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

EFECTO DE LAS ALTAS PRESIONES HIDROSTÁTICAS Y DINÁMICAS SOBRE LAS ISOENZIMAS DEL NÉCTAR DE MANGO ATAULFO

Por:

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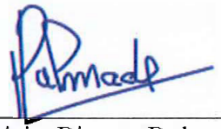

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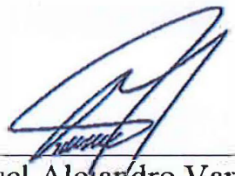

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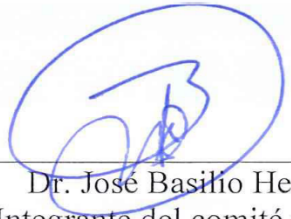

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RESUMEN

El deterioro de atributos de calidad como la textura y el color de productos a base de frutos se relaciona con la actividad de enzimas como pectinasas y oxidoreductasas. El procesamiento con altas presiones supone una alternativa a la pasteurización de bebidas frutales para reducir la actividad enzimática y extender la vida de anaquel. Sin embargo, los sistemas biológicos presentan múltiples isoenzimas con diferente grado de resistencia hacia la alta presión, reduciendo la eficiencia del procesamiento. Este trabajo se enfocó en estudiar éste fenómeno utilizando como modelo de estudio néctar de mango "Ataulfo", variedad endémica de México. El objetivo fue identificar las isoenzimas baroresistentes de enzimas de interés tecnológico, como pectin metilesterasa y polifenol oxidasa en néctar de mango "Ataulfo" procesado por altas presiones hidrostáticas y dinámicas. En los resultados obtenidos, se encontró que la alta presión hidrostática no tuvo un efecto de inactivación sobre las enzimas evaluadas, y en la mayoría de los tratamientos propició un incremento de actividad con respecto a la del control. La alta presión dinámica solo tuvo efecto en la reducción de la actividad de polifenol oxidasa, ya que la pectin metilesterasa resultó tolerante a los tratamientos por ambos tipos de alta presión. La enzima poligalacturonasa se descartó tras los primeros experimentos por su bajo nivel de actividad. Referente a la calidad, ambas tecnologías retuvieron de satisfactoriamente atributos como pH, sólidos solubles (°Brix), viscosidad y color. En el caso de color, la alta presión dinámica incrementó los valores de luminosidad (L*) y b*, en cambio la presión hidrostática incrementó los valores de a*. En cuanto a viscosidad, ambas tecnologías modificaron la viscosidad aparente del néctar, el cual se comportó como fluido no-newtoniano. Finalmente, la utilización de zimografía en una dimensión, se aplicó a la enzima más resistente, que fue pectin metilesterasa. Se identificaron dos isoenzimas denominadas PME1 y PME2 con pesos molecularesde 66 y 33 kDa respectivamente. La isoenzima PME2, se encontró en todos los tratamientos evaluados salvo el de 100 MPa por 5 min en presión hidrostática, cuyo valor de actividad enzimática relativa fue el más bajo (72.6%). Esta información permitió contribuir mediante la generación de conocimiento para entendimiento del fenómeno de baroresistencia de enzimas y comparar el efecto de ambos tipos de alta presión sobre un mismo modelo de estudio. Además, el estudio del fenómeno a nivel proteómico permitirá replicar la información a otras matrices alimenticias para optimizar su preservación por altas presiones.

Palabras claves: Alta presión hidrostática, Alta presión dinámica, Actividad enzimática, Atributos de calidad, Baroresistencia, Néctar de mango

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ABSTRACT

The deterioration of quality attributes, such as the texture and color of fruit-based products, is related to the activity of enzymes such as pectinases and oxidoreductases. High-pressure processing offers an alternative to the pasteurization of fruit beverages to reduce enzyme activity and extend shelf life. However, biological systems present multiple isoenzymes with different degrees of resistance to high pressure, reducing processing efficiency. This work focused on studying this phenomenon using "Ataulfo" mango nectar as a study model, a variety endemic to Mexico. The study aimed to identify the baroresistant isoenzymes of enzymes of technological interest, such as pectin methylesterase, and polyphenol oxidase in "Ataulfo" mango nectar processed by high hydrostatic and dynamic pressures. The results showed that high hydrostatic pressure did not have an inactivating effect on the enzymes evaluated, and in most of the treatments, it increased the activity compared to that of the control. High dynamic pressure only affected the reduction of polyphenol oxidase activity, since pectin methylesterase was tolerant to the treatments by both types of high pressure. The polygalacturonase enzyme was discarded after the first experiments because of its low activity level. Regarding quality, both technologies successfully retained attributes such as pH, soluble solids (°Brix), viscosity, and color. In the case of color, high dynamic pressure increased the values of lightness (L*) and b*, while hydrostatic pressure increased the values of a*. In terms of viscosity, both technologies modified the apparent viscosity of the nectar, which behaved as a non-Newtonian fluid. Finally, the use of one-dimensional zymography was applied to the most resistant enzyme, which was pectin methylesterase. Two isoenzymes named PME1 and PME2 of molecular weights of 66 and 33 kDa, respectively, were identified. The PME2 isoenzyme was present in all the evaluated treatments, except 100 MPa for 5 min under hydrostatic pressure, whose relative enzymatic activity value was the lowest (72.6%). This information made it possible to contribute by generating knowledge for understanding the phenomenon of enzyme baroresistance and to compare the effect of both types of high pressure on the same study model. In addition, the study of the phenomenon at the proteomic level will allow the replication of the information to other food matrices to optimize their preservation under high pressure.

Key words: High hydrostatic pressure, High dynamic pressure, Enzyme activity, Quality attributes, Baroresistance, Mango nectar.

1. SINOPSIS

El procesamiento por altas presiones es una tecnología emergente no térmica (moderadamente térmica en el caso de alta presión dinámica) para la preservación de alimentos que representa una alternativa atractiva para su aplicación en bebidas frutales suaves como método para garantizar su inocuidad microbiológica y vida de anaquel, sin afectar de manera adversa la composición nutrimental, contenido de compuestos fitoquímicos y atributos sensoriales del alimento.

El efecto letal de este procesamiento sobre microorganismos patógenos y deteriorativos ha sido estudiado y demostrado en alimentos ácidos y acidificados (pH < 4.6) como lo son los jugos y néctares elaborados a base de diversos frutos. Sin embargo, su efectividad en cuanto a la reducción de la actividad de algunas enzimas relacionadas con el deterioro durante el almacenamiento de atributos de calidad del producto como la viscosidad, color y separación de fases, ha generado resultados diversos. Esto ha sido atribuido a la presencia de isoformas de las enzimas, las cuales pueden presentar diversos grados de tolerancia a las altas presiones (barotolerancia), requiriendo el efecto sinérgico del calor para elevar la temperatura del producto y conseguir los niveles de inactivación necesarios para que el producto mantenga sus atributos de calidad por el mayor tiempo posible. Se ha estipulado, que cuando el producto alcanza temperaturas mayores a 60 °C en los sistemas de procesamiento por altas presiones, el efecto letal sobre microorganismos y de inactivación de enzimas debe ser atribuido principalmente al calor, en sinergia con el efecto de la presión.

Esto genera un área de oportunidad referente al entendimiento del mecanismo de inactivación enzimática por efecto de la presión. Es por ello, que resulta necesario identificar y estudiar las isoenzimas barotolerantes de pectinmetil esterasa y polifenoloxidasa con el propósito de contribuir al conocimiento del mecanismo de su inactivación mediante procesamiento por altas presiones hidrostáticas y dinámicas usando como modelo néctar de mango ‘Ataulfo’. Este conocimiento generado podrá ser posteriormente aplicado para generar modelos tridimensionales que permitan ver el efecto que tiene la presión sobre la conformación estructural de las enzimas, lo cual puede

ser aprovechado en futuras investigaciones para el diseño de procesos óptimos que garanticen tanto la seguridad microbiológica, como la estabilidad durante el almacenamiento de este tipo de bebidas frutales.

1.1. Justificación.

El Plan Nacional Agrícola 2017-2030 para mango mexicano, contempla dentro de sus puntos estratégicos la transformación y otorgamiento de valor agregado al mango mexicano mediante tecnologías innovadoras, especialmente para el mango producido en las zonas del pacífico sur mexicano, donde la variedad 'Ataulfo', sobresale al tener denominación de origen en el soconusco chiapaneco. De las tecnologías emergentes de preservación con mayor aceptación entre la comunidad científica e industria alimentaria, se encuentran las altas presiones hidrostáticas y existe un interés creciente en las altas presiones dinámicas.

El procesamiento por altas presiones hidrostáticas y dinámicas ha cobrado importancia para procesamiento bebidas frutales por sus ventajas con respecto a los procesos térmicos, que son el estándar de la industria alimentaria, en cuanto a la retención de nutrientes, características sensoriales y prolongada vida de anaquel se refiere. Además, estas tecnologías emergentes de procesamiento tienen la función de asegurar la inocuidad del producto y conferirle estabilidad ante cambios que pudieran ocurrir durante el almacenamiento, sin despreciar que son también amigables con el medio ambiente. En productos alimenticios a base de frutos, como jugos y néctares, se presentan cambios indeseables debidos a la actividad de enzimas como pectinmetil esterasa y polifenol oxidasa que ocasionan modificaciones en la textura, turbidez y color del producto respectivamente.

Los estudios realizados sobre el efecto de las altas presiones en sus dos vertientes sobre la actividad de estas enzimas han arrojado resultados contrastantes, recurriendo en algunos casos a temperaturas mayores a 60 °C para reducir la actividad enzimática lo cual de acuerdo con algunos autores, convierte al calentamiento en el principal mecanismo de inactivación enzimática en lugar de la

presurización. También existe discrepancia en la literatura científica entre los resultados que reportan algunos autores, ya que mientras un grupo reporta inactivación de enzimas por altas presiones en una matriz alimenticia, otro grupo reporta resultados contrarios en la misma matriz. Los estudios que logran reducir actividad enzimática en base al efecto de la presión, se limitan a reportar el porcentaje de reducción, sin profundizar en el efecto de la presión para obtener información a nivel molecular sobre las enzimas o la resistencia hacia la presión de las mismas.

Es debido a esto, que es necesario hacer estudios que profundicen en el efecto de la presión como la única fuerza mecánica actuando sobre la reducción de la actividad enzimática e identificar las causas de la resistencia de éstas hacia la alta presurización.

1.2. Antecedentes

1.2.1. Principales Retos de la Industria Alimentaria para Preservación de Alimentos

Uno de los retos históricos a los cuales se ha enfrentado la industria del procesamiento de alimentos, ha sido el de conferirle suficiente vida de anaquel y estabilidad durante toda su cadena de suministro a los alimentos procesados. En el caso específico de los alimentos procesados a base de frutos y hortalizas, el asegurar la disponibilidad de los mismos en el mercado cuando los ciclos de producción agrícola han finalizado, es otro reto adicional relacionado con la vida de anaquel (Bevilacqua *et al.,* 2019; Singh & Anderson, 2004). Una de las tecnologías de preservación de alimentos con mayor relevancia y prevalencia en la actualidad, es el tratamiento térmico (pasteurización y esterilización comercial, principalmente), que ha sido adoptado por la industria alimentaria como mecanismo de seguridad para retardar el mayor tiempo posible el deterioro de alimentos procesados (Misra *et al.*, 2017). Actualmente, el reto para la industria de procesamiento de alimentos, son las exigencias de los consumidores, quienes demandan productos menos procesados, sin aditivos, a precios competitivos y con suficiente vida de anaquel (Nychas & Panagou, 2011). Con el crecimiento exponencial del desarrollo tecnológico, el interés se ha centrado sobre las denominadas tecnologías emergentes para preservación de alimentos, las cuales

tienen como principal objetivo la disminución de los impactos negativos del tratamiento térmico sobre los atributos del alimento, pero logrando el mismo nivel de estabilidad e inocuidad. Desafortunadamente, la vida de anaquel de los alimentos no es indeterminada, todos los alimentos se deterioran durante su almacenamiento, independientemente de la tecnología que se utilice para preservarlos.

1.2.2. Mecanismos de Deterioro de los Alimentos

Es posible definir el deterioro de un alimento como cualquier proceso que conduzca a la pérdida de la seguridad, calidad sensorial o valor nutricional del mismo, de tal manera que ya no es aceptable para el consumo. Con base a esta definición, existen al menos tres tipos de mecanismos mediante los cuales los alimentos pueden deteriorarse: físico, microbiológico y químico.

1.2.2.1. Mecanismo de deterioro físico. En el caso del deterioro físico, es más fácil de apreciar en alimentos sólidos, en los cuales el daño mecánico puede ocasionar ruptura y pérdida de la forma e integridad del producto. Además, pueden estar relacionados otros fenómenos que alteran la estructura de los alimentos, como pueden ser la cristalización de azúcares, la separación de emulsiones y el colapso de geles (Berk, 2018). Si el deterioro físico es muy severo en el alimento, es altamente probable que sea rechazado por el consumidor.

1.2.2.2. Mecanismo de deterioro microbiológico. Por otra parte, el deterioro microbiológico es ocasionado por el desarrollo de microorganismos, que pueden ser patógenos o no, y que constituyen un riesgo tanto por el consumo de alimentos contaminados o por los cambios que ocasionan a las propiedades de mismo. En lo que respecta a pérdidas económicas por deterioro de alimentos, el ocasionado por los microorganismos es el de mayor impacto (Nychas & Panagou, 2011). Es por ello que se considera el tipo de deterioro más importante en los alimentos, ya que no solo afecta la calidad, sino la seguridad también, ya que, en este tipo de mecanismo, la actividad y/o presencia

de microorganismos en el alimento puede causar deterioro y enfermedades (Singh & Anderson, 2004).

1.2.2.3. Mecanismo de deterioro químico. El mecanismo de deterioro químico, es el que ocurre debido a cambios en los componentes dentro del mismo alimento (Berk *et al.,* 2018; Hernández *et al.,* 2018). Existen diversas reacciones químicas que ocurren entre los componentes de los alimentos, las cuales están catalizadas por enzimas, y se relacionan con el deterioro de atributos de calidad, sensoriales e incluso de compuestos bioactivos. Al enfocarse en el néctar de mango como modelo de estudio, el cual es una bebida frutal, las principales enzimas relacionadas al deterioro de calidad en este tipo de productos destacan las pectinasas (como pectin metilesterasa y poligalacturonasa), asociadas a la pérdida de la nube y turbidez, así como las oxidoreductasas (como polifenol oxidasa), asociadas a reacciones de oscurecimiento (Vásquez-Caicedo *et al.,* 2010; Jolie *et al.,* 2010; Sulaiman *et al.,* 2015).

1.2.3. Enzimas Relacionadas al Deterioro de Calidad en Alimentos de Origen Vegetal

En el caso de alimentos y bebidas a base de frutos, las enzimas endógenas constituyen uno de los criterios de control de calidad más importantes para garantizar la estabilidad del producto durante la vida de anaquel. En los frutos enteros, las enzimas y sus sustratos se encuentran regularmente separados en compartimientos a nivel celular, los cuales se pierden al momento de procesar mecánicamente los frutos para obtener puré de pulpa y jugo. Una vez que las enzimas entran en contacto son sus sustratos, puede ocurrir un deterioro de atributos de calidad en productos alimenticios a base frutos, entre los cuales destacan deterioro de color, sabor, valor nutricional, textura y viscosidad (Augusto *et al.,* 2017; Chakraborty *et al.,* 2014).

Entre las principales enzimas que se consideran deteriorantes de la calidad en este tipo de alimentos, se encuentran las pectinasas como pectin metilesterasa (PME), poligalacturonasa (PG), y oxidoreductasas como polifenol oxidasa (PPO).

1.2.3.1. Pectin metilesterasa. De acuerdo con el criterio de clasificación del Comité de Enzimas (EC), la pectin metilesterasa se encuentra registrada con el número de clasificación EC 3.1.1.11 en función de la reacción que cataliza. Pertenece al grupo de las hidrolasas que actúan sobre enlaces estéricos y su nombre aceptado es "Pectin esterasa", teniendo como nombres alternativos: pectin desmetoxilasa, pectin metoxilasa y pectin metilesterasa.

La reacción que cataliza es:

$$Pectina + n\ H_2O = n\ Metanol + Pectato$$

PME está clasificada como una hidrolasa que actúa sobre enlaces estéricos, específicamente ésteres carboxílicos y estructuralmente, se cataloga como una proteína del tipo β-hélice, ya que cuenta con una estructura media formada por tres β-láminas paralelas cuyo espacio interior es hidrofóbico (Figura 1). El sitio de unión a la pectina se encuentra al costado izquierdo formando una hendidura accesible a los solventes, la cual se constituye de aminoácidos aromáticos como triptófano, fenilalanina y tirosina principalmente. El sitio activo se encuentra dentro de la hendidura y en el intervienen dos residuos de ácido aspártico, dos residuos de glutamina y un residuo de arginina. En base a la estructura cristalina de la PME de zanahoria, se propuso el mecanismo de acción de la enzima. Este implica que uno de los residuos de ácido aspártico forma un enlace mediante puente de hidrógeno con el residuo de arginina el cual, a su vez, ejerce un ataque nucleofílico sobre la unión éster del carboximetilo en la molécula de homogalacturonano (pectina). Los residuos de glutamina dan estabilidad al compuesto intermediario cargado negativamente y el residuo de ácido aspártico restante actúa como donador de protones para la liberación de metanol (Johansson *et al.*, 2002; Jolie *et al.*, 2010; Pelloux *et al.*, 2007).

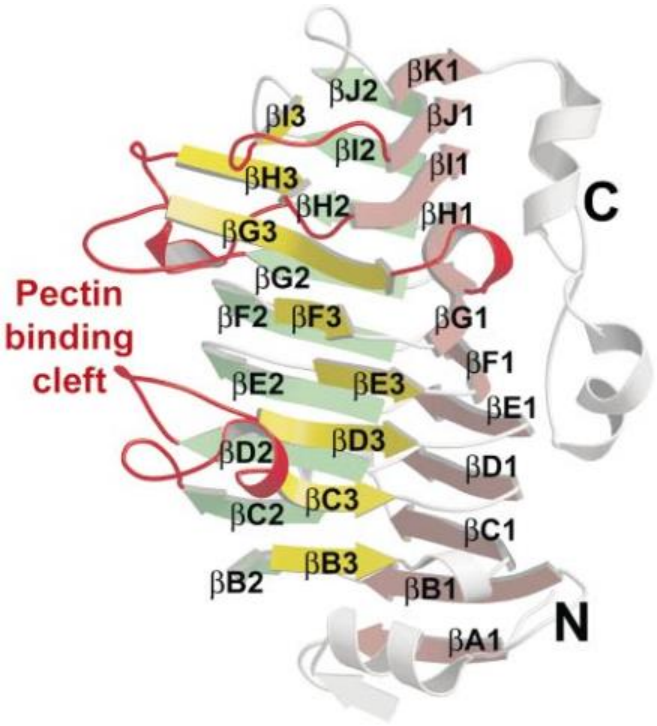

Figura 1. Estructura tridimensional de PME de zanahoria (Tomado de Jolie *et al.*, (2010)).

1.2.3.2. Polifenol oxidasa. La enzima polifenol oxidasa se clasifica con el número EC 1.10.3.1, encontrándose en el grupo de las oxidoreductasas que actúan sobre difenoles y sustancias relacionadas como donadores que tienen al oxígeno como aceptor, es conocida también como catecol oxidasa.

La polifenol oxidasa cataliza la conversión de difenoles en o-quinonas (Figura 2):

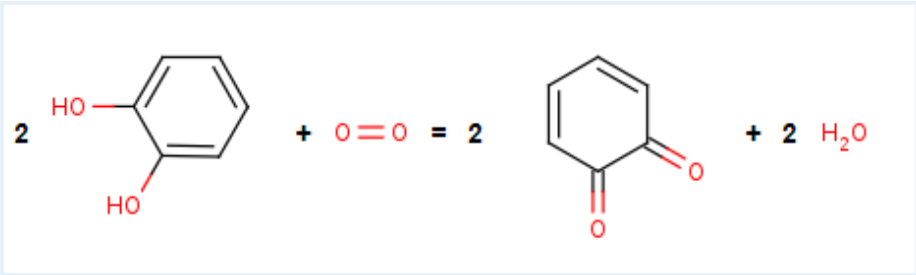

Figura 2. Reacción enzimática catalizada por polifenol oxidasa. Tomado de (*Www.Brenda-Enzymes.Org*, 2022)

Así mismo, existe otra enzima a la cual también se le llama polifenol oxidasa, ésta tiene el código EC 1.14.18.1 y se denomina tirosinasa. La diferencia entre polifenol oxidasa (Catecol oxidasa) y tirosinasa, es que PPO no tiene actividad monofenolasa ni monofenoloxigenasa como tirosinasa. Es debido a esto, que tirosinasa también es considerada como partícipe del oscurecimiento enzimático ya que tiene la finalidad de adicionar grupos OH a monofenoles para convertirlos en difenoles, sobre los cuales actúa catecol oxidasa, para dar inicio a la serie de eventos que desencadenan el oscurecimiento enzimático (Mayer, 2006).

