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Review

Current views on fungal chitin/chitosan, human chitinases, food preservation, glucans, pectins and inulin: A tribute to Henri Braconnot, precursor of the carbohydrate polymers science, on the chitin bicentennial

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ABSTRACT

Two hundred years ago, Henri Braconnot described a polysaccharide containing a substantial percent of nitrogen, later to be called chitin: that discovery stemmed from investigations on the composition of edible mushrooms and their nutritional value. The present interdisciplinary article reviews the major research topics explored by Braconnot, and assesses their importance in the light of our most advanced knowledge. Thus, the value of fungi, seafoods and insects is described in connection with the significance of the presence of chitin itself in foods, and chitinases in the human digestive system. The capacity of chitin/chitosan to depress the development of microbial pathogens, is discussed in terms of crop protection and food preservation. Other topics cherished by Braconnot, such as the isolation of pectin from a large number of plants, and inulin from the *Helianthus* tubers, are presented in up-to-date terms. Acids isolated from plants at that early time, led to enormous scientific advancements, in particular the glyoxylic acid and levulinic acid used for the preparation of soluble chitosan derivatives that paved the way to a number of applications. An opportunity to trace the origins of the carbohydrate polymers science, and to appreciate the European scientific heritage.

Contents

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1. Introduction

This review article intends to revisit the major research topics to which Henri Braconnot devoted his scientific life, on the occasion of the bicentennial of his discovery of chitin in edible fungi. He was always attracted by the alimentary aspects of botany, and his research was most often aimed at alleviating food shortages, not to say famine, that the majority of the French population had to face. Concisely, we present here the current views on the title points to underline the durable validity of Braconnot's interests, experimental approaches and results. The problems faced 200 years ago still persist for the majority of the exceedingly large world's population, notwithstanding the technological advances made. A glimpse back to 1811 would give us an opportunity to appreciate the immense spiritual resources of the western Countries that, in the context of the American and French revolutions, elaborated new scientific interests, methodologies and communication ways. For this Journal, the chitin bicentennial is also an opportunity for tracing the roots of the carbohydrate polymers science.

2. The precursor of the carbohydrate polymer science

Henri Braconnot (1780–1855) laid the foundations of the carbohydrate polymer science: after the discovery of chitin, the first polysaccharide described 30 years earlier than cellulose, he continued with his idea of extracting sugars from edible fungi such as *Agaricus bisporus*, and remarkably extracted inulin from the tubers of *Heliantus tuberosus*. He also studied pectins of various origins, and isolated pure sugars after chemical hydrolysis of straw, wood and cotton; likewise, he isolated trehalose from edible fungi, and refined beet sugar to a white and crystalline substance. Among various biographies, those by Labrude and Becq (2003) and Prévost and D'Amat (1956) deserve mention.

Systematic sulfuric acid treatment of a large number of substances led him to isolate glycine, "glycocolle", from gelatine. Simultaneously with Proust, he described leucine. These discoveries brought him a certain acclaim. He also isolated several vegetable albumins.

Research activities of more chemical flavour were those on the nitration of cellulose, that yielded a hydrophobic and flammable product, "xiloidine", endowed with filmogenic properties: this was a precursor of Celluloid® that in the second half of that century led to "fulmicoton" and other explosive substances that replaced the black powder. From plant tissues, a series of acids of major importance were isolated by Braconnot: namely acetic, malic, maleic, sorbic, gallic, ellagic, pyrogallic, and lactic; the picric acid among others was synthesized.

In 1807 Henri Braconnot was appointed director of the Botanical Garden in Nancy with teaching duties of natural history. The four-century old University of Nancy, as well as the University of Strasbourg, had been suppressed by the Assemblée Générale, and in Nancy the Medical School and the Academy were the only structures for further education. Actually, the Garden was part of the Medical School because of the interest in officinal plants: there, Braconnot started a research programme on the chemistry of vegetal extracts, and his publications attracted the attention of learned societies and eminent chemists, so that King Louis XVIII appointed him as a member of the Royal Medical Academy (1820) to become "Chevalier de la Légion d'honneur" in 1828.

While taking care of the Garden, Braconnot started a large scale cultivation of sugar beet and extraction and purification of sugar with the intention of alleviating food shortage. He studied the cultivation of Italian rice and used starch in various experiments. Braconnot was interested in the definition of the nutritional value of mushrooms: he wrote that poor countrymen considered mushrooms a manna given free as a gift of providence, and eagerly waited for the mushroom seasons.

Management of the Garden and the relevant problems (risky use of gas for heating the hothouses, diatribes against military plans to build barracks inside the Garden) prevented Braconnot from exploiting his chemical discoveries. He was a precursor of Chevreul with his studies on fats, but he had no means of identifying the fatty acids; he brought forward the idea of plant alkali but he could not isolate the alkaloids. Braconnot published 112 papers in the form of memoirs of the Academy of Sciences, Letters and Arts of Nancy, also known as the Academy of Stanislas, the King of Polish origin who ruled the Lorraine region. Other publications are in the *Annales de Chimie et Physique* and the *Journal de Chimie Médicale*.

Braconnot certainly was an eminent chemist, as his successor wrote, but he dedicated most of his energy to botany (Godron, 1872). Actually his teaching followed Linnée's principles, in a period when novel theories on cellular structure, plant sexuality and alternate generations were being brought forward, as a consequence of the studies conducted on enormous collections of previously unknown plants. For instance the Flinders expedition (1801) made available 4000 unknown species of plants from Australia. The Empress Joséphine visited the Nancy Garden and sent a large number of plants to Braconnot, so that in 1852 the 14,100 m^2 Garden had 3452 plant species, including some from New Zealand and Reunion Island.

2.1. The discovery

The discovery of chitin was essentially based on some reactions carried out on raw material isolated from *Agaricus volvaceus*, *A. acris*, *A. cantarellus*, *A. piperatus*, *Hydnum repandum*, *H. hybridum* and *Boletus viscidus*. The fungal material was partially purified by boiling in dilute (potassium) hydroxide [that removed proteins and pigments, to yield the chitin–glucan complex]. The resulting "fungine", when distilled in admixture with KOH, released ammonia, that demonstrated the presence of nitrogen as the fourth element. On the other hand, concentrated sulfuric acid liberated acetic acid

from "fungine" thus demonstrating the presence of acetyl moieties (Braconnot, 1811).

This article, translated into English by Children (1824) became widely known; Lassaigne (1843) purified coleopteran elytra and *Bombyx mori* exuviae, and then treated the residues with potassium at warm thus obtaining potassium cyanide that (no matter the danger) unequivocally demonstrated the presence of nitrogen in chitin. He further stated that chitin was not present in the higher animals. He also underlined that Odier (1823), who coined the name chitin, did not actually demonstrate the chitin identity. Payen (1843) immediately corroborated the Lassaigne's findings and views, and added his own results, demonstrating that ammonia was released from purified arthropod exoskeletons upon heating with sodium hydroxide, and that nitrogen was ~9%. Interestingly, Payen described a number of chemical differences between plant tissues (cellulose) and arthropod exoskeletons (chitin) that could be easily replicated in the laboratory, to become well refined and reproducible in the course of the next 100 years (BeMiller, 1965; Conrad, 1964). The chemical similarity of fungal and animal chitins was assessed at the end of the century by Gilson (1894). Further early reports on chitin and chitosan have been chronologically enumerated in the book "Chitin" (Muzzarelli, 1977).

2.2. The scientific environment

The discovery of chitin took place in a dramatic historical moment: Napoleon was ready to invade Russia with an army of 600,000 soldiers who, the following year (1812), were reduced to less than 10,000 miserable survivors afflicted by famine, wounds, contagious diseases, amputations and parasites. Again, in 1813 the subsequent defeat at Leipzig upset eastern France where masses of French soldiers came back in desperate conditions. The Napoleonic wars depressed the development of chemistry in France: notwithstanding the sudden progress made at the time of the revolution (new polytechnic schools, decimal metric system, teaching of medicine within hospitals) French chemistry lost some of its prestige under Napoleon. The most famous chemist at that time was Claude-Louis Berthollet (1748–1822) who accompanied Napoleon in Egypt, thus losing contact with his European colleagues, among whom Benjamin J. Richter (1762–1807), who elaborated the concept or chemical equivalent in Germany. Joseph L. Proust (1755–1826) contemporary of the physician André Ampère, made important contributions to chemistry, as well as Joseph L. Gay-Lussac (1778–1850) who published his well known laws in 1802.

Those years were crucial for the connections between botany, chemistry and medicine. For example, morphine was isolated by Serturner in 1806, quinine was discovered by Pelletier and Caventon in 1823 and atropine was crystallized in 1833. The discovery of the anaesthetic action of nitrous oxide, diethyl ether and chloroform gave rise to deep innovations in surgery.

Real chemical advances were made at that time by English chemists, among whom Joseph Priestley, Humphrey Davy (1778–1829), William Higgins (1763–1825) and John Dalton; in Sweden Jons J. Berzelius (1779–1848) had a large number of pupils of various nationalities, and finally in Turin (Piedmont, now Italy) Amedeo Avogadro published his famous chemical law in 1811 (which also deserves a bicentennial celebration!).

3. The occurrence of chitin in edible and filamentous fungi

The most recent description of chitin in living organisms is the one by Muzzarelli (2011). As Fig. 1 shows, the repeating structural unit of chitin is the dimer of GlcNAc. Chitin is the characteristic component of the taxonomical groups Zygo-, Asco-, Basidio- and Deuteromycetes.

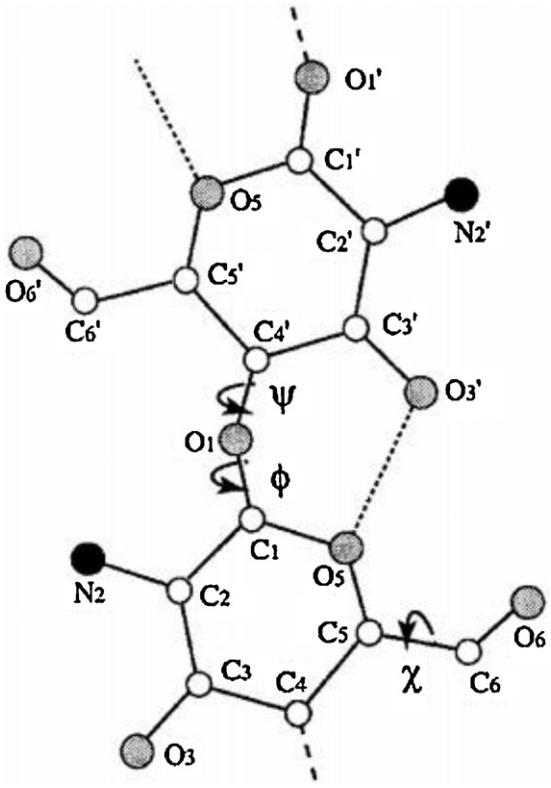

Fig. 1. Chemical structure of a disaccharide segment of chitosan, showing position numbering. The two angles Ψ and Φ define the chain conformation, and the angle χ define the O6 orientation. Dashed lines denote the O3–O5 hydrogen bonds that are crucial for rigidity. Hydrogen bonds connecting various positions of adjacent chains are omitted for sake of clarity.

