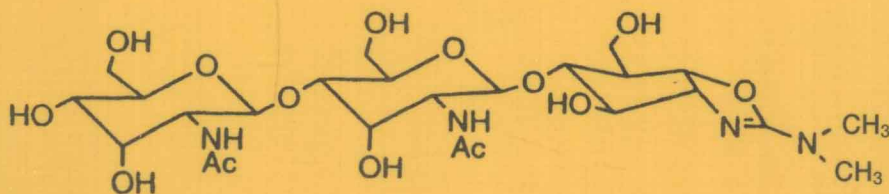


Chitin and Chitinases

Edited by
P. Jollès
R.A.A. Muzzarelli



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Editors

Prof. Dr. P. Jollès
Laboratoire de Chimie
des Substances Naturelles
URA C.N.R.S. No. 401
Muséum National d'Histoire Naturelle
63, rue Buffon
F-75005 Paris
France

Prof. R.A.A. Muzzarelli
Center for Innovative Biomaterials
Faculty of Medicine, University
Via Ranieri 67
I-60100 Ancona
Italy

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List of Contributors

Ichiro Azuma, Institute for the Immunological Sciences,
Hokkaido University, Sapporo 060, Japan

Jeroen Bakkers, Leiden University, Institute of Molecular Plant Sciences,
Wassenaarseweg 64, NL-2333 AL Leiden, The Netherlands;
e-mail: BAKKERS@rulbim.leidenuniv.nl

Graziella Biagini, Center for Innovative Biomaterials, Faculty of Medicine,
University, Via Ranieri 67, I-60100 Ancona, Italy

Gilles Bleau, Département d'Obstétrique-Gynécologie,
Centre Hospitalier de l'Université de Montréal, Hôpital Saint-Luc, 264,
boul. René-Lévesque est, Montréal, Québec, Canada H2X 1P1;
e-mail: bleaug@ere.umontreal.ca

Chantale Boisvert, Département d'Obstétrique-Gynécologie,
Centre Hospitalier de l'Université de Montréal, Hôpital Saint-Luc, 264,
boul. René-Lévesque est, Montréal, Québec, Canada H2X 1P1;
e-mail: boisvertc@ere.umontreal.ca

Ilan Chet, The Hebrew University of Jerusalem,
Otto Warburg Center for Agricultural Biotechnology,
Faculty of Agriculture, P.O. Box 12, Rehovot 76100, Israel

Bice Conti, Department of Pharmaceutical Chemistry, University of Pavia,
Viale Taramelli 12, I-27100 Pavia, Italy

Raul G. Cuero, Prairie View A & M University,
Texas A & M University Systems, CARC, P. O. Box 4079,
Prairie View, TX 77446, USA; e-mail: Olimpa@aal.com

Angel Durán, Instituto de Microbiología Bioquímica,
Consejo Superior de Investigaciones Científicas/
Universidad de Salamanca, Edificio Departamental, Room 219, Avda.
Campo Charro s/n, E-37007 Salamanca, Spain

Yukio Fukumoto, Asa Zoological Park, Asa-Kita,
Hiroshima 731-3355, Japan

Ida Genta, Department of Pharmaceutical Chemistry, University of Pavia,
Viale Taramelli 12, I-27100 Pavia, Italy

Graham W. Gooday, Department of Molecular and Cell Biology,
University of Aberdeen, Institute of Medical Sciences, Foresterhill,
Aberdeen AB25 2ZD, UK; e-mail: g.w.gooday@abdn.ac.uk

Lee A. Hadwiger, Dept. of Plant Pathology, Washington State University,
Pullman, WA 99164-6430, USA; e-mail: chitosan@mail.wsu.edu

Koji Hamada, Ohmu Agricultural Mutual Aid Association, Omu,
Monbetsu 098-1702, Japan

Bernard Henrissat, Architecture et Fonction des Macromolécules
Biologiques CNRS, 31 Chemin Joseph Aiguier,
F-13402 Marseille Cedex 20, France; e-mail: bernie@afmb.cnrs-mrs.fr

