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Isolation and Characterization of a Novel Lignin Peroxidase from the White–rot Basidiomycete *Trametes cervina*

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To isolate lignin peroxidase (LiP), extracellular activity of this type of enzyme was assayed against several white–rot basidiomycetes. *Trametes cervina* was found to show relatively higher lignin peroxidase (LiP) activity in extracellular culture medium. LiP from *T. cervina* (TcLiP) was purified using several chromatographic techniques. The molecular weight and pI value of TcLiP were found to be 37 kDa by SDS–PAGE and 3.55 by the isoelectric focusing, respectively.

TcLiP activity against veratryl alcohol was higher at lower pH. A one–electron oxidation mechanism of TcLiP was spectroscopically confirmed using 1, 4–dimethoxylbenzene as a reducing substrate. Furthermore, TcLiP effectively oxidize a polymeric substrate, ferrocytochrome *c* to ferricytochrome *c*. These results strongly suggested that TcLiP meets criterion for lignin peroxidase established by the study on LiP from *Phanerochaete chrysosporium*. Production of LiP by *T. cervina* is reported for the first time.

INTRODUCTION

Lignin is the most abundant renewable aromatic polymer found in woody cell wall, and is known as one of the most recalcitrant biomaterials on the earth (Crawford *et al.*, 1980; Sarkanen *et al.*, 1971). Lignin degradation process is thought as a rate–determining step of the carbon cycle in the biosphere. Only white–rot basidiomycetes are responsible for the complete mineralization of this polymer. As extracellular ligninolytic enzymes, lignin peroxidase (LiP) and manganese peroxidase (MnP) play central role in initial and nonspecific attack on lignin (Gold *et al.*, 1989; Gold *et al.*, 1993; Higuchi, 1990; Kirk and Farrell, 1987; Schoemaker, 1990; Tien, 1987). MnP (EC: 1.11.1.13) oxidizes Mn^{II} to Mn^{III} and the latter acts as a freely diffusible one–electron oxidizer, nonspecifically reacting with terminal organic substrates (Glenn and Gold, 1985; Gold *et al.*, 1989; Gold *et al.*, 1993; Wariishi *et al.*, 1989a; Wariishi *et al.*, 1989b; Wariishi *et al.*, 1991).

LiP (EC: 1.11.1.14) was isolated for the first time from the extracellular culture filtrate of the white–rot basidiomycete, *Phanerochaete chrysosporium* as a series of isozymes (Glenn *et al.*, 1983; Kuwahara *et al.*, 1984; Tien and Kirk, 1984). Later a number of LiP was isolated from other white–rot basidiomycetes (Camarero *et al.*, 1999; Heifling *et al.*, 1998; Johansson and Nyman, 1993; Mester and Field, 1998). Nucleotide sequences of a number of LiP cDNA and genomic clones and crystal structures of LiP have demonstrated that important peroxidase catalytic residues, including the proximal and distal His, the distal Arg, and an H–bonded Asp to proximal His, are all conserved (Camarero *et al.*, 2000; Edwards *et al.*, 1993; Gold and Alic, 1993; Piontek *et al.*, 1993; Poulos *et al.*, 1994; Ritch *et al.*, 1991; Tien, 1987; Tien and Tu, 1987). Therefore, LiP shares many structural and mechanistic features with other peroxidases, yet it has several unique properties. The enzyme catalyzes the one–electron oxidation of nonphenolic aromatic compounds with a high redox potential through the formation of a substrate cation radical (Kersten *et al.*, 1985; Schoemaker *et al.*, 1985). LiP can directly interact and oxidize polymeric substrates such as lignin and ferrous cytochrome *c* via a long–range electron transfer (Johjima *et al.*, 1999; Wariishi *et al.*, 1994). Since only 20% of aromatic nuclei of polymeric lignin contain free phenolic groups, the ability to oxidize nonphenolic aromatic compounds is advantageous for the biodegradation of lignin. However, a major effort for LiP mechanistic studies has been devoted to only *Phanerochaete chrysosporium* LiP (PcLiP).

To more exclusively understand LiP mechanism, we have initiated to explore for LiP from other white–rot basidiomycetes. It is said that 9,000 genuses and 12,000 species of basidiomycetes were on the earth; however, LiP activity was so far reported from only 20 species.

