$ 7.37	37.17 $\pm$ 7.97	39.77 $\pm$ 8.03	32.15 $\pm$ 6.41	26.27 $\pm$ 7.45	28.17 $\pm$ 8.05
MCV (fL)	59.67 $\pm$ 3.20	60.12 $\pm$ 2.59	62.22 $\pm$ 2.79	61.47 $\pm$ 2.83	58.67 $\pm$ 1.15 ^c	60.96 $\pm$ 2.41
MCH (pg)	17.10 $\pm$ 0.64	16.80 $\pm$ 0.73	17.55 $\pm$ 0.74	17.67 $\pm$ 0.93	16.17 $\pm$ 0.48 ^c	16.40 $\pm$ 2.10
MCHC (g/L)	286.83 $\pm$ 10.15	280.00 $\pm$ 12.03	281.67 $\pm$ 8.21	287.50 $\pm$ 9.09	275.50 $\pm$ 7.87	268.00 $\pm$ 30.47
PLT ($10^9/L$)	291.00 $\pm$ 60.81	282.50 $\pm$ 50.78	305.00 $\pm$ 64.60	518.00 $\pm$ 346.25	171.33 $\pm$ 69.09 ^c	353.57 $\pm$ 205.0
N ($10^9/L$)	0.64 $\pm$ 0.25	0.79 $\pm$ 0.36	0.67 $\pm$ 0.31	0.66 $\pm$ 0.28	0.37 $\pm$ 0.20	0.55 $\pm$ 0.36
L ($10^9/L$)	2.36 $\pm$ 0.86	2.98 $\pm$ 1.22	2.53 $\pm$ 0.64	2.70 $\pm$ 0.97	1.39 $\pm$ 0.28 ^c	2.32 $\pm$ 1.11
E ($10^9/L$)	0.00 $\pm$ 0.01	0.06 $\pm$ 0.06 ^c	0.02 $\pm$ 0.04	0.04 $\pm$ 0.04	0.03 $\pm$ 0.06	0.01 $\pm$ 0.00
M ($10^9/L$)	0.13 $\pm$ 0.05	0.16 $\pm$ 0.06	0.15 $\pm$ 0.03	0.17 $\pm$ 0.12	0.11 $\pm$ 0.04	0.18 $\pm$ 0.07 ^b
B ($10^9/L$)	0.00 $\pm$ 0.01	0.02 $\pm$ 0.03	0.01 $\pm$ 0.02	0.02 $\pm$ 0.02	0.02 $\pm$ 0.03	0.00 $\pm$ 0.00

^b $p < 0.05$ for the experimental group compared with the negative control group.^c $p < 0.05$ for the negative control group compared with the blank control group.**Table 4**The blood biochemistry mean values $\pm$ SD ($n = 6$).

