# Tn-SPECIFIC LECTINS PRODUCTION FROM *Salvia palifolia* and *Hyptis mutabilis* BY CELLULAR SOMACLONAL VARIATION

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## PRODUCCIÓN DE LECTINAS Tn-ESPECIFICAS OBTENIDAS DE *Salvia palifolia* y *Hyptis mutabilis* POR VARIACIÓN SOMACLONAL CELULAR

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## ABSTRACT

Lectins from several genus of Lamiaceae that recognize Tn antigen, present in enzymatically-modified erythrocytes and in cancerous tissue, have arisen considerable interest. In this work, we describe and compared the isolation of a Tn-specific lectin from seeds and in vitro cultured cells of Salvia palifolia and Hyptis mutabilis. For the latter, the somaclones that produced lectin were cultured in media supplied with 2-4 Dichlorophenoxy acetic acid and 6-Benzylaminopurine. The lectin purification scheme included PBS extraction, 75% EtOH precipitation, anion exchange, and affinity chromatography. Lectin activity was monitored by ELISA and agglutination of enzymatically-modified erythrocytes. SDS-PAGE of H. mutabilis lectin revealed two bands with Mr at 55 and 51 kDa; molecular weight determined by gel filtration was 82 kDa, the protein was a glycoprotein with 27.7% neutral sugars. SDS-PAGE of S. palifolia lectin showed two bands at 64 and 58 kDa; by gel filtration native protein showed one band at 77 kDa and has 23.8% sugar. We characterized the Tn-specific lectins with respect to pI, amino acid composition, N-terminal sequence, and carbohydrate inhibitory activity.

**Keywords:** Lamiaceae, Salvia palifolia, Hyptis mutabilis, Tn-antigen, lectins.

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## RESUMEN

Lectinas obtenidas de diferentes géneros de la familia Lamiaceae que reconocen el antígeno Tn, presente en eritrocitos enzimáticamente modificados y en células cancerosas han sido de especial interés. En este trabajo describimos y comparamos la extracción de lectinas Tn específica de semillas y de células cultivadas in vitro de Salvia palifolia e Hyptis mutabilis. Los somaclones que produjeron lectina fueron cultivado en medio suplementado con 2-4-D y 6-BAP, el esquema de purificación de las lectinas incluyo, extracción con PBS, precipitación con etanol al 75%, intercambio iónico, y cromatografía de afinidad. La actividad de le lectina fue monitoreada por el método de ELISA y aglutinación de eritrocitos modificados enzimáticamente. El ensayo de SDS-PAGE de la lectina de H. mutabilis mostro dos bandas con un Mr de 55 y 51 kDa en contraste con el peso molecular determinado por la técnica de filtración en gel de 82 kDa, la proteína es una glicoproteína con 27.7% de azucares neutros. El ensayo de SDS-PAGE de la lectina de S. palifolia mostro dos bandas de 64 y 58 kDa, mientras que por la técnica de filtración en gel se detectó una única proteína con 77 kDa y con un 23.8% de azucares. Caracterizamos las lectinas Tn-especificas con respecto a su pI, composición de aminoácidos, secuencia N-terminal, y actividad de carbohidratos inhibidores.


**Palabras clave:** Lamiaceae, Salvia palifolia, Hyptis mutabilis, Antígeno Tn, lectinas.

### Abbreviations

aBSM, asialo bovine submaxillary mucin; aOSM, asialo ovine submaxillary mucin; BAP, 6-benzylaminopurine; 2-4-D dichlorophenoxy acetic acid; DDCA, diethyldithiocarbamic acid; EME, enzymatically-modified erythrocytes; HML, *Hyptis mutabilis* lectin; MS, Murashige and Skoog; SPL, *Salvia palifolia lectin;* Murashige and Skoog culture medium; RBCs, red blood cells; PBS, phosphate buffer solution.

## INTRODUCTION

Lectins of *Salvia* and *Hyptis* genus that recognize the Tn antigen, GalNAc-α-O-Ser/Thr in a glycoprotein, have been detected in a number of temperate species (Bird and Wingham, 1977; Fernandez-Alonso, et al., 2009; Vega and Perez, 2006). The importance of Tn-specific lectins is related to the fact that this antigen is expressed in the surfaces of tumoral cells (Ghazarian et al., 2010, Robles et al., 2010). The tumor-associated Tn antigen has been investigated extensively as a biomarker and therapeutic target. Cancer vaccines containing the Tn antigen as a single tumor antigen or as a component of a polyvalent vaccine have progressed into phase I and II clinical trials (Li, et al., 2009).