The worldwide production of mushrooms was updated by the Food and Agriculture Organization of the United Nations in 2009. China is the largest producer (>1.5 million metric tons in 2007, with an increment of 65% in 10 years). United States and Canada ranked 2nd and 3rd with 390,000 and 81,500 tonnes, respectively. Israel and India showed the most impressive increase over the said decade, due to consumer awareness of the health benefits of mushrooms, in terms of contents of prebiotics and polysaccharides.

The preferred cultivated mushroom worldwide is *A. bisporus* (button mushroom), followed by *Lentinus edodes* (shiitake), *Pleurotus* spp (oyster mushrooms), *Auricula auricula* (wood ear mushroom), *Flamulina velutipes* (winter mushroom) and *Volvariella volvacea* (straw mushroom) (Aida, Shuhaimi, Yazid, & Maaruf, 2009). The chemical composition and the nutritional value of European species of wild mushrooms has been reviewed by Kalac (2009): carbohydrates usually account for the prevailing component of fruiting bodies: glucose, mannitol and α,α-trehalose are the main representatives of monosaccharides, their derivatives and oligosaccharides, respectively. The contents of trehalose and mannitol decrease considerably during boiling of fresh mushrooms, while drying and freezing results in limited loss. The reserve polysaccharide of mushrooms is glycogen (5–10% dry matter), instead of starch in plants. Anti-nutritional components of edible mushrooms are also indicated.

Notwithstanding the high significance of the structural models of chitin elaborated by experts in X-ray diffraction spectrometry, one should be aware of the multiplicity of forms that chitin assumes to satisfy the requirements of the various chitinous tissues.

3.1. Chitin in edible fungi

The chitin content of pileus and stipes of fruit bodies of cultivated *Agaricus bisporus*, *Pleurotus ostreatus* and *Lentinula edodes* is a stable characteristic of the species and there are no significant differences between the varieties. The chitin levels of pileus and stipes are not significantly different (for *A. bisporus*, 6.68 and 7.25) but they are for *P. ostreatus* and *L. edodes*, for which the pileus has higher chitin content than the stipe. The saprotrophic mushroom *A. bisporus* has higher chitin level than both wood-rotting *P. ostreatus* and *L. edodes*. The chitin of cultivated mushrooms is an important component of their nutritional value (Vetter, 2007).

Evidence for covalent linkages between chitin and β-glucan in *Schizophyllum commune* (Basidiomycete) was provided by Sietsma and Wessels (1979) among others. The isolation of the chitin + glucan complex from the biomass of *Armillariella mellea* and the yellow morel *Morchella esculenta* (classes Basidiomycetes and Ascomycetes, respectively), has been developed by Ivshina, Artamonova, Ivshin, and Sharnina (2009). The four steps were deproteinization, demineralization, depigmentation, and final removal of residual protein. The chitin in the isolated complexes was ca. 80% and 47%, respectively; the degree of acetylation was 0.80 and 0.41, respectively, while the degree of crystallinity was 61 for both; various analytical techniques demonstrated the identity of fungal and arthropod chitin.

The solid-state fermentation of *L. edodes* was found to be an efficient approach to chitosan production, according to Crestini and Giovannozzi-Sermanni (1996); in fact, the chitosan yield was 6.18 g/kg and the acetylation degree was 12.5% at 12 days after inoculation. These data are of particular significance: the solid-state fermentation gave yields 50 times higher than submerged fermentation, and the chitosan had smaller degree of acetylation than crustacean commercial chitosans.

3.2. Chitin and chitosan in filamentous fungi

Yeasts and filamentous fungi contain glucan complexes with chitin or chitosan in their cell walls and septa (Muzzarelli, Tanfani, & Emanuelli, 1981; Smith & Berry, 1977; Trinci, 1978). San-blas and Carbonell (1974) published microphotographs of stained material from the mycelia of *Histoplasma farciminosum* treated with chitinase and β-1,3 glucanase, showing the remaining thick bundles 42 nm wide composed of 5-nm fibres made of β-1,3 glucan and chitin that escaped hydrolysis by said lytic enzymes thanks to their stability.

In the order Mucorales many strains have interesting productivity of chitosan, in particular *Absidia* spp as documented by Kuhlmann et al. (2000): MW as high as 700 kDa and yields of 1.0–1.3 g per liter of submerged culture medium were obtained. For example, the mycelia of cultured *Absidia glauca* var. *paradoaxa* were treated with hot 2% sodium hydroxide to isolate the alkali-insoluble polysaccharides, from which the extraction of chitosan was carried out with 2% acetic acid at room temperature. The chitosan extracted had degree of deacetylation 0.86, and viscosity 4.0 cP at 0.1% chitosan in 0.5% acetic acid that indicates low molecular weight (Rungsardthong, Wonputtanakul, Kongpien, & Chotiwaranon, 2006). Niederhofer and Muller (2004) extracted chitosan with average MW 45 kDa from *Absidia coerulea*.

Chitosan is the most abundant component of both filamentous and yeast-like forms of *Mucor rouxii*: the maximum chitosan levels are 32.7 and 27.9%, respectively, according to Bartnicki-Garcia and Nickerson (1962) while the degree of acetylation of chitosan is 0.19. Chitosan is also accompanied by minor amounts of chitin and glucan. The pathway of chitosan formation in *M. rouxii*, including the description of the role of chitin deacetylase (the enzyme that hydrolyses the acetamido groups of chitin) was reported by Araki and Ito (1975). The biochemistry of *Mucor* was exhaustively treated by Sypherd, Borgia, and Paznokas (1978) and the glyoxylate cycle in *Mucor* was detailed by O'Connor and Paznokas (1980). The production yield of chitosan from *M. rouxii* was greatly improved with the aid of plant growth hormones, viz., indoleacetic acid, indolebutyric acid, kinetin and gibberellic acid: the yield of chitosan increased 34–69%, gibberellic acid being the most potent hormone (Chatterjee, Adhya, Guha, & Chatterjee, 2005; Chatterjee, Chatterjee, Chatterjee, & Guha, 2009).

A. niger, instead, contains only chitin. Amounts of glucan in the range 7.4–39.8% are associated with chitin and are eliminated by extraction. In the *A. niger* mycelium, Wu, Zivanovic, Draughon, and Sams (2004) and Wu, Zivanovic, Draughon, Conway, and Sams (2005) found that the maximum glucosamine level was 11.10% d.w. The degrees of acetylation were determined to be 0.76–0.50. In consideration of the large spent biomass generated during the production of citric acid, many authors characterized the chitin + glucan complex present in the cell walls of *A. niger* with a view at its recovery (Stagg & Feather, 1973) and determined the recoverable amount of chitin. In the earliest report on the isolated chitosan + glucan from *A. niger*, the statistical evaluation of a large set of preparative conditions indicated that a 4-h treatment with 40% NaOH aqueous solution without nitrogen blanketing at 128 °C provided satisfactory yield (44%) and good chitosan content (>32%). These products were not fully soluble in 5% acetic acid, but dispersions were obtained upon prolonged stirring. The infrared spectra taken on the fractions obtained after filtration indicated different ratios of chitosan and glucan. The collection percentages of 8 transition metal ions were definitely higher than the corresponding values for animal chitosan despite the fact that the complex contains nearly one half chitosan by weight, this fact being justified by the much bigger the surface area. The filamentous mycelia shapes were still visible under the microscope in rather frail films obtained thanks to the retained typical filmogenicity of chitosan (Muzzarelli, Tanfani, & Scarpini, 1980). Evidence of the covalent binding of chitin with glucans was confirmed many years later for a variety of fungi by Nwe, Stevens, Tokura, and Tamura (2008), Nwe, Furuike, and Tamura (2010), Vincendon and Desbrieres (2002) and Zamani, Edebo, Sjostrom, and Taherzadeh (2007) among others. The glucans have been determined quantitatively in edible mushrooms by Nitschke et al. (2011).

The chitin + glucan from *A. niger* was later produced industrially: the poly(*N*-acetyl-D-glucosamine) and the *β*(1,3)-D-glucan were present in the w/w ratio 35/65 as determined by ^{13}C-solid state NMR. Said material was studied in an animal model of atherosclerosis by Berecochea-Lopez et al. (2009): it strongly reduced the area of aortic fatty streak deposition by 87–97%, cardiac production of superoxide anion by 25%; on the other hand, it enhanced liver superoxide dismutase activity by 7–45% and glutathione peroxidase activity by 38–120%. These findings support the view that constant consumption of chitin + glucan has potential effects against atherosclerosis, the underlying mechanism being related mainly to improving the antioxidant defenses. No undesirable or toxic effects were detected, nor evidence of other clinical signs, thus at low doses chitin + glucan was deemed to be a safe nutraceutical supplement. Certain aspects of said work were confirmed by Neyrinck et al. (2009) who concluded that fungal chitosan counteracts some inflammatory disorders and metabolic alterations occurring in diet-induced obese mice since it decreases feed efficiency, adipocytokine secretion, fat mass and ectopic fat deposition in the liver and the muscle. The *Absidia* chitosans tested according to ASTM F813-83 and F619-79 with murine fibroblasts were found to be biocompatible in terms of cytotoxicity and lactate dehydrogenase determinations in agreement with Muzzarelli, Ilari, Tarsi, Dubini, and Xia (1994), Muzzarelli, Ilari, Xia, Pinotti, and Tomasetti (1994) and Chung, Schmidt, Hamlyn, and Sagar (1994).

Potato peel was used as a substrate for chitosan production from *Rhizopus oryzae*. The best extraction condition was using 46% sodium hydroxide at 45 °C for 13 h followed by 2% acetic acid at 95 °C for 8 h: the chitosan yield obtained was 10.8 g/kg substrate after 5 days of fermentation at pH 5. The *R. oryzae* chitosan had 86–90% degree of deacetylation, molecular weight of 80–128 kDa and viscosity of 3.1–6.1 mPa s: this set of results qualify this system as a convenient one for fungal chitosan production (Kleekayai & Suntornsuk, 2011).

