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A first attempt to elucidate the amino acid sequence of some lichen lectins

(With 2 Tables & 3 Figures)

El primer intento en determinar la secuencia de aminoácidos de algunas lectinas de líquenes

(Con 2 Tablas y 3 Figuras)

Sacristán M, AM Millanes, B Fontaniella, ME Legaz, C Vicente

Abstract. Secreted arginases from *Evernia prunastri* and *Xanthoria parietina* thalli, exhibiting lectin activity, have been purified to homogeneity. Analyses of both amino acid and glycoside composition are reported. Purified proteins have been subjected to tryptic digestion and some amino acid sequences have been analyzed. An undecapeptide from *Evernia* arginase, which shows some degree of homology with the domain to which Mn^{2+} binds, a heptapeptide and an undecapeptide from *Xanthoria* arginase have been analyzed, and homologies with some arginases from other fungi and plants have been found.

Key words: *Evernia prunastri*, *Xanthoria parietina*, arginases, lectins, structure analysis.

Resumen. Arginasas segregadas por talos de *Evernia prunastri* y *Xanthoria parietina* que exhiben actividad lectina han sido purificadas a homogeneidad. Los resultados del análisis de su composición de aminoácidos y de la fracción glicídica son descriptos en este trabajo. Ambas proteínas purificadas han sido sometidas a digestión trípica y la secuencia de

Department of Plant Physiology, The Lichen Team. Faculty of Biology, Complutense University, JoséAntonio Novais s/n, 28040 Madrid, Spain

Address Correspondence to: Prof. Dr. Carlos Vicente. Department of Plant Physiology, Faculty of Biology, José Antonio Novais st., s/n. Complutense University, 28040 Madrid, Spain. Phone number: 34-1-3944565, Fax number: 34-1-3945034. e-mail: cvicente@bio.ucm.es

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aminoácidos de algunos de sus fragmentos ha sido analizada. De la arginasa de *Evernia* se ha identificado un undecapéptido que muestra algún grado de homología con el dominio al que se une el cofactor metálico Mn^{2+} , mientras que de la arginasa de *Xanthoria* se han analizado un heptapéptido y un undecapéptido que muestran homología con arginasas de otros hongos y plantas.

Palabras clave: *Evernia prunastri*, *Xanthoria parietina*, arginasas, lectinas, análisis de estructuras.

INTRODUCTION

Lichens are symbiotic associations between a fungus and a cyanobacterium (cyano-lichens) or a green alga (chlorolichens), joined to form a new biological entity different from its individual components. Recognition mechanisms used by lichens are based on the production and secretion of fungal lectins as signalling molecules (Kardish et al., 1991), which therefore develop arginase enzymatic activity (Molina et al., 1993). Whereas arginasas retained on the cell wall of fungal hyphae serve for adhering algal cells (contact lectins), secreted enzymes produce adhesion of free-living algal cells (recruitment lectins), which grow near the fungal partner. Both recruitment and contact require the occurrence of receptor sites (lectin ligand) on the surface of the potential photobionts, to which the lectin molecule binds. This receptor has been defined as a cell wall-pelletable, glycosylated urease in *Evernia prunastri* and *Xanthoria parietina* photobionts. Lack of the adequate lectin ligand makes incompatible the photobiont. Consequently, the lectin enters the algal cell, the concentration of polyamines increases (Molina & Vicente, 1995), and these polyamines, mainly putrescine, induce ultrastructure damages such as chloroplast disorganization, hydrolysis of the cell wall and, finally, cell death (Molina et al., 1998). Increases in polyamine concentration are the result of the arginase activity of the signaling protein. On the contrary, the occurrence of the ligand at the algae surface retains the lectin outside the cell, and preserves both the structure and function of the recognized photobiont (Legaz et al., 2004).

Previous results (Molina & Vicente, 2000; Legaz et al., 2001) indicated some similarities between secreted arginasas from *E. prunastri* (SAE) and *X. parietina* (SAX) with respect to their amino acid composition (Table 1). These similarities were:

- Both proteins contained Asx, Cys and Trp in a similar proportion.
- Content of Ala was identical for both arginasas.

- Although Gly and Glu were the most abundant amino acids for both arginases, their percentage value was greater in SAX than in SAE.
- The lowest content of Val was found in SAX.
- SAX contained lower amounts of Ile, Leu and Lys, and higher amounts of His, than SAE.

