RECEIVED JAN 2 6 2004

Petition submitted to NOSB.doc

National Organic Standards Board
C/o Robert Pooler, Agriculture Marketing Specialist
USDA/AMS/TM/NOP
Room 2510-50. Ag stop 0268
P.O. Box 96456
Washington D.C., 20090-6456

Phone 202/720-3252
Fax 202/205-7808
E-mail: nlpetition@usda.gov

Enclosed is an application to request that chitosan (Poly-D-glucosamine) be added to the Nation List of materials approved for use by organic growers. It is being submitted in consultation with Small Planet Foods a organic industry and with the advise from the Washington State Dept of Agriculture.

Expedition of this review would be beneficial to the organic industry since there is currently no effective Potato Late Blight control available for the next growing season. The last two growing seasons we have worked with Steve L. Foss Pesticide information specialist of the Washington State Dept of Agriculture to acquire a Washington State experimental use permit (WSUEP). Last year, 2 of the 4 grower cooperators were unable to test chitosan as an adjuvant (sticker) because the permit had a limitation of 10 acres. Therefore it is hoped that the process can be completed in the time to get chitosan on the National List in time for the grower-cooperators and all other users to be able to help protect their potatoes from Late Blight.

Fortunately, chitosan has had previous applications in nutrition, agriculture and Industry. The literature documenting efficacy, safety etc is quite extensive. Most of the literature appears in proceedings of various world wide symposia and is readily available to me. I have included some of this information in this application but for further information please contact:

Lee A. Hadwiger
Dept of Plant Pathology
Washington State University
Pullman, WA 99164-6430
Phone 509-335-3751
Fax 509-335-9581
Email chitosan@wsu.edu

Lee A Hadwiger
1/23/04

The following naturally occurring compound, chitosan is being petition for inclusion on the national list in the following category:

Non-synthetic substance allowed for use in organic crop production.

1. Common name:
The material is called chitosan or (poly-D-glucosamine) to be sold under the label, "Chito-stik".
2. Manufacturers name, address and telephone no.

Vanson/Halo Source
14716 8th street NE
Redmond, WA 98052
Phone 425-881-6464
Fax 425-882-2476
Email enichols@vanson.com

3. Current use:
Spray adjuvant (sticker) to be used in combination with an organic program-approved fungicide.

4. Crop:
Potato and other agronomic crops.

Rate of Application:
66mg/liter or 0.011 pounds/20 gallons water which is adequate to spray on 1 acre. (Can be used to an advantage with copper sulfate pentahydrate when the latter is applied at a rate of 18 mg/liter or 68mg/gallon or 0.00075 pounds/20 gallons of water to control Potato Late Blight on 1 acre). Chitosan's water solubility properties enables it to be applied by both aerial and ground spray rigs.

Mode of Action:
Chitosan is a β-1-4 linked polymer of the sugar glucosamine. The amino group on this chain bonds with negative groups of any other component and thus has the property of attaching or "sticking" other material to the plant leaf surface. This proposed usage is not to be confused with other chitosan applications involving high rates of application. The abundance of evidence in the literature indicate that when much higher applications are applied to plant tissue some of the chitosan molecules enter the cell and associate with the phosphate groups on the DNA within the plant nucleus, which can activate plant defense responses.

Source of the substance:
Chitosan naturally occurs in fungal cell walls and the shells of crustascian organisms, however the most economical source is from crab and shrimp shells. A bonus to the manufacture of chitosan is that its many commercial uses make the crab shells by product of crab meat processors more valuable and less likely to be discarded; thus less likely to pollute waters close to these processing plants.

. Thus the starting material is crab shells containing some residual meat and the shell contains some minerals inherent to the organism. The following processing occurs in preparing chitosan for its sticking agent function:

Demineralization:
a. Shells are demineralized with hydrochloric acid to remove any potentially undesirable minerals.
b. Demineralized shells are heated in a basic solution (NaOH) to remove residual meats attached to the shells to give pure chitin.

Adjustment of % deacetylation from 20% to 50-80% to give chitosan.

c. Chitin which is 20% deacetylated is adjusted (with stronger base--NaOH) to chitosan. This exposure of additional amino groups provides a potential for water solubility and makes its antimicrobial properties the same as the chitosan which occurs naturally in fungal walls and other locations in nature. The exposed amino groups also provide the binding required for chitosan's use as a "sticker"(See the reference, Kendra et al.that is enclosed).

Adjustment of the pH towards neutrality

d. Extensive water washes of the insoluble chitosan removes the residual base and other impurities.
e. Adjustment of the pure chitosan suspension in water to a pH below neutrality with an organic acid that is NOP-approved such as lactic acid or acetic acid.

Desication:

For purposes of further purification, reducing transport costs and packaging, the chitosan is desiccated, removing all of the water but retaining a readily water-soluble material.

Application:

Chitosan's utilization as a sticker starts at this point and the following processing occurs to prepare it for it sticking agent function. The concentration of chitosan utilized as a sticking agent is 66 micrograms chitosan/ml. When utilized as a sticking agent with copper sulfate pentahydrate this latter compound is utilized at 14 -28 micrograms/ ml. The amount of chitosan applied / acre is ~5 grams when utilized as a sticking agent. As a comparison the chitosan approved for consumption by humans for weight loss or chlolesterol reduction is 1.5 grams daily.

6. Summary of previous reviews:

The Washington State Dept. of Agriculture has twice reviewed applications from Lee A. Hadwiger, Dept of Plant Pathology Washington State University Pullman, WA

99164 for experimental use permits which were approved for the last two growing seasons.
Reference Contact: Steve L. Foss, Pesticide in formation/Biopesticide specialist, Washington State Department of Agriculture, Pesticide Mgmt Div., Registration Section.

7. Information on EPA and state regulatory authority registration.
EPA: Poly-D-glucosamine(chitosan): exemption from the requirement of a tolerance. Federal Register/Vol.60, No.75/Wed April 19, 1995/Rules and Regulations. See attached additional information concerning chitosan documented by the U.S. Environmental Protection Agency. An application for a Washington State Regulatory permit for spray adjuvant registration has been submitted.

8. Chitosan's Chemical Abstract Service (CAS) number: CAS# 9012-76-4
OPP Chemical Code: 128930

Label of the product that contains the petitioned substance, chitosan:
Chito-Stik.

9. Chitosan's physical properties and chemical mode of action :
Chitosan is an organic polymer of glucosamine and N-acetyl-glucosamine, typically 80% glucosamine and 20% N-acetyl-glucosamine. Quality assessments of chitosan are often based on viscosity measurements at concentrations maximally soluble in water following an initial solubilization in dilute organic acid. The longer the chitosan molecule, the more viscous the solution. Pure chitosan solutions are essentially colorless, with only a slight yellow appearance. The prominence of the glucosamine possessing an amino group and contributes a positive charge to the chitosan polymer. Due the β-1,4 linkage of the glucosamine sugars, there is an alternating position of the positive charge along the length of the polymer. The resultant cationic polymer can thus act to attract negative charges it becomes exposed to. In application, the negative charges of copper sulfate and the negative charges of the plant leaf surface are attracted by the chitosan molecule. As a result it appears that the copper sulfate can become secured to the plant leaf surface in such a way that the antimicrobial action of the copper can be dispersed or displayed in an optimal manner. This makes possible a chitosan/copper treatment that has a high efficacy for controlling the pathogen, *Phytophthora infestans*. Additionally the copper is secured in such a manner that the application does not cause copper toxicity to the plant leaf. Finally, the quantity of copper compound required for treatment is 1/40 th that of other copper-containing treatments that have been approved for use by organic growers.

(a) Chemical interactions with other substances used in organic production:
Chitosan's application is anticipated to be in association with copper sulfate and possibly other fungicides that have received approval for use by organic growers.
(b) Toxicity and environmental persistence. Toxicity data are presented in the attached Material Safety Data Sheet. This sheet indicates the Acute oral, LD50 as being

in excess of 10 g/kg. The processing of chitosan for agronomic use and human consumption are essentially the same.

(c) Environmental impacts from its use or manufacture: EPA has declared chitosan exempt from tolerance. Chitosan as component of fungi and crustaceans is very prevalent in nature and thus is subjected to many organisms possessing enzymes such chitinase and chitosanase that can digest the molecule and utilize its sugars metabolically. Thus there are no known or perceived negative impacts on the environment.

(d) Effects on human health. The literature on this aspect of chitosan is very extensive. A recent book covering most of the health aspects is entitled, "Chitosan in Pharmacy and Chemistry" Ed. R.A.A. Muzzarelli and C. Muzzarelli. Atec Edizioni, Via Valtesino, 29, IT-63013 Grottammare (AP), Italy. Further at least 6 international symposia have been published over the last 2 decades, dealing primarily with chitosan and chitin (The index and two papers from one such symposia are enclosed). In summary there are no know negatives effects on human health and more importantly there are many positive effects such as in weight reduction and in lowering cholesterol. Again chitosan as a polycation acts to aggregate positive charges from all sources and these include components in the diet and components of fatty acids and bile material. Subsequently these complexes are removed from the body through the intestinal tract.

(e) Effects on soil organisms, crops or livestock.

Small polymers of chitosan (heptamers) are antifungal and antibacterial(See enclosed article by Kendra et al). They are also capable of activating defense genes in plants. Some of the same benefits of humans are possible with chitosan's use with animals. Chitosan has been approved as an animal feed component (Page 322 of the 2001 official publication of the Association of American Feed Control Officials is enclosed).

10. Safety information about chitosan including a Material Safety Data Sheet. (Enclosed).

11. Research Information about the substance with bibliographies. There are over 20 books on chitosan/chitin and numerous individual reports. Some individual articles are included. There is an index of titles that cover some chitosan processing techniques, physical properties and commercial uses as well as some other agricultural applications. (See symposium Edited by Skjak-Braek, Anthonsen, and Sandford.)

Information on chitosan submitted to address the specific criteria established by NOSB for evaluating petitioned substances (Act 7 U. S. C.6518 (m). There is substantial duplication of information from material submitted above.

1. The potential of chitosan for detrimental chemical interactions with other materials used in organic farming systems.

Chitosan (poly-D-glucosamine) as a naturally occurring cationic polymer has an affinity to all compounds and materials with negative charges. Therefore it possesses an ability to encumber many materials on plants and in the soil, and in the process it can stabilize materials *in situ* until its carbohydrate chain is digested or until the glucosamine sugar residue is hydrolyzed from the chain and further metabolized by adjacent organisms. This affinity to negative charges property of chitosan has been utilized commercially to remove sludge from the wastes of biological processing plants e.g. slaughter houses etc.

Chitosan when associated with equivalent quantities of natural compounds or negative elements can attach to and display the material along the length of the chitosan polymer. A major benefit has been derived with its use as a sticker for materials such as copper sulfate that has been routinely used by organic growers to control *Phytophthora infestans* the causal organism of potato late blight. Rather than being detrimental this property has been beneficial in that when so combined can control this plant disease utilizing $1/40^{th}$ the amount copper sulfate recommended for other control treatments.

2. Toxicity and mode of action of the substance and its break down products or any contaminants and their persistence in areas of concentration in the environment.

Toxicity. Chitosan possesses no inherent toxicity and has obtained a blanket exemption from the EPA . In further support of its safety, chitosan is consumed by humans in large quantities as a dietary supplement. Again the cationic polymer functions to associate with negative charges and as a result encumbers fatty acids, cholesterol and other nutrients within the digestive tract, resulting in the desired effect of reducing weight and lowering cholesterol.

Mode of action: Chitosan is a polymer of glucosamine sugars. Each sugar possesses an amino group which gives the total polymer an intense positive charge. Its mode of action is based on this polymer's length of continuous positive charges. When used as an adjuvant to plant leaf treatments it can attach, both to the negative charges of the leaf and any negative charges of the treatment, thus acting as a "sticker". This action can be optimal at concentrations of 25 μg/ml. When chitosan is applied to plant tissues at concentrations 10-20-fold higher, some polymers that contain 7 or more sugar residues can enter the plant cell. These polymers enter the living plant cell and can attach to the negative charges of the DNA molecule's phosphates in the sugar/phosphate backbone.

An extensive series of reports (For a review see enclosed article, Hadwiger, Chiang, Victory, and Horovitz) indicate that the chitosan/DNA complexing results in the activation of genes called pathogenesis related (or PR) genes. The activation of these PR genes is required to generate disease resistance responses in plants. Because of the need for greater quantities of chitosan to deliver molecules to the nucleus, the use of chitosan

as an external treatment to activate defense genes differs from its purely physical attachment action of complexing copper sulfate and dispersing its effect on the leaf surface. The latter mode of action of chitosan in plant defense is a prophylactic action related to the antimicrobial action of the attached copper.

Breakdown and persistence of chitosan in the environment.

Chitosan in its natural state is typically composed of approximately 80% glucosamine and 20% N-acetyl glucosamine (or chitin like) sugar residues. Chitinase and chitosanases, enzymes found in plant tissue and microbes, have the potential to break the β-1, 3 bonds. However the most efficient digestion of chitosan is related to the ability of chitinase or lysozyme to recognize or hydrolyze the chitin-like portion of the molecule. These latter enzymes are abundant in plant and/or microbial cells.

The use of chitosan as a pesticide sticker requires very small amounts of (5 g per acre) for efficacy. These trace amounts of chitosan become subject to the hydrolysis of the enzymes listed above. The major residues released from chitosan degradation are glucosamine and N-acetyl glucosamine. These sugars can be further metabolized as carbohydrate sources for many microorganisms. These molecules have no adverse effects in human nutrition.

3. The probability of environmental contamination during manufacture, use, misuse or disposal of such substance (chitosan).

The manufacture and commercial use of chitosan provides a financial return for the utilization of crab shell wastes, the primary source of chitosan. As a result this manufacture, has made valuable, wastes that once were disposed of in coastal areas in the vicinity of crab meat processing plants.

. The shells are de-mineralized to enrich the content of chitin the primary material of shell. The chitin mother molecule is composed of 20% glucosamine and 80% N-acetyl-glucosamine at one end of the spectrum of the continuum of these two closely related molecules (chitin and chitosan). Chitosan at the other end of the spectrum is typically 80% glucosamine and 20 % N-acetyl-glucosamine The spectrum is adjusted towards the chitosan spectrum using a basic pH which further de-acetylates the N-acetyl-glucosamine residues and allows chitosan molecules to precipitate because of their insolubility in a basic pH. This process is not synthetic, but only partially degradative in the same manner that chitosan develops from chitin in nature (See enclosed article by Salomon Bartnicki-Garcia). The now insoluble chitosan is rendered soluble by dissolving it in dilute acetic acid or other organic acids, which have been approved for use by organic growers. These organic acids are listed on the national list of substances approved for the organic grower. Thus the chitosan manufacturing process described above does not cause environmental contamination and the chitosan, itself, does not pose a disposal problem.

Chitosan is a naturally occurring compound in nature. It is a polymer of glucosamine (poly-D-glucosamine). Chitosan is biosynthesized in abundance in fungi and crustaceans. Chitosan is released from fungal walls when fungi come in contact with plant cells. Fungal chitosan is reportedly biosynthesized from Uridine diphosphate N-acetyl-glucosamine and initially incorporated into chitin (poly-N-acetyl-glucosamine). In

the next step in nature the chitin polymer is partially de-acetylated to chitosan. The difference between chitin and chitosan is only in the two compound's degrees of acetylation.

Chitosan occurs as long polymers of glucosamine (and some N-acetyl-glucosamine) that constitute molecular weights in excess of a million. The positive charges alternating along the length of the molecule alternate from side to side.

5. The efforts of chitosan on biological and chemical interactions in agroecosystems.

Chitosan is a naturally occurring component of the agroecosystem since it is synthesized as a major cell wall component of Mucors a major group of microorganisms it is also a minor component of the walls of most fungi. The proposed use of chitosan as an adjuvant or "sticker" applied to plant leaves in combination with fungicidal components approved for use by organic growers. Chitosan is an effective sticker when applied at approximately 5-10 grams per acre. At this application rate any accumulation of chitosan applications to soil content of total chitosan would be well below detection levels.

6. Alternatives to using chitosan.

There are a number of chemically synthesized sticking agents commercially available that are not currently, or are not likely to be in the future, approved for use by the National Organic Program.

Currently chitosan has the advantage of 1) being a naturally occurring compound 2) possessing demonstrated efficacy at extremely low levels of application and 3) being biodegradable and 4) actually approved for human consumption. Further it is the only compound capable of dramatically reducing application rates of National Organic Program approved components such as copper sulfate. The latter combination is likely to be the only available treatment effective for controlling the devastation of Late Blight epidemics in organically grown potatoes. A copper hydroxide containing fungicide with the label, Kocide was approved for use during the 2002 growing season. The author of this application was informed by a grower/cooperator that Kocide, a copper hydroxide-containing fungicide, was not approved for the 2003 growing season. If copper hydroxide treatment is no longer available the approval of a chitosan/copper sulfate treatment becomes essential as a protectant against Potato Late Blight for the follow reason. Each grower, organic or conventional, must have access to a level of protection to prevent epidemics. If organic growers do not have a treatment that will adequately suppress inoculum levels, they will put all growers at risk. Such situations can generate legal implications. The spores of *Phytophthora infestans*, the causal agent of Potato Late Blight, are carried by wind and rain for long distances. Thus the disease has no borders within a potato-growing region.

There are cultural practices that help reduce the inoculum levels *of Phytophthora infestans*.

1. Elimination of cull potato piles that may be infected.
2. Crop rotations of 4 years with potatoes planted only one of the years.
3. Eliminate potato plants near the center of a circle irrigation pivot.
4. Space potato plants to aerate the plant canopy.

5. Restrict irrigation to the minimum.
6. Develop good sanitation practices through out the entire potato production system.

Although all of these procedures are helpful they are inadequate to prevent epidemics especially during a cool wet season. Once started a Late Blight epidemic becomes devastating to all unprotected growers within a region. Conventionally-bred and genetically-modified potatoes with improved Late Blight resistance have been publicized and may eventually be adapted. The above alternatives are the only ones that this applicant is aware of currently.

7. The chitosan adjuvant (sticker) is compatible with systems of sustainable agriculture.

Chitosan has no known beneficial or detrimental effect on any insect pest. It has no detrimental effect on the nutrient uptake or development of crop plants. Extremely high concentrations of chitosan have been tested with some positive effects as a material to prevent soil erosion. Chitosan applied to wheat seeds can enhance accumulation of and increase root diameters. Chitosan has been reported to protect stored fruits from deterioration, when sprayed at concentrations from 0.2 mg/ml to 1 mg/ml water. Again these levels are far above the 0.050 mg/ml proposed for its use as an adjuvant (sticker). Note: The term "adjuvant" has been used synonymous with "sticker" to describe its function in securing fungicides to the leaf surface in agronomic applications. The term adjuvant has also been used with a different meaning in the agriculture (animal) literature to indicate chitosan's ability to enhance the immune response (anti-body formation etc.) in animals.

Registation
Reregistration
Pesticide-Producing Establishments
Laws
International Issues
Adverse Effects Reporting
Storage & Disposal
Restricted & Canceled Uses
Pesticide Tolerances
Registration Information Sources

Chitosan; Poly-D-glucosamine (128930)

Fact Sheet	Technical Doc	Products	Registrants	Regulatory Activity	FR Notices	Bibliography

SUMMARY

Chitosan is used primarily as a plant growth enhancer, and as a substance that boosts the ability of plants to defend against fungal infections. It is approved for use outdoors and indoors on many plants grown commercially and by consumers. The active ingredient is found in the shells of crustaceans, such as lobsters, crabs, and shrimp, and in certain other organisms. Given its low potential for toxicity and its abundance in the natural environment, chitosan is not expected to harm people, pets, wildlife, or the environment when used according to label directions.

Issued: 6/03

I. DESCRIPTION OF THE ACTIVE INGREDIENT

Chitosan (poly-D-glucosamine) is one of the most common polymers found in nature. Structurally, it is related to cellulose, which consists of long chains of glucose molecules linked to each other. In chitosan, the building block of the chains is a slightly modified form of glucose. [For another pesticide active ingredient structurally related to chitosan and cellulose, see **chitin**, also called poly-N-acetyl-D-glucosamine.] Like chitin, chitosan is present in the shells of all crustaceans and insects, and in certain other organisms including many fungi, algae, and yeast. Commercially, chitosan is prepared from chitin, which is isolated from the shells of crustaceans after the edible parts have been removed.

OPP Chemical Code: 128930 ; (CAS# 9012-76-4)

II. USE SITES, USES, TARGET PESTS, AND APPLICATION METHODS

Use Sites: Many field crops, ornamentals, and turf grown in fields, home gardens, nurseries, and other sites.

Uses: Plant defense booster; plant growth regulator (enhancer).

Target Pests: Helps plant defend against certain fungal diseases, including early and late blight, downy and powdery mildew, and gray mold.

Application Methods: Spray on leaves throughout growing season, with applications every one to two weeks as needed.

III. ASSESSING RISKS TO HUMAN HEALTH

No risks to humans are expected when products containing chitosan are used according to

label directions. In toxicity tests, the only effect seen was slight skin irritation after chitosan ws applied to skin.

IV. ASSESSING RISKS TO THE ENVIRONMENT

Risks to the environment are not expected because chitosan has not shown toxicity in mammals, it is abundant in nature, and it is used in tiny amounts.

V. REGULATORY INFORMATION

Year registered (licensed for sale) as active ingredient: 1986

Number of end products, February 2001: 4

VI. MANUFACTURERS

August Bjornson, DCV, Inc., 3521 Silverside Rd., Wilmington, DE 19810
SafeScience Products, Inc, 31 St James Avenue, 8th floor, Boston, MA 02116-4101

Agent: Bruce Jaeger, ph 301-261-8491

VII. FOR FURTHER INFORMATION, CONTACT

Brian Steinwand
Biopesticides and Pollution Prevention Division (7511C)
Office of Pesticide Programs
Environmental Protection Agency
1200 Pennsylvania Ave, NW
Washington, D.C. 20460
Phone: 703-305- 7973 (or 308-8712)
Fax: 703-308-7026
e-mail: steinwand.brian@epa.gov

DISCLAIMER: *The information in this Biopesticide Fact Sheet is a summary only. Consult the person listed above or the Biopesticides Web Site for more information.*

and this final action was inappropriate, EPA will either propose or take final action finding that the State has not corrected the original disapproval deficiency. As appropriate, EPA will also issue an interim final determination or a final determination that the deficiency has not been corrected. Until EPA takes such an action, the application of sanctions will continue to be deferred and/or stayed.

This action does not stop the sanctions clock that started for this area on September 29, 1993. However, this action will defer the application of the offsets sanctions and will defer the imposition of the highway sanctions. See 59 FR 39832 (Aug. 4, 1994). If EPA publishes a notice of final rulemaking fully approving the State's submittal, such action will permanently stop the sanctions clock and will permanently lift any applied, stayed or deferred sanctions. If EPA must withdraw the proposed full approval based on adverse comments and EPA subsequently determines that the State, in fact, did not correct the disapproval deficiency, the sanctions consequences described in the sanctions rule will apply. See 59 FR 39832, to be codified at 40 CFR 52.31.

II. EPA Action

EPA is taking interim final action finding that the State has corrected the disapproval deficiency that started the sanctions clocks. Based on this action, imposition of the offset sanctions will be deferred and imposition of the highway sanctions will be deferred until EPA's final action fully approving the State's submittal becomes effective or until EPA takes action proposing or disapproving in whole or part the State submittal. If EPA's proposed rulemaking action fully approving the State submittal becomes final, at that time any sanctions clocks will be permanently stopped and any applied, stayed or deferred sanctions will be permanently lifted.

Because EPA has preliminarily determined that the State has corrected the deficiency identified in EPA's limited disapproval actions, relief from sanctions should be provided as quickly as possible. Therefore, EPA is invoking the good cause exception under the Administrative Procedure Act in not providing an opportunity for comment before this action takes effect.[1] 5 U.S.C. 553(b)(3). EPA believes that notice-and-comment rulemaking before the effective date of this action is impracticable and con ary to the public interest. EPA has revie ved the State's submittal and, through its proposed action is indicating that it is more likely than not that the State has corrected the deficiencies that started the sanctions clocks. Therefore, it is not in the public interest to initially im ose sanctions or to keep applied sancti ns in place when the State has most like y done all it can to correct the deficien ies that triggered the sanctions clocks.

Moreover, it would e impracticable to go through notice-a d-comment rulemaking on a findi g that the State has corrected the defi iencies prior to the rulemaking appro ing the State's submittal. Therefore, PA believes that it is necessary to use t e interim final rulemaking process to temporarily stay or defer sanctions wh le EPA completes its rulemaking proces on the approvability of the S ate's submittal. Moreover, with respe t to the effective date of this action, EP A is invoking the good cause exception o the 30-day notice requirement of the APA because the purpose of this no ice is to relieve a restriction. See 5 U. .C. 553(d)(1).

III. Regulatory Proce s

Under the Regulatc y Flexibility Act, 5 U.S.C. 600 et seq., I PA must prepare a regulatory flexibilit analysis assessing the impact f any proposed or final rule on small en ities. 5 U.S.C. 603 and 604. Alternativel , EPA may certify that the rule will not ave a significant economic impact on substantial number of small enti ies. Small entities include small busine ses, small not-for-profit enterprises, an government entities with jurisdic ion over populations of less t an 50,000.

This action tempo rily relieves sources of an additio al burden potentially placed o them by the sanctions provisions of the Act. Therefore, I certify t at it does not have an impact on any sm all entities.

The Office of Man gement and Budget (OMB) has exempte this action from review under Execut ve Order 12866.

List of Subjects in 4(CFR Part 52

Environmental pr tection, Air pollution control, H drocarbons, Intergovernmental r lations, Reporting and recordkeeping r quirements, Ozone, Volatile organic com pounds.

Authority: 42 U.S.C. 7401–7671q.

Dated: April 12, 199 .

John C. Wise,

Acting Regional Admi istrator.

[FR Doc. 95–9706 File 4–18–95; 8:45 am]

BILLING CODE 6560–50–P

[1] As previously noted, however, by this action EPA is providing the public with a chance to comment on EPA's determination after the effective date and EPA will consider any comments received in determining whether to reverse such action.

40 CFR Part 180

[PP 4F4334/R2114; FRL–4941–2]

RIN 2070–AB78

Poly-D-Glucosamine (Chitosan); Exemption from the Requirement of a Tolerance

AGENCY: Environmental Protection Agency (EPA).

ACTION: Final rule.

SUMMARY: This document establishes an exemption from the requirement of a tolerance for residues of the biochemical growth regulator poly-D-glucosamine (hereafter referred to as chitosan) when used as a seed treatment in or on rice. Based on the nontoxic nature of this chemical, the Agency is also establishing an exemption from the requirement of a tolerance for residues of poly-D-glucosamine when used as a pesticide in the production of any raw agricultural commodities. Vanson L.P requested this exemption.

EFFECTIVE DATE: This regulation becomes effective April 19, 1995.

ADDRESSES: Written objections, identified by the document control number, [PP 4F4334/R2114], may be submitted to: Hearing Clerk (1900), Environmental Protection Agency, Rm. M3708, 401 M St., SW., Washington, DC 20460. A copy of any objections and hearing requests filed with the Hearing Clerk should be identified by the document control number and submitted to: Public Response and Program Resources Branch, Field Operations Division (7506C), Office of Pesticide Programs, Environmental Protection Agency, 401 M St., SW., Washington, DC 20460. In person, bring copy of objections and hearing requests to: Rm. 1132, CM #2, 1921 Jefferson Davis Hwy., Arlington, VA 22202. Fees accompanying objections shall be labeled "Tolerance Petition Fees" and forwarded to: EPA Headquarters Accounting Operations Branch, OPP (Tolerance Fees), P.O. Box 360277M, Pittsburgh, PA 15251.

