Molecular cloning and characterization of an alpha-amylase inhibitor (TkAAI) gene from *Trichosanthes kirilowii* Maxim.

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Abstract *Trichosanthes kirilowii* Maxim taxonomically belongs to the Cucurbitaceae family and *Trichosanthes* genus. Its whole fruit, fruit peel, seed and root are widely used in traditional Chinese medicines. A ribosome-inactivating protein with RNA N-glycosidase activity called Trichosantrip was isolated and purified from the seeds of *T. kirilowii* in our recent previous research. To further explore the biological functions of Trichosantrip, the cDNA of *T. kirilowii* alpha-amylase inhibitor (TkAAI) was cloned through rapid-amplification of cDNA ends and its sequence was analyzed. Also, the heterologous protein was expressed in *Escherichia coli* and its alpha-amylase activity was further measured under optimized conditions. The full-length cDNA of *TkAAI* was 613 bp. The speculated open reading frame sequence encoded 141 amino acids with a molecular weight of 16.14 kDa. Phylogenetic analysis demonstrated that the Alpha-Amylase Inhibitors Seed Storage domain sequence of *TkAAI* revealed significant evolutionary homology with the 2S albumin derived from the other plants in the Cucurbitaceae group. In addition, *TkAAI* was assembled into pET28a with *eGFP* to generate a prokaryotic expression vector and was induced to express in *E. coli*. The *TkAAI*-eGFP infusion protein was proven to exhibit alpha-amylase inhibitory activity against porcine pancreatic amylase in a suitable reaction system. Analysis of gene expression patterns proved that the relative expression level of *TkAAI* in seeds is highest. The results presented here forecasted that the *TkAAI* might play a crucial role during the development of *T. kirilowii* seeds and provided fundamental insights into the possibility of *T. kirilowii* derived medicine to treat diabetes related diseases.

Keywords *Trichosanthes kirilowii* Maxim. · Alpha-amylase inhibitors · Molecular cloning · Enzymatic reaction · Diabetes

Introduction

*Trichosanthes kirilowii* Maxim is a perennial herbaceous liana of the Trichosanthes genus in the Cucurbitaceae family. Its distribution ranges from the eastern Himalayas in India and southern China through to southern Japan, Malaysia and tropical Australia. The fruit, seed and root of the plant have been commonly used as traditional Chinese medicines. Moreover, *T. kirilowii* is considered as one of the 50 fundamental herbs. (Yang et al. 2012a) They also possess a variety of bioactive substances, including trichosanthin, which is a ribosome inactivating protein extracted...
from the root tube. It has been reported to have properties of immunomodulatory, anti-tumor and anti-human immunodeficiency virus (HIV) properties (Shaw et al. 2005). Moreover, it was found that T. kirilowii seeds were energy-rich for diet, containing 62% oil, up to 30% proteins, 2.5% mono and oligosaccharides (Yang et al. 2012b).

Alpha-amylases (α-1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) are hydrolytic enzymes and are widely distributed in nature (Franco et al. 2002). They catalyze the cleavage of the α-1, 4 glycosidic linkages found in starch and other oligosaccharides, hydrolysates and molecules such as dextrin and reducing sugars. Of those molecules the function and structure of porcine pancreatic amylase (PPA) has been comprehensively investigated (Robyt and French 1970; Granger et al. 1975; Prodanov et al. 1984). PPA was originally purified from tissue extracts and nowadays commercial production has been conducted via microorganism (Darnis et al. 1999; Sun et al. 2018). In the process of evolution, cereal seeds stock large amounts of the substrate for these enzymes, making them vulnerable to attack by pests and herbivores. Nonetheless, many plant species have developed defense systems against these attacks, which include the expression of a set of seed inhibitors acting on a range of amylases of different origins (Capocchi et al. 2013). Alpha-amylase inhibitors (AAI) are a type of glycoside hydrolase inhibitors used in this area. AAI were first isolated from wheat in 1933 (Franco et al. 2002). Natural alpha-amylase inhibitors are numerous, mainly including peptidoglycan, alkaloids, glycosides, glycopeptides, polysaccharides, oligosaccharides and other compounds (Coelho et al. 2007), among which flavonoids are a kind of substance with significant inhibitory effect on alpha-amylase activity as well as their inhibitory potential related to the number of hydroxyl groups in compound molecules (Williams et al. 2015).

