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# TYchi, a novel chitinase with RNA N-glycosidase and anti-tumor activities

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## 1. ABSTRACT

Chitinases which catalyze hydrolysis of chitin are believed to be antifungal proteins in plant. Nevertheless, a variety of functions and some new enzymatic activities of chitinases have been found in recent years. We cloned a novel protein from *Trichosanthes kirilowii* Maximowicz (Family Cucurbitaceae) named TYchi. Expression of TYchi gene in *T. kirilowii* plants was induced by *F. oxysporum*, an important cucurbitaceous fungal pathogen, which indicated that TYchi involved in the pathogen-induced plant defense reaction. In addition to its chitin-hydrolytic activity, the recombinant TYchi protein also had RNA N-glycosidase property. In cell-free rabbit reticulocyte lysate system, TYchi inhibited protein synthesis with an $IC_{50}$ of approximate 5 nM. TYchi also exhibited efficient cytotoxicities to leukemia U937 and choriocarcinoma JAR cells with $IC_{50}$ about 54 μg $ml^{-1}$ and 73 μg $ml^{-1}$, respectively. Structure analyses indicated that the putative domain of TYchi is highly similar to the well known active domain of the N-glycosidase trichosanthin (TCS). This bifuntional protein should be useful in diverse applications like RIP-based immunotoxin agent and genetic engineering of plant resistance.

## 2. INTRODUCTION

The plant antifungal proteins play important roles in defense response against fungal infections. Chitinases and ribosome inactivating proteins (RIPs) belong to the best known antifungal proteins (1, 2). Synergistic effects of the two enzymes are observed both *in vitro* and *in vivo* in defense response process (3). RIPs are RNA N-glycosidases that inactivate ribosomes through a site-specific deadenylation of the ribosomal RNA thereby blocking translation and causing cytotoxicity. Because several of RIPs are found to be more toxic to tumor cells than to normal cells, they are considered as potential antitumor agents (4). Therefore, many RIPs are isolated from various plants, and attentions have been paid to their multiple activities, including polynucleotide:adenosine glycosidase, DNase, and RNase activities (5). The classical enzyme activity of chitinases is catalyzing the hydrolysis of beta-1,4-linkages of the N-acetylglucosamine of chitin, a major structural component of fungal cell walls (2). So plant chitinases are thought to play important roles in plant defense responses to fungal pathogens. Chitinases have been cloned or isolated to make studies of their classical enzyme activity, but investigations were less focused on

**Table 1.** List of primers used in different experiments

| Primer name | Sequence |
|---|---|
| AD | forward: 5'-GGAATCGC(C/T)(A/G)T(C/T)TA(C/T)TGGGG(C/T)C-3' |
| | reverse: 5'-CCA(T/C)A(A/G)CAT(G/C)ACTCCTCCATA(G/C)-3' |
| $AD_2$ | 5'-NGTCGA(G/C)(A/T)GANA(A/T)GAA-3' |
| $AD_{2-2}$ | 5'-NGTGCA(G/C)(A/T)GTNT(A/T)GAA-3' |
| group 1 | 5'-GAAGGCGCAACCATTGTTGCTG-3' |
| | 5'-CAAGGTTCAGGACCGGAGTTCG-3' |
| | 5'-TCCGGTGGAACAAGTGAAGGAA-3' |
| group 2 | 5'-GGCAAGGCCAACAATCTCCTGA-3' |
| | 5'-TCTTGGAACCGGTGGTTGTCGT-3' |
| | 5'-GGTTTATTCCGGCAAACGTGCT-3' |
| TYchi-5 | forward: 5'-GCGCCCATGGCCGGAATCGCCAT-3' |
| TYchi-3 | reverse: 5'-CGCGCTCGAGAAGACTGCCTTTGAT-3' |

new enzymatic properties of chitinases. In recent years, such study of multifunctional chitinases has increased, and new enzymatic activities of chitinases have been found including chitosanase activity during symbiotic interaction (6), actin-binding activity in cytoplasmic agglutination (7), ice-binding activity in freezing tolerance (8), and insect amylase suppression activity (1). Moreover, three proteins were isolated and reported had chitinase and ribosome-inactivating activities (9), however, little is known about their sequences, structures, and physiological roles.

In natural condition, Chinese herbal plant *Trichosanthes kirilowii* Maximowicz is seldom infected by pathogen, which was presumed that this plant might possess an efficient resistance mechanism. It is intriguing to research the antifungal proteins involved in this strong pathogen-resistance. And a type-I RIP, trichosanthin (TCS), isolated from the root tuber of *T. kirilowii* has been extensively studied. In this study, we cloned a novel enzyme TYchi from *T. kirilowii*. The research in this enzyme led to unexpected findings that TYchi exhibited not only chitinase activity but RNA N-glycosidase activity. The RNA N-glycosidase enzyme activity of TYchi was confirmed by three-dimensional structure modeling.

