Centro de Investigación en Alimentación y Desarrollo, A.C.

OBTENCIÓN E IDENTIFICACIÓN DE DETERMINANTES ANTIGÉNICOS DE PROTEÍNAS ALERGÉNICAS DEL POLEN DE NOGAL PECANERO (*Carya illinoinensis*) APLICABLES AL DIAGNÓSTICO Y TERAPIA DE LA ALERGIA RESPIRATORIA

Por:

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RESUMEN

La alergia es un problema de salud mundial. La Organización Mundial de la Salud estima que alrededor del 30% de la población padece alergia, y que de esta al menos un 40% es alergia respiratoria (AR). La AR es causada por aeroalérgenos, como el polen de las plantas, cuya fuente principal en el ambiente es el proveniente de los árboles. Sin embargo, pocas fuentes polínicas han sido bien caracterizadas y muchas otras permanecen sin explorar y, por ende, tampoco hay diagnostico disponible para esas ARs. Aunado a esto, la inmunoterapia con alérgenos de las fuentes causantes de AR ha demostrado ser el único tratamiento efectivo y que mejorar la calidad de vida de los pacientes, lo que deja de manifiesto la importancia de la exploración de nuevas y relevantes fuentes alergénicas. Hasta hace poco, el polen de *Carya illinoinensis*, especie ampliamente distribuida en el norte de México por su fruto, la nuez pecana, había permanecido inexplorado. En este trabajo hemos logrado reportar la primera identificación de alérgenos de polen de dicha fuente (17 proteínas únicas) mediante un enfoque inmunoproteómico, utilizando una base de datos específica para *C. illinoinensis* (92,960 proteínas), creada mediante el ensamble de datos de transcriptoma. Por otro lado, seleccionamos dos alérgenos del polen de nogal, profilina y enolasa, obtuvimos sus marcos de lectura, y sus secuencias nucleotídicas se sintetizaron en gBlocks independientes que se clonaron en el vector de expresión pET-28a(+) el cual se usó para transformar células BL21(DE3)pLysS para la sobreexpresión de los alérgenos. Las proteínas recombinantes se purificaron mediante la combinación de IMAC y otras técnicas cromatográficas. La identidad de cada proteína se confirmó empleando cromatografía de líquidos acoplada a espectrometría de masas en tándem. La predicción de epítopes se realizó combinando las herramientas bioinformáticas ABCpred, BepiPred, Immunomedicine y ElliPro. En conjunto, la identificación de proteínas alergénicas, así como la sobreexpresión y predicción epítopes en los alérgenos profilina y enolasa, sientan las bases para el diagnóstico y diseño de nuevas modalidades terapéuticas para la alergia al polen de nogal pecanero.

Palabras clave: alérgenos de polen, *Carya illinoinensis*, predicción de epítopes, sobreexpresión.

ABSTRACT

Allergy is a global health problem. The World Health Organization estimates that around 30% of the population suffers from allergies, being at least 40% respiratory allergy (RA). RA is caused by aeroallergens, such as plant pollen, whose main source in the environment is trees. However, few pollen sources have been well characterized and many others remain unexplored and, therefore, no diagnosis is available for these ARs. In addition, immunotherapy with allergens from the sources that cause RA has been shown to be the only effective treatment and to improve the quality of life of patients, which highlights the importance of exploring new and relevant allergenic sources. Until recently, the pollen of *Carya illinoinensis*, a species widely distributed in northern México for its fruit, the pecan nut, had remained unexplored. In this work we have been able to report the first identification of pollen allergenic proteins (17 unique proteins) from this source through an immunoproteomic approach, using a specific database for *C. illinoinensis* (92,960 proteins), created by transcriptomic data assembly. On the other hand, we selected two pecan nut pollen allergens, profilin and enolase, obtained their reading frames, and their nucleotide sequences were synthesized into independent gBlocks that were cloned into the expression vector pET-28a(+) which was used to transform BL21(DE3)pLysS cells for overexpression of allergens. Recombinant proteins were purified by combining IMAC and other chromatographic techniques. The identity of each protein was confirmed using liquid chromatography-tandem mass spectrometry. Epitope prediction was performed by combining the ABCpred, BepiPred, Immunomedicine, and ElliPro bioinformatics tools. Together, the identification of allergenic proteins, as well as the overexpression and prediction of epitopes in profilin and enolase allergens, lay the foundations for the diagnosis and design of new therapeutic modalities for pecan tree pollen allergy.

Keywords: pollen allergens, *Carya illinoinensis*, epitope prediction, overexpression.

1. SINOPSIS

1.1. Justificación

La Organización Mundial de la Salud (OMS) estima que al menos el 25% de la población a nivel mundial sufre de alergia respiratoria (AR), esta enfermedad consiste en una respuesta inmunológica a elementos ambientales generalmente inocuos transportados por el aire, los que ocasionan reacciones de hipersensibilidad en pacientes susceptibles. Las manifestaciones clínicas de la AR son molestas para quienes las padecen, y en ocasiones pueden ser mortales (Oh, 2018). Las alergias respiratorias son un problema de salud mundial por su alta prevalencia y creciente incidencia, además del elevado costo de los tratamientos farmacológicos (antihistamínicos, corticosteroides) dirigidos a tratar únicamente los síntomas (Bousquet *et al.*, 2008). Se calcula que los aeroalérgenos, de los cuales el polen es el más común, son responsables de hasta el 40% de las enfermedades respiratorias alérgicas y la OMS reconoce que millones de personas que padecen alergia respiratoria no cuentan con un diagnóstico específico (D'Amato *et al.*, 2007).

El diagnóstico común de la alergia ocasionada por el polen se basa en el uso de extractos que por lo general tienen una composición variable e indefinida, donde la cercanía filogenética entre algunas especies ocasiona con frecuencia reactividad cruzada, por lo que el diagnóstico dista de ser específico (Jeong *et al.*, 2016). Para contrarrestar esto y mejorar la especificidad del diagnóstico, se han utilizado alérgenos purificados (tanto de fuentes naturales como recombinantes), y en algunos casos se han modificado alérgenos o se han construido híbridos moleculares, derivados del conocimiento de los determinantes antigénicos (epítopes), mejorando los resultados de la prueba cutánea utilizada como el principal método de diagnóstico (Douladiris *et al.*, 2018). Dichas moléculas pueden emplearse también para inmunoterapia con alérgenos específicos, que es el único tratamiento efectivo para la desensibilización alérgica y que ofrece una mejora en la calidad de vida de los individuos afectados (Asero *et al.*, 2016).

Sin embargo, actualmente solo se conocen algunos alérgenos de las fuentes más comunes, por lo que la exploración de nuevas fuentes alergénicas con relevancia clínica para la identificación de sus alérgenos y epítopes, son de suma importancia para el desarrollo de métodos diagnósticos

específicos y confiables. Además de ser el primer paso para el desarrollo de estrategias terapéuticas (Curin *et al.*, 2018).

Los granos de polen son la fuente más común de alérgenos inhalados que por su tamaño se transportan fácilmente por las corrientes de aire, y se estima que éstos causan alergias respiratorias en entre un 15-30% de la población mundial (Singh & Mathur, 2012). El polen del nogal pecanero (*Carya illinoinensis*) es de relevancia clínica, el INER lo ubica dentro de los primeros 15 pólenes de árboles causantes de alergias respiratorias en los pacientes atendidos en sus instalaciones y es la segunda causa de alergias graves en el estado de Texas, USA (Rachmihl *et al.*, 1996., Mani, 2015). Este árbol tiene un gran valor comercial, ya que produce las nueces pecaneras, por lo que su cultivo, y por ende su polen, están ampliamente distribuidos en América del Norte, principalmente en el Sur de Estados Unidos y el Norte de México (Reyes-Vázquez y Urrea-López 2016). Su amplia distribución geográfica y el aumento de las zonas de cultivo en las últimas tres décadas incrementan la cantidad de partículas de este polen en el aire, causando sensibilización en la población de sectores cercanos a estas áreas, incrementando su importancia epidemiológica y relevancia clínica. Hasta hace poco las proteínas alergénicas del polen de nogal pecanero no habían sido identificadas. Hasta el momento no hay un diagnóstico ni tratamiento específico para esta alergia en particular, el presente trabajo tuvo como objetivo la obtención de las versiones recombinantes de dos proteínas alergénicas presentes en el polen de *Carya illinoinensis*, así como la predicción de epítopes que contribuirán a un diagnóstico específico y al futuro desarrollo de una adecuada inmunoterapia.

1.2. Antecedentes

1.2.1. Alergia Respiratoria

El sistema inmune es el encargado de montar una respuesta de defensa ante las infecciones en el cuerpo. Dicha respuesta se genera regularmente ante agentes infecciosos como bacterias, virus, hongos, protozoos y parásitos. Sin embargo, en ocasiones el sistema inmune de algunos individuos tiene una reacción exacerbada ante agentes generalmente inocuos para la mayoría de las personas,

a dicha reacción se le conoce como alergia (Janeway *et al.*, 2009).

Cuando los agentes causales de la alergia son acarreados por el aire y sensibilizan a personas susceptibles, son denominados aeroalérgenos. En este grupo se enlistan principalmente ácaros, epitelios animales (ej. perro y gato), esporas de hongos, así como el polen de diversos árboles y plantas (Guidos y Almeida, 2005). Las manifestaciones más comunes de la alergia respiratoria son la rinitis, conjuntivitis y urticaria, catalogadas como síntomas leves, por otro lado, entre sus manifestaciones graves se encuentran el, asma y anafilaxia, estas últimas pueden llegar a poner en riesgo la vida de los individuos (Oh, 2018).

1.2.2. Manifestaciones Clínicas de la Alergia Respiratoria

Ha habido un marcado aumento en la prevalencia de alergia a finales del siglo XX, en todos los rangos de edad y severidad. Las tasas de prevalencia varían en el mundo, siendo el más alto en países desarrollados de habla inglesa. Sin embargo, la prevalencia de la alergia a nivel global sigue aumentando, principalmente en los países en desarrollo. Hace 150 años las manifestaciones de la alergia eran raras, pero su notable aumento al pasar de los años, a la par de la civilización urbana le han permitido alcanzar prevalencias de alrededor del 40% (Holgate y Church, 2012). Se ha intentado atribuir este incremento a diversas razones como el aumento de la contaminación, el cambio a un estilo de vida interior, sedentario e higiénico (principalmente en ciudades), el calentamiento global, la falta de vitamina D, exposición a nuevos alérgenos con la globalización del mundo moderno, y al mayor estrés que conlleva la vida actual. Todos estos factores contribuyen a que las diversas manifestaciones clínicas de la alergia respiratoria, principalmente la rinitis alérgica y el asma, sean más incidentes con el pasar de los años, así como otras manifestaciones como la conjuntivitis, urticaria y angioedema, dermatitis atópica e incluso anafilaxia (Holgate y Church, 2012). Todas las manifestaciones clínicas antes mencionadas son diferentes entre si y son de ayuda en el historial clínico para el diagnóstico y manejo de cada paciente, por lo que se describirán brevemente a continuación.

1.2.2.1. Rinitis alérgica. Los pacientes con rinitis alérgica pueden presentar secreción nasal, picazón, estornudos y obstrucción, debidos a una reacción mediada por IgE, ocasionados principalmente por exposición a alérgenos inhalantes como pólenes y ácaros del polvo o epitelio de animales domésticos. Pese a ser considerada como una manifestación leve, frecuentemente es un factor de predisposición para el desarrollo de asma. Así mismo, es una de las manifestaciones más comunes y que representa mayor costo, ya que merma la calidad de vida de los pacientes, dificultando el desarrollo de actividades cotidianas como el estudio y el trabajo, o el tiempo de juego en el caso de los niños (Wheatley y Togias, 2015). Para controlar los malestares ocasionados por la rinitis alérgica, los pacientes deben adquirir constantemente medicamentos que les hagan sus días más llevaderos, representando para algunos, un gasto considerable a lo largo del año. Predomina en el rango de edad de 15 a 25 años, pero en los últimos años muestra mayor incidencia en niños y es frecuente también en adultos. Aproximadamente el 85% de quienes padecen rinitis alérgica presentan alteraciones en el sueño, debidas a la congestión nasal la cual suele empeorar en las primeras horas de la mañana y por las noches. Los pacientes pueden presentar microdespertares del sueño, respiración irregular, ronquidos y apnea obstructiva, teniendo como consecuencia fatiga y menor rendimiento, así como derivar en problemas de salud mental como falta de concentración, irritabilidad, problemas de conducta, ansiedad y depresión (Holgate y Church, 2012).

1.2.2.2. Asma. Es una enfermedad que afecta los pulmones, las vías respiratorias medias se inflaman, estrechan y se producen mayores cantidades de mucosa de lo habitual. Esta afección causa sibilancias, dificultad para respirar, opresión en el pecho y episodios de tos, dificultando la exhalación, en algunos casos puede exacerbarse y conducir a ataques mortales. Esta suele ocurrir cuando las vías respiratorias se encuentran en un estado de inflamación crónica, por lo que la rinitis suele ser un indicativo del estado de inflamación frecuente, causada principalmente por la inhalación de alérgenos (Khan, 2014). Es difícil establecer o predecir la severidad de los padecimientos asmáticos, ya que están influenciados principalmente por la producción de mediadores liberados por eosinófilos, células T y mastocitos. Esta enfermedad es más común en niños, y regularmente va disminuyendo con el pasar de los años. Cuando pacientes mayores a 20 años presentan asma, regularmente es indicativo de alta sensibilidad a alérgenos o mal manejo en edades tempranas (Holgate y Church, 2012).

1.2.2.3. Otras manifestaciones de la enfermedad alérgica. Una de las manifestaciones más comunes es la ocular o conjuntivitis alérgica, consiste en la inflamación alérgica de la superficie ocular en respuesta a un aerolérgeno transitorio, la cual acompaña regularmente a la rinitis, derivando en síntomas desagradables, pero no graves. También existen la queratoconjuntivitis atópica y la queratoconjuntivitis primaveral, que son manifestaciones graves, pero inusuales de conjuntivitis en pacientes atópicos (Holgate y Church, 2012).

Urticaria y angioedema. Estas manifestaciones se deben a la vasopermeabilidad transitoria de la microvasculatura cutánea y submucosa, que tienen como resultado hinchazón. Las hinchazones pueden ser superficiales, describiéndose como manchas rojizas y/o erupciones o ronchas que pueden causar picazón en la piel, en este caso se conoce como urticaria. Por otro lado, si la hinchazón se presenta bajo la piel en vez de en la superficie, es decir en la dermis y tejido subcutáneo, se conoce como angioedema, ocurriendo frecuentemente en ojos y labios (Holgate y Church, 2012).

Dermatitis atópica. En esta variedad de dermatitis es común observar sequedad y comezón en la piel, además de sarpullido en la cara, al interior de los codos y detrás de las rodillas; así como en las manos y los pies. En esta enfermedad se presenta una infiltración de células mononucleares en la dermis, ocasionando claras lesiones en la piel. La dermatitis atópica es una condición crónica, se caracteriza por prurito intenso, con distribución distintiva de lesiones cutáneas eccematosas, puede ser hereditaria, cuando hay antecedentes de familiares atópicos. Suele comenzar en la primera infancia y sigue un curso de remisiones y exacerbaciones, puede llegar a ser muy molesta y a provocar lesiones severas en la piel (Holgate y Church, 2012; Hartmann *et al.*, 2016).

Anafilaxia. La anafilaxia se define como una reacción alérgica severa e inesperada de comienzo rápido y que puede causar la muerte. El diagnóstico de anafilaxia es esencialmente clínico destacando el compromiso de varios sistemas: cardiovascular, cutáneo, respiratorio y gastrointestinal. A nivel cutáneo se presenta como urticaria y angioedema, presentándose en el 90% de los casos, en vías respiratorias se puede manifestar como disnea en grado variable, sibilancias, broncoespasmo y paro respiratorio. A nivel gastrointestinal se puede presentar como dolor abdominal, náuseas, vómitos, pujo y diarrea. A nivel cardiovascular se presenta taquicardia e hipotensión puede progresar a un paro cardiovascular. Pueden presentarse también agitación psicomotora y pérdida de conocimiento, se considera poco frecuente estimando una prevalencia de un 0,05% a un 2% incluyendo todas las etiologías, sin embargo, se cree puede estar subdiagnosticada (Toche, 2011).

1.2.3. Mecanismo de la Alergia Respiratoria

Las reacciones alérgicas constituyen reacciones inmunológicas de desarrollo rápido, que ocurren generalmente minutos después del reconocimiento de un antígeno por la inmunoglobulina E (IgE) unida a mastocitos o basófilos. El reconocimiento entre la IgE y el antígeno desencadena una reacción localizada (rinitis, urticaria, conjuntivitis) o generalizada (anafilaxia), causada por la desgranulación de las células activadas y su efecto sobre los tejidos. Para poder presentar una reacción alérgica inmediata es necesaria una primera etapa, es decir una exposición previa al alérgeno, en la cual se llevó a cabo el proceso de sensibilización, siendo hasta la segunda etapa o reexposición al agente causal cuando se manifiestan los síntomas característicos de la reacción alérgica (Kay; 2001). En la Figura 1 se muestra una representación de las dos fases del mecanismo de la alergia respiratoria.

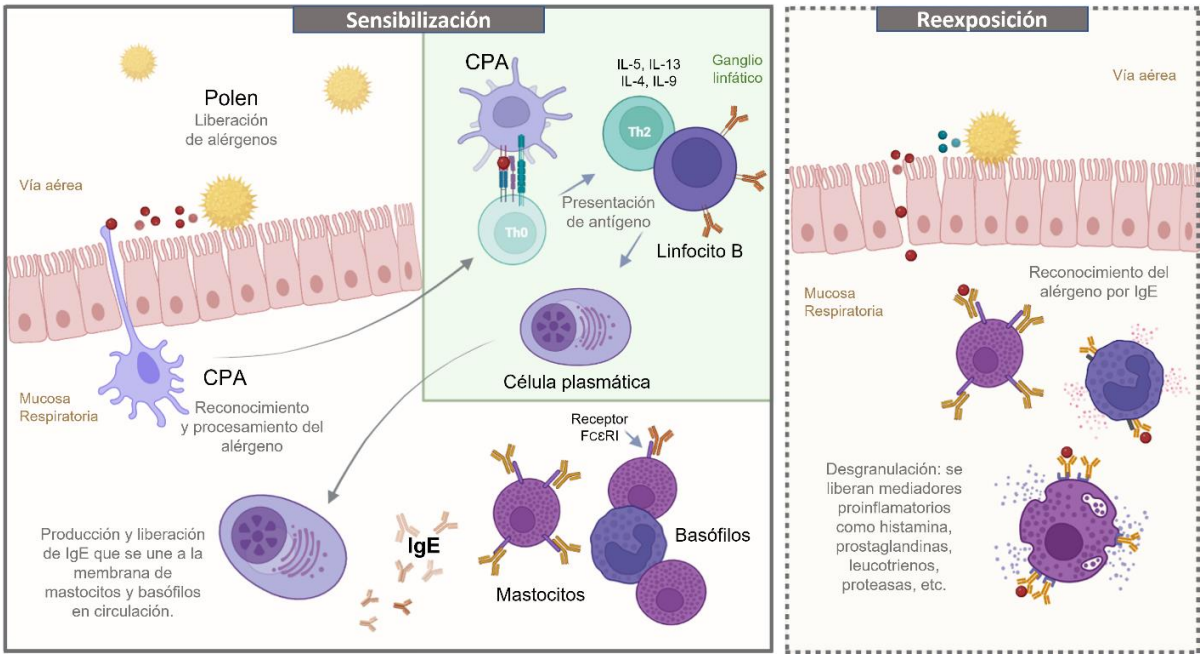

Fig. 1. Mecanismo de la alergia respiratoria.

1.2.3.1. Primera etapa: Sensibilización al alérgeno. La etapa de sensibilización comprende dos pasos importantes, la producción de IgE específica y su posterior unión a receptores específicos en

la membrana de mastocitos y eosinófilos. Los factores principales que contribuyen a la producción y regulación de IgE son la herencia, el ambiente y una respuesta de los linfocitos T cooperadores variedad 2 (Th2), estos factores en conjunto contribuyen al inicio y desarrollo de la enfermedad alérgica (Bruhns *et al.*, 2005).

La sensibilización comienza cuando el aeroalérgeno llega a tejidos como las mucosas respiratorias, donde las células presentadoras de antígenos (CPA) o también llamadas células dendríticas lo capturan y digieren intracelularmente. Los alérgenos son generalmente proteínas, por lo que son digeridas hasta convertirlas en péptidos, que son ensamblados en el complejo principal de histocompatibilidad clase II (MHC II), encargado de sostener a péptidos antigénicos de naturaleza exógena, procesados por endocitosis. Posteriormente las CPA migran a los ganglios linfáticos, donde presentan los péptidos antigénicos a los linfocitos T vírgenes (Th0) mediante su interacción con el Receptor de células T (TCR), lo que los diferencia a linfocitos T cooperadores tipo 2 (Th2). Esta variedad de linfocitos se caracteriza por la secreción de interleucinas como IL-5 e IL-9, que promueven la proliferación de basófilos y mastocitos, respectivamente, así como IL-4 e IL-13, causantes del cambio de isotipo de los linfocitos B para la producción de IgE (Janeway *et al.*, 2009). Los linfocitos B se convierten en células B plasmáticas productoras de IgE, esta inmunoglobulina al entrar en circulación se une, por su región FC, al receptor para IgE de alta afinidad Fcalt épsilon RI (FcεRI), presente en la superficie de los mastocitos y basófilos, concluyendo la etapa de unión a receptores (Bruhns *et al.*, 2005). De aquí en adelante, los mastocitos y basófilos, serán capaces de reconocer al alérgeno en futuras exposiciones, ya que la IgE adquiere la función de receptor específico para el alérgeno (Kay; 2001).

1.2.3.2. Segunda etapa: Reexposición al alérgeno. En esta etapa se desencadena una respuesta inmune, iniciada por la reexposición al antígeno, una posterior activación celular y liberación de mediadores químicos, responsables de los síntomas de la alergia, que puede ocurrir en diversos tejidos del organismo (Oh, 2018).

Cuando el alérgeno vuelve a entrar en contacto con los individuos sensibilizados inicia la activación celular, correspondiente a la reacción de hipersensibilidad tipo 1. El alérgeno es reconocido por las IgE ancladas en la superficie de los mastocitos y basófilos, lo que propicia el entrecruzamiento de los FcεRI, promoviendo la activación de estas células, las cuales liberan inmediatamente mediante

desgranulación, mediadores preformados, los cuales son responsables de la respuesta inflamatoria. Los gránulos contienen principalmente histamina, proteoglucanos y proteasas neutras (triptasa y quimasa), liberadas mediante la fusión de los gránulos con la membrana de las células activadas. Este método de liberación garantiza una respuesta inmediata (Janeway *et al.*, 2009). Los mastocitos están presentes en la mayoría de los tejidos, principalmente en las puertas de entrada a alérgenos, como la piel, el epitelio del tracto respiratorio y gastrointestinal, así como en la conjuntiva, por lo tanto, la vía de contacto será la que muestre las principales manifestaciones clínicas (Kay; 2001).

1.2.4. Métodos Diagnósticos de Alergia Respiratoria

1.2.4.1. Pruebas Cutáneas. Existen dos tipos de pruebas cutáneas utilizadas para el diagnóstico de la alergia, una de tipo puntura (PT) y la otra intradérmica (ID). Ambas consisten en colocar el extracto alergénico en contacto con las células cutáneas en una parte plana y depilada del cuerpo, regularmente el antebrazo o la espalda, donde se encuentran mastocitos que reaccionan liberando mediadores inflamatorios locales que promueven la formación de una pápula con eritema igual o mayor a 3 mm entre los primeros 15-30 minutos posteriores a la aplicación, demostrando la presencia de IgE específica para el alérgeno probado (Arruda-Chavez, 2004).

La prueba cutánea de tipo PT se realiza aplicando extractos glicerinados a través de una punción con lanceta en el tejido cutáneo. Esta técnica se considera el estándar de oro, por ser una prueba segura, sensible, de bajo costo y poco invasiva. Por su parte, en la prueba de tipo ID se emplean extractos acuosos, y se considera menos práctica, por ser más dolorosa, tener mayor riesgo de provocar efectos sistémicos, y requerir una mayor habilidad de ejecución, ya que si se aplica a una mayor profundidad de lo debido puede causar falsos negativos. La seguridad de ambas pruebas está directamente relacionada con la habilidad técnica del profesional que la realiza, la calidad del material utilizado y la correcta interpretación de los resultados (Arruda-Chavez, 2004).

Los extractos comerciales que generalmente se utilizan para realizar las pruebas cutáneas, contienen una mezcla compleja de proteínas y comúnmente, otros compuestos co-extraidos del polen. Dichas mezclas pueden contener proteínas alergénicas ubicuas en otras fuentes de alérgenos, pudiendo presentarse el fenómeno de reactividad cruzada, es decir, un reconocimiento de la IgE

específica para un alérgeno hacia moléculas alergénicas similares, que inducen una reacción alérgica, generalmente de menor magnitud, lo que puede dificultar el diagnóstico del agente causal o generar falsos positivos. Por lo anterior, estos extractos pueden ser poco específicos al emplearse en el diagnóstico clínico. Lo idóneo sería realizar las pruebas cutáneas con moléculas alergénicas purificadas, que garanticen sensibilidad y, sobre todo, especificidad (Curin *et al.*, 2018).

Actualmente se sugiere el uso de entre tres y cuatro alérgenos principales (reconocidos por arriba del 50% de los pacientes alérgicos a ese polen) de la principal fuente sospechosa, acompañados de un panalérgeno, regularmente una profilina, purificados y en concentraciones conocidas, buscando reducir al mínimo las posibilidades de reacciones adversas y a su vez, asegurar un diagnóstico específico. Adicionalmente, se recomienda realizar las pruebas cutáneas con un panel de fuentes alergénicas representativa de la región en donde el paciente habita, para descartar o confirmar polisensibilización. Los paneles de alérgenos estandarizados que se utilizan actualmente en México son adquiridos de Estados Unidos y Europa, sin embargo, la sensibilización a pólenes en pacientes mexicanos es diferente a la reportada en esos países, por lo que los paneles para pruebas cutáneas sugeridos en sus guías de diagnóstico e inmunoterapia (ambas implementadas en nuestro país), no son totalmente adecuados para México (Larenas-Linnemann *et al.*, 2019). Esto último resalta la importancia de la exploración de nuevas y relevantes fuentes de alérgenos, principalmente en países como el nuestro, que no cuentan con paneles diagnósticos bien definidos con base en a su flora.

1.2.4.2. Medición de IgE sérica Total. La técnica de determinación de IgE sérica total se utiliza regularmente como complemento diagnóstico junto a la prueba cutánea para enfermedades alérgicas mediadas por esta inmunoglobulina. Esta prueba indica el cambio en la concentración de IgE sérica, ocasionada por la respuesta a alérgenos, su concentración bajo condiciones normales es un pequeño porcentaje (0.001%) respecto al total de inmunoglobulinas en suero, y se expresa en UI/L (Unidades Internacionales por Litro) considerándose concentraciones mayores a 100 UI/L como un resultado positivo. Sin embargo, esta medición no ayuda en la identificación del alérgeno causal, simplemente indica un incremento en la abundancia de IgE sérica tras la exposición a este (Arruda-Chavez, 2004).