Diabetes is a type of metabolic disorder and it is a global disease and the patients showing a trend in younger age groups is also observed. Targeting enzymes in the metabolic pathway of diabetes and designing specific enzyme inhibitors is a research area to explore for the treatment of diabetes in the future (Obiro et al. 2008). One of the vital enzymes associated with diabetes is alpha amylases. For human beings, alpha-amylase inhibitors effectively restrain the activity of amylase in saliva and pancreas to inhibit the hydrolysis and digestion of carbohydrates in food, thereby reducing the absorption of glucose (Xiao-Ping et al. 2011). In addition, alpha-amylase inhibitors play a role in reducing blood fat levels and controlling food intake (Obiro et al. 2008). Consequently, it is used in clinical medicine for the prevention and treatment of hyperglycemia, hyperlipidemia and diabetes mellitus type II, etc. (Tormo et al. 2006). In other applications, alpha-amylase inhibitors show a wide range of utilisable value in agriculture. AAI has proven to have defense related activity in botanical systems. Through genetic engineering technology to carry AAI into crop cells or into the genome to generate genetically modified (gm) crops (Shade et al. 1994; Lüthi et al. 2013a), resistance of plants could be improved to defend from diseases and pests and reduce the loss of crop yield (Lüthi et al. 2013b).

Recently, trichosanthrip, a novel ribosome-inactivating proteins (RIPs) with a molecular mass of 10,964.617 Da, was purified from the seeds of T. kirilowii, and its enzyme activity was assayed (Shu et al. 2009). Function analysis indicated that trichosan-thrip belongs to the Alpha-Amylase Inhibitors Lipid Transfer and Seed Storage (AAI_LTSS) superfamily and widely exist in the seeds of plants that can inhibit the amylase activity of a protein, lipid transport and storage amongst other properties. In this paper, the alpha-amylase inhibitor gene from T. kirilowii (TkAAI, GenBank accession no. AJQ68010.1.) was cloned and the expression pattern of TkAAI in different tissues of T. kirilowii was investigated. The TkAAI protein sequences, structure and function were characterized by bioinformatics analyses. Protein was obtained via prokaryote expression in Escherichia coli and alpha-amylase activities were assayed. To our knowledge, this is the first alpha-amylase inhibitor gene characterized from T. kirilowii. The present study revealed sectional biological functions and mechanisms of trichosan-thrip/TKAAI protein in T. kirilowii seeds from the perspective of biochemistry, molecular biology and bioinformatics. The intent of this study was to provide a meaningful insight into the characterization and application value of this kind of multifunctional protein.
**Materials and methods**

**Plant materials and chemicals**

The leaves, stems, roots, flowers, fruits and seeds of *T. kirilowii* were collected from the medicinal botanical garden of Huazhong Agricultural University. They were immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction. Alpha-amylase from porcine pancreas (PPA) were purchased commercially. (Yuanye Bio-Technology Co., Shanghai, China).

**RNA isolation and cloning of TkAAI**

Total RNA was isolated via the total RNA Kit (Promega). The extracted RNA was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, San Jose, California, USA) and the integrity was further analyzed by 1% agarose gel electrophoresis. Prior to cDNA synthesis, total RNA was treated with a DNase to remove DNA contamination from the samples. The first cDNA fragment was synthesized by reverse transcription using the Oligo (dT) primer, AA7-F (5’-CCT ACC GCA CCA CTA TCA CCA-3’) and AA7-R (5’-CCA GTG AGC AGA GTG ACG-3’), and 2 mg total RNA as the template according to the instructions of the PrimeScript® 1st Strand cDNA Synthesis Kit (TaKaRa, Japan). A length in 326 bp fragment of TkAAI was amplified from cDNA prepared from seeds. Two Primers AA7-F (5’-CCT ACCGACCACACTATCA CCA-3’) and AA7-R (5’-GAGACCATTGAAAGTCCGATCG-3’) which contained a conserved sequence were designed based on the homology of the *TkAAI* from *M. charantia* (gil21327880) and *Cucurbita cv.* (gil459404). The degenerate PCR reaction was conducted in a total volume of 20 μL mixture containing 12.7 μL of ddH2O, 2.0 μL of 2.5 mM dNTP mixture, 2.5 μL of Ex Taq buffer (Takara), 0.3 U Ex Taq (Takara), 0.5 mM of each primer, and 1.0 μL of cDNA (50 ng/μL). PCR was performed using the thermal cycle profile of 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, with a final extension of 10 min at 72 °C. One amplified product was recovered using the DNA rapid purification kit (Axygen). The purified PCR products were ligated into pMD-18 T vector (Takara) and then transformed to competent *E. coli* DH 5 cells for sequencing. RACE-PCR was processed to obtain the 3’ end and 5’ end of the cDNA sequence of *TkAAI* via terminal deoxynucleotidyltransferase as previously described (Scotto-Lavino et al. 2007a, b). The 3’ RACE: The cDNA first strand was synthesized by reverse transcription using primer QT (5’-CCAGTGAGCAGTGTAGCG AGGACTCGAGTCTAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
A phylogenetic tree was constructed online by itol (https://itol.embl.de/). TkAAI protein tertiary structure was constructed via the SWISS-MODEL (https://swissmodel.expasy.org/interactive) and Robetta (https://robeta.bakerlab.org/). The accuracy of the tertiary structure models was determined by Savesv6.0 (https://saves.mbi.ucla.edu/).

