UNIVERSIDAD DE CUENCA

Facultad de Ciencias Químicas

Maestría en Biociencias Aplicadas

Identificación y clonación de secuencias codificantes de peroxidasas de granos inmaduros de fréjol (*Phaseolus vulgaris*)

Trabajo de titulación previo a la obtención del título de Magíster en Biociencias Aplicadas

Autora:

Karla Esther Espinoza Castro

CI:0104611561

Correo electrónico: karlita_eec25@hotmail.com

Director:

Antonio Javier Vallecillo Maza

CI:0151059417

Cuenca, Ecuador

31-octubre-2020

Resumen

Las peroxidasas son hemoproteínas cuya actividad catalítica se utiliza en pruebas de diagnóstico, desarrollo de biosensores y biorremediación, las más utilizadas son la peroxidasa de rábano picante (*Armoracia rusticana*) o HRP y la peroxidasa de soja (*Glycine max*) o SBP. Actualmente estas enzimas tienen un alto costo, por lo que es necesario buscar alternativas que permitan obtenerlas y disponer de manera inmediata en el país para su aplicación en el desarrollo de herramientas de diagnóstico. En la búsqueda de alternativas a las peroxidasas comerciales, se realizó una revisión de la literatura para identificar secuencias de SBP previamente descritas, las secuencias localizadas se utilizaron para identificar secuencias codificantes ortólogas en fréjol (*Phaseolus vulgaris*), con las secuencias identificadas se diseñaron oligonucleótidos para la generación y amplificación de los ADNc respectivos. Después de obtener el ARN total de la cutícula de granos inmaduros, se llevó a cabo la síntesis del ADNc y la generación de productos de PCR. Los resultados de la secuenciación de los productos de PCR mostraron que ha sido posible obtener secuencias que codifican peroxidasas ortólogas de Ep (NP_001238315.1, NM_001251386 .1) y Prx2 (NP_001237601.1, NM_001250672.2) a partir de fréjol. Las secuencias obtenidas en este trabajo tienen una alta identidad con las secuencias reportadas en *P. Vulgaris*, Ep (PHAVU_006G129900g) con 99.08% y 99.69% para Prx2 (PHAVU_003G078600g). Las secuencias codificantes de peroxidasas clonadas en el vector pET15b fueron inducidas en la cepa de *Escherichia coli* Origami (DE3) y el extracto de proteína total obtenido mostró actividad enzimática sobre los sustratos de peroxidasas 3,3′-diaminobencidina, 4-cloro-1-naftol y siringaldazina. Finalmente, los resultados obtenidos permiten concluir que fue posible obtener dos secuencias codificantes de proteínas con actividad peroxidasa a partir de *Phaseolus vulgaris*.

Palabras clave

Secuencias codificantes. Peroxidasa. Granos inmaduros. *Phaseolus vulgaris*.

Abstract

Peroxidases are hemoproteins whose catalytic activity is used in diagnostic tests, development of biosensors and bioremediation, the most used are Horseradish (*Armoracia rusticana*) peroxidase or HRP and Soybean (*Glycine max*) peroxidase or SBP. Currently these enzymes have a high cost, so it is necessary to find alternatives for obtaining them and have immediate availability in the country for application in the development of diagnostic tools. In the search for alternatives to commercial peroxidase, a literature review was carried out to identify previously described sequences of SBP, the localized sequences were used to identify orthologous coding sequences in *Phaseolus vulgaris* (Common bean), with the identified sequences were designed oligonucleotides for the generation and amplification of the respective cDNAs. After obtaining total RNA from immature grain cuticle, synthesis of the cDNA and generation of PCR products was carried out. PCR products sequencing results showed that it has been possible to obtain sequences encoding the orthologue peroxidase of Ep (NP_001238315.1, NM_001251386 .1) and Prx2 (NP_001237601.1, NM_001250672.2) from common bean. The sequences obtained in this work have a high identity with the reported sequences in *Ph. vulgaris*, Ep (PHAVU_006G129900g) with 99.08 % and 99.69 % for Prx2 (PHAVU_003G078600g). Cloned coding peroxidases sequences in pET15b vector were induced *Escherichia coli* Origami (DE3) and total protein extract obtained showed enzymatic activity on 3,3′-diaminobenzidine, 4-chloro-1-naphthol and syringaldazine peroxidases substrates. Finally, the results obtained allow us to conclude that it was possible to obtain two protein coding sequences with peroxidase activity from *Phaseolus vulgaris*.

Key words

Coding sequences. Peroxidase. Immature grains. *Phaseolus vulgaris.*

Índice del trabajo

Índice de contenidos

Índice de tablas

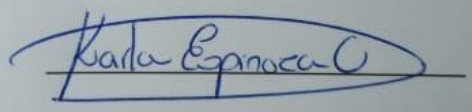
Índice de figuras

Cláusula de licencia y autorización para publicación en el Repositorio Institucional

Yo, Karla Esther Espinoza Castro, en calidad de autora y titular de los derechos morales y patrimoniales del trabajo de titulación "Identificación y clonación de secuencias codificantes de peroxidasas de granos inmaduros de fréjol *(Phaseolus vulgaris)*", de conformidad con el Art. 114 del CÓDIGO ORGÁNICO DE LA ECONOMÍA SOCIAL DE LOS CONOCIMIENTOS, CREATIVIDAD E INNOVACIÓN reconozco a favor de la Universidad de Cuenca una licencia gratuita, intransferible y no exclusiva para el uso no comercial de la obra, con fines estrictamente académicos.

Asimismo, autorizo a la Universidad de Cuenca para que realice la publicación de este trabajo de titulación en el repositorio institucional, de conformidad a lo dispuesto en el Art. 144 de la Ley Orgánica de Educación Superior.

Cuenca, 31 de octubre del 2020.



