Phosphorylation of the 12S Globulin from Rapeseed (*Brassica napus* L.) by Phosphorous Oxychloride: Chemical and Conformational Aspects

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The effect of progressive phosphorylation by phosphorous oxychloride upon the conformation of the 300 kDa storage protein (cruciferin) from rapeseed has been studied using chemical analysis, SDS−PAGE, HPLC, analytical ultracentrifugation, viscometry, fluorescence spectroscopy, and hydrophobicity measurement. The amount of phosphorous in the protein increased with the excess of phosphorous oxychloride and the pH of reaction. The bulk of phosphorus was only loosely bound to the protein and was removed by washing with cold perchloric acid. The more stably bound phosphorus groups after reaction at pH 8 were found to be nearly equally attached to amino and hydroxyl groups, whereas phosphorylation at pH 10−11 led to predominant O-phosphorylation as detected by studying the acid- and alkali-lability of the protein−phosphorous bonds. A 50 kDa component appeared as a product of covalent cross-linking of the constituent α- and β-polypeptide chains. A 2.5S fraction appeared as the main product of dissociation, which takes place after a critical step of modification. The higher the extent of phosphorylation, the larger was the percentage of higher molecular weight products, the percentage of which was most significant after modification under strongly alkaline conditions. They may be attributed both to products of chemical cross-linking and to noncovalently linked aggregates formed by interactions of partially unfolded derivatives exhibiting an increased surface hydrophobicity.

Keywords: *Rapeseed globulin; phosphorylation; conformational changes; protein cross-linking*

INTRODUCTION

Phosphorylation has been recently practiced as a useful tool for improving the functional properties of food proteins (Matheis and Whitaker, 1984; Frank, 1987; Shih, 1992). Taking into account the special physicochemical and functional properties of naturally occurring phosphoproteins such as casein, the phosphorylated proteins are expected to acquire characteristics of the latter, that is, improved surface functional properties and increased gel-forming capacity in the presence of calcium ions.

Both enzymatic and chemical methods have been used to phosphorylate food proteins (Shih et al., 1992; Schwenke, 1997). Enzymatic phosphorylation is highly specific and, thus, limited to the consensus sequences to be recognized by the applied kinases (Kennelly and Krebs, 1991). Moreover, conformational constraints in globular proteins can prevent potent phosphorylation sites from being enzymatically phosphorylated (Chardot et al., 1998).

To achieve high degrees of phosphorylation in proteins, chemical methods, which nonspecifically phosphorylate functional groups, are best suited. From the various phosphorylating compounds proposed to introduce phosphoryl residues in food proteins (Matheis and Whitaker, 1984), phosphorous oxychloride has been shown to be one of the most suitable reagents (Matheis, 1991). It has been used to phosphorylate a number of food proteins both of animal origin, such as casein and lysozyme (Matheis et al., 1983) and β-lactoglobulin (Woo and Richardson, 1983), and of plant origin, such as zein (Chobert et al., 1987) and soy proteins (Hirotsuka et al., 1984; Shih, 1993). The latter papers described the efficient improvement of functional properties of globular plant food proteins by phosphorylation. However, knowledge about the conformational changes in these type of proteins induced by the phosphorylation is lacking. This information is a prerequisite to derive structure−functionality relationships.

Storage proteins from oil seeds and grain legumes are the main components in plant protein isolates. They have a globular oligomeric structure, which undergoes a dissociation and unfolding upon chemical modification by acylation (Schwenke et al., 1990). Corresponding physicochemical changes may occur after phosphorylation. Additionally, phosphorylation by phosphorous oxychloride can induce protein cross-linking (Matheis, 1991). It was, therefore, the aim of the present work to study the chemical and conformational changes of a typical plant storage protein after modification by phosphorous oxychloride under various conditions. Cruciferin, the major storage protein in rapeseed, was used for this study. It is a typical hexameric 12S protein with a molecular mass of 300 kDa (Schwenke et al., 1983).

Hydrodynamic and spectroscopic methods were used besides SDS−PAGE and HPLC to follow the conformational changes of cruciferin after successive phosphorylation.

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MATERIALS AND METHODS

Materials. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was purchased from Sigma, Deisenhofen, Germany; 1-anilino-8-naphthalenesulfonic acid (ANS) was from Serva, Heidelberg, Germany, and buffer substances were from Merck, Darmstadt, Germany. All physicochemical experiments were carried out in standard phosphate buffer, pH 8 (0.05 M phosphate, sodium chloride, $I = 0.5$).

Methods. *Protein Preparation.* Rapeseed flour (*Brassica napus* var. *Lirajet*) was prepared from dehulled meal by defatting with hexane following extraction with diethyl ether and screening (particle size > 315 μm). A micelle protein isolate was prepared by extraction with 0.15 M sodium chloride following concentration by ultrafiltration, dilution by water, and precipitation in cold storage as described by Ismond and Welsh (1992). The micelle isolate, which mainly consisted of cruciferin, was purified by means of ion-exchange chromatography on DEAE-Sepharose CL6B (0.05 M Tris/HCl, pH 8.0, sodium chloride gradient, 0−2 M), which separated small amounts of a low molecular weight protein fraction napin (Schwenke et al., 1973).