Gene expression pattern analysis of TkAAI in various organs

Real-time PCR amplification and analysis was performed on CFX96™ Real-Time PCR System (Bio-Rad, California, USA) via SYBR® Premix Ex Taq™ (Tli RnaseH Plus) (TaKaRa, Japan). Primers (GSP-F 5’- AAGAAGGGCGGTCTTGTGAT-3’, GSP-R 5’-TCTGCTCTCCTGAGCAATCT-3’) used for real-time PCR were designed to amplify 100–120 bp fragments from full-length cDNA of TkAAI. A constitutively expressed housekeeping gene, GAPDH (GAPDH-F 5’-TGCAGTACCAACTGCTACG-3’, GAPDH-R 5’-CCTTCACCAAGTCATCCCCC-3’) from cucumber was used for normalization in the quantification of the gene expression in different tissues. qPCR was performed at a final volume of 20 μl containing 1 μL cDNA, 0.5 μL of each primer diluted to 10 mM, 10 μL SYBR® Premix Ex Taq™ and 7 μL ddH2O. The thermal cycle condition used in real-time PCR was: 94 °C for 1 min, followed by 40 cycles of 94 °C for 10 s, and 56 °C for 20 s. Following the real-time PCR cycles, the specificity of the SYBR green PCR signal was confirmed by using melting curve analysis. Data analysis was performed according to the instructions of the manufacturer of the quantitative real-time PCR instrument CFX96™ management software. The expression level for each sample was calculated as $2^{-\Delta\Delta C(t)}$ where Ct represents the cycle number when the fluorescence signal in each reaction reaches the threshold. All samples were repeated three times.

Protein expression and purification

Firstly, there was amplification of the TkAAI coding sequence and reporter gene eGFP fragments. The prokaryotic expression plasmid pET28a was linearized by PCR. Primers for TkAAI, (TkAAI-F 5’-CTT TAAGAAGGAGATAT-ACCATGGCAAGACTC ACAGGTATCATTG-3’, TkAAI-R 5’-TCTCCTGCCC TTGCTCACTTGCT-GCTGCTGCTGCTGCTG AAGGGCCCATCGTCT-3’) eGFP (eGFP-F 5’-GCC CAGACCGATGCC-CTTCCGACAGCAAGACAG CAGCAATGGTAGCAAGGGGAGGAGCTG-3’, eGFP-R 5’-CTTCCATGGGCTTTGTGAT GGTGCTGTGTTGTGCTTTGCTACAGCTCGC CCATGCCAG-3’) and pET28a (Vector-R 5’-GCA TGGACGAGCTGTAACAGCACCACA-CCA CCACCACTAAGAGGGA-CCAAGGAGGCTG AGT-3’, Vector-F 5’-ATACCTGTGATCTTGCC ATGGTATCTTCTCTTAA-AGTTAAACAAA TTATTTCTAG-3’) were used to amplify these three sequences. Then the PCR products were purified using a QIAquick PCR Purification Kit (Cwbio, China). Three purified TkAAI and eGFP fragments were assembled into linearized pET28a to generate TkAAI-eGFP-pET28a plasmid. The recombinant vector was transformed into E. Coli BL21 (DE3) strain for expression of TkAAI-eGFP fusion protein. Then E. coli BL21 (DE3) cells harbouring the expression vector TkAAI-eGFP-pET28a were cultured in LB broth containing 50 μg/mL kanamycin overnight. The cells were cultured for 12 h with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in 16 °C for induction, they were then harvested and then disrupted by sonication on ice. After centrifugation (12,000×g, 10 min at 4 °C), the supernatant was purified by Ni–NTA affinity column chromatography (TransGen, China) and His-tagged recombinant protein was eluted with a linear gradient of 100–300 mM imidazole in 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris–HCL) buffer (pH 7.9).