Previous work on Lamiaceae species have been carried out in a few species. The lectin that recognize Tn antigen from *Salvia sclarea* seeds (SSL), was the first to be isolated and partially characterised by Piller and collaborators (1986), who established its specific binding to native Tn red blood cells and enzyme-treated, as well as the inhibitory potency of a variety of glycoproteins including aBSM. Medeiros and collaborators (2000) described several additional molecular features of SSL that have helped to define

Tn specificity. Besides recognizing $A^{MM}$ and $O^{NN}$ erythrocytes, the lectin binds strongly to Tn-bearing glycoproteins (Duk et al., 1994) and Tn-bearing lymphocytes (Thurnher et al., 1993). Wang and collaborators (2003) have described a lectin, Gleheda from leaves of *Glecoma hederacea* that readily interacts with O-glycans linked to asialo mucin or asialo fetuin in which Gal/GalNAc are terminally exposed. Fernandez-Alonso and collaborators (2003) studied 40 taxa belonging to 6 genera in Colombian Labiatae nutlets, lectin activity was detected in more than 80% of the species. Vega and Perez (2006) isolated a lectin from *Salvia bogotensis* seeds and they established its molecular properties, also its specificity for Tn, RBCs, and glycoproteins junction.

Hitherto studies about S. palifolia and H. mutabilis lectins and in vitro lectin production have not been reported. This work presents a new approach to obtain lectins, using cell culture methods, as a way to have at one's disposal a permanent source of lectins, avoiding the problem of seeds availability.

## MATERIAL AND METHOLS

### Callus production

Seeds and aerial parts of *S. palifolia* and *H. mutabilis* were collected at Fúquene and Pacho (Cundinamarca) respectively; species were classified by Dr. José Luis Fernandez (Instituto de Ciencias Naturales, Universidad Nacional, Colombia), (Fernandez-Alonso, et al., 2009). Seeds were disinfected in 3% sodium hipoclorite and 95% EtOH and germinated in vitro in MS culture medium (Murashige and Skoog,1962), supplied with 30 g $l^{-1}$ sucrose, 2.5 g $l^{-1}$ Phytagel (Sigma), pH 5.6, 22°C with 18 h photoperiod. Micro plants leave explants were grown in MS supplied with 10 ppm 2-4-D and 2 ppm BAP. The calluses produced were re-cultivated every four weeks in the same medium. Somaclones of these calluses were obtained in this medium by liquid callus cellular culture. Single cells were cultured in the same solid medium, but Phytagel® was replaced by Gel Rite® low melting point (Gifco) 2 g $l^{-1}$. Somaclones that appeared 4 weeks later were re-cultivated The callus produced was recultured each 4 weeks in the same medium and transferred to liquid medium, without Phytagel®. The individual cells were obtained by filtration in 50 mesh filters and were grown ($5x10^2$ cells $ml^{-1}$) in Gel Rite® low melting point with MS supplied with 10 ppm 2-4-D and 2 ppm BAP, during 6 weeks.

### Lectins extraction and purification

In vitro micro plants were established in *ex vitro* greenhouse conditions in a mixture of soil: charcoal (1:1) using a semi-hydroponics system of nutrition. Plants flowered 40-50 days later and produced seeds 45 days after *ex vitro* adaptation. Seeds were defatted with petroleum ether at room temperature and air-dried; 1-2 g seeds or calluses from *S. palifolia* and *H. mutabilis* were extracted (1:10 w/v) with 20 mM phosphate-150 mM NaCl buffer (PBS) pH 7.2; 5 mM EDTA, 0.5% diethyldithiocarbamic acid (DDCA), 4°C,

The sugar minimal inhibitory concentration that inhibited EME agglutination was established.

overnight, with stirring. The suspension was centrifuged at 18,000 rpm, 1h, 4°C and the pellet was re-extracted twice under the same conditions. The pool of supernatants was precipitated overnight with 75% EtOH -150 mM NaCl, 4°C and centrifuged at 18,000 rpm, 1 h, 4°C. The pellet was re-suspended in PBS and after centrifugation the viscous supernatant was applied to a column (2x20 cm) packed with Amberlite IRA-400 equilibrated in twice-deionised $H_2O$ (DDW). An un-retained fraction, which contained the lectin, as assessed by erythroagglutination assays, was eluted with DDW and subsequently was subjected to affinity chromatography on aBSM-Sepharose 4B as described by Vega and Pérez (2006). Briefly the procedure was as follows. The fraction eluted by DDW was concentrated X10 by ultra-filtration on Amicon PM10 and applied to a aBSM-Sepharose column (2x17 cm) equilibrated in PBS. After desorbing the un-retained fraction, the lectin was eluted with 50 mM Tris-OH, pH 11.4 and each fraction was neutralized to pH 7.2 with 2N HCl. Protein elution was monitored by measuring A280 nm and lectin was detected by erythroagglutination; the pool of lectin-containing fractions was exhaustively dialysed against 50 mM $NH_4HCO_3$ and freeze-dried.

