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Inventaire PUB
N° 00002489**Mild method of simultaneous methionine grafting and phosphorylation of soybean globulins improves their functional properties**M. Sitohy¹, Y. Popineau*, J.-M. Chobert and T. Haertlé

Soybean 7S and 11S globulins were phosphorylated to different extent as a function of the molar ratios of POCl_3 used (75, 150 and 300 mol POCl_3 /mol protein) in the presence of equivalent amounts of triethylamine. Simultaneous addition of L-methionine during the phosphorylation yielded methionylated globulins. In some experiments, the basic amino acid arginine was used as the sole base of the reaction, instead of triethylamine, at a ratio of 6 mol arginine/mol POCl_3 . Adding L-methionine in the course of the phosphorylation reaction reduced slightly the extent of protein phosphorylation, with increasing L-methionine content in the phosphorylated globulin up to about 280% of its original level. The use of L-arginine as the reaction base

led to lower phosphorylation yields but could raise simultaneously both L-methionine and L-arginine to about 143% and 155% of their original levels, respectively. Isofocusing electrophoresis showed lower isoelectric points of the phosphorylated samples, compared to the starting aliquots. SDS-PAGE did not show major changes in the subunit distribution of the modified soybean globulins. Phosphorylation and "amino acylation" changed the solubility of the modified samples and also other related functional properties e.g. foaming and emulsifying which were remarkably improved, especially for the samples with grafted L-methionine.

1 Introduction

Most plant storage proteins are characterized by poor solubility especially around the neutral pH. This is generally due to their high contents of nonpolar amino acids, weak charges, hydrophobic aggregations between the protein subunits and amidation neutralizing the charges of the aspartyl and glutamyl residues [1–3]. Mild chemical phosphorylation of proteins could be a good tool for improving their functional properties such as emulsifying and foaming [4, 5].

Functional properties, especially solubility, can be enhanced by chemical phosphorylation [3–11]. Protein solubility is considered an essential feature since other protein functional properties expressed at the interface heavily depend on it. As such, phosphorylation of proteins introduces negative phosphate groups into the protein molecule, bound to lysyl, seryl and threonyl residues and hence increases the net negative charge. As a result, the isoelectric point of the phosphorylated protein is lowered and its solubility at the neutral pH is improved. Consequently, the enhanced hydrophilicity of the modified protein will decrease the hydrophobically driven aggregations so frequently observed in the plant proteins and improve the general protein solubility. Few studies were performed on the phosphorylation of soybean proteins under conditions involving relatively high amounts of a phosphorylating agent and concentrated inorganic bases [3, 12] subjecting the modified proteins to degradation and other uncontrolled changes. The

proteins involved in these studies were mixtures of whole protein isolates.

The aim of this work was to study the phosphorylation of defined soy protein fractions (7S and 11S soybean globulins) under well-defined mild reaction conditions using equivalent amounts of the phosphorylating agent and the organic base used in order to obtain undegraded phosphorylated, phosphoamidated and phosphodiamidated proteins. Moreover, soybean globulins are originally deficient in L-methionine considered as the main extrinsic amino acid [13]. Its grafting was achieved by the addition of free methionine during the phosphorylation reaction. Grafted amino acids are nutritionally preferable to the addition of free amino acids [14]. L-arginine was also used as the base for the phosphorylation reaction to facilitate the reaction.

2 Materials and methods**2.1 Materials**

Soybean protein globulin fractions were prepared from defatted soy flour (Sogip, France) using the method of *Thanh and Shibasaki* [15]. The method consists in a total solubilisation of albumins and globulins followed by an isoelectric precipitation at pH 6.4 and 4.8 for 11S and 7S globulins, respectively. After purification, the amount in protein for 11S and 7S globulins was 89 and 86%, respectively. All other chemicals used were of analytical grade.

