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Tyrosinase-mediated quinone tanning of chitinous materials

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Stable and self-sustaining gels were obtained from tyrosine glucan (a modified chitosan synthesized with 4-hydroxyphenylpyruvic acid) in the presence of tyrosinase. Similar gels were obtained from 3-hydroxybenzaldehyde, 4-hydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde: all of them were hydrolyzed by lysozyme, lipase and papain. Microcapsules were similarly obtained by introducing tyrosinase in a water-in-oil emulsion containing tyrosine glucan in the water phase. No cross-linking was observed for chitosan derivatives of vanillin, syringaldehyde and salicylaldehyde. Collagen–chitosan–tannin mixtures were also studied under the catalytic action of tyrosinase: partially crystalline, hard, mechanically resistant and scarcely wettable materials were obtained upon drying. By contrast, products obtained from albumin, pseudocollagen and gelatin, in the presence of a number of phenols and chitosan under comparable conditions, were brittle.

INTRODUCTION

Quinones control the biosynthesis of lignin and melanoproteins in plants and animals, respectively: for instance, in insects, cross-linking of structural proteins through quinone and quinone methides results in construction of the sclerotized exoskeleton. The chemical modification of biopolymers with quinone is, therefore, important (Muzzarelli, 1976) and the mechanisms have been discussed (Sugumaran, 1988).

Protein cross-linking in biological systems includes addition of ε-amino groups of lysine to quinone. When the addition reaction is followed by the rearrangement of hydrolysis of the azomethine, an aldehyde is generated in a Streicker-type reaction. The aldehyde is itself reactive as both an electrophilic amino group acceptor and a component in aldol condensation. This reaction brings about one of the main structural modifications in collagen and related proteins (Peter, 1989).

Binding of chitin β-alanine is required for the current packing of the macromolecular network in the insect cuticle. The β-alanine mediated browning of chitin results most likely from Maillard-type reactions. The possibility of a copolymerization of diphenols and tyrosine residues of protiens has also been underlined, as an alternative to the involvement of protein amino groups (Peter *et al.*, 1985). The skeletal basis of the black coral *Antipathes fiordensis* is protein (70%), chitin (10%), diphenol (15%) and lipid (5%), the only diphenols being 3-(3,4-dihydroxyphenyl)-DL-alanine (DOPA) and 3,4-dihydroxybenzaldehyde. In the acid extracts of the cuticle of the insect *Manduca sexta*, chitin is 20% and the predominant diphenol is *N*-acyldopamine (Holl *et al.*, 1992).

If chitosan is considered, advantage can be taken from the large availability of primary aliphatic amino groups for carrying out selective reactions with phenols mediated by tyrosinase. Essentially, two reaction paths are possible, a rapid reversible reaction resulting in imine formation, and an irreversible oxidative reaction leading to aminoquinones. Formation of oligomeric phenols is also considered. Quinone absorption on to chitosan was found to be rapid (minutes) and strong (−25 kcal/mol), when they were separated from alcohols and ethers in dilute aqueous media (Payne *et al.*, 1992; Sun *et al.*, 1992; Payne & Sun, 1994).

Modified chitosans such as those from *p*-hydroxyphenylpyruvic acid (tyrosine glucans), described by Muzzarelli *et al.* (1985), would expectedly lend themselves to cross-linking reactions under the catalytic action of tyrosinase.

MATERIALS AND METHODS

Mushroom tyrosinase (E.C. 1.14.18.1) of specific activities 2400, 57 000 and 630 000 U/mg solid, as tyrosinase, polyphenol oxidase and catechol oxidase, respectively, was purchased from Sigma, Milan, Italy. A solution was prepared in 50 mM phosphate buffer (pH 6·80, 0·1 mg/ml). Chitosan was supplied by Aber Technologies, Plouvien, France, and had degree of acetylation 0·13. Analytical grade chemicals were purchased from Aldrich Chimica, Milan, Italy. Bovine collagen fleeces (cosmetic grade) were purchased from Uniderm, Rome, lot no. 02–1133; gelatin (food grade, DGF Stoess) was from Gelita Eberbach/Baden, Germany.

