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Original article

Purification and characterization of two porcine ß-lactoglobulin variants by NaCl salting-out and reversed phase-HPLC

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Summary — Porcine whey proteins have been separated by reversed-phase HPLC and the different eluted fractions were analysed by SDS–PAGE. Two protein fractions lately eluted from HPLC, with an apparent molecular weight of approximately 18 800 had an amino acid composition corresponding to that of porcine β-lactoglobulin. These 2 fractions differed only in their Ala/Val content and consequently may be genetic variants of porcine β-lactoglobulin. In order to obtain larger amounts of β-lactoglobulin, we attempted to adapt to porcine whey a previously published method used for bovine β-lactoglobulin and based on NaCl salting-out at low pH. Each step of purification was monitored by RP-HPLC. After precipitation (14% NaCl), solubilisation in 7% NaCl and dialysis against distilled water, β-lactoglobulin was obtained with a purity of 94%. Final separation of β-lactoglobulin variants can be rapidly achieved by RP-HPLC on a semi-preparative column.

β-lactoglobulin / sow milk / purification

Résumé — Purification et caractérisation de deux variants de la β-lactoglobuline porcine par précipitation fractionnée par NaCl et CLHP en phase inverse. *Les protéines du lactosérum de lait de truie ont été séparées par CLHP en phase inverse et les différentes fractions éluées ont été analysées par électrophorèse en présence de SDS. Les deux fractions les plus retenues sur la colonne, d'un poids moléculaire voisin de 18 800 et ayant une composition en acides aminés correspondant à celle de la β-lactoglobuline porcine, ne diffèrent que par leur teneur en alanine et en valine. Ces deux fractions peuvent correspondre à deux variants génétiques de la β-lactoglobuline porcine. Afin d'obtenir de plus grandes quantités de β-lactoglobuline, nous avons adapté un protocole de précipitation fractionnée par NaCl à pH acide (protocole déjà utilisé pour la β-lactoglobuline bovine). Chaque étape de purification a été analysée par CLHP en phase inverse. En trois étapes, précipitation dans NaCl 14%, redissolution à NaCl 7% et dialyse contre de l'eau distillée, nous avons obtenu de la β-lactoglobuline pure à 94%. La séparation des deux variants peut ensuite être rapidement effectuée par CLHP en phase inverse sur colonne semi-préparative.*

β-lactoglobuline / lait de truie / purification

INTRODUCTION

β-lactoglobulin (BLG) is an abundant component of the whey fraction of the milk of several animal species. Bovine BLG has been studied for a long time and is a well characterized protein (McKenzie, 1971; Creamer *et al,* 1983; Sawyer *et al,* 1985; Papiz *et al,* 1986). The primary structure of porcine BLG was elucidated by Conti *et al* (1986). It is composed of 159 amino acids, in contrast to longer bovine or equine BLGs, containing 162 or 166 amino acids, respectively (Braunitzer *et al,* 1972; Godovac-Zimmermann *et al,* 1985). When compared with bovine BLG, porcine BLG displays 61.8% sequence identity. Porcine BLG was also reported to be monomeric, in contrast to ruminant BLG, and polymorphic (Kessler and Brew, 1970).

BLG is classified in the lipocalin protein "superfamily". Structural studies of retinol binding protein (Newcomer *et al,* 1984), bilin binding protein (Huber *et al,* 1987), insecticyanin (Holden *et al,* 1987) and β-lactoglobulin (Papiz *et al,* 1986; Monaco *et al,* 1987) show that these transporters of hydrophobic molecules share a 3-dimensional structural pattern of β-barrel. Despite abundant data, the biological role of BLG is not well understood (Monti *et al,* 1989; Said *et al,* 1989). Moreover, the exact location of the main retinol binding site of BLG (Futterman and Heller, 1972; Fugate and Song, 1980) is still insufficiently defined (Papiz *et al,* 1986; Monaco *et al,* 1987; North,1989). This task is complicated by the presence of 2 tryptophan chromophores in the molecule of the best-studied bovine BLG, hampering the unambiguous interpretation of fluorescence data. BLG also binds fatty acids and triglycerides (Brown, 1984; Diaz de Villegas *et al,* 1987), alkanone flavors (O'Neill and Kinsella, 1987), hemin (Dufour *et al,* 1990) and ellipticine (Dodin *et al,* 1990).