Actualmente la técnica de ImmunoCAP es la prueba de cuantificación de IgE específica más utilizada. Consiste en un panel de proteínas alergénicas unidas covalentemente a una fase sólida de

celulosa, estos se ponen en contacto con el suero del paciente, que en principio contiene IgE específica que será capaz de reconocer a su antígeno específico. Posteriormente se utiliza un anticuerpo anti-IgE marcado con fluorescencia, emitiendo señal tras su excitación, solo los anti-IgE unidos a IgE que a su vez se unieron a alérgenos, siendo el valor de señal detectada directamente proporcional a la cantidad de IgE unida al antígeno (Johansson, 2004).

1.2.5. Alérgenos Recombinantes para un Mejor Diagnóstico de Alergia Respiratoria.

La alta prevalencia de la alergia respiratoria demanda un estudio más profundo respecto a la identificación de los pólenes alergénicos y a los alérgenos contenidos en ellos. Como se mencionó anteriormente, los extractos de polen que se utilizan para el diagnóstico de la alergia respiratoria son mezclas complejas que pueden llegar a presentar reacción cruzada y, por ende, un diagnóstico poco específico (Saha y Bhattacharya, 2017). La utilización de este tipo de extractos se ha relacionado también con una mayor posibilidad de desarrollar efectos secundarios severos que en algunos casos han llegado a ser fatales (Sircar *et al.,* 2016). Para cumplir los parámetros de seguridad y especificidad, se ha recurrido a la sobreexpresión de alérgenos recombinantes en modelos de expresión como las bacterias, que ofrecen un mayor rendimiento y disponibilidad en comparación de los obtenidos de las fuentes naturales, disponibles solo por una o dos temporadas al año y donde partiendo de grandes cantidades de polen, las concentraciones de los alérgenos en los extractos son diversas y algunas llegan a ser mínimas.

La identificación y purificación de alérgenos provenientes del polen es crucial en el conocimiento certero de las moléculas alergénicas causantes de desencadenar la respuesta alergénica, y ha contribuido a mejorar la especificidad en el diagnóstico (Valenta *et al.,* 2018). Este proceso de exploración de alérgenos también es necesario para poder obtenerlos mediante vías recombinantes, las cuales pueden aplicarse directamente al diagnóstico como en el caso de la variante de ImmunoCAP que consiste en un chip de microarreglos con moléculas alergénicas, conocido como *Multiplex* (Hiller *et al.,* 2002). En esta variante, los chips están conformados por un panel completo de alérgenos que representan a las fuentes alergénicas más comunes. Con la capacidad de evaluar simultáneamente a más de 100 moléculas alergénicas, brindando una visión general y rápida del

perfil de sensibilización de los pacientes (Harwanegg *et al.,* 2003). Esto vino a revolucionar el diagnóstico de la alergia, ya que ayuda a determinar las moléculas alergénicas causales. El uso del sistema *Multiplex* es de gran ayuda en la exploración de los perfiles de sensibilización complejos, principalmente en los pacientes polisensibilizados (Valenta *et al.,* 2018).

Actualmente, diversos estudios sustentan que las moléculas recombinantes no sólo pueden utilizarse con fines diagnósticos, si no que han resultado efectivas como tratamiento para la desensibilización a alérgenos mediante exposiciones paulatinas y en cantidades adecuadas de los mismos (Valenta *et al.,* 2012). Sin embargo, no se conocen todos los pólenes alergénicos, y de muchos no se han identificado sus proteínas alergénicas, por lo que no se cuenta con la posibilidad de un diagnóstico y tratamiento específicos para todos los casos.

1.2.6. Tratamiento de la Alergia Respiratoria

El tratamiento de los enfermos con patología respiratoria producida por alergia al polen, al igual que la causada por otros aeroalérgenos, se basa en tres pilares fundamentales que se complementan: medidas de control ambiental, farmacoterapia e inmunoterapia (Mims, 2017). Cuando una persona se diagnostica como alérgica a uno o varios tipos de polen, se recomienda el control ambiental ante alérgeno, es decir, evitar entrar en contacto directo con él, por lo que los individuos sensibles deben evitar los lugares con gran población de árboles productores del polen causante de la reacción (Lizaso *et al.,* 2003). Sin embargo, en el caso del polen, como la mayoría de los aeroalérgenos de exterior, es muy difícil evitar, cantidades suficientes para sensibilizar y ocasionar síntomas son transportadas por el aire, por lo que el tratamiento farmacológico es el siguiente paso en el manejo de la enfermedad. Este tipo de tratamiento se centra en el control de los síntomas, ya que no ofrece una cura o mejora respecto a la etiología de la enfermedad. Cuando la sintomatología es leve, como es el caso de la rinitis, los principales fármacos utilizados son antihistamínicos y corticoides nasales. Los fármacos actualmente empleados en el tratamiento del asma se pueden dividir en fármacos antiinflamatorios como los corticoides, cromonas y antileucotrienos; así como broncodilatadores (agonistas β_2), los cuales solo pueden ser indicados por un médico especialista. La elección de uno u otro de estos fármacos dependerá de la gravedad de la enfermedad, pudiendo

llegar a representar un gasto considerable para las personas que presentan síntomas fuertes y/o continuos durante todo el año (Mims, 2017).

1.2.7. Inmunoterapia

La inmunoterapia específica es la única estrategia terapéutica que ofrece una mejora de los síntomas y modificación del transcurso de la enfermedad. Desde el primer tratamiento de inmunoterapia alergénica (AIT por sus siglas en inglés) realizado por Noon en 1911, en el cual inyectó repetidamente extractos acuosos de proteínas de pólenes de pastos y registró las dosis aplicadas necesarias para causar reacción alérgica, que fueron cada vez mayores, lo que indicaba una mayor tolerancia. Actualmente la AIT ha evolucionado en diferentes aspectos como el contenido y método de obtención de alérgenos, implementación de diversas vías de administración, uso de adyuvantes y diversos esquemas de aplicación. Todos estos en busca de una AIT que brinde mejores resultados y seguridad a los pacientes alérgicos (Calderón *et al.,* 2012).

Se han explorado varias rutas de administración para la AIT, siendo la vía subcutánea (SC-AIT) y la vía sublingual (SL-AIT) las más utilizadas. La SC-AIT se administra por medio de inyecciones y la SL-AIT se administra oralmente, mediante pastillas o gotas sublinguales, actualmente se estudian rutas como la intranasal e intralinfática, sin embargo, son implementadas con menos frecuencia. En la vía sublingual se utilizan cantidades mayores de alérgenos, respecto a la subcutánea, ya que no todo el alérgeno administrado es absorbido. Varios estudios han tratado de determinar cuál de las rutas es más efectiva, sin embargo, no hay resultados significativos hasta el momento (Pipet *et al.,* 2009). La AIT cuenta con evidencia, respaldada por numerosos casos clínicos y datos experimentales que han sido recabados en los últimos 50 años y lo único definitivo es que la AIT el único tratamiento capaz de brindar una mejora en la sintomatología y una menor sensibilidad al alérgeno por lo menos 7 años posteriores a la conclusión del tratamiento (Asero *et al.,* 2016).

La mayoría de las AIT actuales utilizan extractos con alérgenos de fuentes naturales que muestran severas limitaciones, asociadas a su baja calidad. Los extractos de pólenes naturales muestran una gran variabilidad en su composición y entre los componentes puede haber otros alérgenos que

podrían ocasionar una reacción o sensibilización durante la administración. Este tipo de tratamiento es reservado para los pacientes con síntomas muy fuertes o altamente recurrentes, en los que manifestaciones como asma y anafilaxia están controladas o ausentes, y dado que la AIT no es un procedimiento económico, su relación costo/beneficio debe ser evaluada. Para la prescripción de AIT se requiere un médico alergólogo experimentado e idealmente una identificación específica de los alérgenos causantes de la enfermedad (Curin *et al.,* 2018). Cabe recalcar nuevamente que muchas de las fuentes de alérgenos no han sido caracterizadas, por lo que hay un gran número de pacientes que no pueden obtener una AIT específica a pesar de ser candidatos para su administración, ya que para su segura y correcta implementación es necesario un diagnóstico especifico previo. Esta situación pone de manifiesto, la importancia de la identificación de los alérgenos en los diversos pólenes con relevancia clínica y la continua investigación y desarrollo en el campo de la alergia.

1.2.7.1. Epítopes alergénicas y su impacto en la ITA. Las fuentes de pólenes alergénicos más exploradas a nivel mundial, como los provenientes de los árboles de abedul, olivo, y varios tipos de pastos, son algunos de los cuales mejor se conoce su composición alergénica y sus alérgenos principales, incluso se ha llegado al conocimiento de los principales determinantes antigénicos en algunos de sus alérgenos más representativos (Vadlamudi y Shaker, 2015). Estos últimos son denominados también como epítopes, son secuencias cortas de aminoácidos, que representan una pequeña porción de la macromolécula alergénica, la cual es reconocida por el sistema inmune, específicamente por los anticuerpos. Los epítopes pueden ser de naturaleza conformacional o lineal, esto en función de si su reconocimiento depende de la conformación tridimensional del alérgeno o no, respectivamente (Wang *et al.,* 2018). Conocer las epítopes ha sido de gran importancia en el tratamiento de la enfermedad alérgica, ya que en combinación con técnicas moleculares han impulsado el desarrollo de estrategias inmunoterapéuticas novedosas. Actualmente en la AIT se usan alérgenos naturales o sus equivalentes recombinantes, así como variantes de estos alérgenos entrecruzados o también llamados alergoides (con la finalidad de reducir su actividad alergénica y conservar su inmunogenicidad), pero algunas de las alternativas terapéuticas más efectivas y seguras que se han documentado en la inmunoterapia, son derivadas de conocer las epítopes alergénicas. Este es el caso de las proteínas hipoalergénicas, implementadas

en diversas modalidades como híbridos con múltiples regiones o péptidos antigénicos unidos a moléculas acarreadoras, obtenidos por técnicas recombinantes y ofreciendo alta especificidad, ya que son alérgenos puros, generalmente con alergenicidad atenuada, disminuyendo el riesgo de efectos secundarios y eliminando el problema de la co-sensibilización hacia otros alérgenos (Curin *et al.*, 2018; Tulaeva *et al.*, 2020). En la Figura 2 se muestra una representación de las opciones de ITA actuales que se derivan del conocimiento o predicción de epítopes alergénicas.

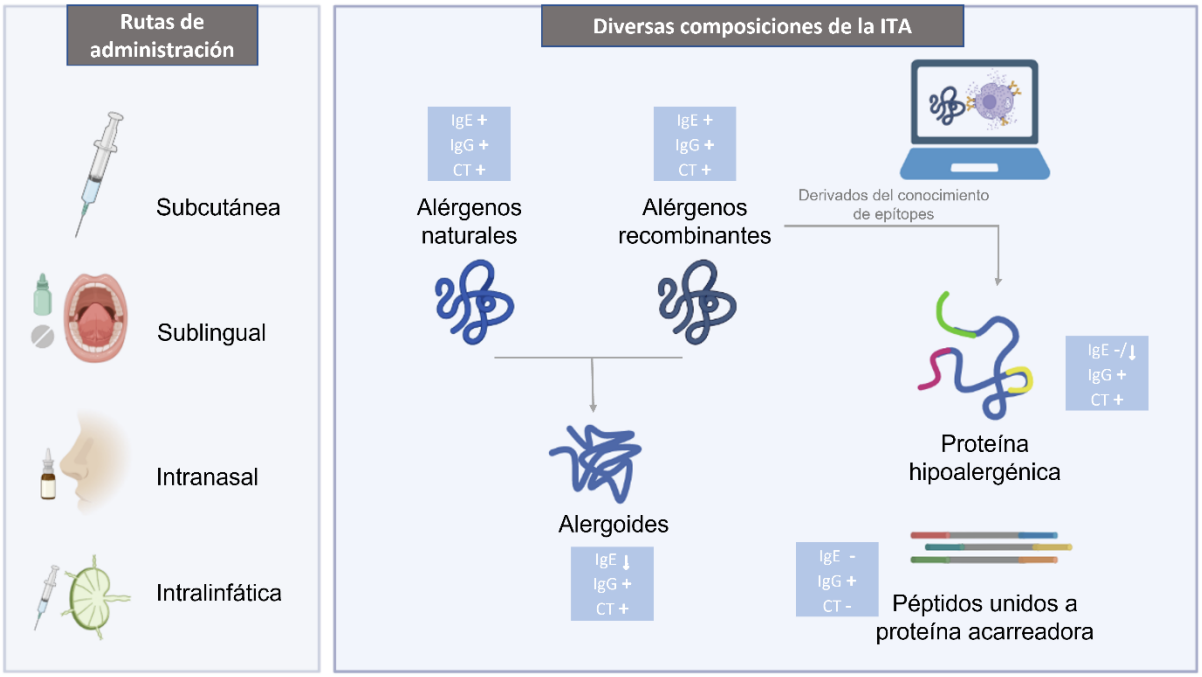

Fig. 2. Infografía que muestra las diversas rutas de administración de inmunoterapia con alérgenos (ITA) y sus formulaciones más comunes en la actualidad.

Entre las estrategias moleculares antes mencionadas se resalta la efectividad del uso de proteínas hipoalergénicas. Estas son proteínas alergénicas a las cuales se les causan mutaciones sitio-dirigidas a sus epítopes, con la finalidad de disminuir, o incluso desaparecer su reconocimiento por IgE, pero conservando su capacidad de estimular la producción de IgG neutralizante. Es decir, se obtienen moléculas con baja o nula alergenicidad, pero su inmunogenicidad es conservada. Un ejemplo de esto, son los resultados reportados para el alérgeno del hongo *Rhizopus oryzae*, que mediante mutaciones en sus epítopes generaron una variante hipoalergénica, comprobando esto mediante la respuesta alérgica de conejos sensibles a Rhi o 1, que al ser expuestos a la molécula

hipoalergénica mostraron síntomas inflamatorios menos adversos, menor reconocimiento de dicha molécula por la IgE en sus sueros y una alta titulación de IgG específica (Sircar *et al.,* 2016). Por otro lado, variantes con péptidos antigénicos, no alergénicos pero si inmunogénicos, es decir no inductores de respuesta por IgE pero si por IgG, y unidos a proteínas acarreadoras se han probado con éxito, llegando a fases avanzadas de pruebas en humanos, como el caso de la vacuna para la alergia al pasto BM32 la cual se encuentra en fase III de pruebas clínicas, y presuntamente será registrada como vacuna oficialmente en Europa, donde se promueve mayormente el uso de alérgenos recombinantes. La formulación de BM32 ha llevado años de trabajo que han sido documentados en diversas publicaciones, en las cuales se demuestra rotundamente que esta vacuna basada en péptidos hipoalergénicos unidos a una molécula acarreadora con propiedades inmunoestimuladoras, es capaz de modular el sistema inmune (Tulaeva *et al.,* 2020; Akinfenwa *et al.,* 2021). Idealmente la ITA debe promover escasa respuesta por IgE (sea por desensibilización o inducida por mutaciones sitio-dirigidas), altos niveles de IgG en suero, induciendo un perfil bajo de citocinas proinflamatorias, favoreciendo una respuesta inmune Th1.

Puesto que la AIT es hasta ahora la única posibilidad de mejorar la calidad de vida de los pacientes, la obtención de alérgenos por vías recombinantes para un mejor diagnóstico y tratamiento son avances importantes en el campo de la alergia, si adicionalmente este conocimiento se complementa con la predicción de epítopes en dichos alérgenos, pueden llegar a desarrollarse opciones terapéuticas como las antes mencionadas.

1.2.8. El Polen como Potente Aeroalérgeno

Por su pequeño tamaño de entre 15-75 μm el polen es fácilmente acarreado por corrientes de aire, lo que le permite viajar grandes distancias y favorece su entrada por las vías aéreas, logrando sensibilizar a individuos susceptibles (Oh, 2018). Una cantidad pequeña de polen es suficiente para causar sensibilización o una respuesta alérgica. Dicha respuesta no es ocasionada por el polen *per se*, sino por algún alérgeno específico (inocuo para las personas no sensibilizadas), que generalmente son proteínas con un peso molecular de entre 10-60 kDa (Guidos y Almeida, 2005). La mayoría de los pacientes sensibilizados al polen presentan reacción ante algún panalérgeno,

estas proteínas están presentes en menor abundancia en el polen, sin embargo, desempeñan funciones relativamente básicas en los organismos, por lo que están muy conservadas y presentan una alta identidad de secuencia en relación con sus homólogos en otras especies y son causa de reacción cruzada entre diversas fuentes de alérgenos. Los principales panalérgenos del polen son las proteínas PR-10, polcalcinas, proteínas de transferencia de lípidos (nsLPT por sus siglas en ingles) y profilinas (Hauser *et al.,* 2010). Sin embargo, dado que el reconocimiento de proteínas generalmente inocuas se considera un fallo en el sistema inmune, la lista de proteínas alergénicas no se limita a los panalérgenos antes mencionados, y muchas proteínas podrían ser potencialmente alergénicas. Los alérgenos en el polen son capaces de estimular una reacción de hipersensibilidad tipo I, descrita previamente, que es mediada por IgE y causante de manifestaciones alérgicas como la rinitis, conjuntivitis, asma y anafilaxia (Oh, 2018). Además de ser el portador de alérgenos, factores como el medio ambiente, la contaminación y su facilidad de viajar largas distancias por medio del aire, han contribuido a que el polen sea el principal agente causante de sensibilización alérgica (Schiavoni *et al.,* 2017).

La mayoría de los alérgenos polínicos con relevancia clínica son producidos por árboles, y pertenecen principalmente a los órdenes Fagales, Lamiales, Proteales y Pinales, estos con una distribución casi mundial. El polen de las Fagales es el principal causante de polinosis en épocas de primavera e invierno, principalmente en zonas de clima templado. El orden de los Fagales está conformado por siete familias, de entre las cuales Betulaceae y Fagaceae son las principalmente implicadas en desencadenar reacciones alérgicas (Pablos *et al.,* 2016). En nuestro caso particular, nos interesa el estudio del polen de nogal pecanero, *Carya illinoinensis*, árbol perteneciente a la familia Juglandaceae, del orden de los Fagales.

1.2.9. *Carya illinoinensis*

El nogal pecanero [*Carya illinoinensis* (Wangenh.) K. Koch] es un árbol con gran valor comercial a nivel mundial, es una especie altamente valorada por sus frutos, las nueces pecaneras. La distribución geográfica de este árbol en el continente americano va desde el estado de California a Carolina del Sur en USA y desde el estado de Missouri en USA hasta Oaxaca en México.

Actualmente la producción de la nuez pecanera proviene principalmente de plantaciones y no de bosques naturales, simplemente en México en los últimos 30 años se ha triplicado el área de siembra, llegando hasta 112,000 Ha y cuadruplicado su producción, principalmente en los estados del Norte como Chihuahua, Coahuila, Sonora, Durango y Nuevo León (Reyes-Vázquez y Urrea-López, 2016). El polen de nogal pecanero se considera altamente alergénico y de relevancia clínica. En el estado de Texas en USA este polen representa la segunda causa de alergias graves ocasionadas por pólenes y existen reportes que lo catalogan como un alérgeno de importancia clínica en países como Israel (White y Bernstein, 2003; Waisel *et al.,* 1994, Rachmihl *et al.,* 1996). El estado de Sonora ocupaba el tercer lugar a nivel nacional en cuanto a territorio de siembra con nogal pecanero, detrás de Chihuahua y Coahuila, en 2020 el volumen de producción de nuez creció de 7,867 toneladas en 2014 a 24,053 toneladas solamente en tierras sonorenses (Retes *et al.,* 2021). Los estudios aerobiológicos en el territorio mexicano son escasos, sin embargo, dos realizados en la ciudad de Monterrey, Nuevo León, reportan el polen del género *Carya* entre los más abundantes en su atmósfera. Cabe mencionar que Nuevo León ocupa el quinto lugar a nivel nacional respecto al área de siembra de nogal (Rocha-Estrada *et al.,* 2008; González-Díaz *et al.,* 2010; Retes *et al.,* 2021). El incremento del área de cultivo de *Carya illinoinensis* aumenta por consecuencia el número de partículas de este polen en el ambiente, confiriéndole importancia epidemiológica y facilitando la sensibilización de personas susceptibles.

Existe evidencia clínica de que el polen de nogal pecanero es agente causal de reacciones alérgicas, sin embargo, hasta el momento no hay un registro de las proteínas responsables de su alergenicidad (Mani, 2015). En nuestro grupo de trabajo se realizó una primera aproximación en busca de las proteínas alergénicas del polen de nogal pecanero. Para ello, se puso en contacto, mediante 2-DE Western-Blot, el extracto proteínas de polen de *Carya illinoinensis* con el suero de pacientes previamente diagnosticados como alérgicos por el Instituto Nacional de Enfermedades Respiratorias (INER). Se lograron inmunodetectar 18 manchas, de las cuales se realizó una identificación de proteínas por homología contra la base de datos para plantas de NCBI. Oficialmente no hay alérgenos de polen registrados para esta esta especie, sin embargo, varias proteínas de reserva del fruto de la nuez pecanera sí están registradas como alérgenos alimenticios importantes, como Car i 1, Car i 2 y Car i 4.

La exploración del polen de *Carya illinoinensis*, y la purificación de proteínas específicas causantes de alergia, permitirá el desarrollo de un diagnóstico clínico y molecular específico, y sentará las bases para el desarrollo de estrategias inmunoterapéuticas.

1.2.10. Inmunoproteómica en la Exploración de Nuevas Fuentes Alergénicas

La proteómica se encarga del estudio a gran escala de un conjunto determinado de proteínas, centrándose en conocer las interacciones entre ellas y construir redes que ayuden a entender el funcionamiento de los seres vivos. Es decir, la proteómica es el conjunto de técnicas usadas para estudiar al proteoma. Dado que las proteínas son moléculas que presentan funciones estructurales, efectoras, reguladoras, así como de ser antigénicas, la proteómica ha resultado ser uno de los enfoques que ha brindado una gran aportación al conocimiento en el estudio de la enfermedad alérgica. Tanto en la exploración de nuevas fuentes alergénicas que eventualmente llegan al diagnóstico y tratamiento, así como en la búsqueda de biomarcadores que indiquen la efectividad de tratamientos inmunoterapéuticos (Fulton *et al.,* 2019). La exploración y caracterización de nuevas fuentes alergénicas requiere de la conjunción de técnicas clásicas de proteómica, como la electroforesis de proteínas en una y dos dimensiones, el análisis e identificación de péptidos por cromatografía de líquidos acoplada a espectrometría de masas (LC-MS), y de técnicas clásicas de inmunología como Western Blot y ELISA, para derivar en una rama denominada inmunoproteómica (Fulton *et al.,* 2019; Pitarch *et al.,* 2018).

La respuesta humoral o producción de anticuerpos es una de las principales estrategias de defensa del sistema inmune. Esta le permite defenderse ante agentes reconocidos como extraños y potencialmente dañinos, estableciendo una memoria ante dichos agentes a largo plazo, y que, como en el caso de la respuesta alérgica, es capaz de desencadenar una respuesta inmediata y altamente específica hacia el agente causal (Janeway *et al.,* 2009; Fulton *et al.,* 2019). Por lo que explorar potenciales fuentes alergénicas empleando el suero de pacientes sensibilizados, facilita el conocimiento de las moléculas diana de los anticuerpos, permitiendo desarrollar posteriores estrategias diagnósticas y terapéuticas, así como profundizar en el entendimiento de la enfermedad alérgica y sus mecanismos, ya que hasta el momento su etiología permanece desconocida (Pitarch *et al.,* 2018). En este sentido la implementación del enfoque inmunoproteómico para el análisis serológico del proteoma (SERPA) culmina regularmente en la identificación de proteínas alergénicas, permitiendo la exploración de fuentes alergénicas nuevas y de relevancia (Kniemeyer *et al.,* 2016; Pitarch *et al.,* 2018).

Para el análisis serológico del proteoma se emplean técnicas proteómicas como la electroforesis de proteínas en una (SDS-PAGE, condiciones desnaturalizantes y reductoras) y dos dimensiones (2-DE), siendo esta última la más utilizada. En la 2-DE las proteínas se separan por dos propiedades físicas, regularmente el punto isoeléctrico (p*I*) y el peso molecular (MW), lo que permite una separación de las proteínas de una mezcla compleja, como un extracto de polen, a un conjunto de manchas, en donde en cada una de ellas hay una composición mucho menos diversa, ya que las proteínas en determinada mancha deben coincidir en ambas propiedades, p*I* y MW. Además, la 2-DE es fácilmente adaptable al trabajo con otras técnicas como Western Blot y espectrometría de masas, ya que las proteínas pueden permanecer intactas para transferirse a una membrana y realizar una posterior inmunodetección o recuperarse del gel para ser posteriormente digeridas y analizadas por cromatografía de líquidos acoplada a espectrometría de masas en tándem (LC-MS/MS), procesos que regularmente se trabajan de forma paralela. Comúnmente, un gel se utiliza para que las proteínas sean transferidas e inmunodetectadas, y así visualizar qué manchas contienen proteínas reconocidas por la IgE (como anticuerpo primario) en el suero de pacientes alérgicos, las cuales se detectan por anticuerpos anti-IgE (anticuerpo secundario) conjugados a enzimas o fluoróforos que permiten evidenciar las manchas/bandas de proteínas reconocidas por los anticuerpos de los pacientes. Una réplica de la electroforesis empleada para para la inmunodetección se usa para teñir las proteínas totales y cortar del gel las manchas de proteína correspondientes a las manchas inmunodetectadas por los anticuerpos de los pacientes. Estas manchas de proteína se destiñen, se digieren el líquido (usualmente con tripsina) y se analizan mediante LC-MS/MS. El empleo bases de datos de proteínas, motores de búsqueda (softwares) especializados y los datos de LC-MS/MS permiten la identificación de las proteínas reconocidas por los anticuerpos IgE (Fulton *et al.*, 2019). Después del análisis de los resultados, comúnmente se obtienen listas de proteínas, de donde se pueden reconocer proteínas pertenecientes a familias bien documentadas como alergénicas o descubrirse alergenos novedosos en las fuentes estudiadas, esto se esquematiza en la Figura 3.

