Effects of Glycosylation on Functional Properties of Vicilin, the 7S Storage Globulin from Pea (*Pisum sativum*)

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Functional properties (solubility, foaming, and emulsifying capacities) of neoglycosylated derivatives of pea vicilin were investigated and compared with unmodified vicilin. Glysosylation was achieved by reductive alkylation of ϵ-amino groups of lysine residues using different carbohydrates (lactose, galactose, glucose, or galacturonic acid). Levels of carbohydrate incorporation ranged from 5 to 13 mol of carbohydrate/mol of vicilin. Solubilities of glycosylated derivatives (notably derivatives modified with glucose or galacturonic acid) were significantly improved in the pH range 5−6 compared to unmodified vicilin. Emulsifying capacity and emulsion stability were also significantly increased in glycosylated vicilin derivatives. Modification with galacturonic acid was the most effective in increasing both emulsifying capacity and stability. Derivatives containing lactose or galactose displayed enhanced foaming capacity relative to unmodified vicilin. However, foam stability was found to decrease by glycosylation of the protein. These results indicate that glycosylation can be used as an efficient method to improve emulsifying capacity and emulsion stability of vicilin.

Keywords: *Vicilin; storage globulins; glycosylation; Pisum sativum*

INTRODUCTION

With the growth of world population there will be an increased demand for direct consumption of plant products in foods. Food ingredients enriched with plant proteins should at the same time be of improved nutritional value and be attractive for consumption. These developments will require proteins with multiple functional properties (Kinsella, 1979), such as good solubilities, emulsifying, gelling, and/or foaming capacities. Functional properties are directly dependent on the physicochemical properties and structures of proteins. Thus, in order to understand and develop strategies for rational modification of functional properties of proteins it is necessary to investigate in detail their structural features, as well as their interactions with solvent and other solutes in different environmental conditions.

Seeds are an abundant and relatively inexpensive source of proteins. In the past two decades, there has been considerable interest in investigating structure−function relationships in seed proteins to be used in the food industry (Kinsella et al., 1979; Wright and Bumstead, 1984; Wright, 1985; Caer et al., 1990; Chambers et al., 1990; Baniel et al., 1992; Colas et al., 1993; Subirade et al., 1994). Studies involving chemical modification of seed storage proteins have revealed variable extents of structural changes induced by derivatization (Brinegar and Kinsella, 1980; Schwenke et al., 1983, 1986, 1990; Prahl and Schwenke, 1986; Kim and Rhee, 1990; Gueguen et al., 1990; Caer et al., 1990; Larré et al., 1992). Several of these studies examined acylation reactions and showed significant structural changes involving partial unfolding/subunit dissociation of the proteins. More recently, covalent attachment of carbohydrate to seed globulins was investigated as a more gentle means to modify their functional properties while preserving their overall structures (Caer et al., 1990; Baniel et al., 1992; Colas et al., 1993; De Felice et al., 1995). Previous studies of glycosylation of proteins of nonvegetable origin such as β-lactoglobulin and casein revealed improved solubilities of the modified proteins (Waniska and Kinsella, 1984; Kitabatake et al., 1985; Courthaudon et al., 1989). Glycosylation may cause changes in hydrophilic/hydrophobic balance or net charge at the protein surface. These changes, in turn, may affect protein−protein or protein−water interactions, producing significant changes in solution and interfacial properties of glycosylated proteins. Baniel et al. (1992) and Colas et al. (1993) reported a detailed investigation of functional properties (solubility, viscosity, foaming and emulsifying capacities) of glycosylated derivatives of pea legumin. These authors concluded that glycosylation improved the functional properties of legumin, and the degree of improvement depended on the specific carbohydrate bound to the protein.