Polifenol oxidasa pertenece a la familia de las cuproproteinas tipo 3 las cuales se caracterizan por tener un sitio activo binuclear compuesto por dos átomos de cobre, cada uno coordinado por tres residuos de histidina conservados entre secuencias (Aguilera *et al.*, 2013). La secuencia de aminoácidos, es la que le otorga diferencias a cada una de las proteínas que pertenecen a esta familia (por ejemplo, la hemocianina en crustáceos y moluscos) ya que de ellos depende su unión al sustrato y la accesibilidad del mismo al sitio activo.

La molécula de polifenol oxidasa tiene dos β-láminas paralelas, y el sitio activo donde se ubican los átomos de cobre se encuentra dentro de un conjunto de cuatro hélices (Figura 3).

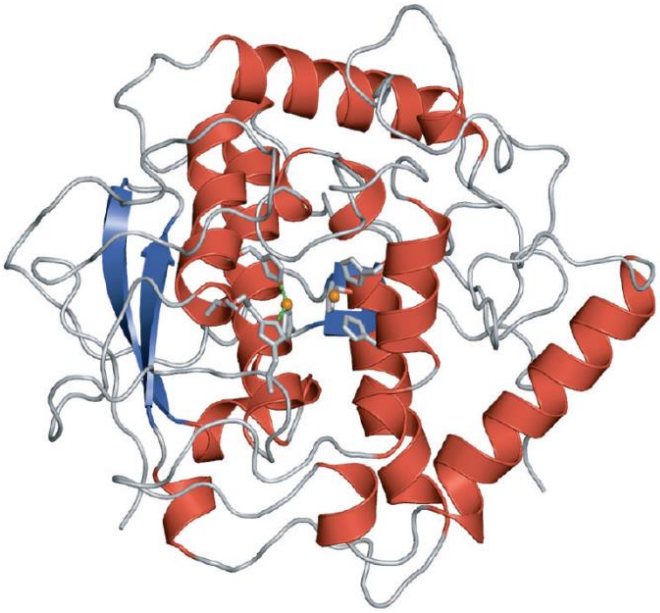

Figura 3. Estructura molecular de polifenol oxidasa de *Vitis vinifera* (Tomado de Virador *et al.*, (2010)).

Una de las propuestas sobre el mecanismo de acción de PPO a nivel molecular contempla que el sitio activo presenta tres estados de oxidación: met-PPO (Cu^{+2}), deoxi-PPO (Cu^{+2}) y oxi-PPO (Cu^{+2}). Se considera que el estado normal es met-PPO (Cu^{+2}), el cual se reduce a la forma deoxi-PPO (Cu^{+2}) con una molecula de difenol y se combina con O_2 para formar un complejo o-difenol-PPO, después, el difenol se oxida a su respectiva o-quinona y PPO se reduce a su forma met-PPO (Cu^{+2}) (Mayer, 2006; Taranto *et al.*, 2017; Yoruk & Marshall, 2003).

1.2.3. Tecnologías Convencionales de Preservación de Alimentos

De manera tradicional en la industria alimentaria, las enzimas catalizadoras de reacciones de deterioro se han controlado mediante la aplicación de tratamientos térmicos. En el caso de productos industrializados a base de jugo y pulpa de frutos, los cuales se catalogan como alimentos de alta acidez (pH < 4.6), los procesos térmicos leves son la estrategia tecnológica más utilizada en la industria para asegurar la calidad microbiológica y vida de anaquel estable a temperatura ambiente de los productos alimenticios, pero causan un deterioro en los atributos sensoriales y afectan la calidad nutricional de los mismos (Miller & Silva, 2012). Actualmente, las principales tecnologías para conservación en base a la alta temperatura son la esterilización comercial y la pasteurización. La esterilización comercial es un proceso térmico severo donde el producto final no debe tener células vegetativas ni esporas viables para desarrollarse bajo condiciones normales de distribución y almacenamiento (Augusto *et al.*, 2018). La pasteurización es un proceso térmico moderado que se utiliza para inactivar bacterias, levaduras y hongos que son relativamente sensibles al calor y ocasionan el deterioro de alimentos. Además, mediante la pasteurización se inactivan enzimas como polifenol oxidasa, lipooxigenasa, peroxidasa, pectinmetil esterasa (que son responsables también de la disminución de la calidad de los alimentos) ya que a temperaturas mayores a 50°C, se induce el desdoblamiento molecular y agregación amorfa de las enzimas, lo cual es irreversible, afectando su actividad catalítica (Ağçam *et al.*, 2017; Dirix *et al.*, 2005).

A pesar de que los tratamientos térmicos para procesamiento de frutos en la industria de alimentos son muy confiables en cuanto al aseguramiento de la inocuidad y vida de anaquel de los productos,

se ha comprobado que dichos procesos térmicos conducen a una pérdida de compuestos bioactivos y nutrimentos en los alimentos tratados (Kaushik *et al.,* 2014; Alline Artigiani Lima Tribst *et al.,* 2011). Esto, sumado a que en tiempos recientes los consumidores demandan productos alimenticios que sean más frescos, con alta calidad, valor nutricional y de preferencia de fácil uso, ha propiciado la búsqueda de tecnologías alternativas de procesamiento que maximicen el contenido de nutrientes, compuestos bioactivos y resulten en productos estables (Misra *et al.,* 2017; Rawson *et al.,* 2011). Esto ha impulsado a la investigación científica para adopción de las denominadas tecnologías emergentes no térmicas para la preservación de alimentos, dentro de las cuales, una que ha contado con mayor grado de adopción, explotación y crecimiento es el procesamiento por altas presiones.

1.2.4. Tecnologías Emergentes de Preservación de Alimentos con Base a Presión

Las tecnologías emergentes son aquellas que se encuentran en desarrollo o que podrían desarrollarse en un periodo de cinco a diez años y que podrían tener impactos económicos, sociales y ambientales significativos (Misra *et al.,* 2017). Sin embargo, muchas tecnologías emergentes requieren décadas para su adaptación y explotación a nivel comercial. En el caso específico del procesamiento de alimentos por altas presiones hidrostáticas y la homogeneización por altas presiones (conocida también como altas presiones dinámicas), son tecnologías emergentes que han sido científicamente evaluadas y se encuentran disponibles comercialmente (Yamamoto, 2017; Zamora & Guamis, 2015). Aunque ambos sistemas de procesamiento tienen como principio la presurización del producto alimenticio, sus fundamentos son diferentes.

1.2.4.1. Procesamiento por altas presiones hidrostáticas. El procesamiento de alimentos por altas presiones hidrostáticas cobró popularidad en Japón a partir de 1990, debido a su efecto similar al de la pasteurización, logrando inactivar microorganismos al destruir su membrana celular, incrementando la vida de anaquel de los productos alimenticios (C. Y. Wang *et al.,* 2016). Este proceso de preservación se basa en el principio de compresión isostática (presión constante) y el

principio de Le Chatelier. El primero hace referencia a que la transmisión de la presión se realiza de manera uniforme e instantánea independientemente de la geometría y tamaño del alimento. El segundo hace referencia a que cualquier fenómeno en equilibrio, ya sea reacción química, transición de fases o cambio en la configuración molecular está acompañado por un decremento en el volumen, lo cual puede ser mejorado por la presión hidrostática (Augusto *et al.,* 2017; Picart-Palmade *et al.,* 2019). Los sistemas de procesamiento por altas presiones hidrostáticas consisten en cámaras aisladas o confinadas (Figura 4), enchaquetadas o no, donde se pueden procesar cualquier tipo de alimentos independientemente de su tamaño, composición, estado de agregación y geometría debido al principio de compresión isostática (Chawla *et al.*, 2011).

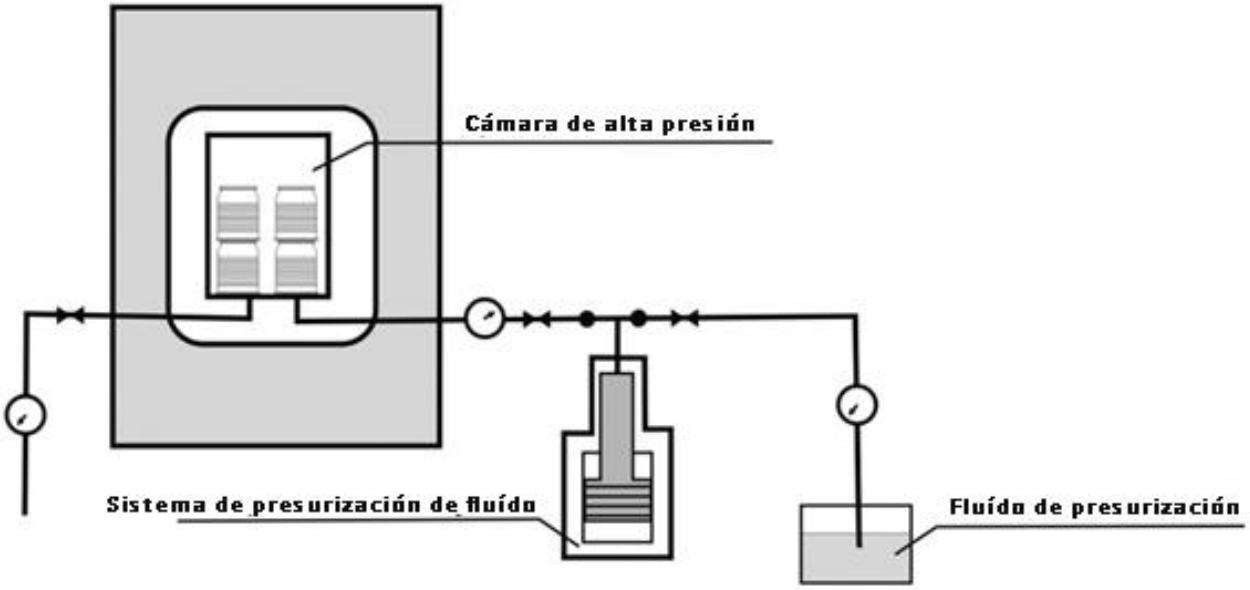

Figura 4. Representación esquemática de un sistema de procesamiento por altas presiones hidrostáticas (Adaptado de Augusto *et al.,* (2017)).

Por lo general, no hay intercambio de calor entre el sistema y sus alrededores, por lo cual el proceso se considera adiabático. Dentro de la cámara, el alimento se encuentra rodeado por un fluido transmisor de la presión (agua, aceite y propilenglicol, entre otros) el cual puede reutilizarse entre procesos, por lo que es una tecnología amigable con el medio ambiente. Una vez que inicia el proceso, se comprime el fluido ya sea con un sistema manual de pistones para incrementar la presión o mediante bombas o intensificadores. La presión se incrementa a razón aproximada de

100 MPa/min hasta alcanzar el estado isoestático de la presión de trabajo, la cual, puede retenerse por tiempo indeterminado. Generalmente los tiempos de residencia varían entre 1–15 min. La liberación de la presión al finalizar el proceso es instantánea y genera una reducción de temperatura en el sistema, por lo que el alimento puede tener una temperatura final menor que su temperatura inicial si el proceso se efectuó en condiciones de estado adiabático (Balasubramaniam *et al.,* 2015).

Esta tecnología de procesamiento presenta varias ventajas comparada con los tratamientos térmicos convencionales. Entre las más destacadas se encuentra la retención del valor nutrimental, nutraceútico y sensorial de los alimentos (Rawson *et al.,* 2011). También, la logística de operaciones en las industrias se ve optimizada, ya que los tiempos de procesamiento son muy cortos, en cuestión de unos minutos se puede procesar un lote completo de producto (Dependiendo el sistema) a comparación de un tratamiento térmico como pasteurización o esterilización donde el calentamiento y enfriamiento pueden tardar varios minutos y comprometer la calidad del alimento. Desafortunadamente el costo de inversión de estos sistemas es muy elevado, se considera que los productos conservados con estos sistemas tienden a catalogarse como productos 'Premium' o tienen nichos de mercado muy exclusivos. En Japón, existen una gran variedad de productos preservados por altas presiones en los mercados, desde jugos de frutas, a productos cárnicos y mariscos (Yamamoto, 2017). Por otra parte, empresas como Coca-Cola y Starbucks, han invertido en marcas como Suja y Evolution Fresh, respectivamente, para competir en el mercado de jugos de frutas prensados en frío conservados por APH. Otro de los productos de gran popularidad es el guacamole tratado por APH, como el de la marca Good Foods. En el caso de Sinaloa, la empresa Grupo Agrícola Doble RR cuenta con dos sistemas de APH para procesar toda una línea de productos alimenticios desarrollados internamente.

1.2.4.2. Procesamiento por altas presiones dinámicas. Por otra parte, una de las tecnologías de efecto térmico moderado para procesamiento de alimentos con mayor potencial comercial y de investigación, es el procesamiento por altas presiones dinámicas, la cual es una vertiente del procesamiento por altas presiones (Picart-Palmade *et al.,* 2019; Velázquez-Estrada *et al.,* 2013). El procesamiento por altas presiones dinámicas, también conocido como homogeneización por altas presiones (HAP), es un proceso físico continuo, moderadamente térmico, aplicado a alimentos

fluidos, que fue introducido en 1980 en el área de alimentos con el propósito de mejorar la eficiencia de homogeneización y emulsificación de productos lácteos y emulsiones (Diels & Michiels, 2006). El principio de operación de los sistemas de homogeneización por altas presiones dinámicas consiste en alcanzar un alto nivel de presión (150 – 400 MPa) en tan solo segundos en el fluido procesado, para después forzar su flujo a través de un orificio muy pequeño, el cual corresponde a la brecha de la válvula de alta presión del sistema, las cuales tienen pocos micrómetros de diámetro interno (Figura 5).

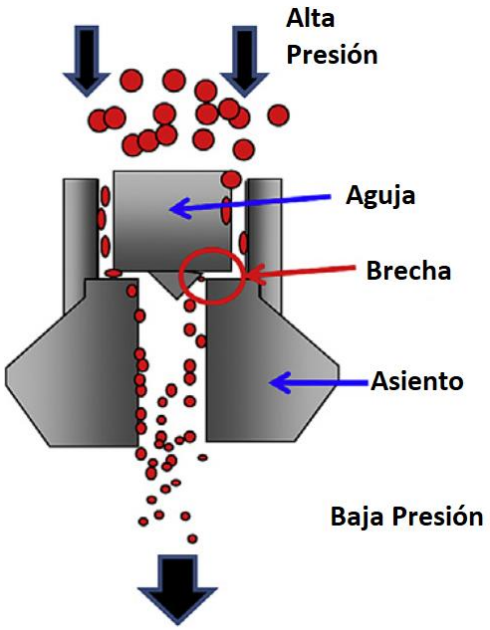

Figura 5. Esquematización de la válvula de alta presión del sistema de homogeneización por altas presiones (Adaptado de Dumay *et al.*, (2013)).

Esto ocasiona una caída de presión que genera intensas fuerzas mecánicas y estrés de elongación en flujo laminar a la entrada de la válvula, y turbulencia, cavitación e impactos con superficies sólidas a la salida de la válvula, las cuales son acompañadas de un incremento en la velocidad del fluido y calentamiento adiabático (1.5 a 2.5°C por cada 10 MPa) por efecto de la intensa fricción (Dumay *et al.*, 2013; Zamora & Guamis, 2015). Finalmente, son estos efectos mecánicos los que ocasionan disrupción en las partículas del fluido, traduciéndose en inactivación de microorganismos y modificación de los componentes constituyentes del alimento (de Castro Leite Júnior *et al.*, 2017).

En el cuadro 1 se integran los resultados de diversos estudios que evaluaron la efectividad del procesamiento por altas presiones hidrostática y dinámicas para inactivar microorganismos tanto patógenos como asociados al deterioro de la calidad en alimentos se origen frutal, demostrando la capacidad de preservación de alimentos de ambas tecnologías.

Cuadro 1 – Efecto de las altas presiones hidrostáticas y dinámicas sobre grupos microbiológicos de interés para la conservación de alimentos de origen frutal.

Sistema de presurización	Matriz alimenticia y grupo microbiológico de interés	Condiciones de proceso	Efecto sobre grupo microbiológico de interés	Referencia
Hidrostática	Cuenta total bacteriana, hongos y levaduras, coliformes en jugo de caña	600 MPa, 6 min, 60°C	Inactivación total de todos los grupos microbiológicos de interés	Chauhan *et al.*, (2017)
Hidrostática	Hongos y levaduras, mesófilos aerobios, coliformes, bacterias ácido lácticas, psicotrofos en pulpa de mango	600 MPa, 5 min, 40°C	<10 UFC/g en todos los grupos evaluados de microflora natural de la pulpa de mango	Kaushik *et al.*, (2014)
Hidrostática	*E. coli* en néctar de mango	275, 345 y 414 MPa, 1, 2 y 4 min, 17°C	3.5 log de reducción a 275 MPa 7 log de reducción a 345 MPa 8 log de reducción a 414 MPa	Bermudez-Aguirre *et al.*, (2011)
Hidrostática	Hongos y levaduras, bacterias aeróbicas totales en puré y jugo de fresa	400-600 MPa, 1.5 y 3 min, 20°C	Valores debajo de límite de detección en UFC/g en todos los tratamientos	Aaby *et al.*, (2018)
Dinámica	*Aspergillus niger* en néctar de mango	100 - 300 MPa	6.24 log de reducción a 300 MPa	Tribst *et al.*, (2009)
Dinámica	Bacterias mesofílicas totales en jugo de plátano	150-400 MPa a 4°C*	<1 UFC/mL en tratamientos de 200 a 400 MPa	Calligaris *et al.*, (2012)
Dinámica	Cuenta total bacteriana, hongos y levaduras en jugo de mango	40 – 190 MPa, 1 – 5 circulaciones, 20-60°C*	Inactivación de la cuenta total bacteriana a 190 MPa, 1-3 pasadas y 60°C <2.0 $\log_{10}$ UFC/mL de hongos y levaduras	Guan *et al.*, (2016)
Dinámica	E. coli 0157:H7 no patogénica en jugo de durazno	80 MPa a 45°C*	5.03 log UFC/mL de reducción	Yildiz, (2019)

*Temperatura de entrada del producto al sistema

1.2.5.- Mecanismos de Inactivación Enzimática por Altas Presiones

Además de la inactivación de microorganismos, el procesamiento por altas presiones hidrostáticas y dinámicas han sido estudiados por su capacidad de inactivar enzimas endógenas (pectinmetilesterasa, polifenoloxidasa, peroxidasa) en bebidas a base de frutas y hortalizas para

garantizar la estabilidad de las mismas durante el almacenamiento (Chakraborty *et al.,* 2015). A nivel molecular, la alta presión hidrostática (<1 GPa) no ocasiona cambios drásticos en las estructuras químicas de las moléculas de los alimentos, ya que solo afecta enlaces no covalentes (Fuerzas de Van der Waals, puentes de hidrógeno e interacciones electrostáticas), por lo cual muchas biomacromoléculas y compuestos bioactivos son conservados casi en su totalidad después del procesamiento (Martín-Belloso *et al.,* 2014; Mújica-Paz *et al.,* 2011; Rojas *et al.,* 2020). Teóricamente, el efecto reductor de la actividad catalítica de las altas presiones hidrostáticas sobre las enzimas se debe a que ocasiona alteraciones a nivel de estructura por la reducción del volumen molecular. Esto resulta en exposición del núcleo hidrofóbico de la proteína, aunado al incremento de la hidrofobicidad superficial, lo que lleva a la desnaturalización. En cambio, la alta presión dinámica incrementa la exposición de grupos sulfhidrilos (SH), promoviendo la formación de nuevos enlaces disulfuro y desdoblamiento de la enzima, además, afecta las proporciones de configuraciones de α-hélice y lámina-β de la estructura secundaria y reduce el número de puentes de hidrógeno intermoleculares (de Castro Leite Júnior *et al.,* 2017; Iqbal *et al.,* 2019; Liu *et al.,* 2009; Alline Artigiani Lima Tribst *et al.,* 2014, 2017).