MATERIALS AND METHODS

Organisms and culture conditions

Bjerkandera adusta WD1, *Grammothele fuligo* WD844, *Trametes cervina* WD550, and *Trametes versicolor* WD1670 were examined. These strains were originally screened for ligninolytic activity at Forestry and Forest Products Research Institute (Takano *et al.*,

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2001). All strains were grown from hyphal inocula at 30 °C in stationary culture under air. The medium used in this study was previously described as an HCLN Kirk's medium with 1% glucose and 1.2 mM ammonium tartrate and buffered with 20 mM sodium 2, 2–dimethyl succinate (pH 4.5–6.0) with a modification such as no addition of manganese ion (manganese free Kirk media) (Kirk *et al.*, 1978). LiP activity was assayed by oxidation of 2.0 mM veratryl alcohol to veratraldehyde (ε_{310}= 9.33 $mM^{-1}cm^{-1}$) in 20 mM sodium succinate pH 3.0, with 0.1 mM H_2O_2 and 200 μL extracellular culture medium as previously reported (Tien and Kirk, 1984).

Semi–large scale incubation of *T. cervina* and purification of LiP

Trametes cervina LiP (TcLiP) was incubated as described above at pH 5.0 for 7 days under stationary conditions, mycelial mat (80 mL culture) was homogenized and re–inoculated to a 500–mL flask containing 250 mL of the above mentioned medium containing 0.1% Tween 80. The flasks were incubated at 30 °C under air on a rotary shaker at 120 rpm for 3 days. The culture fluid was collected by filtering through glass wool, pH adjusted at 7.0, and applied onto DEAE Sepharose Fast Flow (Amersham Biosicence) equilibrated in 20 mM potassium phosphate buffer (pH 7.0). After washing the column with 50 mM sodium succinate (pH 4.5), the active fractions were separated using a 0–1.0 M NaCl gradient in 50 mM sodium succinate (pH 4.5). The active fractions were collected, concentrated using a 10–kDa cut–off centricon (Millipore), then applied to Superdex 200 column and eluted with 50 mM sodium succinate (pH 4.5) containing 0.1 M NaCl. The fractions showing the absorption at both 400 and 280 nm were collected, concentrated, then applied to CIM QA Disk column (BIA Separations), separated using 0.01–1.0 M sodium acetate (pH 6.0). The fractions with the absorbance at both 400 and 280 nm were pooled, concentrated, then charged onto Mono Q HR 5/5 (Amersham Biosicence). The separation was conducted with pH gradient from 4.0 to 3.0 of 50 mM sodium succinate.

Protein concentration was determined using a Bio–Rad protein assay kit based on Bradford method (Bradford, 1976). SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 10% polyacrylamide gel. A molecular weight was estimated using Perfect Protein Marker (Takara) on SDS–PAGE. Isoelectric focusing was performed on 5% polyacrylamide gel with pI range from 2.5 to 5.0 using Pharmalyte (Amersham Biosicence). A pI was estimated using Low pI calibration Kit (Amersham Biosicence). Proteins were visualized using Coomassie Brilliant Blue R (TCI).

Enzyme characterization

Electronic absorption spectra were recorded on Hitachi U–3000 spectrophotometer with a 1–cm quartz cuvette at 25 °C. The pyridine hemochrome of reduced TcLiP was measured as previously described (Appleby and Morton, 1959).

The oxidation reactions with a series of methoxybenzenes as substrates were carried out. The reaction mixtures contained 30 nM TcLiP, 0.2 mM substrates, and 0.1 mM H_2O_2 in 20 mM sodium succinate, pH 3.0. Reactions were initiated by adding H_2O_2. After a 1–h incubation, residual substrate and reaction products were analyzed by HPLC after filtration (0.45 μm) and by GCMS after reduction with excess dithionite, extraction with ethyl acetate at pH 2.0, drying over $MgSO_4$, evaporation under N_2, and derivatization using *N, O*–bis–(trimethilsilyl)–trifluoroacetimide (TMS)/pyridine (2:1, v/v). The spectral shift was monitored during the oxidation of 1, 4–dimethoxybenzene.

