Nematicidal activity of *Remusatia vivipara* lectin expressed in *Escherichia coli*

Remusatia vivipara, commonly known as Hitchhiker Elephant Ear, is found in the Himalayas and Western Ghats of South India where it grows in humus-rich soils and as an epiphyte on shrubs and trees. Mainly its tubers and leaves are edible; also the tubers are used as folk medicine for treating inflammation and arthritis. Our previous study showed that tubers of *R. vivipara* contained lectin to the extent of ~390.0 mg per 100 g tissue and it constituted 26.70% of the total protein[1]. *R. vivipara* lectin (RVL1) agglutinated only rabbit erythrocytes, but not human erythrocytes of A, B and O groups. RVL1 showed strong inhibition by mucin and asialofetuin. Lectin activity was stable up to 80°C and under wide range of pH (2.0–9.3). RVL1 was a tetramer of Mr 49.5 kDa, with subunits differing for mass (12 kDa and 12.7 kDa) and N-terminal sequences. *R. vivipara* lectin gene (*rvl1*) was cloned by RACE–PCR and its sequence showed homology with the mannose-binding lectins from other members of the Araceae family[1,2]. Expression of plant lectins in *Escherichia coli* is important for various applications. An attempt was made to clone *rvl1* into *E. coli* and to express it. RVL1 expressed in *E. coli* was checked for its biochemical properties and nematicidal activity against the common root knot nematode, *Meloidogyne incognita*.

E. coli expression vector was constructed by releasing the *rvl1* gene from pNR64 (pTZ57R/T, a T/A cloning vector carrying *rvl1*), by double digestion with *Nco*I and *Eco*RI. Fragment was cleaned using MiniElute PCR Purification Kit (QIAGEN 28006) and cloned into pET-28a(+), cut with the same enzyme to get pNR72 (Figure 1), which was transferred first into *E. coli* DH5α and later into BL21(DE3)pLysS (Novagen #69388-3). The clone was confirmed by restriction digestion, *rvl1*-specific PCR and sequencing. For expression of RVL1, a 10 ml culture of *E. coli* BL21(DE3)pLysS cells transformed with the pNR72 was grown overnight at 37°C in Luria broth containing 50 mg/ml kanamycin. A 0.5 ml aliquot of this culture was used to inoculate 100 ml L-broth/kanamycin, which was incubated at 37°C until an A_{600} of approximately 0.6 was obtained. The bacteria were induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM, and growth was continued at 37°C for 3 h. After induction, cells were harvested by centrifugation, washed once with 0.5% (w/v) NaCl and stored at –80°C. The pellet was resuspended in 500 μl of phosphate buffer saline (50 mM, pH 7.2). The suspension was sonicated on ice for 1 min for six times at an interval of 1 min. Contents were centrifuged at 13,000 rpm, 4°C for 20 min. The supernatant was collected in a fresh tube and stored at –20°C for the future use. Total protein in the sample was estimated by the method of Lowry *et al.*[3].

The activity and sugar specificity of recombinant protein produced in *E. coli* were determined by haemagglutination assay and Hapten inhibition. Haemagglutination assay was carried out by the method of Lis and Sharon[4] using trypsinized rabbit and human erythrocytes (A, B and O) in an ELISA microtitre plate by the serial two-fold dilution technique of Liener and Hill[5] with some modifications. Inhibition of haemagglutination activity of the RVL1 was tested with a variety of sugars, sugar derivatives, glycoproteins and their glycoconjugates according to the method described by Hariharan and Rao[6]. Expression of *rvl1* in *E. coli* was checked with total protein isolated from induced BL21(D3)pLysS carrying pNR72 and pET-28a(+). Cell extract of *E. coli* with pNR72 contained a total protein of 12.01 mg. *E. coli* expressed RVL1 showed agglutination with only trypsinized rabbit erythrocytes (Figure 2), but not with human, sheep and goat erythrocytes, indicating that its haemagglutination property did not vary from that of native RVL1[1,2]. Minimum concentration of agglutinin required for agglutination was 0.195 μg. The specific activity and total activity was found to be 5.11×10^3 and 6.14×10^4 respectively, with rabbit erythrocytes. Of the nine sugars and three glycoproteins tested for

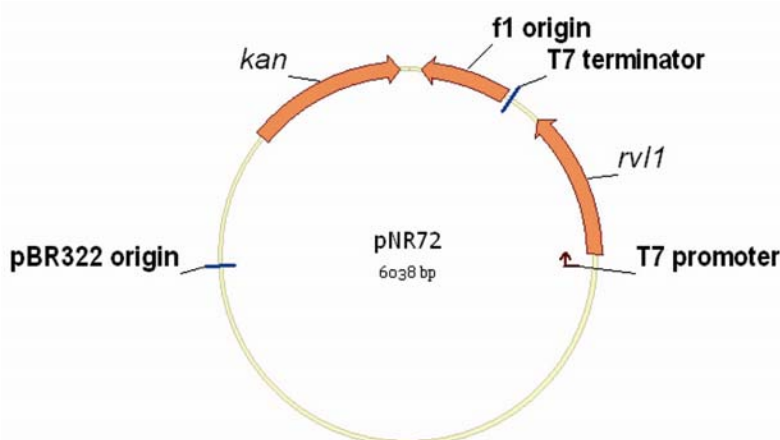

Figure 1. Vector map of pNR72.