1.2.6. Efecto de la Altas Presiones Sobre Actividad Enzimática

En la actualidad, los estudios enfocados a evaluar y explicar las alteraciones que ocasionan las altas presiones a nivel estructural en las enzimas son limitados, debido posiblemente a la complejidad y diversidad en su estructura y capacidad de reaccionar a la presurización. Esto cobra importancia ya que se ha reportado que, de manera natural, los sistemas biológicos como los frutos presentan múltiples formas de la misma enzima, llamadas isoenzimas, las cuales actúan sobre los mismos sustratos y catalizan la misma reacción, pero poseen diferentes grados de tolerancia a la presión (Pelley, 2012). Se ha reportado que las isoenzimas más resistentes pueden llegar a contribuir más del 15% de la actividad enzimática global, por lo que la dificultad de inactivarlas disminuye la eficiencia de los procesos de conservación por altas presiones (Terefe *et al.,* 2017).

Se ha estudiado el efecto del procesamiento por altas presiones sobre la actividad de enzimas

endógenas relacionadas con la calidad y estabilidad durante el almacenamiento de diversas matrices alimenticias a base de frutas. Los resultados de diversos estudios sobre matrices de origen frutal se muestran en el cuadro 2.

Cuadro 2 – Efecto de las altas presiones hidrostáticas y dinámicas sobre la actividad de enzimas relacionadas al deterioro de productos alimenticios en diversas matrices de origen frutal.

Sistema de presurización	Matriz alimenticia y enzima de interés	Condiciones de proceso	Efecto sobre actividad enzimática	Referencia
Hidrostática	PPO y POD en puré de fresa	690 MPa, 20 min, 90°C	23% de inactivación de PPO	Terefe *et al.,* (2010)
Hidrostática	PME, PPO y PG en puré de fresa	600 MPa, 10 min, 80°C, 30% sacarosa	67% inactivación PME 50% inactivación PPO 80% inactivación PG	Chakraborty *et al.,* (2015)
Hidrostática	PME en néctar de mango	275, 345 y 414 MPa, 1,2 y 4 min, 17°C	45% de inactivación a 345 MPA 250% de activación a 414 MPa	Bermudez-Aguirre *et al.,* (2011)
Hidrostática	PPO y POD en rebandas de aguacate	200-600 MPa, 3-10 min	50% de inactivación de POD a 500 y 600 MPa 30% de activación de PPO a 400 MPa	Woolf *et al.,* (2013)
Hidrostática	PME, PPO y POD en pulpa de mango	600 MPa, 20 min, 70°C	78% inactivación PME 85% inactivación PPO 74% Inactivación POD	Kaushik *et al.,* (2017)
Dinámica	PME en jugo de naranja	170 MPa, 25°C	20% de inactivación	Lacroix *et al.,* (2005)
Dinámica	PME en jugo de naranja	250 MPA, 5 pasadas	80% inactivación	Welti-Chanes *et al.,* (2009)
Dinámica	PME jugo de naranja	300 MPa, 95°C*	96% de inactivación	Velázquez-Estrada *et al.,* (2012)
Dinámica	PME y PPO en jugo de manzana	300 MPa**	100% de inactivación de PME 100% inactivación de PPO	Suárez-Jacobo *et al.,* (2012)
Dinámica	PPO en néctar de pera	180 MPa	83% de activación de PPO	Liu *et al.,* (2009)

*Temperatura alcanzada debido al calentamiento adiabático
**Temperatura de procesamiento no reportada

Estos resultados son contrastantes, ya que se han reportados valores de inactivación variables para las mismas enzimas en diferentes matrices. En el caso de altas presiones hidrostáticas, se han empleado temperaturas elevadas como estrategia para incrementar la eficiencia del procesamiento. Para las altas presiones dinámicas la temperatura cobra mayor importancia sobre la actividad enzimática, especialmente si se considera que a presiones mayores a 300 MPa, el producto puede alcanzar temperaturas mayores a 60°C, a partir de las cuales se considera que los efectos de inactivación sobre microorganismos y enzimas son atribuibles al calor y no a las fuerzas mecánicas

como la presión. Adicionalmente, el paso reiterado de producto a través del sistema de HAP para incrementar la efectividad de inactivación enzimática, propicia que se pierda el principio de continuidad del proceso, lo cual podría generar problemas al escalar a nivel industrial (dos Santos Aguilar *et al.*, 2018).

1.2.7. Selección y Definición del Modelo de Estudio

La selección de néctar de mango de la variedad 'Ataulfo' como modelo de estudio para este manuscrito se basa en que el mango (*Mangifera indica* L.) es un fruto bien conocido por su excelente sabor exótico. Este fruto es muy importante económicamente sobretodo en el mercado internacional y es ampliamente cultivado en los trópicos y subtrópicos (Siddiq *et al.*, 2012). Dada su importancia económica a nivel mundial, se reporta que la producción de mango para comercialización se lleva a cabo en 87 países, siendo los más prominentes productores de mango: India en primer lugar, seguido de China, Tailandia, Indonesia y México (FAOSTAT, 2014). México destaca como el principal exportador de mango a nivel mundial con 33,8000 toneladas en el 2013, lo cual representó ingresos mayores a 2,700 miles de dólares americanos, siendo Estados Unidos de América su principal comprador.

Para el año 2015, se tenían cultivadas 191,000 hectáreas del mango en México, lo que correspondió a una producción nacional anual de 1'775,500 toneladas. Esto es consecuencia de que México cuenta con las condiciones climáticas y geográficas adecuadas para que se tenga un desarrollo pleno del cultivo (Ayala-Garay *et al.*, 2009). Dentro del territorio nacional, los estados de Sinaloa, Nayarit, Guerrero, Chiapas, Michoacán y Oaxaca se ubican como los principales productores de mango. Es importante hacer hincapié porque actualmente éstos últimos cuatro estados, los cuales conforman la denominada región del pacífico sur, en términos de producción agrícola representan en conjunto el 49% de la producción anual de mango en el país (SAGARPA, 2015). Los productores de la región pacífico sur han enfrentado varias problemáticas que limitan la rentabilidad y competitividad de su producción de mango, especialmente las pérdidas ocasionadas por la presencia de la mosca de la fruta en las zonas productoras y la reducción del margen de

exportación (Ayala-Garay *et al.*, 2009). Con el propósito de detonar el potencial de desarrollo de los 4 estados anteriormente mencionados, se creó la Alianza para el Desarrollo Sustentable de la Región Pacífico Sur (ADESUR) para beneficiar a todos los involucrados en la cadena productiva de mango y otros productos hortícolas mediante la transferencia de ciencia y tecnología para la creación y aplicación de proyectos de alta prioridad aprovechando las riquezas con la que cuentan estos estados. Debido a esto, se busca potencializar su importancia como cadena productiva con base a principios de sustentabilidad en cada una de sus actividades (campo, empaque, procesamiento y comercialización).

Además de la explotación del mango como fruta fresca, es primordial utilizarlo de forma integral para el desarrollo productos de valor agregado. El fruto de mango se consume principalmente fresco y como varios otros subproductos industriales entre los que se incluyen deshidratados, purés, jugos y néctares. En años recientes, la producción de jugo y pulpa se incrementó a nivel mundial en 13 y 17% respectivamente, en especial la destinada a elaboración de purés y concentrados (Vásquez-Caicedo *et al.*, 2007).

Al tener en consideración los factores antes citados, resulta de gran importancia estudiar a fondo el mecanismo de inactivación enzimática por efecto de las altas presiones, aislando solamente a la presión como factor de interés y estudiando el efecto sobre las enzimas de importancia para la matriz alimenticia. En este caso, se eligió néctar de mango Ataulfo, con el fin de definir condiciones de procesamiento que garanticen la estabilidad del producto durante su almacenamiento.

La presencia de isoenzimas en el fruto de mango, representa un problema para que su procesamiento por altas presiones sea efectivo (de Castro Leite Júnior *et al.*, 2017). En lo que respecta a las enzimas endógenas de mango, existen pocos reportes sobre las isoenzimas que tienen mayor interés para la industria alimentaria. Prasanna et al. (2006), identificaron y caracterizaron tres isoenzimas de poligalacturonasa en mango de la variedad Alphonso, las cuales presentaron pH óptimo de 3.5 y la isoenzima PG III resultó termoestable a 69°C. Similarmente, Singh y Dwivedi (2008), identificaron al menos tres isoenzimas de poligalacturonasa en mango de la variedad Dashehari, encontrando que su pH óptimo se encontraba entre 5.0-7.5 y la isoenzima PG-III resultó termoestable a 70°C. Referente a la variedad de mango ‘Ataulfo’, Cheema y Sommerhalter (2015)

identificaron al menos dos isoenzimas de polifenoloxidasa con alto grado de actividad y una con bajo grado de actividad en extractos de pulpa y cáscara en fruto de estado de madurez avanzado; se encontró que tanto el estado de madurez como la parte del fruto eran factores que afectaban el nivel de actividad y distribución de las isoformas de esta enzima asociada con el oscurecimiento del fruto y sus derivados.

Una estrategia propuesta en este manuscrito para estudiar el efecto de las altas presiones hidrostáticas y dinámicas sobre la estructura y actividad de pectinmetil esterasa, polifenoloxidasa y poligalacturonasa en néctar de mango Ataulfo es a través de la identificación de las isoenzimas baroresistentes mediante zimografía en gel de poliacrilamida en una dimensión.

1.3. Hipótesis

1. La actividad de las enzimas pectinmetil esterasa, polifenol oxidasa y poligalacturonasa en néctar de mango “Ataulfo” se reduce en al menos 15% con el procesamiento por altas presiones hidrostáticas y dinámicas.

2. La alta presión dinámica es más efectiva que la alta presión hidrostática en relación a la inactivación de enzimas y preservación de atributos de calidad en néctar de mango ‘Ataulfo’.

3. Existen al menos una isoenzima baroresistente de pectinmetil esterasa y/o polifenol oxidasa en néctar de mango “Ataulfo” ´procesado por altas presiones hidrostáticas y dinámicas.

4. No hay diferencias en la calidad del néctar de mango “Ataulfo” por efecto del procesamiento por altas presiones hidrostáticas y dinámicas.

1.4. Objetivo General

Identificar las isoenzimas baroresistentes de las enzimas pectinmetil esterasa, polifenol oxidasa y

poligalacturonasa en néctar de mango “Ataulfo” procesado por altas presiones hidrostáticas y dinámicas, y evaluar los efectos sobre los atributos de calidad del néctar.

1.5. Objetivos Específicos

1. Determinar la actividad enzimática de pectinmetil esterasa, polifenol oxidasa y poligalacturonasa en néctar de mango “Ataulfo” procesado por altas presiones hidrostáticas

2. Determinar la actividad enzimática de pectinmetil esterasa y polifenol oxidasa en néctar de mango “Ataulfo” procesado por altas presiones dinámicas

3. Evaluar el efecto de las altas presiones hidrostáticas y dinámicas sobre los atributos de calidad de néctar de mango ‘Ataulfo’, asociados a la actividad de las enzimas.

4. Identificar isoenzimas barorresitentes de pectinmetil esterasa presentes en néctar de mango “Ataulfo” procesado por altas presiones hidrostáticas y dinámicas.

5. Evaluar la calidad física, química y proximal del néctar de mango “Ataulfo” estabilizado mediante el procesamiento por altas presiones hidrostáticas y dinámicas.

1.6. Sección Integradora del Trabajo

La información presente en este manuscrito está dividida en secciones denominadas capítulos y son presentados de la siguiente manera:

La Sinopsis consiste en una introducción concisa del problema de investigación, mencionando de manera generalizada el problema a tratar y el enfoque central de la investigación. Este proyecto de investigación pretende generar información respecto al efecto que tiene el procesamiento por altas presiones, tanto hidrostáticas como dinámicas, en la actividad enzimática utilizando una bebida de

mango como modelo de estudio y considerando solamente el efecto de la presurización. Las implicaciones del proceso sobre los atributos de calidad relacionados con la actividad de dichas enzimas, también fueron evaluados. Esta información puede replicarse a otras matrices alimenticias, que deben tratarse como únicas, cada que se implemente un proceso de preservación en base a la aplicación de altas presiones.

El artículo I consistió en la evaluación del efecto combinado del procesamiento por alta presión hidrostática y el almacenamiento frío sobre la actividad de tres enzimas relacionadas con la reducción de la calidad durante el almacenamiento en productos alimenticios procesados a base de frutos. En esta investigación se utilizó como modelo de estudio al néctar de mango de la variedad 'Ataulfo'. Este estudio permitió valorar la resistencia de las enzimas en un amplio rango de condiciones experimentales y su efecto sobre atributos de calidad, para definir los parámetros y enzimas más importantes para futuros experimentos. Con a los resultados obtenidos, se descartó la enzima poligalacturonasa en experimentos posteriores. También, la sinergia de alta presión hidrostática con el almacenamiento frío, permitió generar información de una combinación de barreras de conservación de alimentos que ha sido poco estudiada en la literatura científica, y que propició resultados relevantes en cuanto a la reducción de la actividad de polifenol oxidasa en néctar de mango procesado por alta presión hidrostática almacenado a 4°C. Este manuscrito fue enviado y se encuentra bajo revisión en la revista CyTA – Journal of Food.

El segundo artículo se publicó en la revista "Molecules" de MDPI, bajo la licencia de acceso libre. Para esta publicación, se desarrolló un diseño experimental que pudiera permitir generar suficientes tratamientos de magnitud similar para comparar ambos procesos; además, el néctar formulado se filtró a través de malla de 250 µm previamente al procesamiento por ambos sistemas de alta presión. Se integra información que compara la eficiencia de ambas tecnologías de procesamiento por alta presión (hidrostática y dinámica) para inactivar las enzimas de interés, así como su efecto en atributos de calidad como el color y viscosidad, que están ampliamente relacionados con la actividad de PPO y PME respectivamente. Con esta publicación se abordó la segunda hipótesis planteada en la investigación.

El tercer artículo se encuentra como borrador, en preparación para su envío en formato de comunicación corta a la revista Food Science and Technology Research. Técnicamente representó un reto, ya que la enzima polifenol oxidasa no pudo ser evaluada mediante zimografía. La causa atribuible es la posible omisión de pasos críticos en las metodologías reportadas para zimografía de PPO. Por otra parte, esta publicación se enfocó en la enzima PME, la cual está considerada como resistente a la presurización. Fue posible utilizar zimografía para identificar *in situ* dos isoenzimas con pesos moleculares aproximados de 63 y 33 kDa de PME en muestras selectas de néctar de mango procesado por altas presiones hidrostáticas y dinámicas.

2. THE COMBINED EFFECT OF HIGH HYDROSTATIC PRESSURE AND COLD STORAGE ON THE ENZYMATIC ACTIVITY OF PECTIN METHYLESTERASE, POLYPHENOL OXIDASE, AND POLYGALACTURONASE AND ON QUALITY ATTRIBUTES IN 'ATAULFO' MANGO NECTAR

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Abstract:

The combined effect of high hydrostatic pressure (HHP) (100 - 400 MPa) and processing time (5 – 25 min) with cold storage (4°C) on the enzymatic activity of pectin methylesterase, polygalacturonase, polyphenol oxidase, and quality attributes in "Ataulfo" mango nectar were evaluated. Relative enzymatic activity was measured after HHP processing and after 20 d at 4°C. The PME relative activity increased between 25 and 55%, the PPO relative activity increased up to 88% but was reduced by HHP combined with cold storage to 22%, and PG relative activity increased between 7 and 48%. The effects of processing conditions on quality parameters were not significant. These enzymes in mango nectar were resistant to HHP, and the combined effect with

cold storage only showed a significant effect on the activity of PPO. Further studies are suggested to provide more information regarding the baroresistance of enzymes in food products.
Keywords: High hydrostatic pressure, Cold storage, Mango nectar, Enzymatic activity, Quality attributes

1. Introduction

For several decades, thermal processing has been the industry standard to ensure food safety and the quality of fruit-based processed foods. However, research has highlighted the downsides of thermal processes due to their detrimental effects on food nutrients, bioactive compounds, and sensory attributes (De Paepe *et al.,* 2014; Petruzzi *et al.,* 2017). Recently, consumers have become more aware of the products they consume and have demanded fresher products with better quality and attributes, pushing research on food processing technologies towards those regarded as "nonthermal technologies." Among these technologies, high-pressure processing has been extensively studied and adopted because pressurization, for the processing of food products, has shown promising results in terms of retention of bioactive compounds, maximization of nutritional content, and preservation of sensory characteristics, while it also preserves the products due to inactivation of microorganisms and spoilage-related enzymes (Augusto *et al.,* 2017; Martín-Belloso *et al.,* 2014). However, compared to thermal processing, high-pressure processing has disadvantages, especially in regard to enzymes related to quality-loss in food products, as literature often mentions the use of mild temperatures or additives to improve enzyme inactivation (Guerrero-Beltrán *et al.,* 2006; Iqbal *et al.,* 2019; Li and Padilla-Zakour, 2021). Enzyme inactivation is considered a requirement in the production of high-quality food products, especially in fruit-based products, where enzymes can lead to undesirable changes in color, texture, and other sensory attributes during storage (Ağçam *et al*, 2017). Some key enzymes related to quality loss in

fruit-based products are pectin methylesterase (PME), polygalacturonase (PG), and polyphenol oxidase (PPO). The activities of PME and PG are highly related to the cloud stability of citrus juices and can be important for other fruit beverages with a high pectin content because pectin and pectic substances have shown potential as prebiotics (Cautela *et al.*, 2018; Chung *et al.*, 2017). Additionally, PPO is an important enzyme in fruit-based products due to the enzymatic browning reactions it catalyzes, which can impact the biological activities of the phytochemicals present in fruit-based products, such as polyphenols, due to oxidation (Szczepańska *et al.*, 2020; Tinello and Lante, 2018).

Several studies on the inactivation of these enzymes in fruit products have reported high tolerance to high hydrostatic pressure in the range of 100-600 MPa (Terefe *et al.*, 2014). To improve the effect of HHP on enzyme inactivation, the hurdle effect with thermal processing has been strategically evaluated. Kaushik *et al*. (2017) evaluated the effect of high-pressure and thermal treatments in the domain of 0.1 - 600 MPa and 40-70°C for 1 s - 90 min on the activity of PME and PPO in mango pulp, achieving enzyme inactivation levels of 68 and 85%, respectively, at 600 MPa/70°C/20 min. Similarly, Morales de la Peña *et al*. (2018) achieved almost 60 and 40% inactivation of PME and PPO, respectively, in mango puree processed at 550 MPa/59°C/16 min. Chakraborty *et al*. (2015) reported up to 80, 67, and 50% inactivation of PG, PME, and PPO, respectively, in strawberry purees in the combined pressure-temperature domain of 200-600 MPa at 40-80°C. Specifically, 600 MPa at 80°C for 10 min was necessary to achieve these levels of inactivation. However, in some cases, the combined high-pressure and high-temperature treatments were insufficient for the complete inactivation of quality loss-related enzymes (Terefe *et al.*, 2010). In addition to the initial effect of high pressure, more knowledge of the storage stability of pressure-treated products is essential for the industrial development of this process. A scarcely studied alternative, especially regarding the control of enzymatic activity, is the hurdle effect of low

temperature or cold storage combined with high-pressure processing of fruit-based products. Bodelón *et al*. (2013) evaluated the effect of high pressures of 100-400 MPa for 15 min at 20 and 50°C on the activity of PME, ascorbic acid, and anthocyanins in strawberry puree stored at 5°C. Their findings showed that the treatments had no significant effects on PME, as it retained its activity during 6 months of storage at 5°C. More recently, Kaushik *et al*. (2018) reported an inactivation ratio of approximately 50% for PME, PPO, and peroxidase (POD) in mango pulp after HHP treatment at 600 MPa for 10 min and 52°C. Their results also suggest that the enzyme activities remained unchanged after 20 d at 5°C, with slight reductions over time. Additionally, Quiroz-González *et al*. (2020) evaluated the effect of HHP and storage at 4°C on the activity of PME in pitaya juice, which was reduced by 63% after HHP treatment and an additional 16% by cold storage after 30 d, indicating good synergy between both preservation mechanisms.

Given the high capital costs of HHP systems operating at 400-600 MPa, especially for incorporation in Mexican processing plants, we aimed to provide information on the effect of cold storage as an additional barrier for controlling the enzymatic activity of fruit-based products in combination with high hydrostatic pressure. We selected mango nectar from the "Ataulfo" variety as an experimental fruit-based product, because of its relevance in terms of production and economy to Mexico. We evaluated the gradual effects of high-pressure level processing time and cold storage on the activity of PME, PPO, and PG, which are quality loss-related enzymes with high tolerance to high-pressure processing, and on some quality attributes of mango nectar.

2. Materials and methods

2.1. Plant material

"Ataulfo" variety mango fruits were harvested from an orchard in Chiapas, Mexico (latitude 15.285056, longitude (92.698389), in ripeness stage 4 (14-16 °Brix and pH = 4.5 - 4.7 in pulp).