FOR FURTHER INFORMATION CONTACT: By mail: Joanne Miller, Product Manager (PM) 23, Registration Division (7505C), Environmental Protection Agency, 401 M St., SW., Washington, DC 20460. Office location and telephone number: Rm. 237, CM #2, 1921 Jefferson Davis Hwy., Arlington, VA 22202, (703)-305-7830; E-mail: miller.joanne@epamail.epa.gov.

SUPPLEMENTARY INFORMATION: In the Federal Register of November 2, 1994 (59 FR 54907), EPA issued a notice that Vanson L.P., 8840, 152nd Ave.,

Northeast, Redmond, WA 98052, had submitted pesticide petition (PP 4F4334) to EPA proposing that an exemption from the requirement of a tolerance be established for residues of the biochemical growth regulator chitosan when used as a seed treatment on rice.

Chitosan is a naturally occurring substance produced from chitin extracts of crustacean shells (e.g., crab, shrimp, and lobster). The product is intended for use in treatment of seed prior to planting. Plant root growth is stimulated and stem strength enhanced, helping to prevent lodging (when the plants fall over because weak stems are unable to support it) in rice. Plants which lodge are difficult to harvest; therefore, yields may be decreased.

The chemical is taken up by plant cells where it enters the nucleus and stimulates messenger RNA and enzyme production. In the case of rice, such enzymes are thought to be responsible for stimulating the plant to produce more lignin in the stems, resulting in stronger stems and decreased lodging.

The Agency considered the following factors in support of this request for exemption from the requirement of a tolerance: Chitosan (1) is not toxic, as demonstrated in acute toxicity studies in mice, rats, and rabbits; (2) is naturally occurring in the environment in large concentrations; (3) has been exempted from the requirement of a tolerance in or on barley, beans, oats, peas, and wheat (40 CFR 180.1072) when used as a seed treatment at an application rate of 4 oz./100 lbs. seed; (4) has been approved by the State of Oregon for use in unrestricted amounts as a soil amendment (fertilizer), a use not regulated by EPA under the Federal Insecticide, Fungicide, and Rodenticide Act. Certain chitin-based products are permitted to be used in foods as hypocholesterolemic agents, as dietary fiber in low-calorie diets, and as agents to increase the specific loaf volume of bread.

Acceptable daily intake (ADI) and maximum permissible intake (MPI) considerations are not relevant to this exemption request. Therefore, the requirement for an analytical method for enforcement purposes is not applicable to this exemption request.

Chitosan is considered useful for the purpose for which the exemption from the requirement of a tolerance is sought. Based on the information considered, the Agency concludes that establishment of the exemption will protect the public health. Therefore, the regulation is established as set forth below.

Based on the nontoxic nature of this chemical, the Agency is also establishing an exemption from the requirement of a tolerance for residues of poly-D-glucosamine when used as a pesticide in the production of any raw agricultural commodities.

Any person adversely affected by this regulation may, within 30 days after publication of this document in the **Federal Register**, file written objections and/or request a hearing with the Hearing Clerk, at the address given above (40 CFR 178.20). A copy of the objections and/or hearing requests filed with the Hearing Clerk should be submitted to the OPP docket for this rulemaking. The objections submitted must specify the provisions of the regulation deemed objectionable and the grounds for the objections (40 CFR 178.25). Each objection must be accompanied by the fee prescribed by 40 CFR 180.33(i). If a hearing is requested, the objections must include a statement of the factual issue(s) on which a hearing is requested, the requestor's contentions on such issues, and a summary of any evidence relied upon by the objector (40 CFR 178.27). A request for a hearing will be granted if the Administrator determines that the material submitted shows the following: There is a genuine and substantial issue of fact; there is a reasonable possibility that available evidence identified by the requestor would, if established, resolve one or more of such issues in favor of the requestor, taking into account uncontested claims or facts to the contrary; and resolution of the factual issue(s) in the manner sought by the requestor would be adequate to justify the action requested (40 CFR 178.32).

Under Executive Order 12866 (58 FR 51735, Oct. 4, 1993), the Agency must determine whether the regulatory action is "significant" and therefore subject to review by the Office of Management and Budget (OMB) and the requirements of the Executive Order. Under section 3(f), the order defines a "significant regulatory action" as an action that is likely to result in a rule (1) having an annual effect on the economy of $ 00 million or more, or adversely and materially affecting a sector of the economy, productivity, competition, jobs, the environment, public health or safety, or State, local, or tribal governments or communities (also referred to as "economically significant"); (2) creating serious inconsistency or otherwise interfering with an action taken or planned by another agency; (3) materially altering the budgetary impacts of entitlements, grants, user fees, or loan programs or the rights and obligations of recipients thereof; or (4) raising novel legal or policy issues arising out of legal mandates, the President's priorities, or the principles set forth in this Executive Order.

Pursuant to the terms of the Executive Order, EPA has determined that this rule is not "significant" and is therefore not subject to OMB review.

Pursuant to the requirements of the Regulatory Flexibility Act (Pub. L. 96-354, 94 Stat. 1164, 5 U.S.C. 601-612), the Administrator has determined that regulations establishing new tolerances or raising tolerance levels or establishing exemptions from tolerance requirements do not have a significant economic impact on a substantial number of small entities. A certification statement to this effect was published in the **Federal Register** of May 4, 1981 (46 FR 24950).

List of Subjects in 40 CFR Part 180

Environmental protection, Administrative practice and procedure, Agricultural commodities, Pesticides and pests, Reporting and recordkeeping requirements.

Dated: April 3, 1995.

Daniel M. Barolo,

Director, Office of Pesticide Programs.

Therefore, 40 CFR part 180 is amended as follows:

PART 180—[AMENDED]

1. The authority citation for part 180 continues to read as follows:

Authority: 21 U.S.C. 346a and 371.

2. Section 180.1072 is revised to read as follows:

§ 180.1072 Poly-D-glucosamine (chitosan); exemption from the requirement of a tolerance.

(a) An exemption from the requirement of a tolerance is established for residues of the biological plant growth regulator poly-*D*-glucosamine when used as a seed treatment in or on barley, beans, oats, peas, rice, and wheat.

(b) An exemption from the requirement of a tolerance is established for residues of the biological plant growth regulator poly-D-glucosamine when used as a pesticide in the production any raw agricultural commodity.

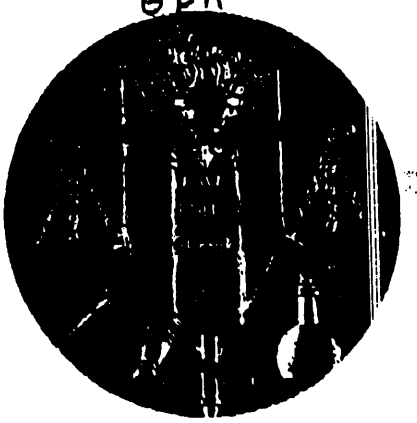
[FR Doc. 95-9165 Filed 4-18-95; 8:45 am]

BILLING CODE 6560-50-F

OFFICIAL PUBLICATION

2001

GDA



Association of

AMERICAN FEED CONTROL OFFICIALS
INCORPORATED

a) does not exceed anhydrous am nonia equivalent to 0.35 percent of the corn plant material,
b) the corn plant material contair 30 to 35 percent dry matter,
c) 75 to 85 percent of the anhydr as ammonia is liquid at ambient pressure during the direct applic tion, and
d) the treated material is used in airy or beef cattle rations.

The labeling of the article must cont in the following information in addition to any other required information:

(1) The name of the article.
(2) The concentration of ammonia.
(3) The maximum percentage of eq ivalent crude protein from nonprotein nitrogen
(4) Directions for use consistent wit 1) (b) and (c), 2) (c), and 3) (d) above, and
(5) A prominent: "Warning--This fe d should be used only in accordance with the directions furnished on t e label. (Proposed 1974, Adopted 1975, Revised 1982, Adopted 19 .) Reg. 573.180

IFN 5-14-511 Ammonia anhydrous

87.12 **Bentonite** is a naturally occu ing mineral consisting primarily of the tri-layered aluminum silicate, montmoril onite. It may contain calcium or sodium as the predominant available or exchan e ion. It is used or intended for use in non-medicated animal feed as an anti-cal ing agent and pelleting aid in an amount not to exceed 2% in total ration. It is not rohibited in medicated animal feed for the same purposes and at the same levels hen it can be demonstrated that it does not interfere with the bioavailability of th medicament to animals and the analysis of the feed for the medicament by acce able methods. It is the manufacturer's responsibility to determine and submit a lequate data to support the conclusion that interference does not occur before u ing it in a feed containing medicaments. Medicaments with which it may current be used are listed in 87.5. (Proposed 1974, Adopted 1975.) Reg. 582.1155

IFN 8-00-695 Bentonite

87.13 **Sodium Bentonite** is a naturall occurring mineral consisting primarily of the tri-layered hydrous aluminum silic te, montmorillonite characterized by a sodium exchange or available ion conter of not less than 1% and not more than 2% of the air dried material. It is used or intended for use in non-medicated animal feed as an anti-caking agent and elleting aid in an amount not to exceed 2% in total ration. To reduce seepage silage, the amount added would not exceed 1% sodium bentonite. It is not p ohibited in medicated animal feed for the same purposes and the same levels w ten it can be demonstrated that it does not interfere with the bioavailability of th medicament to animals and the analysis of the feed for the medicament by acce able methods. It is the manufacturer's responsibility to determine and submit a equate data to support the conclusion that interference does not occur before u ng it in a feed containing medicaments. Medicaments with which it may current be used are listed in 87.5. (Proposed 1974, Adopted 1975, Amended 1983.) R g. 582.1155

IFN 8-14-512 Sodium bentonite

87.14 **Powdered Cellulose** is purifie , mechanically disintegrated cellulose prepared by processing alpha cellulose btained as a pulp from fibrous plant materials. (Proposed 1975, Adopted 197 .)

IFN 1-15-514 Cellulose powdered

87.16 **Chitosan** is a cationic carboi ydrate polymer intended for use as a precipitating agent of proteinaceous ma erial from food processing plants. It is chemically derived by deacetylation of th naturally occurring chitin in crab and

shrimp shells. It may be used in an amount not to exc ed that necessary to accomplish its intended effect. Chitosan when fed as a (omponent of feed to livestock shall be present at no more than 0.1% of the feed. Proteinaceous material coagulated with chitosan must have safety and e ficacy data approved before it can be registered or offered for sale. (Proposed 784, Adopted 1985.)

IFN 8-17-730 Chitosan

87.19 **Urea Formaldehyde Condensation Polymer** is pelleting aid for use in animal feeds, excluding aquatic species. Restrictions: I ot to exceed 0.1 ppm free formaldehyde in the finished pelleted product. (Pro osed 1989, Adopted 1990)

IFN 8-30-422 Urea Formaldehyde Condensation Pol mer

87.17 **Perlite** is the expanded, powdered form of a glassy volcanic rock, consisting essentially of fused sodium potassium aluminiu silicate. It meets the specifications of current edition and supplements of the F d Chemicals Codex. It is used as a filter aid or pressing aid in the processi g of foods and feed ingredients and also may be used as an anti-caking agent. t may not exceed 4% by weight of the product in which it is present as a processir aid. (Proposed 1977, Amended 1978, Adopted 1979.)

IFN 8-26-242 Perlite

Tentative

T87.15 **Formaldehyde Solution** is produced by dis olving about 37% by weight of formaldehyde gas in water usually with 10 to 15 % methanol added to prevent polymerization. (1) It is used to improve the han ing characteristics of animal fat incombination with certain oilseed meals by pro ucing a dry, free-flowing product; an aqueous blend of soybean and sunflower m eals in a ratio of 3:1 is mixed with animal fat in a ratio of 3:2 and formaldehyde (3 % solution) is added at a level of 4% of the dry matter weight of the mixture whic upon drying contains not more than 1% formaldehyde and 12% moisture. Th mixture is used as a component of dry, nonpelleted feeds for beef and nonlac ating dairy cattle. To assure safe use of the additive the label of the mixture sha bear the name of the additive and adequate directions for use providing that fe d as consumed is not to contain more than 25% of the mixture and (2) it is used a the rate of 5.4 pounds (2.5 kilograms) per ton of poultry feed. At this level, it is n antimicrobial agent used to maintain complete poultry feeds salmonella nega ive for up to 14 days. To assure safe use of the additive, in addition to other in ormation required by 21CFR 573.460, the label and labeling shall contain: [a] th name of the additive, [b] a statement that formaldehyde solution which has bee stored below 40 F or allowed to freeze should not be applied to complete oultry feeds, and [c] adequate direction for use including a statement that fo maldehyde should be thoroughly mixed into complete poultry feeds and that th finished poultry feed shall be labeled as contains formaldehyde. (Proposed 1977, Adopted 1978, Amended 1996.)

IFN 8-26-243 Formaldehyde solution

T87.18 **Reed-Sedge Peat** is a natural, partially decon posed plant material, formed from a mixture of reeds, sedges, grasses and ome hypnum mosses occurring in wetlands and containing one third to two thir peat fibers. It should be dehydrated to a moisture content of not more than 15% and be in a state free from all harmful micro-organisms. It is intended for use in animal feed as a carrier for liquid products and premixes or as a nutritional dilu nt for lowered energy diets at a level not to exceed 5% of the total daily ration. (Proposed 1986)

IFN 1-18-898 Peat Whole Dehydrated

Manufacturer Name and Address: **VANSON, INC.** **8840 152nd AvenueNE** **Redmond, Washington 98052** **Emergency Phone: 425-881-6464**	**Material Safety Data Sheet** **CHITOSAN**
1 Product Identification	
Trade name: **Chitosan**	
2 Composition	
Chitosan Chemical names: Source	CAS # 9012-76-4 beta-(1,4)-2-amino-2-deoxy-D-glucose, or poly-D-glucosamine, or poly N-acetyl-D-glucosamine Chitin extracted from recycled crab and shrimp shells.
3 Physical/Chemical Characteristics	
Appearance and odor Solubility Chemical formula Molecular weight Density	Chitosan a fine, off-white; odorless; and tasteless powder. Insoluble in water and alcohols Soluble in dilute organic acids. $C_6H_{11}NO_4$ 161 0.5 - 0.6 g /cc
4 Toxicological Data	
Acute oral, LD50 (mice)	> 10 g / kg

5 Fire & Explosion Hazard Data	
Flash Point	
Flammability	Not Applicable
	Keep away from oxidizing agents and avoid open flames. Product may ignite at temperatures In excess of 400° F.
Unusual Fire and Explosion Hazards	Depending on moisture content, and particle size, airborne dust of Chitosan might explode in the presence of an ignition source. It comparable to flour and wood dust.
Fire Fighting Media	Use water, dry chemicals, carbon dioxide, sand, or foam.
6 Health Hazards Information	
Acute Health Effects - Signs and Symptoms of Exposure, Emergency and First Aid Procedures	EYE CONTACT: Chitosan powder may cause mechanical irritation. Treat powder in eye as foreign object. Flush with water to remove. SKIN CONTACT: The powder can cause irritation or rash. Seek medical help if it persists. INHALATION: Chitosan may aggravate preexisting respiratory conditions or allergies. It may accumulate on linings of the nose and lungs resulting in dryness and coughing. Remove to fresh air. Get medical help if persistent irritation or breathing difficulties occur. INGESTION: Not likely to be hazardous if ingested.
Potential Chronic Health Effects	There is no known effect from chronic exposure to this product.
Carcinogenicity	Not listed by ACGIH, IARC, NIOSH, NTP, or OSHA

7 Personal Protective Equipment	

Respiratory protection	A NIOSH/MSHA-approved respirator is recommended when the dust is airborne
Protective gloves	Not required. However cloth or plastic gloves are recommended to minimize potential mechanical irritation from handling.
Eye protection	Goggles are recommended when there is a high level of airborne dust.
Other protective clothing	Not needed
8 Regulatory	
TSCA	Not listed on the TSCA inventory
SARA Section 302	Does not have an RQ or TPQ
SARA Section 313	Not reportable under Section 313
Clean Air Act	This material does not contain any hazardous air pollutants.
Clean Water Act	Not listed as Hazardous Substance, Priority Pollutant or Toxic Pollutant
OSHA	Not considered hazardous
DOT	Shipping name: Chitosan Class: 50, Not regulated
9 Storage, Handling and Disposal	
Storage	Store in a cool, dry place away from open flames and strong oxidizers.
For spills of Chitosan	The material may be vacuumed or collected for recovery or disposal.
Waste disposal method	Land disposal is acceptable. The material is biodegradable. Follow local, state, and federal regulations.
Work / hygiene practices	Follow good hygienic and housekeeping practices. Clean up areas where Chitosan dust settles to avoid excessive accumulation of this combustible material. Minimize blowdown, sweeping, or other practices that generate high airborne dust concentrations.
Prepared by: Gordon Sargent	**Revision Date:** September 7, 1998

Pool & Spa SEA-KLEAR Waste Water Chitin/Chitosan Nutriceuticals MSDS Index

U.S. Environmental Protection Agency

Pesticides

Contact Us

Results of Searching the "Pesticides" Area of EPA's Web S

We have searched the area of EPA's site related to Pesticides and found the following re: search for the same terms across EPA's entire site.

Searched 8225 files for **chitosan**; displaying results 1 - 10 of 28 total matches.

Rank	Score	Title of Highlighted Document and URL
1	1.00	EPA Chitosan; Poly-D-glucosamine (128930) Factsheet http://www.epa.gov/oppbppd1/biopesticides/ingredients/factsheets/factshee **Summary:** Chitosan is used primarily as a plant growth enhancer, and as a boosts the ability of plants to defend against fungal infections. [For another ingredient structurally related to chitosan and cellulose, see chitin, also cal
2	0.86	EPA Chitin; Poly-N-acetyl-D-glucosamine Factsheet http://www.epa.gov/oppbppd1/biopesticides/ingredients/factsheets/factshee **Summary:** [For another pesticide structurally related to chitin and cellulose also known as poly-D-glucosamine.] Chitin is closely related structurally to t chitosan (poly-D-glucosamine), which shows no toxicity to mammals, and i
3	0.82	US EPA, Office of Pesticide Program, Inerts Ingredients Listing http://www.epa.gov/opprd001/inerts/completelist_inerts.pdf **Summary:** 25750- 84- 9 Acrylic acid, butyl ester, polymer with ethylene 4B Acrylic acid, copolymer with butyl acrylate 4B 141754- 64- 5 Acrylic acid, isc ammonium salt 3 25136- 75- 8 Acrylic acid, polymer with acrylamide and di:
4	0.82	Permanent Tolerances by Pesticide:8-05-2002 http://www.epa.gov/oppsrrd1/tolerance/pdf_files/TolUniv8-05-2002.PDF **Summary:** Permanent Tolerances By Pesticide: Aug 1996 TIS Decision CF Name Source Reassess Date Minor Kids Food? 75- 37- 6) D00021 3 Grou COMMODITIES 180.1001C Number of Tolerances for 1,1- DIFLUOROETH NO. PPM PINEAPPLES, FODD
5	0.82	Permanent Tolerances by Pesticide:8-05-2002 http://www.epa.gov/oppsrrd1/tolerance/newpdf/TolUniv8-05-2002.PDF **Summary:** Permanent Tolerances By Pesticide: Aug 1996 TIS Decision CF Name Source Reassess Date Minor Kids Food? 75- 37- 6) D00021 3 Grou COMMODITIES 180.1001C Number of Tolerances for 1,1- DIFLUOROETH NO. PPM PINEAPPLES, FODD
6	0.82	Permanent Tolerances By Pesticide: 7-23-2002 http://www.epa.gov/oppsrrd1/tolerance/Xpdf_files/TolReassesUniv7-23-200 **Summary:** Permanent Tolerances By Pesticide: Aug 1996 TIS Decision CF Name Source Reassess Date Minor Kids Food? PPM CATTLE, FAT 180.1(CATTLE, KIDNEY 180.169 Yes No1.0 CATTLE, LIVER 180.169 Yes No1.0 (EXC. PPM HOPS, DRIED 185
7	0.80	Status of Pesticides in Registration, Reregistration, and Special Review (Ra Spring 1998 http://www.epa.gov/Rainbow/98rainbo.pdf **Summary:** STATUS OF PESTICIDES IN REGISTRATION, REREGISTRA SPECIAL REVIEW (RAINBOW REPORT) TABLE OF CONTENTS Chapter INTRODUCTION .....
8	0.79	EPA: Pesticides - Registered Biopesticides (PPDC) http://www.epa.gov/oppfod01/cb/ppdc/2002/regist-biopes.htm

Summary: Repellents Dried blood Capsaicin Red Pepper Methyl Salicylate Putrescent whole egg solids Methyl anthranilate Allium sativum (Garlic oil) L Cederwood oil Anthraquinone Iron phosphate . kurstaki EG2348 B. thuringi

9 0.77 EPA - Biopesticide Active Ingredient Fact Sheets
http://www.epa.gov/oppbppd1/biopesticides/ingredients/index.htm
Summary: Bacillus cereus Strain BP01 (119802) Bacillus licheniformis Stra (006492) Bacillus popilliae (054502) Bacillus popilliae & Bacillus lentimorbu pumilus strain GB 34 (006493) Bacillus pumilus strain QST 2808 (Petition &

10 0.77 Report of Pesticide Data Submitters in EPA Files by Chemical
http://www.epa.gov/DataSubmittersList/dslmain.pdf
Summary: * DATA TYPES * EU AT EC FW EF OT XX XX XX XX COMPAI DATA TYPES * EU AT EC FW EF OT XX CHEMICAL CHEMICAL NAME 0 COMPANY# 001021 * DATA TYPES * EU AT EC FW EF OT XX XX COMF DATA TYPES * EU AT EC FW EF OT XX XX COMPA

Pages: 1 2 3 [Next]

New Search: Within Pesticides

Advanced Search Try other options for searching Pesticides

EPA Home | Privacy and Security Notice | Contact Us

Search performed on Tuesday, December 16, 2003

Group 3 Tolerance Reassessment Status

		# Tolerances	# Reassessed	# Raised	# Same	# Lower	#Revoke	#TBD
1,12-DODECANEDIOL DIMETHACRYLATE POLYMER	811666	1	1	0	1	0	0	0
1,6-HEXANEDIOL DIMETHYACRYLATE POLYMER	811668	1	1	0	1	0	0	0
12-HYDROXYSTEARIC ACID-- POLYETHYLENE GLYCOL	800251	1	1	0	1	0	0	0
2-BUTENEDIOIC ACID (Z)-, POLYMER WITH ETHENOL	801508	1	1	0	1	0	0	0
A-(P-NONYLPHENYL)W-HYDROSYPOLY(OXYPROPYLENE	790255	1	1	0	1	0	0	0
A-{P-1,1,3,3- TETRAMETHYLBUTYL)PHENYL} POLY(OXYPROPYLEN	900589	1	1	0	1	0	0	0
A-BUTYL-W- HYDROXYPOLY(OXYPROPYLENE BLOCK POLYMER	900430	1	1	0	1	0	0	0
ACETAMIDE	129051	13	0	0	0	0	0	0
ACETIC ACID ETHENYL ESTER, POLYMER WITH ETHENOL	711195	1	1	0	1	0	0	0
ACRYLAMIDE POTASSIUM ACRYLATE--ACRYLIC ACID COPOLYME	800626	1	1	0	1	0	0	0
ACRYLATE POLYMERS AND COPLOYMERS	999999	1	0	0	0	0	0	0
ACRYLIC ACID--SODIUM ACRYLATE--SODIUM-2-METHYLPROPANI	811242	1	1	0	1	0	0	0
ACRYLIC ACID--STEARYL METHACRYLATE COPOLYMER	911640	2	2	0	2	0	0	0
ACRYLONITRILE--STYRENE--HYDROXYJPROPYL METHACRYLATE	800621	1	1	0	1	0	0	0
A-HYDRO-W-HYDROXPOLY(OXYPROPYLENE)	790272	2	2	0	2	0	0	0
A-HYDRO-W-HYDROXYPOLY(OXYETHYLENE)	790271	1	1	0	1	0	0	0
ALKYL (C12-C20) METHACRYLATE- METHACRYLIC ACID COPOLYI	800613	1	1	0	1	0	0	0
ALLYL ISOTHIOCYANATE	004901	1	0	0	0	0	0	0
AMMONIA	005302	4	0	0	0	0	0	0
AMPELOMYCES QUISUALIS	021007	1	0	0	0	0	0	0
ANAGRAPHA FALCIFERA MULTINUCLEAR POLYHEDROSIS VIRUS	127885	1	0	0	0	0	0	0
aqueous extract of seaweed meal (Cytokinin)	116801	38	38	0	0	0	38	0
Arthropod pheromones	000003	1	0	0	0	0	0	0
Autographa californica NPV	128885	1	0	0	0	0	0	0
Azadirachtin	121701	1	0	0	0	0	0	0
BACILLUS POPILLIAE AND B LENTIMORBUS	054501	2	0	0	0	0	0	0
Bacillus subtilis GB03	129068	1	0	0	0	0	0	0
Bacillus subtilus MBI 600	129082	1	0	0	0	0	0	0
BACILLUS THURINGIENSIS	006401	2	2	0	2	0	0	0
BACILLUS THURINGIENSIS CRYIAC DELTAENDOTOXIN AND THE	006445	1	0	0	0	0	0	0
BACILLUS THURINGIENSIS CRYLAB DELTAENDOTOXIN AND THE	006458	3	0	0	0	0	0	0
BACILLUS THURINGIENSIS CRYLAB DELTAENDOTOXIN AND THE	006400	1	1	0	1	0	0	0
Bacillus thuringiensis subspecies tenebrionis delta endotoxi	006432	1	0	0	0	0	0	0
BACILLUS THURINGIENSIS VARIETY KURSTAKI	006402	1	1	0	1	0	0	0
BACILLUS THURINGIENSIS VARIETY SAN DIEGO	128946	1	0	0	0	0	0	0
BASIC COPPER SULFATE	008101	1	0	0	0	0	0	0
BEAUVARIA BASSIANA	128924	16	0	0	0	0	0	0
BEET ARMYWORM NPV POLYHEDRAL INCLUSION BODIES OF SP	129078	1	0	0	0	0	0	0
BENTAZON	103901	45	4	0	0	0	4	0
BENZALDEHYDE	008601	1	0	0	0	0	0	0
Benzoic acid (Tebufenozide)	009101	2	2	0	2	0	0	0
Biochemical Pesticide Plant Volatile Attractant Compounds	202000	1	0	0	0	0	0	0
Bitertanol	117801	1	0	0	0	0	0	0
BORAX	011102	1	0	0	0	0	0	0
BORIC ACID	011001	1	0	0	0	0	0	0
BORIC OXIDE	011002	1	0	0	0	0	0	0
CADRE	128943	1	0	0	0	0	0	0
CALCIUM HYPOCHLORITE	014701	2	0	0	0	0	0	0
CALCIUM POLYSULFIDE	076702	2	0	0	0	0	0	0
Candida oleophila isolate I-182	021008	1	0	0	0	0	0	0
CARBON DIOXIDE	016601	2	0	0	0	0	0	0
CARBON DISULFIDE	016401	4	4	0	4	0	0	0
CELLULOSE ACETATE	811206	1	1	0	1	0	0	0
CHITIN	128991	1	0	0	0	0	0	0
CHITOSAN	128930	8	0	0	0	0	0	0
CHLORINE	020501	1	0	0	0	0	0	0
CINNAMALDEHYDE	040506	1	0	0	0	0	0	0
CITRIC ACID	021801	2	0	0	0	0	0	0
CLARIFIED HYDROPHOBIC EXTRACT OF NEEM OIL	025007	1	0	0	0	0	0	0
CLOPYRALID (Dichloropyridinecarboxylic Acid Alkanolamine)	117401	40	40	0	39	0	1	0
COLLETOTRICHUM GLOEOSPORIOIDES SPORES	226300	2	2	0	2	0	0	0
COMBUSTION PRODUCT GAS	000001	1	0	0	0	0	0	0
COPPER CARBONATE	022901	2	2	0	2	0	0	0
COPPER HYDROXIDE	023401	1	1	0	1	0	0	0
COPPER METALLIC	022501	1	0	0	0	0	0	0
COPPER OXYCHLORIDE	023501	1	1	0	1	0	0	0