Protein Determination. The protein content was determined using a microbiuret method (Itzhaki and Gill, 1964). Native cruciferin was used for calibration, the protein content of which was determined using the Kjeldahl nitrogen content and the experimentally determined nitrogen−protein conversion factor amounting to 5.70 (Schwenke et al., 1981).

Determination of Phytic Acid and Phosphorus. The content of phytic acid was determined by a modified version of the method described by Rounds and Nielsen (1993). The content of total phosphorus was determined after mineralization with sulfuric acid by means of a phosphomolybdate reaction using a commercial cuvette test (LCK 350, Dr. Lange, Düsseldorf, Germany).

Phosphorylation of the Protein and Determination of Variously Bound Phosphorus. Phosphorylation was performed according to the method of Matheis and Whitaker by dropwise addition of a solution of $POCl_3$ in CCl_4 to a solution of the protein in water (pH 8 or 10) under continuous stirring and ice cooling. The molar ratio of $POCl_3$ to protein varied from 500 to 5000 and the temperature of reaction between 3 and 20 °C. The pH was kept between 8 and 9 and between 10 and 11, respectively, by adding 5 N NaOH simultaneously. Both the CCl_4 and the precipitate formed between the organic layer and the aqueous layer were separated by centrifugation after finishing the reaction and the obtained protein solution was exhaustively dialyzed against 0.1 M KCl and then against H_2O (4−6 °C). Phosphorus analysis was performed in the freeze-dried samples after various treatments as described by Weller (1979) as follows:

To remove inorganic phosphorus, phospholipids, and phytic acid, the phosphorylated protein was successively washed with 0.5 M perchloride acid, ethanol/diethyl ether, and chloroform/methanol at 2−4 °C. The phosphorus bound to the protein after this washing was designated chemically bound phosphorus. Acid-labile phosphorus was determined after 20 h of incubation with 1 N HCl and alkali-labile phosphorus after 20 h of incubation with 1 N NaOH at 37 °C. Shortened names of the phosphorylated cruciferin (C-P) samples are given in the tables and figures, mentioning the pH of modification and the excess of reagent used. Thus, C-P-8-2000 means modification at pH 8 with a 2000-fold molar excess of $POCl_3$.

Gel Electrophoresis. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was performed using a combined Weber−Osborn and Laemmli system. The separation gel and sample gel were prepared according to the method of Weber and Osborn (1969); the electrode buffer and the sample buffer used were those of Laemmli (1970).

Analytical Ultracentrifugation. Sedimentation velocity experiments were performed at protein concentrations of 0.75−3.00 g L^{-1} by means of the Beckman Optima XL-A/I analytical ultracentrifuge (Beckman Instuments, Palo Alto, CA) at 35.000 or 40.000 rpm rotor speed and 20 °C. UV absorption at λ = 280 nm was used for the detection of the sedimentation velocity in a conventional 12 mm analytical cell. The radial position r of the sedimentation boundary was determined for any scan by second-moment analysis, and the mean apparent sedimentation coefficient was calculated according to

$$s_{app} = \frac{1}{\omega^2 r}\frac{dr}{dt} = \frac{d\ln(r)}{d\omega^2 t} \quad (1)$$

The error of determination of the sedimentation coefficient was 5%. The distribution of the sedimentation coefficients [$g(s)$] was calculated from the same absorbance scans by the sedimentation time derivative analysis according to the method of Stafford (1976).

Viscometry. Viscosity measurements were performed at 20 °C in an automatic viscometer type Viskotimer (LAUDA, Königshofen, Germany) using a 1.5 mL microcapillary. The protein content of the starting solution was ∼1%. The reduced viscosity ($\eta_{red} = \eta_{sp}/c$) was plotted against the protein concentration to obtain the kinematic intrinsic viscosity [η]′, which was corrected according to the method of Tanford (1955) using eq 2 to obtain the dynamic intrinsic viscosity:

$$[\eta] = [\eta]' + (1 - \bar{v}\rho_0)/\rho_0) \quad (2)$$

Here, $\bar{v}$ is the partial specific volume of the protein and ρ_0 is the density of the buffer. $\bar{v}$ was determinded after density measurements of variously concentrated protein solutions with a digital precision densimeter DMA 5000 (Anton Paar/Paar Physica, Graz, Austria) at 20.00 ± 0.01 °C using eq 3 (Kratky et al., 1973):

$$\bar{v} = (1 - d\rho/dc)\rho_0 \quad (3)$$

Here, ρ is the density of the protein solutions.

Fluorescence Spectroscopy. Fluorescence emission spectra were obtained after excitation of the protein solutions (0.10 g/L) at 280 and 295 nm with an Aminco Bowman series 2 spectrometer at a scan rate of 0.2 nm/s. The band-pass was 4 nm in excitation and 2 nm in emission; the detector high voltage was 900 V.

