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Cloning of a Novel Trypsin Inhibitor from the Traditional Chinese Medicine Decoction Pieces, Radix Trichosanthis

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Abstract

Most herbs of traditional Chinese medicine (TCM) are used as air-dried decoction pieces that are manufactured and kept at ambient temperature for long periods. Given the ability of some desiccation-tolerant plants to conserve RNA, it could be worthwhile to isolate mRNA from TCM decoction pieces as part of a transcriptomic strategy to identify new substances with potential pharmaceutical application. Here, we report the molecular cloning of a novel trypsin inhibitor (as the probable allelic variants TKTI-2 and TKTI-3) from the decoction piece of Radix Trichosanthis, representing the dried root of \textit{Trichosanthes kirilowii}. From this material, the total RNA was extracted and a cDNA library was constructed from the isolated mRNA from which the cDNAs of two precursors were successfully cloned and sequenced. TKTI-3 showed an amino-acid substitution in the otherwise highly-conserved P1-P1’ reaction site of the mature peptide, which we confirmed to not be an artefact. Subsequent analysis using LC-MS/MS detected the presence of specific tryptic peptides expected from TKTI-3, confirming the presence and expression of this locus in Radix Trichosanthis. More generally, this study indicates that mRNA can persist in decoction pieces and so could present a viable option for the molecular cloning from other TCMs.

Keywords Chinese medicine; \textit{Trichosanthes kirilowii}; trypsin inhibitor peptide; molecular cloning; MS/MS sequencing

Abbreviations

TCM, traditional Chinese medicine; cDNA, DNA complementary to RNA; DTT, dithiothreitol; ORFs, open reading frames; IAA, iodoacetamide; TFA, trifluoroacetic acid; NCE, normalized collision energy

1. Introduction

Traditional Chinese medicine (TCM) has a long history in maintaining the health of the Chinese population. In addition to continuing to fulfil that role today, TCM also represents a promising source
for lead drug discovery as exemplified by the discovery of the anti-malarial drug artemisinin [1]. In this new context, contemporary research has focused on the extraction, isolation and identification of bioactive compounds from TCM followed by an evaluation of their biological activities [2]. Proteins and peptides from the herbs of TCM have attracted the attention of the research community because of their bioactivities, especially in the areas of antimicrobial and anticancer research [3, 4], as well as their potential economic benefits [5].

To date, the discovery of proteins and peptides from TCM derives mainly via chemical extraction using organic solvents [6], although a few studies have demonstrated the discovery of novel proteins and peptides via transcriptomic studies from fresh [7] or snap-frozen fresh plant tissues [8, 9]. However, most herbs of TCM are present in the form of sliced or cropped decoction pieces because they are harvested in their habitats and then air-dried. Although this situation would normally present a hindrance for transcriptomic studies, pioneering work has shown that some desiccation tolerant plants are able to conserve RNA during either rapid or slow dehydration [10, 11] such that transcriptomic studies have been accomplished on desiccation-tolerant plant species using their dehydrated tissues [12, 13]. Altogether, these results suggest that it might be feasible to study the transcriptome using the RNA from dried Chinese medicinal herbs (i.e., from the decoction pieces) purchased from a pharmacy rather than the more labour-intensive option of sampling from the local habitats.

Herein we examined a typical TCM, the root of Chinese Snake Gourd (*Trichosanthes kirilowii*), known as Radix Trichosanthis, to test for potential for the conservation of RNA in dried plant material. This specimen has demonstrated extensive medicinal value in TCM [14], with the dry roots, ripe fruits, ripe seeds and pericarp having been officially recorded for the treatment for different symptoms in the Chinese Pharmacopoeia. For instance, the root is used as an antiphlogistic and an abortifacient, whereas the dry, ripe fruits are used as an antipyretic and in the treatment of constipation [15]. In addition, Radix Trichosanthis is known to contain bioactive proteins and peptides, including the ribosome-inactivating protein trichosanthin [16] and squash trypsin inhibitors [17]. Under this framework, we attempted to extract the total RNA from the decoction pieces of Radix Trichosanthis using the subsequent molecular cloning of the squash trypsin inhibitor peptide-encoded mRNA as a proof of concept. In this, our experiments were successful insofar as we can report the discovery of two variants of a novel squash trypsin inhibitor peptide.

2. Materials and methods

2.1. Specimen biodata

Specimens of *T. kirilowii* in the form of Radix Trichosanthis, which are the dried root pieces of *T. kirilowii*, were obtained from a commercial source (lot number 20086M302P2SW1000; Hebei Anguo Medical Materials Corporation; Anguo, China) and deposited in Nanjing University of Chinese Medicine. The corresponding authors verified the samples as Radix Trichosanthis according to Pharmacopoeia of the People’s Republic of China [18] (see Figure S1 and S2). Before being used, all samples were pulverized before being filtered through a standard sieve of mesh size 250 μm ± 9.9 μm.

