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Functionality of phosphorylated vicilin exposed to chemical and physical agents

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Abstract

The effects of phosphorylation with sodium trimetaphosphate (STMP) on functional and physicochemical properties of pea vicilin, as probed by high hydrostatic pressure and chemical denaturation were evaluated. The isoelectric point of unmodified and phosphorylated vicilin decreased in the presence of 0.5 M NaCl, resulting in a decrease of the solubility at pH 1.0. The gelation capacity of unmodified vicilin in the presence of NaCl decreased approximately 80% when compared with unmodified vicilin without NaCl. Increasing pressure from 0.1 MPa (atmospheric pressure) to 240 MPa significantly decreased the solubility of vicilin phosphorylated with 4% STMP at pH 1.0 and 4.0 by about 30%. Pressure had no effect on solubility of native vicilin. Pressure treatment at 240 MPa improved the gelation capacity of vicilin phosphorylated with 1% STMP. Glycerol decreased the gelation capacity of vicilin and its solubility in the acidic pH range.

Keywords: *Pisum sativum*; Vicilin; Functional properties; Phosphorylation; STMP

1. Introduction

The use of plant proteins in the formulation of new food products or as a replacement for expensive animal proteins in conventional foods has been the focus of various research efforts. In order to develop plant proteins for use as ingredients in the food industry, there is a need to determine the physicochemical and functional properties of these proteins (Adebowale & Lawal, 2004; Chavan, McKenzie, & Shahidi, 2001; Rangel, Domont, Pedrosa, & Ferreira, 2003). Protein hydration and properties related to hydration such as solubility, water holding capacity, oil binding properties, foaming capacity and stability, emulsion capacity and stability, viscosity, and gelation can have a significant impact on the quality of products destined for the food industry.

Chemical modifications such as phosphorylation, succinylation, glycosylation and acetylation have been used to improve the functional properties of seed proteins for food processing (Krause, 2002; Lawal, 2005). Kunsheng, Yangyang, and Yunxia (2007) showed that soy protein isolate phosphorylated with sodium tripolyphosphate had greater solubility than native protein at pH 3.0 and 4.0. Sodium trimetaphosphate (STMP) is an economical and practical reagent for phosphorylating proteins on a large scale (Matheis & Whitaker, 1984), and has been successful as a means of modifying food proteins (Sung, Chen, Liu, & Su, 1983).

Factors such as pH, salts and solvents affect physicochemical properties and interaction between proteins and can also alter functional properties (Mwasaru, Muhammad, Bakar, & Che Man, 2000; Ragab, Babiker, & Eltinay,

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2004). Sodium chloride (NaCl) is an important ingredient in food formulation due to its ability to enhance flavour, prolong shelf life and solubilize proteins (Gimeno, Astiasaran, & Bello, 1999). High-pressure processing, an emerging technology, is also used to preserve food products. High-pressure modifies macromolecules (including proteins) due to disruption of hydrophobic and electrostatic interactions. The protein conformational changes induced by high-pressure bring about significant changes in their functional properties in food (Bouaouina, Desrumaux, Loisel, & Legrand, 2006; Chapleau & de Lamballerie-Anton, 2003).

The functional properties of vicilin, a major storage protein from pea (*Pisum sativum*) seeds, have been investigated (Rangel et al., 2003); however, a literature survey shows that the separate and combined effects of salt, high-pressure and glycerol on its functional properties have not been explored. Vicilin accounts for up to 35% of the total protein content of the seeds and consists of three major subunits of 50 kDa assembled into a 150 kDa oligomer (Gatehouse, Croy, & Boulter, 1984). This study aims to investigate the effects of phosphorylation with STMP (1% and 4%) on functional properties of pea vicilin exposed to physical (hydrostatic pressure) and chemical (salt and glycerol) agents.

