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Extraction and Characterization of *Foeniculum vulgare* Pectins and Their Use for Preparing Biopolymer Films in the Presence of Phaseolin Protein

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Pectins from *Foeniculum vulgare* were extracted under acidic conditions. The obtained pectins were mainly composed of uronic acid but also contained traces of rhamnose, galactose, and arabinose. Extracted pectins were used as a carbohydrate source to prepare biopolymer films in the absence and in the presence of phaseolin protein. The swelling characteristics of the films were examined as a function of ionic strength, pH, and the applied osmotic stress. The swelling behavior was dominated by a Donnan-type effect, which decreases with increasing ionic strength and counterion valency. In all cases the swelling of films containing phaseolin was reduced, suggesting a network formation between protein and pectins. Mechanical property studies have also estimated the validity of the obtained novel biopolymer films in terms of mechanical resistance.

KEYWORDS: Biopolymers; fennel; phaseolin; swelling

INTRODUCTION

Pectin polysaccharides are found in the primary cell wall of higher plants where they have a range of functions, including contributing to the mechanical properties of the cell wall and its hydration characteristics. From the commercial point of view, pectins are traditionally used as gelling agents for jams and jellies; further applications extend to dairy products, desserts, and soft drinks, even though recently their use is proposed also in pharmaceutical production (*1*).

Pectins extracted from various sources exhibit different characteristics (molecular weight, degree of esterification, acetyl content, neutral sugar content, and distribution of the methoxylated carboxyl groups) and therefore have different functional properties. For industrial production the prime sources of pectins are apple pomace, citrus peel, and sugar beet pulp. Alternative sources are currently investigated. In this work we have characterized pectins extracted from fennel (*Foeniculum vulgare*, Mill), a food plant commonly cultivated in the Mediterranean area (*2*). In particular, more than 70000 tonnes of fennel are produced annually in Southern Italy, of which about 30% is waste currently simply disposed of by farmers. Our aim was to enable the development of uses for fennel waste with a focus on the properties of biopolymer films prepared from fennel-extracted pectins. Our studies have established the effectiveness of fennel pectins as carbohydrate components of novel biopolymer films in the presence of phaseolin. This protein, easily obtainable from common beans, has been chosen because it possesses a globular structure (*3*), which is essential for the formation of complexes and coacervates with polyelectrolytes (*4*). Fennel films, obtained in the absence and in the presence of phaseolin, have been characterized for their swelling and mechanical properties, which are useful information for assessing an application of such films either in food protection and/or as biomaterial for drug delivery.

MATERIALS AND METHODS

Extraction of Fennel Pectins. Fennel bulbs (*Foeniculum vulgare*, Mill), from a Southern Italy market, were washed, sliced, frozen in liquid nitrogen, and ground to a fine powder by freeze milling (Freezer/Mill Spex, Certiprep, Metuchen, N.J.). The powder was used as described by Levigne et al. (*5*). The extracts were filtered through GF/C (Millipore, Bedford, MA) and exhaustively dialyzed against distilled water. The extracts were freeze-dried and kept at 20 °C until use.

Characterization of Pectins. The intrinsic viscosity of fennel pectins was measured using an Ubbelohde capillary viscometer (Spinea, Venezia, Italy) in 50 mM acetate buffer at pH 5.6 in dilutions from 0.3 to 1 mg/mL. The viscometer was thermostated to 25 ± 0.1 °C. The intrinsic viscosity [η] was calculated from the extrapolation of specific viscosity measurements to zero concentration. The average molecular weight (MW) was estimated by applying the Mark−Houwink equation reported by Anger and Berth (*6*):

$$[\eta] = 9.55 \times 10^{-2}\,\mathrm{MW}^{0.73} \quad (1)$$

Individual neutral sugars were analyzed as their alditol acetate derivatives by gas chromatography according to Blakeney et al. (*7*). Uronic acids were quantified by using the modified method of

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Blumenkrantz and Asboe-Hansen (*8*). The degree of methyl esterification was determined as described by MacDougall et al. (*9*).

Phaseolin Extraction. The ascorbate−NaCl procedure recommended by Sun and Hall (*3*) was used for the isolation of phaseolin in *Phaseolus vulgaris*. To achieve maximum extraction of phaseolin, the extraction steps were repeated twice, and to maximize precipitation of phaseolin, the samples were kept in the dark at 4 °C for 30 min and then centrifuged for 20 min. The precipitate was then resuspended in a known volume of 0.5 M NaCl and analyzed by 2D electrophoresis; as expected, the purified protein, that in its native state is a trimer made of three almost identical subunits, shows a monomeric molecular mass of ∼50 kDa and isoelectric point of 5.7 (*10*).