### General methods

Protein concentration was determined by a modified Bradford assay (Zor and Selinger, 1996) or by microKjeldahl (Steyermark, 1961). Lectin activity was determined by ELISA using asialo ovine submaxillary mucin (aOSM) linked to the plate (Vega and Pérez, 2006) or by agglutination of enzymatically-treated $A^+$ erythrocytes (Hirohashi et al., 1985) to expose the Tn antigen. PAGE-SDS was done by the method of Laemmli (1970). *Mr* of native protein was assessed by gel filtration; lectin (1 ml, 1 mg $ml^{-1}$) was applied to a BioGel P-150 column (1.2x80cm) equilibrated in 150 mM Tris-HCl-150 mM NaCl buffer, pH 7.2; the same buffer was used for the run (flow, 1 ml/12 min) and protein was detected by $A_{280}$ nm. Protein neutral sugar content was estimated according to Dubois and collaborators (1956). pI was established by Bollag and Edelstein (1991) method; amino acid composition by the Hirs and Stein (1956) method. Tryptophan content was determined according to Spies and Chambers (1984); the N-terminal sequence was determined (Matsudaira, 1987), on bands cut from SDS-PAGE gels. Carbohydrate inhibition assays were done according to Pérez (1984) with *S. palifolia* lectin solution (323 µg $ml^{-1}$) or *H. mutabilis* lectin (HML) solution (141 µg $ml^{-1}$), the sugar being serially diluted.

## RESULTS AND DISCUSSION

### Callus production

*S. palifolia* and *H. mutabilis* seeds produced micro plants on *in vitro* culture using MS basic media without hormone addition. *S. palifolia* and *H. mutabilis* somaclones were obtained using a callus originated from micro plant young leaf explants and cultivated in MS supplied with 2-4-D and BAP. The explants were cultivated during 4 weeks, until the callus cell emerging from the tissue was observed; after obtaining a visible callus, each single cell of them being a somaclon, each of them were assayed for the presence of Tn lectins showing variable amounts production of lectin; amongst them we selected 16 *S. palifoli*a and *24 H. mutabilis* somaclones with the highest lectin activity. Lectin activity was also observed to be present when we cultivated the callus in liquid culture media. The somaclones of *S. palifolia* and *H. mutabilis* released different quantities of phenol compounds to the medium this being a limiting factor to choose the best somaclon that produced Tn lectins.

The *H. mutabilis* somaclones were brown, friable and with a high cellular division rate. The *S. palifolia* somaclones were dark brown, non-friable and with a lower cellular division rate. The lectin activity and protein content of seed extracts, somaclones and exudates in

culture medium are shown in Table 1. The lectin activity and protein concentration were lower both in callus and seeds extracts from *H. mutabilis* than in corresponding *S. palifolia* extracts, whereas there were not significant differences in agglutination ability between callus and seed lectins from the same species or amongst the two species. It is noteworthy that for *H. mutabilis* the lectin activity in cell culture medium (corresponding to secreted lectin), was similar to that of seed extracts and higher than in calluses; on the other hand *S. palifolia* lectin activity was significantly lower in exudates than in callus or seeds. The lectin activities, assessed by ELISA, agree well with the extent of erythroagglutination showed by the different preparations. The fact that callus released Tn-specific lectin to the culture medium suggests that lectin production in a bioreactor would feasible; as far as we are aware there are not any antecedents of lectin obtaining in this manner.

**Table 1.** Protein concentration and lectin activity in *H. mutabilis* and *S. palifolia* extracts from callus and seeds.

| Source | Protein content* $mg.g^{-1}$ | Lectin activity | Agglutination Tn erythrocytes** |
|---|---|---|---|
| *H. mutabilis callus* | 1.87 | 10.7 | 4 |
| *H. mutabilis exudates* | 0.58 | 26.0 | 2 |
| *H. mutabilis seeds* | 6.08 | 23.3 | 4 |
| *S. palifolia callus* | 2.29 | 67.9 | 5 |
| *S. palifolia exudates* | 0.83 | 30.0 | 2 |
| *S. palifolia seeds* | 25.17 | 50.9 | 5 |

* Determined by microKjeldahl | ** Agglutination score: 5: full agglutination; 1: agglutination barely observable

