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# A peptide with HIV-1 reverse transcriptase inhibitory activity from the medicinal mushroom *Russula paludosa*

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ABSTRACT

Hot water extracts of 16 species of mushrooms, including both edible and medicinal mushrooms, were screened for human immunodeficiency virus (HIV)-1 reverse transcriptase (RT) inhibitory activity. Extracts of *Lactarius camphoratus*, *Trametes suaveolens*, *Sparassis crispa*, *Pleurotus sajor-caju*, *Pleurotus pulmonarius*, and *Russula paludosa* elicited over 50% inhibition when tested at the concentration of 1 mg/ml. The extract of *R. paludosa* demonstrated the highest inhibitory activity on HIV-1 RT (97.6%). Fraction SU2, purified from *R. paludosa* extract by anion exchange chromatography on DEAE-cellulose and gel filtration on Superdex 75, exhibited potent inhibitory activity on HIV-1 RT. At the concentrations of 1 mg/ml, 0.2 mg/ml, and 0.04 mg/ml, the inhibition ratios were 99.2%, 89.3%, and 41.8%, respectively, giving an $IC_{50}$ of 11 μM. The molecular mass of SU2 was 4.5 kDa and its N-terminal amino acid sequence was determined to be KREHGQHCEF. The peptide was devoid of hemagglutinating, ribonuclease, antifungal, protease, protease inhibitory, and laccase activities.

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## 1. Introduction

The causative agent of acquired immunodeficiency syndrome (AIDS) is human immunodeficiency virus (HIV) [5]. The symptoms of people infected with HIV encompass immunodeficiency, decline in ability to combat infections, opportunistic infections, malignant tumors, and nerve handicaps [5,10,20,21,24]. The distribution of AIDS is worldwide, and it has become one of the most difficult viral diseases to treat.

In the past 20 years, rapid progress has been made in developing natural products and chemically synthesized compounds as anti-HIV drugs. Through HIV kinetics studies, medicines have been developed that are targeted at many stages, for example, the infection and replication of HIV. These drugs include the following: (a) soluble molecules of CD4, and receptor inhibitors used to block and prevent HIV entry into cells, such as rsCD4, and sulfated glucans, (b) RT inhibitors, comprising nucleotide analogs, such as AZT, ddC, ddT, and ddT, and non-nucleotide analogs such as ramifications of thiazole, (c) integrase inhibitors [11], (d) viral accessorial protein inhibitors, (e) viral nucleoside inhibitors [22], (f) protease inhibitors, (g) glycosidase inhibitors [8], and (h) virus assembly inhibitors [2,23,25].

Currently the prevailing strategy for the therapy of AIDS is combinative application of several therapeutic agents, that is, reverse transcriptase inhibitors, such as nucleotides or non-nucleotides used in conjunction with protease inhibitors [1,3,4,6,7]. The treatment can reduce viral number to such a low level that the virus cannot be detected in blood and lymphoid tissues. But the drugs have toxicity and are extremely

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expensive. Resistance of HIV against the drugs can develop easily. About 90% of the 40 million HIV-infected people in the world cannot afford the substantial sum incurred in treatment. Thus, research and development of drugs with higher efficacy, lower toxicity, and reduced cost are in urgent need.

Chinese medicine is a treasure of China and the world. It is one of the hottest pharmacological issues to screen anti-AIDS drugs from Chinese medicinal materials. Abundant resources exist in edible medicinal mushrooms which possess a multitude of biological activities [9,17,18,26–28,30–35]. The advantages of screening HIV-1 RT inhibitors from edible medicinal fungi are as follows: (i) abundant resources. There are about 10,000 species of fungi which can form large fruiting bodies. Among them, more than 2000 species are edible medicinal mushrooms. The wild fungi can be collected, some of them can be cultivated, and the majority of the species can be cultured by large-scale fermentation to yield mycelia. (ii) Lower toxicity and side effects. The use of edible medicinal fungi has a long history. Generally speaking, natural products extracted from edible medicinal fungi exhibit lower toxicity and fewer side effects than chemical drugs. The natural products can be taken over a prolonged period as medicine, or consumed as tonics by healthy people. (iii) More affordable prices. Because of the abundant resources and ready availability of most of the edible medicinal fungi, natural products extracted from these materials could be produced at a lower cost than those in clinical use. It means that more patients could be treated. RT is one of the key enzymes in HIV replication. HIV replication would be interfered with if the enzyme is inhibited. So RT inhibitors can be used to treat AIDS [22].