In this work we present an investigation of the effects of glycosylation on the functional properties of vicilin, a major storage protein from pea (*Pisum sativum*) seeds. Vicilin corresponds to 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 et al., 1984; Bewley and Black, 1985). Some of the 50 kDa subunits are nicked by proteolysis soon after biosynthesis (Gatehouse et al., 1982, 1983; Lycett et al., 1983), and the resulting peptides (ranging from 12.5 to 33 kDa) remain associated with intact subunits in the native 150 kDa oligomer (Gatehouse et al., 1982). We have recently reported a thermodynamic characterization of protein−protein and protein−solvent interactions of vicilin (Pedrosa and Ferreira, 1994). In order to investigate in more detail correlations between structure and functional properties we have prepared and characterized glycosylated vicilin derivatives (De Felice et al., 1995). Here, we report on the solubilities, emulsifying and foaming capacities of neoglycovicilins,

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S0021-8561(96)00815-1 CCC: $14.00

and these functional properties are discussed in terms of the potential usefulness of glycosylation to improve functionality of seed proteins.

MATERIALS AND METHODS

Materials. Vicilin was purified from pea seeds as described by Pedrosa and Ferreira (1994) and stored in 50 mM ammonium bicarbonate, pH 8, at 4 °C. Sodium cyanoborohydride, carbohydrates, galacturonic acid, and *n*-dodecane were from Sigma (St. Louis, MO). [^{14}C]Carbohydrates were purchased from New England Nuclear (Boston, MA).

Protein Glycosylation. Glycosylation of vicilin was carried out by reductive alkylation by a modification of the procedure described by Caer et al. (1990). Reactions were performed at 37 °C for 50 h in 6.5 mL of 0.1 M sodium phosphate buffer (pH 8.0) containing 7.8 mg of vicilin, 30 mg of sodium cyanoborohydride, and 50 mg of carbohydrate or galaturonic acid. After the reactions, samples were dialyzed against 50 mM ammonium bicarbonate buffer (pH 8.0) for 48 h.

Levels of incorporation of glucose, lactose, or galactose to vicilin were determined using [^{14}C]carbohydrates followed by liquid scintillation counting of dialyzed samples. Incorporation of galacturonic acid was measured fluorometrically by monitoring formaldehyde released upon periodate oxidation of carbohydrate moieties by the Hantzsch reaction (Nash, 1953), as described by Gallop et al. (1981).

Solubility. Solubilities of unmodified or neoglycosylated vicilin samples were determined by a modification of the method of Coffmann and Garcia (1977). Protein was diluted in distilled water at a concentration of 0.5 mg/mL in a final volume of 5 mL. Then the pH of the solution was carefully adjusted in the range 2−11 (at steps of 1 pH unit) by adding 0.1 N HCl or 0.1 N NaOH. Identical pH values were used for unmodified or neoglycosylated vicilin samples. After 60 min of stirring (with a magnetic stirrer at room temperature), the pH of the solutions was again measured and samples were centrifuged for 15 min at 34 000*g*. Protein concentration in the supernatants was determined according to Lowry et al. (1951) and expressed as a percentage of initial total protein concentration.

Emulsifying Properties. Emulsions of unmodified or glycosylated vicilin were prepared as described by Dagorn-Scaviner et al. (1987) by homogenizing 2 mL of *n*-dodecane with 6 mL of protein solution (1.3 mg protein/mL in 50 mM ammonium bicarbonate, pH 8.0), using a Polytron (Brinkmann Instruments, Westbury, NY) PT 10−35 (30 s at 20 000 rpm).

Emulsifying Activity Index. The EAI was estimated according to Pearce and Kinsella (1978). The turbity T ($T = 2.303A/l$; A, absorbance of the emulsion; l, 1 cm, path length of the cuvette) of the emulsion, diluted 250-fold with a solution of 0.1% SDS, 0.1 M NaCl, pH 7.0, was immediately measured at 500 nm. EAI is defined as

$$\mathrm{EAI} = 2T/\phi C \qquad (1)$$

where ϕ is the volume fraction of oil phase (here $\phi = 0.25$) and C is the protein concentration in the aqueous phase. EAI, expressed in m^2/g, is related to the stabilized interfacial area per unit weight of protein (Dagorn-Scaviner et al., 1987; Baniel et al., 1992).