Surface Hydrophobicity. The surface hydrophobicity index S_0 was determined by fluorescence probe (ANS) measurements according to the methods of Kato and Nakai (1980) and Hayakawa and Nakai (1985) with the following modification. An ANS solution (∼5×10^{-3} g/L) in standard phosphate buffer was used. The real ANS concentration was determined by absorption measurements at 350 nm (E = 4950 M^{-1}). The protein concentration amounted to a maximum of 0.5 g/L. The incubation time of the ANS−protein mixture before the measurement was 20−30 min. The measurement was performed in the Aminco Bowman fluorometer with an excitation wavelength of 280 nm, slits of 8 nm, and a high voltage of 600 V. The emission was determined in the range from 420 to 500 nm. S_0 was normalized to an ANS concentration of 10×10^{-3} g/L.

RESULTS

Chemical Analysis. The results of chemical analysis of phosphorylated cruciferin are given in Table 1. Unmodified cruciferin, although purified by ion-ex-

Table 1. Variously Bound Phosphorus Residues in Phosphorylated Cruciferin

sample	P_{total}		CBP (mol/mol)	
	μm/g	mol/mol	acid-labile	alkali-labile
cruciferin nonmodified	4.2 (phytic acid)			
C-P-8-1000	8.7	84.9	6.1	2.0
C-P-10-1000	9.9	95.5	14.0	11.7
C-P-8-2000	9.5	92.1	11.6	10.6
C-P-10-2000	20.2	195.6	22.3	67.9
C-P-8-5000	12.3	118.9	13.7	11.6
C-P-10-5000	19.6	189.4	24.2	47.7

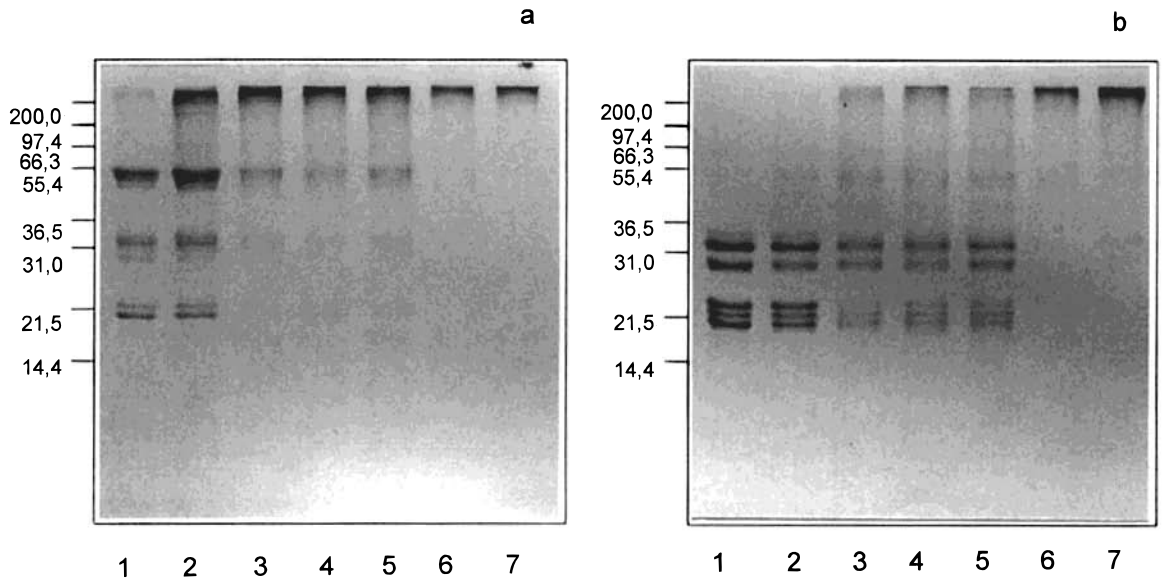

Figure 1. SDS−PAGE of cruciferin and variously phosphorylated cruciferins (a) in the absence and (b) in the presence of a reducing agent (DTE): (lane 1) unmodified cruciferin; (lane 2) C-P-8-1000; (lane 3) C-P-8-2000; (lane 4) C-P-8-5000; (lane 5) C-P-10-1000; (lane 6) C-P-10-2000; (lane 7) C-P-10-5000.

change chromatography, still contained phosphorous, which can be attributed mainly to phytic acid and small amounts of phospolipids. Phosphorylation widely removed the phytic acid bound electrostatically to the protein (Schwenke et al., 1987). However, all phosphorylated samples contain a considerable amount of phosphorous, increasing with the excess of reagent used, which was removed by rapid washing with 0.5 M perchloric acid, ethanol, diethyl ether, and chloroform/methanol/hydrochloric acid at 2−4 °C. Because the modified protein samples were exhaustively dialyzed against 0.1 M KCl and distilled water, the presence of unbound phosphate might be excluded. This phosphorous may be assumed to be loosely attached to the protein through very labile bounds broken by washing with cold perchloric acid and by electrostatic interaction. A minor part of the extracted phosphorous corresponds to phospholipids. The phosphorous that remained in the protein after washing was described as chemically bound phosphorous (CBP). The amount of CBP increased with the excess of reagent up to 2000 and the pH used for the phosphorylation. Maximum phosphorylation achieved was 22−25 and 72−90 phosphorous residues per mole of protein at pH 8 and 10−11, respectively. Raising the excess of reagent from 2000 to 5000 did not increase the amount of CBP. Nearly equal amounts of acid-labile and alkali-labile phosphorous were found under these conditions at pH 8 and at lower excess of reagent (1000) at pH 10−11. In contrast to that, modification at pH 10−11 with a high excess of reagent (2000−5000) led to the introduction of a dominating portion of alkali-labile phosphorous. The latter can be attributed to O−P bonds (mostly P−O-seryl and P−O-threonyl) and the acid-labile phosphorous to N−P bonds (mostly P−ϵ-N-lysine) (Matheis and Whitaker, 1984).