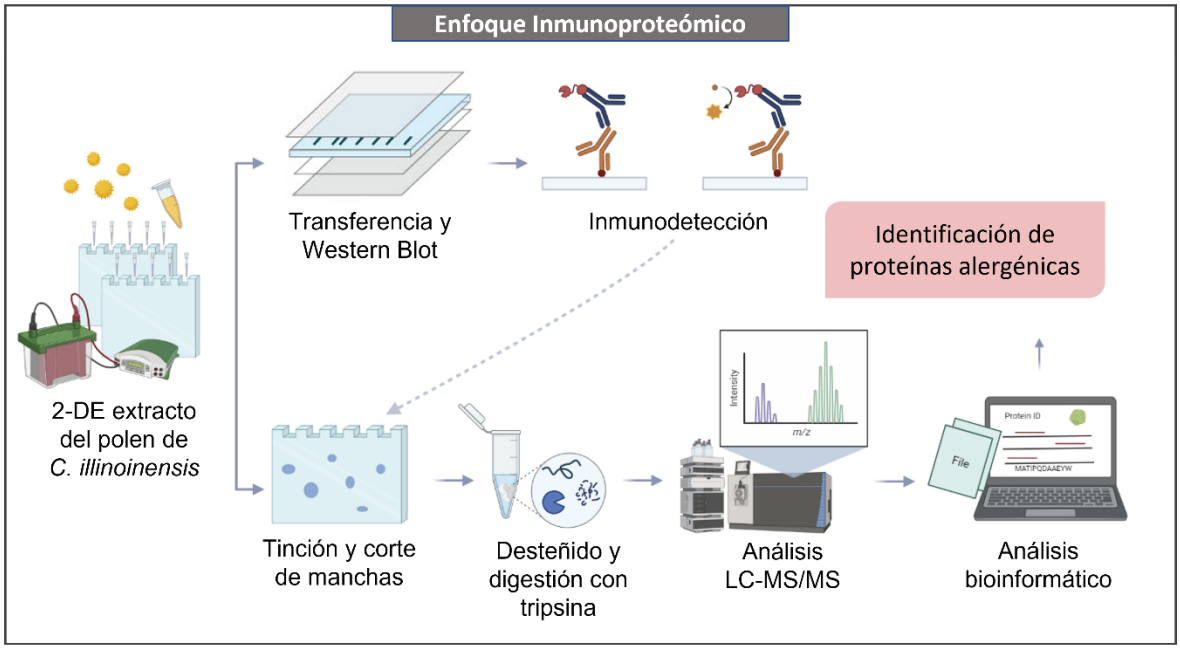

Fig. 3. Representación del análisis serológico del proteoma del polen de *Carya illinoinensis* mediante un enfoque inmunoproteómico clásico.

1.3. Hipótesis

Las proteínas alergénicas puras del polen de nogal pecanero (*Carya illinoinensis*) constituirán una herramienta para el diagnóstico y desarrollo de una estrategia inmunoterapéutica de la alergia a este polen.

1.4. Objetivo General

Obtener dos proteínas del polen de nogal pecanero (*Carya illinoinensis*), confirmar su alergenicidad e identificar sus principales determinantes antigénicos.

1.5. Objetivos Específicos

1. Obtener el marco de lectura abierto de dos proteínas alergénicas y expresarlas mediante técnicas recombinantes.
2. Corroborar el reconocimiento de los alérgenos recombinantes por la IgE en el suero de pacientes alérgicos.
3. Modelar las proteínas alergénicas seleccionadas y predecir sus determinantes antigénicos mediante herramientas bioinformáticas.

1.6. Sección Integradora del Trabajo

La exploración de fuentes nuevas y de relevancia para la enfermedad alérgica es de vital importancia. Primero, en México el panel de alergenos utilizado no está adaptado a la flora predominante de la región, ya que son fuentes alergénicas de especies dominantes en Estados Unidos y Europa. En segundo lugar, dado a que el panel de fuentes bien caracterizadas para diagnóstico es reducido, muchos pacientes no cuentan con un diagnóstico específico para su agente sensibilizador, y esto impide en muchos casos la implementación de una ITA segura y específica para cada paciente. En tercer lugar, es relevante incursionar en la sobreexpresión de alérgenos recombinantes, ya que estos son la puerta de entrada hacia diagnósticos más específicos e ITAs más seguras. Además, permiten seguir evolucionando en la formulación novedosa de ITAs como las proteínas hipoalergénicas, que presentan numerosas ventajas para los pacientes, mencionadas en secciones anteriores.

Por lo anterior, se seleccionó al polen de *Carya illinoinensis* como modelo de estudio y exploración para profundizar en el conocimiento de sus proteínas alergénicas. Dado que es una especie introducida en el territorio mexicano con fines económicos por la producción de su fruto, la nuez pecana, esta especie amplía año con año su extensión territorial y producción. Existe evidencia que reporta a este polen como causa de alergia respiratoria desde hace varios años, sin embargo, este no cuenta con alergenos registrados oficialmente hasta el momento. En nuestro grupo de trabajo se

realizó una primera aproximación para identificar las proteínas alergénicas de esta variedad de polen, mediante inmunodetección y LC-MS/MS, de donde resultó una lista de proteínas alergénicas identificadas por homología, tras un análisis bioinformático. A partir de este antecedente, surge el presente proyecto, que se centra en la obtención de dos proteínas alergénicas del polen de *C. illinoinensis* mediante técnicas recombinantes y la determinación de sus principales epítopes.

Para poder cumplir este objetivo, se seleccionaron dos alergenos de la lista antes mencionada, tras un proceso de revisión se decidió iniciar con las proteínas profilina y enolasa. El primer artículo producto de este proyecto doctoral fue el titulado "The Role of Enolases in Allergic Disease" (sección 2), artículo de revisión con el cual pretendemos resaltar la relevancia de esta proteína como alérgeno, ya que, si bien es un alérgeno bien caracterizado en hongos, también se ha reportado en músculo de animales como peces y gallina, sin embargo en el polen regularmente se había considerado solo como potencial proteína alergénica en diversos estudios inmunoproteómicos, y pocos se habían dirigido a confirmar su alergenicidad. Hasta donde sabemos, ninguna enolasa de polen había sido sobreexpresada, ni se habían predicho sus determinantes antigénicos para ser comparados con los de otras fuentes. En este artículo, se resalta a la enolasa como un alérgeno común entre diversas fuentes, animales, hongos y plantas, así como la alta identidad en las secuencias de algunos de sus representantes alergénicos más representativos.

Para poder cumplir el objetivo uno, y todos los siguientes, una vez seleccionados los alergenos, debíamos tener conocimiento a sus marcos de lectura abiertos. Por lo que a partir de datos del transcriptoma de *Carya illinoinensis* depositados en NCBI, se realizó un ensamble de transcriptoma utilizando el programa Trinity, y tras una anotación funcional mediante Trinotate, se creó una base de datos específica para *C. illinoinensis* con 92, 960 secuencias traducidas o de aminoácidos (ver detalles específicos en la sección 3). Con esta base de datos se analizaron nuevamente los archivos crudos del proceso de MS/MS, mediante el motor de búsqueda Spectrum Mill. Los resultados de este trabajo comprenden el primer artículo original derivado de este proyecto, el cual se titula "Immunoproteomic identification of allergenic proteins in pecan (*Carya illinoinensis*) pollen". En el cual se reportan por primera vez los alérgenos presentes en el polen de nogal pecanero. Este artículo y todas sus especificaciones pueden leerse en la sección 3 del presente documento.

En la sección 4, presentamos el manuscrito de un artículo original titulado "Recombinant expression and epitope prediction of enolase and profilin: allergenic proteins from pecan nut (*Carya illinoinensis*) pollen", el cual comprende los objetivos uno y tres de este proyecto. Los datos

presentados en este artículo consisten en la sobreexpresión y purificación de los alérgenos profilina y enolasa, así como el modelado y predicción de sus determinantes antigénicos. Para esto, las secuencias identificadas correspondientes a los marcos de lectura abiertos para ambas proteínas se optimizaron para su expresión en *E. coli*, y fueron clonadas en el vector pET28pps. Las construcciones obtenidas se utilizaron para transformar células *E. coli* BL21(DE3)pLysS, para posteriormente sobrexpresar y purificar las proteínas recombinantes. La predicción de epítopes lineales y conformacionales se realizó mediante cuatro herramientas bioinformáticas. Por causas ajenas al desempeño y disposición de todos los participantes en este proyecto, relacionadas a la situación mundial durante la pandemia de COVID-19, fue complicado cumplir con el objetivo dos en los tiempos establecidos por el programa educativo. Eventualmente será cubierto con la finalidad de culminar este proyecto y transmitir los resultados derivados del mismo de la mejor manera posible. Sin embargo, el avance hasta el momento se considera adecuado para el proceso de titulación en el posgrado y que los resultados aquí mostrados sentarán las bases para el desarrollo de estrategias inmunoterapéuticas futuras.

2. THE ROLE OF ENOLASES IN ALLERGIC DISEASE

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Clinical Commentary Review

The Role of Enolases in Allergic Disease

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Enolase is one of the most abundant cytosolic enzymes as well as an important glycolytic metalloenzyme highly conserved among organisms from different taxonomical groups. Participation of enolase in processes in which its enzymatic activity is not required has been widely reported. Some of these processes provide special qualities to microorganisms, which favor, in some cases, their pathogenicity. Remarkably, enolase has been reported as an allergen by itself, it is well recognized as allergenic in molds and yeasts, whereas it has also been recognized by the immune system of susceptible individuals acting as a food and inhaled allergen from other diverse sources such as insects, birds, fishes, and plants. To date, 14 enolases have been officially recognized by the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Subcommittee. The use of discovery proteomics has also uncovered novel allergenic enolases, particularly from pollen sources. Here, we review the relevance of enolases as sensitizers and as nonsensitizing cross-reactive allergens in allergic disease. © **2021 American Academy of Allergy, Asthma & Immunology (J Allergy Clin Immunol Pract 2021;■:■-■)**

Key words: *Allergen; Cross-reactivity; Immunoproteomics*

INTRODUCTION

The glycolytic enzyme enolase is an important mediator of the allergic response in susceptible individuals; however, it has been an underestimated allergen. The first enolase associated with allergic disease was identified in *Saccharomyces cerevisiae*, by binding to IgE.[1] However, since then a wide range of allergenic enolase sources have been reported, highlighting its relevance as an elicitor of the allergic response. The present study was based on a bibliographic search conducted in PubMed and Scopus under the topics "enolase- and/or enolase allergy." The search was limited to articles published in English in the last 2 decades.

ENOLASE

Phosphopyruvate hydratase, also known as 2-phospho-D-glycerate hydrolase or commonly called enolase (International Union of Biochemistry and Molecular Biology, Enzyme Commission number 4.2.1.11), is an enzyme that catalyzes the penultimate step in glycolysis, interconverting 2-phosphoglycerate and phosphoenolpyruvate, according to their concentration in the medium.[2] The active site of enolase is found at the carboxylic end of the TIM-type barrel, where the magnesium metal ions act as cofactors and bind to the substrate: 2-phosphoglycerate.[3] The role of enolase is of vital importance in the energy metabolism of many organisms; however, this enzyme has also been reported as a moonlighting protein. Moonlighting proteins are characterized by performing other functions in the cell, in addition to its canonical or main function.[4] Moreover, enolase plays an important role in adhesion, for the subsequent infection of several microorganisms. To date, 3 enolase isotypes have been reported in mammals: α-enolase or ENO-1, which is expressed in most tissues, β-enolase or ENO-3, normally expressed in muscle tissue, and γ-enolase or ENO-2, mainly present in neural tissues. ENO-1 is the protein most differentially expressed among human tissues under different pathologies.[5] There is evidence that enolase from various organisms such as animals, fungi and yeasts, plants, and pollen can cause an IgE-inducing response. Cross-reactivity is another feature of enolases, mainly between fungi and plants, and between diverse food such as fish and chicken.[6,7] There are 14 enolases in the list of the systematic allergen nomenclature approved by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Subcommittee (www.allergen.org), of which 6 correspond to fungi, 2 to pollens, 1 to plants, and 5 to foods of animal origin (Table I).

ENOLASE IN ALLERGIC DISEASE

Enolase protein sequences are highly conserved among taxonomically distant groups or organisms[29] causing respiratory, food, and contact allergies. This degree of evolutionary conservation of their protein sequences supports the high immunologic cross-reactivity of enolases. To date however, the role of enolase in allergic disease has not been fully investigated. The earliest evidence of enolase as an allergen dates to the early 1990s, from fungi and yeast. The combination of modern biochemistry with molecular biology led to the identification of a small number of enolase isoforms from the yeast *Saccharomyces cerevisiae* and *Candida albicans*, and from the fungus *Aspergillus fumigatus*,

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Abbreviations used
IUIS- International Union of Immunological Societies
WHO- World Health Organization

which were purified, sequenced, and expressed as recombinant proteins.[8] However, in the last decades, mass spectrometry, specifically tandem mass spectrometry, has allowed the identification of novel allergens in complex biological samples. In fact, allergen identification in general has benefited from combining proteomics with IgE immunoblotting, also known as "immunoproteomics." Briefly, the allergen source proteome is first subjected to a 2-dimensional electrophoresis; the first dimension is an isoelectric focusing to separate proteins on the basis of their isoelectric point, followed by separation according to their molecular masses (second dimension), which allows proteins to resolve as small spots that are then transferred to a membrane by western blotting. The membrane is exposed to the IgE present in the sera of patients with allergy, which are specific to environmental allergens; highlighting reactive IgE spots are then subsequently characterized by mass spectrometry. In the case of enolase, this approach has allowed its identification in various allergenic sources that are discussed below.

FUNGAL ENOLASE

Enolases are one of the most prominent and conserved allergenic families in fungi. From the 6 fungal enolases officially registered as allergens by IUIS Allergen Nomenclature Subcommittee (see www.allergen.org), 5 correspond to molds and 1 to yeast. Enolases are considered minor allergens; however, some authors consider them as pan-allergens among fungi.[30] It is estimated that around 20% to 30% of people allergic to fungi are sensitized to enolases.[30] Cross-reaction between allergenic enolases from fungal origin has been reported among Asp f 22, Pen c 22, Alt a 6, Rho m 1, Cur l 2, and Cla h 6.[8,9,11,12] Figure 1 shows the identity matrix of multiple amino acid sequence alignment of enolases officially recognized as allergens (WHO/IUIS Allergen Nomenclature Subcommittee), which shows that fungal enolases share a high percentage of identity (72.02%-94.29%) among them, and also high identity percentage is found among plant (86.77%-89.77%) and animal allergenic enolases (81.11%-91.20%), which explains the cross-reaction among these allergens. Some yeast enolases have been reported as potential allergens; however, they were not well characterized. Some of these enolases derive from *Saccharomyces cerevisiae* and *Candida albicans*. Interestingly, they showed cross-reaction between them[13] and with other allergens.[7]

Interestingly, the prevalence of fungal allergy is an important cause of asthma and allergic rhinitis. In 1997, a multicenter European study by the Aerobiology Subcommittee of the European Academy of Allergy and Clinical Immunology reported that 9.5% of patients with suspected respiratory allergy were sensitized to Alternaria and/or Cladosporium.[31] More recently, a study conducted in Spain (Castile and Leon) showed that Alt a1 from Alternaria is the most prevalent allergen in the diagnosis of patients with allergy to fungi in the area (94.4%), followed by enolase Alt a 6 (Alternaria), revealing the importance of these allergens in allergic respiratory disease.[32] Simon-Nobbe et al[30] performed epitope mapping of *Cladosporium herbarum* enolase and showed that Ala124-Leu130, Asp136-Pro143, and Arg163 are the most probable regions for IgE binding in *C herbarum* enolase. The cross-inhibition experiments demonstrated that these IgE-binding epitopes are highly conserved between different fungal species. From the clinical point of view, it is important to point out that the source of different fungal allergens is not identical. For example, Alternaria and Cladosporium species are important outdoor allergens, sensitizing patients to develop asthma and rhinitis, whereas Penicillium and Aspergillus can cause allergic diseases as indoor allergens. It is well established that *A fumigatus* can also colonize the bronchial tract of asthmatic patients, leading to severe persistent asthma, and sometimes causing allergic bronchopulmonary aspergillosis. Malassezia are commensals of skin, and they are associated with atopic dermatitis, particularly on the head and neck, but not with respiratory allergies. The role of fungi as sensitizing agents in allergic disease has been reviewed.[33]

FOOD ENOLASES

Food is an important source of allergens. It is estimated that approximately 10% of the world population suffers from food allergy and that it has been increasing in the last 3 decades.[34] Fish is a common inducer of IgE-mediated food allergy, and it causes severe hypersensitivity reactions. However, many cases of fish allergy are not diagnosed. The prevalence of fish allergy may vary depending on the consumption patterns of the populations studied, ranging from 0% to 8%.[35] Fish sensitization usually starts during childhood, and it may remain into adult life.[36] Clinical symptoms can be mild (oral allergy syndrome, erythema) to moderate (urticaria, vomiting) or severe (angioedema, asthma, anaphylaxis). Among the allergens reported in fish, parvalbumins are recognized as main allergens, whereas β-enolases and aldolases are considered minor allergens. However, only around 0.5% of the fish species have been analyzed at the molecular level, with the species most consumed in Europe being the most studied, such as carp, salmon, trout, tuna, and cod.[37] In 2013, Kuehn et al[24] undertook the task of searching for additional allergens to parvalbumin in protein extracts from widely consumed and highly commercially valuable fish such as cod, tuna, and salmon. They were able to immunodetect the presence of β-enolase in the 3 fish sources using the serum of fish-allergic patients. After purification, they showed that these enolases stimulate the degranulation of basophils and demonstrated that they can inhibit each other, with cod enolase showing the greatest inhibitory capacity. β-enolases of cod (*Gadus morhua*), tuna (*Thunnus albacares*), and salmon (*Salmo salar*) are currently registered in the IUIS as the allergens Gad m 2, Thu a 2, and Sal s 2, respectively. In the same year, Tomm et al[22] reported the identification of allergenic proteins in Nile perch fish (*Lates niloticus*) and *G morhua*, where 66% of the patients allergic to *L niloticus* and 100% of those allergic to *G morhua* were sensitized to β-enolase, indicating that enolases are relevant allergens and a possible cause of cross-reaction.

Oreochromis mossambicus or Mozambique tilapia is a fish of high consumption worldwide due to its pleasant texture and flavor. Interestingly, enolase was identified among the 4 proteins recognized by the serum of patients with a history of allergy to tilapia.[26] Enolase was also found as 1 of the allergenic proteins in Wuchang bream (*Megalobrama amblycephala*).[25] Similarly, the presence of enolase was reported among the allergic proteins of largemouth bass (*Micropterus salmoides*).[26] *O mossambicus,*

TABLE I. Enolases identified as IgE-recognized proteins among unrelated allergenic sources

Source	Allergen	Identification strategy	Reference
Molds and yeasts			
Alternaria alternata	Alt a 6	Serum IgE binding to purified protein on immunoblot	8
Aspergillus fumigatus	Asp f 22	Serum IgE binding to both natural and recombinant protein on immunoblot	9
Cladosporium herbarum	Cla h 6	Positive immunoblot of *C herbarum* extract to Cla h 6. IgE binding to recombinant protein on immunoblot	10
Curvularia lunata	Cur l 2	Recombinant protein was recognized by individual *Curvularia*-positive patient sera (n = 20) in immunoblot and ELISA	11
Penicillium citrinum	Pen c 22	Sera IgE binding to the natural (47-kDa component in protein extract) and recombinant protein on immunoblot	9
Rhodotorula mucilaginosa	Rho m 1	Immunoblotting against recombinant and native (47-kDa) protein	12
Candida albicans	Nonregistered*	Circulating IgE and IgG antibodies were detected in 54 sera with positive IgE RAST result to *C albicans*. IgE antibody against *C albicans* enolase was detected in 20 sera (37%) and IgG antibody in 27 sera (50%)	13
Saccharomyces cerevisiae	Nonregistered*	Skin and RAST testing with purified enolase from *Saccharomyces cerevisiae* and comparisons with the whole yeast extract showed that the enzyme is a major allergenic component of the extract	14
Plants			
Hevea brasiliensis (rubber tree)	Hev b 9	Latex-allergic patients revealed IgE binding to recombinant Hev b 9 on immunoblot	7
Pollen			
Ambrosia artemisiifolia (ragweed)	Amb a 12	Sera from inhalation allergy and sensitized to ragweed proteins had IgE binding to enolase in western blot and dot blot	15
Cynodon dactylon (Bermuda grass)	Cyn d 22	IgE-reactive proteins immunodetected in 2-DE WB and identified by LC-MS/MS	16
Ailanthus altissima (Tree of heaven)	Nonregistered*	IgE-binding proteins immunodetected in 2-DE WB were identified using MALDI-TOF/TOF mass spectrometry and database searching	17
Cocos nucifera (coconut palm)	Nonregistered*	IgE-reactive proteins immunodetected in 2-DE WB and identified by *de novo* sequencing and homology-driven proteomics	18
Ligustrum lucidum (Privet)	Nonregistered*	IgE-reactive proteins immunodetected in 2-DE WB and identified by LC-MS/MS and database searching	19
Quercus rubra (Red oak)	Nonregistered*	IgE-reactive proteins immunodetected in 2-DE WB and identified by LC-MS/MS and database searching	20
Populus deltoides (Poplar)	Nonregistered*	Allergenicity prediction based on protein sequence and structure	21
Fishes			
Gadus morhua (Atlantic cod)	Gad m 2	Sera individuals with fish allergy had IgE that reacted with natural purified protein in ELISA	22
Pangasianodon hypophthalmus (Striped catfish)	Pan h 2	IgE-binding protein immunodetected by SDS-PAGE reducing blots identified by LC-MS/MS and database searching	23
Salmo salar (Atlantic salmon)	Sal s 2	Detection by IgE-immunoblots of salmon extract. Serum IgE reacted with purified natural protein in ELISA. Identified by N-terminal sequencing and by peptide mass fingerprint	24
Thunnus albacares (Yellowfin tuna)	Thu a 2	Detection by IgE-immunoblots of tuna extract. Serum IgE reacted with purified natural protein in ELISA. Identified by N-terminal sequencing and by peptide mass fingerprint	24
Lates niloticus (Nile perch)	Nonregistered*	IgE-reactive proteins were detected by 2D immunoblotting and identified using LC-MS/MS and database searching	22
Megalobrama amblycephala (Wuchang bream)	Nonregistered*	IgE-reactive proteins were immunodetected by 1D (individual serum samples) and 2D SDS-PAGE (pooled serum) along with IgE immunoblot and identified using MALDI-TOF/TOF mass spectrometry and database searching	25
Oreochromis mossambicus (Mozambique tilapia)	Nonregistered*	IgE-reactive proteins were detected by 2D immunoblotting and identified using MALDI-TOF/TOF mass spectrometry and database searching	26
Other			
Gallus domesticus (chicken)	Gal d 9	Case history for chicken and IgE tests and positive SPT result in 26 of 27 subjects, and 4 of 5 by SPT with natural protein. Protein identified by N-terminal sequencing and by peptide mass fingerprint	6
Blattella germanica (cockroach)	Nonregistered*	IgE-reactive proteins immunodetected in 2-DE WB and identified by LC-MS/MS and database searching. Dot Blot against natural purified protein	27

(continued)

TABLE I. *(Continued)*

Source	Allergen	Identification strategy	Reference
Vespa affinis venom (wasp venom)	Nonregistered*	IgE-reactive proteins immunodetected in 2-DE WB and identified by LC-MS/MS and database searching	[28]

2-DE, Two-dimensional electrophoresis; *MALDI*, matrix-assisted laser desorption/ionization; *MS/MS*, tandem mass spectrometry; *RAST*, radio allergo sorbent test; *SPT*, skin prick test; *TOF*, time of flight.
*Nonregistered as allergens by WHO/IUIS Allergen Nomenclature Subcommittee (http://www.allergen.org).

M amblycephala, and *M salmoides* are freshwater fish that are highly consumed in countries such as China, where fish allergy represents a public health problem.[25] Although cross-reaction between various fish species is a common phenomenon, there are also reports of cross-reaction between fish and chicken. The cause of this cross-reaction was thought to be mainly due to the protein parvalbumin, the main allergen in fish that has also been found in chicken.[38] By comparing 2 groups of patients with allergy—one group sensitized to chicken and the other to fish—, it was found that the 7 chicken-allergic patients developed a cross-reaction with the parvalbumin present in a fish extract. In contrast, 6 cross-reacted with fish enolase, and 2 samples, specific to cod enolase, showed 100% inhibition after IgE incubation with purified chicken enolase. These findings give hints of the existence of a fish-chicken syndrome, caused by shared allergens among these species, and chicken enolase was registered at the IUIS as Gal d 9. As we can see in Figure 1, the fish β-enolase (Sal s 2) and chicken β-enolase (Gal d 9) share more than 80% amino acid identity. Other fish (sablefish, zebrafish, among others) have also been reported as possible sources of allergenic β-enolases, and further studies are required to formally register them as allergens.[39]

The presence of enolase in protein sources highly consumed worldwide such as fish and chicken hints at their importance as a group of allergens. In a recent study, Ruethers et al[40] analyzed the variability of allergenic proteins in commercial extracts used for skin prick test for fish allergy. They report that 100% of the extracts tested contained enolase, and that 68.75% of patients with confirmed fish allergy tested positive for this allergen. However, they highlight that some of the extracts used lack some allergens or contain different concentrations of them, which can cause variability in the diagnosis in some individuals. Allergen characteristics such as solubility, stability, and molecular size appear to influence allergenicity, particularly in food allergy. For example, Maillard modification in dried fish was found to exhibit a higher IgE-binding capacity due to the creation of new epitopes being associated with primary sensitization to the glycosylated-tropomyosin in scallop.[41] Purified enolases are large protein dimers sensitive to thermal treatment: Cod and tuna enolase are only detectable in samples heated less than 1 minute at 90°C, whereas salmon enolase can still be detected in samples heated for 5 minutes. Kuehn et al[6] proposed that enolase sensitivity to thermal treatment could either destroy or create new epitopes; however, they concluded that further studies with a larger number of samples should be undertaken. Pan h 2 is a novel enolase allergen identified in raw catfish.[23]

PLANT ENOLASE

Enolases have also been identified as allergens in plant and some pollen sources. Enolase Hev b 9 was reported as allergenic in rubber tree (*Hevea brasiliensis*/latex). Figure 1 shows that this contact allergen shares more than 88% sequence identity with allergenic enolases from the pollens of Common ragweed (*Ambrosia artemisiifolia*, Amb a 12) and Bermuda grass (*Cynodon dactylon*, Cyn d 22).[15,16] However, despite the high level of identity, there are no reports of cross-reactivity between Hev b 9 and these 2 pollen allergens, but cross-reaction with fungal enolases has been reported. A study conducted in 19 patients' serum samples with IgE reactivity to recombinant Hev b 9 showed that 100% and 94.7% exhibited cross-reactivity with the recombinant fungus allergens, rAlt a 6 and rCla h 6, respectively.[7] Rubber tree enolase sequence identities with *C herbarum* (Cla h 6) and *A alternata* (Alt a 6) enolases are 61.38% and 60.74%, respectively (Figure 1). Troncoso-Ponce et al[42] cloned and characterized 3 cDNAs encoding different ENO isoforms from developing sunflower seeds. However, their role in allergic disease remains to be shown.