Western blotting analysis

Purified protein was analysed by 10% SDS–PAGE (80 V, 2.5 h) and transferred to the polyvinylidene difluoride (PVDF) membrane (100 V, 1.5 h). Then, the protein was immersed in the PVDF membrane into 1×TBST (3% (w/v) skimmed milk) for 2 h at room temperature. The membrane was washed by 1×TBST three times, then incubated with primary antibody diluted in 1×TBST (3% (w/v) skim milk, 1:5000) and shaken for 2 h. The membrane was washed as mentioned before. The membrane was then incubated with anti-eGFP secondary antibody diluted in 1×TBST (3% (w/v) skimmed milk (1:10,000) and shaken for 1 h followed by washing as mentioned)

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before. Specific binding was detected with an omnipotent imaging system (Bio-Rad, USA).

Alpha-amylase inhibitory activity assays

The purified protein was concentrated to 0.2 mg/mL by molecular weight cutoff (MWCO) with a 10 kDa ultrafiltration tube (Merk). Twenty μL (4 μg) of TkAAI-eGFP solution and 20 μL of PPA solution (1 units/mL) were incubated at 37 °C for 10 min, then the reaction was started by adding 50 μL 1% (w/v) soluble starch solution to 20 mM phosphate buffer (pH 6.9, containing 6.7 mM sodium chloride) and accurately incubating it for 8 min at 37 °C. The reaction was immediately stopped by adding 50 μL NaOH (1 M) solution. Then it was filled in 50 μL DNS color reagent solution (Solarbio, Beijing) and heated up to 100 °C for color reaction for 10 min. After the reaction, the mixture was cooled to room temperature and 100 μL distilled water was added. The 200 μL samples were taken to measure the absorbance at 540 nm. The formula for calculating the inhibition activity of alpha-amylase inhibition is shown as:

\[
\text{Inhibition(\%)} = 1 - \left( \frac{A_s - A_b}{A_t - A_c} \right) \times 100\%
\]

\(A_s\) is the absorbance of the mixture of TkAAI-eGFP, starch solution, alpha-amylase solution and DNS color reagent of solution. \(A_b\) is the absorbance of the mixture of TkAAI-eGFP, starch solution, phosphate buffer (replace alpha-amylase solution) and DNS color reagent of solution. \(A_t\) is the absorbance of the mixture of phosphate buffer (replace TkAAI-eGFP), starch solution, alpha-amylase solution and DNS color reagent solution. \(A_c\) is the absorbance of the mixture of phosphate buffer (replace TkAAI-eGFP and alpha-amylase solution), starch solution and DNS color reagent of solution.

Results

Cloning the full-length cDNA of TkAAI

The total cDNA was extracted from T. kirilowii seeds. The splicing of RACE PCR products shows full-length sequence of the cDNA consisting of 585 bp nucleotides with a 423 bp open reading frame, encoding a 141 amino acids polypeptide (Supplementary Fig. 1). The deduced amino acid sequence of the cDNA showed that it encoded a polypeptide of approximately 16.14 kDa, with a calculated isoelectric point of 5.36. This gene was designated as TkAAI and the cDNA sequence was deposited in GenBank with accession number AJO68010.1.