Group 3 Tolerance Reassessment Status

		# Tolerances	# Reassessed	# Raised	# Same	# Lower	#Revoke	#TBD
COPPER SULFATE	024401	1	1	0	1	0	0	0
CP ENOLPYRUVYLSHIKIMATEPHOSPHATE AND THE GENETIC MA	817306	1	0	0	0	0	0	0
CRYAC AND CRYC DERIVED DELTAENDOTOXINS OF BACILLUS	006457	1	0	0	0	0	0	0
CRYOLITE	075101	50	50	0	36	0	14	0
CUPRIC OXIDE	042401	1	0	0	0	0	0	0
CUPROUS OXIDE	025601	8	0	0	0	0	0	0
CYDIA POMONELLA GRANULOSIS VIRUS	129090	1	0	0	0	0	0	0
DIATOMACEOUS EARTH	072605	4	0	0	0	0	0	0
DICHLOROPYRIDINECARBOXYLIC ACID (Clopyralid)	117403	5	5	0	5	0	0	0
DICHLOROQUINOLINECARBOXYLIC ACID (Quinclorac)	128974	23	23	0	23	0	0	0
DIFENZOQUAT	106402	22	0	0	0	0	0	0
Dihydro-5-pentyl-2(3H)-furanone	122301	2	0	0	0	0	0	0
DIHYDROAZADIRACHTIN	121702	1	0	0	0	0	0	0
DIMETHYLNAPHTHALENE	054002	1	0	0	0	0	0	0
DIQUAT DIBROMIDE	032201	45	2	0	0	0	2	0
DISODIUM OCTABORATE TETRAHYDRATE	011103	1	0	0	0	0	0	0
DODECENYL ACETATE	129004	1	0	0	0	0	0	0
DODECENYL ACETATE AND ZDODECENOL	128906	4	0	0	0	0	0	0
DRIED FERMENTATION SOLIDS AND SOLUBLES OF MYROTHECIU	119204	1	0	0	0	0	0	0
ETHEPHON	099801	52	7	0	0	0	7	0
ETHYLENE	041901	26	26	0	26	0	0	0
ETHYLENE GLYCOL DIMETHACRYLATE POLYMER	811667	1	1	0	1	0	0	0
ETHYLENE GLYCOL DIMETHYACRYLATE--LAURYL METHACRYLAT	911664	1	1	0	1	0	0	0
FARNESOL	128910	1	0	0	0	0	0	0
FENARIMOL	206600	42	0	0	0	0	0	0
FERBAM	034801	64	37	0	0	0	37	0
FERROUS SULFATE HEPTAHYDRATE	050502	2	0	0	0	0	0	0
FLUMETSULAM	129016	4	0	0	0	0	0	0
flumiclorac-pentyl	128724	5	0	0	0	0	0	0
FUMARIC ACID--ISOPHTHALIC ACID--STYRENE--ETHYLENE PROF	890319	1	1	0	1	0	0	0
GIBBERELLIC ACID	043801	48	48	0	37	0	11	0
GLIOCLADIUM VIRENS G	129000	1	0	0	0	0	0	0
GLYPHOSATE AND ITS METABOLITES	417300	130	130	0	129	0	1	0
GLYPHOSATE ISOPROPYLAMINE SALT	103601	14	14	0	14	0	0	0
GOSSYPLURE (Hexadecadienol acetates)	114103	1	0	0	0	0	0	0
HEPTYLDIHYDROHFURANONE	122302	2	0	0	0	0	0	0
HEXADECENAL (virelure)	120001	1	0	0	0	0	0	0
HEXAZINONE	107201	25	0	0	0	0	0	0
Hydroprene	486300	1	0	0	0	0	0	0
HYDROXYETHYL CELLULOSE	800124	1	1	0	1	0	0	0
HYDROXYPROPYL CELLULOSE	911288	1	1	0	1	0	0	0
HYDROXYPROPYL METHYLCELLULOSE	800125	1	1	0	1	0	0	0
Imidacloprid	129099	45	45	0	45	0	0	0
INDOLEBUTYRIC ACID	046701	34	34	0	0	0	34	0
INERT INGREDIENTS OF SEMIOCHEMICAL DISPENSERS	999998	1	0	0	0	0	0	0
ISOMATEC	129028	2	0	0	0	0	0	0
JOJOBA OIL	067200	1	0	0	0	0	0	0
LACTIC ACID	128929	3	0	0	0	0	0	0
LAGENIDIUM GIGANTEUM MYCELIUM	129084	8	0	0	0	0	0	0
LAURYL METHACRYLATE-- 1,6- HEXANEDIOL DIMETHACRYLATE	811664	1	1	0	1	0	0	0
LEPIDOPTERAN PHEROMONES	000004	1	0	0	0	0	0	0
Limonene	079701	2	0	0	0	0	0	0
MALEIC ACID MONOBUTYL ESTER-- VINYL METHYL ETHER COPO	800728	2	2	0	2	0	0	0
MALEIC ACID MONOETHYL ESTER-- VINLY METHYL ETHER COPO	800727	2	2	0	2	0	0	0
MALEIC ACID MONOISOPROPYL ESTER-VINYL METHYL ETHER	800726	2	2	0	2	0	0	0
MALEIC ANHYDRIDE-- METHYL VINYL ETHER, COPOLYMER	900575	1	1	0	1	0	0	0
MALEIC HYDRAZIDE	051501	3	0	0	0	0	0	0
MEAT MEAL	100628	1	0	0	0	0	0	0
MENTHOL	051601	1	0	0	0	0	0	0
METALAXYL	113501	137	15	0	0	0	15	0
METARHIZIUM ANISOPLIAE STRAIN ESF	129056	3	0	0	0	0	0	0
METHOPRENE	105401	38	1	0	0	0	1	0
Methyl anthranilate	128725	3	0	0	0	0	0	0
METHYL CHLORIDE	053202	1	1	0	0	0	1	0
METHYL FORMATE	053701	2	2	0	0	0	2	0
METHYL METHACRYLATE- 2-SULFOETHYL METHACRYLATE	911160	1	1	0	1	0	0	0

Group 3 Tolerance Reassessment Status

		# Tolerances	# Reassessed	# Raised	# Same	# Lower	#Revoke	#TBD
METHYL METHACRYLATE-- METHACRYLIC ACID-- MONOMETHOX	790370	1	1	0	1	0	0	0
METHYL VINYL ETHER--MALEIC ACID COPOLYMER	800725	2	2	0	2	0	0	0
METHYL VINYL ETHER--MALEIC ACID COPOLYMER CALCIUM SOI	800730	2	2	0	2	0	0	0
METHYLCELLULOSE	900004	2	2	0	2	0	0	0
MINERAL OIL INCLUDES PARAFFIN OIL FROM PETROLEUM	063502	2	0	0	0	0	0	0
NEOMYCINPHOSPHOTOTRANSFERASE II	806303	1	0	0	0	0	0	0
NITROGEN	128934	1	0	0	0	0	0	0
NOSEMA LOCUSTAE	117001	1	0	0	0	0	0	0
OCTADECANOIC ACID, 12-HYDROXY-, HOMOPOLYMER OCTADEC	800257	1	1	0	1	0	0	0
OIL OF LEMON	040518	1	0	0	0	0	0	0
OIL OF ORANGE	040517	1	0	0	0	0	0	0
OXYTETRACYCLINE	006304	2	0	0	0	0	0	0
PARASITOID INSECTS	599990	5	0	0	0	0	0	0
PASTEURIA PENETRANS	006455	1	0	0	0	0	0	0
PELARGONIC ACID	217500	2	2	0	2	0	0	0
PETROLEUM HYDROCARBONS	063503	4	0	0	0	0	0	0
Phosphinothricin acetyltransferase and genetic material	817305	3	0	0	0	0	0	0
PHYTOPHTHORA PALMIVORA	111301	1	0	0	0	0	0	0
PICLORAM	005101	48	48	0	47	0	1	0
PLANT VOLATILES AND PHEROMONE	112401	1	0	0	0	0	0	0
POLY (VINYL PYRROLIDONE)	800163	2	2	0	2	0	0	0
POLY(OXYETHYLENE/OXYPROPYLENE) MONOALKYL(C6-C10)	711683	1	1	0	1	0	0	0
POLY(OXYPROPYLENE) BLOCK POLYMER WITH POLY(OXYETHYL	790274	2	2	0	2	0	0	0
POLY(PHENYLHEXYLUREA), CROSS-LINKED; MINIMUM	811210	1	1	0	1	0	0	0
POLY(VINYLPYRROLIDONE-1- HEXADECENE)	700608	1	1	0	1	0	0	0
POLY(VINYLPYRROLIDONE-1-EICOSENE	846941	1	1	0	1	0	0	0
POLYACRYLIC ACID	900071	1	1	0	1	0	0	0
POLYETHYLENE	800152	2	2	0	2	0	0	0
POLYETHYLENE GLYCOL--POLYISOBUTENYL ANHYDRIDE--TALL	790257	1	1	0	1	0	0	0
POLYETHYLENE, OXIDIZED	900305	1	1	0	1	0	0	0
POLYHEDRAL INCLUSION BODIES OF HELIOTHIS NUCLEAR	107301	2	0	0	0	0	0	0
POLYMETHYLENE POLYPHENYLISOCYANATE, POLYMER	911208	1	1	0	1	0	0	0
POLYOXYETHYLENE	127101	7	0	0	0	0	0	0
POLYPROPYLENE	900397	1	1	0	1	0	0	0
POLYSTYRENE	800145	2	2	0	2	0	0	0
POLYTETRAFLUOROETHYLENE	911358	1	1	0	1	0	0	0
POLYVINYL ACETATE	800146	1	1	0	1	0	0	0
POLYVINYL ACETATE--POLYVINYL ALCOHOL COPOLMER	700167	1	1	0	1	0	0	0
POLYVINYL ALCOHOL	801504	2	2	0	2	0	0	0
POLYVINYL CHLORIDE	800179	3	1	0	1	0	0	0
POTASSIUM SALTS OF FATTY ACIDS C	079021	1	0	0	0	0	0	0
PROMALIN	116901	1	0	0	0	0	0	0
PROPIONIC ACID	077702	40	0	0	0	0	0	0
PSEUDOMONAS CEPACIA TYPE WISCONSIN	006419	1	0	0	0	0	0	0
PSEUDOMONAS FLUORESCENS NATURAL OCCURRING STRAIN	006418	3	0	0	0	0	0	0
Pseudomonas fluorescens Strain NCIB 12089	006420	1	0	0	0	0	0	0
Pseudomonas syringae	006441	1	0	0	0	0	0	0
PUCCINIA CANALICULATA	129085	1	0	0	0	0	0	0
PUTRESCENT WHOLE EGG SOLIDS	105101	1	0	0	0	0	0	0
PYRETHRUM POWDER OTHER THAN PYRETHRINS	069002	1	0	0	0	0	0	0
PYRIDATE	128834	7	7	0	6	0	1	0
RED PEPPER	070703	1	0	0	0	0	0	0
SESAME PLANT GROUND	128970	32	0	0	0	0	0	0
Sodium 5-nitroguiacolate	129075	7	0	0	0	0	0	0
SODIUM BORATE	011104	1	0	0	0	0	0	0
SODIUM CARBONATE	073506	2	0	0	0	0	0	0
SODIUM CARBOXYMETHYLCELLULOSE	790301	1	1	0	1	0	0	0
SODIUM CHLORIDE	013905	2	0	0	0	0	0	0
SODIUM DIACETATE (Acetic Acid)	044008	12	0	0	0	0	0	0
SODIUM HYPOCHLORITE	014703	2	0	0	0	0	0	0
SODIUM METASILICATE	072604	2	0	0	0	0	0	0
Sodium o-nitrophenolate	129076	7	0	0	0	0	0	0
Sodium p-nitrophenolate	129077	7	0	0	0	0	0	0
STEARYL METHACRYLATE--1,6- HEXANEDIOL DIMETHACRYLATE	811669	1	1	0	1	0	0	0
STREPTOMYCES GRISEOVIRIDIS	129069	1	0	0	0	0	0	0
STREPTOMYCIN	006306	5	0	0	0	0	0	0

Group 3 Tolerance Reassessment Status

		# Tolerances	# Reassessed	# Raised	# Same	# Lower	#Revoke	#TBD
STYRENE-2-ETHYLHEXYL ACRYLATE- GLYCIDYL METHACRYLATE	811159	1	1	0	1	0	0	0
SULFONIUM TRIMETHYLSALT WITH NPHOSPHONOMETHYLGLYC	128501	9	9	0	9	0	0	0
SULFUR	077501	2	0	0	0	0	0	0
SULFURIC ACID	078001	3	0	0	0	0	0	0
SULFURIC ACID MONOUREA ADDUCT	128961	1	0	0	0	0	0	0
TARTAR EMETIC	006201	3	0	0	0	0	0	0
TRICHLOROETHANE	081201	1	0	0	0	0	0	0
TRICHODERMA VIRIDE SENSU BISBY	128903	13	0	0	0	0	0	0
TRIDECENYL ACETATE	121901	1	0	0	0	0	0	0
VINYL ACETATE--ALLYL ACETATE-- MONOMETHYL MALEATE	801520	1	1	0	1	0	0	0
VINYL ACETATE--ETHYLENE COPOLYMER	911190	1	1	0	1	0	0	0
VINYL ACETATE--VINYL ALCOHOL ALKYL LACTONE COPOLYMER	801507	1	1	0	1	0	0	0
VINYL ALCOHOL--DISODIUM ITACONATE COPOLYMER	801509	1	1	0	1	0	0	0
VINYL ALCOHOL--VINYL ACETATE-- MONOMETHYL MALEATE	801519	1	1	0	1	0	0	0
VINYL PYRROLIDONE DIMETHYLAMINOETHYLMETHACRYLATE	700839	2	2	0	2	0	0	0
Watermelon mosaic virus-2 coat protein as produced in cucurb	006442	1	0	0	0	0	0	0
XYLENE	086802	1	0	0	0	0	0	0
ZIRAM	034805	55	0	0	0	0	0	0
Zucchini yellow mosaic virus caot protein as produced in cuc	006443	1	0	0	0	0	0	0
Total		1488	679	0	509	0	170	0

Supplemental information

MSDS Solutions Leading Online Management & Support Solutions...

Search Results

Product keyword(s) ..... **CHITOSAN**

Items found **29**

		Product Name	Manufacturer Name	Issue Date	Language	Item No.	
■	21	CHITOSAN, MEDIUM MOLECULAR WEIGHT	Sigma Aldrich Fluka Supelco /Riedel-de Haen(CAN) *	2003-07-01	English	248513	◆
■	22	CHITOSAN OLIGOSACCHARIDE LACTATE	Sigma Aldrich Fluka Supelco /Riedel-de Haen(CAN) *	2003-07-01	English	284408	◆
■	23	Chitosan Practical Grade From Crab Shells	Sigma Aldrich - (Fluka/Ronkonkoma/RdH Laborchemikalien) U.S.A.*	2003-07-01	English	117799	◆
■	24	CHITOSAN PRACTICAL GRADE FROM CRAB SHELLS	Sigma Aldrich Fluka Supelco /Riedel-de Haen(CAN) *	2003-07-01	English	218818	◆
■	25	GLYCOL CHITOSAN	Sigma Aldrich - (Fluka/Ronkonkoma/RdH Laborchemikalien) U.S.A.*	2003-07-01	English	105185	◆
■	26	GLYCOL CHITOSAN	Sigma Aldrich Fluka Supelco /Riedel-de Haen(CAN) *	2003-07-01	English	219304	◆
■	27	METHYLGLYCOL CHITOSAN	Sigma Aldrich - (Fluka/Ronkonkoma/RdH Laborchemikalien) U.S.A.*	2003-07-01	English	157592	◆
■	28	METHYLGLYCOL CHITOSAN	Sigma Aldrich Fluka Supelco /Riedel-de Haen(CAN) *	2003-07-01	English	254186	◆
■	29	RITA CHITOSAN	R.I.T.A. Corporation	1996-07-30	English	314186	◆

Legend : ■ Product ◆ Synonym

Results for Search Question:
RN: 9012-76-4 AND chitosan, adjuvant

Answer Page

Save **these search terms for future use**

Total Hits: **17** [Show Answers By Database]

Too many answers? Refine Your Search Show Duplicates

Clear	**Titles from AGRICOLA** in **Most Recent Order**
☐ 1	Fining treatments of white wines by means of polymeric adjuvants for their stabilization against browning. [$1.65]
	Titles from BIOSIS in **Most Recent Order** | Best Match Order
☐ 2	Biodegradable microspheres of curcumin for treatment of inflammation. [$2.60]
☐ 3	Chitosan and its derivatives in mucosal drug and vaccine delivery. [$2.60]
☐ 4	Chitosan as an adjunct to dietary treatment of obesity. [$2.60]
☐ 5	Influenza vaccine compositions. [$2.60]
☐ 6	Fining treatments of white wines by means of polymeric adjuvants for their stabilization against browning. [$2.60]
☐ 7	Immune stimulating activity of two new **chitosan** containing **adjuvant** formulations. [$2.60]
☐ 8	Chitosan induced immunopotentiation. [$2.60]
☐ 9	Chitosan induced immunopotentiation. [$2.60]
☐ 10	Chitosans as nasal absorption enhancers of peptides: Comparison between free amine chitosans and soluble salts. [$2.60]

[Page 1] [2] [Next]

Display Selection Selected on all pages Display Format Standard Display Style STNEasy

Display Answers

Results for Search Question:
RN: 9012-76-4 AND chitosan, adjuvant

Answer Page

Save **these search terms for future use**

Total Hits: **17** [Show Answers By Database]

Too many answers? Refine Your Search Show Duplicates

Clear	**Titles from BIOSIS** in **Most Recent Order** | Best Match Order
☐ 11	Effects of various **adjuvants** (lactic acid, glycerol, and **chitosan**) on the injectability of a calcium phosphate cement. [$2.60]
☐ 12	A study of embolizing materials for chemo-embolization therapy of hepatocellular carcinoma: Antitumor effect of cis-diamminedichloroplatinum(II) albumin microspheres, containing chitin and treated with chitosan on rabbits with VX-2 hepatic tumors. [$2.60]
☐ 13	IMMUNOADJUVANT PROPERTIES OF CHITOSAN. [$2.60]
	Titles from CAplus (Food & Agriculture focus) in **Most Recent Order** | Best Match Order
☐ 14	Composite natural organic fertilizer [$4.05]
☐ 15	Preparation of multifunctional nutrition agent for animal and plant use [$4.05]
☐ 16	Immunoadjuvant activities by chitin and chitosan [$4.05]
☐ 17	Agrochemical adjuvants containing charcoal fine powders and/or porous inorganic powders, bentonite, and water. [$4.05]

[Prev] [1] **[Page 2]**

Display Selection: Selected on all pages | Display Format: Standard | Display Style: STNEasy

Display Answers

Display without Links | Return to Results

Display Page

Display from REGISTRY

ANSWER 1 © 2003 ACS on STN

CAS Registry Number

9012-76-4 REGISTRY

Deleted Registry Number

57285-05-9

Chemical Name

Chitosan (8CI, 9CI) (CA INDEX NAME)

100D-VL

Amidan

BC 10

BC 10 (polysaccharide)

Biopolymer L 112

Chicol

Chitan, N-acetyl-

Chitin, N-deacetyl-

Chitoclear

Chitoclear 400

Chitofos

Chitolaze

Chitopearl 3510

Chitopearl BC 3000

Chitopearl BCW 2500

Chitopearl BCW 3000

Chitopearl BCW 3500

Chitopearl BCW 3505

Chitopearl BCW 3507

Chitopearl K 20

Chitosan 500

Chitosan CLH

Chitosan EL

Chitosan F

Chitosan FL

Chitosan H

Chitosan LL

Chitosan LL 80

Chitosan LLWP

Chitosan M

Chitosan MP

Chitosan PSH

Chitosan SK 10

Chitosan VL

Chitosan WL-M

Chitosol
Chitosom
Crystan LA-S
CTA 1 Lactic Acid
CTA 4
DAC 50
DAC 70
Daichitosan 100DVL
Daichitosan DVL
Daichitosan P-VL
Daichitosan VL
Daichitosan VLA
Daichitosan W 10
Deacetylchitin
FCM 117

Molecular Formula

Unspecified

CAS REGISTRY

Search for information related to CAS RN **9012-76-4** in these categories:

- Agriculture [$2.00]
- Biotechnology [$2.00]
- Business [$2.00]
- Chemical Catalogs [$2.00]
- Chemical Substances [$2.00]
- Chemistry References [$2.00]

Restricted to: NO RESTRICTIONS

Preparation
Industrial Manufacture
Synthetic Preparation
Formation, Nonpreparative
Purification

- Chemistry, Analytical [$2.00]
- Drug Names [$2.00]
- Drug News [$2.00]
- Engineering, Chemical [$2.00]
- General Science [$2.00]
- Life Sciences [$2.00]
- Paper & Pulp Technology [$2.00]
- Patents [$2.00]
- Patents, Full Text [$2.00]
- Patents, National [$2.00]
- Pharmaceuticals [$2.00]
- Pharmacology [$2.00]
- Physics [$2.00]
- Regulated Chemical Lists [$2.00]
- Regulations, Government [$2.00]
- Toxicology [$2.00]

Results for Search Question:
RN: 9012-76-4 AND chitosan, sticker

Answer Page

Save **these search terms for future use**

Total Hits: **0** [Show Answers By Database]

The query you have entered has generated no answers. Use your browser's Back button to edit your query.

Biomedical

Vanson HaloSource is the world's premier producer of biomedical Chitin and Chitosan and their derivatives. Our CGMP manufacturing capability and our world-class staff of scientists and researchers have helped companies create the most innovative products for biomedical and healthcare applications.

BIOMEDICAL APPLICATIONS

Chitin- and Chitosan-Based Biomedical Applications

Chitosan, a versatile derivative, has been recognized for over 200 years in the treatment of wounds and burns. Chitin wound dressings have been used for burn victims to speed healing and prevent the formation of scar tissue. These chitosan-based products have been used in forms such as gels, powders, and viscous liquids.

Hemostasis

Chitosan can be referred to as a polycation. Since the early 1950s, polycations have been known to bind to red blood cells. Many studies since have shown that polycations are effective cellular agglutinating agents. In the early 1960s, chitosan was investigated for its agglutinating and binding abilities. It was found that chitosan, even at very low concentrations, had the ability to agglutinate red blood cells. This led to chitosan's consideration as a hemostatic agent. The agglutination of red blood cells by polycations is dependent both on polymer structure and molecular weight. Out of six common polycations, only chitosan was able to effectively initiate gel formation of heparinized blood.

Wound Healing

Chitin has been found to have an acceleratory effect on the wound healing process. Regenerated chitin fibers, non-woven mats, sponges, and films show an increase in wound healing. Chitin can also be used as a coating on normal medical materials. Standard silk and catgut sutures coated with regenerated chitin or chitosan show wound-healing activities only slightly lower than the all-chitin fibers. Surgical gauze coated with regenerated chitin demonstrates a substantially greater amount of activity than an uncoated control group.

Burn Treatment

Chitosan is a very attractive candidate for burn treatment. This is true since chitosan can form tough, water-absorbent, biocompatible films. These films can be formed directly on the burn by application of an aqueous solution of chitosan acetate. The solution, although acidic, provides a cool and pleasant soothing effect when applied to the open wounds of burn patients. Another advantage of this type of chitosan treatment is that it allows excellent oxygen permeability. This is important to prevent oxygen-deprivation of injured tissues. Additionally, chitosan films have the ability to absorb water and are naturally degraded by body enzymes. This fact means that the chitosan need not be removed. In most injuries (and especially burns), removing the wound dressing can cause damage to the injury site.

PARTNER WITH US

Vanson HaloSource is always looking for strategic partners to develop new and innovative products for the biomedical markets.

Click here to learn more about how to partner with Vanson HaloSource.

ChemPort® CONNECTION

Here is the document you requested...

Chitosan as an adjunct to dietary treatment of obesity. . International Journal of Obesity, (May, 2001) Vol. 25, No. Supplement 2, pp. S105. print. Meeting Info.: 11th European Congress on Obesity. Vienna, Austria. May 30-June 02, 2001. CODEN: IJOBDP. ISSN: 0307-0565. ; English

- Send a colleague this reference

Journal

- International Journal of Obesity

Publisher

- Nature Publishing Group

- Logoff
- ChemPort Help
- About

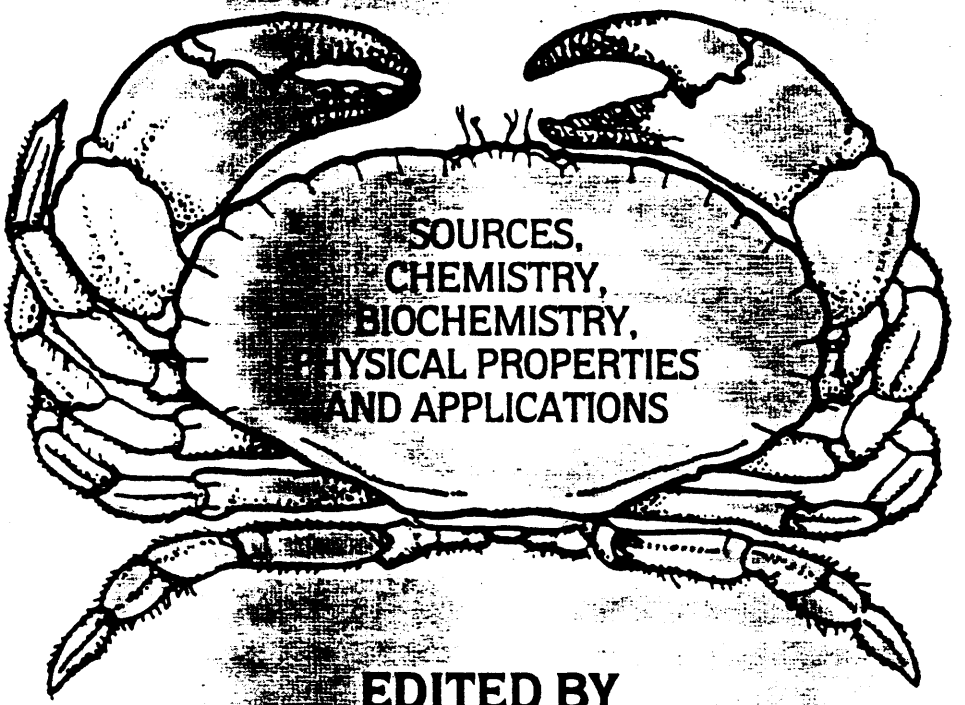
Here are the options for the document you requested...