POLLEN ENOLASES

Pollen is the most common source of inhaled allergens, causing respiratory allergic reactions in 15% to 30% of the world population.[43] Most of the pollens that cause allergic disease come from grasses, flowering trees, and weeds. Hence, pollen can be found almost anywhere. In addition, the introduction of some tree species as ornaments and/or shade plants in cities and private gardens has increased the prevalence of pollen sensitization in recent decades.[44] Similarly, nonnative tree species have been transported worldwide to create or enhance services; however, they can also make people prone to develop pollen allergy.[45] Indeed, once released in the atmosphere they penetrate the human airways, causing disease. Some pollen allergens are classified as pan-allergens including PR-10 proteins, profilins, and polcalcins, which are highly conserved among plants.[46,47] Enolases are not considered pan-allergens in plants but are widely considered fungal pan-allergens.[30] Currently, only 2 pollen enolases are registered in the IUIS database (http://www.allergen.org/; as accessed in July 2020): Cyn d 22 from Bermuda grass and Amb a 12 from ragweed; in this last source, enolase shows signs of being a relevant allergen: 66% of subjects (27 from 41) sensitized to ragweed proteins had IgE binding to enolase in western blot and dot blot. Interestingly, immunoproteomic studies have reported enolases among the proteins recognized by IgE antibodies present in the serum of patients sensitized to several pollens. Bordas-Le Floch et al[15] identified an enolase from ragweed pollen, whereas Mousavi et al[17] immunodetected this allergen in the pollen of the Tree of heaven (*Ailanthus altissima*). Allergy sensitization to ragweed and *A altissima* pollens are major cause of respiratory disease in North America and Europe. Enolase has also been identified in coconut palm (*Cocos nucifera*) pollen: a study conducted in the city of Kolkata, India, reported that this pollen affected 47% of the allergic population suffering from asthma and rhinitis.[48] Identification of pollen allergens from *C nucifera* discovered 12 proteins, including enolase.[18] More recently, we have identified enolase from privet

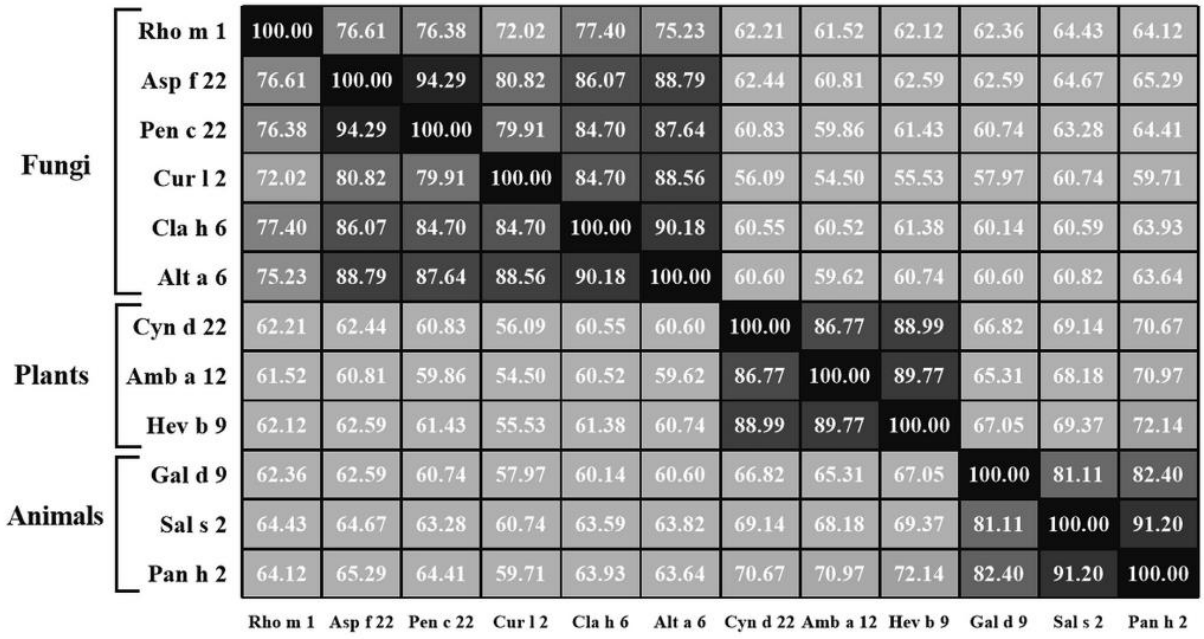

FIGURE 1. Percent Identity Matrix of amino acid sequences of enolases reported as allergens on the Official Allergen List: WHO/IUIS Allergen Nomenclature Subcommittee (http://www.allergen.org).

(*Ligustrum lucidum*)[19] and red oak (*Quercus rubra*)[20] using pools of sera from sensitized patients. These plants are widely distributed worldwide,[49] and they cause allergy sensitization. Wang et al[50] identified several forms of enolase from Chinese white poplar (*Populus tomentosa*) pollen. The genus Populus contains approximately 30 plant species widely distributed in the northern hemisphere. However, it is not known whether they all produce this allergen. Using combinatorial peptide libraries, Shahali et al[51] also demonstrated that common cypress (*Cupressus sempervirens*) also releases an IgE-binding protein such as enolase. Future studies will define the role of pollen enolases in allergic respiratory disease.

OTHER SOURCES OF ALLERGENIC ENOLASES

Enolase has also been reported as an allergen in cockroaches (*Blattella germanica*) and wasp (*Vespa affinis*) venom. Cockroaches have been reported as a major source of indoor allergens in various regions of the world, including America, Europe, South Africa, and Eastern Asia.[52] A study in Taiwan reported that 50.7% of patients with allergic rhinitis and 57.5% of patients with asthma were sensitive to cockroach extract.[53] A subsequent study was devoted to the search for allergens in the cockroach, among which enolase was reported as recognized by the sera of 25% of patients sensitized to cockroach.[27] However, using an immunoproteomic approach, enolase was identified in wasp venom, where it was recognized by the sera from 66.67% of the patients allergic to the sting of this insect, demonstrating an allergenic role.[28]

ENOLASE CROSS-REACTIVITY

Cross-reactivity is attributed to the great structural and immunologic similarity that can exist among homologous allergenic proteins.[54] So far, cases of cross-reaction have been reported mainly between enolases of various fungi and yeasts.[8,30] However, in high consumption foods such as cod, salmon, and tuna fish, this phenomenon also occurs, with cod enolase exhibiting the greatest inhibition capacity compared with the other 2 sources.[24] Similarly, there is a cross-reaction between salmon (Sal s 2) and chicken (Gal d 9) allergens, which, although rare and uncommon, stands out for the apparent phylogenetic distance of its sources; however, Gal d 9 is capable of inhibiting IgE binding to some fish enolases up to 100%.[6] However, this is not the only case in which cross-reaction between species has been reported. This also occurs between the fungal enolases Alt a 6 and Cla h 6 with the plant enolase Hev b 9.[7] To date, the cross-reactivity between pollen enolases has not been investigated.

ENOLASE IN CLINICAL PRACTICE

A wide range of enolases may cause allergic disease. However, there are no reagents available for diagnosis in either the office or the outpatient clinic. Currently, diagnostic tests based on natural allergen extracts are the most common methods for allergy diagnosis. However, they are composed of mixtures of allergic and nonallergic material, making it difficult to identify the disease-eliciting allergen. Fish enolases are the ones that have been investigated most extensively using fish-allergen assays. For example, Sørensen et al[55] performed double-blind, placebo-controlled food-challenge trial with different fish species, evaluating the correlation between clinical reactivity and IgE reactivity to fish-allergen molecules in fish-allergic patients and demonstrated sensitization to enolases in patients with objective symptoms. The component-resolved diagnostics is an approach that was developed to improve the current laboratory diagnoses

of allergic diseases; in this method, allergen molecules are produced either by recombinant expression of allergen-encoding cDNAs or by purification from natural sources. Interestingly, evidence for the role of enolase in allergic respiratory disease derives from a component-resolved diagnostics study showing increased specific IgE fungal enolase in patients sensitized to Alternaria.[56] Cross-sensitization between aeroallergens and food allergens is also common. In some cases, the presence of a respiratory allergy to an allergen with a shared epitope to food may lead to clinically relevant cross-reactivity. Pollen sensitization may lead to "pollen-food syndromes," such as birch-apple or celery-mugwort-spice syndrome. However, pollen-derived enolase has not been associated with any food syndrome. To date, immunoproteomics has been the most successful method for the detection of enolases in allergic disease. However, immunoproteomics is relatively expensive and labor intensive, impeding its use in the clinic. Morever, there is limited access to these kind of tests in developing countries where allergic diseases are prevalent. Proteomic-based miniaturized devices allowing accurate allergen diagnostics are being developed, and they will be available in the near future. Indeed, there is steady progress in the use of new nanoscopic-scale biosensors in allergy.[57] In addition to the use of immunoproteomics to improve allergen diagnosis, purification of native enolases and production of their recombinant versions, particularly in sources of enolases not recognized as relevant for allergic disease (such as pollen, cockroaches, and venoms), is necessary to evaluate their equivalent IgE reactivity through inhibition assays. Cross-reactive and well-recognized allergenic enolases and total extracts of these sources can also be used to evaluate the comparative reactivity of native and recombinant enolases, as well as to evaluate the ability to release allergy mediators by these enolases.

CONCLUDING REMARKS

There is increasing evidence that enolase may play an important role in allergic disease. Indeed, enolases can sensitize individuals orally or via the respiratory tract, and there is evidence that allergen sensitization can also take place via skin. In relation to a possible role of enolases from airborne sources (pollen, molds), it is relevant to stress that most enolases are dimeric proteins and thus larger than expected in relation to sensitization as well as in relation to symptom elicitation via the airways. Enolases derived from fish, fungi, and latex have been extensively investigated, and they have been associated with allergic disease. For example, the studies conducted by Kuehn et al[24] identified enolase as a major allergen in patients suffering fish allergy, with a prevalence of 70% to 80.6% in 3 different clusters of fish-allergic patients. Moreover, there is frequent cosensitization between cod, salmon, and tuna homologues: 70% for the cod enolase, 15% for the salmon enolase, and 20% for the mackerel. Similarly, fungal enolases have been fully characterized, and their role in allergic disease has been well established, particularly in asthma and allergic rhinitis. Interestingly, the latex enolase Hev b 9 exhibits an identity of 60% to various fungal enolases. It has been proposed that enolases are evolutionary proteins that resulted from gene duplications 200 million years to 300 million years ago,[58] which may explain the wide distribution of enolases. The identification of novel serum IgE binding to enolases in many patients suffering from asthma and allergic rhinitis suggests that they may also be involved in the allergic disease. However, their specific contribution to allergic disease is not fully established. The general availability of a few prototypic enolase (eg, fungal or fish enolase) as recombinant proteins and as ImmunoCAP-type reagents would enable testing sera with IgE antibodies reactive to some other enolase for cross-reactivity with these prototypic enolases and help identify the most likely culprits for this patient. The development of specific diagnostic tests for proper identification of the individual or cross-reactive enolases will allow delivering personalized care and prevention.

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REFERENCES

1. Kortekangas-Savolainen O, Lammintausta K, Kalimo K. Skin prick test reactions to brewer's yeast (*Saccharomyces cerevisiae*) in adult atopic dermatitis patients. Allergy 1993;48:147-50.
2. Harris PT, Raghunathan K, Spurbeck RR, Arvidson CG, Arvidson DN. Expression, purification, crystallization and preliminary X-ray studies of *Lactobacillus jensenii* enolase. Acta Crystallogr Sect F Struct Biol Cryst Commun 2010;66:938-40.
3. Gerlt JA, Babbitt PC, Rayment I. Divergent evolution in the enolase superfamily: the interplay of mechanism and specificity. Arch Biochem Biophys 2005;433:59-70.
4. Jeffery CJ. Moonlighting proteins: old proteins learning new tricks. TRENDS Genet 2003;19:415-7.
5. Petrak J, Ivanek R, Toman O, Cmejla R, Cmejlova R, Vyoral D, et al. Deja vu in proteomics. A hit parade of repeatedly identified differentially expressed proteins. Proteomics 2008;8:1744-9.
6. Kuehn A, Codreanu-Morel F, Lehners-Weber C, Doyen V, Gomez-André S-A, Bienvenu F, et al. Cross-reactivity to fish and chicken meat—a new clinical syndrome. Allergy 2016;71:1772-81.
7. Wagner S, Breiteneder H, Simon-Nobbe B, Susani M, Krebitz M, Niggemann B, et al. Hev b 9, an enolase and a new cross-reactive allergen from Hevea latex and molds: purification, characterization, cloning and expression. Eur J Biochem 2000;267:7006-14.
8. Simon-Nobbe B, Probst G, Kajava AV, Oberkofler H, Susani M, Crameri R, et al. IgE-binding epitopes of enolases, a class of highly conserved fungal allergens. J Allergy Clin Immunol 2000;106:887-95.
9. Lai H-Y, Tam MF, Tang R-B, Chou H, Chang C-Y, Tsai J-J, et al. cDNA cloning and immunological characterization of a newly identified enolase allergen from *Penicillium citrinum* and *Aspergillus fumigatus*. Int Arch Allergy Immunol 2002;127:181-90.
10. Achatz G, Oberkofler H, Lechenauer E, Simon B, Unger A, Kandler D, et al. Molecular cloning of major and minor allergens of *Alternaria alternata* and *Cladosporium herbarum*. Mol Immunol 1995;32:213-27.
11. Sharma V, Gupta R, Jhingran A, Singh B, Sridhara S, Gaur S, et al. Cloning, recombinant expression and activity studies of a major allergen "enolase" from the fungus *Curvularia lunata*. J Clin Immunol 2006;26:360.
12. Chang CY, Chou H, Tam MF, Tang RB, Lai HY, Shen H-D. Characterization of enolase allergen from *Rhodotorula mucilaginosa*. J Biomed Sci 2002;9:645-55.
13. Ito K, Ishiguro A, Kanbe T, Tanaka K, Torii S. Detection of IgE antibody against *Candida albicans* enolase and its crossreactivity to *Saccharomyces cerevisiae* enolase. Clin Exp Allergy 1995;25:522-8.
14. Baldo BA, Baker RS. Inhalant allergies to fungi: reactions to bakers' yeast (*Saccharomyces cerevisiae*) and identification of bakers' yeast enolase as an important allergen. Int Arch Allergy Appl Immunol 1988;86:201-8.
15. Bordas-Le Floch V, Le Mignon M, Bouley J, Groeme R, Jain K, Baron-Bodo V, et al. Identification of novel short ragweed pollen allergens using combined transcriptomic and immunoproteomic approaches. PLoS One 2015;10:e0136258.
16. Kao SH, Su SN, Huang SW, Tsai JJ, Chow LP. Sub-proteome analysis of novel IgE-binding proteins from Bermuda grass pollen. Proteomics 2005;5:3805-13.
17. Mousavi F, Majd A, Shahali Y, Ghahremaninejad F, Shoormasti RS, Pourpak Z. Immunoproteomics of tree of heaven (*Ailanthus atltissima*) pollen allergens. J Proteomics 2017;154:94-101.

18. Saha B, Sircar G, Pandey N, Gupta Bhattacharya S. Mining novel allergens from coconut pollen employing manual de novo sequencing and homology-driven proteomics. J Proteome Res 2015;14:4823-33.
19. Mani BM, Huerta-Ocampo JA, Garcia-Sanchez JR, Barrera-Pacheco A, De La Rosa APB, Teran LM. Identification of *Ligustrum lucidum* pollen allergens using a proteomics approach. Biochem Biophys Res Commun 2015;468:788-92.
20. Huerta-Ocampo JÁ, Valenzuela-Corral A, Robles-Burgueño MDR, Guzmán-Partida AM, Hernández-Oñate MÁ, Vázquez-Moreno L, et al. Proteomic identification of allergenic proteins in red oak (*Quercus rubra*) pollen. World Allergy Organ J 2020;13:100111.
21. Zhang J, Wu L-S, Fan W, Zhang X-L, Jia H-X, Li Y, et al. Proteomic analysis and candidate allergenic proteins in *Populus deltoides* CL. "2KEN8" mature pollen. Front Plant Sci 2015;6:548.
22. Tomm JM, Van Do T, Jende C, Simon JC, Treudler R, von Bergen M, et al. Identification of new potential allergens from Nile perch (*Lates niloticus*) and cod (*Gadus morhua*). J Investig Allergol Clin Immunol 2013;23:159-67.
23. Ruethers T, Taki AC, Karnaneedi S, Nie S, Kalic T, Dai D, et al. Expanding the allergen repertoire of salmon and catfish [published online ahead of print August 28, 2020]. *Allergy*, https://doi.org/10.1111/all.14574.
24. Kuehn A, Hilger C, Lehners-Weber C, Codreanu-Morel F, Morisset M, Metz-Favre C, et al. Identification of enolases and aldolases as important fish allergens in cod, salmon and tuna: component resolved diagnosis using parvalbumin and the new allergens. Clin Exp Allergy 2013;43:811-22.
25. Liu R, Krishnan HB, Xue W, Liu C. Characterization of allergens isolated from the freshwater fish blunt snout bream (*Megalobrama amblycephala*). J Agr Food Chem 2011;59:458-63.
26. Liu R, Yang E, Liu C, Xue W. Tilapia (*Oreochromis mossambicus*) allergens characterized by ELISA, SDS-PAGE, 2D gels, Western blotting and MALDI-TOF mass spectrometry. Int J Food Sci Nutr 2012;63:259-66.
27. Chuang JG, Su SN, Chiang BL, Lee HJ, Chow LP. Proteome mining for novel IgE-binding proteins from the German cockroach (*Blattella germanica*) and allergen profiling of patients. Proteomics 2010;10:3854-67.
28. Sookrung N, Wong-din-dam S, Tungtrongchitr A, Reamtong O, Indrawattana N, Sakolvaree Y, et al. Proteome and allergenome of Asian wasp, *Vespa affinis*, venom and IgE reactivity of the venom components. J Proteome Res 2014;13:1336-44.
29. Eremina M, Rozhon W, Yang S, Poppenberger B. ENO 2 activity is required for the development and reproductive success of plants, and is feedback-repressed by A t MBP-1. Plant J 2015;81:895-906.
30. Simon-Nobbe B, Denk U, Pöll V, Rid R, Breitenbach M. The spectrum of fungal allergy. Int Arch Allergy Immunol 2008;145:58-86.
31. D'amato G, Chatzigeorgiou G, Corsico R, Gioulekas D, Jäger L, Jäger S, et al. Evaluation of the prevalence of skin prick test positivity to Alternaria and Cladosporium in patients with suspected respiratory allergy. Allergy 1997;52:711-6.
32. Armentia A, Martín-Armentia S, Moral A, Montejo D, Martin-Armentia B, Sastre R, et al. Molecular study of hypersensitivity to spores in adults and children from Castile & Leon. Allergol Immunopathol 2019;47:350-6.
33. Fukutomi Y, Taniguchi M. Sensitization to fungal allergens: resolved and unresolved issues. Allergol Int 2015;64:321-31.
34. Sicherer SH, Sampson HA. Food allergy: a review and update on epidemiology, pathogenesis, diagnosis, prevention, and management. J Allergy Clin Immunol 2018;141:41-58.
35. Kourani E, Corazza F, Michel O, Doyen V. What we know about fish allergy by the end of the decade? J Investig Allergol Clin Immunol 2019;29:414-21.
36. Sharp MF, Lopata AL. Fish allergy: in review. Clin Rev Allergy Immunol 2014;46:258-71.
37. Kuehn A, Radauer C, Lopata A, Kleine-Tebbe J, Swoboda I. Extract-based and molecular diagnostics in fish allergy. In: Molecular Allergy Diagnostics. Switzerland: Springer International Publishing; 2017:381-97.
38. González-de-Olano D, Bartolomé B, Maroto A, Vivanco F, Pastor-Vargas C. Asthma after chicken consumption due to cross-reactivity between fish and chicken parvalbumin. J Investig Allergol Clin Immunol 2012;22:227-8.
39. Fernandes TJ, Costa J, Oliveira MBP, Mafra I. An overview on fish and shellfish allergens and current methods of detection. Food Agr Immunol 2015;26:848-69.
40. Ruethers T, Taki AC, Nugraha R, Cao TT, Koeberl M, Kamath SS, et al. Variability of allergens in commercial fish extracts for skin prick testing. Allergy 2019;74:1352-63.
41. Nakamura A, Watanabe K, Ojima T, Ahn DH, Saeki H. Effect of Maillard reaction on allergenicity of scallop tropomyosin. J Agr Food Chem 2005;53:7559-64.
42. Troncoso-Ponce MA, Rivoal J, Dorion S, Sánchez R, Venegas-Calerón M, Moreno-Pérez AJ, et al. Molecular and biochemical characterization of the sunflower (*Helianthus annuus* L.) cytosolic and plastidial enolases in relation to seed development. Plant Sci 2018;272:117-30.
43. Singh AB, Mathur C. An aerobiological perspective in allergy and asthma. Asia Pacific Allergy 2012;2:210-22.
44. Castro-Díez P, Vaz AS, Silva JS, van Loo M, Alonso A, Aponte C, et al. Global effects of non-native tree species on multiple ecosystem services. Biol Rev 2019;94:1477-501.
45. Charpin D, Pichot C, Belmonte J, Sutra J-P, Zidkova J, Chanez P, et al. Cypress pollinosis: from tree to clinic. Clin Rev Allergy Immunol 2019;56:174-95.
46. Huertas A, Carreno A, Mérida C, Pajarón-Fernández M, Ramírez-Hernández M, Carnés J. Profilin sensitisation in a Mediterranean population. Allergol Immunopathol 2014;42:387-94.
47. Pablos I, Wildner S, Asam C, Wallner M, Gadermaier G. Pollen allergens for molecular diagnosis. Curr Allergy Asthma Rep 2016;16:31.
48. Karmakar PR, Chatterjee BP. *Cocos nucifera* pollen inducing allergy: sensitivity test and immunological study. Ind J Exp Biol 1995;33:489-96.
49. Robledo-Retana T, Mani BM, Teran LM. Ligustrum pollen: new insights into allergic disease. World Allergy Organ J 2020;13:100104.
50. Wang L, Zhang X, Zhang J, Fan W, Lu M, Hu J. Proteomic analysis and identification of possible allergenic proteins in mature pollen of *Populus tomentosa*. Int J Mol Sci 2018;19:250.
51. Shahali Y, Sutra J-P, Fasoli E, D'Amato A, Righetti PG, Futamura N, et al. Allergomic study of cypress pollen via combinatorial peptide ligand libraries. J Proteomics 2012;77:101-10.
52. Pomes A, Chapman MD, Vailes LD, Blundell TL, Dhanaraj V. Cockroach allergen Bla g 2: structure, function, and implications for allergic sensitization. Am J Respir Crit Care Med 2002;165:391-7.
53. Arruda LK, Chapman MD. The role of cockroach allergens in asthma. Curr Opin Pulmon Med 2001;7:14-9.
54. Pancholi V. Multifunctional α-enolase: its role in diseases. Cell Mol Life Sci CMLS 2001;58:902-20.
55. Sørensen M, Kuehn A, Mills ENC, Costello CA, Ollert M, Småbrekke L, et al. Cross-reactivity in fish allergy: a double-blind, placebo-controlled food-challenge trial. J Allergy Clin Immunol 2017;140:1170-2.
56. Postigo I, Gutiérrez-Rodríguez A, Fernández J, Guisantes JA, Suñén E, Martínez J. Diagnostic value of Alt a 1, fungal enolase and manganese-dependent superoxide dismutase in the component-resolved diagnosis of allergy to pleosporaceae. Clin Exp Allergy 2011;41:443-51.
57. Neethirajan S, Weng X, Tah A, Cordero J, Ragavan K. Nano-biosensor platforms for detecting food allergens—New trends. Sensing Bio-Sensing Res 2018;18:13-30.
58. Piast M, Kustrzeba-Wójcicka I, Matusiewicz M, Banaś T. Molecular evolution of enolase. Acta Biochim Polonica 2005;52:507-13.

3. IMMUNOPROTEOMIC IDENTIFICATION OF ALLERGENIC PROTEINS IN PECAN (*Carya illinoinensis*) POLLEN

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Immunoproteomic identification of allergenic proteins in pecan (*Carya illinoinensis*) pollen

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ABSTRACT

Pecan (*C. illinoinensis*) pollen is an important cause of allergic respiratory disease. Pecan is distributed worldwide as shade, ornamental or cultivation tree. To date three well known pecan food allergens have been reported, however, pollen allergens have not been identified. Here, we describe the first identification of IgE recognized pecan pollen proteins, for which proteins were analyzed by 2-DE and immunoblotting using a pool of 8 sera from pecan sensitive patients as primary antibody. IgE recognized protein spots were analyzed by LC-MS/MS and identified using a database of translated protein sequences obtained by the assembly of *C. illinoinensis* public transcriptomic information. This study has identified 17 IgE binding proteins from pecan pollen including proteins widely recognized as allergens and panallergens. These findings will contribute to develop specific diagnosis and treatment of pecan pollen allergy.

Significance: Pecan is a tree highly valued for its fruits that have a great commercial value. To date three pecan seed storage proteins have been officially recognized by the WHO/IUIS allergen nomenclature subcommittee as food allergens (*Car i* 1, *Car i* 2 and *Car i* 4). Pecan tree pollen is highly allergenic and a clinically relevant cause of allergies in North America (USA and Mexico) and regions where the tree is extensively cultivated (Israel, South Africa, Australia, Egypt, Peru, Argentina, and Brazil). Here, we describe the first identification of IgE recognized pollen proteins using an immunoproteomics approach and a protein database created by the assembly of pecan public transcriptomic information. The findings described here will allow the development of new diagnostic and therapeutic modalities for pecan pollen allergy.

1. Introduction

The World Health Organization (WHO) estimates that at least 25% of the world's population suffers from respiratory allergic diseases including asthma and allergic rhinitis, asthma can sometimes be fatal [1]. It is estimated that pollens are responsible for up to 40% of allergic respiratory diseases and WHO recognizes that millions of people do not have a specific diagnosis [2]. To date however, the allergens from many pollens remains to be identified.

Pecan (*C. illinoinensis*), a tree highly valued for its fruits that have a great commercial value worldwide, is also recognized as a highly allergenic plant [3]. It is endemic to North America and is the most economically important member of the *Carya* Genus, widely distributed in the USA and Mexico, whether as a shade, ornamental or cultivation tree [4,5]. Other regions where pecans are grown commercially but to a lesser extent are Israel, South Africa, Australia, Egypt, Peru, Argentina, and Brazil [5] To date, three pecan seed storage proteins have been officially recognized by the WHO/IUIS allergen nomenclature subcommittee as food allergens (Car i 1, Car i 2, Car i 4) [6–8]. However, in the clinical context, allergenic pecan pollen proteins have not been

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identified. Nevertheless, pecan tree pollen is highly allergenic, resulting in allergic rhinitis, conjunctivitis, and asthma [3]. For example high concentrations of this pollen was reported in an aerobiological study conducted in Caxias do Sul in southern Brazil [9].

In a study in St. Louis, Missouri, USA, skin tests showed that box elder, willow and pecan elicited the highest number of allergic reactions [10]. Similarly, a high prevalence of hypersensitivity to pecan was reported in 371 allergic patients in an area surrounding New York [11]. Interestingly, pecan pollen also sensitized children: a study conducted in 209 military children in Texas demonstrated that 27.8% were skin test-positive to this pollen [12].