Tyrosine glucan was prepared according to Muzzarelli *et al.* (1985); briefly, chitosan (2 g) was suspended in water (196 g) and dissolved with glacial acetic acid (1 g); 4-hydroxyphenylpyruvic acid (1 g; molar ratio of amine to ketone *c.* 2:1) was added to the solution, which was vigorously stirred for 1 h at room temperature until dissolution of the acid was complete, and then for a further 45 min. The resulting pH was 4·0. Finally, $NaBH_4$ (10 ml, 50 g/liter) was added slowly to reach pH 4·8. The product was dialyzed for 70 h against demineralized water and freeze-dried. The FTIR spectrum revealed the presence of the band at 1250 cm^{-1} typical of phenols, and the ^{1}H-NMR analysis indicated a degree of substitution of 0·30.

Chitosan films were prepared as follows: a chitosan solution (10 g/liter in 10 g/liter of acetic acid) in plastic Petri dishes was dried overnight at 50°C. For the preparation of chitosan–protein films, the same chitosan solution was mixed with a gelatin solution, in a chitosan-to-gelatin ratio of 3:7. All polymer solutions were degassed before film casting. As for collagen, the collagen fleece was imbibed with excess of chitosan solution (10 g/liter) as acetate salt, under vacuum, and dried at 50°C. The weight increase was taken as a measure of chitosan uptake, the resulting chitosan-to-collagen ratio being 7:3.

Use of tyrosinase

The tyrosinase solution was mixed with the desired phenol in phosphate buffer and brought in contact with the chitosan film: for instance, the chitosan–collagen film was incubated in a tannin solution (2 g/liter) in phosphate buffer (50 mM, ph 6·8, 25°C, 4 h) to which 1 ml of the tyrosinase solution was added. After washing in demineralized water, the film was soaked in a sodium borohydride solution (10 g/liter) and, after rinsing, was dried at 50°C. The same procedure was followed for other proteins.

In the case of tyrosine glucan, a chitosan film was soaked in the phosphate buffer, and the *p*-hydroxyphenyl pyruvic acid and tyrosinase were added. The tanned film was then soaked in a sodium borohydride solution.

Lysozyme, papain and lipase were used as previously described (Muzzarelli *et al.*, 1994).

Instrumental characterization

Ultraviolet spectrophotometry was performed with a Beckman DU 640 instrument. X-ray diffraction spectra were obtained by using a vertical powder diffractometer; the source was a rotating anode generator (Rigaku Denki RU-300) and Ni-filtered Cu kα radiation (0·154 nm) was used. For the contact angle data, each specimen was assembled in a sandwich of cardboard and white paper. Such sandwiches were plastified to impart rigidity to the film; the test surface was exposed by cutting away the white paper in the area of interest. Chitosan films used in the experiments presented different surface roughness, and the procedure adopted for the preparation of the samples obviated the effects of the surface roughness. The initial contact angles θ, were measured by using 1% NaCl aqueous solution at room temperature. An excellent definition of the drop shape was obtained by positioning the sample between the spot light and the camera (Nikon F-601 lens 60 mm, with bellows focussing attachment PB-6). The contact angle was obtained according to Oshida *et al.* (1993) by measuring the height h and the width d of the drop with the formula: $\theta = 2\arctan(2h/d)$.

RESULTS AND DISCUSSION

Cross-linking of tyrosine glucan

In the course of the preparation of tyrosine glucan, upon mixing chitosan and 4-hydroxyphenylpyruvic acid solutions, a sharp decrease of absorbance at 226 nm was observed within 10 min. The FTIR spectrum of the resulting dialyzed tyrosine glucan showed the presence of novel bands at 820, 1250 and 1500 cm^{-1} for *p*-substituted phenylene groups, the accompanying bands overlapping the most prominent chitosan bands at 1100, 1550 and 1640 cm^{-1} (Fig. 1). A remarkable feature in the ^{1}H-NMR spectrum was a quartet at 6·7696, 6·8113, 7·0993 and 7·1414 ppm, corresponding to a *p*-disubstituted phenylene group. When the same reaction was carried out heterogeneously on chitosan films at pH 6·8, however, it was very slow. A yellow solution of tyrosine glucan, dialyzed against water (pH 5·74, 225 ml, > 10 g/liter), when treated with tyrosinase (5 ml, 1000 U/ml) turned red. When kept at −20°C, the solution gelled and a self-sustaining pink gel was obtained, which did not melt upon heating at 60°C and did not undergo syneresis. Films cast from the solution of cross-linked tyrosine glucan were resistant to traction, but not to folding.