2.2. RNA extraction

Total RNA was extracted from the powdered Radix Trichosanthis using TRIzol RNA extraction method (Invitrogen; Vilnius, Lithuania) with modifications according to Wang et al. [19]. In detail, 200 mg of Radix Trichosanthis was mixed with 400 μl of lysis buffer (1% SDS and 2% β-mercaptoethanol in 100 mM Tris-HCl (pH 9.0)) and incubated at room temperature for 15 min. After
centrifugation at 12,000× g for 10 min at 4 ºC, the supernatant was transferred into 800 µl of TRIzol and incubated at room temperature for 10 min. Thereafter, the total RNA from the aqueous phase was collected after adding 240 µl of chloroform and centrifugation at 12,000× g for 10 min at 4 ºC. Subsequently, the total RNA was precipitated by mixing it with an equal volume of isopropanol and incubating at -20 ºC for 20 min. The RNA pellet was collected by centrifugation for 10 min and resuspended in 400 µl of DEPC-treated water. Additional DNA residue was removed using an equal volume of citrate buffer saturated phenol (pH 4.3) : chloroform (1:1, v/v) after which the aqueous phase was washed using an equal volume of chloroform. The RNA was precipitated again by adding 1/10 volume of 3 M sodium chloride and two volumes of ice-cooled ethanol at -80ºC for 30 min. Afterwards, the RNA pellet was washed using 70% ethanol and finally redissolved in DEPC-treated water. The concentration of extracted RNA was measured by using a NanoDrop™ OneC.

2.3. Molecular cloning

Polyadenylated mRNA was isolated from the extracted, total RNA using a Dynabeads® mRNA DIRECT™ Kit (Invitrogen; Vilnius, Lithuania). In detail, the total RNA was resuspended in 500 µl of lysis/binding Buffer (provided in the kit) and hybridized with the magnetic Dynabeads at room temperature. The mRNA/beads complex was further washed using Washing Buffers A and B (provided in the kit) three times and finally eluted in 20 µl of DEPC-treated water. The mRNA so obtained was then reverse-transcribed into a cDNA library using SMART RACE cDNA Kit (Clontech; Palo Alto, USA) and then subjected to a nested PCR, employing forward (5'-CTTGGTGATGGCAGCTTTTGTAGAGTCT-3') and reverse primers (5'-CAGAAGATCCGACACGGCGGAGT-3') that were designed from the signal peptide and 3'-untranslated regions, respectively, of a trypsin inhibitor encoded cDNA from T. kirilowii (GenBank accession number X82230). The 3'-RACE PCR also served to verify the presence of the polyA tail of the peptide encoding mRNA. The PCR products were purified by an E.Z.N.A.® Tissue DNA Kit (Omega Bio-Tek; Norcross, GA, USA), cloned into a pGEM-T vector (Promega Corporation; Southampton, UK), and sequenced using an ABI3100 automated capillary sequencer (Applied Biosystems; FosterCity, CA, USA). In total, each of the 32 monoclonal DNA samples were sequenced twice.

2.4. Extraction of total protein

Five grams of filtered, powdered Radix Trichosanthis were mixed with 50 ml of 50% ethyl alcohol for 10 h. After removing the ethyl alcohol using an evaporator, the remaining solution was centrifuged at 13,000× g and the supernatant was snap-frozen in liquid nitrogen and lyophilised. One hundred milligrams of this lyophilised extraction was prepared for total protein isolation using the Invitrogen TRIzol Reagent Kit (Invitrogen; Vilnius, Lithuania) according to the manufacturer’s recommendations.

2.5. Digestion, alkylation and trypsin digestion

One hundred micrograms of the extracted proteins were denatured in 8 M urea and 25 mM Tris-HCl. The cysteines were then alkylated using 40 mM iodoacetamide (IAA) over a 30-min incubation period at room temperature and in the dark before being reduced using 20 mM dithiothreitol (DTT) at 60 ºC for 1 h. The alkylation reaction was then quenched by adding an additional 10 mM DTT. Finally, the proteins were trypsinized using 1 µg MS-grade trypsin (Promega Corporation; Southampton, UK) for 16 hours at 37 ºC after which the protein solution was 10-fold diluted by adding 100 mM ammonia...
bicarbonate. The digestion was stopped by adding trifluoroacetic acid (TFA) to yield a final 1% (v/v) solution. The tryptic peptides were desalted using Ziptip (Merck; Darmstadt, Germany) and stored at -20 ºC until MS analysis.