Emulsion Stability. This was determined by following the separation of aqueous phase during aging of the emulsion (Dagorn-Scaviner et al., 1987). Immediately after preparation, the emulsion (8 mL) was poured into a graduated cylinder and destabilization was followed by plotting the volume of separated aqueous phase (V_t) as a function of time (t). According to Dagorn-Scaviner et al. (1987) destabilization can be considered a succession of first-order kinetics which can be analyzed by plotting ln $V_e/(V_e - V_t)$ vs t, V_e being the equilibrium value of V_t after 24 h (Baniel et al., 1992). Each kinetic step is characterized by its rate constant α and duration Δt.

Foaming Properties. These were expressed as foaming capacity and foam stability (Gueguen et al., 1990). Foaming was induced by bubbling a stream of nitrogen (2.5 mL/s) during 15 s through a plastic Millipore filter holder placed at the bottom of a pyrex column containing 3 mL of protein solution (1.0 mg/mL in 50 mM ammonium bicarbonate, pH 8.0). Conductivity of the resulting foam was recorded using a conductivity meter (Cole-Parmer Instrument Co., Chicago, IL). Foam capacity was evaluated by the initial conductivity (C_i, μS/cm). Foam stability was expressed as (1) $t_{1/2}$, the time necessary to obtain a conductivity of $C_i/2$ measured on the experimental curve and (2) the foam stability index (FSI), defined by Kato et al. (1983) as

$$\mathrm{FSI} = C_o \Delta C/\Delta t \ (\mathrm{min}) \qquad (2)$$

where C_o is the conductivity at zero time obtained by extrapolation of the linear part of the C vs t plot and $\Delta C/\Delta t$ is the slope of this linear region. $t_{1/2}$ is related to drainage of liquid which occurs just after foam formation, while FSI describes the stability of the foam when most of the drainage has occurred and is mostly related to coalescence (Kato et al., 1983; Baniel et al., 1992).

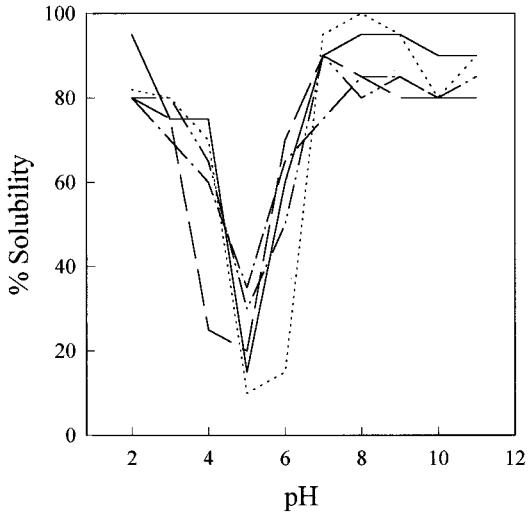

Figure 1. pH dependence of the solubility of native vicilin and glycosylated derivatives. (···) unmodified vicilin; (—··—) galactosylated derivative; (—) lactosylated derivative; (—·—) glucosylated derivative; (- - -) galacturonic acid derivative. Samples contained 0.5 mg of protein/mL in distilled water at the indicated pH values. Values represent averages of five determinations with different preparations of vicilin. Standard deviations ranged from 5% to 25% for all data points.

RESULTS

Vicilin Glycosylation. Modification of vicilin with glucose, lactose, or galactose was determined using [^{14}C]-carbohydrates and measuring covalently bound radioactivity by liquid scintillation. Under our experimental conditions, incorporation levels of 13.4 ± 3.0, 11.1 ± 3.6, and 4.7 ± 0.3 mol of carbohydrate/mol of vicilin (expressed as the 150 kDa oligomer) were obtained with glucose, galactose, and lactose, respectively. Modification with galacturonic acid was measured fluorometrically and found to be 7.5 ± 4.2 mol/mol of vicilin.