Alfredo Herrera-Estrella, Centro de Investigación y Estudios Avanzados,
Unidad Irapuato, A.P. 629, 36500 Irapuato, Gto. Mexico;
e-mail: aherrera@irapuato.ira.cinvestav.mx

Markus Horsch, Department of Plant Biology, University of Zurich,
Zollikerstrasse 107, CH-8008 Zürich, Switzerland

Jan W. Kijne, Leiden University, Institute of Molecular Plant Sciences,
Wassenaarseweg 64, NL-2333 AL Leiden, The Netherlands;
e-mail: KIJNE@rulbim.leidenuniv.nl

Daizo Koga, Laboratory of Biochemistry,
Department of Biological Science, Faculty of Agriculture,
Yamaguchi University, Yamaguchi 753-8515, Japan;
e-mail: koga@agr.yamaguchi-u.ac.jp

Michiko Kono, Fisheries Research Laboratory, Faculty of Agriculture,
The University of Tokyo, Maisaka, Shizuoka 431-0211, Japan;
e-mail: amkono@honga.ecc.u-tokyo.ac.jp

Alfredo D. Martínez-Espinoza, Departamento de Ingeniería Genética,
Unidad Irapuato, Centro de Investigación y de Estudios Avanzados del
Instituto Politécnico Nacional, Apartado Postal 629, Irapuato 36500,
Gto. México; e-mail: amartinez@irapuato.ira.cinvestav.mx

Frédéric Massicotte, Département d'Obstétrique-Gynécologie,
Centre Hospitalier de l'Université de Montréal, Hôpital Saint-Luc, 264,
boul. René-Lévesque est, Montréal, Québec, Canada H2X 1P1;
e-mail: massicottef@ere.umontreal.ca

Masahiro Matsumiya, Laboratory of Marine Products Utilization,
Department of Marine Science and Resources, College of Bioresource
Sciences, Nihon University, Fujisawa, Kanagawa 252-8510, Japan;
email: matsumiya@brs.nihon-u.ac.jp

Monica Mattioli-Belmonte, Center for Innovative Biomaterials,
Faculty of Medicine, University, Via Ranieri 67, I-60100 Ancona, Italy

Yannick Merlen, Département d'Obstétrique-Gynécologie,
Centre Hospitalier de l'Université de Montréal, Hôpital Saint-Luc, 264,
boul. René-Lévesque est, Montréal, Québec, Canada H2X 1P1;
e-mail: merleny@ere.umontreal.ca

Regula A. Merz, Department of Plant Biology, University of Zurich,
Zollikerstrasse 107, CH-8008 Zürich, Switzerland;
e-mail: remerz@botinst.unizh.ch

Saburo Minami, Department of Veterinary Surgery, Faculty of Agriculture,
Tottori University, 4-101 Koyama-Minami, Tottori 680-8553, Japan;
e-mail: minami@pear.agr.tottori-u.ac.jp

Masaru Mitsutomi, Laboratory of Food Chemistry,
Department of Applied Biological Sciences, Faculty of Agriculture,
Saga University, Saga 840-8502, Japan; e-mail: mitsutom@cc.saga-u.ac.jp

Tiziana Modena, Department of Pharmaceutical Chemistry,
University of Pavia, Viale Taramelli 12, I-27100 Pavia, Italy

Arthur F. Monzingo, Institute of Cellular and Molecular Biology,
Department of Chemistry and Biochemistry, University of Texas, Austin,
TX 78712, USA; e-mail: amonzingo@mail.utexas.edu

Riccardo A. A. Muzzarelli, Center for Innovative Biomaterials,
Faculty of Medicine, University, Via Ranieri 67, I-60100 Ancona, Italy;
e-mail: muzzarelli@popcsi.unian.it

Lars E. Nyhlén, Department of Plant Biology, University of Zurich,
Zollikerstrasse 107, CH-8008 Zürich, Switzerland

Yoshiharu Okamoto, Department of Veterinary Surgery,
Faculty of Agriculture, Tottori University, 4-101 Koyama-Minami,
Tottori 680-8553, Japan; e-mail: okamoto@pear.agr.tottori-u.ac.jp