2. Materials and methods

2.1. Materials

Pea (*P. sativum*) seeds were obtained from the Centro Nacional de Pesquisa de Hortaliças, Empresa Brasileira de Pesquisa Agropecuária – EMBRAPA, Brasília, Brazil. Ethylenediaminetetraacetic acid (EDTA), leupeptin, phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI) and pepstatin A were from Sigma–Aldrich (St. Louis, MO, USA). Tris [tris-(hydroxymethyl) aminomethane], boric acid, ammonium sulphate, sodium acetate and sodium citrate were from Reagen (Rio de Janeiro, Brazil). Acetone, glycine and glycerol were from Merck (Darmstadt, Germany). Sodium chloride (NaCl) was from Vetec (Rio de Janeiro, Brazil). Sodium trimetaphosphate (STMP) from Solutia was kindly provided by Dr. V. C. Sgarbieri (ITAL, Campinas, Brazil). All other chemicals were analytical grade.

2.2. Methods

2.2.1. Purification of vicilin

Vicilin was purified from pea seeds as described by Pedrosa and Ferreira (1994) that consists of precipitation at 45% ammonium sulphate prior to isolation of the 75–99% ammonium sulphate pellet. All solutions contained a cocktail of protease inhibitors, including 0.5 μg/mL leupeptin, 0.007 μg/mL pepstatin A, 2 μg/mL PMSF and 0.05 μg/mL SBTI. Vicilin stock solutions (~5 mg/mL) were stored at −18 °C in 50 mM Tris-HCl, pH 10.0. Protein concentration was determined according to Bradford (1976), using bovine serum albumin as a standard.

2.2.2. Protein phosphorylation

Phosphorylation of vicilin was carried out by a modification of the procedure described by Sung et al. (1983). Vicilin (25 mg) was phosphorylated with a solution of STMP (1% or 4%, w/v) in 0.2 M borate buffer, pH 11.0. Reactants were incubated at 37 °C for 3 h, and then dialyzed against 10 L of 10 mM Tris HCl buffer (pH 10.0) for 48 h at 4 °C.

2.2.3. High-pressure treatment

Native or STMP-phosphorylated vicilin (0.5 mg/mL for solubility experiments and 10 mg/mL for gelation capacity) was subjected increasing pressure at a gradient of about 2 MPa/s, and then kept at 240 MPa for 30 min at 23 °C, before descompression to 0.1 MPa (1 atm). After high-pressure treatment, vicilin functional properties were evaluated as described below. Pressure experiments were performed using a high-pressure cell equipped with optical windows (Silva, Silveira, Correa, & Pontes, 1992), manufactured by ISS (Champaign, IL).

2.2.4. Evaluation of functional properties of vicilin

2.2.4.1. Solubility. Solubilities of unmodified or phosphorylated vicilin samples exposed to physical (hydrostatic pressure) or chemical (0.5 M NaCl or 20% glycerol) agents were determined by a modification of the method of Coffmann and Garcia (1977). Protein solutions (0.5 mg/mL) were incubated over a range of pH values (1.0–10.0) using a mixture of buffers (0.1 M each of tris, glycine, sodium acetate and sodium citrate). After 5 min of stirring with a magnetic stirrer at room temperature (25 °C), samples were centrifuged for 15 min at 34,000*g*. Protein concentration in the supernatant was determined according to Bradford (1976) and expressed as a percentage of initial total protein concentration. All analyses were conducted in triplicate with at least three different preparations of vicilin.

2.2.4.2. Gelation capacity. The method of Coffmann and Garcia (1977) was employed with some modifications. For gelation experiments the pH was adjusted to 7.0 with 0.1 M Tris-HCl. Microtubes containing 0.5 mL of unmodified or phosphorylated vicilin (10 mg/mL) exposed to chemical or physical agents were heated for 1 h in a boiling water bath and cooled to 4 °C in an ice bath. After it, the supernatant was discarded and the microtube was weighed. The results are expressed in mg of gellified sample weight. All analyses were conducted in triplicate with at least three different preparations of vicilin.

2.2.5. Statistical analysis

Statistical analysis of the results was done using Prism 4 for Windows (version 4.02) to apply Students' *t*-test. Trends were considered significant when means of compared sets differed at $P < 0.05$.