2.2 Protein phosphorylation

The general procedure of *Sitohy et al.* [16] was followed for protein phosphorylation. Amounts of phosphorus oxychloride (POCl_3) equivalent to 75, 150 and 300 mol/mol protein (based on an average molecular weight of protein subunit of 60,000) were dissolved to make 20%

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solution in carbon tetrachloride. Equivalent amounts of triethylamine (6 mol base/mol POCl_3) were added to a 1% soybean globulin aqueous solution which was kept cold in an ice-bath. The reaction mixture was stirred for about 5 min until complete protein solubilisation before the addition dropwise, over 30 min of the determined amounts of POCl_3 . The reaction medium was left for 10 min to achieve complete separation of the aqueous phase which was recovered by decantation, dialyzed for 4 days against several changes of distilled water, and lyophilized. Its phosphorus content was determined according to the method of Bartlett [17] modified by Sitohy et al. [16]. During L-methionine grafting both 7S and 11S globulins were phosphorylated with a molar ratio of 300 mol POCl_3 /mol protein while different molar ratios of L-methionine (1, 2 and 4 mol amino acid/mol POCl_3) were added dropwise in the course of the phosphorylation reaction. In other experiments, L-arginine was added as a free base at a ratio of 6 mol amino acid/mol POCl_3 in order to replace triethylamine. In this case, POCl_3 was used at a ratio of 300 mol/mol protein and L-methionine was simultaneously added at a ratio of 2 mol/mol POCl_3 .

2.3 Protein solubility

Native and phosphorylated globulins were dispersed in distilled water (0.1% w/w) and the pH was adjusted from 2 to 7 with concentrated HCl or NaOH in order to limit dilution. After 10 min equilibration at room temperature, the dispersion was centrifuged for 15 min at 2700 g and 4°C and the protein content was determined in the supernatant, as previously described by Chobert et al. [18].

2.4 SDS-electrophoresis

SDS-PAGE was performed according to Laemmli [19] on 15% acrylamide running gel and 3.2% acrylamide stacking gel, on a Mini Protean II (Bio-Rad) apparatus. Proteins were stained with 0.2% Coomassie brilliant blue R250.

2.5 Isofocusing electrophoresis

Electrofocusing was performed in the pH range 3–7 on ready-to-use isoelectric focusing gels (FMC, Rockland, USA). A marker kit of pI in the range 3.50–7.35 was used for calibration (Pharmacia, Sweden).

2.6 Amino acid analysis

Acid hydrolysis of protein was realized under vacuum in the presence of 6 N HCl for 24 h, at 110°C in a Pico-Tag station (Waters). Amino acids were derivatized with phenylisothiocyanate (PITC) according to the method described by Bidlingmeyer et al. [20] and quantified by reversed-phase HPLC on a Pico-Tag C_{18} column (3.98 mm i.d. $\times$ 15 cm, Waters).

2.7 Emulsifying properties

Emulsions were realized at pH 7 using 0.1% protein solution and corn oil. Emulsifying activity index (EAI) was determined according to the procedure outlined by Chobert et al. [21]. Emulsion stability kinetics were determined according to the methods described by Dagorn-Scaviner et al. [22] and Loisel and Popineau [23].

2.8 Foaming properties

The foaming properties of native and phosphorylated soybean globulin solutions (0.4 mg/ml) prepared in 0.1 M phosphate buffer, pH 7.0, were measured with a recently described apparatus [24, 25].

3 Results and discussion

3.1 Extent of phosphorylation

Both soybean globulin fractions (7S and 11S) were phosphorylated to considerable extents when using relatively low

Table 1. Extent of soybean globulin phosphorylation with different molar ratios of phosphorus oxychloride.