## 2. Materials and methods

### 2.1. Edible medicinal mushrooms

*Tricholoma robostum*, *Morchella esculenta*, *Pholiota adiposa*, *Schizophyllum commune*, *Sparassis crispa*, *Lentinus tigrinus*, *Trametes suaveloens*, *Grifola umbellatum*, *Pleurotus nebrodensis*, *Pleurotus pulmonarius*, *Pleurotus sajor-caju*, *Russula paludosa*, and *Lactarius camphorates* were gifts from the Laboratory of Edible and Medicinal Fungi at China Agricultural University.

### 2.2. Extraction of mushroom fruiting bodies

Dried fruiting bodies of edible or medicinal mushrooms (5 g) were homogenized in distilled water and then extracted with distilled water at 95 °C for 6 h. The mixture was centrifuged at 4000 × *g* for 10 min, and the supernatant was collected. Three volumes of 95% ethanol were added, and the mixture was centrifuged again. The precipitate was stored at 60 °C. The dried samples were powdered for the next step.

### 2.3. Purification of R. paludosa peptide

A DEAE-cellulose (Sigma) column (1.5 cm × 20 cm) which had previously been equilibrated with 10 mmol/l phosphate buffer (pH 7.5) was used. The extract, prepared from 50 g dried fruiting bodies and dissolved in the same buffer, was chromatographed on the column. After elution of inactive unadsorbed materials (fraction D1), adsorbed materials (fractions D2 and D3) were eluted with a linear concentration gradient of NaCl (0–1 mol/l) in 10 mmol/l phosphate buffer (pH 7.5). The second adsorbed fraction, fraction D3, was dialyzed and further fractionated by FPLC on a Superdex 75 HR 10/30 column (Amersham Biosciences) in 200 mmol/l $NH_4HCO_3$ (pH 8.6). The Superdex 75 column had previously been calibrated with molecular mass markers including bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), ribonuclease A (13.7 kDa), and cytidine (0.246 kDa). The eluate was monitored for HIV-1 RT inhibitory activity [12,15,16].

### 2.4. Assay of HIV-1 RT inhibitory activity of mushroom extract and peptide

The assay was performed as described in the protocol included with the kit (HIV-1 RT assay kit, Boehringer Mannheim, Germany). The extract from a given mushroom species was dissolved in distilled water at the concentration of 4 mg/ml. RT solution (20 μl) was added to 20 μl test sample, and 20 μl lysis buffer. Then 20 μl solution of the reaction mixture was added after 10 min to each well of a 96-well plate of the assay kit. The plate was incubated at 37 °C for 1 h. Washing buffer (250 μl) was added to each well and the wells were washed five times. Antibody (anti-DIG-POD) (200 μl) was added to each well, followed by incubation at 37 °C for 1 h. The wells were then washed again. In the final step, the peroxidase substrate, 200 μl ABTS solution, was added. The plate was left at room temperature for about 30 min. The absorbance of the sample at 405 nm was determined using a microtiter plate reader and is directly correlated to the level of RT activity. The inhibitory activity of the purified peptide was calculated as percent inhibition as compared to a control without the purified peptide [12,15,16].

### 2.5. Molecular mass determination by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by FPLC–gel filtration

SDS-PAGE was carried out in accordance with the procedure of Nielsen and Reynolds [19], using a 12% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was stained with Coomassie brilliant blue. FPLC–gel filtration was carried out using a Superdex 75 column and also on a Superdex peptide column which had been calibrated with molecular mass standards (Amersham Biosciences).

### 2.6. N-terminal amino acid sequence analysis

The N-terminal amino acid sequence of *R. paludosa* peptide was analysed by means of automated Edman degradation. Microsequencing was carried out using a Hewlett Packard 1000A protein sequencer equipped with an HPLC system [9].