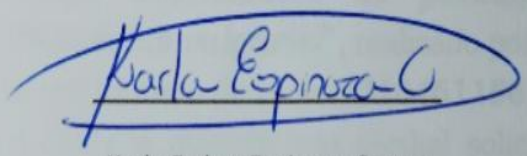
Karla Esther Espinoza Castro

C.I: 0104611561

Cláusula de Propiedad Intelectual

Yo, Karla Esther Espinoza Castro, autora del trabajo de titulación "Identificación y clonación de secuencias codificantes de peroxidasas de granos inmaduros de fréjol *(Phaseolus vulgaris)*", certifico que todas las ideas, opiniones y contenidos expuestos en la presente investigación son de exclusiva responsabilidad de su autora.

Cuenca, 31 de octubre del 2020



Karla Esther Espinoza Castro

C.I: 0104611561

AGRADECIMIENTOS

A mi director, Doctor Antonio Vallecillo, brillante docente e investigador, quien, con su experiencia y vasto conocimiento, me brindo su apoyo permanente para el desarrollo de la investigación.

A la Doctora Nardy Diez, por su valioso aporte académico para la conclusión del proyecto.

A la Dirección de Investigación de la Universidad de Cuenca.

Al programa de postgrados VLIR NETWORK Ecuador.

DEDICATORIA

A Dios y a la Virgen María, mi fortaleza en todo momento.

A mi hijita bella, María Gabriela, mi compañera incondicional, quien llena de alegría mi vida y ha sido mi inspiración siempre para seguir adelante en mi formación profesional.

A mi angelito en la tierra, mi abuelita Esther, quien con sus oraciones ha velado por mí, de quien recibí cariño y cuidado a lo largo de mi vida y ha sido mi pilar fundamental en los momentos difíciles.

A mi esposo Darío, mi mejor amigo y compañero de vida, tú me has alentado siempre para culminar esta meta.

A mis padres, Severo y Julia, quienes me han brindado su apoyo constante, enseñándome con su ejemplo, el valor del esfuerzo y del trabajo.

A mis ñaños queridos, Paola, Juan y Fabiana, si Diosito permite siempre juntos.

Con amor

Karla

IDENTIFICATION AND CLONING PEROXIDASES CODING SEQUENCE FROM IMMATURE GRAINS OF COMMON BEAN (*Phaseolus vulgaris*)

Karla Esther Espinoza Castro[1], Antonio Javier Valecillo Maza[2]
[1] Facultad de Ciencias Químicas
Universidad de Cuenca, karla.espinozac@ucuenca.edu.ec
[2] Facultad de Ciencias Agropecuarias
Universidad de Cuenca, antonio.vallecillo@ucuenca.edu.ec

1. Introduction

Peroxidases are hemoproteins exhibiting catalytic activity (Vlasova, 2018), which use hydroperoxides as electron acceptors to cause oxidative reactions in a wide variety of substrates (Zhang *et al*., 2018). They are widely found in plants, animals and microorganisms (Hiraga *et al*., 2001), and currently they have been defined as the proteins of great biotechnological interest in the twenty-first century because of the large diversity of areas in which they are used, such as bioremediation, proteomics, food manufacturing and processing, therapeutics, immunodiagnostics, among others (Sakharov *et al*., 2010; Steevensz *et al*., 2013; Vdovenko *et al*., 2010a; Zhao *et al*., 2015; Bilal *et al*., 2018).

Current scientific research has focused on the cloning and protein expression of the so-called classic hemo-vegetable peroxidases, such as horseradish (*Armoracia rusticana*) and soybean (*Glicine max*), known as HRP (Horseradish peroxidase) and SBP (Soybean peroxidase) (Sakharov *et al*., 2010), respectively. These peroxidases belong to the group of class III vegetable peroxidases, secreted peroxidases, and, as such, feature similar structural and biochemical characteristics (Hiraga *et al*., 2001). HRP has been isolated from the roots of horseradish, and SBP from the soybean epidermis (Ryan *et al*., 2006; Baig *et al*., 2018).

These are commercially important enzymes and are mainly used as tracers in immunodiagnostic tests for the identification of antigens or antibodies (Grigorenko *et al*., 2015; Tang *et al*., 2018; Vdovenko *et al*., 2010b; Yang *et al*., 2019).

In Ecuador, access to HRP and SBP, for the development of immunodiagnostic tools, is limited because of considerable expenses and time required for importation; therefore, it would be very useful to find other economically viable options to isolate peroxides with similar characteristics from other plant sources (Xue *et al*., 2014).

The activity of peroxidases in the cuticle of common bean grains (*Phaseolus vulgaris*) has been has been associated with their hardening while stored at high temperature and humidity (Rodríguez, 2012; Xue *et al*., 2014); on the other hand, recent studies have reported the presence of genes or orthologous sequences to those of SBP, in the messenger RNA of *P. vulgaris* (Nemli *et al*., 2014); as a result, a protein exhibiting peroxidase activity could probably be obtained from this species, which could be used as a catalytic alternative to the classic ones, soybeans and horseradish, as a tracer enzyme in immunodiagnostic tests and other applications. However, no studies have been conducted for the cloning, expression and protein characterization of the peroxidases found in this species.

This study aims to obtain two protein coding sequences with peroxidase activity from common beans, *Escherichia coli* expressed proteins exhibiting peroxidase activity on 3,3′-diaminobenzidine, 4-chloro-1-naphthol and syringaldazine substrates.

2. Materials and methods

2.1 Bioinformatic analysis and primer design

To obtain sequences encoding enzymes with peroxidase activity from *P. vulgaris*, a literature search was conducted to locate information regarding the sequences reported in soybeans. With the information on the sequences, either coding or of the proteins, the reference sequences for the proteins in the GenBank (https://www.ncbi.nlm.nih.gov/refseq/) were located. Peptide reference sequences were used to locate in the Phytozome 12.1 database (https://phytozome.jgi.doe.gov/pz/portal.html) the possible orthologous sequences in *P. vulgaris*. For the annotated peptide sequences of *P. vulgaris* with higher identity scores, the mature peptide coding sequence of the reference mRNAs sequences were extracted from GenBank (https://www.ncbi.nlm.nih.gov/nuccore/). Coding sequences was employed to design the oligonucleotides necessary to obtain the coding sequence of possible peroxidases from *P. vulgaris* tissue plants by RT-PCR, to complete the design of the primers, restriction sites sequences were added to the NdeI enzyme at the amino terminus and BamHI for the carboxyl terminus, considering the creation of a translational fusion at the amino terminus when possible coding sequences are cloned into pET15b bacterial expression vector with same restriction site.

