THE EFFECT OF CHITOSAN ON HEMOSTASIS: CURRENT WORK AND REVIEW OF THE LITERATURE

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Abstract

Previous studies with chitosan provided evidence of enhanced lingual hemostasis in normal and platelet dysfunctional models. Our current work demonstrates that chitosan enhances hemostasis in anticoagulated (heparinized) models. An *in vivo* experiment investigated the effect of chitosan on bleeding times for tongue incisions in ten New Zealand white rabbits. Using a randomized, blinded experimental design, one incision in each animal was treated with chitosan while the other was treated with control vehicle. Activated coagulation times and extra-oral bleeding times were measured for each animal prior to, during, and after heparin. Intravenous infusion of heparin more than tripled mean activated coagulation time and increased mean systemic bleeding time by 37%. In this heparin model, lingual incisions receiving chitosan showed a 48% improvement in bleeding time as compared to lingual incisions receiving control vehicle ($p=0.003$). An *in vitro* experiment investigated the effect of chitosan on blood flow. Normal human blood was centrifuged and separated into fractions. Samples of blood with and without chitosan were passed through micropore filters (12µ) under negative pressure (20cm water). Blood flow rates were measured for 0.4 ml sample fractions mixed with chitosan or control vehicle. The red blood cell fraction interacted with chitosan to decrease blood flow. Similar reactions were not seen in any other fraction nor in the red cell fraction with vehicle only. Chitosan (400 µg/ml) decreased blood (red cells) flow by 60% compared to all other samples while ≥800 µg/ml chitosan stopped blood flow. Conclusion: Chitosan interacts with red blood cells to form cellular clots and stop blood flow. These results support previous studies suggesting a hemostatic mechanism that is independent of platelets and the normal clotting cascade.

Keywords: Chitosan, heparin, bleeding, hemostasis, erythrocytes, lingual, incision, rabbit model.

Introduction

Chitosan, a polymeric complex carbohydrate derived from chitin, is a biologically derived biopolymer that may have application in medically compromised (bleeding disordered) individuals. Previous studies with chitosan have established its unique attributes as a hemostatic agent functioning independently of platelet and normal clotting mechanisms as presently understood (1,2). Of particular importance may be its putative capacity to induce clot formation in the absence of coagulation factors and/or platelets. The purpose of this study was to evaluate the effect of chitosan as a hemostatic agent in an anticoagulated (heparinized) animal model.

Materials and methods

Experimental design: Anesthesia in ten 5.0-6.0 lb. New Zealand white rabbits (Oryctolagus cuniculus) was induced with IM ketamine hydrochloride (15mg/kg, Bristol Laboratories, Syracuse, NY) and xylazine hydrochloride (2mg/kg, Lloyd Laboratories, Shenandoah, Iowa) and then maintained with 1.5% halothane and 50% nitrous oxide in oxygen via oral endotracheal tube.

Coagulation time was increased by intravenous infusion of heparin sodium (ElkinsSinn, Inc., Cherry Hill, NJ). After a pre-determined level (>400 sec.) of anticoagulation was confirmed by activated coagulation time (ACT) measurements, each animal received an incision along the right and left lateral borders of the tongue, one side was treated with chitosan solution and the other was treated with the control vehicle. Lingual bleeding times were measured up to a maximum of 1200 seconds (20 min.). Following the final lingual bleeding time measurement, systemic bleeding time and ACTs were measured. A single, blinded examiner performed all incisions, applied test substances, and recorded measurements.

Tongue incision model: The mouth was held open with an ocular speculum and a stainless steel stent was sutured to the tongue for stabilizing soft tissues and ensuring consistency in making incisions. Tongue incisions were made on the lateral border with a sterile #15 surgical blade to a depth of 2 mm and a length of 15 mm. Incisions were immediately perfused with 0.2 ml of test solution (chitosan or control) and bleeding time was measured by the filter paper method described below.

Test solutions: Chitosan (Hoechst-Roussel Pharmaceuticals Inc., Somerville, NJ) was used at a concentration of 2 mg/ml in 0.2% glacial acetic acid. Control solution consisted of 0.2% glacial acetic acid without chitosan. Each animal received one syringe of each test solution randomly assigned to right or left sides. Test solution was introduced as a bolus application along the length of the incision with a 20 gauge blunt needle. With the tongue

extruded and secured to the stent, each lateral border was sufficiently isolated to prevent cross-contamination of sites.

Lingual bleeding time measurement: Lingual bleeding time was measured in seconds from the time the test solution application was completed until hemostasis was established or until the incision bled for a maximum of 1200 seconds (20 min.). Hemostasis was determined using the filter paper procedure. Small squares of Whatman No. 1 filter paper (W & R Balston, Ltd., England) were gently blotted near the forming blood clot.

Systemic coagulation time measurement: Activated coagulation times were measured in anesthetized animals before, during and after infusion of heparin sodium using an Hemochron 400 (International Technidyne Corp., Edison, NJ). Blood collected from a femoral catheter was used to fill Hemochron activation tubes. The dosage of heparin sodium used to increase coagulation time was determined and titrated individually by monitoring repeated coagulation time measurements. Each animal was titrated with heparin sodium to an average activated coagulation time that was between 400-600 seconds. The average of two measurements was recorded as the coagulation time for each period.

Systemic bleeding time measurement: Extraoral bleeding times were measured in anesthetized animals before, during and after infusion of heparin sodium. The external aspects of the ears were shaved, washed, dried and an area free of visible blood vessels selected and marked for incision length. Two incisions, 1 cm in length were made using a sterile scalpel blade. Bleeding time was measured using the filter paper method. Measurements were continued to a maximum of 1200 seconds (20 min.). Average bleeding time of the two incisions was used for analysis.

In vitro blood flow experiment: Flow rates were measured for human blood separated into cellular components. Twelve milliliters whole blood was drawn from a healthy volunteer. The whole blood was separated into cellular fractions and mixed with chitosan, glucosamine or vehicle solution. The cellular fractions were re-suspended in 0.05 M phosphate buffer solution (pH 7.4). In order to measure the magnitude of clot formation, a negative pressure apparatus (3) was utilized. Various filter sizes (5-12μm pore) and negative pressures (0-20 cm water) were used. Flow rate tests were performed for the chitosan and control solutions.

Results and discussion

Systemic coagulation time: The heparinization procedure was highly effective and induced more than a three-fold increase in ACT. Prior to heparin administration the coagulation time was 144±22 seconds while after heparin, time was increased to 477±18 seconds. This effect was found to be significant by paired t-test ($p \leq$

0.001) and is consistent with known properties of this drug. The increase in systemic coagulation time was sustained within the range of 400-600 seconds throughout the experimental period.

Systemic bleeding time: The anticoagulation achieved with intravenous administration of heparin correspondingly induced a 40% increase in systemic bleeding time (232±26 sec.) as compared to bleeding time before heparinization (166±11 sec.). This increase was found to be significant by paired t-test ($p \leq 0.01$). Although heparin is primarily known as an inhibitor of blood coagulation via inactivation of coagulation enzymes, a heparin induced increase in bleeding time was anticipated and may be attributed to its effect on platelet aggregation and adhesion.

Lingual bleeding time: Control (368±108 sec.) and chitosan (209±81 sec.) lingual bleeding times differed significantly. Topical administration of chitosan resulted in lingual bleeding times that were 43% lower than control, despite the heparin-induced anticoagulation. The difference between chitosan and control lingual bleeding times was significant by paired t-test ($p \leq 0.001$).

In vitro blood flow experiment: In a separate experiment, flow rates for chitosan, glucosamine and vehicle solutions with and without blood fraction were tested via Millipore filters. Separately, all test solutions and blood fractions passed through in 0.2-0.3 ml/second. This indicated that neither the blood cell fractions nor the test solutions had an effect on flow rate through this apparatus. Flow rates for the red blood cell fraction mixed with chitosan were the most significant. The red blood cell fraction interacted with chitosan to decrease blood flow. Similar reactions were not observed for any other fraction nor the mixture of fractions with test solutions. At 400 µg/ml, chitosan decreased blood flow by 60% while at ≥800 µg/ml, chitosan stopped blood flow. The *in vitro* effect of chitosan on red blood cell morphology and behavior supports the theory that it interacts specifically with erythrocytes causing red cell adhesion and achieving hemostasis by forming a cellular aggregate or plug. (Figures 1,2)

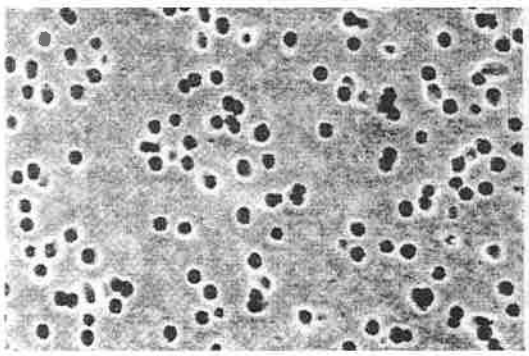

Figure 1. Millipore filter after blood flow test (control). No RBCs or clot formation. (x500)

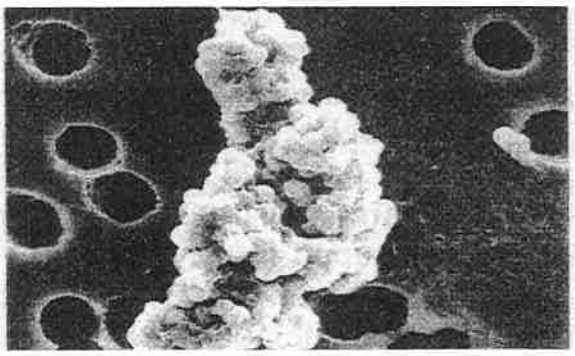

Figure 2. Millipore filter after blood flow test (chitosan). RBCs aggregate to form clot. (x2000)

Potential application for this agent in oral hemostasis was previously examined in normal and platelet dysfunctional rabbits. Chitosan has been shown to decrease lingual bleeding time by 32% in a normal animal model (1). However, its potential therapeutic value may depend on an ability to promote hemostasis in bleeding-disordered and/or therapeutically anticoagulated individuals. Another study showed that chitosan decreased lingual bleeding time by 56% in a model with epoprostenol-induced platelet dysfunction (2). In the present study, chitosan decreased lingual bleeding time by 43% in a model with heparin induced anticoagulation. Heparin was chosen because of the known properties of anticoagulation and because it is a situation perhaps representative of the type of clinical condition in which chitosan's therapeutic properties could be advantageously exploited. Heparin was easily administered and demonstrated a potent anticoagulation.

While chitosan's hemostatic mode of action is not known, it is thought to function independent of platelets and normal coagulation pathways. Several investigators have suggested an interaction of chitosan with cellular components of blood. Malette, et. al. (4) reported chitosan-induced clots with a substrate of washed erythrocytes only and of chitosan's ability to maintain hemostasis in arterial Gore-Tex™ (Gore, Flagstaff, AZ) grafts in the presence of heparinized blood. Brandenberg, et. al. (5) demonstrated microscopically and histopathologically that chitosan was biocompatible with brain tissue and suggested that an interaction with blood cells were responsible for clot formation. Subsequently, Arand and Sawaya (6) cited chitosan's unique mode of action as an attribute for chemical hemostasis in neurosurgery. Van der Lei, et. al. (7,8) showed that chitosan treated PTFE vascular grafts induced a unique red blood cell clot layer on the inner surface of the grafts that enhanced wound healing. Chitosan treated grafts were lined with smooth muscle and endothelium whereas, without chitosan the grafts healed with typical fibrosis. Finally, Rao and Sharma (9) performed extensive testing of chitosan (with various treatments) as a hemostatic biomaterial. They demonstrated that chitosan was non-toxic, biocompatible and that it has hemostatic activity that is independent of the classical coagulation cascade suggesting interaction of chitosan with platelets as well as erythrocytes. They found that chitosan's agglutination potential resulted from its polymeric structure and its polycationic charge. The present *in vivo* and *in vitro* experiments support the findings of other investigators that it is the interaction of chitosan with the red blood cells that is responsible for hemostasis.

Kind, et. al. compared topical chitosan, to topical thrombin and pressure applied to liver lacerations in rats. (10) They

compared each of these modalities with and without the administration of heparin. As in the present study, they found that heparin increased control bleeding by about 40%. However, they reported that chitosan treatment was no better than pressure application or no treatment (control) and that it was inferior to topically applied thrombin. It is possible that liver lacerations behave differently or that the chitosan used in their study differed from that of other studies.

Hemostasis achieved independent of platelets and normal coagulation factors has significant advantages for individuals who are therapeutically anticoagulated or who have coagulopathies. The current work as well as previous studies strongly suggest that chitosan does not require normal function of the classical coagulation pathway nor the presence or function of platelets. Topical application of chitosan to lingual incisions in this heparinized model had the effect of returning bleeding time to normal. If this relationship holds true for human patients with coagulopathies (therapeutic or pathologic), topical chitosan could be a useful adjunct for attaining hemostasis.

Conclusion

This *in vivo* study examines the effectiveness of chitosan as a hemostatic agent in the oral cavity of New Zealand white rabbits whose coagulation function has been impaired by intravenous infusion of heparin sodium. It was demonstrated that topical application of chitosan significantly decreased lingual bleeding time in heparinized rabbits. Individually titrated intravenous infusion of heparin sodium increased systemic bleeding time by 40%. This heparin induced coagulopathy was counteracted by a 43% decrease in lingual bleeding time when chitosan was applied topically to lingual incisions as compared to controls. Topical application of chitosan to lingual incisions in this heparinized model had the effect of returning bleeding time to normal.

The *in vitro* study examined the effect of chitosan on different fractions of whole blood. The red blood cell fraction interacted with chitosan most significantly and stopped blood flow completely at concentrations ≥800 μg/ml. The most probable mechanism of action for chitosan's effect on hemostasis is a cross-linking of erythrocytes to one another, hence forming an artificial cellular clot.

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Acknowledgments

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THE ASSESSMENT OF INFLUENCE OF CHITOSAN ON THE DENTAL PULP IN RATS.

Abstract

The aim of this study was to evaluate the effect of composite with chitosan modified bonding system on the condition of dental pulp in rats in the light of histological examination.The tests were carried out on molar teeth of the 15 albino Wistar rats in 2 experimental groups: a) primer without chitosan, glass-ionomer, b) primer with chitosan, bond, composite ; and in 3 control groups : c) calcium hydroxide, primer without chitosan, glass-ionomer , d) primer without chitosan, glass-ionomer, e) sound teeth.

Comparable conditions were established by preparation of cavities of similar depth, which were filled with above mentioned materials. The procedures were performed under general anaesthesia and local analgesia.

There were two periods of observations: 3 days after and 3 weeks after the procedure. The three molars forming somewhat a complex were cut with a bone for observations of eventual inflammation and its extent. Decalcified histological preparations were examined under a light microscope.

These results suggest that the composite materials with chitosan modified bonding system used in the experiment in the most cases caused slight, reversible pathological changes in the pulp.

Keywords : chitosan, teeth, dentistry bonding system, rats

The dental pulp is a tissue responsible for its vitality, elasticity and durability. It takes a space in a chamber closed by the dentin. Tubular structure of the human dentin enables connection of the pulp chamber with the oral environment following preparation of a caries lesion. Change of physical conditions and necessity of drying cause loss of dentinal fluid which in turn leads to dehydratation of the dental pulp and disorder of the pulp physiology.

Restoration of a tooth is connected with the use of a bonding system, due to which resin tags are formed in the tubules through an inflow of resin and polymerization and enable adhesion to the restorative material. The resin monomers during the polymerization or those which have not been completely polymerized are toxic to the pulp. On filling the dental cavity the outflow of fluid from dentinal tubules is not a favourable factor. It causes dilution of primer, microlaekage and reduction of bonding between the material and the tooth, thus increasing the possibility of breaking the bond due to polymerization shrinkage and

Results and discussion

Out of histological preparations with the same restorative material I have chosen and taken photographs of the cases with more severe and extended changes of the pulp.

Three days following the use of primer without chitosan and glass-ionomer blood extravasation, blood vessels hyperaemia, inflammatory cells infiltration and single small focuses of necrosis were noticed (Fig.1). After 3 weeks blood vessels hyperaemia, baloon cells and a small focus of necrosis in one case were still visible in the same group.

Three days after the application of the bonding system modified by chitosan and Valux composite material resulted in an edema of the pulp, hyperaemia and small necrotic focuses (Fig.2). In the same group blood vessels hyperaemia, baloon cells and single granulocytes were still seen. The area of necrosis had been absorbed (Fig.3).