## 3. MATERIAL AND METHODS

### 3.1. Cloning and sequencing of TYchi gene

The genomic DNA of *T. kirilowii* (plant materials were collected in Yannan Garden of Peking University, Beijing, China) was isolated by the CTAB method (10). To amplify gene sequences from the genomic DNA, two arbitrary degenerate (AD) primers (Table 1) were designed according the conserved regions of chitinase genes from the homologous plants *Cucumis sativus* (GenBank accession no. M24365), *Cucurbita moschata* (GenBank accession no. AF082284), and *Cucumis melo* (GenBank accession no. AF241266). PCR was carried out in a total volume of 50 µl with the following reagents: 5 µl Taq polymerase buffer, 2.5 mM/each dNTPs, 10 pmol of each primer, and 0.5 units of Taq polymerase. PCR cycling was 94°C (2 min); 30 cycles of 94°C (45 s), 55°C (30 s), 72°C (1 min); and 72°C (10 min). The specific PCR products were cloned using pUCm-T vector (Shanghai Sagon Biotechnology Co., China) and sequenced. In order to get the whole information of the specific DNA fragment we got from *T. kirilowii*, thermal asymmetric interlaced-PCR (TAIL-PCR) was performed referring to the method of Yaoguang liu *et al*. (11). The AD primers used in TAIL-PCR were $AD_2$ and $AD_{2-2}$ (Table 1). For 5' and 3' TAIL-PCR, two groups (group 1 for 5' TAIL-PCR and group 2 for 3' TAIL-PCR) of specific nested primers (Table 1) were designed based on the sequences of the pervious PCR products we got. The TAIL-PCR system used in each cycle was: 100 ng of genomic DNA, 4 pmol of specific primer, 20 pmol of $AD_2$ or $AD_{2-2}$ primer, 0.5 mM/each of dNTPs, and 1 U of Taq polymerase. The products from each PCR cycle acted as the template for the next cycle after being diluted into 20 folds.

### 3.2. Southern and northern blot hybridization

The southern blotting was performed as followed: 5 µg genomic DNA was digested by restriction enzymes, separated by 1% agarose gel electrophoresis, transferred onto a Zeat-GT membrane (Bio-Rad Co.), and hybridized with a [$^{32}$P]-labeled DNA probe.

For fungus induction assay, the *T. kirilowii* sterile cultured shoots were punctured and wrapped in sterile paper towels saturated with *Fusarium oxysporum* f.sp. *niveum* dispersed in sterile distilled water. Other stimuli involving wounding, watering, and salicylic acid (SA) treatment as formerly reported (12-14). All tissues were harvested at various time points after treatment and frozen in liquid $N_2$. RNA was isolated from different tissues of sterile plants induced with different stimuli and mature plant grown under natural environment (15). TYchi gene encoding region was used as hybridization probe template, and was radiolabelled by the means of random primer extending. The 18S RNA was used as loading control for northern blot analysis. Hybridization conditions followed the manufacture's instructions of Zeta-GT membrane (Bio-Rad Co.). The pattern was quantified by phosphor-imaging (Molecular Dynamics).

### 3.3. Expression and purification of recombinant TYchi

The TYchi mature protein gene was amplified by PCR reaction with gene-specific primers: TYchi-5 and TYchi-3 (Table 1) contained *Nco*I and *BamH*I sites respectively. The PCR produce was cloned into bacterial expression vector pET22b$^+$ (Novagen Co.) between *Nco*I and *BamH*I sites to fuse a 6-His tag at the C-terminus of expressed protein, and this plasmid was named pTYchi. Each expression plasmid (pET22b$^+$, pTYchi) was transformed into *E. coli* Rosetta and the expression of TYchi was induced by IPTG. The cultures were harvested

by centrifugation and disrupted by sonication. Recombinant TYchi was purified on Ni-NTA agarose column according to the manual of QIA expressionist (QIAGEN Co.).

### 3.4. Chitinase enzymic assay of TYchi

The purified TYchi was run on 12% SDS–polyacrylamide gel containing 0.01% (w/v) glycol chitin. Crude extracts of *E. coli* Rosetta with pET22b$^+$ and pTYchi were run synchronously. Protein bands were detected by staining with Coomassie Brilliant Blue R-250. In addition, renaturation of enzymes in 12% polyacrylamide gels containing 0.01% (w/v) glycol chitin and detection of chitinase activity were performed as described by Michaud and Asselin (16). Samples were mixed with a sample buffer without reducing agent and after electrophoresis, gel was incubated at 37°C for 1.5 h in sodium acetate buffer (0.2 M, pH 5.0) containing 1% (v/v) Triton X-100 to remove SDS. After being washed with distilled water, the gel was stained with Calcofluor white M2R (Sigma).