Sample	Molar ratio [mol POCl_3 / mol protein]	Total P [%]	Bound P [%]	Extent of phosphor- ylation [mol P/mol protein]
7S globulin	0	0.68	0	0
	75	1.35	0.67	9
	150	1.65	0.97	13
	300	1.76	1.08	18
11S globulin	0	0.31	0	0
	75	1.34	1.03	10
	150	1.52	1.21	13
	300	1.65	1.34	21

molar ratios of POCl_3 in the presence of equivalent amounts of triethylamine (Table 1). The extent of phosphorylation was proportional to the molar ratio of POCl_3 , and comparable to that obtained by Hirotsuka et al. [12] for soybean isolate phosphorylated with POCl_3 in the presence of an excess of sodium hydroxide. The use of triethylamine at an amount exactly equivalent to the amount used for POCl_3 was intended to keep the protein molecule in an undegraded form during and after the phosphorylation reaction.

3.2 Methionine grafting and extent of phosphorylation

Methionine was grafted on soybean globulin molecules with considerable yields during the phosphorylation. The quantity of methionine was increased to 281% and 215% of its original amount in 7S and 11S globulins, respectively. The grafted methionine amounted to 2, 4 and 8 mol methionine/mol soybean globulin (7S and 11S) when the amino acid was added to the phosphorylation reaction medium at levels of 1, 2 and 4 mol/mol POCl_3 , respectively. Increasing the amount of added L-methionine increased gradually its grafting while reducing slightly the phosphorylation yield (Table 2). This could be caused by the competition between the NH_2 group of free methionine and the protein molecules for the phosphorylating agent. L-methionine is linked to the protein molecule through two phosphoamide bonds connecting the protein molecule and L-methionine.

When using the phosphorylating agent at 300 mol POCl_3 /mol protein, triethylamine was replaced by free L-arginine at 6 mol/mol POCl_3 as the sole source of base for the phosphorylation reaction. L-methionine was added at a ratio of 2 mol/mol POCl_3 . It resulted in lower phosphorylation yield (14 and 15 mol P/mol protein for 7S and 11S globulins, respectively). The yields of grafted methionine increased only by about 43 and 31% over its original content in 7S and 11S globulins, respectively. This corresponds to 2 mol methionine grafted to one mol globulin. Simultaneously, L-arginine content was increased by about 55% for 7S and 11S globulins, leading to the grafting of 23 arginine on one molecule of soybean globulin. Theoretically, the number of phosphate groups bound to one 7S or 11S globulin (14 and 15 moles P/mol globulin, respectively) is quite sufficient to bind the amino acids. Lower yield of grafted L-methionine seems to be due to the lower phosphorylation, confirming the role of remaining activated phosphate groups in secondary grafting of the amino acids on soybean globulins. Moreover, the presence of both arginine

Table 2. Extent of phosphorylation and methionine grafting on soybean 7S and 11S globulins.

Sample	Molar ratio [mol POCl ₃ / mol protein]	Molar ratio [mol methionine/ mol protein]	Bound P [mol P/mol protein]	Total Met [%]	Bound Met [%]	Bound Met [mol methionine/ mol protein]
7S globulin	0	0	0	1	0	0
	300	1	20	1.37	0.37	2
	300	2	18	2.01	1.01	5
	300	4	15	2.81	1.81	8
11S globulin	0	0	0	1.48	0	0
	300	1	21	2.01	0.53	2
	300	2	18	2.41	0.93	4
	300	4	16	3.21	1.73	8

Figure 1. pH-solubility profiles of native (●) and phosphorylated 7S soybean globulins. (○) 9; (▼) 13 and (▽) 18 mol P/mol protein. **Figure 2.** pH-solubility profiles of native (●) and phosphorylated 11S soybean globulins. (○) 10; (▼) 13 and (▽) 21 mol P/mol protein.

and methionine in the reaction medium leads to a competition between two amino acids to react with the activated phosphate group. This is strengthened by greater reactivity of arginine because of its higher basicity and its presence in a higher concentration in the reaction medium.