Fruits were washed in 200 ppm sodium hypochlorite solution. The pulp was separated from the pericarp, packed, sealed in vacuum bags, and stored at -20°C throughout the experimental phase. Frozen pulp was transferred from Mexico to the facilities of the UMR IATE of the University of Montpellier to perform the experiments.

2.2. Mango nectar formulation

The pulp was thawed overnight at 4°C and blended in a Lidl monsieur cuisine thermomixer (Lidl, Spain) for 120 s at high speed until a smooth paste was formed. The nectar formulation contained 40% w/w pulp paste diluted with reverse osmosis-treated water and mixed by magnetic agitation until fully homogenized. No sugar or additives were added. The following properties were measured on the untreated nectar (control) pH, soluble solids (°Brix), and color (CIEL*a*b*).

2.3. High hydrostatic pressure processing

Pressurization experiments were carried out in a discontinuous 1-liter high-pressure stainless steel vessel from ACB GEC Alsthom (Nantes, France) with a maximum operating pressure of 450 MPa. Samples were treated at pressure levels of 100, 200, 300, or 400 MPa for a gradual screening of enzyme activity, with 5, 15, and 25 min processing times and an initial temperature (Ti) = 10°C using distilled water as a pressure transmitting fluid. For each batch, 300 mL of nectar was packed in polyvinylidene chloride (PVDC) tubings (26 mm diameter, 50 µm thick, Krehalon, Eygalières, France) sealed with two knots at both ends and kept at 4°C for a maximum of 4 h before being processed (control assay). After processing, one set of samples was used to determine the enzymatic activity at two different points in time: (t) = 0 d (immediately after processing) to evaluate only the HHP effect, and the other was packaged in 50 mL polyethylene terephthalate centrifuge tubes and stored at 4°C for determination of the HHP and cold storage effect at (t) = 20

d (after 20 d). Due to the processing conditions and product formulation, we considered the samples to be microbiologically safe after processing.

2.4. Enzymatic activity assays

Nectar samples processed by HHP were diluted with extraction buffer (0.2 M sodium phosphate pH 6.6, 4% polyvinylpyrrolidone, 1% Triton X-100, 1 M NaCl) at a 1:2.5 buffer-to-sample ratio and mixed at 4°C for 1 h. Afterward, samples were centrifuged at 11,000 rpm at 4°C for 30 min in a Sorvall RC 5B Plus (Du Pont, USA) centrifuge (Chakraborty *et al.*, 2015). The supernatant was collected and used as an extract for enzymatic assays.

2.4.1 Pectin methylesterase activity assay

The activity of pectin methylesterase was measured according to Rodrigo *et al*. (2006), and 0.25 mL of extract was added to 30 mL of substrate solution (0.35% apple pectin, 0.12 mM NaCl pH 7.0) to start the reaction. Then, 0.05 N NaOH was automatically added by a 902 titrando automatic titrator fitted with an 800 Dosino Unit (Metrohm, Switzerland) for a reaction time of 6 min. The activity of PME was calculated according to the following equation:

$$PME\ (U) = \frac{V \cdot N \cdot 1000}{V_s \cdot t}$$

Where:

V = Volume of NaOH (mL).

N = Normality of NaOH.

V_s = Sample volume (mL).

t = Reaction time (min).

All assays were performed at 30°C in quadruplicate.

2.4.2 Polyphenol oxidase activity assay

The activity of polyphenol oxidase was measured according to the method by Palma-Orozco *et al*. (2014); 50 µL of enzyme extract was added to 1 mL of substrate solution (0.2 M sodium phosphate buffer pH 6.6, 50 mM pyrocatechol) to initiate the reaction in a 1 cm quartz cell. Then, absorbance was measured every 5 s for 5 min in a Unicam UV2 UV-Vis spectrophotometer (ATi Unicam, United Kingdom) at 420 nm. PPO activity was calculated from the slope of the initial linear portion of the absorbance vs. time curve and expressed in units per mL of extract. All assays were performed at 25°C in quadruplicate.

2.4.3 Polygalacturonase activity assay

The activity of polygalacturonase was determined according to Terefe *et al*. (2009), 0.1 mL of extract was added to 0.3 mL of 0.5% polygalacturonic acid solution and incubated at 35°C for 30 min. The reaction was stopped by adding 2 mL of 0.1 M borate buffer solution (pH 9.0) and 0.4 mL of 1% cyanoacetamide solution. Afterward, the samples were incubated at 100°C for 10 min and immediately cooled in an ice-water bath. After reaching room temperature (25°C), the absorbance of the samples was measured at 276 nm in a Multiskan™ Spectrum multiplate reader (Thermo Fisher Scientific, US). The release of reducing sugars was related to a standard curve of D-galacturonic acid from 0 - 0.2 g/L, from which the PG activity was calculated with the following equation:

$$PG\ (U) = \frac{RGA}{V_s \cdot 194.1 \cdot t}$$

where

RGA = Released galacturonic acid (µg);

V_s = Sample volume (mL);

t = Reaction time (min).

All assays were performed in quadruplicate.

2.5. Relative enzymatic activity calculation

The PME, PPO, and PG activities were calculated as units per mL of extract. For better comparison against the control sample, the values were converted to relative activity regarding the untreated nectar, which was calculated according to the following equation:

$$Relative\ enzymatic\ activity\ (\%) = \frac{A_s}{A_0} \times 100$$

where

A_s = Enzymatic activity in U/mL of the processed sample;

A_0 = Enzymatic activity in U/mL of the untreated sample.

2.6. Quality attributes

The sample color was measured in quintuplicate as L, a*, and b* parameters (CIEL*a*b*), with a DR Lange spectro-color d/8° portable, spectral colorimeter (HACH Lange, Germany). The pH was measured in quadruplicate with a C832 multiparameter analyzer (Consort, Belgium), and total soluble solids were measured in quadruplicate with a manual refractometer with a 0 – 32 °Brix range. All measurements were performed at room temperature. Chroma and °hue were calculated based on the L*, a*, and b* parameters according to the following equations:

$$°Hue = Arc\tan(\frac{b*}{a*})$$

$$Chroma = \sqrt{(a*^2 + b*^2)}$$

2.7. Design of experiments and statistical analysis

A nested three-factor (pressure level, processing time, and cold storage) design of experiments was performed. The levels of the pressure level factor were 100, 200, 300, and 400 MPa. The levels of the processing time factor were 0, 5, 15, and 25 min. This factor was nested in the pressure level, as the control received only one level from this factor as part of the treatments (0 MPa, 0 min). The levels of these two factors were selected to evaluate the gradual effect of high pressure on the enzyme activity, considering the system's maximum operating pressure in combination with other experimental factors. Finally, the cold storage factor levels were 0 and 20 d. All data were analyzed in Minitab 17® statistical software. Analysis of variance (ANOVA) was performed by the general linear model function, and mean comparison tests were performed with the Tukey test. The significance level was set at $\alpha = 0.05$.

3. Results and discussion

3.1. Effect of processing on the relative enzymatic activity of pectin methylesterase

The high-pressure level, processing time, and cold storage had significant effects ($P \leq 0.05$) on the relative activity of PME (Table 1). Despite this significance, no treatment conditions evaluated decreased the activity of this enzyme. The relative activity of PME increased at 300 MPa at 0 d and further increased after 20 d of cold storage at 4°C for all conditions tested, especially at 200 and 300 MPa. It is also quite interesting how the enzyme was preserved and showed increased activity despite the combined effect of HHP with cold storage. The highest relative activity was observed in samples processed at 400 MPa for 5 min, with an increase of 55.04% after 20 d. These

results indicate that PME in this mango variety is baroresistant. PME is considered a very pressure-tolerant enzyme that does not follow first-order inactivation kinetics in most matrices, requiring high-pressure levels exceeding 600 MPa to significantly reduce its activity (Duvetter *et al.*, 2009). Other studies on mango nectars processed by HHP have reported results similar to our findings in the same high-pressure domain, such as Bermúdez-Aguírre *et al*. (2011), who reported that processing mango nectar at 345 MPa decreased the activity of PME by 45%, whereas pressure levels of 414 MPa further activated the enzyme. Other reports of PME inactivation in mango nectar and pulp showed that combined thermal effects were required to reduce the activity. Kaushik *et al*. (2016) reported a reduction in PME activity in mango pulp to 45% by treating the samples at 600 MPa combined with mild heating (52°C) for 10 min. This result indicates that temperature has a more significant impact on PME activity than high hydrostatic pressure itself.

One of the main reasons that can explain the variable behavior regarding the activity of PME in our experiments could be the presence of multiple isoforms of the enzyme. As a ubiquitous enzyme, PME exists as multiple isoenzymes with different biochemical characteristics but the same catalytic activity. These isoforms are present in the sample either at the same location or time, contributing to the high resistance to hydrostatic pressure processing (Jolie *et al.*, 2010). Regarding the combined effect of HHP and cold storage, Cameron *et al*. (2008) identified four isoforms of PME in citrus juice, with different catalytic activities at 30 and 4°C. Their findings suggest that one isoform had a greater effect on cloud loss at 4°C, but its activity decreased at 30°C, which could support our results regarding the increase in PME activity in nectar during cold storage.

Table 1 – Relative activity of pectin methylesterase, polyphenol oxidase and polygalacturonase in 'Ataulfo' mango nectar processed by high hydrostatic pressure in combination with cold storage.

Pressure Level (MPa)	Processing time (min)	Relative enzymatic activity (%)					
		Day 0			Day 20*		
		PME	PPO	PG	PME	PPO	PG
Control	0	100.00±0.73^{j}	102.61±5.69^{h}	99.80±6.31^{fghijk}	138.56±2.49^{b}	22.46±1.50^{l}	89.21±3.72^{k}
	5	101.38±1.06^{ij}	147.33±8.85^{def}	114.59±5.08^{cdef}	122.48±1.52^{f}	23.65±2.62^{l}	98.87±1.73^{fghijk}
100	15	105.79±0.28^{ghij}	181.52±2.33^{ab}	107.21±4.17^{efghij}	135.60±1.91^{bc}	44.19±2.64^{k}	108.58±6.32^{defghij}
	25	109.35±0.54^{g}	148.53±5.06^{de}	111.52±3.32^{cdefghi}	125.28±1.70^{ef}	62.43±1.13^{j}	95.78±2.31^{ijk}
	5	101.28±0.81^{ij}	145.02±3.97^{ef}	98.25±6.52^{ghijk}	154.08±2.86^{a}	60.58±3.09^{j}	146.86±7.26^{a}
200	15	129.18±2.67^{cde}	121.83±2.58^{g}	93.79±0.75^{jk}	155.04±3.38^{a}	82.69±4.09^{i}	136.37±7.26^{ab}
	25	99.83±2.81^{j}	135.11±4.09^{efg}	103.26±2.65^{efghijk}	154.72±1.87^{a}	64.62±2.81^{j}	137.79±2.14^{ab}
	5	125.03±0.84^{ef}	181.75±2.43^{ab}	117.91±9.87^{cde}	133.10±1.18^{bcd}	79.23±7.08^{i}	138.78±7.52^{ab}
300	15	127.49±2.53^{def}	170.93±11.92^{bc}	96.95±5.24^{hijk}	138.80±1.11^{b}	88.27±6.51^{i}	148.06±8.13^{a}
	25	137.11±4.03^{b}	186.90±3.87^{a}	124.88±5.38^{bc}	139.21±0.81^{b}	63.31±4.04^{j}	141.21±3.17^{a}
	5	104.12±0.95^{ghij}	130.58±9.32^{g}	114.74±7.56^{cdef}	155.60±5.39^{a}	80.38±1.83^{i}	123.90±9.48^{bcd}
400	15	102.46±4.00^{hij}	134.57±2.33^{fg}	116.39±8.11^{cde}	107.95±0.91^{gh}	53.85±4.66^{jk}	113.16±5.28^{cdefg}
	25	104.98±0.85^{ghij}	160.67±6.42^{cd}	116.43±1.69^{cde}	106.88±4.87^{ghi}	78.85±5.49^{i}	111.97±8.89^{cdefgh}

Mean comparison by Tukey test (α=0.05). Means that do not share a letter are significantly different.

*Activity evaluated after 20 d at 4°C.

3.2. Effect of processing on the relative enzymatic activity of polyphenol oxidase

Statistical analysis showed that all factors (high-pressure level, processing time, and cold storage) were significant ($P \leq 0.05$) for the PPO relative activity. However, as observed in Table 1, the PPO relative enzymatic activity increased without a clear pattern after each treatment HHP. The highest observed increment was for the sample treated at 300 MPa for 25 min at 0 d; the PPO relative activity was around 84.29% higher than the control sample. Regarding the combined effect of HHP and cold storage, the PPO activity decreased in all samples after 20 d stored at 4°C. The relative PPO activity at 20 d was still higher than that of the control, especially in the range of 200 - 400 MPa. The lowest relative activity in treated samples was 23.65% at 100 MPa for 5 min and 20 d.

Compared to PME, PPO also demonstrated to be baroresistant in this mango variety. Despite the statistical significance, HHP combined with cold storage had a greater effect on PPO stability than pressurization only. PPO from "Ataulfo" mangoes has shown activity in a temperature range of 20-70°C (Palma-Orozco *et al.*, 2014).However, the enzymes from tropical fruits such as mango fruits, can preserve their catalytic activity even at lower temperatures than their optimum, albeit at a much slower rate (Struvay and Feller, 2012). Interestingly, only PPO stability was affected during cold storage; in this regard, Kaushik *et al*. (2018) reported that after 20 d at 6°C, PME and PPO maintained residual activity up to 50% in HHP-treated mango pulp, with a subsequent decrease to approximately 40% after 120 d, while the POD activity increased during cold storage. Xu (2005) reported that PPO from the chestnut epidermis and endosperm maintained up to 30% activity after 180 d at -20°C. The variability in increased activity immediately after processing could be related to the simultaneous presence of labile and resistant isoforms of PPO in the mango nectar. Cheema and Sommerhalter (2015) identified from "Ataulfo" mangoes at least three isoforms of PPO with molecular weights between 53 and 144 kDa, exhibiting the same catalytic activity. Additionally, the release of phenolic compounds due to pressurization increases the availability of substrates for

polyphenol oxidases after HHP processing, which could be related to the increased activities at 0 d (Huang *et al.,* 2013).

3.3. Effect of processing on the relative enzymatic activity of polygalacturonase

The results for the relative activity of PG in mango nectar showed a trend similar to that of PME. All factors were significant ($P \leq 0.05$). As shown in Table 1, the relative activity of PG increased compared to the control sample at 0 d for all conditions tested, but after 20 d at 4°C, a steeper increase was observed between200 - 300 MPa. The highest increase in the relative activity of 48.06% occurred in samples treated at 300 MPa for 15 min and 20 d. The combined effect of HHP and cold storage did not affect the activity, which is similar to the results observed for PME. It must be stated that in terms of pure activity (U/mL), PG showed the lowest values of the three studied enzymes, which could indicate that its increase in activity by 20 d was related to the activity exerted by PME on the pectin content in the nectar by releasing protons (Deytieux-Belleau *et al.*, 2008). From these results, it appears that PG is also resistant to HHP. The pressure-temperature domain for inactivating PG ranges from 300-600 MPa at 20-60°C and holding times of 1-15 min (Terefe *et al.,* 2014). Unlike PME and PPO, there are more reports of isoforms of PG from different mango varieties, which could explain the variability in its relative activity due to the presence of labile and resistant fractions of the enzyme (Prasanna *et al.,* 2006; Singh and Dwivedi, 2008). A report by Dautt-Castro *et al*. (2019) highlighted that at least 49 putative genes annotated with PG activity were found in the transcriptome of ‘Tommy Atkins‘ mangoes grown in Mexico. Additionally, nine PGs were expressed during postharvest ripening and shown to contribute to PG enzymatic activity. These findings suggest that for ripe fruits submitted to technological processing, there could be several isoenzymes simultaneously present that contribute to global enzymatic activity in the final product.

3.4. Effect of processing on pH and total soluble solids (°Brix)

Concerning pH, only the high-pressure level, cold storage, and the interaction between pressure level and cold storage factors were significant ($P \leq 0.05$). As shown in Table 2, the pH slowly decreased during cold storage in all samples under all conditions tested. Specifically, the lowest pH values were those from samples processed at 200 MPa for 15 min (4.03) and 300 MPa for 25 min (4.07) after 20 d. The mean comparison test showed no difference between treatments at 0 d ($P > 0.05$). The initial pH of mango nectar was lower than 4.6, and it was considered an acid food requiring an equivalent pasteurization process to ensure safety for consumption. The significant effect of the interaction between high-pressure level and cold storage is probably related to the increased activity of PME and PG during cold storage, as the de-esterificationof pectin releases methanol and pectate into the medium, lowering pH, and to the release of organic acids by the effect of pressurization (Morales-de la Peña *et al.*, 2018). While microbial growth could contribute to changes in pH, microorganism populations decrease following first-order kinetics in HHP products, and our results indicate higher activity of the pectinases evaluated at 200-300 MPa than at lower pressure levels. Additionally, HHP in the range of 100 - 600 MPa has shown good results for controlling microorganisms in food systems. The reduction of up to 5 log in target groups such as aerobic mesophiles, coliforms, yeasts, molds, specific gram-positive bacteria such as S. aureus, and gram-negative bacteria such as E. coli, have been reported, which supports our consideration of our mango nectar being microbiologically safe after HHP processing (Kaushik *et al.*, 2014; Kultur *et al.*, 2017).

Table 2 – pH and °Brix of ‘Ataulfo’ mango nectar processed by high hydrostatic pressure at different pressure levels in combination with cold storage

Pressure Level (MPa)	Processing time (min)	Day 0		Day 20*	
		pH	°Brix	pH	°Brix
Control	0	4.47±0.08^{a}	6.90±0.11^{abc}	4.16±0.09^{defghi}	6.00±0.13^{e}
	5	4.42±0.12^{ab}	6.97±0.19^{ab}	4.21±0.19^{bcdefghi}	6.63±0.69^{abcde}
100	15	4.39±0.08^{abc}	6.83±0.19^{abcde}	4.25±0.14^{abcdefghi}	6.76±0.69^{abcde}
	25	4.43±0.09^{ab}	6.90±0.33^{abc}	4.32±0.08^{abcdefg}	6.72±0.67^{abcde}
	5	4.29±0.03^{abcdefgh}	6.67±0.10^{abcde}	4.12±0.08^{efghi}	6.10±0.09^{cde}
200	15	4.34±0.10^{abcde}	6.66±0.10^{abcde}	4.03±0.07^{i}	6.07±0.08^{cde}
	25	4.33±0.11^{abcdef}	6.60±0.00^{abcde}	4.10±0.03^{fghi}	6.10±0.00^{cde}
	5	4.37±0.04^{abcd}	6.63±0.08^{abcde}	4.18±0.11^{cdefghi}	6.05±0.05^{de}
300	15	4.34±0.04^{abcde}	6.60±0.00^{abcde}	4.10±0.04^{ghi}	6.13±0.08^{bcde}
	25	4.34±0.02^{abcde}	6.47±0.30^{abcde}	4.07±0.11^{hi}	6.03±0.12^{de}
	5	4.42±0.03^{ab}	6.90±0.21^{abc}	4.21±0.18^{bcdefghi}	7.08±0.62^{a}
400	15	4.35±0.05^{abcd}	6.80±0.22^{abcde}	4.24±0.19^{abcdefghi}	6.83±0.54^{abcde}
	25	4.30±0.06^{abcdefg}	6.80±0.18^{abcde}	4.23±0.19^{bcdefghi}	6.87±0.60 ^{abcd}

Mean comparison by Tukey test (α=0.05). Means that do not share a letter are significantly different.

*Activity evaluated after 20 d at 4°C.

Only the pressure level, cold storage, and their interaction in the ANOVA had a significant impact ($P \leq 0.05$) on the total soluble solids, expressed as °Brix. The highest decrease was observed between 200 and 300 MPa after 20 d of cold storage (Table 2), which could be related to the increased activity of PME and PG during cold storage under these processing conditions, indicating the presence of more suspended solids due to cloud instability and loss of gelling properties of pectin. Despite these results, mean comparison tests showed that all samples at 0 d were similar to the control, and only a few exceptions after 20 d were significantly different. However, at 400 MPa, the °Brix value increased after HHP and remained stable during cold storage. This effect could be related to a minor degree of activity of PME and PG in samples processed at 400 MPa during cold storage and the release/extraction of soluble compounds from vacuoles associated with the effects of high-pressure processing, which at higher pressures is more pronounced for some food matrices

(Varela-Santos *et al.*, 2012).