Characterization

Blastp showed that TkAAI belonged to the TkAAI super-LTSS family and was specifically classified as alpha-amylase inhibitors (AAIs) and seed storage (SS) proteins (Fig. 1a). ProtParam indicated a strong preference that Arg was the richest amino acid in the composition with a frequency of 14.2%. TkAAI did not contain His, Trp, Pyl and Sec. The instability index (II) was computed to be 59.44 which indicated that the protein was unstable. SOPMA was used to analyse the secondary structure of TkAAI and the result revealed that the protein contained 60.99% α-helices, 9.220% extended strand and 29.79% random coils (Fig. 1b). TargetP-2.0 predicted that from the first to 19th amino acid residues were presumed to be the signal from the peptide region and the cleavage site was in 22nd amino residues. This meant that TkAAI protein was located in the secretion pathway and secreted outside the cells in plants (Fig. 1c). In the meantime, the TkAAI protein contained five alpha-amylase binding sites including the 41st, 94th, 95th, 96th and 103rd amino acid residues (Fig. 1a). These results showed that TkAAI protein shared typical characteristics of proteins from alpha-amylase inhibitors. Sequence analysis was performed through InterProScan and the result indicated that TkAAI contained Bifunctional inhibitor (trypsin-alpha amylase inhibitor)/plant lipid transfer protein/seed storage helical domain (55–139 amino acid residues) and belonged to the Protease inhibitor/seed storage/LTP family (55–139 amino acid residues). Also, the AAI_SS conserved domain was found in TkAAI (44–136 amino acid residues) (Supplementary Fig. 2). A comparison with the NCBI database blastp further demonstrated that TkAAI had extremely high homology with the 2S albumin protein in Alpha-Amylase Inhibitors (AAIs) and the Seed Storage (SS) Protein subfamily. Four 2S albumin from different species
which had relatively high homology to TkAAI were selected for amino acid sequence alignment. The result revealed that TkAAI protein had 70.21% to 83.69% sequence identity to C.maxima 2S (XP_022993226.1, Cucurbita maxima), C.moschata2S (XP_022942593.1, Cucurbita moschata), C.sativus2S (XP_011650534.1, Cucumis sativus) and Bh2S1 albumin-like (XP_038904176.1, Benincasa hispida) (Fig. 1d). The gene bank accession numbers and names referred to in this article of 2S seed storage albu-
mins selected from the 30 species are shown in Supplementary Table 1. The phylogenetic tree was clustered into three large branches, and phylogenetic analysis revealed that TkAAI was closely related to 2S albumin from other species in the Cucurbitaceae family, which stemmed from one of the big branches. This involved species that were phylogenetically related (Fig. 1e). The sequence was uploaded to the SWISS-MODEL database employing comparative modelling. A valid three-dimensional model of TkAAI (Fig. 2b) was generated via Brazil Nut 2S albumin Ber e 1 as a template, whose identity value reached 37.37%.

Fig. 1 Characterization of TkAAI. a Conserved domains of TkAAI. b Calculated secondary structure of TkAAI protein, blue area indicates alpha helix, red area indicates extended strand and purple area indicates random coil. c Signal peptide and subcellular localization analysis of TkAAI. d Alignment and of the amino acids sequences between TkAAI and 2S albumin from other four cucurbitaceous species. The con-
served cysteine sequence of 2S albumin was marked with red triangle (Wilding et al. 2009). e Phylogenetic relationship of 2S albumin and 2S albumin-like. The Phylogenetic tree analy-
isis was performed by the MEGA 7.0 program with the neighbor joining method and with 1000 replicates. The phylogenetic
tree was constructed based on the alignment of amino acids
sequences of 30 orthologs proteins, different color indicated to
different species, and TkAAI was marked in red
Nonetheless, the GMQE (Global Model Quality Estimate) value was only 0.44, which means that the modelling of the protein was not sufficiently reliable. Therefore, the Robetta servers were employed to predict the tertiary structures of the TkAAI (Fig. 2a). To estimate the quality of protein models, PROCHECK, Errat and Verify_3D of SAVESv6.0 servers was simultaneously employed to validate the accuracy and quality of the structures. Errat evaluated the three-dimensional structure of proteins based on crystallography to identify incorrect regions of protein. The overall quality factor of Robetta (100.00)’s protein model was apparently higher than from the SWISS-MODEL models (91.01). In addition, VERIFY3D indicated that the percentage of residues with averaged 3D-1D score ≥ 0.2 of protein models from Robetta was 70.21%, which was also higher than from the SWISS-MODEL models (44.12%) (Mahtarin et al., 2020). Besides this set of findings, the results of eight evaluations from PROCHECK likewise supported that the models from Robetta were significantly superior models from the SWISS-MODEL in passage and error rates.