Groups	Male			Female		
	Water	BSA	TaDREB4	Water	BSA	TaDREB4
ALT (U/L)	71.80 $\pm$ 23.53	105.00 $\pm$ 34.31	66.40 $\pm$ 23.05	37.33 $\pm$ 6.28	38.00 $\pm$ 2.16	42.14 $\pm$ 15.61
TP (g/L)	72.65 $\pm$ 7.02	70.18 $\pm$ 5.27	69.03 $\pm$ 5.85	64.72 $\pm$ 7.18	66.13 $\pm$ 4.01	63.77 $\pm$ 5.87
ALB (g/L)	39.17 $\pm$ 1.04	39.22 $\pm$ 1.32	38.43 $\pm$ 1.77	39.22 $\pm$ 2.44	39.77 $\pm$ 1.52	36.67 $\pm$ 2.35
ALP (U/L)	193.50 $\pm$ 49.41	187.17 $\pm$ 39.15	149.00 $\pm$ 31.07	190.17 $\pm$ 42.79	219.67 $\pm$ 36.06	145.43 $\pm$ 21.82 ^{ab}
GGT (U/L)	249.17 $\pm$ 61.69	315.00 $\pm$ 73.12	216.17 $\pm$ 45.39 ^b	173.17 $\pm$ 29.70	179.00 $\pm$ 19.25	197.57 $\pm$ 49.21
GLU (mmol/L)	5.83 $\pm$ 0.80	7.93 $\pm$ 2.77	7.65 $\pm$ 0.92 ^a	4.23 $\pm$ 0.99	4.43 $\pm$ 0.76	5.73 $\pm$ 1.57
BUN (mmol/L)	10.73 $\pm$ 0.73	10.70 $\pm$ 1.41	10.08 $\pm$ 0.50	9.17 $\pm$ 0.52	11.00 $\pm$ 1.42	9.16 $\pm$ 1.69
CR (mmol/L)	27.33 $\pm$ 5.09	29.33 $\pm$ 6.74	28.83 $\pm$ 2.23	25.67 $\pm$ 9.75	26.67 $\pm$ 4.99	27.71 $\pm$ 5.82
CHO (mmol/L)	4.98 $\pm$ 0.99	5.35 $\pm$ 0.72	4.36 $\pm$ 0.69 ^b	3.22 $\pm$ 0.40	3.48 $\pm$ 0.11	3.60 $\pm$ 0.56
P (mmol/L)	4.60 $\pm$ 0.49	4.43 $\pm$ 0.28	4.29 $\pm$ 0.68	3.40 $\pm$ 0.52	4.05 $\pm$ 0.46	3.83 $\pm$ 0.59
LDH (U/L)	1759.83 $\pm$ 383.10	1991.00 $\pm$ 394.33	1472.33 $\pm$ 507.51	1936.00 $\pm$ 213.50	1416.67 $\pm$ 109.55 ^c	1884.86 $\pm$ 244.57 ^b

^a $p < 0.05$ for the experimental group compared with the blank control group.^b $p < 0.05$ for the experimental group compared with the negative control group.^c $p < 0.05$ for the negative control group compared with the blank control group.

immunoglobulin E (IgE) (FAO/WHO, 2001; Codex Alimentarius Commission, 2003a, b).

Although the FAO/WHO proposed to reduce the contiguous amino acid sequence similarity from eight to six amino acids in 2001, available data suggest that this would result in a great number of false positive predictions (König et al., 2004). In the present study, we used the identity of eight contiguous amino acids to search for potential allergenicity. The results showed that the TaDREB4 protein has two hits (EAAAAAAT, AAAAAATP) with the

thioredoxin h1 protein from *Zea mays*. Although there was uncertainty in the 8 contiguous amino acid search, the lack of a high sequence homology in the full-length protein or in an 80 amino acid sequence allowed us to preliminarily view TaDREB4 as safe. If we want to further identify its potential cross-reactivity, targeted sera screening is needed.

Because most food allergens are stable and resistant to digestive processes (Astwood et al., 1996; Hérouet et al., 2005), the digestive stability of exogenous proteins is an important factor in safety

assessments. The digestive stability test showed that the TaDREB4 protein can be digested thoroughly in a short period of time (less than 15 s) in SGF and SIF. The bioinformatic analysis and the digestive stability test demonstrated that the protein is not resistant to human digestion.

To date, there is no uniform standard about the doses that should be used in acute toxicity tests. The OECD provides 2000 mg kg⁻¹ BW as the upper fixed dose, but 5000 mg kg⁻¹ may be considered when justified by specific regulatory needs (OECD, 2001). China sets 5000 mg kg⁻¹ BW as the practically nontoxic grade (Chinese Standard GB15193.13-2003). In the present study, we conducted the acute toxicity test following the Chinese Standard. The test showed that the mouse acute oral LD₅₀ was >5000 mg kg⁻¹ BW, which suggests that TaDREB4 belongs to the practically nontoxic grade. KM mice fed TaDREB4 protein did not show any significant differences ($p < 0.05$) in body weight compared with the control groups. Although several organs and hematology indices showed a difference, the differences may not be related to the TaDREB4 protein.