2.2 Samples of common bean plant tissues and total RNA extraction

Common bean grains were purchased at a local supermarket and were used to obtain plants under greenhouse conditions at the facilities of the Agricultural Sciences school of the University of Cuenca. According to the referred in the literature, the tissues of the plants where the sequences of the soybean peroxides have been obtained is of the immature grains

testa, so the bean plants were maintained until they had grains 4 weeks after flowering (Chen & Vierling., 2000).

Of the immature grains harvested 100 mg of testa tissue was obtained and the total RNA was purified with TRIreagen solution (Sigma Aldrich), following the manufacturer's recommendations.

2.3 Coding sequences amplification, cloning and sequencing

The total RNA obtained was used in the synthesis of cDNA to the different coding sequences with oligonucleotides $d(T)_{18}$ in a reaction volume of 50 µl using the enzyme SuperScript™ III system (SuperScript™ III First-Strand Synthesis System, Invitrogen) following the manufacturer's instructions.

The coding sequence was amplified from synthesized cDNA by PCR using the specific designed primers, PCR reaction was conducted in a 25 µl volume, mix containing 1X PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl), 2 mM $MgCl_2$, 800 µM dNTP mix, 0.8 µM of each of the specific forward and reverse primers, 1 U of *Taq* DNA polymerase (Invitrogen) and 5 µl cDNA reaction synthesis. The temperature profile and amplification times were initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, alignment between 52 to 58 °C (Tm curve) for 25 s and extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Once the amplification process was completed, the PCR reaction were resolved in agarose-TAE gel electrophoresis Ethidium bromide stained.

PCR products obtained were purified with the GenJet kit (Thermo scientific) from PCR reaction and 20 ng of them were ligated into plasmid pCR2.1 (TA Cloning™ kit, Invitrogen), the ligation reaction was employed to transform TOP10F´ *E. coli* strain by thermal shock and the selection of the transformants was performed by incubation overnight at 37 °C in static

in LB medium with 100 µg/ml Ampicillin and the overlayer addition of 40 µl of a solution of IPTG (100 mM) and 40 µl of a solution of X-Gal (40 mg/ml). Four white colonies of each construction were inoculated in 5 ml of LB broth with 100 µg/ml of Ampicillin and incubated overnight at 37 °C with constant agitation (200 r.p.m.), extracted plasmids from bacterial cultures (GeneJet plasmid miniprep kit, Thermo Scientific) were digested with EcoRI restriction enzyme to verify the cloning of the PCR products. Finally, plasmids with PCR´s product cloned were submit to Sanger´s sequencing (Macrogen, Korea).

2.4 Subcloning in bacterial expression vector, induction and peroxidase activity assay

Coding sequences from sequenced clones were liberated from pCR2.1 vector with NdeI-BamHI restriction enzyme and ligated in the pET15b bacterial expression vector linearized with the same restriction enzymes. The ligation reaction was used to transform the TOP10F´ strain, the transformants were selected by incubation overnight at 37 °C in LB medium with 100 ug/ml of Ampicillin. In four transformants obtained by each coding sequence the correct cloning in the expression vector was verified by restriction mapping, the correct plasmids obtained pET15-Ep and pET15-Prx2 were used for the transformation of the Origami (DE3) *E. coli* expression strain by thermal shock.

In order to show peroxidase activity in bacterial culture transformed with different construction were preformed in a 10 ml volume LB medium-100 ug/ml of Ampicillin with addition 1:100 of an overnight preculture from one selected colony, after incubation for two hours at constant agitation IPTG and Hemin solution were added to a final concentration of 100 µM and 12 µM, respectively. Bacterial culture non-induced, induced, with and without Hemin were incubated for additional 5 h in described conditions. Concluded incubation period bacterial biomass were collected by centrifugation (12,000 x *g* by 10 min at room

temperature) and cells pellets were sonicated (Q125 Sonicator, Qsonica) in PBS with 0.05% of Tween 20 (100 mM Potassium phosphate buffer, pH 7.4 at 23 °C). The obtained lysate was spotted (2.5 µl) onto a piece of Whatman paper grade 1, and enzymatic activity was evidenced by moistening the paper with sufficient quantity of the solutions of PBS with 0.5% of Tween and different substrates, DAB (0.5 mg/ml of 3,3′-diaminobenzidine tetrahydrochloride), 4CN (0.6 mg/ml of 4-chloro-1-naphthol) and syringaldazine (5 µM of 4-Hydroxy-3,5-dimethoxybenzaldehyde azine) plus 1:10,000 of 30% solution of hydrogen peroxide.

3. Results and discussion

3.1 *In silico* identification of peroxidase coding sequences in common bean

In the literature review, reports of six peroxidases sequences were found (Gijzen, 1997; Gijzen, *et al*., 1999; Vierling, 1999; Chen & Vierling, 2000; Henriksen, *et al*., 2001). With the corresponding reference sequences (GenBank), the most identified protein sequences recorded in *P. vulgaris* were located in the Phytozome database, the sequence identity of the possible orthologous proteins varies from 88% (Ep) to 97% (Prx2) (Table 1).

Table 1. Soybeans (*G. max*) described peroxidases and common beans (*P. vulgaris*) possible orthologous peroxidase identified *in silico* analysis.

Protein *	***Glycine max* RefSeq GenBank ‡**		***Phaseolus vulgaris* RefSeq GenBank ‡**		**Ident. (%) †**	**Reference**
	Protein	**CDS &**	**Protein**	**CDS &**		
Ep (1FHF) (SBP)	NP_001238315.1	NM_001251386.1	XP_007147501.1	XM_007147439.1	**88**	Gijzen, 1997; Henriksen, *et al*., 2001
Prx2 (Cationic)	NP_001237601.1	NM_001250672.2	XP_007153945.1	XM_007153883.1	**97**	Gijzen, *et al*., 1999
SepA1	XP_003540316.1	XM_003540268.3	XP_007149959.1	XM_007149897.1	**90**	Vierling, 1999; Chen & Vierling, 2000;
SepA2	XP_006594890.1	XM_006594827.2	XP_007149959.1	XM_007149897.1	**90**	
SepB1	XP_003517158.1	XM_003517110.3	XP_007157875.1	XM_007157813.1	**89**	
SepB2	NP_001304547.1	NM_001317618.1	XP_007157875.1	XM_007157813.1	**89**	

* Another protein nomination in revised literature.
‡ Number of reference sequence in GenBank.
& Coding sequence.
† Identity percentage.