Gene expression pattern analysis of TkAAI in various organs

The mRNA expression level of TkAAI in T. kirilowii was investigated via real-time PCR. The results verified that TkAAI was detected in stems, fruits, roots, flowers and seeds. It was found that TkAAI expressed extensively in stems and fruits. Transcripts were relatively more abundant in seeds (**P < 0.01) (Fig. 3), followed by detection in stems and fruits. Consequently, the following investigation of the TkAAI expression could be focused on different developing periods of seeds. In the early stage of seed germination, the activity of alpha-amylase was enhanced to catalyze the hydrolysis of starch stored in the endosperm to produce small molecular reducing sugars (Stanley et al. 2011). The decomposition of these sugars provides a direct source of energy for the elongation of the seed coleoptile (Daussant et al. 1983). Hence, it was deduced from this that TkAAI directly affected the activity of alpha-amylase in endosperm to regulate the formation, maturation and germination of T. kirilowii seeds.
Optimal design of heterologous expression of TkAAI

As a type of reporter gene, eGFP was introduced downstream of TkAAI. In the meanwhile, the 6-His-tidine tag was introduced to C-terminus to purify fusion protein. In the *E. coli* expression system, the adjustment of induction temperature (°C), induction time (h) and inducer IPTG concentration (mM) all affected the expression of TkAAI protein. Hence, the present experiment adopts three significant factors affecting prokaryotic expression. Using SPSS Statistics 19 to design (final concentration of IPTG (0.1 mM, 0.5 mM, 1 mM), induction temperature (16 °C, 28 °C, 37 °C), induction time (4 h, 8 h, 12 h)) a three factor and three-level orthogonal experiment, a total of nine treatments were carried out. The three-factor and three-level design and the combination of experimental treatments is shown in Table 1.

The results of the range analysis demonstrated that the primary and secondary order of factors affecting the relative fluorescence intensity of TkAAI-eGFP bacterial solution was induction temperature, IPTG concentration, and induction time. This hence indicated that induction temperature played a significant role in expression efficiency of the TkAAI-eGFP prokaryotic expression system. After inducing for 12 h under 0.5 mmol final IPTG concentration and 16 °C inducing expression temperature, the relative fluorescence intensity of TkAAI-eGFP fusion protein was the highest and significantly higher than other treatment groups (*P < 0.05). The relative fluorescence intensities of the induced bacterial solution under 16 °C (Trials1, 2, 3) were also significantly varied from other trials with different temperature.

![Relative expression level of TkAAI in different tissues of T. kirilowii.](image)

Optimal design of heterologous expression of TkAAI

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Table 1 L9 (3³) orthogonal design table and experimental results, relative fluorescence intensity values are shown as mean ± SD

| Combination | A Induction temperature (°C) | B Induction time (h) | C Concentration of IPTG (mM) | Relative fluorescence intensity |
|-------------|-----------------------------|---------------------|-----------------------------|--------------------------------|
| 1           | 16                          | 4                   | 0.1                         | 0.45 ± 0.081c                  |
| 2           | 16                          | 8                   | 1                           | 0.76 ± 0.034b                  |
| 3           | 16                          | 12                  | 0.5                         | 0.95 ± 0.043a                  |
| 4           | 28                          | 4                   | 0.5                         | 0.31 ± 0.0043d                 |
| 5           | 28                          | 8                   | 0.1                         | 0.28 ± 0.042d                  |
| 6           | 28                          | 12                  | 1                           | 0.19 ± 0.016c                  |
| 7           | 37                          | 4                   | 1                           | 0.069 ± 0.038f                 |
| 8           | 37                          | 8                   | 0.5                         | 0.027 ± 0.032f                 |
| 9           | 37                          | 12                  | 0.1                         | 0.035 ± 0.0057f                |
| S1          | 2.16                        | 0.83                | 0.77                        |
| S2          | 0.78                        | 1.06                | 1.29                        |
| S3          | 0.13                        | 1.18                | 1.02                        |
| K1          | 0.72                        | 0.28                | 0.26                        |
| K2          | 0.26                        | 0.35                | 0.43                        |
| K3          | 0.04                        | 0.39                | 0.34                        |
| R           | 0.68                        | 0.12                | 0.17                        |

Fluorescence analysis of TkAAI-eGFP production by using Duncan’s multiple range test

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Simultaneously, observation of the bacterial liquid by super resolution laser confocal microscope (Nikon) could yield fluorescence intensity of the cells treated with IPTG to be higher than the blank control. It indicated that IPTG could significantly up-regulate the expression level of protein in *E. coli* (Supplementary Fig. 3).

The molecular weight of TkAAI-eGFP infusion protein was estimated to 44 kDa according to the encoding cDNA. The results of SDS-PAGE analysis demonstrated that there were obvious target bands in the 44 kDa region in lane 2 and lane 3. It is also to be noted that after purification by Ni–NTA column, a certain purity target band could be detected (Fig. 4a).