3.5. Effect of processing on color (L, chroma, and °hue)*

The effects of all conditions tested on the L* parameter, except for cold storage, were not significant ($P > 0.05$). All the samples showed a decrease in L* values (Table 3) after 20 d at 4°C, which could be associated with the remaining activity of PPO. In this study, the samples' luminosity results agree with previously reported L* values for 'Ataulfo' mango mesocarp (Ornelas-Paz *et al.*, 2008). Regarding the values of °hue (Table 3), only the pressure level and processing time factors were significant ($P \leq 0.05$). The results indicate that the global color saturation of the samples was near 80°, representing a yellow coloration (90°). It is important to state that color values in ripe mango varieties are diverse; Ibarra-Garza *et al*. (2015) reported °hue values ranging from 84 – 87° in 'Keitt' mangoes, and Osuna-Enciso *et al*. (2012) reported 81° for the 'Tommy Atkins' variety. Both of these varieties are also grown in Mexico. Regarding chroma values (Table 3), only cold storage showed a significant effect ($P \leq 0.05$). In all samples, the chroma values decreased during cold storage. Our results showed that color saturation was retained in all samples, but dullness increased over time. This effect could be attributed to the remnant PPO activity, even when it was the only evaluated enzyme affected by the combined effect of HHP and cold storage, indicating that browning reactions continued, albeit at a slower rate under cold storage conditions. The reported chroma values for mango-based products range between 60 and 65 (Yao *et al.*, 2020), which are very high compared to our results. Aaby *et al*. (2018) reported similar findings regarding the effect of HHP on °hue and chroma in strawberry juice and puree during cold storage. Their results suggested that °hue remained stable during storage at 6°C, but chroma decreased slightly, which supports our results. Concerning the changes in °Brix during cold storage, a possible cause for the reduction in total soluble solids in some of the mango nectar samples during

storage is the enzymatic browning and oxidation of polyphenolic compounds, which contribute to the total soluble solids quantification. According to Persic *et al*. (2017) there was a high correlation between changes in the total phenolic content during storage, enzymatic browning, and PPO activity in apple slices. Additionally, Szczepánska *et al*. (2020) found that in carrot juice, which is another carotenoid-rich source, processed at 300, 450, and 600 MPa, the remnant activity of PPO and POD and lowered pH contributed to the darkening of the product during preservation by enzymatic and non-enzymatic pathways. However, the POD activity was not evaluated in our study.

Table 3 –Color parameters (L*, °hue and chroma) of 'Ataulfo' mango nectar processed by high hydrostatic pressure in combination with cold storage.

Pressure Level (MPa)	Processing time (min)	Day 0			Day 20*		
		L*	Hue	Chroma	L*	Hue	Chroma
Control	0	31.25±0.24^{abc}	80.96±1.15^{a}	13.35±0.33^{ab}	29.97±0.21^{abc}	83.32±1.07^{a}	11.75±0.44^{ab}
	5	31.35±1.37^{ab}	79.84±1.87^{a}	13.57±0.86^{ab}	29.55±1.63^{abc}	78.01±1.92^{a}	11.19±2.10^{ab}
100	15	33.48±3.17^{a}	81.95±1.15^{a}	14.93±1.81^{a}	28.20±0.48^{bc}	80.83±0.18^{a}	9.35±0.63^{b}
	25	31.08±0.79^{a}	78.09±0.44^{a}	13.36±0.61^{ab}	29.41±0.98^{abc}	77.00±0.55^{a}	11.09±1.15^{ab}
	5	31.10±1.22^{abc}	80.82±0.78^{a}	13.34±1.53^{ab}	26.35±1.88^{c}	77.55±5.12^{a}	9.88±1.26^{ab}
200	15	30.50±0.85^{abc}	80.03±1.52^{a}	12.58±0.96^{ab}	28.88±1.44^{abc}	81.61±2.40^{a}	10.51±2.11^{ab}
	25	31.48±0.59^{ab}	81.18±0.34^{a}	13.51±0.43^{ab}	29.93±1.31^{abc}	81.32±1.19^{a}	10.71±1.64^{ab}
	5	32.68±0.37^{ab}	80.03±2.31^{a}	13.58±2.06^{ab}	29.56±1.44^{abc}	82.99±0.94^{a}	11.62±2.01^{ab}
300	15	32.82±0.08^{ab}	80.62±0.87^{a}	14.55±0.22^{ab}	29.12±0.26^{abc}	82.99±3.42^{a}	11.23±0.34^{ab}
	25	31.02±0.17^{abc}	80.25±1.01^{a}	13.09±0.33^{ab}	29.15±1.40^{abc}	82.63±1.42^{a}	10.64±1.04^{ab}
	5	29.54±1.50^{abc}	80.21±0.50^{a}	11.40±1.99^{ab}	28.85±0.32^{abc}	82.12±1.70^{a}	10.25±0.34^{ab}
400	15	32.19±1.71^{ab}	80.04±0.39^{a}	14.42±1.13^{ab}	29.69±1.06^{abc}	82.81±1.78^{a}	11.43±1.35^{ab}
	25	29.77±0.69^{abc}	79.26±0.92^{a}	11.95±0.86^{ab}	29.53±0.10^{abc}	77.98±.14^{a}	11.43±0.40^{ab}

Mean comparison by Tukey test (α=0.05). Means that do not share a letter are significantly different.

*Activity evaluated after 20 d at 4°C.

Conclusions

From the results obtained in this study, high-pressure processing in the domain of 100-400 MPa combined with cold storage is not a good alternative for preserving the quality and stability associated with enzymatic reactions of fruit-based beverages such as mango nectars. The efficacy of their combined effect on enzyme inactivation might vary among products and processing conditions. Our findings suggest that the combined effect of high hydrostatic pressure and cold storage only significantly reduced the enzyme stability of PPO in 'Ataulfo' mango nectar, but increased the activity of PME and PG. Additionally, the presence of isoforms of enzymes in biological systems such as mangoes complicates the application of HHP for preservation in substitution of thermal processing, which is why further studies on isoenzymes, their baroresistance, and their stabilities are required to design target-specific processes based solely on high pressure and hurdle technologies with HHP.

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Conflicts of Interest

The authors declare no conflict of interest.

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3. COMPARISON OF THE EFFECT OF HYDROSTATIC AND DYNAMIC HIGH-PRESSURE PROCESSING ON THE ENZYMATIC ACTIVITY AND PHYSICOCHEMICAL QUALITY ATTRIBUTES OF ´ATAULFO´ MANGO NECTAR

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Comparison of the Effect of Hydrostatic and Dynamic High Pressure Processing on the Enzymatic Activity and Physicochemical Quality Attributes of 'Ataulfo' Mango Nectar

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Abstract: The effects of hydrostatic (HHP) and dynamic (HPH) high-pressure treatments on the activity of pectin methylesterase (PME) and polyphenol oxidase (PPO) as well as the physicochemical quality attributes of 'Ataulfo' mango nectar were assessed. HHP reduced PME relative activity by 28% at 100 MPa for 5 min but increased PPO activity almost five-fold. Contrarily, HPH did not affect PME activity, but PPO was effectively reduced to 10% of residual activity at 300 MPa and at three passes. Color parameters (CIEL*a*b*), °hue, and chroma were differently affected by each type of high-pressure processing technology. The viscosity and fluid behavior were not affected by HHP, however, HPH changed the apparent viscosity at low dynamic pressure levels (100 MPa with one and three passes). The viscosity decreased at high shear rates in nectar samples, showing a shear-thinning effect. The results highlight how different effects can be achieved with each high-pressure technology; thus, selecting the most appropriate system for processing and preserving liquid foods like fruit beverages is recommended.

Keywords: hydrostatic high pressure; high-pressure homogenization; enzymatic activity; color; rheology; mango nectar

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1. Introduction

In the production of high-quality food products, like fruit-based beverages, enzyme inactivation is considered a requirement for sufficient shelf life. Fruit-based beverages contain indigenous enzymes like pectinases and oxidoreductases, which are considered as the main causes of undesirable changes in color, texture, and other sensory attributes during storage [1,2]. Thermal processing has been so far the most effective preservation technology in the industry for the inactivation of enzymes like pectin methylesterase (EC 3.1.1.11) and polyphenol oxidase (EC 1.10.3.1) in fruit-based beverages, although it affects the nutritional content and sensory attributes of food products [3]. Alternatively, non-thermal processing technologies like hydrostatic high pressure (HHP) or high-pressure homogenization (HPH) are becoming more popular for their advantages over conventional preservation technologies [4,5]

HHP is a technology that does not cause significant changes in molecules because it only affects non-covalent bonds, unlike thermal processing, and it has a superior capacity for the retention of nutrients, nutraceutical compounds, and sensory attributes of foods [6]. The main enzyme inactivation mechanism associated with HHP is the loss of their native

tridimensional configuration (tertiary and quaternary structures), which are stabilized by non-covalent bonds. These conformations are disrupted when hydrostatic pressure is applied in an aqueous medium [7]. Unlike HHP, HPH is mostly used for the processing of liquid foods with a very limited range of suspended particle sizes. At a molecular and structural level, the shear stress induced by HPH and the thermal effect by adiabatic heating (temperature increases around 20 °C per 100 MPa) cause disruption of the particles in the food system; hence, molecules like proteins are unfolded and aggregated by the effect of homogenization, which could lead to losing their catalytic activity and increasing the product's shelf life [8–10].

There are numerous reports of the effects of HHP on the enzymatic activity in fruit-based food products, often with variable results in terms of enzyme inactivation. However, HHP in combination with thermal treatments has been evaluated to increase process efficiency for enzyme inactivation in fruit-based food products [11–13]. Reports on HPH, by contrast, have been focused on the inactivation of PME in citrus juices [14–16]. In terms of a comparison between these technologies, there are scarce reports providing information on HHP and HPH on the same matrix in a similar high-pressure range. HHP has been compared with sonication, thermal treatments, and pulsed electric fields in terms of enzyme inactivation efficiency [17–19]. Meanwhile, the direct comparison of these pressure-based processing technologies has been limited to a few studies, such as the one on egg white lysozyme by Tribst et al. [20]. This provides an opportunity for comparing the effect of these technologies on fruit-based products and their attributes.

Considering the importance of the residual activity of enzymes such as PME and PPO in the overall quality and shelf life of fruit-based products, the objective of this study was to compare the effect of HHP and HPH on the activity of both enzymes using 'Ataulfo' mango nectar as a study model. The physicochemical parameters often associated with the activity of these enzymes, such as color and viscosity, were also evaluated, as they are important quality attributes in this type of product.

2. Results and Discussion

2.1. Effect of High Hydrostatic and Dynamic Pressure on the Relative Enzymatic Activity of Pectin Methylesterase

Concerning the effect of high hydrostatic pressure processing on PME relative activity, the Analysis of Variance (ANOVA) showed that all the experimental factors, with the exception of hydrostatic pressure level, were significant ($p \leq 0.05$), with a significant interaction between the hydrostatic pressure level and the processing time experimental factors. Our results showed an interesting trend, where the relative enzymatic activity of PME was reduced to 72.6% at 100 MPa and 5 min, in contrast with higher pressure levels and processing times, where activity remained above 90% (Table 1). For analyzing the effect of dynamic pressure processing on the relative enzymatic activity of PME, the outlet temperature after the high-pressure valve of the homogenization system (Table 2) was included as a covariate in the ANOVA. Adjusting the terms of the factorial regression model showed that the dynamic pressure level and outlet temperature were significant as a linear model ($p \leq 0.05$). The Variance Inflation Factor (VIF = 15.31) showed a high correlation between dynamic pressure level and outlet temperature on PME relative activity, despite the fact that it was mostly unaffected and partially increased (up to 119.9%) by dynamic pressure processing. In comparison, neither of the high-pressure processing systems was efficient for reducing PME activity under the studied processing conditions. Although the outlet temperature was significantly correlated to dynamic pressure, the holding times at those temperatures during processing might not be sufficient to synergize with the pressure to reduce PME activity. It is commonly stated in the literature that PME is a highly baroresistant enzyme, as it can withstand hydrostatic pressure levels higher than 600 MPa [21]. Previously reported results of the HHP effect of PME in mango nectar by Bermudez-Aguirre et al. [22] showed that at 345 MPa, activity was reduced by 45%, but at 414 MPa, PME increased its activity. Regarding the effect of high dynamic pressure on

PME, Welti-Chanes et al. [23] reported 75% inactivation of PME in orange juice treated at 250 MPa with an initial temperature of 45 °C. Their results might suggest that the thermal effects from adiabatic heating were mainly responsible for the higher level of inactivation, as opposed to our results, where we kept the initial temperature at 10 °C to reduce thermal effects as much as possible. Similarly, Velázquez-Estrada et al. [16], in orange juice, achieved 95% inactivation of PME at 300 MPa, with a final temperature of 95 °C. Likewise, Navarro et al. [15] reported 10% residual PME activity in clementine juice processed at 150 MPa and 68 °C. These previous works support our claims that thermal effects might be more pronounced and impactful than dynamic pressurization on enzyme inactivation.

Table 1. Effect of hydrostatic and dynamic high-pressure treatments on the relative activity of pectin methylesterase and polyphenol oxidase in "Ataulfo" mango nectar.

High Hydrostatic Pressure (MPa)	Processing Time (min)	Relative Enzymatic Activity (%)	
		Pectin Methylesterase	Polyphenol Oxidase
Control	0	100.00 ± 4.84 ^{a,*}	100.00 ± 7.09 ^{d}
100	5	72.60 ± 1.79 ^{c}	337.04 ± 48.02 ^{c}
	25	96.79 ± 6.27 ^{ab}	382.64 ± 37.66 ^{bc}
250	15	94.92 ± 2.76 ^{ab}	481.36 ± 23.99 ^{a}
250	15	98.19 ± 2.80 ^{a}	448.26 ± 49.81 ^{ab}
250	15	99.2 ± 2.76 ^{a}	398.23 ± 34.78 ^{bc}
400	5	90.05 ± 3.03 ^{b}	380.87 ± 33.24 ^{bc}
	25	89.28 ± 2.92 ^{b}	421.02 ± 18.88 ^{ab}
High Dynamic Pressure (MPa)	**Number of Passes**	**Relative Enzymatic Activity (%)**	
		Pectin Methylesterase	**Polyphenol Oxidase**
Control	0	100.00 ± 4.84 ^{ab}	100.00 ± 7.09 ^{c}
100	1	104.98 ± 4.93 ^{ab}	158.80 ± 7.60 ^{ab}
	3	93.13 ± 3.44 ^{b}	149.87 ± 11.16 ^{b}
200	2	97.25 ± 4.93 ^{b}	112.65 ± 7.57 ^{c}
200	2	101.08 ± 7.78 ^{ab}	142.34 ± 14.43 ^{b}
200	2	95.13 ± 9.89 ^{b}	179.43 ± 5.33 ^{a}
300	1	119.95 ± 14.09 ^{a}	26.74 ± 3.25 ^{d}
	3	99.53 ± 4.36 ^{b}	10.73 ± 1.13 ^{d}

* Mean pairwise comparisons by Tukey's test (α = 0.05). Means that do not share a letter are significantly different. Grouping information corresponds to each column.

Table 2. Temperature of mango nectar samples processed by high-pressure homogenization after the high-pressure valve.

Treatment	Temperature (°C) *
100 MPa/1P	31.8 ± 1.1
100 MPa/3P	37.5 ± 1.4
200 MPa/2P	58.3 ± 1.1
200 MPa/2P	55.1 ± 1.7
200 MPa/2P	52.6 ± 0.9
300 MPa/1P	65.3 ± 3.4
300 MPa/3P	73.3 ± 1.2

* Temperature values are means ± standard deviation.

2.2. Effect of High Hydrostatic and Dynamic Pressure on Relative Enzymatic Activity of Polyphenol Oxidase

Regarding the effect of high hydrostatic pressure processing on PPO relative activity, none of the experimental factors were significant ($p > 0.05$). As shown in Table 1, the relative enzymatic activity of PPO was highly increased between 337.0 and 481.3% among all treatments. The ANOVA of the effect of dynamic pressure processing on the relative activity of PPO showed that outlet temperature was significantly correlated ($p \leq 0.05$,

VIF = 72.78) with dynamic pressure level and possibly had a greater effect on PPO activity, as pressure level and the number of passes were not significant (p = 0.067 and 0.055, respectively). As observed in Table 1, the relative enzymatic activity of PPO increased at 100 and 200 MPa; however, processing at 300 MPa for one and three passes reduced the relative activity to 26.7 and 10.7%, respectively. To discard any unwanted effect in enzyme activity due to possible variations in protein content between samples, the average protein content in the samples treated by HHP and HPH, respectively, was 10.7 ± 1.0 and 9.9 ± 0.4 mg/mL, as quantified by the BCA assay. There was no significant difference between the protein content of the nectar samples; hence, the differences in enzymatic activity between processing conditions might not relate to changes in protein content. In comparison with HHP, the reduction of PPO activity in HPH treated samples could be attributed to the possible synergy between high dynamic pressure and the thermal effects due to adiabatic heating in the system, since temperatures of 65 and 73 °C were reached at the HP valve outlet after one and three passes, respectively, and their correlation was statistically significant. However, due to the function of the cooling system in the HPH device, the treated samples stayed at these high temperatures for holding times of less than one second. Our results suggest that HPH might be more effective than HHP for reducing the activity of PPO in fruit-based beverages, as long as the dynamic pressure synergizes with high temperatures. In the literature, PPO is considered resistant to HHP at 100–600 MPa, and some cases of increased activity have been observed within these pressure levels [24]. Unlike PME, there are fewer reports of PPO inactivation by high dynamic pressure. Bot et al. [25] reported a reduction of PPO activity in apple juice by 50% after 10 passes at 150 MPa. These conditions, however, are not feasible in an operational environment for pilot or commercial-scale production. In addition, Marszalek et al. [26] reported that the combination of dynamic pressure and heat increases enzyme inactivation at >200 MPa in dynamic pressure systems, which supports our results showing how PPO activity was reduced significantly at 300 MPa for one and three passes due to the correlated effect of dynamic pressure and high outlet temperature from adiabatic heating.

2.3. Effect of High Hydrostatic and Dynamic Pressure on Color Parameters

In relation to the effect of high hydrostatic pressure processing on the color parameters of mango nectar, the ANOVA showed that the experimental factors were not significant, except for the two-way interaction between hydrostatic pressure level and processing time, which significantly affected a* values ($p \leq 0.05$). As shown in Table 3, the samples processed by HHP retained the luminosity (L*) and b* values, while a* increased slightly in comparison to the control sample. The total color difference values (ΔE) concerning the JND (Just Noticeable Difference) ranges indicated that only an experienced observer could notice differences between samples [27]. The effect of processing conditions on °hue and chroma is shown in Figure 1A. The °hue of samples is near to the yellow color (90°), and the highest chroma value was observed in samples treated at 100 MPa for 5 min. The lower chroma values indicate a duller color saturation when combined with the color tone (°hue), and considering the slight decrease in L* in HHP samples, these effects might be related to the highly increased activity of PPO after processing. It is known in the literature that HHP is a preservation technology that efficiently retains sensory attributes like color. Aaby et al. [28] reported good color retention in strawberry puree processed by HHP. Moreover, our results showed that the samples processed by HPH experienced increases in L* and b* values, while a* was slightly reduced compared to the control sample, despite the fact that the ANOVA showed no significant effects from the experimental factors. The effect of HPH on sample color was slightly different from that of HHP, as the ΔE values were higher, indicating a clear difference of color in comparison to the control sample. In addition, the results of °hue and chroma values (Figure 1B) indicated that the mango nectar processed by HPH became lighter in color (higher L* values from Table 3), and the yellow coloration increased as the dynamic pressure level increased. This could be attributed to the reduced PPO activity in HPH processed samples compared to HHP, and the disruptive

effect of HPH on cells, as it can lead to the release of pigments into the serum phase of the product [29]. Guan et al. [30] reported an increase in carotenoids from mango juice treated by HPH. These pigments relate to the characteristic yellow coloration of 'Ataulfo' mangoes. Kruszewski et al. [31] reported a similar behavior in the color parameters of blackcurrant juice processed by HPH. Processing slightly increased L* and b* values, while a decrease in a* was observed. These effects were attributed to a partial loss of the main pigment (decreased a* values) and non-enzymatic browning (increased b* value). In our results, the increase in b* value is a desirable trait, because carotenoids are the main pigments in 'Ataulfo' mangoes and can be associated with a more intense yellow coloration.

Table 3. Effect of high hydrostatic and dynamic pressures on color parameters (CIEL*a*b*) and total color difference in 'Ataulfo' mango nectar.