Postek (6) reported in her investigations that there were some differences among composite materials in inducing irritation of the pulp. She carried out the comparative study of pulp reactions in dogs to selected composite materials. Histopathological examinations revealed that composite materials caused reversible pathological changes in the pulp. The author suggests that the macrofilled composite is characterised by lower polymerization shrinkage, thus causing smaller marginal microleakage. These features result in less intense pathological changes in the pulp.

In the third group 3 days after using calcium hydroxide, primer without chitosan, glass-ionomer blood vessels hyperaemia, their damage, edema, inflammatory infiltration from granulocytes and focuses of necrosis were observed (Fig.4). After 3 weeks vessels hyperaemia, inflammatory infiltration from limphocytes and small focuses of necrosis were still found (Fig.5).

Three days after the teeth had been filled with glass-ionomer a large necrotic focus occurred in the pulp adjacent to the restoration (Fig.6). No results were obtained after 3 weeks because the fillings went out.

The performed investigations are preliminary. Further development of the method of cutting composites and hybride layer will allow me to assess the range and nature of changes in relation to marginal sealing. The achieved results encourage to carry out further investigations. A change of chitosan percentage in the primer might give different results. The loss of fillings in many cases inclines me to use crowns or bony glues as protection in my future research.

Conclusion

1. Application of a composite material with the bonding system modified by 2% chitosan induced reversible pathological changes in the pulp.
2. Pathological changes in the rat dental pulp were more intense after using the bonding system wiyhout chitosan.
3. Chitosan addition to the primer in quantity of 2% improved biocompatibility of the primer with the pulp.

mastication. Microlaekage leads to penetration of bacteria and their toxins into the pulp chamber and finally to pulpal inflammation (2,3). Another problem encountered in the process of dental restoration is impermeability of adhesive resin tags, formed in the dentinal tubules, to ions and water which are necessary to nourish the tooth.

The dentin dilutes or binds noxious substances but its action is not always satisfactory. A decrease in dentin permeability may by obtained through chitosan bonding of the dentinal fluid (organic biohydrogel) which would reduce its flow.

Chitosan can seal dentinal tubules (4,5) and the hydrogel that is formed reduces dentin permeability but maintains diffusion of ions and water. Reduction in dentin permability and buffer capacities of chitosan may increase defensive function of tubular contents in relation to the pulp. Linden (4) also reports that the complex of chitosan with a native biohydrogel binds with tubular walls.

A basic substance of intertubular dentin consist of long carbohydrate chains (glycosaminoglycans or GAG's) attached to protein core. Carbohydrate chains easily maintain and bind water. They work as springs due to their chemical properties and are responsible for gel form of the basic substance. This substance is highly resistant to compression and that is why the dentin is elastic. Chitosan has been examined in the natural environment containing GAG's. They form complexes which are highly resistant to acid action (1).

The aim of the study was to evaluate the influence of a bonding system modified by chitosan on the rat pulp using histopathological examinations.

Materials and methods

The in vivo investigations were performed in first mandibular molars of 15 white Wistar rats weighting 270 g in two groups studied: a) primer without chitosan, glass-ionomer, b) bonding system modified by chitosan and composite material; and in two controls: c) calcium hydroxide, primer without chitosan, glass-ionomer, d) glass-ionomer, e) an intact tooth.

Dental cavities were prepared on the occlusal surface about 1 mm deep using a high-speed drill with water cooling in order to obtain similar conditions of the procedures. The enamel and the dentin were etched with 37% phosphoric acid for 30 s. After rinsing and drying of the cavities a light-cured polimer modified by chitosan (2% chitosan, 40% HEMA in 10% of maleic acid by weight) was applied. Calcium hydroxide agent - Life was inserted into the control teeth. Finally the teeth were restored with Valux composite material or Vitremer glass-ionomer. The dental procedures were performed in general anaesthesia. Local anaesthesia using 0,8 ml of 2% lignocaine was additionally injected.

Histopathological examinations were carried out after 3 days and 3 weeks. Dental blocks along with a bone were cut off to observe possible inflammation and its extent. The specimens were embedded in paraffin, decalcified in 40% formic acid and stained with hematoxylin and eosin. The material was fixed (through perfusion) with 6% glutaraldehyde neutralised by sodium cacodylate. The prepared specimens were observed in the light microscope. Type and extent of pathological changes in the pulp were determined.

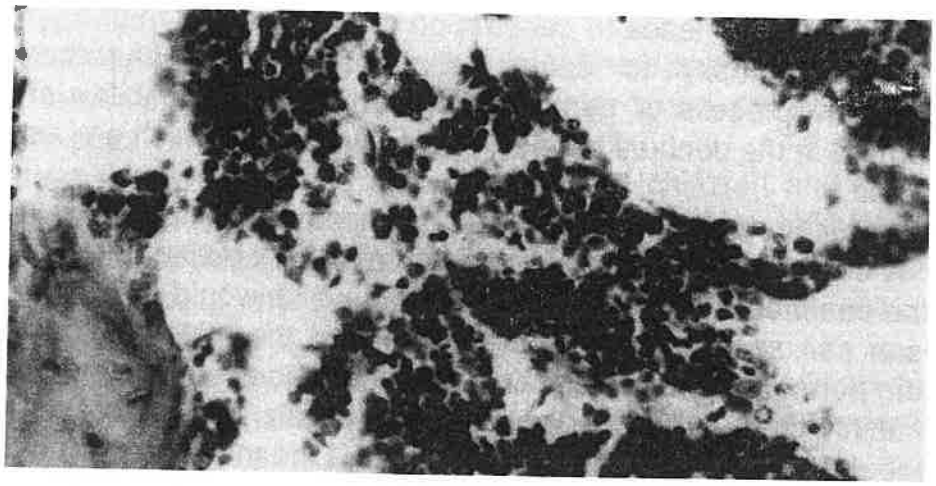

Fig. 1. Blood extravasation, blood vessels hyperaemia, inflammatory cells infiltration and single small focuses of necrosis. (x 250)

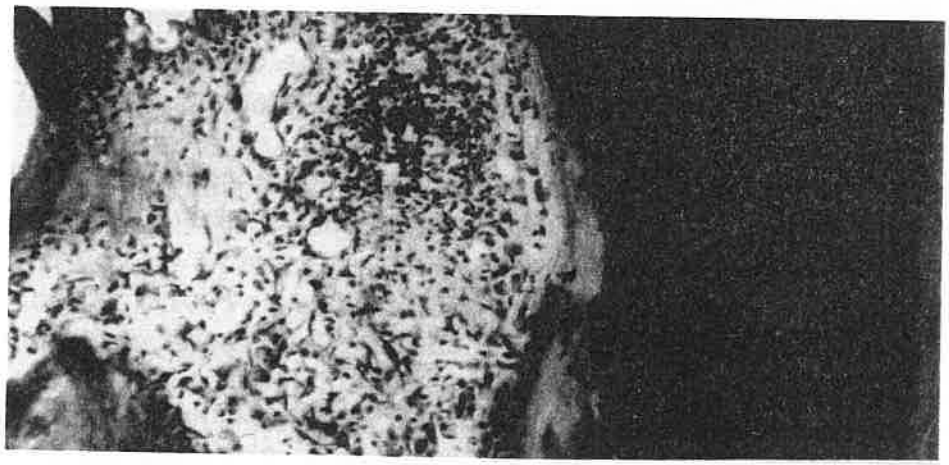

Fig. 2. Edema of the pulp, hyperaemia and small necrotic focuses. (x 120)

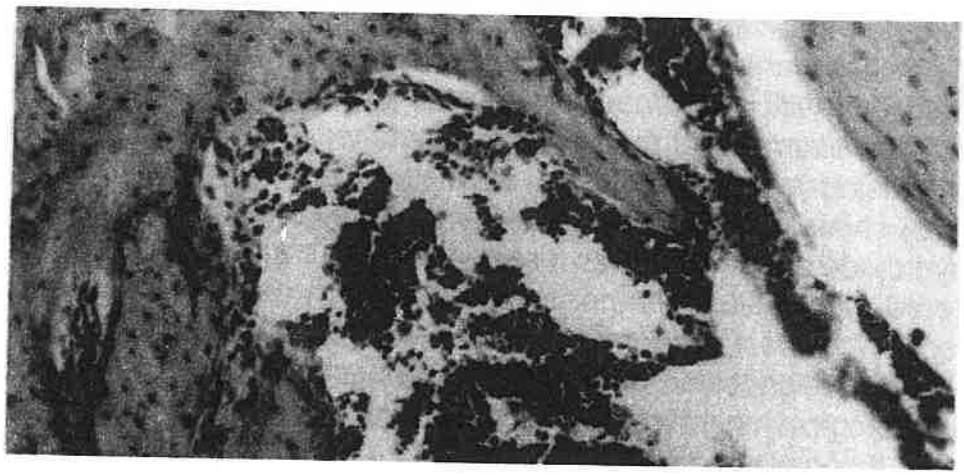

Fig. 3. Blood vessels hyperaemia, baloon cells and single granulocytes, necrosis had been absorbed. (x 250)

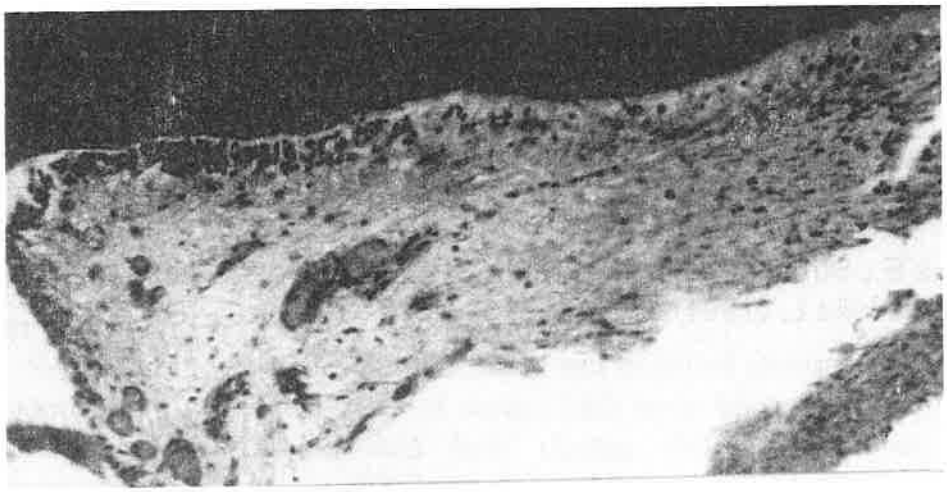

Fig. 4. Blood vessels hyperaemia, their damage, edema, inflammatory infiltration from granulocytes and focuses of necrosis. (x 120)

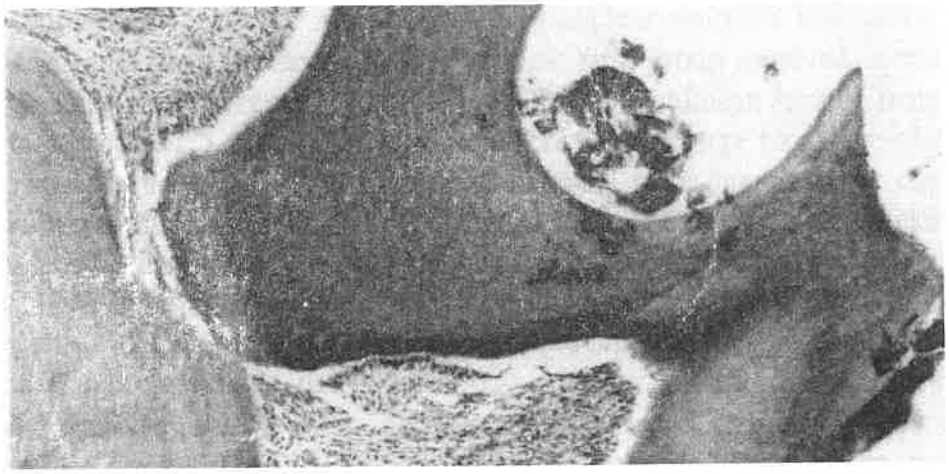

Fig. 5. Vessels hyperaemia, inflammatory infiltration from limphocytes and small focuses of necrosis.(x 65)

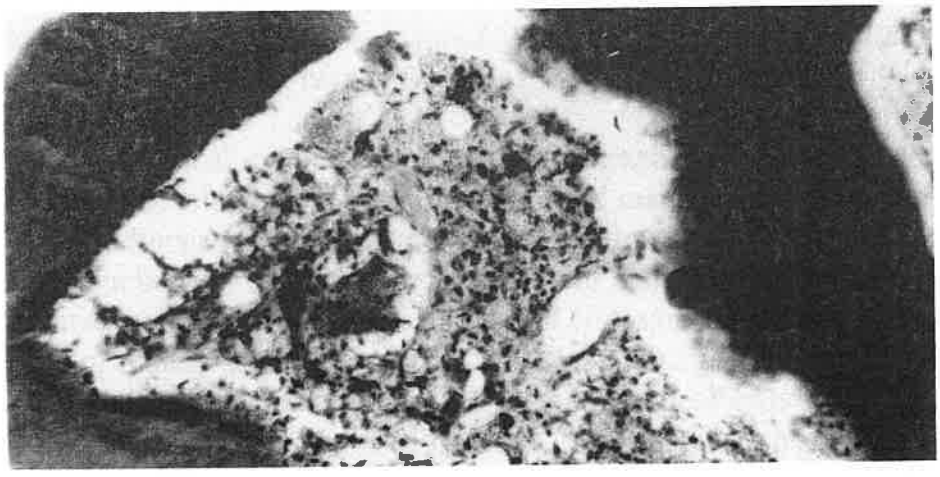

Fig. 6. Large necrotic focus occurred in the pulp adjacent to the restoration (x 120).

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CLINICAL APPLICATION OF UNMODIFIED AND MODIFIED CHITOSANS IN BONE REPAIR

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Abstract

Bone healing properties of unmodified and modified chitosan has been studied by in situ application, in clinical cases of severe limb bone fractures in canines. Chitosan without modification was obtained from shrimp shell (s.s.-chitosan) as partially deacetylated chitosan powder (PDC) whereas chitosans meant for modification were obtained from highly deacetylated shrimp shell chitosan (s.s.-chitosan) and cuttle fish chitosan (c.f.-chitosan). Both forms of highly deacetylated chitosan were modified into the 5-methyl pyrrolidinone chitosan derivatives by chemical coupling. The chemical structure of these chitosan preparations was confirmed by nuclear magnetic resonance (NMR).

Animals were divided into four groups: first group received unmodified α-PDC powder, second group received 5-methyl pyrrolidinone chitosan sponge from c.f.-chitosan, third group received 5-methyl pyrrolidinone chitosan sponge from s.s.-chitosan and the fourth group served as a control group. All animals were examined by clinical, physical and radiographic means on the first week, third week and during 6-15 weeks after surgery, to assess the status of bone healing. Application of all three types of preparations seemed to exhibit bone healing property but were accompanied by exudate formation in four out of nineteen cases (21.5%) during the first week of application. The incidence of exudate formation was seen in three out of seven cases applied with unmodified α-PDC, and one out of six cases applied with modified s.s.-chitosan but none from the modified c.f.-chitosan. The degree and speed of healing was enhanced in chitosan treated cases as compared with controls. Modified c.f.-chitosan was found to perform better than the unmodified α-PDC and modified s.s.-chitosan.[10]

Keywords: *Chitin, Partially deacetylated chitosan, 5-methyl pyrrolidinone chitosan, nuclear magnetic resonance, osteogenesis,and callus.*

Introduction

The biomedical properties of chitin, chitosan and chitosan derivatives have already been extensively utilized in skin and wound management products. Chitosan and 5-methyl pyrrolidinone derivative have also been implicated to speed up the healing process of broken bone by accelerating the growth of bone forming cells and thereby filling the gaps created by loss of bone tissue. Success of chitosan compounds for healing holes in the leg bones of rodents has been reported. As a long, charged polymer, chitosan seems to attracts macrophages to the bone. It was suggested that these cells may liberate messenger chemicals that cause immature bone cells nearby to mature into specialized bone cells. [1]

A marked osteogenic effect of chitosan was investigated in the endochondral long bones of the of the rabbit extremity by making defects of 10 mm bilaterally in the fibulas and third metacarpals of each rabbit. Chitosan reportedly induced osteogenesis in all elements of the adjacent bone structure. Soft tissue surrounding long bone defects

appeared to contribute to osteoinduction in some instances with chitosan treatment. Chitosan stimulated proliferative osteogenesis with closure of the bony defects in 8-12 weeks.[2] Bone tissue regeneration was also noticed following removal of 2-3 × 15 mm bone portions from the tibiae and subsequent medication with freeze-dried methyl pyrrolidinone chitosan.[3] In dogs, saline sponge exhibited the classic callus formation that undergo osteoblastic-osteoclastic sequence with periosteal bone formation and bone formation in the marrow cavity whereas the chitosan treated wound simply reformed cortical bone without going through this sequence. [4]

Methyl pyrrolidinone chitosan (MPC) when applied at the site of wisdom tooth avulsion promoted osteoconduction. The space left was filled with newly formed bone tissue.[5] MPC is a substituted chitosan where the nitrogen atom is simultaneously part of the methyl pyrrolidinone moiety and of the glucosamine repeating unit. Lyophilized MPC is soluble in distilled water and gelifies with biological fluids. It is susceptible to the hydrolytic action exerted by lysozyme even when chitosan used is highly deacetylated. [6-7]

Chitosan and its MPC derivative were expected to shorten the healing period when applied in conjunction with hardware implants compared to the usage of plaster cast or hardware implants alone. The main aim of the present study is to study the effect of chitosan and its derivative on bone healing in clinical cases of fractures in canines.

