

**SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES**

2020
Bogotá D.C.,

Abogado
JUAN PABLO CADENA SARMIENTO

ASUNTO	Radicación	09 063 560
	Trámite	011
	Evento	379
	Actuación	519
	Oficio	
	Folios	8

14080

Patente de Invención



Patente de Modelo de Utilidad

Vía Nacional



Vía PCT (Fase Nacional)



Capítulo I

Capítulo II

No. Solicitud Internacional

PCT/EP2007/062968

Fecha de Presentación Internacional

28/Noviembre/2007

Como resultado del Examen de Fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Folios 8 a 25	18/Junio/2009
Reivindicaciones:	Folios 26 a 27	18/Junio/2009
	Folios 90 a 91	18/Junio/2009
Prioridad:	EP 06124918.1	28/Noviembre/2006

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque esta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

Título de la solicitud: "Composición que comprende oligogalacturonanos y sacáridos policatiónicos"

El problema planteado en esta solicitud consiste en proveer una composición para uso agrícola que tenga menor impacto ambiental y permita reducir la cantidad de productos químicos sintéticos que deben ser usados en las cosechas.

Para resolver este problema técnico la solicitud en estudio proporciona una composición que comprende uno o más oligogalacturonano(s) con un grado de Expediente 09 063 560 1er Art 45

polimerización comprendido entre 9 y 20 y que tiene una configuración dimérica de huevera estabilizada además por uno o más sacáridos policatiónicos.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61L26/00, C08L5/06.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicación 18 no es patentable de acuerdo con el Art 20 de la Decisión 486 dado que se refiere al uso de la composición reclamada "en el tratamiento de cicatrización de heridas", lo cual constituye un método de tratamiento humano o animal.

5. Reivindicaciones relativas a un uso o a un segundo uso (Art 14 y 21 D 486)

Los usos reclamados en las reivindicaciones 13 – 17 no son patentables, de acuerdo con el Art 14 de la Decisión 486.

6. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

El capítulo reivindicatorio no es claro ni conciso porque se emplean expresiones como "uno o más", "superior a", "inferior a", "inferior al" que son de carácter no limitativo e impiden establecer el alcance de las reivindicaciones. Se sugiere eliminar dichas expresiones del pliego reivindicatorio y reemplazarlas por rangos concretos con base al soporte proporcionado en la descripción.

Además se aclara al solicitante que no es aceptable definir los componentes de una composición por su actividad, función o por medio de generalizaciones como "sacáridos policatiónicos", "oligosacárido", "polisacárido", "sacáridos", "reguladores o factores de crecimiento", "minerales", "iones", "nutrientes", "aditivos alimenticios", "potenciadores de sabor", "colorantes", "vitaminas", "fitonutrientes", "molécula bioactiva". El uso de dichas denominaciones origina un número indefinido de posibles composiciones para las cuales no existe soporte en la descripción, en consecuencia las reivindicaciones 1 y 3 a 8 carecen de soporte en la descripción, por lo tanto se sugiere al solicitante restringir el alcance del pliego reivindicatorio de acuerdo al soporte y evidencia de efecto técnico suministrado en la descripción (folios 17-25; págs. 10 -18).

En la reivindicación 1 se incluye el siguiente fragmento "que tiene una configuración dimérica de huevera estabilizada", de acuerdo a la descripción la configuración dimérica de huevera es una configuración inducida por la presencia de iones bivalentes como Ca^{2+} o monovalentes como Na^+ , por lo tanto es necesario que el solicitante incluya el contenido de dichos iones en la composición ya que son indispensables para obtener el efecto técnico reclamado.

En cuanto a la "estabilización" de la estructura de huevera al ser combinada con "sacáridos policatiónicos" esta constituye un resultado a alcanzar por medio de la composición reclamada y como tal no debe incluirse en la caracterización de la composición puesto que en realidad equivaldría a definir el problema técnico a resolver y el alcance de la reivindicación, incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese

resultado. Por lo tanto se requiere al solicitante eliminar dicha información del alcance de la reivindicación 1.

La reivindicación 2 no es aceptable ya que se caracteriza la composición porque “la configuración de huevera es detectada por el anticuerpo 2F4”, se aclara al solicitante que la metodología de caracterización estructural de una composición no es una característica esencial de la composición y no puede debe incluirse dentro de las reivindicaciones. Se requiere al solicitante retirar la reivindicación 2.

Las reivindicaciones 9 y 10 no son aceptables porque se define la composición por el uso que puede dársele como “composición farmacéutica”, “nutracéutica”, “alimenticia”, “pienso”, “producto fitosanitario”, “estimulante”, “fertilizante”, “adyuvante”, “reemplazo de fitohormona”, “mejorador de fitohormona”, “modulador de de fitohormona” o “fungicida”. Una composición no puede ser caracterizada por la aplicación en la que ha de emplearse ya que esto no constituye una característica esencial de la composición.

En conclusión, se recuerda al solicitante que una composición debe reivindicarse estableciendo sus características esenciales por medio de una definición concreta de los componentes y de las proporciones de los mismos en la composición con base al soporte presentado en la descripción. Se sugiere entonces modificar el capítulo reivindicatorio de acuerdo a las observaciones señaladas.

Descripción (Art 28 D 486)

La Descripción no es clara porque la información que contiene establece la utilidad de la composición en campos técnicos diversos, lo cual dificulta comprender el objeto específico de la invención y el aporte de la invención.

Adicionalmente se le recuerda al solicitante que cuando se mencionan marcas registradas en el capítulo descriptivo p. ej. “Pectinex ultra SPL” debe especificarse la composición del producto al cual se hace referencia.

Por último, se llama la atención del solicitante sobre el hecho que la invención no ha sido divulgada de manera suficiente para dar validez al uso de términos generales como “sacáridos policationicos”, “oligosacárido”, “polisacárido”, “sacáridos”, “reguladores o factores de crecimiento”, “minerales”, “iones”, “nutrientes”, “aditivos alimenticios”, “potenciadores de sabor”, “colorantes”, “vitaminas”, “fitonutrientes”, “molécula bioactiva” en el pliego reivindicatorio. Se requiere al Solicitante hacer las aclaraciones necesarias.

7. Determinación del Estado de la Técnica

El Estado de la Técnica se determinó con base en la Fecha de Presentación de la Solicitud de Prioridad: Noviembre 28 de 2006 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Item	Documento	Título	Fecha de Publicación*
D1	WO2006085075 (A2)	Colonic delivery of active agents	17/Agosto/2006
D2	Chang K L B et al , Carbohydrate Polymers Vol. 43, No 2. Págs. 163–169	Swelling behavior and the release of protein from chitosan–pectin composite particles	Octubre de 2000

D3	Munjeri O et al, <i>Journal of Controlled Release</i> Vol. 46, No. 3, Págs. 273-278	Hydrogel beads based on amidated pectins for colon-specific drug delivery: the role of chitosan in modifying drug release	2/Junio/1997
D4	Atyabi F et al, <i>Carbohydrate Polymers</i> Vol. 61, No 1, Págs. 39-51	In vitro evaluation and modification of pectinate gel beads containing trimethyl chitosan, as a multi-particulate system for delivery of water-soluble macromolecules to colon	Julio de 2005
D5	Messiaen J. et al <i>Journal of Cell Science</i> , Vol.104, Págs. 365-371	Cell wall oligogalacturonides increase cytosolic free calcium in carrot protoplasts	1993
D6	Messiaen J. et al <i>Plant and Cell Physiology</i> , Vol. 35, No. 4, Págs. 677-689	Pectic Signal Transduction in Carrot Cells: Membrane, Cytosolic and Nuclear Responses Induced by Oligogalacturonides	1994
D7	Rodriguez A. T. et al, <i>Revista Mexicana de fitopatología</i> , Vol. 24, No. 001, Págs. 1-7.	Efecto de la quitosana en la inducción de la actividad de enzimas relacionadas con la defensa y protección de plántulas de arroz (<i>Oryza Sativa</i> L.) contra <i>Pyricularia Grisea</i> SACC	7/Ferebro/2006

D1

<http://worldwide.espacenet.com/publicationDetails/description?CC=WO&NR=2006085075A2&KC=A2&FT=D&ND=4&date=20060817&DB=EPODOC&locale=en> EP divulga (Pág. 3 lin. 18 – Pág. 4 lin. 5) una composición para liberación de medicamentos en el colon en forma de gránulos de pectina (metilada, no metilada, aminada, no aminada) entrecruzados con calcio. Se divulgan composiciones tales que incluyen además quitosana (Tabla 6, Pág. 49).

D2 <http://www.sciencedirect.com/science/article/pii/S0144861700001454> divulga (Resumen, Tabla 1, párrafo 2.2) una composición para liberación controlada de medicamentos que contiene pectina, quitosana y calcio. Se divulga además que la pectina empleada contiene un 50-70% de ácido galacturónico y la enzima empleada es pectinasa (Párrafo 2.1).

D3 <http://www.sciencedirect.com/science/article/pii/S0168365996016070> divulga (Resumen) una composición para liberación de medicamentos (indometacin, sulfametoxazol) que consiste en un sistema gelificado de pectina en presencia de calcio. La formación de los gránulos gelificados se realiza por adición de una solución acuosa de pectina y el medicamento sobre una solución que contiene cloruro de calcio y quitosana (Párrafo 2.2).

D4 <http://www.sciencedirect.com/science/article/pii/S0144861705000573> divulga (Resumen) una composición en gránulos de pectina que contiene quitosana (cloruro de trimetilquitosana) para liberación controlada y dosificación de medicamentos. Los estudios fueron realizados en composiciones que contienen pectina, quitosana en relación 1:8, 1:6 y 1:4 y adicionalmente un catión bivalente seleccionado entre Zn^{2+} y Ca^{2+} (Sección 2.3.1).

D5

http://jcs.biologists.org/content/104/2/365.abstract?ijkey=54789750f3e3b08b4ac22597dc216730f2fca79b&keytype=tf_ipsecsha divulga (resumen, Págs. 365-366, 369-370) la actividad estimuladora de pared celular de plantas, la activación de mecanismos de defensa y modificación de las cascadas de transducción por elevación de concentración de Ca^{2+} causadas por el ácido poligalacturónico u oligogalacturonanos con grado de polimerización mayor a 9. También divulga que en presencia de calcio se induce la formación de la conformación conocida como "huevera" en oligopectatos con grado de polimerización entre 10-14.

D6

http://pcp.oxfordjournals.org/content/35/4/677.abstract?ijkey=c3aa329fe9d2e10b9f27fa7a230445f5bea33994&keytype=tf_ipsecsha divulga (Resumen) una composición de oligogalacturonanos con grado de

polimerización de 9-16 que contiene NaCl y CaCl₂ y su efecto como estimulador de respuestas de defensa en plantas p. ej. actividad de PAL. Se divulga la fuerte correlación de los efectos en la actividad de PAL en función del grado de polimerización de los oligogalacturonanos (Pág. 682 fig. 4) y de su concentración (Pág. 683 Fig. 5).

D7 <http://redalyc.uaemex.mx/src/inicio/ArtPdfRed.jsp?iCve=61224101> divulga (introducción) la actividad estimuladora de defensas de plantas de la quitosana y se divulga que puede ser empleada para obtener dos efectos inhibición de crecimiento de hongos e inducción de mecanismos de defensa en la plantas. La quitosana es aplicada en semillas de arroz comen forma de solución acuosa a diferentes concentraciones 100-1000g/mL y los resultados del estudio demuestran la activación de enzima PAL (Pág. 3), β-1,3 glucanasa y quitinasa (Pág. 4).

8. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en una composición como se describe en el folio 90.

Comparación de las características técnicas **esenciales**, con las del Estado de la Técnica:

Características esenciales	D1	D2	D3	D4
Una composición	Una composición	Una composición	Una composición	Una composición
Que comprende	Que consiste en	Que contiene	Que consiste en	Que contiene
Uno o más oligogalacturonano(s) con un grado de polimerización comprendido entre 9 y 20 que tiene una configuración dimérica de huevera estabilizada	Gránulos de pectina entrecruzados con calcio	Péctina con un 50-70% de ácido galacturónico	Un sistema gelificado de pectina en presencia de calcio	Gránulos de pectina entrecruzados con Zn ²⁺ y Ca ²⁺
Uno o más sacáridos policatiónicos.	Quitosana	Quitosana	Quitosana	Quitosana

Las características **esenciales** de la composición de la reivindicación 1 habían sido divulgadas previamente en los documentos D1 a D4.

D1 enseña (Pág. 3 lin. 18 – Pág. 4 lin. 5) a una composición para liberación de medicamentos en el colon en forma de gránulos de pectina (metilada, no metilada, aminada, no aminada) entrecruzados con calcio. Se divulgan composiciones tales que incluyen además quitosana (Tabla 6, Pág. 49).

D2 da a conocer (Resumen, Tabla 1, párrafo 2.2) una composición para liberación controlada de medicamentos que contiene pectina, quitosana y calcio. Se divulga además que la pectina empleada contiene un 50-70% de ácido galacturónico y la enzima empleada es pectinasa (Párrafo 2.1).

D3 divulga (Resumen) una composición para liberación de medicamentos (indometacin, sulfametoxazol) que consiste en una sistema gelificado de pectina en presencia de calcio. La formación de los gránulos gelificados se realiza por adición de una solución acuosa de pectina y el medicamento sobre una solución que contiene cloruro de calcio y quitosana (Párrafo 2.2).

D4 da a conocer (Resumen) una composición en gránulos de pectina que contiene quitosana (cloruro de trimetilquitosana) para liberación controlada y dosificación de

medicamentos. Los estudios fueron realizados en composiciones que contienen pectina, quitosana en relación 1:8, 1:6 y 1:4 y adicionalmente un catión bivalente seleccionado entre Zn^{2+} y Ca^{2+} (Sección 2.3.1).

Ninguno de los documentos hace referencia al grado de polimerización de los oligogalacturonano(s) o los sacáridos policatiónicos, sin embargo no existe indicación alguna de que los oligómeros empleados en las composiciones de D1 a D4 no tuvieran las características descritas aunque no hayan sido determinadas o divulgadas.

En consecuencia, la reivindicación 1 carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 2 – 9 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

Las reivindicaciones 11 y 12 que se refieren a productos que incluyen la composición descrita en la reivindicación 1 carecen de novedad puesto que la composición no es novedosa.

Se le recuerda al solicitante que según el Art. 21 de la Decisión 486 los productos comprendidos en el estado de la técnica, de conformidad con el artículo 16 de la presente Decisión, no serán objeto de nueva patente, por el simple hecho de atribuirse un uso distinto al originalmente comprendido por la patente inicial.

Nivel Inventivo (Art18 D486)

La reivindicación 1 consiste en una composición como se describe en el folio 90.

Comparación de las características técnicas esenciales , con las del Estado de la Técnica:			
Características esenciales	D5	D6	D7
Una composición	Una composición	Una composición	Una composición
Para estimular respuestas de defensa en plantas			
Que comprende	Que comprende	Que comprende	Que comprende
Uno o más oligogalacturonano(s) con un grado de polimerización comprendido entre 9 y 20 que tiene una configuración dimérica de huevera estabilizada	Oligogalacturonanos con grado de polimerización entre 10-14 que en presencia de ión calcio adquieren la conformación de "huevera"	Oligogalacturonanos con grado de polimerización entre 9 y 16. y NaCl y $CaCl_2$	No se menciona
Uno o más sacáridos policatiónicos.	No se menciona	No se menciona	Quitosana

Los documentos D5 a D7 son considerados el estado de la técnica cercano a la invención definida en la reivindicación 1 porque:

El documento D5 (resumen, Págs. 365-366, 369-370) da a conocer la actividad estimuladora de pared celular de plantas, la activación de mecanismos de defensa y modificación de las cascadas de transducción por elevación de concentración de Ca^{2+} causadas por el ácido poligalacturónico u oligogalacturonanos con grado de polimerización mayor a 9. También divulga que en presencia de calcio se induce la formación de la conformación conocida como "huevera" en oligopectatos con grado

Expediente 09 063 560 1er Art 45

de polimerización entre 10-14. Dentro de los estudios realizados se muestra a estimulación de la actividad de PAL en protoplastos de zanahoria (Pág. 369 fig. 4).

El documento D6 da a conocer (Resumen) una composición de oligogalacturonanos con grado de polimerización de 9-16 que contiene NaCl y CaCl_2 y su efecto como estimulador de respuestas de defensa en plantas p. ej. actividad de PAL. Se divulga la fuerte correlación de los efectos en la actividad de PAL en función del grado de polimerización de los oligogalacturonanos (Pág. 682 fig. 4) y de su concentración (Pág. 683 Fig. 5). Los autores concluyen que la actividad inductora de los oligogalacturonanos depende de su tamaño y sugieren que la estructura de "huevera" podría ser la especie activa reconocida por las células que desencadena las cascadas de reacción de defensa (Pág. 687) y que por lo tanto los oligogalacturonanos con grado de polimerización superior a 9 con conformación inducida por calcio son los responsables de la estimulación de mecanismos de defensa.

El documento D7 da a conocer (introducción) la actividad estimuladora de defensas de plantas de la quitosana y se divulga que puede ser empleada para obtener dos efectos inhibición de crecimiento de hongos e inducción de mecanismos de defensa en la plantas. La quitosana es aplicada en semillas de arroz comen forma de solución acuosa a diferentes concentraciones 100-1000g/mL y los resultados del estudio demuestran la activación de enzima PAL (Pág. 3), β -1,3 glucanasa y quitinasa (Pág. 4). En el documento se concluye que es posible tratar de manera efectiva con quitosana cultivos de arroz contra la enfermedad P. Grisea y se señalan las ventajas del tratamiento con quitosana como inocuo para el ambiente y no tóxico para el hombre o los animales (Pág. 6).

La diferencia entre la invención, definida en la reivindicación 1, y las composiciones divulgadas en D5-D7 radica en que en la presente invención se divulga una composición que contiene tanto oligogalacturonanos como quitosana, en tanto que los documentos D5 a D7 hace referencia a composiciones para inducción de defensa en plantas que contienen únicamente uno de los dos oligo/polisacáridos.

La combinación de oligogalacturonanos y quitosana no parece conceder un efecto técnico inesperado dado que la capacidad inductora de mecanismos de defensa por parte de los oligogalacturonanos en presencia de ión calcio (D5-D6) y de la quitosana (D7) se encuentra anticipadas en el estado de la técnica, por lo tanto era totalmente predecible el obtener mayor efecto de activación de defensa al usar los componentes mezclados. El solicitante reclama un efecto sinérgico inesperado, sin embargo se aclara que dicho efecto se considera comprobado solo si se demuestra que el efecto de activación de defensas provocado por la combinación es superior a la suma de los efectos individuales de cada componente actuando por separado.

Por lo tanto, el Problema Técnico Objetivo que pretende resolver esta invención se puede formular así: "cómo modificar las composiciones para estimulación de defensas en plantas conocidos en D5 a D7 para obtener composiciones más efectivas".

Sin embargo, una persona normalmente versada en la materia, con el objetivo de obtener composiciones para estimulación de defensas en plantas, habría inferido con gran expectativa de éxito la posibilidad de modificar las composiciones de oligogalacturonanos y cloruro de calcio empleadas para tal fin que se encuentran divulgadas en D5 y D6, incluyendo en ellas otros sacáridos con tal actividad

estimuladora según se divulga en D7 para la quitosana, llegando así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Las reivindicaciones 2-12 no aportan ninguna característica inventiva a la composición descrita en la reivindicación 1 y por lo tanto se considera que también carecen de nivel inventivo.

Conclusión:
Las reivindicaciones 1 – 9 y 11 – 12 no cumplen el requisito de novedad (Art. 16), y las reivindicaciones 1– 12 pero no cumplen con el requisito de nivel inventivo (Art. 18).

9. Conclusión

Los requisitos de Patentabilidad de la presente Solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO2006085075 (A2)	1 – 9 y 11 – 12	Novedad
D2	Chang K L B et al, <i>Carbohydrate Polymers</i> Vol. 43, No 2, Págs. 163–169	1 – 9 y 11 – 12	Novedad
D3	Munjeri O et al, <i>Journal of Controlled Release</i> Vol. 46, No. 3, Págs. 273–278	1 – 9 y 11 – 12	Novedad
D4	Atyabi F et al, <i>Carbohydrate Polymers</i> Vol. 61, No 1, Págs. 39–51	1 – 9 y 11 – 12	Novedad
D5	Messiaen J. et al <i>Journal of Cell Science</i> , Vol.104, Págs. 365-371.	1-12	Nivel Inventivo
D6	Messiaen J. et al <i>Plant and Cell Physiology</i> . Vol. 35, No. 4, Págs. 677-689	1-12	Nivel Inventivo
D7	Rodriguez A. T. et al, <i>Revista Mexicana de fitopatología</i> , Vol. 24, No. 001, Págs.1-7.	1-12	Nivel Inventivo

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (C)

El anterior requerimiento fue notificado por fijación en lista, de fecha
14 AGO. 2012

Elaboró Laura Carolina Pardo
Revisó Jacqueline Alfonso.
Expediente 09 063 560 1er Art 45

OK

Swelling behavior and the release of protein from chitosan–pectin composite particles

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Received 3 November 1998; received in revised form 15 November 1999; accepted 25 November 1999

Abstract

Pectin solution gelled with chitosan and Ca^{2+} ion produced composite particles with a double layer structure. Response surface analysis revealed that the particle swelling at pH 7.4 was influenced significantly by chitosan and Ca^{2+} concentrations. Chitosan and Ca^{2+} concentrations also significantly influenced the swelling in pH 7.4 buffer at 1 h, whereas chitosan and pectin concentrations significantly influenced the swelling in pH 7.4 at 12 h. Results from swelling experiment and statistical analysis suggested that complex formation between chitosan and Ca^{2+} , or between chitosan and pectin might have occurred. Negligible protein was released from the chitosan–pectin particles in pH 1.4 buffer. The chitosan–pectin particles could sustain the release of model protein until after 24 h in pH 7.4 buffer. Pectin degrading enzyme increased the protein release from 39 to 63% within 12 h in pH 7.4 buffer. These characteristics of the chitosan–pectin particles would be useful for colon-specific delivery of bioactive macromolecules. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Chitosan; Pectin; Swelling; Protein; Controlled release

1. Introduction

In recent years, the advances in biotechnology have made available many novel drugs consisting of proteins, peptide, or nucleotides. Researchers have been constantly searching for more effective ways of delivering these drugs to patients for various therapeutic purposes (Langer, 1990, 1998). Among various drug carriers for proteins, polysaccharide hydrogels have attracted a lot of interest because they are natural, biodegradable, and nontoxic after degradation (Chen, Jo & Park, 1995).

Chitosan is a cationic polysaccharide made from alkaline N-deacetylation of chitin, the second most abundant natural polymer on earth (Chang, Tsai, Lee & Fu, 1997; Knorr, 1984). Chitosan consists of N-acetyl-glucosamine and glucosamine residues. It has biocompatible, biodegradable, nontoxic, and mucoadhesive characteristics (Hirano, Seino, Akiyama & Nonaka, 1990; Luefen et al., 1997). Pectin is an anionic polysaccharide that may provide colon-specific delivery (Ashford, Fell, Atwood, Sharma & Woodhead, 1993; Rubinstein, Radai, Ezra, Pithak & Rokem, 1993) for several drugs. The polymeric chain of pectin contains galacturonic acid, rhamnose, arabinose and galactose. The

properties of interpolymeric complex gel beads (Meshali & Gabr, 1993; Munjeri, Collett & Fell, 1997), films (Yao, Liu, Cheng, Lu, Tu, & Silva, 1996) or laminated films (Hoogland & Paris, 1996) composed of chitosan and pectin have been reported recently. The complex formation changed the drug release behavior of hydrogel beads, modified their pH-sensitive swelling behavior, but retained the colon-specific characteristic of pectin. In addition, pectin improved the storage and loss moduli of laminated chitosan films. These features have proved the feasibility of combining chitosan and pectin to form new carriers for drug delivery. The swelling behavior of chitosan–pectin particles, however, has not been examined in detail. Moreover, the feasibility of using chitosan–pectin particles for protein delivery has never been explored.

The objectives of this study were to investigate the pH-sensitive swelling behavior of composite gel particles made of chitosan and pectin and to examine the release pattern of a model protein from these particles.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Ohka Enterprise Inc. (Kaohsiung, Taiwan). The degree of N-deacetylation,

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Table 1
Central composite design conditions for the preparation of chitosan–pectin composite particles

Treatment	Variable 1 coded	Variable 2 coded	Variable 3 coded	X ₁ (pectin concentration (%))	X ₂ (Ca ²⁺ concentration (%))	X ₃ (chitosan concentration (%))
1	–1	–1	–1	6.01	7.24	7.24
2	–1	–1	1	6.01	7.24	16.76
3	–1	1	–1	6.01	8.99	16.76
4	–1	1	1	6.01	8.99	16.76
5	1	–1	–1	6.01	6.01	7.24
6	1	–1	1	6.01	6.01	16.76
7	1	1	–1	6.01	8.99	7.24
8	1	1	1	6.01	8.99	16.76
9	–1.682	0	0	5	12	12
10	1.682	0	0	10	12	12
11	0	–1.682	0	7.5	6.01	5
12	0	1.682	0	7.5	6.01	10
13	0	0	–1.682	7.5	7.5	7.5
14	0	0	1.682	7.5	7.5	7.5
15–20	0	0	0	7.5	7.5	7.5

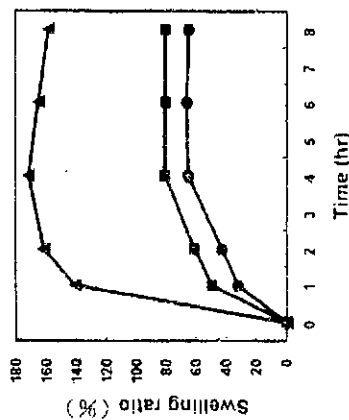


Fig. 1. The swelling ratio of chitosan-pectin composite particles in pH 1.4 buffer solution. Particle formulation (1:1 chitosan/Ca²⁺/pectin in solution): ● 0.84/16.76/8.99; ■ 0.61/27.5; ▲ 0.84/27.4/6.0.

determined by colloid titration (Chang et al., 1997) and IR spectroscopy (Baxter, Dillon, Taylor & Roberts, 1992), was 64%. The viscosity averaged molecular weight, determined by using the correlation of Wang, Bo, Li and Qin (1991), was 6.5×10^5 . Low methoxyl pectin (unmodified, containing 3.0–7.0% methoxyl group and 50–70% galacturonic acid) and reagent grade calcium chloride were obtained from Wako Pure Chemicals (Osaka, Japan) as received. Other chemicals used were reagent grades supplied by Merck (Darmstadt, Germany) or Wako Pure Chemicals SIGMA (St. Louis, MO, USA) supplied the pectinase (EC 3.2.1.15; from *Aspergillus niger*).

2.2. Preparation of chitosan-pectin composite particles

Chitosan and calcium chloride (CaCl₂) were dissolved in 5% acetic acid solution. Pectin was dissolved in deionized water. The wt.% of chitosan, CaCl₂, and pectin (Table 1) were determined by using a three-variable central composite experimental design (Myers & Montgomery, 1995). The pectin solution was pumped by a metering pump (Pharmacia LKB Pump P-1, Sweden) and dropped through a plastic tubing (1.0 mm ID) into the chitosan-Ca²⁺ solution to form composite gel particles. The particles were cured in 0.2 N NaOH (in water/ethanol (v/v) = 8:2) solution for 15–30 min. The gel particles were then rinsed repeatedly with distilled water until neutrality and dried at $\pm 40^\circ\text{C}$ in an air-circulated oven.

2.3. Measurement of pH sensitivity *in vitro*

The pH sensitivity of chitosan-pectin particles was determined under simulated gastric condition in pH 1.4 hydrochloric acid buffer solution or simulated intestine condition in pH 7.4 phosphate buffer solution (PBS) (Dong & Hoffman, 1991). The solution was maintained at 37°C in a thermostated shaker rotating at 50 rpm. The initial particle

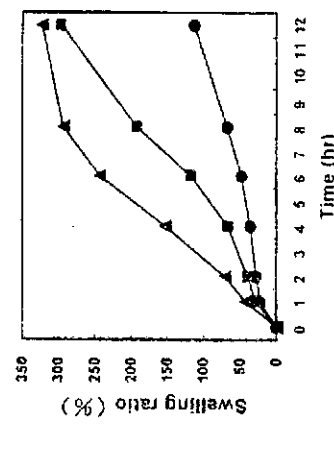


Fig. 2. The swelling ratio of chitosan-pectin composite particles in pH 1.4 phosphate buffer solution. Particle formulation (1:1 chitosan/Ca²⁺/pectin in solution): ● 0.84/16.76/8.99; ■ 0.61/27.5; ▲ 0.84/27.4/6.0.

was lower than that of Ca²⁺. The above factors led to a softer core and a more resilient surface layer for freshly formed gel beads.

When dried chitosan-pectin particles were put into the pH 1.4 buffer, the particles swelled considerably. The weight swelling ratio leveled off to ca 70–170% (Fig. 1) within 4 h. The diameter increased in parallel with the weight increase. However, the plateau of dimension increase occurred within ~ 2 h, before the weight increase plateau. This suggested that the particle expansion proceeded faster than the water uptake at pH 1.4. The phenomenon might be caused by the double-layer nature of the composite particles. Chitosan in the surface layer became positively charged and charge repulsion helped the swelling of the particles. The expansion in the positively charged chitosan surface layer might therefore be faster than water uptake. In addition, pectin became a polyacid at pH 1.4. The carboxyl groups of pectin were protonated and became neutral in charge. The hydrogen bonds and hydrophobic forces between or within pectin molecules increased the network elasticity. This would lead to

Table 2

RSREG regression results of the swelling ratio (swelling ratio (%) = $C_0 + C_1 \times X_1 + C_2 \times X_2 + C_3 \times X_3 + C_4 \times X_1 \times X_2 + C_5 \times X_1 \times X_3 + C_6 \times X_2 \times X_3 + C_7 \times X_1 \times X_2 \times X_3$) for chitosan-pectin composite particles after 4 h in pH = 1.4 buffer

Intercept	Degrees of freedom	Parameter estimate $C_0, C_1, C_2, C_3, C_4, C_5, C_6, C_7$	Probability > t
137.3336	1	C_0	0.3871
-30.3420	1	C_1	0.3405
-5.0187	1	C_2	0.5307
364.0563	1	C_3	0.0407
1.8257	1	C_4	0.3270
1.0924	1	C_5	0.4974
0.1598	1	C_6	0.9902
-25.5348	1	C_7	0.1221
-14.6435	1	C_8	0.0114
30.8741	1	C_9	0.6725
0.7377	1	C_{10}	0.6725

* $P < 0.05$

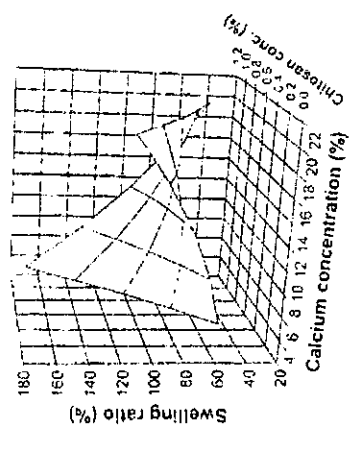


Fig. 3. The swelling ratio of chitosan-pectin composite particles after 4 h in pH 1.4 buffer solution for a pectin concentration of 7.5%.

make the inner core, compressed mainly of pectin, harder to absorb water.