Preparations of Films. Phaseolin was dissolved into distilled water at a concentration of 13 mg mL^{-1}. NaOH (0.1 M) was added to the solution until pH 5.8 was reached. Pectins were dissolved in water at a concentration of 16 mg mL^{-1}.

Hence, two different kinds of films were prepared: films made of pectins and films made of pectins and phaseolin in the ratio 2:1. Films were cast by pouring the solution into 8 cm diameter polystyrene Petri dishes. Solutions were allowed to dry in air at 20 °C for 14 h. The films were peeled from the Petri dishes and stored at 20 °C.

Film thickness was measured using a micrometer with sensitivity of ±10 μm (Mitutoyo Corporation, Minato-ku, Tokyo). Mean thickness (μm) of the films was determined from the average of measurements at 10 locations.

Swelling Experiments. Disks of freshly prepared films were cut out with a punch and bisected with a razor. The length of the straight edge was measured with a traveling microscope to 10 μm (PTI Liss, Hampshire, England). The polymer volume in the swollen state is a measure of fluid imbibed and retained by the film. Solutions of various concentrations of polyethylene glycol (PEG) (20000) were prepared to provide a range of osmotic pressures from 0 to 20 MPa using reference data (*11*). The relation between PEG 20000 and osmotic pressure (at $T = 30$ °C) fit by the following equation (*12*),

$$\mathrm{Log}[\Pi_{20}(w;30°\mathrm{C})] = 0.161 + 0.272w^{0.21} \quad (2)$$

where Π_{20} is in N/m^2 and w is the weight percent polymer.

The pectin films were pre-equilibrated in 3 MPa PEG + 1 M NaCl. To investigate the effect of pH on swelling, solutions were prepared in 0.4 MPa PEG, 20 mM NaCl adjusted to the required pH with 0.1 M HCl.

Six independent determinations for each swelling experiment have been carried out.

Mechanical Properties. The tensile modulus of films (1 cm × 0.4 cm × 0.005 cm) was determined from force versus deformation measurements in simple extension using a Stable Microsystem Texture Analyser (TAXT2i, Godalming, Surrey, UK). The films were glued to metal supports with a cyanoacrylate adhesive (Cyanolit 223-F, Settingbourne, Kent, UK). The mechanical properties were examined in uniaxial extension as a function of deformation, up to a maximum of 0.03, at a rate of 0.02 mm s^{-1}. Six independent tests for each film were performed.

Statistical Analysis. Microsoft Excel-2002 was used for all statistical analyses. The data were subjected to the analyses of variance and the means were compared using Student *t*-test. Differences were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Fennel Pectins Characterization. The characteristics of fennel pectins are summarized in **Table 1**. Such pectins, extracted by an acidic method, are rich in uronic acid but also contain rhamnose, galactose, and arabinose. They exhibit a composition similar to that of sugar beet pulp pectins studied by Levigne et al. (*5*).

The degree of methyl esterification, which is one of the most important characteristic of pectins, was equal to 48%. The galacturonic acid content, which was equal to 800 μg mg^{-1}, is typical of cell-wall pectic polysaccharides. The value of intrinsic viscosity was 254 mL g^{-1}, suggesting that the pectins adopt an expanded conformation in aqueous solution (*13*).

Table 1. Characteristics of Fennel Pectins[a]

yield (mg g^{-1})	η (mL g^{-1})	uronic acid (μg mg^{-1})	degree of methyl esterification[b] (%)	rhamnose (μg mg^{-1})	arabinose (μg mg^{-1})	galactose (μg mg^{-1})
22 ± 2.9	254 ± 33	800 ± 87	48 ± 5	16 ± 2.4	14 ± 2.5	44 ± 7

[a] The compounds are expressed as μg per mg of pectin dry weight. Results are expressed as means of 6 replicates ± standard deviation. [b] With respect to the uronic acid content.

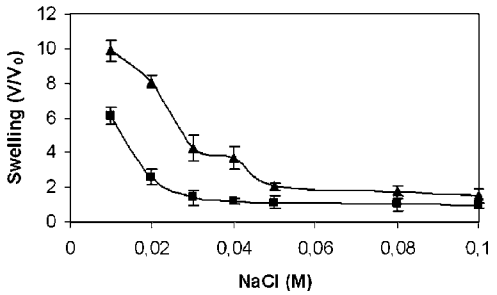

Figure 1. Swelling of fennel pectin (▲) and fennel pectin−phaseolin (■) films as a function of salt concentration at 0.4 MPa osmotic stress and pH 5.7.