Effect of Glycosylation on the Solubility of Vicilin. Solubilities of neoglycovicilins in aqueous solution were measured and compared to unmodified vicilin. Figure 1 shows solubilities as a function of pH in the range 2−11. The solubility of native vicilin displayed a sharp pH dependence, being very poor at pH 5−6 and increasing markedly below or above these values. Glycosylated samples exhibited similar pH profiles, but significantly higher solubilities than native vicilin at pH 5−6: at pH 6, for example, the solubilities of galacturonic acid or glucose derivatives averaged

Table 1. Emulsifying Properties of Neoglycovicilins[a]

			first phase		second phase	
exogenous carbohydrate	EAI (m^2/g)	V_e (mL)	Δt_1 (min)	α_1 (10^{-4} s^{-1})	Δt_2 (min)	α_2 (10^{-4} s^{-1})
unmodified vicilin	85 ± 3	4.9 ± 0.3	5.1 ± 0.3	27 ± 2	11 ± 2.5	7.0 ± 0.2
lactose	142 ± 7	4.3 ± 0.4	5.5 ± 0.9	19 ± 3	14 ± 0.1	4.1 ± 0.4
glucose	68 ± 3	4.0 ± 0.3	5.3 ± 0.3	36 ± 1	16 ± 0.1	4.2 ± 0.2
galactose	157 ± 3	4.1 ± 0.4	5.5 ± 0.1	29 ± 1	16 ± 0.9	5.6 ± 0.3
galacturonic acid	180 ± 8	4.3 ± 0.3	10 ± 0.1	16 ± 6	35 ± 0.1	4.1 ± 0.1

[a] Emulsions were prepared using unmodified vicilin or vicilin modified with the indicated exogenous carbohydrates. EAI, emulsifying activity index measured at 1.3 mg of protein/mL; V_e, aqueous phase volume at equilibrium; Δt_i, duration of kinetic phase i; α_i, rate constant for phase i.

about 70% and 65%, respectively, compared to 15% for unmodified vicilin (Figure 1). In addition to improving solubility, glycosylation caused an acid shift of the solubility profile (relative to unmodified vicilin), possibly reflecting the decrease in positive charge density of the protein by derivatization of lysine residues. This effect was more pronounced with galacturonic acid, which shifted the insolubility range to pH 4−5 (likely due to the incorporation of additional carboxylate groups to the protein).

Emulsifying Properties of Neoglycovicilins. Emulsifying capacity and emulsion stability were determined for glycosylated vicilin samples. Emulsifying capacity was expressed in terms of the emulsifying activity index (EAI), which measures the ability of a protein to help dispersion of an oil phase into an aqueous medium (Dagorn-Scaviner et al., 1987; Baniel et al., 1992). Native vicilin displayed an EAI of 85 m^2/g (Table 1), whereas lactosylated, galactosylated, and notably galacturonic acid vicilin derivatives had significantly higher EAI than native vicilin (at confidence levels $p < 0.001$ in Student's *t*-tests; Snedecor, 1962).

For all derivatives, the volume of separated aqueous phase at equilibrium (V_e) was smaller ($p < 0.025$) than the volume for unmodified vicilin (Table 1), indicating increased emulsion stability with glycosylated vicilin samples. Direct measurements of emulsion stability were carried out by following the kinetics of separation of aqueous phase following vigorous stirring of the emulsion. Figure 2 shows the kinetics obtained for unmodified and glycosylated vicilin samples. In the absence of added vicilin, the emulsion became rapidly unstable, and an equilibrium value of separated aqueous phase of 5.3 mL was achieved within 3 min (data not shown). Addition of unmodified vicilin conferred some stability to the emulsion, and glycosylated derivatives provided further stabilization (Figure 2). The kinetics of emulsion destabilization were analyzed in terms of sums of exponential phases. For unmodified vicilin and glycosylated derivatives the kinetics could be well described in terms of two consecutive exponential phases, each described by a characteristic rate constant (α) and duration (Δt) (Table 1). Examination of the fit parameters obtained for the first kinetic phase indicated that lactosylated and galacturonic acid derivatives had significantly lower rate constants than unmodified vicilin ($p < 0.01$ in Student's *t*-test). On the other hand, modification with glucose led to a significant ($p < 0.001$) increase in rate constant (α_1) for emulsion destabilization, whereas modification with galactose did not have much effect (Table 1). A significant increase ($p < 0.001$) in duration of the first kinetic phase was also observed with the galacturonic acid derivative. For the second, slower kinetic phase the effect of glycosylation was more marked (Table 1), with a significant decrease in rate constant observed for all neoglycovicilins ($p < 0.001$). Furthermore, the duration of the second phase was also extended in glycosylated samples relative to unmodified vicilin ($p < 0.001$). Taken together, these results indicate that glycosylation resulted in an increase in the capacity of vicilin to stabilize an oil-in-water emulsion.