Dried chitosan-pectin particles swelled to a greater extent in pH 7.4 PBS (Fig. 2). In addition, both the weight and diameter increased continuously. Swelling ratio at 24 h ranged from 280 to 445% (data not shown). This indicated that water uptake occurred simultaneously with structure expansion at pH 7.4. These results indicated that the pH sensitivity of chitosan-pectin particles differed significantly in simulated physiological conditions (pH 1.4 and 7.4) due to the difference in the sample formulations. Several factors might influence the swelling behavior of prepared particles. Water diffusion from the solution into the gel and the electrostatic repulsion among the $-\text{NH}_3^+$ groups along chitosan chains contributed to the swelling in acidic solution. On the contrary, the complex interaction between chitosan and pectin, hydrogen bonding, hydrophobic interactions, and the network elasticity within the particles restricted them from further expansion. The balance of these phenomena led to a plateau in particle swelling after a short period of time in pH 1.4 buffer. In contrast, chitosan-pectin particles

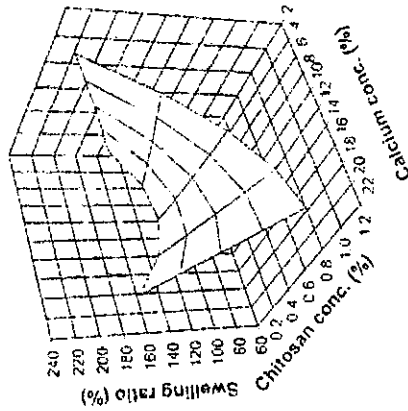


Fig. 4. The swelling ratio of chitosan-pectin composite particles after 4 h in pH 7.4 buffer solution at a pectin concentration of 7.5%.

could swell to a larger extent at pH 7.4 because the fully negatively charged $-COO^-$ groups in pectin molecules (Zhong, Williams, Keenan, Goodall & Rolin, 1997) repelled one another. Apparently this repulsion force was greater than the attractive forces that tend to reduce the volume of the particles.

The swelling ratios of chitosan-pectin particles after 4 h at pH 1.4 were regressed with respect to the preparation condition using the RSREG procedure (SAS, 1985). The regression results were shown in Table 2. The regression equation was plotted in Fig. 3. These results indicated that the swelling of these particles at pH 1.4 were influenced significantly by the interaction term between chitosan and Ca^{2+} concentrations, and the first order term of chitosan concentration. At low Ca^{2+} concentration, the swelling

ratio increased with increasing chitosan concentration. At high Ca^{2+} concentration, the interaction between chitosan concentration and Ca^{2+} concentration was more significant than the effect of chitosan concentration. Slightly more swelling occurred at the condition with high Ca^{2+} and low chitosan concentration. These results suggested statistically that chitosan might interact with Ca^{2+} .

The RSREG regression results for the swelling ratios of chitosan after 4 and 12 h in PBS (pH 7.4) could be obtained similarly. They are as follows:

Swelling ratio (%), 4 h in : pH 7.4

$$= 133.2 + 1.546X_1 - 15.17X_2 + 272.4X_3 + 0.5418X_1 \times X_1 + 0.8391X_1 \times X_2 + 0.4328X_2 \times X_2 + 26.01X_3 \times X_3 + 12.02X_3 \times X_2 + 6.041X_1 \times X_3$$
$$R^2 = 0.7671$$

Swelling ratio (%), 12 h in : pH 7.4

$$= -501.2 + 175.7X_1 + 19.63X_2 + 583.5X_3 + 0.549X_1 \times X_1 - 2.656X_1 \times X_2 - 0.7019X_1 \times X_3 - 40.12X_3 \times X_3 + 17.17X_3 \times X_2 - 469.9X_3 \times X_3$$
$$R^2 = 0.7512$$

These regression equations corresponded to the 3D response surfaces in Figs. 4 and 5. The regression results indicated that the second order term of Ca^{2+} and the interaction term between chitosan concentration and Ca^{2+} concentration had more significant effect on the swelling of particles after 4 h in pH 7.4 PBS. The lower the Ca^{2+} concentration, the larger the swelling ratio. The second order term of chitosan concentration and the pectin concentration term had more significant influence on the swelling ratio after 12 h in pH 7.4 PBS. Higher swelling ratios occurred at intermediate to lower chitosan and pectin concentrations. These results suggested that chitosan might interact with Ca^{2+} in the gel matrix and influenced the initial swelling of the particles in pH 7.4 PBS. After 12 h of exposure to pH 7.4 PBS, the Ca^{2+} had mostly diffused out of the gel matrix. Therefore, the concentrations of chitosan and pectin that formed the polymeric matrix of the gel particles showed more significant influence on the swelling behavior. These statistical results revealed that complex might have been formed between chitosan and Ca^{2+} , and between chitosan and pectin. This is contrary to prior knowledge, that chitosan does not form complex with the ions of alkaline earth metals (Roberts, 1992). It is possible that complex formation occurred in this work because much higher Ca^{2+} concentration was used during the particle preparation.

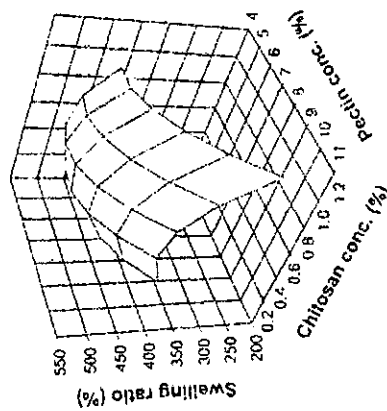


Fig. 5. The swelling ratio of chitosan-pectin composite particles after 12 h in pH 7.4 buffer solution at a Ca^{2+} concentration of 7.5%.

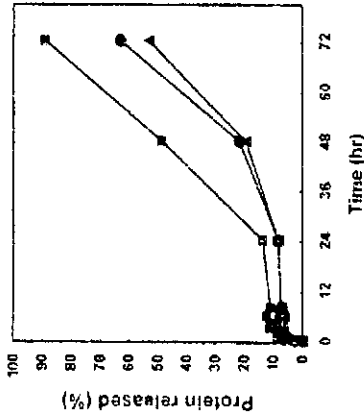


Fig. 6. Protein release from chitosan-pectin composite particles in pH 7.4 buffer solution. Particle formulation (% chitosan/ Ca^{2+} /pectin in solution): ● 12/87/3; ■ 14/87/5; ▲ 0.6/12/6.

3.2. In vitro release of BSA from chitosan-pectin composite particles

A potential carrier of protein or peptide drugs for oral colon-specific delivery should preferably provide minimal swelling in stomach (pH ~1.4) and maximal swelling in colon (pH ~7.4). These optimal conditions for preparing protein-loaded gel particles should be close to the conditions for the minimum swelling in Fig. 3, or for the maximums in Figs. 4 and 5. These conditions were used to prepare chitosan-pectin particles containing BSA. Test results showed that practically no protein was released from the gel particles after 6 h in pH 1.4 buffer. The reason that there was a lack of release of protein might be that BSA molecules were denatured at pH 1.4. Presumably they could aggregate, became insoluble, or too bulky to diffuse out of the

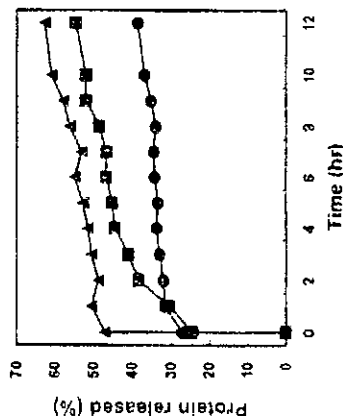


Fig. 7. Protein release from chitosan-pectin composite particles in pH 7.4 buffer solution in the presence of 100 IU pectinase. Particle formulation (% chitosan/ Ca^{2+} /pectin in solution): ● 12/87/3; ■ 14/87/5; ▲ 0.6/12/6.

double-layered chitosan-pectin gel particle in the buffer solution.

Approximately 10% of the total protein were released after additional 24 h of exposure in pH 7.4 PBS (Fig. 6). This indicated a gradual diffusion of protein from the swollen particles. The rate of protein release increased dramatically after 24 h in pH 7.4 PBS. The particles prepared by chitosan to pectin ratio of ca 1–10 seemed to swell less and release less protein in pH 7.4 buffer. This ratio is the same as the ratio that yielded the lowest viscosity of chitosan-pectin solution as reported in Meshali and Gabr (1993). Thus it is probable that a weight concentration ratio of ca 1–10 could produce the best stoichiometric binding or complex between chitosan and pectin and form the most stable composite particles.

Total amounts of protein released after 72 h ranged from 53 to 99%. The sudden increase in the release rate corresponded with the erosion of the gel particles after prolonged exposure to the pH 7.4 PBS. The chitosan-pectin particles prepared with the lowest Ca^{2+} concentration released the largest amount of protein. This suggested that Ca^{2+} contributed to the cross-linked structure of the particles. The higher the Ca^{2+} concentration, the more the cross-linking bonds between Ca^{2+} and chitosan or pectin. More cross-linked particles would slow down the erosion process, make the diffusion of BSA from the swollen particles more difficult, and result in a slower rate of protein release. The release rate of BSA from chitosan-pectin composite particles was much lower than that from chitosan-alginate microcapsules (Okamoto, Amstutz, Chu & Goosen, 1996) under simulated gastric condition. This characteristic may help protect large biological molecules in stomach and make chitosan-pectin particles more effective as the oral delivery carrier of various macromolecular drugs under development.

Fig. 7 showed the release of protein from the chitosan-pectin particles into PBS containing pectinase. Pectinase dramatically increased the rate of release. In the presence of the enzyme, ~25–47% of total protein were released within 0.5 h. The amounts released in 2 h ranged from 32 to 50%. After 12 h of exposure to pH 7.4 PBS with pectinase, up to 39–63% of BSA could be released. The particles prepared with lower chitosan and pectin concentrations showed the fastest release rate. The release rate of protein from the particles prepared in this study was slower than that of indomethacin and sulphamethoxazole from chitosan-coated pectin hydrogel beads (Manjori et al., 1997). The difference in the release rate of encapsulated material could be attributed to the lower solubility and lower diffusivity of protein. Nevertheless, our data indicated that the protein release rate could be increased substantially by pectinolytic enzymes *in vitro*. It has been shown that calcium pectinate or pectin is degraded by commercial pectinolytic enzymes or microbes from rat cecal contents and human colon (Rubinstein et al., 1993; Salyers, Verechhetti, West & Wilkins, 1977). As a consequence, these results proved that chitosan-pectin

particles provided an enzyme controlled release system. This property would make these particles good potential colon-specific carriers for large molecules such as protein drugs.

4. Conclusion

Chitosan-pectin composite particles containing BSA could be prepared under mild temperature from aqueous solutions. The chitosan-pectin particles swelled slightly in pH 7.4 buffer solution; they swelled considerably in pH 7.4 PBS. The swelling of these particles under physiological pH could be adjusted by changing the concentrations of chitosan, Ca^{2+} and pectin. At a pH of 7.4, BSA gradually diffused through the swollen gel network in the first few hours. After ca. 24 h of exposure, the swollen gel matrix started to erode. This could be due to the diffusion and dissolution of Ca^{2+} , the dissolution of pectin, or a breakdown of the cross-linked 'egg-box' structure between pectin and Ca^{2+} . Consequently, the release of protein was a combined result of swelling, diffusion, and erosion processes. In the presence of pectinase, the glycoside bonds in pectin were degraded. The cross-linked networks in the chitosan-pectin particles rapidly broke into pieces, releasing a considerable amount of protein in a short time. The release of protein was mainly the result of swelling and enzymatic degradation. This research suggested that chitosan-pectin particles were able to sustain the release of BSA under simulated gastric condition and release it under simulated condition in colon. These particles might be good oral controlled release carriers for large molecules such as vaccines, peptides, nucleotides, or proteins.

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References

- Ashford, M., Fell, J., Anwood, D., Sharma, H., & Woodhead, P. (1995). An evaluation of pectin as a carrier for drug targeting to the colon. *Journal of Controlled Release*, 26, 213–220.
- Baxter, A., Dillm, M., Taylor, K. D. A., & Roberts, G. A. F. (1992). Improved method for IR determination of the degree of N-acetylation of chitosan. *International Journal of Biological Macromolecules*, 14, 166–169.
- Cheng, K. L. B., Tsai, G., Lee, J., & Yu, W. R. (1997). Determination of N-deacetylation of chitin in alkaline solution. *Carbohydrate Research*, 303, 327–332.
- Crem, J. to S., & Paul, S. (1985). Polysaccharide hydrogels for protein drug delivery. *Carbohydrate Polymers*, 28, 69–76.
- Ding, L., & Robinson, A. S. (1991). A novel approach for preparation of pH-sensitive hydrogels for enteral drug delivery. *Journal of Controlled Release*, 13, 141–152.
- Hirano, S., Senoo, H., Akiyama, Y., & Nomura, I. (1990). Chitosan, a biocompatible material for oral and intravenous administrations. In G. G. Gebeken & R. I. Dennis (Eds.), *Progress in biomedical polymers* (pp. 283–289). New York: Plenum.
- Huazhang, P. D., & Parris, N. (1996). Chitosan/pectin laminated films. *Journal of Agricultural and Food Chemistry*, 44, 1915–1919.
- Kisor, D. (1981). Use of chitosan polymers in food. *Food Technology*, 29 (1), 85–97.
- Kinger, R. (1990). New methods of drug delivery. *Science*, 249, 1527–1533.
- Laugel, R. (1981). Drug delivery and targeting. *Nature*, 292, 5–10.
- Leary, O. H., Riechling, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, 194, 265–275.
- Lusk, H. J., Renel, C. O., Kozak, A. E., Labe, C. M., & Best, A. G. (1997). Microcapsule polymers in personal peptide drug delivery. IV. Polycarboxyl and chitosan as potential enhancers of peptide transport across intestinal mucosa in vitro. *Journal of Controlled Release*, 45, 15–23.
- Mesbah, M. M., & Gahr, K. E. (1993). Effect of interpolymer complex formation of chitosan with pectin or acacia on the release behaviour of cefepime/maize DCl. *International Journal of Pharmaceutics*, 89, 177–181.
- Mueller, O., Cohen, J. H., & Bell, J. T. (1997). Hydrogel beads based on amidated pectins for colon-specific drug delivery: the role of chitosan in modifying drug release. *Journal of Controlled Release*, 46, 273–278.
- Myers, K. H., & Montgomery, D. C. (1995). *Response surface methodology: Principles and product optimization using designed experiments*. New York: Wiley (pp. 279–380).
- Othman, A. U., Amden, B., Chu, W., & Gansen, M. F. A. (1996). Modulation of protein release from chitosan-alginate microcapsules using the pH-sensitive polymer hydroxypropyl methylcellulose acetate succinate. *Journal of Microencapsulation*, 13, 497–508.
- Roberts, G. A. F. (1992). *Chitin, chitinase*. London: Macmillan (pp. 201–249).
- Roberts, G. A. F., Kaba, R., Ezra, M., Pataki, S., & Rokem, J. S. (1993). In vitro evaluation of calcium pectinate: a potential colon-specific drug delivery carrier. *Pharmaceutical Research*, 10, 258–263.
- Sajers, A. A., Verrelli, J. R., West, S. E. H., & Wilkins, T. D. (1977). Fermentation of mucin and plant polysaccharides by strains of *Bacteroides* from the human colon. *Applied and Environmental Microbiology*, 33, 319–322.
- SAS User's Guide: Statistics, Version 5. SAS Institute Inc.: Cary, NC, USA, 1985.
- Wang, W., Bo, S. J., & Qin, W. (1991). Determination of the Maillard-Horvink equation for chitosans with different degrees of N-deacetylation. *International Journal of Biological Macromolecules*, 13, 281–285.
- Yan, K. D., Lin, J., Cheng, G. N., Lu, A. D., Tu, H. L., & Silva, H. A. L. D. (1996). Swelling behavior of pectin/chitosan emulsion films. *Journal of Applied Polymer Science*, 60, 279–285.
- Zhang, H. J., Williams, M. A. K., Keenan, R. D., Goodall, D. M., & Rolin, C. (1997). Separation and quantification of pectins using capillary electrophoresis: a preliminary study. *Carbohydrate Polymers*, 32, 27–32.

Hydrogel beads based on amidated pectins for colon-specific drug delivery: the role of chitosan in modifying drug release

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Abstract

A multiparticulate system with the potential for site-specific delivery to the colon has been investigated. Gelation of droplets of amidated pectin solutions in the presence of calcium is the basis of the method of preparation. Two drugs, indomethacin and sulphamethoxazole were successfully incorporated into the beads. Drug release from the beads was a function of media pH and drug loading. In simulated gastric and small intestinal conditions, drug release was greater with the more soluble sulphamethoxazole, but release of both drugs could be reduced to satisfactory levels by the formation of a chitosan polyelectrolyte complex around the beads. All the preparations released drug in simulated colonic conditions within 135 min.

Keywords: Hydrogel beads; Drug delivery; Chitosan; Amidated pectins

1. Introduction

Pectin (polygalacturonic acid) has been investigated as a potential excipient for colon-specific delivery [1-3]. These investigations used single unit preparations which may exhibit delays at the ileo-caecal junction leading to drug loss prior to entry in the colon [1]. This problem may be overcome by the use of a particulate dosage form, the particles of which may pass more freely through the various junctions. The methods available for colonic-delivery include pH-sensitive coatings, the use of pro-drugs [4] and enzymically biodegradable matrices [5-7]. Control by utilizing the pH of the GI tract is subject to inter-individual variations [8] and the pro-drug

approach is limited in that the chemical nature of the drug is modified. Biodegradable matrices relying on enzymic breakdown by colonic bacteria almost exclusively confined to the colon show considerable promise.

Amidated pectins are low-methoxyl pectins in which some of the carboxylic acid groups are amidated. They are more tolerant of pH variations and calcium levels than conventional pectins which could make them useful in colonic delivery systems. Gelation of droplets in the presence of calcium may provide a valuable approach to the formation of a multiparticulate system for colonic delivery. The properties of such amidated pectin beads may be altered by the formation of a polyelectrolyte complex membrane around the bead using cationic polymers such as chitosan, in a similar manner to that used with alginate gel beads [9, 11].

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The aim of this research was to investigate the suitability of hydrogel beads based on amidated pectin for potential use in colon-specific drug delivery matrices using indomethacin and sulphamethoxazole as model insoluble and relatively soluble drugs respectively.

2. Experimental

2.1. Materials

Amidated low-methoxyl pectin (AP), degree of methylation DM 23; degree of amidation DA=24) was a donation from Buhner Pectin UK. Chitosan (C), indomethacin (I) and sulphamethoxazole (S) were used as received (Sigma Chemical, UK). Two buffers were used, McIlwaine's citric acid-phosphate buffer (pH 3.0 and pH 5.0) and Sorcensen's phosphate buffer (pH 7.4). Pectinex Ultra SP-1, (26 600 PG/ml at pH 3.5) was obtained from Novo Ferment, Switzerland.

2.2. Preparation of drug-loaded beads

Amidated pectin/indomethacin (API) and amidated pectin/sulphamethoxazole (APS) beads were made by dissolving AP in distilled water, with agitation, followed by the drug, to give pectin/drug weight (g) ratios of (4:1); (4:2) and (4:4) in 100 ml of the final solution for bead production. This procedure gives theoretical loadings in the dried beads of 20%, 33% and 50% w/w. Chitosan solution, 1% w/v, was made by adding the chitosan to distilled water containing 5% v/v glacial acetic acid and stirring for 60 min in a covered flask. Individual droplets of the AP/drug mixture were produced by pumping the liquid through a 2-mm diameter tube at 1.6 ml/min. The droplets were allowed to fall into 500 ml agitated calcium chloride solution, 2% w/v, containing 0 or 0.1% w/v chitosan, to give uncoated (API and APS) and chitosan-coated (APIC and APSC) gel beads with specified drug loadings. The beads were dried overnight in a fan assisted oven at 50°C.

2.3. Swelling studies, *in vitro* release and drug loading

The release of drugs from the beads (500 mg) into 600 ml of media was studied using the BP Method H apparatus (Caleva SST, UK) at 37°C with a paddle speed of 100 revs. min. The media chosen were a compromise between the pH conditions pertaining in the GI tract and the need to obtain valid comparisons between the model drugs. At pH 1, no release of I was detected so pH 3, where some release occurred, was chosen to represent acidic conditions in the GI tract. Conditions in the small intestine were represented by pH 7.4 and pH 5 was a compromise between the pH of the colon and the optimum pH for the pectinolytic enzymes, added at a level of 2 ml/600 ml. Samples (3 ml) were withdrawn at specified time intervals and assayed for drug content using a Perkin Elmer 550 spectrophotometer at the wavelength of maximum absorption (I, pH 3.0 and 5.0, 304 nm; pH 7.4, 279 nm; S, pH 3.0 and 5.0, 265 nm; pH 7.4, 256 nm). The same volume of buffer was replaced to maintain a constant volume.

The swelling behaviour of the beads was studied by measuring the diameter of the beads, using a calchometer, after exposure to pH 3.0, 5.0 and 7.4 using the same conditions as for the release studies. Beads were removed for measurements at specified time intervals. The actual drug loading of the beads was determined by UV spectroscopy after allowing complete dissolution of the drug from a known weight of beads.

2.4. Drug solubility

This was determined by placing excess drug in 100 ml of the buffers used in the dissolution studies, maintained at 37°C, and shaken continuously. Samples were removed at time intervals until the absorbance readings, taken at the appropriate wavelength of maximum absorbance, remained constant. The results are the mean of two determinations.

2.5. Scanning electron microscopy

The surface morphology of the drug-free and drug loaded beads was examined using a Cambridge 600

Scanning Electron Microscope (Cambridge Instruments, UK).

3. Results

The beads produced all had good spherical geometry. There was no significant variation in particle sizes between the bead types, the mean diameter being 2.0±0.1 mm (n=20). The surface of drug-free beads was smooth but particles were observed on the surface when beads were prepared from mixtures containing the drugs. The solubilities of the drugs in the buffers used in the dissolution studies are shown in Table 1. Consideration of the drug loadings, the volume of dissolution fluid and the drug solubility show that the dissolution studies were performed under sink conditions.

The swelling behaviour of the beads was independent of the bead formation. Typical results are shown in Fig. 1. The greatest swelling occurred at pH 5.0. When pectinolytic enzymes were added at this pH, the beads initially increased in size but were totally degraded within 135 min. Less swelling occurred at pH 3.0 and 7.4. At pH 7.4, the size changes were a balance between swelling and erosion.

The loading efficiency, expressed as a percentage of the actual loading to the theoretical loading, varied between 67 and 82% for S and 82-98% for I. The lower loading efficiency for S will be a reflection of greater loss of the more soluble drug during bead formation.

The percentages of the drugs released from the beads after 2 h exposure to pH 3.0 are given in Table 2. There is little release of I at this pH from any of the bead formulations despite the degree of swelling that takes place. This is a reflection of the relative insolubility of I in acidic media. A greater release of

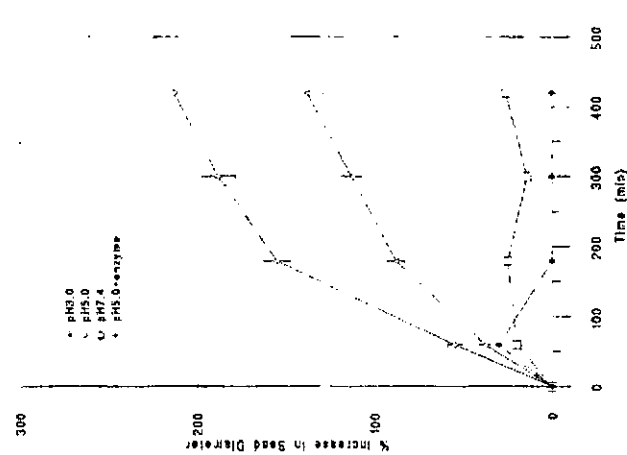


Fig. 1. Swelling of drug-free annealed pectin hydrogel beads in aqueous buffers. Each point represents the mean±S.D. (n=3).

S was observed commensurate with its greater solubility. Drug release decreased with an increase in drug loading. The addition of chitosan reduced the amount of drug released. Similar release data at pH 7.4, for 3 h, are given in Table 3, the greater release for both compounds being a reflection of their greater solubilities at this higher pH. Again the amount released is reduced by the formation of a chitosan membrane.

Fig. 2 shows the release of the drugs at pH 5 in

Table 1
Solubilities of indomethacin and sulphamethoxazole in aqueous buffers

pH	Indomethacin solubility (mg/100 ml)	Sulphamethoxazole solubility (mg/100 ml)
1.0	0.5	56.0
3.0	1.5	52.0
5.0	3.0	50.0
6.8	100.0	315.0
7.4	130.0	500.0

Table 2
Percentage drug released after 2 h in pH 3.0 Methane's citric-acid-phosphate buffer

Sample	API 20	API 33	API 50	API 50	API 50	API 50	API 50	API 50
% Drug released	<1.0	<1.0	<1.0	31.0	26.0	21.0	27.0	18.0
S.D.	1.1	1.5	1.2	2.4	2.2	3.8	1.5	2.3

S.D., standard deviation (n=3).

API, annealed pectin/indomethacin (uncoated); API, chitosan-coated; API, annealed pectin/sulphamethoxazole (uncoated); API, chitosan-coated.

Table 3
Percentage drug released after 3 h in pH 7.4 Sorensen's phosphate buffer

Sample	API 20	API 33	API 50	API 50	API 50	API 50	API 50	API 50
% Drug released	6.5	11.0	16.0	26.5	11.5	31.5	37.0	38.0
S.D.	1.1	1.5	1.2	2.0	1.7	2.4	2.2	3.1

S.D., standard deviation (n=3).

See Table 2 and text for abbreviations.

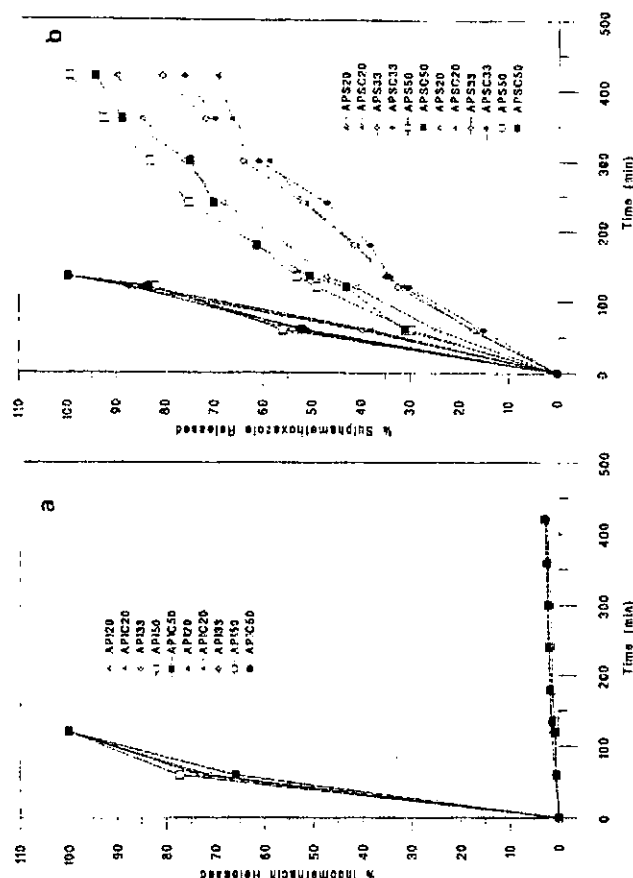


Fig. 2. Percentage of drug released into pH 5.0 Methane's citric-acid-phosphate buffer in the presence (—) and absence (---) of pectinolytic enzyme (Pectinase Ultra SPL). Each point represents the mean±S.D. (n=3). a, Indomethacin; b, Sulphamethoxazole.

109

the presence and absence of pectinolytic enzymes. The enzymes degrade the beads in all cases leading to a rapid release of drug.

4. Discussion

Matrix systems designed for specific drug delivery to the colon are naturally prone to drug release in the upper gastro-intestinal tract. This release may be minimal in the case of a relatively insoluble compound, 1 at pH 3 for example. If the compound is soluble, release will take place as from a normal hydrophilic matrix system, exacerbated by the higher surface area of the multiparticulate formulation. Restriction of release can be achieved by decreasing one or more of swelling, erosion or diffusion. In the present study, complexation with chitosan was investigated as to its value in restricting release. No attempt to maximise the benefit of this approach was made, but a significant reduction in release was achieved.

The effectiveness of matrices based on amidated pectins for retarding the release of drugs will be governed by the gel structure and its resistance to erosion. Calcium induces cross-linking between pectin chains to form a stronger, less open gel, often known as an 'egg-box' structure. The sites of this cross-linking will be disrupted by the bulky methoxyl groups, but will be relatively unaffected by the smaller amide group. However, the sites for cross-linking may be further limited as the amide groups tend to be distributed in blocks, with a random distribution of carboxyl and methylated groups [12]. The gel may, therefore, be relatively open, allowing free drug diffusion. The structure will be changed by environmental pH and the presence of chitosan. A low pH will limit the ionisation of the pectin, reducing coulombic repulsion and favouring a closer gel network. The opposite will be true at higher pH values. The interaction between the pectin and the chitosan will be favoured at low pH. The above arguments suggest that the approach adopted will be more successful at lower pH values. The gel structure formed at pH 7.4 allows observable erosion which was not noted at pH 3.