Fee-based document services

- Order Document

One of the symposia that occur world-wide about twice each year

CHITIN AND CHITOSAN



EDITED BY
GUDMUND SKJÅK-BRÆK
THORLEIF ANTHONSEN
PAUL SANDFORD

ELSEVIER APPLIED SCIENCE

CHITIN AND CHITOSAN

Sources, Chemistry, Biochemistry, Physical Properties and Applications

The importance of chitin and chitosan has grown partly because they represent a renewable and biodegradable source of materials, and partly because of the recent increased understanding of their functionality in biology and in technological, biotechnological and medical applications. As the second most abundant natural polymer, chitin, and its derivative chitosan, represent a great challenge both to the scientific community and to industry.

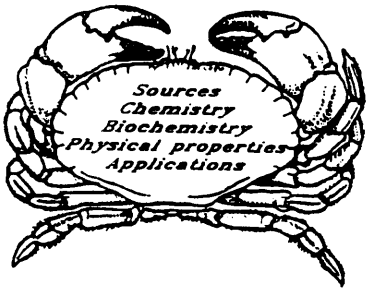
The previous three International Conferences on Chitin and Chitosan were held at MIT, Cambridge, USA (1977), Sapporo, Japan (1982) and Ancona, Italy (1985). The 4th International Conference on Chitin and Chitosan was held on 22–24 August 1988 at the University of Trondheim, Norway. The response to the 4th International Conference on Chitin and Chitosan was overwhelming. The total number of scientific contributions exceeded the maximum possible number of oral presentations for a three-day meeting with two parallel sessions.

Since the 3rd conference, new chitin/chitosan production capacity has appeared both in the USA and Japan and many new forms of chitosan are now commercially available. This increased interest in commercial applications has stimulated research both at academic and industrial laboratories. Commercialization of chitosan-containing products is stimulating the finding of new sources of chitinous materials and has led to the finding of better ways of recovering chitin from existing sources.

New medical and biotechnology applications have required high purity forms of chitin and chitosan to be made commercially. Currently, commercial suppliers and users are formulating specifications and standards to ensure that their products will meet the criteria of *in vivo* use. New derivatives of chitin and chitosan continue to appear, providing new solubilities and useful properties. With the increasing availability of commercial products, coupled with chitin/chitosan's variety of forms (powder, solution, gel, films, fibre, bead, derivatized), the need for continued research on the basic and applied aspects of chitin/chitosan will inevitably increase. This volume will provide the researcher with the most up-to-date information on this important and rapidly expanding area of research.

ISBN 1 85166 395 9

Proceedings from the 4th International Conference on Chitin and Chitosan
held in Trondheim, Norway, August 22–24 1988



ORGANIZING COMMITTEE

Olav Smidsrød
Thorleif Anthonsen
Paul Sandford
Mentz Indergaard
Sissel Hertzberg
Kjell Morten Vårum
Gudmund Skjåk-Bræk

ADVISORY COMMITTEE

G. Allan
C. J. Brine
L. Hadwiger
C. Jeuniaux
R. Muzzarelli
M. Rinaudo
S. Tokura
P. R. Austin
G. W. Gooday
S. Hirano
D. Knorr
C. Rha
G. Roberts

1989

CHITIN AND CHITOSAN

Sources, Chemistry, Biochemistry, Physical Properties and Applications

Edited by

GUDMUND SKJÅK-BRÆK

Division of Biotechnology, The Norwegian Institute of Technology, Trondheim, Norway

THORLEIF ANTHONSEN

Department of Chemistry, The University of Trondheim, Trondheim, Norway

PAUL SANDFORD

Protan, Inc., Bio Applications Group, Woodinville, Washington, USA

ELSEVIER APPLIED SCIENCE
LONDON and NEW YORK

ELSEVIER SCIENCE PUBLISHERS LTD
Crown House, Linton Road, Barking, Essex IG11 8JU, England

Sole Distributor in the USA and Canada
ELSEVIER SCIENCE PUBLISHING CO., INC.
655 Avenue of the Americas, New York, NY 10010, USA

WITH 178 TABLES AND 313 ILLUSTRATIONS

British Library Cataloguing in Publication Data

International Conference on Chitin and Chitosan (4th: 1988: Trondheim, Norway)
Chitin and chitosan.
1. Chitin. Chitosan
I. Title II. Skjak-Braek, Gudmund III. Anthonsen, Thorleif IV. Sandford, Paul
547.7'82

ISBN 1-85166-395-9

Library of Congress CIP data applied for

Printed in Northern Ireland by The Universities Press (Belfast) Ltd.

CONTENTS

In Chitin and Chitosan, sources, Chemistry Biochemistry, Physical Properties and applications Eds. G. Skjak-Braek T. Anthonsen + P. Sandford Elsevier Applied Science London N.Y. 1989

The Molecular Biology of Chitosan in Plant/Pathogen Interaction and Its Application in Agriculture

Chitosan in fungal-plant interactions?

L. A. Hadwiger, C. Chiang, S. Victory and D. Horovitz

Washington State University

Department of Plant Pathology

Pullman, WA 99164-6430

In nature, chitosan is a component of the walls of some pathogenic fungi. After we found chitosan to possess biological properties crucial for resistance in some plant/pathogen interactions, our efforts have been to develop practical utilizations for these properties and concomitantly generate demand for this compound which is commercially available from the chitin of crustacean shell wastes.

The quickest approach for full utilization of chitosan's biological properties probably resides in research directed towards understanding how chitosan, as a polycationic polymer, interacts with a multiplicity of cellular components to influence cell regulation. However, some progress in developing chitosan seed treatments has been realized by observing beneficial effects via direct application trials (6).

The interactions between pea endocarp tissue and certain formae species of Fusarium solani (which are plant pathogenic fungi) naturally release chitosan which can accumulate in both the fungal cell and the adjacent host plant cells. Chitosan, when applied to Fusarium solani spores or mycelia, inhibits

germination and mycelial growth at less than 10 μg/ml (5). When applied to pea tissue in advance of inoculation with F. solani f. sp. pisi, chitosan induces a host response which develops a complete immunity of the pea to this pathogen, a pathogen which normally grows well on peas. Host processes induced by chitosan include activation of disease resistance response genes, increase in resistance response associated proteins (13), increases in the enzymes chitinase, ß-glucanase (14) and phenylalanine ammonia lyase, and accumulations of pisatin (an antifungal antibiotic) (5), callose and lignin (Fig. 1). The combined responses following chitosan application appear to both aid the vitality of nearby host cells and enhance the plant's potential to degrade the walls of fungi which results in the release of additional chitosan. Virulent pathogens must eventually suppress these host responses, reduce the vitality of host cells and tolerate chitosan, pisatin, lignin and callose accumulations to successfully infect pea tissue.

Chitosan appears to be the major inducer of host immunity. Therefore, the plant genes and their regulatory segments induced by chitosan or non-virulent pathogen spores are probably responsible for developing immunity. Some of these induced genes were cloned in preparation for transfer to other plant species such as potatoes which are also parasitized by some specific potato pathogens. These potato pathogens are readily suppressed when inoculated onto pea plants. The development of immunity is related to the speed at which some of the genes operating in the

(5). When
F. solani f.
lops a
ogen which
by chitosan
es, increase
eases in the
ammonia
tibiotic)
nses follow-
ity of
o degrade
iitional
s these host
:ate chito-
:essfully

immunity.
its induced
ly respon-
genes were
:ies such as
otato
sed when
ty is
ing in the

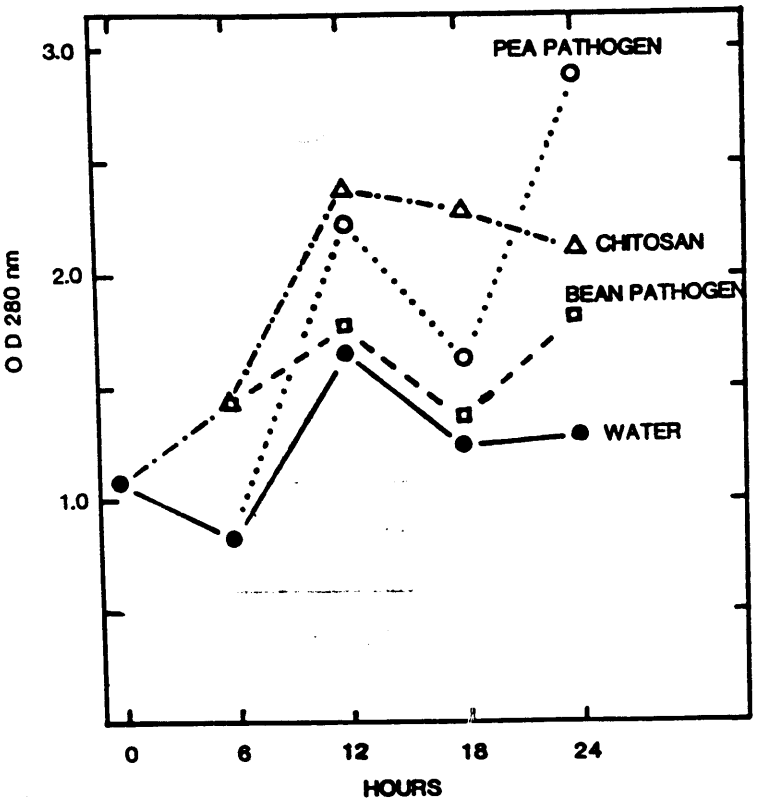


Figure. 1. Relative content of lignin in excised pea pods following treatment with Fusrarium solani or chitosan. The OD_{280} values indicate the relative lignin determined by the lignin thioglycolic acid assay. Two g of pod tissue was prepared in methanol, dried, ground to powder in liquid N_2. The dessicated powder (60 mg) was assayed according to Hammerschmidt (8).

plant response are induced subsequent to inoculation. Thus, the mode of gene induction was investigated. Evidence previously

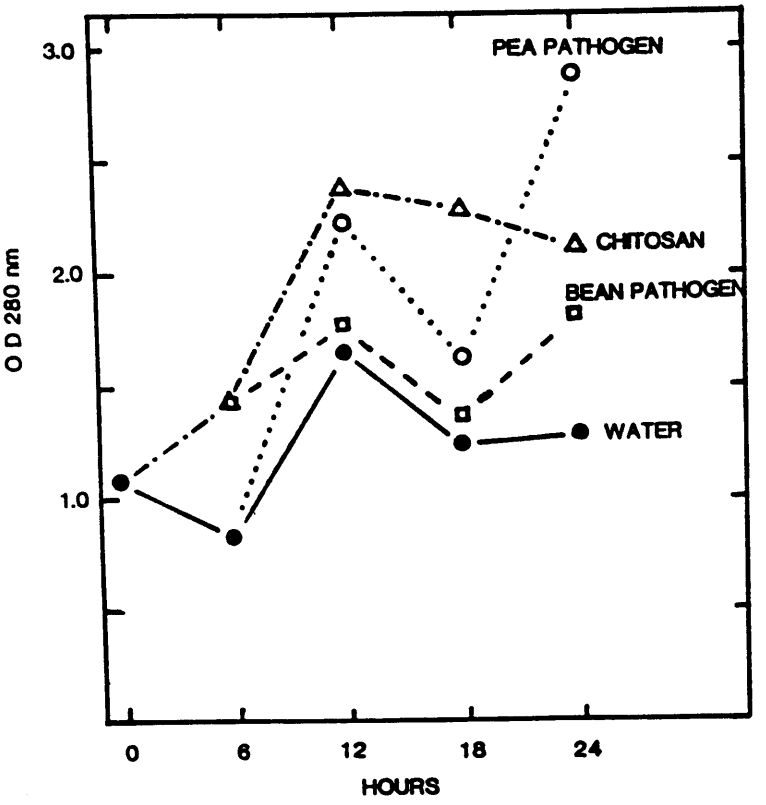


Figure. 1. Relative content of lignin in excised pea pods following treatment with Fusrarium solani or chitosan. The OD_{280} values indicate the relative lignin determined by the lignin thioglycolic acid assay. Two g of pod tissue was prepared in methanol, dried, ground to powder in liquid N_2. The dessicated powder (60 mg) was assayed according to Hammerschmidt (8).

plant response are induced subsequent to inoculation. Thus, the mode of gene induction was investigated. Evidence previously

(10) obtained on 1) cellular localization of chitosan, 2) circular dichroism (CD) spectra and molecular modeling of chitosan\DNA complexes, and 3) sequence analyses of 5' regions of chitosan-induced genes encouraged us to determine if chitosan may directly associate with chromatin. When labelled fungal cell-

Table 1. Localization of glucose-^{3}H labeled F. solani f. phaseoli-cell wall chitosan 5 h after application to pea pods.

Fraction	Total CPM	% of isotope taken up
Applied to pods	389,140	
Wash of pods	174,000	
Adsorbed to pod	215,140	
Taken up by pea cells	184,480	100
Non-particulate aqueous	118,100	64
Microsomal-membrane	11,958	6.4
Chloroplast-mitochondrial	9,751	5.1
Nuclear	35,300[a]	19.1

[a] Counts in nuclear fraction were present as large oligomers which upon hydrolysis yielded primarily glucosamine-^{3}H. Similar percentages were observed in the nuclear fraction when cold chitosan was present in the original extraction buffer.

wall chitosan is applied to pea endocarp tissue 19% of the label taken up by a pea cell localizes in the nucleus (Table 1). This chitosan uptake is associated with nuclear changes usually observed cytologically (4). Chitosan-^{3}H taken up by the cell also accumulates in other cellular fractions. This uptake of cationic polymer can also be expected to also alter membrane components of the cell (11). A possible explanation was that the trauma of chitosan entree into the cell alone may initiate a cascade effect which in turn would activate the beneficial resistance responses in peas. Since the induced responses are able to suppress fungal growth within 4-6 hours after inoculation, events within this period were studied. Applications of high concentrations of chitosan (1 mg/ml) were found to reduce cell viability and eventually alter membrane leakage in pea endocarp tissue, however, the detectable alterations caused by physiological levels of chitosan occurred subsequent to the expression of disease resistance (11). In the absence of detectable traumatic changes, chitosan may alternately affect cell function via the release of secondary messenger signals. The intermediate action of calcium following chitosan application has been investigated by Young and Kauss (25). They propose that chitosan applied to cell suspensions of Glycine max or phaseolus vulgaris causes the release of Ca^{2+} from the cell wall and/or plasma membrane thus altering local Ca^{2+} concentrations releasing intercellular Ca^{2+} which may function as a secondary messenger to trigger gene expression. We found that changes in exogenous Ca^{2+}

levels, blockage of Ca^{2+} channels or inhibition of calmodulin did not alter assayable disease resistance responses induced by chitosan in pea endocarp tissue (12). Obviously many cellular processes subject to alteration by chitosan remain to be investigated.

We have observed the same enhanced induction of a pattern of "disease resistance response" proteins synthesized in peas with either chitosan or DNA specific compounds, such as actinomycin D which intercalates DNA molecules. Thus, the bias of our research direction is toward the possibility that chitosan can directly influence chromatin structure. Again, both direct and indirect influences are possible. Chitosan can readily complex with negative charged cellular proteins or more negatively charged nucleic acids (Fig. 2), either of which could conceivably influence chromatin structure within the nucleus. Some elementary testing has shown that chitosan readily complexes with DNA (5) and, depending on the prevalence and arrangement of base pairs, can alter the circular dichroism spectra which is indicative of conformational changes. Such changes, though somewhat different, are also observed with a related cationic polymer of galactosamine (Fig. 3). Interestingly the polygalactosamine polymer is very inefficient in eliciting pisatin accumulations in peas in comparison to chitosan (Table 2).

Further, it is evident from computer modeling predictions that the structures of these two polycationic molecules have significant steric differences which would likely influence their

modulin did
ced by
cellular
be invest-

a pattern of
peas with
tinomycin D
our research
directly
d indirect
x with
charged
ably in-
elementary
n DNA (5)
ase pairs,
icative of
t different,
galacto-
polymer is
n peas in

edictions
es have sig-
nce their

fit in the minor groove of a DNA molecule (Fig. 4), a likely site for interfacing between DNA and the two polycationic polymers Many DNA regions within the nucleus of untreated cells are attached to native proteins and initially are not fully available for such interaction with carbohydrate molecules. Thus, the presence of chitosan within the nucleus could effect some DNA-protein complexes in addition to any direct effects on DNA.

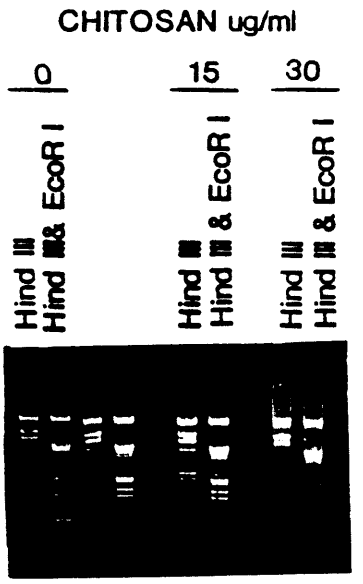


Figure 2. Hind III or Hind III and EcoR I digested phage were electrophoresed in agarose-ethidium gels containing 0, 15, or 30 µg/ml of high M.W. chitosan (unlabelled lanes are repeat separation of lanes 5 and 6.). The disappearance of fragments of lower molecular wt with progressively higher contents of chitosan in the gel, indicates the ability of the gel stabilized chitosan to complex DNA.

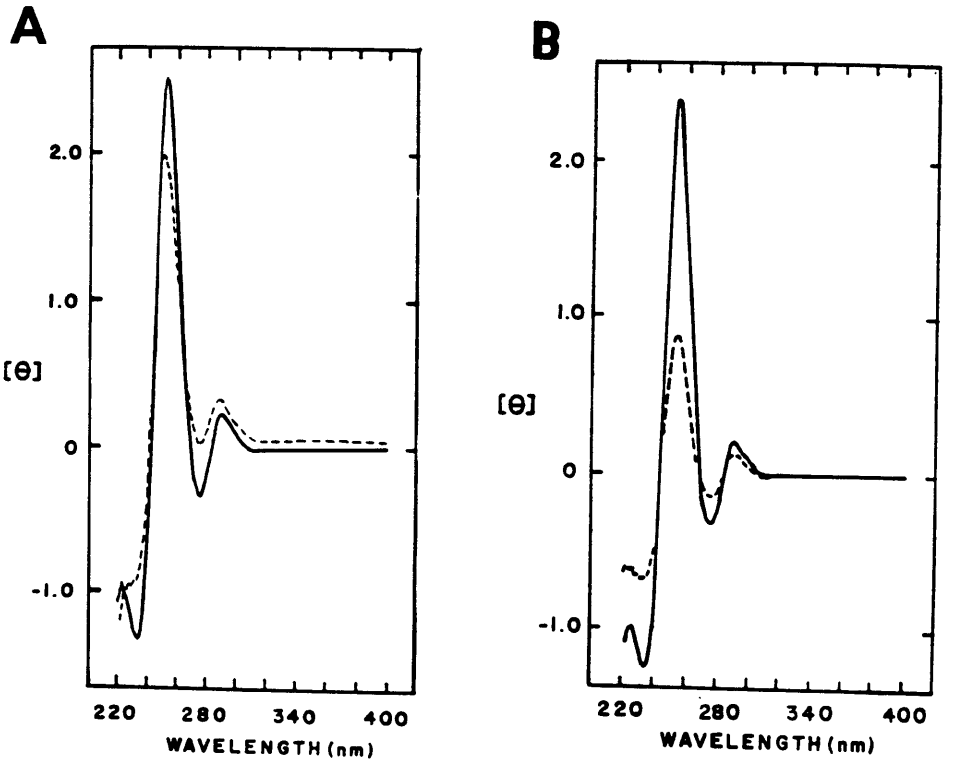


Figure 3. Conformational changes in synthetic DNA indicated by CD spectra in Dichrograph Mark III CS spectrophotometer following addition of polycationic polymers.

The graphs A and B are CD spectra of poly(dG) poly(dC) sequences of DNA (50 μg in 2.6 ml of 0.1% SSC)(solid line) altered by the addition; A of 18 μl of a 1 mg/ml fungal wall chitosan solution (dashed line); B of 18 μl of a 1 mg/ml polygalactosamine solution (dashed line).

Table 2. Relative induction of phytoalexin accumulation (a disease resistance response) in pea endocarp tissue within 24 h by the polycationic molecules chitosan (poly ß 1,4-glucosamine) and poly α 1,4-galactosamine.

Treatment	Concentration Applied mg/ml	Pisatin Accumulation µg/g fr. wt.
H_2O	--	0
Polygalactosamine polymer PF101[a]	2	30 ± 7
	1	23 ± 3
	0.5	15 ± 8
	0.25	15 ± 5
	0.125	13 ± 7
Polyglucosamine (shrimp chitosan)	2	125 ± 5
	1	119 ± 40
	0.5	38 ± 4
	0.25	33 ± 7
F. solani f.sp. phaseoli	1 X 10^6 spores	157 ± 13

[a] Polygalactosamine polymer PF101 was extracted from Paecilomyces sp. I-1d (21) and kindly supplied by Professor Kiyoshi Kadowaki, Higeta Shoyu Co. Ltd. Tokyo, Japan.

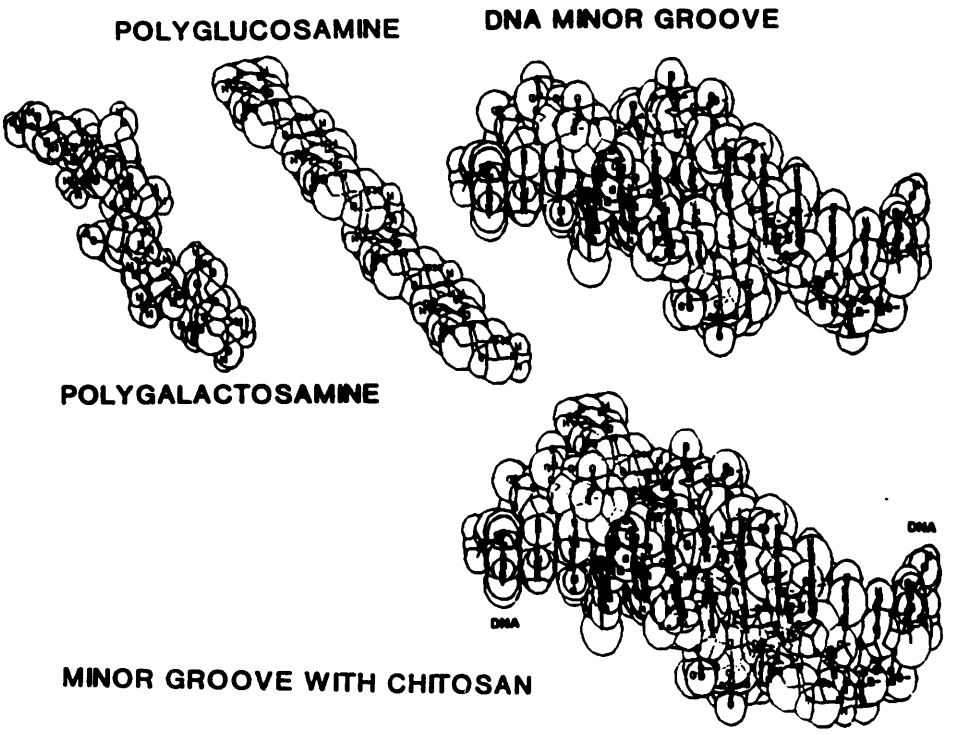


Fig. 4. Computer modeling of galactosamine and glucosamine polymers and a segment of DNA showing the minor groove a potential attachment site for chitosan. The lower model shows that there is apparently room within the minor groove for the computer to interface a 8 sugar unit of the polyglucosamine polymer. (Courtesy of Dr. Susan Johns, CADAM Laboratory, Washington State University.) The distorting effects of the resulting intercharge attractions have not been determined.

Much of the recent work on the initiation of DNA transcription has dealt with poised states of chromatin. Some, but apparently not all gene transcription is dependent on the presence of super helical states of DNA (9) in regions near or sometimes at significant distance from the target gene. Thus, gene regulation is conditioned both by specific DNA sequences and their specific complexing proteins in 5' regions adjacent to the structural gene, and by enhancer regions sometimes a kilobase or greater from the gene. Indeed as a recent report contends, the enhancer region may not need to be on the same chromosome (2). Recent information on nuclear structure brings to mind even more potential nuclear effects possibly associated with the application of highly charged molecules such as chitosan to pea cells. The eucaryotic interphase nucleus is presently visualized as a well organized structure, with specific DNA sequences dictating sites of attachment to the nuclear envelope and to the nuclear super structure called the nuclear matrix or scaffold (15). Chromosomes are oriented with centromeres attached to the nuclear envelope at one pole of the nucleus and telomeres attached to the envelope at the opposite pole. The chromosomes are highly contorted and very closely packed within the nucleus but do not loop around each other. Certain specific chromosomal loci are frequently found attached to the nuclear envelopes. In polytene chromosomes such loci occur almost exclusively at positions of intercalary heterochromatin. These sites are relatively evenly distributed along each chromosome arm, on the average every 10^6

b.p. Chromatin itself is organized in loop domains. Following removal of the majority of chromatin's proteins with high salt, the nuclear DNA remains anchored to a residual proteinaceous scaffold that retains the basic morphological organization of the nucleus. Two proteins, ScI, a 170 Kd protein (topoisomerase II) and ScII, a 135 Kd protein, are major components of the Scaffold of the interphase nuclei. The DNA (a single duplex strand) is attached to this scaffold at numerous sites forming loop. The organization of chromatin into loop domains is stable and conserved feature of interphase and metaphase chromosomes. These DNA sequences for scaffold attachment can be preliminarily identified by looking for clusters of DNA consensus sequences for topoisomerase (16).

The possibility that chitosan can effect the topology of this nuclear organization has been difficult to test because it has not been possible to relate general nuclear changes with the genes actually induced by chitosan. Recent 5' sequence analysis of the chitosan inducible gene PG49 indicates a cluster of sequences closely homologous to the consensus topoisomerase II cleavage sequence GTNA/TAT/CATTNATNNA (16), about 800 bp from a TATA box (C. Chiang amd L. A. Hadwiger, unpublished). DNA segments encompassing these clusters appear to have an affinity for scaffolding preparations from pea nuclei. We are interested in assessing any role (direct or indirect) that chitosan may have in influencing the topology of chromatin regions proximal to the structural sequences whose transcription is enhanced.

Several lines of evidence suggest that DNA topology is important in eukaryotic gene expression (15,17,18,23). Transcriptionally active chromatin segments are hypersensitive to DNase I. When such DNA segments are transferred to super coiled plasmids the corresponding regions remain nuclease sensitive, suggesting that the chromatin DNA which is transcriptionally active contains such super helical regions. DNase-hypersensitive regions can contain binding sites for DNA topoisomerase II. Since topoisomerase II is found in the nuclear scaffold to which the "chromatin loops" are anchored, topoisomerase might have an important role in the regulation of the topology and concomitantly the transcription within these loops (2,9). It has been repeatedly (10,13) observed that super coiled DNA is more highly expressed than linear DNA. The final state of super coiling of intracellular DNA may thus be controlled by two opposing topoisomerases, topoisomerase I (which can interfere with or relax super coiling) and DNA polymerase II (which with ATP and the proper factor promotes negative super coiling) (18).