In the present study, we have investigating IgE binding proteins in pecan pollen using an immunoproteomics discovery approach. The identification of novel IgE binding proteins may lead to the development of novel diagnostic and therapeutic interventions.

2. Material and methods

2.1. Selection of patients and healthy volunteers

Eight polysensitized allergic patients to pecan pollen and four healthy control subjects were recruited from the outpatient allergy clinic at the Instituto Nacional de Enfermedades Respiratorias (INER), Mexico. The protocol was approved by the human ethics and research committees at the INER. The study was conducted in accordance with the ethical principles of the 1975 Declaration of Helsinki (as revised in 1983), and it was consistent with Good Clinical Practice Guidelines. Volunteers were enrolled in the study between January 2019 and February 2020. None of these patients were taking specific allergen immunotherapy, corticosteroids, or antihistamines at the time of the study. Skin prick tests were performed with a small drop of commercial allergenic extracts (ALK-Abelló, Round Rock, TX). Aeroallergens tested included *Dermatophagoides pteronyssinus,* dog hair, feather mix, cat fur, grass mix, tree mix, weed mix, *C. illinoinensis* and mold mix. A skin reaction characterized by ≥3 mm wheal and erythema within 20 min indicated that the subject was positive. Histamine induced reaction was used as positive control, and saline solution was used as negative control. In addition to be allergic to *C. illinoinensis*, 5 patients were positive to *Dermatophagoides pteronyssinus* and 3 to cat fur. Serum collected from blood samples was stored at −80 °C until use.

2.2. Pollen collection and pollen soluble protein extraction

Fresh inflorescences were collected from pecan trees (April 2018) in an orchard on the coast of Hermosillo, Sonora, Mexico. Once dried, anthers were selected from the inflorescence by passing through a 2 mm mesh. Pollen grains were separated using two USA standard test sieves (250 and 63 μM, VWR International, Radnor, Pennsylvania USA.). Pollen soluble proteins were extracted using a modified phenolic extraction [13]. Briefly, 200 mg of pollen was washed with cold acetone by centrifugation at 10,000 rpm for 10 min at 4 °C. The acetone wash was repeated prior to air drying the pellet at room temperature. The pellet was then dissolved in 3 mL of SDS buffer (30% sucrose, 2% SDS, 50 mM Tris-HCl, 5% 2-Mercaptoethanol, and 1 mM PMSF) and incubated at 4 °C with shaking for 10 min. One volume of phenol (equilibrated with 10 mM Tris-HCl) was added to the above solution, mixed and centrifuged. The phenolic phase was recovered in a new tube and mixed with 0.1 M ammonium acetate and incubated overnight at −20 °C. After 20 min centrifugation at 13,000 rpm and 4 °C, the pellet was washed with acetone and dried at room temperature. The resulting pellet was suspended in 400 μL of rehydration buffer (8 M urea, 2% CHAPS, 20 mM DTT, 0.002% Bromophenol blue, 0.5% IPG buffer pH 3–10) (Bio-Rad Laboratories, Hercules, California, USA). Protein concentration was determined with the RC/DC Protein Assay (Bio-Rad) using BSA as standard.

2.3. Two-dimensional electrophoresis

Pollen proteins (900 μg) were loaded onto 7 cm linear pH 3–10 IPG strips (Bio-Rad). Passive rehydration was carried out at room temperature during 16 h. IEF was conducted at 50 mA per IPG strip and 20 °C in an Ettan IPGphor system 3 (GE Healthcare, Piscataway, New Jersey, USA). The IEF conditions were: (1) 500 V gradient until 0.01 kVh, (2) 4000 linear gradient until 5.6 kVh, and (4) Constant 5000 V until 2.5 kVh. After IEF, the strips were equilibrated by shaking for 15 min in SDS equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl buffer pH 8.8, 0.002% bromophenol blue) the first time containing 1% dithiothreitol and the second time equilibration was performed in the same solution now containing 2.5% iodoacetamide instead of dithiothreitol. Equilibrated strips were transferred to a vertical SDS-PAGE gel, sealed with agarose and the second dimension was performed using the Mini-PROTEAN® Tetra cell (Bio-Rad). Preparative (for Coomassie stain) and analytical gels (for immunodetection with sera from controls and atopic patients) were run by triplicate. Preparative gels were stained with Coomassie blue and once distained, scanned with the Typhoon FLA 9500 (GE Healthcare). Image analysis was performed with PDQuest 2-D Analysis Software v8.0 (Bio-Rad) and experimental molecular mass of each protein spot was estimated by comparison with molecular weight standards (Invitrogen, Carlsbad, California USA). Experimental *pI* was determined by migration of protein spots on the IPG linear gradient strips. Pollen proteins separated in analytical gels were electrotransferred onto PVDF sheets (Immun-Blot, Bio-Rad) by using a Mini Trans-blot Cell (Bio-Rad). After electrotransference, membranes were rinsed in PBS pH 7.5 and non-specific binding was blocked by incubating for 2 h in sodium azide-free BSA solution (5%).

2.4. Immunodetection analysis

PVDF membranes were incubated overnight with pooled sera (for controls and atopic patients) diluted to 1:50 in PBS. After washing (3 times for 5 min with PBS-Tween 20, plus one wash with PBS), the membranes were incubated for 1 h with a mouse monoclonal (B3102E8) horseradish peroxidase-conjugated anti-human IgE-Fc antibody (ABCAM Laboratories, Cambridge, Massachusetts, USA) diluted to 1 μg/mL in PBS and membranes were washed again. The enzyme-substrate reaction was performed by incubating the membrane in peroxidase substrate for enhanced chemiluminescence (Clarity Western ECL Substrate, Bio-Rad) and imaged by Chemidoc MP Imaging System (Bio-Rad). Exposition parameters were automatically optimized by Image Lab software (Bio-Rad).

2.5. In-gel protein digestion and LC-MS/MS analysis

Protein spots were excised from corresponding position on Coomassie-stained gels, reduced, alkylated, and trypsin digested, as previously reported [13]. Tryptic peptide separation was performed using the 1290 Infinity LC System (Agilent Technologies, Santa Clara, California, U.S.A.) equipped with an analytical column ZORBAX 300SB-C8 (5 μm × 2.1 mm × 150 mm, Agilent Technologies), and MS/MS analysis was performed by a 6530 Accurate-Mass Quadrupole Time-of-Flight LC/MS system (Agilent Technologies) as previously reported [14].

2.6. Assembly of publicly available C. illinoinensis transcriptomic datasets

Free access transcriptomic datasets (PRJNA342905, PRJNA413769, PRJNA431042, PRJNA504494, and PRJNA533506) from various pecan tree tissues, ≈ 150 RNAseq (fastq) files equivalent to ≈ 133 GB of information, were assembled using Trinity [15] program obtaining about 170,000 unique transcripts. Functional annotation was performed by Trinotate [16]. Trinotate uses Blastn, Blastp, and HMMER tools, besides the Pfam and UniProt databases to obtain a specific database for

C. illinoinensis containing 92,960 protein sequences.

2.7. Protein identification

MS/MS raw data files (.d files) were processed in the Spectrum Mill MS Proteomics Workbench server (Agilent Technologies) and Data Extractor tool was used to obtain .mzXML files that were used for protein identification using the Spectrum Mill MS/MS Search Tool. Searches were conducted against the translated protein database obtained from the assembly of publicly available *C. illinoinensis* transcriptomic datasets (92,960 sequences, October 2019). Trypsin was used as the specific protease, allowing one missed cleavage. Mass error tolerance for precursor and fragment ions was set to 20 ppm and 0.1 Da, respectively. Oxidation of methionine was specified as variable modification whereas carbamidomethylation of cysteine was set as fixed modification. Individual peptide ion scores $\geq$ 9 and score peak intensities $\geq$60 thresholds were considered for suitable peptide matches, whereas a protein score $\geq$ 25 and at least two peptides were necessary for confident protein identification.

2.8. Serum IgE measurements

Total IgE was measured in serum samples using a Human IgE Elisa kit (OriGene, Rockville MD, USA) following the manufacturer's protocol. The lower limit of detection was 5 IU.

3. Results

3.1. Patients

Clinical features of the pecan allergic patients and the non-atopic subjects that participated in the study are shown in Table 1. The two groups were almost the same average age (39 ± 4 years vs 30 ± 3.2 years, respectively). Total IgE levels in allergic patients were higher when compared with healthy controls (median 285, range: 90–1330 IU/mL vs median 63 range: 20–86, respectively).

3.2. 2-DE profile of pecan pollen proteins and immunodeteccion of IgE-binding pollen proteins

The 2-DE protein profile of pecan pollen was resolved into around 350 protein spots (Fig. 1A). Interestingly, two-dimensional immunodetection using pool sera from atopic patients revealed 18 IgE binding protein spots (Fig. 1B) that were mainly distributed in 4 regions. The first region was in the molecular range of 51–54 kDa and isoelectric points of 5.2–5.7 (spots 1, 2 and 3). The second region, from 38 to 41 kDa and p*I* of 6.45–6.85 (spots 4, 5 and 6), the third region, 30–37 kDa and p*I* of 5.1–5.9 (spots 7 to 12) and the fourth region contained spots of 28–31 kDa and p*I* of 6.2–6.5 (spots 13, 14 and 15). Finally, three unique spots were observed (16, 17 and 18) with molecular masses below 25 kDa and p*I* in the range of 4.0–7.0 (Fig. 1). In contrast, the sera from healthy volunteers did not exhibit strong IgE binding protein spots (Supplementary Fig. 1).

Table 1
Clinical characteristics of allergic and non-atopic subjects.

	Allergic patients	Control subjects
No. subjects	8	4
Age (years)	39 ± 4	30 ± 3.2
Females	5	3
Males	3	1
FEV_1%	84% (80–95)	106% (99–115)*
Total IgE (U/dL)	285 (90–1330)	63 (20–86)*
Atopy	Yes	No
Asthma	1	0
Asthma + Allergic rhinitis	4	0
Allergic rhinitis	3	0

FEV1 = Forced expiratory volume at the end of the 1st second.
* $p < 0.05$. Statistical analysis by Mann Whitney *U* test.

3.3. Protein identification by tandem mass spectrometry and homology database search

Homology database search of MS/MS datasets from protein spots analyzed against the pecan protein database using the Spectrum Mill search engine allowed the identification of 17 unique proteins among 17 of the 18 immunoreactive spots (Table 2 and Supplementary Table S1). Spot 16 was not identified.

4. Discussion

Pecan pollen allergy is clinically relevant where this tree is cultivated extensively. Allergic rhinitis caused by pecan pollen is frequently treated with steroids and antihistamines, however they do not cure the disease. Allergy immunotherapy is the only therapeutic strategy that induces immunological tolerance providing prolonged symptom alleviation in allergic rhinitis patients, it also prevents the development of asthma in children. So far, the allergens from pecan pollen remain unknown. In this study, we investigated IgE binding proteins from this pollen using 2-DE immunoblotting and MS.

We have identified 17 IgE binding proteins which are known to be involved in several cellular processes, including carbohydrate metabolism, electron transport chain, lipid oxidation, anaerobic energy metabolism, among others. Xylose isomerase (spot 3) is an oxidoreductase that catalyzes the reversible transformation between aldose D-xylose and ketosa D-xylulose through the transposition of a hydrogen atom between two atoms in neighboring positions, although it can also carry out the same transformation between other bio-sugars of the same types, particularly D-glucose and D-fructose. Organisms that do not have this enzyme, such as yeasts, require two different reactions to carry out the same conversion, catalyzed by xylose reductase and xylitol dehydrogenase, respectively. This protein has already been reported as an IgE binding protein in date palm pollen [17]. We also identified two different enolases: enolase 1 (spots 2 and 3) and a chloroplastic enolase (spot 3). Enolase is a glycolytic enzyme and an important mediator of the allergic response in susceptible individuals. The first enolase associated with allergic disease was identified in *Saccharomyces cerevisiae*, by binding to IgE [18]. Since then, several allergenic enolase sources have been reported, emphasizing its significance as an elicitor of the allergic response [19]. Enolases were also recognized by IgE from patients allergic to tree of heaven, privet, and red oak pollen [20–22]. Interestingly, a protein homologous to enolase 1 is the major latex allergen Hev b 9 [23].

Fructokinase (spots 7 and 8), another protein related to carbohydrate metabolism, may participate in the regulation of pollen germination by providing fructose-6-phosphate for glycolysis, or by conversion to UDP-glucose to sustain the biogenesis of cell wall raw material for pollen tube growth [24]. Coconut pollen fructokinase has also been reported as IgE recognized by allergic patients [25]. Other proteins related to carbohydrate metabolism identified in this study [glyceraldehyde 3 phosphate dehydrogenase (spot 6), and cytosolic fructose-bisphosphate aldolase (spots 5)] have been previously described as allergenic in the pollen of ribgrass (*Plantago lanceolata*) and sunflower [26,27].

Triosephosphate isomerase is a homodimeric enzyme that functions in a non-linear step of glycolysis, converting dihydroxyacetone phosphate into glyceraldehyde 3-phosphate. Spots 14 and 15 contained this protein that has been described as a IgE-binding latex protein [28]. Malonyl-CoA-acyl carrier protein transacylase (spot 9) is involved in the fatty acid biosynthesis pathway. It catalyzes the transfer of a malonyl moiety from malonyl-CoA to the free thiol group of the phosphopantetheine arm of the mitochondrial ACP protein. To our knowledge, this is the first report of transacylase as an IgE-reactive pollen protein. The

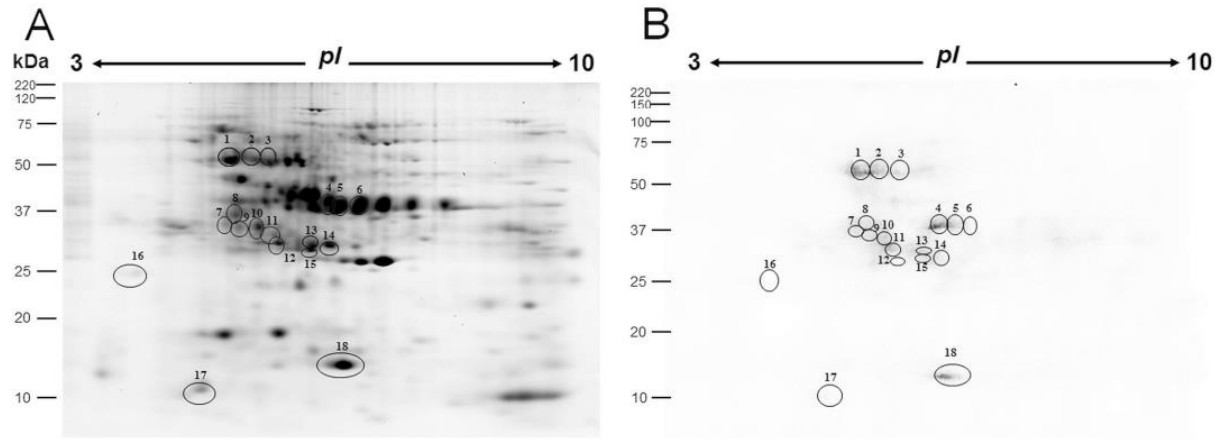

Fig. 1. 2-DE and Immunoblotting using pollen proteins from Pecan nut. A) 2-DE of total soluble proteins stained with Coomassie blue. B) Immunoreactive protein spots detected with pooled sera from allergic patients using 2-DE immunoblotting.

cytosolic L-ascorbate peroxidase (spots 13 and 15) is a major hydrogen peroxide-scavenging enzyme in plant cells, and it removes hydrogen peroxide via the ascorbate-glutathione cycle. Mus a 6, the most recent reported banana allergen, is an ascorbate peroxidase of 27 kDa according to the WHO/IUIS Allergen Nomenclature Sub-Committee (www.allergen.org), whereas a pollen ascorbate peroxidase was previously reported as a coconut allergen [25].

Two oxidoreductases: malate dehydrogenase (spots 4 and 5) and chloroplastic malate dehydrogenase (spots 10 and 11) were also IgE reactive. Malate dehydrogenase homologs were reported as allergens in the pollen of *Senecio jacobaea* [29], whereas an IgE binding protein band (36 kDa), detected in watermelon extract and recognized among the three major allergens involved in watermelon allergy, was identified as malate dehydrogenase [30]. Proteoforms of the alpha (spots 2 and 3) and beta subunits (spots 1 and 2) of ATP synthase were also identified from pecan pollen as IgE binding proteins in this study. They have recently been reported as allergens in the pollen of common lantana (*Lantana camara*), privet (*Ligustrum lucidum*), date palm (*Phoenix sylvestris*), and red oak (*Quercus rubra*) [17,20,21,31]. Carbonic anhydrases (spots 12, 13 and 14) are ubiquitous zinc enzymes encoded by five distinct, evolutionarily unrelated gene families, present in prokaryotes and eukaryotes. They catalyze the interconversion between carbon dioxide and the bicarbonate ion, and are therefore implicated in key physiological processes [32]. Carbonic anhydrases serve also as signaling molecules to control anther cell differentiation in plants [33]. This enzyme has been identified as a protein recognized by IgE in date palm pollen [17].

Nucleoside diphosphate kinase B (NDPK-B, spot 18) has been reported as an allergen in largemouth bass (*Micropterus salmoides*) [34]. In the present study, NDPK-B (spot 18) was detected as an IgE binding protein. To our knowledge, NDPK-B has not been previously reported as a pollen protein recognized by IgE.

Isoflavone reductase (spot 5) and profilin (spot 17) were also IgE reactive in pecan pollen. Two food [Dau c 5 from Carrot (*Daucus carota*), and Pyr c 5 from Pear (*Pyrus communis*)] and two airway [Cor a 6 from Hazelnut (*Corylus avellana*) and Ole e 12 from Olive (*Olea europaea*)] allergens recognized by the WHO/IUIS Allergen Nomenclature Sub-Committee (www.allergen.org) are isoflavone reductases. Additionally, isoflavone reductase was identified as a pollen allergen in date palm, coconut, and olive pollen [17,25,35]. Profilin has been found in several pollen species including olive, birch, hazelnut, timothy grass, and maize [36,37]. According to the literature, profilins 1, 2 and 3 are commonly expressed in all plant tissues, but types 4 and 5 are expressed mainly in floral tissues and reproductive organs such as pollen [38]. Profilins are panallergens with clinical relevance since they cause cross-reaction between various pollens and fresh foods of plant origin. Thus, profilin cross-reactivity can occur between pollens and fruit profilins, causing the oral allergy syndrome in susceptible people [38–41].

Pecan tree, *C. illinoinensis* (Jugalndaceae) belong to the order Fagales that comprise 8 families: Betulaceae, Casuarinaceae, Fagaceae, Juglandaceae, Myricaceae, Nothofagaceae, Rhoipteleaceae, and Ticodendraceae. Bet v 2, a birch (Betulaceae) profilin, may result in cross-reactivity with other pollen (mugwort, ragweed, timothy grass) or food (anise, apple, carrot, celery, coriander, cumin, fennel, hazelnut, mango, paprika, peach, peanut, potato and tomato) profilins [42,43]. Similarly, cross-reactivity between species of *Carya* and *Juglans* genera is expected, and to a lesser extent with another Fagales because amino acid sequence. For example, the identity between pecan and walnut profilin is 98.50%, whereas that between birch (Bet v2) and pecan profilin is 90.23%.

5. Conclusions

We have identified 17 IgE binding proteins from pecan pollen including proteins widely recognized as allergens and panallergens. To date this is the first study demonstrating that pecan pollen produces IgE binding proteins. These findings may lead to the development of new diagnostic and therapeutic interventions for pecan allergy. For example, the component-resolved diagnostics (CRD) use recombinant protein immunoassays rather than extracts to establish the specific proteins the patients are allergic to. Similarly, the development of recombinant hypoallergenic immunotherapy has been proposed as a replacement for whole allergen extracts in allergen immunotherapy.

Author contributions

Conceptualization, Luis M. Teran and José Ángel Huerta-Ocampo; Data curation, Cesaré Ovando-Vázquez; Formal analysis, Martha Beatriz Morales-Amparano, Alejandra Valenzuela-Corral, Abraham Escobedo-Moratilla, Guillermo Pastor-Palacios and José Ángel Huerta-Ocampo; Investigation, Martha Beatriz Morales-Amparano, Alejandra Valenzuela-Corral and José Ángel Huerta-Ocampo; Software, Cesaré Ovando-Vázquez; Supervision, Gabriela Ra-mos-Clamont Montfort, Luz Vázquez-Moreno, Luis M. Teran and José Ángel Huerta-Ocampo; Writing original draft, Martha Beatriz Morales-Amparano, Alejandra Valenzuela-Corral, Luis M. Teran and José Ángel Huerta-Ocampo; All authors reviewed and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Table 2
Allergenic proteins identified in pecan pollen.

Spot [a]	Protein	Accession number [b]	Exper. Mr./pI [c]	Theor. Mr./pI [d]	PM / score [e]
1	ATP synthase subunit beta, mitochondrial	DN362_c0_g1_i4.p1	53.6/ 5.20	57.6/ 5.28	14/ 224
2	ATP synthase subunit beta, mitochondrial	DN362_c0_g1_i1.p1	52.2/ 5.52	59.9/ 5.75	8/121
2	ATPase subunit alpha, mitochondrial	DN17467_c0_g1_i4. p1	52.2/ 5.52	55.6/ 5.84	8/98
2	Enolase 1	DN65134_c0_g1_i1. p1	52.2/ 5.52	48.3/ 5.60	7/68
3	ATPase subunit alpha, mitochondrial	DN17467_c0_g1_i4. p1	51.4/ 5.69	55.6 /5.84	5/76
3	Enolase 1	DN65134_c0_g1_i1. p1	51.4/ 5.69	48.3/ 5.60	6/74
3	Enolase 1, Chloroplastic	DN1916_c0_g1_i1. p1	51.4/ 5.69	52.5 /6.23	5/61
3	Xylose isomerase	DN3168_c0_g1_i1. p1	51.4/ 5.69	53.5/ 5.57	2/28
4	Malate dehydrogenase	DN1199_c0_g1_i1. p1	41.0/ 6.45	35.8/ 6.32	5/76
5	Malate dehydrogenase	DN1199_c0_g1_i1. p1	39.0/ 6.61	35.8/ 6.32	8/116
5	Isoflavone reductase	DN799_c0_g1_i1.p1	39.0/ 6.61	34.0/ 6.20	7/89
5	Fructose-bisphosphate aldolase, cytosolic	DN38_c0_g1_i2.p1	39.0/ 6.61	38.8 /8.41	5/62
6	Glyceraldehyde-3-phosphate dehydrogenase	DN46_c0_g1_i2.p1	38.5/ 6.85	37.1/ 7.82	7/86
7	Fructokinase-4	DN330_c6_g3_i1.p1	34.4/ 5.15	35.7/ 5.27	6/72
8	Fructokinase-4	DN330_c6_g3_i1.p1	36.5/ 5.27	35.7/ 5.27	6/83
9	Malonyl-CoA-ACP transacylase,	DN5246_c0_g1_i3. p1	31.0/ 5.32	44.3/ 8.52	2/26
10	Malate dehydrogenase, chloroplastic	DN689_c0_g1_i1.p1	33.9/ 5.57	43.8/ 7.80	3/40
11	Malate dehydrogenase, chloroplastic	DN689_c0_g1_i1.p1	32.8/ 5.75	43.8/ 7.80	5/67
12	Carbonic anhydrase 2-like	DN45949_c0_g1_i1. p1	30.2/ 5.83	20.9/ 6.35	3/40
12	Carbonic anhydrase 2-like	DN3629_c0_g1_i5. p1	30.2/ 5.83	29.0/ 7.85	3/36
13	L-ascorbate peroxidase, cytosolic	DN1667_c0_g1_i1. p1	30.7/ 6.25	27.5/ 5.84	5/76
13	Carbonic anhydrase 2-like	DN3629_c0_g1_i5. p1	30.7/ 6.25	29.0/ 7.85	4/64
13	Carbonic anhydrase 2-like	DN45949_c0_g1_i1. p1	30.7/ 6.25	20.9/ 6.35	2/40
14	Triosephosphate isomerase cytosolic	DN576_c0_g1_i1.p1	29.7/ 6.45	27.5 /6.24	6/96
14	Carbonic anhydrase 2-like	DN3629_c0_g1_i5. p1	29.7/ 6.45	29.0/ 7.85	4/89
15	L-ascorbate peroxidase cytosolic	DN1667_c0_g1_i1. p1	28.6/ 6.23	27.5/ 5.84	7/92
15	Triosephosphate isomerase cytosolic	DN576_c0_g1_i1.p1	28.6/ 6.23	27.5/ 6.24	5/63
16	Not identified	–	24.5/ 4.01	–	–
17	Profilin	DN10658_c0_g1_i2. p1	11.3/ 4.82	14.5/ 5.00	2/31
18	Nucleoside diphosphate kinase B	DN181_c0_g2_i1.p2	15.0/ 6.65	16.45/ 6.31	2/33

[a] Spot numbers as indicated in Fig. 1.
[b] Accession numbers according to *Carya illinoinensis* protein database obtained by the assembly of public transcriptomic information.
[c] Experimental molecular mass (kDa) and *pI*.
[d] Theoretical mass (kDa) and *pI*.
[e] Peptides matched/ Spectrum Mill Protein Score (Scores ≥25 and at least two peptides were necessary for confident protein identification).

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2021.104348.