Hydrostatic Pressure Level (MPa)	Processing Time (min)	L*	a*	b*	ΔE
Control	0	$34.93 \pm 0.37^{a,*}$	2.48 ± 0.31^{a}	14.68 ± 0.50^{b}	0.00^{d}
100	5	33.15 ± 0.52^{b}	1.75 ± 0.08^{b}	14.14 ± 0.53^{b}	2.18 ± 0.30^{b}
	25	35.96 ± 0.36^{a}	3.04 ± 0.32^{a}	11.38 ± 0.56^{c}	3.28 ± 0.43^{a}
250	15	34.97 ± 1.06^{a}	2.48 ± 0.56^{a}	13.64 ± 1.34^{b}	1.30 ± 0.24^{c}
250	15	34.96 ± 0.45^{a}	2.81 ± 0.09^{a}	13.62 ± 0.74^{b}	1.13 ± 0.15^{c}
250	15	32.75 ± 0.98^{b}	2.68 ± 0.26^{a}	16.30 ± 1.15^{a}	3.25 ± 0.55^{a}
400	5	33.62 ± 0.17^{b}	2.55 ± 0.20^{a}	15.22 ± 0.19^{ab}	1.54 ± 0.06^{bc}
	25	33.36 ± 0.51^{b}	1.87 ± 0.12^{b}	14.56 ± 0.66^{b}	1.67 ± 0.21^{bc}
Dynamic Pressure Level (MPa)	**Number of Passes**	**L***	**a***	**b***	**ΔE**
Control	0	34.93 ± 0.37^{d}	2.48 ± 0.31^{ab}	14.68 ± 0.50^{d}	0.00^{d}
100	1	38.66 ± 0.48^{ab}	1.78 ± 0.18^{cd}	13.96 ± 0.58^{d}	4.10 ± 0.30^{bc}
	3	37.02 ± 1.27^{bcd}	2.00 ± 0.35^{bc}	16.13 ± 1.12^{bcd}	3.42 ± 0.40^{c}
200	2	38.43 ± 0.73^{abc}	2.79 ± 0.15^{a}	15.32 ± 1.01^{cd}	3.73 ± 0.40^{c}
200	2	39.87 ± 0.95^{a}	1.97 ± 0.26^{bc}	14.57 ± 0.46^{d}	5.34 ± 0.60^{a}
200	2	36.95 ± 1.61^{bcd}	1.36 ± 0.41^{d}	17.59 ± 1.74^{abc}	3.71 ± 0.27^{c}
300	1	36.60 ± 1.71^{bcd}	1.30 ± 0.38^{d}	18.22 ± 2.21^{ab}	3.92 ± 0.19^{c}
	3	36.36 ± 0.26^{cd}	1.59 ± 0.08^{cd}	19.15 ± 0.27^{a}	4.78 ± 0.17^{ab}

* Mean pairwise comparison by Tukey's test ($\alpha = 0.05$). Means that do not share a letter are significantly different. Grouping information corresponds to each column.

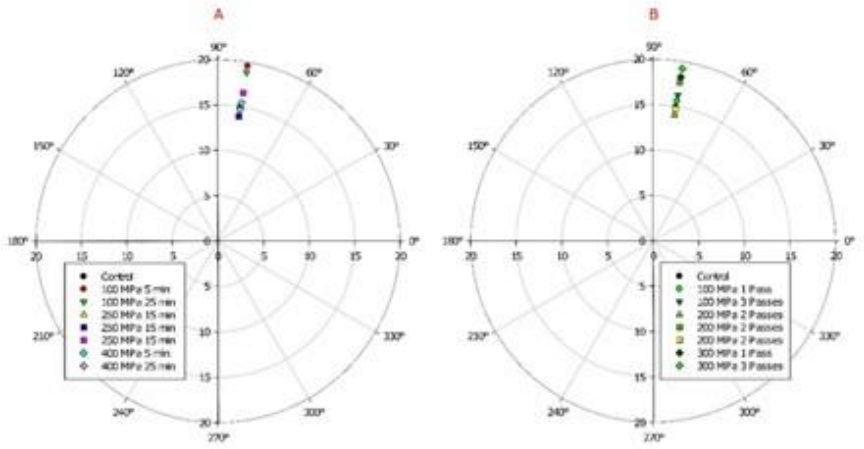

Figure 1. Polar plot of °hue vs. chroma of 'Ataulfo' mango nectar processed by high pressure: (**A**) hydrostatic pressure, (**B**) dynamic pressure.

2.4. Effect of High Hydrostatic and Dynamic Pressure on Rheological Parameters

The rheological behavior of mango nectar samples processed by HHP and HPH showed a difference between both processing technologies only at the 100 MPa pressure level, especially at low shear rates. The changes in apparent viscosity as a function of the shear rate of the samples processed by HHP or HPH are presented in Figure 2. All nectar samples presented a shear-thinning behavior, associated with pseudo-plastic non-Newtonian fluids. The rheology of nectar samples after HHP compared to the control sample was not significantly affected, independently of the processing conditions (hydrostatic pressure level and processing time). Liu et al. [32] reported that HHP had no significant effect on the viscosity of mango nectars, unlike a high-temperature short-time treatment (HTST), which decreased viscosity. The effects in sample rheology in HHP may depend on the relation between the pressure level and holding time with the total soluble solids content of the processed sample [33]. In our results, HPH had a greater impact on fluid behavior and viscosity than HHP. An interesting phenomenon was observed in nectar samples treated by HPH at 100 MPa (one and three passes) and low shear rates, as their viscosity was higher than that of the control sample. In contrast, samples treated at 300 MPa by HPH at the same shear rates did not show the same effect, as apparent viscosity was significantly lower than the control samples at high shear rates (Table 4). This behavior could be attributed to the effect of HPH on pectins and the other polysaccharides present in mango pulp, as it might reduce the apparent viscosity of the serum phase of the nectar, altering consistency and texture [7]. In addition, HPH modifies the particle size and distribution, which could increase the particle–particle and particle–serum interactions, resulting in variable pulp stability [34]. It is important to mention that the effects on viscosity could also be product-dependent, as Leite et al. [35] reported that HPH reduced the consistency of apple juice and claimed that the response of each product to HPH could be difficult to predict.

Table 4. Viscosity at select shear rates of nectar samples processed by high hydrostatic pressure and high pressure homogenization.

	Nectar Viscosity (mPa·s) at Low Shear Rate (7 s^{-1})	Nectar Viscosity (mPa·s) at High Shear Rate (53 s^{-1})
High Hydrostatic Pressure		
Control	174.7 ± 8.1 ^{a,*}	36.2 ± 0.2 ^{ab}
100 MPa 5 min	183.0 ± 20.8 ^{a}	36.6 ± 0.4 ^{b}
100 MPa 25 min	201.5 ± 31.8 ^{a}	40.3 ± 7.2 ^{a}
250 MPa 15 min	173.9 ± 12.4 ^{a}	38.7 ± 0.4 ^{ab}
250 MPa 15 min	172.7 ± 11.8 ^{a}	41.7 ± 1.6 ^{ab}
250 MPa 15 min	177.3 ± 9.1 ^{a}	37.5 ± 2.8 ^{ab}
400 MPa 5 min	189.4 ± 16.7 ^{a}	44.3 ± 0.6 ^{a}
400 MPa 25 min	187.1 ± 29.5 ^{a}	32.2 ± 0.4 ^{b}
High Pressure Homogenization		
Control	174.7 ± 8.1 ^{b,*}	36.2 ± 0.2 ^{b}
100 MPa 1P	281.2 ± 14.3 ^{a}	39.4 ± 0.2 ^{a}
100 MPa 3P	221.0 ± 5.9 ^{ab}	26.5 ± 0.6 ^{c}
200 MPa 2P	172.4 ± 7.3 ^{b}	25.6 ± 0.7 ^{c}
200 MPa 2P	165.2 ± 6.2 ^{b}	24.8 ± 0.4 ^{c}
200 MPa 2P	179.7 ± 5.8 ^{b}	26.3 ± 0.5 ^{c}
300 MPa 1P	166.6 ± 7.7 ^{b}	26.6 ± 0.8 ^{c}
300 MPa 3P	185.8 ± 8.6 ^{b}	24.9 ± 0.1 ^{c}

* Mean pairwise comparison by Tukey's test ($\alpha = 0.05$). Means that do not share a letter are significantly different. Grouping information corresponds to each column.

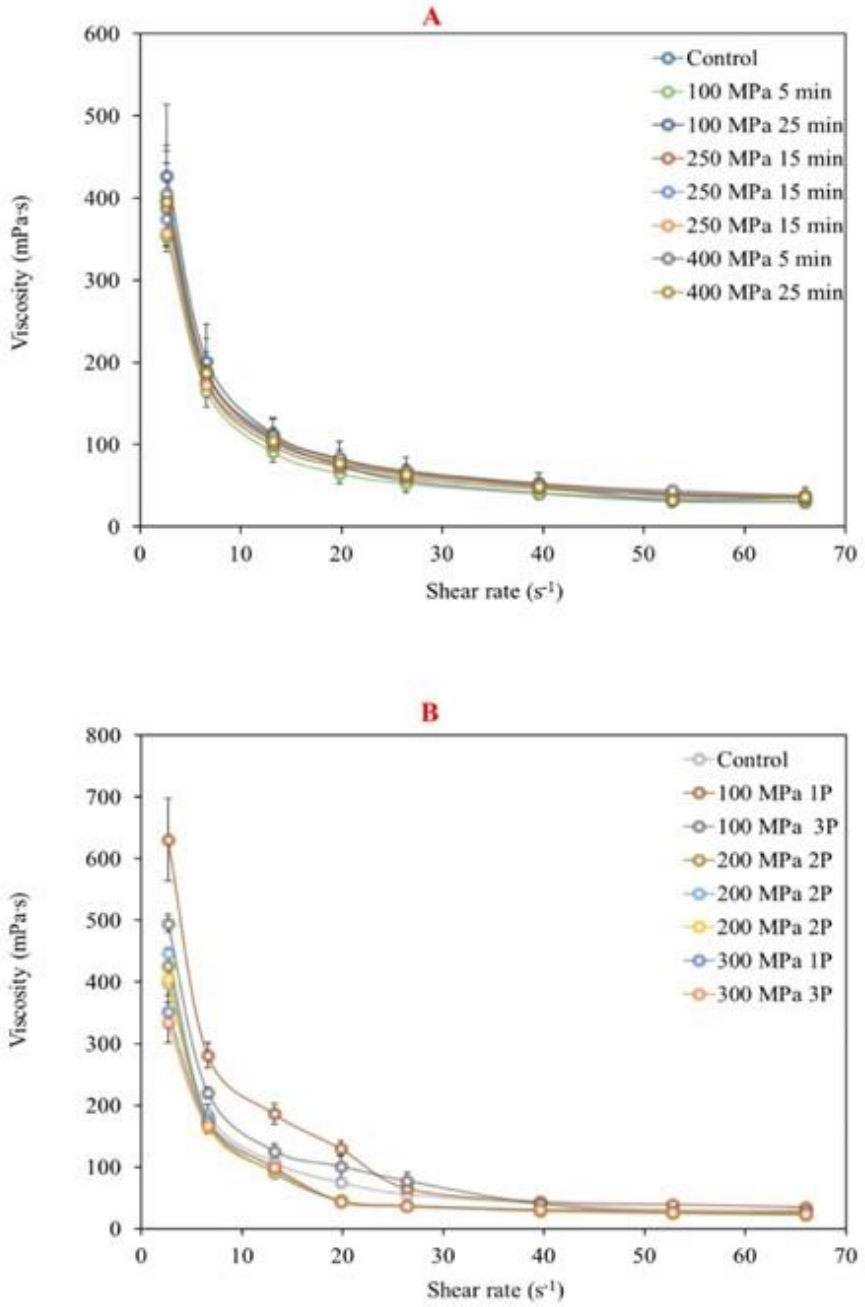

Figure 2. Apparent viscosity as a function of shear rate in mango nectar processed by high pressures: (**A**) hydrostatic pressure, (**B**) dynamic pressure. Bars show standard errors.

3. Materials and Methods

3.1. Plant Material

'Ataulfo' variety mango fruits were harvested from an orchard in Chiapas, Mexico (latitude 15.285056, longitude −92.698389) at ripeness stage 4 (14–16 °Brix and pH = 4.5–4.7 in pulp). Fruits were washed in a 200 ppm sodium hypochlorite solution. Pulp was separated from the pericarp, packed, sealed in vacuum bags, and stored at −20 °C. Frozen pulp was

transferred to the facilities of the UMR IATE of the University of Montpellier to perform the experiments. Mango pulp was stored at −20 °C throughout the experimental phase.

3.2. Mango Nectar Formulation

The pulp was thawed overnight at 4 °C and blended in a thermomixer for 120 s at high speed until a smooth paste was formed. The nectar formulation contained 40% *w*/*w* pulp paste diluted with reverse-osmosis-treated water and mixed by magnetic agitation until fully homogenized. No sugar or additives were added. The nectar was filtered through a sieve of 250 µm mesh before high-pressure processing, and the pH, soluble solids (°Brix), and color (CIEL*a*b*) of the untreated filtered nectar (control) were measured.

3.3. High Hydrostatic Pressure Processing

Pressurization experiments were carried out in a 1 L capacity high-pressure stainless steel vessel from ACB (Nantes, France), with a maximum operating pressure of 450 MPa. Samples were treated at pressure levels of 100, 250, or 400 MPa for a gradual screening of enzyme activity, with 5, 15, and 25 min processing times and an initial temperature (Ti) = 10 °C, using distilled water as a pressure transmitting fluid. For each batch, 300 mL of nectar was packed in polyvinylidene chloride (PVDC) tubings (26 mm diameter, 50 µm thick, Krehalon, Eygalières, France), sealed with two knots at both ends, and kept at 4 °C for a maximum of 4 h before being processed (control assay). After processing, one set of samples was used for the determination of the enzymatic activity.

3.4. High Dynamic Pressure Processing

High-pressure homogenization experiments were carried out in a Stansted Fluid Power UHP homogenization system (Essex, UK), which operates at a maximum dynamic pressure of 350 MPa. Nectar samples were stored in a sampling stainless steel tank at 10 °C and treated at 100, 200, and 300 MPa for 1, 2, and 3 passes. Temperatures were recorded before and after the HP valve. After processing, one set of samples was used for the determination of the enzymatic activity immediately after processing.

3.5. Enzymatic Activity Assays

3.5.1. Enzyme Extraction

Nectar samples processed by HHP and HPH were diluted with extraction buffer (0.2 M sodium phosphate pH 6.4, 4% *w*/*w* polyvinylpolypyrrolidone, 1 M NaCl) at a 4:1 buffer-to-sample ratio and mixed at 4 °C for 1 h. Afterward, samples were centrifuged at 37,750× *g* at 4 °C for 30 min in a Sorvall RC 5B Plus (Du Pont, Wilmington, DE, USA) centrifuge [36,37]. The supernatant was collected and used as an extract for enzymatic activity assays. Protein content in enzyme extracts was determined by the bicinchoninic acid (BCA) assay.

3.5.2. Pectin Methylesterase Activity Assay

The activity of pectin methylesterase was measured according to Rodrigo et al. [38], and 0.25 mL of extract was added to 30 mL of substrate solution (0.35% apple pectin, 0.12 mM NaCl pH 7.0) to start the reaction. Then, 0.05 N NaOH was automatically added by a 902 Titrando automatic titrator fitted with an 800 Dosino Unit (Metrohm, Herisau, Switzerland) for a reaction time of 6 min. The activity of PME was calculated according to the following equation:

$$\text{PME (U)} = \frac{V \cdot N \cdot 1000}{V_s \cdot t} \quad (1)$$

where

V = volume of NaOH (mL);
N = normality of NaOH;
V_s = sample volume (mL);
t = reaction time (min).

All assays were performed at 25 °C in triplicate.

3.5.3. Polyphenol Oxidase Activity Assay

The activity of polyphenol oxidase was measured according to the method by Palma-Orozco et al. [39]; 50 µL of enzyme extract was added to 1 mL of substrate solution (0.2 M sodium phosphate buffer pH 6.6, 50 mM pyrocatechol) to initiate the reaction in a 1 cm quartz cell. Then, absorbance was measured every 5 s for 5 min in a Unicam UV2 UV-Vis spectrophotometer (ATi Unicam, Cambridge, UK) at 420 nm. PPO activity was calculated from the slope of the initial linear portion of the absorbance vs. time curve and expressed in units per mL of extract. All assays were performed at 25 °C in triplicate.

3.5.4. Relative Enzymatic Activity Calculation

The activities of PME and PPO were calculated as units per mL of extract. For better comparison against the control sample, the values were converted to relative activity regarding the untreated nectar, which was calculated according to the following equation:

$$\text{Relative Enzymatic Activity } (\%) = \frac{A_s}{A_0} \times 100 \quad (2)$$

where

A_s = enzymatic activity in U/mL of the processed sample;
A_0 = enzymatic activity in U/mL of the untreated sample.

3.6. Color Parameters

Color (CIELa*b*) was measured in all samples to determine changes after processing compared to the control sample. The color was measured in quintuplicate as L*, a*, and b* with a Dr. Lange portable colorimeter. The chroma and °hue values were calculated with Equations (3) and (4), respectively. °Hue values were converted from radians to degrees and plotted using SigmaPlot v12.0 (Systat Software, Inc., San Jose, CA, USA).

$$\text{Chroma} = \sqrt{(a*^2 + b*^2)} \quad (3)$$

$$^{\circ}\text{Hue} = \arctan\left(\frac{b*}{a*}\right) \quad (4)$$

3.7. Rheological Parameters

The apparent viscosity of processed mango nectar was estimated by application of linearly increasing shear rate values with a Brookfield DV-III ULTRA Programmable Rheometer (Brookfield Engineering Laboratories, Stoughton, MA, USA) fitted with a No. 18 spindle. A total of 10 mL of sample was added to the sample cup and maintained at 10 °C ± 0.1 with a recirculating water bath, using a mixture of distilled water and glycol. Shear rate values were variated linearly (2.64–66 s^{-1}) to progressively measure the corresponding shear stress and viscosity. The apparent viscosity for each sample was reported in mPa·s.

3.8. Design of Experiments and Statistical Analysis

A 2^k experimental design was used for each type of high-pressure processing. For hydrostatic pressure, a 2^2 design with pressure level (100 and 400 MPa for low and high levels, respectively) and processing time (5 and 25 min for low and high processing times, respectively) as experimental factors was utilized (Figure 3A). For dynamic pressure, a 2^2 design with pressure level (100 and 300 MPa for low and high levels, respectively) and the number of passes (1 and 3 for low and high number of passes, respectively) as experimental factors was utilized (Figure 3B). The center point was independently replicated three times for each design. All data were analyzed in the Minitab 17 statistical software (Minitab, LLC, State College, PA, USA).

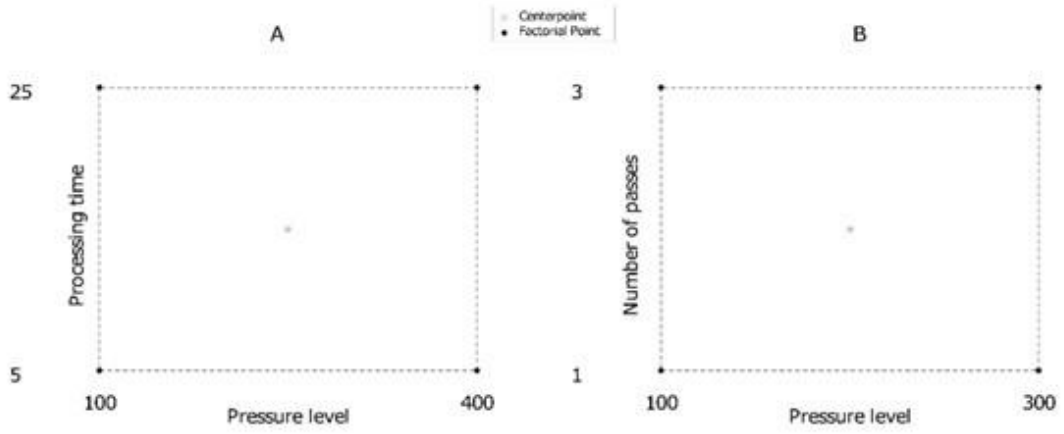

Figure 3. Experimental domain for hydrostatic and dynamic pressure treatments: (**A**) hydrostatic, (**B**) dynamic.

4. Conclusions

High hydrostatic and dynamic pressure processing are two interesting technologies for the preservation of fruit-based beverages like mango nectar. Regarding enzyme inactivation, our results suggest that neither is suitable for controlling the activity of pectin methylesterase without the assistance of thermal processing. Regarding polyphenol oxidase activity, high-pressure homogenization showed a good activity reduction at 300 MPa with three passes, with a possible synergy of high temperature due to adiabatic heating, even for very short holding times. For retention of quality attributes, both technologies showed good results concerning color retention. In terms of viscosity and fluid behavior, high-pressure homogenization showed interesting results at low-pressure levels, which could be useful as a tool for modifying the texture and consistency of fruit-based beverages with high pulp content. Despite these results, other relevant emergent technologies suitable for liquid food preservation such as pulsed electric fields, high-power ultrasound, high-pressure carbon dioxide, and ultraviolet irradiation could be compared in terms of effectiveness to high-pressure technologies. Additionally, the combination (hurdle effect) and optimization of emergent technologies might be a future trend for the food industry to produce the best food products with the best quality attributes and shelf-life.