Materials And Methods

Preparation of Chitosan Samples

Chitosan was obtained from shrimp shells as PDC powder with about 50-55% degree of deacetylation (DD) whereas chitosans meant for modification were obtained from highly deacetylated s.s.-chitosan with about 86 % DD and c.f.-chitosan with about 82% DD. Both forms of highly deacetylated chitosan were modified into the 5-methyl pyrrolidinone chitosan derivative by chemical coupling.[6-7] The chemical structure of these chitosan preparations was confirmed by ^{1}H NMR and ^{13}C NMR spectroscopy. [8-9] (Figure 1-2). The quartet centred at 3.679-3.733 ppm and doublets at 2.10-2.146 and 1.596-1.1619 and 1.05-1.238 ppm were typical for lactam ring formation.

Unmodified PDC powder from shrimp shells intended for clinical application was sterilized by ethylene oxide, and modified 5-methyl pyrrolidinone chitosan sponges were sterilized by irradiating with gamma rays using Cobalt ^{60} isotope to a radiation dose of 25 KGy for 115 minutes in Gammacell-220 (Nordion).

Clinical Application of Chitosan Samples

Bone repair, in twenty six dogs ranging from 3 months to 2 years old, submitted with limb bone fractures was investigated in this study. The clients were informed about the clinical study. All the surgical corrections were done under general anaesthesia using standard surgical protocol. Animals were divided into four groups: first group (n=7) received unmodified α-PDC powder, second group (n=6) 5-MPC sponge from c.f.-chitosan, third group (n=5) 5-MPC sponge from s.s.-chitosan and the fourth group (n=7) served as the control. All three types of chitosan biomaterials were applied once, after internal fixation of fractured bones by open reduction technique using hard ware implants. The main purpose of internal fixation was to achieve anatomical alignment of the broken bones and thereby setting a proper environment for chitosan and its derivative to bring about ordered callus formation in a shorter time. To have more homogeneity, this study

excluded young animals of less than three months old and animals above two years of age and all pregnant females. Similarly, highly debilitated animals, animals with old fracture and fractures other than limb bones were not considered.

Results

Animals of all the three treatment groups and one control group were examined by clinical, physical and radiographic means on the first week, third week and at six to eighth week and until 15 weeks after surgery, to assess the status of bone healing. Based on the radiographic interpretation, the degree and onset of callus formation, disappearance of fracture line, and restoration of original histoarchitecture of the bones were studied. Physical examination was performed to determine the presence of pain, swelling and early recovery of limb functions. The qualitative observations made in all the four groups of animals were transformed into quantitative measurements by assigning scores based on the healing responses. Healing response was assessed mainly based on radiographic, clinical and physical examinations. Score of 5 was the maximum and 1 was the least (Table 1).

Animals applied with α-PDC showed exudate formation in 3 out of 7 cases from the site of skin sutures during the first week but did not show any signs of systemic illness. Appetite was normal in all the cases. Bone healing was complete by five weeks in Case 11, and an appreciable amount of callus was obvious on third week in Case 7. Mal-alignment of the bones due to migration of pin in 3 cases (Cases 13, 14 and 18) hindered the process of bone healing and hence necessitated its early removal. Very excellent healing was obvious by 6 weeks in Case 8 and had its plate and screws removed on 9 weeks. In other two cases, migration of pin caused unstability and hence were dropped from the study. In Case 16, very good healing was noticed by 7 weeks and the pin was removed by 10 weeks.

In animals applied with MPC from c.f.-chitosan, Case 22 showed a very good healing in which there was complete disappearance of fracture line in a matter of 6 weeks. Initiation of callus development was clearly seen by third week of operation. Similarly, there was satisfactory amount of callus in Case 21. Cases 29 and 36 showed very satisfactory amount of callus in 4 and 3 weeks of time respectively. In Case 23, complete healing with resumption of normal trabacular structure was obvious on radiograph taken at 9 weeks of chitosan application.

Among the dogs treated with MPC from shrimp chitosan, excepting Case 37, there was no exudate formation. In Cases 25 (Plates 25A-E) and 35, a good amount of callus formation was noticed by third week and fracture line was disappeared completely on the radiograph taken at 6 weeks in the former animal. In Case 34, radiograph taken at second week did not reveal callus but the animal was able to bear weight at times and radiograph taken 7 weeks later revealed a very good amount of callus and complete recovery of limb function. Case 35 which had the fractures of both right femur and left tibia showed formation of new bone tissues on radiograph taken at third week. Case 28 had exhibited a typical atrophic changes of right fore limb not treated with chitosan but development of good amount of ordered callus in the left radius ulna that was treated with MPC. The plate and screws implanted in the right fore limb has been removed under general anaesthesia to prevent further complications. Case 37 had shown chronic sero-sanguinous discharge for a quite long period. On about 3 to 4 weeks time, minimal callus development was obvious. On clinical examination, animal was found to be able to bear weight without difficulty. The callus formed by 8 weeks, had united the broken pieces of ulna completely with disappearance of fracture line, but still was evident in the radius.

Table 1 Scoring based on healing responses with different chitosan samples

SN	Case	Wt (Kg)	Sex	Age	Fracture location	Results	Score in 5.00
α-PDC							
1	8	2.6	F	6m	Rt. radius-ulna	Excellent healing	4.59
2	11	5.5	M	4m	Rt. tibia-fibula	Bone healed *	4.315
3	14	3.5	M	8m	Rt. femur	Delayed union	3.665
4	16	4	M	15m	Rt. femur	Bone healed	4.09
Case No. 7, and 18 showed exudate and had no complete data and hence dropped; Case 13 had problems of instability due to hardware and not taken up for scoring.							
MPC from c.f.-chitosan							
1	21	2.5	F	11m	Rt. radius-ulna	Bone healed	4.3
2	22	10	F	7m	Rt. radius-ulna	Excellent healing	4.695
3	23	3.7	M	6m	Lt. radius ulna	Bone healed	4.33
4	29	5	M	3m	Rt. femur	Bone healed	4.55
5	36	9	F	6m	Lt. femur	Bone healed	4.475
Case No. 30 was dropped due to incomplete observations.							
MPC from s.s.-chitosan							
1	25	3.5	F	5m	Lt. radius	Excellent healing	4.64
2	28	1.95	M	5m	Lt. radius-ulna	Bone healing	4.23
3	34	3	F	1 yr	Lt. radius-ulna	Bone healing	4.39
4	35	4	F	6m	Lt. tibia-fibula	Good healing	4.55
5	37	16	M	2 yr	Rt. radius -ulna (comminuted)	Union in progress *	3.59
Case 24 was not shown due to incomplete data. Case 28 which also had a fracture of right radius-ulna, showed atrophic degeneration.							
Control							
1	9	4	M	3m	Rt. femur	Bone healed	4.165
2	10	5	M	4m	Lt. femur	Bone healed	4.06
3	12	2.4	M	7m	Lt. radius-ulna	Bone healing **	3.81
4	20	10	M	6m	Rt. tibia-fibula	Bone healed	4.23
5	27	7	F	5m	Lt. radius-ulna	Bone healing	3.845
6	32	11	F	7m	Rt. femur	Incomplete union	3.465
7	33	3.5	M	6m	Rt. radius-ulna	Bone healed	4.025
**: Healing was delayed in Case 12 due to wreckage of plate.							

Legends: * : exudate, M : Male, F : Female, yr: Year, m : month, Lt.: Left, Rt.: Right

Out of seven cases selected for control, Cases 9 and 10 showed complete healing by 7 weeks of operation. In Case 27, callus development was seen by fifth week but was not adequate to close the gap and by 14 weeks, fracture line was not seen in the lateral view of the radiograph. Very good healing was seen by 6 weeks of operation in Case 20. Closure of the fracture line was obvious in the radiograph taken at 47 days but on by one month (Plates 33A-33D) in Case 33. Remodeling was still due to complete. Case 27 showed the gap on the radiographs taken at 5 weeks and 9 weeks but not at 14 weeks. Unstable pin in Case 32, hindered the normal bony reunion process although callus development was seen at day 30 and hence, the pin was removed on 33rd day.

Discussion

Based on the clinical, physical and radiological examinations of the animals, it was concluded that both modified and unmodified chitosans have osteogenic activity. Out

of nineteen animals that were applied with chitosan, exudation was seen in four animals (21.5%), at the site of sutures. However, there were no signs of systemic illness in all cases except two, in which transient fever lasted for a day. Generally, healing seemed to be complete by six weeks in chitosan treated cases compared to seven weeks in control cases. The more appropriate amount for intra-osseous application was seemed to be 10 to 30 mg per kg body weight for α-PDC powder. There might be so many unexplained reasons for the variations and some of them could be due to different nature of fracture, age and nutritional levels of the animal, post operative care, etc. However, it can be concluded that α-PDC was highly biocompatible inside the osseous tissues.

With the 5-methyl pyrrolidinone chitosans, exudate formation was almost not seen in both type of derivative except in one case applied with MPC from shrimp chitosan which was applied at the lowest dose rate of 2.25 mg/kg body weight. The exudate formation could be due to the reaction of pin or plate to the bone or to the reaction to chitosan. The more appropriate amount of MPC for intra-osseous application seemed to be 5 to 10 mg per kg body weight. The trials showed formation an appreciable amount of new bone tissue in about three to four week. The mode of application of sponge sample was also comparatively easier than the unmodified powder.

Slight exudation was seen in animals applied with α-PDC for the fractures of radius-ulna or tibia-fibula (bones that have less musculature than other parts of the limb) except in Case 8. The exudate was not of infectious nature which was confirmed by cytology for the presence wound invaders, but resulted from the accumulation of polymorphonuclear cells at the site of chitosan application. In Case 8, chitosan applied at the dose of 30 mg/kg gave best results and the healing was complete within 6 weeks. Although there was an appreciable amount of exudate in Case 11 during the first few days, very good bone healing with restoration of normal bony trabculae was noticed within five weeks. This could be due to liberation of messenger chemicals from the white blood cells accumulated at the site of exudation and thereby causing immature bone cells nearby to mature into specialized bone cells.[1] The peculiar feature in control group of animals was free from exudation during the first week of chitosan application. The onset of callus formation was slightly delayed which might be due to absence of osteoconducting materials like chitosan. Healing was slightly delayed by one week or so. Fracture was repaired by classic osteoblatic-osteoclasitc sequence but not by ordered deposition of callus.

None of the animals applied with MPC from cuttle fish chitosan developed exudate formation and the healing was found to be very good with rapid onset of organized callus. Loose molecular arrangement of c.f.-chitosan might make the derivative more susceptible to depolymerization and thereby conferring greater biocompatibility, biodegradability and osteogenicity. In dogs treated with MPC from shrimp chitosan, Case 35 showed quicker healing of femur (control) than in tibia as there was more severe fracture in the tibia (MPC). In Case 28, the reason for the atrophic degeneration of right fore limb (control) could not be traced out but might be due to impairment of blood supply and the left fore limb (MPC) showed excellent healing by 6 weeks. Chronic sero-sanguinous discharge in Case 37, from the site of skin incision might be due to reaction of bone to hardware or creation of dead space or due to reaction of chitosan although the amount of chitosan applied for this case was the least in whole groups. The reason for the development of transient fever in this animal was obscure.

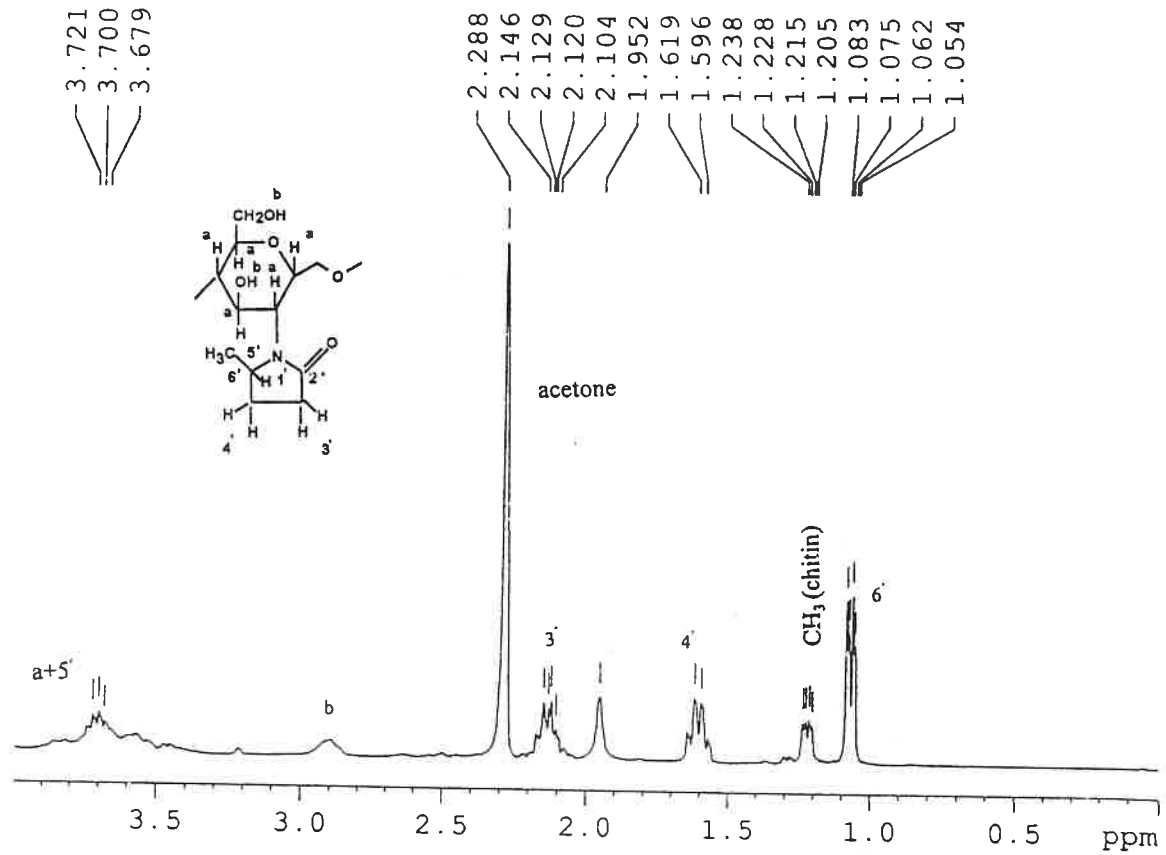


Fig.1 ^{1}H NMR spectrum of 5-methyl pyrrolidinone chitosan from cuttle fish chitosan (solvent, D_2O; at room temperature)