References

[1] J.-W. Oh, Pollen Allergy in a Changing World, Springer, Singapore, 2018.
[2] W. Eder, M.J. Ege, E. von Mutius, The asthma epidemic, N. Engl. J. Med. 355 (21) (2006) 2226–2235.
[3] M. Rachmiel, H. Verleger, Y. Waisel, N. Keynan, S. Kivity, Y. Katz, The importance of the pecan tree pollen in allergic manifestations, Clin Exp Allergy 26 (3) (1996) 323–329.
[4] M.G. Cervantes-Vázquez, I. Orona Castillo, C. Vázquez Vázquez, M. Fortis Hernández, J.D.J. Espinoza Arellano, Análisis comparativo de huertos de nuez pecanera (Carya illinoensis Koch) en la Comarca Lagunera, Rev. Mexic. Cien. Agrícol. 9 (1) (2018) 25–35.
[5] T.E. Thompson, P.J. Conner, Pecan, in: M.L. Badenes, D.H. Byrne (Eds.), Fruit Breeding, Springer US, Boston, MA, 2012, pp. 771–801.
[6] G.M. Sharma, A. Irsigler, P. Dhanarajan, R. Ayuso, L. Bardina, H.A. Sampson, K. H. Roux, S.K. Sathe, Cloning and characterization of 2S albumin, Car i 1, a major allergen in pecan, J. Agric. Food Chem. 59 (8) (2011) 4130–4139.
[7] G.M. Sharma, A. Irsigler, P. Dhanarajan, R. Ayuso, L. Bardina, H.A. Sampson, K. H. Roux, S.K. Sathe, Cloning and characterization of an 11S Legumin, Car i 4, a major allergen in pecan, J. Agric. Food Chem. 59 (17) (2011) 9542–9552.
[8] Y. Zhang, B. Lee, W.-X. Du, S.-C. Lyu, K.C. Nadeau, L.J. Grauke, Y. Zhang, S. Wang, Y. Fan, J. Yi, T.H. McHugh, Identification and Characterization of a New Pecan [*Carya illinoinensis* (Wangenh.) K. Koch] Allergen, Car i 2, J. Agric. Food Chem. 64 (20) (2016) 4146–4151.
[9] S.M. Vergamini, R.M. Valencia-Barrera, B.C. Antoni Zoppas, C. Pérez Morales, D. Fernández-González, Pollen from tree and shrub taxa in the atmosphere of Caxias do Sul (Rio Grande do Sul, Brazil), Aerobiologia 22 (2) (2006) 141–148.
[10] W.H. Lewis, W.E. Imber, Allergy epidemiology in the St. Louis, Missouri, area: II grasses, Ann. Allergy 35 (1) (1975) 42–50.
[11] R.Y. Lin, A.E. Clauss, E.S. Bennett, Hypersensitivity to common tree pollens in New York City patients, Allergy Asthma Proc. 23 (4) (2002) 253–258.
[12] C.W. Calabria, J. Dice, Aeroallergen sensitization rates in military children with rhinitis symptoms, Ann. Allergy Asthma Immunol. 99 (2) (2007) 161–169.
[13] J.Á. Huerta-Ocampo, J.A. Osuna-Castro, G.J. Lino-López, A. Barrera-Pacheco, G. Mendoza-Hernández, A. De León-Rodríguez, A.P. Barba de la Rosa, Proteomic analysis of differentially accumulated proteins during ripening and in response to 1-MCP in papaya fruit, J. Proteom. 75 (7) (2012) 2160–2169.
[14] M.B. Morales-Amparano, G. Ramos-Clamont Montfort, I. Baqueiro-Peña, M.D. R. Robles-Burgueño, L. Vázquez-Moreno, J.Á. Huerta-Ocampo, Proteomic response of Saccharomyces boulardii to simulated gastrointestinal conditions and encapsulation, Food Sci. Biotechnol. 28 (3) (2019) 831–840.
[15] M.G. Grabherr, B.J. Haas, M. Yassour, J.Z. Levin, D.A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Z. Chen, E. Mauceli, N. Hacohen, A. Gnirke, N. Rhind, F. di Palma, B.W. Birren, C. Nusbaum, K. Lindblad-Toh, N. Friedman, A. Regev, Full-length transcriptome assembly from RNA-Seq data without a reference genome, Nat. Biotechnol. 29 (7) (2011) 644–652.
[16] D.M. Bryant, K. Johnson, T. DiTommaso, T. Tickle, M.B. Couger, D. Payzin-Dogru, T.J. Lee, N.D. Leigh, T.-H. Kuo, F.G. Davis, J. Bateman, S. Bryant, A.R. Guzikowski, S.L. Tsai, S. Coyne, W.W. Ye, R.M. Freeman Jr., L. Peshkin, C.J. Tabin, A. Regev, B.

J. Haas, J.L. Whited, A tissue-mapped axolotl De novo transcriptome enables identification of limb regeneration factors, Cell Rep. 18 (3) (2017) 762–776.
B. Saha, S.G. Bhattacharya, Charting novel allergens from date palm pollen (*Phoenix sylvestris*) using homology driven proteomics, J. Proteom. 165 (2017) 1–10.
O. Kortekangas-Savolainen, K. Lammintausta, K. Kalimo, Skin prick test reactions to brewer's yeast (Saccharomyces cerevisiae) in adult atopic dermatitis patients, Allergy 48 (3) (1993) 147–150.
M.B. Morales-Amparano, J.Á. Huerta-Ocampo, G. Pastor-Palacios, L.M. Teran, The role of enolases in allergic disease, J. Allergy Clin. Immunol. 9 (8) (2021) 3026–3032.
J.Á. Huerta-Ocampo, A. Valenzuela-Corral, M.D.R. Robles-Burgueño, A. M. Guzmán-Partida, M.Á. Hernández-Oñate, L. Vázquez-Moreno, G.F. Pavón-Romero, L.M. Terán, Proteomic identification of allergenic proteins in red oak (Quercus rubra) pollen, World Allergy Organiz. J. 13 (3) (2020) 100111.
B.M. Mani, J.A. Huerta-Ocampo, J.R. Garcia-Sanchez, A. Barrera-Pacheco, A. P. Barba de la Rosa, L.M. Teran, Identification of Ligustrum lucidum pollen allergens using a proteomics approach, Biochem. Biophys. Res. Commun. 468 (4) (2015) 788–792.
F. Mousavi, A. Majd, Y. Shahali, F. Ghahremaninejad, R. Shokouhi Shoormasti, Z. Pourpak, Immunoproteomics of tree of heaven (*Ailanthus atltissima*) pollen allergens, J. Proteom. 154 (2017) 94–101.
S. Wagner, H. Breiteneder, B. Simon-Nobbe, M. Susani, M. Krebitz, B. Niggemann, R. Brehler, O. Scheiner, K. Hoffmann-Sommergruber, Hevb9, an enolase and a new cross-reactive allergen from Hevea latex and molds, Eur. J. Biochem. 267 (24) (2000) 7006–7014.
L. Karni, B. Aloni, Fructokinase and hexokinase from pollen grains of bell pepper (*Capsicum annuum* L.): possible role in pollen germination under conditions of high temperature and CO2 enrichment, Ann. Bot. 90 (5) (2002) 607–612.
B. Saha, G. Sircar, N. Pandey, S. Gupta Bhattacharya, Mining novel allergens from coconut pollen employing manual de novo sequencing and homology-driven proteomics, J. Proteome Res. 14 (11) (2015) 4823–4833.
N. Ghosh, G. Sircar, B. Saha, N. Pandey, S.G. Bhattacharya, Search for allergens from the pollen proteome of sunflower (*Helianthus annuus* L.): a major sensitizer for respiratory allergy patients, PloS One 10 (9) (2015), e0138992.
R. Sousa, H. Osório, L. Duque, H. Ribeiro, A. Cruz, I. Abreu, Identification of Plantago lanceolata pollen allergens using an immunoproteomic approach, J. Investig. Allergol. Clin. Immunol. 24 (3) (2014) 177–183.
A. Posch, Z. Chen, M.J. Dunn, C.H. Wheeler, A. Petersen, G. Leubner-Metzger, X. Baur, Latex allergen database, Electrophoresis 18 (15) (1997) 2803–2810.
O. Luengo, R. Mollá, C. Gámez, V. Cardona, E. Lopez, B. Sastre, Y. Waisel, J. Belmonte, A. Cadahia, C. Lahoz, Allergenicity and cross-reactivity of Senecio pollen: identification of novel allergens using the immunoproteomics approach, Clin. Exp. Allergy 38 (6) (2008) 1048–1060.
[30] C. Pastor, J. Cuesta-Herranz, B. Cases, M. Pérez-Gordo, E. Figueredo, M. de las Heras, F. Vivanco, Identification of major allergens in watermelon, Int. Archiv. Allergy Immunol. 149 (4) (2009) 291–298.
[31] K. Ghosal, B. Saha, S. Gupta Bhattacharya, Clinical and immuno-proteomic approach on Lantana camara pollen allergy—a major health hazard, Allergy Asthma Clin. Immunol. 12 (1) (2016) 33.
[32] H. Sahin, R. Aliyazicioglu, O. Yildiz, S. Kolayli, A. Innocenti, C.T. Supuran, Honey, polen, and propolis extracts show potent inhibitory activity against the zinc metalloenzyme carbonic anhydrase, J. Enzyme Inhibit. Med. Chem. 26 (3) (2011) 440–444.
[33] J. Huang, Z. Li, G. Biener, E. Xiong, S. Malik, N. Eaton, C.Z. Zhao, V. Raicu, H. Kong, D. Zhao, Carbonic anhydrases function in anther cell differentiation downstream of the receptor-like kinase EMS1, Plant Cell 29 (6) (2017) 1335–1356.
[34] C.-Y. Liu, S. Tao, J.-Y. Xue, H. Zhang, W.-T. Xue, F.-S. Chen, Identification and purification of a novel fish allergen from largemouth bass (Micropterus salmoides), Food Agric. Immunol. 25 (1) (2014) 70–81.
[35] L. Castro, J.F. Crespo, J. Rodríguez, R. Rodríguez, M. Villalba, Immunoproteomic tools are used to identify masked allergens: Ole e 12, an allergenic isoflavone reductase from olive (*Olea europaea*) pollen, Biochim. Biophys. Acta 1854 (12) (2015) 1871–1880.
[36] J.C. Jimenez-Lopez, S. Morales, A.J. Castro, D. Volkmann, M.I. Rodríguez-García, J.D.D. Alché, Characterization of profilin polymorphism in pollen with a focus on multifunctionality, PLoS One 7 (2) (2012), e30878.
[37] J. Quiralte, E. Llanes, P. Barral, J.M. Arias de Saavedra, B. Sáenz de San Pedro, M. Villalba, J.F. Florido, R. Rodríguez, C. Lahoz, B. Cárdaba, Ole e 2 and Ole e 10: new clinical aspects and genetic restrictions in olive pollen allergy, Allergy 60 (3) (2005) 360–365.
[38] A. Santos, R. Van Ree, Profilins: mimickers of allergy or relevant allergens? Int. Arch. Allergy Immunol. 155 (3) (2011) 191–204.
[39] S.E. Edwards, F.F. Halton, Pollen food syndrome, Paediatr. Child Health 29 (3) (2019) 137–141.
[40] A.J. Huertas, A. Carreño, C. Mérida, M.J. Pajarón-Fernández, M. Ramírez-Hernández, J. Carnés, Profilin sensitisation in a Mediterranean population, Allergol. Immunopathol. 42 (5) (2014) 387–394.
[41] A. Price, S. Ramachandran, G.P. Smith, M.L. Stevenson, M.K. Pomeranz, D. E. Cohen, Oral allergy syndrome (pollen-food allergy syndrome), Dermatitis 26 (2) (2015).
[42] R. Hirschwehr, C. Heppner, S. Spitzauer, W.R. Sperr, P. Valent, U. Berger, F. Horak, S. Jäger, D. Kraft, R. Valenta, Identification of common allergenic structures in mugwort and ragweed pollen, J. Allergy Clin. Immunol. 101 (2) (1998) 196–206.
[43] E.A. Pastorello, C. Ortolani, L. Farioli, V. Pravettoni, M. Ispano, Å. Borga, A. Bengtsson, C. Incorvaia, C. Berti, C. Zanussi, Allergenic cross-reactivity among peach, apricot, plum, and cherry in patients with oral allergy syndrome: an in vivo and in vitro study, J. Allergy Clin. Immunol. 94 (4) (1994) 699–707.

4. RECOMBINANT EXPRESSION AND EPITOPE PREDICTION OF ENOLASE AND PROFILIN: ALLERGENIC PROTEINS FROM PECAN NUT (*Carya illinoinensis*) POLLEN

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Abstract

The World Health Organization estimates that pollen is responsible for up to 40% of respiratory allergies. However, the number of well-characterized allergenic sources is limited; being identification and purification of allergens, the basis for developing diagnostic and immunotherapeutic strategies. Three pecan nut (*Carya illinoinensis*) seed proteins are recognized as allergens, but so far none from pollen, which has been reported as a cause of severe allergies in Texas, USA, and whose allergens were recently identified. We aimed to overexpress and purify two recombinant allergenic proteins from this pollen and predict their linear and conformational epitopes. We selected the *Carya illinoinensis* pollen allergens, profilin and enolase, obtained their open reading frames, and their nucleotide sequences were synthesized into independent gBlocks that were inserted into the expression vector pET-28a(+) which was used to transform BL21(DE3)pLysS cells for overexpression of allergens. After purification by chromatographic techniques, identity of recombinant proteins was confirmed using LC-MS/MS. Linear and conformational epitope prediction was performed by combining the ABCpred, BepiPred, Immunomedicine, and ElliPro bioinformatics tools, taking into account the coincidence of the same sequence predicted by 3 of the 4 tools, as well as lengths between 5 and 25 amino acids for linear epitopes. We obtained a total of 4 and 13 linear epitopes, as well as 2 and 3 conformational epitopes predicted for profilin and enolase, respectively. This lays the groundwork for the design of new therapeutic modalities for pecan pollen allergy.

Keywords: Pecan allergens, epitope prediction, overexpression, *Carya illinoinensis*.

1. Introduction

The World Health Organization (WHO) estimates that at least 25% of the world's population suffers from respiratory allergy (RA), which represents a global health problem due to its high prevalence and increasing incidence (Bousquet *et al.,* 2008). It is estimated that 40% of allergic diseases are of respiratory origin, caused by aeroallergens, the most common of which is pollen, and the WHO recognizes that millions of people suffering from respiratory allergy do not have a specific diagnosis (D'amato *et al.,* 2007). On one hand, RA has no cure, and then pharmacological treatments are aimed at treating only the symptoms. On the other hand, there is the option of immunotherapy, which consists of repeated and gradual exposure to the allergens, employing extracts from the allergenic source. This results in the desensitization of individuals to allergens, making symptoms less frequent and/or less intense (Asero *et al.,* 2016). Generally, both the diagnosis and treatment of pollen allergy are based on the use of extracts that often have a variable and undefined composition, so the diagnosis is far from being specific (Jeong *et al.,* 2016). In addition, the number of explored and well-characterized allergen sources is small. To improve the specificity of diagnosis and treatment, purified allergens have been used (both from natural and recombinant sources), and in some cases, allergens have been modified or molecular hybrids derived from the knowledge of the antigenic determinants have been constructed, improving the results of diagnosis using skin testing and immunotherapy (Asero *et al.,* 2016; Douladiris *et al.,* 2019).

There are currently three registered *Carya illinoinensis* allergens (Car i 1, Car i 2, and Car i 4) derived from the pecan nut fruit, but so far, none from its pollen, which has shown to be of clinical relevance in regions such as the state of Texas, USA, where it has been reported as the second cause of severe pollen allergy (White & Bemstein, 2003; Geiselhart *et al.,* 2018). Immunodetection and identification of allergenic pecan pollen proteins were recently published (Morales-Amparano *et al.,* 2021). Profilin is a highly conserved protein among plants, being a cause of cross-reactivity between different pollens. However, this phenomenon occurs not only between profilins of pollens but also with profilins of fresh plant foods such as fruits and vegetables, which is known as oral allergy syndrome, causing swelling and itching in the mouth (Scheurer *et al.,* 2000; Santos & Van Ree, 2011). Enolase as a pollen allergen has been little explored, although it is frequently included in the list of allergenic proteins identified in several pollen sources studied under an

immunoproteomic approach. However, only three pollen-derived enolases are registered in the official list of allergens (http://www.allergen.org/), belonging to *Cynodon dactylon* (Cyn d 22), *Ambrosia artemisiifolia* (Amb a 12) and *Platanus acerifolia* (Pla a 6). In the last 30 years, purified allergens have been obtained, which are not only used to improve diagnostic procedures, they are useful in the implementation of safer, more controllable, and effective immunotherapies (Popescu & Vieru, 2018). In recent years, with the emergence of bioinformatic tools it is possible to predict epitopes of unexplored allergens; assess mutations of specific amino acids in already known epitopes, in the process of reducing their allergenicity and modulating the immune response (Raoufi *et al.*, 2019). The aim of this work was the recombinant expression and epitope prediction of two allergenic proteins from the pollen of the pecan nut tree *Carya illinoinensis*, enolase and profilin, as an initial step to evaluate its potential diagnostic and therapeutic use.

2. Materials and methods

2.1 In silico obtaining of nucleotide sequences of allergenic proteins

As previously reported (Morales-Amparano *et al.*, 2021), open-access transcriptomic data sets from various pecan nut tissues (PRJNA342905, PRJNA413769, PRJNA431042, PRJNA504494, and PRJNA533506) were used, ≈ 150 RNAseq (fastq) files equivalent to ≈ 133 GB of data were assembled using Trinity (Grabherr *et al.*, 2011), obtaining approximately 170 000 transcripts. Functional annotation was performed by Trinotate using tools such as Blastn, Blastp and HMMER, in addition to the Pfam and UniProt databases to obtain a specific database for *Carya illinoinensis* that resulted in a total of 92,960 protein sequences. Using the BioEdit tool, we performed local blast for each of the proteins, using the aforementioned database and as Query, the peptides used for the identification by mass spectrometry of enolase and profilin (Morales-Amparano *et al.*, 2021). We also used the *Carya illinoinensis* genome at the scaffold level (GenBank ASM1103780v1) and the TBLASTn tool, using as query the protein sequences obtained by the local Blast analysis mentioned above.

The open reading frame of both proteins was localized using the ExPASy Translate tool, and the sequences were optimized for expression in *E. coli* by complementarily using the GenScript and IDT websites (https://www.genscript.com/tools/rare-codon-analysis and

https://www.idtdna.com/site/account/login?returnurl=%2FCodonOpt, respectively). Finally, the sequences were analyzed by NEBcutter (http://nc2.neb.com/NEBcutter2/) to corroborate the absence of the recognition sites for the restriction enzymes NdeI and HindIII.

2.2. Cloning and expression of recombinant proteins

Optimized nucleotide sequences of profilin and enolase were synthesized by Integrated DNA Technologies (IDT) as gBlocks, which were cloned into the expression vector pET28pps between the restriction enzyme sites NdeI and HindIII, generating a construct for each of the proteins, which were used to transform chemically competent BL21(DE3) pLysS cells by heat shock, which were grown in one liter of LB medium with kanamycin and chloramphenicol at 37 °C under constant agitation, until an optical density of 600 nm was obtained. Overexpression was induced with 0.5 mM IPTG for 3 h, and cells were harvested by centrifugation.

2.3. Isolation by nickel pseudo affinity chromatography

Thirty milliliters of binding buffer composed of 50 mM NaH2PO4, and 300 mM NaCl, spiked with 1 mM DTT and 1 mM PMSF was used to suspend the bacterial pellet and lysed by sonication on ice and centrifuged at 13 000 x g, keeping the soluble fraction. The supernatant was filtered through a 0.45 μm membrane and transferred to a Ni-NTA agarose column with 2 mL as bed volume, previously equilibrated with 20 mL of binding buffer. Later, the column was washed with 20 mL of wash buffer 1 (binding buffer plus 20 mM imidazole), subsequently with 20 mL of wash buffer 2 (binding buffer plus 50 mM imidazole), and finally elution buffer (binding buffer plus 300 mM imidazole). A sample from each step of the process was collected and resolved by SDS-PAGE. Histidine tail cleavage reaction was performed with PreScission protease (GE Healthcare, Piscataway, New Jersey, USA) according to the manufacturer's instructions.

2.4. Ion exchange chromatography

Before performing ion exchange chromatography, a buffer change was made from elution buffer to buffer A (50 mM Tris, 0.5 mM DTT, pH 8.0). Both samples were brought to a volume of 15 mL

each, filtered on a 0.22 µm membrane to be injected onto an anion exchange column in an FPLC-AKTA (GE Healthcare, Piscataway, New Jersey, USA). Samples were eluted in a linear gradient of buffers A and B (buffer B: Tris 50 mM, NaCl 1M, DTT 0.5 mM, pH 8), from 0-100% buffer B for 150 min, on a column previously equilibrated with buffer A. Elution in both cases was detected by absorbance at 280 nm and the different fractions were visualized with SDS-PAGE gels. Enolase fractions were combined and lyophilized. The same procedure was done with profilin fractions. Finally, a portion of the proteins was suspended in deionized water and visualized by SDS-PAGE gels stained with Coomassie blue. Bands with the expected molecular weights of enolase and profilin, around 50 and 15 kDa, respectively, were removed from the gel digested with trypsin and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as detailed below.

2.5. Molecular exclusion chromatography

Lyophilized recombinant proteins were suspended in 50 mM Tris pH 8.0 and quantified by the Bradford method. An amount of 600 µg of r-Enolase and 300 µg of r-Profilin, both in a volume of 200 µL, were injected onto a Superdex 200 column, using 50 mM Tris pH 8.0 as eluent in an FPLC-AKTA (GE Healthcare, Piscataway, New Jersey, USA). The runs were 60 min, both at a constant flow rate of 0.5 mL/min, 1 mL fractions were collected, and according to their chromatograms, the total elution volume was divided into different pools, which were desalted, lyophilized, and suspended in deionized water. Protein concentration in each pool was quantified and visualized on SDS-PAGE by loading 5 µg of each pool.

2.6. In-gel protein digestion and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

Protein bands excised from the SDS-PAGE gels corresponding to r-Enolase and r-Profilin were chopped, destained, and subsequently reduced with 10 mM DTT in 25 mM ammonium bicarbonate followed by protein alkylation with 55 mM iodoacetamide. Protein digestion was carried out overnight at 37 °C with mass spectrometry-grade trypsin (Pierce™ Thermo Scientific. Rockford, IL, USA). Tryptic peptide separation was performed using the 1290 Infinity LC System (Agilent Technologies, Santa Clara, California, USA) equipped with an analytical column ZORBAX

300SB-C8 (5 µm x 2.1 mm x 150 mm, Agilent Technologies), and MS/MS analysis was performed by a 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS system (Agilent Technologies) at the Institutional Analytical Platform from the Research Center for Food and Development A.C. (Hermosillo, Sonora, Mexico) as previously reported (Morales-Amparano *et al.*, 2019).

2.7. Protein identification

Protein identification was performed using the MS/MS raw data (.d files) and the Spectrum Mill MS Proteomics Workbench server (Agilent Technologies), Data Extractor tool was used to obtain .mzXML files and proteins were then identified using these files in the MS/MS Search tool. Searches were conducted against the .FASTA files with the sequences of both recombinant proteins. Trypsin was used as the specific protease, allowing one missed cleavage. Mass error tolerance for precursor and fragment ions was set to 20 ppm and 0.1 Da, respectively. Oxidation of methionine was specified as variable modification whereas carbamidomethyl cysteine was set as fixed modification. Protein identifications were considered successful when at least two peptides and a significant protein score (>25) was obtained.

2.8. Prediction of linear and conformational epitopes

Allergen epitope prediction was performed by combining several bioinformatic tools. The protein amino acid sequences were introduced in the following predictors of linear epitopes: ABCpred (https://webs.iiitd.edu.in/raghava/abcpred/ABC_submission.html), BepiPred (https://services.healthtech.dtu.dk/service.php?BepiPred-2.0), Immunomedicine (http://imed.med.ucm.es/Tools/antigenic.pl), and ElliPro (http://tools.iedb.org/ellipro/). ElliPro was used for the prediction of conformational epitopes, for the use of this tool and for the visualization of the spatial arrangement of the linear epitopes in PyMol, modeling by homology was carried out using Swiss Model (https://swissmodel.expasy.org/interactive) to generate the required .PDB file, and as templates the structures of Bet v 2 (SMTL ID: 1cqa.1) for profilin and human alpha-enolase (SMTL ID: 2psn.1). For linear epitopes, we selected sequences from 5 to 25 amino acids, predicted by three of the four computational tools used. The parameters recommended

by each of the tools were used. After reviewing the data, totally linear epitopes that appeared in the results, that had fewer than 5 amino acids involved and/or that had scores below 0.65 were discarded, this score value was also used as a cut-off value for the selection of conformational epitopes.

3. Results and discussion

3.1. Overexpression and isolation by chromatographic techniques

Overexpression and isolation of enolase and profilin were successful. Fig.1 includes SDS-PAGE analysis of fractions corresponding to the overexpression of r-Enolase and r-Profilin and their subsequent purification process by nickel pseudo affinity chromatography. Fig. 1A shows the elution fractions corresponding to r-Enolase in 12 % SDS-PAGE, where the protein predominates in lanes 6 and 7 (eluted with 300 mM imidazole binding buffer) in the expected mass range of approximately 50 kDa. In Figure 1B, r-Profilin overexpression on a 15% SDS-PAGE (migrated as a predominant band of approximately 15 kDa) in lanes 5, 6, and 7.

Subsequently, both proteins were analyzed by ion exchange chromatography using a linear salt gradient. The elution chromatograms and fractions collected from each protein are shown in Fig. 2. r-Enolase eluted at 230 to 380 mM NaCl, while r-Profilin at 340 to 450 mM NaCl. After lyophilization of both recombinant proteins fractions (Fig. 2E) were suspended in deionized water and analyzed by 13% SDS-PAGE, where both allergens were predominant in their corresponding fractions.

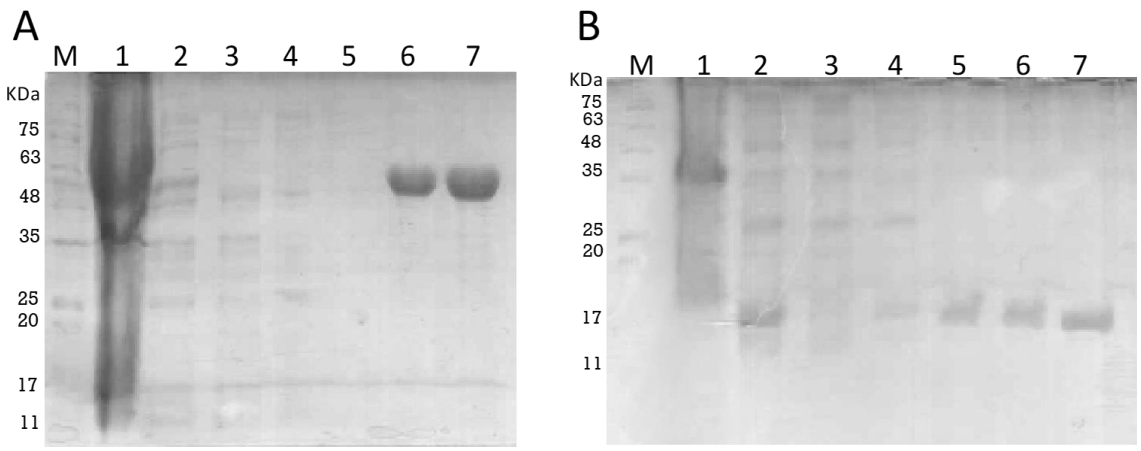

Fig. 1 SDS-PAGE gels (12% and 15 %) corresponding to the overexpression of A) r-Enolase and B) r-Profilin, respectively, and their subsequent purification process by nickel pseudo affinity chromatography. Each of the lanes, in both gels, was loaded with 10 µL of each of the samples in the following order: M) molecular mass marker, 1) insoluble fraction from extraction, 2) soluble fraction from extraction, 3) flow through, 4) wash with 20 mM imidazole binding buffer, 5) wash with 50 mM imidazole binding buffer, 6 and 7) elutions with 300 mM imidazole binding buffer.