3.3. Filamentous fungi in traditional foods

While there is much interest in growing filamentous fungi in order to produce fungal chitin and chitosan, it should be noted that the microbial cells (algae, bacteria, yeasts and fungi) have a place in world food supply from both standpoints of animal feedstuff and human food applications, and that this will continue in the future. It should not be expected, however, that the portion of edible protein of microbial origin will be quite large.

Filamentous fungi in fact are present in traditional sources of food in many regions of the world. The Indonesian tempe (or tempeh), for example, consists of soybean fermented by the zygomycete *Rhizopus oligosporus* and contains a fraction of glucosamine that can reach important concentrations, from 51 to 111 g/kg dry biomass. Values of glucosamine as high as 97 and 93 g/kg of dry biomass for submerged cultures of *Rhizopus japonicus* and *Mucor racemosus*, respectively were reported. The glucosamine content of the mycelium can vary significantly, being strongly influenced by the growth medium; it increases in old mycelia where the chitin fraction is larger (Sasaki, Kodama, Uchida, & Yoshino, 1985; Sparringa & Owens, 1999).

Moreover Roubos-van den Hil, Rob Nout, van der Meulen, and Gruppen (2010) demonstrated tempe to play a bioactive function against toxigenic *Escherichia coli*. This effect of soybean tempe is obtained by inhibiting the adhesion of enterotoxigenic *E. coli* to intestinal brush border cells, thus preventing the most frequent cause of childhood diarrhea in developing countries, and of traveler's diarrhea. Moreover, other foods such as barley, chick pea, cowpea, groundbean, horsebean, pea, oats, sorghum and wheat are also good substrates for growing chitin-bearing fungi.

4. Human chitinases in nutrition and metabolism

Chitinases hydrolyze the β1–4 bonds of the chitin chain down to the N-acetyl-D-glucosamine dimer (Jollès & Muzzarelli, 1999). This is an extraordinary performance because the substrate chitin in most instances is an insoluble and crystalline solid that assumes a large variety of forms depending on the role of the living tissue, an example being the one in Fig. 2.

These enzymes are highly preserved through species and kingdoms (Boot et al., 2001; Gianfrancesco & Musumeci, 2004) and exert various functions in many living organisms. For example, in yeast and fungi they take part in morphogenesis (Cohen-Kupiec and Chet, 1998), and in plants they have an important role against pathogens; they are also implicated in the processes of development and growth, i.e. during insect ecdysis (Kaspresewska, 2003).

Until 1994 the human organism was not thought to produce chitinases, because of the absence of endogenous chitin in human tissues, which means no need for enzymatic turnover and remodeling of chitin structures. Therefore nutritionists have indicated chitin as a non-digestible polymer in the human organism (Bukkens, 2005; DeFoliart, 1992) although chitin-containing foods such as mushroom, crustaceans and insects are largely consumed, especially in tropical countries. This assertion, however, has been recently contradicted by reports describing up to nine mammalian

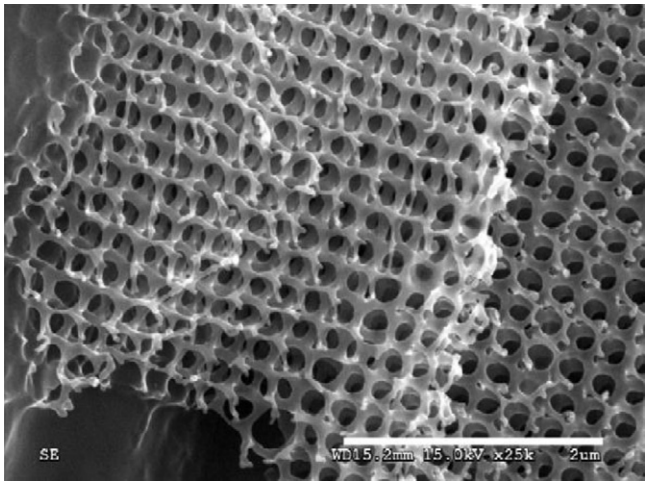

Fig. 2. Scanning electron micrographs of different regions within a single wingscale of *Callophrys rubi*. The scale of the structure is commensurate with the wavelength of visible light, with an edge of the conventional cubic unit cell of the parallel-Gyroid of approximately 310 nm. In the butterfly, the templating is achieved by the lipid–protein membranes within the smooth endoplasmic reticulum (while it remains in the chrysalis), that likely form membranes folded according to the form of the Gyroid. The subsequent formation of the chiral rigid chitin framework is driven by the gradual polymerization of the chitin precursors; the material is optically active, that is, it rotates the polarisation of incoming light.
Source: From Schroder-Turk et al. (2011). Elsevier Science, San Diego.

chitinases and chitinase-like genes that are present in man and other mammals, all belonging to the glycosyl hydrolase 18 family (Bierbaum et al., 2005; Boot et al., 2001; Fusetti et al., 2002; Lee, Waalen, Crain, Smargon, & Beutler, 2007; Seibold et al., 2009).

The first human chitinase was detected by Hollak, van Weely, van Oers, and Aerts (1994) who found high chitinolytic activity in serum of patients affected by Gaucher disease. Because of its capacity to hydrolyze chitotriose, it was called chitotriosidase. This protein is specifically expressed by activated macrophages and has an optimum pH of 6. Subsequent studies proved and quantified the chitinolytic activity of chitotriosidase on colloidal chitin and on chitin of cell wall of *Candida albicans*. In the early investigations, a catalytic deficiency of chitotriosidase was described and named Allele H. The complete sequencing of chitotriosidase gene was also described (Barone, Simpore, Malaguarnera, Pignatelli, & Musumeci, 2003; Boot, Renkema, Strijland, van Zonneveld, & Aerts, 1995; Boot et al., 1998; Renkema, Boot, Muijsers, Donker-Koopman, & Aerts, 1995).

In search of other chitinases that could compensate chitotriosidase deficiency, Bleau, Massicotte, Merlen, and Boisvert (1999) and Chang et al. (2001) identified and characterized 4 mammalian chitinase-like enzymes that, because of mutations in their highly conserved catalytic sites, maintain chitin-binding capability but not the catalytic activity. Boot et al. (2001) discovered another chitinase showing high catalytic activity at pH 2 and named it acidic mammalian chitinase (AMCase); it is expressed in gastric epithelia, in lung macrophages and in the pulmonary epithelia during asthmatic inflammation (Zhu et al., 2004).

4.1. Role of human chitinases

Since the earliest discoveries, scientists hypothesized that mammalian chitinases take part in the innate immune response to parasites, with the function of binding and digesting chitin structures such as cell walls, egg shells and cysts produced by many organisms including fungi, protozoans and nematodes (Fusetti et al., 2002; Malaguarnera et al., 2003 among others).

Barone et al. (2003) detected higher chitotriosidase activity in plasma of African children affected by acute malaria with respect to reference values obtained in age-matched African children. Moreover, chitotriosidase activity was found to be higher in African children than in Caucasian children matched for age. Musumeci et al. (2005) obtained similar results by comparing chitotriosidase concentrations in colostrum in African and Caucasian populations.

Furthermore, AMCase is adapted to different tissue-specific functions and Boot et al. (2005) discussed the possibility that this chitinase might have a dual function in immune defense and in food digestion. *Nature* published the study of Reese et al. (2007), who, using a murine model of infection with the nematode *Nippostrongylus brasiliensis*, induced alternative macrophages activation and recruitment of Th2 cells, eosinophils and basophils in different tissues. Moreover, in the same experiment they demonstrated that when chitin was pre-treated with a recombinant AMCase, or in transgenic mice over-expressing active AMCase, the inflammatory responsiveness to chitin was reduced. Further data are in Lee et al. (2011).

4.2. Chitin digestion

The state of the art of human activity of collecting, cultivating and consuming chitin-bearing food such as crustaceans, insects and mushrooms has been the object of several comprehensive ecological and ethnobiological investigations (DeFoliart, 1992; Paoletti & Bukkens, 1997; Paoletti, 2005). The importance of entomophagy has been also remarked by FAO, which stressed the great diversity and accessibility of this class of alimentary resources, being a source of inorganics, proteins, vitamins and poly-unsaturated fatty acids (Bukkens, 2005; Fontaneto et al., 2011).

More than 2000 species of arthropods are mostly used as food by many populations worldwide, as extensively described and discussed by Posey (1984) for Amazonia, Ramos-Elorduy et al. (1997) for Central America, Malaisse (1997) for central Africa, Zhi-Yi (2005) for China, and Mitsihashi (2005) for Japan. For example, within the Tukanoan population living in the Colombian Vaupes Amazon rain forest, arthropods represent a fundamental source of food, providing 12–24% of total protein intake (Paoletti, Buscardo, & Dufour, 2000).

Chitin represents 5–20% of total dry-weight in adult crustaceans and insects. For this reason, Paoletti, Norberto, Damini, and Musumeci (2007) and Paoletti, Norberto, Cozzarini, and Musumeci (2009) suggested that a chitin-rich diet may influence chitinase variability in human populations. Consequently they proposed that the reduction of chitin intake in western diet may have reduced selective pressure on the chitinase genes, probably resulting in the loss of catalytic efficiency.

Chitin digestion seems to depend on AMCase, the main chitinase synthesized by gastric tissues and, therefore, adapted to extremely acidic pH conditions (Boot et al., 2005). Human chitinases hydrolyze chitin to N-acetyl-glucosamine that can be easily metabolized by N-acetylglucosamine kinase, an enzyme present in all human tissues including gastric mucosa and intestinal epithelium, as described by Gindzienski, Głowacka, and Zwierz (1974). Also the commensal human bacteria *Clostridium paraputrificum* was isolated in human faeces and was found to have chitinolytic activity (Simunek, Kopecny, Hodrova, & Bartonova, 2002).

Genetic and histochemical analyses of mammalian chitinase expression, conducted by Suzuki et al. (2002) and Goto, Fujimoto, Nio, Iwanaga, and Kawasaki (2003), respectively, reported the presence of AMCase in the human and murine gastric mucosae. Shortly afterwards, Boot et al. (2005) confirmed elevated expression of mammalian chitinases in the glandular portion of the stomach, in chief cells at the bottom of the gastric glands. Interestingly, pyloric glands in the antrum of the stomach do not express chitinase mRNA: likewise AMCase was not detected in the intestine. On the other hand, chitotriosidase is synthesized also in duodenum and other parts of the small intestine, in specialized secretory cells called Paneth cells, probably with a defensive role against parasites. Boot et al. (2005) discussed the possibility that gastrointestinal chitinases might have a dual function, in immune defense and in food digestion, thus supporting the observations by Dobson, Prager, and Wilson (1984).