### 3.5. Assay for RNA N-glycosidase activity

Rat liver ribosomes were isolated as described by Shuangli Mi (17). Total ribosomes (60 μg) were incubated respectively with recombinant TYchi and TCS purified by our laboratory in 100 μl (final volume) of binding buffer (25 mM Tris/HCl, pH 7.6, 25 mM KCl, 5 mM $MgCl_2$, 30 mM beta-Mercaptoerhanolat) at 37°C for 15 min. After adding 10 μl of 10% SDS solution to the reaction mixture in an ice-bath, ribosomal RNAs were extracted with phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v) and precipitated by ethanol. After acidic aniline treatment at 60°C for 10 min, ribosomal RNAs were electrophoresed on 8 M urea-denatured 5% polyacrylamide gel at a constant voltage of 100 V for 1 h) and stained with ethidium bromide.

### 3.6. Assay for cell-free translation-inhibiting activity

Rabbit reticulocyte lysate was prepared and the protein synthesis in rabbit reticulocyte lysate was performed according to the Rabbit Reticulocyte Lysate System Technical Manual (Promega Co.) with slight modifications. Various amounts of proteins (10 μl) were added to 40 μl of radioactive working rabbit reticulocyte lysate mixture containing 1 μl [$^3$H]leucine. Incubation proceeded at 37°C for 30 min before addition of 1 M NaOH and 2% $H_2O_2$. Further incubation for 10 min allowed decolorization and tRNA digestion. One volume of the reaction mixture was then added to 40% trichloroacetic acid with 2% casamino acids in a 96-well plate to precipitate radioactively labeled protein. The precipitate was collected on Whatman GF/A filter and [$^3$H]leucine incorporation was counted in an LS6500 Beckman liquid scintillation counter.

### 3.7. Assay for cytotoxic activity on human tumor cells

The cytotoxic activity of the recombinant TYchi against tumor cells was assayed on two different cell lines based on the method described by Shuangli Mi *et al*. (17). Histiocyte lymphoma U937 and choriocarcinoma JAR cell were diluted $1\times10^4$ cells per well in 96-well plates. Then filtrated proteins were added at a designed concentration with the final volume of 100 μl. After incubation in 5% $CO_2$ at 37°C for 24 h, 10 μl of the MTT solution (5 mg ml$^{-1}$, Sigma) was added to each well and the culture was reincubated for 5 h, and then 100 μl of 10% acid SDS solution containing 0.01 M HCl was added. The $OD_{540}$ values were measured with a Microplate Autoreader (Bio-Tek Instruments).

### 3.8. Modeling of the three-dimensional structure of TYchi

The three-dimensional structure of TYchi was modeled using Swiss-Model protein-modeling server (Version 3.5; http://www.expasy.ch/swissmod/SWISS-MODEL.html) (18) and compared with the known 3-D structure of TCS (19). The residues on the 3-D structure of TYchi probably contributing to the N-glycosidase active sites were analyzed.

## 4. RESULTS

### 4.1. Cloning and characterization of TYchi genes

By using PCR with AD primers, we obtained approximately 750 bp specific DNA fragment from *T. kirilowii* genomic DNA. The full-length gene sequence (879 bp) of TYchi (GenBank accession no. AF404590) was obtained by TAIL-PCR. With a typical signal peptide sequence at the N-terminus, the deduced protein shared about 70% identity with chitinases of *C. melo*, *C. sativus*, and *C. moschat* (Figure 1). But no significant sequence similarity to the known RIPs was detected.

In the genomic southern blots, at least four hybridization bands were seen (Figure 2), indicating that TYchi gene represented a small multigene family. TYchi mRNA was found to be specially accumulated in the storage root tubers (Figure. 3A). The transcription of TYchi gene was also only induced by the infection of *F. oxysporum* (Figure. 3B).

### 4.2. Protein expression and chitinase activity analysis

Mature TYchi was successfully expressed in *E. coli* host strain Rosetta. The $His_6$-tagged chitinase protein was expressed in a partially soluble form in cytosol. SDS-PAGE was carried out under non-reducing condition on a gel containing glycol chitin (Figure. 4A). At the same time, chitinase activity of TYchi was analyzed in the gel (Figure. 4B). After staining with Calcofluor White M2R, lytic zones consistent with chitinase activity appeared as dark bands against fluorescent background (Figure. 4B). The lytic zones were also detected in positive control (commercial *Trichoderma viride* chitinases) but not in negative control (TCS). The gel was then stained with Coomassie blue and a band corresponding to calculated molecular mass (30.720 kDa) of the recombinant TYchi was observed in SDS-PAGE.

### 4.3. RNA N-glycosidase, cell-free translation-inhibiting, and cytotoxic activity analysis

As shown in figure 5A, after aniline-catalyzed hydrolysis of phosphodiester bonds on the depurinated site, a clearly specific bands, standing for the released small RNA fragment of approximately 450nt (R fragment), was detected in both TYchi and TCS line on the urea-PAGE.