Affinity chromatography profiles of callus lectin from *H. mutabilis* and *S. palifolia* were very similar (Fig. 1). *H. mutabilis* yielded, besides the un-retained protein, devoid of lectin activity, a fraction eluted by pH 11.4, which showed by ELISA and by erythroagglutination lectin activity (74.1% and +5, respectively); in the case of *S. palifolia* the retained fraction, also showed activity both by ELISA (81.5%) and by erythroagglutination. Similar elution profiles were obtained

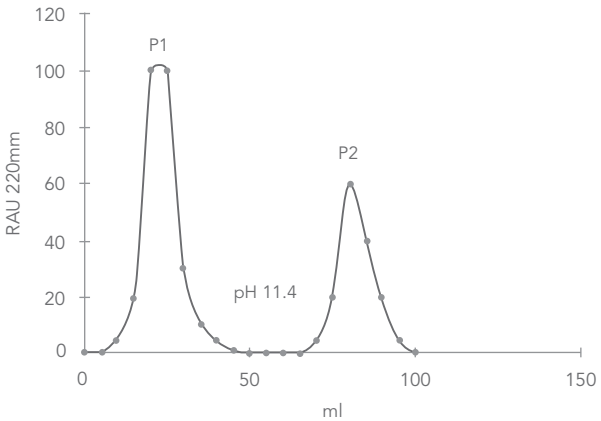


**Fig. 1.** Affinity chromatography of *H.mutabilis* and *S. palifolia* callus lectin on aBSM-Sepharose 4B. Elution procedure as described in methods. Lectin no-active and non-retained fraction (P1), lectin active fraction (P2) eluted with buffer Tris-OH 50mM, pH 11.4. Relative absorbance unit (RAU).

with seed extracts from both species (results not shown), demonstrating the usefulness of aBSM-Sepharose as an affinity support for Lamiaceae lectins.

### Lectin's molecular properties

Assessment of the sugar content showed that *S. palifolia* and *H. mutabilis* lectins from callus and seeds were glycoproteins. In *H. mutabilis* lectin, neutral sugars amounted to 27.7% whereas in *S. palifolia* lectin they were 23.8%. By SDS-PAGE *H. mutabilis* lectin presented two bands at 55 and 51 kDa; *S. palifolia* lectin showed two bands at 64 and 58 kDa. These values agree well with those observed for Lamiaceae lectins. *Salvia bogotensis* lectin showed two major bands at 72.6 kDa (33% total protein) and 38.8 kDa (54.2%) and a minor band (12.6%) at 35.7 kDa by Tricine-PAGE (Vega

and Perez, 2006), in reducing/non-reducing conditions; the two close bands were likely monomeric lectin glycoforms and the 72.6 kDa component corresponds to the dimeric form. In the case of SSL MALDI-TOF MS showed a 60-61 kDa protein (Medeiros et al., 2000) considered to be the protein's dimmer form.

The Mr of native protein, determined by BioGel P-150, is significantly higher both for *H. mutabilis* lectin (82 kDa) and for *S. palifolia lectin* (77 kDa) and probably these values result from unspecific interactions with the support. This behavior had been observed in seed and callus lectin cases. The isoelectrofocusing assay revealed for *H. mutabilis* lectin two bands with pI 7.5 and 6.7; *S. palifolia* lectin showed two bands with pI 7.4 and pI 6.8, It appears therefore that the two lectins have isoforms which can be observed by SDS-PAGE and by electrofocusing.

**Table 2.** Amino acid composition of *S. palifolia* and *H. mutabilis* Tn lectin, obtained from seeds and callus. Calculation is based on Mr 65 kDs for *H. Mutabilis* and 58.6 kDs for *S. palifolia.*

| | g/100g protein | | Calculated residues mol$^{-1}$ | |
|---|---|---|---|---|
| **AA** | **Hyptis** | **Salvia** | **Hyptis** | **Salvia** |
| Asp | 5.61 | 12.49 | 24.9 | 55.0 |
| Glu | 3.48 | 4.62 | 14.0 | 18.4 |
| Ser | 1.80 | 12.64 | 10.1 | 70.5 |
| Gly | 1.11 | 7.10 | 8.7 | 55.5 |
| His | 15.06 | 2.91 | 46.6 | 8.9 |
| Arg | 10.09 | 11.40 | 28.4 | 31.7 |
| Thr | 3.39 | 4.07 | 16.8 | 20.0 |
| Ala | 2.38 | 2.45 | 15.8 | 16.1 |
| Pro | 3.15 | 4.58 | 16.2 | 23.3 |
| Tyr | 9.14 | 2.06 | 29.9 | 6.6 |
| Val | 6.37 | 1.26 | 32.2 | 6.3 |
| Met | 1.01 | 3.46 | 3.3 | 11.2 |
| Cys | 1.70 | 2.46 | 6.1 | 8.9 |
| Ile | 3.82 | 5.50 | 17.2 | 24.6 |
| Leu | 7.12 | 2.94 | 32.1 | 13.1 |
| Phe | 10.74 | 5.50 | 31.5 | 15.9 |
| Lys | 10.63 | 5.20 | 34.5 | 16.7 |
| Trp | 0.49 | 0.11 | 1.4 | 0.4 |