The sequence of the Prx2 peroxidase described by Gijzen *et al* (Gijtzen, *et al*., 1999) was the second identified in immature soybeans, by means of a Northern blot test, a greater expression of Prx2 was evidenced in the grain testa in contrast to Ep, additionally the Prx2 transcripts were found in other tissues of the plant.

In the case of the SepA1, SepA2, SepB1 and SepB2, sequences of possible peroxidases were found in *P. vulgaris* with an identity of 89 to 90%. The sequences SepA1, SepA2, SepB1 and SepB2 were described by Chen and Vierling, showing a differential expression in various tissues of the soybean plant (Chen & Vierling, 2000). In the same work, Chen and Vierling were able to demonstrate the enzymatic activity of one of the sequences expressed in *E. coli* (Chen & Vierling, 2000).

Finally, for the sequence of the peroxidase Ep, a possible ortholog protein was found in *P. vulgaris* with 88% identity. Ep also called SBP by Soybean peroxidase, from which the crystallographic structure has been obtained (Protein Data Bank code: 1FHF) (Henriksen, *et al*., 2001), this soy enzyme is the best characterized to functional level. It has been observed that it is functional at a higher temperature and pH range compared to HRP, 5.5 being the pH value where Ep shows the greatest catalytic activity and conformational stability (Kamal & Behere, 2002; Kamal & Behere, 2003).

After obtaining the nucleotide reference sequences (CDS) of the genes that code for these proteins, specific primers were designed for each of them, considering amplifying the coding sequence for the mature protein, that is, without the signal peptide, which is encoded within the open reading frame (ORF) since these proteins are secreted, which was identified through the use of on-line SignalP-5.0 software (http://www.cbs.dtu.dk/services/SignalP/; on the other hand, in the sequences to be amplified it was verified that there were no cut sites for the restriction enzymes NdeI and BamHI (CATATG and GGATCC sequences, respectively) that will be placed to direct the cloning, by using the Serial Cloner 2.6 software. 1, as indicated in Table 2.

Table 2. Designed oligonucleotides for different possible coding sequence of *P. vulgaris*. Selected annotated coding sequences (CDS) with identified secretion signal peptide, size peptide of mature proteins and expected RT-PCR product with designed oligonucleotides.

CDS *	**Signal peptide** (Amino acids)	**Mature protein** (Amino acids)	**Oligonucleotides designed** (5´-3´) ‡	**RT-PCR product size** (Base pairs)
XM_007147439.1	26	326	**CATATG**CAGCTTAGTCCTACGTTCTAC **GGATCC**TTAAGATTGAGAAACCATGTTTTG	990
XM_007153883.1	25	310	**CATATG**GAGGAAGGCCAAGATAATG **GGATCC**TTAAGGCTCCTCATGGTGCTT	945
XM_007149897.1	21	303	**CATATG**CAACTTCAGTTAGGATTTTATGC **GGATCC**TTAGCTATTTACAAATGCACAATG	924
XM_007157813.1	21	295	**CATATG**TCACTGAGCTTAAACTACTACG **GGATCC**TTAATTGACCACTCTGCAG	900

* GenBank identification number.
‡ Underline and in bold sequences correspond to added restrictions sites.

3.2 Cloning of peroxidase coding sequences in immature grains of common bean

With the total RNA extracted from the testa of immature grains of bean plants obtained from a product marketed in a local supermarket, the coding sequences of proteins with possible peroxidase activity were amplified by RT-PCR. As RT-PCR reactions products of the sequences XM_007153883.1 and XM_007147439.1 of *P. vulgaris* corresponding in soybeans to the peroxidases Prx2 and Ep (SBP), the size of the amplicons obtained were very close to that expected (Figure 1A). In the case of the coding sequence XM_007149897.1 corresponding in soybean to the SepA1 peroxidase, there was no amplification which probably could be due to the fact that at the time the total RNA was extracted it had not transcribed in the testa tissues or because the primers did not have the ability to hybridize due to that there is no homology and another possibility is that the range in which the temperature curve was developed did not allow them to hybridize in the PCR assay (Figure 1B). Finally, the coding sequence XM_007157813.1 corresponding in soybeans to the peroxidase SepB1, a PCR product obtained was greater than expected, as a result of

sequencing, the presence of a sequence corresponding to an intron was evidenced, which explains the difference in size with respect to the expected one. On the other hand, results of the sequencing assays of the RT-PCR products of XM_007153883.1 and XM_007147439.1 sequences, showed a high identity with the sequences reported in *P. vulgaris*, Ep (PHAVU_006G129900g) with 99.08% and 99.69% for Prx2 (PHAVU_003G078600g).