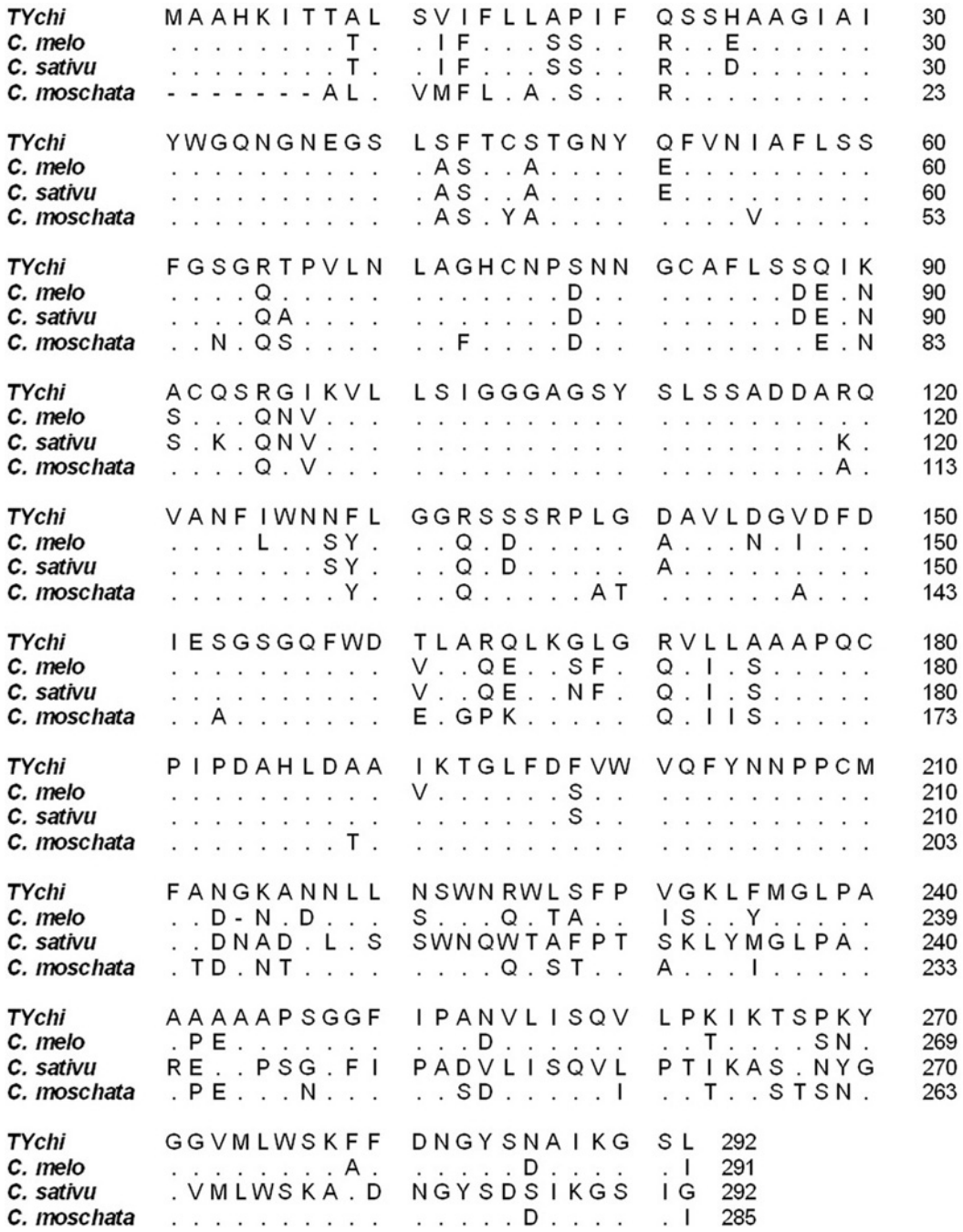


**Figure 1.** Alignment of the deduced amino acid sequence of TYchi with other class III chitinases. The other class III chitinases were *C. Melo* (GenBank accession no. AF241266), *C. sativus* (GenBank accession no. M24365), and *C. moschata* (GenBank accession no. AF082284). The residues matching with TYchi were indicated by dots. The absent residues were denoted by short lines.

However, the commercial *T. viride* chitinases could not release the R-fragment even with much higher amount used (7.5 fold higher than TYchi and TCS) (Figure. 5A).

In the cell-free system, the recombinant TYchi inhibited protein synthesis with $IC_{50}$ of about 5.0 nM (Figure. 5B). It was also found that recombinant TYchi had strong toxicity against U937 and JAR cells. The $IC_{50}$ of recombinant TYchi on U937 and JAR were 54 $\mu g.ml^{-1}$ and 73 $\mu g.ml^{-1}$ in 24 hours respectively (Figure. 6).

