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Original Research Article

Antibacterial Effect of Peptide Compound Isolated from Horseradish (*Armoracia rusticana)* Tissue Culture

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ABSTRACT

Antimicrobial peptides are an abundant group of proteinaceous compounds widely produced in the plant kingdom. Here, we report the purification and partial characterization of a novel antimicrobial peptide compound from horseradish tissue culture (*Armoracia rusticana*), named Ar-AMP, with bactericidal activity against both Gram-positive and Gram-negative bacteria. The production of antimicrobial peptides was induced with fungi *Aspergillus ochraceus*. Ar-AMP was isolated by precipitation with amonium sulphate and separated on SPE and RP-HPLC and then characterized by tricine electrophoresis and mass spectrometry. The molecular mass of the Ar-AMP was determined to be 4 828.61 Da. The activity of isolated peptides was tested by agar-well diffusion test and the MIC was estimated with the apparatus Bioscreen C. The Ar-AMP exerted antibacterial activity towards *E. coli*, *P. aeruginosa, S. enteritidis* and *B. megatherium* at tested concentration 100 µg/ml. The. MIC was estimated 50 µg/ml for *E. coli* and 100 µg/ml for *B. megatherium*.

Keyword: Antibacterial activity; peptide; *Armoracia rusticana*; tissue culture

INTRODUCTION

As antibiotic resistance increases worldwide, pressure rises to develop novel classes of antimicrobial compounds to fight infectious diseases. Peptide therapeutics represents a novel class of therapeutic agents [1]. Plants are continuously exposed to different kinds of stress, ranging from environmental conditions, such as temperature, salt and humidity variation, to pest and pathogen predation, which includes insects, nematodes, viruses, bacteria and fungi. In response to abiotic and biotic stress, plants have developed numerous

defense mechanisms during their evolution to minimize detrimental effects of such stress [2, 3]. Production of antimicrobial peptides (AMPs) is a widely used defense strategy. AMPs are pivotal components of innate immunity constituting an ancient defense mechanism found in diverse organisms including microorganisms [4], arthropods [5], plants [3, 6] and animals [7]. In plants antimicrobial peptides play an important role in defense against bacterial and fungal infections. The presence and expression of genes encoding antimicrobial peptides in a wide variety of plant species investigated thus far, demonstrations of their bactericidal and fungicidal activity *in vitro* and correlations between expression levels of peptides and susceptibility to a given pathogen or the extent of resistance of a particular bacterium to plant-derived peptides and its virulence have been observed [8].

The first isolated antibacterial peptide from a plant species was the purothionin from wheat flour (*Triticum aestivum*) [9]. Almost 40 years later, several additional peptides with antibacterial activity have been characterized, represented not only by thionins, now named defensins, but also by other groups of proteins such as cyclotides, glycine-rich proteins, snakins, 2S albumins, and hevein-type proteins [3, 10-18]. Peptides have been isolated from roots, seeds, flowers, stems, and leaves and have demonstrated activities towards phytopathogens, as well as against bacteria pathogenic to humans [1, 10, 12, 19]. Antibacterial peptides have become an interesting tool for the development of new techniques in control of crop losses and in the production of novel antibiotics for treatment of diverse human infections [20, 21].

Plant extracts and essential oils are known for their antibacterial, antifungal and antiinsecticidal effects [22, 23]. Although numerous studies have been carried out to extract peptides with antimicrobial effect from the family *Brassicaceae*, none of them was isolated from horseradish. Horseradish (*Armoracia rusticana*), a perennial herb, is an important Brassica crop in United States and Northern Europe. Most of the commercial crop is used fresh as a food additive for its pungent flavor [23]. Horseradish is a source of many biologically active compounds. Many of its chemical substances, mostly enzymes and essential oils, were isolated from the root where the main focus was given to peroxidases [24-26], myrosinases [27] and isothiocyanates [28, 29]. Plant cell cultures of *Armoracia rusticana* have been studied for their secondary metabolites production and their protein content and were found to produce and release into the culture medium several proteins, primarily peroxidase isoenzymes [25]. However, no antimicrobial protein or peptide from *A. rusticana* have been described or identified to date.

This work describes some biochemical and functional aspects of a new peptide with antibacterial activity isolated from *Armoracia rusticana* tissue culture after induction with a fungal pathogen *Aspergillus ochraceus*.