AMCase-dependent chitinolytic activity was finally proved in humans and quantified by Paoletti et al. (2007) who analyzed the gastric juice of 25 patients submitted to gastroscopy. The AMCase activity was tested *in vitro* on two artificial substrates, 4-methylumbelliferyl-β-D-N,N′-diacetylchitobiose and/or fluorescein isothiocyanate chitin. Out of the 25 patients as many as 20 showed a detectable activity, ranging from 0.21 to 36.27 nmol/ml/h and 8881–1,254,782 fluorescence emission units, with the highest chitinolytic activity observed at pH 2, as expected. In subsequent experiments realized on human gastric juices, Cozzarini et al. (2009) associated increased expression of chitotriosidase, but not of AMCase, to *Helicobacter pylori* and other gastric diseases.

Subsequent studies were performed to test *in vitro* the ability of human gastric juices to hydrolyze natural chitin model obtained from the wings of *Calliphora vomitoria*. Fly wings were found to be only moderately digested by gastric juices even though AMCase levels were high, thus no significant results were obtained from these unpublished studies: it might be supposed however that in the wings the chiral rigid chitin framework shown in Fig. 2 can withstand the enzymatic attack better than other chitinous substrates.

The most complete study on AMCase genetic variability, stemming from an inter-institutional investigation on a large cohort of Latin-American and Afro-American asthmatics and their relatives, has been published by Seibold et al. (2009). Interestingly, they identified a particular haplotype codifying for an isoform that revealed, by biochemical characterization, an enhanced efficiency and adaptability at three different pH values, those characteristic of stomach, lung and lysosomes, thus confirming the hypothesis of a tissue-specific adaptation of AMCase. The consistent increase in enzymatic activity at pH 2.2 (4-fold higher) and 7.0 (10-fold higher), typical of stomach and lung, respectively, is extremely relevant because these organs are intensively exposed to chitin through ingestion and inhalation, respectively. This functional variant has a relevant frequency in the whole sample, specifically: 24.1% in AfroAmerindians, 12.5% in Mexicans and 8.4% in Puerto Ricans.

Thus the expression of AMCase in the human gastrointestinal tract can represent a reminiscence of a past nutritional function (Boot et al., 2005), and, therefore, AMCase expression in western population now can be a redundant phenomenon due to the reduction of chitin in modern diet (Paoletti et al., 2007). To test this hypothesis, the University of Padua, in collaboration with the University of Trujillo (Peru), is currently performing a study on chitinase genetic variability in Amerindian populations living in the Amazonian basin, where dependence on chitinous food may still represent a strong selective factor for chitinase functionality. Two intriguing ethnobiological findings that expect to be assessed and confirmed, emerged recently from the first fieldwork in those regions: the traditional use, as an anti-asthmatic treatment, of a syrup produced from suri palm-worm (*Rhynchophorus palmarum*, Coleoptera) and the popular belief that states: people eating insects do not suffer from asthma.

4.3. Deficiency of chitotriosidase

A relevant question has been whether chitinases activity in humans is just an evolutionary remnant or they still have an

Table 1
Frequencies of Allele H: the 24 bp duplication causing catalytic deficiency in chitinase in different populations. This mutation could have a positive correlation with malaria endemicity but not with filarial nematodes. References are in parentheses.

Population	Subjects	% wt/wt	% wt/mut	% mut/mut	Wt allele frequency	Mut allele frequency
Ashkenazi Jews (1)	68	60	34	6	0.77	0.23
Basque Country (2)	60	76.7	23.3	0	0.883	0.17
Benin (3)	100	100	0	0	1.00	0.00
Burkina Faso (3)	100	98	2	0	0.98	0.02
Chin. Han-Taiwan (4)	82	15	55	30	0.42	0.58
Corsica (2)	194	74.8	24.2	1.0	0.869	0.131
France, continental (2)	128	59.4	31.3	9.3	0.75	0.25
Holland (1)	171	59	35	6	0.77	0.23
India, Southern (5)	67	31	58	10	0.60	0.40
Italy, continental (2)	99	63.7	34.3	2.0	0.808	0.192
Morocco (2)	90	75.6	20.0	0.0	0.81	0.105
Papua New Guinea (6)	906	77	22	1	0.88	0.12
Portugal (7)	295	60	37	3	0.78	0.22
Sardinia (3)	107	64	32	4	0.78	0.21
Sicily (3)	100	51	43	6	0.73	0.27
Spain (2)	103	61.2	32.0	6.8	0.772	0.228
Turkey (2)	95	66.3	29.5	4.2	0.811	0.189

References: (1) Boot et al. (1998); (2) Piras et al. (2007); (3) Malaguarnera et al. (2003); (4) Chien et al. (2005); (5) Choi et al. (2001); (6) Hise et al. (2003).

adaptive function in man. Gianfrancesco and Musumeci (2004) utilized a phylogenetic approach by comparing the chitotriosidase gene sequences of primates, rodents and man. Results revealed that the Allele H mutation is not present in primates, suggesting that this polymorphism may be a post-speciation event. Indeed, many authors have investigated a possible adaptive function of chitotriosidase enzymatic deficiency (Table 1).

The first data were from studies in tropical malarial regions, where 58 and 55% of mutant heterozygosis were found in South Indians and in Chinese population, respectively, by Choi et al. (2001) and Chien, Chen, and Hwu (2005). The high occurrence of the catalytic deficiency for chitotriosidase in the South Indian population was reported to be associated with a susceptibility to infection by *Wuchereria bancrofti*, a filarial parasite endemic in the study area, whose microfilarial sheath contains chitin. However, subsequent studies by Hise et al. (2003) showed no correlation between filarial distribution in a population from Papua New Guinea and Allele H frequency. Nor any difference was found between subjects living in endemic areas and people living in salubrious areas, where any filaria-driven selective pressure on chitotriosidase genetic variability should be absent. Similarly, Hall et al. (2007) found no evidence for a protective effect of chitotriosidase against hookworm infection in Papua New Guinea and looking at 279 unrelated individuals, found genotype frequencies which did not differ from those expected under the Hardy–Weinberg equilibrium. This suggests that there is no selective advantage of possessing the wildtype gene in this population.

Interesting data came from Malaguarnera et al. (2003) who investigated the distribution of the Allele H mutation in Mediterranean and Sub-Saharan populations and found an high frequency of chitotriosidase catalytic deficiency in Mediterranean regions (44.54% of heterozygote mutants in Sicily and 32.71% in Sardinia), where malaria has been eradicated very recently. Contrarily, this mutation is almost absent in Sub-Saharan populations: 0 and 2%, in Benin and Burkina Faso, where malaria is endemic. The most significant observation was the great difference in the heterozygote mutant allele frequency between African (0–2%) and in Asian populations (55–58%).

Piras et al. (2007) carried out new studies on European and Mediterranean populations, and concluded that heterozygosis frequency in these regions could not represent an adaptation to the very recent *Plasmodium* eradication. Considering that a higher Allele H frequency is present in Asia, they finally proposed an Asian origin of the mutation and its subsequent migration to Europe and, perhaps, to the Americas.

In fact, more recently, Musumeci and Paoletti (2009) suggested the mechanism by which chitinases expressed in human blood help *Plasmodium* complete its life-cycle in the mosquito. When the vector bites man, the ingested infected blood remains trapped in the peritrophic membrane of the insect stomach that is mainly made of chitin. As a consequence, *Plasmodium* produces chitinases (Huber, Cabib, & Miller, 1991) to get out of the stomach and migrate to the salivary glands of mosquito and, therefore, human chitotriosidase present in blood may facilitate this process. In conclusion, people expressing unfunctional chitotriosidase may be advantaged in escaping malaria (Musumeci, Giansanti, & Musumeci, 2009).

5. Crop protection and food preservation

The tremendous increase in crop yields associated with the green revolution has been made possible in part by the utilization of chemicals for pest control. However, concerns over the impact of pesticides on human health and the environment has led to new pesticide registration procedures, that have prohibited a number of synthetic pesticides, including the chitin synthesis inhibitors that posed a real threat to the environment (Muzzarelli, 1986). Among new natural pesticides, chitosan is not only an antimicrobial agent, but also an effective elicitor of plant systemic acquired resistance to pathogens, including phytoalexin synthesis. Presumably, plants have evolved a receptor/signal system to sense fungal pathogens in order to initiate biochemical defenses. Preparations of chitin/chitosan from either crustacean exoskeletons or dried *Saccharomyces cerevisiae* hydrolysate are commercially available (Dayan, Cantrell, & Duke, 2009).

The extraordinary interest of chitosan in agriculture, horticulture. environmental science, industry, and microbiology is attested by over 17,000 papers on this subject, according to Scopus database in early 2011. In fact, chitosan has a direct effect on the morphology of the chitosan-treated microorganism reflecting its fungistatic or fungicidal potential. In addition to its direct antimicrobial activity, other studies indicate that chitosan induces a series of plant defense reactions intended to increase the production of glucanohydrolases, phenolic compounds and synthesis of specific phytoalexins with antifungal activity; it further reduces macerating enzymes such as polygalacturonases and pectin metil esterase. In addition, chitosan induces structural barriers for example by inducing the synthesis of lignin-like material. Due to its ability to form a semipermeable coating, chitosan extends the shelf life of treated fruit and vegetables by minimizing the rate of respiration and reducing water loss. As a nontoxic biodegradable material,

as well as an elicitor, chitosan has the potential to become a new class of plant protectant, assisting towards the goal of sustainable agriculture (Bautista-Banos et al., 2006).

5.1. Applications in crop protection

Attack by various fungi to certain seeds may result in decreased germination (Donald & Mirocha, 1977; Sharma, Fisher, & Webster, 1977). Chitosan-based coatings exerting antifungal activity help preserve the quality of the stored seeds. The treatment consists in seed immersion in a chitosan suspension (up to 4%) followed by drying. Chitosan molecular weight, presence of a surfactant, pH value, and thickness (number of coating layers) are parameters acting on the seed germination, as well as on the fungal activity and vegetative growth. Generally, chitosan treatments reduce the number of type of fungi and promote plant growth. Mazaro et al. (2009) treated tomato and beet seeds by immersion in a chitosan suspension. Seeds were then sowed in trays with a substrate infected with *Rhizoctonia* spp. and maintained in greenhouse for 14 days. Chitosan induced seedling resistance against *Rhizoctonia* and reduced damping-off. It increased the phenylalanine ammonialyase activity and interfered with the total proteins and total and reduced sugars rates in the leaves. The partially hydrolyzed chitosans lead to better results in terms of microbial contamination and germination.

