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Original article

Functional properties of β-lactoglobulin phosphorylated in the presence of different aliphatic amines

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Summary — Phosphorylation of β-lactoglobulin was carried out with 20, 40 and 80 mol $POCl_3$/mol protein in the presence of different aliphatic amines. The physicochemical properties of the modified proteins changed according to the nature of amine (tertiary or primary; shorter or longer aliphatic chain). The use of butylamine during the phosphorylation of β-lactoglobulin with $POCl_3$ resulted in a low phosphorylation yield (2–4 mol P/mol protein), conferring, however, excellent foaming and emulsifying properties to the phosphobutylated protein. The use of longer aliphatic primary amines (8–12 C) as bases and nucleophiles during the phosphorylation of β-lactoglobulin gave rise to low phosphorylation, reduced solubility, poor foaming and emulsifying properties.

β-lactoglobulin / phosphorylation / aliphatic amine / functional properties

Résumé — **Propriétés fonctionnelles de la β-lactoglobuline phosphorylée en présence de différentes amines aliphatiques.** *La β-lactoglobuline a été phosphorylée en utilisant différents rapports molaires d'oxychlorure de phosphore/mole de protéine (20, 40 et 80) et en présence de différentes amines aliphatiques. Les propriétés physicochimiques des protéines modifiées varient en fonction du type de l'amine utilisée (tertiaire ou primaire; chaîne aliphatique plus ou moins longue). L'utilisation de butylamine pendant la phosphorylation de la β-lactoglobuline par $POCl_3$ a conduit à un faible taux de phosphorylation (2–4 mol P/mol protéine), mais à d'excellentes propriétés moussantes et émulsifiantes de la protéine phosphobutylée. L'utilisation d'amines primaires aux plus longues chaînes (8–12 C) comme bases et agents nucléophiles durant la phosphorylation de la β-lactoglobuline a donné naissance à des protéines faiblement phosphorylées, moins solubles et présentant de moins bonnes propriétés moussantes et émulsifiantes.*

β-lactoglobuline / phosphorylation / amine aliphatique / propriétés fonctionnelles

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INTRODUCTION

There is an increasing demand for proteins with changed functional properties either for food or non-food applications. Chemical phosphorylation may provide a powerful tool for a change of the physicochemical properties of the proteins (Yoshikawa *et al,* 1981; Hirotsuka *et al,* 1984; Huang and Kinsella, 1987; Chobert *et al,* 1989a; Matheis, 1991). It may be very efficient if the reaction conditions are carefully designed (Medina *et al,* 1992; Sitohy *et al,* 1994). Recently, it was found possible to phosphorylate proteins under mild conditions avoiding oligomerization (Sitohy *et al,* 1994, 1995). These conditions included the use of low molar ratio of phosphorus oxychloride ($POCl_3$) and stoichiometric addition of a nucleophile amidating the phosphoryl groups of the modified proteins. The aim of this work was to compare the physicochemical and functional properties of β-lactoglobulin phosphorylated with $POCl_3$ in the presence of different aliphatic amines in order to reveal the effects of aliphatic chain length.

MATERIALS AND METHODS

Materials

β-Lactoglobulin (a mixture of variants A and B) was purchased from Laiteries Triballat (Noyal/Vilaine, France) and purified according to the procedure of Mailliart and Ribadeau Dumas (1988). All other chemicals were reagent grade.

Phosphorylation

The procedure of Sitohy *et al* (1994) was applied as follows: $POCl_3$ was dissolved in carbon tetrachloride at 20%; amounts of phosphorus oxychloride equivalent to molar ratios (MR) of 20, 40 and 80 mol $POCl_3$/mol protein were added dropwise to 1% aqueous β-lactoglobulin solution during 30 min. Six-fold molar excess of aliphatic amines (triethylamine -TEA, butylamine, hexylamine, octylamine, decylamine or dodecylamine) per mol $POCl_3$ (if otherwise stated) were added to protein solutions before reaction start except for dodecylamine, which because of its poor solubility in water was dissolved in small aliquot of ethyl alcohol before being added to the reaction medium. Six-fold excess of this base was requested to neutralize protons generated during the hydrolysis of $POCl_3$. This simple method makes it possible to control the pH during the reaction. The reaction was carried out in an ice-bath (0–5°C). The reaction medium was left for 10 min for complete separation of the organic phase from the aqueous layer, which was then recovered and dialysed against several changes of distilled water for 4 d and freeze-dried. Phosphorus content was determined in both native and modified β-lactoglobulin according to Bartlett (1959) as modified by Sitohy *et al* (1994). The extent of phosphorylation was determined by subtracting the phosphorus content in the native from that in the modified protein.