Gels were immediately obtained when tyrosinase was added to tyrosine glucan solutions (> 10 g/liter). These

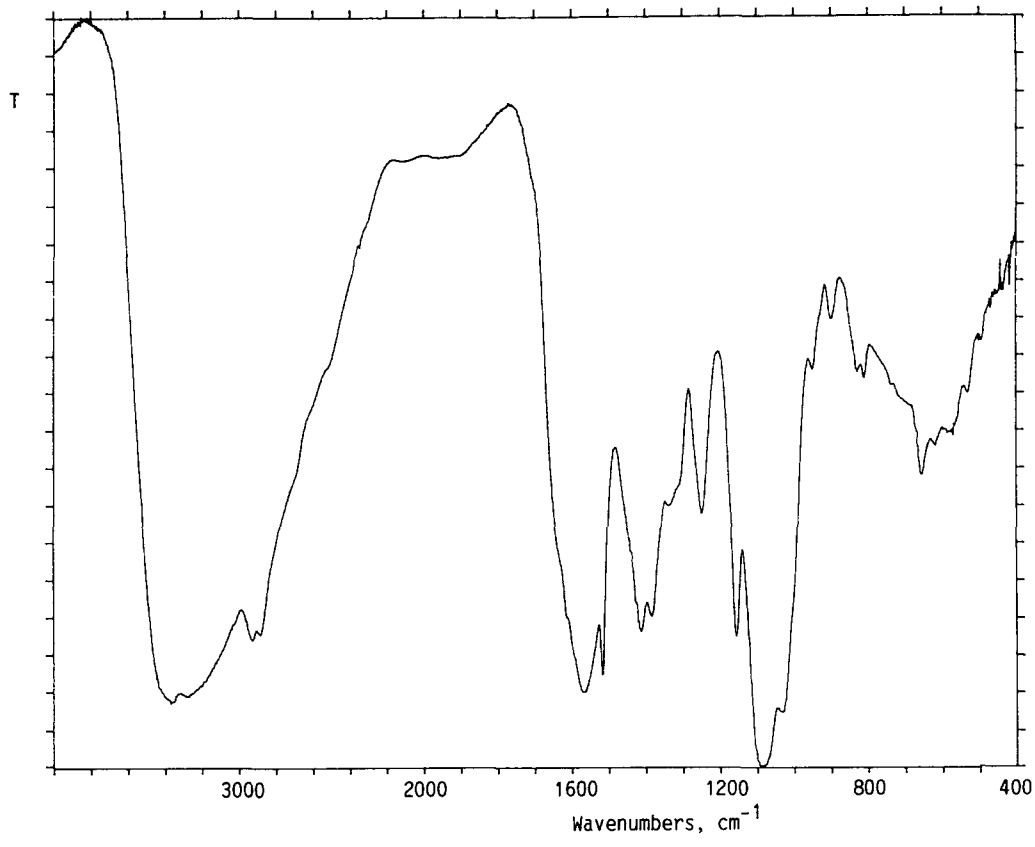

Fig. 1. Infrared spectrum (FTIR) for tyrosinase-treated tyrosine glucan showing bands at 820, 1250 and 1500 cm^{-1} for *p*-substituted phenylene groups.

gels were very stable in the course of a 4 month observation period under normal laboratory conditions (21°C, exposure to air, no protection against contamination, moisture 70%) and did not undergo microbiological spoilage. Table 1 summarizes the results for the compounds tested. The cross-linked gels obtained from chitosan modified with 3-hydroxybenzaldehyde, 4-hydroxybenzaldehyde or hydroxyphenylpyruvic acid were susceptible to the hydrolytic action exerted by hen egg white lysozyme, papain and lipase, which brought about immediate viscosity drops as illustrated in Fig. 2. The freeze-dried gels swelled but did not dissolve in water; in the presence of the hydrolytic enzymes they dissolved.