### 2.7. Assay of HIV-1 integrase inhibitory activity

#### 2.7.1. Expression and purification of recombinant HIV-1 integrase

The plasmid used, pT7-7-His(Y|TX)-HIV-1-IN, expressed His-tagged wild-type HIV-1 integrase. To express the protein, a 1 l

culture of *E. coli* BL21(DE3) cells containing the expression plasmid was grown at 37 °C until $OD_{600}$ reached 0.7–0.8. Cells were induced by addition of 0.8 mM IPTG and harvested after a 4 h incubation by centrifugation at 6000 × *g* for 10 min at 4 °C. Cells were suspended at a concentration of 10 ml/g wet cell paste in 20 mM Tris–HCl (pH 8.0), containing 0.1 mM EDTA, 2 mM β-mercaptoethanol, 0.5 M NaCl, and 5 mM imidazole. Lysozyme was added to a concentration of 0.2 mg/ml. After 1 h incubation at 4 °C, the lysate was sonicated and centrifuged at 40,000 × *g* at 4 °C for 20 min. The pellet was homogenized in 50 ml buffer A (20 mM Tris–HCl, pH 8.0, 2 M NaCl, 2 mM β-mercaptoethanol) containing 5 mM imidazole. The suspension was rotated at 4 °C for 1 h and cleared by centrifugation at 40,000 × *g* at 4 °C for 20 min. The supernatant was loaded onto a 1 ml chelating Sepharose column charged with 50 mM imidazole. The column was washed with five column volumes of buffer A containing 5 mM imidazole and the protein was eluted with three column volumes of buffer A containing 200 mM and 400 mM imidazole, respectively. Protein-containing fractions were pooled and EDTA was added to a final concentration of 5 mM. The protein was dialyzed against buffer B (20 mM HEPES, pH 7.5, 1 mM EDTA, 1 M NaCl, 20% glycerol) containing 2 mM β-mercaptoethanol and then against buffer B containing 1 mM dithiothreitol. Aliquots of the protein were stored at −70 °C [12,15].

#### 2.7.2. *HIV-1 integrase assays*

A non-radioactive ELISA-based HIV-1 integrase assay was performed according to the DNA-coated plates method as detailed by Ng et al. [12]. In this study, 1 μg of *Sma*I-linearized p Bluescript SK was coated onto each well in the presence of 2 M NaCl as target DNA. The donor DNA was prepared by annealing VU5BR (5′-biotin-GTGTGGAAAATCTCTAGCAGT-3′) and VU5 (5′-ACTGCTAGAGATTTTCCACAC-3′) in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, and 0.1 M NaCl at 80 °C followed by 30 min at room temperature. Integrase reaction was performed in 20 mM HEPES (pH 7.5), containing 10 mM $MnCl_2$, 30 mM NaCl, 10 mM dithiothreitol, and 0.05% Nonidet-P40 (Sigma). After the integrase reaction, the biotinylated DNA immobilized on the wells was detected by incubation with streptavidin-conjugated alkaline phosphatase (Boehringer Mannheim) followed by colorimetric detection with 1 mg/ml *p*-nitrophenyl phosphate in 10% diethanolamine buffer, pH 9.8, containing 0.5 mM $MgCl_2$. The absorbance due to the alkaline phosphatase reaction was measured at 415 nm [12,15].

### 2.8. *Assay of hemagglutinating activity*

The isolated peptide was assayed for lectin (hemagglutinating) activity in view of the report that some lectins exhibited HIV-1 reverse transcriptase inhibitory activity [27]. A serial two-fold dilution of a solution of *R. paludosa* peptide in microtiter U-plates (50 μl) was mixed with 50 μl of a 2% suspension of rabbit red blood cells in phosphate buffered saline (pH 7.2) at 20 °C. The results were read after about 1 h when erythrocytes in the blank had fully sedimented and formed a dot at the bottom of the well. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was reckoned as one hemagglutination unit. Specific activity is the number of hemagglutination units per milligram of protein [9].