3. Results and discussion

3.1. Effects of NaCl on solubility

Sodium chloride had significant effects on the solubility of both unmodified and phosphorylated vicilin (Fig. 1). The solubility of vicilin is low in the pH range of 4.0–5.0. The phosphorylation of vicilin with 4% STMP improved its solubility from 27% to 61% at pH 4. The solubility of phosphorylated vicilin with 1% STMP was lower than unmodified vicilin at almost all pH (Fig. 1a). NaCl significantly decreased the solubility of the protein, both unmodified and phosphorylated at pH 1.0. The presence of 0.5 M NaCl led to an increase in vicilin solubility in the pH range of 4.0–5.0, around its isoelectric region (Fig. 1b). The solubility at pH 4.0 increased from 27% to 68% ($P < 0.05$) for unmodified vicilin, 26–59% ($P < 0.05$) for vicilin phosphorylated with 1% STMP, and 61–69% ($P \geqslant 0.05$) for vicilin phosphorylated with 4% STMP. From pH 7.0 to 10.0, the solubility of vicilin was not affected by NaCl. For unmodified and phosphorylated vicilin, solubility in 0.5 M NaCl was maximal at alkaline pH. In 0.5 M NaCl, the lowest solubility was observed at pH 1.0 for unmodified vicilin (33%), followed by vicilin phosphorylated with 1% STMP (38%) and with 4% STMP (37%). Without NaCl at pH 1.0, solubility of the controls was 100%, 85% and 100%, respectively.

Mwasaru et al. (2000) have shown that above pH 4.0, the solubility of pigeonpea and cowpea protein isolates increases with increasing ionic strength; at pH < 4.0, the effect of NaCl diminishes. Our findings are similar to those of previous investigations on the effect of NaCl on the pH dependence of the solubility of protein isolates from beach pea (Chavan et al., 2001) and cowpea (Ragab et al., 2004). The effects of ionic strength on protein solubility presumably involve several phenomena, including electrostatic interactions, solvation, salting-in and salting-out (Kinsella, 1979). Aluko and Yada (1995) have proposed that at low pH, all carboxyl groups are protonated and the protein acquires a net positive charge, resulting in decreased repulsion of the Cl^- ions and enhanced hydrophobic interactions leading to the formation of insoluble aggregates. At high pH values the increased negative charge on the proteins, combined with the salting-in effect of NaCl, serve to dissociate the protein aggregates and thus increase solubility. Vicilin showed good solubility at alkaline pH, and the presence of salts enhanced its solubility at around neutral pH, which is an important characteristic for food formulations, like milk enriched with proteins. Besides the solubility can influence other functional properties such as foaming capacity, emulsion stability and gelation capacity that can interfere in the characteristics of food products (Idouraine, Yensen, & Weber, 1991).

3.2. Effects of NaCl on gelation properties

The effects of ionic strength on gelation capacity of unmodified and phosphorylated vicilin are shown in Fig. 2. Phosphorylation with 1% and 4% STMP caused a decrease of about 80% in the gelation capacity of vicilin ($P < 0.05$), and there was no change when 0.5 M NaCl was added. The gelation capacity of unmodified vicilin in the presence of 0.5 M NaCl decreased approximately 80% when compared with unmodified vicilin without NaCl ($P < 0.05$). Ragab et al. (2004) suggested that gelation is related to the type of protein as well as to the non-protein components and protein solubility. Akintayo, Oshodi, and Esuoso (1999) had earlier reported ionic strength-dependent gelation properties for *Cajanus cajan* protein. In their report, the gelation capacity of flour improved in the presence of 0.5 M NaCl, while it diminished at higher ionic strength (1.0 M NaCl). They attributed improvement in gelation capacity at low ionic strength to enhanced protein solubilisation which created an effective overlapping of the

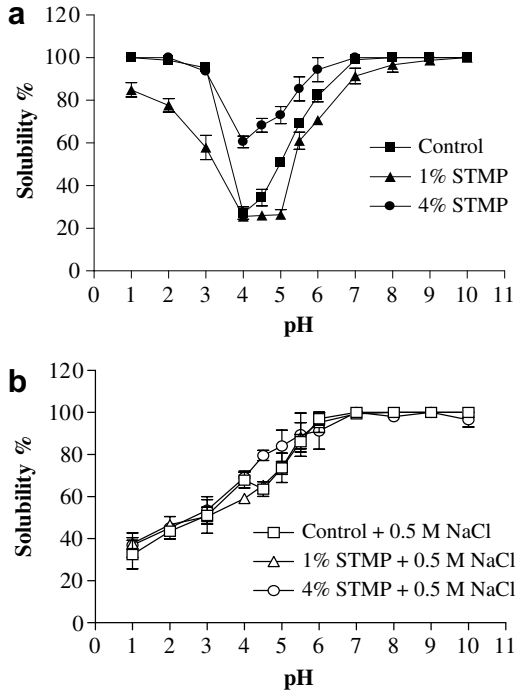

Fig. 1. Effect of NaCl on the solubility of unmodified and phosphorylated vicilin.