Substantial differences in the amino acid composition of the purified lectins were expected for SAE, SAX, and *Dictyonema glabratum* (*Atheliaceae*) on the basis of their phylogenetical distance (Elifio et al., 2000). This was because of (1) the presence of the amino acid Phe, (2) the fact that the content of Gly and Ser was lower than that found for SAE and SAX and, (3) the absence of Tryp. Only the low amount of Met in *D. labratum* lectin was similar to that found in SAX.

The glycosidic moiety of SAX contained equimolar amounts of both D-galactose and D-glucose (Molina & Vicente, 1996), whereas that of SAE contained 280 residues of glucose, 85 of mannose and 27 of fructose per molecule of protein (Planelles & Legaz, 1987). The lectin of *D. glabratum* contained mainly galactose and xylose and minor amounts of glucose, mannose, glucosamine and galactosamine (Elifio et al., 2000).

Binding of fungal arginase to glycosylated urease in the algal cell wall implies an affinity mechanism between some specific amino acid residues of the lectin and specific sugar residues of the glycoside moiety of the ligand (Table 2). This is a polygalactose chain attached to the urease polypeptide in *Evernia prunastri* (Pérez-Urria & Vicente, 1989). This polysaccharide moiety results to be an α - (1,4) polygalactoside, as found by Sacristán et al. (2006). Nevertheless, other receptors exist different from α -(1,4) polygalactosyl urease. This is because concanavalin A, the lectin from *Canavalia ensiformis*, is able to bind to the cell wall of algal cells recently isolated from *Evernia prunastri* and *Xanthoria parietina* thalli (Fontaniella et al., 2004). Concanavalin A of *C. ensiformis* also develops arginase activity. This binding involves a ligand, probably a glycoprotein containing mannose, which can be isolated by affinity chromatography. Concanavalin A also binds to the cell wall of *Nosotoc* from several *Peltigera* species (Marx & Peveling, 1983). In addition, a new lectin has been isolated from an aqueous extract of the symbiotic phenotype of *Dictyonema glabratum* and its cyanobacterial photobiont *Scytonema* sp. (Elifio et al., 2000). The lectin is a glycoprotein with a low degree of glycosylation, with both N- and O-linkages. The receptor the lectin binds to contains N-acetylgalactosamine as the most active monosaccharide for affinity relationship.

In this work, we analyzed the amino acid sequence of secreted arginases from *E. prunastri* and *X. parietina* to find homologies that support the function of both lectins produced by two phylogenetically unrelated lichen species.

Table 1. Quantitative composition in amino acids (mol/mol) of *Evernia prunastri* (SAE) and *Xanthoria parietina* (SAX) according to Legaz et al. (2001) and Molina & Vicente (2000), respectively.

Tabla 1. Composición cuantitativa de aminoácidos (mol/mol) de *Evernia prunastri* (SAE) y *Xanthoria parietina* (SAX) siguiendo a Legaz et al. (2001) y Molina & Vicente (2000), respectivamente.

Amino acid	SAE	SAX
C (Cys)	0.8	1.13
D + N (Asx)	11.6	9.88
T (Thr)	6.0	3.56
S (Ser)	9.8	14.58
E + Q (Glx)	14.9	18.04
P (Pro)	4.8	2.41
G (Gly)	15.4	22.39
A (Ala)	8.4	7.13
V (Val)	5.6	2.72
M (Met)	1.0	0.17
I (Ile)	3.2	1.45
L (Leu)	5.6	2.1
Y (Tyr)	1.8	1.07
F (Phe)	2.3	0.34
H (His)	1.4	6.28
K (Lys)	4.6	2.87
R (Arg)	2.4	4.0
W (Trp)	1.2	1.02

Table 2. Monosaccharide residues per molecule of secreted arginases purified from *E. prunastri* (SAE) and *X. parietina* (SAX).

Tabla 2. Residuos de monosacáridos por molécula de argininas segregadas, purificadas de *E. prunastri* (SAE) y *X. parietina* (SAX).

Monosaccharide	SAE	SAX
Glucose	280	8
Mannose	85	-
Fructose	27	-
Galactose	-	4

MATERIALS AND METHODS

Plant material and chemicals

Evernia prunastri (L.) Ach. (Parmeliaceae) containing *Trebouxia excen-trica* as a photobiont, and *Xanthoria parietina* (L.) Th.Fr. (Teloschistaceae) containing *Pseudotrebouxia aggregata* as a photobiont, were collected during January 2003 in La Quinta (El Pardo, Madrid, Spain). Both species grow on *Quercus rotundifolia* Willd. They were dried under air flow, and stored in the dark for no longer than two weeks. All chemicals used in this work were from Sigma Chemical Company (St.Louis, MO, USA) unless stated otherwise.