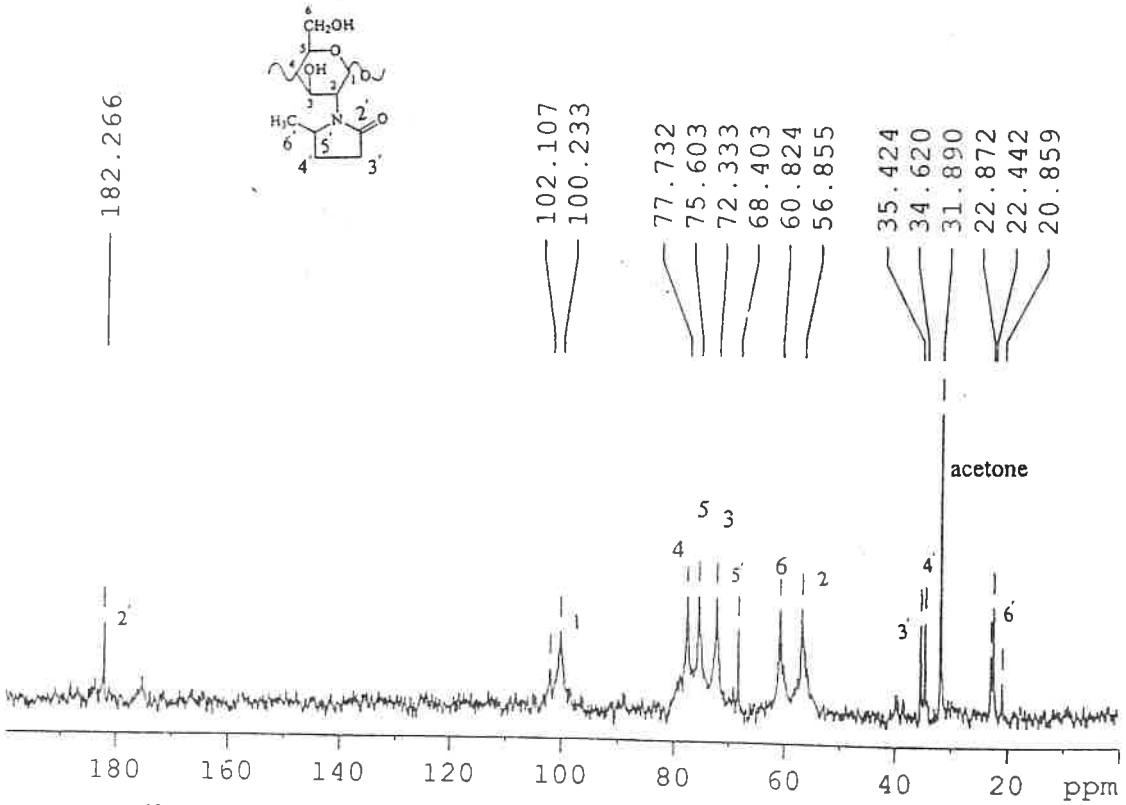
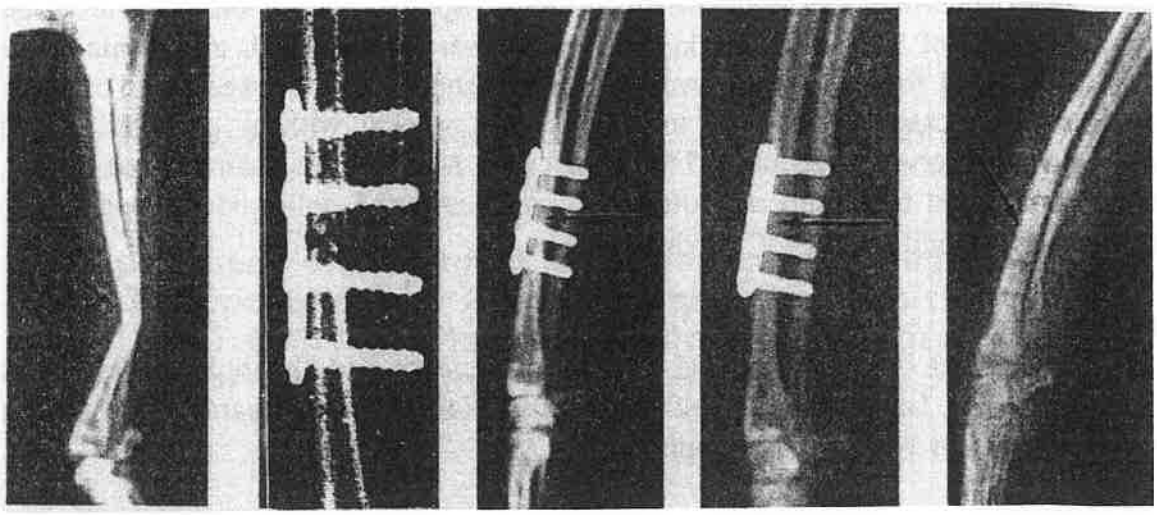


Fig.2 ^{13}C NMR spectrum of 5-methyl pyrrolidinone chitosan from cuttle fish chitosan (solvent, D_2O; at room temperature)

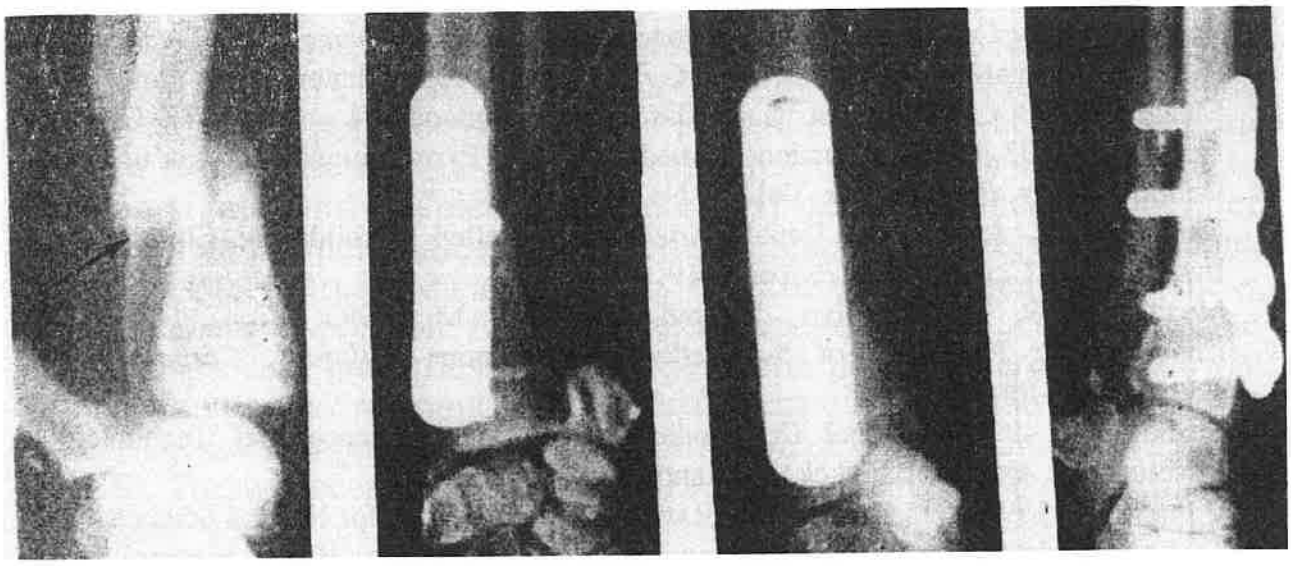
Plates Showing Serial Radiographs of Fractures Treated with MPC



25 A 25 B 25 C 25 D 25 E

Plate 25A, Lateral view of the left radius and ulna of a 5 months old, male Poodle dog with transverse fracture of the distal third of the radius. 25B, Post operative radiograph of the case shown in Plate 25A. 25C, Post operative radiograph taken three weeks after chitosan application, showing bridging of the bones. 25D, Post operative radiograph taken after 6 weeks showing primary union of the radius and complete bridging of the fractured fragments with restoration of original architecture of the bone. 25E, Radiograph taken after 9 weeks showing plate removal.

Plates Showing Serial Radiographs of Fractures in Control Animal



33A 33B 33C 33D

Plate 33A, Lateral view of the right radius and ulna of an year old, male Poodle, with a simple transverse fracture of the distal third of the radius and ulna. 33B, AP view of the post operative radiograph of the case. 33C, AP view of the radiograph taken at 25 days showing callus formation but insufficient for bridging. 33D, Lateral view of the radiographs taken at 44 days showing complete union of the radius and bridging of the ulna by callus, however, restoration of original architecture of the ulna not fully attained.

Conclusions

Both modified and unmodified chitosans, applied locally on the site of bone fracture at a dose of 5 to 30 mg per kg body weight seemed to exhibit osteogenic activity. The time required for bone repair was apparently shorter by one week (5-6 weeks) in chitosan treated cases as opposed to control (6-7 weeks) without chitosan. Exudate formation was observed in about 21.5 % of the cases. Modified chitosan derived from c.f.-chitosan was found to be superior among all samples tested, followed by modified s.s.-chitosan and unmodified α-PDC, in that order.

Acknowledgment

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ENZYME-BASED MODIFICATION OF CHITOSAN

by

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INTRODUCTION

Synthetic polymers are an integral part of modern society and serve a variety of functions. There are however growing concerns with current state of polymers and polymer processing. First, the majority of polymers are derived from petroleum, a non-renewable resource which is expected to become significantly depleted over the next century. Second, polymer synthesis and functionalization reactions generally involve hazardous conditions (e.g. free radical reactions or hazardous reagents) and these operations are responsible for a substantial fraction of the accidents in the chemical process industries. Finally, synthetic polymers are only slowly degraded in the environment and this recalcitrance has become an important concern given that polymers are a large fraction of input to landfills (20% in the US). These concerns have stimulated considerable research on alternative strategies which: exploit renewable resources; employ safer and more environmentally-friendly processing steps; and yield products which are more readily biodegraded.

Chitosan offers a diverse range of properties and has gained considerable attention for various applications. We believe chitosan has several features which offer interesting possibilities for its use as an industrial polymer. First, chitosan is derived from chitin, a renewable resource which is found in the cuticle of crustaceans and in fungal cell walls. Second, the rich amine functionality of chitosan is unique with respect to biopolymers and this functionality is useful due both to its polycationic nature and its

nucleophilicity. Third, chitosan is soluble in dilute acetic acid and can be cast into various forms without the need for complex organic solvents (in contrast to cellulose). Finally, it is conceivable that products derived from chitosan (especially when enzymes are used to generate linkages common in nature) may be more readily degraded in the environment.

In our studies we are examining an enzymatic approach to modify chitosan. In general, there are several enzymatic approaches which could be considered. After briefly describing the various enzymatic approaches, we will discuss the potential advantages and disadvantages for using the enzyme tyrosinase to modify chitosan.

POSSIBLE ENZYMATIC APPROACHES

Although several enzymatic approaches to modify chitosan could be envisioned, we will only consider approaches which employ simple substrates and do not require complex, physiological cofactors (i.e. we will not consider reactions involving sugar nucleotides or sugar phosphates). Further, enzymes which hydrolyze the glycosidic linkage (e.g. chitinases) will not be considered. Rather, we focus on the use of enzymes capable of attaching side chains onto the intact chitosan polymer.

Enzymatic Acyl-Tranfser. It is conceivable that side chains could be attached to chitosan using enzymes capable of transferring acyl groups either to chitosan's amine or hydroxyl groups. Interest in enzymatic acyl-transfer was stimulated by the recognition that enzymes can function in nonaqueous environments (Zaks and Klibanov 1985) and thus hydrolytic enzymes could be "reversed" to catalyze condensation reactions as illustrated by the amide-forming reaction:

$$\text{RCOOH} + \text{H-Enzyme} \leftrightarrow \text{RCO-Enzyme} + \text{H}_2\text{O}$$

$$\text{RCO-Enzyme} + \text{H}_2\text{NR}' \leftrightarrow \text{RCONHR}' + \text{H-Enzyme}$$

The use of enzymes in non-aqueous environments also eliminates the requirement for water-solubility and permits enzyme processing to be expanded to a wider range of hydrophobic substrates.

With respect to chitosan, it is possible to envision the use of "hydrolytic" enzymes such as proteases and lipases to convert chitosan's primary amine to an amide, or to form an ester with the hydroxyl of the C-6 position. Although we are unaware of such studies with chitosan, Bruno et al. (1995) reported the use of a protease to transfer an acyl group to a hydroxyl group of the insoluble sugar amylose.

Although enzyme-catalyzed condensation reactions have attracted considerable attention, there are several limitations to this enzymatic approach. First, enzyme-catalyzed condensation reactions can yield only a handful of possible linkages (e.g. amides, esters and glycosides). We believe that enzymatic strategies which can yield alternative linkages merit attention. Second, as illustrated in the above reaction, the nucleophile attacks the covalently-bound acyl-intermediate at the active site of the enzyme. The need for both the acyl intermediate and nucleophile to be present at the enzyme's active site imposes substantial steric limitations to the reaction and this limitation can be particularly severe for large molecular weight reactants such as polymers. Finally, water has a substantial impact on enzyme-catalyzed condensation reactions because water of hydration appears to be important for enzyme function, while excess water can shift the reaction toward hydrolysis. For instance, when enzymes are used to synthesize esters, it is generally necessary to employ trans-esterification (rather than condensation) reactions in order to avoid equilibrium and kinetic constraints.

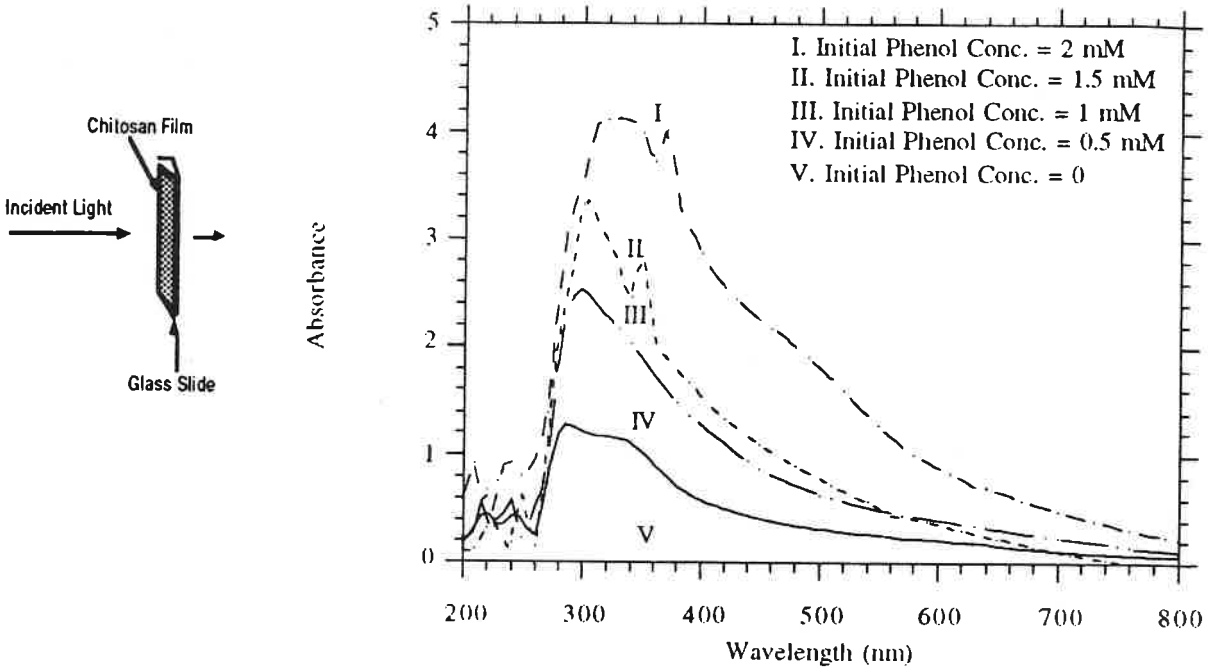
Tyrosinase-Catalyzed Reactions. Tyrosinase is a copper-containing enzyme which is ubiquitous in nature. One unique feature of this enzyme is that it can catalyze two distinct reactions, the hydroxylation of a monophenol to an *o*-diphenol and the oxidation of the *o*-diphenol to *o*-quinone. As shown, tyrosinase-catalyzed reactions use molecular oxygen as the oxidant and there are no requirements for complex co-factors.

The tyrosinase-generated quinones are reactive electrophiles and can undergo reaction with various nucleophiles - including the nucleophilic amines of chitosan (Sun et al. 1992).

To demonstrate that tyrosinase-generated quinones could be used to modify chitosan, we conducted a series of studies. In one study, we contacted several chitosan films with solutions containing tyrosinase and various concentrations of phenol. After incubation overnight and washing with distilled water, the UV-visible spectra of the various films were measured and referenced against the absorbance of the same film prior to reaction (i.e. difference spectra were measured). As shown in Figure 1, the

absorbance of the chitosan films increased in a nearly proportional manner with the level of phenol used for modification (Payne et al. 1996).