MATERIALS AND METHODS

Bacterial and fungal strains

The bacterial strains *Escherichia coli DBM 3001*, *Pseudomonas aeruginosa* DBM 3084, *Salmonella enteritidis* DBM 3026, *Bacillus megatherium* DBM 3045, *Enterococcus faecalis* DBM 3075 and *Staphylococcus aureus DBM 3002* were obtained from the collection of Institute of Chemical Technology in Prague together with the fungal strain *Aspergillus ochraceus* DBM 4241.The mycelia of *Aspergillus ochraceus* were prepared from a stock plate. The spores were transferred to a fresh plate with Sabouraud agar and incubated at 28 °C for 72 hours before use.

Armoracia rusticana tissue culture propagation and infection with *Aspergillus ochraceus*

The small part of horseradish root tissues were propagated *in vitro* on a solid Murashige and

Skoog (MS) nutrient medium containing 8.5 g/l of agar and 30 g/l of glucose without any growth regulator. Culture conditions were: 24 °C, 16-hour light/8-hour dark period. Tissues were subcultured every three weeks. Two weeks after growing on a solid medium, 4 g of inoculum with leaves was aseptically transferred into 250 ml Erlenmeyer flasks, containing 100 ml of MS liquid nutrient medium containing 30 g/L of glucose without addition of growth regulators [30]. Culture conditions were: 24 °C, 16-hour light/8-hour dark period and 140 RPM orbital shaker. After 3 weeks, the half of tissue culture was collected manually, air-dried and frozen (non-infected tissues), the second half was infected with *Aspergillus ochraceus* fungal spores. The infected plants were collected manually after 2 days of induction, air-dried and frozen at – 20 °C.

Extraction and protein isolation

Collected plant material of *Armoracia rusticana* from non-induced and induced tissue culture (200g) was ground into fine powder with mortar and pestle in liquid nitrogen. Peptides and proteins were extracted overnight at 4°C in a cold buffer (50 mM Tris-HCl, 2 mM EDTA, 1,5 % PVPP, pH 7,5) with mixture of protease inhibitors for plant cell and tissue extracts (SIGMA). Crude extract was centrifuged at 12.000 g for 30 min at 4°C and the supernatant was precipitated with amonium sulphate added to 30 % and 90 % saturation (added slowly while continously stirring on ice). The precipitate was collected by centrifugation at 13.000 g for 10 min at 4 °C and resuspended and dialyzed against distilled water in 1000 Da cut-off dialysis tubing. After dialysis, samples were lyophylized and resuspended in 0.1% trifluoroacetic acid (TFA) in water.

Solid-Phase Extraction on Chromabond C-18 Cartridges

Dialysed samples in 0.1% (v/v) aqueous TFA were fractionated into hydrophilic and hydrophobic fractions using Chromabond C-18 cartridges (Macherey-Nagel, Germany), activated by 80% (v/v) aqueous acetonitrile, rinsed with water and the samples were loaded repeatedly. Hydrophilic fractions were obtained using 0.1% TFA elution. Hydrophobic fractions were eluted from cartridges by 80 % aqueous acetonitrile. All fractions were concetrated using Speed-Vac.

Reverse-phase chromatography purification (RP-HPLC)

Samples were lyophilized and dissolved in MilliQ water solution of 0.1 % TFA to concentration 1 mg/ml, then separated using HPLC (Hewlett Packard model 1100, Chemstation software). HPLC semi-preparative procedure employed Discovery® BIO Wide Pore C8 250 mm x 10 mm x 5 µm column (Sigma). Elution was performed at 3 ml/min using gradient of acetonitrile from 0.8 to 80% in 0.1% TFA (v/v). Elution times of the hydrophilic and hydrophobic fractions were 45 and 75 minutes, respectively. The absorbance was monitored at 214 nm. Fractions were manually collected, concentrated with Speed-Vac, lyophilized and reconstituted to final concentration to 100 µg/ml in MilliQ water before analysis of antimicrobial activity.

SDS-PAGE Analysis

SDS-tricine gel electrophoresis was performed according to the method of Schägger [31], using Mark12™ Protein Standard from Invitrogen (2.5–200 kDa).