The super coiling is thought to facilitate the formation of active template by increasing the affinity or stability of DNA-protein or protein-protein interactions. As transcription proceeds the DNA in front of the transcription ensemble (the polymerase, its nascent RNA and RNA-associated proteins) becomes positively super coiled and the DNA behind the ensemble becomes negatively super coiled (24), thus, the state of super coiling is strongly modulated by transcription.

In following the regulatory control of disease resistance responses in peas, pisatin accumulation or phenylalanine ammonia lyase, an enzyme in pisatin synthesis, can be rapidly assayed to estimate the influence of externally applied compounds such as chitosan on the total pea response. Pisatin accumulation and related responses are also induced with compounds (e.g., actinomycin D) which intercalate into DNA (7), or intercalate and form covalent bounds (psoralen derivatives) with pyrimidine bases (3). Chitosan's action on DNA has been established through mutagenesis assays (10). The precise nature of its association is not resolved, but it is likely that it will be attracted by the PO_4 groups and associated with available minor grooves in a manner anologous to other poly cations such as poly-L-lysine, histones, protamine, spermine, etc. Since response genes such as PG49 may have 5' regions stabilized by scaffold attachment, the topography of these genes in the chromosomal loop may well be influenced by localized DNA conformational changes. Additionally, the conformation of the entire loop can be influenced at the point of attachment by the topoisomerases in the scaffold. The topoisomerases can enhance negative super coiling or relax super helicity depending on the enzyme functioning and the availability of ATP (22). This level of control would be in addition to the promoter regions and the nuclear proteins immediately 5' of the structural gene. Various promoters require various amounts of super coiling for optimal function (9). Negative super coiling is the DNA property that is thought to play an important role in

the modulation of specific DNA-protein interactions in many biological processes (22).

Although the control of host responses so outlined appear to be many faceted, their induction by chitosan or intact fungi is remarkably reproducible in pea endocarp tissue. The pisatin accumulation data (Table 3) demonstrates that one or more components, hypothetically effecting the topography of the chromosomal loop, can act synergistic. First novobiocin, an inhibitor of topoisomerase II, is ineffective in inducing pisatin accumulations except when applied prior to chitosan or actinomycin D. Surprisingly novobiocin treatments in advance of chitosan, actinomycin D or direct fungal treatment are able to enhance the pisatin accumulation by all of these elicitors. Presumably, this level of control is localized in the scaffold attachment region. Also low levels of chitosan and low levels of actinomycin D, which individually cannot induce pisatin, when combined (not shown) can induce high accumulations of pisatin. If indeed, chitosan acts in plant cells by altering chromosome loop topology the effects on transcription will be pleiotrophic because of the complexity of gene regulation. Logically, the activity of chitosan is likely to be unpredictable when acting on the differently assembled genomes of other genera. Chitosan has been shown to induce different responses in other plant genera (19). Since the effects of chitosan treatments have been shown to be beneficial on several crop plants, the more information acquired

Physiol. 47:346-351.

8. Hammerschmidt R. 1984. Rapid deposition of lignin in potato tuber tissue as a response to fungi non-pathogenic on potato. Physiol. Plant Pathol. 24:33-42.

9. Hirose, S. and Y. Suzuki. 1988. In vitro transcription of eukaryotic genes is affected differently by the degree of DNA super coiling. Proc. Nat'l. Acad. Sci. USA 85:718-722.

10. Kendra, D. F., B. Fristensky, C. H. Daniels and L. A. Hadwiger. 1987. Disease resistance response genes in plants: expression and proposed mechanisms of induction. In. Molecular Strategies for Crop Protection. Ed. C. A. Ryan and C. Arntzen, Alan R. Liss, Inc. p. 13-24.

11. Kendra, D. F. and L. A. Hadwiger. 1986. Cell death and membrane leakage are not associated with the induction of disease resistance in peas by chitosan. Phytopathology

12. Kendra, D. F. and L. A. Hadwiger. 1987. Calcium and calmodulin may not regulate the disease resistance and pisatin formation responses of Pisum sativum to chitosan or Fusarium solani. Molecular and Physiol. Plant Path. 31:337-348.

13. Loschke, D. C., L. A. Hadwiger and Wendy Wagoner. 1983. Comparison of mRNA populations coding for phenylalanine ammonia lyase and other peptides from pea tissue treated with biotic and abiotic phytoalexin inducers. Physiol. Plant Path. 23:163-173.

14. Mauch, F., L. A. Hadwiger and T. Boller. 1984. Ethylene:

symptom not signal for the induction of chitinase and ß-1,3-glucanase in pea pods by pathogens and elicitors. Plant Physiol. 76:607-611.

15. Newport, John W. and D. J. Forbes. 1987. The nucleus: structure, function and dynamics. Ann. Rev. Biochem. 56:535-565.

16. Phi-Van, L. and W. H. Stratling. 1988. The matrix attachment regions of the chicken lysozyme gene co-map with the boundaries of the chromatin domain. EMBO J. 7:655-664.

17. Pruitt, S. C. and R. H. Reeder. 1984. Effect of topological constraint on transcription of ribosomal DNA in Xenopus oocytes. J. Mol. Biol. 174:121-139.

18. Richet, E., P. Abcarian and H. A. Nach. 1986. The interaction of recombination proteins with super coiled DNA: defining the role of super coiling in lambda integrative recombination. Cell 46: 1011-1021.

19. Ryan, C. A. 1987. Oligosaccharide signalling in plants. Ann. Rev. Cell Biol. 3:295-318.

20. Ryoji, M. and A. Worcel. 1984. Chromatin assembly in Xenopus oocytes: <u>in vivo</u> studies. Cell 37:21-32.

21. Takagi, H. and K. Kadowaki. 1986. Polygalactosamine produced by a microorganism. In. Chitin and nature and technology. Eds. R. Muzzarell and G. W. Gooday. Plenum Press. NY and London. p. 121-128.

22. Wang, J. C. 1985. DNA topoisomerases. Ann. Rev. Biochem. 54:665-697.

23. Weintraub, H. 1985. Assembly and propagation of repressed and derepressed chromatin states. Cell 42:705-711.

24. Wu, H.-Y., S. Shyy, J. C. Wang and L. F. Liu. 1988. Transcription generates positively and negatively super coiled domains in the template. Cell 53:433-440.

25. Young, D. H. and H. Kauss. 1983. Release of calcium from suspension cultured Glycine max cells by chitosan, other polycations, and polyamines in relation to effects on membrane permeability. Plant Physiology 73:698-702.

degradation of chitosan (large polymers and release in nature

Physiological and Molecular Plant Pathology (1989) **35**, 215–230

Chitosan oligomers from *Fusarium solani*/pea interactions, chitinase/β-glucanase digestion of sporelings and from fungal wall chitin actively inhibit fungal growth and enhance disease resistance

DAVID F. KENDRA†, DAVID CHRISTIAN and LEE A. HADWIGER‡

Molecular Biology of Disease Resistance Laboratory, Department of Plant Pathology, Washington State University, Pullman, WA 99164–6430, U.S.A.

(*Accepted for publication February 1989*)

Chitosans which had been: (1) derived chemically from the chitin of fungal cell walls, (2) accumulated in *Fusarium solani*/pea interactions or (3) released from chitinase and β-glucanase digestion of sporelings, were used to determine if these fungal polymers had the biological activity of the chitosan chemically derived from crustaceans. The biological activity of the cell wall chitin-derived chitosan from *F. solani* f. sp. *phaseoli* mimicked that of shrimp chitosan and was somewhat superior to that from f. sp. *pisi*. *F. solani* f. sp. *phaseoli* chitosans inhibited germination of *F. solani* macroconidia at concentrations as low as 8 μg ml^{-1}. 100 μg ml^{-1} of this chitosan provided protection against *F. solani* f. sp. *pisi* in pea pod tissue for periods of at least 5 days while 10 μg ml^{-1} could only maintain resistance for up to 3 days. In comparisons of chitosan-like oligomers released from the ff. sp. *pisi* and *phaseoli*, greater proportions of [^{3}H]-N-acetylglucosamine labelled chitosan fraction (heptamer or larger) could be recovered both from f. sp. *phaseoli*/pea interactions and from f. sp. *phaseoli* germlings in contact with a chitinase and β-glucanase-rich basic pea protein fraction. The results indicate that the chitosan heptamer-plus fraction readily recovered from these plant–fungal interactions is able to function as a major biological signal in pea/–*Fusarium* interactions while the chitosan pentamer which preferentially accumulates in f. sp. *pisi* had less detectable biological activity.

INTRODUCTION

Chitosan, a mostly deacetylated β-1,4 linked D-glucosamine polymer, is a component of the fungal walls of many fungi, including *Fusarium solani* [*2*, *4*, *10*]. Chitosan has been implicated as a regulatory component in the *Pisum sativum–Fusarium solani* host–pathogen interaction [*8*, *10*, *11*]. Hexosamine-containing oligomers are apparently released from the fungal cell, since they enter and/or accumulate within both the host and fungal cells within 15 min following inoculation. Chitosan also accumulates within the host cell following application of high molecular weight [^{3}H]-chitosan to the pea pod endocarp tissue surface [*11*]. Shrimp or crab chitosan, when applied to pea endocarp tissue at low concentrations, activates multiple biological processes in the host tissue,

† Present address: Department of Biology, Plant Science Institute, University of Pennsylvania, Philadelphia, Pennsylvania 19104, U.S.A.

‡ To whom all correspondence should be addressed: Department of Plant Pathology, Washington State University, Pullman, WA 99164–6430, U.S.A.

0885-5765/89/090215+16 $03.00/0

which are identical to those observed following inoculation with *Fusarium solani* f. sp. *phaseoli*, an incompatible pathogen of peas [*8*, *12*, *13*]. Chitosan induces the formation of phenylalanine ammonia lyase (PAL), an enzyme which is considered to be the key to the phenyl propanoid pathway [*19*, *20*] and affects the synthesis of at least 20 other pea proteins [*31*], including chitinase and β-glucanase [*22*, *23*, *25*]. Chitosan exceeds many of the known elicitors in inducing lignification in wounded wheat leaves [*3*]. Chitosan has also been shown to activate disease resistance response genes in peas, some of which have been assayed by hybridization with cDNA probes from cloned genes [*8*, *28*] and others by *in vivo* and *in vitro* translation of the mRNAs from elicitor induced pea tissue [*14*]. Chitosan applied externally to *F. solani* macroconidia can inhibit germination and growth [*1*] and reduce incorporation of [^{3}H]-uridine into RNA [*12*].

The mechanism(s) by which chitosan can both activate plant genes and inhibit fungal growth is not presently known. However, it is known that the optimal glucosamine polymer for both functions must have a degree of polymerization (DP) of seven units or greater [*16*]. The alternating positive charges along the length of the polymer, due to the alternating orientation of the glucosamine units, and its affinity for DNA indicate that chitosan may be partially responsible for the structural changes in pea cell nuclei observed within 30 min following cell to cell contact of the host tissue with *F. solani* macroconidia [*9*, *16*, *17*].

The present study was conducted to examine the biological activity and actual polymer sizes of chitosan produced from the plant–fungus interaction or produced from the deacetylation of chitin synthesized by the pathogen. Chitosans from shrimp or crab shell had been used in previous investigations. Because chitosan is highly active at low concentrations and can complex to many cellular components, an abundance of free chitosan oligomers was not expected. Since naturally occurring fungal chitosan is biosynthesized via chitin, a source adequate for experimentation was derived chemically from fungal chitin. The work of Davis & Bartnicki-Garcia [*6*] and Araki & Ito [*2*] indicates that N-acetyl glucosamine not glucosamine is the precursor of chitosan. The transfer of the N-acetyl glucosamine moiety from UDP-N-acetyl glucosamine into chitin is catalysed by chitin synthetase. Thus chitin deacetylase subsequently converts chitin oligomers (but not monomers or dimers) to chitosan. In cell free extracts of *Mucor rouxii* deacetylation occurs after a chitin chain is formed, however the enzymatic deacetylation of chitin already deposited in the wall was not observed [*6*]. We have investigated the possibility that the availability and size of chitosan oligomers so generated in *forma speciales* of *F. solani* can be influenced by the interaction with pea endocarp tissues and their hydrolytic enzymes. More importantly, we have been able to recover a biologically active chitosan oligomer (heptamer or larger) from these fungi. To be inclusive, we bioassayed the chitosan derived both by chemical deacetylation of the wall chitin from *F. solani* f. sp. *pisi* and f. sp. *phaseoli* and by rigorous extraction of that actually present in the host–parasite interactions.

MATERIALS AND METHODS

Materials

The compatible pathogen isolates, *F. solani* f. sp. *pisi* strain P-A (American Type Culture Collection 38136) and strain 50 as well as the incompatible pathogen, *F. solani*

f. sp. *phaseoli* strain W-8 (American Type Culture Collection 38135) and strain W10 were used throughout this study and were maintained on potato dextrose agar plates supplemented with pea shoots (*F. solani* with reported perfect stages has been renamed *Nectria haematococca*). Pods were produced on greenhouse grown *Pisum sativum* variety Alaska.

Crab and shrimp chitosan, approximately 80–90% deacetylated, was supplied by Bentech Laboratories, Inc., Clackamas, Oregon and Sigma Chemical Company, respectively. Mixed chitosan oligomer sample containing dimer, trimer, tetramer, pentamer, hexamer and over was obtained from Katakura Chikkarin Comp., Ltd., Tokyo.

Isolation and characterization of chitosan derived from chitin of the fungal wall

The protocol used was modified from that of Ride & Drysdale [*27*]. Shake cultures of Vogels media [*30*] (50 ml) were inoculated with 1×10^7 macroconidia of *F. solani* f. sp. *pisi* or *F. solani* f. sp. *phaseoli* and incubated at room temperature on an orbital shaker in continuous light at 22 °C for 4 days. The mycelium was filtered through a double layer of Mira-cloth and then washed extensively with distilled water. The residue was then suspended in distilled water and disrupted in a Virtis homogenizer 4500 at full speed for five 1-min pulses. Homogenization was repeated three times or until no cellular contaminants could be detected microscopically. The resulting tissue was then ground to a fine powder in liquid nitrogen. Following extensive washing with glass distilled water, the tissue was treated with proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, U.S.A.) for 12 h followed by chloroform–methanol (1 : 1 v/v) extraction. The fungal wall chitin was then converted to chitosan as previously described [*27*]. The resulting chitosan, which will subsequently be termed chitin-derived chitosan, was dialysed to pH 7·0, lyophilized, and stored at −20 °C. The fungal cell wall chitin-derived chitosan oligomers were separated on a Fractogel TSK-HW 50(S) gel filtration column (90 × 2·5 cm) as described [*16*], some of the separations were conducted at 22 °C and others at 45 °C. Fractions were detected by absorbance $UV_{650\,nm}$ (detection of glucosamine with the nitrous acid- 3-methyl-2-benzothiazolinone hydrazone (MBTH)-$FeCl_2$ assay according to Ride & Drysdale [*27*]).

Carbohydrate composition following acid hydrolysis of the resulting fungal cell wall chitin-derived chitosan was assayed by thin-layer chromatography using monosaccharide standards of sugars previously shown to be components of fungal cell walls [*4*]. The glucosamine content of the fungal cell wall-derived chitosan was determined on an amino acid analyser by the Washington State University Bioanalytical Center. Infrared spectra of the chitosans were made on an IBM IR 98 Infra-red spectrophotomer. Molecular weights were determined as before [*16*].

Chitosan released from sporelings

Chitosan was released directly from spores germinated in complete Vogel's, from spores pre-germinated 4 h in shake culture at 22 °C and recovered on a 25 μm pore size filter prior to contact with pea endocarp tissue. Alternately these pre-germinated spores were treated with pea β-glucanase and chitinase. Since in fungi chitosan is synthesized from N-acetyl-glucosamine via chitin, rather than directly from glucosamine, the accumulation of chitosan was followed by prelabelling spores with N-acetyl-glucosamine (sp. act. 30–60 Ci $mMol^{-1}$) (20 μCi per 1×10^7 spores). The N-acetyl-glucosamine was

tritiated on ring carbons 1 and 6. The isotope was mixed directly into a sporeling pellet or filter cake. An uptake period of 45 min was required for incorporation of adequate counts to identify chitosan oligomer peaks. Germination and label incorporation continued in a complete Vogels (300 µl) on a shake culture with or without hydrolytic enzymes. Similarly pre-germinated spores, prelabelled 45 min, were distributed to the endocarp surface of 5 g of split pea pods in 160 mm petri plates. Chitosan biosynthesis by the spores in shake culture was terminated and the oligomers dissociated from complexes with other macromolecules by an overnight treatment with 1 % proteinase K and 0·5 % SDS. Spores recovered from the pod surface after 4 h were recovered in 10 ml of water and pelleted by centrifugation 5000 ***g*** and the volume reduced to 0·6 ml prior to proteinase K and 0·5 % SDS treatments. Chitosan was solubilized away from chitin and other macromolecules by making the proteinase digest 1 % acetic acid. Following 5 min of centrifugation in a microfuge tube the supernatant was separated on a 2·2 × 83 cm fractogel column maintained at 45 °C. In separate runs a high molecular weight chitosan, a commercial oligomer mixture, [^{3}H] glucosamine, and [^{3}H]-N-acetyl-glucosamine were utilized as markers to determine the elution pattern of oligomers. The individual oligomer sizes were calculated on the basis of prior published separations [*16*]. Fractions in major peaks were combined, lyophilized and assayed with *F. solani* f. sp. *phaseoli* or f. sp. *pisi* for determination of fungicidal activity.

Source of hydrolytic enzymes for digestion treatments

Pure β-glucanase and chitinase were obtained as described previously [*22*]. The basic protein fraction of peas used was essentially that derived from the DEAE column in the same purification scheme [*22*] and included the entire complement of proteins subsequently resolved on the chromatofocusing column. The basic protein was recovered after 18 h from challenged and unchallenged pea tissue under comparable conditions, and adjusted to equal volumes. The protein concentration of the basic protein preparation from the f. sp. *phaseoli* [*17*] challenged tissue was 74 µg ml^{-1} and from unchallenged tissue was 6 µg ml^{-1} and 100 µl of each preparation were utilized per treatment.

Chitosan inhibition of F. solani *germination and growth*

Macroconidia were suspended in Vogel's minimal medium [*30*] and used to inoculate microdilution plate wells (approx. 40 spores per well) in which the chitosans had been serially diluted in H_2O [*9*]. Growth was scored at 24 and 48 h. The lack of growth in wells appearing clear was verified microscopically.

Pisatin assay

Pisatin was isolated from treated pea pods as previously described [*10*]. Purity of the extracted pisatin was evaluated by UV spectra and by silica gel TLC separation using a chloroform solvent phase [*16*].

Protection of pea pods against F. solani *f. sp.* pisi *by shrimp or fungal cell wall chitin-derived chitosans*

Shrimp or fungal wall chitin-derived chitosans (10, 100 or 1000 µg ml^{-1}) were coinoculated with a macroconidial suspension of *F. solani* f. sp. *pisi* (1 × 10^7 macroconidia

per ml) on pea endocarp tissue. Host tissue and fungal growth viability were observed cytologically after 24 and 48 h using the vital stains, fluorescein diacetate (FDA) and phenosafranin [*17*] or 1% cotton blue, respectively. Resistance was evaluated by examining approximately 30 spores. If the growth of the germ tube did not exceed the length of the spore (approx. 5 μm), the fungus was considered to be suppressed by a resistant reaction. In a partial-resistance reaction, a few germ tubes grow in excess of the spore length. In a susceptible reaction, the growth of most of the spores was prolific, forming a mat of mycelia.

RESULTS

Characterization of fungal cell wall chitin-derived chitosan

The general biological properties of fungal chitosan were determined using the larger quantities available from that chemically converted (see methods) from cell wall chitin. Gel filtration separations of the cell wall chitin-derived chitosans of *F. solani* f. sp. *pisi* and *F. solani* f. sp. *phaseoli* indicate only minor differences in oligomer size distribution (not shown). F. sp. *pisi* oligomers contained a larger proportion of oligomers smaller than the heptamer. However, most of the chitosan was eluted in the void fraction which does not accurately resolve oligomers containing ten or more sugar residues. Thin layer chromatography separations of the acid-hydrolysed chitosans yielded only a single band which co-migrated with the glucosamine standard ($R_F = 0{\cdot}34$; solvent system: pyridine–2-pentanol–H_2O 1:1:1). No other reducing sugars were observed. No residual amino acids or amino sugars were observed following ninhydrine staining. Infra-red spectra of fungal chitosans (not shown) derived from alkali treatment of walls of f. sp. *pisi* and *phaseoli* were essentially identical to those of the shrimp chitosan [Fig. 1(a)]. However, the spectra are distinctly different from those of chitin [Fig. 1(d)] [*26*].

Inhibition of F. solani *macroconidial germination by chitosan*

Shrimp chitosan inhibited germination of macroconidia of *F. solani* f. sp. *pisi* and *F. solani* f. sp. *phaseoli* at concentrations of 8 and 4 μg ml^{-1}, respectively, while *F. solani* f. sp. *phaseoli* cell wall chitin-derived chitosans inhibited both formae speciales at 12 and 8 μg ml^{-1}, respectively (Table 1). Chitosan derived from *F. solani* f. sp. *pisi* chitin was inhibitory only at higher concentrations.

Fungal cell wall chitin-derived chitosan as elicitors of pisatin formation

Fungal cell wall chitin-derived chitosans effectively induce pisatin formation in pea endocarp by 24 h (Table 2). Pisatin formation at 6 h, a time when growth of both the compatible and incompatible fungi are suppressed [*11*], was negligible for all chitosan treatments as well as for fungal macroconidial inoculations. By 24 h the pisatin level had substantially increased above the 6 h level (Table 2). Pisatin levels induced by shrimp, *F. solani* f. sp. *phaseoli* and *F. solani* f. sp. *pisi* chitin-derived chitosans were nearly identical to those induced by inoculation with *F. solani* f. sp. *phaseoli* macroconidia (Table 2).

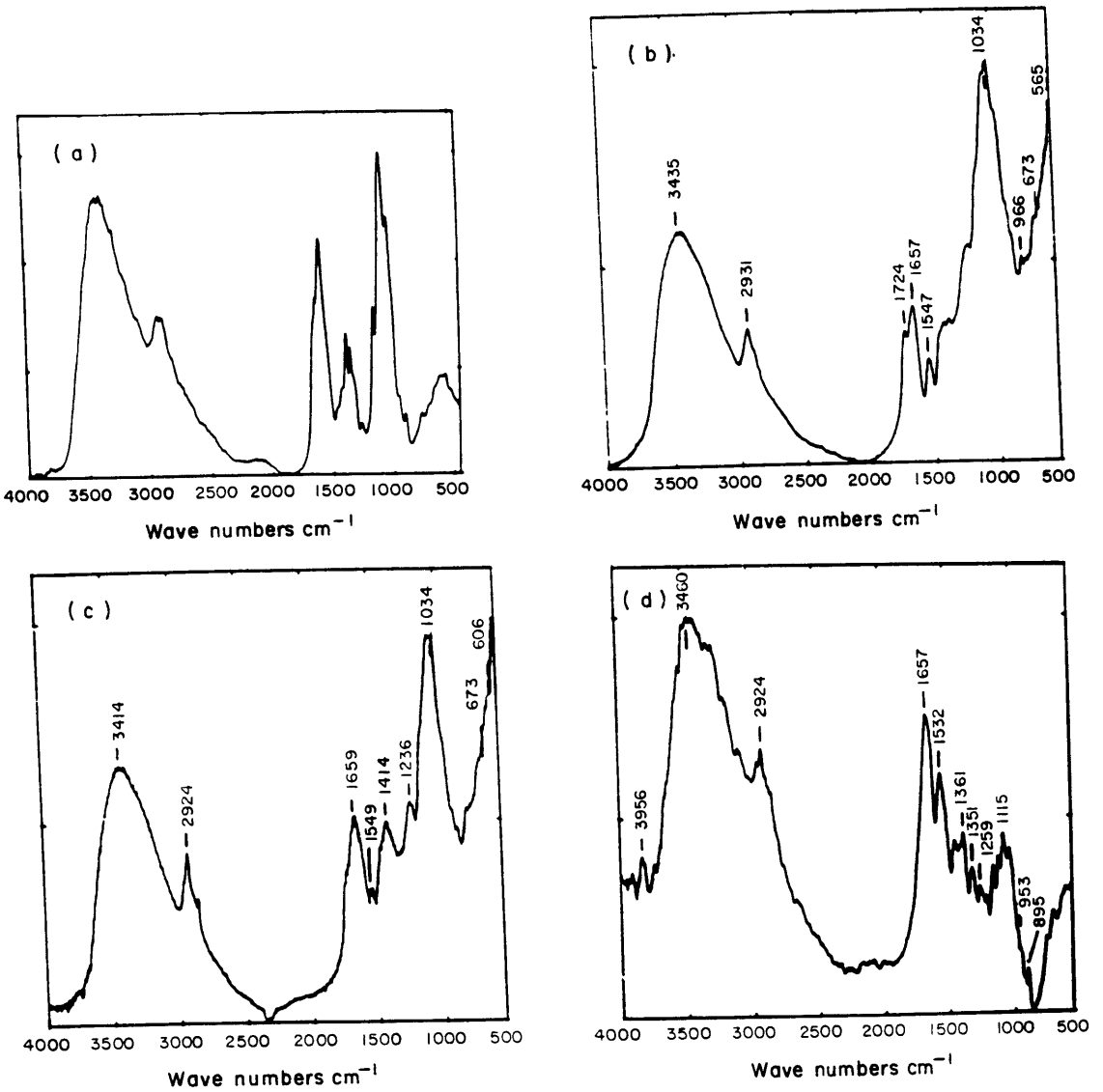


FIG. 1. Infrared spectra of fungal cell wall-derived and shrimp chitosans. (a) Shrimp chitosan; (b) chitosan heptamer from *F. solani* f. sp. *pisi*; (c) chitosan heptamer from *F. solani* f. sp. *phaseoli*; (d) chitin from crab shells.

Protection in pea tissue against F. solani *f. sp.* pisi *by chitosan treatment.*

Since pretreatment of pea pod tissue with shrimp chitosan at concentrations as low as 2 μg ml^{-1} can enhance protection against *F. solani* f. sp. *pisi* [*10*], the influence of fungal cell wall chitin-derived chitosans on the germination and growth of *F. solani* f. sp. *pisi* and *F. solani* f. sp. *phaseoli* and pea endocarp cell viability was assessed. Shrimp and *F. solani* f. sp. *phaseoli* cell wall chitin-derived chitosans at a concentration as low as 10 μg ml^{-1} were able to protect the pea endocarp tissue temporarily from *F. solani* f. sp. *pisi* growth (Table 3). Higher concentrations of the *F. solani* f. sp. *pisi* cell wall chitin-derived chitosan were required to develop protection. At a concentration of 100 μg ml^{-1}, the cell wall-derived chitosans from both formae speciales and the shrimp

TABLE 1
Fungal growth inhibition by F. solani *cell wall chitin-derived chitosans and shrimp chitosan*

	Minimum concentration (μg ml^{-1}) at which no growth was detected *F. solani*	
	f. sp. *pisi*	f. sp. *phaseoli*
F. solani f. sp. *pisi* cell wall chitosan	125[a]	125
F. solani f. sp. *phaseoli* cell wall chitosan	12	8
Shrimp chitosan	8	4

[a] Values are an average of three replications of duplicate dilutions.