Moreover, the average molecular weight was estimated as 50000 g/mol, calculated by the Mark−Houwink equation (1).

Swelling of Fennel Pectin Films. Studies addressed at swelling properties were carried out, taking into account that factors affecting the swelling of polyelectrolytes include the following: the affinity of the polymer for water; the mobile counterions associated with the fixed charges of the polymer which generate an osmotic swelling force, known as the Donnan effect (*14*); the presence of cross-linking that may occur in the presence of oppositely charged proteins (*15*).

The swelling of pectin films was investigated in the absence and in the presence of phaseolin, a globular protein that represents the major storage protein in the cotyledons of the bean *Phaseolus vulgaris*. Many published scientific reports and patents refer to hydrocolloid films constituted by globular proteins (*16*). In this paper, for the first time, the use of phaseolin as a protein component to prepare hydrocolloid films has been described. Before study of swelling properties, the thickness of both pectin films and pectin−phaseolin films was measured, being equal to 33.5 ± 3.6 μm and 62.8 ± 7.5 μm, respectively. It was not possible to measure the thickness of films made by only phaseolin since this protein does not itself exhibit film-forming properties.

It was established that the films swelled isotropically. The swelling data have been expressed as the hydrated volume of the film, V, in relation to that of the dry film, V_0. The swelling of the films was examined at 20 °C as a function of NaCl salt concentration, osmotic stress, and pH. Swelling experiments as a function of NaCl concentration were carried out at a constant osmotic pressure of 0.4 MPa (**Figure 1**). From 0.05 to 0.1 M NaCl swelling is very restricted for both kinds of films. In contrast, at lower NaCl concentrations, the swelling behavior exhibited by the films was different, showing a significantly marked increase with decreasing salt concentration, which was more marked for the simple pectin films. The swelling of the pectin-based film increased from 2.1 V/V_0 to 11.5 V/V_0, as the ionic strength of monovalent counterion (Na^+) decreased from 0.1 to 0.01 M. For the pectin−phaseolin film the corresponding increase was from 1 V/V_0 to 6.1 V/V_0. At lower ionic strengths

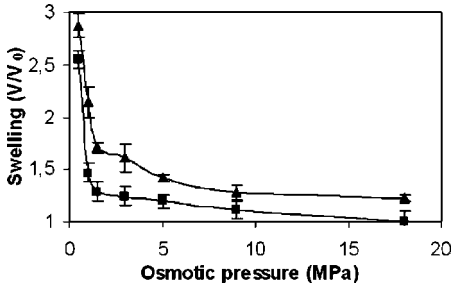

Figure 2. Swelling of fennel pectin (▲) and fennel pectin–phaseolin (■) films as a function of osmotic stress at 1 M NaCl and pH 5.7.

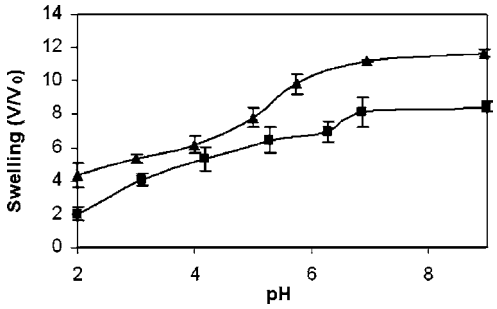

Figure 3. pH dependence of fennel pectin (▲) and fennel pectin–phaseolin (■) films swelling at 0.4 MPa osmotic pressure and 0.02 M NaCl.

the films began to lose their integrity. The observed increase in swelling with decreasing ionic strength is characteristic of polyelectrolyte swelling through a Donnan-type effect, which is principally driven by an imbalance in the distribution of mobile couterions between the film and the surrounding solution.

The reduced swelling for the mixed pectin/phaseolin films could have two physicochemical origins. First, the phaseolin could act as a cross-linking agent. At the pH of the experiment, phaseolin carries an average net charge of +5.5 (as determined by turbidimetric titration of the protein) (*17*). Second, the replacement of monovalent counterions with a polyvalent counterion would reduce the magnitude of the Donnan effect as the protein is positively charged.

Further evidence of the importance of the protein component in influencing swelling is shown in **Figure 2**, which examines the swelling of the films as a function of applied osmotic pressure when NaCl concentration is kept at 1 M. As shown, swelling increases when osmotic pressure is reduced, with the mixed pectin−phaseolin films having a reduced swelling.