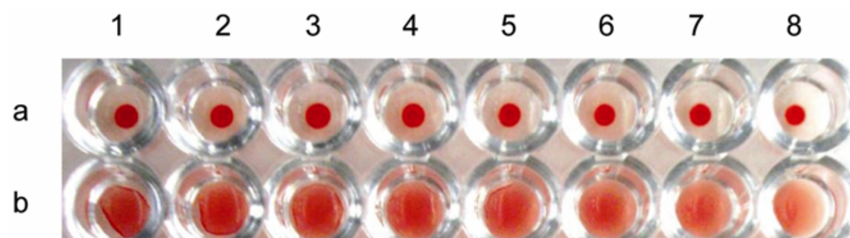

Figure 2. Haemagglutination assay of RVL1 with rabbit erythrocytes. a1–8, *Escherichia coli* cell extract without RVL1; b1–8, *E. coli* cell extract with RVL1.

Table 1. *Meloidogyne incognita* juvenile mortality (%) caused by *Escherichia coli* expressed RVL1

Time (h)	*E. coli* protein with RVL1 (12.01 mg) 1 : 25	1 : 50	1 : 75	1 : 100	*E. coli* protein without RVL1 (0.64 mg)	PBS	Distilled water
3	0	0	0	0	0	0	0
6	20	15	10	3	0	0	0
12	48	41	35	19	0	0	0
24	77	75	69	61	0	0	0
48	90	86	81	80	0	0	0

sugar specificity of the *E. coli* expressed RVL1, asialofetuin and mucin could inhibit haemugglutination with rabbit erythrocytes, indicating that there was no shift in the sugar specificity compared to tuber lectin (native). The minimum concentration inhibition (MCI) required was 3.125 and 1.56 μg for asialofetuin and mucin respectively. Several plant lectins have been successfully expressed in heterologous hosts such as *E. coli*, *Saccharomyces cerevisiae*, *Pichia pastoris*, *Spodoptera frugiperda* and COS7 cells for various applications[7–12]. Large number of lectins expressed in heterologous hosts was similar to that of native in terms of blood group and sugar specificity[7,8,11,13]. To the best of our knowledge, this is the first report on expressing RVL1 in *E. coli*.

In order to determine the growth inhibitory role of recombinant RVL1 on the common root knot nematode, *Meloidogyne incognita*, an *in vitro* bioassay was conducted by incubating 50 juveniles (stage 2; J2) in four dilutions (1 : 25, 1 : 50, 1 : 75 and 1 : 100) of 12.01 mg total protein from containing recombinant RVL1. Crude protein (0.64 mg) from *E. coli* BL21(D3)pLysS containing pET-28a(+), PBS and distilled water were used as the negative controls. Number of inactive juveniles was recorded at 3, 6, 12, 24 and 48 h and expressed in percentage. Juveniles not regaining activity even after 2 h in water were considered dead. Recombinant RVL1 showed nematicidal activity starting from 6 h of incubation and it increased with time and RVL1 concentration. Maximum mortality of 90% was noticed with 1 : 25 dilution after 48 h (Table 1). However, other dilutions (1 : 50, 1 : 75 and 1 : 100) also showed high mortality after 48 h. Juveniles placed in PBS, water and crude extract of *E. coli* BL21(D3)pLysS containing pET-28a(+) did not show any mortality.

Mechanism of lectin toxicity to nematodes has been well discussed[1,14,15]. Two lines of mechanisms have been recognized. When the diet containing lectin passes through the digestive tract of nematodes, lectin binds to the glycoproteins present all along the tract as found in insects[15]. In fact, our previous study with native RVL1 demonstrated that the lectin ingested by the nematode specifically interacted with gut lining, and the binding increased with time[1]. This interaction of lectins with the receptors can result in anti-feedant effect, or retarded growth or mortality[16–18]. Alternative mechanism involves interference in the sensory perception and the ability to establish feeding cells by the binding of lectins to glycoproteins localized on the surface of the nematodes or chemoreceptors present in the amphids and amphidial secretions. A glycoprotein surface receptor has been characterized from the nematode, *Panagrellus redivivus*[19]. RVL1 shows nematicidal activity both in its native and recombinant form in *E. coli*.

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