The oxidation of ferrocytochrome *c* was performed as previously described (Wariishi *et al.*, 1994). A stock solution of ferrocytochrome *c* were prepared by reducing ferricytochrome *c* with adding sodium dithionite and removing excess sodium dithionite utilizing Sephadex G–25 with deionized water as an elutant. The oxidation of ferrocytochrome *c* was monitored with decrease in the absorbance at 550 nm.

Instrumentation

GCMS was performed at 70 eV on a JEOL AMII–15A equipped with a 30–m fused silica column (DB–5, J & W Scientific). The oven temperature was programmed from 80 to 320 °C at 8 °C/min. Product was identified by a comparison of their retention times on GC and HPLC and of mass fragmentation patterns with an authentic standard.

RESULTS AND DISCUSSION

All strains except *G. fuligo* showed LiP activities in their extracellular culture mediums under the growth conditions utilized in this study. Maximal LiP (VA oxidation) activity in extracellular culture medium was observed 7 days after inoculation under stationary culture conditions for all strains. VA oxidation activities found in fungal cultures are listed in Table 1. Since *T. cervina* exhibited relatively high LiP activity among fungi examined and LiP activity of *T. cervina* was observed for the first time, TcLiP was isolated and investigated for more detail.

Table 1. LiP activity of certain white–rot fungi.

Fungus	VA oxidation activity (μmol/min/mL culture)
Phanerochaete chrysosporium	0.13
Bjerkandera adusta	0.038
Trametes cervina	0.084
Trametes versicolor	0.024
Grammothele fuligo	0

To isolate TcLiP, shaking culture in a larger scale was attempted. *T. cervina* showed the highest LiP activity in the HCLN Kirk medium without manganese ion supplement at pH 5.0. No effect of VA addition and

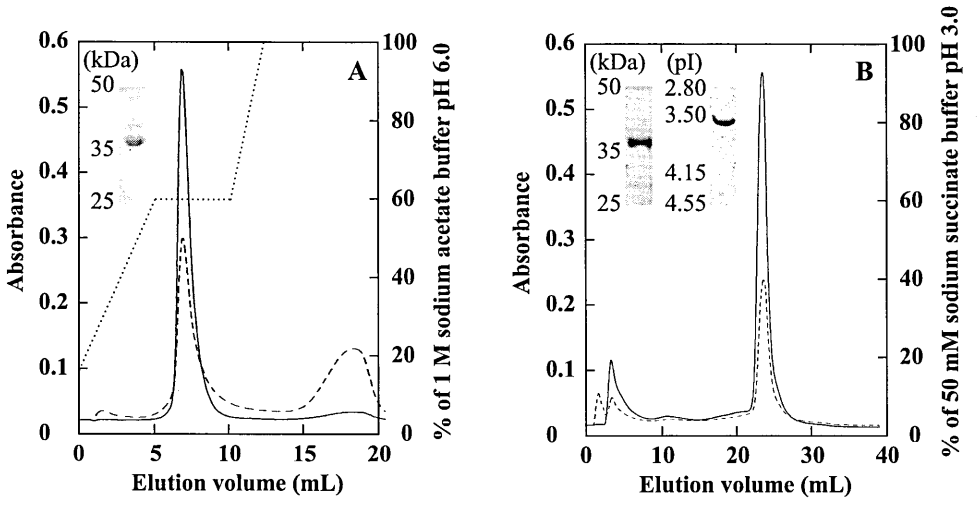

Fig. 1. Elution profile of TcLiP using (A) CIM QA and (B) Mono Q chromatography. Solid lines show the profile monitored by the absorbance at 400 nm and dashed lines show the profile monitored by the absorbance at 280 nm. Dotted line in A indicates % of 1 M sodium acetate, pH 6.0 in 50 mM sodium acetate, pH 6.0, and that in B indicates % of 50 mM sodium succinate, pH 3.0 in 50 mM sodium succinate, pH 4.0. Inset in A shows SDS–PAGE of TcLiP fractions obtained from CIM QA column chromatography. Instets in B shows SDS-PAGE (left) of TcLiP obtained from Mono Q column chromatography and IEF (right) of TcLiP obtained from Mono Q column chromatography.

Table 2. Purification of TcLiP from *T. cervina* culture medium.