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4. ONE DIMENSIONAL ZYMOGRAPHY AS A TOOL FOR IDENTIFICATION OF PRESSURE-TOLERANT ISOFORMS OF PECTIN METHYLESTERASE IN MANGO NECTAR PROCESSED BY HIGH-PRESSURE

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Artículo en borrador.

Abstract:

Zymography is a proteomic technique for the *in situ* identification of enzymes. It represents an interesting alternative for evaluating the efficiency of food preservation processes by the identification of tolerant isoforms. To identify tolerant pectin methylesterase (PME) in mango nectar processed by high hydrostatic pressure (HHP) and high-pressure homogenization (HPH), zymography with ruthenium red was performed. Two high-pressure tolerant isoforms, labeled PME1 and PME2 (66 and 33 kDa respectively) were identified in the processed samples and the control. The activity of the isoforms in the zymogram became more evident as the pressure level increased in the treatments. Our results indicate that these particular PME isoforms in mango nectar might be overly activated by HHP and HPH.

Keywords: Pectin methylesterase, zymography, high-pressure processing, isoenzymes, ruthenium red.

1. Introduction

The inactivation of enzymes in food processing is crucial regarding the preservation of quality attributes during storage, especially of plant-based foods. In the case of fruit-based beverages, such as nectars, there are several enzymes related to the deterioration of quality attributes during storage such as color, flavor, and texture (Terefe *et al.,* 2014). Concerning the texture of fruit-based beverages, one of the most important enzymes in food processing is pectin methylesterase (PME) (EC: 3.1.1.11), a hydrolase that catalyzes the de-esterification of pectin, resulting in the loss of turbidity, consistency, and pulpiness in fruit beverages (Aghajanzadeh & Ziaiifar, 2018). For decades, thermal processing such as pasteurization has been employed in food processing facilities for the inactivation of enzymes like PME in fruit-based beverages. However, extensive research has demonstrated the negative effects of thermal processing on the nutritional, sensory, and functional properties of food products and their components (Petruzzi *et al.,* 2017). As such, the evaluation of non-thermal processing technologies for food preservation has gained popularity in the past decades. One of the most relevant non-thermal technologies for food preservation is high-pressure processing, which is considered to only affect non-covalent bonds in food components, thus retaining the organoleptic, nutritional, and bioactive properties of foods (Mújica-Paz *et al.*, 2011; Yamamoto, 2017).

Currently, two high-pressure processing technologies are commercially available for food preservation: high hydrostatic pressure (HHP) and high-pressure homogenization (HPH, also known as dynamic high-pressure) systems. Despite their fundamental differences, both technologies employ high-pressure levels to preserve foods and have been extensively studied about their effect on enzyme activity especially in fruit-based beverages, where variable results ranging from mild inactivation to increased activity of key enzymes involved in quality loss during storage have been reported (Augusto *et al.,* 2018; de Castro Leite Júnior *et al.,* 2017). One of the main findings for the heterogeneous results for enzyme inactivation by high-pressure technologies is that in complex biological systems such as fruits, different molecular forms of certain enzymes

(known as isoenzymes or isoforms) exist. These catalyze the same reactions, but have different amino acid compositions, sequences, and consequently, possess different degrees of tolerance to pressurization (Pelley, 2012; Plaza *et al.*, 2007; Terefe *et al.*, 2014). The identification of isoenzymes in biological systems, such as fruits, has been performed by techniques such as gel electrophoresis (agarose and polyacrylamide), isoelectric focusing, column chromatography, and zymography. Zymography is a very interesting technique to analyze the *in situ* effects of an enzyme after electrophoresis is performed (Scadden & Naaby-Hansen, 2001).

There are few reports on the utility of zymography to identify isoforms of quality-related enzymes in fruits and vegetables. In Ataulfo mango pulp and peel, Cheema and Sommerhalter (Cheema & Sommerhalter, 2015), identified three isoforms of polyphenol oxidase (PPO), an enzyme related to browning and color deterioration, by SDS-PAGE and zymography with catechol, 3-metyhlcatechol, and pyrogallol as substrates, reporting isoforms with molecular weights of 53, 112 and 144 kDa. Similarly, Escalante-Minataka et al. (2018), studied banana pulp with varying ripeness degrees and identified one to six PPO isoforms (molecular weights of 25, 35, 49, 66, 95, and 199 kDa) by zymography with 0.2 M catechol as substrate. In the specific case of PME, zymography has been performed to identify PME isoforms in model organisms such as *Arabidopsis thaliana* (Guénin *et al.*, 2011), grape pomace (Zocca *et al.*, 2007), and strawberry fruits (Draye & Van Cutsem, 2008). However, there are no reports on PME isoforms identified by zymography on mango fruits or mango-based products. This represents an opportunity to provide information regarding the utilization of zymography as a tool for evaluating the effectiveness of food preservation processes, like high-pressure processing, regarding enzyme inactivation. Also, the identification of possible PME isoforms, which is considered an enzyme with high tolerance to high-pressure processing, using 'Ataulfo' mango nectar as a study model.

2. Materials and Methods

2.1. Plant material

"Ataulfo" variety mango fruits were harvested from an orchard from Chiapas, México (latitude 15.285056, longitude -92.698389), in ripeness stage 4 (14-16 °Brix and pH = 4.5 - 4.7 in pulp).

Fruits were washed in a 200 ppm sodium hypochlorite solution. The pulp was separated from the pericarp, packed, sealed in vacuum bags, and stored at -20°C. Frozen pulp was transferred to the facilities of the UMR IATE of the University of Montpellier to perform the experiments. Mango pulp was stored at -20°C throughout the experimental phase.

2.2. Mango nectar formulation

The pulp was thawed overnight at 4°C and blended in a thermomixer for 120 s at high speed until a smooth paste was formed. The nectar was formulated with 40% w/w mango pulp paste diluted with reverse osmosis-treated water and mixed by magnetic agitation until fully homogenized. No sugar or additives were added. The nectar was filtered through a sieve of 250 µm mesh before high-pressure processing.

2.3. High-pressure processing

The processing conditions of the mango nectar, the high-pressure systems used, and enzymatic activity assays are thoroughly described in Uranga-Soto *et al.*, (2022). Briefly, six treatments (100 MPa/5 min, 250 MPa/15 min, and 400 MPa/25 min for HHP, and 100 MPa/1 pass, 200 MPa/2 passes, and 300 MPa/3 passes for HPH) and the control sample were selected for the identification of PME isoforms. The high-pressure range selected for each system was similar in magnitude to compare their effects. The nectar samples were lyophilized, packed, and sealed in vacuum bags. Finally, the samples were transferred from the facilities of the UMR IATE at the University of Montpellier to the Proteomics laboratory of CIAD, in Culiacan, México, to perform the experiments.

2.4. Enzyme extraction and purification

A 3 g of lyophilized mango nectar sample was transferred to a round bottom high-speed centrifuge tube (previously conditioned at -20°C) and extracted with cold extraction buffer (0.1 M sodium

phosphate pH 7.0, 2% polyvinylpolypyrrolidone (PVPP), 0.5% Triton X-100, 0.05 M NaCl) at 1:6 sample to buffer ratio. The sample-extraction buffer mixture was homogenized using a T 25 digital ULTRA TURRAX® (IKA, Germany), in two non-consecutive intervals of 30 s, with a resting interval of 30 s. Afterward, samples were centrifuged at 11,000 *x g* at 4°C for 30 min in a Sorvall LYNX 4000 super speed centrifuge (ThermoFisher Scientific, USA). The supernatant was collected, and stored in 15 mL conical tubes, and a small aliquot was stored in 0.6 mL Eppendorf tubes, which was immediately used for protein quantification. All samples were kept in ice at all moments throughout the experimental phase.

2.5. Protein quantification

The protein content in the extracts was quantified based on the Quickstart Bradford assay (Bio-Rad, USA), with Bradford reagent and bovine serum albumin as protein standard (Bio-Rad, USA). The standard assay protocol for microplates was used, with a BSA standard curve of 0–1.0 mg/mL. The absorption measurements were performed in an Epoch microplate spectrophotometer (BioTek, USA) at 595 nm in 96-well microplates.

2.6. Semi-denaturing SDS-PAGE

The extracted samples were diluted accordingly with 2X native sample loading buffer (Bio-Rad, USA), and a proper volume of 10% SDS solution was added to contain 1% of SDS in the prepared sample. No reducing agents were added, and samples were not heated. 25 µL of the prepared sample (2 µg protein/µL extract) were loaded into each well of a 1 mm thick, polyacrylamide discontinuous mini gel (4% stacking and 10% polyacrylamide concentration in separation gels respectively). Two identical gels were prepared (29:1 polyacrilamyde:bis solution at 40%, 1.5 M Tris-HCl pH 8.8, 10% sodium dodecylsulphate solution, 10% ammonium persulphate solution, TEMED, ultra-pure water) according to the Laemmli method. The 2-D SDS-PAGE Standard (MW: 17.5 – 76 kDa) was used as a protein ladder for the estimation of the molecular weights of the PME isoforms (Bio-Rad, USA). The separation of proteins was performed in a Mini-PROTEAN cell, using Tris-glycine running buffer (3.03 g Tris, 14.4 g glycine, and 1 g of SDS per liter), at constant

voltage (50V), 3.5 hours. To maintain a low temperature during the electrophoresis, the mini-PROTEAN cell was placed inside a water bath with ice. Finally, one gel required for protein identification by silver nitrate staining was stored overnight in 35 mL of fixing solution (10% glacial acetic acid, 30% ethanol and 60% distilled water). The second gel was used for the identification of PME isoforms by zymography.

2.7. Pectin methylesterase zymography

After the 1D Semi-denaturing SDS-PAGE, the gel was immediately rinsed with distilled water, and treated according to Thonar et al. (2006), with few modifications to reveal PME activity. The gel was washed for 30 min in 1% v/v Triton X-100 solution to remove SDS. Then, it was incubated in 20 mM Tris-HCl, 5 mM EDTA, pH 8.5 for 30 min at room temperature. Then, the gel was incubated in 1% apple pectin solution (Sigma-Aldrich, USA) with 20 mM Tris-HCl, 5 mM EDTA, and 160 mM NaCl pH 7.6 for 40 min at room temperature with subsequent rinsing in distilled water to remove the excess of the substrate. Finally, PME activity was revealed by incubation in 0.04% Ruthenium red solution (30 min of incubation at room temperature), and overnight discoloration in distilled water. PME activity appeared as fuchsia-colored bands in the electrophoresis gel.

2.8. Silver nitrate protein staining.

The staining of proteins in the mango nectar extracts was performed according to the short silver nitrate staining protocol by Chevallet *et al.*, (2006), with some adjustments. The fixated gel was washed twice, for 10 min each, in 35 mL of rinsing solution (20% ethanol). Then it was washed twice, for 10 min each, in 50 mL of distilled water. Afterward, the gel was sensitized by soaking in 35 mL of a 0.8 mM sodium thiosulfate solution for 1 min. Then it was washed twice for 1 min each in 35 mL of distilled water. Afterward, the gel was soaked for 1 h in a 12 mM silver nitrate solution. After the impregnation with silver nitrate, the gel was washed for 15 s in distilled water and soaked in developing solution (3% w/v potassium carbonate, 37% formaldehyde solution (250 µL per liter), and 10% sodium thiosulfate solution (125 µL per liter). After the protein bands were adequately stained, the gel was transferred into 35 mL of stopping solution (4% w/v Tris, 2% v/v

glacial acetic acid). Finally, the gel was stored in distilled water until image analysis was required.

2.9. Image analysis of zymogram and silver nitrate stained gels

The analysis of the PME zymogram and silver-stained polyacrylamide gel was performed with Image Lab 6.0 (Bio-Rad, USA) to determine the molecular weight of the PME isoforms.

3. Results and discussions

Several tests for extraction, separation, and identification of PME isoforms from mango nectar samples were performed to find the most suitable conditions (see supplementary material). The critical steps identified for reproducibility were the low protein concentration in the extract due to the nature of the sample, along with the long-term stability under storage at 4°C. The addition of a protease inhibitor like phenylmethylsulfonyl fluoride (PMSF) did not improve protein stability in the extracts. Regarding the electrophoretic separation of the enzymes in the nectar samples, several polyacrylamide concentrations of the separation gels were evaluated, where 10% polyacrylamide in the resolving gel provided the best separation of samples and molecular weight markers under semi-denaturing conditions. As observed in Figure 1, the zymogram for PME activity in mango nectar samples processed by high hydrostatic pressure (HHP) showed the presence of at least two isoforms of PME stained with ruthenium red solution. The PME 1 isoform with an approximate molecular weight of 63 kDa was present in all the HHP processed samples. The PME 2 isoform (approximate molecular weight = 33 kDa) was only present in the samples processed at 200 and 400 MPa for 15 and 25 min respectively. However, the PME 2 isoform was present in the control sample (Figure 2). By comparing the enzymatic activity of PME in the processed mango nectar samples, treatment 100 MPa for 5 min showed the lowest PME activity (figure 3). Possibly, the PME 2 isoform was partially inactivated at the lowest processing conditions, and a further increase in pressure level and processing time, promoted activation of the isoenzyme, as has been previously reported in mango nectar processed at 414 MPa (Bermúdez-Aguirre *et al.,* 2011). Terefe et al. (2015), suggested that while enzymes may experience conformational changes during HHP processing, it may not always imply the reduction of catalytic activity, but the opposite, an

increment. Regarding the molecular weights of the PME isoforms in this study, they are similar to other isoforms characterized from other fruits. Ciardello *et al.,* (2004), reported a PME isoform in kiwi fruits with a molecular weight of 50 kDa. Also, Hou *et al.*, (1997) isolated two isoforms of PME in 'Valencia' orange, describing a thermostable PME with a molecular weight of 53 kDa. Which might be similar in MW to PME1 in our samples. Regarding the isoforms of smaller MW, Kotnala *et al.*, (2018) characterize a PME from papaya fruits, with a MW of 27 kDa. Additionally, Ciardello *et al.,* (2004) reported a PME with MW = 37 kDa from persimmon fruit, and Hou *et al.*, (1997), isolated a thermolabile PME with MW = 37.5 kDa from 'Valencia' orange. These two isoforms of PME are similar in molecular weight to the PME2 in our zymogram. Concerning the high molecular weight PME isoforms, Wang *et al.,* (2007) identified banana PME isoforms by SDS-PAGE within the range of 49.3 – 78.2 kDa, which supports the finding of PME1 in this study.

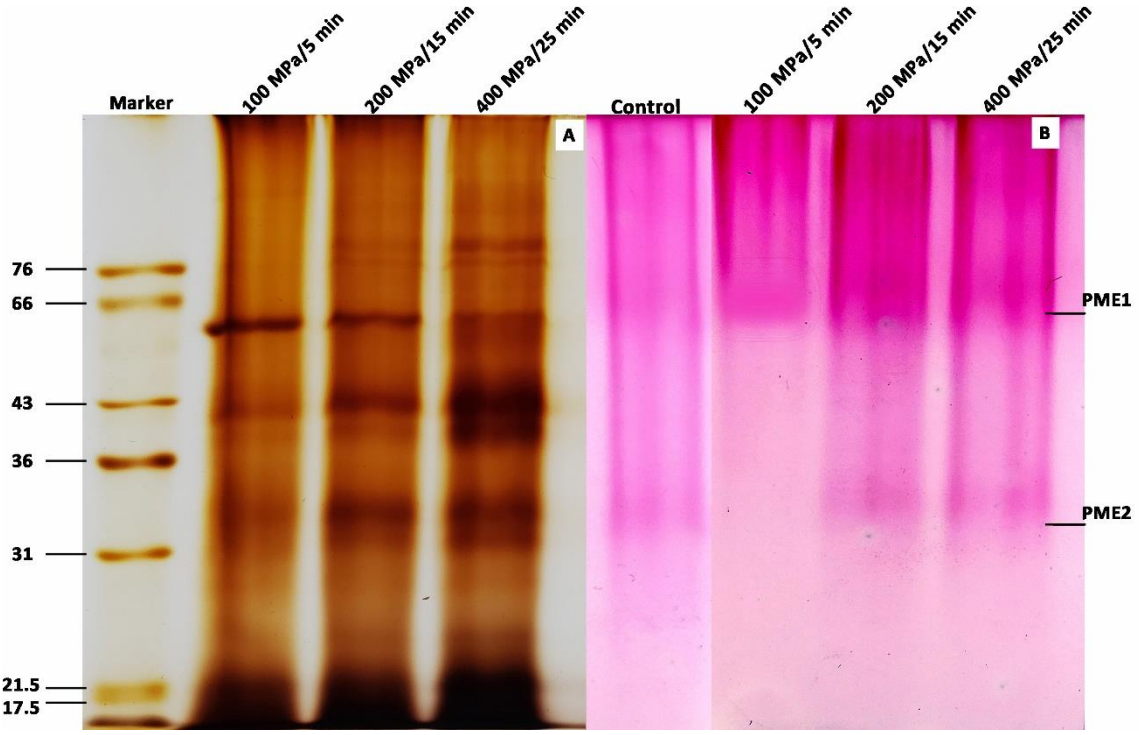

Figure 1. Silver nitrate staining and PME zymogram of enzyme extracts of 'Ataulfo' mango nectar processed by selected high hydrostatic pressure conditions. A) Silver nitrate stain of crude extract proteins. B) PME zymogram with apple pectin as substrate.

Regarding the nectar samples processed by high-pressure homogenization (Figure 2), both PME 1

and PME 2 isoforms were active in all processing conditions, which were similar to the control sample. In terms of enzyme activity, HPH promoted a slight increment in PME activity compared to the minor reductions provided by HHP (figure 3). This could be attributed to the additional mechanical forces that synergize with dynamic pressure in HPH systems, where particle size is more uniform and possibly enzymes have better interactions with their substrates for increased catalytic activity (dos Santos Aguilar *et al.*, 2018). Comparing both technologies, the PME zymograms showed no difference regarding the isoforms contributing to the global enzymatic activity in the nectar, as both PME 1 and PME 2 were present in all but one of the processed mango nectar samples.

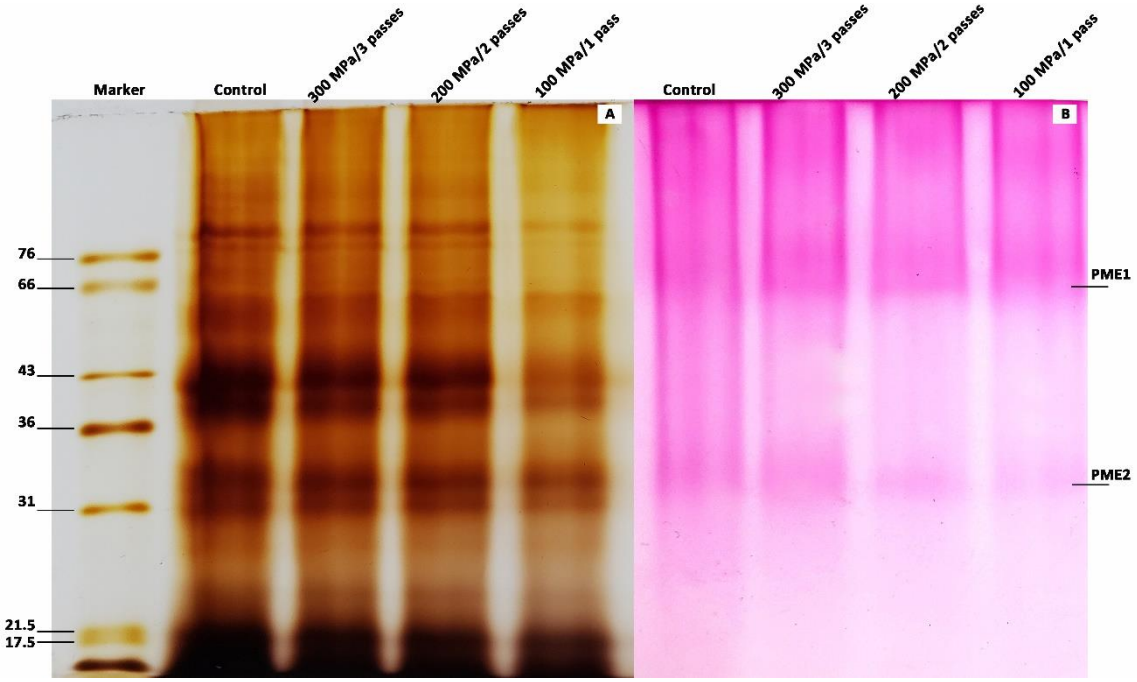

Figure 2. Silver nitrate staining and PME zymogram of enzyme extracts of Ataulfo' mango nectar, untreated and processed by selected high-pressure homogenization conditions. A) Silver nitrate stain of crude extract proteins. B) PME zymogram with apple pectin as substrate.