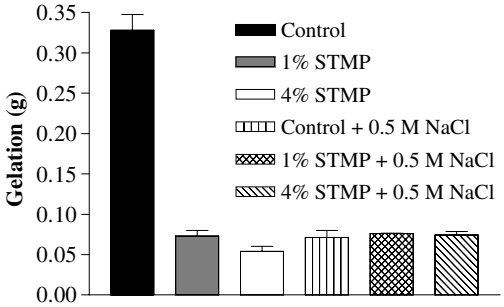

Fig. 2. Effect of NaCl on gelation capacity of unmodified and phosphorylated vicilin.

functional groups between adjacent protein molecules, a condition necessary for a network gel formation. Mwasaru et al. (2000) showed that increasing NaCl from 0.1 to 0.5 M resulted in a significant reduction in the gelation capacity of pigeonpea and cowpea protein isolates. A similar effect was observed by Adebowale and Lawal (2004), who showed that an increase in ionic strength of the protein solution reduced the gelation capacity of mucuna bean flour compared with the control. Arntfield, Murray, and Ismond (1990) reported that optimal network characteristics were obtained with 0.3 M NaCl for ovalbumin and 0.2 M NaCl for faba bean vicilin; they suggested that higher salt concentrations may disrupt the gel network due to the masking of protein charges by the salt. A decrease in the gelation capacity of vicilin in the presence of NaCl may be due to a decrease in protein unfolding induced by heating, limiting access to reactive side groups within the protein molecules, a condition that is necessary for the formation of a three-dimensional network structure as seen in protein gels.

3.3. Effects of high-pressure treatment on solubility

The combined effects of high-pressure treatment and phosphorylation on the solubility of vicilin are shown in Fig. 3. No significant changes were observed in the solubility of vicilin phosphorylated with 1% STMP after treatment at 240 MPa, when compared with Fig. 1a. Pressure treatment significantly decreased the solubility of vicilin phosphorylated with 4% STMP. Its solubility decreased from 100% to 68% at pH 1.0 and from 61% to 44% at pH 4.0. Nevertheless, after pressure treatment the solubility of this phosphorylated vicilin remained higher around its isoelectric point (pH 4.0–5.0) than its control, pressure-treated but not phosphorylated. From pH 7.0 to 10.0, the solubility of the vicilins treated with high-pressure did not change relative to the untreated samples. Chapleau and de Lamballerie-Anton (2003) showed that the solubility of lupin proteins was not affected below 400 MPa. Between 400 and 600 MPa, the proteins lost 25% of their solubility. Molina, Papadopoulou, and Ledward (2001) observed that the solubility of the high-pressure-treated soy protein isolate was affected in a complex way because of differences in the two main globulins (7S and 11S). At 200 MPa, solubility was not affected, but it dropped at 400 MPa. Generally, high-pressure processing induces the rupture of non-covalent interactions within protein oligomers (Silva & Weber, 1993), and this can lead to formation of new complexes between proteins by means of intra- and inter-molecular bonds. A decrease in volume due to the hydration of hydrophobic regions of the protein is usually observed for globular proteins subjected to high-pressure treatment (Collins, Hummer, Quillin, Matthews, & Gruner, 2005; Silva & Weber, 1993). Pressures greater than 200 MPa modify electrostatic and hydrophobic interactions and lead to structural modifications such as protein aggregation induced by changes in tertiary and secondary structures (Masson, 1992). In this scenario, we consider the possibility that an increase in the exposure to solvent of hydrophobic patches of vicilin due to pressure treatment would be responsible for the decrease in solubility.