TABLE 2
Pisatin-inducing potential of F. solani *cell wall chitosans, shrimp chitosan or macroconidia*

Treatment of pea tissue[a]	Pisatin assayed at: 6 h	24 h
	(μg g^{-1} pod tissue)[b]	
F. solani f. sp. *pisi* cell wall chitosan	4[b][c]	356 f
F. solani f. sp. *phaseoli* cell wall chitosan	16 g	370 f
Shrimp chitosan	12 g	377 f
F. solani f. sp. *pisi* macroconidia	24 h	705 g
F. solani f. sp. *phaseoli* macroconidia	22 h	363 f

[a] All chitosan treatments were 1000 μg ml^{-1}. Macroconidial suspensions were 1×10^7 ml^{-1}.
[b] Values are an average of three replications.
[c] The mean values in each column followed by the same letter are not significantly different, $P = 0{\cdot}05$.

chitosan protected the pea endocarp tissue for at least 3 days (Table 3). However, resistance was dissipating within 3 days in the *F. solani* f. sp. *pisi* chitosan-treated pod tissue as was indicated by reinitiation of fungal growth. The tissue treated with either the shrimp chitosan or *F. solani* f. sp. *phaseoli* chitosan remained resistant through 5 days. All chitosans tested at a concentration of 1000 μg ml^{-1} protected the pea endocarp tissue for at least 5 days.

No significant decrease in viability (as assessed by fluorescein diacetate and phenosafranin staining) was observed in pea cells which during the first 6 h were treated with any of the chitosans or with inoculum of *F. solani* f. sp. *pisi* macroconidia. By 24 h, FDA fluorescence had greatly diminished in the tissues treated with chitosan at 1000 μg ml^{-1} or inoculated with macroconidia of *F. solani* f. sp. *pisi*. Pea cells continued to fluoresce with some attenuation at 24 h following chitosan treatments of 10 and 100 μg ml^{-1}. Fluorescence was attenuated in all cells directly in contact with, and adjacent to macroconidia and hyphae of *F. solani* f. sp. *pisi* after 12 h.

TABLE 3

Effect of cell wall chitin-derived chitosans on induced resistance against Fusarium solani *f. sp.* pisi *in pea endocarp tissue*

Chitosan applied	Reaction[a] 1 day	3 day	5 day
None	−	−	−
F. solani f. sp. *pisi*			
Cell wall chitosan			
10 μg ml^{-1}	−[b]	−	−
100 μg ml^{-1}	+	±[c]	−
1000 μg ml^{-1}	+	+	+
F. solani f. sp. *phaseoli*			
Cell wall chitosan			
10 μg ml^{-1}	+	±	−
100 μg ml^{-1}	+	+	+
1000 μg ml^{-1}	+	+	+
Shrimp chitosan			
10 μg ml^{-1}	+	±	−
100 μg ml^{-1}	+	+	+
1000 μg ml^{-1}	+	+	+

[a] + = resistant reaction; ± = partial resistance, some macro conidia had single cell germ tubes;— = susceptible reaction. To be considered positive for growth, the germ tube had to be at least 5 μm in length [8].

[b] No observable difference in fungal growth as compared to spores grown in Vogel's medium lacking chitosan.

[c] Apical and foot cells of numerous conidia (~ 75 %) were swollen or had a small germ tube projecting. No hyphal development was observed.

Chitin-derived chitosans were obtained from other isolates of *F. solani* f. sp. *phaseoli* (strain W-10) and *F. solani* f. sp. *pisi* (strain 50). Their actions were indistinguishable from their respective formae speciales discussed above in assays of enhanced resistance to *F. solani* f. sp. *pisi* in the pea endocarp tissue, inhibition of macroconidia *in vitro* and elicitation of pisatin.

*Chitosan accumulating in the pea/*Fusarium *interaction*

The chitosan actually present with or produced by *F. solani* was not released in quantity without treatment with proteinase K in the presence of SDS. The chitosan oligomers released were initially identified as metabolites of [^{3}H]-N-acetylglucosamine. Since chitosan is synthesized from N-acetylglucosamine via chitin it was also necessary to recover chitosan oligomers which are very soluble in dilute acetic acid from chitin oligomers which are insoluble, except for monomers through tetramers. The fractogel column (Fig. 2) at 45 °C clearly resolves the chitosan oligomers (up to heptamer size). An oligomer series is extractable directly from pregerminated macroconidia of *F. solani* f. sp. *phaseoli* (Fig. 2). Although the IR spectra differed [Figs 1(b),(c)] significantly from those of chitin [Fig. 1(b)], the presence of some acetylation and residual protein in these fractions cannot be excluded.

The predominant oligomer sizes separated and assayed were the heptamer-plus and pentamer peaks (Fig. 2). Because of the limitations of the column resolution the

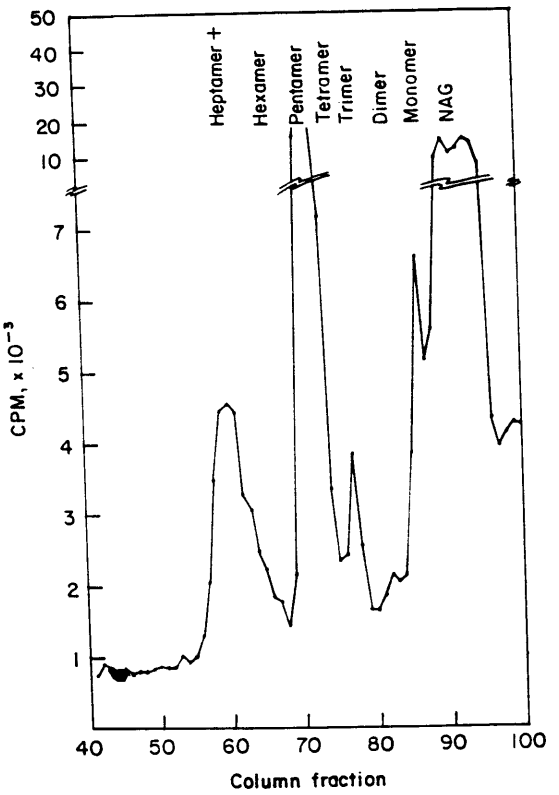


FIG. 2. Acetic acid soluble metabolites of [^{3}H]-N-acetyl glucosamine synthesized by *F. solani* f. sp. *phaseoli* separated on a 2·5 × 75 fractogel column at 45 °C. Pregerminated (4 h) macroconidia were labelled with [^{3}H]-N-acetyl glucosamine for 45 min then cultured for two additional hours. All oligomers were solubilized with proteinase K (1 μg ml^{-1} culture solution) and SDS (1 %). The 1 % acetic acid soluble material containing chitosan oligomers was fractionated. The oligomer sizes were estimated from previously reported column separations with oligomer standards. Each individual column was standardized with N-acetyl glucosamine and high molecular weight chitosan to estimate high molecular weight exclusion fractions and low molecular elution fractions, and with mixed chitosan oligomers from the Katakura Chikkarin Company.

heptamer peak may contain some larger oligomers and thus is termed the heptamer-plus peak. The chitosan heptamer from shrimp has been shown to be highly effective in inducing host responses and inhibiting fungal growth [*16*], while the pentamer is less active and the smaller oligomers are totally inactive. Consequently our investigations of the chitosan accumulating in the pea/*Fusarium* interactions are based on pentamer and larger oligomers. The IR spectrum [Figs. 1(b),(c)] of the heptamer-plus fractions utilized in the experiments to follow were similar to comparable chitosan heptamer fractions (not shown) chemically derived from the chitin of fungal cell walls. The IR spectra of the heptamers were also similar to those of the highly deacetylated chitosans described by Domard & Rinaudo [*7*] and clearly different from those of chitin [Fig. 1(d)].

Repeated fractogel column separations showed that the representations of N-acetylglucosamine-labelled oligomers differed between *F. solani* f. sp. *pisi* and *F. solani* f. sp. *phaseoli* (Fig. 3). There was consistently a greater representation of the newly labelled heptamer peak from *F. solani* f. sp. *phaseoli* when these fungi were grown in the

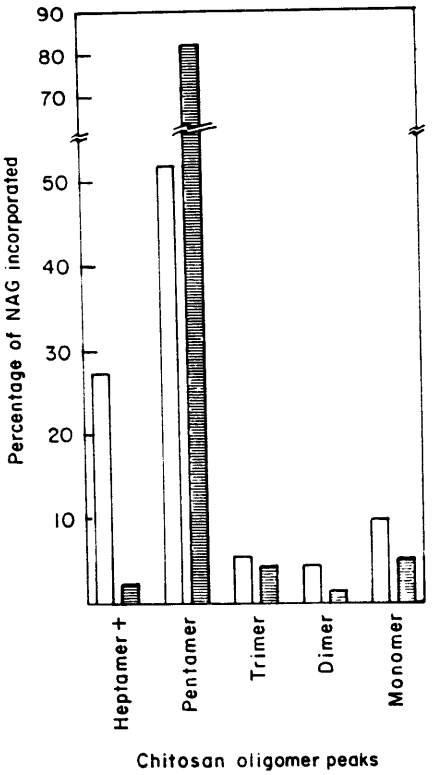


FIG. 3. A comparison of the relative proportion of [H^3] N-acetyl glucosamine incorporated into oligomers by *F. solani* f. sp. *phaseoli* (open bars) with that of *F. solani* f. sp. *pisi* (hatched bars). Sporelings (1×10^7) were pre-germinated for 4 h in complete Vogel's medium and were labelled with [^{3}H]-N-acetyl glucosamine 45 min and incubated in Vogels medium for 2 h. (Peaks correspond to those in Fig. 2.)

absence of the host. The proportion of the more active heptamer fraction was up to ten times that of the heptamer fraction from *F. solani* f. sp. *pisi*.

*N-acetyl glucosamine labelled chitosan oligomers present in the pea endocarp/*F. solani *interaction*

The heptamer fraction labelled in the pea/f. sp. *pisi* interaction is reproducibly half of that recoverable from the pea/f. sp. *phaseoli* interaction (Fig. 4). The proportion of pentamer is similar in the two interactions.

Effect of pure pea hydrolytic enzymes on the proportional accumulation of N-acetyl glucosamine labelled oligomers

Since previous studies [*21–23*] on chitinase and β-glucanase in pea tissue indicate these enzymes increase as the host–parasite interaction progresses, enzyme treatments were applied to pre-germinated macroconidia to determine their effect on oligomer accumulation (Fig. 5). A mixture of previously purified pea chitinase and β-glucanase [*22*] had little effect on the relative distribution of chitosan oligomers labelled with N-acetylglucosamine, possibly because of the high level of enzyme, the overall accumulation of labelled heptamer decreased.

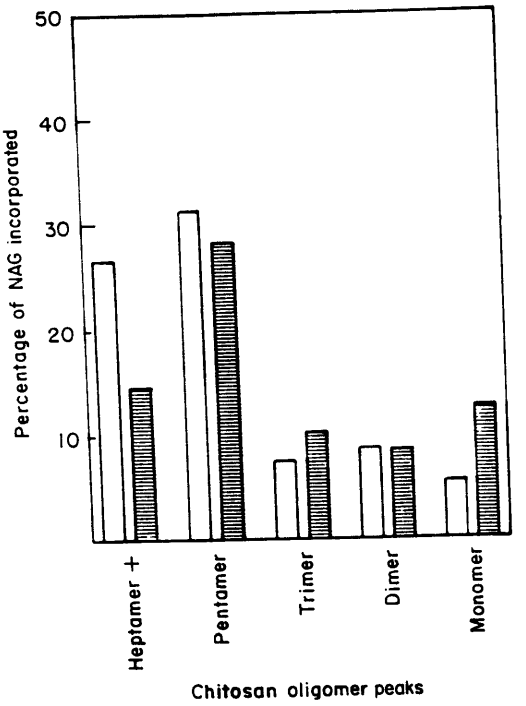


FIG. 4. A comparison of the proportion of [^{3}H]-N-acetyl glucosamine incorporated into chitosan oligomers by *F. solani* f. sp. *phaseoli* (open bars) and *F. solani* f. sp. *pisi* (hatched bars) sporelings inoculated onto pea endocarp tissue. Following a 45-min labelling period sporelings (1×10^7) were in contact with the pea endocarp surface for 4 h. (Oligomer peaks correspond to those in Fig. 2.)

A more representative accumulation of the basic hydrolytic enzymes present in the interaction or in healthy tissue is present in the protein fraction recovered by the ion exchange chromatography step reported in the purification of the major β-glucanases and chitinases of peas. A highly concentrated preparation (1 mg ml^{-1}) of these basic proteins, when in direct contact with pre-germinated sporelings, did not appreciably change the proportion of label in f. sp. *pisi* heptamers and f. sp. *phaseoli* heptamers (Fig. 6). However, moderate concentrations (6–74 μg protein ml^{-1}) of these basic pea proteins when isolated from induced or non-induced pea tissues were shown to enhance the actual rate of incorporation of N-acetylglucosamine into the heptamer fraction by *F. solani* f. sp. *phaseoli*. For example, two separate preparations of these basic protein fractions increased the rate of incorporation of N-acetylglucosamine into heptamer 5–6 fold (within a 3 h pulse period) over that in treatments without added enzymes (not shown).

Large scale basic pea-protein extracts containing chitinase and β-glucanase activity from fungal induced plant tissue were applied to macro preparations of sporelings and the resultant oligomer fractions (separated by Fractogel columns) were evaluated as antifungal components. Table 4 indicates that the heptamer peak is effective in inhibiting growth of *F. solani* at levels approaching those of pure shrimp chitosan heptamer [*16*].

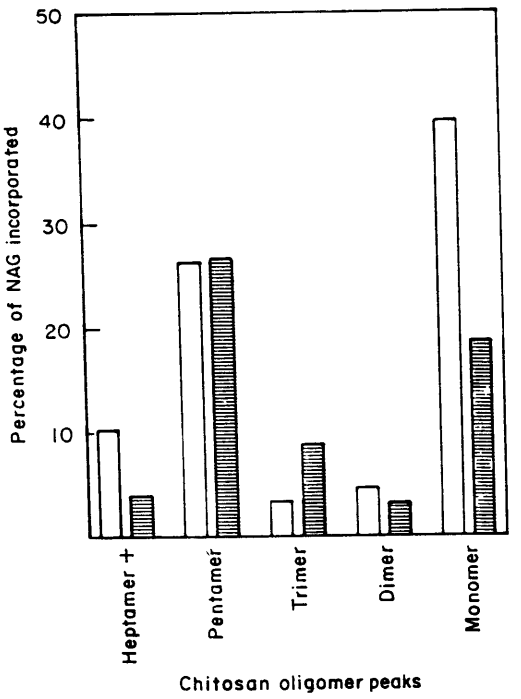


FIG. 5. A comparison of the relative proportion of [^{3}H] N-acetyl glucosamine incorporated into chitosan oligomers by *F. solani* f. sp. *phaseoli* sporelings (open bars) with that of *F. solani* f. sp. *pisi* (hatched bars) treated with a mixture of pure pea chitinase (1·3 μg ml^{-1}) and β-glucanase (2·6 μg ml^{-1}) [*21*]. Spores (1×10^7) were pregerminated for 2·5 h, label was applied for 45 min followed by 2 h exposure to the enzyme mixture. Peaks correspond to those in Fig. 2.

DISCUSSION

Differences in fungal cell wall composition and structure have been shown by Bartnicki-Garcia [*4*] to vary between taxonomically diverse fungal groups, including plant pathogenic fungi. The cell walls of the formae speciales of *Fusarium solani* are composed primarily of chitin and glucan [*4*]; however, about 1 % of the wall material exists as glucosamine [*10*] detectable by a glucosamine-specific colorimetric analysis [*27*]. In this study cell wall chitin was chemically converted to chitosan in order to demonstrate that fungal cell wall derived chitosan had biological properties similar to commercial chitosans chemically derived from shrimp or crab shell chitins which were used in previous investigations [*1*, *8*, *10–18*, *20*, *22*, *23*, *30*]. Identical procedures were used to derive the cell wall chitosans from the two formae speciales of *F. solani*. Although the infrared spectra were essentially identical, the chitosan recovered from the f. sp. *pisi* wall was not as enriched with a biologically active component. The relative intensities of the infra-red peaks for the fungal cell wall derived chitosans did not differ appreciably from those of shrimp chitosan suggesting that difference in the degree of acetylation was probably not the major factor responsible for the differences in the observed biological activities. Other complicating factors were not assessed such as varying polymorphic forms [*21*] and some possible impurities not detectable with infrared spectrophotometry.

Some minor differences between the formae speciales in the oligomer size distributions

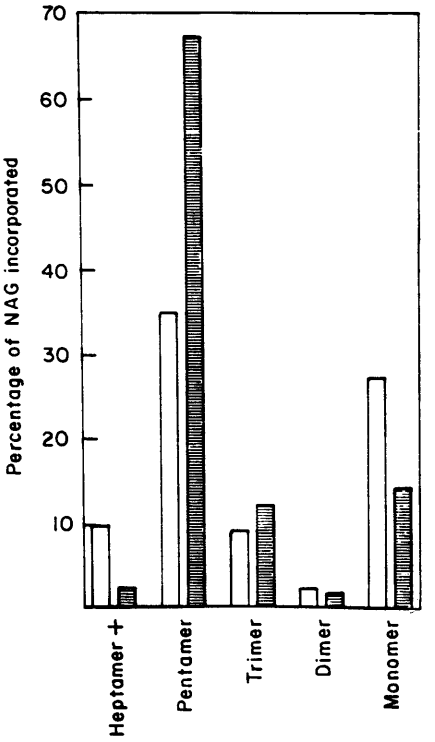


FIG. 6. A comparison of relative proportion of [^{3}H] N-acetylglucosamine incorporated into oligomers fractionated on fractogel column (Fig. 2) by *F. solani* f. sp. *phaseoli* (open bars) with that of f. sp. *pisi* (hatched bars) in the presence of basic pea proteins. Pregerminated sporelings (1×10^7) (4 h) were labelled for 45 min and then cultured for 3 additional hours in the presence of basic proteins [*2*] from pea endocarp tissue inoculated 28 h with *F. solani* f. sp. *phaseoli*. (Peaks correspond to those in Fig. 2.)

TABLE 4

Approximate concentrations of isolated F. solani [^{3}*H*]*-chitosan which inhibit growth of* Fusarium solani *f. sp.* phaseoli *macroconidia cultured in Vogel's medium*[a]

Chitosan (mg ml^{-1})	Chitosan from *F.s.* f. sp. *phaseoli*		Chitosan from *F.s.* f. sp. *pisi*	
	Heptamer	Pentamer	Heptamer	Pentamer
0·500	ND[b]	(0)	ND	(+)
0·250	0	(0)	0	(+)
0·125	0	(0)	0	(+)
0·060	(+)	(+)	0	(+)
0·030	(+)	(+)	+	+
0·015	+	+	+	+
0·007	+	+	+	+
0·003	+	+	+	+
0·001	+	+	+	+

[a] Free chitosan isolated from *F. solani* did not appear as white as that derived from fungal wall chitin, thus indicating some of the weight could be derived from inert material.

[b] ND = No data, 0 = total inhibition of macroconidial growth prior to 10 h, (0) = detectable regrowth at 20, + = growth of macroconidia at 10 h (comparable to control), (+) = limited growth at 10 h.

of cell wall-derived chitosan were consistently observed, indicating that the differences were not artifactual. Since the cell wall-derived chitosan of *F. solani* f. sp. *phaseoli* triggers the same response(s) in the pea endocarp tissue as do high molecular weight shrimp or crab shell chitosans ([*15*] and Tables 1, 2, and 3), the abundance of polymer sizes above a heptamer actually released in the interaction may be important in elicitation of the host responses.

We reported that the minimum oligomer size necessary to elicit the host response and inhibit fungal germination maximally and growth had a degree of polymerization (DP) of approximately seven (or greater) and was essentially non-acetylated [*16*]. A portion of the N-acetyl glucosamine labelled chitosan actually produced by the fungus falls within this range. Since both the anti-fungal and host inducing properties decreased proportionally as the DP is decreased, the relative accumulation of biologically active heptamers in comparison with less active pentamers and non-active monomers, dimers and trimers is important.

The pea enzymes, β-glucanase and chitinase, which increase following inoculation have the potential to degrade fungal walls may influence chitosan oligomer size accumulation and release from the fungal cell. These two enzymes also have been implicated directly in host defences [*22–25*, *29*] against plant pathogenic fungi in lysing cells [*24*], presumably attacking chitin and β-1,3-glucan, the major components of the cell walls. Chitinase has no known function in the plant's own metabolism because there is no known chitin-like substrate present in higher plants [*4*] and thus a chitinase function in plant defence is an attractive hypothesis. Paradoxically, in *Fusarium solani* inoculated pea tissue the chitinase and β-1,3-glucanase activities increase similarly both in compatible and in incompatible reactions or in chitosan-treated pea endocarp tissue [*23*] up to 24 h after treatment, even though within this time the compatible pathogen has resumed prolific growth while growth of the incompatible pathogen is still arrested [*8*].

The percentage of N-acetyl glucosamine-labelled chitosan heptamer fragments recoverable from the two *F. solani* forma speciales in the absence of host tissue is almost ten fold greater for f. sp. *phaseoli*. This proportional advantage of the f. sp. *phaseoli* is influenced but not reversed by the presence of the pea endocarp tissue and by either the chitinase/β-glucanase rich basic pea proteins or the pure pea enzymes. Probably the most authentic measure of heptamer present is that actually accumulating in pre-germinated fungi in contact with the endocarp tissue, which again shows a higher proportion of heptamer in the incompatible interaction.

The measurable biological activity observed for heptamers accumulating from the digestion with chitinase/β-glucanase-rich proteins derived from *F. solani* f. sp. *phaseoli* challenged pea tissues suggest that these enzymes may be important in the release of chitosan. Further, the disproportionately low accumulation of the heptamer and high accumulation of the pentamer suggests an inherent difference exists in the potentially active oligomers available in incompatible and compatible fungi. This oligomer size difference may be important in the recovery period essential for resumption of active growth by the pea pathogen. Since the chitosan heptamers can also develop immunity in pea tissue [*16*], their greater accumulation in the incompatible reaction could influence the intensity of the entire host response including the induction of phenylalanine ammonia lyase, chitinase, β-glucanase, other disease resistance response proteins, disease resistance response genes and accumulations of lignin and pisatin.

Thus chitosan oligomers function as previously inferred by experiments conducted with shrimp or crabshell chitosan. Some of the oligomers with optimal biological activity appear to be present in pea/*Fusarium* interactions.

This research was supported in part by Agriculture Research Center, Washington State University, Pullman, WA, U.S.A. Washington Sea Grant R/X-13 and National Science Foundation Grant DMB-8414870. Scientific Paper No. 7575/ We thank Dr Kerry W. Hipps and S. Gurusiddaiah for IR and amino acid analyses, respectively and Ruby Latham for manuscript preparation.

REFERENCES

1. Allan, C. R. & Hadwiger, L. A. (1979). The fungicidal effect of chitosan on fungi of varying cell wall composition. *Experimental Mycology* **3**, 285–287.
2. Araki, U. & Ito, E. (1975). A pathway of chitosan formation in *Mucor rouxii* enzymatic deacetylation of chitin. *European Journal of Biochemistry* **55**, 71–78.
3. Barber, M. S. & J. P. Ride. (1988). A quantitative assay for induced lignification in wounded wheat leaves and its use to survey potential elicitors of the response. *Physiological and Molecular Plant Pathology* **32**, 185–197.
4. Bartnicki-Garcia, S. (1968). Cell wall chemistry, morphogenesis and taxonomy of fungi. *Annual Review of Microbiology* **22**, 87–108.
5. Boller, T., Gehri, A., Mauch, F. & Vogeli, U. (1983). Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. *Planta* **157**, 22–31.
6. Davis, L. L. & Bartnicki-Garcia, S. (1984). A model for the mechanism and regulation of chitosan synthesis in *Mucor rouxii*. In *Structure. Function and Biosynthesis of Plant Cell Walls*, Ed. by W. M. Dugger & S. Bartnicki-Garcia, pp. 400–408 S. American Society of Plant Physiologists, Rockville, MD.
7. Domard, A. & M. Rinaudo. (1983). Preparation and characterization of fully deacetylated chitosan. *International Journal of Biological Macromology* **5**, 49–52.
8. Fristensky, B., Riggleman, R. C., Wagoner, W. & Hadwiger, L. A. (1985). Gene expression on susceptible and disease resistant interactions of peas induced with *Fusarium solani* pathogens and chitosan. *Physiological Plant Pathology* **27**, 15–28.
9. Hadwiger, L. A. & Adams, M. J. (1978). Nuclear changes associated with the host–parasite interaction between *Fusarium solani* and peas. *Physiological Plant Pathology* **12**, 63–72.
10. Hadwiger, L. A. & Beckman, J. M. (1980). Chitosan as a component of pea–*Fusarium solani* interactions. *Plant Physiology* **66**, 205–211.
11. Hadwiger, L. A., Beckman, J. M. & Adams, M. J. (1981). Localization of fungal components in the pea–*Fusarium* interaction detected immunochemically with anti-chitosan and anti-fungal cell wall antisera. *Plant Physiology* **67**, 170–175.
12. Hadwiger, L. A., Kendra, D. F., Fristensky, B. W. & Wagoner, W. (1986). Chitosan both activates genes in plants and inhibits RNA synthesis in fungi. In *Chitin in Nature and Technology*, Ed. by R. A. A. Muzzarelli, C. Jeuniaux & G. W. Gooday. pp. 209–214. Plenum Press, New York.
13. Hadwiger, L. A. & Loschke, D. C. (1981). Molecular communication in host–parasite interactions: Hexosamine polymers (chitosan) as regulatory compounds in race-specific and other interactions. *Phytopathology* **71**, 756–762.
14. Hadwiger, L. A. & Wagoner, W. (1983). Electrophoretic pattern of pea and *Fusarium solani* proteins synthesized *in vitro* and *in vivo* which characterize the compatible and incompatible interactions. *Physiological Plant Pathology* **23**, 153–162.
15. Kendra, D. F., Fristensky, B., Daniels, C. H. & Hadwiger, L. A. (1986). Disease resistance response genes in plants: expression and proposed mechanisms of induction. *UCLA Symposia on Molecular Strategies for Crop Protection*. Ed. by C. J. Artzen & C. A. Ryan.
16. Kendra, D. F. & Hadwiger, L. A. (1984). Characterization of the smallest chitosan oligomer that is maximally antifungal to *Fusarium solani* and elicits pisatin formation in *Pisum sativum*. *Experimental Mycology* **8**, 276–281.
17. Kendra, D. F. & Hadwiger, L. A. (1986). Cell death and membrane leakage are not associated with the induction of disease resistance in peas by chitosan. *Phytopathology* **77**, 100–106.
18. Kendra, D. F. & Hadwiger, L. A. (1987). Calcium and calmodulin are not involved in the chitosan or *Fusarium solani* interaction with *Pisum sativum*. *Physiological and Molecular Plant Pathology* **31**, 337–348.