Subba Reddy Palli, Rohm and Haas Research Laboratories,
727 Norristown road, P.O. Box 904, Spring House, PA 19477, USA

F. Pavanetto, Department of Pharmaceutical Chemistry,
University of Pavia, Viale Taramelli 12, I-27100 Pavia, Italy

Paola Perugini, Department of Pharmaceutical Chemistry,
University of Pavia, Viale Taramelli 12, I-27100 Pavia, Italy

Armanda Pugnali, Center for Innovative Biomaterials,
Faculty of Medicine, University, Via Ranieri 67, I-60100 Ancona, Italy

Dora M. Rast, Department of Plant Biology, University of Zurich,
Zollikerstrasse 107, CH-8008 Zürich, Switzerland;
e-mail: rast@botinst.unizh.ch

Arthur Retnakaran, Natural Resources Canada, Canadian Forest Service,
Great Lakes Forestry Centre, 1219 Queen Street East, P.O. Box 490,
Sault Ste. Marie, Ontario P6A 5M7, Canada; e-mail: aretnak@nrcan.gc.ca

Jon D. Robertus, Institute of Cellular and Molecular Biology,
Department of Chemistry and Biochemistry, University of Texas,
Austin, TX 78712, USA; e-mail: jrobertus@mail.utexas.edu

César Roncero, Instituto de Microbiología Bioquímica,
Consejo Superior de Investigaciones Científicas/
Universidad de Salamanca, Edificio Departamental, Room 219,
Avda. Campo Charro s/n, E-37007 Salamanca, Spain;
e-mail: crm@gugu.usal.es

José Ruiz-Herrera, Departamento de Ingeniería Genética,
Unidad Irapuato, Centro de Investigación y de Estudios Avanzados del
Instituto Politécnico Nacional, Apartado Postal 629, Irapuato 36500,
Gto. México; e-mail: jruiz@irapuato.ira.cinvestav.mx

Hildgund Schrempf, FB Biologie/Chemie, Universität Osnabrück,
Barbarastrasse 11, D-49069 Osnabrück, Germany;
e-mail: schrempf@mail.biologie.uni-osnabrueck.de

Mohammed Shahabuddin, Medical Entomology Section,
Laboratory of Parasitic Diseases, National Institute of Allergy and
Infectious Diseases, National Institutes of Health,
4 Center Drive MSC 0425, Bethesda, MD 20892-0425, USA;
e-mail: mshahabudd@nih.gov

Yoshihiro Shigemasa, Department of Materials Science,
Faculty of Engineering, Tottori University, 4-101 Koyama-Minami,
Tottori 680-8552, Japan

Herman P. Spaink, Leiden University, Institute of Molecular Plant Sciences, Wassenaarseweg 64, NL-2333 AL Leiden, The Netherlands; e-mail: SPAINK@rulbim.leidenuniv.nl

Klaus-Dieter Spindler, Universität Ulm, Abteilung Allgemeine Zoologie, Albert-Einstein-Allee 11-13, D-89069 Ulm, Germany; e-mail: klaus-dieter.spindler@biologie.uni-ulm.de

Margarethe Spindler-Barth, Heinrich-Heine-Universität Düsseldorf, Lehrstuhl für Hormon- und Entwicklungsphysiologie, Universitätsstr. 1, D-40225 Düsseldorf, Germany; e-mail: spindler@rz.uni-duesseldorf.de

Hiroshi Tamura, Faculty of Engineering, Kansai University and HRC, Suita, Osaka 564-8680, Japan

Seiichi Tokura, Faculty of Engineering, Kansai University and HRC, Suita, Osaka 564-8680, Japan

M. Henar Valdivieso, Instituto de Microbiología Bioquímica, Consejo Superior de Investigaciones Científicas/ Universidad de Salamanca, Edificio Departamental, Room 219, Avda. Campo Charro s/n, E-37007 Salamanca, Spain

Joseph M. Vinetz, WHO Collaborating Center for Tropical Diseases, Department of Pathology and Division of Infectious Diseases, University of Texas Medical Branch, Keiller 2.138, 301 University Blvd., Galveston, TX 77555-0609, USA

Preface

Chitin, the insoluble polymer of N-acetylglucosamine, is the most abundant nitrogen-bearing organic compound found in nature, present in insect exoskeletons, crustacean shells and fungal cell walls.