Mass spectrometry analysis

Mass spectra of the isolated fractions were recorded using a hybride FT-HR mass spectrometer LTQ Orbitrap XL (Thermo). Samples dissolved in acetonitrile/0.1% formic acid in water (1:1, v/v) were injected into the mobile phase of the same composition at a flow rate of 100 µl/min. The electrospray ion source was operated in positive ion mode with ESI

voltage and capillary voltage at 4500 and 30 V, respectively. Nitrogen was used as the sheath, auxiliary, sweep and collision gas. The capillary temperature was 280°C. In the MS mode, the collision energy was set at 10 V. Fractions were MS de novo sequenced using CID fragmentanion. Fragmentation mass spectra were measured using by in FT mass spectrometry mode with resolution 100 000. Fragmentation spectra were processed by "peak" sofware (Thermo).

Antibacterial and haemolytic bioassays

Relative antimicrobial activity was determined using the agar-well diffusion method [32]. For antimicrobial activity evaluation, each bacterial strain was grown in LB medium till stationary stage. Bacterial cells were washed with PBS and resuspended to a final optical density of 0.1 at OD 600 nm. 1 ml of bacterial suspension was spread onto the surface of Mueller-Hinton agar or Mueller-Hinton blood agar and the excess liquid was removed with a pipette. Afterwards, 3 mm diameter wells were punched into the agar and filled with 30 µl of the tested peptide solution and the plates were then incubated at 37 °C for 12 hours before inhibition zones were measured. Control wells were filled with deionized water. All experiments were carried out in triplicates. The antimicrobial activity of the samples was compared with commercially available antibiotics kanamycin for Gram-negative bacteria and vancomycin for Gram-positive bacteria at tested concentrations 1 mg/ml, 0.1 mg/ml and 0.01 mg/ml. Haemolytic activity was measured qualitatively after observation of brighter zone on blood agar.

The MIC of the purified peptide was determined by measuring the growth inhibition of *E. coli* and *B. megatherium* using Bioscreen C apparatus, a computer-controlled incubator evaluating turbidity of the samples (Growth Curves LtD., Finland). Turbidimetric measurements were taken during the course of the 24 h run in a testing volume of 330 µl. The MIC was defined as the lowest final concentration of the compound at which no growth was observed during exponential phase of the bacterial growth.

RESULTS AND DISCUSSION

Previously observed antimicrobial activity of the horseradish lysate led to the further examination of the responsible active compounds. Therefore the horseradish leaf cells grown in culture and infected with the fungus *Aspergillus ochraceus* to induce the expression of antimicrobial compounds. Non-infected control samples were also included to identify molecules produced as a response to fungal infections. Tissue lysates were prepared and proteins precipitated with ammonium sulfate and analyzed by tricine electrophoresis (Fig. 1A). The increased abundance of protein was observed in the sample from the induced culture. The supernatant obtained after amonium sulphate precipitation displayed antimicrobial activity against *B. megatherium* at 1 mg/ml of total protein concentration (Fig. 1B). Further fractionation was performed by two chromatographic methods. Firstly, the SPE column was employed to separate the hydrophilic and hydrophobic fraction. The eluted fractions were then concentrated by Speed-vac evaporation and we obtained 0.34 and 2.67 mg of protein in the hydrophilic and hydrophobic fractions, respectively (using 200 g starting material). The hydrofobic fraction showed some antimicrobial activity against *Pseudomonas aeruginosa, Bacillus megatherium* and *Enterococcus faecalis* at a concentration of 0.5 mg/ml (data not shown), antimicrobial activity was not observed with the hydrophilic fraction. The hydrophobic fractions from infected and non-infected horseradish tissue cultures were further analysed by RP-HPLC