In the present study, recombinant TaDREB4 protein that was expressed in *E. coli* was used to conduct the safety assessment. The results of the MALDI-TOF-MS/MS explained that the TaDREB4 protein produced from *E. coli* was equivalent to the protein that existed in the GMOs. The protein bioinformatic analysis and the digestive stability test showed a low level of risk for potential allergenicity. The oral acute study also demonstrated that TaDREB4 proteins will not be toxic to mammals, even at a high dose (5000 mg kg⁻¹ BW). The results revealed that according to the present experiment, no unfavorable factors of the TaDREB4 protein to animals and humans were found.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgement

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Appendix A. Supplementary data

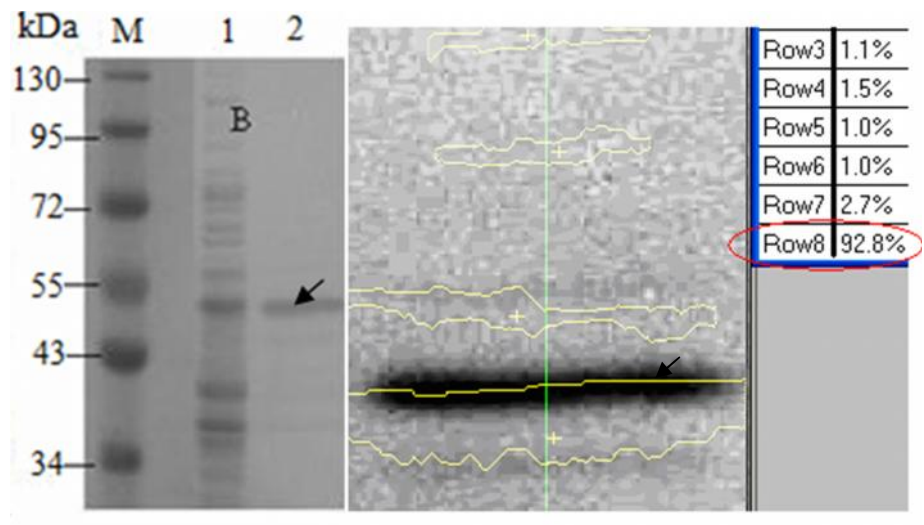
Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.fct.2012.06.026>.

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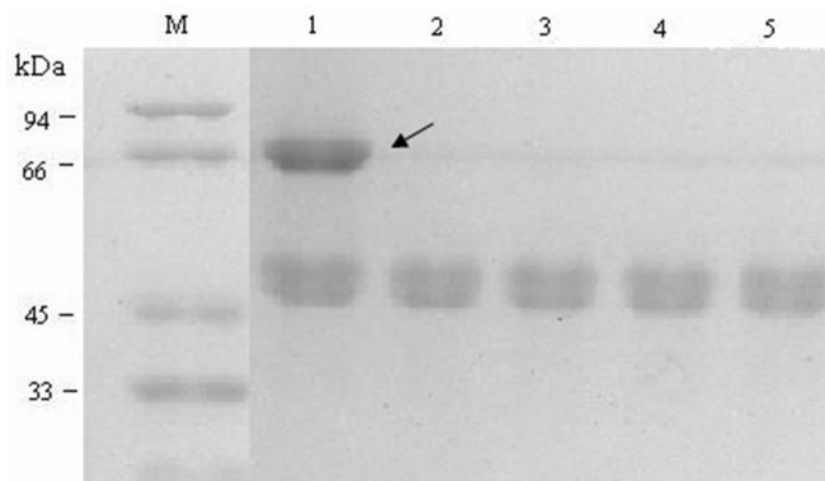
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Fig S1 The result of the purity percentage