3.4. Effects of high-pressure treatment on gelation properties

The effects of high-pressure treatment on gelation capacity of unmodified and phosphorylated vicilin are shown in Fig. 4. The gelation properties of unmodified vicilin and vicilin phosphorylated with 4% STMP were not altered by pressure ($P \geqslant 0.05$). Although the treatment at 240 MPa increased the gelation capacity of vicilin phosphorylated with 1% STMP by a factor of two ($P < 0.05$), the maximum phosphorylation of vicilin occurs with 2% STMP (not shown). Probably, when the vicilin is phosphorylated with 1% STMP, many serine and lysine residues are not phosphorylated. These results suggest that the high-pressure treatment increased the gelation capacity due to the presence of these unphosphorylated residues, which contribute less to the increase of repulsive forces within proteins than phosphorylated residues. The gel capacity and the type of gel formed depend on protein concentration, temperature, pH, and the formation and rupture of disulfide bonds and of hydrophobic and electrostatic

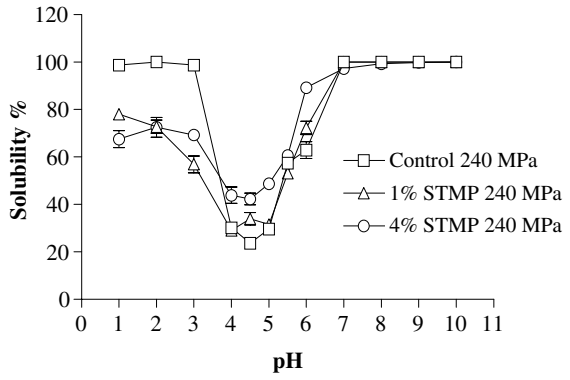

Fig. 3. Effect of high-pressure treatment on the solubility of unmodified and phosphorylated vicilin. 0.1 MPa = 1 atm.

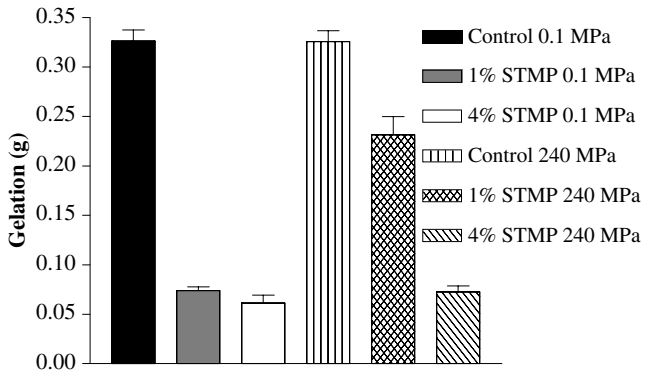

Fig. 4. Effect of high-pressure treatment on gelation capacity of unmodified and phosphorylated vicilin. 0.1 MPa = 1 atm.

interactions (García, Torre, Marina, & Laborda, 1997). Many studies have been carried out to investigate the gels induced by high-pressure (Molina, Defaye, & Ledward, 2002; Zhang, Li, Tatsumi, & Isobe, 2005). Zhang et al. (2005) showed that tofu gel with a coagulant ($CaCl_2$ – calcium chloride) was formed with pressures up to 400 MPa, but these gels displayed very little strength. Molina et al. (2002) observed that the gel network that results from high-pressure treatment of soy 11S subunits involves SH/SS interchange. The –SH residues of the polypeptide chains are exposed during pressure treatment, and after pressure release in the presence of oxygen, they interact to form stable intra- or inter-molecular S–S bonds that help to form the gel matrix. Higher pressures produced gels of greater strength. Heat primarily affects hydrogen-bonded networks, while pressure more effectively disrupts hydrophobic and electrostatic interactions. Thus, pressure treatment before heating improves the gelation capacity, as demonstrated in this work.