3.3 Protein solubility

The solubility of soybean globulins was highly improved by phosphorylation especially in the pH-ranges 4.5–7 and 5–7 for 7S and 11S globulins, respectively (Figures 1 and 2). This increase of protein solubility is not only due to the shift of the isoelectric points towards acidic side but also to the enhanced hydrophilicity resulting from the addition of negatively charged phosphate groups to the protein. However, at lower pHs (2.0–3.5) solubility of the phosphorylated samples was greatly reduced especially for the highest substitution yields. This may result from the protonation of the free phosphate groups which are responsible for the protein solubility.

3.4 Electrophoretic patterns of phosphorylated globulins

SDS-PAGE patterns of phosphorylated soybean 7S and 11S globulins are not different from the native corresponding samples (Fig. 3) indicating that the mild reaction conditions used did not degrade notably any of the modified proteins.

The isofocusing electrophoresis of the modified samples (Fig. 4) shows a decrease of the pI values of the phosphoryl-

ated samples, compared to the starting material. The magnitude of the modification of the pI values is directly proportional to the extent of phosphorylation. This shows clearly that the introduction of a negative phosphate group into the protein molecule can directly change the net negative charge and also the isoelectric point of the modified protein. The systematic decrease of the isoelectric point according to the phosphorylation yield confirms the covalent character of the modification of the protein molecule and can be also used as an indicator of the extent of phosphorylation.

The isoelectrofocusing of the phosphorylated soybean globulins samples with grafted L-methionine (Fig. 5) show also the decrease of pI, according to the extent of phosphorylation. The relatively low amount of attached L-methionine molecules (2–8 molecules/protein molecule) compared with the bigger quantity of attached phosphate groups (15–21 mol/mol protein) did not significantly affect the negative charges of the bound phosphate groups. The same trend was observed in case of both 7S and 11S globulins indicating that the change of the electrophoretic patterns is a function of the extent of phosphorylation only. The use of L-arginine as the sole base of the reaction did not change the trend of the electrophoretic pattern except that the magnitude of the reduction of the pI value was lower than for the other phosphorylated samples. This could be interpreted as a consequence of a relatively lower extent of phosphorylation (Fig. 5 and Tab. 2). The grafted arginyl residues neutralized the negative charges of the protein bound-phosphate groups on which they were bound. This is illustrated

Figure 3. SDS-PAGE of 7S and 11S soybean globulins. S: Molecular weight marker; 1: Native 7S globulin; 2–4: 7S globulins phosphorylated at different extent (9, 13 and 18 mol P/mol protein, respectively); 5: Native 11S globulin; 6–8: 11S globulins phosphorylated at different extent (10, 13 and 21 mol P/mol protein, respectively).

Figure 4. Isoelectrofocusing of 7S and 11S globulins. 1: Native 7S globulin; 2–4: 7S globulins phosphorylated at different extents (18, 13 and 9 mol P/mol protein, respectively); 5: Native 11S globulin; 6–8: 11S globulins phosphorylated at different extents (21, 13 and 10 mol P/mol protein, respectively).

by the slight reduction in the isoelectric point of the arginine-grafted samples compared with the other phosphorylated samples (Fig. 5).

3.5 Foaming properties

The foaming properties of phosphorylated soybean 7S and 11S globulins are presented in Table 3. Native 7S globulin displays very low foaming capacity since its maximal hydration, as measured by the liquid volume, is 1.6 ml in 50 ml foam. The foaming capacity of the native 11S globulin is even worse

Figure 5. Isoelectrofocusing of 7S and 11S globulins. N1: Native 7S globulin; N2: Native 11S globulin; S: pI marker; 1–3: Phosphorylated 7S globulins with grafted methionine (20, 18 and 15 mol P, and 2, 5 and 8 mol methionine/mol protein, respectively); 4: Phosphorylated 7S globulin with grafted methionine and arginine (14 mol P, 2 mol methionine and 23 mol arginine/mol protein); 5–7: Phosphorylated 11S globulins with grafted methionine (21, 19 and 16 mol P, and 2, 4 and 8 mol methionine/mol protein, respectively); 8: Phosphorylated 11S globulin with grafted methionine and arginine (15 mol P, 2 mol methionine and 23 mol arginine/mol protein).

as it did not form any foam at all. The stability of the foam obtained with native 7S globulin was very poor as demonstrated by the half-life time (128 sec). These poor foaming properties may be due to the low protein solubility of native soybean globulins around the neutral pH range what impedes the process of foam hydration.