19. Loschke, D. C., Hadwiger, L. A., Schroeder, J. & Hahlbrock, K. (1981). Effects of light and of *Fusarium solani* on synthesis and activity of phenylalanine ammonia lyase in peas. *Plant Physiology* **68**, 680–685.
20. Loschke, D. C., Hadwiger, L. A. & Wagoner, W. (1983). Comparison of mRNA populations coding for phenylalanine ammonia lyase and other peptides from pea tissue treated with biotic and abiotic phytalexin inducers. *Physiological Plant pathology* **23**, 163–173.
21. Muzzarelli, R. A. A. (1986). Chitin. In *The Polysaccharides*. Ed. by G. O. Aspinall, Volume 3. pp. 417–450. Academic Press, New York.
22. Mauch, F., Hadwiger, L. A. & Boller, T. (1988). Antifungal hydrolases in pea tissue. I. Purification and characterization of two chitinases and two β-1,3-glucanases differentially regulated during development and in response to fungal infection. *Plant Physiology* **87**, 325–333.
23. Mauch, F., Hadwiger, L. A. & Boller, T. (1984). Ethylene: symptom, not signal for the induction of chitinase and β-1,3-glucanase in pea pods of pathogens and elicitors. *Plant Physiology* **76**, 607–611.
24. Mauch, F. C. & T. Boller. 1988. Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-glucanase. *Plant Physiology* **88**, 936–942.
25. Nichols, E. J., Beckman, J. M. & Hadwiger, L. A. (1980). Glycosidic enzyme activity in pea tissue and pea-*Fusarium solani* interactions. *Plant Physiology* **66**, 199–123.
26. Pearson, F. G., Marchessault, R. H. & Liang, C. Y. (1960). Infra-red spectra of crystalline polysaccharides V. Chitin. *Journal of Polymer Science* **17**, 101–116.
27. Ride, J. P. & Drysdale, R. B. (1972). A rapid method for the chemical estimation of filamentous fungi in plant tissue. *Physiological Plant Pathology* **2**, 7–15.
28. Riggleman, R. C., Fristensky, B. & Hadwiger, L. A. (1985). The disease resistance response in pea is associated with increased levels of specific mRNAs. *Plant Molecular Biology* **4**, 81–86.
29. Schlumbaum, A., Mauch, F., Vogeli, U. & Boller, T. (1986). Plant chitinases are potent inhibitors of fungal growth. *Nature* **324**, 365–367.
30. Vogel, H. J. (1956). A convenient growth medium for *Neurospora*. *Microbial and Genetic Bulletin* **13**, 43–44.
31. Wagoner, W., Loschke, D. C. & Hadwiger, L. A. (1982). Two-dimensional electrophoretic analysis of *in vivo* and *in vitro* synthesis of proteins in peas inoculated with compatible and incompatible *Fusarium solani*. *Physiological Plant Pathology* **20**, 99–107.

Supplemented information:

Reviews

Chitosan as Antimicrobial Agent: Applications and Mode of Action

Entsar I. Rabea,[†] Mohamed E.-T. Badawy,[†] Christian V. Stevens,[*,‡] Guy Smagghe,[†] Walter Steurbaut[†]

[†] *Department of Crop Protection,* [‡] *Department of Organic Chemistry, Faculty of Agricultural and Applied Biological Sciences, Ghent University, Coupure Links 653, B-9000 Gent, Belgium.*

1. Introduction

Chitosan is a polycationic polymer with a specific structure and properties.[1] It contains more than 5000 glucosamine units and is obtained commercially from shrimp and crabshell chitin (a *N*-acetyl- glucosamine polymer) by alkaline deacetylation[2-4] (NaOH , 40-50%) (*Fig.*1). Recent advances in fermentation technology suggest that the cultivation of fungi (*Aspergillus niger*) can provide an alternative source of chitosan.[5-6]

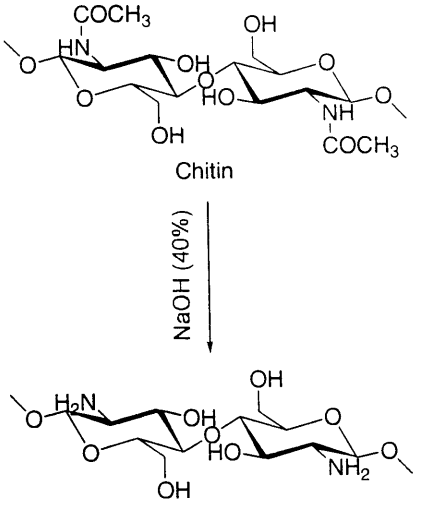


Fig. 1: Preparation of chitosan from chitin

Chitosan is insoluble in most solvents but is soluble in dilute organic acids such as acetic acid, formic acid, succinic acid, lactic acid, and malic acid. The use of chitosan is limited because of its insolubility in water, high viscosity and tendency to coagulate with proteins at high pH. Many efforts to prepare functional derivatives by chemical modifications have been reported in order to increase the solubility in water.[7-15]

Chitosan is the *N*-deacetylated derivative of chitin, although the degree of this *N*-deacetylation is almost never complete. A sharp nomenclature border has not been defined between chitin and chitosan based on the degree of *N*-deacetylation.

*Corresponding author. Chris.Stevens@rug.ac.be

Chitosan and chitin are commercially interesting compounds due to their high nitrogen content (6.89%) compared to synthetically substituted cellulose (1.25%). This makes chitosan a useful chelating agent. As most of the polymers are synthetic materials, their biocompatibility and biodegradability are much more limited than those of natural polymers such as cellulose, dextrose, chitin, chitosan and their derivatives. However, these naturally abundant materials are also limited in their reactivity and processability.

The characteristics of chitosan that may be varied as required for a particular application are the degree of deacetylation (compared to chitin) and the molecular weight. The viscosity of solutions containing chitosan is affected by the degree of deacetylation, the molecular weight, the concentration, the ionic strength, the pH and the temperature. Generally, an increase in temperature causes a decrease in the viscosity of the solution. The effect of the pH on the viscosity depends on the particular acid used. Native chitosan is soluble in organic acids when the pH is < 6, and insoluble in water, in alkaline medium, or organic solvents. However, water soluble salts of chitosan may be formed by neutralization with acids such as hydrochloric acid, acetic acid, lactic acid, or formic acid.

In this review the potential applications and mode of actions of chitosan and its derivatives as antimicrobial compounds will be described.

2. Biological activity

Chitosan is inexpensive, non-toxic and possesses reactive amino groups. It has been shown to be useful in many different areas as antimicrobial compound in agriculture, as potential elicitor of plant defence responses, as flocculating agent in waste water treatment, as additive in food industry, as hydrating agent in cosmetics and more recently as pharmaceutical agent in biomedicine.[16-25]

In this context, the antimicrobial activity of chitosan and its derivatives against different groups of microorganisms, such as bacteria and fungi has received considerable attention in recent years.

2.1. Fungicidal applications of chitosan and its derivatives

The anti-microbial activity of chitosan was observed against a wide variety of micro-organisms including fungi, algae and some bacteria. However, the anti-microbial action is influenced by intrinsic factors such as the type of chitosan, the degree of chitosan polymerisation, the host, the natural nutrient constituency, the chemical and/or nutrient composition of the substrates and the environmental conditions (e.g. substrate water activity and/or moisture). Although both native chitosan and its derivatives are effective as anti-microbial agents, there is a clear difference between them. Their different anti-microbial effect is mainly exhibited in live host plants. The fungicidal effect of *N*-carboxymethyl chitosan (NCMC) is also different in vegetable as compared to graminea hosts. In addition, oligomeric chitosan (pentamer and heptamer) have a better antifungal effect than larger units. The chitosan antimicrobial activity is more immediate on fungi and algae, than on bacteria.[26]

Chitosan has been shown to be fungicidal against several fungi.[4] *(Table 1).*

Table 1. MIC of native chitosan against fungi.[4]

Fungi	MIC[a] (ppm)
Botrytis cinerea	10
Fusarium oxysporum	100
Drechstera sorokiana	10
Micronectriella nivalis	10
Piricularia oryzae	5000
Rhizoctonia solani	1000
Trichophyton equinum	2500

[a]MIC : minimum growth inhibitory concentration.

The minimum inhibitory concentrations (MICs) reported for specific target organisms range from 0.0018 to 1.0 % and are influenced by a multitude of factors such as the pH of the growth medium, the degree of polymerization of chitosan, and the presence/absence of interfering substances such as lipids and proteins.[27-35]

The inhibitory effect of chitosan was also demonstrated with soilborne phytopathogenic fungi.[36] The inhibitory activity of chitosan was higher at pH 6.0 (pKa value of chitosan = 6.2) than at pH 7.5, when most amino groups are in the free base form.[36] The maximal antifungal and pisatin-inducing activities of chitosan were exhibited by chitosan oligomers of seven or more residues.[37] The soil born phytopathogenic fungi *F. solani* and *Colletotrichum lindemuthianum* were inhibited by chitosan and *N*-carboxymethyl chitosan.[36,38,39]

Chitosan has been utilized in soil amendment, in seed treatment, and as a foliar treatment to control the fungus *F. oxysporum.* Chitosan concentrations ranging from 0.1 to 1 mg/ml indicated that higher protection occurred when seed coating and soil amendment were performed with concentrations of 0.5 and 1 mg/ml. Although chitosan at a concentration of 0.1 mg/ml induced a delay in disease development (root lesions visible by 4 days after inoculation), emergence of wilting symptoms occurred between 7 and 10 days post-inoculation while death of about 80% of the plants was recorded one week later.[40]

F. acuminatum, *Cylindrocladium floridanum,* and other pathogens of interest in forest nurseries were inhibited by chitosan in vitro.[41] Similarly, *Aspergillus flavus* was completely inhibited in field growing corn and peanut.[42,43]

Five chemically modified chitosans were tested for their antifungal activities against *Saprolegnia parasitica* by the fungal growth assay in chitosan-bearing broth. Results indicated that as for the chitosan-bearing broth assay, *S. parasitica* did not grow normally; on the first day for methylpyrrolidinone chitosan and *N*-phosphonomethyl chitosan and on the second day for *N*-carboxymethyl chitosan, a tightly packed precipitate was present at the bottom of the test tubes, instead of the fluffy fungal material as in the control. On the contrary, *N*-dicarboxymethyl chitosan seemed to favour fungal growth, while dimethylaminopropyl chitosan did not significantly differ from the control data.[44]

The use of bioactive substances such as chitosan to control post-harvest fungal disease has attracted much attention due to imminent problems associated with chemical agents, which include development of public resistance to fungicide-treated produce, an increasing number of fungicide tolerant post-harvest pathogens and a number of fungicides that are still under observation.[45,46] Chitosan (1 mg/ml) reduces the *in vitro* growth of numerous fungi with the exception of Zygomycetes, *i.e* the fungi containing chitosan as a major component of its cell walls.[47]

Hence, chitosan has the potential as an edible antifungal coating material for post-harvest produce. Recent investigations on chitosan coating of tomatoes have shown that it delayed ripening by modifying the internal atmosphere which reduced decay.[45,46]

Also, the effect of chitosan coating on decay of strawberry fruits held at 13°C was investigated.[45] Strawberry fruits were inoculated with spore suspensions of *B. cinerea* or *Rhizopus stolanifer* and were

subsequently coated with chitosan solutions (10 or 15 mg/ml). After 14 days of storage, decay caused by *B. cinerea* or *R. stolanifer* was markedly reduced by chitosan coating. An other important benefit of chitosan is the increased crop yield when used as a coating on wheat seeds. The authors further confirm the importance of a large number of alternating positively charged groups along the length of the polymer chain because low antifungal activity was observed with *N,O*-carboxymethyl chitosan compared to that of chitosan itself.[46]

Controlling the seed-borne infection *F. graminearum* by chitosan was able to increase the crop yield by 20%.[48] After seed treatment of wheat, peas and lentils during a 5 year trial, plant yield increased 20-30%, and the potential use of chitosan in post-harvest preservation of fruits and vegetables was proposed.[49,50]

Chitosan treatment (2-8 mg/ml) of wheat seeds significantly improved seed germination to recommended seed certification standards (>85%) and vigour at concentrations >4 mg/ml, in two cultivars of spring wheat (Norseman and Max), by controlling seed-borne *F. graminearum* infection. The germination was <80% in the control and >85% in benomyl- and chitosan-treated seeds. The reduction of seed-borne *F. graminearum* was >50% at higher chitosan treatments compared to the control.[51]

An *in vivo* study[52] reported signs of infection in chitosan-coated fruits after 5 days of storage at 13°C compared to 1 day for the control treatment. After 14 days of storage, the chitosan coating at 15 mg/ml reduced the decay of strawberries caused by the same fungi by more than 60%. It was also observed that coated fruits ripened normally and did not show any apparent sign of phytotoxicity. In another study,[53] the preservative effect of chitosan was shown on low-sugar candied kumquat (fruit). The growth of *A. niger* was inhibited by the addition of chitosan (0.1–5 mg/ml) to the medium (pH 5.4), whereas at less than 2 mg/ml, chitosan was not effective in inhibiting mold growth and aflatoxin production by *A. parasiticus*. In a similar study,[38] *N*-carboxymethyl chitosan reduced the aflatoxin production in *A. flavus* and *A. parasiticus* by more than 90% while fungal growth was reduced to less than half. It was reported that apples coated with chitosan reduced the incidence of molds occurring on the apples over a period of 12 weeks.[54] A study carried out on chitosan coating for the inhibition of Sclerotinia rot on carrot showed that the incidence of rotting was significantly reduced (from 88 to 28%) by coating carrot roots with 2 or 4% chitosan.[55]

The antifungal properties of chitosan are of interest in the food industry especially because chitosan is a safe biopolymer suitable for oral administration.[56] In apple juice, 15 yeasts and molds associated with food spoilage including *Mucor racemosus* and *Byssoclamys* spp. were inactivated by chitosan at various concentrations, pH values and temperatures.[31] Similar results were obtained with *A. niger* and *A. parasiticus* in oriental food.[53] Some inhibitory effect on the fish pathogenic Oomycete *S. parasitica* has been reported.[57] The hyphae affected by chitosan at 500-600 mg/l shrunk markedly and contracted.

Chitosan has been successfully used as food wraps.[58] The use of *N,O*-carboxymethyl chitin films to preserve fruits over long periods has been approved in both Canada and the USA.[59] Due to its ability to form a semi-permeable film, chitosan coating is suggested to modify the internal atmosphere as well as decrease the transpiration loss[60,61] and delay the ripening of fruits.[45,61] Rigid chitosan films can be formed using cross linking agents such as glutaraldehyde,[62] divalent metal ions, polyelectrolytes,[63] or even anionic polysaccharides.[64] The preparation of chitosan and chitosan laminated films with other polysaccharides has been reported by various authors; these include chitosan films,[65-67] chitosan/pectin laminated films,[64] and chitosan/methylcellulose films.[68] Several approaches have been used to form these edible films or coatings, including simple coacervation, where a single hydrocolloid is transferred from aqueous suspension or caused to change its phase by evaporation of the solvent. In addition, complex coacervation, where two solutions of oppositely ionized hydrocolloids are united, leads to interaction and precipitation of the polymer complex. Also simple cooling of a warm hydrocolloid suspension inducing a sol–gel transformation, has been practized.

Chitosan films are tough, long lasting, flexible and very difficult to tear. Most of their mechanical properties are comparable to many medium-strength commercial polymers.[65] It was reported that chitosan films have moderate water permeability values and could be used to increase the storage life of fresh produce and foodstuffs with higher water activity values.[67] However, extremely good barriers were observed for the permeation of oxygen, while exhibiting relatively low vapour barrier characteristics.[65,69] Extension of the storage life and the better control of decay of peaches, Japanese pears and kiwi fruits by application of chitosan film has been documented.[70] Similarly, cucumbers, and bell

peppers,[60] strawberries,[60] and tomatoes[52] could be stored for long periods after coating with chitosan. These results may be attributed to decreased respiration rates, inhibition of fungal development and delaying of the ripening due to the reduction of ethylene and carbon dioxide evolution.[45,60,70]

Chitosan and chitosan-laminated films containing antimicrobial agents provide a type of active package so that the preservatives released from the film deposit on the food surface and inhibit the microbial growth.[68,71] The sorbate-loaded edible barrier for mold inhibition on food surfaces was evaluated,[72] and advocated the use of glucose oxidase/glucose as a dip for the extension of the shelf life of fish.[73] The presence of preservatives in chitosan films reduces the intermolecular electrostatic repulsion in the chitosan molecules and facilitates formation of intramolecular hydrogen bonds.[74] It was observed that the packaging film prepared from methylcellulose, chitosan and preservatives possesses antimicrobial activity.[68]

2.2. Bactericidal applications of chitosan and its derivatives

Chitosan inhibits the growth of a wide variety of bacteria[4] *(Table 2).*

Table 2. MIC of chitosan against bacteria[4]

Bacteria	MIC[a] (ppm)
Agrobacterium tumefaciens	100
Bacillus cereus	1000
Corinebacterium michiganence	10
Erwinia sp.	500
Erwinia carotovora subsp.	200
Escherichia coli	20
Klebsiella pneumoniae	700
Micrococcus luteus	20
Pseudomonas fluorescens	500
Staphylococcus aureus	20
Xanthomonas campestris	500

[a]MIC : minimum growth inhibitory concentration

Chitosan has been studied in terms of bacteriostatic/bactericidal activity to control growth of algae and to inhibit viral multiplication.[75-78] Moreover, chitosan has several advantages over other type of disinfectants, because it possesses a higher antibacterial activity, a broader spectrum of activity, a higher killing rate, and a lower toxicity toward mammalian cells.[79,80]

Chitosan derivatives containing quaternary ammonium salts, such as *N,N,N*-trimethyl chitosan, *N,N*-propyl-*N,N*-dimethyl chitosan and *N*-furfuryl-*N,N*-dimethyl chitosan were prepared and tested for their activity against *E.coli.*[11] It was shown that the antibacterial activity of quaternary ammonium chitosan in acetic acid medium is stronger than in water. Their antibacterial activity increased as the concentration of acetic acid is increased. It was also found that the antibacterial activity of quaternary chitosan against *E. coli* is stronger than that of chitosan itself.[11]

Certain antibacterial activities of diethylaminoethyl chitin, diethylaminoethyl chitosan, and triethylaminoethyl chitin were evaluated. The triethylaminoethyl chitin was the most active agent. It had a greater activity against *S. aureus* than against *E. coli.* A concentration of 500 ppm was needed to kill all *S. aureus* within 120 minutes. Different molecular weight hydrolysates of diethylaminoethyl chitin showed a dependence of the antibacterial activity on molecular weight of the hydrolysate.[81]

In addition, the carbohydrate-branched derivatives 1-deoxyglucit-1-yl chitosan and 1-deoxylactat-1-yl chitosan had activity against *Bacillus circulans*, but not against *E. coli,* while chito-oligosaccharides of varying degree of polymerizations (DP's) showed an activity against *E. coli*, but not against *B. circulans.*[82]

A much higher concentration of chitosan (1–1.5%) is required for complete inactivation of *S. aureus* after two days of incubation at pH 5.5 or 6.5.[29] Furthermore, chitosan concentrations of 0.005% were sufficient to elicit complete inactivation of *S. aureus.*[83] This was in accordance with the findings[84] on the effect of chitosan in meat preservation. The antimicrobial effect on different cultures of bacteria on raw shrimp, with different concentrations of chitosan was studied and variations in their degree of susceptibility to chitosan were observed.[25,85] According to these findings, *B. cereus* required chitosan concentrations of 0.02% to display a bactericidal effect, while *E. coli* and *Proteus vulgaris* showed minimal growth at 0.005%, and complete inhibition at 0.0075%. It was also reported that, *B. cereus* was inhibited by chitosan. However, much lower concentrations (0.005%) were required, perhaps due to the low molecular weight (35KD) of chitosan used in this experiment.[83]

Numerous studies have also shown the effect of chitosan on *E. coli* inhibition. Complete inactivation was observed after a 2-day incubation period with concentrations of 0.5 or 1%, at pH 5.5. Complete inactivation could be reached even after the first day, if the chitosan concentration is more than 1% in the broth.[29] Meanwhile, a concentration of 0.1% was required to inhibit *E. coli* growth[84] and only

0.0075% chitosan was needed to inhibit the growth of *E. coli*.[85] These variations were suggested to be due to the existing differences in the degree of acetylation of chitosan; chitosan with a degree of acetylation of 7.5% was more effective than chitosan with a degree of acetylation of 15%.

The antimicrobial effect of water-soluble chitosans[28] such as chitosan lactate, chitosan hydroglutamate and chitosan derived from *Absidia coerulea* fungi was determined on different bacterial cultures. It was observed that chitosan glutamate and chitosan lactate were bactericidal against both gram-positive and gram-negative bacteria in the range of one to five log cycle reduction within one hour. In that same study the authors reported that chitosan was no longer bactericidal at pH 7 due to two major reasons, namely the presence of a significant proportion of uncharged amino groups and poor the solubility of chitosan. These results are in agreement with findings of a similar study[86] where a concentration of 0.2 mg/ml chitosan lactate appeared most effective against *E. coli* with a corresponding population drop of 2 and 4 log cycles within 2 minutes and 1 hour exposure, respectively. These authors observed that chitosan glutamate was also effective against yeast cultures such as *Saccharomyces cerevisiae* and *Rhodotorula glutensis* and inactivation was rapid and complete within 17 minutes when exposed to 1 mg/ml chitosan lactate. This was in contrast to the results[28,86] where, chitosan hydro-glutamate was a more effective antagonist than chitosan lactate.

In another study,[30] the antibacterial effects of 69% deactivated shrimp chitosan, 0.63% sulfonated chitosan (SC1), 13.03% sulfonated chitosan (SC2) and sulfobenzoyl chitosan on oyster preservation were reported. Except in the case of *B.cereus*, bacterial growth was effectively inhibited by at least one of the above four compounds tested at 200 ppm. Even though the sulfonation increased the solubility of chitosan, totally different antibacterial activities were observed for SC1 and SC2. For most of the bacterial cultures SC1 had a very pronounced minimal inhibitory concentration (MIC) effect even at 200 ppm level, with SC2 exhibiting no antibacterial effect at concentrations below 2000 ppm. It was suggested that since SC2 has more sulfonyl groups, it carries a higher negative charge than SC1, thus there would be a greater repulsive force between negatively charged SC2 molecules and bacterial cell walls.[30]

Chitosan derivatives were claimed as antimicrobials for fish and shellfish against infection from *Vibrio anguillarum*, *Edwardsiella tarda*, *Pasteurella piscicida* and several bacteria, in agreement with data obtained on brook trout.[87,88] On the other hand chitin and chitosan are accepted diet supplements for cultured fish.[89]

3. Mode of action of chitosan

3.1. Antimicrobial activity

Because of the positive charge on the C-2 of the glucosamine monomer below pH 6, chitosan is more soluble and has a better antimicrobial activity than chitin.[30] The exact mechanism of the antimicrobial action of chitin, chitosan and their derivatives is still unknown, but different mechanisms have been proposed. Interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents.[27,30,53,90,91] Chitosan acted mainly on the outer surface of the bacteria. At a lower concentration (<0.2 mg/ml), the polycationic chitosan does probably bind to the negatively charged bacterial surface to cause agglutination, while at higher concentrations the larger number of positive charges may have imparted a net positive charge to the bacterial surfaces to keep them in suspension.[28,86]

Chitosan interacts with the membrane of the cell to alter cell permeability. For example, fermentation in bakers' yeast is inhibited by certain cations, which act at the yeast cell surface to prevent the entry of glucose. UV-absorption studies indicated that chitosan caused considerable leakage of proteinaceous material from *Pythium oaroecandrum* at pH 5.8.[92]

Chitosan also acts as a chelating agent that selectively binds trace metals and thereby inhibits the production of toxins and microbial growth.[38] It also activates several defense processes in the host tissue, [45] acts as a water binding agent and inhibits various enzymes. Binding of chitosan with DNA and inhibition of mRNA synthesis occurs through chitosan penetration towards to the nuclei of the microorganisms and interfering with the synthesis of mRNA and proteins.[28]

It has been proposed that when chitosan is liberated from the cell wall of fungal pathogens by plant host hydrolytic enzymes, it then penetrates to the nuclei of fungi and interferes with RNA and protein synthesis.[93]

Chitosan, however, shows its antibacterial activity only in an acidic medium because of its poor solubility above pH 6.5. Thus, water-soluble chitosan derivatives (soluble in both

acidic and basic physiologic circumstances) may be good candidates as a polycationic biocide. Chitosan also inhibits toxin production by *A. alternata* and macerating enzyme production by *Erwinia* in addition to eliciting phytoalexin production.[94,95]

The effects of chitosan on growth inhibition of fungi such as *B. cinerea* in tomato and strawberries was correlated with the reduction of aflatoxin, elicitation of phytoalexin and phenolic precursors, enhanced production of chitinases, and other factors relevant to the plant defenses; direct contact of *A. flavus* with chitosan was reported to produce weakening and swelling of the hyphae. The fungistatic properties of chitosan against *R. stolonifer* were related to its ability to induce morphological changes in the cell wall.[45,46]

The effect of the molecular weight on some antibacterial and antifungal activities has been explored.[96] Chitosan with a molecular weight ranging from 10,000 to 100,000 would be helpful in restraining the growth of bacteria. In addition, chitosan with an average molecular weight of 9,300 was effective in restraining *E. coli* while that with a molecular weight of 2,200 accelerated growth.[97] Moreover, the antibacterial activity of chitosan is influenced by its degree of deacetylation, its concentration in solution, and the pH of the medium. Antibacterial activities were also found to be increasing in the order: *N,O*-carboxymethylated chitosan, chitosan, and *O*-carboxymethylated chitosan.[4]

Quaternary ammonium polymers have previously been considered bacteriostatic, not bactericidal, because they require long contact times to kill microorganisms, and generally they do not have a broad spectrum activity. Some of these polymers have been reported to have antimicrobial activity.[98] The antimicrobial action is believed to occur when the compounds are absorbed onto the bacterial cell surface, increasing the permeability of the lipid cell membrane and causing death through the loss of essential cell materials. In addition, these derivatives of chitosan are generally more active against gram-positive bacteria than their corresponding monomers particularly. This effect is believed to be due to adsorption of the polymers onto the bacterial cell surface and membrane, with subsequent disruption of membrane integrity. Antimicrobial activity generally increases as the content of the quaternary ammonium moiety increases. It has been unexpectedly discovered that 3-trimethylammonium-2-hydroxypropyl-*N*-chitosan and related chitosan derivatives exhibit antimicrobial activity at concentrations as low as 10-20 ppm. The other is one order of magnitude lower than the concentrations at which any previous chitosan derivative has been reported to exhibit antimicrobial activity. These chitosan derivatives may be included in formulations where it is desirable to minimize bacterial attack.[99,100]

The antibacterial activities of quaternary ammonium chitosan salts were evaluated against *S. aureus*, and *E. coli* , gram-positive and gram-negative bacteria, respectively. It was found that the antibacterial activity increased with increasing chain length of the alkyl substituent, and this was attributed to the contribution of the increased hydrophobic properties of the derivatives. These results clearly demonstrated that hydrophobicity and cationic charge of the introduced substituent strongly affect the antibacterial activity of quaternary chitosan derivatives.[101]

In addition, to the formation of gas permeable films, chitosan has a dual function: (a) to direct the interference of fungal growth and (b) to activate several defence processes.[102] These defence mechanisms include accumulation of chitinases, synthesis of proteinase inhibitors, and lignification and induction of callous synthesis.[46] When applied on wounded wheat leaves, chitosan induced lignification and consequently restricted the growth of non-pathogenic fungi in wheat. Chitosan inhibited the growth of *A. flavus* and aflatoxin production in liquid culture, pre-harvest maize, and groundnut, and it also enhanced phytoalexin production in germinating peanut.[38,39] Chitosan also inhibited growth and toxin production by *A. alternata* f. sp. *lycopersici* in culture.[94,95]

3.2. Elicition of plant defence mechanisms by chitosan

Elicitors are substances that can induce defence responses when applied to plant tissues or cultured plant cells (oligosaccharides, glycoproteins, peptides and lipids). The well-studied oligosaccharide elicitors include oligoglucan, oligochitin, oligochitosan and oligogalacturonic acid. When a plant that has developed a resistance mechanism is challenged by a pathogen, rapid and highly localized cell death (hypersensitive cell death) occurs at the attempted infection sites and a variety of biochemical defence responses occur in the surrounding cells. These include the production of reactive oxygen species, structural changes in the cell wall, accumulation of defence-related proteins and phytoalexin biosynthesis.