The results of the amino acid analysis of the two lectins showed (Table 2) different proportions of charged, hydrophobic and polar residues. Whereas 10.5% of residues in *H. mutabilis* are acidic, against 18.3% in Tn lectin of *S. palifolia,* 29.6% in *H. mutabilis* were basic against 14.3% in *S. palifolia.* MW and amino acid composition data suggest that *S. palifolia* lectin encompasses about 400 residues; similarly 370 amino acids would be present in *H. mutabilis* lectin.

N-terminal sequences of *S. palifolia* and *H. mutabilis* lectins from seeds showed that for *H. mutabilis* 56 kDa band the sequence was Asn-Arg-Pro-Glu and Asn-Ile-Pro-Ser for the 48 kDa band. The N-terminal sequence of *S. palifolia* 60 kDa band was Glu-X-Ala-Phe-Phe-Lys-Lys-Lys-Gly-Leu; 50 kDa bands were not analyzed due to insufficient material. The lack of similarity between the N-terminal sequences of the two lectins points to the different molecular character that the lectins likely present.

The sugar minimal inhibitory concentration that inhibited EME agglutination was established. Amongst the tested sugars (*ca.* 30) only Gal derivatives inhibited *S. palifolia* and *H. mutabilis* seed lectins although to different extents. p-Nitro Phenyl Gal was the most

**Table 3.** Minimal concentration of sugar Tn lectin agglutination inhibitors.

| **Carbohydrate** | **Minimal concentration of inhbitors** | |
|---|---|---|
| | ***S. palifolia*** | ***H. mutabilis*** |
| N-Acetyl D-Galactosamine | 0.15M | 0.037M |
| *D-galactose* | - | - |
| *Metyl α-D-galactose* | 0.075M | 0.037M |
| *Metyl β-D-galactose* | 0.075M | 0.037M |
| *p-NitroPhenyl β-D-galactose* | 0.15mM | 0.037mM |

potent inhibitor (Table 3) for both lectins which suggests that hydrophobic interactions take place at or near the lectin's carbohydrate–binding site; the high GalNAc concentration needed to inhibit *S. palifolia* lectin indicates a weak binding to the lectin.

Sugar type and inhibitory concentrations vary widely for the hitherto described Lamiaceae lectins. *S. bogotensis* lectin is inhibited only by GalNAc (37.5 mM), (Vega and Pérez, 2006) Erythroagglutination by *Salvia sclarea* lectin and *Moluccella laevis* lectin was inhibited by lower GalNAc concentrations (0.1 mM and 0.03mM, respectively) and by p-NO2 Phenyl -α/β D-GalNAc (0.12/0.06 mM and 1.75/0.87 mM, respectively), (Piller et al., 1986 and Lis et al., 1988) whereas Gleheda inhibition assays with trypsin-treated human RBCs showed 50% inhibition at 25 mM GalNAc (Wang et al., 2003).

Although *S. palifolia* and *H. mutabil*is belong to not closely related Lamiaceae genus their Tn-specific lectins present similar biochemical characteristics. Both lectins recognize the Tn antigen in affinity chromatography, ELISA and A+ erythrocytes treated to expose the Tn antigen. However the lectins differ in some respects as MW, isoelectric point and sugar content. In this work we demonstrated that somaclonal selected callus is a good source of Tn lectin. We compared the seeds and callus Tn lectins of both species and we did not find any differences.

Lectin that recognise Tn antigen that is overexpressed in various tumour cell types on breast, ovary, and colon have remarkable importance. Measurement of Tn antigen and its expression, is a general problem in the field. The lack of reliable, consistent expression information is a fundamental problem for basic research, biomarker development, and therapeutic development (Li, et al., 2009). In the present study, we demonstrate that cell culture is a convenient source of lectins with similar properties to those shown by seed lectins. Furthermore the quantities of lectin presents on the liquid media will be an excellent source of them. Those result permit to think in the possibility to produce the lectins in a bioreactor facility.

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