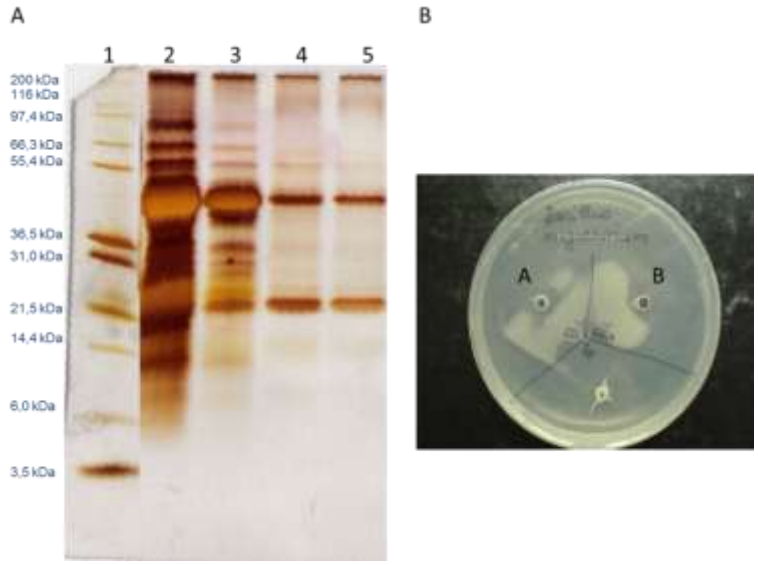

Fig.1 A: Tricine - SDS PAGE of non-induced and induced crude extract: Lane 1: Marker, Lane 2: Crude extract from induced culture 10 µg, Lane 3: Crude extract from induced culture 5 µg, Lane 4: Crude extract from non-induced culture 10 µg, Lane 5: Crude extract from non-induced culture 5 µg

Fig.1 B: Example of inhibition effect of crude extract from non-infected tissue culture (A) and infected tissue culture (B) on *B. megatherium*

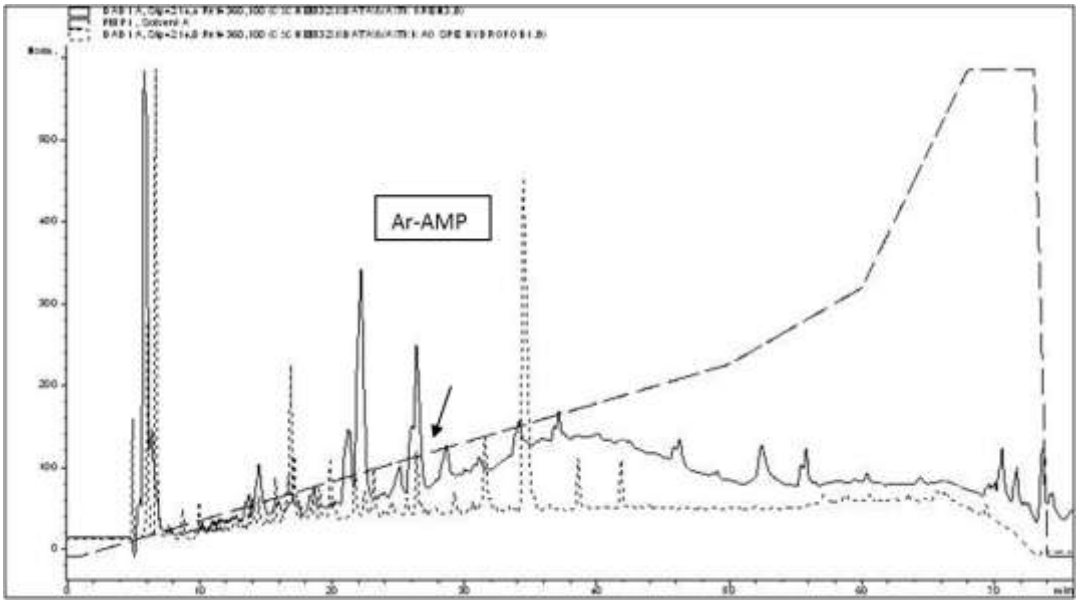

Fig. 2: Reverse-phase chromatogram profile of hydrophobic fraction from horse radish tissue culture extracts. Hydrophobic fractions from infected (dotted line) and non-infected (full line) tissue cultures were separated on by HPLC using the Discovery® BIO Wide Pore C8 column. The diagonal line showes the linear acetonitrile gradient (0 – 80%). The peak representing the fraction with antimicrobial activity is indicated

HPLC. We observed 18 different peaks, 8 of them were present only in the samples obtained from infected plant cultures or were increased in abundance compared to non-infected cultures (Fig. 2). All 18 fractions were separated and concentrated to final concentration 100 µg/ml, and tested for antimicrobial activity towards *E. coli, S. enteritidis, P. aeruginosa, B. megatherium, E. faecalis* and *S. aureus*. The fraction with the highest antimicrobial activity (fraction HP-12) was eluted with 19 % of acetonitrile (Fig. 2). Fraction HP-12 contained 0.2 mg protein based on Bradford analysis. There are several reports describing isolation of antimicrobial peptides of plant origin using reverse phase HPLC, where the peptides were eluted below 50 % concentration of acetonitrile gradient [33-36].