Phosphorylation of 7S globulin highly enhanced its foaming capacity by increasing its foam hydration nearly four times the value obtained for the native globulin, and by doubling its foam stability as deduced from its half-life time. This improvement of the foaming properties may be due to an enhanced solubility of the phosphorylated soybean 7S globulin and more precisely to the presence of bound phosphate groups on the protein molecules enhancing protein hydrophilicity and consequently playing a major role in the foam hydration. Foam collapse is delayed because of the coulombic repulsions between the strong negative charges of the free phosphate groups attached to the modified globulin molecules. This agrees with the previous observation that low extent of phosphorylation is beneficial for the foaming properties of casein [26]. The foaming capacity and stability values attained by phosphorylated 11S globulin samples were comparable with those obtained with phosphorylated 7S globulin since the phosphorylation yields were nearly identical in both cases.

Phosphorylated 7S and 11S globulins containing grafted L-methionine achieved foaming capacity levels higher from those obtained without amino acid grafting (Table 4). The stability of phosphorylated samples with grafted methionine was nearly twice bigger from that obtained in case of samples phosphorylated only. This additive improvement of foaming properties is due merely to additional modification induced by the secondary grafting of amino acid. The grafted methionine

Table 3. Foaming properties of phosphorylated soybean globulins.

Protein sample	1 TF [s]	2 FV max [ml]	3 LV max [ml]	4 LV 20 min [ml]	5 HL [s]
Native 7S globulin	196	50	1.6 ± 0.02	0.4 ± 0.02	128 ± 4
Phospho-7S globulin					
9 mol P/mol protein	189	50	6.4 ± 0.06	1.6 ± 0.05	285 ± 11
13 mol P/mol protein	189	50	6.1 ± 0.06	1.3 ± 0.05	268 ± 10
18 mol P/mol protein	189	50	6.7 ± 0.06	1.3 ± 0.05	275 ± 10
Native 11S globulin	0	0	0	0	0
Phospho-11S globulin					
10 mol P/mol protein	192	50	6.3 ± 0.06	2.1 ± 0.08	373 ± 11
13 mol P/mol protein	191	50	6.3 ± 0.06	1.6 ± 0.05	299 ± 10
21 mol P/mol protein	188	50	5.6 ± 0.06	1.2 ± 0.04	218 ± 9

1: Time of foaming; 2: Maximum foam volume; 3: Maximum liquid volume in foam; 4: Liquid volume after 20 min; 5: Half-life of foam.

Table 4. Foaming properties of phosphorylated and methionine-grafted soybean globulins.

Protein	1 TF [s]	2 FV max [ml]	3 LV max [ml]	4 LV 20 min [ml]	5 HL [s]
Native 7S globulin	196	50	1.6 ± 0.02	0.4 ± 0.02	128 ± 4
Phospho-7S globulin					
20 mol P/mol protein + 2 mol Met/mol protein	189	50	7.2 ± 0.07	2.0 ± 0.08	550 ± 17
18 mol P/mol protein + 5 mol Met/mol protein	189	50	7.2 ± 0.07	2.5 ± 0.09	576 ± 18
15 mol P/mol protein + 8 mol Met/mol protein	189	50	7.5 ± 0.07	2.5 ± 0.09	580 ± 18
12 mol P/mol protein + 2 mol Met/mol protein + 23 mol Arg/mol protein	189	50	8.1 ± 0.08	2.3 ± 0.08	660 ± 21
Native 11S globulin	0	0	0	0	0
Phospho-11S globulin					
21 mol P/mol protein + 2 mol Met/mol protein	192	50	7.2 ± 0.07	1.8 ± 0.06	540 ± 17
18 mol P/mol protein + 5 mol Met/mol protein	191	50	7.1 ± 0.07	1.6 ± 0.06	540 ± 17
16 mol P/mol protein + 8 mol Met/mol protein	188	50	6.9 ± 0.07	1.7 ± 0.06	540 ± 17
18 mol P/mol protein + 2 mol Met/mol protein + 23 mol Arg/mol protein	190	50	7.9 ± 0.08	2.3 ± 0.08	650 ± 20