Molecular exclusion chromatography was used to further purify the recombinant proteins. The elution of each sample was divided into pools corresponding to the absorbance peaks at 280 nm present in each one of them, both molecular exclusion chromatograms are shown in Fig. 3 (A and C). For r-Enolase three protein pools were obtained and SDS-PAGE analysis (Fig. 3 C) showed characteristic bands of about 50 kDa. On the other hand, the purification of r-Profilin resulted in a single predominant peak, corresponding to pool 4, which showed the expected band close to 15 kDa (Fig. 3 D). In both experiments, recombinant proteins fractions show higher purity after size exclusion chromatography in comparison to ion exchange chromatography.

Pollen enolase has been reported as an allergen in several immunoproteomic studies derived from different sources, such as *Cocos nucifera, Ligustrum lucidum,* and *Quercus rubra* (Mani *et al.,* 2015; Saha *et al.,* 2015; Huerta-Ocampo *et al.,* 2020). Allergenic enolases from fungi have been overexpressed, as for *Rhodotorula mucilaginosa*, *Penicillium citrinum*, *Aspergillus fumigatus,* and *Curvularia lunata* (Chang *et al.,* 2002; Lai *et al.,* 2002; Sharma *et al.,* 2006), which retained their IgE binding capacity of serum from sensitive patients. Recently, a recombinant enolase from *Platanus acerifolia* pollen (Pla a 6) was expressed and purified from *E. coli*. Allergenicity of this novel allergen was characterized by enzyme linked immunosorbent assay (ELISA), Western blot, inhibition ELISA, and basophil activation test. However, epitope prediction has not been performed (Jiao *et al.,* 2022).

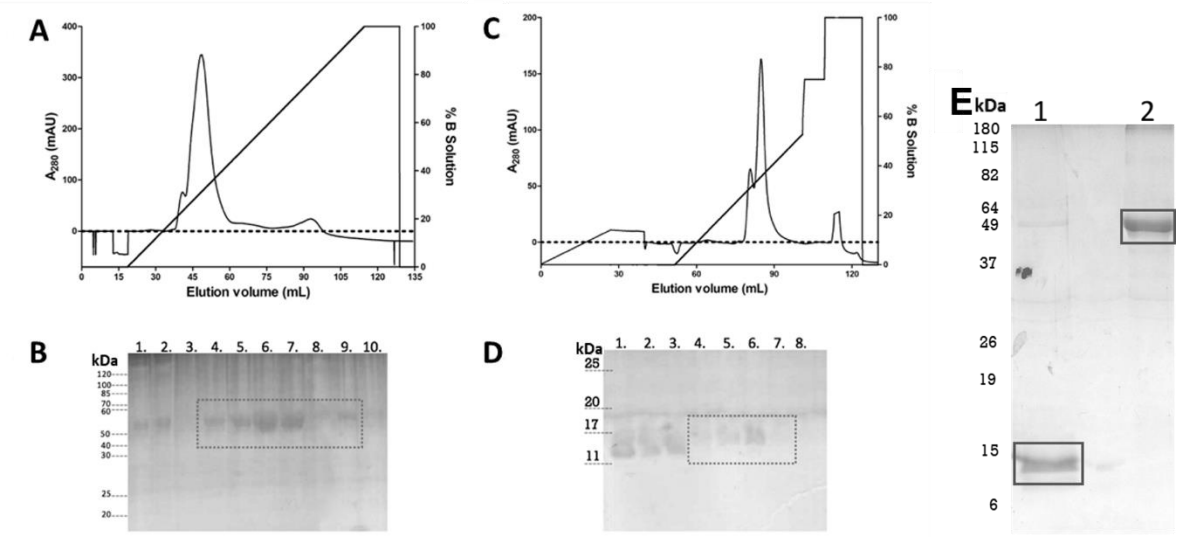

Fig. 2 Fig. 2 Ion exchange chromatography for recombinant pecan nut allergens, in a linear NaCl gradient. Chromatogram of recombinant r-Enolase (A) and r-Profilin (C). SDS-PAGE 12% for enolase (B) and 15% for profilin (D), corresponded to the various elution fractions, each lane contained 5 μL of each of the samples. In both gels lane 1) shows the protein obtained by nickel pseudo-affinity chromatography, line 2) protein in 50 mM tris pH 8.0, and following lanes show anion exchange elution fractions. Fractions framed with the dotted line were lyophilized. E) SDS-PAGE 13% with 5 µg of 1) r-Profilin and 2) r-Enolase suspended in deionized water. Boxed regions were excised out for in-gel trypsin digestion and LC-MS/MS analysis.

Profilin is a widely studied pollen panallergen, and several recombinant profilins from pollen have been used for this purpose, such as rGly m 3, rChe a 2, and rOle e, recombinant parallels of profilins from *Glycine max*, *Chenopodium album*, and *Olea europaea* (Asturias *et al.,* 1997; Rihs *et al.*, 1999; Barderas *et al.*, 2004). The exploration of profilins as allergens has made it possible to understand the cross-reactivity and to include them in diagnostic allergen panels. Furthermore, being able to express them on demand has made it easier to obtain the required quantities in a faster and more efficient way, concerning natural source allergens (Pablos *et al.,* 2016). This allergen has also been linked to oral allergy syndrome, and some recombinant profilins have been used to study the cross-reactivity of pollen profilins, mainly Bet v 2, with profilins present in fresh plant foods such as celery, pear, and cherry (Scheurer *et al.*, 2001); increasing its clinical relevance in allergic disease.

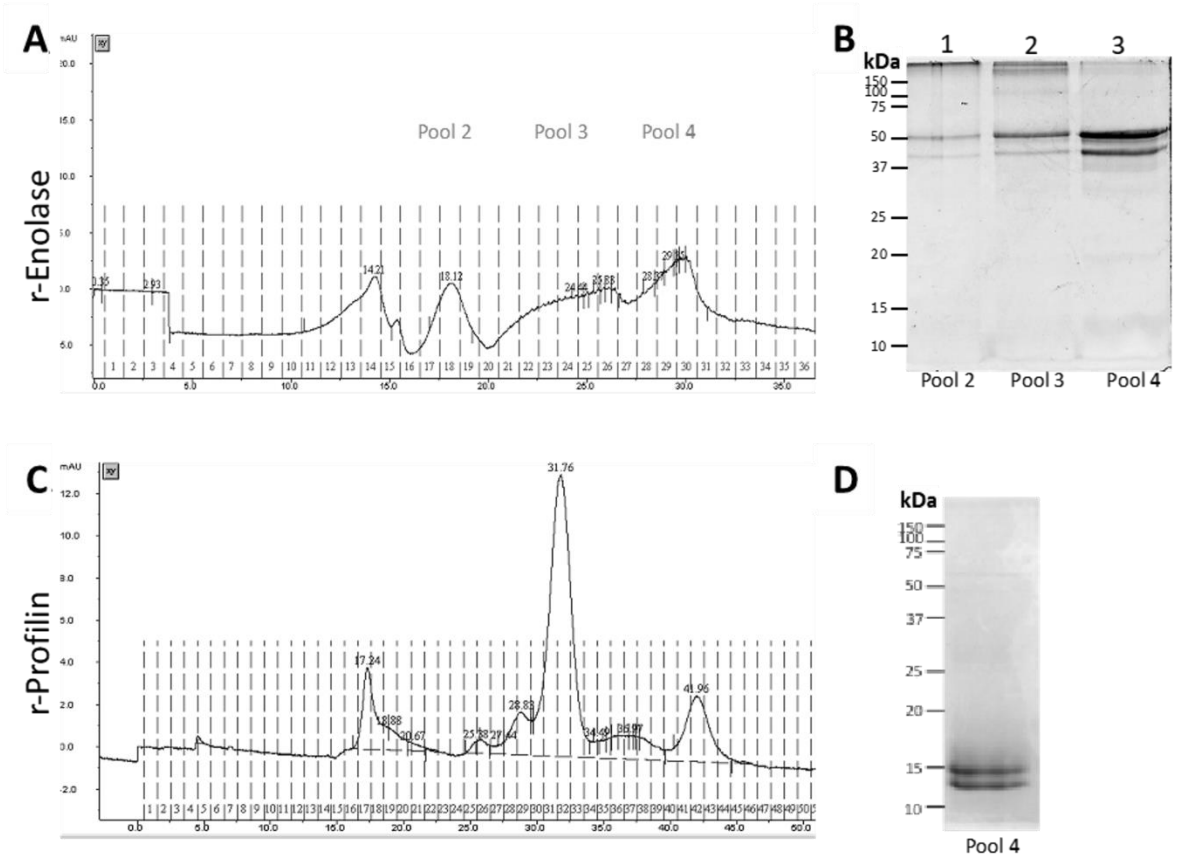

Fig. 3 Molecular exclusion chromatography on Superdex 200 matrix. Chromatograms corresponding to A) r-Enolase and C) r-Profilin. B) 13% SDS-PAGE loaded with 5 µg of protein from 1) pool 2, 2) pool 3 and 3) pool 4 corresponding to r-Enolase. D) 13% SDS-PAGE loaded with 5 µg of protein from pool 4 corresponding to r-Profilin.

3.2 Protein identification by tandem mass spectrometry and homology database search

A homology database search of MS/MS data sets from protein bands analyzed against the recombinant protein sequences, using the Spectrum Mill search engine allowed the confirmation of both proteins. Table 1 shows the peptides for their identification, this corroborates the identities of the overexpressed proteins as r-Enolase and r-Profilin from *Carya illinoinensis*.

Table 1. Identification of r-Enolase and r-Profilin

Accession number	Theoretical Mr(kDa)/*pI*	PM*/ % coverage	Score●	Unique peptides
Enolase_recombinant_Carya	48.28/5.72	20/ 56.7	400.69	AAVPSGASTGVYEALELR DGGSDYLGK IIGPALIGK LGANAILAVSLAICK KIPLYQHIANLAGNK LVLPVPAFNVINGGSHAGNK MGVEVYHNLK YGQDATNVGDEGGFAPNIQENK EGLELLK TYDLNFKEENNDGSQK ISGDALINLYK LTAEIGEK VQIVGDDLLVTNPK VEKAIKEK SCNALLLK VNQIGSVTESIEAVK AGWGVMASHR SGETEDTFIADLSVGLATGQIK YNQLLR IEEELGSEAVYAGANFR GPH(m)ATIQLVK
Profilin_recombinant_Carya	14.34/5.02	5/ 39	81.88	DFEEPGHLAPTGLHLGGTK YMVIQGEAGAVIR KGSGGITIKK KGSGGITIK LGDYLADQGL

* PM: peptides matched

● Spectrum Mill Protein Score (Scores ≥25 and at least two peptides were necessary for confident protein identification).

3.3 Prediction of linear epitopes

Bioinformatics analysis of the properties of allergens has progressed greatly in the last few years, in this work analysis of amino acid sequences of the two allergenic proteins from pecan pollen using several bioinformatic tools (ABCpred, Immunomedicine, BepiPred, and ElliPro) allowed to identify 4 potential linear epitopes for profilin and 13 for enolase (Fig.4 A and B). Characteristics of the -R groups of amino acids such as the presence of positive or negative charges, hydrophobicity, flexibility, access to the surface and ability to interact with solvents contribute to the allergenicity of an amino acid sequence. These features are considered by computational tools to predict epitopes (Guo *et al.,* 2020). PyMol was used to make a three-dimensional representation of the recombinant proteins with the predicted epitopes numbered and colored as shown in Fig. 4 C and D. For both proteins, the predicted epitopes were exposed to the surface, facilitating their interaction with the solvent, and therefore easily accessible for IgE recognition.

Prediction of linear epitopes for profilin match with those reported by Radauer et al, 2006. They reported the prediction of a linear epitope (EEPGHLAPT) shared and similar in profilins Api g 4, Hev b 8, and Bet v 2 corresponding to food, contact and airway allergens, respectively.

Consistently, linear epitopes 1, 2, and 4 are among those reported by Jimenez-Lopez et al, (2014) for diverse profilins and their isoforms, all shared with Bet v 2, linear epitope 1 differing only in one amino acid, while epitopes 2 and 4 are 100% identical. On the other hand, there are also evidence from profilin epitopes obtained experimentally, as in the case of the epitope FPQFKPQ predicted in *C. illinoinensis* (Fig. 4A), which match with the region recognized by the IgE of patients allergic to *Betula verrucosa* profilin (Bet v2) (Wiedemann *et al.,* 1996), this shares 90,23 % of amino acid total sequence identity with *C. illinoinensis* profilin (data not shown).

For enolase, experimental allergenic sequences have also been reported, mainly for fungi, such as the *C. albicans* hexapeptide (ALELRD) recognized by IgE (Ito *et al.,* 1995), which is identical to pecan nut pollen enolase and joins the first two regions predicted by bioinformatics tools. Evenly, a region predicted for *C. illinoinensis* enolase (KKLVLPVPA) shares over 66% identity with an epitope conserved in various fungi (KPYVLPVPF) such as *Paracoccidioides brasiliensis, Coccidioides immitis, Blastomyces dermatitidis, Cryptococcus neoformans, Aspergillus fumigatus, and Candida albicans*. This peptide was synthesized and experimentally tested, yielded promising results as a potential candidate for a protective vaccine against various types of fungi (Kischkel *et al.*, 2021).

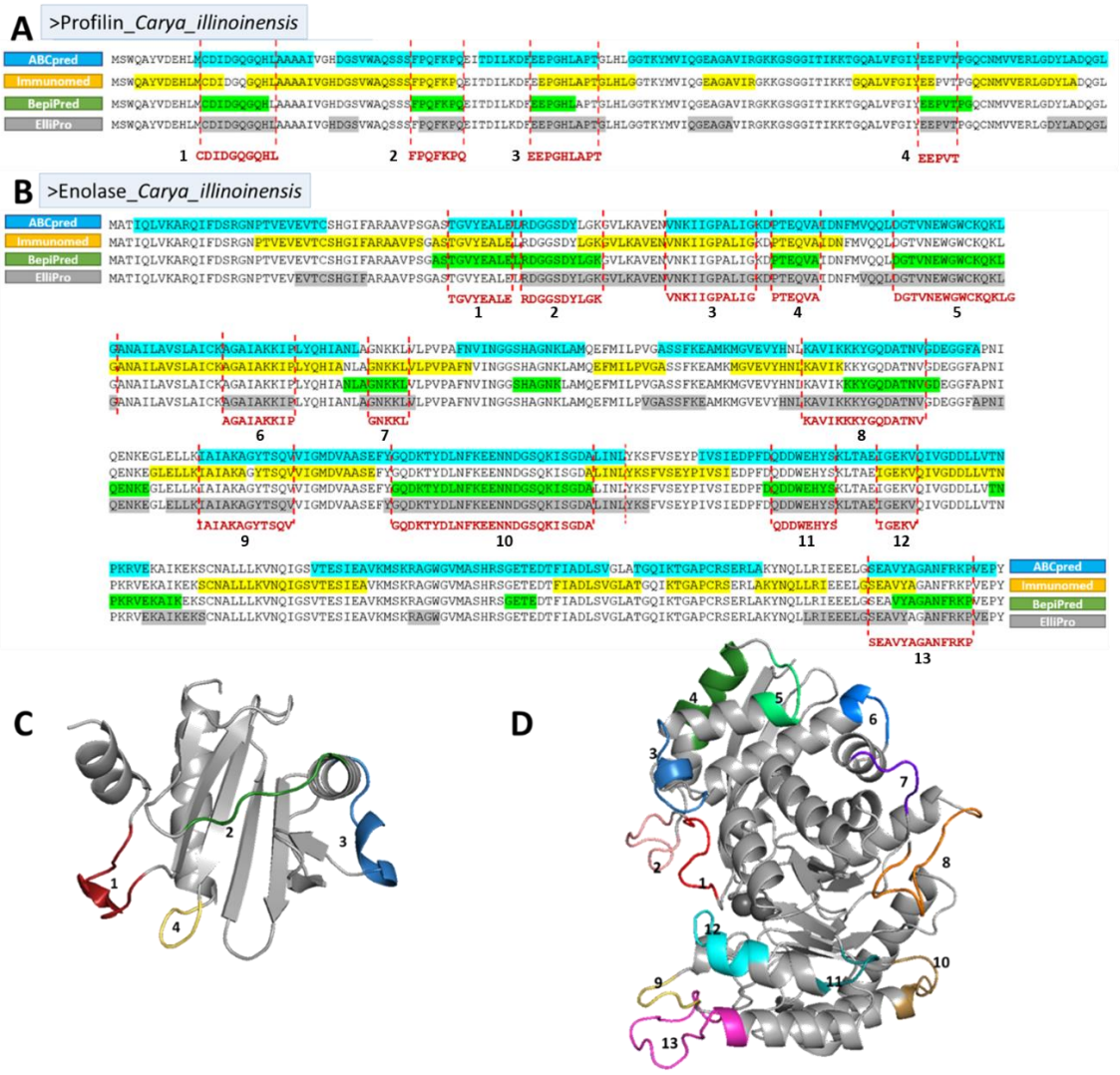

Fig. 4 Linear epitopes predicted using bioinformatic tools. Amino acid sequences of the epitopes of A) profilin and B) enolase and their structural visualization in PyMol, C) profilin, and D) enolase (monomer).

Exposing allergens to IgE after denaturing conditions, such as in a Western Blot may result in little or no binding of the antibody, but when testing it under more gentle conditions, such as in a prick test or ELISA, the binding to IgE appears or increases. This occurs due to the conformational nature of some epitopes, so it is important to consider this when exploring the allergenicity of novel allergens (Ivanciuc *et al.*, 2009). Important linear and conformational epitopes have been described for relevant food allergens in nuts such as Jur r 1 (walnut), Ber e 1 (Brazil nut), Ara h 2 and Ara h 6 (peanut) (Moreno *et al.*, 2005; Downs *et al.*, 2014; Chen *et al.*, 2016). Profilin is classified as a food allergen in fresh foods of plant origin, such as fruits and vegetables. However, the main cause of sensitization in relation to oral allergy syndrome is pollen. Profilin is considered a weak food allergen, as it is easily denatured by heat or other denaturing conditions, due to the existence of conformational epitopes (Saunders and Platt, 2015). This is clearly exemplified by our results where the terminal ends of the profilin were not predicted as linear epitopes, even though there are numerous reports of its allergenicity, these regions only appear as allergenic when analyzed from a conformational perspective (Wiedemann *et al.*, 1996; Mares-Mejía *et al.*, 2020).

Table 2 shows the data corresponding to the amino acids that make up the discontinuous epitopes for the pecan pollen allergens, two for profilin (PC1 and PC2) and three for enolase (EC1, EC2 and EC3), the representation of these conformational epitopes in the three-dimensional structure of both proteins are shown in Figures 5 and 6.

Table 2. Conformational epitopes predicted by ElliPro to profilin and enolase.

Profilin			
Epitope No.		**No. of AAs**	**Score**
PC1	A:H30, A:T99, A:G100, A:Q101, A:Y127, A:A129, A:D130, A:Q131, A:G132, A:L133	10	0.72
PC2	A:S2, A:W3, A:Q4, A:A5, A:Y6, A:D8, A:E9, A:H10, A:C13, A:D14, A:I15, A:D16, A:G17, A:Q18, A:G19, A:Q20, A:H21, A:L22, A:A23, A:S38, A:S39, A:S40, A:K89, A:G90, A:S91, A:E109, A:E110, A:P111, A:V112	29	0.687
Enolase			
Epitope No.		**No. of AAs**	**Score**
EC1	A:N166, A:K167, A:Q223, A:Y257, A:G258, A:Q259, A:D260, A:K261, A:T262, A:Y263, A:D264, A:N266, A:K268, A:E269, A:E270, A:N271, A:N272, A:D273, A:G274, A:S275, A:Q276, A:K277, A:I278, A:S279, A:G280, A:D281, A:A282, A:N285, A:K288	29	0.814
EC2	A:T3, A:I4, A:Q5, A:L6, A:V7, A:T26, A:C27, A:S28, A:H29, A:G30, A:I31, A:F32, A:E68, A:N69, A:N71, A:K72, A:I73, A:G75, A:P76, A:A77, A:L78, A:I79, A:G80, A:K81, A:D82, A:P83, A:T84, A:E85, A:F92, A:Q95, A:Q96, A:D98, A:G99, A:T100, A:V101, A:N102, A:E103, A:W104, A:G105, A:W106, A:C107, A:Q109, A:K110, A:L111	44	0.81
EC3	A:G179, A:A180, A:S181, A:S182, A:K184, A:E185, A:K188, A:E192, A:N196, A:K198, A:A199, A:V200, A:I201, A:K202, A:K203, A:K204, A:Y205, A:G206, A:Q207, A:D208, A:A209, A:T210, A:N211, A:V212, A:F218, A:A219, A:P220, A:N221, A:I222, A:A237, A:K238, A:A239, A:G240, A:Y241, A:T242, A:S243, A:Q244, A:V245	38	0.755

Allergenicity was not predicted in two regions widely reported as IgE epitopes on profilin, one towards the amino-terminus and the other to the opposite terminal end of the protein. These regions are part of important discontinuous epitopes, and since they are in a nonlinear conformation and interact with other amino acids in the tertiary structure of the protein, they have the capacity to be recognized as allergenic. Regions of this nature are revealed in predictions of conformational epitopes. The results indicate that PC2 epitope contains in its composition most of the sequence MSWQAY, shaded in gray in Table 2, located towards the amino terminus, which is identical to epitopes reported in other allergens such as Phl p 12, Hel a 2, Bet v 2, Cuc m 2 and Ara h 5, the last two corresponding to melon and peanut, respectively (Cabanos *et al.,* 2010). Likewise, PC1 contains the allergenic region towards the carboxyl-terminal, with most of the sequence LADQGL (also shaded in Table 2), which is similar in Bet v 2 (LIDQGL) and Cuc m 2 (LIEQGL). These conformational epitopes on the pecan pollen profilin that are shared with food allergens may be involved in oral allergy syndrome as is the case with other profilins.

Table 2 also highlights the amino acid sequences involved in conformational epitopes, for profilin and enolase, which in turn were predicted as likely linear epitopes (underlined). All conformational epitopes except PC1 contain more than one of the predicted linear sequences, e.g., EC2 contains three of those sequences, emphasizing that at the end of antigenic processing, the sequences presented to generate memory are short peptides (Kucuksezer *et al.,* 2020). The study of conformational epitopes can provide valuable information regarding their linear counterparts and be a guide in the selection of regions important for protein allergenicity. Identification of IgE-binding epitopes is important for allergen identification, clinical diagnosis, analysis of potential immunotherapeutics and better understanding of the mechanisms of allergy, this includes exploring linear and conformational epitopes (Sun *et al.,* 2014; Guo *et al.,* 2020).

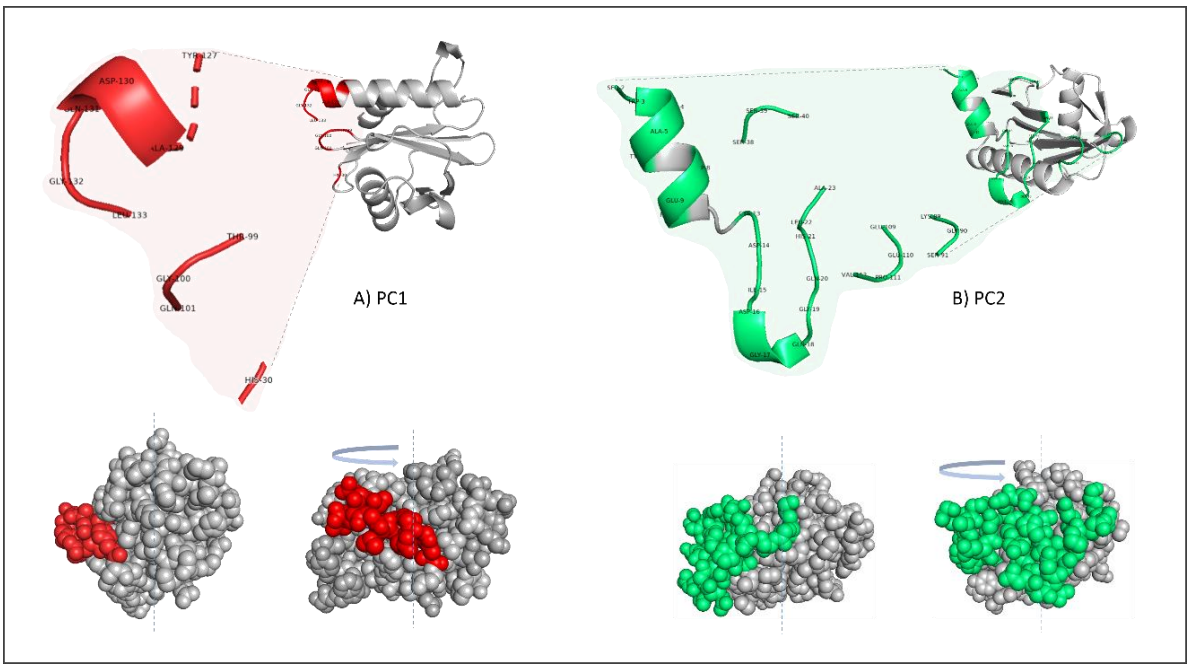

Fig 5. 3D representation of the predicted conformational epitopes for the *C. illinoinensis* profilin, visualization of the model in cartoon and spheres by PyMol. A) PC1, and B) PC2.

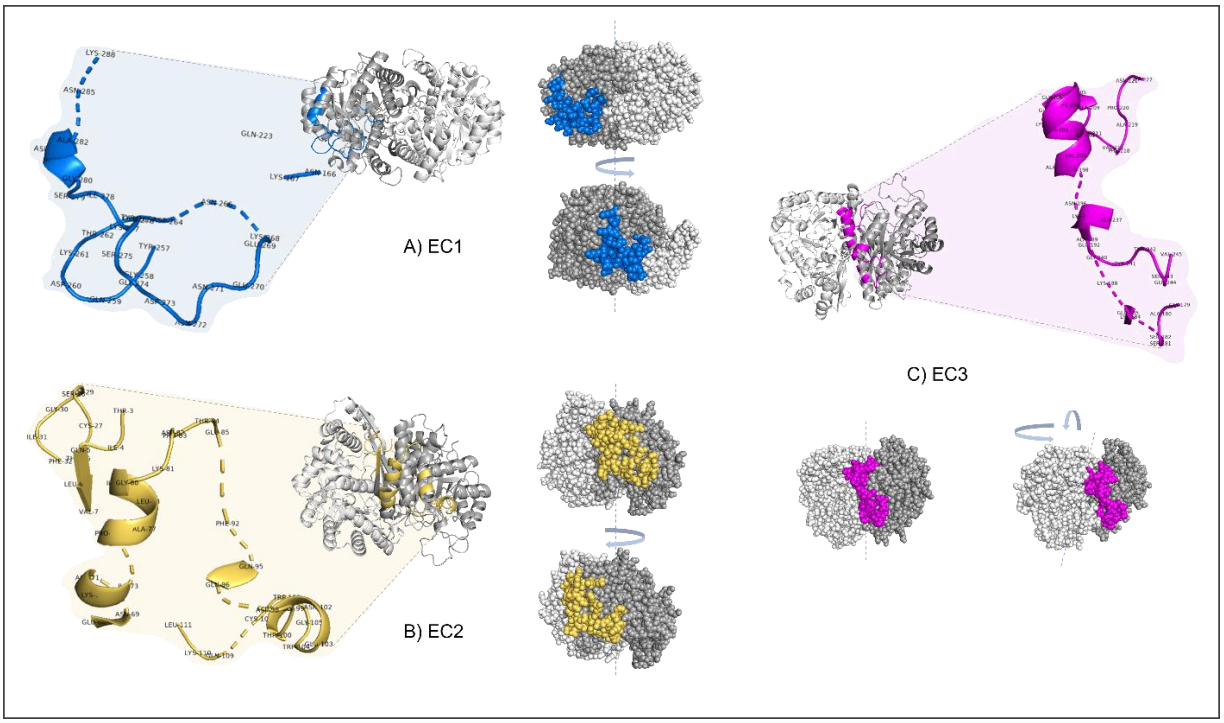

Fig 6. 3D representation of the predicted conformational epitopes for the *C. illinoinensis* enolase, visualization of the model in cartoon and spheres by PyMol. A) EC1, B) EC2, and C) EC3.