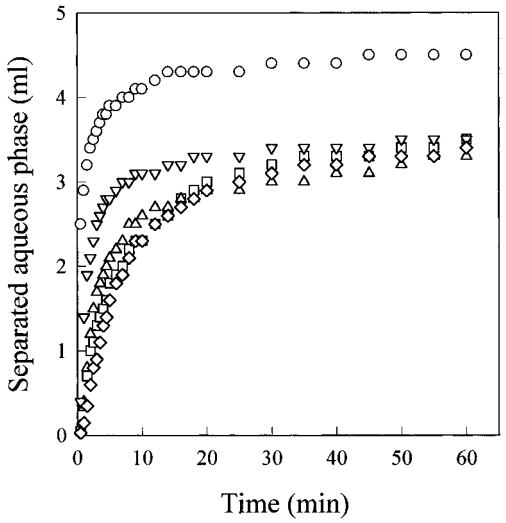

Figure 2. Kinetics of emulsion destabilization. Emulsions of *n*-dodecane in aqueous buffer were prepared and the volume of separated aqueous phase was measured at different time intervals. (○) Unmodified vicilin; (△) galactosylated derivative; (□) lactosylated derivative; (▽) glucosylated derivative; (◇) galacturonic acid derivative. Symbols represent averages of four experiments using different preparations of vicilin, and standard deviations ranged from 0.25 to 0.65 mL for all data points.

Table 2. Foaming Properties of Neoglycovicilins[a]

exogenous carbohydrate	C_i (μS/cm)	$t_{1/2}$ (s)	FSI (min)
unmodified vicilin	1300 ± 206	115 ± 10	120 ± 1
lactose	2376 ± 250	35 ± 5	43 ± 3
glucose	1240 ± 120	55 ± 5	85 ± 1
galactose	1750 ± 218	50 ± 5	53 ± 2
galacturonic acid	1490 ± 218	80 ± 6	97 ± 2

[a] Foaming was induced by bubbling a stream of nitrogen in a solution containing 1.0 mg of protein/mL. C_i, initial conductivity; $t_{1/2}$, time corresponding to $C_i/2$; FSI, foam stability index.

Foaming. Foaming capacity and foam stability were compared for unmodified vicilin and neoglycovicilins. Foam capacity was estimated from the value of initial conductivity (C_i) measured immediately following foam formation by bubbling gas into the protein solution (Kato et al., 1983). Table 2 shows that the initial conductivity was significantly higher ($p < 0.001$) for the lactosylated derivative, and somewhat higher for the galactosylated derivative than for unmodified vicilin. Both galacturonic acid and glucosylated derivatives had initial conductivities similar to control vicilin. The kinetics of foam destabilization were also examined for unmodified and glycosylated vicilin samples (Figure 3).