Figure 1. *Tyrosinase catalyzed chitosan modification (Adapted from Payne et al. 1996).*



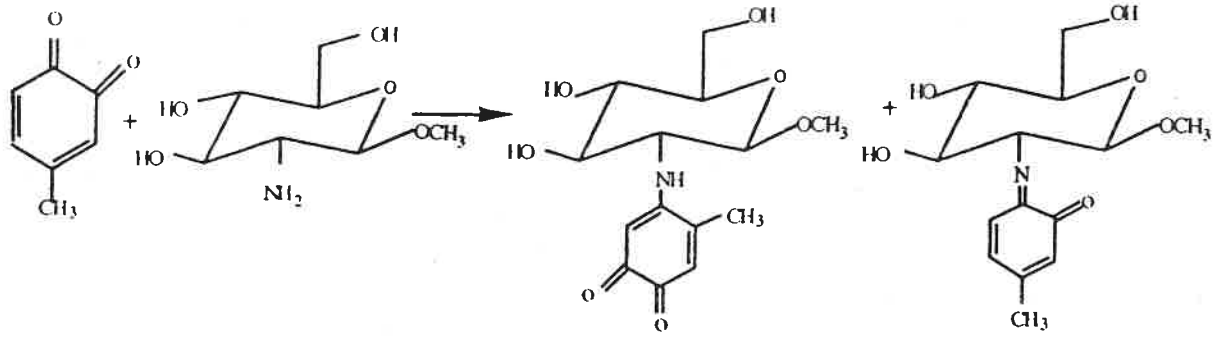
There are several reasons why we believe tyrosinase-catalyzed modification of chitosan may offer interesting opportunities. Because tyrosinases have a broad substrate range for phenolics, it is possible to envision the attachment of a variety of functionalities to chitosan. In previous studies, we were able to modify chitosan with the hydrophobic, anionic and zwitterionic phenolics, tert-butylcatechol, *p*-hydroxyphenoxyacetic acid and dihydroxyphenylalanine, respectively (Payne et al., 1996, Lenhart et al. 1997). Important to note is that since tyrosinase enzymes can function in non-aqueous environments (Kazandjian and Klibanov 1985; Estrada et al. 1993; Burton et al. 1993), reactions with water-insoluble reactants are possible. Also, the ability of tyrosinase to oxidize tyrosyl residues (Ito et al. 1984; Marumo and Waite 1986) suggests the potential for enzymatically grafting peptides and proteins onto chitosan. Possible advantages of a tyrosinase-catalyzed grafting procedure is the specificity of the method (i.e. the enzyme only reacts with tyrosyl residues), and the mild conditions used for reaction.

An additional reason why we believe tyrosinase is an interesting enzyme for chitosan modification is that the reaction is rapid under simple conditions. In contrast to enzymatic acyl-transfer reactions, tyrosinase catalyzes an oxidative reaction in which the reaction is essentially irreversible (i.e. equilibrium is not so dependent on the water concentration). Also, the electrophilic quinone generated from the tyrosinase-catalyzed oxidation is a freely-diffusible intermediate, and nucleophilic attack of the quinone does not need to occur at the active site of tyrosinase. As a result, tyrosinase-generated quinones can rapidly react with various nucleophiles in solution (and even with insoluble chitosan gels) under facile conditions. Although advantageous with respect to minimizing steric limitations of the enzyme, there is a disadvantage to the generation of a freely-diffusible reactive intermediate. Specifically, the quinone intermediate can undergo undesired reactions with various nucleophiles which may be present in the reaction medium. Fortunately, it is possible to limit undesired reactions of the quinone by using non-aqueous solvents (Kazandjian and Klibanov 1985) and/or by a judicious choice of pH.

CONCLUSIONS AND FUTURE WORK

In summary, we believe tyrosinase provides interesting potential opportunities for grafting various functional groups onto chitosan. Potential benefits of this approach are that it may: permit a renewable natural polymer to be tailored for a variety of commercial applications depending on the added functionality; allow modification under simple and environmentally-more-friendly conditions; and yield linkages which are amenable to biodegradation after the polymer's use.

Although we believe tyrosinase offers interesting possibilities for chitosan modification, there are two unanswered questions. First, it is necessary to understand the chemical linkages formed between quinones and chitosan. We are currently using the β-methylglycoside of glucosamine as a soluble model of chitosan to determine the linkages resulting from reactions with tyrosinase-generated quinones. Although, we have not yet determined the structures of the reaction products we anticipate two types of reaction products, Michael's adducts and Schiff bases. In various studies, Michael's adducts and Schiff bases have been reported as products of reactions between quinones and amines (Davies and Frahn 1977; Parris 1980; Sugumaran 1988; Peter 1989; Thorn et al. 1996).



Michael's adduct Schiff base

A second unanswered question is what are the functional properties (and therefore potential applications) for chitosan polymers modified by tyrosinase-generated quinones.

ACKNOWLEDGEMENTS

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Characterization of a chitinolytic enzyme from rice (*Oryza sativum L)* bran

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Abstract

The tissue specific patterns of chitinase isoforms were observed in the crude enzyme preparations from the bran and hull of rice, cell culture and cell culture treated with the elicitor. An isoform of acidic chitinases from rice bran was purified and characterized.

Keywords: rice brans, hulls, cell culture, tissue specificity, acidic chitinase and purification

Introduction

Chitinases, which catalyze the hydrolysis of chitin, are present in many species of plants. There is increasing evidence that chitinases in plants are important not only in defending plants but also in plant development. The presence of starch and storage proteins in ungerminated grain renders the grain very susceptible to invasion by microorganisms and insects. Biochemical machineries like chitinases and other antifungal proteins in cereal grains may be constitutive against possible attacks[1-6]. Athough total chitinolytic activity in rice grain has been reported[7,8], little attention is focused on their composition and their biological functions in this cereal itself. The objectives of this investigation are to determine activities of chitinases in the bran and hull of rice, and to isolate and characterize an isoform of the enzymes.

Materials and Methods

1. Materials

Whole grains of rice were purchased from the market immediately after the harvest and stored at a constant temperature and humidity. Dehulling and polishing of the grains were done in a commercial milling. Rice cell culture and the treatment with a mixture of *N*-acetylchitooligomers were carried out as previously described[9].

2. Extraction and purification procedures

White rice and hulls were crushed and they were passed throurgh a 20-mesh sieve. Brans, hulls and cell culture media were extracted by the method previously described [10]. The protein extract was successively centrifuged at 3,000 *g* and 20,000 *g*. The supernatants of 20,000 *g* were loaded onto an anion exchanger column (Sources Q,

Pharmacia) and eluted with a stepwise gradient of NaCl from 0 to 1.0M. Those fractions containing the enzyme activity were pooled, and $(NH_4)_2SO_4$ was added to the solution with the final concentration of 1M. The mixture was chromatographed on a hydrophobic column(phenyl-Sepharose, Pharmacia). The eluted chitinase was pooled, lyophilized and further purified with a gel filteration column(Superdex 200 , Pharmacia) with 20mM Tris-HCl buffer(pH 7.5). The fractions containing the chitinase activity were pooled and dialyzed against 25mM Bis-Tris buffer (pH 6.3), and they were appplied onto a chromatofocusing column of Mono P(Pharmacia) and the column was eluted with Polybuffer74(pH 4.0). The fractions of interest were pooled and dialyzed against 20mM Tris HCl buffer (pH7.5) for subsequent affinity chromatography on a regenerated chitin column. The column was eluted successively with the buffers having pH 8.4, 5.4 and 3.3, respectively.

3.Electrophoresis and Detection of enzymes on the gel

SDS-PAGE was performed in the presence of 7.5% glycol chitin according to Laemmli [11] and the enzyme activity was detected with Calcofluor white M2R after treatment of the gel with Triton X-100. After non-denaturing gel electrophoresis by the method of Davis [12], the gel was overlayed with a gel containing 0.01% glycol chitin and stained as described by Trudel[13].

4.Enzyme Assays

β-*N*-acetylglucosaminidase assay was carried out by adding the enzyme solution containing 1 mg of protein to 2mg the colloidal chitin in 4 ml 40mM citrate/Na_2HPO_4 buffer, pH 5.2 at 50°C for 2 h. The reaction mixture was boiled for 10 minutes and centrifuged at 3,000 *g* for 20 minutes. The supernatant was taken for measurement of the β-*N*-acetylglucosaminidase activity by the coloimetric detemination of *N*-acetylglucosamine with ρ-dimetylaminobenzaldehyde[14]. When [^{3}H]-chitin was substrate, the measurement was performed by the method described by Molano et al [15]. For the measurement of endochitinase activity, 500μl of the supernatant, which was previously taken for the β-*N*-acetylglucosaminidase, was mixed with 100μl of snail gut β-glucuronidase(Sigma), and held at 37°C for 1 h, during which the β-glucuronidase completely converted the water-soluble oligomers into *N*-acetylglucosamine [10]. The difference between the activities before and after the treatment of the β-glucuronidase was taken as the activity of the endochitinase.

Chitosanase activity was measured with colloidal chitosan in stead of colloidal chitin at pH 6.0. The liberated hexosamine was determined with the method of Tsuji et al [16], and β-1,3-glucanase activity was assayed with laminarin using the method of Fink[17] .

Results and Discussion

1. Some hydrolytic enzyme activities of rice bran and hull

In the bran of rice, the endochitinase activity was 5 times higher than that of β-*N*-acetylglucosaminidase (Table 1.). However, the activity of β-*N*-acetylglucosaminidase in the hull was 3.6 times higher than that of endochitinase. The substantially high activity of β-*N*-acetylglucosaminidase in the hull needs further investigation since most plant chitinases are known to be endochitinases[18].

These crude enzyme preparations showed also β-1,3-glucanase activity. Indeed, this

observation is in good agreement with a recent finding that a β-1,3-glucanase was purified from the bran of rice [19]. In addition to the above three enzymes, significant amount of chitosanases activity was found in both hull and bran (Table 1.). Therefore, it is suggested that these crude enzyme preparations may contain chitosanases.

Table 1. Distribution of chitinase, chitosanase and β-1,3-glucanase activity in the bran and hull of rice.

	β-*N*-acetylglucosaminidase (μg/mg/2h)	endochitinase (μg/mg/2h)	β-1,3-glucanase (μg/mg/2h)	chitosanase (μg/mg/2h)
Bran	13.4	63.6	10.9	57.7
Hull	85.6	23.9	43.5	87.9

These values were obtained from two independent determinations and variations were with 15% of the range.

2. Comparison of chitinase isoforms of rice bran, hull and cell culture

As seen in Fig.1, relatively high levels of basic chitinases activity were observed in the hull, cell culture and cell culture treated with elicitor. On the other hand, a weak activity of acidic chitinases was observed in the medium of cell culture. On further examination of the patterns of acidic chitinases, the activity of RAC1 was found to be higher than RAC2 in rice bran and cell culture with elicitor. In the hull, both RAC1 and RAC2 enzymes showed the same level of activity. These different patterns of chitinases found in the bran, the hull and the cell culture demonstrate that there is a tissue specific regulatory mechanism. This type of differences in the patterns of the chitinases has been well investigated with rice plant[20-22], but not in the rice bran nor in the hull. Furthermore, RAC1 in the bran seems to comigrate with the induced chitinase in cell culture treated with elicitor, therefore, we have focused our efforts on the purification of this enzyme RAC1.

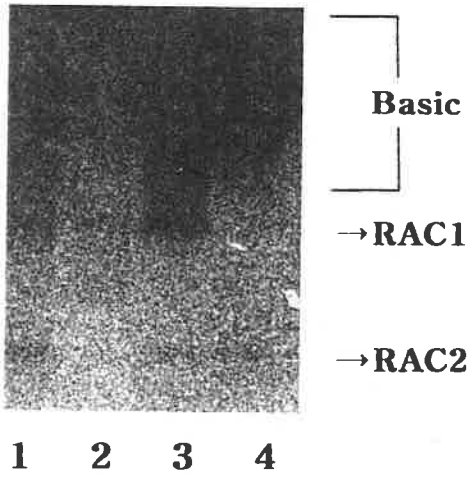

Fig.1. Non-denaturing gel electrophoresis of crude enzyme preparations from rice bran, hull and rice cell culture.
Lane1: Hull. Lane2: Bran. Lane3: Rice cell culture treated with N-acetyl chitooligomers as elicitor Lane4: Rice cell culture medium.
After overlaying the gel with a gel containg glycol-chitin it was incubated and detected with Calcofluor white M2R.

3. Purification of the chitinase

Total protein, specific activity, yield and fold-purification are listed in Table 2. The activity of RAC1 was most highly purified by the choromatofocusing(Table 2). However after the step of chromatofocusing it was very difficult to detect the protein or the enzyme activity on the SDS-PAGE gel(data not shown).

Table 2. Purification of RAC1 from rice bran

Purification step	Total protein (mg)	Specific activity (μmole/ mg protein/2h)	Total activity (μmole/2h)	Purification (-fold)	Yield (%)
Crude extract	4,796	0.3	1669	1	100
Anion exchange	343	1.5	518	4.3	31
HIC	83	4.0	334	11.6	20
Gel filtration	21	8.9	186	25.9	11
Chromotofocusing	0.27	155.5	42	469	2.5

Chitinase activities were determined in 40mM citrate/Na_2HPO_4 buffer(pH 5.2) with [^{3}H[-chitin.

Column chromatographic profiles are presented in Fig. 2. As seen in the chromatofocuing(Fig. 2D), it is confirmed that RAC1 is an acidic chitinase whose pI is below 4. The poor binding of RAC1 to the regenerated chitin (Fig. 2E) may suggest that this enzyme is a class II chitinase, according to the classification of Collinge et al(18). Apparent molecular weight obtained by gel filteration (Superdex 200) was 130 kDa(Fig. 3). SDS-PAGE patterns of the enzyme preparations at various stages of the purification are shown in Fig. 4. Molecular weight determined by SDS-PAGE (Fig. 4) shows the value of 140 kDa. These data indicate that RAC1 exists in solution as a monomer. Obviously, these values are significantly higher than those published by others [18] who reported the molecular weight of 25 to 35 kDa of plant chitinases. The properties of RAC1 were listed in Table 3. This chitinase hydrolyzed [^{3}H]-chitin into trimer of *N*-acetylglucosamine as major reaction product(Table 3).

Table 3. Properties of the purified RAC1.

Acidic/ basic	Endo/ Exo	chitosanase activity	β-1,3-glucanase activity	Lysozyme activity	MW (kDa)	Major products
Acidic	Endo type	UD	UD	UD	130~ 140	Trimer

UD: Undetectable under our experimental conditions. Enzyme activities were measured as described in Materials and Methods. Analysis of reaction products was carried out on a column of Biogel P2(BioRad, USA) by eluting the column with H_2O. These values were obtained from two independent determinations and variations were with 15% of the range.

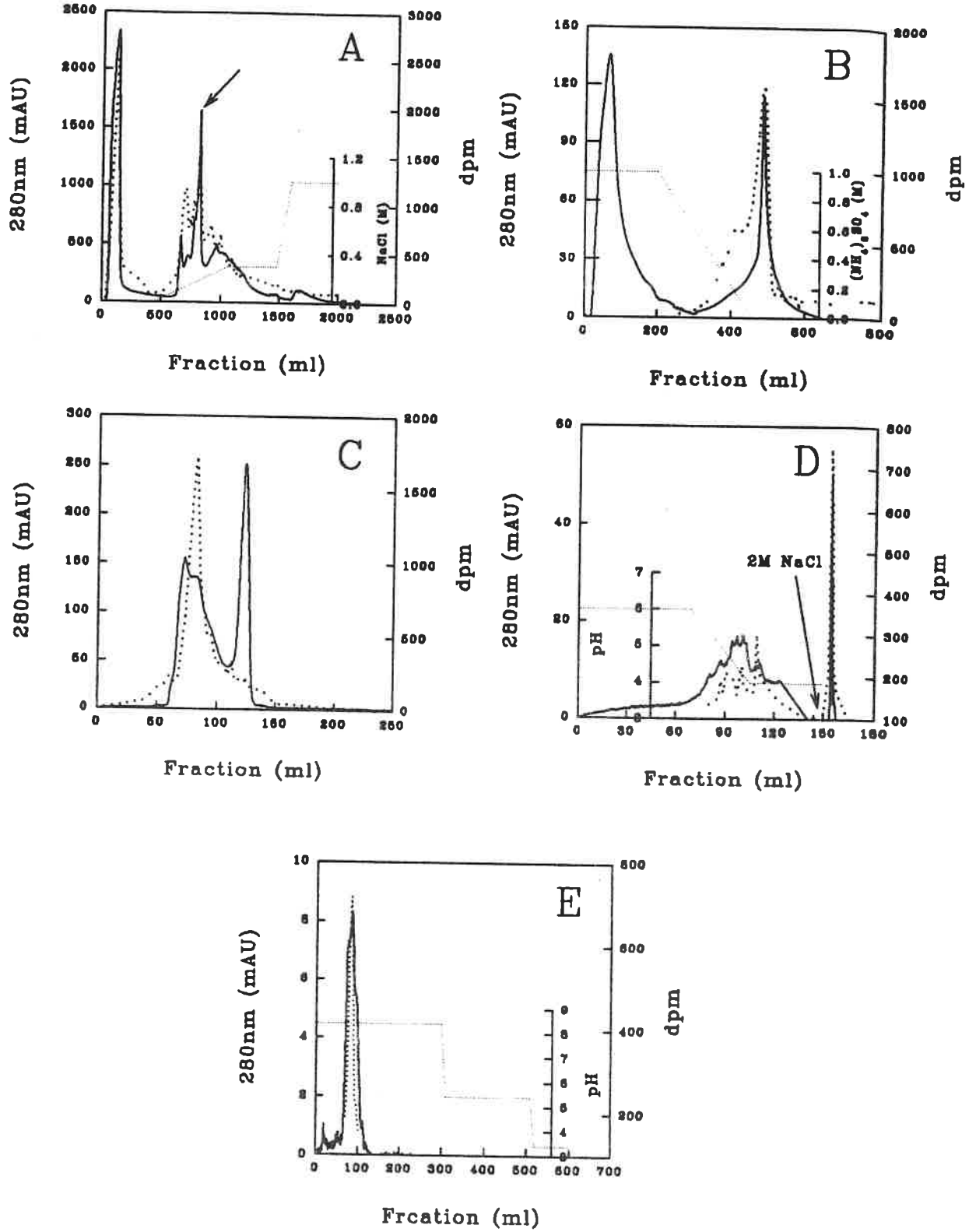

Fig.2. Purification of RAC1 by different chromatographic methods.
2A: Anion exchange chromatography on SourceQ, 2B: Hydrophobic interaction chromatography on Phenyl-Sepharose, 2C: Gel filtration on Superdex 200, 2D: Chromatofocusing with polybuffer74, 2E: Chitin affinity chromatography
——— : protein, -------- : chitinase activity