Zeng and Shi (2009) obtained another safer, cheaper and more environmentally friendly seed coating agent using chitosan combined with plant growth regulators and other additives. Such a novel seed coating agent significantly enhanced sprout growth in regard of the traditional agents. It stimulated the seedling growth of rice, advanced the growth of root, improved root activity and increased the crop yield in the germination test and field trial. Compared with the traditional rice seed coating agent, the crop yield of coated seeds increased by 5%, and at 25% lower cost. The fungal inhibition test of this seed coating agent showed that it has an evident fungal inhibitory effect and a higher safety index during usage and disposal.

As noted by El Hadrami, Adam, El Hadrami, and Daayf (2010), since chitin and chitosan fragments are known to have eliciting activities leading to a variety of defensive responses in host plants against microbial infections (e.g. accumulations of phytoalexins, pathogen-related proteins and proteinase inhibitors, lignin synthesis and callose formation) the use of chitin and chitosan derivatives has increased, because they allow to increase host plant defenses in agricultural systems and to reduce the negative impact of diseases on yield and quality of crops.

5.2. Applications in food technology

The main applications of chitosan in the food area are in regard of its flocculating and antibacterial activities (Vargas and González-Martínez (2010)). In fact, chitosan and its derivatives are characterized by great versatility in flocculation and adsorption capacity mainly because of their cationicity and macromolecular structure. Chitosan is used as a flocculant in various domains and particularly in food technology (Zheng, Wu, & Xu, 2009), for example for fruit juice clarification in eliminating pectin and carbohydrates, as an aid for the separation of suspended particles from beverages (Chatterjee, Chatterjee, Chatterjee, & Guha, 2004). Such juice clarifications have been successfully carried out for apple, carrot, grape, lemon, orange and pineapple juices (Rungsardthong et al., 2006).

Moreover since chitosan has a good affinity for polyphenolic compounds such as catechins, proanthocyanidins, cinnamic acid, and their derivatives, it can be used to remove polyphenolic components which allows to reduce juice color (Oszmianski & Wojdylo, 2007; Spagna et al., 1996) or to change the initial color of a solution. With the aid of xanthan, the removal of haze precursors becomes feasible and has some advantages in terms of time saving in comparison to conventional fining methods (Fang, Zhang, Tao, Sun, & Sun, 2006). Chitosan treatment, can also clarify leave infusions (e.g. green tea), thus avoiding the formation of haze during low-temperature storage. In order to understand better the coagulation process, some studies have also been performed to analyze the interaction between chitosan and proteins (Boeris, Micheletto, Lionzo, da Silveira, & Pico, 2011; Hiorth, Skoien, & Sande, 2010; Marudova, McDougall, & Ring, 2004).

The antibacterial activity of chitosans is well known, since the publication of a study involving 298 strains (Muzzarelli et al., 1990). In fact the antibacterial activity of chitosans depends on the chitosan characteristics and on the bacterial strains (Fig. 3). Water-soluble chitosans can inhibit bacterial growth very sharply. However, this effect is less remarkable than for acid-soluble chitosans: minimum inhibitory concentration values of acid-soluble chitosans were much lower (0.03–0.1%) than those of water-soluble chitosans (0.05–0.8%) (Jung et al., 2010). The acid-soluble chitosan with deacetylation degree 0.99 and low viscosity is most effective in inhibiting bacterial growth (Friedman & Juneja, 2010). Important studies have also been done by other authors such as Rhoades and Roller (2000), Sagoo, Board, and Roller (2002a), Sagoo, Board, and Roller (2002b), whereas other reviews are giving interesting informations on this aspect (Goy, De Britto, & Assis, 2009; Nejati Hafdani & Sadeghinia, 2011; No, Park, Lee, & Meyers, 2002) (Figs. 4 and 5).

The antibacterial activity is exerted by chitosan in other physical forms than solutions, such as suspensions, powders, non-vowens, microspheres, films and coatings. Since chitosans are active against foodborne pathogens, spoilage bacteria, pathogenic viruses and fungi, they are useful for ameliorating foodborne illness. They are used for antimicrobial activities in fruit (citrus, grape, tomato), fruit juices (orange, etc.), eggs, dairy (milk), cereal, meat products (bacon, beef, pork, poultry, etc.), and seafood products. Note also that a mixture of chitosan and mint allowed to get a new preservative for meat combining the antioxidant and antimicrobial properties of these compounds (Kanatt, Chander, & Sharma, 2008). The antioxidative and metal chelating activities or effects of chemically modified chitosans and of nanochitosans should be also noted. The relevant literature suggests that the low-MW chitosans at pH < 6.0 are in optimal conditions for exerting desirable antimicrobial, antioxidative and preservative actions in foods. Chitosans have also antiviral activities and therapeutic properties and textiles or fabrics can be treated with them in order to confer antimicrobial properties for food preservation (Friedman and Juneja, 2010). The influence of the molecular weight of chitosans on the efficacy against several bacteria (enterobacter, bacillus, bifidobacterium) and also *Candida* has been studied by Gerasimenko, Avdienko, Bannikova, Zueva, and Varlamov (2004).

5.3. Applications in food packaging

Chitosan applications in food packaging are mainly justified by their antimicrobial and antifungal activities against pathogenic and spoilage microbes. Such chitosan-based active films allow to extend food preservation and to reduce the use of chemical preservatives (Aider, 2010). Their manufacture includes first an extraction of chitosan, then incorporation into plastic or polymeric matrices. This allows to obtain films with antimicrobial properties which qualify for active packaging. Such films can be laminated with pectin films (Lehr, Bouwstra, Schacht, & Jungiger, 1992), optionally in the presence of lactic acid (Hoagland & Parris, 1996).

The facile preparation of the films included dissolution of chitosan in acetic acid with 5% surfactant (Tween 20 and Span 80): the solutions were poured into Petri dishes and allowed to dry at 60 °C and 20% relative humidity in a climatic chamber. The degree of

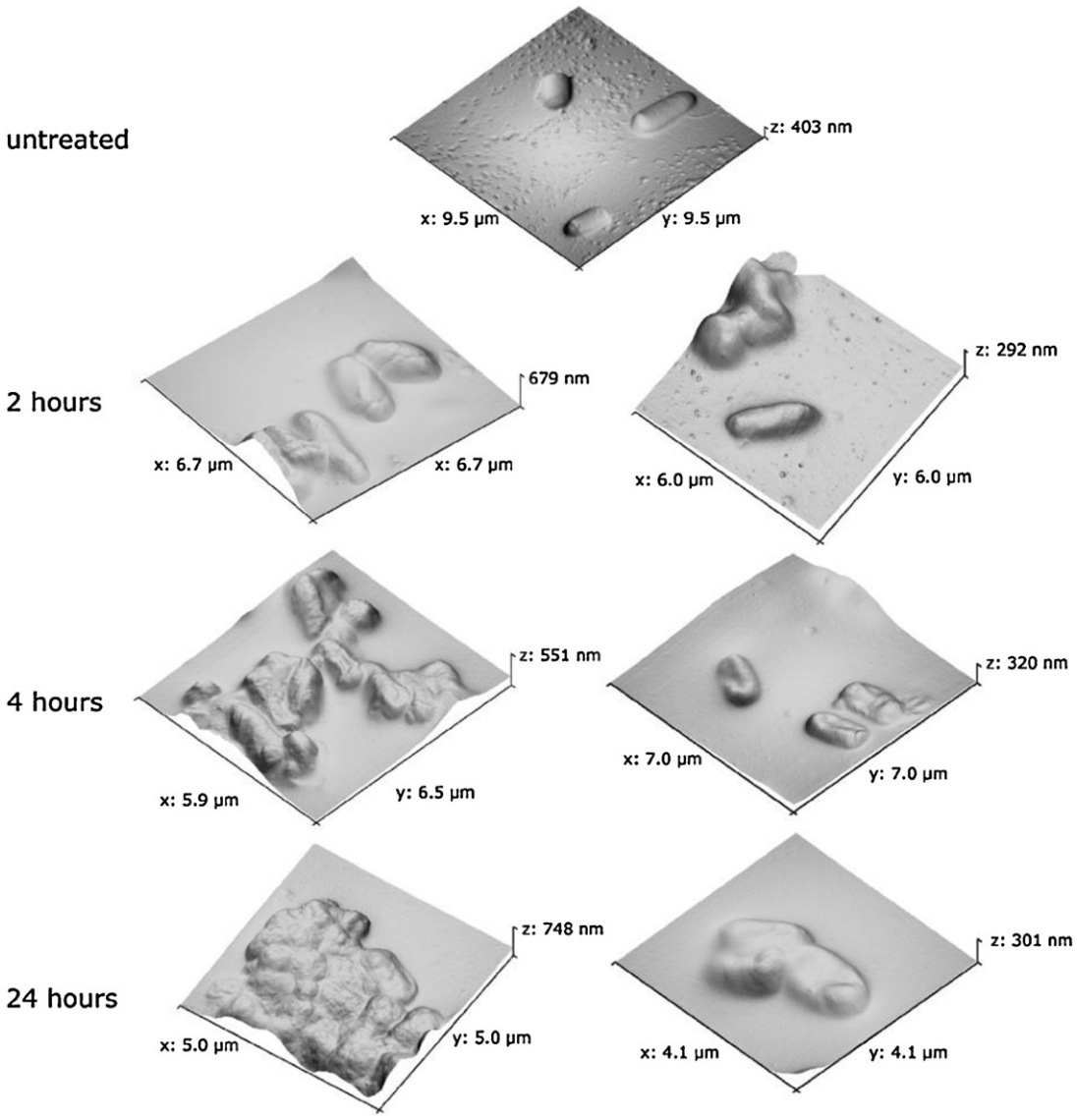

Fig. 3. *Escherichia coli* treated with chitosan oligomers (left), and with chitosan (right), for 2, 4 and 24 h. Upper frame, control. Progressive decay is visible, particularly with oligomers that penetrate *E. coli* and probably inhibit the DNA transcription. Similar results (not shown) for *Staphylococcus aureus*.
Source: From Eaton et al. (2008). Elsevier Science B.V.

deacetylation of chitosans is one of the parameters which allow to control some of the physical characteristics of the bioactive films. For example, if the degree of deacetylation is low (e.g. 0.60) the tensile strength and elongation can be high. The introduction of glycerol, a plasticizer, provides more elasticity and more permeability accompanied by lower mechanical resistance. The presence of Tween 20 may improve the wettability and affect the mechanical, optical and barrier properties of such films. The use of chitosan with high degree of deacetylation and the use of glycerol as a plasticizer resulted in higher crystallinity of the films. The chitosan antifungal action was evaluated against *Alternaria alternata*, *Aspergillus niger*, and *Rhyzopus oryzae* (Ziani, Fernandez-Pan, Royo, & Mate, 2009).