Purification procedures

Secreted arginase from *Evernia* (SAE) was purified according to Legaz et al. (2001). Samples of 50 g of air-dried thalli of *E. prunastri* were floated on 1250 ml of buffer (10 mM Tris-HCl, pH 9.15) containing 40 mM L-arginine for 8 h at 26 °C in the dark. Then, the medium was filtered through Whatman 3MM filter paper (W. and R. Balston Ltd, Maidstone, UK), concentrated under air flow up to 300 ml, filtered through Millipore GS filters (0.22 mm diameter, Millipore Corporation, Billerica, MA, USA) and immediately precipitated with ammonium sulphate at 50% (w/v). The mixture was stored for 2 h at 4 °C, thereafter centrifuged for 1h at 43,000 x g at 2 °C and subjected to dialysis. Protein in the dialysate was then adsorbed on calcium phosphate gel. The eluate from 0.14 M Tris-HCl buffer (pH 9.1) contained the highest arginase activity. This eluate was chromatofocused by using a LKB 8100 column (LKB, Bromma, Sweden). The pH gradient was made with 1% (w/v) ampholines (Servalyt, Serva Feinbiochemica Co., Heidelberg, Germany), pH 3.5-10, and the density gradient was prepared from 5 to 50% sucrose solutions. The electrode solutions were 1.0 M NaOH at the cathode, and 1.0 M H₃ PO₄ at the anode. The cooling temperature was 4 °C. Fractions of 2.0 ml were collected and assayed for arginase activity and pH value. The fraction eluted at pH 5.6 contained the highest arginase activity.

Alternatively, samples of 15 g of *X. parietina* thalli were floated on 150 ml 10 mM Tris-HCl buffer (pH 9.1) for 1h at 26 °C in the dark. Secreted arginase (SAX) was purified from the incubation media according to Molina & Vicente (2000). This was made by precipitation with ammonium sulphate at 50% saturation, adsorption on calcium phosphate gel (SAX was desorbed with 220 mM Tris-HCl) and filtration through a column of Sephadex (Pharmacia, Uppsala, Sweden) G-150 (30 cm x 3.0 cm i.d.). To test the

homogeneity of the protein, 10 µg of SAE and 6.0 µg SAX in 150 µl were mixed with 75 µl glycerol. This solution was applied onto 12% polyacrylamide gels, and subjected to standard electrophoresis, employing 50 mM Tris-glycine (pH 8.3) as running buffer. The mixture SeeBlue 2 plus (Invitrogen, Carlsbad, CA, USA) was used as molecular mass marker.

SDS-PAGE

Both proteins (SAE and SAX), purified at homogeneity, were analyzed by SDS-PAGE according to Laemmli (1970). Proteins were redissolved in Laemmli buffer containing 50 µM 2-mercaptoethanol. Samples were clarified by centrifugation at 20,000 x *g* during 15 min at 2 °C. Supernatants were boiled for 5 min and loaded onto 15% (w/v) polyacrylamide gel and developed by SDS/PAGE. Fifty milimols Tris/0.1 M Gly containing 0.1% (w/v) SDS was used as running buffer. Protein was revealed with colloidal Coomassie blue (Gel Code Blue stain reagent, Pierce, Rockford, IL, USA), the gel was dried in vacuum and scanned. Colloidal dye was selected since its sensitivity in revealing protein bands is similar to that obtained with silver stain (Neuhoff et al., 1988), about 6.0 ng protein (2.5 ng for the silver method using bovine serum albumin). The mixture SeeBlue 2 plus, from Novex Innogenetics was used as molecular marker.