Characterization at a molecular level of the binding site(s) seems particularly interesting. Fluorescence spectroscopy is an ideal technique for such an investigation, provided the system has well-defined fluorophores (Dufour *et al,* 1990). Porcine BLG, which has only one tryptophan residue in a position conserved in all BLG sequences, seems to be quite appropriate for such a study. In this paper we report the purification of porcine BLG variants in large amounts by 2 single steps.

MATERIALS AND METHODS

Materials

Organic solvents used for HPLC were from Carlo Erba (Italy). Trifluoroacetic acid (TFA), 6 N HCl, methane sulfonic acid and amino acid standards were obtained from Pierce chemicals (Rockford, IL, USA). All other reagents were of analytical grade. Buffers and solvents were filtered through Millipore 0.45-μm filters (Millipore, Bedford, MA) and degassed under vacuum before use. Porcine milk was kindly provided by L Aumaitre (INRA, St-Gilles, France).

Methods

Preparation of acid whey and acid whey protein analysis by reversed phase–HPLC (RP-HPLC)

Porcine colostrum obtained from one sow was defatted by centrifugation at 3 000 *g* for 30 min. After removal of caseins by precipitation at pH 4.6 with 1 N HCl, the acid whey protein fraction was dialysed and lyophilised.

The analysis of porcine whey proteins was performed by RP–HPLC on a Nucleosil C-18 column (4.6 mm id x 25 cm, SFCC, Gagny, France), equilibrated in 70% solvent A (0.11% TFA in H_2O) and 30% solvent B (40% H_2O, 60% CH_3CN, 0.09% TFA). Elution was obtained by using linear gradients, up to 70% solvent B in the first 15 min, then 74% solvent B in the next 18 min. The flow rate was 1 ml/min and the absorbance was recorded at 214 nm.

Purification of BLG was performed on a semi-preparative Nucleosil C-18 column (7.2 mm id x 25 cm, SFCC, Gagny, France) using the same solvents as above. The column was equilibrated with 30% solvent A and 70% solvent B and the elution obtained by using a linear gradient from 70 to 74% solvent B in 18 min, at a flow rate of 1.5 ml/min. The different fractions were collected and dried in a Speed Vac concentrator.

Electrophoresis

SDS–PAGE was performed according to Laemmli (1970) on a 15% acrylamide gel with a 3.2% acrylamide stacking gel.

Amino acids analysis

After acid hydrolysis under vacuum in the presence of 6 N HCl for 24 h at 110 °C in a Pico-Tag Station (Waters Associates, Millford, MA, USA) amino acids were derivatized with PITC according to Bidlingmeyer *et al* (1984) and analysed on a C-18 Pico-Tag column (3.9 mm id x 15 cm, Waters). Dried samples were dissolved in 95% 2 mmol/l Na_2HPO_4, pH 7.4 5% acetonitrile. The HPLC column was equilibrated in solvent A (94% 0.14 mol/l CH_3COONa containing 3.59 mmol/l triethylamine pH 6.4 6% acetonitrile) and the elution performed with a convex gradient from solvent A to 46% solvent B (40% H_2O/60% acetonitrile) in 10 min, at a flow rate of 1 ml/min. Both the column and solvents were maintained at 38 °C and the absorbance recorded at 254 nm.

For tryptophan determination, proteins were hydrolysed in the presence of 4 mol/l methane sulfonic acid containing 0.2% tryptamine, according to Simpson *et al* (1976). After 24 h at 110 °C, the samples were neutralized with 4 N KOH and derivatized according to Cohen *et al* (1989). HPLC analysis was performed using the previously described solvents, and elution obtained using a convex gradient from 0 to 50% B in 10 min.

Separation of whey proteins by NaCl salting-out at low pH

Separation was performed according to Mailliart and Ribadeau Dumas (1988) with modifications (fig 1). Whey proteins were dissolved in water (10%, w/v) and the pH adjusted to 2 with HCl. Then, NaCl was added to give a 14% (w/v) final concentration. After 20 min at 20 °C, the suspension was centrifuged at 10 000 *g* for 20 min. The precipitate (P1) was suspended in 14% (w/v) NaCl, pH 2, then diluted to a final concentration of 7% (w/v) NaCl and, after holding for 20 min at 20 °C, centrifuged at 10 000 *g* for 20 min. The resulting supernatant (S2) was dialysed against water, centrifuged at 10 000 *g* for 20 min and the supernatant (S3) lyophilised. All the precipitates and supernatants were analysed by RP–HPLC.

The amounts of proteins were calculated from their HPLC peaks areas. The proportions of BLG were expressed as percentages of the total amount of eluted proteins considered as 100.