**4.4. TYchi 3-D structure analysis**

The sequence alignment of the N-glycosidase activity domain showed four conserved residues clustered

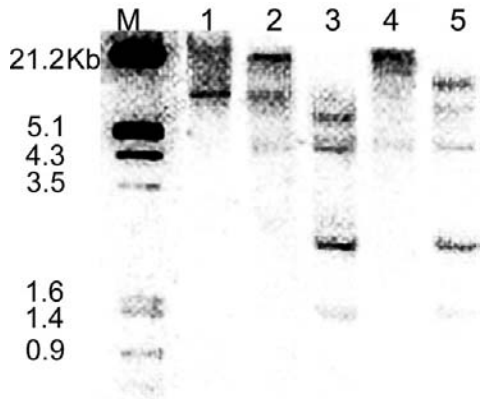


**Figure 2.** Southern blot analysis of TYchi genes. Lanes 1~5 showed: restriction digests of genomic DNA: *Bam*H I, *Bam*H I+*Hind* III, *Bam*H I+*Xba* I, *Hind* III, and *Hind* III +*Xba* I respectively. Lane M showed λDNA *Eco*R I+*Hind* III marker. The molecular weight of marker was shown on the left.

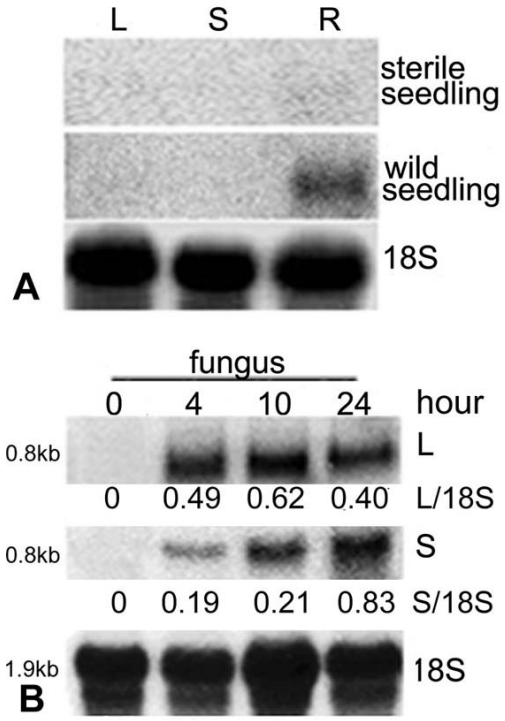


**Figure 3.** Northern blot analysis of TYchi in different tissues and different stimuli in *T. Kirilowii*. TYchi gene encoding region was used as hybridization probe template. (A) Accumulation of TYchi mRNA in different tissue of mature plants grown under natural environment. L: leaves; S: stems; R: roots. (B) Accumulation of TYchi mRNA after treatment with *F. oxysporum* for 0, 4, 10, and 24 h. L: leaves; L/18S: the ratio of amount of TYchi mRNA of leaves to amount of 18S RNA; S: stems; S/18S: the ratio of amount of TYchi mRNA of stems to amount of 18S RNA. The molecular weights of TYchi mRNA and 18S RNA were shown on the left. The relative amount of TYchi mRNA of leaves and stems were assessed by densitometric scanning.

within the cleft located on the protein surface, including Tyr 70, Tyr 111, Glu 160, Arg 163 (TCS numbering) (19). Similar to TCS, TYchi contained a potential N-glycosidase active site (Tyr50, Tyr284, Glu38, Lys278, TYchi numbering) in its 3-D model surface, except converting Arg to another basic amino acid Lys (Figure. 7B).

## 5. DISCUSSION

A TAIL PCR-based procedure was used to isolate TYchi gene from *T. kirilowii*. The deduced protein exhibited a low homology to other chitinases (31-70%). Plant chitinases are believed to play important roles in plant defense against fungi infection. In *T. kirilowii*, TYchi gene existed in multigene family, which was consistent with the phenomenon that in response to the rapid evolution of pathogens the evolution of resistance genes generally excels those of other plant genes and results in multigene family in plant genome (20). The expression of TYchi gene was remarkable induced by *F. oxysporum* or constitutive in tissue that is vulnerable to pathogen attack. Furthermore, wounding or SA could not induce the TYchi gene expression. This result implied that the elicitors of the resistance TYchi involved in might mainly be certain cell wall components of fungi. From these results, considering the chitinase activity of TYchi, we presumed that TYchi should participate in deterrent to fungal invading, and hydrolyzing the chitin on of the fungus cell wall maybe the first step in its antifungal process.