Some forms of chitosan, mostly chitosan salts, are known to be very water sensitive which decreases their interest in food packaging or food coating applications, particularly for high moisture food. In some cases it is also useful to add water-resistant proteins such as gliadin isolated from wheat gluten. Incorporation of chitosan into an insoluble biopolymer matrix was revealed as a method to generate novel chitosan-based antimicrobial materials with applicability in food packaging. In this case both plain chitosan and its blends with such proteins presented significant antimicrobial activity, which allow to increase the possibility of chitosan composite formulations. The protein–chitosan blends can show also good transparency and film-forming properties and sometimes better water resistance than pure chitosan. Dissolution of the biocide glucosamine units of the chitosan water soluble fractions can also increase with the amount of chitosan present in the blend (Fernandez-Saiz, Lagaron, & Ocio, 2009). These authors showed also that gliadin-chitosan acetate films have good antimicrobial properties, and confer extended shelf life to packaged food.

It should be also noted that the antimicrobial properties and strength depend upon the casting temperature of the films. For example, Fernandez-Saiz et al. (2009) found that this capacity highly decreased if temperature is high (120 °C). These films should be stored at low temperature under dry conditions, which are crucial: Mayachiew, Devahastin, Mackey, and Niranjan (2010) found that ambient drying, low-temperature hot air drying led to films with higher antimicrobial activity and higher degree of swelling due to lower intermolecular interaction. Moderate electric fields can have an influence on the structure of chitosan films, cristallinity

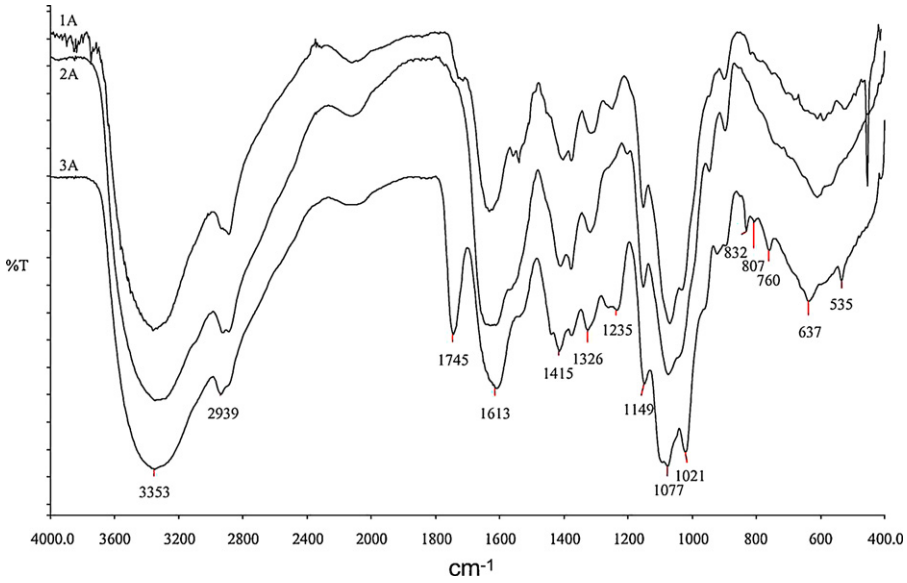

Fig. 4. FTIR spectra of the complexes of chitosan with: 1A, xanthan; 2A, hyaluronan; 3A, pectin. The remarkable band at 1745 cm^{-1} is assigned to the methyl ester groups typical of pectin.
Source: From Muzzarelli et al. (2004), Elsevier Science Publ., Oxford.

index and morphology. For example Souza et al. (2010) found that after such applications the structure are more regular.

According to Elsabee, Abdou, Nagy, and Eweis (2008) polypropylene films treated with acidic solutions of chitosan and so having antifungal and antibacterial properties can adhere to carboxymethyl films leading to carboxymethyl chitosan and chitin films. The antifungal and antibacterial properties of these derivatives were found to be even superior to the chitosan itself. Chitosan and pectin can interact at pH 5.6, however the gel behavior depends upon the degree of esterification of the pectin. In fact the polyelectrolyte complex formation requires ionized carboxylate groups of pectin and protonated amino groups of chitosan (Lehr et al., 1992). Chitosan can also form complex compounds with pectin allowing to build up stable multilayered structures on these film surfaces to produce a much better antimicrobial films which can be used to fabricate very good packaging materials for post-harvest crop protection; for a review, see Zhang, Li, and Liu (2011).

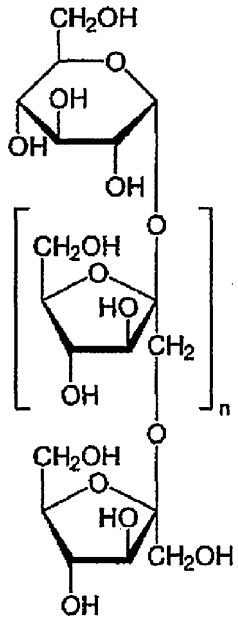

Fig. 5. Structure of inulin: the number of repeating units, n, is in the range 0–60 in chicory. Inulin is both a dietary fibre and a prebiotic.

6. Pectins and inulin

6.1. Pectins

Braconnot described for the first time the occurrence of an acid universally present in all vegetables. He detected this acid in carrots, apples, pears, grains and onions, and noted its gelling characteristics. Hence he coined the term pectic acid ("acide pectique"), from the Greek word πήκτίζ for coagulum. The title of this article is of impressive clarity: "Research on a novel acid universally present in all plants" (Braconnot, 1825a). He described its properties in another publication where he emphasized its gelling characteristics, and suggested that it might be a good antidote for heavy metal poisoning because of the insolubility of the compounds formed as an anticipation of the chelating capacity of the polysaccharides systematically described nearly 150 years later (Braconnot, 1825b; Muzzarelli, 1973).

It is now known that pectin is a major component of the plant cell wall and the most complex macromolecule in nature (Voragen, Coenen, Verhoef, & Schols, 2009). It is surprising to see that so many years after its discovery, the structure of pectins still is not fully unravelled. This is partly due to the fact that it depends on plant species, cellular location, and plant development stage, but also on its very complex molecular architecture.

6.2. The chemical complexity of pectins

The following main monosaccharides can be found in pectins: D-galacturonic acid, D-xylose, L-rhamnose, L-arabinose and D-galactose. They constitute the repeating units of the different structural elements. These can be described as homogalacturonan (HG), xylogalacturonan, rhamnoglacturonan type I and type II (RG I and II), arabinan and arabinogalactan. Whereas the structure of these elements is fairly well known, the precise nature of the linkages between them is still uncertain.

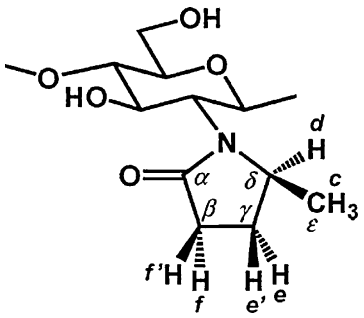

Fig. 6. The 5-methylpyrrolidinone functional group of chitosan, spontaneously formed upon reaction of chitosan with levulinic acid, with substitution degree up to 41%. The annotations refer to NMR assignments. Levulinic acid is industrially obtained from fructose.

The α-(1–4)-linked D-galacturonic acid forms the linear backbone of HG, while substituted galacturonans are characterized by the presence of saccharide residues (such as D-xylose in the case of xylogalacturonan) branching from the backbone of D-galacturonic acid residues. RG-I contains a backbone of the repeating disaccharide: 4)-α-D-galacturonic acid-(1,2)-α-L-rhamnose-(1. From many of the rhamnose residues, side chains of various neutral sugars branch off. The neutral sugars are mainly D-galactose, L-arabinose and D-xylose, the types and proportions of neutral sugars varying with the origin of pectin. RG-II, which is a less frequent complex, is a highly branched polysaccharide with a backbone of D-galacturonic acid units. It contains four different side chains with unusual sugar moieties, such as aceric acid and apiose.

In arabinan, the L-arabinose units are linked through α-(1,5) bonds in the backbone and substituted with side chains of the same sugar. Arabinogalactan comes in two forms. AGI consist of a β-(1,4) linked D-galactose backbone branched with L-arabinose side chains at the O-3 of the galactosyl residues, whereas AGII is built up from a β-(1,3) linked D-galactose backbone with short side chains consisting of a disaccharide L-arabinose-β-(1,6)-D-galactose.

Currently two models describe the pectin structure: the smooth and hairy region model and the RG backbone model. The difference is the way the HG and RG elements are linked together. In the first model hairy regions are formed of RG I with neutral sugar side chains, interspersed with smooth regions of HG. In the second model RGI has a side chain of HG. Isolated pectin has a molecular weight of typically 60–130,000 g/mol, varying with origin and extraction conditions. For reviews and more detailed discussions, see Porta, Mariniello, Di Pierro, Sorrentino, and Giosafatto (2011), Schols, Coenen, and Voragen (2009) and Voragen et al. (2009).

6.3. Usefulness of pectins

Nowadays, this polysaccharide is of great importance for the pharmaceutical and food industry as one of the main natural gelling additives. Low methoxyl pectins are of particular interest in drug delivery as they can form gels with calcium ions which have potential applications especially in nasal formulations. Current and potential drug delivery systems are based on the unique properties of both chitosans and pectins. Remarkably, chitosan and pectin can form polyelectrolyte complexes (Grabnar & Kristl, 2010; Hoagland & Parris, 1996; Muzzarelli, 1973, 1990). In their review article, Morris, Kok, Harding, and Adams (2010) discuss the physico-chemical properties of chitosans and pectins and how these translate to current and potential drug delivery systems.

The FTIR (KBr) spectrum for the pectin–chitosan complex as prepared by Muzzarelli, Stanic, Gobbi, Tosi, and Muzzarelli (2004) included the very sharp 1745 cm^{-1} band assigned to the methyl ester groups of pectin (Fig. 6). The XRD spectra showed that the combination of chitosan with pectin led to a nearly amorphous product. In spite of the chemical differences (alcohol groups in guaran, carboxyl groups in xanthan, partially esterified carboxyl groups in pectin) these three polysaccharides, once combined with chitosan in the microspheres are able to bring chitosan into solution. This is particularly interesting if one considers that these three polysaccharides of which the solubility in water is well known, have important applications in the food and pharmaceutical industries, where they are amply used as food additives, excipients and emulsifiers for the preparation of ice-creams, soups, puddings, cakes, tooth-pastes and spray-dried fixed flavours.