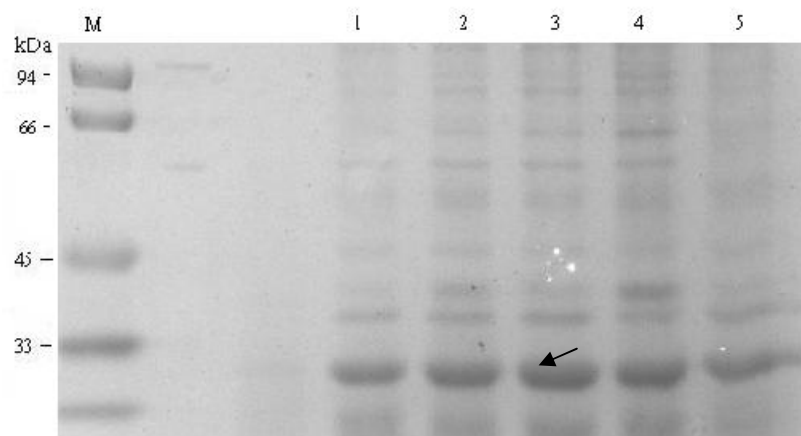
Line 1: The total protein; Line 2: The purified protein.

Fig S2a SDS-PAGE of digestion assays of BSA in stimulated gastric fluid (negative control) .



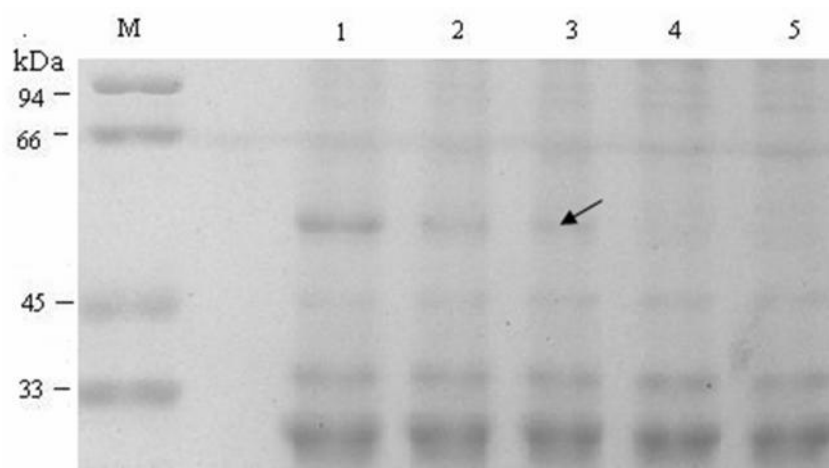
The BSA protein (shown by the arrow) was not digested in SGF at 0 seconds (lane 1). At 15 s, 2 min, 30 min and 60 min (lanes 2-5) the BSA protein was digested totally.

Fig S2b SDS-PAGE of digestion assays of STI in stimulated gastric fluid (positive control)



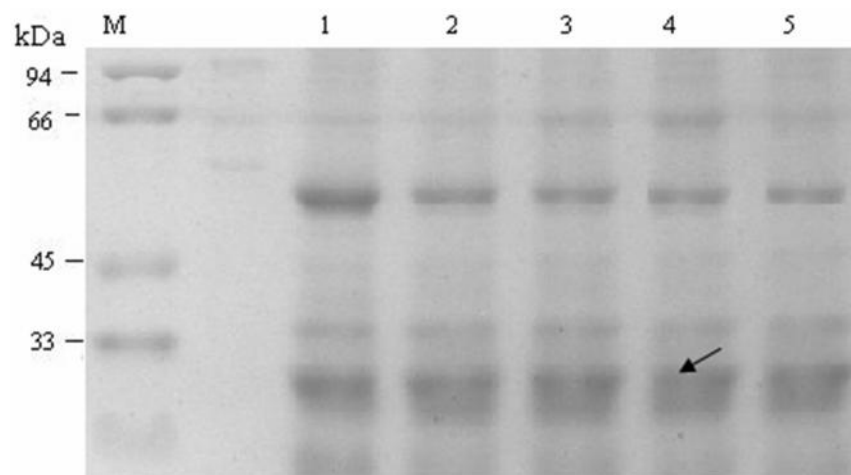
The STI protein (shown by the arrow) was not digested in SGF for 0 s, 15 s, 2 min, 30 min and 60 min(lanes 1-5), respectively.