2.6. Identification and structural analysis of the predicted mature peptide

MS and MS/MS characterization and detection of the tryptic peptides derived from Radix Trichosanthis was performed using an LCQ ion-trap mass spectrometer (Thermo Inc., San Jose, California, USA) by means of electrospray ionization (ESI). A gradient elution of solvents A (water containing 0.1% FA) and B (acetonitrile with 2% water and 0.1% FA) was applied with a flow rate of 200 µl/min as follows: 0-5 min, 0% solvent B; 5-80 min, 0%-50% solvent B; 80-105 min, 50%-100% solvent B; and 105-120 min, 100% B. The nitrogen sheath and auxiliary gas flow were maintained at 20 and 5 arbitrary units, respectively. The heated capillary temperature was 300 ºC and the spray voltage was set to 5 kV in positive ion mode. The three ions with the highest intensity were subjected to MS/MS fragmentation under a normalized collision energy (NCE) of 30 and the resulting fragment ion profiles were then trawled using the Protein Discoverer 1.0 (Thermo Finnigan; San Jose, California, USA) against a customized FASTA database. Only peptides with sufficient confidence (i.e., probability > 40) were retained.

3. Results

The total RNA was successfully extracted from Radix Trichosanthis at a concentration of 15.4 ± 4.4 µg/g dried sample weight (n = 5). The A260/A280 and A260/A230 ratios were 1.96 ± 0.06 and 1.00 ± 0.17, respectively. The subsequent isolation of the mRNA and the amplification of the squash trypsin inhibitor-encoded cDNA was confirmed via observation of the appropriate DNA band on the agarose gel (Figure 1). Finally, two novel trypsin inhibitors precursor-encoded cDNAs were cloned from the constructed cDNA library (Figure 2), with the presence of polyadenylated tails for both cDNAs being verified by 3'-RACE (Figure S3). The open-reading frames of both precursors consisted of 65 amino-acid residues and showed a high degree of identity with the trypsin inhibitor known from same species (GenBank accession number X82230; here named TKTI-1). A comparison of the nucleotide sequences of the cloned cDNAs (TKTI-2 and TKTI-3, respectively) with that of TKTI-1 reveals one and three differences, respectively (Figure 3a), resulting in amino-acid changes at positions 38 (A/G) and, for TKTI-3 only, 42 (I/V) (Figure 3b); only the latter change is within the putative mature peptide (Figure 2). A further, broader comparison with other known squash trypsin inhibitors in the Uniprot protein database from Sicyoeae, a tribe in the subfamily Cucurbitaceae to which T. kirilowii belongs, shows the high degree of conservation among all mature peptides as well as the same disulphide bridge motif (Figure 4). The I/V mutation at the N-terminal domain of TKTI-3 is unique, however. Both cDNA encoding precursors have been deposited into GenBank with the accession numbers MF770981 and MF770982, respectively.
Figure 1. The PCR gel electropherogram of trypsin inhibitor precursor-encoded cDNAs from the cDNA library of the decoction pieces of Radix Trichosanthis. In the negative control, the PCR was performed without the cDNA template.
Figure 2. Nucleotide sequences and translated open reading frames of cloned cDNAs encoding the biosynthetic precursors of (A) TKTI-2 and (B) TKTI-3 from *T. kirilowii*. The putative mature peptide sequences as determined from [20] are underlined.
Figure 3. Alignments of nucleotide sequences (A) and translated open reading frames (B) of cloned cDNAs encoding the biosynthetic precursors of TKTI-2 and TKTI-3 and the previously identified trypsin inhibitor (TKTI-1) from *T. kirilowii* (accession number X82230). Identical nucleotides are colored in red.
Figure 4. Alignment of the sequences of trypsin inhibitor peptides known from the tribe Sicyoeae and present in the UniProt database against the two novel peptides discovered in this study. The pattern of disulphate bridges are indicated by the lines 1, 2 and 3. Sites of amino acid identity are indicated by asterisks.

LC-MS/MS analysis of the tryptic peptides from the extracted total proteins of Radix Trichosanthis (Figure S4) identified the specific tryptic peptides from TKTI-1 and the protein trichosanthin (Table S1) as well as VLMPCKVNDCLR from TKTI-3. The latter was also confirmed through the corresponding MS/MS spectrum (Figure 5) to support the expression of two TKTI variants in Radix Trichosanthis.