Electrophoresis

Urea-PAGE was carried out according to the procedure described by Chobert *et al* (1989b). SDS-PAGE was performed according to Laemmli (1970) on 15% acrylamide running gel and 3.2% acrylamide stacking gel. Electrofocusing was performed in the pH range of 3–7 on ready-to-use isoelectric focusing gels (FMC, Rockland, USA). A marker kit of pI in the range of 3.5–7.35 was used (Pharmacia LKB, Uppsala, Sweden).

Surface hydrophobicity measurement

Surface hydrophobicity was measured according to Halpin and Richardson (1985) in native and modified protein solutions (10^{-6} mol/l) prepared in 0.01 mol/l phosphate buffer, pH 7, using 1-anilinonaphthalene-8-sulphonate (ANS) as hydrophobic probe.

Protein solubility and emulsification properties

Protein pH solubility curves and emulsifying activity were assayed in the pH range of 2–7 according to the procedure outlined by Chobert *et al* (1991). Emulsifying stability was measured according to Chobert *et al* (1991) with slight modifications.

Foaming properties

The foaming properties of native and phosphorylated β-lactoglobulin solutions (0.4 mg/ml) prepared in 0.1 mol/l phosphate buffer, pH 7.0, were measured with a recently described apparatus (Guillerme *et al*, 1993; Loisel *et al*, 1993).

RESULTS AND DISCUSSION

Extent of phosphorylation

The phosphorylation yield of β-lactoglobulin increased proportionally to the molar ratio of $POCl_3$/protein, especially in the presence of tertiary or short chain primary amines (table I). Triethylamine addition was the most efficient for the phosphorylation reaction followed by hexylamine then butylamine. This is due to the different basicity and reactivity of these 3 amines. Triethylamine plays a scavenger role in this reaction by binding generated HCl which otherwise would hydrolyse phosphoamide bonds. Other studied amines with long aliphatic chains (8–12 C) were associated with poor phosphorylation yields, possibly due to their high hydrophobicities which might make phosphoamidated protein molecules less soluble and hence less accessible for further phosphorylation. Poor solubility was observed for proteins phosphorylated in the presence of octylamine, decylamine and dodecylamine. The convenience of using the tertiary amine TEA as a base for the phosphorylation reaction may be due to its high basicity, making it possible to quench the excess of produced HCl, a relatively low hydrophobicity which leads to products well soluble in the reaction medium and hence more accessible to further phosphorylation.

Electrophoretic patterns

In general, the urea-PAGE patterns of the phosphorylated β-lactoglobulins showed increased migration towards the anode due to their increased contents of the negatively charged phosphoryl groups. The migration rate of the samples phosphorylated in the presence of butylamine increased propor-

Table I. Extent of β-lactoglobulin phosphorylation (mol P/mol protein) with different molar ratios of $POCl_3$ in the presence of different aliphatic amines (6 mol amine/mol $POCl_3$).
Étendue de la phosphorylation de la β-lactoglobuline, à différents rapports molaires de $POCl_3$ et en présence de diverses amines aliphatiques (6 mol amine/mol $POCl_3$).

Molar ratio ($POCl_3$/protein)	*Triethyl-amine*	*Butyl-amine*	*Hexyl-amine*	*Octyl-amine*	*Decyl-amine*	*Dodecyl-amine*
			(mol P/mol protein)			
20	7	2	2	2	2	2
40	8	3	5	3	3	3
80	9	4	7	3	3	3

tionally to the molar ratio of $POCl_3$/protein or amine/$POCl_3$; this was due to the increase of the phosphorylation yield and amidation (fig 1A).