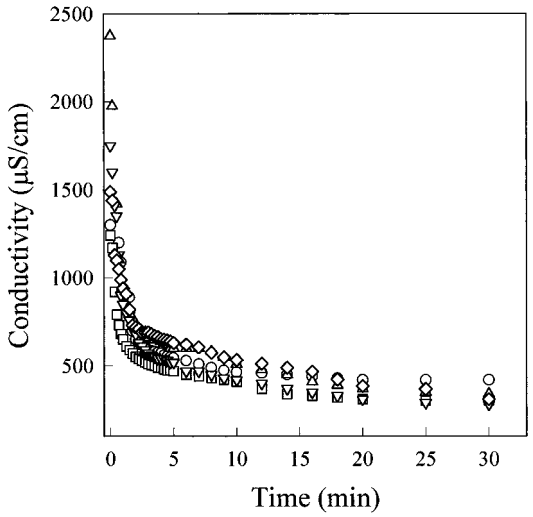

Figure 3. Kinetics of foam destabilization. Foaming of protein solutions was induced and conductivities were measured at different times after foaming. (○) Unmodified vicilin; (▽) galactosylated derivative; (△) lactosylated derivative; (□) glucosylated derivative; (◇) galacturonic acid derivative. Symbols represent averages of four experiments using different preparations of vicilin, and standard deviations ranged from 26 to 41 μS/cm for all data points.

For all neoglycovicilins, reduced $t_{1/2}$ values were found relative to unmodified vicilin (Table 2), suggesting that they were less effective in stabilizing foam. This was further indicated by the decrease in foam stability index (FSI; Table 2) observed with neoglycosylated samples ($p < 0.001$).

DISCUSSION

The increased research activity on seed globulins in recent years can be partly explained by their important roles in the food economy of the world. Recurrent forecasts of increased usage of vegetable protein as an alternative for animal proteins (Kinsella, 1979; Wright, 1985) have brought considerable attention to the functional properties of those proteins which are of interest to the food industry. As pointed out by Wright (1985), utilization of seed globulins as functional agents will depend on a number of factors, among which are whether our understanding of their properties is sufficient to predict their behavior in food products and whether the seed globulins can meet a range of desired functional properties. Clearly, understanding the functional properties of seed globulins requires detailed understanding of their underlying physicochemical properties and structure−function relationships. This should also lead to development of strategies for manipulation or modification of their physicochemical and functional properties.

The results reported here indicate that modification of vicilin amino groups with neutral or charged carbohydrates improved the functional properties of the protein. Levels of incorporation averaged 13.4, 11.1, 4.7, and 7.5 mol of carbohydrate/mol of vicilin with glucose, galactose, lactose, and galacturonic acid, respectively. These are relatively low values compared to the total 93 amino groups of vicilin (90 lysine residues + 3 N-terminal residues per trimer; Watson et al., 1988). These low levels of modification probably do not cause major conformational/structural changes of vicilin. Indeed, circular dichroism and fluorescence spectroscopic studies of glycosylated vicilin derivatives revealed structural preservation of the protein (Pedrosa, Trisciuzzi, De Felice, Tetin, and Ferreira, manuscript in preparation). Furthermore, the quaternary structure of glycosylated vicilin derivatives was also preserved, and intersubunit interactions appear stronger in glycosylated samples than in unmodified vicilin (De Felice et al., 1995). These observations indicate that changes in functional properties upon glycosylation are not caused by partial unfolding or structural alterations and are probably related to changes in protein−solvent and protein−protein interactions.

In order to obtain optimum functionality in applications where emulsifying or foaming activities are required, a highly soluble protein is desired (Kinsella, 1979). Therefore, it seemed of interest to examine the effects of carbohydrate incorporation on the solubility of vicilin. All neoglycosylated vicilin derivatives exhibited higher solubilities than unmodified vicilin, specially at pH 6 (i.e, near the isoelectric point; confidence levels $p < 0.001$). A previous study on neoglycosylated casein has shown increased solubility relative to unmodified protein (Courthaudon et al., 1989). To our knowledge, the only other seed globulin which has been studied in neoglycosylated form is legumin, the 11S storage protein from pea (Caer et al., 1990; Baniel et al., 1992; Colas et al., 1993). These authors showed that glycosylation also increased the solubility of legumin.