To estimate the portion of phosphorylated amino groups of the total amount of blocked amino groups, the content of total amino groups before and after phosphorylation was determined. The results are given in Table 2. It is easily seen that the maximum decrease of amino groups amounting to 40−50% occurred at medium and high modifications. Only 56−65% of these blocked amino groups correspond to N-phosphorylated groups. The remaining part of blocked amino groups could be attributed to those involved in chemical cross-linking via isopeptide bonds (Matheis, 1991). They were negligible or not detectable, when the modification was performed with a high excess of reagent at pH 10−11.

Table 2. Blocking of Amino Groups in Phosphorylated Cruciferin

sample	blocked NH_2 total mol/mol	blocked NH_2 total %	NH_2 involved in P−N bonds[a] (%)
C-P-8-1000	15.9	15.4	38.4
C-P-10-1000	21.4	43.4	65.4
C-P-8-2000	19.4	39.4	59.8
C-P-10-2000	23.4	47.5	95.3
C-P-8-5000	24.5	49.7	55.9
C-P-10-5000	21.5	43.8	88.8

[a] Calculation based on the data of acid-labile phosphorus in Table 1.

SDS−PAGE. SDS−PAGE patterns of variously phosphorylated cruciferins are given in Figure 1. The nonmodified protein shows three major polypeptide groups corresponding to molecular masses around 50, 30 (α), and 20 kDa (β) when studied under nonreducing conditions (Figure 1a). The 50 kDa fraction, representing the disulfide-linked α−β-polypeptide pair, disapppeared under reducing conditions that liberate additional α-chains (27−31 kDa) and β-chains (18−21 kDa) (Figure 1b). The α- and β-chains, which are not disulfide-linked, form an α−β-subunit in the native cruciferin by noncovalent interaction. This polypeptide pattern, elucidated by Dalgalarrondo et al. (1986), is unusual in 11S globulins, the subunits of which are normally composed of a disulfide-linked α−β-polypeptide pair (Derbyshire et al., 1976).

Progressive phosphorylation resulted in a decreased intensity of the constituent polypeptide chains. This effect is most pronouced for the free α-chains and β-chains. Reduction of the α−β-dimer in medium phosphorylated samples gave a rather constant pattern of α- and β-chains. Their decreased intensity, when compared with the pattern of the nonmodified protein, might be due to the partial loss of free α- and β-chains. In place of them, a 50 kDa zone appeared under

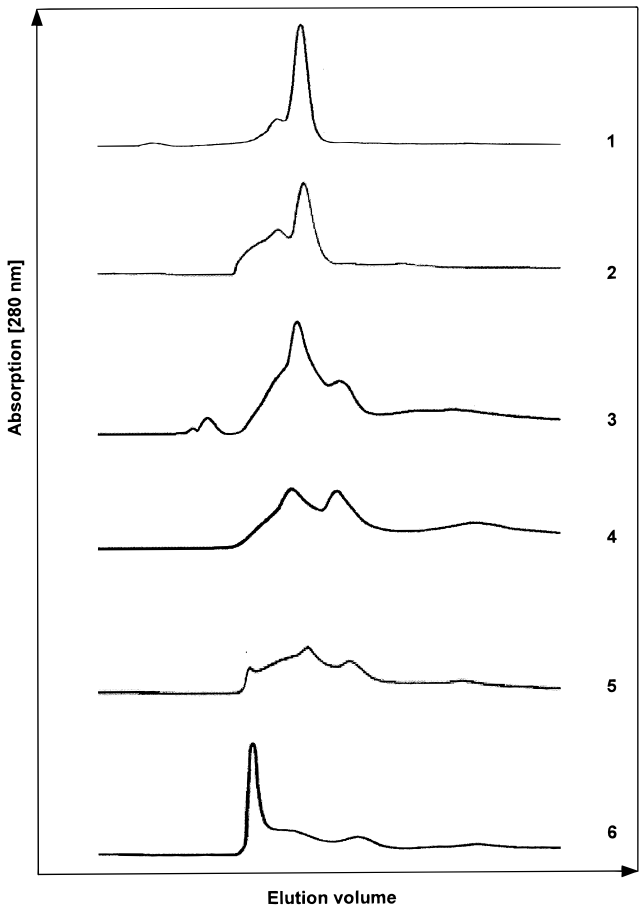

Figure 2. Gel filtration patterns of cruciferin at increasing state of phosphorylation: (1) unmodified cruciferin; (2) C-P-8-1000; (3) C-P-8-2000; (4) C-P-8-5000; (5) C-P-10-1000; (6) C-P-10-5000.