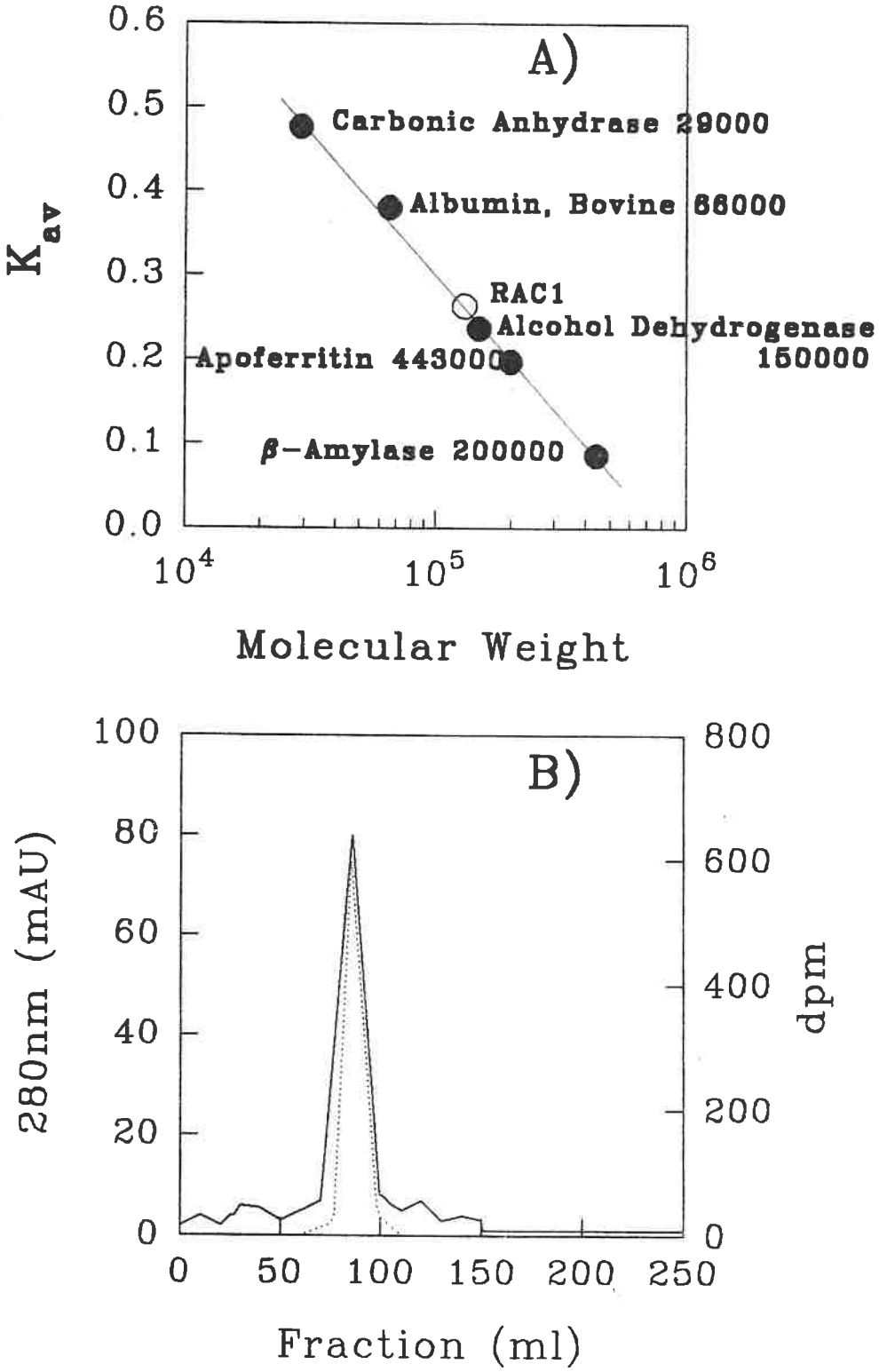

Fig.3. Determination of molecular weight of RAC1 by gel-filtration.

The RAC1 obtained by chromatofocusing was applied onto Superdex 200 and eluted with 20mM Tris-HCl buffer(pH 7.5).

A) Standard curve, B) Chromatogram of gel filtration

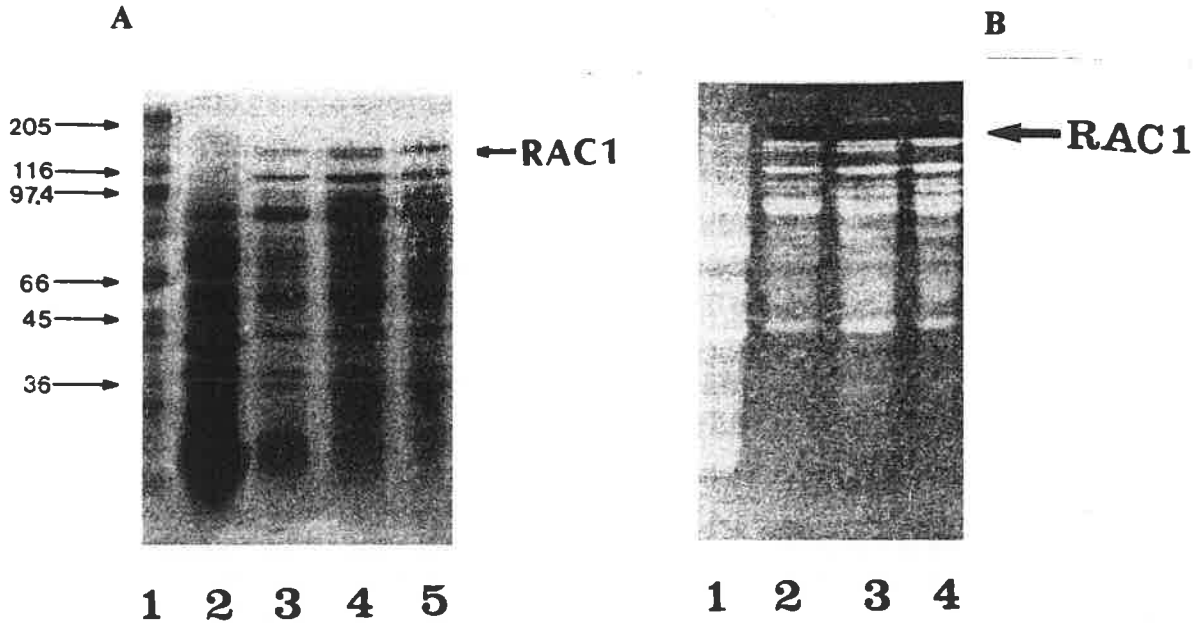

Fig. 4. SDS-PAGE analysis of the enzyme fractions during the purification of RAC1.
A: Protein staining by Coomassie Blue.
Lane1:molecular weight marker, Lane2: Crude extract, Lane 3:Source Q, Lane4: HIC, Lane 5: Superdex Fractions
B: Enzyme activity detected with Calcofluor white M2R under UV.
Lane1: Crude extract, Lane2: Source Q, Lane3: HIC, Lane4: Superdex Fractions

Conclusions

In this report we examined tissue specific patterns of chitinase isoforms in the bran and hull of rice, and cell culture. We also purified and charactierized an isoform of acidic chitinases from rice bran. High levels of basic chitinases activity were observed in the hull, cell culture and cell culture treated with elicitor. On the other hand, a weak activity of acidic chitinases was found in the medium of cell culture. On further examination of the pattern of acidic chitinases, the activity of RAC1 was found to be higher than RAC2 in rice bran and cell culture with elicitor. In the hull, both RAC1 and RAC2 enzymes showed the same level of activity. The purified RAC1 has a molecular weight of 130~140kDa and hydrolyzed [^{3}H]-chitin into trimer of N-acetylglucosamine as main reaction product. In addition RACI did not show the activity of chitosanase, lysozyme nor β-1,3-glucanase.

Acknowledgments

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Induction of matrix metalloproteinase(MMP) 2 and 9 in skin and subcutaneous tissue by implanted chitin in rats.

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Abstract

Chitin was found to induce matrix metalloproteinases(MMP) activity in rat skin and subcutaneous tissue. Sponge type chitin(22.5mg) was implanted in subcutaneous tissue of 8- weeks old rats by skin incision. After operation, MMP activity was more increased in the chitin-treated skin and subcutaneous tissue than that of only the incision until 2.5th days. Gelatin zymography revealed that the induced MMPs had the molecular mass at 92 and 72 kDa corresponding to MMP-9 and MMP-2, respectively. We discussed about the mechanism of the MMP induction by chitin.

Keywords: Chitin, matrix metalloproteinase(MMP), induction, zymography, rat, skin and subcutaneous tissue

Materials and methods

Preparation of chitin

Chitin sponge from squid pen (Sunfive Inc., Japan) with >90% deacetylation were used.

Animals

Forty-two 8-weeks-old male Wistar rats weighing 270-290g were used. They were divided equal into two groups by implanted chitin or without. They were applied general anesthesia. Their back was opened and the chitin(22.5mg) was implanted or not and sutured. Each group divided into seven groups by the day of after chitin implanted. Each three rats were euthanasied at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 7.0 days after implanted and the sample were collected.

Preparation of enzyme solution

Rat skin and subcutaneous tissue were cut at 3 by 3 cm and stripped. The tissues were cut in pieces and homogenized with a 50mM Tris phosphate buffer (pH 7.5) containing 0.01M $CaCl_2$ and 0.25% Triton-X. The homogenates were centrifuged at 8,000 rpm for 15 min at 4°C and aspirated supernatants. The supernatants were further centrifuged 50,000 rpm for 15 min at 4°C and supernatants were collected for analysis. The protein concentration was measured by Lowry method.

MMPs Assay

MMP was routinely assayed using Azocoll as substrate. Enzyme was adjusted to a volume of 1 ml with assay buffer containing 0.05M Tris-HCl, pH 7.5, 0.2M NaCl, 0.01M $CaCl_2$, 0.02% NaN_3, and 0.05% Briji-35. Azocoll, 1mg in 0.25ml buffer was added with a wide-mouthed pipette. Assay tubes were capped and shaken in a horizontal position at 37°C. Aminophenylmercuric acetate(0.5mM) was used to activate latent enzyme and blanks were prepared with 1mM 10-phenanthroline. Azocoll was omitted from the assay buffer as blank solution. Enzyme reaction was stopped by filtration with glass wool to remove the substrate azocoll. Then, the absorbancy of digested azocoll in the filtrates was determined spectrophotometrically at 520 nm.

Zymography

Gelatin substrate gels were prepared by incorporating gelatin (1mg/ml) into 10% polyacrylamide gels containing 0.1 % SDS. Equal amounts of enzyme solution (45μg protein) were loaded onto the gels. After electrophoresis under nonreducing conditions, the gels were washed for 30 min at room temperature in 2.5% Triton X-100 and subsequently incubated twice for 30 min at room temperature in a Ca^{2+} assay buffer consisting of 1 mM $CaCl_2$ in 0.1M Tris-HCL(pH 7.5). And then, they were incubated for 12 h at 37°C in the Ca^{2+} assay buffer. Gels were stainted with Coomassie brilliant blue for 60 min and destained in 10% acetic acid/25% ethanol. Clear zone of lysis against a blue background indicated MMP activity.

Results and discussion

Activity of MMPs

The level of MMP using chitin were accelerated after implanted 0.5, 1, 1.5, 2 days and then decreased at 2.5, 3, 7 days. In the controls without chitin, they were also increased after implanted 0.5, 1, 1.5, 2 days and then decreased at 2.5, 3, 7 days(Table 1).

The activity of MMPs with chitin were markedly higher than control after implanted 0.5, 1, 1.5, 2, 2.5 days(Fig. 1).

Table 1. The level of MMPs

Days after treatment	Control mean ± S.E.	Chitin mean ± S.E
0.5	0.00534±0.00059	0.00920±0.00044
1.0	0.00765±0.00069	0.01032±0.00056
1.5	0.00761±0.00076	0.01163±0.00011
2.0	0.00922±0.00069	0.01361±0.00102
2.5	0.00678±0.00125	0.00878±0.00040
3.0	0.00520±0.00035	0.00569±0.00081
7.0	0.00479±0.00060	0.00405±0.00025

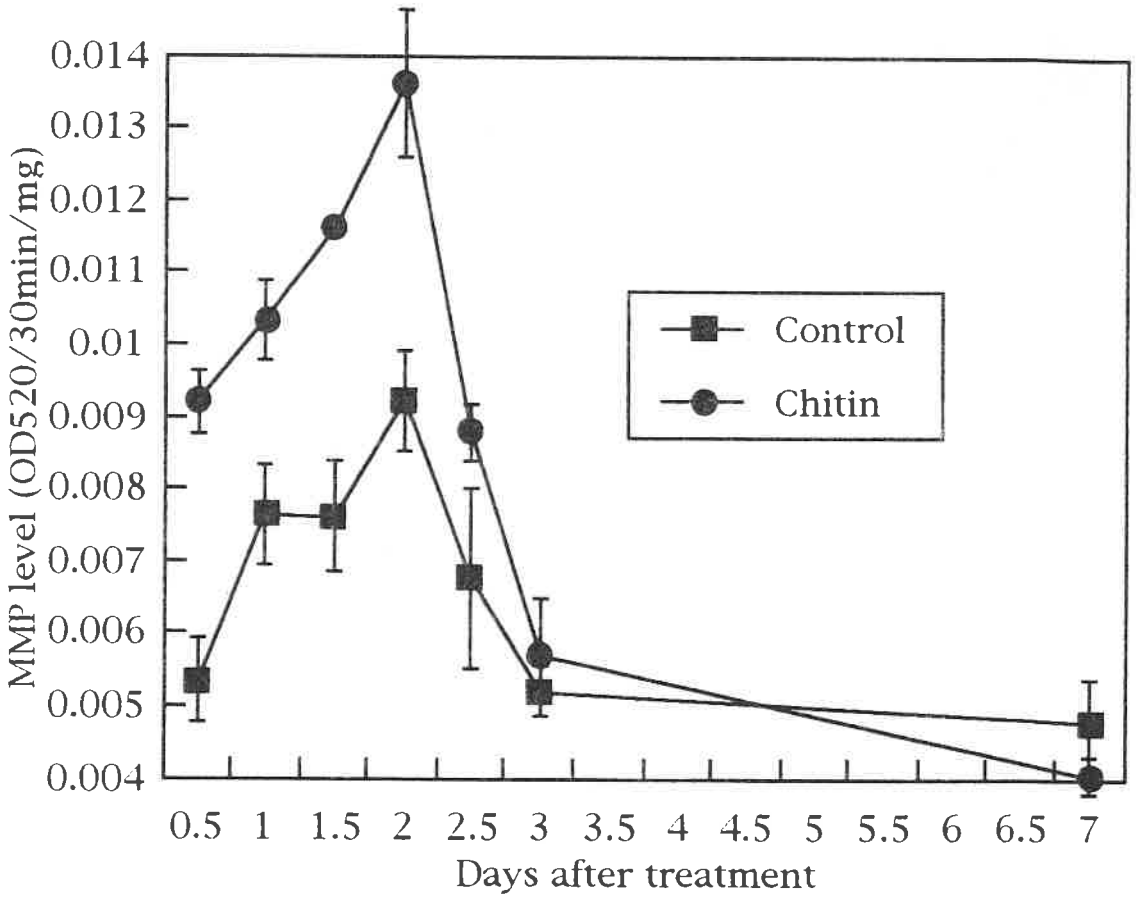

Fig.1 Change of MMPs activity

Zymography
Gelatin zymograhy demonstrated the MMP9(92kDa) and MMP2(72kDa) in using chitin were highly expressed than control on 0.5th, 1st, 1.5th days after implanted. They were strongly expressed than control on 2nd day(Fig.2). MMP9(92kDa) was slightly expressed on 2.5th day. There is no difference in MMP9(92kDa) and MMP2(72kDa) on 3rd and 7th day.