### 2.9. *Assay of laccase activity*

The isolated peptide was assayed for this activity in view of a report that some laccases possessed HIV-1 reverse transcriptase inhibitory activity [17]. Laccase activity was assayed by measuring the oxidation of 2,7′-azinobis[3-ethylbenzothiazolone-6-sulfonic acid]diammonium salt (ABTS). A modification of the method of Ng and Wang [17] was used. An aliquot of a solution of the peptide was incubated in 1.3 ml of 67 mM sodium acetate buffer (pH 4.5) containing 1.54 mm ABTS at 30 °C. One unit of enzyme activity was defined as the amount of enzyme required to produce an absorbance increase at 405 nM of $1\ min^{-1}\ ml^{-1}$ of reaction mixture under the aforementioned condition.

### 2.10. *Assay of ribonuclease activity*

Some ribonucleases exhibited HIV-1 reverse transcriptase inhibitory activity [26]. Hence the activity of the purified peptide toward yeast tRNA (Sigma) was assayed by determining the generation of acid-soluble, UV-absorbing species with the method of Wang and Ng [31,33]. The peptide was incubated with 200 μg tRNA in 150 μl of 100 mM MES buffer (pH 5) at 37 °C for 15 min. The reaction was terminated by introduction of 350 μl of ice-cold 3.4% perchloric acid. After leaving on ice for 15 min, the mixture was centrifuged (15,000 × *g*, 15 min) at 4 °C. The $OD_{260}$ of the supernatant was read after appropriate dilution. One unit of enzymatic activity is defined as the amount of enzyme that brings about an increase in $OD_{260}$ of $1\ min^{-1}$ in the acid-soluble fraction per milliliter of reaction mixture under the specified condition.

### 2.11. *Assay of antifungal activity*

Some antifungal peptides inhibited HIV-1 reverse transcriptase [18,29]. The assay of the purified peptide for antifungal activity toward *Mycosphaerella arachidicola* and *Physalospora piricola* was carried out in 100 mm × 15 mm petri plates containing 10 ml of potato dextrose agar. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rim of the mycelial colony. An aliquot (15 μl) of a solution of the purified peptide was added to a disk. The plates were incubated at 23 °C for 72 h until mycelial growth had enveloped the disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity [16,33].

### 2.12. *Assay of protease activity*

A solution of casein, which was used as substrate in the protease assay, was freshly prepared as follows. To 0.1 g casein 10 ml 200 mM phosphate buffer (pH 7.5) were added. Subsequently, the solution was heated at 60 °C for 30 min. The precipitate was removed and the resulting solution could be used. The test sample or trypsin solution (25 μl) was mixed with 140 μl of the above casein solution and the reaction mixture was incubated at 37 °C for 15 min. Subsequently, 600 μl 5% trichloroacetic acid (TCA) was added. The reaction mixture was allowed to stand at room temperature for 30 min

before centrifugation at 8000 × *g* for 15 min. The absorbance of the supernatant was read at 280 nm against water as blank. Protease activity was calculated based on the activity of trypsin (7900 BAEE units/mg according to Sigma) in the protease assay using casein as substrate [28].

### 2.13. Assay of protease inhibitory activity

Some protease inhibitors inhibit HIV-1 reverse transcriptase [14,27]. Thus, the isolated peptide was assays for protease inhibitory activity. The assay was similar to the assay of protease activity except for the use of trypsin as the protease. The activity of trypsin toward casein was determined in the presence and also in the absence of the isolated peptide.

## 3. Results

### 3.1. Extraction of mushrooms fruiting bodies

Extracts were prepared from 16 mushrooms according to the following steps: hot water extraction, centrifugation, ethanol precipitation, centrifugation, precipitate drying, and homogenization.

### 3.2. HIV-1 RT inhibition activity of mushroom extracts

The HIV-1 RT inhibition ratio of samples from 16 edible medicinal mushrooms ranged from 9.2% to 97.6% when the extracts were tested at the concentration of 1 mg/ml (Table 1). The inhibition ratio of the following five extracts was above 50%: *Lactarius camphorates*, *Rametes suaveloens*, *S. crispa*, *P. sajor-caju*, *P. pulmonarius*, and *R. paludosa*. Peptide from *R. paludosa* demonstrated the highest inhibition ratio of 97.6% at 1 mg/ml. When the concentration was reduced to 0.2 mg/ml and 0.04 mg/ml, the inhibition ratios dropped to 42.6% and 14.3%, respectively. The $IC_{50}$ value was 0.25 mg/ml.