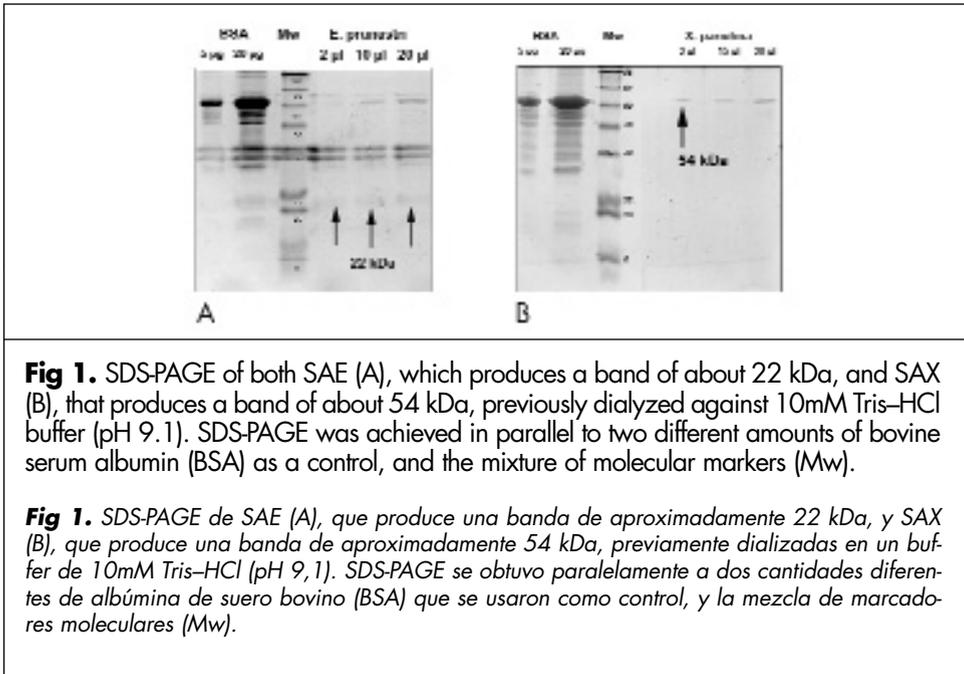
Enzymatic digestion and sequence analysis

Individual bands of SAE and SAX from polyacrylamide gels were digested with trypsin, analysing then extracted peptides in a MS spectrometer 4700 Reflector Spectrometer (Thermo) in tandem type MALDIT OF/TOF. From a first analysis, peptidic trends, after filtering the results to remove peaks corresponding to trypsin, were used to achieve a screening for peptide identification using MASCOT as a screening motor and all trypsin digested of protein stored in the SWISSPROT database. A peptide of molecular mass 1224.62, obtained from SAE and those of molecular masses 927.5 and 1478.78 from SAX were used to obtain a probable sequence de novo using a DENOVO TOOL program from Applied Biosystems.

RESULTS AND DISCUSSION

Tryptic digestion of individual bands of SAE and SAX in SDS-PAGE (Fig.1) produced different peptides. Their sequence was analyzed as described in methods. The sequence of the fragment of a molecular mass value of 1224.62, obtained after tryptic digestion of SAE, was revealed as PPG-

GHNKAKYR (Pro-Pro-Gly-Gly-His-Asn-Lys-Ala-Lys-Tyr-Arg). The tripeptide PPG also occurred in arginases from *Arabidopsis thaliana* (Perozich et al., 1998), *Schizosaccharomyces pombe* (Van Huffel et al., 1994) and *Pinus taeda* (Todd & Gifford, 2003), whereas GHN was repeated in arginases from *A. thaliana* and *G. max*, KAK in those from *Saccharomyces cerevisiae* (Kawamoto et al., 1987), and GGH in those from *P. taeda* (Fig. 2 and 3).



Tryptic digestion of SAX produced two different peptides. One of them, with a molecular mass of 927.5, had a sequence YLYELAR (Tyr-Leu-Tyr-Glu-Leu-Ala-Arg) in MS-MS. The tripeptide ELA was repeated in the sequences of *A. thaliana*, *S. pombe* and *G. max*. The second fragment, with a molecular mass of 1479.78 and a sequence KNGLKFGTSTR (Lys-Asn-Gly-Leu-Lys-Phe-Gly-Thr-Ser-Thr-Arg), contained the tripeptides TST and KFG, repeated in arginase from *G.max* and KGN, repeated in arginase from *Agaricus bisporus* (Wagemaker et al., 2005). No homologies were found between SAE and SAX with arginase from *Neurospora crassa* (Marathe et al., 1998).

It has been proposed that His141 of rat liver arginase directly binds arginine, acting as a catalytic proton shuttle (Canales et al., 2001). Glu277 is also involved in arginine binding, which implies the corrected orientation

of the substrate for the nucleophilic attack of Mn^{2+} -bridging water (Kanyo et al., 1996). Very similar conformation can be found in the active center of yeast arginase (Sumrada & Cooper, 1984), and soybean arginase (Goldraj et al., 1998). In soybean arginase, the tripeptide Gly-His-Asn, which contains the active rest of His for the association to the substrate, is clearly repeated in the undecapeptide obtained from SAE. However, Tyr rest found in the heptapeptide obtained from SAX did not have any equivalence to Tyr rests involved in the site for Ca^{2+} binding, required to develop lectin activity of secreted arginases in the recognition of compatible photobionts (Legaz et al., 2004). A motif Tyr100, Asp208 and Arg228 in the well-known legume lectin, concanavalin A, is required to Ca^{2+} binding (Shoham et al., 1979) and repeated in Tyr173, Asp280 and Arg301 from soybean arginase (Goldraj et al., 1998). Curiously, concanavalin A also binds to mannose-containing ligands in the cell wall of lichen photobionts, as it has been found by Fontaniella et al. (2004).