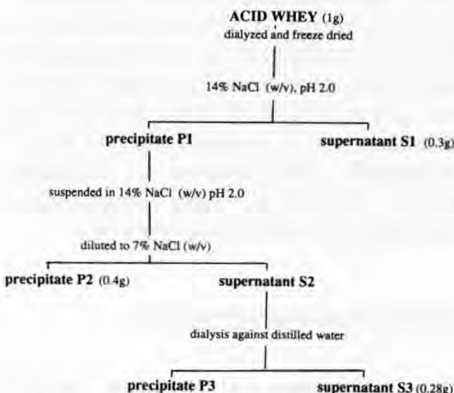


Fig 1. Purification procedure of porcine BLG by NaCl salting-out at low pH.
Protocole de purification de la BLG porcine par précipitation fractionnée à différentes concentrations en NaCl à pH 2.

RESULTS AND DISCUSSION

Whey proteins analysis by RP-HPLC and electrophoresis

The 8 fractions separated by RP–HPLC (fig 2A) were analysed by SDS–PAGE (fig 2B). Electrophoresis pattern of crude acid whey showed numerous bands: proteins of molecular weight > 94 000 which rapidly destained, 4 major proteins of apparent molecular weight 67 000, 58 000, 18 800 and 14 400. The last 2 corresponded to porcine β-lactoglobulin and α-lactalbumin, respectively (Erhardt, 1989). Minor bands of various molecular weight were also observed.

The first 4 fractions obtained by RP–HPLC corresponded to large and hydrophilic proteins of molecular weight > 94 000. Fraction 5 contained a protein of molecular weight 16 000. This was also observed by Erhardt (1989) who supposed it to be a soluble fraction of casein. Fraction 6 was essentially constituted by α-lactalbumin. The last 2 fractions (7 and 8) showed the same electrophoretic pattern: proteins of molecular weight 18 800, corresponding to BLG. As determined by chromatographic peak area, porcine whey protein content was about 44% BLG (21% and 23% for variants 1 and 2, respectively), 17.5% α-lactalbumin, 30% proteins of molecular weight > 94 000 (probably immunoglobulins) and 8.5% other minor proteins.

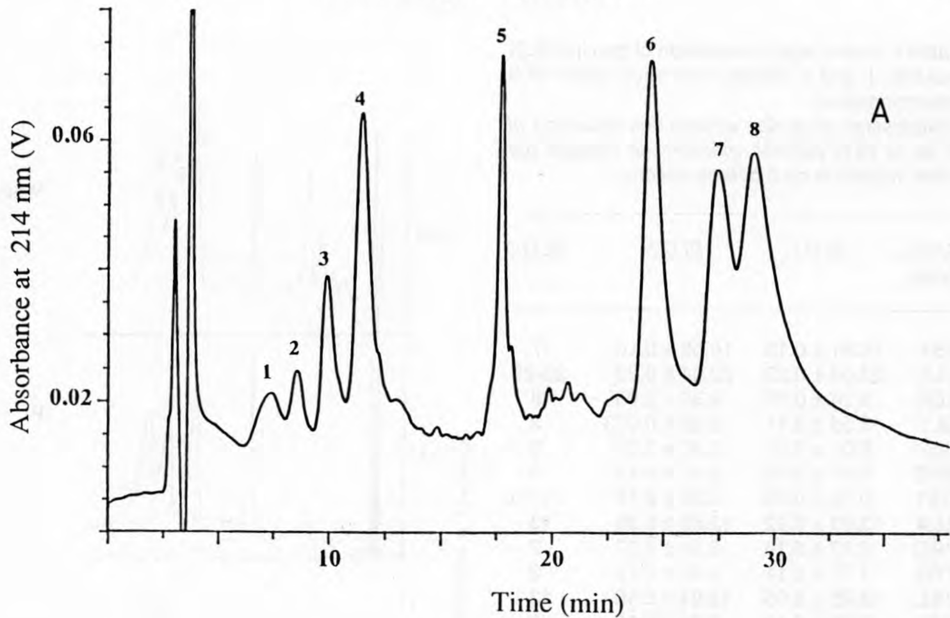
Porcine BLG characterization

The 2 fractions of molecular weight 18 800, named BLG1 (fraction 7) and BLG2 (fraction 8) were purified by semi-preparative RP-HPLC. The amino acid compositions of these 2 fractions (table I) are very comparable and correspond to those described by several authors (Bell *et al*, 1981; Conti *et al*, 1986) for porcine BLG. These 2 fractions differ only in their Ala/Val contents (BLG1: 13 Ala, 12 Val; BLG2: 12 Ala, 13 Val). Kalan *et al* (1971) have published the amino acid composition of 2 porcine BLG variants named A and B which seems to correspond to our BLG2 and BLG1 variants, respectively. Variant A has also been described by Bell *et al* (1981). As expected, each variant of porcine BLG has only one Trp residue.