Table 2 and Table 3 show that drug loss from amidated pectin beads in conditions mimicking the

stomach and small intestine can be limited by the formation of a complex between chitosan and the amidated pectin. Even at pH 7.4, when the complex is less stable and erosion takes place, a reduction in release is achieved. The total amount of drug released during the complete 5 h testing varies from 7.5% to 27.5% for 1 and 33% to 65% for 5. The loss for the relatively insoluble indomethacin is acceptable whereas that for the sulphamethoxazole is not. Hence a more positive approach to protection from loss is required for soluble drugs, whereas simple matrix systems are acceptable for less soluble compounds.

Pectinolytic enzymes can still attack the beads despite the cross-linking and the presence of chitosan. The presence of calcium may be a positive attribute as it has been shown that it enhances the action of the enzymes [13]. The chitosan complex clearly does not influence degradation as drug release is effectively identical from chitosan-treated or untreated beads.

The results of the investigation indicate that a multiparticulate system with suitable drug release characteristics for site-specific colonic delivery can be prepared.

References

[1] M. Ashford, J. Fell, D. Atwood, H. Sharma and P. Woodhead, An evaluation of pectin as a carrier for drug targeting to the colon, *J. Control. Release* 26 (1993) 213–226.
[2] A. Rubinstein, R. Radzi, M. Ezra, S. Padhak and J.S. Rikem, In vitro evaluation of calcium pectinate: a potential colon-specific drug delivery carrier, *Pharm. Res.* 10 (1993) 258–263.
[3] R. Radzi and A. Rubinstein, In vitro and in vivo analysis of colon specificity of calcium pectinate formulations, *Proc. Int. Symp. Control. Release Biotech. Mater.* 20 (1993) 330–331.
[4] D.R. Friend, S. Phillips and J.N. Tozer, Colon-specific drug delivery from a glucoside prodrug in the guinea-pig: efficacy study, *J. Control. Release* 15 (1991) 47–54.
[5] M.A. Mateescu, Y. Dumoulin, G. Delmas, V. Lemaire and L. Carlier, Crosslinked amylose: a new matrix for drug controlled release. Molecular insights, *Proc. Int. Symp. Control. Release Biotech. Mater.* 20 (1993) 290–293.
[6] Y. Dumoulin, L. Carlier, V. Lemaire and M.A. Mateescu, Cross-linked amylose: enzymatically controlled drug release (ECDR) system, *Proc. Int. Symp. Control. Release Biotech. Mater.* 20 (1993) 306–307.

[7] V. Lemaire, Y. Dumoulin and M.A. Mateescu, Controlled release of theophylline from cross-linked amylose tablets, *J. Control. Release* 15 (1991) 39–46.
[8] M. Ashford, J.T. Fell, D. Atwood and P.J. Woodhead, An investigation into the suitability of pH dependent polymers for colonic targeting, *Int. J. Pharm.* 91 (1993) 241–245.
[9] T. Yotsuyanagi, T. Ohkubo, T. Ohbuchi and K. Ikeda, Calcium-induced gelation of alginate and pH-sensitive swelling of dried gels, *Chem. Pharm. Bull.* 35 (1987) 1555–1563.
[10] Y. Munia, T. Macchi, E. Miyamoto and S. Kawashima, Preparation of chitosan-reinforced alginate gel beads: effects of chitosan on gel matrix erosion, *Int. J. Pharm.* 96 (1993) 139–145.
[11] A. Polk, B. Arvidson, K. De Yao, T. Peng and M.F.A. Goosen, Controlled release of albumin from chitosan-agarose microcapsules, *J. Pharm. Sci.* 83 (1994) 178–185.
[12] J. Rocafoe, J.-F. Thibault, J.C.F. Rasmussen and W. Palay, Properties of amidated pectins. II. Polyelectrolyte behaviour and calcium binding of amidated pectins and amidated pectin acids, *Biopolymers* 28 (1989) 1435–1448.
[13] M. Ashford, J.T. Fell and D. Atwood, Enhanced pectin degradation in the presence of calcium: implications for colonic delivery, *Proc. Int. Symp. Control. Release Biotech. Mater.* 21 (1994) 750–751.

In vitro evaluation and modification of pectinate gel beads containing trimethyl chitosan, as a multi-particulate system for delivery of water-soluble macromolecules to colon

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Abstract

Pectinate beads containing trimethyl chitosan chloride (TMC) as an absorption enhancer were prepared using Geomassie Brilliant Blue G 250 (G B) as a relatively high molecular weight water-soluble model drug. Effects of different formulation variables, such as cross-linker type, cross-linking time, cross-linker concentration, TMC: pectin ratio, pectin concentration and volume of the bead generator, were assessed on in vitro bead characteristics by release and swelling–erosion studies. The bead formulation was optimized by factorial design. Some measures were taken to improve the bead characteristics and prolong their integrity during the gastrointestinal transit, such as biomimeticization of the beads or coating them with high-methoxy pectin (HMP) or Eudragit L20 D 55 (EU). Possible CB-TMC complexation was investigated by Job's Plot method. The suitable system was obtained by coating the optimized core formulation with HPM or EU. TMC was found to form a complex with CB as a model anionic drug. Therefore, TMC drug interactions can be used to modify the release characteristics of dosage forms.

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Keywords: Pectinate bead; Swelling–erosion study; Trimethyl chitosan; Colon-specific delivery; Water-soluble drugs

1. Introduction

In recent years colon-specific drug delivery has received much attention. This is in part due to the spiraling trend of introduction of new peptide and protein drugs to the market and the need for their oral delivery (Zhang, Akorn, & Neau, 2002). However, so far it has not been possible to administer these new and effective entities via the oral route due to their sensitivity to the acidic environment of the stomach and degradation by the proteolytic enzymes of the upper GI tract. Above all, owing to their high molecular weights and hydrophilic nature, peptides and protein drugs are scarcely absorbed.

Colon is a relatively mild environment with reduced proteolytic activity and decreased fluid and motility

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Pectin is one of the most widely investigated polysaccharides in the colon-specific drug delivery. It is predominantly a linear polymer of mainly α -D-4-linked D-galacturonic acid residues. It has been used in different dosage forms for colon-specific drug delivery. Pectin can be broken down by pectinase enzymes produced by anaerobic bacteria of colon and control the drug release by this principle. It can also act via the pH- and time-controlled mechanisms. However, due to water solubility and swelling pectin is not capable of shielding the drug effectively during the passage through the stomach and small intestine. Therefore, less water-soluble forms of pectin should be used in this regard (Sinha & Kumria, 2001). For instance, low-methoxy pectin (degree of methoxylation <50%) can form gel beads with an egg-box configuration, with reduced solubility and swelling, in the presence of many divalent cations (Wellner, Kacurakova, Malovikova, Wilson, & Helton, 1998). Moreover, introduction of amide groups into low-methoxy pectin molecule tends to make the pectin less hydrophilic, increasing the tendency to form gels (CP Kelco information sheet; Wackerly, Fell, Atwood, & Perkins, 1997). As a result, by utilizing amidated low-methoxy pectin, more resistant beads can be prepared.

Beads have several advantages over single-unit dosage forms: they are multiple-unit dosage forms with established merits such as predictable and reproducible gastrointestinal transit time (especially for gastric residence time which is the major variable in overall gastrointestinal transit time), more consistent blood levels and improved bioavailability of the embedded drug, less localized gastrointestinal disturbances and greater product safety (Mathiowitz, 1999; Pillay & Fassihi, 1999a). They are prepared easily and quickly in ranges of 50–2000 μ m with perfect spherical shape and narrow size distribution, without any use of organic solvents and harsh ingredients and time-consuming procedures.

Some researchers have studied different aspects of pectinate beads; Munjeri, Collett, and Fell (1997) investigated the release of indomethacin and sulphamethoxazole from calcium pectinate beads (uncoated or coated with chitosan) in different pH values. Sriramalak and Nanthanid (1998) and Sriramalak (1998, 1999) studied the characteristics of pectinate beads containing indomethacin as a water-insoluble model drug or albumin. Pillay et al. (1999a,b) examined the behavior of calcium alginate, calcium pectinate and calcium-alginate-pectinate in various buffers using diclofenac sodium as a model drug. El-Gibaly (2002) investigated delayed-release zinc pectinate ketoprofen-loaded beads formed into single-unit tablets. However, few studies have addressed the inclusion of relatively high molecular weight and water-soluble drugs (such as the situation encountered with peptide drugs) in the beads. Moreover, with the exception of the study by Pillay and Fassihi (1999a), in the previous studies continuous pH changes, simulating gastrointestinal pH variation, have not

been used. Besides, in these studies the capability of the beads to reach colon intact and as a multiple-unit dosage form is not investigated. Sometimes the release data alone can be misleading with regards to preservation of the physical shape of a dosage form; for instance the beads may lose their spherical structure and consistency and become a shapeless single-unit gel in the simulated gastrointestinal media, but at the same time they may have kept the major amount of the drug unreleased. Therefore, apart from the release studies, evaluation of swelling behavior of beads is essential to determine their in vivo fate and integrity of shape and structure throughout the gastrointestinal tract and upon reaching colon. On the other hand the swelling studies elucidate the mechanisms of drug release. During the swelling studies, the weight changes do not always follow an increasing trend and upon release of drug and erosion of the polymeric matrix, negative changes are also observed, hence the term swelling–erosion ratio (SER) was coined and employed throughout this study. This work was designed to assess the feasibility of pectinate beads to protect and deliver a water-soluble high molecular weight model drug to colon in a multi-particulate form.

Oral drug delivery of high-molecular weight hydrophilic agents needs to be coupled with the use of an absorption-enhancing agent, which is safe and biodegradable. Chitosan is an established mucoadhesive and absorption-enhancing polymers, but it has a pK_a of 5.5, thus it is only soluble in acidic environments, where most of the amino groups are protonated. Recent studies have shown that only protonated soluble chitosan opens the tight junctions, facilitating the paracellular transport of hydrophilic compounds. To overcome this problem a chitosan derivative, *N,N*-dimethyl chitosan chloride (TMC), has been synthesized and characterized. TMC shows higher solubility than chitosan in a broader pH range and is proved to be non-toxic (Thunert, Kotz, Verhoeff, Bruesee, & Junginger, 2000). Therefore, in this study, TMC was included in the beads and its reciprocal effect on the pectinate beads characteristics was investigated.

This work focuses on in vitro behavior of pectinate beads with different formulation variables and modification of these beads to optimize their characteristics for colon-specific drug delivery of water-soluble drugs.

2. Experimental

2.1. Materials

Low-methoxy amidated pectin type GENU[®] pectin LAM-104 AS-FS (PLMA) (D.M. = 287, D.A. = 20%) was purchased from CP Kelco (Denmark). Chitosan type Chitoclear FG95 (D.D. > 95%) was supplied by Primex (Norway). Pectinex Ultra SP1 (pectinase from *Aspergillus aculeatus*, activity 26000 PGU/ml at pH 3.5) and high-methoxy pectin from citrus fruits (PHN) were purchased

2.3. Preparation of pectinate gel beads

PLMA and CB, with or without TMC, were dissolved in distilled water at the desired concentrations indicated in each experiment. The gel mixture was completely deaerated under vacuum (fast Vac™ vacuum pump, J/B Industries, USA) and employed to prepare the beads by isotropic gelation using an electrostatic bead generator (Nisco encapsulator VAR V-1, Switzerland) equipped with a syringe pump (Kd Scientific, USA). The mixture was dropped from a syringe into a cross-linking solution (either Zn acetate or Ca chloride), at the rate of 10 ml/h. The nozzle diameter was either 0.7 or 1.1 mm. The dripping distance was 5 cm. Volume of the cross-linking solution was 25 ml for every 10 ml of the gel mixture. The prepared beads were cross-linked for the period stated in each experiment, then washed twice with distilled water and dried overnight at room temperature.

2.3.1. Rationale for selection of ingredients and processes; preliminary studies

Some preliminary studies were first carried out to determine the appropriate formulation variables for the preparation of beads.

PLMA was chosen for this study because of its capability to form gels (compared to PLGA). Moreover the introduction of amide group into its structure makes it less hydrophilic, increasing the tendency to form gels (compared to conventional low-molecular weight) (CP Kelco Information sheet; Wakely, Fell, Atwood, & Parkins, 1997). PLMA concentrations of 3.5, 5 or 7% (w/v) were initially studied for the preparation of beads. CB (M = 854.03 g/mol) was chosen as a model drug for relatively high molecular weight and water-soluble therapeutic agents. CB concentration of 0.3% (w/v) in the PLMA gel was selected, based on the experiments of limit of quantification of CB with spectrophotometric method, and the volumes of the cross-linking solution and release media, for easy assay of the drug.

Zn acetate or Ca chloride at the concentrations of 0.15 or 0.6 M were chosen as cross-linker for the beads and were compared in each other. To assess the safety of application of Zn acetate, some measures were taken. In order to assess the amount of Zn²⁺ retained in the beads, precise 10-ml volumes of PLMA gel mixture of 3.5 or 5% were used to prepare the beads with Zn acetate 0.15 M as the cross-linker. The obtained beads were washed with distilled water and completely dried until no further weight change was observed. The beads were then weighed with an analytical balance with readability of 0.0001 g (OHAUS (GAZ00, Germany), and their weight was compared with the dry content of pectin in the initial gel volume.

Cross-linking times 0.2, 0.4 or 2.4 h were investigated. In addition different TMC:PLMA ratios (1/8, 1/6, and 1/4) were investigated and the suitable ratio was selected based on the preliminary studies.

from Sigma, USA. Calcium chloride (Ca chloride), zinc acetate (Zn acetate), *n*-methyl-2-pyrrolidone (NMP), methyl iodide, sodium iodide (NaI), Coomassie Brilliant Blue G 250 (CBB), di-sodium hydrogen phosphate (Na₂HPO₄), potassium hydrogen phthalate, polyethylene glycol (600 (PEG6000) and trimethyl citrate (TEC) were obtained from Merck, Germany. Eudragit L 30-D 55 (EU) was a gift from Rohm Pharma Polymers, Germany and Akbarieh Co., Iran. All other chemicals used were of reagent grade.

2.3.2. Synthesis of TMC

TMC was synthesized according to the method by Sieval et al. with slight modifications (Sieval, Thanou, Kotzé, Brasseur, & Junginger, 1998). Briefly, 2 g of chitosan was dissolved in 80 ml of NMP and stirred in a 2-necked flask in a constant temperature water bath at 60 °C. The flask was connected to a condensation column. 11 ml of 15% NaOH solution was added to the flask and it was followed by addition of 11.5 ml of methyl iodide (as the methylation agent) and 4.8 g of NaI. The mixture was stirred for 75 min and precipitated with addition of 200 ml of ethanol, centrifuged, washed with acetone on a sintered glass filter and dried. In the second stage, 80 ml of NMP was added to the precipitate (mainly dimethyl chitosan iodide) and the mixture was stirred at 60 °C. Then 1 ml of 15% NaOH, 7 ml of methyl iodide and 4.8 g NaI were added successively and the mixture was stirred for 30 min. An additional 2 ml of methyl iodide and 0.6 g pellets of NaOH were added and stirring was continued for 1 h. This mixture was precipitated with 200 ml of ethanol, centrifuged and the solid substance was filtered on a sintered glass filter and washed with acetone to obtain a powdery substance, which is trimethyl chitosan iodide. To exchange iodide with chloride, trimethyl chitosan iodide was dissolved in 40 ml solution of 10% NaCl. The solution was precipitated with 200 ml of ethanol. Excess ethanol should be avoided because it will also precipitate NaCl. The mixture was centrifuged and the supernatant containing the excess NaCl was completely removed. The precipitate, which was TMC, was filtered and washed with acetone, dried and milled to obtain an off-white water-soluble powder. To determine the degree of quaternization (% DQ), ¹H NMR spectrum of TMC was measured in D₂O using a 400 MHz spectrometer (Varian 400 Unityplus spectrometer, USA). The %DQ was calculated from Eq. (1):

$$\%DQ = \frac{[TMC]_{H^+} - [H]}{[TMC]_{H^+} + [H]} \quad (1)$$

Where [TMC] is the integral of the trimethyl amino group (quaternary amino) peak at 3.3 ppm on the ¹H NMR spectrum, and [H] is the integral of the ¹H peaks between 4.7 and 5.7 ppm on (Snyman, Hamman, Kotzé, & Rollings, 2002).

The beads were prepared with an electrostatic bead generator. Voltage of the bead generator can be changed based on the desired bead characteristics. Since the electrode, which is placed in the cross-linking solution, has a positive charge, it might repel Zn²⁺ or Ca²⁺ and decrease the local concentration of the cross-linker and consequently diminish the cross-linking of the beads. Voltages of 0 or 2 kV were chosen to prepare the beads of the same size and compared them to determine the effect of the encapsulator voltage on the beads.

2.3.2. Experimental design and optimization of beads

Based on the results of the preliminary experiments (see Section 3.1, a 3rd full factorial design was used to prepare the optimized beads and assess the effect of PLMA concentration, Zn acetate concentration and voltage of the bead generator on bead characteristics (Table 1).

2.4. Determination of loading efficiency

Loading efficiency (LE) of the beads for CB was determined by the indirect method, i.e. exact volumes of PLMA gel mixture containing CB were employed to prepare beads and the amount of CB lost in 25 ml of cross-linking solution was assayed spectrophotometrically at 578.6 nm (Schimco, S-3100 spectrophotometer, Korea). LE of the beads for CB was calculated by Eq. (2):

$$LE_{CB} = \frac{[M_1 - M_2] \times 100}{M_1} \quad (2)$$

Where M_1 is the theoretical amount of CB in the beads and M_2 is the amount of CB lost in the cross-linking and washing solutions.

LE of the beads for TMC was determined by a simple gravimetric method, using Eq. (3):

$$LE_{TMC} = \frac{(W_{TMC} - W_{TMC0}) \times 100}{W_{TMC0}} \quad (3)$$

Where W_{TMC} is the theoretical dry weight of TMC used in the beads preparation and W_{TMC0} is the weight of the loaded

Table 1
2nd factorial design utilized for preparation of pectinate beads

Formulation code ^a	Independent variables		Dependent variables	
	PLMA concentration (% w/v)	Zn acetate concentration (M)	Voltage (kV)	Rel. LE ^b (%)
F1	3.5	0.15	0	19.2 ± 5.1
F2	5	0.15	0	6.17 ± 2.8
F3	3.5	0.6	0	26.1 ± 4.3
F4	5	0.6	0	86 ± 15.2
F5	3.5	0.15	2	4.8 ± 1.2
F6	5	0.15	2	15 ± 2
F7	3.5	0.6	2	2.9 ± 1.3
F8	5	0.6	2	146.6 ± 75.7
				Mean ± S.D., n = 3
				3.1 ± 0.4
				65.3 ± 15.2

^a F1, F2, TMC:PLMA ratio 1/8, nozzle diameter 0.7 mm

^b Cumulative percent CB released in 4 h from beads in phosphate buffer pH 7.4. Mean ± S.D., n = 3.

^c Swelling-croton ratio (SECR) of beads in 4 h in phosphate buffer pH 7.4. Mean ± S.D., n = 3.

TMC, W_{TMC0} in turn is calculated from Eq. (4):

$$W_{TMC0} = W_0 + W_{TMC} \quad (4)$$

W_0 is the observed weight of beads and W_{TMC} is the sum of dry weight of pectin and dry weight of loaded CB (calculated by the above-mentioned method)

2.5. Drug release behavior of beads in simulated gastrointestinal media

In standard methods of dissolution experiments in pharmacopoeias, due to the large volumes of the vessels, relatively large amounts of multi-particulate dosage forms are needed; whereas in our study, the beads could not be prepared in large amounts due to the limitations of the laboratory-scale bead generator and shortage of the ingredients, particularly trimethyl chitosan, which was synthesized by us. On the other hand if small amounts of beads in a standard dissolution tester were to be used, very high amounts of the model drug, Coomassie Brilliant Blue G 250 in the beads, should have been incorporated to the beads, to enable the assay the amount of drug released, in the high volumes of the buffers. This was not either feasible, because it adversely affected the beads' characteristics. Therefore, in this study, a modified alternative method, used by some other research groups for their dissolution experiments of multi-particulate dosage forms, intended for oral delivery (Zhang, Alsara, & Nuan, 2002), was employed.

In one set of experiments, fixed amounts of beads were placed in test tubes containing 10 ml of USPXXIII acidic buffer medium (KCHCl, pH 1.5) in an oscillating water-bath (Menmert, Germany) at 37 °C (for 2 h). Then the medium was replaced with 10 ml of USPXXIII phosphate buffer solution (KH₂PO₄/NaOH, pH 7.4) and was further oscillated in the water-bath for 4 h. In another set of experiments, the beads were placed directly in phosphate buffer medium (pH 7.4) for 4 or 5 h to assess the beads' characteristics in simulated intestinal environment alone. In the third set of experiments the beads were first brought in

contact with 10 ml of acidic buffer solution (pH 1.5) for 2 h, followed by 4 h in 10 ml of phosphate buffer medium (pH 7.4). Afterwards, the beads were transferred to USPXXIII phosphate buffer media (pH 5) with or without 50 µl of Pectinase (Ultra SPL) and the experiments were continued for another 3 h. A pH of 5 was chosen for colonic medium, as a compromise between colonic pH (6.4–7.0) and the optimum pH for enzyme activity (3–5) (Mackert, Fell, & Cullen, 1999). With regards to the low pH value chosen for colon, pH values, as low as 5.5–5.7 are also reported for colon; therefore a pH value of 5 is not very far from the actual colonic pH (Semić, Mees, Devleeschouwer, & Auzan, 2002; Fillingim, 1999). Some researchers have used this pH to assess the effect of pectinolytic enzymes on pectin-containing dosage forms, intended for colon-specific drug delivery (Mackert, Fell, & Cullen, 1999; Semić et al., 2002), but one should note that these conditions may somewhat weaken the relevance to colonic environment. The periods of time, chosen for exposure of beads to each media, are almost the gastrointestinal condition encountered *in vivo* (Coto et al., 2004; Raghavan, Muthulingam, Jeyan, & Ravi, 2002).

One test tube was assigned to each time point. The samples of 5 ml were withdrawn at desired time points, centrifuged at 4000 rpm for 10 min, and assayed for CB against the respective blank buffers. Sink condition was maintained throughout the experiments (based on solubility of CB in the respective media). The samples were assayed spectrophotometrically at 578.6 nm and CB concentration was determined using calibration curves in the respective media.

2.6. Swelling/erosion behavior of the beads

In these experiments, pre-weighed amounts of dry beads were placed in the same conditions, started for the release studies. One test tube was assigned to each time point. The beads were removed at every time point, blotted on filter paper to remove the excess water and weighed. The swelling–erosion ratio (SER) was calculated by Eq. (5) (5)

$$SER = \frac{W_t}{W_0} = \frac{W_t - W_0}{W_0} \times 100 \quad (5)$$

where W_t is the beads' weight at the given time point, W_0 is the initial weight of the dry beads.

Positive SER and upward trend denote overall swelling and weight gain of beads with water absorption, while negative SER and downward trend demonstrate the erosion/loss of drug release of beads. SER = 100% is when the beads are in a form of a shapeless loose gel, which cannot be manipulated for weighing, or have dissolved completely.

2.7. Modification of pectinate beads

To improve the stability of beads in phosphate buffer after exposure to acidic medium, some measures were adopted as follows. Release studies and swelling–erosion

experiments were performed on these modified beads. These beads were prepared based on the optimized core formulation obtained from the results of the factorial design.

2.7.1. Biomineralization of beads

Biomineral-inspired beads have been prepared with alginate/chitosan with enhanced mechanical strength and reduced permeability in deionized water (Leveque, Rhodes, & Mann, 2002). The concept of biomineralization was exploited to prepare biomineralized Zn phosphate pectinate beads.

In situ precipitation of phosphate salts around pectinate beads was carried out. Na_2HPO_4 at 50 or 100 mM was added to pectin gels and the beads were prepared as described in Section 2.3. Upon preparation of beads, *in situ* spontaneous precipitation of $\text{Zn}(\text{H}_2\text{PO}_4)_2$ membranes occurred around the beads.

2.7.2. Coating the beads with PHM

PHM is known to swell less than PLMA, because the ester ($\text{COO}(\text{CH}_3)$) groups present in PHM are less hydrophilic than the acid (COO^-) groups of PLMA (CPKelco's information sheet; Vanhamme et al., 2002). Therefore, in order to protect the core beads from exposure to acidic environment and control their swelling, the beads were coated with 3 or 5% (w/v) solutions of PHM. Briefly the dry beads were added to solutions of PHM upon stirring and after 15 min they were removed, washed gently with distilled water and dried at room temperature overnight.

2.7.3. Coating the beads with EU

The beads were coated with EU, a pH-dependent enteric polymer (which dissolves at pH values over 6) by a suspension-salting-out method (SSO) designed in our laboratory. EU is known to coagulate in presence of Mg stearate (Rohm Pharma Polymer's Literature). We exploited this knowledge and used NaCl (a neutral salt) to design a method for coating beads with EU.

Briefly the dried beads were pre-treated with 25% (w/v) solutions of NaCl (an agent that induces the salting out of EU). The slightly damp beads were suspended in a solution of PVA 1% (w/v) (a protective colloid which prevents the beads from adhering together) (Liu, Chen, & Teng, 1999) and stirred under the propeller mixer (Velp Scientifica, Italy) for 1 min. Then 5 ml dispersion of EU containing 5% (w/v) PEG 600 and 5% (w/v) TEAC (as plasticizers), was added and the suspension was stirred for 5 min. The coated beads were washed with distilled water and dried.

2.8. Assessment of CB-TMC complexation

During the studies it was noted that inclusion of TMC in the formulations increases the loading efficiency and modifies the release of CB, while TMC is a water-soluble ingredient and believed at first to increase the release and disintegration of the beads. Therefore, the possibility of

formation of a complex between TMC and CB was considered. In order to examine TMC–CB interaction, a continuous variation technique (Job's plot) was performed to determine the spectral changes of CB (Goold & Yashburgh, 1942). Separate solutions of 30 µg/ml CB and TMC were prepared in phosphate buffer (pH 7.4). These solutions were added to each other in 11 test tubes with increasing volumes of TMC (0–10 ml) and decreasing volumes of CB (10–0 ml). The test tubes were centrifuged and the spectrophotometric absorbance of supernatant was determined at 578.6 nm for determination of free amount of CB. In order to check whether the changes in the spectrophotometric absorbance is due to pH changes upon addition of TMC, pH of the test tubes were determined.

2.9. Statistical analysis

Based on the 2^4 full factorial design (Table 1), linear multiple regression models for the data were calculated. After constructing these models, assumptions underlying these tests, such as absence of co-linearity, homogeneity of variance, as well as normality of the data were tested and found to be met. P value of less than 0.05 was considered as significant. Statistical analysis of the results for all eight formulations was performed to determine the best formulation. SPSS 11.0 software for Windows was used for statistical analysis.

3. Results and discussion

3.1. Synthesis and characterization of TMC

TMC was successfully synthesized by the two-step method. NMR spectrum of TMC showed 65% degree of quaternization (Fig. 1). The synthesized TMC was used in the preparation of beads.

3.2. Rationale for selection of ingredients and processes

Beads prepared with PLMA concentrations less than 3.5% had a weak consistency and shrank significantly upon drying and lost their spherical shape and became somewhat flat.

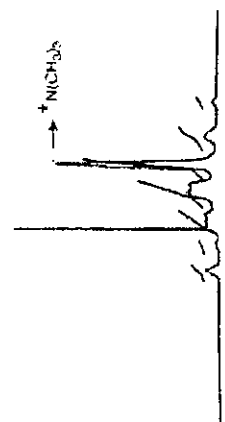


Fig. 1. ^1H NMR spectrum of N-vinyl-2-pyrrolidone (NVP). The peak at 6.5 ppm (shown by arrow) denotes the ^1H (CH_2) groups.

On the other hand, pectin concentration of more than 5% produced a very viscous gel and the beads prepared with it had an undesirable drop-like shape; thus in the factorial design final concentrations of 3.5 or 5% were chosen for PLMA.

Based on the following preliminary studies, Zn acetate was used as an alternative cross-linker to Ca chloride. Wellner et al. (1998) assessed pectinate gels (not beads), formed by various divalent cations and compared their physical state. In their study it is stated that the size and charge density of ions are important parameters in the gel properties. They observed that Zn^{2+} forms gels even with pectin with degree of methoxylation of 59.1% (where high ester groups eliminate the negative charge on pectin chain and may also sterically hinder the formation of chain aggregates), while Ca^{2+} does not; therefore Zn^{2+} is a stronger cross-linker than Ca^{2+} . Our studies confirmed this observation as well. Beads cross-linked by Ca chloride had a slightly lower loading efficiency compared to those cross-linked by Zn acetate (Table 2, formulations Ia and Ib). The effect of cross-linker type (Zn acetate or Ca chloride) on the release behavior and SER of beads is shown in Fig. 2. As seen in Fig. 2a and b, Ia releases CB much faster than Ib and it has completely eroded in 1.5 h in phosphate buffer (Fig. 2c).

With regards to the safety of application of Zn acetate, the weight of the pectinate beads (without TMC or CB) was compared to the weight of the dry content of pectin in the initial gel volume (350 or 500 mg/10 ml, respectively). The weight of the beads was the same as the weight of the dry content of pectin. Thus it can be concluded that the amount of Zn^{2+} in the beads is less than 1 mg/350 or 500 mg bead (possible daily dosage of the beads). Zinc is an essential micronutrient with recommended dietary allowance (RDA) of 10 mg (NIH's Office of Dietary Supplements, 2002). Rats ingesting up to 25.5 mg Zn/kg/day (as zinc acetate) in drinking water for 47 weeks exhibited no overt toxic effects (Drinker, Thompson, & Marsh, 1927). Therefore, the amount of less than 1 mg Zn^{2+} in the beads is surely safe. Consequently, Zn acetate was chosen as the cross-linker for optimization of beads.