The most important industrial sources for pectins are apple pomace or citrus peel, but also other pectins are produced for specific purposes, e.g. pectins from sugar beet show interesting emulsifying properties. In nature, ca. 80% of carboxyl groups of galacturonic acid are in the methyl ester form. This proportion is decreased during pectin extraction. The ratio of esterified to non-esterified galacturonic acid determines the behavior of pectin in food applications. This is why pectins are classified as high- or low-ester pectins (or methoxyl pectins) with more or less than half of all the galacturonic acid esterified. It also shows that by changing the processing conditions the functional properties of pectins can be modified, and adapted to specific application needs. Next to these functional properties it is also becoming evident that pectins exhibit beneficial physiological effects. These include a lowering of serum lipids (cholesterol) relevant for the prevention of cardiovascular diseases, immune regulatory effects in the intestine and lowering the postprandial glucose response. Since they are also dietary fibres, being non-digestible dietary carbohydrates, they also show effects on bowel movement.

From this description it is clear that the original observation by Braconnot (1825b) on the novel acid in plants has delivered an immense wealth of interesting research data on the structure of this component, on the applications and on the health benefits. Clearly, something that this attentive scientist could not envisage when he extracted pectins for the first time.

Table 2
Inulin content of various crops.

	Inulin content, % fresh weight	Degree of polymerization
Onion (*Allium cepa*)	1–8	2–12
Leek (*Allium ampeloprasum*)	3–10	n.a.
Garlic (*Allium sativum*)	9–16	2–50
Wheat (*Triticum aestivum*)	1–4	2–8
Barley (*Hordeum vulgare*)	0.5–1.5	n.a.
Banana (*Musa cavendishii*)	0.3–0.7	2.5
Globe artichoke (*Cynara scolymus*)	2–3	2–250
Jerusalem artichoke (*Helianthus tuberosus*)	16–20	2–50
Chicory (*Cichorium intybus*)	15–20	2–60

Based on Van Loo, Coussement, De Leenheer, Hoebregs, and Smits (1995) and unpublished data from Sensus.

6.4. Inulin from Helianthus tuberosus

The term inulin was adopted in the early 19th century when Rose in 1804 isolated a substance from *Inula helenium* (elecampane) that was called inulin in 1811 by Thomas (Suzuki, 1993). Its presence in *Helianthus tuberosus* was reported by Braconnot (1824). He found that it occurred at the concentration of 16% in the tubers of this crop and that it closely resembled the substance described as dahline by Payot (1824), who confirmed that the two samples had the same identity. We know now that inulin is present in the tubers of these and many other crops as a reserve carbohydrate (Table 2).

Helianthus tuberosus, also known as topinambour or Jerusalem artichoke, originates from North America and was introduced in Europe in the early 17th century. It seems that a famine in New

Canada in 1612 was the basis for its reputation as the Canadians survived on these tubers (Barwald, 2008). Shortly thereafter Samuel de Champlain, the founder of Quebec City, sent the first samples to France. There it was cultivated as a vegetable, but later it was also grown in the Mediterranean area as a staple crop before it was superseded by the potato in the middle of the 18th century. It was and still is also used as feed material for animals. Jerusalem artichoke was consumed in Europe in the 16th century and later (Clayton & Rowbotham, 2008) and in the early 20th century inulin was already known as a carbohydrate suitable for diabetics, as it did not give rise to a glycemic response. Also in the early post-World War II years *H. tuberosus* was grown in France and Germany as a staple crop due to the scarcity of potato at that time. In these countries this crop continues to be grown as a vegetable.

Its staple characteristics originate from the high inulin content as was already found by Braconnot (1824). The structure of inulin as a β-(2,1) linked fructan with or without a terminal glucose residue was established by Haworth and collaborators in the early 1930s (Suzuki, 1993). Later studies showed that branches may occur through β-(2,6) linkages (De Leenheer & Hoebregs, 1994) and that the structure depends on the plant species.

The industrial production of inulin from chicory roots is concentrated in the Netherlands and Belgium. *H. tuberosus* turns out to be less suitable as raw material as it produces a substantial amount of structural stem material with a concomitantly lower inulin yield per ha (Meijer, Mathijssen, & Borm, 1993). Also the yield is less constant and the quality of the inulin is somewhat less (it exhibits a lower DP) than with chicory. Finally the fact that even a small piece of tuber when left in the ground will grow, makes this crop difficult to handle in crop rotation schemes. Nevertheless Jerusalem artichoke is used in some countries for small scale inulin production, such as in China and Hungary. Furthermore it continues to be grown as feed material and it is still being considered as a crop to produce biomass (both in the form of tubers and of stems), e.g. for bioethanol production (e.g. Li et al., 2010b).

The relevance of inulin for modern food industry is based on its ever increasing use as a fat and sugar replacer, as a texturizer especially in low fat dairy products (Meyer, De Wolf, & Olivier, 2007) and as a means to enhance the health characteristics of a food product: therefore the properties as dietary fibre and prebiotic are important (Tungland & Meyer, 2002). Since the β-2,1-bond in inulin cannot be digested by the human digestive system, inulin is a dietary fibre and has physiological effects such as for improved bowel movement and a lowering effect on serum lipids. In this respect inulin resembles pectin. Moreover, inulin is a prebiotic (Gibson, Probert, & van Loo, 2004) as it specifically stimulates growth of health bacteria in the colon which has beneficial effects for the host. Many studies have shown the specific increase in faecal bifidobacteria in humans following inulin consumption (Meyer & Stasse-Wolthuis, 2009). The beneficial effects associated with these changes in faecal microbiota are the subject of many investigations: they include an enhancing effect on calcium absorption (with potential benefits for bone health), a positive effect on resistance to infections, a stimulation of the immune system and the capacity to increase satiety and reduce food intake (Roberfroid et al., 2010). Besides the applications in food, inulin is also applied in pet food and feed (Van Loo, 2007), whereas chemical derivatives are used in non-food applications such as for anti-scaling in waste water treatment (Martinod, Neville, Euvrad, & Sorbie, 2009).

7. Plant aldehydoacids, ketoacids and phenols

When chitosan is dissolved in diluted glyoxylic acid, CHO·COOH, it undergoes spontaneously the Schiff reaction leading to the aldimine; the latter, upon hydrogenation with borohydride, $NaBH_4$, yields N-carboxymethyl chitosan, otherwise called glycine glucan, that combines in itself the backbone of chitin and the functionality of glycine, i.e. the two major compounds discovered by Braconnot (Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1982).

7.1. N-carboxymethyl chitosan (glycine glucan)

The covalent combination of glycine and chitosan into N-carboxymethyl chitosan has progressively assumed key roles in the exploitation of chitosan in a number of areas: the glycine moiety imparts amphiphilic behavior that enhances the solubility over a continuous and extended pH range that includes alkaline values. The N-carboxymethyl chitosans are today the most studied and cited modified chitosans, along with their homologous O- and N,O-carboxymethyl chitosans, that however require Na chloroacetate under severe alkaline conditions and high temperature for the ester synthesis with the hydroxyl groups at C3 and C6. Theoretical and experimental studies on the chemical structure of carboxymethyl chitosans have been published by An, Thien, Dong, and Dung (2009), Chen and Park (2003), De Abreu and Campana-Filho (2009), Di Colo, Zambito, Burgalassi, Nardini, and Saettone, 2004, Jayakumar et al. (2010), Lu et al. (2011), Mourya, Inamdar, and Tiwari (2010), Muzzarelli, Tanfani, Emanuelli, and Bolognini (1985), Muzzarelli and Tanfani (1982a), Muzzarelli and Tanfani (1982b). Carboxymethyl chitin and hydroxyethyl chitin were originally developed and commercialized in Japan for a variety of applications as described in an early review by Muzzarelli (1988).

According to Song, Zhang, Gao, and Ding (2011), N-carboxymethyl chitosan can be synthesized from chitosan in water with chloroacetic acid as well, provided that NaOH is omitted, permitting the selective reaction at the nitrogen atom with degree of substitution up to 1.32, under the optimal reaction conditions (90 °C, 4 h, and ratio of chloroacetic acid to chitosan 5:1). The N-carboxymethyl chitosans were characterized in terms of water solubility and isoelectric point for different degrees of substitution.

Verheul, van der Wal, and Hennink (2010) provided an elegant example of the versatility of N-carboxymethyl chitosan obtained with glyoxylic acid and sodium borohydride. The remaining primary amines were quantitatively dimethylated with formaldehyde and sodium borohydride and then quaternized with iodomethane in N-methylpyrrolidone (Muzzarelli & Tanfani, 1985; Verheul et al., 2008). Subsequently, these partially carboxylated trimethyl chitosans dissolved in water were reacted with cystamine at pH 5.5 using carbodiimide as a coupling agent: thiolated TM-chitosans were obtained, varying in degree of quaternization (25–54%) and degree of thiolation (5–7%). All thiolated TM-chitosans showed rapid oxidation to yield disulfide-crosslinked trimethyl chitosan at pH 7.4.

A as an alternative, a water soluble amphiphilic O-carboxymethyl-N-trimethyl chitosan chloride was also synthesized by Li et al. (2010a): it was compared to chitosan, N-trimethyl chitosan chloride, and O-carboxymethyl chitosan, in terms of turbidity and COD lowering in sugar refinery suspensions at pH 5, 7 and 9 at dosage up to 8 mg/l. The water soluble amphiphilic O-carboxymethyl-N-trimethyl chitosan showed the best performance. According to the opposite approach, N-trimethyl chitosan was carboxymethylated with chloroacetic acid to obtain N-trimethyl-O-carboxymethyl chitosan (Xu, Xin, Li, Huang, & Zhou, 2010). These derivatives were the most frequently studied in 2010.

An impressive biochemical activity of the carboxymethylated chitins and chitosans is the inhibition of matrix metalloproteinase-2 and -9 (MMP-2 and -9) accompanied by antioxidative effect.