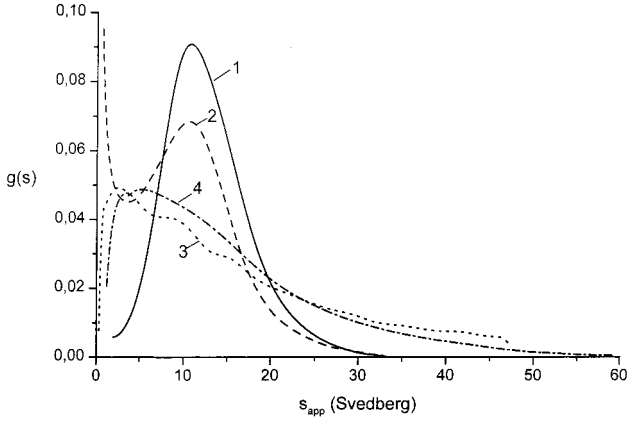

Figure 3. Ultracentrifugal patterns of native and progressively phosphorylated cruciferins: (1) unmodified cruciferin, $c = 0.75$ g/L, 35000 rep/min; (2) C-P-8-2000, $c = 0.52$ g/L, 35000 rep/min; (3) C-P-8-5000, $c = 0.75$ g/L, 40000 rep/min; (4) C-P-10-5000, $c = 0.52$ g/L, 35000 rep/min.

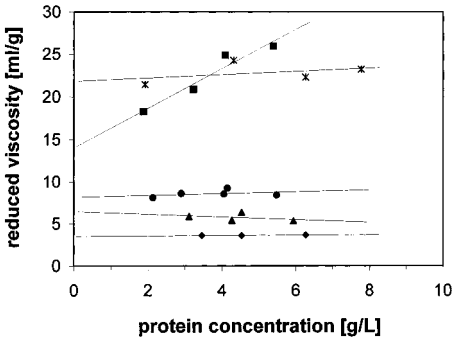

Figure 4. Concentration dependence of the reduced viscosity of native and progressively phosphorylated cruciferin: (◆) unmodified cruciferin; (▲) C-P-8-1000; (●) C-P-8-2000; (∗) C-P-8-5000; (■) C-P-10-2000.

reducing conditions, obviously a product of covalent cross-linking of the α- and β-chains to a stable dimer. Additionally, slower migrating zones mainly remaining at the start indicate cross-linking to higher molecular weight products. They increased at the expense of the α- and β-chains when an extremely high excess of phosphorous oxychloride (5000) was used for the modification.

Liquid Chromatography and Ultracentrifugation. Figure 2 shows the elution diagrams of the gel chromatography of native and variously phosphorylated cruciferins. Cruciferin appeared as a 300 kDa peak fraction beside a higher molecular weight aggregate. The former decreased with progressive phosphorylation in favor of higher and lower molecular weight fractions. When modified at pH 8, the phosphorylated protein showed dissociation products only at a higher excess of reagent. Dissociation into subunits became evident at pH 8 and a $POCl_3$/protein ratio of 2000−5000. Both dissociation and aggregation were observed when the modification was carried out at pH 10−11 and moderate or high excess of reagent (1000−5000).

Sedimentation velocity analysis was carried out with protein samples phosphorylated with an excess of reagent of 2000 and 5000. The distribution functions of the sedimentation coefficients derived from the UV absorption scans are given for native cruciferin and three modified samples in Figure 3. Cruciferin gives a maximum at an apparent sedimentation coefficient of $s = 11.3$ S. It did not show a significant dependence on the protein concentration. A considerable amount of slowly sedimenting components indicates the presence of dissociation products after phosophorylation with a 2000-fold excess of reagent, whereas the major component has the sedimentation coefficient of cruciferin. When the excess of reagent was increased to 5000, dissociation products dominated. The resulting distribution curve gives a maximum at $s = 2.5$ S, indicating dissociated subunits (Gueguen et al., 1990). Intermediary and higher sedimenting components are also present. A broad distribution of sedimentation coefficients was obtained after phosphorylation at pH 10−11 with a 5000-fold excess of reagent. A maximum around 5 S indicates the presence of dissociation products as major components. As in the case of phosphorylation at pH 8 with a comparable amount of reagent (C-P-8-5000), higher molecular mass components comprise a distribution between 10 and 50 S.

Comparison of the results obtained by both methods verifies the higher resolution power of the chromatographic technique used. However, the high molecular mass aggregation products formed after phosphorylation and appearing with the exclusion volume of the chromatographic column could not be detected by ultracentrifugation due to their very quick sedimentation.