Beads were cross-linked with Zn acetate for 2, 4, or 24 h to determine the effect of t_c on beads' characteristics (Fig. 3a–c). The release characteristics and SER of these

Table 2
Loading efficiency (LE) of pectinate beads prepared in the preliminary studies

Formulation Code	Cross-linker type	TMC/PLMA ratio (w/w)	t_c (h)	LE (%)
Ia	Ca	1/6	2	99.2
Ib	Zn	1/6	2	100
Ic	Zn	1/6	4	100
Id	Zn	1/6	24	100
Ie	Zn	1/6	2	95.9
If	Zn	1/8	2	99.9
Ig	Zn	1/4	2	100

PLMA concentration: 3.5% (w/v); cross-linker concentration: 0.15 M; nozzle diameter: 1.1 mm; encapsulation voltage: 2 kV.

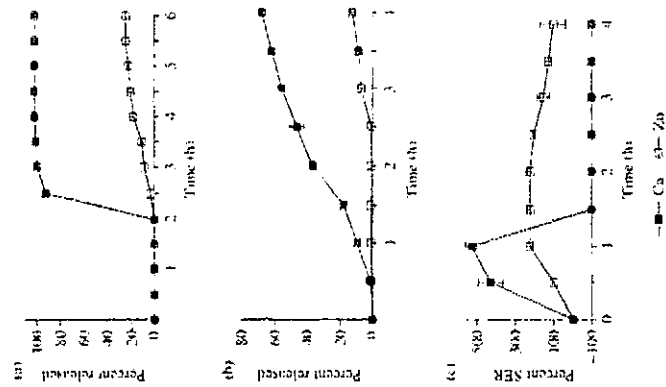


Fig. 2. Influence of cross-linker type on beads' characteristics. In vitro behavior of TMC-pectinate beads cross-linked either with Zn acetate or Ca chloride (mean $\pm$ S.D., $n = 3$). (a) release profile of CB from beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h). (b) release profile of CB from beads in phosphate buffer pH 7.4 (4 h). (c) swelling erosion ratio (SER) of beads in phosphate buffer pH 7.4 (4 h). PLGA concentration = 3.5%, crosslinker concentration = 0.15 M, $t_c = 2$ h, TMC:PLGA ratio = 1:6, encapsulation voltage = 2 kV, nozzle diameter = 1.1 mm.

formulations show that there is no significant difference between the release behavior of beads cross-linked for 2, 4 and 24 h. Thus t_c of 2 h was selected.

TMC is said to be an effective absorption enhancer at concentrations of 0.5–1% (w/v) in the absorption site (Hamann, Schultz, & Kotzé, 2003; Jonker, Hamann, & Korte, 2002). Therefore, it was included in the formulations. The higher the concentration of TMC in a formulation, the lower is the amount of the formulation that has to be administered, for the absorption enhancer to be in the effective range. However, excessive amounts of TMC in the formulations led to impaired beads characteristics (Fig. 4a–c). Therefore, TMC concentration was fixed at the optimum level of 1.6 of PLGA concentration.

3.3. Experimental design and optimization of beads

Zn pectinate beads were prepared based on the 2^3 full factorial design (Table 1). The loading efficiency of these

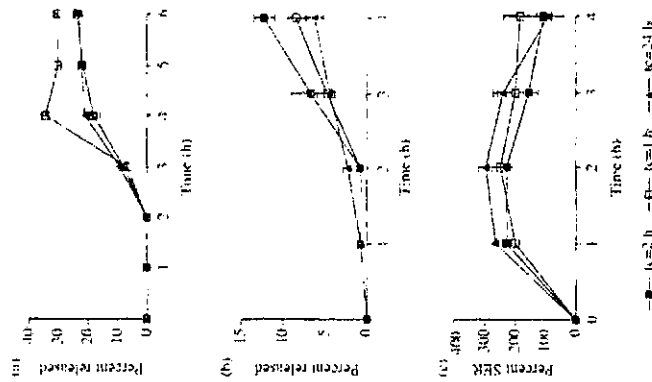


Fig. 3. Influence of cross-linking time (t_c) on beads' characteristics. In vitro behavior of TMC-pectinate beads cross-linked with Zn acetate buffer for 2, 4 or 24 h (mean $\pm$ S.D., $n = 3$). (a) release profile of CB from beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h). (b) release profile of CB from beads in phosphate buffer pH 7.4 (4 h). (c) swelling erosion ratio (SER) of beads in phosphate buffer pH 7.4 (4 h). PLGA concentration = 3.5%, Zn acetate concentration = 0.15 M, TMC:PLGA ratio = 1:6, encapsulation voltage = 2 kV, nozzle diameter = 1.1 mm.

formulations for both, CB and TMC was 100%. The release studies were carried out with these beads in simulated gastrointestinal conditions (Fig. 5a and b). The SER studies were also performed on these beads (Fig. 5c). From the results of Table 1, the following linear regression models were derived Eqs. (6) and (7):

$$\text{Rel}_{4h} = 32.506(\pm 8.218) - 5.44(\pm 1.904)(\text{CPLMA}) + 81.444(\pm 18.792)(\text{CZnaacetate}) - 17.407(\pm 4.354)(\text{CPLMA} \times \text{CZnaacetate})$$

$$P \text{ value: } (0.001) (0.01) (0.000) (0.001) \quad \text{AR}^2 = 88.2\% \quad (6)$$

$$\text{SER}_{4h} = 30.556(\pm 13.442)(\text{CPLMA}) - 420.378(\pm 44.806)(\text{CZnaacetate}) - 24.583(\pm 10.081)(\text{Voltage})$$

$$P \text{ value: } (0.034) (0.000) (0.024) \quad \text{AR}^2 = 77\% \quad (7)$$

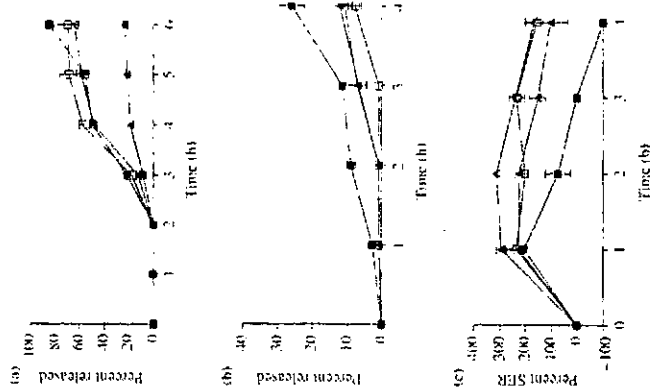


Fig. 4. Influence of TMC:PLGA ratio on beads' characteristics. In vitro behavior of TMC-pectinate beads with TMC:PLGA ratio of 1:3, 1:6, 1:4 (mean $\pm$ S.D., $n = 3$). (a) release profile of CB from beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h). (b) release profile of CB from beads in phosphate buffer pH 7.4 (4 h). (c) swelling erosion ratio (SER) of beads in phosphate buffer pH 7.4 (4 h). PLGA concentration = 3.5%, Zn acetate concentration = 0.15 M, $t_c = 2$ h, encapsulation voltage = 2 kV, nozzle diameter = 1.1 mm.

Rel_{4h} is cumulative percent of CB released in 4 h from beads in phosphate buffer (pH 7.4) and SER_{4h} is SER of beads in 4 h in phosphate buffer (pH 7.4). CPLMA is PLGA concentration in the beads (% w/w). CZnaacetate is concentration of Zn acetate in cross-linking solution (mM). Voltage is voltage of the bead generator (kV). The P value of each term in the equations is subscripted under the term.

As clearly seen in Fig. 5a and b, and based on the results of Eqs. (6) and (7), PLGA concentration has a dramatic effect on beads' characteristics. Higher PLGA concentrations in the beads, lead to formation of denser matrix, which improves the beads' release behavior and retards the CB release from the beads. Moreover, we can deduce from Eq. (7) that beads with higher PLGA, are favored with regards to preserving their multi-particulate structure.

We can conclude from the Eqs. (6) and (7) and Fig. 5, that release and swelling-erosion behavior of beads are dependant on Zn acetate concentrations. Excessive Zn

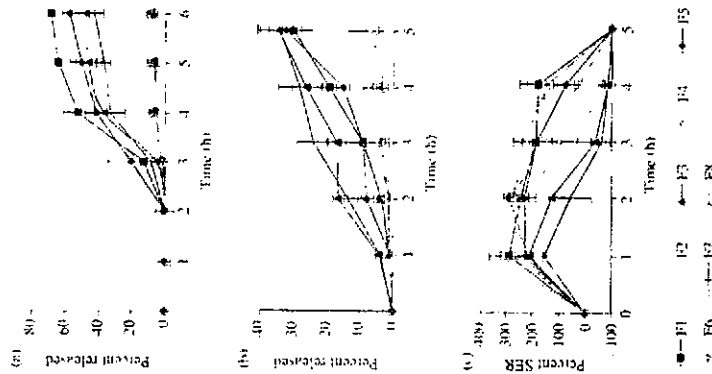


Fig. 5. Effect of PLMA concentration, Zn acetate concentration and encapsulation voltage on beads' characteristics. In vitro behavior of TMC-pectinate beads prepared based on the 2^3 factorial design (Table 1) (mean $\pm$ S.D., $n = 3$). (a) release profile of CB from beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h). (b) release profile of CB from beads in phosphate buffer pH 7.4 (4 h). (c) swelling-erosion ratio (SER) of beads in phosphate buffer pH 7.4 (4 h). $t_c = 2$ h, TMC:PLMA ratio = 1:6, nozzle diameter = 0.7 mm.

acetate increases the CB release, in formulations with lower PLMA concentration. On the other hand, it causes an early phase of swelling, followed by rapid erosion of the beads. We used high concentrations of Zn acetate to obtain higher cross-linking of the PLGA matrix, but on the contrary, in case of beads with lower PLMA concentration, the beads' characteristics were impaired. This observation can be explained by the fact that Zn acetate is a slightly acidic salt (pH of 5% solution is 6). Therefore, its excessive application as a cross-linker might tend to transform some of COO⁻ groups of PLGA into COOH groups (which are unavailable for cross-linking). Moreover, once the dried beads are placed in the aqueous media, high Zn acetate concentration entrapped in the beads exerts an osmosis phenomenon and absorbs the water into the beads. Thus the swelling of the beads is accompanied by erosion and drug release. On the other hand, the interaction of Zn acetate

concentration and PLMA concentration is also observed to be significant in Eq. (6). In case of formulations with higher PLMA concentration, increasing Zn acetate concentration causes a very slight decrease in drug release (Table 1). However, this decrease is infinitesimal and within the standard deviation range of the data and not statistically significant; therefore in these formulations, Zn acetate concentration seems not to be influential in bead's release behavior. This might be due to the dense pectin matrix of the beads with higher PLMA concentration, which overshadows the above-stated adverse effects of excessive Zn acetate concentration on release. Consequently, since excessive Zn concentration either has a negative or no effect on bead characteristics, it should be used in optimized concentrations to render enough cross-linking to the beads, yet its unwarranted high concentrations should be avoided in bead preparation.

Voltage of the bead generator did not have any effect on the R_{eff} of the beads Eq. (6); however, it inversely affected the SER of the beads Eq. (7). Nevertheless the regression coefficient for voltage (-24.583) is trivial compared to the coefficient of Zn acetate concentration (-420.370). Moreover, the P value of this independent variable is 0.037, which is higher than the P value of Zn acetate concentration. Therefore the effect of encapsulator voltage is less pronounced than the effect of Zn acetate concentration. Consequently based on the results of Eqs. (6) and (7), and Fig. 5, it was concluded that the beads can be prepared with electrostatic bead generator without impaired characteristics.

Based on the overall results of the one-way analysis of variance (ANOVA) and multiple comparison (Duncan's test), for release and SER of the F1–8 formulations, P6 was chosen as the optimized core formulation. Moreover, nozzle diameter of 1.1 mm was chosen for further experiments, because beads prepared with 1.1 mm nozzle had better sphere-like shape and were easier to coat.

Although F1–8 formulations showed release of 20–30% in phosphate buffer (pH 7.4) after 5 h, by referring to the swelling-erosion data, it is noted that their SER is declining, and consequently in 5 h, SER is $\sim 100\%$ for all formulations. This low percent of release (20–30%), might seem perplexing, when at the same time SER value is $\sim 100\%$. In this regard, one should bear in mind that a SER value of $\sim 100\%$ does not necessarily mean that the beads have completely dissolved. In some cases SER value of $\sim 100\%$ shows that beads are no longer in the multi-particulate form (see Section 2.6); thus it is possible (as microscopically observed by us in such cases), that beads keep some of their drug unrelieved. Actually, in such cases the beads turned into a blue shapeless gel, which retained some of the drug. Besides, it is possible that some of the CB in the beads, form a complex of decreased solubility, with TMC (see Sections 2.8 and 3.6). This complexation modifies the ultimate drug release from beads. Therefore, the part of CB, entrapped in the insoluble complex with TMC, is in the form of

a precipitate and is not included in the withdrawn samples, and cannot be detected by spectrophotometer.

These experiments show that although the release data alone seem satisfactory, the beads could not retain their spherical and multi-particulate structure. Therefore, they could not fulfill our goals of colon targeting along with maintaining their multi-particulate form. Hence, the beads had to be further modified.

3.4. Rationale for modification of beads

The release and swelling-erosion data of the beads, in all our experiments showed that while no drug release and swelling occurs in 2 h of acid exposure (pH 1.5), pre-exposure to acidic medium drastically affects the beads' behavior and decreases the resistance in phosphate buffer (pH 7.4). The possible explanation of this phenomenon is that in the acidic medium the carboxylic group of pectin in the surface of the beads, turns into COOH , because the pH of the medium (1.5) is less than the pK_a of pectin (3.5) (CP Kelco information sheet). Therefore, at this pH, due to less hydrophilicity and lack of repulsion between carboxylic groups, Zn pectinate (or even cross-linked pectin) is in the form of a relatively insoluble and tightly packed gel. Thus no swelling and release is observed. At the same time, at this pH, displacement of Zn^{2+} with H^+ or K^+ occurs in the outer layers of beads via diffusion. This way the egg-box structure is depleted of its cross-linking agent, but since pectin is insoluble in acidic environment the beads keep their spherical structure in macroscopic observation. Thereafter upon change of medium and entrance into pH 7.4, the COOH groups once again turn into COO^- , which repel each other. Since some cross-linker is depleted from the beads, the structure is loose and a dramatic swelling and subsequent erosion and drug release occurs. These findings compelled us to modify the beads to obtain the desired profiles.

A conventional way to protect the beads from exposure to harsh gastric environment is to put them into capsules or shape them into a tablet form and enteric-coat the single unit dosage form. Nevertheless, by the conventional method stated above, the advantages of the multiple-unit dosage form are lost (see Section 1). The gastric emptying time is the most important source of variation in the gastrointestinal transit time. The multiple-unit dosage forms act reproducibly and predictively in this regard. Therefore, in this study it was imperative to keep the multiple-unit dosage forms throughout the modification experiments. These beads were prepared by 1.1-mm nozzle because with this nozzle they were more spherical in shape and the coating of the beads were easier.

3.5. Characterization of modified pectinate beads

3.5.1. Characteristics of bio mineralized beads

Bio mineralization was not a successful approach for improvement of beads, and it even worsened the beads'

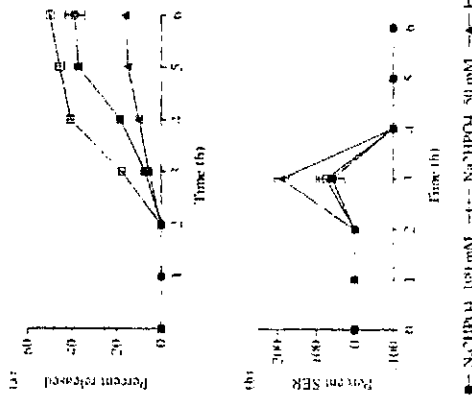


Fig. 6. In vitro behavior of bio mineralized TMC-pectinate beads (mean $\pm$ S.D., $n=3$). (a) release profile of CB from bio mineralized beads in acidic buffer (pH 1.5) followed by phosphate buffer pH 7.4 (4 h). (b) swelling-erosion ratio (SER) of bio mineralized beads in acidic buffer (pH 1.5) (2 h) followed by phosphate buffer pH 7.4 (4 h). Based on 16 formulation of Table 1, PLMA concentration = 5%, Zn acetate concentration = 0.15 M, TMC:PLMA ratio = 1:6, $V=2$ h, encapsulator voltage = 2 kV, nozzle diameter = 1.1 mm, Na₂HPO₄ concentration = 50 or 100 mM.

behavior (Fig. 6a and b). This may be due to the displacement of some of Zn^{2+} by Na^+ from Na_2HPO_4 . Sodium pectinate and other monovalent salts of pectin are in the form of a non-viscous liquid and do not form gel at the pH of a non-viscous liquid and do not form gel at the neutral environment (Wellner et al., 1998). Moreover, the discrepancy between our results and the results obtained by Levesque et al. (2002), might arise from the fact that in the latter study, the release profile of the beads was tested in deionized water. This can affect the results in two ways: (a) pH value of deionized water is 6. At this pH value the solubility of pectin is lower than in pH 7.4. (b) As there is no ion present in deionized water, no sequestration or displacement of crosslinker occurs between the beads and the medium.

3.5.2. Characteristics of PHM-coated beads

The results of release studies on PHM-coated beads show somewhat higher release (yet below 30%), compared to uncoated beads. However, the SER data demonstrates that despite this higher release, the PHM coat is capable of keeping the integrity of beads, while the uncoated beads loose their structure within 4 h in acidic medium and a successive 2 h in phosphate buffer, pH 7.4 (Fig. 7a and b). The higher CB release from PHM coated beads might be due to prolonged exposure of the core to a PHM layer of local most viscous gel during the coating process and also in release environment, which withdraws some of the drug

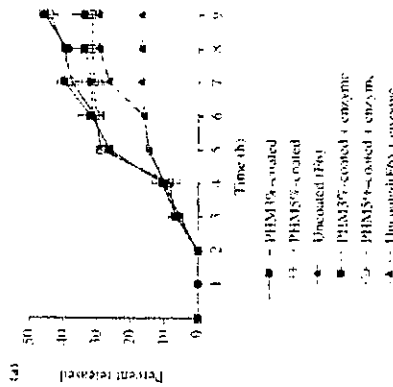


Fig. 7. In vitro behavior of PHM-coated TMC-pectinate beads (mean $\pm$ S.D., $n=3$). (a) release profile of CB from PHM-coated beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h) (b) swelling-erosion ratio (SER) of PHM-coated beads in acidic buffer (pH 1.5) (2 h) followed by phosphate buffer pH 7.4 (4 h). The core formulation is 16 formulation of Table 1, PLMA concentration = 5%, Zn acetate concentration = 0.15 M, TMC:PLMA ratio = 1:6, $V=2$ h, encapsulator voltage = 2 kV, nozzle diameter = 1.1 mm, PHM concentration = 3 or 5%.

to the surface, and upon erosion and dissolution of the PHM coat, creates a burst-like release. Upon the change of medium to phosphate buffer (pH 7.5) with Pectinex Ultra SPL, PHM-coated beads crumbled to solid pieces and the SER could not be further determined. Surprisingly, in the presence of enzyme, no further CB release was observed both for 1/6 and PHM-coated formulations. On the other hand, in the absence of pectinolytic enzyme, the PHM-coated beads were initially in the form of gel like beads, which completely eroded within 3 h and continued to release CB.

With regards to the paradoxical phenomenon observed with the pectinolytic enzyme, it was doubted that the enzyme might reduce the solubility of CB and impair its release from the beads. However, this hypothesis was ruled out by determining the spectrophotometric absorption of CB in solutions of known concentration, in phosphate buffer, either with or without the enzyme. The enzyme had no effect

on the solubility of CB. From the macroscopic inspection of the beads exposed to the pectinolytic enzyme, it appeared that the pectinolytic enzyme, suppresses further water-uptake of the beads (possibly by hydrolysis of glycosidic linkages, breaking the polymer chain, and reducing its gel-forming properties). Less water-uptake of the beads might be partially implicated in the inhibition of CB release from pectinate beads. Such results are also reported by Sendi, Amighi, Deleesschouwer, & Moes (2000). They observed that pectinolytic enzymes slowed down the theophylline release from the pellets coated with ethylcellulose or polymethacrylate polymers, containing 10% w/w of PHM or calcium pectinate. They proposed that in the presence of pectinolytic enzymes, pectin is rapidly degraded and leached out of the film coating, therefore the swelling and the hydration of the film-coatings is decreased. Moreover, the film-coating restructures and plugs up the pores (formed by leaching of pectin), slowing down the drug release. On the other hand, in the absence of pectinolytic enzyme, pectin rapidly absorbs the surrounding water, since PHM and particularly calcium pectinate are hydrophilic and good gel-forming polymers. The pectin, which is now hydrated, can swell and induce a further increase of the swelling and the hydration of the film-coatings, the formation of hydrated pectin channels and probably, the appearance of distensions in the film-coatings. As a result, the diffusion of hydrophilic drugs such as theophylline through the film-coatings is improved. Such mechanisms might be involved in our system as well.

Nonetheless, the colonic pectinolytic enzymes may act differently, *in vivo*. Moreover, as discussed earlier (see Section 2.5), we chose a pH value of 5 for colonic medium, to optimize the enzymatic activity. This issue may weaken the relevance to the colonic environment, *in vivo*, and the above argument might be only of academic value. Therefore, further studies either *in vivo*, or with a fecal content should be carried out to clarify the actual effect of pectinolytic enzymes on the beads behavior.

3.5.3. Characterization of EU-coated beads

EU-coated beads exhibited satisfactory characteristics with regards to release and swelling–erosion studies. The optimized coating process ensures that a flawless coat encloses the beads, which is impermeable to acidic medium. The coat dissolves within one hour in phosphate buffer (pH 7.4), releases the cores in intestinal medium in intact state (Fig. 8a and b), which will then reach colon. As observed for PHM-coated beads, when EU-coated beads, (whose EU coat was then completely dissolved), were transferred to phosphate buffer (pH 5) with Pectinex Ultra SPL, they crumbled and their SIER could not be determined. In the same way, no further CB release from these formulations, occurred in the presence of enzyme. However, in the phosphate buffer (pH 5) alone, EU-coated beads started to swell and release the model drug.

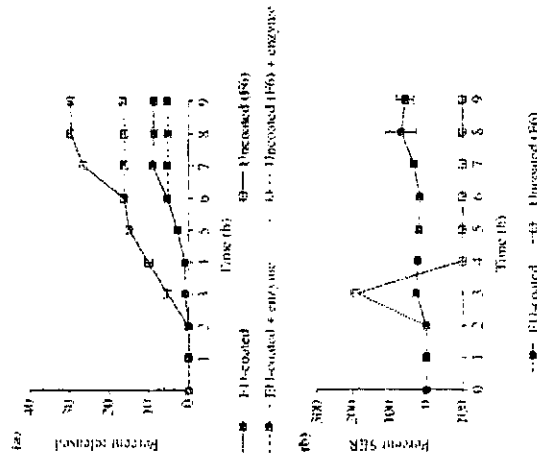


Fig. 8. In vitro behavior of EU-coated TMC pectinate beads (mean $\pm$ S.D., $n = 3$). (a) Release profile of CB from EU-coated beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h) and phosphate buffer pH 5 without or without 50 μ l of Pectinex Ultra SPL (3 h). (b) Swelling–erosion rate (SIER) of EU-coated beads in acidic buffer pH 1.5 (2 h) followed by phosphate buffer pH 7.4 (4 h). The core formulation is 10% formulation of Table 1. PLGA concentration = 5%, Zn acetate concentration = 0.15 M, TMC:PLGA ratio = 1.6, 4, 2 h, encapsulation voltage = 2 kV, nozzle diameter = 1.1 mm.

The dependence of PHM- and EU-coated pectinate beads on microbially triggered mechanisms, should be further studied. However, apart from the microbially triggered mechanisms, these systems appear to be time- and pH-controlled.

3.6. CB-TMC complexation

Comparison of Fc, Ff, Fb and Fg formulations (TMC:PLGA ratio of 0, 1.8, 1.6 and 1.4, respectively), show that increasing amounts of TMC in formulations led to slightly higher (although not significant) loading efficiency of beads for CB (Table 2). Fig. 3 also illustrates that

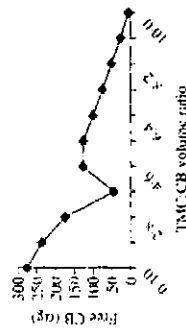


Fig. 9. Job's plot of CB-TMC in phosphate buffer pH 7.4, investigated by spectrophotometric absorption of CB at 578.6 nm (mean $\pm$ S.D., $n = 3$).

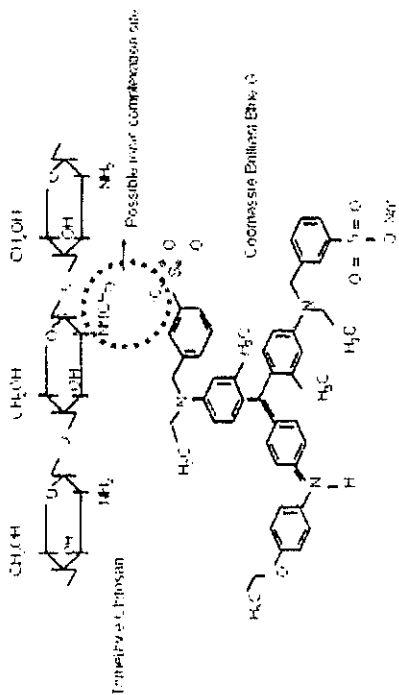


Fig. 10. Structure of trimethyl-chitosan and Coomassie Brilliant Blue G 250 and their possible ionic complexation.

the increase in TMC amount modifies CB release and SIER of beads.

Fig. 9 illustrates the Job's plot for CB-TMC. Upon increasing TMC:CB volume ratio, which in fact corresponds to increasing TMC:CB weight ratio, at some point a steep decrease in the free CB concentration is observed, which does not conform to the rest of the curve. This point (TMC:CB = 2:3) denotes the optimum weight ratio of complex formation between CB and TMC. In macroscopic inspection, at this point a blue precipitate was observed in the test tube. The pH values of the test tubes were measured and it was observed that the values had not changed from that of the diluting buffer (pH 7.4). Therefore, the possibility that changes in pH, upon addition of TMC, might have caused this phenomenon, was ruled out. This interesting finding can be exploited to inhibit premature release of drugs in any dosage form, including the beads prepared in this study. Some studies have proposed that the ionic interactions can be used for controlled delivery and release of drugs. Benkirp-Schmitz, Schlubauer, Clausen, and Hanel (2004), developed a sustained-release dosage form for α -lipase acid based on ionic interactions between the cationic polymer chitosan and the anionic drug, Shalaby, Jackson, and Moreau (2001) invented a sustained release composition, which features polyester containing free COOH groups ionically conjugated with a biologically active poly(ethylene glycol) composed of at least one effective, ionogenic amine.

CB is reported to have three pK_a s with values of 1.15 and 1.82 and 12.4, with, respectively, red, green and blue colors (Chial, Thompson, & Spilgerger, 1993). At the pH of PLGA gel mixture (pH ~4), CB is in the blue color and it is in the deprotonated form. Thus the $N(CH_3)_3$ groups of TMC form ionic bonds with SO_3^- moieties on the CB (Fig. 10). Nevertheless, the formulations, which contain TMC, do not release 100% of their loaded CB in the soluble

form, even after the beads are completely degraded. Therefore, CB-TMC complex may alter the final amount of released drug from the dosage forms, to mask the absorption-enhancing effect of TMC. Therefore, further in-depth investigation is required to elucidate this phenomenon and assess its feasibility.

4. Conclusion

Pectinate-TMC beads can be promising, multi-particulate dosage forms for colon-specific drug delivery. However, they should be protected from gastric acidic environment. The optimized system was designed by coating the beads with either PHM or EU. *In vitro* studies confirmed that this system is capable of delivering the water-soluble high molecular weight drug together with TMC as an absorption enhancer, to colon. This multi-particulate system appears to be both, time- and pH-controlled and possibly microbially triggered. Further studies are underway to assess the *in vivo* feasibility of these systems for peptide and protein drugs.

References

Benkirp-Schmitz, A., Schlubauer, U., Clausen, A. E., & Hanel, R. (2004). Development of a sustained release dosage form for α -lipase, acid design and *in vitro* evaluation. *Drug Investigation and Industrial Pharmacy*, 30(1), 27–31.

Chial, H. J., Thompson, H. R., & Spilgerger, A. G. (1993). A spectral study of the charge forms of Coomassie blue G. *Analytical Biochemistry*, 209(2), 258–266.

CP Beta information sheet, <http://www.sphelco.com/infocp/beta.html>

Dewker, K. R., Thompson, P. K., & Marsh, M. (1977). An investigation of the effect upon rate of long continued ingestion of zinc compounds with a special reference to the relation of zinc excretion to zinc intake. *American Journal of Physiology*, 234, 284–306.