3.5. Effects of glycerol on solubility

Fig. 5 shows the effect of 20% glycerol on the solubility of unmodified vicilin and vicilin phosphorylated with 4% STMP ($P < 0.05$). Glycerol decreased vicilin solubility in the acidic range (pH 1.0–3.0). The solubility of vicilin phosphorylated with 4% STMP decreased from 61% without glycerol to 50% with glycerol at pH 4.0, near the isoeletric point of vicilin. In spite of this decrease, the solubility of vicilin phosphorylated with 4% STMP was significantly higher than that of unmodified vicilin. There was no change in vicilin solubility at pH values above 7.0. Glycerol and other polyolic cosolvents have been shown to enhance protein stability (Gekko & Timasheff, 1981; Ruan et al., 2003). It has been proposed that this phenomenon is due to "preferential hydration" of the protein, i.e. exclusion of the cosolvent molecules from the protein surface, which creates a tendency of the protein to minimize its surface (Gekko & Timasheff, 1981). The nonpolar groups on the protein surface would be expected, then, to react unfavorably to contact with the mixed solvent. Surface hydrophobic groups would prefer to migrate into the interior of the protein, out of contact with solvent, in order to relieve this situation. Thus, this could decrease the surface hydrophobicity of the protein (Gekko & Timasheff, 1981). Tolstoguzov (2003) showed that the aggregation of insoluble complex particles is mainly due to electrostatic and hydrophobic interactions. The decrease in solubility of vicilin in the acidic range (pH < 7.0) observed in our study may be due to an increase in hydrophobic interactions because of the decrease in surface hydrophobicity. In addition, the preferential binding data indicate that the chemical potential of a protein (or its activity coefficient) increases with increasing glycerol concentration. An increase in the activity coefficient of a solute corresponds to a decrease in its concentration at constant activity or a decrease in its solubility (Gekko & Timasheff, 1981). A detailed mechanistic explanation of the effects of glycerol on the structural and functional properties of vicilin will require further investigation.

3.6. Effects of glycerol on gelation properties

The gelation capacity of vicilin in the presence of glycerol is presented in Fig. 6, which shows that glycerol significantly decreased the gelation of both unmodified and phosphorylated vicilin. Gelation mechanism and gel appearance are fundamentally controlled by the balance between attractive hydrophobic interactions and repulsive electrostatic interactions. The repulsive forces are due to surface charges and the attractive forces are due to various functional groups exposed by the thermal unfolding of the protein (Kojima & Nakamura, 1985). Denaturation, or unfolding, involves an increase in the surface of contact between protein and solvent, and in particular exposes additional hydrophobic residues to contact with the solvent. Glycerol would increase the thermodynamically unfavorable situation and require the use of more free energy for unfolding than in water. As a result, the presence of glycerol would tend to favor the more folded, or native, state (Gekko & Timasheff, 1981). Thus, like NaCl, glycerol would decrease protein unfolding, limiting access to reactive side groups within the protein molecules, which

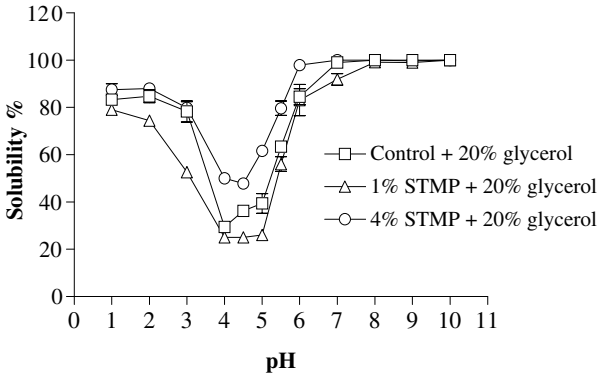

Fig. 5. Effect of glycerol on the solubility of unmodified and phosphorylated vicilin.

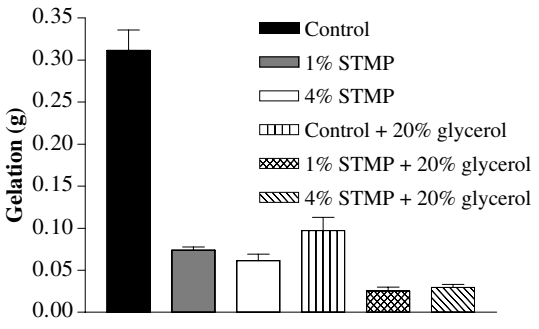

Fig. 6. Effect of glycerol on gelation capacity of unmodified and phosphorylated vicilin.

is a condition necessary for the formation of protein gels. It is important to emphasize that this is the first study of the effect of glycerol on functional properties of vicilin.

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