Step	Total protein (mg)	Total activity (μmol/min)	Specific activity ($\mu\mu$mol/min/mg)	yield (%)	purification fold
Extracellular culture	159.6	57.8	0.4	–	–
DEAE Sepharose	5.4	57.0	10.6	98.6	29.3
Superdex 200	3.0	46.7	15.5	80.9	42.9
CIM QA	1.2	27.4	23.1	47.5	63.8
Mono Q (pH 4.0–3.0)	0.7	14.0	21.2	24.2	58.6

O_2 purge on LiP activity was observed, although these are known to be compulsory for PcLiP production (Faison and Kirk, 1985; Tonen and Odier, 1988). In the shaking culture, the activity of LiP began to be observed 56 h after inoculation and reached maximum at 72 h; thus, TcLiP was purified from a 72–h–old extracellular culture medium. The purification of TcLiP is summarized in Table 2. The initial total activity in the medium (2.5 L) was 57.8 μmol min^{-1}. After removal of mycelium, the pH of the medium was adjusted to 7.0, and the medium was applied on DEAE Sepharose Fast Flow column. The TcLiP fraction recovered from the Sepharose showed a yield of 98.6% with a purification factor of 29.3. After passing through a Superdex 200 column, the yield was 80.9% with a purification factor increased to 42.9. In the next step, anion–exchange chromatography on CIM QA column was conducted using 0.01–1.0 M sodium acetate gradient (pH 6.0). In the elution profile from CIM QA column, sharp peak with absorbance at both 400 nm and 280 nm was observed and VA oxidation activity was detected in these fractions (Fig. 1A). Thus, they were pooled as TcLiP. However, an SDS–PAGE analysis exhibited an existence of a minor band with a major band at 37 kDa (Fig. 1A, inset). Therefore, further purification step using Mono Q column with a pH gradient from 4.0 to 3.0 in 50 mM sodium succinate was attempted (Fig. 1B). As a result, a single band of TcLiP on SDS–PAGE was obtained (Fig. 1B, inset). The yield and purification factor was 24.2% and 58.6. Specific activity of TcLiP was calculated to be 21.2 μmol·min^{-1}·mg^{-1}. Since the specific activity was decreased during this final step, a contaminant was thought to be a minor isozyme of TcLiP. The molecular weight of TcLiP was found to be 37 kDa by SDS–PAGE and pI of 3.55 was estimated by the isoelectric focusing (Fig. 1B, inset).

The electron absorption spectrum of TcLiP showed the peaks at 406, 504, and 644 nm (Fig. 2). The sharp Soret peak strongly suggests its feature of a hexa–coordinate high spin ferric iron state of the heme. A molar extinction coefficient at 406 nm was determined to be 147 mM^{-1} cm^{-1} with a pyridine hemochrome method (Fig. 2, inset).

The effect of pH on VA oxidation activity of TcLiP was investigated at pH ranging from 2.5 to 6.0. TcLiP exhibited higher VA oxidation activity at lower pH (Fig.

3). The pH dependency for VA oxidation of TcLiP was very similar to that of PcLiP (Gold *et al.*, 1989; Kirk and Farrell, 1987). PcLiP is known to possess a high redox potential, for which a low pH optimum is advantageous (Oyadomari *et al.*, 2003).

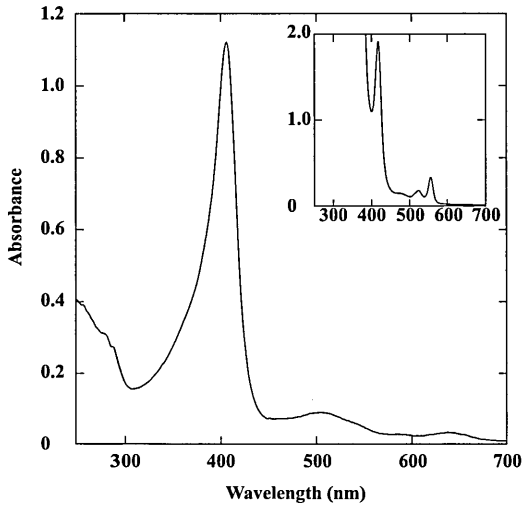

Fig. 2. Electronic absorption spectrum of TcLiP. Solid line shows the spectrum of TcLiP in the resting state. Inset shows the spectrum of pyridine hemochrome of reduced TcLiP.