Viscometry. The concentration dependence of the reduced viscosity of cruciferin before and after phosphorylation with increasing amounts of reagent is given in Figure 4. The resulting values of the intrinsic viscosity [η] are compiled in Table 3, which also shows

Table 3. Intrinsic Viscosity ([η]), Partial Specific Volume ($\bar{v}$), and Viscosity Increment (ν) of Cruciferin and Variously Phosphorylated Cruciferins

sample	[η] (mL/g)	$\bar{v}$ (mL/g)	ν	ν/2.5
cruciferin	3.76 ± 0.15	0.724 ± 0.010	5.19	2.08
C-P-8-1000	6.76 ± 0.27	0.687 ± 0.011	9.84	3.94
C-P-8-2000	8.49 ± 0.43	0.657 ± 0.011	12.92	5.17
C-P-8-5000	22.11 ± 1.11	0.682 ± 0.012	32.42	12.97
C-P-10-2000	14.36 ± 0.72	0.695 ± 0.013	20.66	8.26

the data of the partial specific volume $\bar{v}$, the viscosity increment $\nu = [\eta]/\bar{v}$, and the ratio ν/2.5 indicating the deviation of the molecular shape from a compact sphere (Creeth and Knight, 1965).

The intrinsic viscosity of cruciferin ([η] = 3.76 mL/g) was found to be slightly smaller than that previously observed amounting to [η] = 4.1 ± 0.2 mL/g (Schwenke et al., 1986). Progressive phosphorylation at pH 8 resulted in a continuous rising of [η] with a drastic increase of the latter to ~20 when the excess of reagent was enhanced from 2000 to 5000. Modification at pH 10−11 resulted in higher values of [η] at lower $POCl_3$/protein ratios, when compared with the data obtained at pH 8. Moreover, a considerable change of the slope of the η_{red} versus $c_{protein}$ plot was observed.

Phosphorylation decreased the partial specific volume to values between 0.657 and 0.695, which did not show a clear dependence on the extent of phosphorylation. The increase of ν and ν/2.5 indicates a progressive conformational change of cruciferin with increasing extent of phosphorylation being most pronounced at the transition to the largely dissociated state of C-P-8-5000 or C-P-10-2000.

Fluorescence Spectra and Surface Hydrophobicity. Fluorescence emission spectra of cruciferin and variously phosphorylated cruciferins after excitation at 280 nm (Tyr plus Trp excitation) are given in Figure 5 for the modifications at both pH 8 and 10−11. Cruciferin exhibits maximum fluorescence intensity at 321 nm under these conditions. Excitation at 295 nm (Trp excitation) gave a fluorescence maximum at 325 nm (not shown). These are characteristic of the fluorescence maxima of tryptophan residues (Chen et al., 1969). Phosphorylation resulted in a red shift of the fluorescence maxima, which increased continuously with progressive phosphorylation at pH 8 (Figure 5a). Phosphorylation at pH 10−11 caused the most pronounced red shift to 330 nm at the highest extent of modification (C-P-10-2000, C-P-10-5000). Similar results were obtained after excitation at 295 nm. This shift of the fluorescence maximum indicates conformational changes observed during the unfolding of proteins (Chen et al., 1969; Teale, 1960).

Phosphorylation of cruciferin also induced a strong quenching of the fluorescence, which proceeded with increasing extent of modification at pH 8 (Figure 5a). However, when the phosphorylation was performed at pH 10−11, a drastic drop of the fluorescence intensity was observed already at the lowest level of modification, changing only to a small extent at higher modification (Figure 5b).

Figure 6 shows the dependence of the hydrophobicity index S_0 on the excess of reagent used for the phosphorylation at pH 8 and 10−11. A continuous increase of S_0 occurred with increasing extent of phosphorylation at pH 8. Modification under strongly alkaline conditions resulted in a much higher increase of S_0 characterized by a jump at the transition from the medium stage of phosphorylation (C-P-10-1000) to the high stage of phosphorylation (C-P-10-2000) (Figure 6).

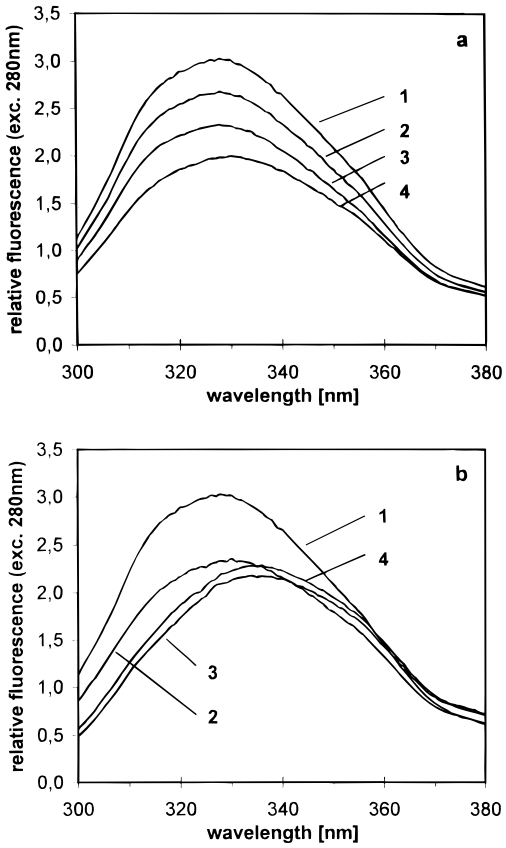

Figure 5. Uncorrected fluorescence emission spectra of native and progressively phosphorylated cruciferins: (a) modification at pH 8 [(1) unmodified cruciferin; (2) C-P-8-1000; (3) C-P-8-2000; (4) C-P-8-5000]; (b) modification at pH 10−11 [(1) unmodified cruciferin; (2) CP-10-1000; (3) C-P-10-2000; (4) C-P-10-5000].