It is worth noting that swelling values at the chosen salt conditions (1 M) are rather low. In fact, pectin films and pectin−phaseolin films reach a swelling degree equivalent to only 2.8 V/V_0 and 2.5 V/V_0, respectively.

The pH dependence of the swelling at a constant osmotic pressure of 0.4 MPa and a salt concentration of 0.02 M is shown in **Figure 3**. As expected, a marked change in swelling occurs over the pH range 2−9, with the effect appearing to approach a plateau at pH 9. Again, it is possible to establish the effect of phaseolin on the swelling due to a protein network into which fennel pectins are entrapped. Reduction of the pH below 5 results in a dramatic decrease in film water sorption. As a matter of fact, at pH 2 fennel pectin films swelling becomes ∼4 V/V_0, while pectin−phaseolin films swelling becomes ∼2 V/V_0. A dependence of swelling on pH is a typical feature of covalently cross-linked polyelectrolytes (*14*). For weakly charged polyelectrolytes, a change in pH which reduces the dissociation of the ionizable groups will reduce the charge on the polymer, and consequently reduce the magnitude of the Donnan effect

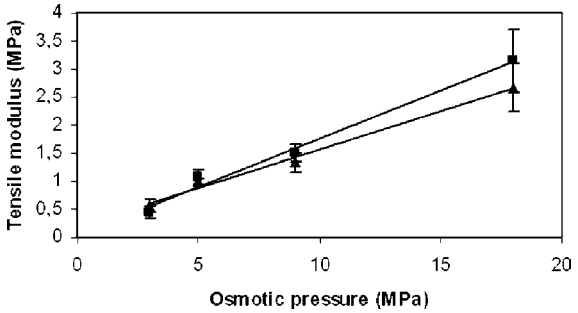

Figure 4. Tensile modulus (MPa) of fennel pectin (▲) and fennel pectin–phaseolin films (■) as a function of osmotic stress.

and lead to reduced swelling. In fact, as the degree of ionization increases (increased system pH), the number of fixed charges augments, resulting in more numerous electrostatic repulsions between the chains. This, in turn, results in extended hydrophilicity of the network, and greater swelling ratios (*14*). On addition of phaseolin, reduction in pH resulted in a more marked decrease in swelling. Such behavior could be explained by the amphoteric property of the protein. In fact, fennel pectins and phaseolin are attracted to each other or repulsed, as the electrostatic charges of pectins and proteins may be opposite or similar depending on the pH (*18*). As the pK_a for the ionization of the carboxyl group of the galacturonic acid of pectins is 3.25, a fall in pH from 5 to 4 would reduce the charge on the polymer and hence the magnitude of the Donnan contribution to cell-wall swelling.

Hence, our results assess the validity of fennel pectins as polyelectrolytes. It is worthy of note that swelling behavior of both fennel pectin and fennel pectin−phaseolin based films are in accordance with results previously reported in the literature. In particular, many research studies have focuses on the study of the dynamic swelling of synthetic (*14*, *19*−*21*) and natural polymers (*15*, *21*−*23*). Some of them report the behavior of natural polymers in the presence of globular proteins (*22*) or peptides (*15*), assessing the importance of these components in conferring higher stability properties to the systems.

Tensile Modulus. Among mechanical properties, the tensile modulus was chosen to characterize fennel films. As expected, for both pectin and pectin−phaseolin films there was a linear relationship between osmotic pressure and tensile modulus (**Figure 4**). However, statistical analysis assesses that tensile modulus value differences between the two kinds of films are not experimentally significant. Thus, the presence of globular protein phaseolin, while is important for decreasing the network hydrophilicity, is ineffective in influencing film stiffness in the conditions chosen in this study. Nevertheless, tensile modulus values observed are in accordance with data reported by Zsivanovitis et al. (*13*), which refer to films made of apple pectins.

Conclusions. With this work, a novel use for both fennel pectins and phaseolin is proposed, in particular, to obtain biopolymer films. In fact, fennel pectins have demonstrated that they possess film-forming properties, while phaseolin is a useful component that confers stability to the obtained novel biopolymer films. As a matter of fact, swelling properties of films containing phaseolin were reduced, limiting their response to moisture, an important property of biopolymer films for assessing application of such films. Moreover, mechanical property studies have also estimated the validity of the obtained novel biopolymer films in terms of mechanical resistance. Permeability properties to gases (i.e., oxygen, carbon dioxide,

and water vapor) could also be investigated to further characterize fennel pectin based films.

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