The HP-12 fraction significantly inhibited the growth of the Gram-negative bacteria *E. coli*, *S. enteritidis*, *P. aeruginosa* and the Gram-positive *B. megatherium* at a concentration of 100 µg/ml in the agar-well diffusion assay. Example of antibacterial effect on selected microorganism is shown in Fig. 3. No growth inhibition of *E. faecalis* and *S. aureus* was observed at this concentration (Table 1).

Table 1: Antibacterial activity of Ar-Amp (100 µg/ml) in agar-well diffusion method

Bacterial strain	Zone diameter (mm)
E. coli	27±0.5
P. aeruginosa	18±0.7
S. enteritidis	26±0.5
B. megatherium	14±0.4
E. faecalis	-
S. aureus	-

The results are the mean of three independent determinations

Fraction HP-12 showed no hemolytic activity on blood agar (Fig. 3). A minimal inhibitory concentration of the peptide (MIC) for *E. coli* and *B. megatherium* was measured as a lowest concentration of the peptide inhibiting the growth of bacteria in liquid culture and the MIC values were determined to 50 and 100 µg/ml, respectively.

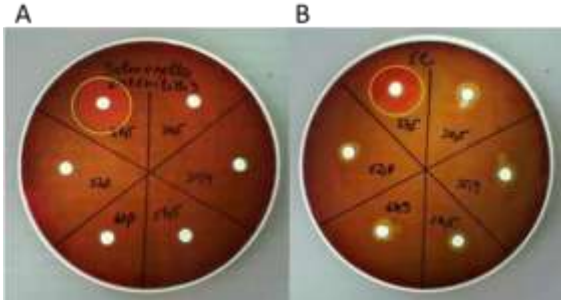

Fig. 3: Example of inhibition effect of fraction HP-12 obtained after RP-HPLC on *S. enteritidis* (A) and *E. coli* (B)

Fraction HP-12 was further analysed by Tris-Tricine SDS-PAGE which revealed a major band migrating with an apparent molecular weight of 6 kDa. Based on SDS-PAGE the purity of the isolated peptide was estimated above 98% (Fig. 4). Mass spectrometric analysis of the HP-12 fraction revealed a major peak with a molecular mass of 4828.61 Da (Fig. 5); we named this peptide Ar-AMP: *Armoracia rusticana*-derived Anti-Microbial Peptide. Proteins with different molecular mass were also detected in low amounts, most likely representing the minor contaminants seen in the Tris-Tricine PAGE.

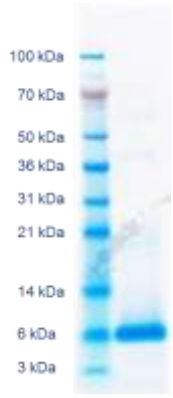

Fig. 4 SDS-PAGE analysis of the Ar-Amp: Lane 1; Marker, Lane 2: 5 µg of purified Ar-Amp

Hence, the acetonitrile concentration for elution and molecular mass of Ar-AMP show