We have selected the most recent and sophisticated chitin-related advances in life sciences, and approached chitin from an original standpoint: the prompt and enthusiastic response of the colleagues invited to collaborate is gratefully acknowledged.

The first part of this book, after a short presentation of chitin in the environment, is devoted to chitin biosynthesis. Successively, we discuss the biochemistry of chitin synthase and the state of knowledge of chitin synthesis *in vitro*, chitin biosynthesis and structural organization *in vivo*, and the chitin synthases of yeasts and fungi. The role of chitin oligosaccharides in plant morphogenesis, and biochemical aspects of inhibitors of chitin synthesis, are also approached. Some chitin-binding proteins are reviewed.

The second part is devoted to chitinases, which split the β -1,4-glucosidic bonds of chitin as, in a less pronounced manner, lysozymes also do. Biochemical, structural and evolutionary aspects concerning chitinases are discussed. Chitin-containing organisms produce chitinases, but some organisms deprived of chitin, such as a wide variety of bacteria and higher plants, also produce chitinases for their defence. These aspects are reviewed in a series of chapters. Some enzyme inhibitors are also mentioned. Newly characterized mammalian chitinase-like proteins are presented. Aspects concerning N-acetyl- β -D-glucosaminidases, enzymes releasing N-acetylglucosamine monomers from chitin, are also discussed in relation with their growing medical importance.

The third part of the book is devoted to chitosan, a family of deacetylated chitins. The agricultural, food, cosmetic and pharmaceutical industries more and more frequently use this polysaccharide in the form of threads, fibers, films, gels, microspheres and liposomes. Exciting applications are discussed in a series of chapters that emphasize the fact that chitosan applications based on its biological significance often depend on its biodegradability.

We are confident that this book will provide a stimulating background for further fruitful research on chitin in the biochemical and biological area.

January 1999

Pierre Jollès and
Riccardo A. A. Muzzarelli

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Native, industrial and fossil chitins

Riccardo A. A. Muzzarelli

*Center for Innovative Biomaterials, Faculty of Medicine, University, Via Ranieri 67,
I-60100 Ancona, Italy*

Summary. Countless living organisms continuously synthesize and degrade chitin enzymatically, for nutritional, morphogenetic and defensive or aggressive purposes. Chemically modified chitins are important in the light of their biochemical significance in medicine and crop protection; their environmentally friendly behaviour permits industrial exploitation of the huge chitinous biomasses generated by fishing activities and biotechnology. Chitin is promptly metabolized in sediments, and fossil chitin is not frequently encountered.

Being convinced that this book will attract many readers not fully acquainted with chitin, I deem it appropriate to provide a brief introduction. The reader is referred to a large body of information on chitin available in a number of books: a selection is listed below [1–20].

Chitin, (1-4)-linked 2-acetamido-2-deoxy- β -D-glucan, is widely distributed among invertebrates. At least 10 gigatons (1.10^{13} kg) of chitin are synthesized and degraded each year in the biosphere. It is found as α -chitin in the calyces of hydrozoa, the eggshells of nematodes and rotifers, the radulae of mollusks and the cuticles of arthropods, and as β -chitin in the shells of brachiopods and mollusks, cuttlefish bone, squid pen, and pogonophora tubes. Chitin is found in exoskeletons, peritrophic membranes and the cocoons of insects. Chitin is ubiquitous in fungi: the chitin in fungal walls varies in crystallinity, degree of covalent bonding to other wall components, mainly glucans, and degree of acetylation.

The polymorphic forms of chitin differ in packing and polarities of adjacent chains in successive sheets; in the β -form all chains are aligned in parallel manner, whereas in α -chitin they are antiparallel. The molecular order of chitin explains its physiological role and tissue characteristics, for instance in the insect cuticle and tendon (α -chitin) and in the pen of Cephalopoda (β -chitin). The grasping spines of *Sagitta* are made of pure α -chitin because they should be suitably hard to hold a prey. Also, solubility and reactivity are different.