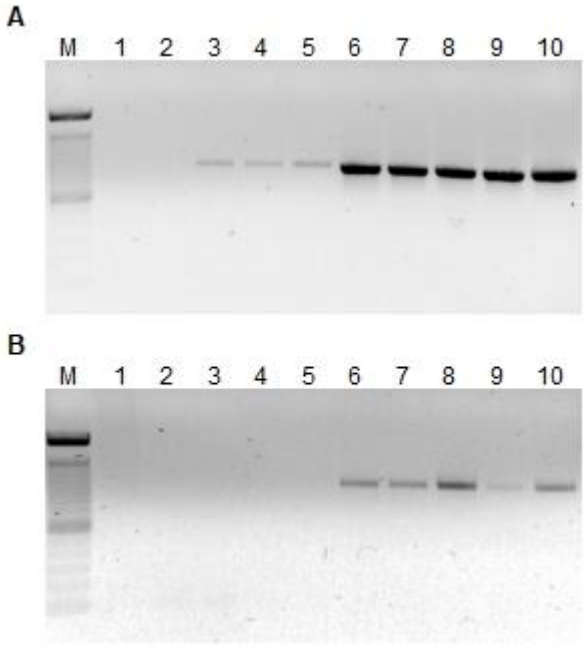


Figure 1. Agarose-TAE buffer electrophoresis image of obtained RT-PCR products from purified total RNA of immature grains testa tissue of *P. vulgaris*. **A**: Lane M, Molecular weight marker (100 bp DNA ladder, Invitrogen™); Lanes 1, 2, 3, 4 and PCR products of *P. vulgaris* Ep orthologous sequence; Lanes 6, 7, 8, 9, and 10, PCR products of *P. vulgaris* Prx2 orthologous sequence generated to primers temperature hybridization in a 52 - 58 °C interval. **B**: Lane M, Molecular weight marker (100 bp DNA ladder, Invitrogen™); Lanes 1, 2, 3, 4 and PCR products of *P. vulgaris* SepA1 orthologous sequence; Lanes 6, 7, 8, 9, and 10, PCR products of *P. vulgaris* SepB1 orthologous sequence generated to primers temperature hybridization in a 52 - 58 °C interval.

3.3 Enzymatic activity of peptide sequence expressed in bacterial cells

Obtained sequences PhavEp and PhavPrx2 of *P. vulgaris* orthologous to soybean peroxidases Ep and Prx2 were cloned in the bacterial expression vector pET15b, both genetics construction pET15b-Ep and pET15b-Prx2 were employed for transform Origami (DE3) *E.*

coli expression strain. Protein extract from obtained recombinant bacterial strains were assayed for identification enzymatic activity of peroxidase on three substrates. In both case, sequences PhavEp and PhavPrx2 of *P. vulgaris* expressed in *E. coli* cultures with Hemin generate protein extract that show peroxidase activity on DAB (3,3′-diaminobenzidine tetrahydrochloride), 4CN (4-chloro-1-naphthol), Syr (syringaldazine) substrates (Figure 2). Signal of peroxidase activity is absent when hydrogen peroxide is removed from the assay. In all cases, the peroxidase activity signal is low, so it is necessary to optimize the induction conditions or carry out the modification of the expression strain according to that described by Liu, *et al* (Liu, *et al*., 2017), which will allow the generation of a sufficient amount of enzymes to perform the characterization and evaluation of their enzymatic capacities and thus be able to suggest their use as tracer enzymes in immunoenzymatics assays.

Peroxidase substrate *		**DAB with H_2O_2**				**DAB without H_2O_2**				**4CN with H_2O_2**				**Syr with H_2O_2**			
Culture condition	**IPTG ‡**	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
	Hemin	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+
Non protein																	
His tag-PhavEp																	
His tag-PhavPrx2																	

Figure 2. Activity peroxidase assay. Peroxidase activity over different substrate visible in extract from bacterial culture obtained with inductor (IPTG) and peroxidase cofactor (Hemin), no activity is observed in extract from bacterial culture of non-transformer Origami (DE3) E. coli expression strain.

* DAB (3,3′-Diaminobenzidine tetrahydrochloride), 4CN (4-chloro-1-naphthol), Syr (Syringaldazine).
‡ IPTG (Isopropyl β-D-1-thiogalactopyranoside).

4. Conclusions

Thus, this work clearly demonstrated the identification and cloning peroxidase coding sequence expressed in immature grains of commons beans (*P. Vulgaris)*. Additionally, peptide sequences expressed in bacterial cells display enzymatic activity over diverse chemical substrates. But more research is necessary to optimize the expression and purification of these proteins to evaluate their enzymatic activity for future evaluation as tracer enzymes, which could be used as input in the development of immunodiagnostic tools.

5. Acknowledgments

The authors are grateful for the support granted by the Direction of investigation of Universidad of Cuenca and the Master in Applied Biosciences postgraduate program of VLIR Network Ecuador for the realization of this work.

References

Baig, M. A., Ahmad, J., Bagheri, R., Ali, A. A., Al-Huqail, A. A., Ibrahim, M. M., & Qureshi, M. I. (2018). Proteomic and ecophysiological responses of soybean (*Glycine max* L.) root nodules to Pb and hg stress. BMC Plant Biol, 18(1), 283. DOI:10.1186/s12870-018-1499-7

Bilal, M., Rasheed, T., Zhao, Y., & Iqbal, H. M. N. (2018). Agarose-chitosan hydrogel-immobilized horseradish peroxidase with sustainable bio-catalytic and dye degradation properties. Int J Biol Macromol. DOI:10.1016/j.ijbiomac.2018.11.220

Chen, H., & Vierling, R.A. (2000). Molecular cloning and characterization of soybean peroxidase gene families. Plant science, 150(2), 129-137. DOI: 10.1016/S0168-9452(99)00165-X.

Gijzen, M. (1997). A deletion mutation at the ep locus causes low seed coat peroxidase activity in soybean. Plant J. 12(5):991-998.

Gijzen, M., Miller, S. S., Bowman, L. A., Batchelor, A. K., Boutilier, K., & Miki, B. L. (1999). Localization of peroxidase mRNAs in soybean seeds by *in situ* hybridization. Plant Mol Biol. 1999 Sep;41(1):57-63. DOI: 10.1023/A:1006244500951.

Grigorenko, V. G., Andreeva, I. P., Rubtsova, M. Y., & Egorov, A. M. (2015). Recombinant horseradish peroxidase: Production and analytical applications. Biochemistry (Mosc), 80(4), 408-416. DOI:10.1134/S0006297915040033

Henriksen A., Mirza O., Indiani C., Teilum K., Smulevich G., Welinder K.G. & Gajhede M. (2001). Structure of soybean seed coat peroxidase: A plant peroxidase with unusual stability and haem-apoprotein interactions. Protein Sci. 10(1):108-115. DOI: 10.1110/ps.37301

Hiraga, S., Sasaki, K., Ito, H., Ohashi, Y., & Matsui, H. (2001). A large family of class III plant peroxidases. Plant Cell Physiol, 42(5), 462-468.