In contrast with previous methods used by other authors to purify porcine BLG, *eg* gel filtration and ion exchange chromatography (Bell *et al*, 1981) or preparative electrophoresis and electroelution (Conti *et al*, 1986), RP–HPLC allows separation of porcine BLG variants in a single step. However, using this procedure, only small amounts of BLG are loaded on the column and 30 min cycles are needed. For this reason, a preliminary separation of BLG from the other proteins was necessary as a first step before the semi-preparative RP–HPLC purification.

BLG purification by NaCl salting-out at low pH (figs 1 and 3)

BLG precipitated at pH 2 in 14% NaCl. This allowed proteins of molecular weight higher than 94 000 which remained in the supernatant to be discarded (results not shown). The precipitate (P1) contained about 55% BLG. Dilution to 7% NaCl released BLG in the supernatant (S2) which was comprised of 88% BLG, residual α-lactalbumin and some other minor proteins. A small amount of BLG (about 8% of the total BLG) remained in the precipitate, P2. After dialysis of S2 against distilled water and centrifugation, the resulting supernatant (S3), depleted of precipitating α-lactalbumin, contained 94% BLG. Porcine



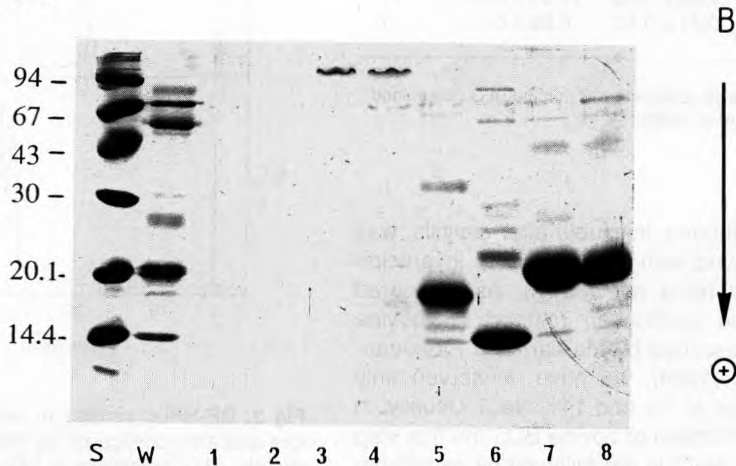


Fig 2. (A): RP–HPLC profile of porcine whey proteins. (B): SDS–PAGE of whey (W) proteins and RP–HPLC fractions (1–8). S: reference proteins (from top to bottom): phosphorylase b, MW 94 000; bovine serum albumin, MW 67 000; ovalbumin, MW 43 000; carbonic anhydrase, MW 30 000; soybean trypsin inhibitor, MW 20 100; α-lactalbumin, MW 14 400.

Profils en CLHP phase inverse des protéines de lactosérum de truie. (B) : Électrophorèse sur gel de polyacrylamide en présence de SDS des protéines de lactosérum (W) et des différentes fractions (1 à 8) obtenues par CLHP; S : protéines de référence (du haut vers le bas) : phosphorylase b, PM 94.000; sérum albumine bovine, PM 67 000; ovalbumine, PM 43 000; anhydrase carbonique, PM 30 000 30 000; inhibiteur trypsique de soja, PM 20 100 et α-lactalbumine, PM 14 400.

Table I. Amino acid composition of porcine BLG variants 1 and 2 (residue per mol; mean of 5 determinations).
Composition en acides aminés des variants 1 et 2 de la BLG porcine (nombre de résidus par mole; moyenne de 5 déterminations).