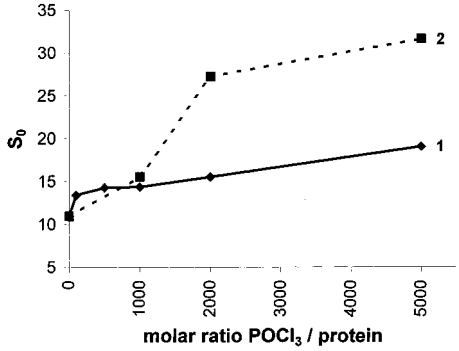

Figure 6. Dependence of the surface hydrophobicity (S_0) of phosphorylated cruciferin on the extent of phosphorylation: (1) modification at pH 8; (2) modification at pH 10−11.

DISCUSSION

To estimate the level of phosphorylation attained in this work by reaction of cruciferin with phosphorous oxychloride, a comparison with the results obtained by Hirotsuka et al. (1984) on soybean protein isolates is indicated.

The authors reported on the incorporation of ~150 mol of P_i/mol of protein after the reaction with a 1000−3000-fold molar excess of phosphorous oxychloride at pH 11, whereas 103 mol of P_i was incorporated at pH 8 and reagent excess of 1500 mL/mol. These data are to

be compared with those of the total amount of phosphorous given in Table 1 of this paper, because the determinations of phosphorous were performed in both cases after an exhaustive dialysis (or gel filtration) of the phosphorylated proteins. However, as the data in Figure 1 show, the content of total phosphorous in the modified proteins dropped drastically after the proteins were washed with cold 0.5 M perchloric acid and polar organic solvents. The latter removes minor amount of phospholipids, the content of which is independent of the extent of phosphorylation. The following possibilities for the nature of phosphorous binding to the protein, which increased with the excess of phosphorous oxychloride used and split by the washing procedure, can be discussed: (1) electrostatic interactions of phosphate residues [or polyphosphate formed under the conditions of the reaction (Matheis, 1991)] with positively charged groups of the protein; and (2) formation of carbonic acid/phosphorous acid anhydrides, which are energy-rich and rather sensitive to hydrolysis. These kinds of activated groups could be intermediates for the following cross-linking reaction (Matheis, 1991). They can also serve for the attachment of essential amino acids to the protein as described by various authors (Matheis, 1991; Sitohy et al., 1999).

The reaction of cruciferin with phosphorous oxychloride led to the reduction of free amino groups, partially by formation of N-phosphoryl bonds and partially by their participation in cross-links. However, in the case of phosphorylation under strongly alkaline conditions (pH 10−11) and a high excess of reagent (2000−5000-fold), the amino acid blocking occurred practically completely due to the formation of N-phosphoryl bonds (Table 2). That means that the cross-linking observed under these conditions should be realized without the contribution of amino groups. The cross-linking can, therefore, be thought to occur via phosphoryl groups bridging protein hydroxyl group (Matheis, 1991).

In contrast to the succinylation of plant 11S globulins, which can result in a nearly complete blocking of protein amino groups (Schwenke et al., 1990), phosphorylation of cruciferin led to a limited modification of the amino groups amounting to a maximum blocking of 40−50% under extreme conditions.

Attachment of negatively charged groups such as succinyl residues to functional groups of oligomeric proteins induces the dissociation of the latter to the constituent subunits (Means and Feeney, 1971). Progressive succinylation of plant 11S globulins resulted in a stepwise dissociation into the 3S subunit via the 7S half-molecule (Schwenke et al., 1992). At a critical step of succinylation, characterized as the beginning succinylation of hydroxyl groups additionally to succinylated amino groups, a sudden unfolding of the dissociated subunits has been observed in various succinylated 11S globulins (Schwenke et al., 1990, 1998). In the case of succinylated rapeseed globulin (cruciferin), the critical degree of N-succinylation amounted to 60−70% (Schwenke et al., 1986). Analogous structural changes ought to be expected in the case of phosphorylation.

As revealed from gel filtration studies (Figure 2), an increasing portion of higher molecular weight products appeared with proceeding phosphorylation before any dissociation products were observed. They may consist both of chemically cross-linked products, as revealed by SDS−PAGE (Figure 1), and of aggregates. Dissociation of cruciferin requires a minimum amount of attached phosphoryl residues; that is, at least 20−30% of the amino groups should be phosphorylated. However, the highest structural changes are accomplished by the synergistic effect of N- and O-phosphoryl residues. Thus, ~10 N-attached plus ~10 O-attached phosphoryl residues, detected in cruciferin after phosphorylation at pH 8 and a 2000 or 5000 molar excess of reagent, are sufficient to induce a large dissociation. As revealed by ultracentrifugal studies (Figure 3), the major product of the dissociation is a 2.5S component corresponding to the monomer subunit of the hexameric globulin. Due to cross-linking reactions, this dissociated subunit is partially stabilized by covalent bonds between the α- and β-chains (Figure 1).