Kamal J.K. & Behere D.V. (2002). Thermal and conformational stability of seed coat soybean peroxidase. Biochemistry. 41(29):9034-9042. DOI: 0.1021/bi025621e.

Kamal J.K. & Behere D.V. (2003). Activity, stability and conformational flexibility of seed coat soybean peroxidase. J Inorg Biochem. 94(3):236-242. DOI: 10.1016/s0162-0134(03)00004

Liu, C., Zheng, K., Xu, Y., Stephen, L. T., Wang, J., Zhao, H., . . . Liu, H. (2017). Expression and characterization of soybean seed coat peroxidase in *Escherichia coli* BL21(DE3). Prep Biochem Biotechnol, 47(8), 768-775. DOI:10.1080/10826068.2017.1342258

Nemli, S., Kaya, H. B., & Tanyolac, B. (2014). Genetic assessment of common bean (*Phaseolus vulgaris* L.) accessions by peroxidase gene-based markers. J Sci Food Agric, 94(8), 1672-1680. doi:10.1002/jsfa.6477

Ryan, B. J., Carolan, N., & O'Fágáin, C. (2006). Horseradish and soybean peroxidases: comparable tools for alternative niches? Trends Biotechnol, 24(8), 355-363. DOI:10.1016/j.tibtech.2006.06.007

Vierling R.A. (1999). Soybean peroxydase gene family and assay for detecting soybean peroxidase activity. United State Patent. Patent number: 5,866,695.

Vlasova, I. I. (2018). Peroxidase activity of human hemoproteins: Keeping the fire under control. molecules, 23(10). DOI:10.3390/molecules23102561

Zhang, Z., Lai, J., Wu, K., Huang, X., Guo, S., Zhang, L., & Liu, J. (2018). Peroxidase-catalyzed chemiluminescence system and its application in immunoassay. Talanta, 180, 260-270. DOI:10.1016/j.talanta.2017.12.024

ANEXOS

ANEXO 1. Sanger sequencing results

1.- Secuencia codificante Ep2:

>190509-004_A01_Ep2_M13F.ab1 1178

GATAACTCGTATAGGGCGATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGC
AGAATTCGGCTTGGATCCTTAAGATTGAGAAACCATGTTTTGTTTAGAATTTTTGGACGTCATACTAGCTAG
TCCAAAGGAGTCTCCATTCACAAAATTACACTGTAACCGAATTTCTCCTTCACCCCCCGTCAGCACTCCAAT
ATTTCCCATTTTTATCATTGACGCCCTAAAGTTGTCAAAGAAAGTGTTTTGGTTACTGCTGAAGCTATTGACA
ATGGGAATGGTATCAGCAGCAGGAGTAGAGAATAGTTCTTGGTCGCTCTGAAGTAAGCCATTGAGTTGCTG
AAGATTGGAGTAATATTTGTTATCAAATTGATCAGGAGTGGTGAGGTCCAAATTGGTGAGGTTGTTCCCAGT
TGCATTCTGGGGGCATATCTGACGCAGTGTTGCTAAGTAAACTGTATTCAGAGTTGGATCAGGGTTTCCAGT
GTTGTTGAAGTCGTATAATCGGTTATTGAATGTACTGCAGTGAGCTCTTCCAAACGTATGAGCACCTGAGAG
TGCAACTAAATCAGTGGTGTTGAGACCTTGAACAGCAAATGATGCTTTAAGTTGAGTGAGGTTGAAGAAAG
GAGGAGGAAGGTTTTGATTTGCAAGGGTTCTGTTTGCAGTTAAACTATCTTTTCTTCCTAATGGAACTAACC
ATCCTGGACCTCCTCCCGGAATAGAACCTATTTCAGCTGCAATAGTAAGAATATCAGCACAAGAAACTGTC
CCCGGACAACTATTTTCCACCGCTGTCTTGATATTATTGACCACATCCAAACCCCTTATTGAATTGTTATTTG
GAATTGCATCTTGTTCGCTTTCTATAATAGCAGTGTTGTTCAGTAAAACTGATGCATCACAACCTTGAACAA
AGCAATCATGAAAATGAAGCCTGACGAGGCTAGCCCCTATTCGGGGATCGGTTAAGGAAGCTTGAAAGATG
ACTTGAAACACAATATTGAATAGATTTGGACATGTTTCTCTGTAGAACGTAGGACTAAGCTGAATTCCACCA
CACTGGGGGGCCGTTACTAATGGGATCCCAACTCGGGACCAAACTTGGCGGAATCATGGGGCAAAACTGTT
TTCCGGGGGGAAAATGTTTTCCGCTCCCAA

>190509-004_G01_Ep2_M13R.ab1 1183

AAAATGAAATGATTACGCCAGCTTGGTACCGAGCTAGGATCCACTTAGTAACGGCCGCCAGTGTGCTGGAA
TTCAGCTTAGTCCTACGTTCTACAGAGAAACATGTCCAAATCTATTCAATATTGTGTTTCAAGTCATCTTTCA
AGCTTCCTTAACCGATCCCCGAATAGGGGCTAGCCTCGTCAGGCTTCATTTTCATGATTGCTTTGTTCAAGGT
TGTGATGCATCAGTTTTACTGAACAACACTGCTATTATAGAAAGCGAACAAGATGCAATTCCAAATAACAA
TTCAATAAGGGGTTTGGATGTGGTCAATAATATCAAGACAGCGGTGGAAAATAGTTGTCCGGGGACAGTTT
CTTGTGCTGATATTCTTACTATTGCAGCTGAAATAGGTTCTATTCCGGGAGGAGGTCCAGGATGGTTAGTTC
CATTAGGAAGAAAAGATAGTTTAACTGCAAACAGAACCCTTGCAAATCAAAACCTTCCTCCTCCTTTCTTCA
ACCTCACTCAACTTAAAGCATCATTTGCTGTTCAAGGTCTCAACACCACTGATTTAGTTGCACTCTCAGGTG
CTCATACGTTTGGAAGAGCTCACTGCAGTACATTCAATAACCGATTATACGACTTCAACAACACTGGAAACC
CTGATCCAACTCTGAATACAGTTTACTTAGCAACACTGCGTCAGATATGCCCCCAGAATGCAACTGGGAAC
AACCTCACCAATTTGGACCTCACCACTCCTGATCAATTTGATAACAAATATTACTCCAATCTTCAGCAACTC
AATGGCTTACTTCAGAGCGACCAAGAACTATTCTCTACTCCTGCTGCTGATACCATTCCCATTGTCAATAGC
TTCAGCAGTAACCAAAACACTTTCTTTGACAACTTTAGGGCGTCAATGATAAAAATGGGAAATATTGGAGT
GCTGACGGGGGGTGAAGGAGAAATTCGGTTACAGTGTAATTTTGTGAATGGAGACTCCTTTGGACTAGCTA
GTATGACGTCCAAAAATTCTAAACAAAACATGGTTTCTCAATCTTAAGGATCCAAGCCAAATTCTGCAGAAT
TCCTTACACTGGGGGCCGCTCGAACATGCATCTAGAGGGCCCAATTCGCCCCATAAGGAGTCGAATTACAA
TTCCTTGGGCGGCGGTTTTAAAAAGCCGGGACTGGG