Amino acids	*BLG1*	*BLG2*	*BLG* *
ASX	16.61 ± 0.13	16.59 ± 0.16	17
GLX	23.04 ± 0.23	23.04 ± 0.23	22-23
SER	9.24 ± 0.12	9.30 ± 0.05	8
GLY	3.55 ± 0.11	3.30 ± 0.073	3
HIS	3.01 ± 0.01	3.00 ± 0.06	3
ARG	5.11 ± 0.23	5.07 ± 0.15	5
THR	9.10 ± 0.23	9.26 ± 0.18	11-10
ALA	**13.63 ± 0.22**	**12.63 ± 0.20**	**13**
PRO	8.67 ± 0.16	8.41 ± 0.07	7
TYR	1.73 ± 0.11	1.83 ± 0.12	2
VAL	**12.05 ± 0.05**	**13.04 ± 0.10**	**13**
ILE	6.08 ± 0.12	5.94 ± 0.15	6
LEU	23.32 ± 0.16	23.39 ± 0.18	25
PHE	3.10 ± 0.07	3.05 ± 0.05	3
LYS	11.00 ± 0.20	11.19 ± 0.21	11
TRP	0.71 ± 0.10	0.68 ± 0.12	1

* Amino acid composition of porcine BLG determined by Conti *et al* (1986).

α-lactalbumin, insoluble after dialysis, was also found with other proteins in precipitate P3 (data not shown). As compared with the purification method for bovine BLG described by Mailliart and Ribadeau-Dumas (1988), we have conserved only the steps at 7% and 14% NaCl. Usually, in the purification of bovine BLG the first step at 14% NaCl is not followed by centrifugation. Centrifugation is essential, however, during the purification of porcine BLG in order to eliminate the immunoglobulins present in high amounts in the colostrum. The step at 25–30% NaCl used during bovine BLG preparation in order to concentrate BLG and remove minerals, lactose

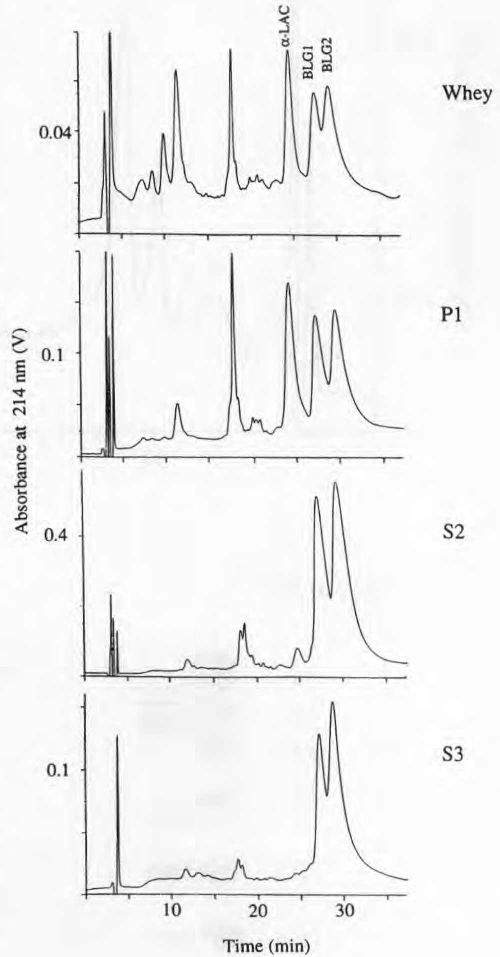


Fig 3. RP-HPLC profiles of whey proteins before and after purification by NaCl salting-out at low pH. (P1): precipitate in 14% NaCl: (S2): supernatant in 7% NaCl; (S3): supernatant after dialysis against distilled water.
Profils en CLHP phase inverse des protéines du lactosérum avant et après purification par précipitation fractionnée. (P1) : précipité formé dans NaCl 14%; (S2) : surnageant dans NaCl 7%; (S3) : surnageant après dialyse contre de l'eau distillée.

and vitamins, was inefficient in the case of porcine BLG which precipitated only partially under these conditions. In the case of bovine BLG, the pH plays an important role in association–dissociation phenomena. At acid pH, bovine BLG is perfectly soluble and exists as a monomer, while it associates to different degrees at higher pH values. This property is exploited during pH treatments proposed by Mailliart and Ribadeau Dumas (1988). Since porcine BLG was reported to be monomeric irrespective of the pH (Kessler and Brew, 1970), the pH role is likely to be less important in the purification of porcine BLG.

The adapted procedure of NaCl salting-out at low pH is suitable for rapid purification of porcine BLG in large amounts and with a high degree of purity. A subsequent step of purification by RP–HPLC allowed the separation of 2 BLG variants. These variants are actually used in our laboratory to study interactions of BLG with various hydrophobic ligands having nutritional and physiological interest.

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