**Western blotting analysis**

Western blotting displayed that non-specificity binding in lane 1 (negative control), and for lanes 2 to 6, there were specific binding bands aggregating for the range of molecular weights from 27 to 44 kDa. Interestingly, the predicted molecular weight of eGFP protein was 27 kDa. Accordingly, we deduced that the fusion protein may undergo protease cleavage leading to the separation of TkAAI and eGFP peptides, or the mechanical fracture of the fusion protein caused by the external force (Shear force generated by ultrasonication) applied during the extraction of the separated protein, which caused the fragmented eGFP to accumulate in the 27 kDa region (Fig. 4b).

**Alpha-amylase inhibition assay**

The qualitative alpha-amylase inhibitory activity measurement of TkAAI-eGFP fusion protein (4 μg) on PPA (1 unit/mL) was performed under a solution condition of pH 6.9 and 37 °C. The experimental result manifested that the TkAAI-eGFP fusion protein represented 71.3 ± 4.4% (mean ± SE values (n = 3)) alpha-amylase inhibitory activity against PPA.

**Discussion**

The full-length cDNA of TkAAI was cloned from the seeds of *T. kirilowii* by RACE PCR. The gene expression pattern indicated that the transcript of TkAAI in seeds was the most abundant and the expression levels were significantly higher than the other tissues. Blast alignment annotates that 40–136 amino acids in the TkAAI protein sequence were AAI_SS domains. Annotation display members of the AAI_SS subfamily involve cereal-type alpha-amylase inhibitors and seed storage protein. TkAAI protein contained multiple functional domains, which played a significant role in plant response to different stresses such

![Fig. 4 Detection of heterologous expression of TkAAI. a 10% SDS-PAGE gel electrophoresis of TkAAI-eGFP. Lane M indicated marker. Lane 1 indicated total protein from *E. coli* carrying pET28a. Lane 2 indicated total protein from *E. coli* carrying TkAAI-eGFP-pET28a with IPTG induction. Lane 3 indicated supernatant of ultrasonic treated bacterial solution from *E. coli* carrying TkAAI-eGFP-pET28a with IPTG induction. Lane 4 indicated flow-through fluid. Lane 5 indicated 10 mM Imidazole washing solution. Lane 6, 7, and 8 indicated Ni–NTA column purified TkAAI-eGFP. b Western blotting detection of TkAAI-eGFP by eGFP antibody. Lane M indicated marker. Lane 1 indicated total protein from *E. coli* carrying pET28a. Lane 2 indicated supernatant of ultrasonic treated bacterial solution from *E. coli* carrying TkAAI-eGFP-pET28a without IPTG induction. Lane 3 indicated total protein from *E. coli* carrying TkAAI-eGFP-pET28a with IPTG induction. Lane 4 indicated supernatant of ultrasonic treated bacterial solution from *E. coli* carrying TkAAI-eGFP-pET28a with IPTG induction. Lane 5 and 6 indicated Ni–NTA column purified TkAAI-eGFP](image)
as insects, microorganisms and other environmental factors.

NJ phylogenetic tree analysis revealed a high ortholog of TkAAI with 2S albumin. Moreover, 2S albumin was reported to be a member of the alcohol-soluble superfamily. Most of these small molecules with a molecular weight of about 12–15 kDa come from dicotyledonous plants. 2S albumin had eight or more conservative cysteine patterns Cys-(X)n-Cys55-(X)n-Cys86-Cys87-(X)n-Cys97-X-Cys99-(X)n-Cys-(X)n. We further confirmed this feature in the alignment of amino acid sequences (Fig. 1d). The conservative cysteine pattern of TkAAI is Cys43-(X)n-Cys132-(X)n-Cys139. We further confirmed this feature in the alignment of amino acid sequences (Fig. 1d). The conservative cysteine pattern of TkAAI is Cys43-(X)n-Cys55-(X)n-Cys86-Cys87-(X)n-Cys97-X-Cys99-(X)n-Cys132-(X)n-Cys139. However, only six cysteine residues in this domain may have formed three pairs of disulfide bonds (Fig. 2d). The conservation of these cysteine sequences and disulfide bond pairs may be related to the specific binding of lipids in plant seeds, thereby protecting the development and reproduction of plant embryo sacs (Zhang 2009). The Robetta server was employed to guarantee the accuracy of the tertiary structure modeling of the tertiary structure of TkAAI (Kim et al. 2004).