Chitosan has been extensively evaluated to determine its ability to elicit natural plant defence responses. Physiological and

biochemical changes, which occur within plants due to elicitation by chitosan have been reported.[103-109] one primary physiological change that has been observed when plants are treated with chitosan is the reduction of stomatal aperture, reducing fungal access to the inner leaf tissues. Guard cells in plant leaves, produce H_2O_2 which mediates the elicitor (chitosan)-induced decrease of stomatal apertures in response to chitosan treatment.[103,104]

The synthesis of phenolic acids is stimulated in primary leaves following chitosan treatment, and levels of these phenolic acids, especially ferulic acid, increased significantly with increasing chitosan concentration. The lignin content of primary leaves also showed a similar pattern. The synthesis of precursors of lignin such as *p*-coumaric, ferulic, sinapic acids and phenolic acids having antimicrobial activity was also stimulated by chitosan treatment. The induction of phenolic acids and lignin was significantly lower in cultivar of spring wheat Max compared to Norseman. Chitosan also inhibited fungal transmission to the primary roots of germinating seedlings. Results suggest that chitosan controlled seed-borne *F. graminearum* infection and increased the resistance in seedlings by stimulating the accumulation of phenolics and lignin.[105]

In addition, chitosan oligosaccharides elicit the accumulation of lignin, callose, phytoalexin, "or" or "and" protease inhibitors in various plant tissues and induce early cellular responses. The mechanisms of action by which chitosan induces this lignification has been studied in a wide range of crops.[106-109]

The elicitors of the fungal cell wall are released from the wall by cell wall-degrading enzymes (chitinases, glucanases) secreted by the plants upon infection *(Fig. 2)*. On the other hand, oligogalacturonic acids are degradation products of the plant cell wall, the result of microbial pectic enzymes secreted by pathogens.[110] In addition, oligogalacturonide (DP10 to 12) induces phytoalexin formation in soybean tissues and stimulates lignin and protease inhibition in several other plants. Plants are capable of responding not only to fragments of the pathogen but also to their own cell wall components produced by the enzymes of the pathogen. It has been clearly proved that oligosaccharides elicitates the plant-pathogen interactions.[110]

The culture filtrate of *Phytophthora megasperma sojae,* a fungal pathogen of soybean, has the ability to stimulate the accumulation of a soybean phytoalexin in soybean tissues. The elicitor turned out to be a component of the mycelia cell wall of the pathogen. The minimum structure required for the eliciting activity was a branched 1-3,1-6 *hepta beta*-glucoside. The maximum concentration necessary for the elicitation was 1nM. This concentration suggests that the *hepta beta*-glucoside acts as an infection signal and soybean has a mechanism to perceive and transduce this specific signal. The elicitor is effective not only in soybean but also in several other leguminous plants.[110-112]

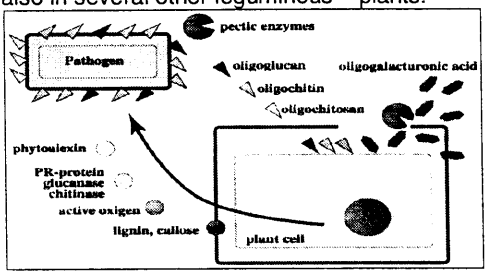


Figure 2. Production mechanism of oligosaccharide elicitors in plant cell.[110]

Oligosaccharides are effective in nanomolar to micromolar concentrations, giving them a true plant growth regulator status.[113,114] It has also been found that some oligosaccharide compounds affect only certain species in a predicted way.[111]

Oligosaccharins is the name given to a small group of oligosaccharides that cause hormonal effects on plants. It seems natural that a plant would differently respond to an attack from one organism than it would to another. The defence mechanism towards insects is probably not going to be effective against bacteria. When a plant is attacked by a fungus, an oligosaccharin, *beta*-glucanase, is biosynthesized by the plant cell wall. This substance then proceeds to degrade the fungal cell wall, fragments from which amplify the effects. Some of these fragments are *beta*-glucans, another oligosaccharin, which initiates the production of phytoalexins, leading to lignin synthesis, and to the promotion of ethylene formation.[114,115]

It has been suggested that specific structural features are needed in order for the oligosaccharins to bind and to produce phytoalexin.[116] It has also been shown that the size of the oligosaccharin can play a role in the kind of effect it has on the plant. The effect of chitin with a degree of polymerisation (DP) of less than 4 does not seem to be biologically important while those with a DP > 6 appear to be the most active. The induction of phytoalexins appears to require an oligosaccharin with a DP between 3 and 15, but this ranges depending on the species. The lignin production is started by those with a DP

in the range of 8 to 15. This seems to be a fairly steady range for most species that are known to utilize lignin for defensive purposes. Proteinase inhibitors can be elicited by some of the smaller oligosaccharins, even by those with a DP as low as 2.[111,117]

Chitin and chitosan are cell wall structural components found in many fungi. When oligosaccharins are released from these compounds, it has been shown to elicit phytoalexin accumulation in pea pods, to lead to accumulation of proteinase inhibitors in tomato and potato leaves, and to the synthesis of a *beta*-1,3-glucan, callose, in parsley.[111,114]

If, as suggested above, oligosaccharin receptors are located in the cell membranes of plants, there must be some mode of action for a signal to be sent out and to start the production of the various chemicals used for defense. Some of the events that take place occur very quickly and affect the cell membrane in such a way making them less susceptible to attack. The speed with which this happens suggests that there may be a direct link between the oligosaccharins and the cell membrane. One of the actions that may cause this is the rapid depolarization of the membrane caused by the oligosaccharin. Fungal elicitors from the material of two plant pathogens began depolarization of root cells within seconds after application. About a minute later, a steady state was reached that lasted for 25 minutes before returning to their original state. When the elicitors were removed before the 25 minutes were over, repolarization began immediately. Another response to the elicitor also involves depolarization, but includes another step. 10 Minutes after depolarization, the membrane became hyperpolarized, and this seems to have an influence in the production of phytoalexins.[113]

At present, the signaling pathway from the point at which the oligosaccharin contacts the cell surface to where the final response is realized is not well conducted. Moreover, when the oligosaccharin binds to a protein on the cell surface it is followed by an unknown step that is thought to activate more than one defence response.[118] This unknown step triggers a specific gene activity. This gene activity begins the production of proteins involved in the synthesis of phenylpropanoid enzymes, HO-cinnamoyl-CoA and HO-cinnamoyl-polymers. The phenyl propanoids are an initial step in the formation of soluble coumarin phytoalexins which can be exported out of the cell. The HO-cinnamoyl-CoA leads to polymers similar to lignin. The HO-cinnamoyl-polymers are exported to the cell wall and are thought to be possible phytoalexins that are derived from the cell wall.[118]

Chitosan leads directly to 1,3 *Beta*-glucan synthase that leads to callose deposition in the cell wall. Some proposed that this callose deposition may include a change in protein phosphorylation/dephosphorylation, an increase of internal calcium concentration , a decrease in internal potassium concentration, an decreasing internal pH, and a change in the jasmonic acid production. It is unclear which combination of these events and how much of each is necessary to cause the desired response.[118,119]

Preliminary studies have been performed to examine the importance of calcium concentration in cells in connection with oligosaccharin mechanisms. When a calcium blocker was added to carrot cell cultures, the normal accumulation of phytoalexins was no longer observed following the infection of the cells. It was concluded that the calcium concentrations are important in phytoalexin elicitor responses. An increase in intercellular levels of cyclic adenosine mono-phosphate (cAMP) was noticed when the cells were exposed to an elicitor. It is proposed that calcium and cAMP may act as second messengers in phytoalexin synthesis.

Another study[120] investigated the effect of pH on the defence responses. Oligosaccharins with a DP from 6 to 15 were used to elicit responses from tobacco cells in culture. Following the application of the elicitors, the cell culture medium became alkaline, reaching maximum levels after 50 minutes. The pH slowly returned to its original state after another 150 to 200 minutes. In addition, after application of the elicitor, the external potassium levels was increased. The intercellular cytosolic levels of potassium, however dropped after exposure to elicitors but then rose again in the following hours. This data suggests a possible place for both potassium concentration and pH level changes as second messengers on the oligosaccharin pathway.

3.3. Antiviral activity of chitosan

Antiviral activity of chitosan depends on the average degree of polymerization, the degree of *N*-deacetylation, the positive charge value, as well as the character of the chemical modifications of the molecule. Possible mechanisms of suppressing viral infections by chitosan are also discussed.[121-126]

Major factors of suppressing phage infections by chitosan are phage particles inactivation and inhibition of bacteriophage reproduction at the cellular level. Evidently

chitosan may be used for induction of phagoresistance in industrial microorganism cultures in order to prevent undesirable phagolysis caused by inoculum contamination by virulent bacteriophages or by spontaneous prophage induction in lysogenic culture.

Chitosan possesses an antiviral activity by its ability to induce resistance toward viral diseases in plants, to inhibit viral infections in animal cells and to prevent the multiplication of bacteriophages in infected cultures of microorganisms.[121,122]

The ability of chitosan to suppress viral plant infections does not depend on the virus type; chitosan affects the plant itself inducing resistance to the viral infection in plants. Imitating the contact of the plant with a phytopathogen, chitosan induces a wide spectrum of protective reactions in the plant, which limits a systemic spread of the viruses and viroids over the plant and lead to the development of the systemic acquired resistance.

Chitosan applied by spraying or inoculating leaves protected various plant species against local and systemic infection caused by alfalfa mosaic virus (ALMV), tobacco necrosis virus (TNV), tobacco mosaic virus (TMV), peanut stunt virus (PSV), cucumber mosaic virus (CMC) and potato virus X (PVX). The efficiency of chitosan to inhibit viral infections depends on the host-virus combination, chitosan concentration and the application method.[123,124]

Using chitosan or its salts acetate and hydrochloride prevents phage infection of an *E. coli*. It was shown that chitosan inhibited the productive infection caused by the bacteriophage, the efficiency of inhibition of bacteriophage depending directly on the final concentration of chitosan in the medium. Neither chitosan nor its salts significantly inhibited the growth of the bacterial culture. Chitosan added to nutrient media inhibits the multiplication of virulent bacteriophages in infected microbial cultures thus preventing lysis of microorganisms.[125]

In mammals, application of chitosan can stimulate the immune response to virus antigens. The ability to induce the interferon production could be supposed as another mechanism of antiviral activity of chitosan in animals. In addition, certain sulphated derivatives of chitosan were shown to inhibit the reproduction of some retroviruses *in vitro*.[126]

Chitosan's impact on viral infections in animals is determined by its ability to affect both the inductive phase of the immune response in animals and numerous effector mechanisms of the immune system. Chitosan's ability to induce interferon production may represent another important factor of antiviral resistance. Chitosan sulfated derivatives, specifically inhibiting retroviruses reproduction, were also synthesized.[126]

4. Conclusion

Even though, chitosan and its derivatives have been considered as versatile biopolymers in agricultural applications, its potential uses as antimicrobial compound and its mode of action need to be further studied in depth.

In that sense, research and development is interest in developing novel derivatives of chitosan to increase the antimicrobial activity in accordance with low mammalian toxicity. Most physiological activities and functional properties of chitosan and its derivatives clearly depend upon their molecular weight. It is a current matter of discussion as to whether these biopolymers may have the potential to influence physiological functions or metabolism functions in microorganisms.

Therefore, a significant increase in the number of scientific studies can be expected.

References

(1) Muzzarelli, R. A. A. *'Chitin', Pergamon Press, Oxford. UK,* **1977.**

(2) Goosen, M. F. A. *Technomic Publishing Co.,* Inc., Lancaster, Pa. **1997.**

(3) Han, L. K.; Kimura, Y.; Okaauda, H. *Int. J. Obes. Relat. Metab. Disord.* **1999**, 23,174.

(4) Liu, X. F.; Guan, Y. L.; Yang, D. Z.; Li, Z.; Yao, K. D. *J. Appl. Polym. Sci.* **2001,** 79,1324.

(5) Teng, W. L.; Khor, E.; Tan, T. K.; Lim, L. Y.; Tan, S. C. *Carbohydr. Res.* **2001,** 332, ,305.

(6) Pochanavanich, P.; Suntornsuk, W. *Lett. Appl. Microbiol.* **2002**, 35, 17.

(7) Muzzarelli, R. A. A. *Carbohydr. Polym.* **1992,** 19, 321.

(8) Muzzarelli, R. A. A.; Ilari, P. ; Tomasetti, M. *Carbohydr. Polym.* **1993,** 20, 99.

(9) Muzzarelli, R. A. A.; Ilari, P. *Int. J. Biol. Macromol.* **1994**, 16, 177.

(10) Heras, A.; Rodriguez, N. M.; Ramos, V. M.; Agullo, E. *Carbohydr. Polym.* **2001,** 44, 1.

(11) Jia, Z.; Shen, D.; Xu, W. *Carbohydr. Res.* **2001,** 333, 1.

(12) Ding, W.; Lian, Q.; Samuels, R. J.; Polk, M. B. *Polym.* **2003**, 44, 547.

(13) Kurita, K.; Ikeda, H.; Yoshida, Y.; Shimojoh, M.; Harata, M. *Biomacromol.* **2002**, 3, 1.
(14) Ramos, V. M.; Rodriguez, N. M.; Diaz, M. F.; Rodriguez, M. S.; Heras, A.; Agullo, E. *Carbohydr. Polym.* **2003**, 52, 39.
(15) Ronghua, H.; Yumin, D.; Jianhong, Y. *Carbohydr. Polym.* **2003**, 51, 431.
(16) Hudson, S. M. In: Domard, A.; Roberts, G. A. F.; Varum, K. M. (Eds.), *Advances in Chitin Science*, **1997**, II, 590.
(17) Kim, C. H.; Cho, J. W.; Chun, H. J. *Polym. Bull.* **1997**, 38, 387.
(18) Aiedeh, K.; Taha, M. O. *Eur. J. Pharm. Sci.* **2001**, 13, 159.
(19) Ishii, T.; Okahata, Y.; Sato, T. *Biochem. Biophys. Acta.* **2001**, 1514, 51.
(20) Pascual, E.; Julia, M. R. *J. Biotechnol.* **2001**, 89, 289.
(21) Peter, G. S.; Martinez, M. L. *Vaccine* **2001**, 19, 661.
(22) Baba, Y.; Noma, H.; Nakayama, R.; Matsushita, Y. *Anal. Sci. **2002**,* 18, 359.
(23) Ikinci, G.; senel, S.; Akincibay, H.; Kas, S.; Ercis, S.; Wilson, C. G.; Hincal, A. A. *Int. J. Pharm.* **2002**, 235, 121.
(24) Li, Z.; Zhuang, X. P.; Liu, X. F.; Guan, Y. L.; Yao, K. D. *Polym.* **2002**, 43, 1541.
(25) Strand, S. P.; Varum, K. M.; Ostgaard, K. *Colloids and surfaces B: Biointerfaces* **2003**, 27, 71.
(26) Savard, T.; Beauliu, C.; Boucher, I.; Champagne, C. P. *J. Food Protection* **2002**, 65, 828.
(27) Seo, H. J.; Mitsuhashi, K.; Tanibe, H. *In Advances in Chitin and Chitosan* ed. Brine, C. J., Sandford, P. A. and Zikakis, J.P.**1992**, pp.34-40. NewYork: Elsevier Applied Science.
(28) Sudarshan, N. R.; Hoover, D. G.; Knorr, D. *Food Biotechnol.* **1992**, 6, 257.
(29) Wang, G. J. *J. Food Protection* **1992**, 55, 916.
(30) Chen, C. S.; Liau, W. Y.; Tsai, G. J. *J. Food Protection* **1998**, 61, 1124.
(31) Roller, S.; Covill, N. *Int. J. Food Microbiol.* **1999** , 47, 67.
(32) Roller, S.; Covill, N. *J. Food Protection* **2000**, 63, 202.
(33) Tsai, G. J.; Su, W. H. *J. Food Protection* **1999**, 62, 239.
(34) Rhoades, J.; Roller, S. *Appl. Environ. Microbiol.* **2000**, 66, 80.
(35) Knowles, J. R.; Roller, S. *J. Food Protection* **2001**, 64, 1542.
(36) Stossel, P.; Leuba, J. L. *Physiopathol.* **1984**, 111, 82.
(37) Kendra, D. F.; Hadwiger, L. A. *Exp. Mycol.* **1984**, 8, 276 .
(38) Cuero, R. G.; Osuji, G.; Washington, A. *Biotechnol. Lett.* **1991**, 13, 441.
(39) Cuero, R.; Duffus, E.; Osuji, G.; Pettit, R. *J. Agric. Sci.* **1991**, 117, 165.
(40) Benhamou, N.; Lafotaine, P. J.; Nicole, M. *Phytopathol.* **1994**, 84, 1432.
(41) Laflamme, P.; Benhamou, N.; Bussieres, G.; Dessureault, M. *Can. J. Bot.* **1999**, 77, 1460.
(42) El Ghaouth, A. J.; Asselin, A. *In Advances in Chitin and chitosan*, Brine, C. J.; Sandford, P. A.; Zikakis, J. P., Eds., Elsevier, Amsterdam, **1992**, 440.
(43) El Ghaouth, A. J.; Wilson, C. L. PCT, Wo **1996**, 96, 13985,.
(44) Muzzarelli, R. A. A.; Muzzarelli, C.; Tarsi, R.; Miliani, M.; Gabbanelli, F.; Cartolari, M. *Biomacromol.* **2001**, 2, 165.
(45) El Ghaouth, A.; Arul, J.; Asselin, A.; Benhamou, N. *Mycol. Res.* **1992**, 96, 769.
(46) El Ghaouth, A.; Smilanick, J. L.; Brown, G. E.; Ippolito, A.; Wisniewski, M.; Wilson, C. L. *Plant Dis.* **2000**, 84, 243.
(47) Allan, C. R.; Hadwiger, L. A. *Exp. Mycol.* **1979**, 3, 285.
(48) Bhaskara, M. V. *J. Agric. Food. Chem.* **1999**, 47, 1208.
(49) Freepons, D. *IN Applications of Chitin and Chitosan*; Goosen, M. F. A., Eds.; Technomic: Basel, Switzerland, **1997**, 129.
(50) Hadwiger, L. A.; Fristensky, B.; Riggleman, R. In: *Chitin, Chitosan and Related Enzymes*; Zikakis, J. P.; Ed.; Academic Press: New York, **1984**, 292.
(51) Bhaskara, M. V.; Arul, J.; Angers, P.; Couture, L. *J. Agric. Food Chem.* **1999**, 47, 1208.
(52) El Ghaouth, A.; Pannampalam, R.; Castaigne, F.; Arul, *J. Hortscience* **1992**, 27, 1016.
(53) Fang, S. W.; Li, C. F.; Shin, D. Y. C. *J. Food Protection* **1994**, 57, 136.
(54) Savage, P. J.; Savage, G. P. *Proceeding of the Nutriton Society of New Zealand* **1994**, 19, 129.
(55) Cheah, L. H.; Page, B. B. C. New Zealand *J. Crop Horticultural Sci.* **1997**, 25, 89.
(56) Muzzarelli, R. A. A., *Ed. Chitosan per os:* from dietary supplement to drug carrier, Atec: Grottammare, Italy, **2000**.
(57) Min, H. K.; Hatai, K.; Bai, S. *Fish Pathol.* **1994**, 29, 73.
(58) Muzzarelli, R. A. A. *Chitin in nature and technology,* Plenum Press, New York, London **1986**.
(59) Davies, D. H.; Elson, C. M.; Hayes, E. R. *Chitin and Chitosan* (Skjak-Braek, G.;

Anthonsen, T.; Sandford, P., Eds.), 467-472, Elsevier Applied Science, London, UK.
(60) El Ghaouth, A.; Arul, J.; Pannampalam, R. *J. Food Proc. Preserv.* **1991**, 15, 359.
(61) Jiang, Y.; Li, Y. *Food Chem.* **2001**, 73, 139.
(62) Uragami, T.; Matsuda, T.; Okuno, H.; Miyata, T. *J. Membrane Sci.* **1994**, 88, 243.
(63) Dutkiewiez, J.; Tuora, M.; Judkiewicz, L.; Ciszewski, R. In: *Advances in Chitin and Chitosan* (Brine, C. J.; Sandford, P. A.; Zikakis, J. P., Eds.), **1992**, 496-505, Elsevier Applied Science, Oxford, UK.
(64) Hoagland, P. D.; Parris, N. *J. Agric. Food Chem.* **1996**, 44, 1915.
(65) Butler, B. L.; Vergano, P. J.; Testin, R. F.; Bunn, J. N.; Wiles, J. N. *J. Food Sci.* **1996**, 61, 953.
(66) Chen, R. H.; Hwa, H. *Carbohydr. Polym.* **1996**, 29, 353.
(67) Kittur, F. S.; Kumar, K. R.; Tharanathan, R. N. *Z lesbensm. Unters Forsch.* A. **1998**, 206, 44.
(68) Chen, M.; Yeh, G. H.; Chiang, B. *J. Food Proc. Preserv.* **1996**, 20, 379.
(69) Wong, D. W. S.; Gastineau, F. A.; Gregorski, K. A.;Tillin, S. J.; Pavlath,A. E. *J. Agric. Food Chem.* **1992**, 40, 540.
(70) Du, J.; Gemma, H.; Iwahori, S. *J. Japan Soc. Hort. Sci.* **1997**, 66, 15.
(71) Labuza, T. P.; Breene, W. M. *J. Food Proc. Preserv.* **1989**, 13, 1.
(72) Torres, J.; Motoki, M.; Karel, M. *J. Food Proc. Preserv.* **1985**, 9, 75.
(73) Field, C.; Pivarnik, L. F.; Barnett, S. M.; Rand, A. *J. Food Sci.* **1986**, 51, 66.
(74) Rinaudo, M.; Domard, A.; Skjak-Braed, G.; Anthonsen, T.; Sandford, P., eds), **1989**, 71-86, Elsevier Applied Science, London, UK.
(75) Cuero, R.; Lillehoj, E. *Biotechnol. Lett.* **1990**, 4, 275.
(76) Muzzarelli, R. A. A.; Tarsi, R.; Filippini, O.; Giovanetti, E.; Biagini, G.; Varaldo, P. E. *Antimicr. Agents Chemother.* **1990**, 34, 2019.
(77) Sosa, M. A.; Azley, F.; Koch, J. A.; Vercellotti, S. V.; Ruprecht, R. M. *Biochem. Biophys. Res. Comm.* **1991**, 174, 489.
(78) Jolles, P.; Muzzarelli, R. A. A. *Eds. Chitin and Chitinases*; Birkhauser: Basel, Switzerland, **1999**.
(79) Franklin, T. J.; Snow, G. A. *In Biochemistry of Antimicrobial Action*; chapman and Hall: London **1981**.
(80) Takemono, K.; Sunamoto, J.; Askasi, M. Polymers and Medical Care; Mita: Tokyo; **1989**, Chapter IV.
(81) Kim, C. *Polymers for Advanced Technologies*, **1997**, 8, 319.
(82) Yalpani, M. In C. Brine *et al.* (Ed.), *Advances in Chitin and Chitosan* **1992**, pp. 543.
(83) Chang, D. S.; Cho, H. R.; Goo, H. Y.; Choe, W. K. *Bull. Korean Fish Soc.* **1989**, 22, 70.
(84) Darmadji, P.; Izumimoto, M. *Meat Sci.* **1994**, 38, 243.
(85) Simpson, B. K.; Gagne, N.; Ashie, I. N. A.; Noroozi, E. *Food Biotechnol.* **1997**,11, 25.
(86) Papineau, A. M.; Hoover, D. G.; Knorr, D.; Farkas, D. F. *Food Biotechnol.* **1991**, 5, 45.
(87) Anderson, D. P.; Siwicki, A. K. *Prog. Fish-Cult.* **1994**, 56, 258.
(88) Kawai, T.; Naito, T.; Matadaira, Y.; Yura, H. *Jpn. Kokaitokkyo Koho JP* **1999**, 11, 441.
(89) Golovin, P. P.; Golovina, N. A.; Guseva, N. V.; Gamygin, E. A.; Romanova, N. N.; Suskov, I. D. *In Chitin and Chitosan*; Varlamov, P., Ed.; VINRO: Moscow, **1999.**
(90) Hadwiger, L. A. *J. Cell Biochem.* **1986,**1S-S Suppl. 10C.
(91) Jung, B.; Kim, C.; Choi, K.; Lee, Y. M.; Kim, J. *J. Appl. Polym. Sci.* **1999**, 72, 1713.
(92) Leuba. S.; Stossel, P. In: *Chitin in Nature and technology*, (Muzzarelli, R. A. A.; Jeuniaux, C. eds.), Plenum: New York. **1985**, P 217.
(93) Hadwiger, L. A.; Kendra, D. F.; Fristensky, B. W.; Wagoner, W. In: *Chitin in Nature and Technology,* (Muzzarelli, R. A. A.; Jeuniaux, C.; Gooday, G. W., eds.) Plenum Press, New York, USA. **1985**, pp. 209.
(94) Dornenburg, H.; Knorr, D. *J. Agric. Food Chem.* **1997**, 45, 4173.
(95) Bhaskara, M. V.; Arul, J.; Essaid, A. B.; Anger, P.; Richard, C.; Castaigne, F. *Biocontrol Sci. Technol.* **1998**, 8, 33.
(96) Chen, T. *In Product and Nature Biological Medicine :* Beijing, China, **1998**, pp. 282.
(97) Tokura, S.; Miuray, Y.; Johmen, M.; Nishi, N.; Nishimura, S. I. *J. control. Rel.* **1994**, 28, 235.
(98) Daly, W. H. *United States Patent*, **2001**, 6,306,835.
(99) Lang, G. In: *G. Skjak-Braek et al. (eds.), Chitin and Chitosan, Sources, Chemistry, Biochemistry, Physical*

RECEIVED JAN 2 6 2004

From: VALERIE L AKINS (509)335-9542
DEPARTMENT OF PLANT PATHOLOGY
JOHNSON HALL ROOM 345

PULLMAN, WA, 99164

REVENUE BARCODE

FedEx.

To: Robert Pooler (202)720-3252
National Organic Standards Board
USDA/AMS/TM/NOP, Rm2510-So
Ag Stop 0268
Washington, DC, 200906456

SHIP DATE: 23JAN04
WEIGHT: 2 LBS

Ref:

DELIVERY ADDRESS BARCODE(FEDEX-EDR)

TRK # 7925 5618 5590 FORM 0201

FedEx **STANDARD OVERNIGHT**

MON
AA
Deliver by:
26JAN04

IAD

20090-DC-US **XC WASA**

Shipping Label: Your shipment is complete

1. Use the 'Print' feature from your browser to send this page to your laser or inkjet printer.
2. Fold the printed page along the horizontal line.
3. Place label in shipping pouch and affix it to your shipment so that the barcode portion of the label can be read and scanned.

Warning: Use only the printed original label for shipping. Using a photocopy of this label for shipping purposes is fraudulent and could result in additional billing charges, along with the cancellation of your FedEx account number.