1: Time of foaming; 2: Maximum foam volume; 3: Maximum liquid volume in foam; 4: Liquid volume after 20 min; 5: Half-life of foam.

moieties diminish the strong negative charges of the phosphate groups on the modified protein molecule.

The soybean globulin samples phosphorylated in the presence of L-arginine and L-methionine – and containing both grafted amino acids – exhibited the highest foaming capacity and stability among all the phosphorylated samples. This again shows that amino acids grafted on the phosphate groups of the modified samples improved further their foaming properties by neutralizing the excessive negative charge of the phosphate groups while maintaining partial charges still big enough to induce the hydration of the protein foam. It can also be concluded that the grafted arginine had more powerful modifying effect than methionine because of its chemical character. As a dibasic amino acid, arginine binds to the phosphate group by primary amino group while still having a free guanidine and a free carboxyl group. Consequently, the final result of grafting is the replacement of one negative charge on the free phosphate groups by a grafted amino acid with a zero net charge,

but displaying a great hydrophilic capacity. The influence of grafted arginine on the net negative charge can be seen on the isofocusing patterns (Fig. 5). These products may be quite interesting from a nutritional point of view since they are prepared without the use of the organic base triethylamine unacceptable for alimentary purposes and which otherwise should be removed from the final products.

3.6 Emulsifying properties

The phosphoamidated with methionine 7S and 11S globulins showed improved emulsifying activity and stability when compared with the native globulins (Fig. 5 and 6). The soybean globulins phosphorylated in the absence of methionine exhibited similar emulsifying behavior (data not shown). Hence, this improvement in emulsifying properties is due mainly to the presence on the globulin of the phosphates conferring bigger hydrophilicity to globulin molecule and counter-

Figure 6. Emulsion stability curves for 7S globulins. (●) Native; Phosphorylated and methionine grafted [(■): 22 mol P and 2 mol methionine/mol protein; (▼) 15 mol P and 8 mol methionine/mol protein].

Figure 7. Emulsion stability curves for 11S globulins. (●) Native; Phosphorylated and methionine grafted [(○): 21 mol P and 2 mol methionine/mol protein; (▼): 16 mol P and 8 mol methionine/mol protein].

balancing its original hydrophobicity. Methionine grafted on the globulin phosphate groups did not change their activity in promoting formation of emulsion and increasing its stability. Generally, the improvement of the emulsifying properties of the phosphorylated proteins agrees well with the results of Sung et al. [3], Hirotsuka et al. [12] and Huang and Kinsella [6]. These authors attributed this improvement to the increase of number of negative charges on the phosphorylated proteins which tends to induce strong electrostatic repulsions between the negatively charged emulsion surfaces reducing thus the coalescence of the emulsions.

4 Conclusion

The extent of soybean 7S and 11S globulins phosphorylation can be controlled by varying the reaction conditions, also by the simultaneous addition of L-methionine which can divert the reaction towards methionylated globulins. Surprisingly, the

electrophoretic patterns of the phosphorylated proteins did not show major changes in the subunit distribution of the modified soybean globulins. The functional properties of the modified proteins strongly depend on the extent of phosphorylation and amino acylation as for example the foaming and emulsifying properties which get improved remarkably, especially in case of the samples with grafted L-methionine. As can be seen, this simple approach using nutritionally acceptable reagents can constitute an efficient way to modify the functional properties of these classes of proteins. Since it is of a general character, it is clear that the application of this simple method can be extended to other proteins.

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