If the expression rate of heterologous protein was too high and there was no molecular chaperone to assist protein folding in the expression system, the newly formed protein may not fold correctly to form a reasonable conformation, resulting in the formation of soluble and inactive inclusion bodies, which is not conducive to the follow-up study of the biological activity of the protein (Baneyx and Mujacic 2004). Reports have shown that within a condition of a low temperature range, the folding process of the protein came to be true and the rate of the formation of inclusion body may be reduced (Donovan et al. 2000). We confirmed the favorable effect of low temperature (16 °C) on the soluble expression of the TkAAI-eGFP fusion protein, together with appropriate IPTG concentrations ranging around 0.5 mM, which was rapidly detected via determining the relative green fluorescence intensity signal of the bacterial solution under different treatments.

With the rapid development of maturity of molecular cloning technology, plenty of plant-derived alpha-amylase genes have been reported in recent years (Bunyatang et al. 2016; Bhide et al. 2017; Yu et al. 2017). Interestingly, the tertiary structure of AAI from Amaranthus hypochondriacus contained three conserved cysteine residues which were the same as TkAAI, and cysteine residues were reported to maintain the stability of these types of proteins (Lehmann et al. 2006). However, the difference was embodied in the number of remaining conservative disulfide bonds between AhAI (1) and TkAAI (2), which revealed the conservation and tendency of variation of AAI during evolution (Zhang 2009). It was worth mentioning that the methanolic extract of T. kirilowii revealed a high anti-amylase activity (Uchholz et al. 2016). Results of enzyme assays and previous reports have demonstrated that T. kirilowii, as a traditional Chinese medicine (TCM), possessed the ability to produce strong alpha-amylase inhibitory substances. Generally, alpha-amylase inhibitors naturally-derived from the plants had few negative effects on humans, so the prospect of alpha-amylase inhibitors peptides could be developed as a treatment for diabetes mellitus type II by alleviating abnormal absorption of carbohydrates (Quintero-Soto et al. 2021).

To our knowledge, TkAAI was the first alpha-amylase inhibitor gene extracted from T. kirilowii. Inhibitory activity of TkAAI against PPA was preliminarily measured. Interestingly, the alpha-amylase inhibition mechanism of the cereal-type inhibitor superfamily had been reported (Strobl et al. 1998). Ragi bifunctional alpha-amylase/trypsin inhibitor (RBI) competed with the substrate for the amylase active site (Asp185, Glu222 and Asp287) in alpha-amylase yellow mealworm alpha-amylase (TMA). Due to the high homology of TMA and PPA in binding to substrate and binding to inhibitor sequences (Strobl et al. 1997), we speculated that TkAAI, which also belonged to cereal-type inhibitors and contained a similar domain with RBI, had a similar competitive inhibitory pattern to PPA. Subsequent experiments were necessary to be designed for more mammalian alpha-amylases to further explore the inhibitory pattern between TkAAI and alpha-amylase.

Our study qualitatively investigated the alpha-amylase inhibitory activity in T. kirilowii. Our further investigations included giving the kinetic value of the inhibition as IC50 (half maximal inhibitory concentration). In the meantime, it was also worth designing the enzymatic kinetic experiments expanded around the multi-site mutants of TkAAI, such as the as-before mentioned five alpha-amylase binding sites including Glu61, Asp94, Glu95, Glu96 and Glu103 amino acid residues (Rydberg et al. 2002). It is also
worth mentioning that plant hormones such as gibberellin (GA) and abscisic acid (ABA) jointly regulated the expression and activity of alpha-amylase genes by antagonizing or promoting effects to control the process of seeds from embryogenesis to germination (Gómez-Cadenas et al. 2001). Whether TAA1 and plant hormones co-regulate the processes of T. kirilowii seeds at the above given period is also an interesting direction of research.

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Author contributions All authors contributed to the study conception and design. ZYP participated in design of the study, performed the clone, and identified the gene, analyzed the data and drafted manuscript; HQY helped designed data and drafted manuscript; SSH helped designed enzymatic reaction. WKY contributed to enzymatic reaction. All authors agree with the policies in the ethical Statement.

Data availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no financial or proprietary interests in any material discussed in this article.

Ethical approval The present article does not involve intervention on a population of humans and/or animals directly and all authors agree with the policies in the ethical Statement.

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