Furthermore, TYchi displayed the RNA N-glycosidase activity in addition to the chitin hydrolyzation activity, which was seldom reported in other chitinases previously. As a mixture with multiple classes of chitinase, the commercial *T. viride* chitinases were in absence of RNA N-glycosidase activity, which excluded the universality of this property in chitinase families. In the reported case that the chitinase purified from plant possessed ribosome inactivating activities, there is a controversy because it cannot be excluded that the observed activities are due to contaminants during target protein isolation (5). In our study, since TYchi was expressed in *E. coli* Rosetta, the chance of contaminantion was very unlikely. Some chitinase-like proteins separated from pinto bean (21) and field beans (22) have translation-inhibiting activity with micromolar-range $IC_{50}$ values, which is deduced for a consequence of RNase activity. Unlike these chitinase-like proteins, TYchi had a much higher translation-inhibiting activity ($IC_{50}$ is about 5.0 nM) that should arise from its RNA N-glycosidase activity. It is believed that proteins with translation-inhibitory function have higher antifungal activity because they can inactivate fungal ribosomes (21). Based on this mechanism, after hydrolyzed the cell wall of fungi, TYchi might subsequently enter the cell and inhibit fungi protein synthesis, thus TYchi could protect plant from the pathogen invasion more efficiently than usual chitinases.

To better understand the relationship between N-glycosidase's function and its structure, we developed a 3-D structure model of TYchi protein. In general, the 3-D structures of the different RIPs are well conserved in their

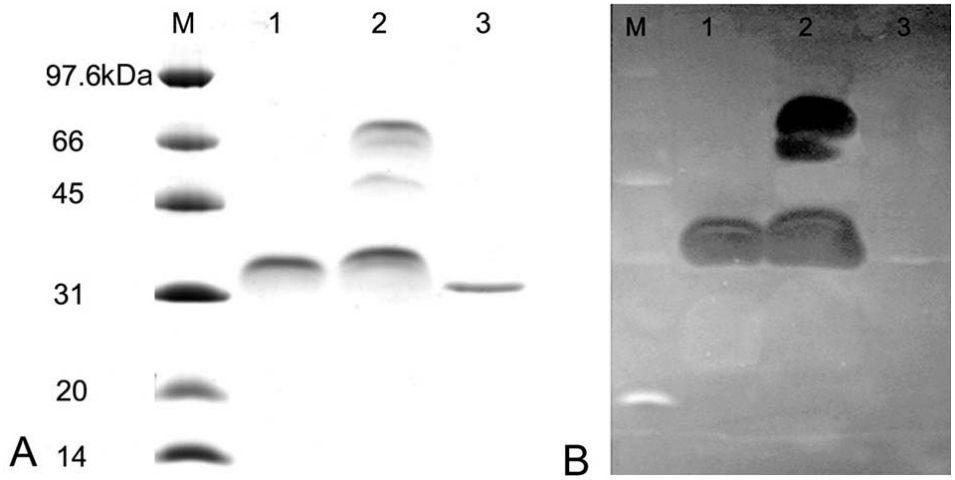


**Figure 4.** Electrophoretogram the recombinant TYchi expressed in *E. coli* Rosetta and detection of chitinase activity by glycol chitin assay. The recombinant TYchi was separated on 12% SDS-PAGE incorporated with glycol chitin as chitinase substrate, and stained with (A) Coomassie Blue or (B) Calcofluor white M2R respectively. Lane M, protein molecular-mass markers. The molecular weight of marker was shown on the left. Lane1: purified recombinant TYchi from *E. coli* Rosetta. Lane2: commercial *T. viride* chitinases. Lane3: TCS.