In the areas of fisheries, textiles, food and ecology, scientists and industry people were urged to upgrade chitin in order to exploit renewable resources and to alleviate waste problems. Today chitins and chitosans from different animals are commercially available.

Chitin isolates differ from each other in many respects, among which are degree of acetylation, typically close to 0.90; elemental analysis, with

nitrogen content typically close to 7%, and N/C ratio 0.146 for fully acetylated chitin; molecular size; and polydispersity. The average molecular weight of chitin *in vivo* is probably in the order of the MDa, but chitin isolates have lower values due to partial random depolymerization occurring during chemical treatment and depigmentation steps. Polydispersity may vary depending on such treatments as powder milling and blending of various chitin batches.

Isolated chitin is a highly ordered copolymer of 2-acetamido-2-deoxy- β -D-glucose, the major component, and 2-amino-2-deoxy- β -D-glucose. As a point of difference from other abundant polysaccharides, chitin contains nitrogen. Chitobiose, O-(2-amino-2-deoxy- β -D-glucopyranosyl)-(1-4)-2-amino-2-deoxy-D-glucose, is the structural unit of native chitin. Bound water is also a part of the structure.

Chitin is easily hydrolyzed by acids, but is stable to dilute alkali; in warm concentrated alkali it is oxidized by air. Chitin hydrolysates can be prepared by adding chitin to concentrated HCl at 4°C and stirring at 40°C. Excess acid is then removed with ion-exchange resin, and the product is resuspended to prepare the so-called colloidal chitin, which remains stable for several weeks when stored at 4°C. In the wet state it is degraded by a number of microorganisms, which produce chitinolytic enzymes or other enzymes with unspecific activity towards chitin. Colloidal chitin is being used since the 1950s for the study of chitinases.

The solubility of chitin is remarkably poorer than that of cellulose because of the high crystallinity of chitin, supported by hydrogen bonds mainly through the acetamido group. Ethanol-containing calcium chloride, dimethylacetamide containing 5–9% LiCl (DMAc/LiCl) and N-methyl-2-pyrrolidinone/LiCl are systems where chitin can be dissolved up to 5%. The main chain of chitin is rigid at room temperature, so that mesomorphic properties may be expected at a sufficiently high concentration of polymer. Circular dichroism of Congo red bound to the chitin films, obtained by moisture uptake from DMAc/LiCl solutions, reveals a cholesteric structure, having an organization similar to that naturally occurring in the chitin cuticle.

Chitosans

Chitosan indicates a family of deacetylated chitins. In general, chitosans have a nitrogen content higher than 7% and a degree of acetylation lower than 0.40. The removal of the acetyl group is a harsh treatment usually performed with concentrated NaOH. Protection from oxygen, with a nitrogen purge or by addition of sodium borohydride to the alkali solution, is necessary in order to avoid undesirable reactions such as depolymerization and generation of reactive species. Commercial chitosans may contain insoluble highly acetylated fractions that come from the core of the

granules submitted to heterogeneous deacetylation. The acetyl groups in the acid-soluble fractions are randomly distributed, whilst the insoluble fractions contain relatively long sequences of acetylated units.

The presence of a prevailing number of 2-amino-2-deoxyglucose units in a chitosan facilitates bringing the polymer into solution by salt formation. Chitosan is a primary aliphatic amine that can be protonated by selected acids, the pK of the chitosan amine being 6.3. The following salts, among others, are water-soluble: formate, acetate, lactate, malate, citrate, glyoxylate, pyruvate, glycolate and ascorbate.

Despite the alteration due to deacetylation, chitosan from crab tendon possesses a crystal structure showing an orthorhombic unit cell. The unit cell comprises four glucosamine units; two chains pass through the unit cell with an antiparallel packing arrangement. Main hydrogen bonds are O3...O5 (intramolecular) and N2...O6 (intermolecular). The crystal structures of salts and derivatives have also been determined, for instance for chitosan ascorbate.