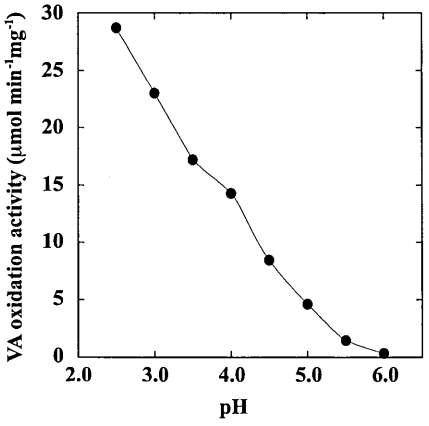

Fig. 3. pH dependency of TcLiP–catalyzed VA oxidation.

A one–electron oxidation of methoxybenzenes forms their stable aryl cation radicals which are distinguishable on the absorption spectrum. Thus, the oxidation of methoxybenzene derivatives is more suitable substrate to characterize LiP–catalyzed one–electron oxidation reactions. PcLiP was reported to directly oxidize methoxybenzenes with $E_{1/2}$ lower than 1.49 V. On the other hand, plant peroxidase (horseradish peroxidase) hardly oxidizes methoxybenzenes with $E_{1/2}$ higher than 1.24 V (Kersten *et al.*, 1990). Table 3 summarizes TcLiP oxidation of several methoxybenzenes. 1, 2–Dimethoxybenzene and 1, 4–dimethoxybenzene, which have $E_{1/2} < 1.45$, were effectively oxidized by TcLiP. But methoxybenzene ($E_{1/2} = 1.76$) and 1, 3, 5–trimethoxybenzene ($E_{1/2} = 1.49$) were hardly oxidized by TcLiP. Oxidation of 1, 4–dimethoxybenzene was further investigated to characterize LiP–catalyzed oxidation mechanism. Spectral analysis revealed the immediate increase of absorbance at 433 and 457 nm, indicating the formation of 1, 4–dimethoxybenzene aryl cation radical (Fig. 4) (Kersten *et al.*, 1990). These peaks

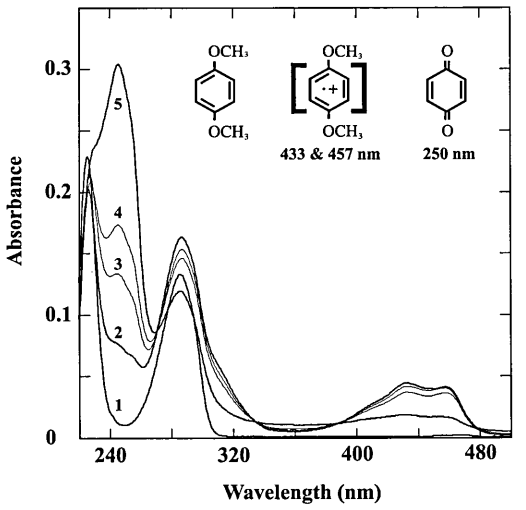

Fig. 4. Oxidation of 1, 4–dimethoxybenzene by TcLiP. Reaction mixture consists of TcLiP (30 nM), 1,4–DMB (0.2 mM), and H_2O_2 (0.1 mM) in 20 mM succinate buffer pH 3.0. The reaction was initiated by H_2O_2 addition. (1); Spectra of 1,4–dimethoxybenzene before adding H_2O_2. (2); immediately after H_2O_2 addition, (3); after 10 minutes, (4); after 20 minutes, (5); after 1 hour. Inset; proposed oxidation pathway by TcLiP/ H_2O_2.

Table 3. Oxidation of methoxybenzes by TcLiP and PcLiP (LiPH8).

Substrate	$E_{1/2}$ (V)[1]	Residual substrate after treatment by TcLiP (%)	Residual substrate after treatment by PcLiP (%)
Methoxybenzene	1.76	—[2]	—
1, 3, 5-Trimethoxy benzene	1.49	ND[3]	ND
1, 2-Dimethoxy benzene	1.45	41.2 ± 3.3	50.3 ± 1.5
1, 4-Dimethoxy benzene	1.34	23.1 ± 2.2	41.2 ± 1.1

[1] Literature values (Kersten *et al.*, 1990)
[2] – indicates that neither spectral nor quantitative changes were observed.
[3] ND indicates that slight spectral change was observed but quantitative change could not be determined by HPLC.