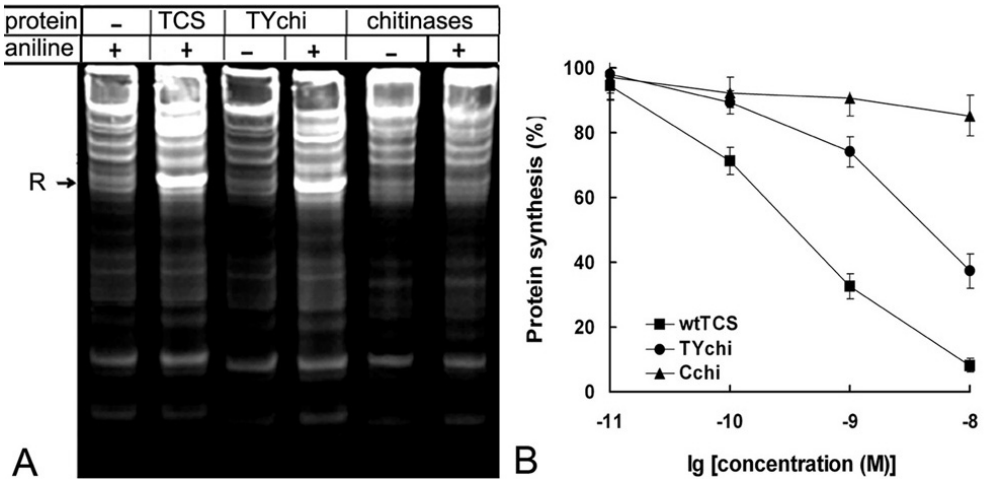


**Figure 5.** N-glycosidase activity and cell-free translation-inhibitory activity of recombinant TYchi. (A) 5% urea PAGE analysis of rat liver ribosome RNA treated with purified recombinant TYchi and stained with ethidium bromide. Lane 1: treated with aniline only (negative control); Lane 2: treated with 20 ng TCS and aniline (positive control); Lane 3 and 4: treated with 20 ng recombinant TYchi with or without aniline; Lane 5 and 6: treated with 150 ng commercial *T. viride* chitinases with or without aniline. The arrow indicates the R-fragment released as a consequence of ribosome inactivating protein action after acid-aniline treatment. (B) Inhibitory effect on cell-free translation in a rabbit reticulocyte lysate**.** TCS was used as positive control. Commercial *T. viride* chitinases (Cchi) were also detected as a mixture of diverse families' chitinases. Data represent as x(mean)+/-s, n=3.

N-glycosidase domain and predicted to have an analogous active-site cleft. Molecular modeling confirmed that TYchi has similar active-site cleft comparing to TCS except converting Arg to another basic amino acid Lys. According to the systematic deletion analysis of RIPs, the mutations of Arg with Lys in the N-glycosidase catalyses cleft can relatively retain its activity (23). In putative active site of TYchi, moreover, the four important residues were distributed in an almost identical manner with that of TCS. So we assumed that in the 3-D structure, TYchi had RIPs-like active domain which were concerned with the RNA N-glycosidase activity.

It is known that some RIPs can cause tumor cell death by inhibiting protein synthesis and inducing apoptosis (2). In this study, we also found that TYchi had strong toxicity to JAR and U937 cells. Since chitin is not found in vertebrates, the cytotoxicity of TYchi was not due to its chitinase activity but its RNA N-glycosidase activity. Compared with TYchi, commercial *T. viride* chitinases did

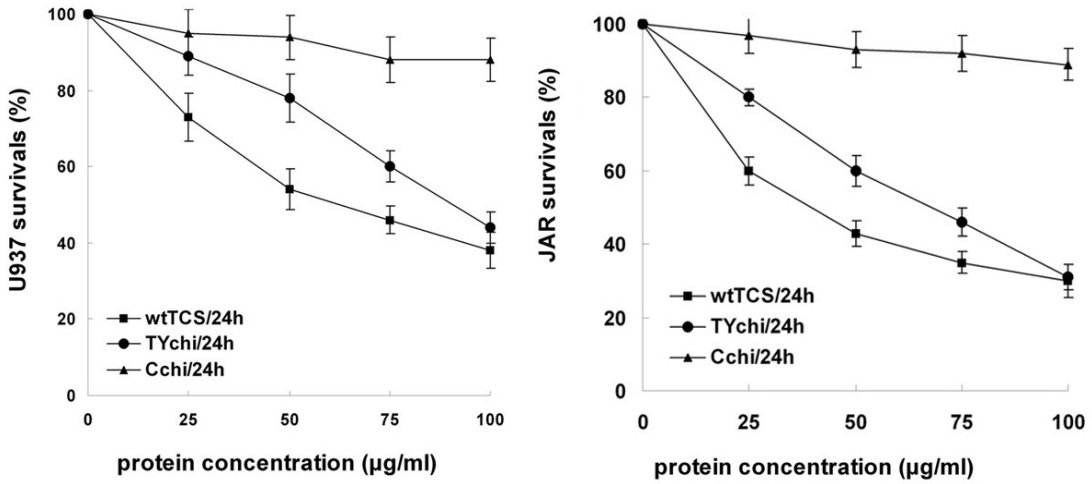


**Figure 6.** The cytotoxic activity analysis of tumor cell lines U937 and JAR. The two cell lines were both treated with recombinant TYchi and TCS respectively for 24 h, and the surviving cells were measured by MTT method. Commercial *T. viride* chitinases (Cchi) were also detected as a mixture of diverse families' chitinases. In above figures each point was the mean value of two parallel experiments with the deviation less than 10%.