- Ek-Gohdy, I. (2002). Oral delivery-release system based on Zn-pectinate gel (ZAPG) microcapsules as an alternative carrier for calcium pectinate beads for colon drug delivery. *International Journal of Pharmaceutics*, 253(1–2), 209–211.
- Fallick, D. J. (1999). Intraluminal pH of the human gastrointestinal tract. *Human Medical Bulletin*, 46(2), 183–196.
- Gault, R. R., & Vasharph, W. C. (1992). Complex ions III: A study of some complex ions in solution by means of the spectrophotometer. *Journal of American Chemical Society*, 64(7), 1630–1633.
- Ghosh, C., Lohita, N., Vasharph, T., Sato, S., Ball, O. J., Whiting, I. R., & Jayaram, B. (2004). Pharmaceutical design of a novel colon-targeted delivery system using two-layer coated tablets of three different pharmaceutical formulations, supported by clinical evidence in humans. *Journal of Controlled Release*, 97(1), 31–42.
- Hamman, J. H., Smith, C. M., & Kozak, A. F. (2003). Chitosan Chloride: optimum degree of quaternization for drug absorption enhancement across epithelial cells. *Drug Development and Industrial Pharmacy*, 29(7), 161–172.
- Kozak, A. F., Hamman, J. H., & Kozak, A. F. (2002). Ingestible pericellular permeation enhancer agent with quaternized chitosan: in situ and in vitro evaluation. *International Journal of Pharmaceutics*, 250(1–2), 205–213.
- Revergne, J., Riviere, K. H., & Miao, S. (2002). Biomimetic-inspired fabrication of semi-permeable calcium phosphate-polysaccharide microcapsules. *Journal of Materials Chemistry*, 12, 2178–2180.
- Lin, S. Y., Chen, A. S., & Teng, H. H. (1999). Protective colloids and polybasic acid co-affecting the polymeric vesicle form and crystallinity of indomethacin encapsulated in microspheres. *Journal of Microencapsulation*, 16(4), 769–776.
- Machalski, G. S., Parr, J. T., & Cobden, J. H. (1999). An in vitro investigation into the potential for buccal drug release from pectin-chitosan/HPMC-coated tablets. *International Journal of Pharmaceutics*, 189(1), 11–18.
- Mathew, E. (1999). In vivo, Mathew, E. (1997). *Oral drug delivery: small intestine and colon. Encyclopedia of controlled drug delivery* (Vol. 2) (pp. 717–728). New Jersey: Wiley.
- Munjan, O., Collett, J. H., & Parr, J. T. (1997). Hydrogel beads based on anhydrous pectin for colon-specific drug delivery: the role of chitosan in modifying drug release. *Journal of Controlled Release*, 46(3), 273–278.
- NIH's office of dietary supplements. (2002). Facts about dietary supplements. <http://web.nid.nih.gov/dietarysupplements.htm>.
- Pillay, V., & Jayash, R. (1999). In vitro release modulation from crosslinked pectin for site-specific drug delivery in the gastrointestinal tract I. Comparison of pH-responsive drug release and associated kinetics. *Journal of Controlled Release*, 59(2), 229–242.
- Pillay, V., & Jayash, R. (1999). In vitro release modulation from crosslinked pectin for site-specific drug delivery in the gastrointestinal tract II. Physicochemical characterization of calcium-alginate, calcium pectinate and calcium-alginate-pectinate pellets. *Journal of Controlled Release*, 59(2), 243–256.
- Raghavan, C. V., Muthulingam, C., Kedia, J. A. I., & Rao, T. R. (2002). An in vitro and in vivo investigation into the suitability of bacterially triggered delivery system for colon targeting. *Chemical and Pharmaceutical Bulletin*, 50(3), 392–395.
- Rafan Thomas Publisher's literature, Section 1.3: Examples of enteric coating processes for tablets, pp. 17. <http://www.rafan.com/Pharmaceuticals.html>
- Sawicki, R., Angioli, R., & Vasharph, M. J., & Allen, A. J. (2000). Effect of pectinase enzymes on the theophylline release from pellets coated with water insoluble polymers containing pectin HMI in calcium pectinate. *International Journal of Pharmaceutics*, 19(1), 21, 167–179.
- Sawicki, R., Miles, A. J., Dreyer, M. J., & Angioli, R. (2005). Synthesis and enzymatic degradation of epiphenylhydrazide cross-linked pectines. *Drug Development and Industrial Pharmacy*, 31(2), 203–213.
- Shah, S. W., Wilson, S. A., & Morcan, I. P. (2003). Ions, molecular recognition of biodegradable polymers and bioactive polypeptides. *United States Patent*, 227, 958.
- Serfaty, A. R., Thano, M., Kozak, A. F., Verheul, J. C., Broese, J., & Jongschaap, H. E. (1998). NMR preparation characterization of highly substituted N-trimethyl chitosan chloride. *Carbohydrate Polymers*, 34(2–3), 157–165.
- Serfaty, A. R., & Kozak, A. F. (2001). Polysaccharides in colon-specific drug delivery. *International Journal of Pharmaceutics*, 224(1–2), 59–68.
- Seymour, D., Hamman, J. H., Kozak, J. S., Rollings, J. E., & Kozak, A. F. (2002). The relationship between the absolute molecular weight and the degree of quaternization of N-trimethyl chitosan chloride. *Carbohydrate Polymers*, 50(2), 143–150.
- Srinivasan, R. P., & Nambial, J. (1998). Calcium pectinate gel beads for controlled drug delivery: I. Preparation and in vitro release studies. *International Journal of Pharmaceutics*, 169(2), 207–212.
- Srinivasan, R. P. (1998). Investigation of pectin as a carrier for oral delivery of proteins using calcium pectinate gel beads. *International Journal of Pharmaceutics*, 169(2), 213–220.
- Srinivasan, R. P. (1999). Effect of calcium concentration, hardening agent and drying condition on release characteristics of oral proteins from calcium pectinate gel beads. *European Journal of Pharmaceutics*, 82(3), 221–227.
- Thano, M. M., Kozak, A. F., Scharfinghausen, P., Lueken, H. J., de Boer, A. G., Verheul, J. C., & Jongschaap, H. E. (2000). Effect on degree of quaternization of N-trimethyl chitosan chloride for enhanced transport of hydrophilic compounds across intestinal Caco-2 cell monolayers. *Journal of Controlled Release*, 64(1–3), 15–25.
- Vasanthakumari, Th. E., Kozak, A. F., Charoian, C. A., Chaudhry, J. C. (1992). The use of polysaccharides to target drugs to the colon. *Carbohydrate Polymers*, 16(3), 219–231.
- Wakabayashi, Z., Ieda, J., Alkassab, D., & Parkins, D. (1997). Studies on anhydrous pectin as potential carriers in colon drug delivery. *Journal of Pharmacy and Pharmacology*, 49(6), 622–625.
- Wolfe, N., Kozak, A. F., Makowski, A., Wilson, R. H., & Belton, P. S. (1998). FT-IR study of pectate and pectinate salts formed by disodium calcium. *Carbohydrate Research*, 308, 123–131.
- Yang, L., Chu, J. S., & Fu, J. A. (2002). Colon-specific drug delivery: New approaches and in vivo evaluation. *International Journal of Pharmaceutics*, 239(1–2), 1–15.
- Zhang, J. L., Alkassab, D. A., & Niu, S. H. (2002). An in vitro evaluation of a chitosan-containing multi-particulate system for macromolecule delivery to the colon. *International Journal of Pharmaceutics*, 239(1–2), 397–405.

Cell wall oligogalacturonides increase cytosolic free calcium in carrot protoplasts

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SUMMARY

The mode of action of cell wall elicitors in the induction of various plant cell responses, such as the activation of host defence mechanisms, is unknown, but signal transduction through cytosolic free calcium ($[Ca^{2+}]_i$) has been suggested. This paper shows that polygalacturonic acid or oligogalacturonides cause a prolonged increase in $[Ca^{2+}]_i$ of carrot protoplasts within 20 min of induction. Our data support the view that a special conformation of the oligogalacturonides possessing >9 residues is necessary to induce an elevation in $[Ca^{2+}]_i$. The localization of $[Ca^{2+}]_i$ elevation around the periphery of protoplast cytoplasm and the inhibition of the response with verapamil suggest that exogenous Ca^{2+} is the major source for the rise in $[Ca^{2+}]_i$.

Key words: oligogalacturonide, pectin, calcium, *Eranthis carotifolia*, fluorescence ratio imaging

INTRODUCTION

Pectin is an acidic polysaccharide constituent of the cell wall. It is a polymer of α -1,4-linked galacturonic acid residues with neutral sugars, including α -1,2-linked rhamnosyl residues. Pectin can exist in either a sol or gel form and the type of gel it forms may be concerned with the regulation of cell wall growth. Cell wall pectin is structurally associated with Ca^{2+} , which can crosslink pectin chains, causing them to cluster and gel (Jarvis, 1982). Binding of Ca^{2+} and the extent and type of gel formation are modified in a sensitive fashion by either acetylation or methylesterification of uronic acid, or by the attachment of longer, neutral side chains.

Many physical studies have shown that binding of Ca^{2+} leads to conformational changes in pectin chains. This is generally believed to result in the "egg box" structure, with Ca^{2+} (the "egg") sandwiched between "egg box" pectin chains in dimeric or multimeric forms (Fig. 1A, B and C). This conformation can also be induced by Mn^{2+} . Structural analyses have indicated that the egg box conformation requires oligopeptides with >10-12 residues (Thibault, 1980; Morris et al., 1982; Powell et al., 1982; Irwin et al., 1984; Thibault and Renaudo, 1985). Monoclonal antibodies have recently been raised to this supramolecular structure (Liners et al., 1989).

Invasion of a resistant plant host by a pathogen triggers a set of well-defined defence mechanisms (Horsfall and Cowling, 1980; Lamb et al., 1989). These host responses

are necessary to induce an elevation in $[Ca^{2+}]_i$. The localization of $[Ca^{2+}]_i$ elevation around the periphery of protoplast cytoplasm and the inhibition of the response with verapamil suggest that exogenous Ca^{2+} is the major source for the rise in $[Ca^{2+}]_i$.

Key words: oligogalacturonide, pectin, calcium, *Eranthis carotifolia*, fluorescence ratio imaging

may involve the production of mechanical barriers, hydrolytic enzymes or specific chemicals such as phytoalexins designed to impair pathogen growth (Lamb et al., 1989; Mith et al., 1986; Dietrich et al., 1990; Forrest and Lyon, 1990). In a number of cases, the eliciting signal is believed to be oligogalacturonides released from the plant cell wall. Optimal elicitation activity has been derived from oligogalacturonides with 9-14 residues (Bruce and West, 1989; Davis et al., 1986; Jin and West, 1984; Horn et al., 1989; Forrest and Lyon, 1990). Oligogalacturonides of the same size have also been found to result in protein phosphorylation (Farmer et al., 1991), as well as signalling other biological responses (Aldington et al., 1991).

How signals provided by cell wall fragments are transduced to initiate host defence mechanisms is still unknown, but recent information suggests that cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) might be involved (Cramer et al., 1985; Kurosaki et al., 1987; Stab and Ethel, 1987). The critical analysis (i.e. measurements of $[Ca^{2+}]_i$ in living cells after elicitor treatment) is still unavailable. As a result we decided to examine the effects of oligogalacturonides on $[Ca^{2+}]_i$ using fluorescence ratio imaging of the dye Indo-1. Because of difficulties in loading walled cells with Ca^{2+} -sensitive dyes (Callaghan and Hepler, 1991; Read et al., 1992), we used carrot protoplasts to study the effects of cell wall oligogalacturonides produced by *Eranthis carotifolia* atropsecta endopolygalacturonase. Since only small quantities of some of the elicitor preparations were available, our measurements were limited to just fluorescence ratio imaging of $[Ca^{2+}]_i$.

in single protoplasts. Our results indicate that oligogalacturonides with a degree of polymerization (DP) of 9 induce a prolonged increase in $[Ca^{2+}]_i$. Furthermore, the data show that the oligogalacturonides need to have a Ca^{2+} -induced conformation in order to elevate $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Culture conditions and protoplast isolation

Eranthis carotifolia cells were cultured in liquid medium containing 4.4 g l⁻¹ Murashige and Skoog salts (Sigma Chemical Co.), 30 g l⁻¹ sucrose and 1 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (pH 5.7). 50 ml cell suspensions were grown at 25°C in 250 ml flasks under constant agitation (150 revs per min) in the dark. The cells were subcultured every week by a 1:4 dilution into fresh medium.

The culture medium was supplemented with 100 g l⁻¹ mannitol, 15 g l⁻¹ peptone (Sigma) and 5 g l⁻¹ cellulase RS (Onozuka) for protoplast isolation. 1 g of *E. carotifolia* cells per 10 ml of enzyme cocktail was used for each experiment. The enzymatic digestion of the cell walls was carried out overnight, with mild agitation at 25°C. Protoplasts were then successively filtered through 200 μ m and 30 μ m nylon filters and centrifuged at 100 g for 5 min. The pellet was resuspended in enzyme-free medium and centrifuged at 200 g for 3 min. The supernatant was discarded and the washing procedure repeated 3 times to remove the enzymes.

Dye loading

The Ca^{2+} -sensitive dye Indo-1 was loaded by electroporation, using a gene pulser (BioRad) (Gilroy et al., 1986). Protoplasts (10⁶ ml⁻¹) were permeabilized in 0.4 cm cuvettes in Murashige and Skoog medium supplemented with 100 g l⁻¹ mannitol, pH 6.5, and containing 50 μ M Indo-1 (free acid, Molecular Probes Inc., Eugene, OR, USA). For electroporation, the field strength was 1200 V cm⁻¹, the time constant 100 μ s and 3 pulses were delivered at 5 s intervals. The protoplasts were kept on ice for 10 min before and after electroporation. The electroporated protoplasts were maintained in the dark for 90 min at room temperature before analysis. After this period no long term effects of protoplasting, low temperature or electroporation were evident and, unless stimulated, the protoplasts maintained constant $[Ca^{2+}]_i$ levels for further periods in excess of 30 min. Further details of dye loading and validation of $[Ca^{2+}]_i$ measurements in carrot protoplasts can be found in Gilroy et al. (1986, 1987, 1989).

Fluorescence ratio imaging

Fluorescence ratio imaging was performed using equipment and techniques previously described in detail (Gilroy et al., 1991; Read et al., 1992). Loaded cells were analysed using a Nikon CF Fluor DI, 40 $\times$ 0.55 NA objective on a Nikon Diaphot inverted microscope equipped with epifluorescence optics. Samples were excited at 350 nm with a 75 W xenon light source. Fluorescence was detected at 405 nm and 480 nm using 10 nm bandwidth interference filters mounted in a computer-controlled filter wheel (Newcastle Photonic Systems, Newcastle-Upon-Tyne, UK). Digitized images were captured at 10 min intervals using an Extended ISIS CCD camera (Photonic Science, Kent, UK) connected to a Synapse frame store (Synographics Ltd., Cambridge, UK). Image capture was controlled by the Scimper 6 Plus image processing language run on a Dell 310 microcomputer equipped with a 80386 microprocessor, maths coprocessor and 40 Mb hard disc. Autofluorescence and dark current were subtracted from each image prior to ratio image calculation. Because autofluorescence could not be imaged in the same cell before and after electroporation, an average pixel value of autofluorescence at each wavelength was

obtained from 10 unloaded protoplasts. Ratio images were subsequently colour-coded to represent different Ca^{2+} concentrations determined after *in vivo* calibration. This was carried out using a set of buffers containing 3 μ M Indo-1, 100 mM KCl, 1 mM MgCl₂, 10 mM HEPES (pH 7.0), 10 mM EGTA and variable amounts of $CaCl_2$ to give appropriate free Ca^{2+} concentrations (Grynkiewicz et al., 1985). Hard copies of images were produced with a Mitsubishi CP 100B video copy processor.

Vital staining of Indo-1 loaded protoplasts with trypan blue demonstrated that they retained viability before, during and after $[Ca^{2+}]_i$ imaging (data not shown).

Conventional fluorescence microscopy, light microscopy and photography

Dye loading was assessed by conventional fluorescence microscopy and phase contrast light microscopy using a Zeiss Photomicroscope III. A 350/10 nm excitation filter, 380 nm dichroic mirror and a 400 nm long pass barrier filter were used for fluorescence microscopy. Photographic images were recorded on Kodak 1 MAX 400 35 mm film.

Production and use of oligogalacturonides

Protoplasts were mixed with polygalacturonic acid (PGA, Sigma Chemical Co.) or oligogalacturonides of different lengths with different conformations.

Oligogalacturonides (Fig. 1B) were produced by digestion of PGA using a purified endopolygalacturonase from *Eranthis carotifolia* (Stark et al., 1980). Digestion products were purified by gel permeation on Bio-gel B6 (Hibbard, 1980). Two fractions were used in experiments: oligogalacturonides <9 residues long and oligogalacturonides >9 residues long. The choice of these two fractions was dictated by the ability of these oligogalacturonides to be recognized by the anti-pectin dimer 2/4 monoclonal antibody described by Liners et al. (1989, 1992).

The dimeric conformation of either PGA or oligogalacturonides was produced in 10% mannitol with either 150 mM NaCl and 0.5 mM $CaCl_2$ or 150 mM NaCl and 0.5 mM $MnCl_2$ (Irwin et al., 1984). The single chain conformation was maintained in solutions of 10% mannitol containing 150 mM NaCl.

For PGA treatments the protoplasts were adhered to a polylysine-coated slide before treatment and perfused with the indu-

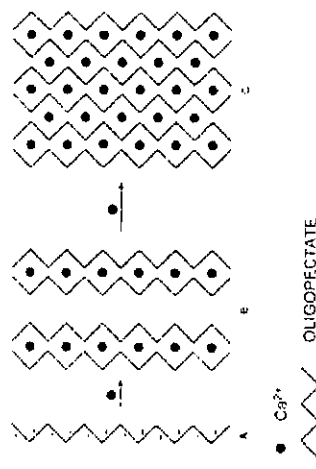


Fig. 1. Diagrammatic representation of polygalacturonic acid (PGA) in the extended single chain conformation (A) and in the dimeric and multimeric "egg box" conformations produced in the presence of Ca^{2+} (B and C) or Mn^{2+} . PGA chains exist as dimers only when in a solution containing either 150 mM NaCl and 0.5 mM $CaCl_2$ or 150 mM NaCl and 0.5 mM $MnCl_2$ (Liners et al., 1989, 1992).

ing solution. Since only very small amounts of the purified oligogalacturonides were available, in these experiments protoplasts were mixed first and then allowed to settle before imaging. Thus in these experiments the earliest images were only obtained after 3–4 min. No effect of immobilization or of 150 mM NaCl and 0.5 mM Ca^{2+} on $[\text{Ca}^{2+}]_i$ was detected in controls (data not shown). In some cases, the sucrose concentration was reduced to keep osmolality constant. Again, we found this had no effect on $[\text{Ca}^{2+}]_i$ (data not shown).

Photobleaching and dye leakage from the cells limited the available time for imaging to approximately 30 min. We considered this satisfactory for our purposes because other studies have demonstrated that oligogalacturonides induce membrane depolarization during this period (Thoin et al., 1990; Malbecq et al., 1991). All experiments were repeated at least four times to establish a common pattern of response. Representative experiments only are illustrated here.

Phenylalanine ammonia lyase (PAL) activity

To demonstrate that cells remained viable and capable of responding to elicitor treatment, we measured PAL activity according to the assay method described by Lamb et al. (1979). The concentrations of elicitors used were the same as those used for imaging $[\text{Ca}^{2+}]_i$. For measuring PAL activity, 0.5 ml of crude protoplast extract was incubated for 1 h at 40°C in a final reaction mixture of 3.0 ml containing 30 μM sodium borate, pH 8.8, and 30 μM phenylalanine. Absorbance at 286 nm was read against an identical mixture except that 30 μM of D-phenylalanine was substituted for L-phenylalanine.

RESULTS

The protoplasts used in this investigation were highly vacuolate (Fig. 2A). Only cells in which dye was clearly loaded in the cytoplasm (amounting to approx. 10% of protoplasts) were used in the analyses. Organelles such as mitochondria, the endoplasmic reticulum and vacuoles (Bush et al., 1989; Schroeder and Hedrich, 1989; Rizzuto et al., 1992) possess free Ca^{2+} concentrations $>5 \mu\text{M}$, which are significantly higher than that found in the

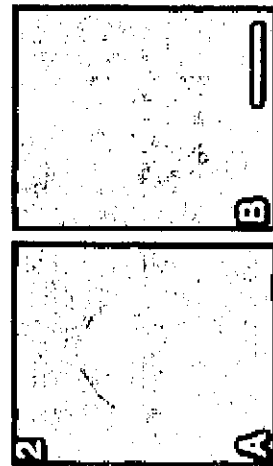


Fig. 2. Fluorescence micrographs of carrot protoplasts loaded with Indo-1. (A) Cytoplasmic loading of dye. This protoplast possesses a single large vacuole surrounded by a thin rim of cytoplasm allowing a very clear distinction between the cytoplasm and vacuole. Note that punctate fluorescence (indicative of dye uptake into organelles) is lacking. The majority of protoplasts possessed two or more vacuoles and more cytoplasm than that shown here (B). Vacuolar loading of dye. Bar, 5 μm .

cytosol. Because Indo-1 has a high affinity for free Ca^{2+} ($K_d \sim 250 \text{ nM}$; Grynkiewicz et al., 1985), any dye compartmentalized within these organelles will be Ca^{2+} -saturated and thus maximally fluorescent. Punctate fluorescence or localized high ratio values were not evident in unstimulated cells (Figs 2A, 3B,C,D and F), indicating that significant dye sequestration within organelles was absent. Cells which exhibited vacuolar loading were obvious (Fig. 2B) and were rejected for analysis. Autofluorescence detected with the narrow 10 nm bandwidth emission filters used for fluorescence ratio imaging was negligible but was, nevertheless, subtracted at each emission wavelength during the production of the ratio images. The resting level of $[\text{Ca}^{2+}]_i$ ranged from 180 nM to 300 nM in different protoplasts, which is in good agreement with measurements we have made previously on carrot protoplasts (Gilroy et al., 1986, 1987).

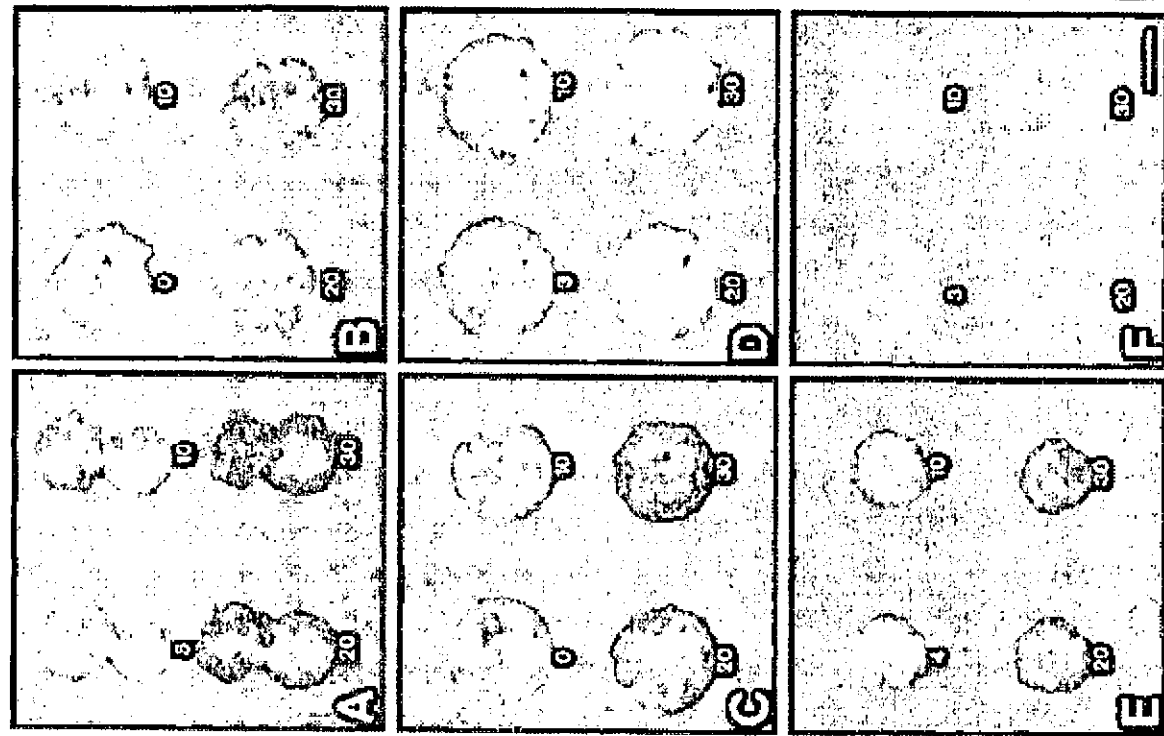
Fig. 3A shows that incubation of Indo-1-loaded protoplasts with 1 mg ml $^{-1}$ PGA plus Ca^{2+} (i.e. with PGA in the dimeric conformation) leads to increases in $[\text{Ca}^{2+}]_i$. The increase in $[\text{Ca}^{2+}]_i$ typically commenced between 5 and 10 min and reached concentrations over 1 μM by 30 min. No $[\text{Ca}^{2+}]_i$ increase was detected in any protoplast before 5 min following exposure to PGA ($n = 6$). The observed increases in $[\text{Ca}^{2+}]_i$ varied between protoplasts, even within the same preparation (Fig. 3A). The highest concentration of $[\text{Ca}^{2+}]_i$ commonly appeared around the periphery of a protoplast with a gradient (red-yellow-green-blue) which seemed to propagate towards the protoplast interior (e.g. see Fig. 3C). This is circumstantial evidence for Ca^{2+} entering the cytosol from outside rather than being mobilised from internal Ca^{2+} reserves. Further evidence that Ca^{2+} channels located in the plasma membrane may be involved in the $[\text{Ca}^{2+}]_i$ increase is shown in Fig. 3B. In the presence of the Ca^{2+} channel blocker verapamil, PGA plus Ca^{2+} failed to induce the $[\text{Ca}^{2+}]_i$ increase shown in Fig. 3A. The increase in $[\text{Ca}^{2+}]_i$ was observed at a concentration ranging from 0.2 mg ml $^{-1}$ up to 1 mg ml $^{-1}$ (data not shown). Below 0.2 mg ml $^{-1}$ no increase was evident. Concentrations higher than 1 mg ml $^{-1}$ were not tested.

Fig. 3C and D further indicates that PGA must have the dimeric conformation to be active. PGA plus Mn^{2+} increases $[\text{Ca}^{2+}]_i$ with a similar time course (Fig. 3C) to PGA plus Ca^{2+} (Fig. 3A). Again the location of $[\text{Ca}^{2+}]_i$ increase is clearly in the cytoplasm with the concentration gradient in $[\text{Ca}^{2+}]_i$ decreasing away from the plasma membrane in the focal plane imaged. Fig. 3D shows that the presence of Ca^{2+} or Mn^{2+} in solution is necessary for the PGA-induced increase in $[\text{Ca}^{2+}]_i$. PGA chains which are not in a dimeric conformation are therefore inactive.

The effects of oligogalacturonides, with chain lengths shorter and greater than 9 residues, were each tested (Fig. 3E and F). Changes in $[\text{Ca}^{2+}]_i$ were only observed with oligogalacturonides >9 residues in length (for DP >9 , $n = 4$; for DP <9 , $n = 6$). The time course of $[\text{Ca}^{2+}]_i$ increase in Fig. 3E commenced within 20 min but the changes in $[\text{Ca}^{2+}]_i$ were not as dramatic as those observed after the addition of PGA dimers (Fig. 3A and C).

PAL activity increased in carrot protoplasts treated with oligogalacturonides with DP >9 , or dimeric PGA in the

Fig. 3. Fluorescence ratio images of carrot protoplasts after exposure to various elicitor and non-elicitor treatments. The numbers beneath each protoplast represent the time (in min) after exposure to each treatment.
(A) Addition of polygalacturonic acid (PGA) (1 mg ml $^{-1}$) plus Ca^{2+} (dimeric egg box form).
(B) Addition of PGA (200 μg ml $^{-1}$) plus Ca^{2+} (dimeric egg box form) after a 15 min pre-incubation with 10 μM verapamil.
(C) Addition of PGA (200 μg ml $^{-1}$) plus Mn^{2+} (dimeric egg box form).
(D) Addition of PGA (1 mg ml $^{-1}$) in single chain form.
(E) Addition of oligogalacturonides with >9 residues (1 mg ml $^{-1}$) plus Ca^{2+} (dimeric egg box form).
(F) Addition of oligogalacturonides with <9 residues (1 mg ml $^{-1}$) plus Ca^{2+} (single chain form). Dark regions within pseudocoloured protoplasts represent the thinnest cytoplasm over vacuoles where the signal strength was insufficient to provide precise ratio values. The reason why a thin rim of cytoplasm surrounding a black vacuole is not seen is because, firstly, all of the protoplasts shown were multivacuolate (unlike Fig. 2A) and, secondly, out-of-focus cytoplasm has contributed to the ratio values obtained in cellular regions which were predominantly vacuolate. Bar, 5 μm .



presence either Ca^{2+} or Mn^{2+} . Large increases in PAL activity were not observed until 18 h after elicitor addition. Significant increases in PAL activity did not occur after treatment with oligogalacturonides with DP <9 , monomeric

PGA, or dimeric PGA in the presence of verapamil (Fig. 4).

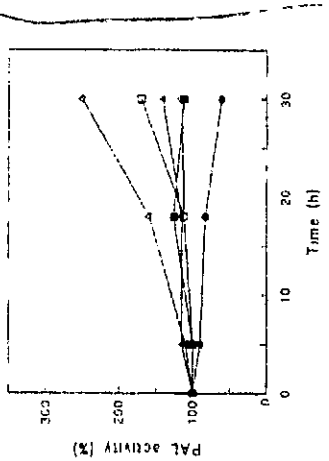


Fig. 4. PAL activity in carrot protoplasts after exposure to oligogalacturonides with ≥ 9 residues (1 mg ml⁻¹) plus Ca²⁺ (1 mM) (filled circles), PGA (200 µg ml⁻¹) plus Ca²⁺ (1 mM) (open squares), PGA (200 µg ml⁻¹) plus Ca²⁺ (1 mM) plus EGTA (2 mM) (filled squares), and PGA (200 µg ml⁻¹) plus Ca²⁺ (1 mM) plus EGTA (2 mM) (open circles). Error bars represent standard deviation.

DISCUSSION

The data shown here indicate that PGA and oligogalacturonides released from the plant cell wall increase [Ca²⁺]_i in a conformation-dependent manner. The addition of PGA in the presence of either Ca²⁺ or Mn²⁺ initiates [Ca²⁺]_i increases commencing some 5–20 min after exposure. Calcium and Mn²⁺ are known to induce a supra-molecular conformation in PGA (Morris et al., 1982; Powell et al., 1982; Irwin et al., 1984). Evidence supporting the inductive character of the dimeric conformation was obtained by treating protoplasts with oligogalacturonides, greater or less than 9 residues in length, with appropriate concentrations of monovalent and divalent cations to induce the dimeric conformation (Fig. 1; Liners et al., 1989, 1992). Our data show that only oligomers of DP ≥ 9 in the dimeric conformation (i.e. the pectic fraction recognized by the 2F4 antibody) induced an elevation in [Ca²⁺]_i within 30 min of addition. Because the dye photobleaching and leakage prevented [Ca²⁺]_i increasing beyond 30 min, we cannot exclude the possibility that dimeric oligomers of DP ≤ 9 do not induce a delayed [Ca²⁺]_i elevation. Whether the multimer has the same inductive effect on [Ca²⁺]_i is not known. However, we consider it possible that the multimer would not be active in plants because the large size of multimers would restrict their diffusion across the cell wall.