Hexylamine gave rise to similar electrophoretic patterns while the amines with longer aliphatic chains (8–12 C) did not give clear-cut results because of their poor solubility (data not shown). The SDS-PAGE patterns of the samples phosphorylated in the presence of butylamine showed negligible cross-linking (fig 1B), and the same was visible in the case of hexylamine (data not shown). The isoelectrofocusing patterns of the samples phosphorylated in the presence of either butylamine (fig 2A) or hexylamine (fig 2B) demonstrated 2 separated regions of bands with different isoionic points distinct from samples phosphorylated in the presence of triethylamine (fig 2B). This separation is not only due to a low extent of phosphorylation which gives rise to heterogenous products but also to the covalent binding of aliphatic amine to the protein phosphoryl groups by phosphoamide bonds. Primary amines may bind to protein phosphoryl groups either through amide or salt bonds while tertiary amines bind exclusively through quaternary salt bonds. The modified proteins with phosphoamide bonds display smaller negative charges. The soluble fractions of the proteins phosphorylated in the presence of longer aliphatic amines (fig 2C) showed only 1 group of bands with small

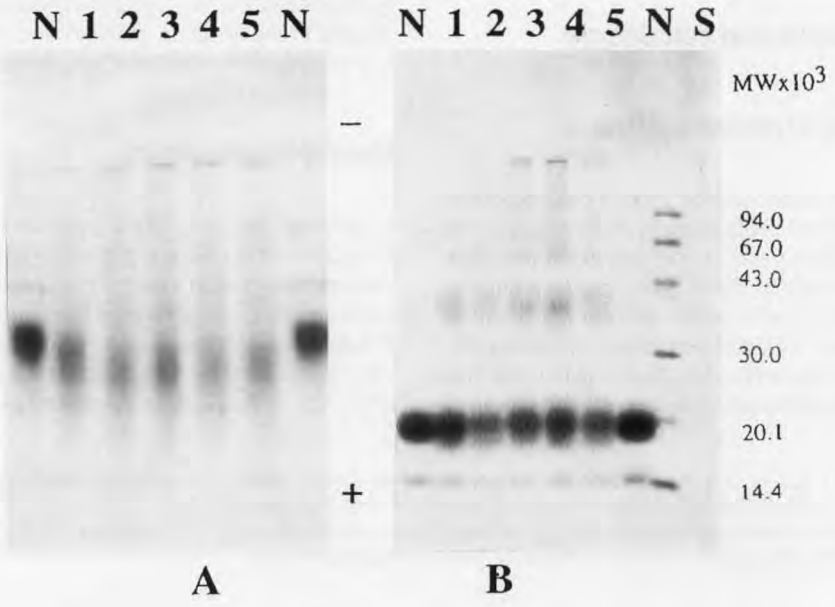

Fig 1. Urea- (A) and SDS-PAGE (B) electrophoretic patterns of native (N) and phosphorylated β-lactoglobulin with 20, 40 and 80 mol $POCl_3$/mol protein in the presence of 6 mol butylamine/mol $POCl_3$ (samples 1, 2 and 3, respectively) or with 80 mol $POCl_3$/mol protein in the presence of 5 and 4 mol butylamine/mol $POCl_3$ (samples 4 and 5, respectively). Sample S is molecular weight marker kit.
Profils électrophorétiques en présence d'urée (A) et de SDS (B) de la β-lactoglobuline native (N) ou phosphorylée avec 20, 40 ou 80 mol $POCl_3$/mol protéine, en présence de 6 mol butylamine/mol $POCl_3$ (échantillons 1, 2 et 3, respectivement) ou avec 80 mol $POCl_3$/mol protéine en présence de 5 et 4 mol butylamine/mol $POCl_3$ (échantillons 4 et 5, respectivement). Échantillons S : marqueurs de poids moléculaires.