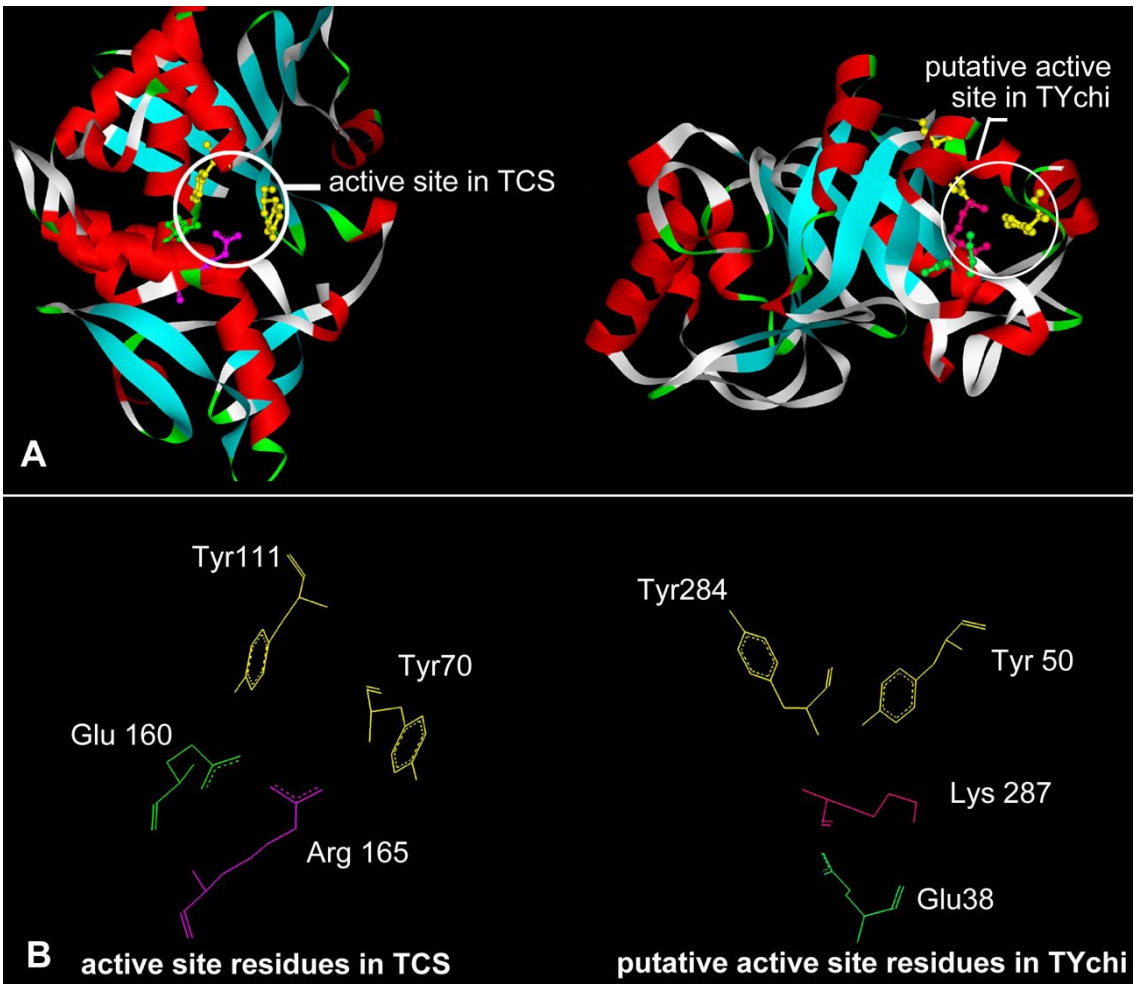


**Figure 7.** Structure modeling of TYchi and TCS. The prominent clefts that were proposed to be the active sites were shown in loops. (A) similarly active sites for TCS and TYchi. (B) active site residues for TCS and TYchi. The figure was constructed with the Swiss Pdb. Viewer (Swiss Institute of Bioinformatics, Geneva).

not exhibit RNA N-glycosidase activity, so they were devoid of cytotoxic activities. Interests in RIPs with anti-tumor functions have been growing since they can be used as components of 'immunotoxins'. However, there are some problems in the application of immunotoxins, such as poor stability, immunogenicity and promotion of vascular leak syndrome (5, 24). Accordingly, looking for new proteins with high anti-tumor activity would make sense to select the best candidates for cancer therapy. TYchi might be developed into a very potential anti-tumor agent.

Despite its preliminary character, our study indicated that TYchi was bifunctional protein with both chitinase and RNA N-glycosidase activity. Moreover, possessing cytotoxic activity and ribosome inactivating activity, the TYchi is extremely attractive in diverse applications, especially for developing RIP-based immunotoxin agent and genetic engineering of plant resistance. However, the catalytic mechanism of the bifunctional enzyme is still an intriguing issue which needs further efforts to resolve.

## 6. ACKNOWLEDGMENTWS

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**Abbreviations:** RIPs: ribosome inactivating proteins, TCS: trichosanthin, CTAB: cetyltriethylammonium bromide, AD primer: arbitrary degenerate primer, TAIL-PCR: thermal asymmetric interlaced-PCR, SA: salicylic acid

**Ke Words**: Chitinase; RNA N-Glycosidase; Ribosome Inactivating Protein; Cytotoxicity; Three-Dimensional Structure

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