2.- Secuencia codificante Prx:

>190509-004_C01_Prx_M13F.ab1 1230

GACTGACTCTATAGGGCGATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGC
AGAATTCGGCTTGGATCCTTAAGGCTCCTCATGGTGCTTGTTGGCAACATTGCACTGCTTTCTGATCTCCCCC
TTTGTGCCAGTGAGAGGATTGTTTTCAGAGAGCAAAGCAATGGCTCTAGAAAACTCCTTGAAGAAATAGTC
TTGGCTCTTGGCCATTTTCTTCACATAAGGCTTGGTCCTCTTGTCATTGGCTAGTTGGTGATCCACTATCAAC
AACCCCTTGTTGTCCAATATGTTCCTGTAGTAGTTGTTGTCTAGAATCATAGGGGTGCCACGGTCGTTTCTCA
CGTACTGCACGGCCTTGGGGTCTGGGATGGCATCAGGGCACTTCTTCAGCATGTGAGGGACATGGTCAGGG
TTCAGCGCTGAATCAATCTCTGGGTACAAACGGTGCACCAACTTCACACAATGGGTTCTACCAACACTGTGT
GCTCCAAGCAATGCAACCACCCCGGGAGTGTCAATTCCCATGGCACCAAACTTGTCAAGAACTGAAGAAAT
GGACTCATTGTGGTCTGGGAGGAACTCTTCCACCACATCAGCTCTGCTCCTTCTACCATCCCTTCTTCCAGTT
TTAAGAGGGATATGGGGGCCTCCTAGCGAAACAATGCCATCTCTGGCAGAGAGAACGAGTATATCAGCACA
GGAAACAACTCCTGGGCACTCCCTTTCCACAGCTTCTTTGATGGTCTCAATGTACCTGAAATTCCTCAAACC
AAAGCTTCTGTCTGTCTCCTTCTCAGACAAGCTCCTTCTTGTGGAGTCCAGCAAGAGTGAAGCATCACAACT
CTGAACAGCACAGTCATGGAAGATGTTTCTGAGCCAGGAGAAAGCAGTGTTCTTGTGGCGTTTGTAGAGAA
GCTTGACTTGTTCTTTGATGATATCTTCAGCCTGAGGACATGTTTCCTTGTAGAAGTTCATGACAAGGCCATT
ATCTTGGCCTTCCTCCATATGAAGCCGAATTCCAGCACACTGGGGGGCCGTTACTAATGGGATCCCAACTCG
GGACCAAACTTGGGGGAAATCATGGGGCATAACTGGTTTCCCGGGGGGGAGAATGGTTATCCGCTCCCCAAT
TTCCCCCAAACATACCAAACCGGAAGACATAAAAAGGAAAGACCCGGGGGGGGCCTAAGGAAGGGAGCAA
ACTCCCCCTA

>190509-004_I01_Prx_M13R.ab1 1208

CGGGAAGTGAATGATTACGCCAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGA
ATTCGGCTTCATATGGAGGAAGGCCAAGATAATGGCCTTGTCATGAACTTCTACAAGGAAACATGTCCTCA
GGCTGAAGATATCATCAAAGAACAAGTCAAGCTTCTCTACAAACGCCACAAGAACACTGCTTTCTCCTGGC
TCAGAAACATCTTCCATGACTGTGCTGTTCAGAGTTGTGATGCTTCACTCTTGCTGGACTCCACAAGAAGGA
GCTTGTCTGAGAAGGAGACAGACAGAAGCTTTGGTTTGAGGAATTTCAGGTACATTGAGACCATCAAAGAA
GCTGTGGAAAGGGAGTGCCCAGGAGTTGTTTCCTGTGCTGATATACTCGTTCTCTCTGCCAGAGATGGCATT
GTTTCGCTAGGAGGCCCCCATATCCCTCTTAAAACTGGAAGAAGGGATGGTAGAAGGAGCAGAGCTGATGT
GGTGGAAGAGTTCCTCCCAGACCACAATGAGTCCATTTCTTCAGTTCTTGACAAGTTTGGTGCCATGGGAAT
TGACACTCCCGGGGTGGTTGCATTGCTTGGAGCACACAGTGTTGGTAGAACCCATTGTGTGAAGTTGGTGCA
CCGTTTGTACCCAGAGATTGATTCAGCGCTGAACCCTGACCATGTCCCTCACATGCTGAAGAAGTGCCCTGA
TGCCATCCCAGACCCCAAGGCCGTGCAGTACGTGAGAAACGACCGTGGCACCCCTATGATTCTAGACAACA
ACTACTACAGGAACATATTGGACAACAAGGGGTTGTTGATAGTGGATCACCAACTAGCCAATGACAAGAGG
ACCAAGCCTTATGTGAAGAAAATGGCCAAGAGCCAAGACTATTTCTTCAAGGAGTTTTCTAGAGCCATTGCT
TTGCTCTCTGAAAACAATCCTCTCACTGGCACAAAGGGGGAGATCAGAAAGCAGTGCAATGTTGCCAACAA
GCACCATGAGGAGCCTTAAGGATCCAAGCCGAATTCTGCAGATATCCATCACACTGGCGGCCGCTCGAGCA
TGCATCTAGAGGGCCCAATTCGCCCTATAAGGAAGTCGTATTACAATTCCTTGGCCGCCGTTTTTCAACGTC
CGGAATGGGGAAAAACCCGGGGTTACCCAACTTTATTCCCTTTGGGAAAATCCCCCTTTTTCCCC