Table 1. Compounds that lead or do not lead to gel formation upon Schiff reaction, hydrogenation and tyrosinase-promoted cross-linking in dilute solutions (chitosan 10 g/liter, 20°C)

Gel formation	Color of self-sustaining gel
4-Hydroxyphenylpyruvic acid	Dark red
3,4-Dihydroxybenzaldehyde	Light brown
4-Hydroxybenzaldehyde	Pink
3-Hydroxybenzaldehyde	Pink
No gel formation	
Salicylaldehyde, syringaldehyde, vanillin, *o*-vanillin, 3-nitro-4-hydroxybenzaldehyde, 5-nitro-2-hydroxybenzaldehyde, 2-hydroxynaphthaldehyde, veratraldehyde	

In another series of experiments, tyrosinase was used to oxidize the 4-hydroxyphenylpyruvic acid. The tyrosinase solution (1 ml, 1000 U/ml) was added to the 4-hydroxyphenylpyruvic acid (50 ml, 0·5 mM) and left overnight. The spectrophotometric analysis was performed at 226 nm, after contacting the above solution with a chitosan film (1·0 g), and the optical density decrement was recorded as a function of time. The control solution was a similar one where tyrosinase was absent and was examined at 221 nm: the reaction was found to occur very slowly (Fig. 3).

Other phenols

Quinones obtained by oxidation of the phenols under the catalytic action of tyrosinase, immediately react with a chitosan film under heterogeneous conditions, as well as with dissolved chitosan salts. For instance, catechol (100 mg/liter) in phosphate buffer was easily measured by spectrophotometry, based on its absorption band centered at 275 nm: the absorbance decreased with time upon addition of tyrosinase as a consequence of quinone tanning.

When these reactions were carried out on precast chitosan films, immediate development of color (reddish brown) took place in the films for all of the phenols

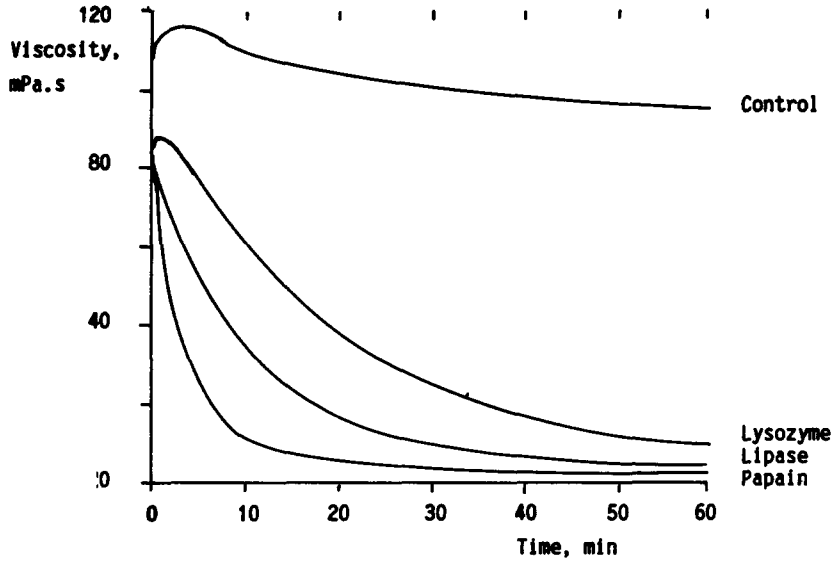

Fig. 2. Viscosity decrease of a gel of chitosan modified with 4-hydroxybenzaldehyde (2·2 g/liter) and cross-linked with tyrosinase, in the presence of lysozyme, lipase or papain (37°C, pH 5·0). Shear rate program: 30 s from 0 to 200 s^{-1} for 59 min, and 30 s from 200 to 0 s^{-1} (Rotovisco M5–NV).