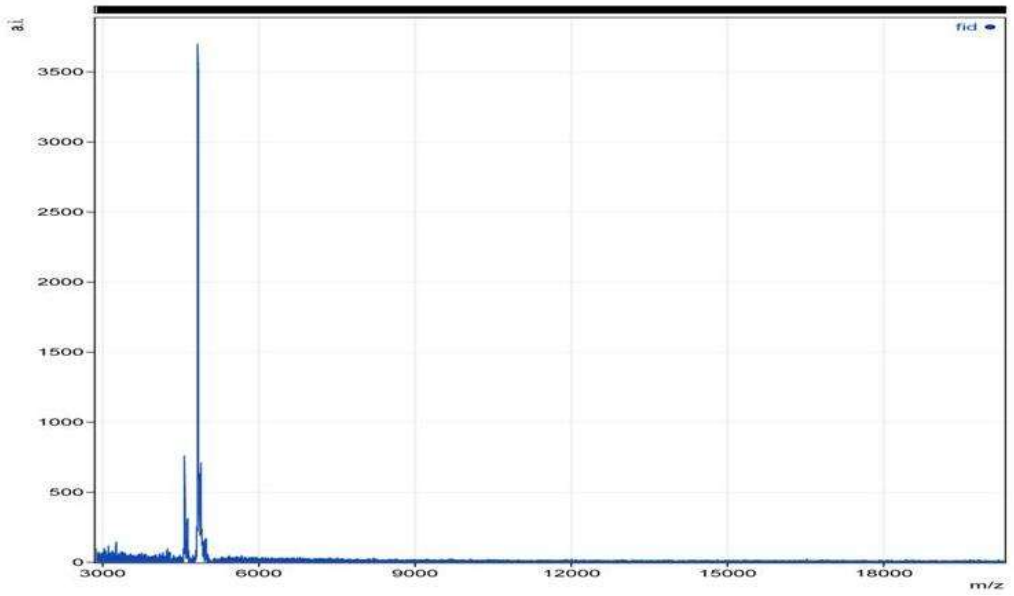

Fig. 5 MALDI-TOF spectrum of purified Ar-AMP from RP-HPLC

similarity to defensins, as well as the observed antibacterial activity.

Defensins are probably the most ancient antimicrobial peptides, present in the whole Eukaryote Domain [7]. Most defensins that are active against bacteria belong to animals or fungi. At the beginning of the 1990s the first plant defensins were characterized. As well as being the most ancient, defensins are among the most basic peptides, showing the size between 5 and 7 kDa [3]. The mature defensin peptide has eight conserved cysteine residues that are responsible for structural stabilization. In plants, they are normally found in abundance in seeds, but also appear in other tissues including leaves, pods, tubers, fruits, roots, bark and floral tissues [37]. This peptide class shows deleterious inhibitory activity against Gram-positive and Gram-negative bacteria, but the mechanism of action is not completely understood [38]. The minimal inhibition concentration of Ar-Amp was estimated 50 µg/ml for *E. coli* and 100 µg/ml for *B. megatherium*. Numerous known defensins act as highly toxic to Gram-negative pathogens, and this is supposed to be due to the primary binding of the charged peptides to the surface lipopolysaccharide. Franco et al. showed that the defensin Cp-thionin II isolated from *Vigna unguiculata* acts in a lethal action against *S. aureus* and *E. coli* and shows minimal inhibition concentration 128 µg/ml for Gram-positive *S. aureus* and 64 µg/ml for Gram-negative *E. coli* [39]. Zhang and Lewis demonstrated that fabatin has clear activity against the Gram-negative bacterium *P. aeruginosa*, but only slight toxicity against *E. coli*, suggesting that the action mode of this peptide is unlike that of other defensins [40]. The peptide with antibacterial activity was isolated from seeds of *Crotalaria pallida* and inhibited growth of *E. coli* by concentration of 32 µg/ml [35]. Defensins with similar molecular weight were isolated from different plants from the family *Brassicaceae*, including species *Arabidopsis halleri* [41], *A. thaliana, Brassica napus, B. rapa*, *Sinapsis alba* [42], *B. oleracea* [43], *Raphanus sativus* [19, 44] and *Lepidium meyenii* [45], but this is the first example of peptide with antibacterial effect isolated from *Armoracia rusticana*. Terras et al. showed, that Br-AFP_2 presented inhibitory activity against *B. megatherium* at the concentration of 52 µg/ml [42].

Our results underline the potential commercial application of horseradish peptides as plant defence agents against two bacterial groups that differ in the cell coat composition, because antimicrobial peptides probably develop their activity in the microbial cell surface.

CONCLUSION

This study is the first report on purification of a peptide with antibacterial effect from horseradish (*Armoracia rusticana*) tissue culture. This peptide named Ar-Amp inhibited the growth of both Gram-negative and Gram-positive bacteria. These compounds could represent a new strategy in biotechnology development and further investigation of the active metabolites should be performed to find new drugs against resistant microorganisms.

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CONFLICT OF INTEREST STATEMENT

None Declared

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