We have also examined the ability of glycosylated vicilin to enhance formation and stabilization of emulsions. Oil-in-water emulsions are thermodynamically unstable, and stabilization of emulsified droplets is achieved by formation of a layer of hydrophilic or charged solutes (e.g., protein) around the droplets. This effect may be enhanced by formation of an effective hydration layer around the interfacial material. The interfacial properties of proteins depend on their abilities to diffuse to the oil/water interface, and then undergo partial unfolding and rearrangement at the interface. Improved emulsifying capacities (as evidenced by higher EAI) were obtained with vicilin modified with lactose and, notably, galactose and galacturonic acid. For the latter two derivatives, an approximately 2-fold increase in EAI was found relative to unmodified vicilin (Table 1). The stability of emulsions formed in the presence of neoglycovicilins was also greater than emulsions formed with unmodified vicilin: the total volume of separated aqueous phase at equilibrium (V_e) was smaller ($p < 0.025$) and the kinetics of emulsion destabilization were slower with glycosylated samples than with native vicilin (Table 1). These effects were specially marked with galacturonic acid derivatives, and are possibly explained by increased capacity to form a heavily hydrated interfacial layer due to the increased density of hydrophilic and/or charged groups at the protein surface.

Dagorn-Scaviner et al. (1987) investigated the emulsifying properties of both pea legumin and vicilin as well as of mixtures of the two globulins. These authors concluded that vicilin was significantly more effective than legumin in terms of its emulsifying capacity and emulsion stabilization, which may be partly due to the lower molecular weight (and thus higher diffusion coefficient) of vicilin. It is interesting to note that Dagorn-Scaviner et al. (1987) reported an EAI of 111 m^2/g for vicilin, whereas the value we found was 85 m^2/g. However, as pointed out by Pearce and Kinsella (1978), the EAI depends on the exact procedures and equipment used to produce the emulsion. More re-

cently, an investigation of the emulsifying capacity of glycosylated legumin derivatives showed that these did not present improved performances relative to unmodified legumin (Baniel et al., 1992). Nevertheless, glycosylated legumin appeared somewhat more effective than native legumin in stabilizing emulsions (Baniel et al., 1992).

Foaming reflects the capacity of proteins to form stable layers surrounding gas droplets in a liquid phase. Proteins with good foaming properties should be soluble in the aqueous phase, diffuse and concentrate at the air/water interface, partially unfold to form a cohesive layer around the gas bubbles, and possess sufficient viscosity and mechanical strength to prevent rupture and coalescence (Kinsella, 1979). Vicilin derivatives with galactose and lactose exhibited significantly higher ($p < 0.012$) foaming capacities than unmodified vicilin (Table 2), whereas modification with glucose or galacturonic acid had no effect on foaming capacity. This is an intriguing finding, because soy protein solubility appears closely related to foaming (Kinsella, 1979), and glucose and galacturonic acid modification were very effective in increasing the solubility of vicilin (Figure 1). The stability of foams was decreased by neoglycosylation of vicilin (as indicated by the decrease in halftime for foam destabilization and by FSI; Figure 3 and Table 2). For glycosylated legumin derivatives (Baniel et al., 1992) higher foaming capacities and stability have been reported relative to unmodified legumin.

In conclusion, our results show that glycosylation appears useful to enhance functional properties of vicilin, namely, solubility (specially in the acid pH range), emulsifying capacity, emulsion stability, and foaming capacity. However, foam stability was decreased by glycosylation, which may not be desirable for certain types of applications. The improvement of functional properties does not appear related to significant structural changes, but rather to changes in protein−protein and protein−water interactions. In conjunction with other studies of chemical modification of seed globulins by acylation or carbohydrate incorporation (Schwenke et al., 1983, 1986; Prahl and Schwenke, 1986; Caer et al., 1990; Baniel et al., 1992; Colas et al., 1993; De Felice et al., 1995), our results suggest that neoglycosylated storage globulins may find interesting applications as functional agents in the food industry.

ABBREVIATIONS USED

EAI, emulsifying activity index; FSI, foam stability index; SDS, sodium dodecyl sulfate.

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Received for review October 25, 1996. Accepted February 27, 1997.[⊗] This work was supported by grants from Financiadora de Estudos e Projetos (FINEP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/PADCT), and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ).

JF960815K

[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1997.