The inhibition of a dimeric PGA-induced [Ca²⁺]_i elevation by verapamil suggests a requirement for Ca²⁺ channel activity for the response to occur. Although we considered using LaCl₃ as an additional channel blocker, La³⁺ can displace Ca²⁺ from oligogalacturonides and results would therefore be difficult to interpret. Several of the images we presented indicate a gradient of [Ca²⁺]_i from the plasma membrane inwards. This suggests the involvement of a plasma membrane Ca²⁺ channel. The opening of Ca²⁺-

significantly elevated at 10⁻⁴ M. A similar oligogalacturonide concentration induced membrane depolarization in tomato leaf cells (Tham et al., 1990). This apparently high concentration, however, may be similar to the local concentration of oligogalacturonides released from the cell wall at the site of pathogen invasion.

The ability of oligogalacturonides to induce [Ca²⁺]_i elevation in a size- and conformation-dependent way suggests that Ca²⁺ signalling may be involved in the triggering of plant defence genes. Indeed, phytoalexin accumulation (Lin and West, 1989) and lignification (Bruce and West, 1989) are induced by the same pectic oligomers that elevate [Ca²⁺]_i levels in our carrot protoplasts. Furthermore, Kurosaki et al. (1987) have shown that the addition to cultured carrot cells of the Ca²⁺ ionophore A23187, in the presence of extracellular Ca²⁺, induces the synthesis of the phytoalexin 6-methoxymellein.

Dimeric PGA and polygalacturonides with a DP ≥ 9 highly increased [Ca²⁺]_i and PAL activity in carrot protoplasts. In contrast, PGA and polygalacturonides with a DP ≤ 8 and dimeric PGA in the presence of verapamil did not. PAL is the first enzyme involved in the synthesis of isoflavonoid phytoalexins (Lamb et al., 1989). Our results, therefore, support the hypothesis that Ca²⁺ is a second messenger in the signal transduction pathway which links oligogalacturonide elicitors with the response of phytoalexin synthesis. However, we found that the quantitative effects of the active elicitors on [Ca²⁺]_i and PAL activity were not the same (e.g. dimeric PGA caused the greatest elevation in [Ca²⁺]_i whilst polygalacturonides with a DP ≥ 9 induced the highest level of PAL activity). The reason for this is not known but may indicate that more than one signalling pathway is involved. In this respect, Kurosaki et al. (1987) found that the addition of a crude elicitor preparation to cultured carrot cells increased intracellular cAMP levels. However, whether cAMP is a second messenger in plant cells is still somewhat controversial (Trewavas and Gilroy, 1991).

In a previous paper we showed that cell wall elicitors from yeast and the filamentous fungus *Gliocladium* both induced increases in [Ca²⁺]_i (Knight et al., 1991). However, further evidence is still necessary to establish clearly whether oligogalacturonides or other elicitors which regulate the expression of specific genes (e.g. in a defence response) are directly mediated by [Ca²⁺]_i.

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REFERENCES

- Atkinson, S., McDougall, G. J. and Fry, S. C. (1991). Structure-activity relationships of biologically active oligosaccharides. *Plant Cell Environ.* 14, 625–636.
- Berredge, M.J. and Irvine, R.F. (1989). Inositol phosphates and cell signalling. *Nature* 341, 197–205.
- Bruce, R.J. and West, C.A. (1989). Elicitation of legum biosynthesis and isoprenoidase activity by pectic fragments in suspension cultures of carrot cells. *Plant Physiol* 91, 850–857.
- Burb, D.S., Bowers, A.K. and Jones, R.L. (1989). Gibberellin-acid-

- stimulated Ca²⁺ accumulation in endoplasmic reticulum of barley aleurone cells: transport and steady-state levels. *Plant J.* 1, 111–120.
- Chabrier, P.A. and Depler, P.K. (1991). Measurement of free calcium in plant cells. In *Calcium in Plants: A Practical Approach* (ed. J.G. McManus and P.J. Scholfield), pp. 383–412. Oxford University Press, Oxford.
- Cramer, C.L., Bell, J.A., Ryder, T.R., Bailey, W.A., Schuch, W., Reinhold, G.P., Robbins, M.P., Dixon, R.A. and Lamb, C.J. (1985). Coordinated synthesis of phytoalexin biosynthetic enzymes in biologically-stressed cells of bean (*Phaseolus vulgaris* L.). *EMBO J.* 4, 285–289.
- Bray, K.R., Durall, A.G., Albertsson, P. and Dell, A. (1989). Host-pathogen interactions: ALX oligogalacturonides released from sodium polyacrylate by endoplasmic reticulum acid base are elicitors of phytoalexin in soybean. *Plant Physiol* 80, 568–571.
- Dierich, A., Mayer, J.E. and Halbrook, K. (1990). Fungal elicitor triggers rapid, transient and systemic protein phosphorylation in parsley cell suspension cultures. *J. Biol. Chem.* 265, 6346–6348.
- Edel, J. (1989). Phytoalexin synthesis: the biochemical analysis of the induction process. *Int. Rev. Physiol.* 24, 235–264.
- Farmer, E.E., Madsen, T.D., Saxton, M.J. and Ryan, C.A. (1991). Oligosaccharide signalling in plants. Specificity of oligogalacturonide-enhanced plasma membrane protein phosphorylation. *J. Biol. Chem.* 266, 3140–3145.
- Forrest, R.S. and Lyon, G.D. (1980). Substrate degradation patterns of polygalacturonase and lyase from *Erwinia carotovora* and *Bacterium pumilus* and release of phytoalexin-eliciting oligosaccharides from potato cell walls. *J. Exp. Bot.* 31, 38–48.
- Gilroy, S., Fricker, M.D., Read, N.D. and Trewavas, A.J. (1991). Role of calcium in signal transduction of *Commelina* guard cells. *Plant Cell* 3, 333–344.
- Gilroy, S., Hughes, W.A. and Trewavas, A.J. (1986). The measurement of intracellular calcium levels in protoplasts from higher plant cells. *FEBS Lett.* 199, 217–221.
- Gilroy, S., Hughes, W.A. and Trewavas, A.J. (1987). Calmodulin antagonists increase free cytosolic calcium levels in plant protoplasts in vivo. *FEBS Lett.* 212, 133–137.
- Gilroy, S., Hughes, W.A. and Trewavas, A.J. (1989). A comparison between plant and animal calmodulins as indicators of cytoplasmic calcium levels in higher plant cell protoplasts. *Plant Physiol* 90, 482–491.
- Gilroy, S. and Jones, R.L. (1992). Gibberellin acid and abscisic acid covalently regulate cytoplasmic calcium and secretory activity in barley aleurone protoplasts. *Proc. Natl. Acad. Sci. USA* 89, 3591–3595.
- Gronke, G., Pante, M. and Tsien, R. (1985). A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260, 3440–3445.
- Hahn, S.H. and Simonsen, M.J. (1991). Cycloheximide increases intracellular Ca²⁺ in *Fusarium* detection with Indo-1. *Cell Calcium* 12, 675–681.
- Hepler, P.K. and Wayne, R.D. (1985). Calcium and plant development. *Ann. Rev. Plant Physiol.* 36, 397–439.
- Hess, P. (1990). Calcium channels in vertebrate cells. *Ann. Rev. Neurosci.* 13, 337–356.
- Horn, M.A., Heinemann, P.F. and Low, P.S. (1989). Receptor-mediated endocytosis in plant cells. *Plant Cell* 1, 1003–1009.
- Horvath, J.C. and Cowling, E.B. (1989). *Plant Disease*, Vol. 5. Academic Press, New York.
- Irwin, P.J., Scallan, M.D. and Shieh, J.J. (1983). FSR evidence for potential to elicit calcium binding in higher plant cell walls. *Biochim. Biophys. Acta* 745, 180–190.
- Jarvis, M.C. (1982). The proportion of calcium-bound pectin in plant cell walls. *Plant J.* 1, 441–446.
- Jin, D.F. and West, C.A. (1984). Characteristics of galacturonic acid oligomers as elicitors of carbonic dehydratase activity in carrot bean seedlings. *Plant Physiol* 74, 989–992.
- Knight, M.R., Campbell, A.K., Smith, S.M. and Trewavas, A.J. (1991). Transgenic plant activation suppresses the effects of touch and cold-shock and elicits on cytoplasmic calcium. *Nature* 352, 524–526.
- Kurosaki, K., Tsurusawa, Y. and Nishi, A. (1987). The elicitation of phytoalexins by J⁺ and cyclic AMP in corn cells. *Phytochemistry* 26, 1919–1923.
- Lamb, C.J., Lawton, M.A., Dixon, R.A. and Ryan, C.A. (1989). Signals and transduction mechanisms for activation of plant defences against microbial attack. *Cell* 56, 215–224.
- Lamb, C.J., Almer, T.K. and Butt, V.S. (1979). Synthesis and removal of

phenylalanine-aminomethylase activity in illuminated discs of potato tuber peroxisomes. *Biochim Biophys Acta* 582, 186-212.

Thibault, J.-F., Thibault, J.-F. and Van Cutsem, P. (1992) Influence of the degree of polymerization of oligogalacturonate and of esterification patterns of pectins on their recognition by monoclonal antibodies. *Plant Physiology* (in press).

Laurer, F., Lefebvre, J.-L., Didenbourg, C. and Van Cutsem, P. (1989) Monoclonal antibodies against pectin. Recognition of a conformation induced by calcium. *Plant Physiol* 91, 1419-1424.

Mathias, Y., Korkel, A., Xia, H., Guern, J., Koller, A., Spitz, M.D., O'Neill, M., Allersheim, P. and Darvill, A. (1991). Membrane responses induced by oligogalacturonides in suspension-cultured tobacco cells. *Plant J* 1, 333-343.

Mitch, H., Spith, V. and Fabel, J. (1986). Phytoalexin synthesis by isolated soybean protoplasts. *Z Naturforsch* 41c, 193-201.

Morris, E.R., Powell, D.A., Gidley, M.J. and Rees, D.A. (1982). Conformations and interactions of pectins. I. Polymorphism between gel and solid states of calcium polygalacturonate. *J. Mol Biol* 155, 507-516.

Powell, D.A., Morris, E.R., Gidley, M.J. and Rees, D.A. (1982). Conformations and interactions of pectins. II. Influence of residue sequence on chain association in calcium pectate gels. *J. Mol Biol* 155, 517-533.

Reid, N.D., Allan, W.T.G., Knight, H., Knight, M.R., Nallio, R., Russell, A., Shacklock, P.S. and Trewavas, A.J. (1992) Imaging and measurement of cytosolic free calcium in plant and fungal cells. *J Microsc* 166, 57-66.

Rizzuto, R., Simpson, A.W.M., Brink, M. and Pozzan, T. (1992) Rapid changes of intracellular Ca^{2+} revealed by specifically targeted fluorescent indicators. *Nature* 358, 324-327.

Ryan, C.A. and Farmer, E.E. (1991). Oligosaccharide signals in plants: a critical assessment. *Annu. Rev. Plant Physiol Mol Biol* 42, 651-674.

Schroeder, J.I. and Hedrich, R. (1989) Involvement of ion channels and active transport in osmoregulation and signalling of higher plant cells. *Trends Biochem* 14, 187-192.

Shacklock, P.S., Reid, N.D. and Trewavas, A.J. (1992). Cytosolic free calcium mediates red light-induced photomorphogenesis. *Nature* 358, 754-755.

Stack, J.P., Mount, M.S., Bernath, P.M. and Hubbard, J.P. (1989). Pectic enzyme complexes from *Erwinia carotovora*: a model for degradation and assimilation of host pectic fractions. *Phytopathology* 79, 267-272.

Stals, M.R. and Fabel, J. (1987). Effects of Ca^{2+} on phytoalexin induction by fungal elicitor in soybean cells. *Arch Biochem Biophys* 257, 416-423.

Thain, J.F., Doherty, H.M., Bowles, D.J. and Willson, D.C. (1990) Oligosaccharides that induce proteinase inhibitor activity in tomato plants cause depolarization of tomato leaf cells. *Plant Cell Environ* 13, 569-574.

Thibault, J.-F. (1989). Separation of α -D-galacturonate and oligomers by chromatography on polyacrylamide gel. *J Chromatogr* 194, 315-322.

Thibault, J.F. and Rinaudo, M. (1985). Chain association of pectic molecules during calcium-induced gelation. *Biopolymers* 25, 455-468.

Trewavas, A.J. and Gilroy, S. (1991). Signal transduction in plant cells. *Trends Micro* 7, 186-191.

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Pectic Signal Transduction in Carrot Cells: Membrane, Cytosolic and Nuclear Responses Induced by Oligogalacturonides

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Oligogalacturonides were applied at a concentration of 0.2 mg ml⁻¹ to *Daucus carota* cells and protoplasts. Fractions containing oligomers of degree of polymerisation 9 to 16 in the presence of 150 mM NaCl and 0.5 mM CaCl₂ and pure oligomers of degree of polymerisation 9 to 13 in the same ionic medium triggered a cytosolic calcium mobilisation, membrane depolarisation and cytosolic acidification. These membrane and cytosolic events coincided with the accumulation of transcripts encoding phenylalanine ammonia-lyase, catalytic activation of the enzyme and *p*-hydroxybenzoic acid accumulation in the cell wall of the elicited cells. The effect of a calcium ionophore, calcium channel blockers, calmodulin inhibitors, phosphatidyl inositol metabolism modulators and H⁺-ATPase modulators were investigated on oligogalacturonide-induced phenylalanine ammonia-lyase enzyme and gene activation in suspension-cultured carrot cells.

Key words: Cytosolic calcium — Cytosolic pH — *Daucus* — Elicitor — 1,4,5-Inositol triphosphate — Phenylalanine ammonia-lyase.

Pectins play an important role in different physiological processes in plants. As structural polysaccharides, pectins determine the plant cell wall strength and plasticity of non-lignified organs and ripening fruits. Pectic polysaccharides are the first targets of enzymes released by fungal or bacterial invading pathogens (Paget and Heitefuss 1990).

Many bacteria and fungi secrete pectolytic enzymes during the first phase of host-pathogen interactions, leading to tissue maceration and release of pectic endogenous elicitors (Allen et al. 1987). Jin and West (1984) showed that pectic fragments enhance caseinase synthase in castor bean seedlings. Caseinase synthase is activated by oligopectates with a degree of polymerisation (DP) higher than 8, the dodecamer being the most active. Forrest and Lyon (1990) reported similar results with unsaturated oligopectates released by a *Bacillus polymyxa* pectate lyase. Pectic fragments also initiate hyperlignification in cucumber (Robertsen and Svalheim 1990) and in castor bean (Bruce and West 1989). Here again, the maximum effect on lignification was observed with oligomers having an average DP of 7 to 15. Farmer et al. (1991) reported an oligogalacturonide-induced modification of the *in vitro* phosphory-

Abbreviations: IP₃, 1,4,5-inositol triphosphate; PAL, phenylalanine ammonia-lyase; PGA, polygalacturonic acid; DP, degree of polymerisation; Ex, excitation; Em, emission; d, day(s).

cytosolic events coincided with the activation of phenylalanine ammonia-lyase (PAL) as well as *p*-hydroxybenzoic acid accumulation in the cell wall of elicited cells. PAL activation was further studied in the presence of signal transduction modulators.

Materials and Methods

Chemicals and bacterial strains.—Indo-1, SNARF-1 AM and DiBAC₄ (3) were from Molecular Probes, Inc. (Eugene, Oregon, U.S.A.). Polygalacturonic acid (PGA), Br-A23187, verapamil, chlorpromazine, trifluoperazine, calmidazolium, nifedipine, vanadate, neomycin, lithium chloride, fusococcine, 2,4-dichlorophenoxyacetic acid and Murashige and Skoog (MS) salts were from Sigma (St Louis, U.S.A.). Cellulase Onozuka RS and Macerozyme RS were from Yakult Pharmaceutical Ind. Co., Ltd. (Tokyo, Japan). Plasmids containing the coding sequence for PAL and polyubiquitin respectively were maintained in MC1061 *Escherichia coli* strain.

Cell culture and protoplasts.—Carrot (*Daucus carota* L.) cell suspensions, initiated from carrot roots, were maintained in supplemented MS (Murashige and Skoog 1962) medium that contained 4.708 g liter⁻¹ MS salts, 30 g liter⁻¹ sucrose and 1 mg liter⁻¹ 2,4-dichlorophenoxyacetic acid (pH 5.7) in 2 liter flasks, at 25°C under constant agitation on a VKS 75 Edmund Bühler orbital rotator (110 rpm, Van Hoplynus, Brussels, Belgium) in the dark. Cells were subcultured every two weeks.

Protoplasts were prepared overnight at 25°C from one gram of 6-d old cells in the supplemented MS medium to which were added 100 g liter⁻¹ mannitol, 15 g liter⁻¹ cellulase Onozuka RS and 5 g liter⁻¹ macerozyme RS. Protoplasts were pelleted by centrifugation at 100 g for 5 minutes and resuspended in 0.4 M sucrose. After centrifugation at 100 × g for 5 minutes, floating protoplasts were carefully removed using a Pasteur pipette and resuspended in MS-Mannitol medium prepared from MS salts, sucrose and 2,4-dichlorophenoxyacetic acid as for supplemented MS, with the addition of 100 g liter⁻¹ mannitol. Protoplasts were further washed three times in supplemented MS-Mannitol medium.

Oligogalacturonide production.—Oligogalacturonides were produced by acid-hydrolysis (pH 3.2) of a 10 g liter⁻¹ polygalacturonic acid solution during 10 h at 100°C. Oligogalacturonides were fractionated by HPLC (high performance liquid chromatography) on a semi-preparative TSK DEAE SPW column (Hewlett Packard, Amsterdam, The Netherlands) as described by Forrest and Lyon (1990) and detected by pulsed amperometry (Dionex, Farnborough, U.K.). DPs <9 and DPs 9-16 were pooled. Pure DPs 7 to DP 13 were also collected separately. Each fraction was dialyzed (1000 MW_{co}) against distilled water, evaporated to dryness before being resuspended in 150 mM

NaCl + 0.5 mM CaCl₂ (high calcium) or 150 mM NaCl + 0.5 μM CaCl₂ (low calcium) ionic solutions to obtain a final oligogalacturonide concentration of 1 mg ml⁻¹. The oligogalacturonides used for video imaging were resuspended in low or high calcium ionic solutions containing 100 g liter⁻¹ mannitol.

Cell and protoplast stimulation.—Cells and protoplasts were washed several times with calcium-free supplemented MS and calcium-free supplemented MS-Mannitol medium respectively before being resuspended in new supplemented MS or MS-Mannitol, both containing either 150 mM NaCl + 0.5 mM CaCl₂ (high calcium) or 150 mM NaCl + 0.5 μM CaCl₂ (low calcium). Oligogalacturonide fractions were applied at a final concentration of 200 μg ml⁻¹ as previously described by Messiaen et al. (1993). Signal transduction modulators were prepared as 100 × stock solutions and filter sterilized.

Dye loading.—Indo-1 (1-[2-amino-5-(6-carboxyindol-2-yl)-phenoxy]-2-(2'-amino-5'-methylphenoxy)-ethane-*N,N,N',N'*-tetraacetate; 10 μM) was loaded into carrot protoplasts using the pH method as described by Guiragossian Kiss et al. (1991). DiBAC₄ (3) (bis-(1,3-dibutylbarbituric acid-(5)) trimethineoxonol; 15 μM) was loaded by simple diffusion through the plasma membrane (Bräuner et al. 1984). SNARF-1 AM (Semi naphtho rhodafuor acetomethyl ester; 85 nM) was loaded into carrot protoplasts by simple diffusion (Roos 1992). Indo-1 was loaded by electroporation using a *GenePulser* (BioRad, Eke, Belgium) and a 0.4 cm cuvette (E = 1,250 V cm⁻¹; C = 25 μF). Protoplasts exhibiting vacuolar dye loading were systematically discarded before image acquisition. Vital staining was performed during each measurement using trypan blue as previously described (Messiaen et al. 1993).

Video imaging.—A Nikon TMD-Diaphot inverted microscope (Nikon Corporation, Tokyo, Japan) with high transmittance fluoride optics mounted with epifluorescence was used. The objective was a CF Fluor 40X, N.A. = 0.85 (spring loaded) with cover-glass correction. Excitation was achieved by a Nikon Xenon discharge lamp (100 W). Excitation (Ex) and emission (Em) filters, and dichroic mirror (DM; Instruments s.a., Alphen a/d Rijn, The Netherlands) used in the experiments were for DiBAC₄ (3): Ex = 488 ± 5 nm, Em = 535 ± 15 nm, DM = 505 nm; for indo-1: Ex = 365 ± 5 nm, Em1 = 405 ± 5 nm, Em2 = 488 ± 5 nm, DM = 380 nm and for SNARF-1 AM: Ex = 514 ± 5 nm, Em1 = 580 ± 5 nm, Em2 = 640 ± 5 nm, DM = 545 nm. Filters were mounted in LAMBDA-10 filter wheels (Sutter Instruments, Novato, California, U.S.A.). Images were grabbed by an ISIS-M extended CCD camera (Photonic Science, Milham, U.K.) fitted on the emission port of the microscope. Filter selection and image acquisition were controlled by a 386SX 16 MHz PC/AT compatible computer fitted with an Adaptec AHA-154013 multitasking interface and a DT-2861 arithmetic frame grabber (Data Translation, Brussels,

Belgium).

Protoplasts were deposited on a 0.17 × 24 × 50 mm glass slide in a 2 ml drop of MS-Mannitol containing either 150 mM NaCl + 0.5 mM CaCl₂ (high calcium) or 150 mM NaCl + 0.5 μM CaCl₂ (low calcium), and allowed to settle for 10 minutes on the stage of the inverted microscope. No cover slip was used to avoid mechanical stimulation and allow aeration of the protoplasts. A first set of images was grabbed at 'time 0' (control images). Immediately after, the elicitor and/or modulators were carefully added (70 μl from a 6 mg ml⁻¹ stock solution) avoiding any movement of the protoplasts. Simultaneously, the image acquisition program was started and images of the same protoplast were acquired during 30 minutes. Acquisition procedures were limited to 30 minutes which corresponded to a minimum photobleaching and leakage of the dyes.

Image analysis itself was carried out by *Image 1.41 b23*, a public domain program for Macintosh IIx fitted with a 68882 Motorola coprocessor (40 MHz). Calcium ratio images were calculated and calibrated as described (Messiaen et al. 1993). Cytosolic pH images were calibrated using a set of KH₂PO₄/K₂HPO₄ buffers (pH 5.8 to 7.5). Mean cytosolic calcium, pH or membrane potential were calculated by integrating pixel values of fluorescence images over the whole surface of the protoplasts using *Image 1.41 b23*.

Assay for PAL.—PAL activity was determined as described by Hahlbrock et al. (1981) and was measured 24 h after each treatment.

Assay for hydroxybenzoic acid.—Hydroxybenzoic acid was extracted as described by Schnitzler and Seitz (1989). Phenol measurements were performed 50 h after elicitor treatment.

RNA dot-blot analysis.—One single batch of cells was subdivided into 5 ml suspensions to which the subsequent elicitor and modulator treatments were applied. Cells were treated for 6 h in the dark before RNA extraction. Extraction of total RNA from elicited cells was as described by DeVries et al. (1988).

The plasmid containing the coding sequence for PAL (Lois et al. 1989) and polyubiquitin (internal control, Somssich et al. 1989) were labelled with 1.85 mBq (110 TBq mmol⁻¹) α³²P-dCTP (Amersham, Buckinghamshire, U.K.) using a random primed DNA labelling kit (Boehringer, Mannheim, Germany).

Total RNA (10 μg dot⁻¹) was absorbed on nylon filters (Hybond-N, Amersham, Buckinghamshire, U.K.). Prehybridization and hybridization with the radiolabelled PAL and polyubiquitin cDNAs were done according to Chappell and Hahlbrock (1984).

Results

Membrane and cytosolic responses in elicited carrot

protoplasts.—All pectic fractions tested induced a membrane depolarisation i.e. an accumulation of the negatively charged dye into the protoplasts (Fig. 1A). The magnitude of the depolarisation was clearly size-dependent (Fig. 2A). Similar results were obtained by Thain et al. (1990) in tomato leaf cells, and by Mathieu et al. (1991) in tobacco cell suspensions using microelectrodes.

In the low calcium solution, none of the oligogalacturonide treatments induced a modification of the mean cytosolic pH (Fig. 1B). In the high calcium solution, the DP₉ < 9 mixture and the pure DP 7 and 8 had no effect on cytosolic pH. Conversely, the DP₉ 9–16 mixture and the pure DP 9 to 13 induced a mean cytosolic acidification of 0.3 pH unit (pH differences up to 0.5–0.6 pH unit were recorded locally in the cytoplasm). PGA had only a very faint effect on cytosolic pH. Acidification of the cytosol occurred 20 minutes after elicitor addition (Fig. 2B) i.e. 10–15 minutes after membrane depolarisation and cytosolic calcium mobilisation. Cytosolic acidification was accompanied by an alkalinization of the external culture medium (data not shown) as already reported by others (Mathieu et al. (1991).

In the low calcium solutions, none of the oligogalacturonide treatments induced a modification of the cytosolic free calcium (Fig. 1C and 2C). In the high calcium solution, the DP₉ < 9 mixture and the pure DP 7 and 8 had no effect on cytosolic free calcium. However, the DP₉ 9–16 mixture and the pure DP 9 to 13 triggered a significant elevation in mean free cytosolic calcium [Ca²⁺]_i from ~130 nM up to ~200 nM within ~5 minutes after addition of the elicitor (Fig. 1C and 2C), and local free calcium concentrations up to 500 nM were observed. No cytosolic calcium transient was detected and this time pattern of calcium mobilisation was highly reproducible. Only 30–40% of the protoplasts did increase [Ca²⁺]_i on elicitor application, but no cytosolic calcium decrease has been observed on treatment with galacturonic oligomers. Polygalacturonic acid did also increase [Ca²⁺]_i, but this calcium mobilisation was much slower and less pronounced. To see whether calcium was mobilised via the opening of voltage-gated calcium channels, indo-1 loaded protoplasts were depolarised by 80 mM KCl in the absence of the elicitor. Such an artificial membrane depolarisation by KCl was completely unable to reproduce the rapid calcium mobilisation induced by the elicitor: the rise in cytosolic free calcium [Ca²⁺]_i was linear and very slow (Δ[Ca²⁺]_i = 20–30 nM within 30 minutes, Fig. 3). This suggests that, if calcium channels are effectively involved in oligogalacturonide-induced calcium mobilisation, they are not of the voltage-gated type, but could be of the receptor-operated type.

PAL activity and phenolic compound accumulation in elicited carrot cells and protoplasts.—PAL is the first enzyme of the phenylpropanoid pathway and its activation has been used in different systems to follow cellular defense

the receptor-operated type.