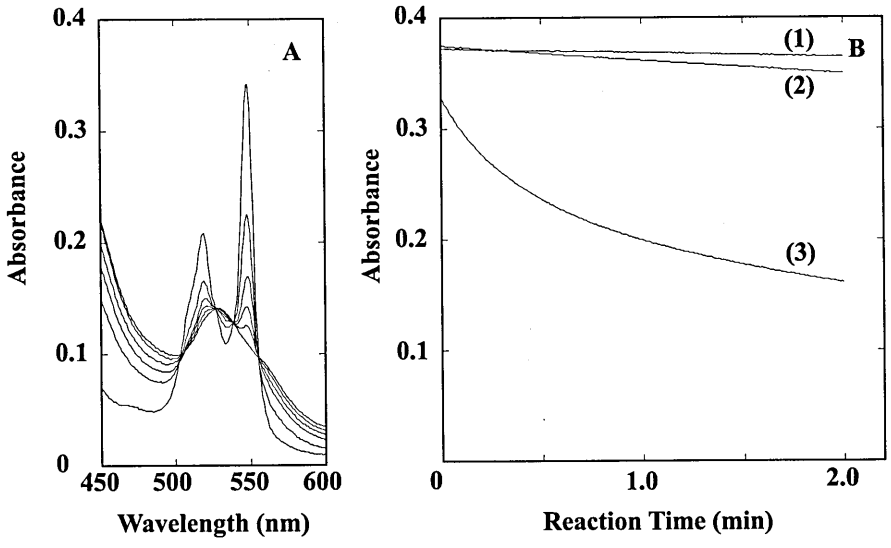

Fig. 5. Oxidation of ferrocytochrome *c*. (A) Spectra were recorded at 1 min intervals. Reaction mixtures consisted of TcLiP (8 nM), ferrocytochrome *c* (14 mM), H_2O_2 (0.1 mM) in sodium succinate buffer, pH 4.5. (B) Ferrocytochrome *c* oxidations were monitored at 550 nm under aerobic conditions. Trace 1 (spontaneous autoxidation): ferrocytochrome *c* (14 mM) in sodium succinate buffer, pH 4.5. Trace 2: Same as trace 1 with addition of 0.1 mM H_2O_2. Trace 3: Same as trace 2 with addition of TcLiP (16 nM).

gradually decreased and a peak at 250 nm appeared, suggesting quinone formation (Fig. 4). HPLC and GCMS analyses also indicated the decrease of 1, 4–dimethoxybenezene and corresponding formation of 1, 4–benzoquinone. The formation of benzoquinone was confirmed as 1, 4–dihydroxybenezene after reduction (diTMS derivatives; 254 (M+, 64), 239 (100), 112 (26), 73 (94)). These results were well consistent with PcLiP (LiPH8) oxidation of methoxybenezenes (Kersten *et al.*, 1990), suggesting that the oxidized intermediates of TcLiP possess a high redox potential as that of PcLiP.

Unique structural characteristics of LiP are found in its substrate oxidation site being on the surface of the protein, which enables the enzyme to oxidize polymeric substrates such as lignin and ferrocytochrome *c* (Johjima *et al.*, 1999; Wariishi *et al.*, 1994). Fig. 5 shows the oxidation of ferrocytochrome *c* by TcLiP. During the course of the reaction, the bands at 550 and 520 nm indicative of ferrocytochrome *c* decreased and a new band appeared at 528 nm, showing the formation of ferricytochrome *c* (Fig. 5A). Without adding TcLiP, ferrocytochrome *c* was stable against autoxidation (Fig. 5B). These results strongly suggested that TcLiP also possesses the oxidation site on the protein surface.

The results in the present study indicated that TcLiP meets the criterion for lignin peroxidase catalytic mechanism. However, our recent studies on TcLiP did not clarify the molecular mechanism of a one–electron oxidation, such as the determination of the oxidation site. Further studies utilizing a chemical modification and a site–directed mutagenesis of certain surface amino acids are now underway as well as obtaining a full–length cDNA coding TcLiP.

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