3.- Secuencia codificante SepB:

>190509-004_E01_SepB_M13F.ab1 1204

GGATGACTCTATAGGGCGATTGGGCCCTCTAGATGCATGCTCGAGCGGCCGCCAGTGTGATGGATATCTGC
AGAATTCGGCTTCATATGTCACTGAGCTTAAACTACTACGAAAATACATGCCCTAAAGTGGAGTGCATTGTT
GCCAAGGCAGTGAAGGATGCCACTGCTAAGGACAAAACTGTTCCTGCAGCTCTGCTTCGAATGCACTTCCA
CGATTGTTTCATTCGGGTAACAGAATTTTGACTCCCTCCTTTCCTCTAGACTGTTTGTTTCTGCTTCATTCGAT

GTGTCATTAATAAAATCAATTTTTCTTATTTTTTCTCCTATTTTCTTGAAGGGGTGTGATGCCTCCGTGCTGTT
AAATTCAAAAGGAAACAGCAAAGCAGAAAAAGATGGGCCACCAAATGTTTCTTTGCATGCATTCTATGTCA
TTGATGCAGCAAAGAAAGCATTAGAAGCCGCATGCCCTGGTGTGGTCTCTTGCGCTGATATCCTAGCTCTGG
CAGCAAGGGATGCAGTTTTTCTGGTAAGTAATCTATTAAAGTACCAAATTTCTCATCAATAAGATTCTAAAC
AATTGTTATGTTATGTCTTTGAGAATTGATAACTGCAGCTACGGTAAATTGTATCTGCAAGTCTTAACTAAT
GTTATAAATATATTCTACAGTCTGGAGGACCTACATGGGATGTTTCTAAAGGAAGAAAGGATGGAAGAACA
TCTAAGGCCAGTGAAACCATACAGTTACCAGCTCCAACCTTCAACTTATCACAACTGCGGCAAAGCTTCTCT
CAAAGAGGACTGTCTGGGGAAAACTTGGTAGCTCTGTCAGGTAAGTTCTCCCTAAAGTCAAAACTGCCAAA
ACTTGTCATTTTTCATTTATTTTAATTTATATCTAAATAGTGTTTAGATTAAAATATCTTTTAACTCGTGTAGG
GGGGCATACTTTGGGTTTTTCTCACTGCTCATCTTTCCGGAGCAGACTCCACAACTTCAGTGCTACACATGAT
GTTGACCCTTCATTAAATCCGACATTTGCAGCAAAACTGAAATCAATTTGTCCAGTAAAAAATCAGGCAAA
AAAAGCAGGGACCACTATGGACCCTTCTTCGCAACTTTTCAAAAAACATTTTACAGGGGGATCCTCCCAGA
AAAAAGGGTTGTGTTCTTTTTGATAAAACTTTGTTTTGGAAAACCCAGAACC

>190509-004_K01_SepB_M13R.ab1 1215

TCGCATGGACATGATTACGCCAGCTTGGTACCGAGCTCGGATCCACTAGTAACGGCCGCCAGTGTGCTGGA
ATTCGGCTTGGATCCTTAATTGACCACTCTGCAGTCCTTCCTAACCTCTTGTCCCCCGTTGATGCTACTCATT
TCGATCATGGACTCAGCGAAAGCCTGATAAAAAGCCTTTTGTGAGGTGGCAAACTTAGTAACCAGATTCTT
GGTGTCTGGGTTATCAAGCAAAGCTTGATCAGAAGAAAACAAGCCTTTATCTTGGAGGATCAACCTGTAAT
ATGTATTATCGAAAGTTGCTGAAGAAGGGTCCATAGTGGTGCCTGCTTTTTTTGCCTGATTTTTTACTGGACA
AATTGATTTCAGTTTTGCTGCAAATGTCGGATTTAATGAAGGGTCAACATCATGTGTAGCACTGAAGTTGTG
GAGTCTGCTCCGGAAAGATGAGCAGTGAGAAAAACCCAAAGTATGCCCCCCTACACGAGTTAAAAGATATT
TTAATCTAAACACTATTTAGATATAAATTAAAATAAATGAAAAATGACAAGTTTTGGCAGTTTTGACTTTAG
GGAGAACTTACCTGACAGAGCTACCAAGTTTTCCCCAGACAGTCCTCTTTGAGAGAAGCTTTGCCGCAGTTG
TGATAAGTTGAAGGTTGGAGCTGGTAACTGTATGGTTTCACTGGCCTTAGATGTTCTTCCATCCTTTCTTCCT
TTAGAAACATCCCATGTAGGTCCTCCAGACTGTAGAATATATTTATAACATTAGTTAAGACTTGCAGATACA
ATTTACCGTAGCTGCAGTTATCAATTCTCAAAGACATAACATAACAATTGTTTAGAATCTTATTGATGAGAA
ATTTGGTACTTTAATAGATTACTTACCAGAAAAACTGCATCCCTTGCTGCCAGAGCTAGGATATCAGCGCAA
GAGACCACACCAGGGCATGCGGCTTCTAATGCTTTCTTTGCTGCATCAATGACATAGAATGCATGCAAAGA
AACATTTGGTGGCCCATCTTTTTCTGCTTTGCTGGTTCCTTTTGAATTTAACAGCACGGAGGCATCACACCCC
TTCAAGAAAATAGGAGAAAAAATAAGAAAAATTGATTTTTTTATGGACACATCGAAGGAAGCCAAAACAA
ACCGTCTAGAGGAAAGGAGGGAGTCAAAATTTTGTTACCCCAATGAGACAAACCGGGGAAGGGGTT