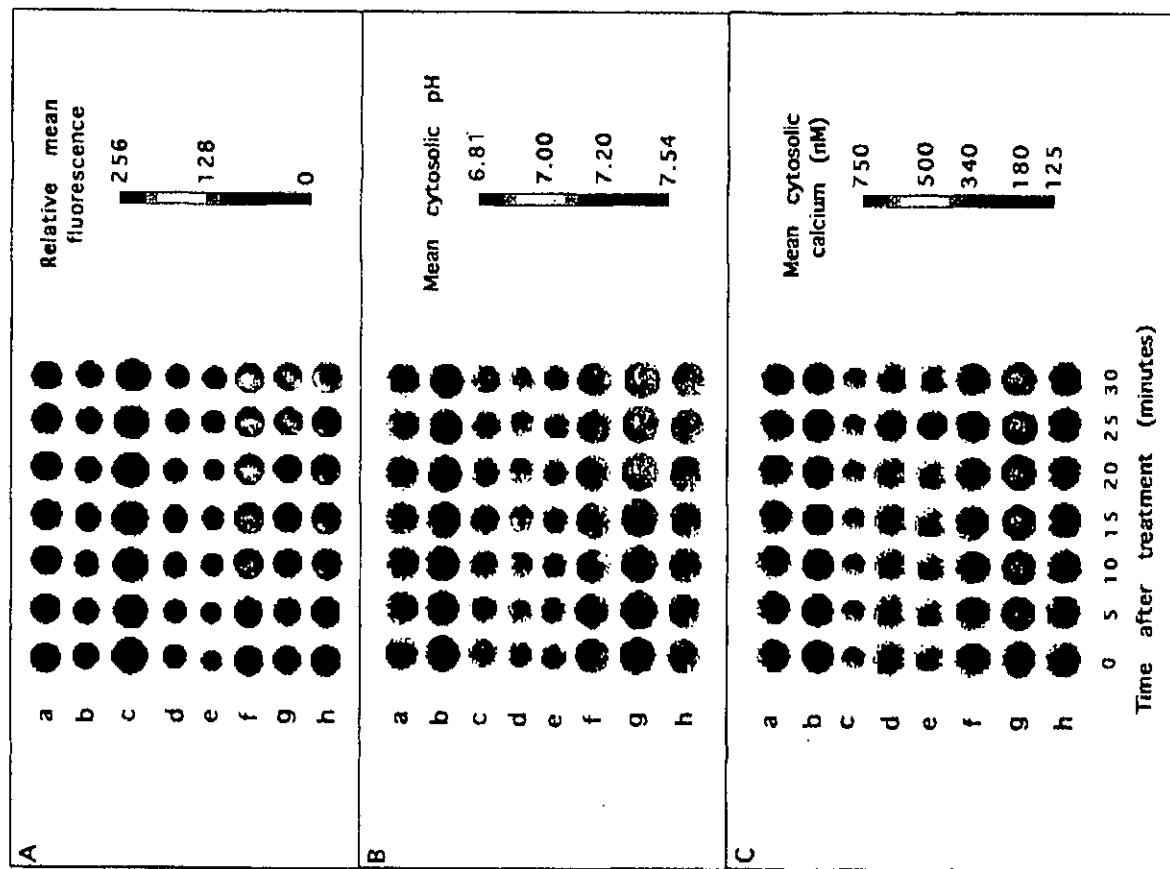


Fig. 1 Representative video images of membrane potential, cytosolic pH and calcium in carrot protoplasts treated by oligogalacturonides. Protoplasts exhibiting vacuolar dye-loading were not used for video imaging. The apparent fluorescence coming from the central vacuole of the protoplast is due to the fluorescence originating from cytosolic regions that are out of focus. A. Changes in membrane potential in DiBAC₄ (3)-loaded carrot protoplasts. B. Changes in cytosolic pH in SNARF-1 AM loaded protoplasts and C. Changes in cytosolic calcium in Indo-1-loaded protoplasts challenged for 30 minutes by pectic oligosaccharides and polysaccharides, each at 0.2 mg ml⁻¹. (a) water control; (b) low calcium control; (c) high calcium control; (d) DP₉ < 9 (low calcium); (e) DP₉ 9–16 (flow calcium); (f) DP₉ 9–16 (high calcium) and (h) PGA (high calcium).

reactions (Kurosaki et al. 1986, Ishizuka et al. 1991). PAL activity of cells was enhanced by DPs 9–16 (high calcium), but not by DPs 7–8 (low calcium), DPs <9 (high calcium), DPs 9–16 (low calcium), pure DPs 7 to 13 (low calcium) and pure DPs 7–8 (low calcium), even after 50 hours exposures (Fig. 4). A minimum

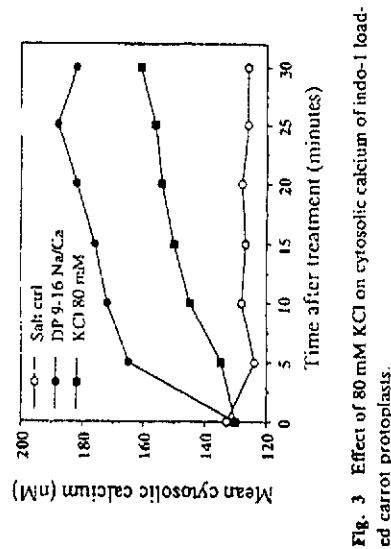
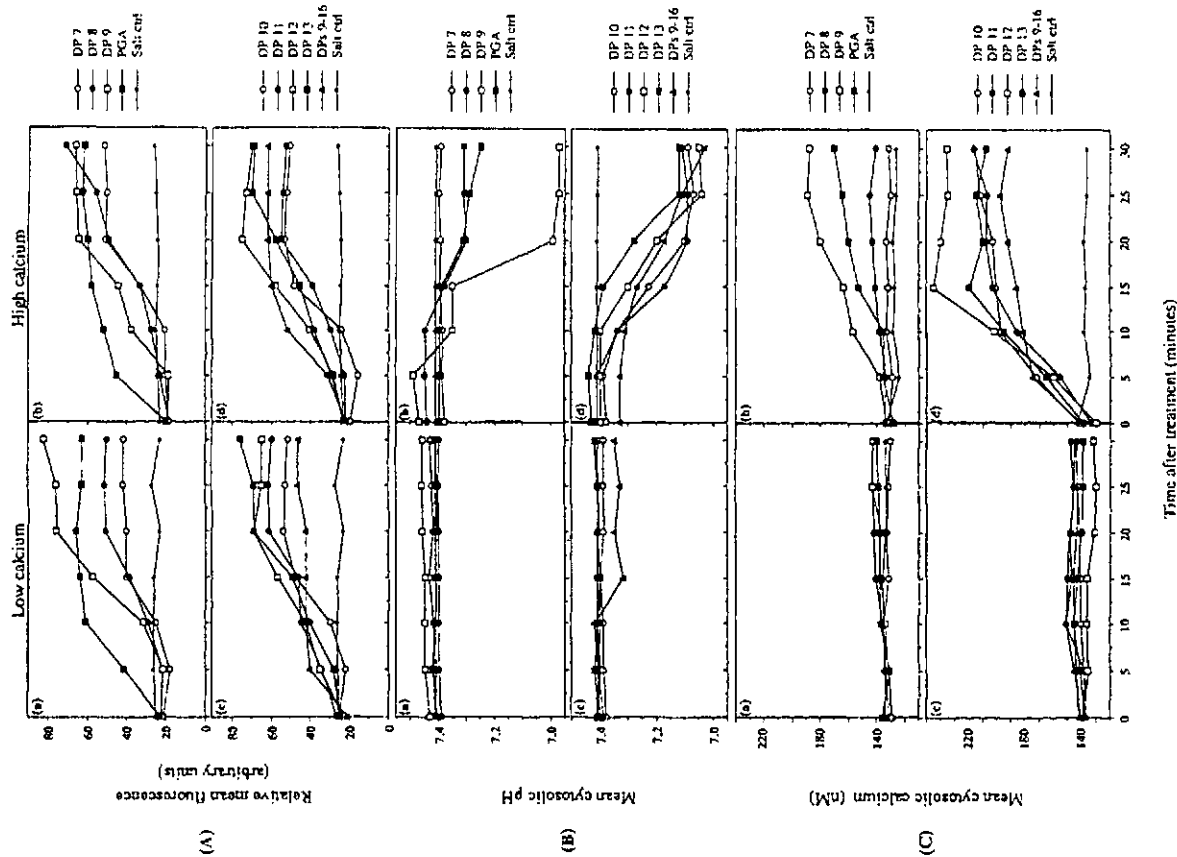


Fig. 3 Effect of 80 mM KCl on cytosolic calcium of indo-1 loaded carrot protoplasts.

mic acid $\mu\text{g protein}^{-1} \text{h}^{-1}$), but much more reproducible within one batch of cells (the standard deviation was reduced down to $<1 \mu\text{mol cinnamic acid } \mu\text{g protein}^{-1} \text{h}^{-1}$ between samples of a single batch of cells).

Protoplasts similarly responded to oligogalacturonide treatments, but the basal PAL activity level was higher than in cells because of the protoplasting step (Fig. 7). When they were further treated for 24 h with the test samples just after isolation, their background PAL activity was still higher than in cells, but they exhibited a significant PAL induction upon elicitor treatment. The size of oligogalacturonides and the ionic requirements needed to activate PAL in protoplasts were the same as in cells.

Hydroxybenzoic acid accumulated in the cytosol and/

Fig. 2 (A) Calculated mean membrane potential in carrot protoplasts challenged for 30 minutes by pectic oligosaccharides and polysaccharides at a final concentration of 0.2 mg ml^{-1} in a low calcium solution ((a) and (c)) or in a high calcium solution ((b) and (d)). The average membrane potential of a protoplast was calculated by integrating pixel values of fluorescence images over the whole surface of the protoplast. Each point is the mean of n protoplasts from independent experiments. (a) and (b): Changes in membrane potential in DPBAC₄ (3)-loaded carrot protoplasts treated with DP 7, DP 8, DP 9 and PGA ($n=5$); (c) and (d): Changes in membrane potential in DPBAC₄ (3)-loaded carrot protoplasts treated with DP 10, DP 11, DP 12, DP 13 and DPs 9–16 mixture ($n=5$). Control protoplasts were treated with the corresponding ionic solutions in absence of pectic oligomers. Membrane potential measurements had a standard deviation (S.D.) <15 arbitrary fluorescence units. (B) Calculated mean cytosolic pH in carrot protoplasts challenged for 30 minutes by pectic oligosaccharides and polysaccharides at a final concentration of 0.2 mg ml^{-1} in a low calcium solution ((a) and (c)) or in a high calcium solution ((b) and (d)). The average cytosolic pH of a protoplast was calculated by integrating pixel values of fluorescence images over the whole surface of the protoplast. Each point is the mean of n protoplasts from independent experiments. (a) and (b): Changes in cytosolic pH in SNARF-1 AM loaded protoplasts treated with DP 7, DP 8, DP 9 and PGA ($n=10$); (c) and (d): Changes in cytosolic pH in SNARF-1 AM loaded protoplasts treated with DP 10, DP 11, DP 12, DP 13 and DPs 9–16 mixture ($n=10$). Control protoplasts were treated with the corresponding ionic solution in absence of pectic oligomers. Cytosolic pH measurements had a standard deviation <0.1 pH unit. (C) Calculated mean cytosolic calcium in carrot protoplasts challenged for 30 minutes by pectic oligosaccharides and polysaccharides at a final concentration of 0.2 mg ml^{-1} in a low calcium solution ((a) and (c)) or in a high calcium solution ((b) and (d)). The average cytosolic calcium activity of a protoplast was calculated by integrating pixel values of fluorescence images over the whole surface of the protoplast. Each point is the mean of n protoplasts from independent experiments. (a) and (b): Changes in cytosolic calcium in Indo-1-loaded protoplasts treated with DP 7, DP 8, DP 9 and PGA ($n=10$); (c) and (d): Changes in cytosolic calcium in Indo-1-loaded protoplasts treated with DP 10, DP 11, DP 12, DP 13 and DPs 9–16 mixture ($n=10$). Control protoplasts were treated with the corresponding ionic solution in absence of pectic oligomers. Cytosolic calcium measurements had a standard deviation $<15 \text{ nM}$.

Fig. 4 PAL enzymatic activation in elicited carrot cells. PAL catalytic activation in carrot cell suspension challenged for 24 h with different pectic fractions. Each pectic fraction was tested in low and high calcium solutions.

or in the cell wall 50 h after elicitor treatment, but was not released into the culture medium (Fig. 8). Maximum accumulation was obtained with oligogalacturonides with DPs 9–16 (high calcium) and with pure DP 9 to 13 (high calcium). Hydroxybenzoic acid accumulation resulted in browning of the carrot cell suspensions.

Effect of signal transduction modulators on PAL enzymatic and gene activation in elicited carrot cells—Since

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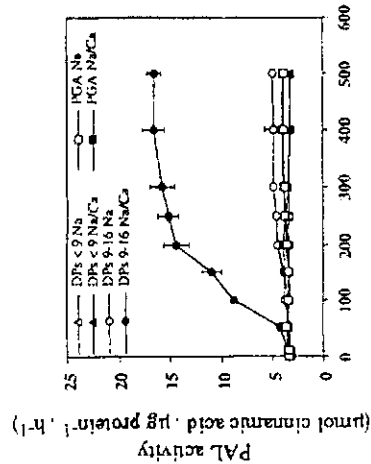


Fig. 5 Dose-response curve of oligogalacturonides. Carrot cells were stimulated by different concentrations of each pectic fraction. PAL activity was assayed after 24 h (n=5).

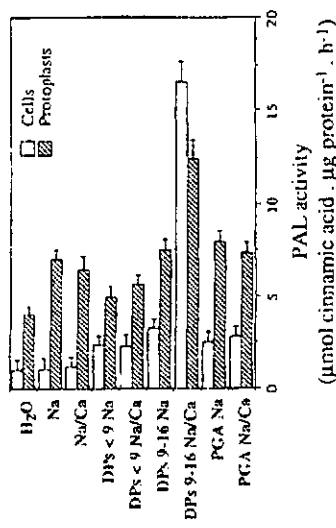


Fig. 7 PAL activity in carrot protoplasts and cells after treatment with different pectic oligosaccharides and polysaccharides. Each pectic fraction was tested in low (Na) and high (Na/Ca) calcium solutions.

PAL enzymatic and gene activation (Messiaen and Van Cutsem 1993) coincided with the synthesis of *p*-hydroxybenzoic acid, they were further used as markers of elicitation to study the effect of different concentrations of signal transduction modulators. Besides the possible non-specific side effects of some modulators in plant cells, the measurement of PAL gene activation after 6 h, and PAL enzymatic activity after 24 h in the presence of these modulators must integrate possible repair mechanisms that might help to

overcome the effect of the drug. However, keeping these considerations in mind, the use of signal transduction modulators is the most straightforward approach for studying an unknown signal transduction chain in cell suspensions. Since the pure oligomers tested (DP 9 to 13) did not have a much stronger elicitor activity than the DPs 9-16 oligomer mixture, the latter was used in the following experiments. Signal transduction modulators and oligogalacturonides were always applied simultaneously to the carrot cells.

The Br-A23187 calcium ionophore (10-100 μM) added simultaneously with the elicitor to the cells led to a slightly

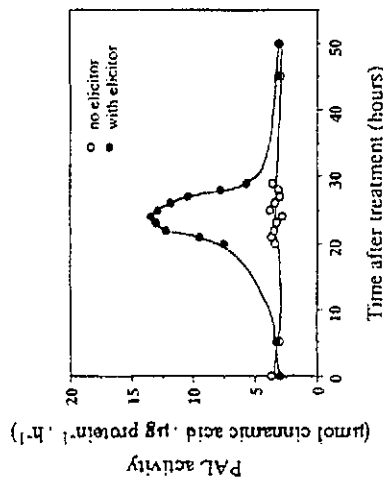


Fig. 6 Time course of PAL activity in carrot cell suspensions treated with oligogalacturonides of DPs 9-16 (high calcium, 0.2 mg ml⁻¹). Control cells were treated with the high calcium solution without pectic fragments. Maximum PAL induction was variable from one cell batch to another (15 ± 3 μg protein⁻¹ · h⁻¹). However, within one single cell batch, S.D. was < 1 μg protein⁻¹ · h⁻¹.

Fig. 8 Hydroxybenzoic acid accumulation in elicited carrot cells. Hydroxybenzoic acid content of carrot cells was measured 50 h after treatment with different pectic fractions. Each pectic fraction was tested in low (Na) and high (Na/Ca) calcium solutions.

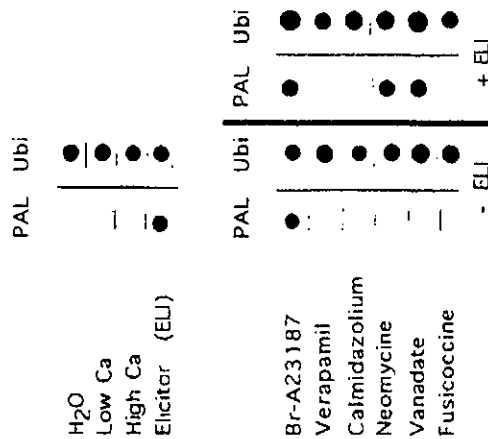
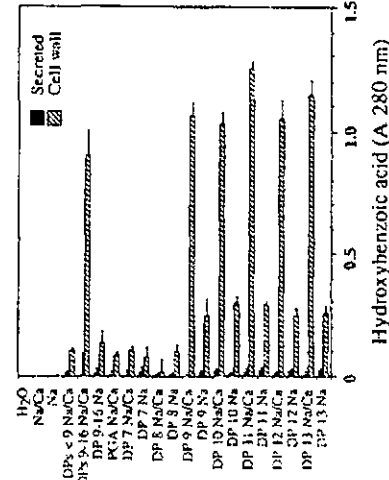


Fig. 9 Effect of 50 μM Br-A23187, 50 μM verapamil, 10 nM calmidazolium, 20 μM neomycin, 150 μM vanadate and 7.5 μM fusicoccin on PAL gene activation in absence and presence of DPs 9-16 (Na/Ca). Control cells were treated with water, low and high calcium ionic solutions (negative controls) and with DPs 9-16 high calcium (positive control, ELI). The polyubiquitin cDNA probe (Ubi) was used as internal control.

stronger activation of PAL catalytic activity. Br-A23187 alone induced only a 3-fold increase of the basal PAL enzymatic activity, indicating that a rise of cytosolic calcium alone was not sufficient to trigger a full PAL activation (Table 1) although PAL gene activation was complete (Fig. 9). This suggests a possible post-translational activa-

tion of PAL enzymes that might be independent of cytosolic calcium. As expected the calcium ionophore (50 μM) alone increased significantly the cytosolic free calcium (Fig. 10C), although it had no effect on membrane potential nor on cytosolic pH (Fig. 10A, B). This also indicates that cytosolic calcium mobilisation does not induce the aforementioned membrane depolarisation and cytosolic acidification.

Verapamil (25-100 μM) or nifedipine (0.1-2 μM), two cell permeant calcium channel blockers, inhibited PAL catalytic activity (Table 1) and gene activation (Fig. 9) down to the basal level, and prevented also cytosolic calcium mobilisation, confirming the importance of cytosolic calcium in cell activation. However, they had no effect on the elicitor-induced membrane depolarisation and on the elicitor-induced cytosolic acidification, establishing that cytosolic calcium mobilisation is independent of these two responses. Verapamil (50 μM) and nifedipine (0.1 μM) used in the absence of the elicitor did not affect the resting membrane potential, cytosolic free calcium nor cytosolic pH (Fig. 10A, B, C). These results are consistent with the involvement of calcium channels in the signal transduction chain triggered by pectic oligomers.

Numerous calcium-regulated processes are mediated by calmodulin (Ladror and Zielinski 1990, Neuhaus et al. 1993). Trifluoperazine (50-400 μM) and chlorpromazine (100-400 μM) largely inhibited the PAL catalytic activation (Table 1) induced by the elicitor, supporting the hypothesis that calmodulin mediates the oligogalacturonide-induced signal transduction. Trifluoperazine and chlorpromazine are, however, known to have some side effects on other physiological functions: inhibition of protein kinase C in animal cells (Bosser et al. 1990) and partial inhibition of IP₃ release in carrot (Rincon and Boss 1987). Therefore, we used the specific calmodulin-inhibitor, calmidazolium. At

Fig. 10 (A) Effect of signal transduction modulators on membrane potential of carrot protoplasts in absence (a) and (c) or in presence (b) and (d) of DPs 9-16 (high calcium) at a final concentration of 0.2 mg ml⁻¹. The average membrane potential of a protoplast was obtained by integrating pixel values of fluorescence images over the whole surface of the protoplast. Each point is the mean of 10 protoplasts from independent experiments. (a) and (b) Effect of A23187 (50 μM), verapamil (50 μM), nifedipine (0.1 μM) and calmidazolium (10 nM); (c) and (d) Effect of neomycin (20 μM), lithium chloride (1 mM), vanadate (150 μM) and fusicoccin (7.5 μM). Control protoplasts were treated with the high calcium solution. Membrane potential measurements had a standard deviation < 15 arbitrary fluorescence units. (B) Effect of signal transduction modulators on cytosolic pH of carrot protoplasts in absence (a) and (c) or in presence (b) and (d) of DPs 9-16 (high calcium) at a final concentration of 0.2 mg ml⁻¹. The average cytosolic pH of a protoplast was obtained by integrating pixel values of fluorescence images over the whole surface of the protoplast. Each point is the mean of 10 protoplasts from independent experiments. (a) and (b) Effect of A23187 (50 μM), verapamil (50 μM), nifedipine (0.1 μM) and calmidazolium (10 nM); (c) and (d) Effect of neomycin (20 μM), lithium chloride (1 mM), vanadate (150 μM) and fusicoccin (7.5 μM). Control protoplasts were treated with the high calcium solution. Cytosolic pH measurements had a standard deviation < 0.1 pH unit. (C) Effect of signal transduction modulators on cytosolic calcium of carrot protoplasts in absence (a) and (c) or in presence (b) and (d) of DPs 9-16 (high calcium) at a final concentration of 0.2 mg ml⁻¹. The average cytosolic calcium of a protoplast was obtained by integrating pixel values of fluorescence images over the whole surface of the protoplast. Each point is the mean of 10 protoplasts from independent experiments. (a) and (b) Effect of A23187 (50 μM), verapamil (50 μM), nifedipine (0.1 μM) and calmidazolium (10 nM); (c) and (d) Effect of neomycin (20 μM), lithium chloride (1 mM), vanadate (150 μM) and fusicoccin (7.5 μM). Control protoplasts were treated with the high calcium solution. Cytosolic calcium measurements had a standard deviation < 15 nM.

125

nM concentrations, calmidazolium inhibited PAL gene activation (Fig. 9) and PAL catalytic activation (Table 1) to the same extent as chlorpromazine and trifluoperazine. Calmidazolium did not disturb the elicitor-induced cytosolic calcium mobilisation (Fig. 10C). However, calmidazolium prevented largely the elicitor-induced membrane de-

polarisation and cytosolic acidification (Fig. 10A, B), suggesting a calmodulin-dependent mechanism. In animal cells, cytosolic calcium mobilisation is modulated by the phosphatidylinositol pathway (Berridge and Irvine 1989), and it appears also to be the case in higher plants (Boss 1989). Neomycin (5–40 μ M) and LiCl

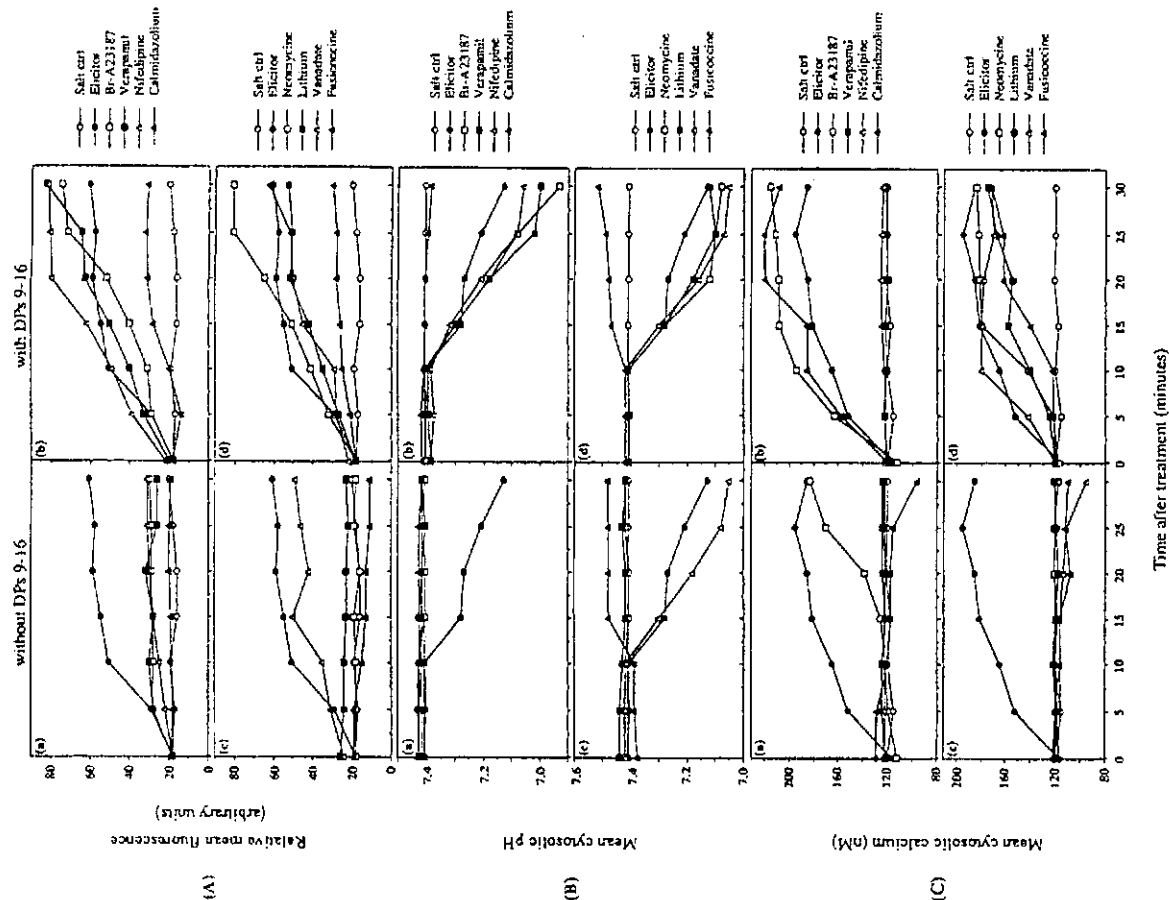


Table 1 Effect of Br-A23187, verapamil, nifedipine, chlorpromazine, trifluoperazine, calmidazolium, neomycin, lithium, fusicoccine and vanadate on PAL activity in carrot cells in presence or absence of the elicitor (DPs 9–16, high calcium, 0.2 mg ml⁻¹)

Treatment of <i>Daucus carota</i> cell suspension	Concentration range of modulators	PAL activity ($\mu\text{mol cinnamic acid } \mu\text{g protein}^{-1} \text{ h}^{-1}$)	
		Absence of elicitor	Presence of elicitor
<i>Calcium ionophore</i>			
- A23187	10-100 μM	2.2 $\pm$ 0.2	13.1 $\pm$ 0.5
+ A23187		6.4 $\pm$ 0.2	15.0 $\pm$ 0.5
<i>Calcium channel blockers</i>			
- Nifedipine	0.1-2 μM	1.2 $\pm$ 0.1	13.0 $\pm$ 1.3
+ Nifedipine		1.4 $\pm$ 0.2	1.9 $\pm$ 0.1
- Verapamil	25-100 μM	2.2 $\pm$ 0.0	13.1 $\pm$ 0.5
+ Verapamil		1.4 $\pm$ 0.0	1.6 $\pm$ 0.0
<i>Calmodulin inhibitors</i>			
- Chlorpromazine	100-400 μM	2.7 $\pm$ 0.6	17.6 $\pm$ 0.6
+ Chlorpromazine		4.7 $\pm$ 0.4	3.3 $\pm$ 0.3
- Trifluoperazine	50-400 μM	2.7 $\pm$ 0.6	17.6 $\pm$ 0.6
+ Trifluoperazine		6.1 $\pm$ 0.4	2.6 $\pm$ 0.1
- Calmidazolium	5-80 nM	2.7 $\pm$ 0.6	17.6 $\pm$ 0.6
+ Calmidazolium		4.0 $\pm$ 0.2	3.5 $\pm$ 0.1
<i>Phospholipid turn-over modulators</i>			
- Neomycine	5-40 μM	2.7 $\pm$ 0.6	17.6 $\pm$ 0.6
+ Neomycine		2.5 $\pm$ 0.1	9.0 $\pm$ 0.1
- Lithium	0.1-10 mM	3.5 $\pm$ 0.3	18.0 $\pm$ 0.5
+ Lithium		3.6 $\pm$ 0.2	9.9 $\pm$ 0.9
<i>Proton pump modulators</i>			
- Fusicoccine	4-20 μM	2.2 $\pm$ 0.2	13.1 $\pm$ 0.5
+ Fusicoccine		3.8 $\pm$ 0.1	3.5 $\pm$ 0.1
- Vanadate	0.1-2 mM	2.2 $\pm$ 0.2	13.1 $\pm$ 0.5
+ Vanadate		2.1 $\pm$ 0.1	10.8 $\pm$ 1.1

Values correspond to the mean PAL activity measured after 24 h from 5 independent experiments. Three controls were used: positive control = elicitor alone; negative control 1 = no elicitor, no modulator, high calcium; negative control 2 = modulator alone, high calcium. Results are expressed as activity ± S.D.

(0.1–10 mM), respectively an inhibitor of phospholipase C and an inhibitor of phospholipid recycling in animal and plant cells, both slowed down the elicitor-induced cytosolic calcium mobilisation rate (Fig. 10C), induced a 50% inhibition of PAL catalytic activity in the presence of the elicitor (Table 1) without affecting elicitor-induced PAL gene activation (Fig. 9). These results are consistent with the reported involvement of inositol phosphates in carrot and pea cells respectively during phytoalexin synthesis (Kurosaki et al. 1987, Toyoda et al. 1992).

Fusicoccine (4–20 μM), an activator of plasma membrane H⁺-ATPase activity, induced a slight membrane depolarisation (Fig. 10A), a cytosolic alkalinization (Fig. 10B), inhibited the elicitor-induced PAL catalytic activity (Table 1) and gene activation (Fig. 9), showing that cytosolic acidification is necessary for PAL activation. On the con-

trary, vanadate (0.1–2 mM), an inhibitor of H⁺-ATPase activity, did not change the elicitor-induced PAL activity (Table 1) nor the ion fluxes normally observed in elicited protoplasts (Fig. 10A, B, C). In absence of the elicitor, these two H⁺-ATPase modulators affected both membrane potential and cytosolic pH as expected: fusicoccine slightly hyperpolarised and alkalinised the protoplasts; vanadate depolarised and acidified the protoplasts (Fig. 10A, B), but this vanadate-induced artificial cytosolic acidification was unable to increase PAL activity on its own (Table 1).

Discussion

In this study, we found that oligolacturonides induced a membrane depolarisation, a cytosolic acidification, a cytosolic calcium mobilisation, PAL gene and en-

zyme activation, and *p*-hydroxybenzoic acid accumulation. Except for membrane depolarisation, these responses were only observed when pectic elicitors were applied to the cells, or protoplasts, in a 150 mM NaCl + 0.5 mM CaCl₂ ionic solution as already reported for defense gene activation in carrot (Messiaen and Van Cutsem 1993). It is now clear that the elicitor activity of pectic oligomers depends on their size and on the presence of calcium. Farmer et al. (1991) studying membrane protein phosphorylation, suggested that calcium and pectic elicitors may form supramolecular complexes of the "egg-box" type (Morris et al. 1982). This supramolecular conformation induced by a proper calcium concentration would be the active species recognized by the cells that triggered the whole cascade of defense reactions in carrot cell suspensions.

The role of calcium as second messenger in signal transduction in plants is now well documented (Knight et al. 1991, Scheel et al. 1991, Messiaen et al. 1993). In this study, we showed that calcium mobilisation plays a central role in the signal transduction cascade activated by pectic oligomers in carrot cells and protoplasts, and that it is clearly independent of membrane depolarisation and cytosolic acidification. Pectic fragments were still able to depolarise the cell and acidify the cytosol in absence of cytosolic calcium mobilisation. The way calcium is actually mobilised in the cytosol is still not clear. From our results on the effect of signal transduction modulators on elicited cells, voltage-independent calcium channels are the best candidates for mediating calcium mobilisation in carrot cells. Calcium mobilisation, as well as PAL gene and enzyme activation, were blocked by calcium channel antagonists. Although the location of such calcium channels is unclear since verapamil and nifedipine can cross the plasmalemma and inhibit vacuolar calcium channels (Hille 1992), these results are consistent with the purification of a high-affinity binding site of verapamil in carrot plasma membranes (Graziana et al. 1988, Thuleau et al. 1990). This binding site has been reconstituted in vitro and exhibited significant calcium gating activity.