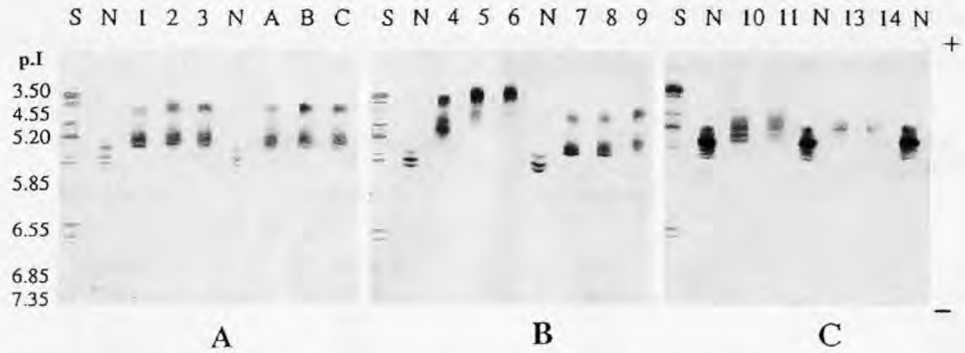

Fig 2. Isofocusing electrophoretic patterns of β-lactoglobulin phosphorylated with $POCl_3$ in the presence of different aliphatic amines. (A) Butylamine; samples 1–3 prepared with 20, 40 and 80 mol $POCl_3$/mol protein and 6 mol amine/mol $POCl_3$, respectively, and samples A–C prepared with 80 mol $POCl_3$/mol protein in the presence of 4, 5 and 6 mol amine/mol $POCl_3$. (B) Triethylamine (samples 4–6) and hexylamine (samples 7–9) prepared with 20, 40 and 80 mol $POCl_3$/mol protein, respectively. (C) Octylamine (samples 10 and 11, prepared with 40 and 80 mol $POCl_3$/mol protein); decylamine (sample 13) and dodecylamine (sample 14) prepared with 80 mol $POCl_3$/mol protein. Samples N and S are native β-lactoglobulin and isofocusing marker, respectively.

Électrofocalisation de la β-lactoglobuline phosphorylée par $POCl_3$ en présence de différentes amines aliphatiques. (A) Butylamine ; échantillons 1 à 3 respectivement préparés avec 20, 40 et 80 mol $POCl_3$/mol protéine et 6 mol amine/mol $POCl_3$; échantillons A à C respectivement préparés avec 80 mol $POCl_3$/mol protéine en présence de 4, 5 et 6 mol amine/mol $POCl_3$. (B) Triéthylamine (échantillons 4 à 6) et hexylamine (échantillons 7 à 9) préparés respectivement avec 20, 40 et 80 mol $POCl_3$/mol protéine. (C) Octylamine (échantillons 10 et 11 préparés avec 40 et 80 mol $POCl_3$/mol protéine) ; décylamine (échantillon 13) et dodécylamine (échantillon 14) préparés avec 80 mol $POCl_3$/mol protéine. Échantillon N : β-lactoglobuline native. Échantillon S : marqueurs de pI.

change in isoionic point from the native protein, which may indicate high content of phosphoamide bonds. It is evident that long chain aliphatic amines confer considerable hydrophobicity to the product.

Surface hydrophobicity

Surface hydrophobicity was reduced by phosphorylation in the presence of aliphatic amines (table II) as a result of introducing an excess of phosphate groups. Phosphorylation in the presence of triethylamine reduced the most β-lactoglobulin surface hydrophobicity in comparison with primary aliphatic amines able to amidate phosphorylated protein. This is due to higher phosphorylation yields in the presence of TEA and amide formation between the primary amines and the phosphoryl groups, as can be seen on the isoelectrofocusing patterns of the modified proteins. This conclusion is confirmed by the observation that a sample phosphorylated in the presence of TEA shows higher reduction in surface hydrophobicity than a protein with the same extent of phosphorylation (7 mol P/mol protein) in the presence of hexylamine (tables I, II). It can be concluded that the aliphatic amine can considerably increase the hydrophobicity of the phosphorylated protein.

Table II. Surface hydrophobicity of β-lactoglobulin phosphorylated with $POCl_3$ in the presence of different amines (6 mol amine/mol $POCl_3$).
Hydrophobicité de surface de la β-lactoglobuline phosphorylée par $POCl_3$ en présence de différentes amines (6 mol amine/mol $POCl_3$).