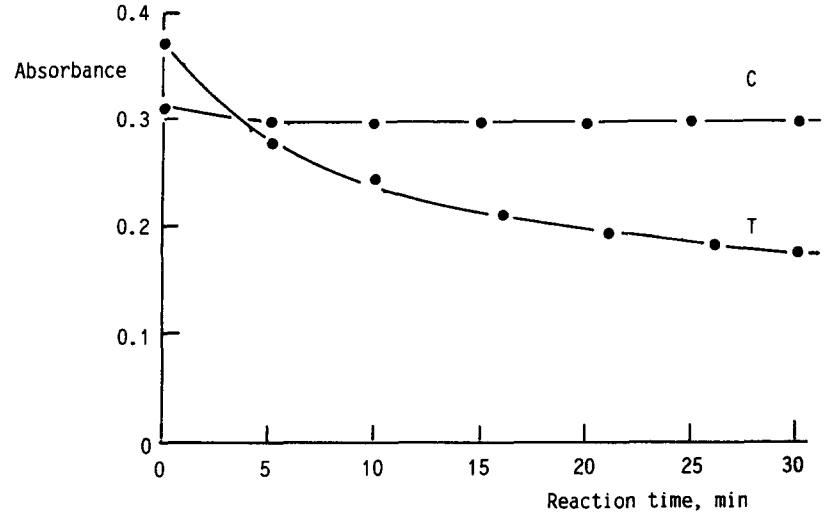

Fig. 3. Absorption decrease with time during the reaction of (T) 4-hydroxyphenylpyruvic acid (50 ml, 0·05 mM) and a chitosan film (0·5 g) in the presence of tyrosinase (1000 U) measured at 226 nm; (C) control, in the absence of tyrosinase. Temperature: 21°C.

(phenol, catechol, *p*-cresol, tannin, gallic acid, sesamol, caffeic acid, guaiacol, 3,4-dimethoxyphenol and 4-hydroxy-3-methoxycynnamic acid ethyl ester), and the integrity of the films was preserved. Once dry, however, the films became brittle. This was verified even for 3,4-dimethoxyphenol in spite of the presence of the methoxy groups.

Chitosan and proteins

When films made of chitosan and protein were considered, the reaction with quinone obtained from tannin in the presence of tyrosinase yielded films whose macroscopic characteristics are listed in Table 2. Albumin and pseudocollagen yielded fragile and brittle films even in the presence of plasticizers, whilst gelatin and collagen fleeces yielded leather-like materials, which were thermoformable when wet, unaffected by acidic media, insoluble, but swellable in aqueous media, and hard and rigid when dry.

The dramatic change in the macroscopic characteristics of the chitosan–protein films confirms that important cross-linking reactions are promoted by tyrosinase. For instance, the chitosan–gelatin films are highly hydrated and, therefore, very difficult to handle; they became tough and rigid after the enzymic treatment.

Examination of the chitosan–collagen cross-linked films by X-ray diffraction spectrometry indicated that, in spite of the presence of a large quantity of amorphous protein and cross-linking agents, the chitosan structure was not completely lost. In fact, peaks at 9 and 20 2θ

Table 2. Composition and macroscopic characteristics of chitosan–protein–tannin films obtained in the presence of tyrosinase

Protein	Protein (%)	Other (%)	Dry film
Albumin	20	0	Fragile
Pseudocollagen	95	0	Brittle
	87	Glycerol (9)	Brittle
	67	Glycerol (30)	Brittle
Gelatin	60–95	0	Thermoformable, leather-like, insoluble
	60	Paraffin (5)	Dishomogeneous
Collagen fleece	30	0	Thermoformable, leather-like, resistant to folding and traction

Table 3. Contact angles (°) measured for various chitosans

System	Water (0 min)	Aqueous NaCl solution (9 g/liter)			
		0 min	10 min	20 min	30 min
Chitosan–gelatin (hydrogenated)	59·0	86·5	nd[a]	nd	nd
(Chitosan–gelatin)–HPP–tyrosinase	69·0	77·5	65·0	57·3	45·6
Chitosan–mucin	94·6	75·9	78·9	75·7	75·3
Chitosan–(mucin–tyrosinase)	96·0	87·9		Highly swollen	
Chitosan–HPP (tyrosine glucan)	84·2	85·6	79·8	57·8	nd
Chitosan–(HPP–tyrosinase)	108·3	90·9		Highly swollen	
Chitosan–veratraldehyde (reduced)	109·6	110·4	103·0	82·4	82·4
Chitosan vanillin (reduced)	60·3	69·9	60·9	49·4	38·0

[a]nd· not determined.

values were closely reminiscent of the original chitosan values, and peaks at 16·2 and 18·1 2θ values were new features of the diffraction spectrum.