Protein samples (700 pmol) were applied to an amino acid sequencer, but an N-terminal sequence could not be assigned, suggesting that the N-terminal amino acid is possibly blocked and involved in the glycosylation of the protein.

Fig 2. Peptidic thread of SAE used to select the significant peak of 1224.62 Da to be sequenced.

Fig 2. Enlaces peptídicos de SAE utilizados para seleccionar el pico significativo de 1224,62 Da que va a ser secuenciado.

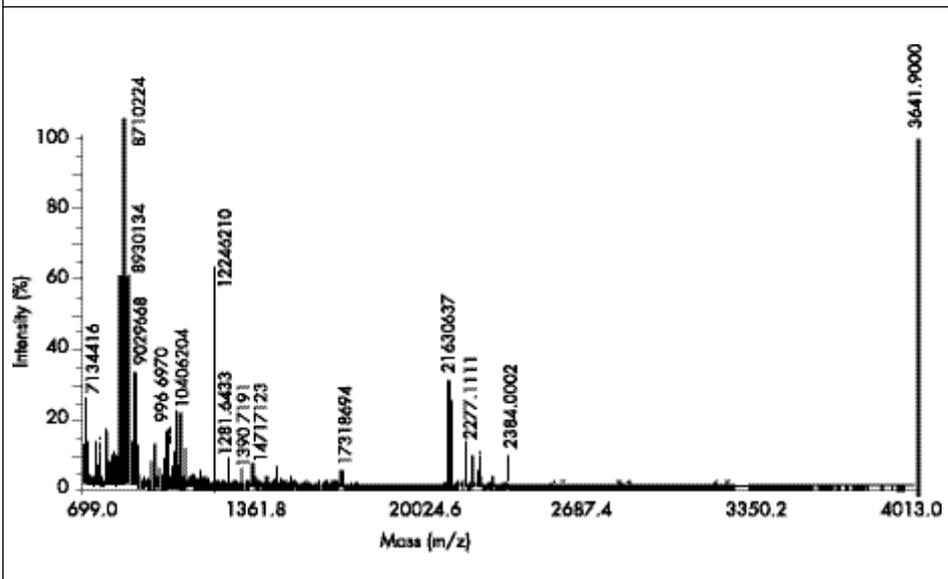
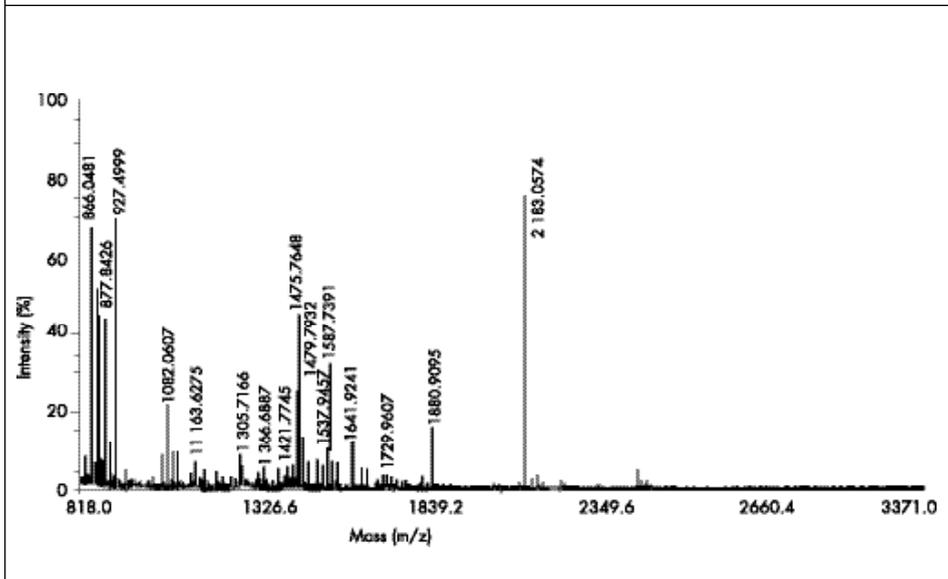


Fig 3. Peptidic thread of SAE used to select the significant peak of 1479.78 Da to be sequenced.

Fig 3. Enlaces peptídicos de SAE utilizado para seleccionar el pico significativo de 1479,78 Da que va a ser secuenciado.



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