Viscometric studies were performed to show the transition from the compact native globulin to the more or less expanded or unfolded phosphorylated samples. Under these conditions, about equal portions of the attached phosphoryl residues were linked to amino groups and hydroxyl groups (Tables 1 and 2). Therefore, the degree of N-blocking can be taken as an equivalent of modification. As can be seen in Table 3, a 2-fold increase of the viscosity increment ν was observed already after a modification of <10%. A drastic increase of ν occurred when the N-blocking increased from 21 to 44%. This change can be due to both a molecular unfolding/expansion and the formation of aggregates, the shape of which may deviate considerably from a sphere. The highest observed value for the intrinsic viscosity amounting to ~22 mL/g corresponds to that of a denatured protein (Diep et al., 1982). It was also observed for the exhaustively succinylated 11S globulin (legumin) from faba beans (Schwenke et al., 1998), whereas the highly succinylated cruciferin gave an intrinsic viscosity of ~15 mL/g (Schwenke et al., 1986), similar to that of succinylated pea legumin (Schwenke et al., 1990). The change of hydrodynamic properties of these succinylated proteins has been discussed to be caused by a molecular unfolding or expansion due to electrostatic repulsion. Even the formation of molten globulin-like structure has been taken into consideration (Schwenke et al., 1998). In the case of phosphorylation, the molecular unfolding may be limited by cross-linking processes. Therefore, a contribution of aggregates to ν can also be assumed. The ratio of ν/2.5 may allow a comparison of the obtained data with those of other well-described proteins (Creeth and Knight, 1965). Thus, unmodified cruciferin can be thought to be compact, similar to bovine serum albumin, whereas medium phosphorylated cruciferin gave ν/2.5 values comparable to those of macroglobulins. Highly phosphorylated cruciferins are very similar to the rodlike fibrinogen with regard to the ν/2.5 ratio.

Arguments for conformational differences between phosphorylated cruciferin modified at lower or higher pH may be obtained from the differences in the concentration dependence of η_{red} as shown in Figure 4. The large slope in the η_{red} versus $c_{protein}$ plot of the sample C-P-10-2000 points to both strong hydrodynamic interactions and intermolecular attraction (Cragg and Bigelow, 1955) despite electrostatic repulsions of phosphoryl residues. This may be due to the interaction of hydrophobic patches exposed after the modification resulting in an increasing aggregation at higher protein concentration.

The results of fluorescence spectroscopic measurements (Figure 5), which revealed a considerable red shift after phosphorylation, indicate an unfolding of the tertiary structure (Chen et al., 1969), although the protein was stabilized by cross-links. These changes are most pronounced after modification under strongly alkaline conditions that already induce protein unfolding (Figure 5b). The strong fluorescence quenching in the spectra of phosphorylated cruciferin resembles that found for highly succinylated 11S globulins (Schwenke et al., 1990, 1998). However, it can hardly be attributed only to molecular unfolding processes, because both an increase and a decrease of the fluorescence intensity have been observed for unfolding proteins (Chen et al., 1969; Kronman and Holmes, 1971). The quenching of tyrosyl fluorescence by phosphate has been proved (Chen and Cohen, 1966). We can, therefore, assume that the decrease in fluorescence intensity in phosphorylated cruciferin is due to the attached phosphoryl residues. Although the presence of O−Tyr−phosphoryl bonds was not studied in this work, their formation (Frank, 1987) cannot be excluded. This could be an additional factor that influences the fluorescence of the modified protein.

The conformational changes established in phosphorylated cruciferin resulted in an increasing surface exposure of hydrophobic clusters leading to an increased surface hydrophobicity (S_0). The latter is thought to be responsible for the formation of aggregates increasing with the extent of phosphorylation and the S_0 value. Similarly to succinylation (Schwenke et al., 1998), the applied ANS fluorescence probe did not give a response to the introduced hydrophilic residues, but to the secondary process of unfolding induced by the latter.

In conclusion, progressive phosphorylation of cruciferin by phosphorous oxychloride results in varied derivatives differing both in the extent and nature of chemical modification and in the conformational state. The latter is characterized by aggregation, dissociation, unfolding, and chemically cross-linked states. These different derivatives can serve for studying the dependence of functional properties such as emulsifying and foaming activity and stability on the structural state. The results of first rheological studies (Krause and Schwenke, unpublished data) provide arguments for the stabilizing effect of phosphorylation-induced cross-links in thermotropic gels. Compared with the unmodified protein, chemically phosphorylated cruciferin represents thus a new quality of gel-forming biopolymer, the properties of which may increase the possibilities for the nonfood use of rapeseed proteins.

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