Treatment of HT1080 human fibrosarcoma cells with OCM-chitosan and CM-chitin suppressed the formation of intracellular reactive oxygen species, protein oxidation and lipid peroxidation in a concentration-dependent manner. In addition, a protective effect against oxidative damage of purified genomic DNA was observed in the presence of OCM-chitosan and CM-chitin. The latter exhibited higher MMPs inhibitory effect than OCM-chitosan through transcriptional down-regulations of activator protein-1 and nuclear factor kB (NF-kB). These findings underline the nutraceutical value of OCM-chitosan and CM-chitin as potent antioxidants and MMP inhibitors thanks to the alleviation of the radical-induced oxidative damage (Kim & Kim, 2006; Kong, Kim, Ahn, Byun, & Kim, 2010; Wu, Jingyuan, Chen, & Du, 2004). The delivery of NCM-chitosan via intra-articular injection, for the suppression of MMP-1 and MMP-3 expression in cartilage, was described by Liu, Qiu, Chen, Peng, & Du (2005). CM-Chitin is also seen as a good drug carrier (Dev et al., 2010).

The formation of bioadhesive and gastroprotective hybrid particles was obtained after the polymerization reaction of acrylic acid on N-carboxymethyl chitosan (D'Agostini, Petkowicz, Couto, de Andrade, & Freitas, 2011) previously reported as an intestinal and transdermal permeation enhancer for macromolecular drugs significantly decreasing the trans epithelial electric resistance and increasing paracellular intestinal absorbtion of low molecular weight heparin at concentrations in the range 3–5% (w/v) (He, Guo, Xiao, & Feng, 2009).

The primary normal human dermal fibroblasts exhibited appropriate cytocompatibility with N-carboxymethyl chitosan and appeared to be safe biomaterials for potential wound healing applications without overproduction of extracellular matrix and fibroblast hyperproliferative activity (Rasad et al., 2010) as a confirmation of previous pre-clinical works. As an additional advantage when used as a wound dressing, NCM-chitosan possesses antimicrobial activity (Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Chitosan and its derivatives are able to interact with anionic groups present on the microbial wall, thus producing flocculation and modifying the membrane permeability.

The glycine functionality of NCM-chitosan, where the nitrogen atom is part of both the glucosamine unit and the glycine function, confers better chelating capacity for transition metal ions to the point that epichlorhydrin crosslined NCM-chitosan, a transparent and rigid hydrogel, could be used to remove tiny quantities of radioelements from industrial waters from nuclear plants. Therefore it could be a potential tool for the treatment of drinking water and wastewater or as a chromatographic support for the isolation of metals (Bhatnagar & Sillanpaa, 2009; Dobetti & Delben, 1992; Muzzarelli, Weckx, Filippini, & Lough, 1989; Muzzarelli, Weckx, Filippini, & Sigon, 1989; Sun & Wang, 2006; Wan Ngah, & Liang, 1999; Wang et al., 2010; Wang, Xu, Yang, & Gao, 2011). Current cosmetic applications of NCM-chitosan are based on properties such as high moisture retention, UV protection, viscosifying action, and anti-aging of particular value for hydrating and protective creams (Muzzarelli, Cucchiara, & Muzzarelli, 2002) corroborated by the mentioned antimicrobial activity and by the inherent filmogenicity.

Besides the aldehydo-acid glyoxylic acid, other aldehydes have been used in connection with the derivatization of chitosan, in particular salicylaldehyde interesting for its metal chelates, veratraldehyde that forms very resistant biodegradable films, 4-hydroxybenzaldehyde (*vide infra*), and cinnamaldehyde.

7.2. Chitosans derivatized with the aid of ketoacids

Likewise, a series of chitosan derivatives bearing aminoacid moieties, was prepared by reacting ketoacids with the primary amino groups of chitosan. The Schiff reaction carried out in a moderately acidic medium at room temperature produces ketimines that are optionally submitted to hydrogenation with $NaBH_4$ to yield secondary amines. When using a highly deacetylated chitosan, the structure of such derivatives is equivalent to that of aminoacid glucans, i.e. polysaccharides carrying aminoacid units as side branches covalently linked to C2. The nitrogen atom is simultaneously part of the aminoacid moiety and the glucosamine repeating unit. Some of these derivatives are well known, in particular the N-carboxybutyl chitosan that in cyclic form is known as 5-methylpyrrolidinone chitosan amply used in wound dressings; and the 4-hydroxyphenylpyruvate chitosan derivative, better known as tyrosine glucan. Pyruvate itself can be used to derivatize chitosan to N-carboxyethyl chitosan, otherwise called alanine glucan.

Several authors reported conjugation of chitosan with other aminoacid moieties, describing improvement of a series of properties. It is the case of cysteine-grafted-chitosan, where the presence of thiol group through the polysaccharide chain potentiates the mucoadhesiveness of the chitosan (Bernkop-Schnurch, Hornof, & Guggi, 2004).

Moreover chitosan ability to transfect nucleic acid (e.g. DNA, siRNA) into the cells, by forming polyplexes, has been described to be enhanced by grafting arginine moiety through the polycation backbone (Gao et al., 2008; Liu et al., 2009; Zhu et al., 2007). More recently, it has been reported the grafting of chitosan by poly(aminoacids), e.g. polylysine (Yu et al., 2007, 2011) and polyarginine (Noh et al., 2010), which not only decrease their toxicity but increase gene transfection ability.

7.3. Phenols, tyrosinase and quinones

In nature, certain quinones, generated enzymatically, react immediately with amines to produce complex biomaterials that have been difficult to analyze in the past. For example, the adhesion of marine animals to submerged surfaces involves a protein with high dihydroxyphenylalanine (DOPA) content: the DOPA units are oxidised by tyrosinase and undergo instant cross-linking with the amino groups of the protein, thus conferring cohesive strength to the mussel glue (Yamada et al., 2000). Another important example in nature is the quinone tanning in the sclerotization of insect tissues: during this process tyrosinase converts low molecular weight phenols into quinones that immediately react with proteins in the outer integument (Muzzarelli, 1976).

Tyrosinases, laccases and phenol oxidases have therefore been used to generate nascent quinones *in situ*: for example, chitosan was modified with the aid of 4-hydroxyphenylpyruvic acid (or likewise with other carbonyl compounds carrying phenolic moieties) to yield the so-called tyrosine glucan. Gels were immediately obtained when tyrosinase was added to tyrosine glucan solutions: those pink gels were very stable for months and did not undergo microbiological spoilage. Albumin, mucin, pseudocollagen and gelatin, as well as veratraldehyde- or vanillin-modified chitosans were also submitted to this enzymatic reaction (Muzzarelli, Ilari, Tarsi, et al., 1994; Muzzarelli, Ilari, Xia, et al., 1994).

Polytyrosine was used as a cross-linking agent in the presence of tyrosinase by Muzzarelli, Littarru, Muzzarelli, and Tosi (2003). Chitosan (0.5 g), water (49.3 g), acetic acid (0.2 g), tyrosinase (0.6 ml 8800 U/ml) and polytyrosine (20 mg in 2 g H_2O, M.W. 25 kDa) were reacted under stirring for 24 h. Centrifugation at 15,000 rpm for 10 min yielded a water-insoluble white product. For practical applications, it is of interest to react phenols with chitosan in the presence of tyrosinase and atmospheric oxygen: immediate perception of the reaction can be obtained by observing the browning of a chitosan film or the viscosity increase of a chitosan salt solution leading to gel formation.

When laccase and polyphenol oxydase were immobilised on chitin, chitosan and chitosan-coated polysulphone, the gel supporting the enzyme was able to react with the quinones

generated by the enzymatic action on phenols. Chitosan could also be coupled to polymeric substances in this way, in particular to proteins, gelatin and polyhydroxy styrene (Vazquez-Duhalt, Tinoco, D'Antonio, Topoleski, & Payne, 2001). In one decade, these studies had fantastic developments in the area of synergy between microelectronics and biotechnology (Liu et al., 2010).

The phenols taken into consideration in the cited studies were volatile phenols such as p-cresol, gallate esters such as dodecyl gallate, phenol, hexyloxyphenol, chlorogenic acid, 1,2-benzenediol, *tert*-butylcathecol, p-hydroxyphenoxyacetate, olive-mill wastewater phenolics, and polyhydroxystyrene: the attention was directed to chemical models of industrial importance. In addition several compound of recognized biomedical/biochemical importance were also examined.

Vitamin K quinones and ubiquinone/ubiquinol actually are compounds of major importance from the biochemical standpoint, being involved in modification of blood coagulation factors and in the electron/proton transport, respectively. Menadione, 2-methyl-1,4-naphthalenedione is a synthetic naphthoquinone derivative having the physiologic properties of vitamin K, the latter being a collective name including phylloquinones K1, menaquinones K2 and menadione K3. Phylloquinone, 2-methyl-3-(3,7,11,15-tetramethyl-2-hexadecenyl)-1,4-naphthoquinone, is actually a photosynthetic electron carrier synthesized in green plants, a major dietary source of protrombogenic vitamin K. Other compounds of interest are plumbagin, 5-hydroxy-2-methyl-1,4-naphthoquinone, an antimicrobial very toxic agent from *Plumbago indica*, and bupravacone, an antibiotic used in clinical experiments against *Cryptosporidium parum*.

The derivatization of chitosan made by Muzzarelli, Weckx, Filippini, and Lough (1989) and Muzzarelli, Weckx, Filippini, and Sigon (1989) with the aid of levulinic acid (4-oxo-pentanoic acid, i.e. a γ-ketoacid obtained from fructose that Broussignac might have handled) deserves a mention here in consideration of the major significance that it had in recent research in experimental surgery, wound management and healing, bone regeneration, repair of lesions of the meniscal cartilage, decubitus ulcer treatment, dentistry, coated prostetic materials, urology, and cosmetology. Most recently the preparation protocol was refined as follows: pH 4.5–5.0. Molar ratios of the primary amino groups, $NaBH_3CN$ and levulinic acid 1:10:3, the substitution degree was close to 41% for 5-methylpyrrolidinone chitosans, after 72 h of reaction at room temperature; no depolymerization occurred, and the NMR spectra were cleaner (Kurita & Isogai, 2010).

8. Conclusion

Well, we are back from our journey to the past. We dreamed to have met Henri Braconnot in his Botanical Garden for an exciting exchange of views, and to have been admitted to his laboratory, where he used to work alone, to the point that he had no disciples. Actually he did not even get married. Even though he attended courses of chemistry, natural history and pharmacy in Strasbourg and Paris whenever possible because of recurrent military obligations, he was never concerned about getting an academic title. The personal qualities that we appreciate in him as a scientist are the clarity of ideas, the scrupulous planning and execution of the experiments, the concise and very efficacious writing, and the total devotion to his duties. We see today that his work was highly anticipatory in character, and opened up new routes in botany, pharmacy and chemistry as we demonstrate in this concise assay. After two centuries, the main subjects of his research work are still pivotal and proficuous for envisaging sustainable green solutions for modern economies.

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