Chitosan can be obtained from fungi, easily cultured on simple nutrients. Chitosan is present in the cell wall of Mucorales and can be isolated from the accompanying glucans by extraction with either acetic acid or alkali, the latter being preferred when glucans are to be dissolved. The final molecular weight is in the order of 500 kDa, and the degree of acetylation is around 0.10.

Chitin and chitosan derivatives

In the past, chitin has been often considered as an intractable biopolymer due to the difficulties encountered in dissolving and reacting it. As soon as the molecular association is prevented or depressed, chitin lends itself to many reactions, affording a wide choice of modified chitins. On chitosan, the reactions of the primary amino group and primary and secondary hydroxyl groups can be easily performed. The chemical modifications of chitin and chitosan, carried out under mild conditions in order to protect glycoside and acetamido linkages, yield more soluble polymers. The latter have higher biodegradability in animal bodies and physical properties of interest for applications in the solid state or in solution.

Chitin treated with NaOH yields alkali chitin, a widely used unstable intermediate which reacts with 2-chloroethanol to yield O-(2-hydroxyethyl) chitin, known as glycol chitin: this material was probably the first derivative to find practical use and to be recommended as a substrate for lysozyme. The reaction of alkali chitin with sodium monochloroacetate gives O-carboxymethyl chitin sodium salt, soluble in water. The latter compound has been and still is one of the protagonists of chitin chemistry, together with N-carboxymethyl chitosan; both have found applications in a variety of fields. Chitosan can be reacylated with acetic anhydride to obtain water-soluble partially reacylated chitin. Countless chitin deri-

vatives have so far been prepared; the present trend is to exert control over the modification reactions in order to achieve the best performance or to enhance the biological significance. For example, upon regiospecific oxidation, chitin yields 6-oxychitin, a fully water-soluble compound [21]; regioselective synthesis affords chitin sulfates endowed with anti-HIV-1 activity [22].

Fossil chitin

Whilst chitinous materials are relatively resistant to degradation under certain conditions, for instance suspension in seawater, and desert sand, they are promptly degraded in other environments such as ocean sediments.

A huge number of mineralized skeletal structures containing chitin-protein complexes settle into deep-sea sediments: there, extraction of organic matter is performed by fungi, algae and bacteria, which use it as a nutrient. Extracellularly secreted enzymes hydrolyze the organic polymers of the skeletal remains colonized by the microorganisms. Because those chitinases seem to be particularly stable and effective under deep-sea conditions, sediments contain little chitin.

Detection of chitin in fossils is not frequent: there are reports of fossil chitin in pogonophora, and insect wings from amber, but fossils of crustacea were found to contain only traces of chitin, and no chitinlike microfibrils were detected by electron microscopy.

Terrestrial arthropods have a fossil record that reaches back to 420 megayears ago (Upper Silurian): their remains are preserved as cuticle fragments. Arachnids were found preserved by precipitation of silica; millipeds occur as calcified remains; coleoptera fossils were recovered from buried peat. The fossil cuticles revealed alkanes and alkenes, indicating substitution of chitin by more resistant organic compounds [23–25].

Chitin is not generally preserved as such, but some of the most spectacular examples of soft part preservation involve replication in calcium phosphate: apatite minerals inhibit the decay of organic compounds. Although proteins have a short survival time even within CaCO_3 crystals, it is possible that phosphate salts provide protection from degradation. Replication of soft tissues is more rapid in calcium phosphate than in any other mineral, and therefore preserves the highest fidelity in detail, such as in the case of insect eyes. Interestingly, chitin associations with calcium phosphate are being studied today for bone regeneration purposes.

Conclusion

Chitin is amply distributed in the biosphere where it constitutes the exoskeletons of many organisms, but also provides delicate structures and

tissues such as the flying apparatus of insects, and tendons of crustaceans. Chitin nitrogen is recycled by biodegradation operated by microbial genera which exist in just about every conceivable environment. Chitin therefore represents a widely distributed organic compound of nitrogen.

The nitrogen present in chitin imparts unique properties to this class of polysaccharides. Goods manufactured from chitin are environmentally important items; chitosan solutions are used to recover proteins and to preserve cereal seeds, or to prepare biomaterials such as wound dressings and biodegradable packaging.

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