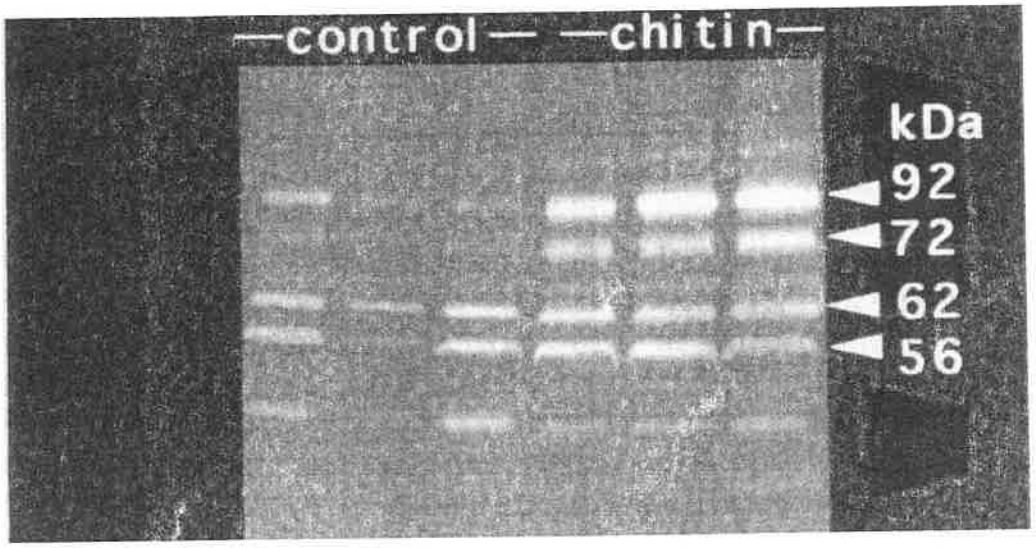

Fig.2 Zymography on 2nd day after implanted. MMP9(92kDa) and MMP2(72kDa) were strongly expressed than control.

Chitin has medically utilizable biological activities including the polynuclear leukocyte infiltration, macrophage, fibroblast, endothelium, newly formed capillaries proliferation, acceleration of granulating tissue formation and enhancing wound healing [1, 2, 3, 4, 5, 6].
MMP9 was produced by neutrophil and macrophage, MMP2 was produced by macrophage, fibroblast and endothelium[7].
Three stages of wound healing were inflammation, granulation and cicatrization. Chitin activates the neutrophil and macrophage infiltration, and then macrophage release PDGF, TGF-β, FGF, TGF-α. They were triggered infiltration of inflammatory cells, subsequently, accelerated proliferation of fibroblast and endothelium. Proliferation of fibroblast infiltrated to injury part, produced collagen, fibronectin, and formed extracellular matrix and neovascularization.
As mentioned above, chitin was found to induce MMP activity in rat skin and subcutaneous tissue.

Conclusion
The activity of MMPs in using chitin were markedly higher than

control on 0.5th, 1st, 1.5th, 2nd, 2.5th days after implanted. MMP9(92kDa) and MMP2(72kDa) were highly expressed on 0.5th, 1st, 1.5th, 2nd, 2.5th days after chitin implanted.

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α -CHYMOTRYPSIN IMMOBILIZED ON CHITIN. HYDROLYTIC ACTIVITY, STABILITY AND PEPTIDES' SYNTHESIS.

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ABSTRACT

α-Chymotrypsin was immobilized on chitin from various natural sources by means of glutaraldehyde. The physico-chemical properties of chitin from different sources were studied by IR spectroscopy and scanning electron microscopy, and its degree of acethylation was determined. The obtained derivatives were tested for activity against the hydrolysis of substrate N-Benzoyl-L-Tyrosine ethyl ester (BTEE) in aqueous and homogeneous aqueous-organic media. The behaviours of the obtained derivatives with respect to temperature were studied. Results showed highest stabilization versus free enzyme at high temperature (37°C and 45°C).

The relationship between the kinetic constant of hydrolysis, the structure, degree of acethylation and aquaphilicity of chitin was also studied.

α-Chymotrypsin immobilized on chitin from prawn was found to be the best derivative in kinetic terms.

Keywords: chitin, α-chymotrypsin, immobilization, hydrolytic activity, stability, peptides' synthesis.

INTRODUCTION

Immobilized biocatalysts have the potential for future industrial and commercial use in many areas of food and fodder, pharmaceutical and chemical industries and chemical specialities in processes that have no current equivalents. For the numerous potential applications of immobilized enzymes the preparation technique should be easy and the cost low. In this regard chitin is considered to be an appropriate support for immobilization of enzymes.

Chitin is a constituent of the outer structure of various living forms including insects, fungi and crustaceans.

Chitin is also significant because of its relationships to source components of food of animal and fungal origin, as well as by its medical and pharmaceutical potential (1).

Thus, it makes an excellent supporting material for immobilization of enzymes as it offers a high mechanical stability, appropiate density and a low solubility in most solvents. The use of chitin as support for immobilized enzymes has lately aroused increasing interest. As a rule, enzymes are immobilized onto chitin by physical adsorption from an aqueous solution or covalent binding with the aid of a cross-linking agent (e.g. an aldehyde). The literature abounds with examples of enzyme immobilization on chitin (2,3)

The biocatalytic system involving enzymes dissolved in water-inmiscible organic solvents opens up promising prospects for development of a wide range of reactions including regioselective transesterification (4) and peptide syntheses (5), that would normally not take place under physiological conditions. However, most reactions involving one-phase conditions (aqueous hydrophilic organic solvents) provide lower yields than physiological conditions and the water-miscible organic solvent deactivates the enzyme by over 50% (6,7).

In previous works, the influence of the support on the microenvironment of immobilized α-chymotrypsin was discused (8-10). The hydrolytic and synthetase activity, as well as their stability, were found to depend on the derivative composition, the physical treatment to which the support was subjected and how the derivative were stored. The effect was favourable or adverse depending on the type of support structure and its interaction with the partially organic medium used (8).

The aim of this work was to study the influence of the structure of chitin from three natural sources (prawn, Spanish crayfish, American crayfish) and commercial chitin used as supports on the behaviour of derivatives obtained from them. The effect is a function of type of chitin structure (α or β) and the degree of acetylation (11). So, we studied the potential influence and relationships between the properties of the support used and the hydrolytic and synthetase activity of the four chitin-immobilized α-chymotripsin derivatives, the kinetic parameters for the hydrolysis reactions, stability versus temperature and the deactivation curves.

MATERIALS AND METHODS

Materials.

Chitin was obtained from crustacean shells of three different natural sources including Prawn (Penaeus caramote), Spanish Crayfish (Astecus fluviabilis) and American Crayfish (Astecus Cambarus).

Commercially available chitin, α-chymotrypsin, benzoyl tyrosine ethyl ester (BTEE) and L-leucinamide hydrochloride were all purchased from Sigma, whereas glutaraldehyde, acetonitrile, sodium bicarbonate, sodium carbonate, sodium dihydrogen phosphate and disodium hydrogen phosphate were supplied by Merck.

All reagents used were analytical grade.

Methods.

Isolation and physico-chemical characterization.

The procedures used for isolation and physico-chemical characterization of chitin from different sources have been described elsewhere (11).

Aquaphilicity of different chitins, were determined according with Mattiasson method (12).

Immobilization of α-chymotrypsin on chitin.

The immobilization procedure has been described by us in a previous paper (13)

Determination of the activity of α-chymotrypsin immobilized on chitin.

Hydrolytic activity

The hydrolytic activity of chitin from the different sources was measured by monitoring proton release in a 10^{-3} M phosphate buffer of pH 7.8 by means of a pH-stat, using 10^{-3} M BTEE at 30°C as substrate. Released protons were neutralized with 10^{-3} M NaOH.

For calculating the kinetic constant Km and Kcat, different standard essays were carried out within a substrate (BTEE) concentration range varying from $2x10^{-5}$ to $2x10^{-3}$ M.

Stability.

The stability of derivatives at 37°C and 45°C was studied by measuring hydrolitic activity as a function of time using a pH-stat.

Peptide synthesis.

Enzymatic reactions were carried out in 0.2M carbonate buffer pH 9 containing $2x10^{-2}$ M BTEE and using L-leucinamide as nucleophile with variable concentrations ($4x10^{-2}$ to $1x10^{-1}$M). The reaction mixture was placed in a thermostatted water bath at 37°C equipped with a stirring mechanism. Samples were analyzed by Waters HPLC in acetonitrile containing 10^{-3}M indole, which was used as an internal standard. HPLC conditions were: Gilson C18 Nucleosil column, 1:1acetonitrile/0.03M phosphate buffer of pH 4.58 as eluent, flow 1.2 ml/min. and UV detection λ=280nm. Samples were injected with a Gilson Auto-sampling Injector.

RESULTS AND DISCUSSION

While the four α-chymotrypsin derivatives studied were immobilized on chitin, the supporting material was obtained from different sources (prawn, Spanish crayfish and American crayfish) and commercial chitin. The preparation and immobilization procedures used were described in detail elsewhere (11, 12). The yield of immobilized α-Chymotrypsin was 90% for all the samples. This suggests that the differences in the behaviour of the four derivatives must be ascribed to some fact other than the amount of the bound enzyme(*e.g.* the support structure, the enzymatic behaviour).

The four chitins used as support were obtained on purpose, except the commercial chitin; they were characterized by IR spectroscopy and scanning electromicroscopy and its degree of acetylation was determined as stated in previous papers (11-13). The results obtained therein will be taken into account for the behaviour of each derivative and their later analysis in this section.

Behaviour in aqueous medium

Hydrolytic activity in an aqueous medium

Table 1. *Aquaphilicity of derivatives and activity of α-chymotrypsin immobilized on different chitin supports.*

Derivative	Aquaphilicity	Hydrolytic activity (u/g support)
P	4.33	7.0 ± 0.491
SC	2.79	3.6 ± 0.252
AC	2.80	4.2 ±0.294
CO	3.23	5.3 ± 0.371

a) Derivative P:α-Chymotrypsin immobilized on chitin from prawn; b)derivative SC: α-Chymotrypsin immobilized on chitin from Spanish crayfish; c)derivative AC: α-Chymotrypsin immobilized on chitin from American Crayfish ; d) CO :α-Chymotripsin immobilized on commercial chitin.

The hydrolytic activity of each derivative was determined in phosphate buffer of pH 7.8 by using the pH-stat method. The results obtained are listed in Table 1 together with aquaphilicity values. As can be seen, the derivative immobilized on chitin from prawn shows the highest aquaphilicity and it is the most active.

Substrate specificity and kinetic properties.

The effect of subtrate concentration on α-chymotrypsin activity was examined with BTEE using the conditions of the assay described in the Materials and Methods section. With this substrate the enzyme follows the Michaelis-Menten equation. Kinetic parameters are shown on table 2.

***Table 2** . Kinetic parameters of hydrolysis of BTEE by α-chymotrypsin immobilized on chitin. T= 30 ℃. Phosphate buffer pH 7.8. Substrate : BTEE*

Derivative	Km (mM)	Kcat ($10^{-3}s^{-1}$)
P	13.2	18.4
SC	36.1	1.92
AC	33.0	2.06
CO	21.3	3.29

As can be seen, derivative P shows the best behaviour in aqueous media. The chitin from prawn exhibits hight values of deacetylation degree, aquaphilicity, and also exhibits some granular structure under the scaning electron microscope (11), being reportedly a chitin-protein complex (14) ; however, no such complex is apparent from the IR spectra, where the bands for these samples are consistent with an α-structure identical with that of chitin from lobster (11). Derivative P has the best kinetic parameter values for the hydrolytic activity; this can be ascribed to a more productive action of α-Chymotrypsin on the substrate. In view of the results of Kcat, it is possible to see that derivative P has the highest. This indicates that the enzyme not only recognizes better the substrate in this derivative, but also that the final product conversion is the biggest, resulting thereby in better Km and Kcat values relative to the other three derivatives.

Stability.

The stability of the four derivatives at 37° and 45°C was studied. The results of the variation of hydrolytic activity as a function of time are shown in figure 1 and 2.

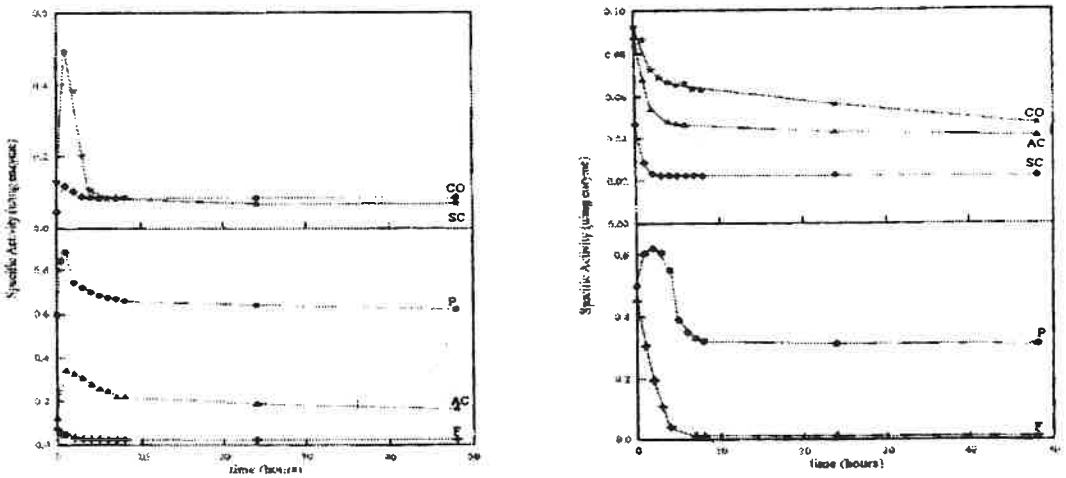

Fig. 1 and 2. Stability of the hydrolitic activity as a function of time of incubation at 37°C and 45°C. P (Prauwn); SC(Spanish Crayfish); AC(American Crayfish); CO(Commercial Chitin)

These figures show the deactivation profiles according to the theoretical models proposed by Henley and Sadana (15). As can be seen, the hydrolytic activity of all four derivatives increased after 120 minutes of incubation of 37°C ; however, each derivative behaved differently after the first two hours. The thermal deactivation curves obtained at 37°C for the four derivatives show higher than initial activities between 1 and 3 hours, depending on the derivative, which is consistent with the model developed by Henley and Sadana (15).

After 48 hours, at both 37° and 45°C, immobilized derivatives are still more active than the free enzyme, wich retains only 35% of the initial activity at 37°C (fig. 1), and it is practically inactive at 45°C (fig. 2). Consequently, this leads us to believe that alterations in enzyme microenvironment produced by immobilization are such that at these temperatures this modification has a favourable influence on the enzyme activity, since immobilized derivatives continue to be more active than free α-Chymotrypsin.

Again derivative P shows the best behaviour with regard to stability even when its activity decreases by half after 10 hours at 45 °C.

Behaviour in aqueous-organic medium

Peptide synthesis.

The peptide synthetase activity of immobilized α-Chymotrypsin derivatives was measured in 0.1M carbonate buffer of pH 9 containing 70% 1,4 butanediol, because this solvent shows the more appropiate physical-chemical properties for this reaction (16), by using the procedure described in (12). The results obtained for different derivatives are shown in figure 3 (a,b,c,d). As can be seen, the highest yield was achieved with α-Chymotrypsin immobilized on prawn chitin, wich thus made the most suitable derivative. This activity was maximal (90%) at 120 min., after which the proportion of peptide synthesized decreased to 60% at 300 min. This suggests that the enzyme is synthetically active and preserves its activity as long as enough nucleophilic agent is present in the medium. After the nucleophile has been depleted (at 120 min.; fig 3) the enzyme continues to be active hydrolytically, and acts on peptide formed, which is present at a high concentration by that time.