Molar ratio $POCl_3$*/protein*	*Maximum fluorescence*		
	Triethylamine	*Butylamine*	*Hexylamine*
0	0.73	0.73	0.73
20	0.40	0.52	0.67
40	0.31	0.48	0.62
80	0.31	0.45	0.55

Protein solubility

The solubility curves of various preparations of β-lactoglobulin phosphorylated in the presence of aliphatic primary amines showed a consequent shift of the isoelectric points towards the acidic pHs (fig 3). The extent of this shift relates well with the phosphorylation yield (table I). The minimal solubility of the modified proteins was reduced considerably when compared to the native β-lactoglobulin. The conformational changes due to the denaturation and additional negative charges of grafted phosphates may account for this phenomenon (Sitohy *et al*, 1995). In addition, the pH range of the minimal solubility was larger for the phosphorylated β-lactoglobulins. The samples phosphoamidated with 80 mol $POCl_3$/mol protein in the presence of butylamine showed broad solubility minimum in the pH range of 2.5–5.5. This may be due to significant heterogeneity induced by low phosphorylation yield (4 mol P/mol protein)

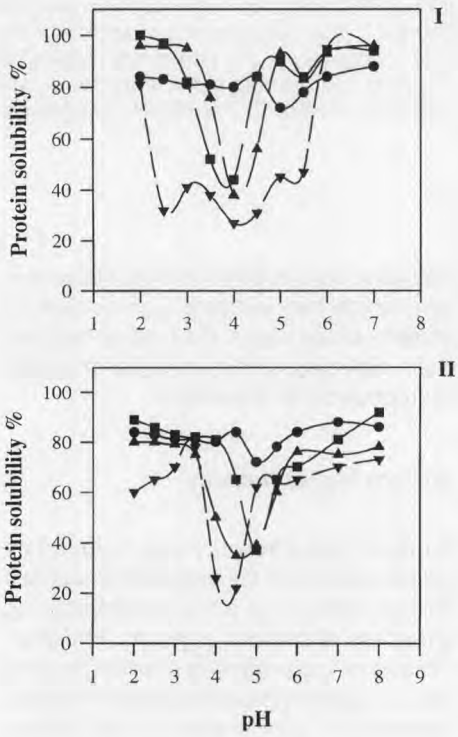

Fig 3. Protein pH solubility curves for native (●) and phosphorylated β-lactoglobulin with 20 (■), 40 (▲) and 80 (▼) mol $POCl_3$/mol protein, respectively, in the presence of butylamine (I) or hexylamine (II) added as 6 mol amine/mol $POCl_3$.
Profils de solubilité en fonction du pH de la β-lactoglobuline native (●) et phosphorylée respectivement par 20 (■), 40 (▲) et 80 (▼) mol $POCl_3$/mol protéine, en présence de butylamine (I) ou d'hexylamine (II) à raison de 6 mol amine/mol $POCl_3$.

and several possible modes of substitution of 8 remaining reactive mixed anhydride groups by primary amines (4 x -NH-$POCl_2$). However, β-lactoglobulins phosphorylated in the presence of butylamine were soluble at neutral pHs (6–7), while those phosphorylated in the presence of hexylamine showed 15–20% reduction in solubility due to higher polymerization visible in the SDS-PAGE electrophoregrams of these proteins. The use of higher aliphatic amines (8–12 C) in the phosphorylation and amidation of β-lactoglobulin resulted in higher reductions in solubility (60–80%) at the neutral pHs (data not shown) as a result of the grafting of long aliphatic chains.

Emulsifying properties

As expected, the pH curves of the emulsifying activity index (EAI) of β-lactoglobulin phosphorylated in the presence of TEA (fig 4 III) was characterized by a displacement of the minimal EAI towards the acidic pHs following the displacement of the isoionic points (fig 2B). Consequently, EAI was improved in the pH range of 4–5.5, while the samples phosphorylated in the presence of either butylamine or hexylamine showed EAI enhancement at all pHs and the most in the pH range of 6–7. This distinction in EAI may be due to different substitutions of phosphoryls either hydrated to mono-dihydro-phospho-monoamides or subject to secondary amidation with primary aliphatic amines. The short chain primary amines modify slightly the charges of the phosphates forming phosphoamide bonds. This decreases the negative charges of the protein phosphates enough to enhance their interfacial properties. The electrostatic charges should not be too high, since they might delay oil–protein interaction. The phospho-amidation of β-lactoglobulin with amines (8–12 C) leads to considerable deterioration in the EAI due to the reduced sol-