![Figure 5](image_url)

Figure 5. Identification of the specific tryptic peptide VLMPCKVNDCLR derived from TKTI-3. (A) The singly- and doubly-charged b-ions (red) and y-ions (blue) that arise from MS/MS fragmentation of the peptide. (B) The annotated MS/MS spectrum of the tryptic peptide, VLMPCKVNDCLR.
4. Discussion

For research involving the molecular cloning of proteins and peptides from TCMs, the availability of good-quality RNA is arguably most significant factor influencing the success of the research. However obtaining fresh specimens is often challenging and time-consuming because the herbs used for TCM are widely-distributed within China, growing in both urban areas as well as in remote localities. In addition, the harvesting period for different plants, and even different parts from the same specimen, can vary. A viable alternative, therefore, might be to perform the transcriptomic studies using the dried plant material given the finding that useful RNA can still be extracted from the dehydrated plant tissue of a desiccation-tolerant moss [10]. Moreover, some plants exhibit vegetative desiccation tolerance when their tissues undergo a rapid or slow dehydration [22] such that their intracellular RNA is protected from degradation and can be used for protein synthesis during rehydration [23]. However, to our knowledge, the potential of performing transcriptomic studies using RNA derived from the dried herbs of TCMs has not been well investigated. Instead, the more common method remains the use of fresh material (e.g., the discovery of the ribosome inactivating protein as well as TKTI-1 from the mRNA in the fresh root tube of T. kirilowii [21]), usually by flash-freezing the tissues using liquid nitrogen and the extracting the mRNA from the ground or lyophilized samples [24].

To facilitate our proof of concept that mRNA could be extracted from the dried, decoction pieces of Radix Trichosanthis, we used 3'-RACE to demonstrate the presence of polyadenylated mRNA tails in our extracts. Moreover, our use of two specific primers targeted to a trypsin inhibitor sequence (TKTI-1) known for this species revealed that the mRNA is also intact. Interestingly, however, instead of recovering the TKTI-1 sequence, the two slightly modified and novel trypsin inhibitor peptides TKTI-2 and TKTI-3 were found, thereby also demonstrating the potential of investigating such material for new sequences. As demonstrated by a previous study [25], variants of squash trypsin inhibitors have been isolated from the same specimen with different mRNA expression levels in the same plant tissue, suggesting that the TKTI variants probably represent different alleles of the same gene. Reinforcing this hypothesis is the fact that the 3'-UTR regions are also identical between TKTI-2 and TKTI-3.

Together with TKTI-1, these new trypsin inhibitor peptide variants all possess the highly-conserved structural motif of the squash inhibitor family, and especially from those in the tribe Sicyoeae, which consists of 27-33 amino-acid residues containing six cysteine residues that form three cross-linked disulphate bridges [26]. Unlike TKTI-1, both prepropeptides of TKTI-2 and TKTI-3 contain a glycine instead of an alanine immediately before the mature peptide domain. However, this could not be confirmed via MS/MS because the glycine is not within the mature peptide domain. TKTI-3 also presents a second mutation unique within the squash inhibitor family in which a valine is present at the conservative P1' position of these Bowman-Birk-type inhibitors instead of isoleucine (Figure 4) [26], a finding that we independently confirmed by an MS/MS analysis of the total proteins to exclude the possibility of an artefact (Figure 5). Although this change does take place at a conservative active site, we assume that the trypsin inhibitory activity could still remained largely unchanged because the P1' position is not essential for activity, although small effects on affinity cannot be ruled out [27].

In summary, our results document the success of molecular cloning of mRNA from the commonly-used TCM decoction pieces of Radix Trichosanthis by the identification of the corresponding cDNA and encoded peptides from a known member of the squash trypsin inhibitor
family. Thus, although RNA can be extracted from such dried plant material, we also observed that less total RNA was extracted compared to values from the literature [19]. We therefore speculate that the extraction method might need further modifications specific for the decoction pieces of Radix Trichosanthis or other TCMs. However, it cannot be ruled out that RNA could be partially degraded in such plant material given that the manufacturing process for TCMs is probably not performed in an “RNA friendly” manner. Nevertheless, if our result does indeed hold more generally, our study presents a cost-effective and labour-saving option to extract the mRNA from the herbs used in TCM via their decoction pieces.

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Compliance with ethical standards

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

Additional Supporting Information may be found in the online version of this article.

Figure S1. Appearance of the Radix Trichosanthis specimen, including (a) the whole cross section, (b) the enlargement of part of (a), and (c) clear radially arranged xylems.

Figure S2. The powder microstructure of pulverized Radix Trichosanthis. (a) Starch grains. (b) Vessel fragment. (c) Sclereid.

Figure S3. Nucleotide sequences and the translated open reading frame amino-acid sequences of cloned cDNAs encoding the biosynthetic precursors of two novel trypsin inhibitor peptide variants from Radix Trichosanthis with the presence of polyadenylated tails.

Figure S4. Total ion currency of mass spectrometry analysis of the digestive peptides mixture of the proteins extracted from Radix Trichosanthis.

Table S1. Identified peptide fragments from the trypsin digestion of the alkylated proteins from Radix Trichosanthis.

References


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