There is little information about plant allergenic enolases, obtaining one of the first recombinant allergenic enolases and the prediction of its linear and conformational epitopes presented in this work, open the way to new knowledge about the role of this protein in allergic disease. It is worth mentioning that the epitopes predicted by computational tools are of great help in understanding allergic reactions, however, what occurs *in vivo* may be different. Allergens can go through various processes of denaturation, digestion or the same variability of the antigenic processing of each individual can lead to different IgE epitopes. More studies are required to confirm allergenicity and the main epitopes of r-Profilin and r-Enolase, from *Carya illinoinensis* pollen.

4. Conclusions

Two pecan nut pollen allergenic proteins, r-Enolase and r-Profilin were successfully overexpressed and purified from *E. coli*. For enolase, three conformational (EC1, EC2 and EC3) and 13 linear epitopes were predicted. Likewise, two conformational and four linear epitopes were predicted to profilin. The advances presented here for the first recombinant allergens from *Carya illinoinensis* pollen, lays base for the development of a specific diagnosis and treatment. However, further experiments are required to confirm their allergenicity by IgE antibody binding and epitope confirmation, as a prerequisite to start the development of new diagnostic and therapeutic strategies in this type of allergy.

CRediT authorship contribution statement

Morales-Amparano Martha Beatriz: Conceptualization, Investigation, Writing - original draft, Visualization. **Pastor-Palacios Guillermo:** Formal analysis, Supervision. **Cárdenas-Conejo Yair:** Software, Formal analysis, Data Curation. **Terán-Juárez Luis Manuel:** Conceptualization, Resources, Writing - review & editing, Supervision. **Huerta-Ocampo José Ángel:** Conceptualization, Formal analysis, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

None.

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BIBLIOGRAPHY

Asero, R., Mistrello, G., & Amato, S. (2016). Detection of pan-allergens in commercial pollen extracts for allergen immunotherapy. *Annals of Allergy, Asthma and Immunology*, *117*(2), 180–185. https://doi.org/10.1016/j.anai.2016.05.010

Asturias, J. A., Arilla, M. C., Gómez-Bayón, N., Martínez, J., Martínez, A., & Palacios, R. (1997). Cloning and expression of the panallergen profilin and the major allergen (Ole e 1) from olive tree pollen. *Journal of Allergy and Clinical Immunology*, *100*(3), 365–372. https://doi.org/10.1016/S0091-6749(97)70250-1

Barderas, R., Villalba, M., & Rodríguez, R. (2004). Recombinant expression, purification and cross-reactivity of chenopod profilin: rChe a 2 as a good marker for profilin sensitization, *385*(8), 731–737. https://doi.org/10.1515/BC.2004.089

Bousquet, J., Khaltaev, N., Cruz, A. A., Denburg, J., Fokkens, W. J., Togias, A., Van Weel, C. (2008). Allergic rhinitis and its impact on asthma (ARIA) 2008. *Allergy*, *63*, 8–160.

Chang, C. Y., Chou, H., Tam, M. F., Tang, R. Bin, Lai, H. Y., & Shen, H. Der. (2002). Characterization of enolase allergen from *Rhodotorula mucilaginosa*. *Journal of Biomedical Science*, *9*(6 Pt 2), 645–655. https://doi.org/10.1159/000067279

Chen, X., Negi, S. S., Liao, S., Gao, V., Braun, W., & Dreskin, S. C. (2016). Conformational IgE epitopes of peanut allergens Ara h 2 and Ara h 6. *Clinical & Experimental Allergy*, *46*(8), 1120-1128.

D'amato, G., Cecchi, L., Bonini, S., Nunes, C., Annesi-Maesano, I., Behrendt, H., Van Cauwenberge, P. (2007). Allergenic pollen and pollen allergy in Europe. *The Authors Journal Compilation*. https://doi.org/10.1111/j.1398-9995.2007.01393.x

Douladiris, N., Garib, V., Focke-Tejkl, M., Valenta, R., Papadopoulos, N. G., & Linhart, B. (2019). Detection of genuine grass pollen sensitization in children by skin testing with a recombinant grass pollen hybrid. *Pediatric Allergy and Immunology*, *30*(1), 59–65. https://doi.org/10.1111/PAI.12991

Downs, M. L., Semic-Jusufagic, A., Simpson, A., Bartra, J., Fernandez-Rivas, M., Rigby, N. M., & Mills, E. C. (2014). Characterization of low molecular weight allergens from English walnut (*Juglans regia*). *Journal of agricultural and food chemistry*, *62*(48), 11767-11775.

Geiselhart, S., Hoffmann-Sommergruber, K., & Bublin, M. (2018). Tree nut allergens. *Molecular Immunology*, *100*, 71–81. https://doi.org/10.1016/j.molimm.2018.03.011

Grabherr, M. G., Haas, B. J., Yassour, M., Levin, J. Z., Thompson, D. A., Amit, I., Regev, A. (2011). Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology 2011 29:7*, *29*(7), 644–652. https://doi.org/10.1038/nbt.1883

Guo, X., Jiang, S., Li, X., Yang, S., Cheng, L., Qiu, J., & Che, H. (2020). Sequence analysis of digestion-resistant peptides may be an efficient strategy for studying the linear epitopes of Jug r 1, the major walnut allergen. *Food chemistry*, *322*, 126711.

Holgate, Stephen T.; Church, Martin K. 2012. Allergy, expert consult online and print. Sauders. 4th edition. China. 399. ISBN: 9780723436584

Huerta-Ocampo, J. Á., Valenzuela-Corral, A., Robles-Burgueño, M. D. R., Guzmán-Partida, A. M., Hernández-Oñate, M. Á., Vázquez-Moreno, Terán, L. M. (2020). Proteomic identification of allergenic proteins in red oak (*Quercus rubra*) pollen. *World Allergy Organization Journal*, *13*(3), 100111. https://doi.org/10.1016/j.waojou.2020.100111

Ito, K., Ishiguro, A., Kanbe, T., Tanaka, K., & Torh, S. (1995). Characterization of IgE-binding epitopes on *Candida*

albicans enolase. *Clinical & Experimental Allergy*, *25*(6), 529-535.

Ivanciuc, O., Schein, C. H., Garcia, T., Oezguen, N., Negi, S. S., & Braun, W. (2009). Structural analysis of linear and conformational epitopes of allergens. *Regulatory toxicology and pharmacology*, *54*(3), S11-S19.

Jeong, K. Y., Son, M., Park, J. H., Park, K. H., Park, H. J., Lee, J. H.,Park, J. W. (2016). Cross-reactivity between oak and birch pollens in korean tree pollinosis. *Journal of Korean Medical Science*, *31*(8), 1202–1207. https://doi.org/10.3346/jkms.2016.31.8.1202

Jiao, Y. X., Song, L. B., Xu, Z. Q., Zhu, D. X., Yang, Y. S., Tian, M., & Wei, J. F. (2022). Purification and characterization of enolase as a novel allergen in Platanus acerifolia pollen. *International Immunopharmacology*, *113*, 109313.

Jimenez-Lopez, J. C., Rodríguez-García, M. I., Alché, J. D. (2014) Correction: Analysis of the Effects of Polymorphism on Pollen Profilin Structural Functionality and the Generation of Conformational, T- and B-Cell Epitopes. *PLOS ONE, 9*(1): 10.1371/annotation/3008c3af-753f-4085b85e5ae0c34a7889.

https://doi.org/10.1371/annotation/3008c3af-753f-4085-b85e-5ae0c34a7889

Kischkel, B., Boniche-Alfaro, C., de Godoy Menezes, I., Rossi, S. A., Angeli, C. B., de Almeida, S. R., & Taborda, C. P. (2021). Immunoproteomic and Immunopeptidomic Analyses of *Histoplasma capsulatum* Reveal Promiscuous and Conserved Epitopes Among Fungi With Vaccine Potential. *Frontiers in immunology*, *12*. 764501. Doi: 10.3389/fimmu.2021.764501

Kucuksezer, U. C., Ozdemir, C., Cevhertas, L., Ogulur, I., Akdis, M., & Akdis, C. A. (2020). Mechanisms of allergen-specific immunotherapy and allergen tolerance. *Allergology International*, *69*(4), 549-560.

Lai, H. Y., Tam, M. F., Tang, R. Bin, Chou, H., Chang, C. Y., Tsai, J. J., & Shen, H. Der. (2002). cDNA cloning and immunological characterization of a newly identified enolase allergen from P*enicillium citrinum* and *Aspergillus fumigatus*. *International Archives of Allergy and Immunology*, *127*(3), 181–190. https://doi.org/10.1159/000053862

Mani, B. M., Huerta-Ocampo, J. A., Garcia-Sanchez, J. R., Barrera-Pacheco, A., De La Rosa, A. P. B., & Teran, L. M. (2015). Identification of Ligustrum lucidum pollen allergens using a proteomics approach. *Biochemical and Biophysical Research Communications*, *468*(4), 788–792. https://doi.org/10.1016/j.bbrc.2015.11.033

Mares-Mejía, I., García-Ramírez, B., Torres-Larios, A., Rodríguez-Hernández, A., Osornio-Hernández, A. I., Terán-Olvera, G., & Rodríguez-Romero, A. (2020). Novel murine mAbs define specific and cross-reactive epitopes on the latex profilin panallergen Hev b 8. *Molecular Immunology*, *128*, 10-21.

Morales-Amparano, M. B., Ramos-Clamont Montfort, G., Baqueiro-Peña, I., Robles-Burgueño, M. del R., Vázquez-Moreno, L., & Huerta-Ocampo, J. Á. (2019). Proteomic response of *Saccharomyces boulardii* to simulated gastrointestinal conditions and encapsulation. *Food Science and Biotechnology*, *28*(3), 831–840. https://doi.org/10.1007/s10068-018-0508-9

Morales-Amparano, M. B., Valenzuela-Corral, A., Ramos-Clamont Montfort, G., Vázquez-Moreno, L., Escobedo-Moratilla, A., Pastor-Palacios, G., Huerta-Ocampo, J. Á. (2021). Immunoproteomic identification of allergenic proteins in pecan (*Carya illinoinensis*) pollen. *Journal of Proteomics*, *248*, 104348. https://doi.org/10.1016/J.JPROT.2021.104348

Moreno, F. J., Mellon, F. A., Wickham, M. S. J., Bottrill, A. R., & Mills, E. N. C. (2005). Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physiologically relevant in vitro gastrointestinal digestion. FEBS Journal, 272, 341–352. https://doi.org/10.1111/j.1742-4658.2004.04472.x.

Pablos, I., Wildner, S., Asam, C., Wallner, M., & Gadermaier, G. (2016). Pollen Allergens for Molecular Diagnosis. *Current Allergy and Asthma Reports*, *16*(4). https://doi.org/10.1007/s11882-016-0603-z

Popescu, F. D., & Vieru, M. (2018). Precision medicine allergy immunoassay methods for assessing immunoglobulin

E sensitization to aeroallergen molecules. *World Journal of Methodology*, *8*(3), 17. https://doi.org/10.5662/WJM.V8.I3.17

Raoufi, E., Hemmati, M., Eftekhari, S., Khaksaran, K., Mahmodi, Z., Farajollahi, M. M., & Mohsenzadegan, M. (2020). Epitope prediction by novel immunoinformatics approach: a state of the art review. *International Journal of Peptide Research and Therapeutics*, *26*(2), 1155-1163. https://doi.org/10.1007/s10989-019-09918-z

Radauer, C., Willerroider, M., Fuchs, H., Hoffmann-Sommergruber, K., Thalhamer, J., Ferreira, F., Scheiner, O. and Breiteneder, H. (2006), Cross-reactive and species-specific immunoglobulin E epitopes of plant profilins: an experimental and structure-based analysis. *Clinical & Experimental Allergy*, *36*: 920-929. https://doi.org/10.1111/j.1365-2222.2006.02521.x

Rihs, H. P., Chen, Z., Ruëff, F., Petersen, A., Rozynek, P., Heimann, H., & Baur, X. (1999). IgE binding of the recombinant allergen soybean profilin (rGly m 3) is mediated by conformational epitopes. *Journal of Allergy and Clinical Immunology*, *104*(6), 1293–1301. https://doi.org/10.1016/S0091-6749(99)70027-8

Saha, B., Sircar, G., Pandey, N., & Gupta Bhattacharya, S. (2015). Mining novel allergens from coconut pollen employing manual de novo sequencing and homology-driven proteomics. *Journal of Proteome Research*, *14*(11), 4823–4833. https://doi.org/10.1021/acs.jproteome.5b00657

Santos, A., & Van Ree, R. (2011). Profilins: Mimickers of allergy or relevant allergens? *International Archives of Allergy and Immunology*, *155*(3), 191–204. https://doi.org/10.1159/000321178

Saunders, S., & Platt, M. P. (2015). Oral allergy syndrome. *Current opinion in otolaryngology & head and neck surgery*, *23*(3), 230-234.

Scheurer, S, Wangorsch, A., Haustein, D., & Vieths, S. (2000). Cloning of the minor allergen Api g 4 profilin from celery (Apium graveolens) and its cross-reactivity with birch pollen profilin Bet v 2. *Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology*, *30*(7), 962–971. https://doi.org/10.1046/J.1365-2222.2000.00821.X

Scheurer, Stephan, Wangorsch, A., Nerkamp, J., Stahl Skov, P., Ballmer-Weber, B., Wüthrich, B., Vieths, S. (2001). Cross-reactivity within the profilin panallergen family investigated by comparision of recombinant profilins from pear (Pyr c 4), cherry (Pru av 4) and celery (Api g 4) with birch pollen profilin Bet v 2. *Journal of Chromatography B: Biomedical Sciences and Applications*, *756*(1–2), 315–325. https://doi.org/10.1016/S0378-4347(01)00090-1

Sharma, V., Gupta, R., Jhingran, A., Singh, B. P., Sridhara, S., Gaur, S. N., & Arora, N. (2006). Cloning, recombinant expression and activity studies of a major allergen "enolase" from the fungus *Curvularia lunata*. *Journal of Clinical Immunology*, *26*(4), 360–369. https://doi.org/10.1007/s10875-006-9032-4

Sun, N., Zhou, C., Zhou, X., Sun, L., & Che, H. (2014). *Use of a rat basophil leukemia (RBL) cell-based immunological assay for allergen identification, clinical diagnosis of allergy, and identification of anti-allergy agents for use in immunotherapy. Journal of Immunotoxicology, 12(2), 199–205.* doi:10.3109/1547691x.2014.920063

White, J. F., & Bemstein, D. I. (2003). Key pollen allergens in North America. *Annals of Allergy, Asthma and Immunology*, *91*(5), 425–435. https://doi.org/10.1016/s1081-1206(10)61509-8

Wiedemann, P., Giehl, K., Almo, S. C., Fedorov, A. A., Girvin, M., Steinberger, P., & Valenta, R. (1996). Molecular and structural analysis of a continuous birch profilin epitope defined by a monoclonal antibody. *Journal of Biological Chemistry*, *271*(47), 29915-29921.

5. CONCLUSIÓN

Este trabajo deja como contribución al conocimiento el primer reporte de proteínas alergénicas del polen de nogal pecanero *Carya illinoinensis*, se logró la sobreexpresión y la predicción de epítopes lineales y conformacionales de las primeras dos proteínas alergénicas de este polen. Una de ellas es la profilina, panalérgeno importante entre los pólenes de los árboles y alimentos frescos de origen vegetal, y la otra es la enolasa, enzima glucolítica reportada como alérgeno en diversas fuentes y no caracterizada en polen, respecto a la composición de sus epítopes lineales y conformacionales. Estos conocimientos sientan las bases para el desarrollo de estrategias inmunoterapéuticas futuras, como el desarrollo de proteínas hipoalergénicas.

6. RECOMENDACIONES

Se recomienda corroborar la alergenicidad de las proteínas recombinantes rProfilina y rEnolasa, para validar su equivalencia con sus contrapartes de la fuente natural. Una vez hecho esto puede considerarse en perspectivas futuras la evaluación de estos alérgenos recombinantes como herramienta diagnóstica y partiendo de las epítopes predichas se podría incursionar en el desarrollo de proteínas hipoalergénicas derivadas del polen de nogal pecanero.

BIBLIOGRAFÍA

Akinfenwa O, Rodríguez-Domínguez A, Vrtala S, Valenta R, Campana R. 2021. Novel vaccines for allergen-specific immunotherapy. Current Opinion in Allergy and Clinical Immunology. 21(1):86-99. doi: 10.1097/ACI.0000000000000706. PMID: 33369572; PMCID: PMC7810419.

Arruda-Chaves E. Pruebas diagnósticas en alergia y su utilidad clínica. 2004. Revista Médica Herediana. 15(2): 113-117.

Asero R., Mistrello G. and Amato S. 2016. Detection of pan-allergens in commercial pollen extracts for allergen immunotherapy. Annals of Allergy, Asthma & Immunology. 117(2): 180-185.

Bousquet J., Khaltaev N., Cruz A.A., Denburg J., Fokkens W.J., Togias A., Zuberbier T., Baena-Cagnani C.E., Canonica G.W., Van Weel C. and Agache I. 2008. Allergic Rhinitis and its Impact on Asthma (ARIA). Allergy. 63(86):8-160.

Bruhns P., Frémont S. and Daëron, M. 2005. Regulation of allergy by Fc receptors. Current Opinion in Immunology. 17(6): 662-669.

Calderón M., Cardona V. and Demoly P. 2012. EAACI 100 Years of Immunotherapy Experts Panel. One hundred years of allergen immunotherapy European Academy of Allergy and Clinical Immunology Celebration: Review of Unanswered Questions. Allergy. 67(4): 462-476.

Curin M., Khaitov M., Karaulov A., Namazova-Baranova L., Campana R., Garib V. and Valenta, R. 2018. Next-generation of allergen-specific immunotherapies: molecular approaches. Current Allergy and Asthma Reports. 18(7): 39.

Douladiris N., Garib V., Tejkl M.F., Valenta R., Papadopoulos N.G. and Linhart B. 2018. Detection of genuine grass pollen sensitization in children by skin testing with a recombinant grass pollen hybrid. Pediatric Allergy and Immunology. (Accepted/In press).

Fulton, K. M., Baltat, I., & Twine, S. M. 2019. Immunoproteomics Methods and Techniques. Immunoproteomics, 25–58. doi:10.1007/978-1-4939-9597-4_2

Guidos G.A. and Almeida V. M. 2005. Polinosis y aeroalérgenos. Alergia, Asma et Inmunología Pediátricas. 14(2): 52-55.

Hartmann, K., Escribano, L., Grattan, C., Brockow, K., Carter, M. C., Alvarez-Twose, I., and Valent, P. 2016. Cutaneous manifestations in patients with mastocytosis: consensus report of the European Competence Network on Mastocytosis; the American Academy of Allergy, Asthma & Immunology; and the European Academy of Allergology and Clinical Immunology. Journal of Allergy and Clinical Immunology, 137(1), 35-45.

Harwanegg C., Laffer S., Hiller R., Mueller M.W., Kraft D. and Spitzauer S. 2003. Microarrayed recombinant allergens for diagnosis of allergy. Clinical and Experimental Allergy. 33(1): 7–13.

Hiller R., Laffer S., Harwanegg C., Huber M., Schmidt W.M., Twardosz A. 2002. Microarrayed

allergen molecules: Diagnostic gatekeepers for allergy treatment. The FASEB Journal. 16(3): 414–416.

Janeway J.A., Travers P., Walport M., Shlomchik M.J. 2009. Immunobiology. The immune system in health and disease. 7th edition. Garland Publishing. USA.

Jeong K.Y., Son M., Park J.H., Park K.H., Park H.J., Lee J.H., Hong C.S. and Park J.W. 2016. Cross- reactivity between oak and birch pollens in korean tree pollinosis. Journal of Korean Medical Science. 31(8):1202-1207.

Johansson S. 2004. *ImmunoCAP® S*pecific IgE test: an objective tool for research and routine allergy diagnosis. Expert Review of Molecular Diagnostics. 4(3): 273–279.

Kay A.B. 2001. Allergy and allergic diseases. New England Journal of Medicine. 344(1): 30-37.

Khan, D.A. 2014. Allergic rhinitis and asthma: epidemiology and common pathophysiology. In Allergy & Asthma Proceedings (Vol. 35, No. 5).

Kniemeyer, O., Ebel, F., Krüger, T., Bacher, P., Scheffold, A., Luo, T., and Brakhage, A. A. 2016. Immunoproteomics of *Aspergillus* for the development of biomarkers and immunotherapies. PROTEOMICS–Clinical Applications, 10(9-10), 910-921.

Larenas Linnemann, D., Luna Pech, J. A., Rodríguez Pérez, N., Rodríguez González, M., Arias Cruz, A., Blandón Vijil, M. V., & Medina Ávalos, M. A. 2019. GUIMIT 2019, Guía Mexicana de Inmunoterapia. Guía de diagnóstico de alergia mediada por IgE e inmunoterapia aplicando el método ADAPTE. Revista Alergia México, 66(5), 1.

Lizaso M.T., García B.E., Gómez B., Zabalegui A., Rodríguez M.J. and Tabar A.I. 2003. Tratamiento de la alergia a hongos. In Anales del sistema sanitario de Navarra. 26: 129-137. Gobierno de Navarra. Departamento de Salud.

Mani B.M. 2015. Proteomic Analysis of Allergens in *Ligustrum lucidum*. Tesis Doctoral. Escuela Superior de Medicina, Sección de Estudios de Posgrado. Instituto Politécnico Nacional. México.

Mims J.W. 2017. Advancements and Dilemmas in the Management of Allergy. Otolaryngologic Clinics of North America. 50(6): 1037–1042.

Oh, J.W., 2018. Allergy and Pollen. In: Pollen Allergy in a Changing World. Springer, Singapore. 1-8 pp.

Pablos I., Wildner S., Asam C., Wallner, M. and Gadermaier G. 2016. Pollen allergens for molecular diagnosis. Current allergy and asthma reports. 16(4): 31.

Pipet A., Botturi K., Pinot D., Vervloet D. and Magnan A. 2009. Allergen-specific immunotherapy in allergic rhinitis and asthma. Mechanisms and proof of efficacy. Respiratory Medicine. 103(6): 800-812.

Pitarch, A., Nombela, C., & Gil, C. 2018. Diagnosis of invasive candidiasis: from gold standard methods to promising leading-edge technologies. Current Topics in Medicinal Chemistry, 18(16), 1375-1392.

Rachmihl, M., Verleger, H., Waisel, Y., Keynan, N., Kivity, S. and Katz, Y. 1996. The importance of the pecan tree pollen in allergic manifestations. Clinical & Experimental Allergy, 26(3), 323-329.

Reyes-Vázquez N.C. y Urrea-López R. 2016. Retos y oportunidades para el aprovechamiento de la nuez pecanera en México. CIATEJ. Primera edición. Guadalajara. 111 pp.

Rocha-Estrada A., Alvarado-Vázquez M.A., Torres-Cepeda T.E., Foroughbakhch-Pournavab R. and Hernández-Piñero J.L. 2008. Airborne pollen of *Carya*, *Celtis*, *Cupressus*, *Fraxinus* and *Pinus* in the metropolitan area of Monterrey Nuevo Leon, Mexico. Annals of Agricultural and Environmental Medicine. 15(2).

Saha B. and Bhattacharya S.G. 2017. Charting novel allergens from date palm pollen (*Phoenix sylvestris*) using homology driven proteomics. Journal of Proteomics. 165: 1-10.

Santos A. and Van Ree R. 2011. Profilins: mimickers of allergy or relevant allergens? International Archives of Allergy and Immunology. 155(3): 191-204.

Schiavoni G., D'Amato G. and Afferni, C. 2017. The dangerous liaison between pollens and pollution in respiratory allergy. Annals of Allergy, Asthma & Immunology. 118(3): 269-275.

Singh A.B. y Mathur C. 2012. An aerobiological perspective in allergy and asthma. Asia Pacific Allergy. 2(3): 210-222.

Sircar G., Jana K., Dasgupta A., Saha S. and Bhattacharya S.G. 2016. Epitope mapping of Rhi o 1 and generation of a hypoallergenic variant a candidate molecule for fungal allergy vaccines. Journal of Biological Chemistry. *291*(34): 18016-18029.

Toche, P. P. (2011). Anafilaxia. Revista Médica Clínica Las Condes, 22(3), 265-269.

Tulaeva, I., Kratzer, B., Campana, R., Curin, M., van Hage, M., Karsonova, A., & Valenta, R. 2020. Preventive allergen-specific vaccination against allergy: mission possible? Frontiers in Immunology, 11, 1368.

Vadlamudi A and Shaker M. 2015. New developments in allergen immunotherapy. Current Opinion in Pediatrics. 27(5): 649-655.

Valenta R., Campana R., Marth K. and Van Hage M. 2012. Allergen-specific immunotherapy: from therapeutic vaccines to prophylactic approaches. Journal of Internal Medicine. 272(2): 144-157.

Valenta R., Karaulov A., Niederberger V., Gattinger P., van Hage M., Flicker S., Pickl W.F. 2018. Molecular Aspects of Allergens and Allergy. Advances in Immunology. 195–256.

Valenzuela-Corral, A. 2018. Proteínas y glicoproteínas alergénicas del polen de *Quercus rubra* y *Carya illinoinensis*: Identificación mediante un enfoque inmunoproteómico. Tesis de maestría. Centro de Investigación en Alimentación y Desarrollo A. C. Hermosillo, Sonora.

Waisel Y., Keynan N., Gil T., Tayar D., Bezerano A., Goldberg A. and Levy I. 1994. Allergic responses to date palm and pecan pollen in Israel. Harefuah. 126(6): 305-310.

Wang D.W., Ni W.W., Zhou Y.J., Huang W., Cao M.D., Meng L. and Wei J. F. 2018. Expression, purification, and epitope analysis of Pla a 2 allergen from *Platanus acerifolia* pollen. Molecular Medicine Reports. 17(1): 394-399.

Wheatley, L. M., and Togias, A. 2015. Allergic rhinitis. New England Journal of Medicine, 372(5), 456-463.

White J.F. and Bernstein D.I. 2003. Key pollen allergens in North America. Annals of Allergy, Asthma & Immunology. 91(5): 425-435.