The contact angle data in Table 3 refer to three systems: (a) chitosan–gelatin, treated with tyrosinase; (b) chitosan–mucin, treated with tyrosinase; and (c) tyrosine glucan, treated with tyrosinase. In all cases the contact angles for pure water remarkably increased upon cross-linking under the effect of tyrosinase. In particular, the tyrosine glucan with contact angle 84·2°, once treated with tyrosinase, had 108·3°, indicative of scarce wettability. For 9 g/liter NaCl solutions, lower contact angle values were expected in consideration of the lower surface tension for a solution: actually, the angles became much lower in the course of the first 30 min as a consequence of the spreading of the drops. While the chitosan–mucin values became stable at 75·3°, tyrosine glucan showed progressively higher wettability. The corresponding tyrosinase-treated films, however, swelled after 10 min, thus preventing further measurements. The tyrosinase-promoted cross-linking led to scarcely wettable films in water, but to swellable films in NaCl solutions (9 g/liter). Table 2 also contains data for comparison purposes on the veratraldehyde chitosan and vanillin chitosan prepared according to Muzzarelli (1994): the former compound was poorly wettable in water (109°) and in NaCl (110·4°) with limited spreading (82.·4°); the latter was wettable with evident spreading.

Microcapsule formation

Water-in-oil emulsions were found to be suitable for the preparation of microcapsules. The continuous organic phase consisted of soya oil (200 g) and the emulsifier was glyceryl stearate (Glicmonos, Comiel, Milan, 2·2 g); the dialyzed tyrosine glucan solution (75 ml, 10 g/liter) was added while stirring with an emulsifier.

Membrane formation was initiated by the addition of tyrosinase solution (0·5 ml) to the emulsion, stirring was protracted at modest speed for 1 min. The emulsion was left in the beaker for a few hours, with occasional mixing to promote oxygen uptake, then settling of the red microspheres took place, while the emulsion broke as a consequence of the cross-linking. At the optical microscope, the microspheres were found to be *c.* 100 μm in diameter. The microspheres could be recovered after washing with oil at 40°C, adding demineralized water and filtering.

CONCLUSIONS

This is the first report on cross-linked chitosans obtained by enzymic routes for preparative purposes. While this work was originally undertaken with the intention of mimicking the quinone tanning of the chitin–protein in the insect cuticle, it appears that the use of chitosan instead of chitin permits one to exploit certain chemical routes which are not fully accessible *in vivo*, due to scarce availability of glucosamine units in the native biopolymer.

Chitosans modified with certain carbonyl compounds carrying phenolic moieties are cross-linked by tyrosinase and yield gels susceptible to hydrolysis under the action of lysozyme, lipase and papain.

The characteristics of the products obtained from chitosan and phenols in the absence of proteins are very far from those of the sclerotized cuticle: this result points to the high rigidity of quinone cross-linked chitosans. On the other hand, the products obtained via enzymic cross-linking from chitosans carrying tyrosine moieties (tyrosine glucans) and those obtained from blends of chitosan and collagen or gelatin are quite resistant from mechanical and chemical standpoints; they would be biodegradable materials of technological interest and a starting point for a deeper investigation into the enzymic modifications of chitosans.

The relevance of these results to the cosmetic field is also apparent: partially hydrolyzed chitosan *p*-hydroxybenzoate salt was proposed by Saruno (1992), as a lotion for the depression of melanin formation. Prevention of the enzymic browning of fruit juices by chitosan (Sapers, 1992) could be explained accordingly.

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