**Table 1 – HIV-1 RT inhibitory activity of extracts from 16 edible medicinal mushrooms when tested at 1 mg/ml**

| Mushroom | Inhibition (%) |
|---|---|
| *Grifola umbellate* | 45.8 |
| *Lactarius camphorates* | 53.5 |
| *L. tigrinus* | 46.7 |
| *M. esculenta* | 9.2 |
| *Pholiota adipose* | 18.7 |
| *Pleurotus eryngii* | 20.2 |
| *Pleurotus nebrodensis* | 40.5 |
| *P. pulmonarius* | 70.8 |
| *P. sajor-caju* | 70.8 |
| *Polyporus umbellatus* | 45.8 |
| *Rubella esculenta* | 10.6 |
| *R. paludosa* | **97.6** |
| *S. commune* | 22.3 |
| *S. crispa* | 70.3 |
| *T. suaveloens* | 69.0 |
| *Tricholoma gambosay* | 46.5 |

Bold value represents highest inhibitory activity.

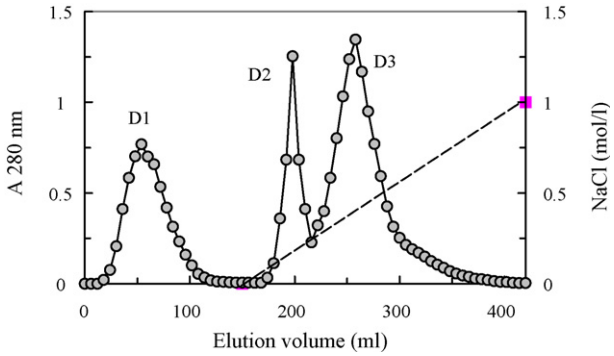


**Fig. 1 – Ion exchange chromatography of *R. paludosa* extract on a DEAE-cellulose column (1.5 cm × 20 cm) in 10 mmol/l phosphate buffer (pH 7.5). The slanting broken line across the chromatogram represents the linear concentration (0–1 mol/l) gradient of NaCl used to elute the adsorbed peaks. Buffer flow rate: 2 ml/min; fraction size: 6 ml/tube.**

### 3.3. Purification and characterization of *R. paludosa* peptide

The extract of *R. paludosa* exhibited strong absorption at 280 nm and was selected for fractionation because it showed the most potent HIV-1 RT inhibitory activity. DEAE-cellulose was employed to purify a peptide from the extract. Three absorbance peaks, designated as D1, D2, and D3, respectively, were obtained (Fig. 1). Only D3 distinctly showed HIV-1 RT inhibitory activity, At the concentrations of 1 mg/ml, 0.2 mg/ml, and 0.04 mg/ml, it inhibited 98.4%, 75.2%, and 28.4% of HIV-1 RT activity, respectively. The $IC_{50}$ value of D3 was 0.086 mg/ml.

Fraction D3 was subjected for further purification by gel filtration on an FPLC-Superdex 75 column. Two peaks, SU1 and SU2, resulted (Fig. 2). SU2, with a molecular mass of 4.5 kDa, demonstrated potent HIV-1 RT inhibitory activity. At the concentrations of 1 mg/ml, 0.2 mg/ml, and 0.04 mg/ml, it inhibited 99.2%, 89.3%, and 41.8% of HIV-1 RT activity,

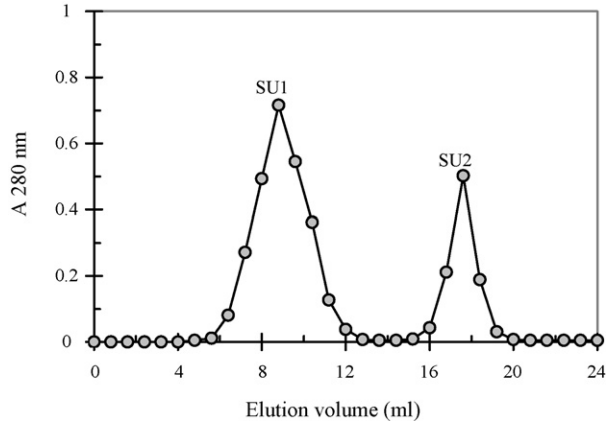


**Fig. 2 – FPLC–gel filtration of fraction D3 on an FPLC Superdex 75 HR 10/30 column. Buffer: 200 mmol/l $NH_4HCO_3$ (pH 8.6); flow rate: 0.4 ml/min; fraction size: 0.8 ml.**