On the contrary, calmodulin antagonists had no effect on calcium mobilisation, but were able to suppress PAL gene and enzyme activation. The involvement of calmodulin in the signal transduction pathway is thus clearly downstream of the cytosolic calcium mobilisation. This is not surprising since calmodulin antagonists act on calmodulin-calcium complexes rather than on isolated calmodulin. The inhibition of PAL activation by calmodulin antagonists was accompanied by a reduced cytosolic acidification, suggesting a calmodulin-dependent mechanism of regulation of cytosolic pH.

The involvement of the phosphatidylinositol pathway in mobilising cytosolic calcium in elicited cells is more and more advocated in different plant systems. In carrot cells, phytoalexin accumulation was shown to be preceded by

an increase in cytosolic IP₃ (Kurosaki et al. 1987). In parsley, IP₃ and IP₂S₃, a non-hydrolysable analogue, were also shown to enhance furanocoumarin synthesis in a dose-dependent manner (Rendel et al. 1993). We showed that neomycin and lithium salts, in the presence of pectic oligomers, reduced the rate, but not the amplitude, of cytosolic calcium mobilisation. This effect is consistent with an inhibition of the turn-over of membrane phospholipids. The reported membrane depolarisation and cytosolic acidification are two cellular responses clearly independent of cytosolic calcium mobilisation. However, it is not clear from our results if membrane depolarisation and cytosolic acidification are linked or two independent processes. On one hand, small oligogalacturonides (DPs < 9) were, indeed, able to induce a membrane depolarisation without affecting cytosolic pH (nor cytosolic calcium). On the other hand, elicitor-induced membrane depolarisation and cytosolic acidification were similarly affected by calmodulin antagonists.

The putative coupling of membrane potential and cytosolic pH will depend on the molecular mechanism underlying membrane depolarisation and cytosolic acidification. Several mechanisms have been proposed in the literature: (i) H⁺-ATPase inhibition and anion effluxes have been found to be responsible for the membrane depolarisation reported in blue-light stimulated cucumber hypocotyls (Spalding and Cosgrove 1992). In that case, inhibition of the plasmalemma proton-pump would be responsible for both membrane depolarisation and cytosolic acidification. (ii) A calmodulin-dependent Ca²⁺-ATPase recently discovered in carrot (Chen et al. 1993) might be involved in cytosolic recycling of H⁺-ATPase extruded protons via an electroneutral Ca²⁺/2H⁺ exchange (Ladror and Zielinski 1990). This would account for cytosolic acidification, but not for membrane depolarisation (iii) Proton influx might also be coupled directly or indirectly to K⁺ efflux in oligogalacturonide-treated tobacco cell suspensions (Mathieu et al. 1991). The involvement of these mechanisms in oligogalacturonide-treated carrot cell suspensions is, however, unknown.

In conclusion, pectic elicitors with a DP ≥ 9 in a calcium-induced conformation induce cytosolic calcium mobilisation in carrot. Calcium is probably mobilised via the opening of voltage-independent calcium channels. The opening of these channels might be mediated by IP₃ release. Pectic elicitors also induce a calcium-independent, but calmodulin-dependent membrane depolarisation and cytosolic acidification. The exact role of membrane depolarisation and cytosolic acidification in the elicitation of defense reactions is unknown, but are not consequences of cytosolic calcium mobilisation.

The involvement of other ion fluxes (anion fluxes), other second messengers (H₂O₂, methyljasmonate) and other signalling pathways (endocytosis) in oligogalacturonide-

treated carrot cells have not been assessed in this study. Further studies are presently in progress to better characterize the complex cellular signal generated by the elicitor treatment.

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References

Alken, C., George, H., Yang, Z., Lacy, G.H. and Mount, M.S. (1987) Molecular cloning of an endo-pectate lyase gene from *Erwinia carotovora* subsp. *atraseptica*. *Physiol. Mol. Plant Pathol.* 31: 325-335.

Berridge, M.J. and Irvine, R.F. (1989) Inositol phosphates and cell signalling. *Nature* 341: 197-205.

Boss, W.F. (1989) Phosphoinositide metabolism: its relation to signal transduction in plants. In: Second messengers in plant growth and development. Edited by Boss, W.F. and Morré, D.J. pp. 29-56. Liss, New York.

Bossen, M.E., Kendrick, R.E. and Vredenberg, W.J. (1990) The involvement of a G-protein in phytochrome-regulated, Ca²⁺-dependent swelling of etiolated wheat protoplasts. *Physiol. Plant.* 80: 55-62.

Branca, C., De Lorenzo, G. and Cervone, F. (1988) Competitive inhibition of the auxin-induced elongation by α-D-oligogalacturonides in pea stem segments. *Physiol. Plant.* 72: 499-504.

Brauner, T., Hüter, D.F. and Strasser, R.J. (1984) Comparative measurements of membrane potentials with microelectrodes and voltage-sensitive dyes. *Biochim. Biophys. Acta* 771: 208-216.

Brecht, J.K. and Huber, D.J. (1988) Products released from enzymatically active cell wall stimulate ethylene production and ripening in prechimeric tomato (*Lycopersicon esculentum* Mill.) fruit. *Plant Physiol.* 88: 1037-1041.

Bruce, R.J. and West, C.A. (1989) Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. *Plant Physiol.* 91: 889-897.

Chappell, J. and Hahlbrock, K. (1984) Transcription of plant defense genes in response to UV light or fungal elicitor. *Nature* 311: 76-78.

Chen, F.H., Ratterman, D.M. and Sze, H. (1993) A plasma membrane-type Ca²⁺-ATPase of 120 kilodaltons on the endoplasmic reticulum from carrot (*Daucus carota*) cells. *Plant Physiol.* 102: 651-661.

De Vries, S., Hoge, H. and Bisseling, T. (1988) Isolation of total and polysomal RNA from plant tissues. In: *Plant Molecular Biology Manual*. Edited by Gelvin, S.B., Schilperoort, R.A. and Verma, D.S. pp. B6 1-13. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Farmer, E.E., Moloshok, T.D., Saxton, M.J. and Ryan, C.A. (1991) Oligosaccharide signalling in plants. Specificity of oligouronide-enhanced plasma membrane protein phosphorylation. *J. Biol. Chem.* 266: 3140-3145.

Forrest, R.S. and Lyon, G.D. (1990) Substrate degradation patterns of polygalacturonic acid lyase from *Erwinia carotovora* and *Bacillus polymyxa* and release of phytoalexin-eliciting oligosaccharides from potato cell walls. *J. Exp. Bot.* 225: 481-488.

Graziana, A., Fosset, M., Ranjeva, R., Hetherington, A.M. and Lazdunski, M. (1988) Ca²⁺ channel inhibitors that bind to plant cell membranes block Ca²⁺ entry into protoplasts. *Biochemistry* 27: 764-768.

Guragossian Kiss, H., Evans, M.L. and Johnson, J.D. (1991) Cytosolic calcium levels in protoplasts from the cap and elongation zone of maize roots. *Protoplasma* 163: 181-188.

Hahlbrock, K., Lamb, C.J., Purwin, C., Ebel, J., Fautz, E. and Schäfer, E. (1981) Rapid response of suspension cultured parsley cells to the elicitor from *Phytophthora megasperma* var. *sojae*. Induction of enzymes of general phenylpropanoid metabolism. *Plant Physiol.* 67: 768-773.

Hille, B. (1992) Mechanisms of block. In: *Ionic Channels of Excitable Membranes* (2nd edition). Edited by Hille, B. pp. 390-423. Sinauer Associates Inc., Sunderland, Massachusetts, U.S.A.

Ishizuka, M., Yamada, F., Tanaka, Y., Takeuchi, Y. and Imaseki, H. (1991) Sequential induction of mRNAs for phenylalanine ammonia-lyase in slices of potato tuber. *Plant Cell Physiol.* 32: 57-64.

Jin, D.F. and West, C.A. (1984) Characteristics of galacturonic acid oligomers as elicitors of chitinase synthetase activity in castor bean seedlings. *Plant Physiol.* 74: 989-992.

Keefe, D., Hinz, U. and Meins, F., Jr. (1990) The effect of ethylene on the cell-type-specific and intracellular localization of β-1,3-glucanase and chitinase in tobacco leaves. *Planta* 182: 43-51.

Knight, M.R., Campbell, A.K., Smith, S.M. and Trevaras, A.J. (1991) Transgenic plant acquirin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* 352: 524-526.

Kurosaki, F., Tashiro, N. and Nishi, A. (1986) Induction of chitinase and phenylalanine ammonia-lyase in cultured carrot cells treated with fungal mycelial walls. *Plant Cell Physiol.* 27: 1387-1391.

Kurosaki, F., Tsurusawa, Y. and Nishi, A. (1987) Breakdown of phosphatidylinositol during the elicitation of phytoalexin production in cultured carrot cells. *Plant Physiol.* 85: 601-604.

Ladror, U.S. and Zielinski, R.E. (1990) Effect of Ca²⁺ and calmodulin on dPH formation in tonoplast vesicles from corn roots. *Plant Physiol.* 92: 850-854.

Lois, R., Dietrich, A., Hahlbrock, K. and Schulz, W. (1989) A phenylalanine ammonia-lyase gene from parsley: regulation and identification of elicitor and light cis-acting elements. *EMBO* 8: 1641-1648.

Mafra, V., Collin, D.J., Eberhard, S., Mohnen, D., Darvill, A.

- and Albersheim, P. (1991) Oligogalacturonides are able to induce flowers to form on tobacco explants. *Plant J.* 1: 217-225.
- Mathieu, Y., Kurkdjian, A., Xia, H., Guern, J., Koller, A., Spiro, M.D., O'Neil, M., Albersheim, P. and Darvill, A. (1991) Membrane responses induced by oligogalacturonides in suspension-cultured tobacco cells. *Plant J.* 1: 333-343.
- Messiaen, J. and Van Cutsem, P. (1993) Defense gene transcription in carrot cells treated with oligogalacturonides. *Plant Cell Physiol.* 34: 1117-1123.
- Messiaen, J., Read, N.D., Van Cutsem, P. and Trewas, A.J. (1993) Cell wall elicitors increase cytosolic free calcium in plant cells. *J. Cell Sci.* 104: 365-371.
- Morris, E.R., Powell, D.A., Gidley, M.J. and Rees, D.A. (1982) Conformation and interactions of pectins. 1. Polymorphism between gel and solid states of calcium polygalacturonate. *J. Mol. Biol.* 155: 507-516.
- Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15: 473-497.
- Neuhäus, G., Bowler, C., Kern, R. and Chua, N.-H. (1993) Calcium/calmodulin-dependent and -independent phytochrome signal transduction pathways. *Cell* 73: 937-952.
- Page, W. and Heitefuss, R. (1990) Enzyme activities in soft rot pathogenesis of potato tubers: effects of calcium, pH, and degree of pectin esterification on the activities of polygalacturonase and pectate lyase. *Physiol. Mol. Plant Pathol.* 37: 9-25.
- Renelt, A., Collings, C., Hahlbrock, K., Nürnberger, T., Parker, J.E., Sacks, W. and Scheel, D. (1993) Studies on elicitor recognition and signal transduction in plant defence. *J. Exp. Bot.* 44: 257-268.
- Ridge, I. and Osborne, D.J. (1970) Hydroxyproline and peroxidases in cell walls of *Pisum sativum*: regulation by ethylene. *J. Exp. Bot.* 21: 843-856.
- Rincon, M. and Boss, W.F. (1987) Myo-inositol trisphosphate mobilize calcium from fusogenic carrot (*Daucus carota* L.) protoplasts. *Plant Physiol.* 83: 395-398.
- Robertsen, B. and Svalheim, O. (1990) The nature of lignin-like compounds in cucumber hypocotyls induced by α -1,4-linked oligogalacturonides. *Physiol. Plant.* 79: 512-518.
- Roos, W. (1992) Confocal pH topography in plant cells—acidic layers in the peripheral cytoplasm and the apoplast. *Bot. Acta* 105: 253-259.
- Scheel, D., Colling, C., Hedrich, R., Kawaletz, P., Parker, J.E., Sacks, W.R., Somssich, I.E. and Hahlbrock, K. (1991) Signals in plant defense gene activation. In *Advances in Molecular Genetics of Plant-Microbe Interactions* (Vol. 1). Edited by Henneke, H. and Verma, D.P.S. pp. 373-380. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Schnitzler, J.P. and Seitz, H.U. (1989) Rapid responses of cultured carrot cells and protoplasts to an elicitor from the cell wall of *Pythium aphanidermatum* (Edson) Fitzp. *Z. Naturforsch.* 44c: 1020-1028.
- Spalding, E.P. and Cosgrove, D.J. (1992) Mechanism of blue-light-induced plasma-membrane depolarization in etiolated cucumber hypocotyls. *Planta* 188: 199-205.
- Somssich, I.E., Bollmann, J., Hahlbrock, K., Kombrink, E. and Schultz, W. (1989) Differential early activation of defense-related genes in elicitor-treated parsley cells. *Plant Mol. Biol.* 12: 227-234.
- Thain, J.F., Doherty, H.M., Bowles, D.J. and Wildon, D.C. (1990) Oligosaccharides that induce proteinase inhibitor activity in tomato plants cause depolarization of tomato leaf cells. *Plant Cell Environ.* 13: 569-574.
- Thuleau, P., Graziana, A., Canut, H. and Ranjeva, R. (1990) A 75 kDa polypeptide, primarily located at the plasma membrane of carrot cell-suspension cultures, is photoaffinity labelled by the calcium channel LU49888. *Proc. Natl. Acad. Sci. USA* 87: 10000-10004.
- Toyoda, K., Shiraishi, T., Yoshioka, H., Yamada, T., Ichinose, Y. and Oka, H. (1992) Regulation of polyphosphoinositide metabolism in pea plasma membranes by elicitor and suppressor from pea pathogen, *Mycosphaerella pinodes*. *Plant Cell Physiol.* 33: 445-452.

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Efecto de la Quitosana en la Inducción de la Actividad de Enzimas Relacionadas con la Defensa y Protección de Plántulas de Arroz (*Oryza sativa* L.) contra *Piricularia grisea* Sacc.

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Resumen. En los últimos años se han incrementado los estudios acerca del empleo de productos naturales como sustitutos de herbicidas y plaguicidas, debido a las posibles ventajas ecológicas y económicas que muchos de estos compuestos ofrecen, especialmente, en la protección de cultivos. Entre estos productos naturales, se encuentran los inductores de resistencia, que son compuestos con la capacidad de estimular los mecanismos de defensa en las plantas. En este trabajo se utilizó una quitosana obtenida en el Instituto Nacional de Ciencias Agrícolas en la provincia La Habana, Cuba. Durante la investigación se determinó el efecto *in vivo* de este compuesto aplicado a la semilla, en la estimulación de la actividad de algunas enzimas de defensa como la fenilalanina amonio liasa (PAL), β -1,3 glucanasa, quitinasa y quitosinasa en plántulas de arroz de la variedad J-104, susceptible al ataque del hongo *Piricularia grisea*. Se observó un incremento de todas las actividades enzimáticas evaluadas en las plantas tratadas con quitosana con relación a las plantas no tratadas. En general, la mayor concentración de quitosana aplicada (1000 mg/L) fue la que indujo la mayor actividad enzimática. En las plantas tratadas con quitosana también se observó una menor severidad de la enfermedad provocada por *P. grisea* con respecto a las plantas testigo.

Palabras clave adicionales: Compuestos naturales, inducción de resistencia, inductores.

Abstract. In recent years, there has been an increase in studies related to the use of natural products as substitutes of

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RELACIONADAS CON LA DEFENSA Y PROTECCIÓN DE PLÁNTULAS DE ARROZ
(ORYZA SATIVA L.) CONTRA PYRICULARIA GRISEA SACC

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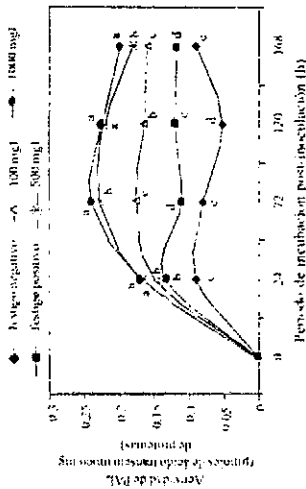


Fig. 1. Cinética enzimática de la quitinasa en plántulas de arroz (*Oryza sativa*) tratadas con diferentes concentraciones de quitosano e infectadas con *Piricularia grisea*.

todas las plantas tratadas con las concentraciones de quitosano, el mayor valor de estimulación se produjo a las 72 h después de la inoculación fúngica, así como que las plantas del testigo infectado provocaron una estimulación de la actividad PAL significativamente mayor que las del testigo no infectado. Este resultado concuerda con los obtenidos por Boonchaisrikul *et al.* (1998) que observaron en hojas de arroz el valor máximo de actividad de PAL al tercer día después de la inoculación con *P. grisea*.

Determinación de la actividad β -1,3 glucanasa. La enzima α -1,3 glucanasa cataliza la hidrólisis del β -1,3 glucano, uno de los componentes mayoritarios de la pared celular de los hongos fitopatógenos. Los resultados de la Figura 2 muestran que las plantas obtenidas de semillas tratadas con las diferentes dosis de quitosano y posteriormente infectadas, exhibieron mayores valores de actividad β -1,3 glucanasa que las de los tratamientos testigo con diferencias significativas entre ellas. Este comportamiento corrobora que la actividad

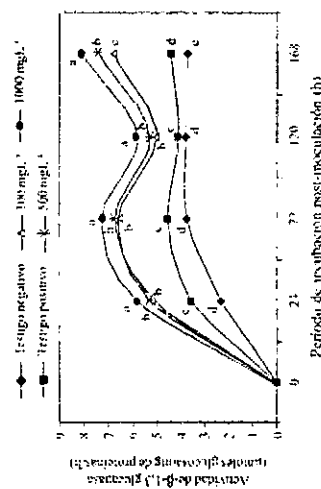


Fig. 2. Cinética enzimática de la β -1,3 glucanasa en plántulas de arroz (*Oryza sativa*) obtenidas de semillas tratadas con diferentes concentraciones de quitosano e infectadas con *Piricularia grisea*.

de la enzima β -1,3 glucanasa es estimulada en las plantas, en respuesta al ataque de patógenos y a la presencia de inductores (Hellebrand *et al.*, 1998; Leubner-Metzger, 2003). Para el caso de las plantas tratadas con quitosano e infectadas con *P. grisea*, la concentración de 1000 mg/L estimuló mayormente la actividad de β -1,3 glucanasa en todas las evaluaciones. Además, puede plantearse que no se observó una tendencia definida en los valores de actividad enzimática en ninguna de las concentraciones, aunque en todas ellas las mayores valores se registraron en las últimas evaluaciones.

Determinación de la actividad de quitinasa. Teniendo en cuenta que la actividad de la enzima quitinasa está también relacionada con la patogénesis, se pueden apreciar en la diferencia en cuanto a los valores alcanzados por la enzima debido a los tratamientos, el comportamiento gráfico de la actividad en el tiempo y las dosis que lograron la mayor actividad (Fig. 3). Se puede señalar que los testigos mantuvieron los menores valores de actividad, siendo el testigo negativo el que mostró los valores más bajos de actividad de quitinasa. Analizando la actividad de quitinasa en plantas de arroz tratadas con quitosano se observó que los valores de actividad enzimática fueron 2-3 veces superiores a los correspondientes de los testigos. Este comportamiento confirma que la enzima quitinasa en las plantas puede ser estimulada después de la aplicación de quitoligosacáridos y después de la inoculación por hongos y bacterias (Leubner-Metzger, 2003; Robert y Schirrmikoff, 1988). Respecto a la actividad de la enzima en las plantas tratadas con quitosano, su tendencia fue aumentar en el tiempo, observándose los mayores valores de actividad quitinasa a las 168 h, en todos los casos, siendo la concentración de 1000 mg/L la que mantuvo los valores mayores en todas las evaluaciones realizadas.

Determinación de la actividad de quitosana. La quitosana es otra de las enzimas de defensa analizadas en este trabajo. Las plantas tratadas con quitosano en diferentes dosis, tuvieron una actividad de la enzima quitosana

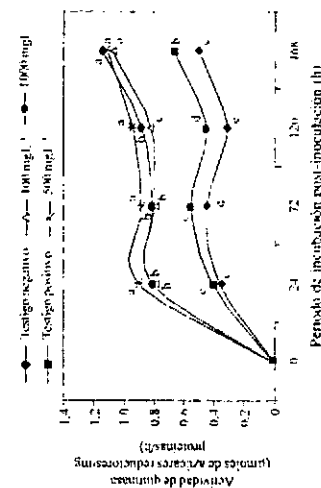


Fig. 3. Cinética enzimática de la quitinasa en plántulas de arroz (*Oryza sativa*) obtenidas de semillas tratadas con diferentes concentraciones de quitosano e infectadas con *Piricularia grisea*.

Cuadro 2. Evaluación de la resistencia de las plántulas de arroz (*Oryza sativa*) tratadas con diferentes concentraciones de quitosano (Q) ante la infección en hojas por el hongo *Piricularia grisea*.

Tratamiento	Días después de la inoculación			
	7	14	21	28
Testigo negativo	0 a	0 a	0 a	0 a
Testigo positivo	2 b	4 d	4 d	4 d
Q-100 mg/L	1 b	2 c	2 c	2 c
Q-500 mg/L	0 a	0 a	0 a	0 a
Q-1000 mg/L	0 a	0 a	0 a	0 a

* Letras diferentes dentro de una misma columna, indican diferencias significativas (Duncan $p < 0.05$).

0 = ninguna lesión; 1 = lesiones pardas pequeñas del tamaño de un alfiler o grandes sin centro esporádico; 2 = pequeñas lesiones redondeadas a ligeramente alargadas; manchas necróticas grises, cerca de 1-2 mm de diámetro con margen pardo; y 4 = lesiones típicas parecidas a 2 pero un número significativo de lesiones están sobre las hojas superiores.

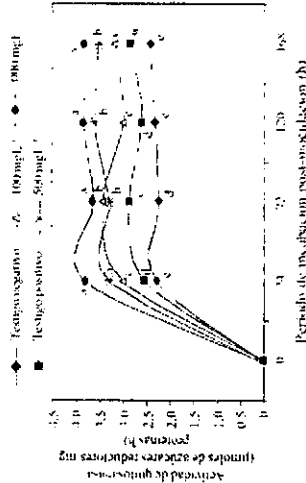


Fig. 4. Cinética enzimática de la quitinasa en plántulas de arroz (*Oryza sativa*) obtenidas de semillas tratadas con diferentes concentraciones de quitosano e infectadas con *Piricularia grisea*.

significativamente mayor que las plantas provenientes de los tratamientos testigo (Fig. 4). Por su parte, dentro de las plantas de los tratamientos testigo, las que fueron inoculadas con el hongo mostraron una mayor actividad enzimática que las sanas. La concentración que logró la mayor actividad de quitosana fue la de 1000 mg/L a las 168 h después de la inoculación, lo cual hace suponer una estrategia integral de defensa vegetal con el desencadenamiento al mismo tiempo de diferentes enzimas defensivas. En ese sentido, se ha demostrado que las enzimas quitinasas y β -1,3 glucanasas tienen un efecto sinérgico, ya que degradan la pared celular de los hongos (Holler, 1993). Una vez demostrada la capacidad de la quitosana de estimular mecanismos de defensa en las plantas de arroz, tanto en ausencia como en presencia de patógeno, se evaluó si este compuesto protegía a plantas de arroz susceptibles, contra la infección de *P. grisea*.

Resistencia en las plántulas inoculadas. El comportamiento de las plántulas de arroz provenientes del tratamiento testigo (positivo), que si bien los primeros síntomas de infección se apreciaron a los 7 días, es a los 14 días donde se observó el grado 4 de la escala (Cuadro 2). Las plantas tratadas con las diferentes concentraciones de quitosano no presentaron ninguna lesión o síntoma típico de infección en la primera evaluación, excepto para la quitosana a la concentración de 100 mg/L, que tuvo sólo grado 1. En la segunda evaluación, a los 14 días se observó una tendencia al incremento de la infección con relación a la primera evaluación; exceptuando los tratamientos de 1000 mg/L, en la que no se apreció ninguna lesión. Sin embargo, en el tratamiento testigo (positivo) se incrementó el grado de infección al igual que en las plántulas tratadas con 100 y 500 mg/L. Este comportamiento se puede atribuir a que en concentraciones menores de 1000 mg/L, la protección es incompleta debido a que la estimulación o activación enzimática no es suficiente para proteger la planta de la infección. Este resultado puede deberse también a que

en las plantas tratadas en comparación con las no tratadas. Los resultados obtenidos en este trabajo sugieren la posibilidad de aplicar la quitosana en condiciones de campo para el control de la enfermedad *P. grisea* en el cultivo del arroz; ya que además de los beneficios que tendría al ser un compuesto biodegradable e inocuo para el medio ambiente, no es tóxico para la salud del hombre y de los animales y se ha comprobado que también aumenta los rendimientos de los cultivos.

LITERATURA CITADA

- Aricapa, G. y Correa-Victoria, F. 1993. Integración de fitopatología, mejoramiento y biología molecular para el desarrollo de resistencia estable al anillo del arroz (*Pyricularia grisea*) Taller DNA fingerprints de *Pyricularia grisea*. Cui. Colombia CIAT. 13 p.
- Bautista-Bañes, S., Hernández-Lauzardo, A. N., Velásquez-del Valle, M.G., Hernández-López, M., Ali Barka, E., Bosquez-Molina, E., and Wilson, C. I. 2006. Chitosan a potential natural compound to control pre- and postharvest diseases of horticultural commodities. *Crop Protection* 25:108-118.
- Boller, T. 1993. Antimicrobial functions of the plant hydrolases chitinases and β 1,3 glucanases. In: B. Frig and M. Legrand (eds.), *Mechanisms of Plant Defense Responses* (Developments in Plant Pathology). Kluwer Academic Publishers, Dordrecht, The Netherlands. 508 p.
- Boonchaisirikul, Ch., Wasano, K., and Nose, A. 1998. Activities of phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) in young rice panicles inoculated with *Pyricularia grisea*. *Japanese Journal of Tropical Agriculture* 42:39-45.
- Cárdenas, R., Cristo, E. y Pérez, N. 2002. Variedades rubanas de arroz promisorias para la provincia de Pinar del Río tolerantes al tizón de la hoja (*Pyricularia grisea* Sacc.). *Cultivos Tropicales* 23:53-56.
- Díaz, D. 2001. Potencialidades Biológicas de la Quitosana y sus Hidrolizados Enzimáticos. Trabajo de Diplomación, Universidad de La Habana, Facultad de Biología. La Habana, Cuba. 58 p.
- Fabregat, M. 1984. Caracterización de Patótipos de *Pyricularia grisea* en la Región Occidental. Tesis de Doctor en Ciencias Agrícolas, Centro Nacional de Sanidad Agropecuaria, La Habana, Cuba. 100 p.
- Ge, Y. and Li, D. 1996. A molecular study on tomato induced resistance to *Alternaria solani*. 8th International Congress of Molecular Plant-Microbe Interaction. Knoxville, TN, USA. Abstract pp. 14-19.
- Guimaraes, E., Yofita, O. y Correa-Victoria, F. 1996. Empleo del concepto de líneas de *Pyricularia grisea* para escoger genotipos de arroz resistente. *Fitopatología Venezolana* 1:2-6.
- Hammerschmidt, R. 1999. Phytoalexins: What have we learned after 60 years? *Annual Review of Phytopathology* 37:285-306.
- Hellwald, S., Banw, G., Helingheri, L., Vasseu, J., and Hilbert, J. 1998. Extracellular β 1,3 glucanases are induced during early somatic embryogenesis in *Cucurbitur*. *Planta* 205:56-63.
- Instituto Técnico del Arroz. 2002. Ministerio de la Agricultura. Habana, Cuba. 24 p.
- Inui, H., Yamaguchi, Y., and Hirano, S. 1997. Inductor actions of N-acetylchitooligosaccharides and laminarioligosaccharides for chitinase and L-phenylalanine ammonia-lyase induction in rice suspension culture. *Bioscience Biotechnology and Biochemistry* 61:975-978.
- IRRI (International Rice Research Institute). 2002. Standard Evaluation System for Rice (SES). Los Baños, Philippines. 56 p.
- Koga, J., Oshima, K., Ogawa, N., Ogasawara, N., and Shimura, M. 1998. A new bioassay for measuring inductor activity in rice leaves. *Annals of the Phytopathological Society of Japan* 64:97-101.
- Kuc, J. 1995. Phytoalexins, stress metabolism and disease resistance in plants. *Annual Review of Phytopathology* 33:275-297.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* 193:265-275.
- Lawrence, C., Joosten, M., and Tuzius, S. 1996. Differential induction of pathogenesis-related in tomato by *Alternaria solani* and the association of basic chitinase isozyme with resistance. *Physiological and Molecular Plant Pathology* 48:361-366.
- Leubner-Metzger, G. 2003. Functions and regulation of β 1,3-glucanases during seed germination, dormancy release and after-ripening. *Seed Science Research* 13:17-35.
- MINAGRI (Ministerio de la Agricultura). 1999. Cuba. Instituto de Suelos. Nueva versión de Clasificación Genética de los Suelos de Cuba. La Habana. AGROINFOR. 64 p.
- Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry* 153:375-380.
- Paz-Lago, D., Cabrera, G., Ramírez, M., Pombo, R. y Gutiérrez, A. 1999. Influencia de derivados de quitina en la interacción tomate-*Fusarium oxysporum* f. sp. *licopersici* a nivel de bioensayo. *Cultivos Tropicales* 20:59-66.
- Paz-Lago, D., Gutiérrez, A. y Luzardo, L. 1998. Relevancia biológica de la enzima β 1,3 glucanasa en la activación de la resistencia sistémica del tabaco inducida por la pared celular fúngica. *Cultivos Tropicales* 19:25-27.
- Ramírez, M.A., Cabrera, G., Gutiérrez, A. y Rodríguez, T. 2000. Metodología de obtención de quitosana a partir de quitina de langosta. *Cultivos Tropicales* 21:81-84.
- Robert, W., and Schreierminkoff, C. 1988. Plant and bacterial chitinases differ in antifungal activity. *Journal of Genetic Microbiology* 169:169-176.

Takeshi, Y., Bo, Y., and Shibuya, N. 2000. Oligosaccharide inducers and their receptors for plant defense responses. *Trends in Glycoscience and Glycobiology* 12:113-112.