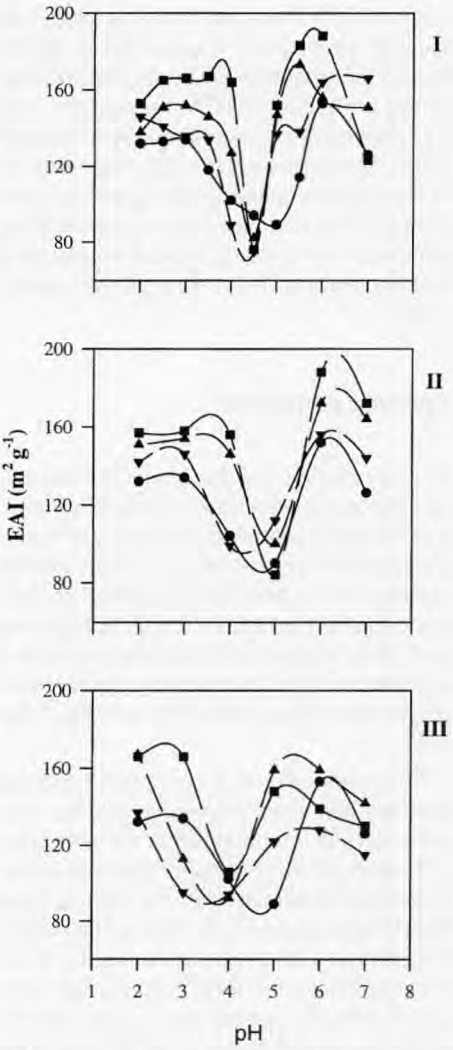

Fig 4. Emulsifying activity index (EAI) profiles of native (●) and phosphorylated β-lactoglobulin with 20 (■), 40 (▲) and 80 (▼) mol $POCl_3$/mol protein, respectively, in the presence of butylamine (I), hexylamine (II) or triethylamine (III) added as 6 mol amine/mol $POCl_3$. Mean of 4 determinations.

Index d'activité émulsifiante de la β-lactoglobuline native (●) et phosphorylée respectivement par 20 (■), 40 (▲) et 80 (▼) mol $POCl_3$/mol protéine, en présence de butylamine (I), d'hexylamine (II) ou de triéthylamine (III) à raison de 6 mol amine/mol $POCl_3$. Moyenne de 4 déterminations.

ubility of the modified proteins (data not shown). However, the emulsifying stabilities of the β-lactoglobulin phosphorylated in the presence of TEA, butylamine and hexylamine were exceptionally improved (fig 5). The enhanced charge and hydration of the phosphorylated proteins can give rise to negatively charged thick interfacial films which are essential for good emulsifying stability (Halling, 1981; Huang and Kinsella, 1987).

Foaming properties

An improvement of the foaming capacity, as indicated by the maximal liquid content, can be observed when proteins are phosphorylated in the presence of triethylamine, butylamine or hexylamine (table III). The largest effect is obtained with butylamine and for all aliphatic amines with the lowest substitution yield. No further improvement is observed when molar ratio is higher than 20.

Foam stability is principally estimated from the evolution of the foam volume. Volume stability is enhanced in the presence of β-lactoglobulin phosphorylated or phosphoamidated with butylamine. On the other hand, the amidation of grafted on the protein phosphoryl with hexylamine results in an intensive foam destabilization. For the highest substitution yields, the foam is almost completely destroyed after 20 min. The largest decrease of the rate of liquid drainage is also obtained for the lowest substitution yields in the presence of phosphorylated or phosphoamidates with butylamine β-lactoglobulin. The best foaming properties could be obtained when protein phosphorylation yield was relatively low (2–4 mol P/mol protein) and short chain primary amine (butylamine) was grafted during the reaction. The use of butylamine kept the phosphorylation relatively low and reduced the charge of the phosphates, inducing good foaming properties. The increase of the phosphorylation extent in the presence of tertiary amine gives rise to high negative charges which deteriorate the foaming properties because of strong electrostatic repulsion. This was observed in the case of β-lactoglobulin phosphorylated with 80 mol $POCl_3$/mol protein in the presence of TEA