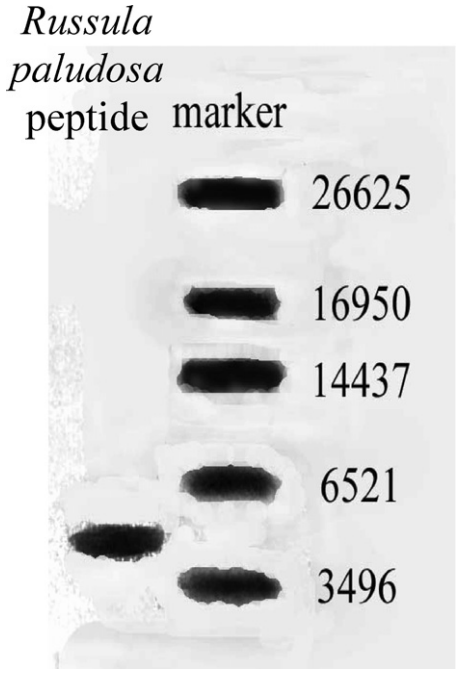


**Fig. 3 – SDS-PAGE of *R. paludosa* peptide. Left lane: *R. paludosa* peptide; right lane: molecular mass markers.**

respectively. The $IC_{50}$ of SU2 was 0.05 mg/ml (11 μM). There was five-fold of purification from extract to the purified peptide with regard to inhibition of HIV-1 reverse transcriptase. The N-terminal amino acid sequence of SU2 was KREHGQHCEF. It was dissimilar to sequences reported in the literature. Its molecular mass was estimated to be 4.5 kDa in SDS-PAGE (Fig. 3) and gel filtration on Superdex 75 and Superdex Peptide columns (data not shown). The peptide was devoid of HIV-1 integrase inhibiting, hemagglutinating, ribonuclease, antifungal, laccase, protease, and protease inhibitory activities.

## 4. Discussion

There are some inconveniences and problems in researching on anti-HIV medicinal materials because HIV is infectious and also because of lack of a proper, easily accessible animal model. Screening of inhibitors to the key enzymes of HIV life cycle is currently a strategy to search for anti-HIV drugs [1–4,6–8,10,11].

Edible and medicinal mushrooms produce compounds including polysaccharides and proteins without overt cytotoxicity, and they represent a resource for seeking new natural drugs [9,17,18,26–28,30–35]. *R. paludosa* is a wild edible mushroom collected from Heilongjiang Province, China. Its fruiting bodies are abundant in the summer. Its mycelia can be obtained in bulk quantities by deep fermentation at any time. The crude extract from *R. paludosa* exhibited an inhibitory effect on HIV-1 RT, and fraction SU2 derived from it by DEAE-cellulose anion exchange chromatography and gel filtration on Superdex 75 exhibited potent inhibitory activity on HIV-1 RT. Some antifungal proteins [18], lectins [27], ribonucleases [26], and laccases [30] of mushroom origin are known to inhibit HIV-1 reverse transcriptase. It is noteworthy that the peptide isolated in the present study does not belong to any of these classes of these proteins because it does not possess any of the bioactivity specific to these proteins. In fact its N-terminal sequence does not bear any resemblance to published sequences. Its HIV-1 RT inhibitory potency is within the range of potencies exhibited by other anti-HIV natural products [13]. It exerts its inhibitory action probably by protein-protein interaction, just like the inhibition of HIV-1 RT by HIV-1 protease [ ]. It is noteworthy that it does not inhibit HIV-1 integrase. The lack of other biological activities in the isolated peptide indicates that it is a new type of peptide.

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