The ruthenium red staining has been used in gel diffusion, and the pectoplate methods for the identification of PME isoforms and other pectinases (Downie *et al.*, 1998; Lionetti, 2015). In polyacrylamide gels, Thonar et al. (2006), identified three PME isoforms from chicory root extract using a ruthenium red solution. The identification of PME activity by this dye is possible because ruthenium red can bond with the acidic groups of polyuronic acid of pectin, and de-esterified pectin

can generate better staining patterns in PME zymograms. In this study, we demonstrate the possibility of at least two isoforms of PME with tolerance to high-pressure processing that contribute to global enzymatic activity in mango nectar after processing.

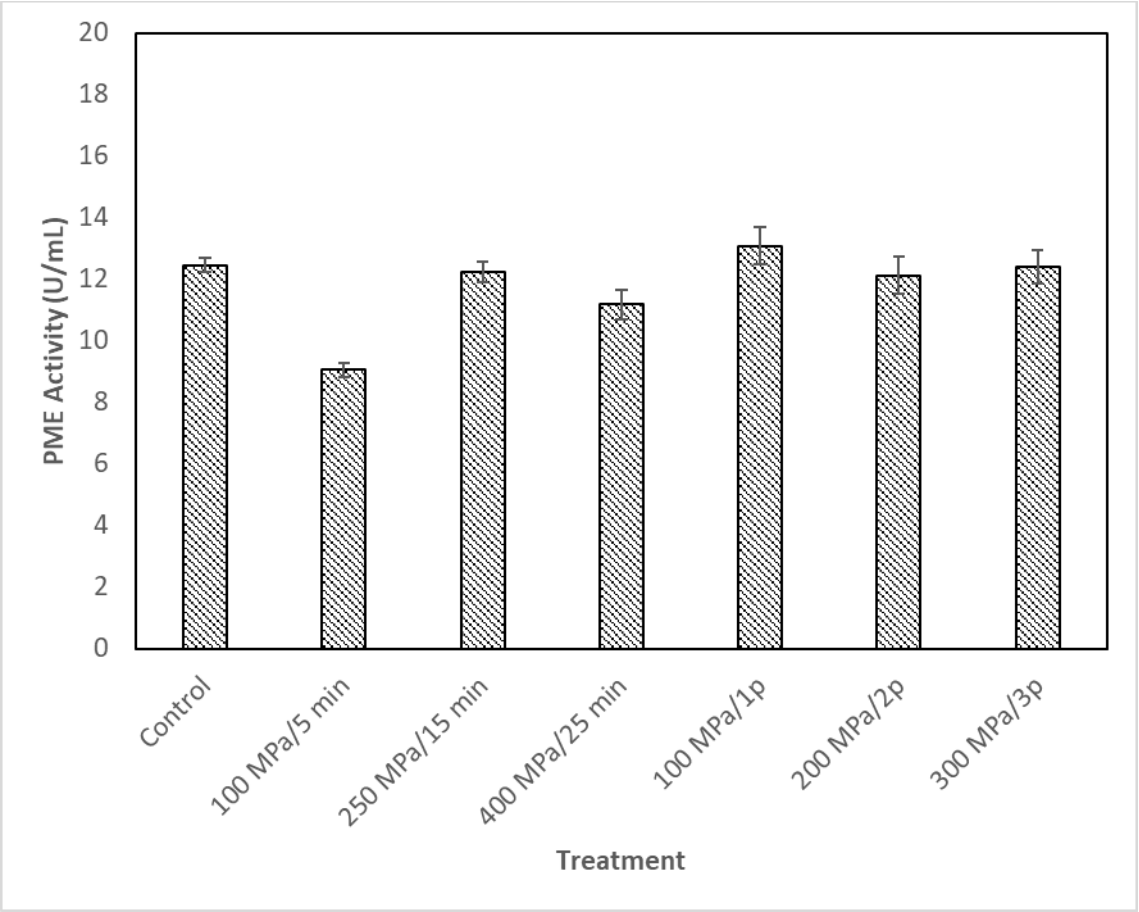

Figure 3. Enzymatic activity of PME in mango nectar samples processed by high hydrostatic and dynamic pressure under selected processing conditions.

4. Conclusions

Pectin methylesterase has been regarded in literature as a very tolerant enzyme to high-pressure inactivation. A possible cause is the presence of multiple isoforms of PME in food products, which could possess different degrees of tolerance to pressurization. Zymography is a proteomic technique that could be applied to differentiate between the isoforms of enzymes related with quality loss, such as PME in high-pressure processed food products such as fruit juices and nectars. By staining with ruthenium red, at least two tolerant isoforms of PME were present in 'Ataulfo' mango nectar processed by high hydrostatic pressure and high-pressure homogenization, which

might contribute to global enzymatic activity and quality loss during storage. This might be one of the first reports regarding the identification of PME isoforms present in a beverage (mango nectar), especially after the application of high hydrostatic and dynamic pressure processing, highlighting the importance of pressure-tolerance of these molecules. It is recommended to separate the isoenzymes of mango nectar by a second dimension (isoelectric point) to identify more isoforms that might possess similar molecular weights.

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary material.

Supplementary Table – Parameters and conditions assayed for the standardization of zymography for the identification of pectin methylesterase isoenzymes in mango nectar samples processed by high hydrostatic and dynamic pressures.

Methodology Step	Tested parameters	Findings	References
Enzyme extraction	a) Extraction buffer composition: Sodium phosphate Molarity:0.1, 0.2 M pH: 5.8, 6.4, 6.6, 7.0 Additives: • NaCl (0.05, 0.1 and 1.0 M) • Triton X-100 (0.5 and 1.0% v/v) • Polyvinylpolypyrrolidone (1, and 4% w/v) • Protease inhibitor (PMSF): 1 mM b) Extraction time: 0.5 h, 1h, overnight c) Homogenization type: Ultraturrax, blender, vortex d) Homogenization time: 30 s, 60 s e) Centrifugation: • Speed (×g): 37,750, 16,500, 11,000 • Time. 30 min • Temperature: 4°C	The most critical step. Mango pulp has a low protein conent (<1%). The best extraction conditions were those reported in the materials and methods section. Parameters such as buffer molarity and pH showed no difference between tests. The protease inhibitor was not significant in preserving enzyme integrity during storage at 4°C for more than a few days. The presence of NaCl improved enzyme extraction, but higher concentrations affected the resolution of PAGE. Centrifugation speed did not show difference between all the tests. A crucial consideration is to maintain the samples at 4°C during the whole process to avoid protein degradation.	Chakraborty *et al.,* (2015) Cheema and Sommerhalter, (2015) Laing and Christeller, (2004) Palma-Orozco *et al.,* (2014)
Protein precipitation	a) Acetone precipitation: 5 volumes of cold acetone per volume of extract. b) Ammonium sulphate precipitation: • Fractionated precipitation: 0-30%, 30-60%, 30-70%, 60-80% saturation	Acetone precipitation was discarded as no bands were observed in the electrophoresis gels or zymograms. Ammonium sulphate	Palma-Orozco *et al.,* (2014) Wingfield, (1998) Wu *et al.,* (2014)

	• Direct precipitation: 0-60%, 0-80% saturation	precipitation showed the best results in the fraction of 30-70%, with increased protein concentration, and clearer bands in the polyacrylamide gels. However, extensive dialysis was required to remove the salt excess.	
Dialysis	Buffer: Ammonium sulphate 0.1, and 0.2 M • Temperature: 4°C • Dialysis time: 6, 12 and 24 h • Membrane: Cellulose MWCO 12 kDa • Buffer exchange: 3 times • Buffer to sample ratio: 100:1	Despite the several conditions tested, dialysis was complicated step. The several buffer exchanges were not sufficient to reduce the excess of ammonium sulphate in the precipitate. Additionally, samples diluted even more after dialysis, forcing the introduction of an additional concentration step.	Guven *et al.,* (2017) Palma-Orozco *et al.,* (2014) Phillips and Signs, (2004) Wingfield, (1998)
Protein concentration	Pierce™ centrifuge protein concentrators MWCO : 3 kDa • Sample volume: 5, and 10 mL • Temperature: 4°C • Centrifugation speed (×g): 6000, 5500, 5000 • Centrifugation time: 8 or more cycles of 15 min each	Dialysate concentration with the Pierce concentrators was not satisfactory overall. Membrane clogging was a recurrent issue, despite performing the process in several steps of 15 min with constant sample homogenization in the concentrator tubes. The expectation of additional removal of salts and other smaller molecules was not met, and no significant results were obtained from this step. Overall, the best option	Phillips and Signs, (2004)

		for the final experiments was to run the PAGE as soon as the crude enzyme extract was obtained, despite the low enzyme concentration in it.	
Polyacrylamide gel electrophoresis (PAGE)	a) SDS PAGE: • Discontinous gels: o Stacking gel concentration: 4% o Resolving gel concentration: 15, 12, 10, 8% o Voltage: 150, 120, 100, 80, 50 V o Amperage: free (Constant voltage) o Run time: dependent on voltage (1h at 150V, 3+ h at 50 V) o Coomassie blue R-250 stain b) Non-Denaturing PAGE: • Discontinous gels: o Stacking gel concentration: 4% o Resolving gel concentration: 15, 12, 10, 8% o Voltage: 150, 120, 100, 80, 50 V o Amperage: free (Constant voltage) o Run time: dependent on voltage (1h at 150V, 3+ h at 50 V) o Silver nitrate stain • Continuous gels: o Gel concentration: 15, 12, 10, 8% o Voltage: 150, 120, 100, 80, 50 V o Amperage: free (Constant voltage) o Run time: dependent on voltage (1h at 150V, 3+ h at 50 V) o Silver nitrate stain c) Semi-Denaturing PAGE*: • Discontinous gels: o Stacking gel concentration: 4% o Resolving gel concentration: 15, 12, 10, 8% o Voltage: 150, 120, 100,	SDS-PAGE was discarded due to the difficulty of refolding enzymes after the electrophoresis. Non-Denaturing (Native) PAGE, provided good results in terms of evaluating the enzyme activity on the zymograms, and band resolution. However, despite several attempts to improve protein separation, especially of the molecular weight marker, by modifying different parameters, a decision was made regarding native PAGE as the best option for this study. Thus, Semi-denaturing PAGE turned into an option, as the incorporation of only SDS (without other denaturants or reducing agents) into the electrophoresis and sample loading buffers, and gels, improved protein migration and separation significantly compared to Native PAGE. However,	Brunelle and Green, (2014) Cheema and Sommerhalter, (2015) Chevallet *et al.,* (2006) Gallagher, (2018) Guven *et al.,* (2017) Klodman and Lewejohann (2011) Weiland *et al.,* (2014) Wittig *et al.,* (2006)

	80, 50 V ○ Amperage: free (Constant voltage) ○ Run time: dependent on voltage (1h at 150V, 3+ h at 50 V) ○ Silver nitrate stain	the best conditions for performing the Semi-denaturing PAGE involved a low constant power (50 V) which increased the time to more than 3 h. Temperature was controlled by placing the Mini PROTEAN chamber in a water/ice bath, and using cold electrophoresis buffer. Regarding the staining methods, Coomassie blue R-250 was used initially, but the low protein concentration in the samples forced the change into a more sensitive method, such as silver nitrate stain. This improved band resolution significantly for image analysis.	
PME Zymography	a) SDS-Removing solution (for semi-denaturing page): • 1% v/v Triton X-100 • 30 min b) Gel conditioning solution: • 20 mM Tris-Hcl • 5 mM EDTA • pH 8.5 • 30 min c) Substrate solution: • 1% apple pectin • 20 mM Tric-HCl • 5 mM EDTA • 160 mM NaCl • pH 7.6 • 40 min d) Gel washing:	The most crucial step in the PME zymography, was the staining with ruthenium red solution. Different concentrations were tested, and the lower concentration of 0.04% was selected due to the difficulty to destain the gel. Several changes of distilled water with increased destain times were required to remove excess coloration. Additional washing steps to remove the pectin	Downie *et al.,* (1998) Lionetti, (2015) Thonar *et al.,* (2006)

	• Ultrapure water • Time: 15 s, 30 s, 60 s e) Ruthenium red solution (PME activity stain): • Ruthenium red concentration: 0.05%, 0.04% • Stain time: 15, 30, 60 min d) Gel destain: • Distilled water • Time: 1 h, 24 h, 48 h	solution were introduced before ruthenium red staining. However, as proteins were not fixed to the gel, short washing times in water were used to avoid resolution issues from PME activity stains.	
PPO Zymography	a) Conditioning solution: • Sodium phopsphate, potassium phosphate buffer • Molarity: 0.1, 0.2 M • pH: 5.8, 6.4, 6.6, 7.0 • Time: 5 min, 10 min • Temperature: Room (25°C) B) Activity stain solution: • Sodium phopsphate buffer • Molarity: 0.1, 0.2 M • pH: 5.8, 6.4, 6.6, 7.0 • Time: 5, 10, 15, 60 min • Substrate: pyrocatechol • Substrate concentration: 10, 25 mM; 0.1, 0.2 M • Temperature: Room (25°C)	PPO showed significant activity during the spectrophotometric assays. However, despite several methodologies tested to evaluate in gel activity, no band was observed under any combination of the evaluated assay conditions for zymography. Buffer composition, molarity, pH, incubation time did not improved the results on the conditioning or staining step. Substrate concentration had no effect neither. The prolongation of exposure time to substrate solution resulted in a polyacrylamide gel turning dark-brown in color, but no activity bands were appreciated on any lane under any condition or sample used. Trials with different samples such as avocado or apple crude extract	Cheema and Sommerhalter, (2015) Escalante-Minataka *et al.,* (2017) Guven *et al.,* (2017) Noda *et al.,* (2017) Rescigno *et al.,* (1997) Rescigno *et al.,* (1997)

		showed the same results. We believe there are crucial steps being omitted from the reported methodologies regarding the zymography of PPO, or the enzyme is easily degraded by proteases after the extraction step.	

5. CONCLUSIONES GENERALES

El procesamiento de bebidas frutales, como el néctar de mango, para su preservación por altas presiones hidrostáticas y dinámicas, constituye una alternativa atractiva para productores e inversionistas de la industria de alimentos en México. En comparación con un tratamiento térmico como la pasteurización, ambos tipos de alta presión son insuficientes en lo que respecta a inactivación de enzimas como PME, PG y PPO, las cuales están estrechamente relacionadas con el deterioro de atributos de calidad como la textura y color durante el almacenamiento de productos tales como bebidas frutales. Fue posible determinar, que PME, PG y PPO, son enzimas con elevada resistencia a la alta presión tanto hidrostática como dinámica en néctar de mango 'Ataulfo'. En el caso de la presión hidrostática, solamente en combinación con almacenamiento frío se logró reducir de forma relevante la actividad de PPO en néctar procesado. En comparación entre ambos sistemas de procesamiento por alta presión, la alta presión dinámica logró reducir significativamente la actividad de PPO, aunque considerando el calentamiento adiabático. A pesar de ello, PME permaneció como la más resistente de las tres enzimas inicialmente consideradas.

En lo que respecta a atributos sensoriales del néctar, ambos sistemas tuvieron efectos sobre la viscosidad aparente, especialmente las altas presiones dinámicas que proporcionan un efecto de adelgazamiento del néctar a niveles de presión muy elevados. Atributos como pH y °Brix se preservaron tras el procesamiento. En el caso de color, ambos sistemas afectaron de manera ligeramente diferente a los parámetros L*, a* y b*. De manera general, las altas presiones dinámicas preservaron de mejor manera los valores de L* y b*, incluso con incrementos en algunos tratamientos. En contraste, la presión hidrostática retuvo de mejor manera los valores de a*, presentando también incremento en algunos tratamientos. En lo que respecta a isoformas tolerantes a la presurización de las enzimas de interés, el enfoque se centró en PME, debido a que ésta enzima presentó mayor tolerancia a ambos sistemas de presurización a diferencia de PPO, cuya actividad fue significativamente reducida por la alta presión dinámica. En el caso de PG, se descartó en experimentos posteriores al del primer manuscrito debido al bajo nivel de actividad enzimática específica (U/mL de extracto) en comparación con PME y PPO. Aplicando zimografía en una dimensión, se identificaron dos isoenzimas de PME, presentes tanto en el control como en seis de

los siete tratamientos evaluados. Estas isoenzimas presentaron pesos moleculares aproximados de 63 y 33 kDa . En el caso del tratamiento de 100 MPa por 5 min en presión hidrostática, solo se observó en el zimograma la isoenzima de 66 kDa, lo que sugiere que la isoenzima de menor peso molecular sufrió una inactivación parcial, pero el estímulo de niveles de presión más elevados, propiciaron su activación.

Es evidente que la aplicación de estas técnicas puede ser útil en la evaluación de la eficiencia de procesos de preservación de alimentos basados en altas presiones con respecto a la activación enzimática. La identificación de isoenzimas resistentes, permitiría un diseño óptimo de condiciones de proceso, al generar conocimiento sobre sus características.

6. RECOMENDACIONES

Derivado de los resultados y hallazgos obtenidos de este proyecto de tesis doctoral, se emiten las siguientes recomendaciones, las cuales podrían dar a futuro oportunidad para desarrollar nuevos proyectos de investigación.

La estandarización de condiciones experimentales reproducibles para realizar la zimografía de las enzimas polifenol oxidasa y poligalacturonasa, tanto en una y dos dimensiones. En el caso de polifenol oxidasa, existen reportes de condiciones para detectar su actividad por zimografía, sin embargo, en este trabajo de investigación no pudieron reproducirse los mismos resultados, lo cual fue indicativo de la omisión de algún paso crítico en la metodología reportada.

La estandarización de condiciones experimentales para realizar la zimografía en dos dimensiones, primero separando las enzimas por punto isoeléctrico y después por peso molecular. El principal reto técnico relativo a esta propuesta, es la incompatibilidad del enfoque isoeléctrico, dado a que se requieren reactivos que afectan de manera significativa la estructura de la enzima (desnaturalizándola), lo cual podría afectar la evaluación de la actividad *in situ* después de la electroforesis.

La evaluación del efecto por alta presión *in situ*, utilizando enzimas comerciales como modelo de estudio (por ejemplo, tirosinasa de champiñón, pectin esterasa de cáscara de naranja), para determinar cómo afectan los diferentes niveles de alta presión, a temperatura controlada, la estructuras cuaternaria, terciaria y secundaria de las enzimas, a fin de determinar los cambios a nivel molecular que les confieren su resistencia o inactivación.

Se recomienda la evaluación y comparación de los efectos de las altas presiones hidrostática y dinámicas sobre otras enzimas como peroxidasa, β-glucosidasa, lipoxigenasa, especialmente en matrices de origen vegetal como frutas y hortalizas y productos procesados a base de éstos. El enfoque sobre altas presiones dinámicas es sugerido, ya que existen menos reportes de sus efectos sobre diversas familias de enzimas que en el caso de la alta presión hidrostática.

Se recomienda la purificación de las enzimas de néctar de mango o pulpa de mango, mediante cromatografía de afinidad y/o exclusión de tamaño, para su posterior caracterización de parámetros cinéticos basados en la ecuación de Michaellis-Menten, y determinar el orden de reacción que siguen estas enzimas cuando son inactivadas por altas presiones, sobre todo altas presiones dinámicas.

Una propuesta ambiciosa e interesante, es la producción de alguna de éstas enzimas de interés como proteína recombinante, utilizando alguna de las siguientes plataformas para su producción: microalgas, cultivos celulares vegetales, células bacterianas, maíz transgénico. La finalidad de producción de enzimas recombinantes sería con propósito de obtener una fuente confiable y constante de producción de alguna enzima que pudiera ser utilizada en procesos industriales como pectin metilesterasa para clarificación de jugo de manzana. Además, las enzimas recombinantes podrían servir como modelos de estudio para diversidad de investigaciones, evaluando los efectos sobre su estabilidad no solo mediante la aplicación de altas presiones, si no otras tecnologías emergentes de preservación de alimentos como campos eléctricos pulsados, plasma frío, dióxido de carbono de alta presión, ultrasonicación, radiación ionizante, radiación ultravioleta. E incluso tecnologías térmicas emergentes como calentamiento óhmico y por microondas.

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