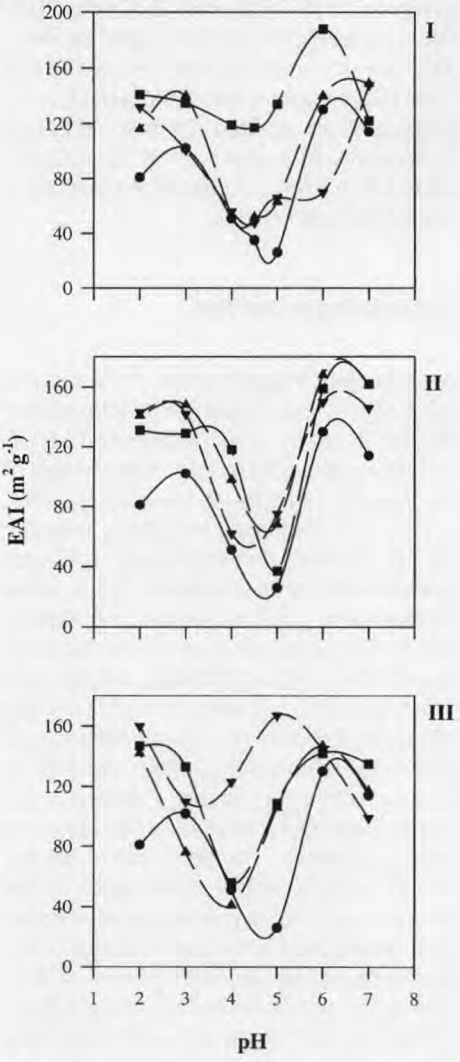

Fig 5. Emulsifying stability profiles for the samples indicated in figure 4.
Stabilité des émulsions caractérisées en figure 4.

Table III. Foaming properties of β-lactoglobulin phosphorylated with $POCl_3$ in the presence of different aliphatic amines (6 mol amine/mol $POCl_3$).
Propriétés moussantes de la β-lactoglobuline phosphorylée en présence de différentes amines aliphatiques (6 mol amine/mol $POCl_3$).

Molar ratio $POCl_3$/protein	*LV max*			*FV*			*HD*		
	A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
0	2.24 ± 0.02	2.24 ± 0.02	2.24 ± 0.02	44.6 ± 0.11	44.6 ± 0.11	44.6 ± 0.11	422 ± 15	422 ± 15	422 ± 15
20	4.85 ± 0.05	7.97 ± 0.09	6.16 ± 0.08	49.0 ± 0.15	47.6 ± 0.16	38.0 ± 0.12	640 ± 20	685 ± 25	450 ± 15
40	4.37 ± 0.04	7.85 ± 0.11	6.06 ± 0.12	47.8 ± 0.22	47.0 ± 0.18	15.0 ± 0.18	572 ± 11	647 ± 15	445 ± 11
80	4.29 ± 0.05	7.85 ± 0.14	4.44 ± 0.05	47.2 ± 0.30	43.6 ± 0.21	15.0 ± 0.13	550 ± 12	560 ± 16	420 ± 10

A: triethylamine; B: butylamine; C: hexylamine; LV max: maximum liquid volume in foam (ml); FV: foam volume after 20 min (ml); HD: half-life of foam(s).
Mean of 4 determinations.

(9 mol P/mol protein). Contiguous foam bubbles mutually repel each other at a critical distance due to the electrostatic repulsion between negative charged proteins on the surface films, minimizing the coalescence (Graham and Philips, 1976; Kinsella, 1981). The charge increase may repel the proteins in the surface films beyond the critical distance and enhances foam coalescence. As expected, the use of longer primary amines during the phosphorylation of β-lactoglobulin resulted in bad foaming properties (data not shown). This resulted from their poor solubility since only soluble proteins can contribute to foaming (Halling, 1981).

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