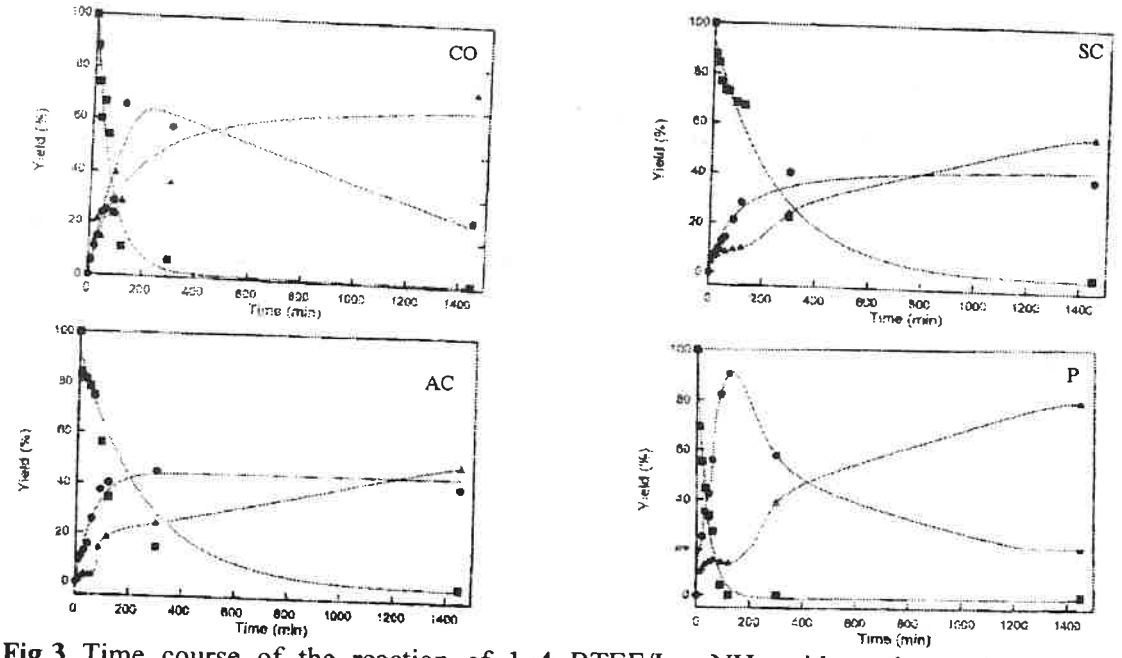

Fig.3 Time course of the reaction of 1 :4 BTEE/Leu-NH_2 with α-chymotripsin immobilized on different chitin supports. (•) Bz-Tyr-Leu-NH_2 ; (Δ) Bz-Tyr-OH ; (■) BTEE.

A comparison of curves in figure 1 and figure 3, which shows the deactivation curves and the variation of peptide yield as a function time, reveals that the activity of the four derivatives, particularly that of P, increases up to a maximum at *ca* 120 min. The increase in amount of peptide formed by derivative P until that time can be assigned not only to the factors described above, but also to the potential effect of incubation at 37°C on the derivative. Thus, the synthetic reaction encompasses both effects, resulting in a higher product yield at *ca.* 120 min.

The kinetics of disappearance of the substrate (BTEE) follows the same trend as the activity of the derivatives on hydrolytic activity. The process is first-order, with rate constant listed on table n°3. As can be seen derivative P is again the most active, followed by CO, AC and finally by SC. It should be noted that these values correspond to the overall rates of disappearance, i.e., they encompass both the hydrolysis and the synthesis process.

Table 3. *Apparent kinetic constants of disappearance of the substrate (BTEE). α-Chymotrypsin immobilized on chitin derivatives P, SC, AC, and CO, in 70 :30 1,4 butanediol/ cabonate buffer of Ph 9.*

Derivative	K ($10^{-3}min^{-1}$)
P	24.10
SC	4.51
AC	5.47
CO	12.10

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Antimicrobial activities of shrimp chitosan and chitosan derivatives and their application on food preservation

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Abstract

Antimicrobial activities of shrimp chitosan, and sulfonated- and sulfobenzoyl- chitosan on various pathogens and food spoilage microorganisms were investigated. The potential application of these products on seafood preservation was also evaluated. In general, chitosan with higher degree of deacetylation (DD) demonstrated better antimicrobial activity. Significant increases of antibacterial activities, due to sulfonation, against *Bacillus cereus*, *Salmonella typhimurium*, *Aeromonas hydrophila*, and *Candida albicans* were observed. Sulfobenzoyl chitosan, as compared to chitosan, also showed the improved inhibitory effects against *Shigella dysenteria* and *A. hydrophila*. Three molds (*Aspergillus parasiticus*, *A. fumigatus*, and *Fusarium oxysporum*) were tested, and chitosan and its derivatives showed the best inhibitory activity against *F. oxysporum*. The counts in oyster stored at 5°C for aerobic plate count, coliforms, *Pseudomonas*, *Salmonella*, *Aeromonas* and *Vibrio* were significantly decreased by adding 2000 ppm chitosan or sulfobenzoyl chitosan.

Keywords: Sulfonated chitosan, sulfobenzoyl chitosan, antimicrobial activity, oyster preservation, shrimp chitosan

Introduction

Chitin is an abundant biomass in nature that's second to cellulose [1]. Chitosan is the product from deacetylation of chitin. Due to chitosan's polycationic and bio-degradable characteristics, it has wide applications, such as, waste water treatment, enzyme immobilization, packaging materials, edible fibers, artificial skin [2].

Researchers discovered, through their studies on pea and its pathogenic fungi (*Fusarium solani*), that once the plant was infected, the enzymes (chitosanase, β-glucanase) of the plant will hydrolyze the cell walls of the pathogens and release chitosan, which in turn, will trigger the synthesis of phytoalexin [3-5]. Consequently, the growth of the fungi was suppressed by phytoalexin. Hadwiger et al. found that chitosans of different number of glucosamine units (i.e. tetramer, hexamer and octamer) have different antifungal effects [6].

The antimicrobial effect of chitosan is influenced by the degree of deacytylation, molecular weight, concentration, pH, and specie of microbes. Ghaouth et al. reported that 3 mg/ml is the minimum concentration to suppress the sporulation of *Botrytis cinerea*, and that for chitosan concentration of 5 mg/ml, only 76% of growth inhibition against *Rhizopus stolonifer* was observed [7]. Hirano and Nagao discovered that low M.W. chitosans have better inhibitory effect than high M.W. chitosans against phytopathogens [8]. Wang observed different antibacterial effect of chitosan when pH was different [9]. Seo et al. pointed out that chitosan has 100 times higher antimicrobial effect in liquid culture than in solid culture [10].

Solubility in water is an important factor in the application of chitosan. Chemical modification in this respect involved alkoxylation, carboxymethylation, acylation, graft polymerization etc. [11]. Many chitosan derivatives have been studied, in terms of their antimicrobial activities, such as, carboxymethyl chitosan, 1-deoxy-1-glucit-1yl chitosan, 1-deoxy-1-lactit-1-yl chitosan, chitosan hydroglutamate, chitosan lactate, sulfuryl chitin, phosphoryl chitin, N-trimethyl chitosan etc. [2, 12-14]. The purpose of this research is three fold, to study antimicrobial activity of sulfonated and sulfobenzoyl chitosan, to compare them with un-derivatized chitosan and the application on oyster preservation.

Materials and methods

Chitosan and Derivatives. Chitosan derived from shrimp, 43% degree of deacetylation (DD43) was purchased from Kau-Hsiung Applied Chemical Company, Taiwan. Raw material was purified by first dissolving in dilute hydrochloric acid, then centrifuged to remove undissolved impurities, followed by neutralization to collect the chitosan gel which was then freeze dried for later use. Purified chitosan was further deacetylated in 40% NaOH, at 100°C, for 90 minutes to get DD69 chitosan.

Sulfonated chitosan (SC) was prepared according to Okiei et al. [15] and Wolfrom and Shen Han [16]. Sulfobenzoyl chitosan (SBC) was prepared through N-acylation reaction, according to Hirano et al. [17]. Degree of deacetylation was determined according to Toei and Kohara [18].

Antimicrobial Test. Flasks with 10 ml NB (or NB+3% NaCl), added with various amounts of chitosan or chitosan derivatives and adjusted the pH value of 6.0, were inoculated with the tested bacteria. After incubation at 37°C or 28°C (for *A. hydrophila*) for 2 days, 0.1 ml of decimal dilutions of samples was spread on nutrient agar (NA) plate for plate counting. For antifungal test, similar protocol was used except that 50 ml YM broth (yeast-malt extract broth) was used and incubated at 25°C for 2 days (*Candida albicans*) and 6 days (molds) before colony count on YM agar plate or mycelium mass was measured. The mold cultures were filtered

through a pre-weighed filter (Whatman No. 1). The mycelia on the filter were washed with deionized water five times, dried in an oven at 100°C for 24 h, and weighed.

Oyster Preservation. Oyster was purchased from local market. Oysters were put into sterilized Erlenmeyer flasks in ratio of 2 ml of sterilized water (containing chitosan/ derivatives of desired concentrations) per gram of oysters (about 25 gram oysters in 50 ml water) and stored at 5°C. Samples were taken every other day (0, 2, 4, 6, 8, 10, 12, 14 days). Before taking samples, oysters were gently machine padded (Stomacher, Model 400, Seward) for 1 minute. 1 ml of sample was diluted at 10X intervals, 0.1 ml of each dilution was spread on various selective agar plates, according to Pan et al. [19] for colony count. Experiments were carried out in triplicates and taken averages.

Results and discussion

Sulfonated and Sulfobenzoyl Chitosan. The presence of the absortion peak at 1610 cm^{-1} demonstrated the attachment of sulfonyl and sulfobenzoyl groups to the $-NH_2$ group of chitosan [17] (data not shown). The result of elemental analysis of SBC was C:H:N:S = 40.61 : 5.56 : 4.44 : 5.63, the calculated degree of substitution was 0.56 sulfobenzoyl group per deacetylated nitrogen. Two different levels, by controlling reaction time, of sulfur content (0.63%, SC1 and 13%, SC2) of sulfonated chitosan were synthesized, elemental analyses were C:H:N:S = 36.45 : 7.14 : 6.36 : 0.63 and 36.45 : 7.12 : 6.35 : 13.03, respectively.

Antimicrobial Activity. Table 1 compared the minimal inhibitory concentration (MIC) of DD43, DD69 chitosan, and sulfonated and sulfobenzoyl chitosan against some common pathogenic and spoilage bacteria and fungi.

Sulfonation increased the solubility of chitosan, therefore, it was expected that upon sulfonation, SC1 (sulfonated chitosan of 0.63% sulfur content) and SC2 (sulfonated chitosan of 13% sulfur content) both will show improved antibacterial effects. Interestingly, SC1 and SC2 showed totally different antibacterial capabilities. SC1 has lower MIC than DD69 chitosan against five of the bacteria studied (*Shigella dysenteria*, *Aeromonas hydrophila* YM1, *Aeromonas hydrophila* , *Salmonella typhimurium* and *Bacillus cereus*), while for SC2, no antibacterial effect was observed at concentration below 2000 ppm (Table 1).

Although SBC was far more soluble than DD69 chitosan, it has the same MICs with DD69 chitosan against three (*E. Coli*, *V. parahaemolyticus*, *L. monocytogenes*) of the bacteria tested. However, in some cases, SBC has lower MIC than DD69 chitosan (*S. dysenteria*, *A. hydophila* YM1, *A. hydrophila*, *S. typhimurium*). Against the bacteria : *V. cholerae*, *P. aeruginosa*, *B. cereus* and *S. aureus*, SBC demonstrated weaker inhibitory effect than DD69 chitosan.

Both DD43 chitosan and DD69 chitosan at 200 ppm could effectively inhibit the growth of *F. oxysporum* (Table 1). SC1 and SBC at 500 ppm also had similar effect. However, SC2 (13 % S) did not show any inhibitory effect at 2000 ppm. All tested samples at 2000 ppm did not completely inhibit the growth of *A. fumigatus* and *A. parasiticus* in liquid culture.

Table 1 Minimal inhibitory concentration (MIC) of chitosan and chitosan derivatives against various microorganisms.

Microorganisms	MIC (ppm)				
	DD43	DD69	SC1	SC2	SBC
Aeromonas hydrophila YM1	nt	500	200	>2000	200
Aeromonas hydrophila	nt	2000	200	>2000	500
Escherichia coli	>200	100	100	nt	100
Pseudomonas aeruginosa	nt	200	200	>2000	2000
Shigella dysenteria	nt	200	100	>2000	100
Salmonella typhimurium	nt	>2000	200	>2000	2000
Vibrio cholerae	nt	200	>2000	>2000	2000
Vibrio parahaemolyticus	nt	100	100	>2000	100
Bacillus cereus	2000	1000	500	nt	>2000
Listeria monocytogenes	nt	100	100	>2000	100
Staphylococcus aureus	nt	100	100	>2000	200
Aspergillus fumigatus	>2000	>2000	>2000	>2000	>2000
Aspergillus parasiticus	>2000	>2000	>2000	>2000	>2000
Candida albicans	2000	1000	500	nt	1000
Fusarium oxysporum	200	200	500	>2000	500

DD43, 43% deacetylated chitosan ; DD69, 69% deacetylated chitosan ; SC1, Sulfonated chitosan (0.63% S) ; SC2, sulfonated chitosan (13% S) ; SBC, sulfobenzoyl chitosan ; nt, not tested.

Application of SBC on Oyster Preservation. Excellent solubility of SBC, along with its antibacterial effect, elevated the potential of SBC, as a natural preservative, on oyster preservation. Table 2 listed the effect of SBC on the growths of certain bacteria during oyster storage at 5 $^{\circ}$C. For the aerobic plate count (APC), initial cell concentration was 5.59 log cycle which gradually increased to 9.97 on the 14th day of storage. When 2000 ppm SBC or DD69 chitosan was employed, APC remained below 10^7 CFU/g within 6 days, which increased to 8.39 or 8.97 log cycle on the 14th day. For the five groups of bacteria tested (Coliforms, *Pseudomonas*, *Salmonella*, *Aeromonas* and *Vibrio*), growths were retarded. For Coliforms and *Pseudomonas*, only minor growths were observed on the 6th day of storage. More effective inhibition against *Salmonella* and *Aeromonas* and *Vibrio* were observed. Their counts for SBC or DD69 chitosna added were apparently much lower than those of control.

Conclusion

This research demonstrated that N-sulfonyl (SC1) and N-sulfobenzoyl chitosan (SBC) have, at least, comparable antibacterial effect to that of DD69 chitosan in some of the cases studied. In some cases, they have superior antibacterial effect to that of DD69 chitosan. However, the degree of sulfonation is an important factor that affects antibacterial capability of sulfonated chitosan. Successful application of SBC on oyster preservation has been performed. Good solubility of sulfonated and sulfobenzoyl chitosan made their application on food preservation easy and effective. These two chitosan derivatives deserve more attention in the field of food protection.

Table 2 Antibacterial effect of chitosan or sulfobenzoyl chitosan against certain groups of bacteria on oyster stored at 5℃

		Bacterial Count (log CFU/g)							
		0 d	2 d	4 d	6 d	8 d	10 d	12 d	14 d
APC	Control	5.59	6.53	8.46	9.14	9.60	9.82	9.39	9.97
	DD69	4.16	4.88	6.74	6.63	7.38	8.04	8.10	8.97
	SBC	4.63	5.88	5.70	6.95	7.28	8.04	8.27	8.39
Coliforms	Control	4.17	6.34	6.55	8.67	9.32	9.03	8.97	10.0
	DD69	3.17	4.70	4.78	4.84	5.68	8.20	6.68	8.97
	SBC	4.12	5.12	6.18	6.45	6.98	7.78	7.61	8.60
Pseudomonas	Control	4.78	4.92	5.62	5.85	6.04	7.11	6.11	6.40
	DD69	2.40	3.05	3.81	4.76	4.00	6.14	5.11	5.14
	SBC	3.55	3.95	4.60	5.65	6.23	6.21	5.56	5.88
Salmonella	Control	3.18	4.85	4.48	4.95	4.93	4.70	5.47	5.93
	DD69	3.40	3.18	4.06	3.40	3.95	3.95	4.23	4.54
	SBC	2.88	3.48	3.67	4.7	2.78	2.54	5.57	5.02
Aeromonas	Control	5.11	5.85	6.60	6.52	8.61	8.88	8.03	8.23
	DD69	2.93	4.16	4.30	5.42	5.74	5.97	5.24	5.26
	SBC	3.69	5.16	5.02	5.74	5.30	6.36	5.67	5.18
Vibrio	Control	<2	<2	<2	<2	3.30	4.42	4.11	4.40
	DD69	<2	<2	<2	<2	<2	3.83	3.13	3.33
	SBC	<2	<2	<2	<2	<2	3.00	2.98	3.4

Control, no chitosan or derivative added; DD 69, 69% deacetylated chitosan added at 2000 ppm; SBC, sulfobenzoyl chitosan added at 2000 ppm

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