Fig S2c SDS-PAGE of digestion assays of α -casein in stimulated intestinal fluid (negative control)



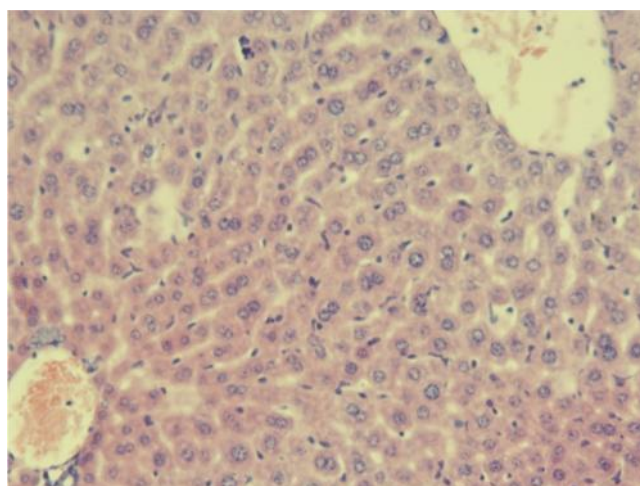
The α -casein protein (shown by the arrow) was digested in SIF progressively (lanes 1-3) .

Fig S2c SDS-PAGE of digestion assays of STI in stimulated intestinal fluid (positive control)

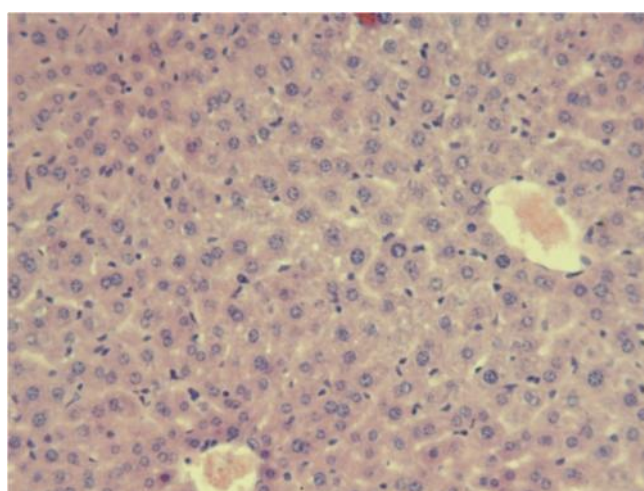


The STI protein (shown by the arrow) was not digested in SIF for 0 s, 15 s, 2 min, 30 min and 60 min (lanes 1-5).

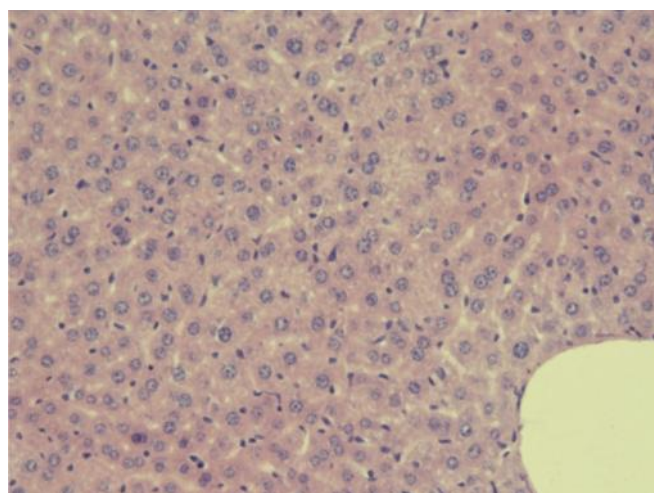
Fig S3 state of liver of all the groups



Blank control (water) group ($\times 200$)



Negative control (BSA protein) group ($\times 200$)



The TaDREB4 protein group ($\times 200$)

Pathological analysis showed that (blank control group, negative control group and TaDREB4 protein group) presented a normal state of liver